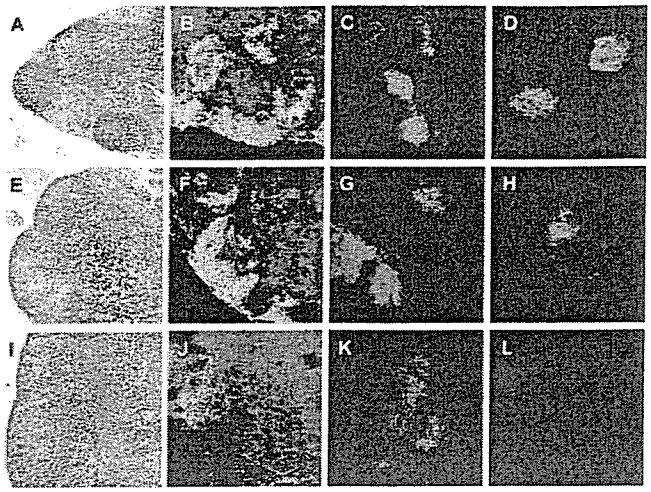

FIGURE 1. Effects of TNF/LTα or LT$\alpha\beta$ inhibition on MLN architecture. Groups of mice were orally immunized with TT plus CT as mucosal adjuvant three times at weekly intervals. These mice were also injected i.p. with TNFR55-Ig or LTβR-Ig at weekly intervals during the immunization period from day -7 to day $+14$ relative to the day of immunization. H&E staining of mice treated with control-Ig (*A*), TNFR55-Ig (*E*), or LTβR-Ig (*I*) is shown. To compare the segregation of T and B cell zones, the sections were stained for CD3 (red) and B220 (green). Shown are the MLN from control-Ig (*B*)-, TNFR55-Ig (*F*)-, or LTβR-Ig (*J*)-treated mice. Formation of GCs in mice treated with control-Ig (*C*), TNFR55-Ig (*G*), or LTβR-Ig (*K*) was stained with PNA (red). The FDC clusters in mice treated with control-Ig (*D*), TNFR55-Ig (*H*), or LTβR-Ig (*L*) were stained with CR1 (red).

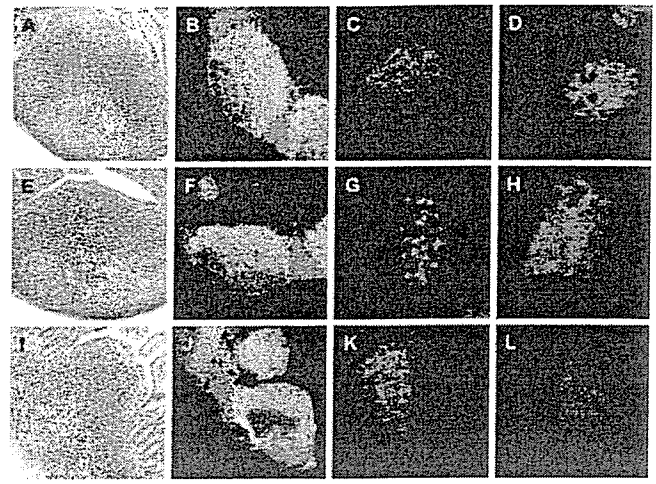

FIGURE 2. Effects of TNF/LTα or LT$\alpha\beta$ inhibition on PP architecture. Groups of mice were orally immunized with TT plus CT as mucosal adjuvant and were treated with TNFR55-Ig or LTβR-Ig during the immunization period, as described in Fig. 1 legend. H&E staining of mice treated with control-Ig (*A*), TNFR55-Ig (*E*), or LTβR-Ig (*I*) is shown. To compare the segregation of T and B cell zones, the sections were stained for CD3 (red) and B220 (green) expression. Shown are the MLN from control-Ig (*B*)-, TNFR55-Ig (*F*)-, or LTβR-Ig (*J*)-treated mice. GC formation in mice treated with control-Ig (*C*), TNFR55-Ig (*G*), or LTβR-Ig (*K*) was stained with PNA (red). The FDC clusters in mice treated with control-Ig (*D*), TNFR55-Ig (*H*), or LTβR-Ig (*L*) were stained with CR1 (red).

IgG-, or TNRF55-Ig-treated mice formed clearly defined PNA$^+$ germinal centers (GCs) and CR1$^+$ FDC clusters in MLN (Fig. 1, *C*, *D*, *G*, and *H*). In contrast, the intensity of FDC clusters was markedly reduced in LTβR-Ig-treated adult mice, although PNA$^+$ B cells resembling GCs were detected (Fig. 1, *K* and *L*). These results indicate that signaling provided via the LTβR plays an important role in the maintenance of the MLN microarchitecture.

Previous studies have shown that blockade of LT$\alpha\beta$ signaling in utero results in the failure to generate PP (6, 22); it was important to assess the contribution of the TNFR55- and LTβR-dependent events in the maintenance of the organized PP structure. There was no discernable change in numbers or size of PP when TNFR55-Ig was administered to adult mice to inhibit TNF/LTα signaling. Furthermore, the segregation of T and B cell areas was maintained, and clear GC formation and FDC clusters were also detected in PP of mice treated with TNFR55-Ig (Fig. 2, *E–H*) just as was seen in the PP of control-Ig-treated mice (Fig. 2, *A–D*). In contrast, the size of PP was reduced in LTβR-Ig-treated mice, as reported earlier (36) (Fig. 2*I*). Furthermore, the numbers of visible PP were reduced to an average of 2–4 per mouse in LTβR-Ig-administered mice when compared with an average of 6–10 visible PP in control-Ig-treated mice. Immunohistological analysis of LTβR-Ig-treated PP showed the maintenance of segregated T and B cell areas and GC formation (Fig. 2, *J* and *K*); however, FDC clusters were slightly less intense than those seen in the control, IgG-treated mice (Fig. 2, *D* and *L*). Thus, the cellular microarchitecture appears to be maintained in the visible PP remaining in LTβR-Ig-treated adult mice.

Previous studies showed that the treatment of mice in utero with LTβR-Ig ablated PP development, but did not impact the development of ILF, recently identified in the mouse small intestine (28, 29). These findings suggested that ILF developed postnatally, and were not affected by the in utero blockade of LTβR-mediated signals. We therefore assessed the effects of TNFR- or LTβR-dependent signaling on the established ILF in adult mice. TNFR55-Ig-treated mice possessed significantly decreased numbers of ILF when compared with those seen in control-Ig-treated mice (Fig. 3, *A*, *B*, and *D–F*). Furthermore, essentially no ILF were seen in the small intestine of adult mice treated with LTβR-Ig (Fig. 3, *C* and *F*).

Influence of postnatal treatment with TNFR55-Ig or LTβR-Ig on the generation of IgA Ab responses

Based upon the alteration of the mature GALT network by blockade of TNFR55- or LTβR-dependent signaling, it was important to examine the influence of these cytokine-mediated signals for the generation of mucosal IgA Ab responses in the intestinal lumen. Immunohistochemical analysis revealed that significant numbers of IgA$^+$ plasma cells were seen in the intestinal lamina propria of TNFR-Ig- or LTβR-Ig-treated, adult mice (Fig. 4*A*). Analysis of total numbers of IgA AFC supported the immunohistochemical study and showed that identical numbers of total IgA-producing cells were detected in TNFR55-Ig- or LTβR-Ig-treated, adult mice when compared with those seen in the control, IgG-treated mice (Fig. 4*B*). These findings suggest that neither TNFR55-Ig nor LTβR-Ig treatment influenced the maintenance of IgA-producing cells in the intestinal lamina propria.

In contrast, blockade of selected inflammatory cytokine signals showed some influence on Ag-specific IgA responses. Mice that received TNFR55-Ig during oral immunization with TT plus CT had TT-specific IgA Ab titers that were comparable to those seen in control, IgG-treated mice. In contrast, oral TT plus CT elicited significant TT-specific IgA Ab responses in adult mice treated with LTβR-Ig; however, the levels were significantly lower than those induced in control mice (Fig. 5*A*). The presence of TT-specific IgA Abs was further confirmed by the analysis of Ag-specific IgA AFCs where significant, but lower numbers of TT-specific IgA AFCs were detected in mononuclear cells isolated from intestinal lamina propria of LTβR-Ig-treated adult mice when compared with control mice (Fig. 5*B*). These findings indicate that loss of ILF and/or alterations in the microarchitecture of MLN resulting from blockade of

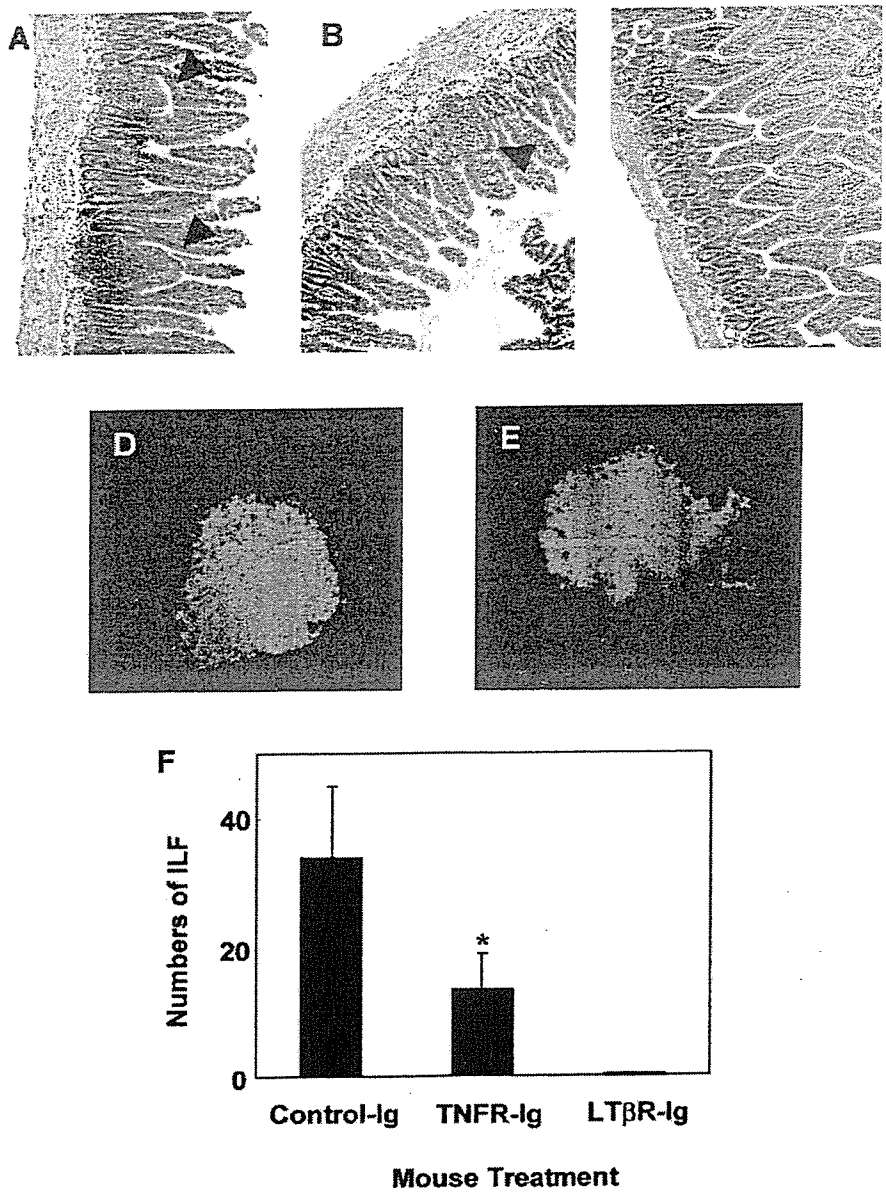

FIGURE 3. Numbers of ILF in the small intestine of mice treated with TNFR55-Ig or LTβR-Ig. Groups of mice were orally immunized with TT plus CT as mucosal adjuvant and were treated with TNFR55-Ig or LTβR-Ig during the immunization period, as described in Fig. 1 legend. The small intestine was divided into four equal parts from the pylorus downward, and segments (2 cm long) were collected from each part. The segments were stained by H&E (*A–C*) or PE anti-B220 mAb (*D* and *E*). Shown are ILF (pointed out by arrowheads) in control-Ig (*A* and *D*)-, TNFR55-Ig (*B* and *E*)-, or LTβR-Ig-treated mice (*C*). Total numbers of ILF from four segments are shown (*F*). The results are representative of three separate experiments containing four to six mice in each group/experiment. *, $p < 0.05$ when compared with control-Ig-treated mice.

LTβR-dependent signaling can directly influence Ag-specific intestinal IgA Ab responses.

ILF are not the essential inductive sites for initiation of the Ag-specific intestinal IgA response

Our previous study had shown that oral immunization of PP-null mice elicited intestinal mucosal IgA Ab responses after oral immunization (6). Other studies have shown that the structure of ILF resembles that of PP (28, 29). Taken together, these studies imply that ILF may act as an additional and compensatory inductive site for regulation of Ag-specific intestinal IgA responses in the absence of other GALT such as PP. Thus, we next investigated the possible role of ILF in the regulation of Ag-specific intestinal IgA Ab responses. For this purpose, mice were treated with both TNFR55-Ig and LTβR-Ig in utero, because the treatment of pregnant mice with both TNFR55-Ig and LTβR-Ig resulted in the lack of both PP and MLN in the progeny, whereas the fusion protein treatment in the gestation period did not influence the development of ILF in the offspring of treated dams (24, 28, 29). Treatment of pregnant mice with both TNFR55-Ig and LTβR-Ig resulted in elimination of both PP and MLN in the progeny, although some mice still possessed MLN-like tissues (data not shown). When numbers of ILF were examined in mice that completely lack both MLN and PP, higher numbers of ILF were detected when compared with control-Ig-treated mice (Fig. 6). Furthermore, total IgA levels were normal in mice treated in utero with TNFR55-Ig and LTβR-Ig (Table I). However, no Ag-specific IgA Ab responses were detected (Table I). These results indicate that ILF are unlikely to be the alternative inductive sites for the induction of intestinal Ag-specific IgA Ab responses following oral immunization.

Discussion

Our previous studies have shown that Ag-specific IgA Ab responses are induced in the small intestinal lumen, despite the lack of an organized PP (6). In the present study, we have further analyzed the involvement and role of the GALT network represented by PP, ILF, and MLN in the induction and regulation of Ag-specific intestinal IgA Ab responses when a protein Ag is given orally with mucosal adjuvant. Our results provide direct evidence for several roles of TNF/LTα and LTαβ signaling pathways in the

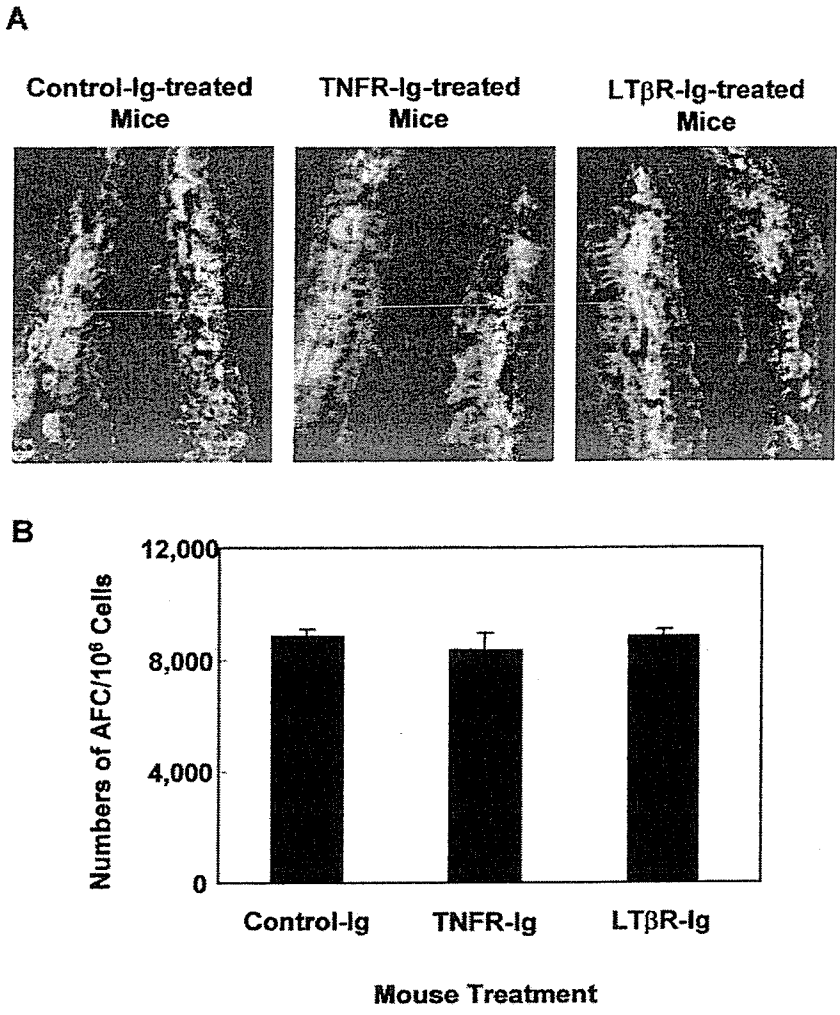

FIGURE 4. Immunofluorescence staining of IgA^+ plasma cells (*A*) and IgA AFCs (*B*) in the small intestinal lamina propria of mice treated with TNFR55-Ig or LTβR-Ig. Groups of mice were orally immunized with TT plus CT as mucosal adjuvant and were treated with TNFR55-Ig or LTβR-Ig during the immunization period, as described in Fig. 1 legend. One week after the last immunization, mononuclear cells were isolated from the small intestinal lamina propria, and total IgA AFCs were determined by ELISPOT. The results are representative of three separate experiments containing four to six mice in each group/experiment.

maintenance of the GALT network that subsequently influence the induction of Ag-specific intestinal IgA Ab responses. First, this study has shown that the signaling pathway through LTβR, but not through TNFR55, plays a key role in the maintenance of MLN microarchitecture, including the segregation of T and B cell areas as well as the presence of FDC clusters in adults. In this regard, blockade of LTαβ, but not the TNF/LTα, pathway after birth resulted in alterations in the splenic white and red pulp (15, 18, 22, 37, 38). These studies, together with our present results, indicate that the LTαβ signaling pathway plays a critical role in the maintenance of the MLN microarchitecture, including the segregation of T and B cell areas, as well as the presence of FDC clusters. Other studies have reached somewhat conflicting conclusions as to the role of TNFR55 and LTβR signaling in maintaining the LN cellular organization. For example, blockade of the TNF/LTα signaling pathway with TNFR55-Ig fusion protein in adult mice only resulted in the dissolution of B cell follicles if the LN were in a quiescent state, that is, not under Ag challenge (24, 39). Similarly, in this study, postnatal LTβR-Ig treatment was found to inhibit the FDC network in LN, as well as disrupt the organization of T and B cell zones, whereas a previous study failed to demonstrate disruption of T and B cell zones in LN (24). The basis for this discrepancy is not known. Despite this, it now appears that FDC networks are particularly sensitive to LTβR antagonism, both in the spleen and LN. Collectively, these results suggest that maintenance of the MLN architecture, e.g., T and B cell segregation and FDC clustering, is not programmed during development, but is dependent upon stimuli provided by the LTαβ pathway.

The GCs, with their prominent clusters of FDCs, are thought to provide a primary venue for the development of Ag-specific Ab responses. However, in this study, the formation of GCs was detected in the MLN of LTβR-Ig-treated, adult mice, despite a reduction in FDC clusters. In this regard, previous studies have shown that LTβ-deficient mice form GC clusters, despite the absence of an FDC network (40, 41). Thus, although the LTαβ pathway is required for generation of the FDC network, PNA^+ B cells resembling GCs can be formed in the absence of this type of FDC network.

Previous studies have shown that $LT\alpha^{-/-}$ mice (15, 37), $TNFR55^{-/-}$ mice (42, 43), and $LT\beta^{-/-}$ mice (16, 18) lack PP development. Furthermore, mice treated with LTβR-Ig in utero lack PP (6, 22). In contrast, others have reported that $TNFR55^{-/-}$ mice have PP; however, these mice only have, on average, 2–4 PP when compared with 8–10 PP in normal mice (44). Furthermore, those PP appear flattened due to a lack of B cell follicle structures (44). Finally, an interesting study has shown that $LT\alpha^{+/-}LT\beta^{+/-}$, but not $LT\alpha^{+/-}$ or $LT\beta^{+/-}$ mice specifically lack PP (45). These studies suggest that both TNFR55 and LTβR pathways are involved in the development of PP. However, the PP seen in adult mice treated with TNFR55-Ig or LTβR-Ig have a normal follicular structure. Immunohistochemical analysis revealed that T cell areas were clearly segregated from B cells, and GC formation and FDC clustering were shown to be normal. These results indicate that the

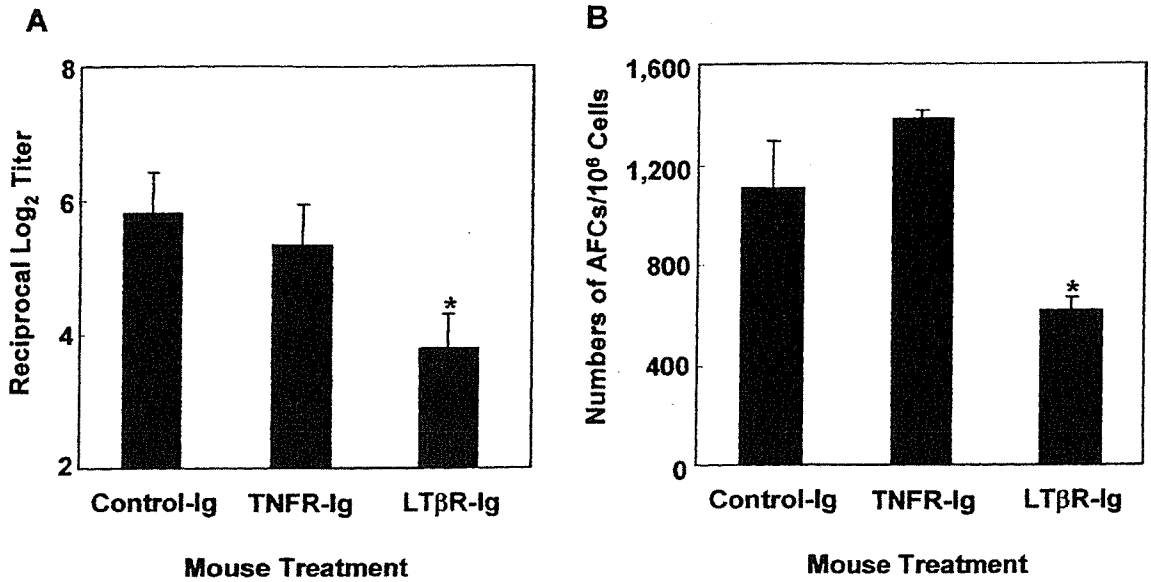

FIGURE 5. The TT-specific fecal IgA Ab titers (*A*) and IgA AFCs in lamina propria (*B*) are shown. Groups of mice were orally immunized with TT plus CT as mucosal adjuvant, and were also treated with LTβR-Ig or both TNFR55-Ig and LTβR-Ig during the immunization period, as described in Fig. 1 legend. One week after the last immunization, fecal samples were collected and examined for TT-specific Abs by ELISA. Mononuclear cells were isolated, and TT-specific IgA AFCs were examined by ELISPOT. The results are representative of three separate experiments containing four to six mice in each group/experiment. *, $p < 0.05$ when compared with control-Ig-treated mice.

TNFR55 and LTβR pathways are not essential for the maintenance of a PP microarchitecture. Importantly, however, in adult mice treated with LTβR-Ig, but not TNFR55-Ig, the PP were small in appearance, and their numbers were reduced. This finding further confirms a previous study (36). In this regard, the PP have been shown to reach their fully developed size and appearance only after stimulation with Ags that initiate a chronic GC reaction within a follicular B cell compartment (46). In this study, although mice were orally immunized with TT plus the strong mucosal adjuvant, CT, the size and numbers of PP were reduced after treatment with LTβR-Ig during the immunization period. Thus, the appearance of a reduced size and numbers of PP may be explained by their inability to undergo expansion of their B cell compartment after oral Ag challenge.

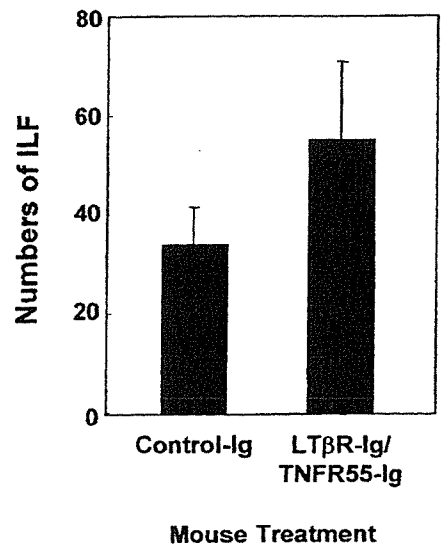

FIGURE 6. Numbers of ILF in the small intestine of mice treated in utero with TNFR55-Ig and LTβR-Ig. Groups of mice were treated with control-Ig or both LTβR-Ig and TNFR55-Ig in utero. The small intestine was divided into four equal parts from the pylorus downward, and segments (2 cm long) were collected from each. The segments were opened longitudinally and sectioned consecutively, and then stained by H&E or PE anti-B220, and numbers of ILF from four segments were counted. The results are representative of three separate experiments containing four to six mice in each group/experiment.

The small intestine has a large number of lymphoid follicles, termed ILF, throughout the length of the antimesenteric wall (28, 29, 47–49). Our previous study showed that ILF consist of a large B cell area, including a GC, and epithelia overlying these ILF contain M cells (28). Thus, ILF are similar to the follicular units that comprise the PP. However, the ILF are not detectable until postnatal life, while PP genesis is already initiated before birth (28, 50). Furthermore, the present results and other studies showed that in utero treatment with LTβR-Ig or both TNFR55-Ig and LTβR-Ig abrogates the development of PP, leaving the development of ILF unaffected (28). Conversely, blockade of TNFR55-mediated signaling in adult mice resulted in significant reductions in ILF formation. Furthermore, blockade of LTβR-dependent events led to a complete ILF deficiency, while the PP remained intact with a normal microarchitecture, although their overall size was reduced. In this regard, it has been shown that while prenatal treatment with LTβR-Ig enhanced the formation of ILF, mice treated pre- and postnatally failed to develop those lymphoid follicles (29). Furthermore, TNFR55$^{-/-}$ mice lacked ILF (29). These studies together with our results indicate that ILF and PP have a different developmental program, although both types of lymphoid tissues

Table I. *Intestinal mucosal IgA Ab responses*

Mice[a]	Total IgA (μg/ml)	Ag-Specific IgA (reciprocal $\log_2$)
Control-Ig	52.4 ± 11.7	7.3 ± 0.4
LTβR-Ig/TNFR55-Ig	43.4 ± 14.4	< 3

[a] Pregnant mice were injected i.v. with LTβR-Ig and TNFR55-Ig on gestational days 13 and 16.

require the LTβR signaling pathway. Thus, LTβR-dependent events in embryonic intestine are essential for organogenesis of PP, whereas postnatal LTβR signaling is required for development and maintenance of ILF. Furthermore, TNFR55-dependent events in adulthood are also important for ILF formation.

The structural similarity of ILF and PP led us to query whether ILF could serve as inductive sites for intestinal IgA Ab responses. In this regard, a previous study reported that isotype switching from $B220^+$ IgM^+ B cells to IgA^+ plasma cells may occur in situ via direct interactions with lamina propria stromal cells (26). Furthermore, LTβR signaling on the intestinal lamina propria stromal cells has been shown to be important for IgA production (27). Moreover, our separate studies have demonstrated that the activation-induced cytidine deaminase- and Iμ-Cα-specific mRNA were detected in lymphocytes isolated from ILF, but not in diffuse connective tissue of the intestinal lamina propria (51). Thus, ILF could be an additional IgA inductive tissue in the GI tract. However, our present study showed that oral immunization of TNFR55-Ig-treated mice, which possess significantly lower numbers of ILF, induced identical levels of Ag-specific IgA Ab responses when compared with control-Ig-treated mice. Furthermore, LTβR-Ig-treated, ILF-null mice elicited significant Ag-specific IgA Ab responses after oral immunization. These results indicate that ILF are not required for the induction of intestinal IgA Abs. In this regard, a recent study has shown that in utero LTβR-Ig-treated, PP-null mice have mature ILF, which contain an overlying epithelium, M cells, GCs, and $CD4^+$ T cells resembling PP, while nontreated mice have immature ILF formed by $B220^+$ cells only (29). This study implies that ILF are alternative inductive tissues that compensate for the function of PP. Thus, ILF may act as inductive sites for IgA Ab responses to orally administered Ag in the absence of PP, whereas these lymphoid follicles do not fully develop and are not strictly required for Ag-specific intestinal IgA responses in the presence of PP. However, our present results showed that mice treated with both TNFR55-Ig and LTβR-Ig in utero that retain ILF, but not PP and MLN, failed to induce TT-specific IgA responses after oral immunization. These findings indicate that ILF are not essential inductive sites for initiation of IgA Ab responses to orally administered Ag, although these lymphoid follicles could be an important source of IgA B cell development.

Alternatively, development of ILF may be induced by luminal bacterial Ag in the small intestine. In this regard, it has been shown that alterations in the bacterial flora by antibiotic treatment abolished ILF hyperplasia, which was provoked by the deficiency of activation-induced cytidine deaminase (30). Furthermore, mature ILF were not found in in utero LTβR-Ig-treated germfree mice; however, the ILF were reorganized when those mice were conventionalized (29). These studies, together with our results, suggest that formation of ILF is induced in response to microenvironmental bacterial Ags, but not to an orally administered protein. An interesting study has shown that IgA can be generated in peripheral LN of μMT mice lacking Ig μ and γ gene expression (52). Furthermore, those IgA Abs bind the protein of commensal intestinal bacteria and are induced by *Salmonella* infection (52). Because ILF can be formed in response to commensal intestinal bacterial Ag, IgA produced without μ- or δ-chain expression in developing B cells may be induced in ILF. These issues are currently under investigation in our laboratories.

It is important to note that postnatal treatment with LTβR-Ig results in the alteration of an MLN microarchitecture, including disorganized T and B cell zones and a diminished FDC network. Furthermore, although Ag-specific mucosal IgA responses were induced in mice treated with LTβR-Ig, the levels were significantly lower than those induced in control-Ig-treated mice. In contrast, ILF alone are not sufficient for the induction of Ag-specific mucosal IgA responses after oral immunization. These results suggest that the MLN microarchitecture at least in part plays an important role in the regulation of intestinal IgA Ab responses to orally administered Ag. In this regard, the MLN is considered to be one of the important components of GALT (2, 53, 54). Indeed, Ag-specific $CD4^+$ T cells are induced in the MLN with subsequent intestinal IgA Ab responses in the absence of PP (6), implying that the MLN is somewhat more important than PP for the generation of Ag-specific intestinal IgA immunity. In the lymphoid tissues, the presence of primary and secondary lymphoid follicles that contain FDC is thought to be required for a mature T cell-dependent, B cell response, and is associated with B cell isotype switching, affinity maturation, and development of Ab-secreting cells (55–57). Thus, it is likely that the disorganized follicular structure, T and B cell zones, and diminished FDC clusters in MLN of LTβR-Ig-treated, adult mice negatively impact Ag-specific mucosal immunity and lead to reductions in IgA Ab responses.

Our results clearly indicated that LTβR-Ig-treated mice that lack ILF induced intestinal IgA Ab responses to orally administered Ag. Thus, we at the least can conclude that ILF are not essential for induction of Ag-specific IgA responses. However, because the responses induced in the mice lacking ILF were significantly lower than those seen in the control mice, ILF may contribute to the generation of maximum IgA responses. In this regard, our previous study has shown that Ag-specific intestinal IgA Abs were induced after oral immunization in mice made deficient in PP, but not ILF and MLN, by in utero treatment with LTβR-Ig; however, the responses were also significantly lower than those seen in the control mice, suggesting a contribution by PP for maximum IgA responses (6). This finding together with our present results suggest that aggregated lymphoid follicles residing in the intestinal lumen, e.g., PP and ILF, and draining MLN may comprise an integrated regulatory network for the induction of IgA Ab responses to orally administered Ag. This interesting possibility is currently under investigation in our laboratories.

In summary, our study has demonstrated that LTβR-dependent events contribute importantly to the maintenance of a normal MLN microarchitecture and to the size and numbers of PP. In contrast, the microarchitecture of the PP was not controlled by LTβR signaling. Furthermore, postnatal blockade of the LTβR pathway resulted in a lack of ILF. Oral immunization of LTβR-Ig-treated mice with TT plus CT elicited significant TT-specific mucosal IgA Abs; however, the responses were significantly lower than those of control mice. In contrast, in utero TNFR55-Ig- and LTβR-Ig-treated mice that have ILF, but not PP and MLN, failed to induce intestinal IgA Abs to orally administered Ag. Taken together, our findings have addressed the roles of TNF/LTα and LTαβ pathways for the establishment of Ag-specific mucosal IgA Ab responses. Furthermore, ILF are not required for the induction of IgA Ab responses to orally administered protein Ag. Finally, the microarchitecture of the MLN plays a critical role in the induction and regulation of IgA Ab responses to orally administered Ag.

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Non-toxic Stx derivatives from *Escherichia coli* possess adjuvant activity for mucosal immunity

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Received 6 November 2003; received in revised form 9 February 2004; accepted 2 March 2004

Available online 22 April 2004

Abstract

Both B subunit of Shiga toxin 1 (Stx1-B), which mediates the binding of toxin to the membrane, and mutant Stx1 (mStx1), which is a non-toxic double-mutated Stx1 harboring double amino acid substitutions in the A subunit, possess potent mucosal adjuvant activity. Nasal immunization of mice with ovalbumin (OVA) plus the Stx1-B or mStx1 induced OVA-specific serum IgG and mucosal IgA responses. IgG subclass analysis revealed that mStx1 and Stx1-B as mucosal adjuvants supported Ag-specific IgG1 followed by IgG2b Abs. The co-administration of either mStx1 or Stx1-B with OVA enhanced the production of IL-4, IL-5, IL-6 and IL-10 with low IFN-γ, by OVA-specific $CD4^+$ T cells. To better elucidate the mechanisms underlying mStx1's and Stx1-B's adjuvant activity, we next sought to examine whether or not dendritic cells (DC) residing in the nasopharyngeal-associated lymphoreticular tissue (NALT) were activated by nasal administration of Stx1-B or mStx1. We found that mice nasally administered with Stx1-B or mStx1 showed an up-regulation in the expression of CD80, CD86 and especially CD40 on NALT DCs. Taken together, these results suggest that non-toxic Stx derivatives could be effective mucosal adjuvants for the induction of Th2-type, $CD4^+$ T cell mediated, antigen-specific mucosal IgA and systemic IgG Ab responses, and that they likely owe their adjuvant activity to the up-regulation of co-stimulatory molecules including CD80, CD86 and CD40 on NALT DCs.

Keywords: Stx; Adjuvant

1. Introduction

Adjuvants are key to the development of effective mucosal vaccine because they can compensate for the often poorly immunogenic nature of orally and nasally administered vaccine antigens by inducing vaccine antigen-specific humoral and/or cellular immune responses. Much of the protection available at mucosal surfaces such as the respiratory, gastrointestinal and urogenital tracts is provided by the production of secretory IgA (S-IgA) antibodies (Abs) which are effectively produced only when vaccine is administered by a mucosal route [1]. In an effort to develop new strategies to curb global infection, researchers in the field are currently trying to develop novel adjuvants which can be nasally or orally co-administered with vaccine antigen to maximize the induction of protective S-IgA antibodies.

Thus far, several bacterial enterotoxins including cholera toxin (CT) of *Vibrio cholerae* and heat labile enterotoxin (LT) of enterotoxigenic *Escherichia coli* have been identified as possessing strong immunoenhancing activity against co-administered protein antigen when given by the oral or nasal routes [2–5]. By eliciting antigen (Ag)-specific Th2-type $CD4^+$ T cell responses with high levels of IL-4 and IL-5 production, mucosally co-administerated CT enhances the generation of Ag-specific systemic IgG1, IgE and mucosal S-IgA responses [6]. In contrast, LT induces a mix of IFN-γ-producing $CD4^+$ Th1-type and IL-4-, IL-5-, IL-6-, and IL-10-secreting Th2-type cells for subsequent induction of serum IgG1, IgG2a, and mucosal S-IgA Ab

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doi:10.1016/j.vaccine.2004.03.034

responses [7]. Other bacterial toxins such as pertussis toxin and PT-9K/129G, the genetically detoxified derivative of pertussis toxin have also been shown to possess mucosal adjuvant activities [8,9]. Pertussis toxin potentiates both Th1 and Th2 responses to antigen co-injected via the systemic route [10]. The mucosal presentation of a chimeric molecule composed of the gp 120V3 loop region of the MN strain of HIV-1 and a non-toxic form of *Pseudomonas* exotoxin resulted in strong mucosal and systemic immune responses to an integrated HIV-Ag [11]. However, Shiga toxin (Stx) was found to possess immunogenicity but not adjuvant activity when given via the oral route [12].

Stx, which is generated by Stx-producing *E. coli* (STEC), is one of the major virulence factors for STEC infectious diseases. Stx is a holotoxin composed of an A subunit measuring approximately 32-kDa in non-covalent association with a pentameric ring of an identical B subunit, each with a molecular mass of 7.7-kDa [13]. The A subunit is the enzymatic component of the toxin and acts as a highly specific *N*-glycosidase enzyme hydrolyzing the bond between ribose and a single adenine residue found on a prominent loop structure in the 28S rRNA component of eukaryotic ribosomes [14,15]. The B subunits mediate the binding of toxin to the neutral glycolipids of cell membranes, globotriaosylceramide and globotetraosylceramide [16]. Stx is classified into two groups: Stx1, the amino acid sequence of which is identical to that of Shiga toxin: Stx2, which is immunologically distinct from Stx1 [17].

In an effort to develop a candidate for a vaccine against infectious diseases caused by enterohemorrhagic *E. coli*, we have previously used site-directed mutagenesis to generate E167Q & R170L (mStx1), a double mutant of Stx1, harboring double amino acid substitutions in its RNA *N*-glycosidase active center [18]. Due to these mutations, mStx1 lacks RNA *N*-glycosidase activity, cytotoxicity and mouse lethality [18]. In the present study, we have addressed whether or not mStx1 and Stx1-B elicit mucosal adjuvant activity when co-administered nasally with protein antigen. Furthermore, we have assessed the capability of nasally administered mStx1 and Stx1-B to activate dendritic cells (DC) in nasopharyngeal-associated lymphoreticular tissue (NALT). Our results suggest that both mStx1 and Stx1-B are effective mucosal adjuvants for the induction of Ag-specific Ab responses in both mucosal and systemic compartments. Moreover, when applied nasally, they are also capable of up-regulating co-stimulatory molecules including CD80, CD86 and CD40 on NALT DCs.

2. Materials and methods

2.1. Mice

C57BL/6 mice purchased from SLC (Shizuoka, Japan) or Cler Japan, Inc. (Tokyo, Japan) were maintained and bred in the experimental animal facility at Osaka University under pathogen-free conditions in microisolator cages. All mice were provided sterile food and water ad libitum. C57BL/6 mice were used in this study at 8–12 weeks of age.

2.2. Bacterial toxins

The mutant of Stx1 (mStx1), native (n)Stx1 and nStx2 were purified from *E. coli* MC 1061 strain M 23, strain 87-27 and strain Tp 8, respectively [18,19]. As described previously, purification steps included ion-exchange, chromatofocusing and HPLC as described previously [19]. The B subunit of Stx1 (Stx1-B) was derived from *Bacillus brevis* pNU212-VT1B and was purified by the use of ion-exchange and gel filtration [20].

The amount of endotoxin was measured in the toxin preparation with an Endospec-SP test (Seikagaku Co., Tokyo, Japan). The nStx1, nStx2, mStx1 and Stx1-B used in this study contained 7.03, 9.52, 34.0 and 3.05 pg of *lipopolysaccharide* (LPS) per 10 μg of protein, respectively. The range of these LPS contents (e.g. 3-35 pg/10 μg protein) has been shown to have no biological effect on the immune system [21,22].

2.3. Immunization protocol and sample collection

A standard nasal immunization protocol was used in this study [23]. Mice were nasally immunized on days 0, 7 and 14 with a 10 μl aliquot (5 μl per nostril) containing 100 μg of ovalbumin (OVA; Sigma, St. Louis, MO) alone or combined with various doses of mStx1, Stx1-B, nStx1 or nStx2 as mucosal adjuvants [23]. Saliva was obtained from mice following i.p. injection with 100 μl of 1 mg/ml pilocarpine (Sigma). Nasal washes were collected by gently flushing the nasal passage with 100 μl of sterile PBS [23].

2.4. Analysis of antibody responses

Ag-specific Ab titers in serum, saliva, and nasal washes were determined by ELISA as described previously [6,24]. Briefly, plates were coated with OVA (1 mg/ml) and blocked with 1% BSA in PBS. After the plates were washed, serial dilutions of serum, saliva, or nasal washes were added in duplicate. Following incubation, the plates were again washed and peroxidase-labeled goat anti-mouse μ, γ or α heavy chain-specific Abs [Southern Biotechnology Associates (SBA), Birmingham, AL] were added to appropriate wells. Finally, 3,3′,5,5′-tetramethylbenzidine (TMB) with H_2O_2 was added for color development.

For IgG subclass analysis, biotinylated rat monoclonal anti-mouse γ1 (G1-7.3), γ2a (R19-15), γ2b (R12-3) or γ3 (R40-82) heavy chain-specific Abs (BD PharMingen, San Diego, CA) and streptavidin-conjugated peroxidase (Vector Laboratories, Inc., Burlingame, CA) were employed. For the analysis of total IgE antibodies, OptEIA ELISA for IgE was used (BD PharMingen). Endpoint titers were expressed

as the reciprocal $\log_2$ of the last dilution giving an optical density at 450 nm (OD_{450}) of ≥ 0.1 above negative control.

2.5. Detection of Ag-specific Ab-forming cells (AFCs) by the enzyme-linked immunospot (ELISPOT) assay

In the ELISPOT assay, numbers of Ag-specific AFCs from various tissues including salivary glands, nasal passages and spleens were determined by direct counting of spots as previously described in detail [4,24]. Ninty-six-well nitrocellulose-based plates (MultiScreen-HA, Millopore Co., Bedford, MA) were coated with 1 mg/ml of OVA diluted in PBS for enumeration of Ag-specific AFC. Wells were blocked with RPMI1640 medium containing 10% FCS, HEPES buffer (15 mM), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) (complete medium). Cells at various dilutions were added and incubated for 6 h at 37 °C in 5% CO_2 in moist air. Antigen-specific AFCs were detected with peroxidase-labeled anti-mouse μ, γ, or α Ab (SBA) and then visualized by adding the chromogenic substrate, 3-amino-9-ethylcarbazole (Moss. Inc., Pasadena, MD). Spots were counted with the aid of a dissecting microscope (SZH Zoom Stereo Microscope System, Olympus, Lake Success, NY).

2.6. OVA-specific $CD4^+$ T cell responses

$CD4^+$ T cells were purified from cervical lymph node (CLN) and splenic cell suspensions by use of the magnet-activated cell sorter system (Miltenyi Biotec) [24]. Cells were added to a nylon wool column (Polysciences, Warrington, PA) and incubated at 37 °C for 1 h to remove adherent cells. The $CD4^+$ T cell subset was then obtained by positive sorting using a magnetic bead separation system consisting of anti-CD4 monoclonal (m)Ab (clone GK1.5)-conjugated microbeads (MACS; Miltenyi Biotec). Purified $CD4^+$ T cells (>98% purity) were cultured at a density of 4×10^6 cells/ml with OVA (1 mg/ml) with T cell-depleted, irradiated (3000 rads) splenic feeder cells (8×10^6 cells/ml) and rIL-2 (10 units/ml; PharMingen) in complete medium [23]. The $CD4^+$ T cell cultures were incubated for 3 days at 37 °C in 5% CO_2 in air. Culture supernatants were then harvested for quantitation of secreted IFN-γ, IL-4, IL-5, IL-6 and IL-10 by a commercial AN'LYZA immunoassay kit (R&D Systems, Minneapolis, MN). To measure the levels of Ag-specific T cell proliferation, 0.5 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech) was added to individual cultures 18 h before termination, and the uptake of [^{3}H]thymidine in counts per minute (cpm) was determined by scintillation counting [25].

2.7. FACS analysis

Cells were analyzed by FACS (FACS Calibur & CellQuest; Becton Dickenson Co. Inc., San Jose, CA) using the following antibodies from BD PharMingen: fluorescerin isothiocyanate (FITC)-conjugated anti-mouse CD11c (clone HL3), phycoerythrin (PE)-conjugated anti-mouse CD80 (clone 16-10A1), PE-conjugated anti-mouse CD86 (clone GL1), PE-conjugated anti-mouse I-A^b (clone AF6-120.1), and PE-conjugated CD40 (clone 3/23).

2.8. Isolation of NALT DC

NALT was isolated and then rinsed in complete medium [23] before being digested with collagenase D (400 Mandl units/ml; Roche, Indianapolis, IN), as previously described [26]. Briefly, NALT was incubated with collagenase D (400 Mandl units/ml) and DNase I (200 μg/ml; Roche) for 35 min at 37 °C in RPMI 1640 medium, and EDTA at a final concentration of 5 mM was added during the last 5 min of incubation. For the enrichment of DC, released cells were layered over a metrizamide gradient column (Accurate, Westbury, NY; 14.5 g of metrizamide added to 100 ml of complete medium) and centrifuged, and the low-density fraction was collected as DCs [27]. The enriched DC cells were counted and then stained with the appropriate monoclonal antibodies, as described above for FACS analysis.

2.9. Statistical analysis

The results are reported as mean ± one standard error (S.E.). Statistical significance ($P < 0.05$) was determined by Student's *t*-test and by the Mann–Whitney *U*-test of unpaired samples.

3. Results

3.1. Induction of Ag-specific systemic Ab responses by nasal administration of OVA and mStx1 or Stx1-B

We began by assessing whether nasal co-administration of newly developed non-toxic Stx1 derivatives such as mStx1 or Stx1-B would provide mucosal adjuvant activity for the induction of Ag-specific Ab responses (Fig. 1A). Mice were nasally immunized with an optimal dose of OVA in the presence or absence of different concentrations of the adjuvant candidates. Although all doses of mStx1 tested in this study (e.g. 0.1–20 μg) provided the adjuvant activity, administration of 0.5 μg of mStx1 resulted in the highest OVA-specific IgM and IgG Ab responses among several doses tested (data not shown). Of all the dosages of Stx1-B tested (e.g. 0.1–20 μg), the administration of 5 μg of Stx1-B produced the most impressive serum IgM and IgG anti-OVA Ab responses. Although a dosage of 0.5 μg of the native form of Stx1 (nStx1) resulted in some adjuvant activity, high doses (e.g. 2 μg) of nStx1 proved universally lethal to mice ($n = 5$) (data not shown). Due to its lethality, native Stx1 does not make a practical mucosal adjuvant candidate. That same lethality also makes nStx1 unsuitable for subcutaneous co-administration, as we found in an earlier study (unpub-

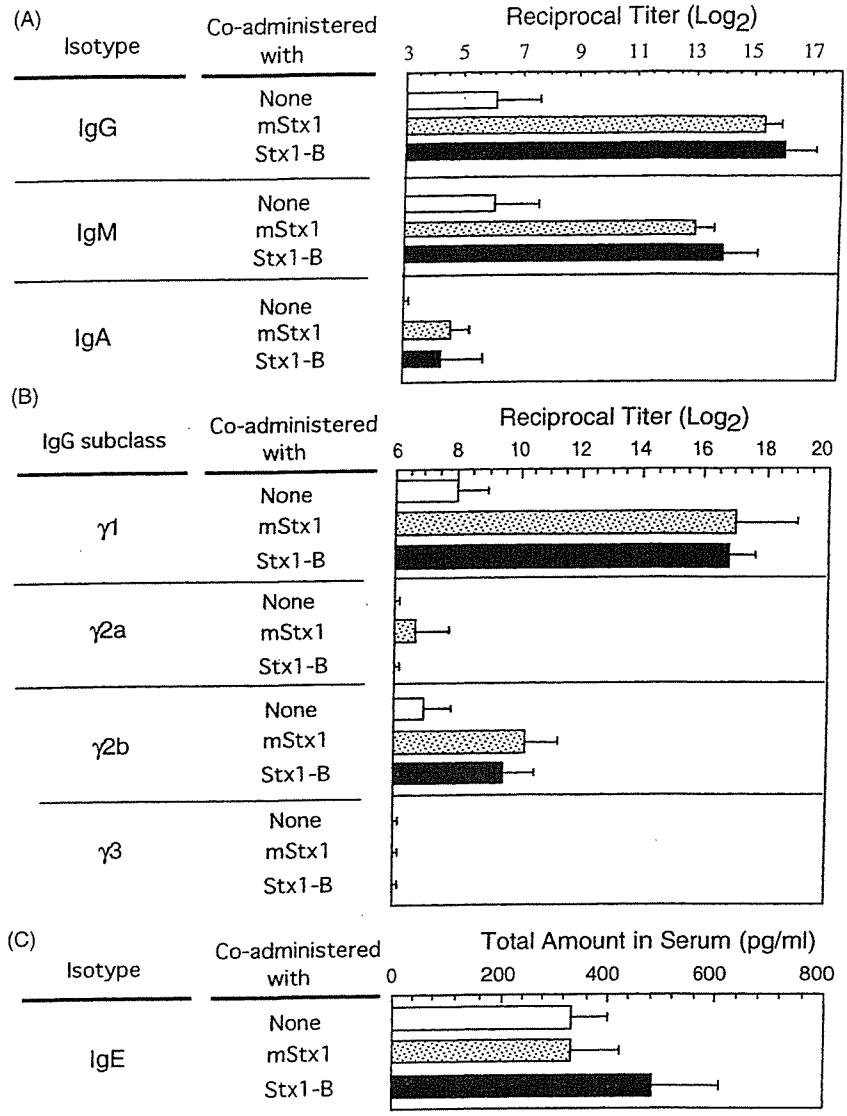


Fig. 1. Mucosal adjuvant activity of Stx1 derivatives for the induction of systemic antibody responses. OVA-specific serum IgM, IgG and IgA Ab responses (A), IgG subclass Ab responses (B) and total IgE responses (C) were examined and compared in serum of mice nasally immunized with OVA plus mStx1 or Stx1-B. Groups of C57BL/6 mice were nasally immunized with 100 μg of OVA plus 0.5 μg of Stx1 mutant (dotted bar) (E167R & R170L; mStx1), or 5 μg of Stx1-B (black bar) as mucosal adjuvant or with OVA alone (white bar) on days 0, 7 and 14. Serum samples were collected at day 21 and examined for OVA-specific IgM, IgG and IgA Abs, OVA-specific IgG subclass Ab responses and total IgE by ELISA. The results are expressed as the mean ± S.E.M. from five to six mice per group and from a total of three separate experiments.

lished data). In summary, we found mStx and Stx1-B to be the best candidates for possible mucosal adjuvants, since an optimal dose of 0.5 or 5 μg, respectively, induced serum IgM, IgG and relatively low IgA anti-OVA Ab responses (Fig. 1A). Consequently, the remainder of our experiments focused on the mucosal adjuvant activity of these two forms of non-toxic derivatives. As expected, antigen-specific Ab responses were low after nasal immunization with OVA alone (Fig. 1A). Analysis of OVA-specific IgG subclass responses revealed that co-administration of mStx1 or Stx1-B resulted in a major IgG subclass response with IgG1 subclass appearing, followed by IgG2b (Fig. 1B). The levels of total IgE were not statistically changed between mice immunized with OVA and Stx1 derivatives and those administered OVA alone (Fig. 1C). Further, nasal immunization of OVA and Stx1 derivatives did not mount for antigen-specific IgE antibodies (Stx1-B: 6.85 ± 0.05 and mStx1: 6.80 ± 0.02) when compared with OVA alone (<6.0) (data not shown).

When antigen-specific IgG antibody forming cells (AFC) in the spleen and cervical lymph node (CLN) of mice nasally immunized with OVA plus Stx1 derivatives were analyzed, significant numbers of OVA-specific IgG AFC were detected, confirming the results obtained by the characterization of OVA-specific serum Ab responses. In contrast, low numbers of OVA-specific IgG AFC were seen in spleen and CLN of mice given OVA alone (Fig. 2A).

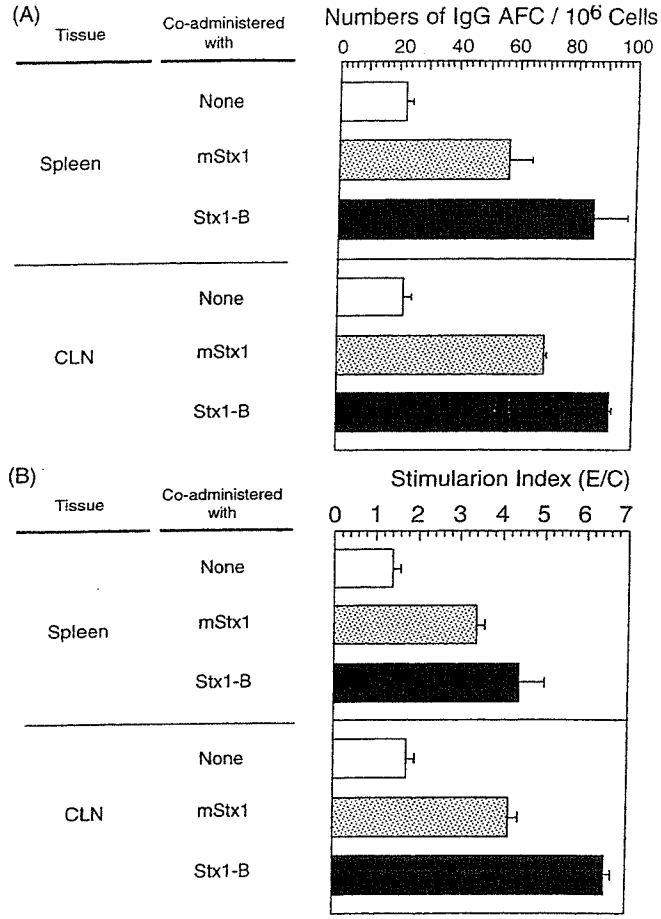


Fig. 2. Analysis of OVA-specific antibody-forming cells (AFC) and OVA-specific CD4$^+$ T cell proliferative responses in mice nasally immunized with OVA and Stx1 derivatives. Mice were nasally immunized with OVA (100 μg) plus 0.5 μg of mStx1 (dotted bar), 5 μg of Stx1-B (black bar) or OVA alone (white bar). Mononuclear cells were isolated from spleen of nasally immunized mice at day 21 and examined by Ag-specific ELISPOT (A). Purified splenic CD4$^+$ T cells were co-cultured at a density of 2×10^6 cells/ml with 1 mg/ml of OVA, T cell-depleted, irradiated splenic feeder cells (4×10^6 cells/ml) in the presence of rIL-2 (10 U/ml) in complete medium for 3 days for the proliferation assay (B). A control culture consisting of CD4$^+$ T cells, feeder cells and rIL-2 (10 U/ml) resulted in a [^{3}H] thymidine incorporation of 160 ± 19 cpm. The results are expressed as the mean of stimulation index ± S.E.M. from five to six mice per group and from a total of three experiments.

3.2. Ag-specific mucosal IgA immune responses induced by the co-administration of mStx1 or Stx1-B as a mucosal adjuvant

It is important to note that nasal co-administration of mStx1 or Stx1-B supported the induction of OVA-specific IgA Ab responses in mucosal secretions. Thus, nasal immunization with OVA and an optimal dose of mStx1 resulted in the induction of antigen-specific IgA Abs in saliva and nasal washes (Fig. 3A). Furthermore, co-administered Stx1-B also induced the secretion of OVA-specific IgA antibodies in saliva and nasal washes. In contrast, IgA Abs were not induced in mice by nasal immunization with OVA alone (Fig. 3A). Analysis of antigen-specific AFC further demonstrated that nasally co-administered mStx1 or Stx1-B supported the induction of OVA-specific IgA AFC in nasal passages and salivary glands (Fig. 3B). These findings further support the notion that the nontoxic Stx1 derivatives possess mucosal adjuvanticity for the generation of antigen-specific mucosal IgA responses following nasal administration.

3.3. Non-toxic Stx1-derivatives induced OVA-specific CD4$^+$ T cell responses

When CD4$^+$ T cells from spleen and CLN of mice nasally immunized with OVA plus mStx1 or Stx1-B were restimulated with the antigen in vitro, the levels of OVA-specific proliferative responses were increased (Fig. 2B). Antigen-specific CD4$^+$ T cell responses were the highest in mice nasally immunized with OVA and Stx1-B, and next highest in those immunized with OVA and mStx1. In contrast, there was virtually no Ag-specific proliferation in CD4$^+$ T cells isolated from mice given OVA alone (Fig. 2B). These results suggest that Stx1-B and mStx1 are potential adjuvants for the induction of antigen-specific CD4$^+$ T cells in both mucosal (e.g., CLN) and systemic (e.g., spleen) tissues.

In the subsequent experiment, the production of IL-4, IL-5, IL-6, IL-10 and IFN-γ production by antigen-specific CD4$^+$ T cells was analyzed at the protein level (Fig. 4). Increased levels of IL-4, IL-5, IL-6 and IL-10 production with low IFN-γ were seen in OVA-stimulated CD4$^+$ T cell cultures prepared from CLN and spleen of mice nasally immunized with OVA plus Stx1-B. Similarly, CD4$^+$ T cells isolated from mice nasally immunized with OVA and mStx1 also resulted in the induction of IL-4, IL-5, IL-6 and IL-10 production. Mice co-administered with Stx1-B always showed higher levels of Th2-type cytokine production than did those co-administered with mStx1. Splenic and CLN CD4$^+$ T cells from mice given OVA alone produced neither IL-4 nor IL-5, and only minimal amounts of IL-6 and IL-10. Taken together, these results show that nasal administration of OVA plus Stx1-B or mStx1 as mucosal adjuvant induced antigen-specific Th2-type cytokine responses which in turn led to the generation of OVA-specific mucosal IgA as well as predominant serum IgG1 Ab responses.

3.4. Up-regulation of co-stimulatory molecules and CD40 on NALT DCs following nasal application of Stx1-B or mStx1

To examine whether the increased Ag-specific Th cell and B cell responses seen in these nasally immunized mice were associated with NALT DC activation, we next analyzed the expression of costimulatory molecules, MHC class II and CD40 on NALT DCs by flow cytometry 24 h after nasal administration of naïve mice with Stx1-B or mStx1 (Fig. 5). Approximately 65–75% of NALT DCs isolated from non-treated mice constitutively expressed the

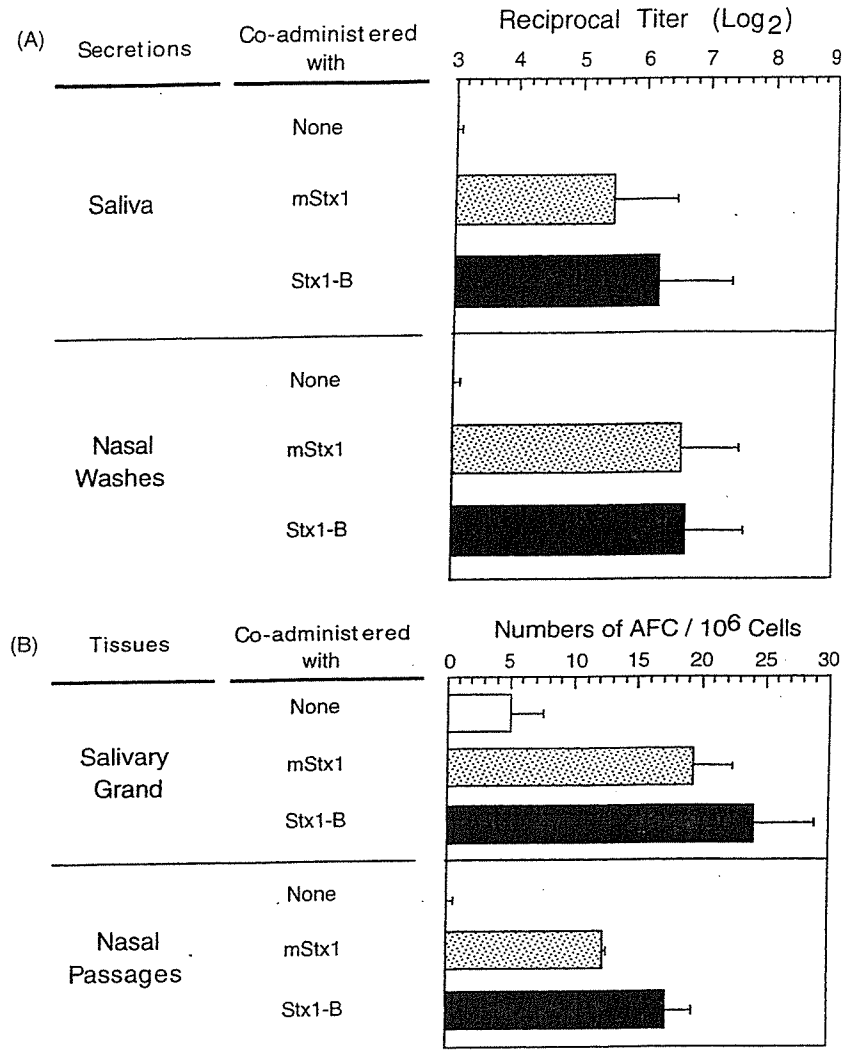


Fig. 3. Mucosal adjuvant activity of Stx1 derivatives for the enhancement of Ag-specific IgA responses. Induction of OVA-specific mucosal IgA Ab responses in saliva and nasal washes (A) and of OVA-specific IgA AFCs in salivary glands and nasal passages (B) of mice nasally immunized with OVA and mStx1 or Stx1-B was examined. Groups of mice were nasally immunized with OVA plus 0.5 μg of Stx1 mutant (dotted bar), 5 μg of Stx1-B (black bar) or OVA alone (white bar). External secretions were collected at day 21 and examined for OVA-specific IgA Ab responses by ELISA. Mononuclear cells were isolated from salivary glands and nasal passages of nasally immunized mice at day 21 and examined for OVA-specific IgA AFCs by ELISPOT. The results are expressed as the mean ± S.E.M. from five to six mice per group and from a total of three experiments.

co-stimulatory molecules CD80 and CD86 (Fig. 5). Further, most of these NALT DC expressed MHC class II (data not shown), with approximately 55% of cells positive for CD40. Following nasal administration of mStx1 or Stx1-B, the levels of CD80, CD86, and CD40 expression on NALT DCs were increased. These findings suggest that nasally co-administered mStx1 and Stx1-B trigger partially activated NALT DC to fully activate.

4. Discussion

In this study, we have assessed a mutant form of Stx1 (E167Q & R170L; mStx1) and the B subunit of Stx1 (Stx1-B) as possible mucosal adjuvants for the induction of antigen-specific mucosal and systemic immune responses. Nasal co-administration of non-toxic Stx1-B or mStx1 as mucosal adjuvant induced high levels of mucosal anti-OVA IgA as well as serum IgG anti-OVA Ab responses. These two distinct forms of non-toxic Stx1 derivative preferentially induced antigen-specific Th2-type $CD4^+$ T cells which in turn generated OVA-specific IgG1 and IgA antibodies in the systemic and mucosal compartments, respectively. Our finding that mStx1 and Stx1-B enhanced CD80, CD86 and CD40 expression on NALT DCs also supports the mucosal adjuvanticity of these two forms of non-toxic Stx1 derivative for the induction of antigen-specific immune responses.

If practical application of the mucosal adjuvanticity of these two forms of non-toxic Stx1 derivative is to be realized,

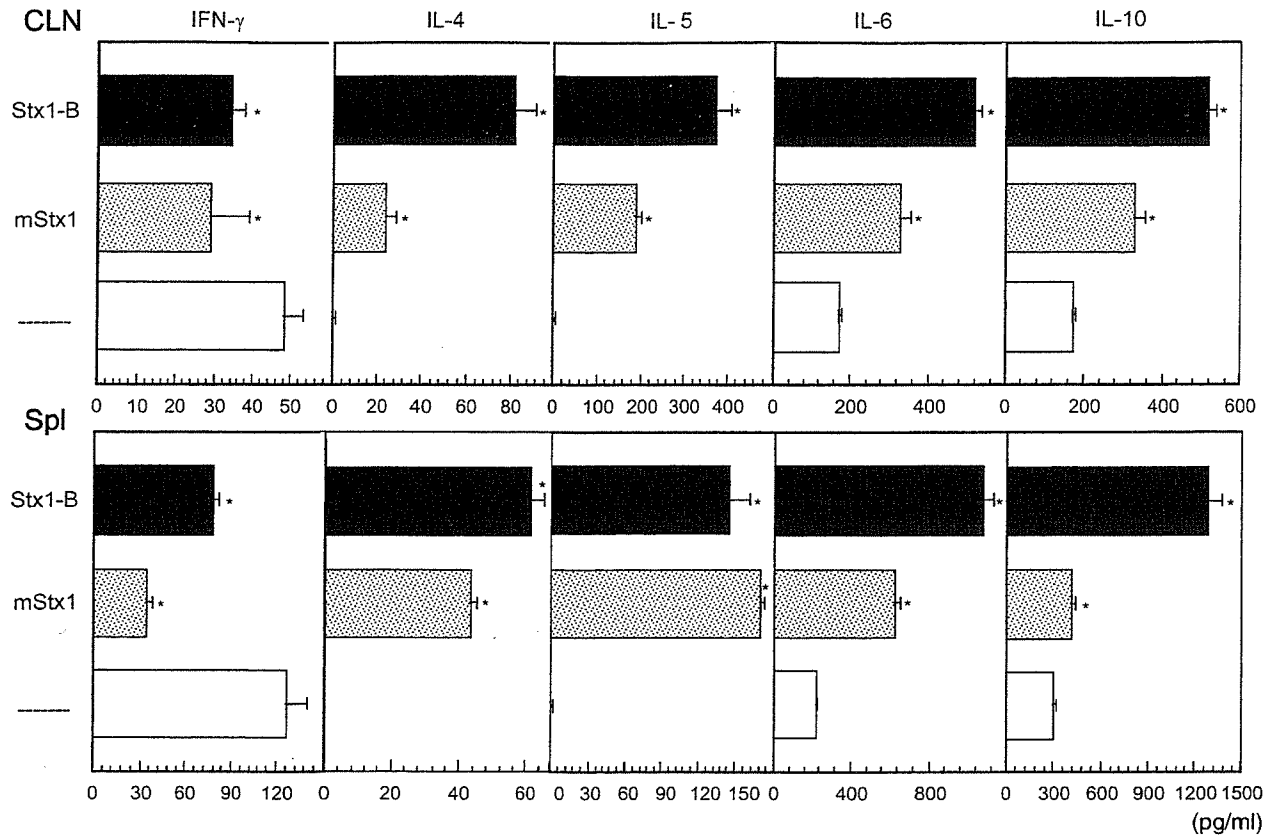
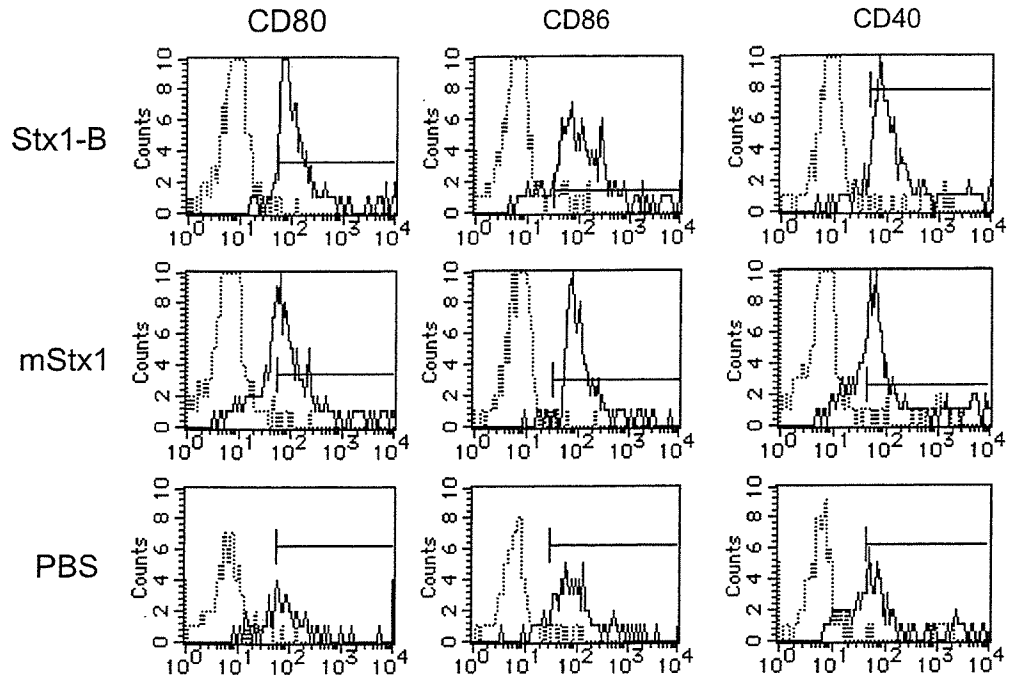


Fig. 4. Analysis of Th1 (IFN-γ) and Th2 (IL-4, IL-5, IL-6 and IL-10) cytokine synthesis by antigen-specific $CD4^+$ T cells isolated from mice nasally immunized with OVA and Stx1 derivative. $CD4^+$ T cells were isolated from CLN and spleen of mice nasally immunized with OVA plus 0.5 μg of mStx1 (dotted bar), 5 μg of Stx1-B (black bar) or OVA alone (white bar). Purified splenic $CD4^+$ T cells were co-cultured at a density of 2×10^6 cells/ml with 1 mg/ml of OVA, T cell-depleted, irradiated splenic feeder cells (4×10^6 cells/ml) for 5 days. Culture supernatants were harvested and then analyzed for cytokine secretion using the IFN-γ-, IL-4-, IL-5-, IL-6- and IL-10-specific ELISA. The results are expressed as the mean ± S.E.M. from five to six mice per group and from a total of three experiments. $^*P < 0.05$ when compared with mice immunized with OVA alone.

safety must be made a priority. The non-toxicity of these Stx1 derivatives had already been previously demonstrated by us both in vitro and in vivo studies. [18,28]. For example, our recent results showed that both native Stx1 (nStx1) and native Stx2 (nStx2) but not Stx1-B induced apoptosis in human monocytic THP-1 cells of eukaryotic cells [29]. Another our recent studies also suggested that nStx1 and nStx2 induced apoptosis in murine bone marrow (BM) cultures while both mStx1 and Stx1-B supported maturation and activation of DC from BM cultures (our unpublished data). As described above in Section 3, nStx1 possessed some nasal adjuvant activity at a dosage of 0.5 μg but proved lethal at higher doses (e.g., 2 μg). Moreover, mice given as little as 0.1 μg of nStx2 did not survive (data not shown). In contrast, all mice given a dose as high as 20 μg of mStx1 or Stx1-B as mucosal adjuvant survived and exhibited high levels of co-administered antigen-specific systemic IgG and mucosal IgA responses. Further, mice given doses as high as 20 μg of mStx1 or Stx1-B showed no sign (e.g., weight loss) of mucosal toxicity (data not shown). These findings further demonstrated the non-toxicity of mStx1 and Stx1-B as mucosal adjuvants.

When given nasally, mStx1 and Stx1-B supported the generation of antigen-specific $CD4^+$ Th2 type responses via the production of IL-4, IL-5, IL-6 and IL-10 and thereby enhanced Ag-specific serum IgG1 and mucosal IgA responses (Figs. 1 and 3). Based on these results, both of these nasally delivered non-toxic derivatives of Stx1 could be categorized as Th2 inducer type adjuvants. Although the exact mechanism by which these non-toxic Stx1 derivatives induce Th2-type T cell responses remains to be elucidated, it is interesting to note that such Th2-type T cell responses are also preferentially induced by oral or nasal administration of CT [4,30]. One obvious explanation would be that the tendency of a toxin-related adjuvant to favor Th1 and/or Th2 cell-mediated immune responses would be affected by the quality of antigen-presenting cells associated with mucosal compartments. For example, mucosally administered CT preferentially enhances B7-2-mediated Ag presentation by B cells and/or macrophages [31,32]. To this end, Th1 or Th2 polarizing factors could be mainly classified into three categories: (1) the anatomical and histological location of DC and T cells, (2) the nature of microbial products (adjuvant) and (3) the nature of the antigen used for the im-



% of CD positive cells with high intensity

	CD80	CD86	CD40
Stx1-B	91.2 ± 7.6*	86.8 ± 3.2*	87.5 ± 1.0*
mStx1	80.8 ± 2.5*	93.9 ± 5.2*	74.7 ± 3.5*
PBS	73.5 ± 3.1	70.2 ± 5.1	56.5 ± 1.7

Fig. 5. Enhancement of CD40, CD80 and CD86 expressions on mucosal DC by nasal administration of non-toxic Stx1 derivatives. 24 h following nasal administration of an optimal dose of the Stx1 derivatives (0.5 μg of mStx1/mouse and 5 μg of Stx1-B/mouse), DC were examined by FACS to determine any alterations in CD40, CD80 and CD86 expression. The dotted line gives the background stain, omitting only the mAb used in the specific stain. Data are expressed as the mean ± S.E.M. and are obtained from a total of three independent experiments. *$P < 0.05$ when compared with control mice administered with PBS.

munization. For instance, the major T cell population in the lungs has been shown to be predominantly of the Th2 type [33], and Peyer's patches has been shown to be a site in which a high proportion of resting lymphocytes prefentially develop into Th2-type cells [34]. DCs in PP possess the ability to induce the differentiation of $CD4^+$ T cells into the Th2 pathway [35]. Thus, NALT DCs may also possess a similar ability to guide $CD4^+$ T cells to differentiate into Th2-type cells. Our data demonstrate that the expression of both CD80 and CD86 was enhanced on DC isolated from NALT of mice nasally administered with mStx1 or Stx1-B (Fig. 5). This pattern of co-stimulatory molecule expression suggests that mStx1- or Stx1-B-treated DC possess at least a capability for supporting Th2 type responses. Since PP, which along with NALT are the best- characterized mucosal inductive tissues, have been shown to contain three distinct subsets of DC including lymphoid, myeloid and double negative subsets [35], it is important to carefully examine the influence of the nasally administered Stx1 derivatives on different subsets of NALT DC and to determine the role of that influence in the creation of an environment conducive to Th2-type cell responses. To understand the importance of

the nature of the microbial compounds, one need only note that toxins such as CT and pertussis toxin have been shown to induce the development of DC type2 [36] and DC type1 [37], respectively, for the generation of Th2 and Th1 polarization, respectively [6,38]. In contrast, LT has been shown to support both Th1- and Th2-type responses [7]. Finally, the nature of the co-administered antigen itself may contribute to outcome of Th1 and/or Th2 polarization. Although CT is reported to be a potent Th2-inducing adjuvant, nasal vaccination with fimbriae and CT induced both Ag-specific Th1/Th2-type responses in $CD4^+$ T cells in mucosal effector tissues [39], while co-administration of tetanus toxoid or OVA resulted in the generation of antigen-specific Th2-type responses [30]. We still do not fully understand the exact molecular/cellular mechanisms by which nasal administration of Stx derivatives enhances Th2-type responses. However, given the promise and potency of non-toxic Stx derivatives as mucosal adjuvants, we are vigorously pursuing the elucidation of these mechanisms.

It is interesting to note that Stx1-B possessed a mucosal adjuvant activity equally as good as that of mStx1. Since Stx1-B binds to the Gb cell surface receptor, the signaling pathways through Gb3 may have immunomodulating activities. In this regard, it is well known that CT-B binds to GM1 ganglioside [40]. Further, other studies have demonstrated that GM1-mediated signaling enhances endocytosis as well as antigen presentation of APC including macrophages and DC. Further, glycosphingolipids such as GM1 have also been implicated as a delivery site for immune-enhancing signals [41]. This contention is supported by findings that rCT-B provides some mucosal adjuvant activity when given nasally [42,43]. Taken together, these findings suggest that interactions between Stx1-B and Gb3, like those between CT-B and GM1, may enhance immune responses. However, other studies including ours have reported that rCT-B does not appear to be as effective as the native form (e.g., holotoxin) in enhancing antigen-specific immune responses [30,44]. Thus, in general higher doses of rCT-B than of the native form have been used to demonstrate mucosal adjuvant activity [42,43]. Taken together, these previous studies along together with our results suggest that the immunomodulating activities induced by Stx1-B/Gb3 interactions may be much higher than those induced by CT-B/GM1 interactions. Studies are underway to elucidate this interesting possibility.

The results of this study have also shown that the administration of mStx1 harboring two mutations at the active site of RNA *N*-glycosidase activity fostered some adjuvant activity. High levels of serum Ag-specific IgG Ab and mucosal IgA Ab responses were induced in mice nasally immunized with OVA and mStx1. In order to develop a safe mucosal immunoenhancer, one that is non-toxic but retains adjuvanticity, a number of non-toxic mutant derivatives of CT or LT have been developed and characterized [25,30,32,45]. Early studies have suggested that ADP-ribosyltransferase activity is essential for the adjuvanticity of both LT and CT [44]. However, single amino acid-substituted mutants of CT S61F, E112K [25,30], P106S, S63K [46] and LT R7K [47], S63K [48], A72R [49] and R192G [50], which lack ADP-ribosyltransferase activity, were shown nevertheless to retain their adjuvanticity. Our results here support the latter group of findings by suggesting that toxic and adjuvant activities are separated from each other in the enzyme activity cleft of the bacterial toxins, including Stxs, CT and LT.

In the past, several different non-toxic mutant derivatives of CT or LT have been developed and characterized for their adjuvanticity [25,30,32,45]. In our separate study, it was shown that mutant CT (mCT of E112K and S61F) possessed adjuvanticity for supporting Th2 mediated IgA antibody responses [25,30]. Since these different forms of mutant CT and LT retain their adjuvanticity, we could consider to use one of these different forms of mutant CT and LT as a gold standard for mStx1 and Stx1-B. However, we have to realize the fact that Stx1 is completely different toxin molecule when compared with CT and LT in terms of biological activity and primary structure. For example, Stx has RNA *N*-glycosidase activity and cleaves a specific *N*-glycosidic bond in the 28S rRNA, thereby inhibiting the peptide chain elongation step of protein synthesis and ultimately causing cell death [51]. On the other hand, CT catalyzes ADP ribosylation of the G protein, Gsα which activates adenylate cyclase, resulting in elevation of intracellular cAMP levels, which causes secretion of water and chloride ions from epithelial cells into the small intestine [40]. The eukaryotic cell surface receptors for the members of Stx and CT families are globotriaosylceramide and GM1 ganglioside, respectively [40,51]. Thus, it might not be appropriate to compare Stx1 type and CT (or LT) type toxin-based adjuvants.

It has been suggested that the dose of adjuvant used for immunization can influence the adjuvant activity [52]. Thus, a simple notion is that adjuvanticity of manipulated toxin-based molecules can be demonstrated for every toxin-based adjuvant by increasing dose used for immunization. In the case of Stx1 derivatives, when a dose of 0.5 μg was employed for both Stx1-B and mStx1, the levels of serum Ag-specific Ab responses in Stx1-B-treated mice were lower than those seen in mStx1-treated groups (data not shown). However, the titer of Ag-specific Ab responses in Stx1-B-treated group was elevated in accordance to the increase of dose used (e.g., 5 μg) (data not shown). The adjuvanticity of mStx1 reached to plateau level at the dose of more than 0.5 μg (data not shown). Our data at least suggested that the dose effect of adjuvanticity was most obvious for Stx1-B when compared with that of mStx1. Inas much as a major purpose of our present study was to elucidate mucosal adjuvant activity of Stx1 derivatives including Stx1-B and mStx1, the optimal dose for these two molecules (5 and 0.5 μg, respectively) were determined and then employed for this study. Based on the present finding, our next goal is to determine the exact molecular mechanisms involved in mStx1 and Stx1-B mediated adjuvant activities. To investigate the differences between the mechanisms of adjuvanticity of mStx1 and Stx1-B, one possible

and interesting experiment would be to compare the induction and regulation of Th2 associated signaling events including GATA-3, c-maf or SLAT by mStx1 or Stx1-B since our result suggested that both Stx1-based adjuvants resulted in the induction of Th2 cell associated Ag-specific IgG1 and IgA responses (Figs. 1 and 3). To this end it has been shown that these signaling molecules are specifically associated with the Th2 cell differentiation [53,54].

In summary, this study has shown that nasal immunization with protein Ag plus mStx1 or Stx1-B as mucosal adjuvant elicits Ag-specific $CD4^+$ Th2 cells in both mucosal and systemic tissues, and these cells in turn induce antigen-specific IgA and IgG Ab responses in the mucosal and systemic compartments, respectively. Non-toxic Stx derivatives could then be considered as new and promising candidates for mucosal adjuvants.

Acknowledgements

We appreciate the constructive comments regarding this work made by members of the Division of Immunology and Medical Zoology at Niigata University Graduate School of Medical and Dental Sciences and members of the Department of Mucosal Immunology, Research Institute for Microbial Diseases, Osaka University.

This study was supported by a grant from the Ministry of Health, Labor and Welfare, the Ministry of Education, Science, Sports and Culture, CREST, JST in Japan, and Special Coodination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government.. M.O. is supported by the Japanese Human Health Science Foundation.

All experiments described herein were approved by the appropriate local authorities. All procedures were in agreement with NIH guidelines for the handling of laboratory animals.

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Clinical Immunology 113 (2004) 326–339

CLINICAL IMMUNOLOGY

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Biological role of Ep-CAM in the physical interaction between epithelial cells and lymphocytes in intestinal epithelium

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Received 13 August 2004; accepted with revision 24 August 2004
Available online 29 September 2004

Abstract

The mucosal epithelium including intestinal epithelial cells (IECs) and intraepithelial lymphocytes (IELs) provide a first line of defense in the gastrointestinal tract. However, limited information is currently available concerning the nature of the physical interaction molecule that interconnects IECs and IELs. Among the several monoclonal antibodies (mAbs) generated by immunizing porcine IECs, mAb (5-15-1) was shown to strongly react with IELs in addition to IECs. MALDI-TOF-MS and tandem MS analysis suggested that the antigen belongs to a family of human homophilic epithelial cell adhesion molecule (Ep-CAM). The amino acid sequence of porcine Ep-CAM showed 82.8%, 78.1%, and 76.8% homology compared to human, mouse, and rat Ep-CAM. Moreover, 5-15-1 specifically reacted with transfectant of porcine Ep-CAM. These data suggest that the Ep-CAM may act as a physical homophilic interaction molecule between IELs and IECs at the mucosal epithelium for providing immunological barrier as a first line of defense against mucosal infection.

Keywords: Mucosa; Epithelial cell; Intraepithelial lymphocyte; Adhesion molecule; Cell surface molecule; Cell trafficking

Introduction

Intestinal epithelial cells (IECs) originate from and are maintained by a small number of pluripotent stem cells in crypts and have a life span of 2–4 days [1]. IECs have long been known to absorb nutrients into the circulation. More recently, IECs have also been found to act as an important immunological barrier against pathogens or nonself antigens and thus to comprise a major compartment of the mucosal immune system. They are equipped with mucus and antibacterial enzymes (e.g., lysozyme), which act as innate barriers, and secretory IgA (S-IgA) and T cell-mediated immunity, which serve as acquired barriers [2]. To preserve the integrity of the first line of defense against pathogens, IECs are welded together by a number of adhesion mechanisms. For example, tight junctions consisting of occlaudin [3] and the family of claudin [4,5] have been shown to play a central role in sealing the intracellular space between epithelial cells [6–8]. E-cadherin, another adhesion molecule expressed by epithelial cells, homophilically regulates Ca^{2+}-dependent interactions at the site of the tight junction [9]. The extracellular domain of E-cadherin is composed of five repeats and contain Ca^{2+} binding motifs [10]. Cadherin forms tight complexes with three cytoplasmic proteins, α-, β-, and γ-catenin, linked to the actin cytoskeleton [11]. This unique cell-to-cell adhesion system

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doi:10.1016/j.clim.2004.08.013

of IECs helps maintain a physiologically normal intestinal epithelium by serving as both a physical and immunological barrier against the invasion of undesirable foreign substances. On the other hand, under the condition of sever mucosal inflammation (e.g., inflammatory bowel diseases), the expression of these epithelial intercellular junction proteins was down-regulated by transmigrating neutrophils from intestinal lamina propria to lumen [12]. Thus, a group of these adhesion molecules associating with the formation of mucosal epithelium is directly influenced by the process of development of inflammation.

Most intraepithelial lymphocytes (IELs), which are present in large numbers in the intestinal epithelium, are $CD3^+$ T cells and are expressed by heterodimer chains of either αβ or γδ T cell receptors (TCR). In mice, IEL expression is almost evenly divided between αβ and γδ TCR [13]. Although their exact immunological functions remain unknown, these IELs have been shown to be involved in both the innate and acquired phases of mucosal immunity [14]. IELs bearing γδTCR (γδIELs) possess several features that distinguish them from αβIELs. For example, γδIELs exclusively express the CD8αα homodimer or doubly negative for CD4 and CD8 and are thought to be developed extrathymically [15]. In addition, γδIELs are absent in mice lacking a common cytokine receptor γ chain, which is a subunit of the receptor for IL-2, IL-4, IL-7, IL-9, and IL-15 [16]. The unique immunological and microbiological environment of the intestinal epithelium perhaps contributes to the creation of subsets of IEL-associated T cells distinct from the thymus-originated T cells located in the peripheral lymph nodes.

A number of recent findings suggest that a reciprocal dependency exists between IECs and IELs and that it is under the regulation of a group of intestinal epithelium-associated cytokines, including IL-7, IL-15, and SCF [17–19]. IECs are capable of producing IL-7 and IL-15, important cytokines for the stimulation and development of γδIELs expressing the corresponding receptor [20–22], while γδIELs have been shown to produce SCF, which stimulates the growth of IECs [19]. These results clearly indicate that an array of interactions between cytokines and their corresponding receptors play an important role in the formation and maintenance of the monolayer of the mucosal epithelium. Further, it has been suggested that IECs and IELs form mucosal intranet and provide a first line of defense at mucosal epithelium [2]. IEC-derived IL-15 has been shown to be a key regulatory molecule for the generation of IELs including γδT cells-mediated immunological barrier function [23]. On the other hand, overproduction of IL-15 at the mucosal epithelium resulted in the development of intestinal inflammation [24]. A similar aberrant condition was also triggered by the overproduction of IL-7 at mucosal epithelium [25]. These findings further emphasize the importance of mucosal intranet operated by IECs and IELs for the control of inflammation and infection. However, minimal information is currently available regarding the cellular and molecular mechanisms underlying the physical cell-to-cell interactions between IECs and IELs via the cell surface adhesion molecule. To address this gap in the data, we have sought in this study to elucidate the molecular basis for the physical cell-to-cell interactions between IECs and IELs by generating monoclonal antibodies (mAbs) to react with the cell surface of both IECs and IELs.

Materials and methods

Animal

Female Balb/c mice (6 weeks old) were purchased from CREA (Tokyo, Japan) and maintained in the experimental animal facility at the Institute of Medical Science, the University of Tokyo. The tissues of male and female three-way cross-bred pigs were purchased from Tokyo Shibaura Zouki (Tokyo, Japan). All of the tissues were obtained from 6-month-old pigs. In some cases, the tissues were kindly provided from Chugai Research Institute for Medical Science (Nagano, Japan).

Isolation of porcine IECs from the small intestine

IECs were physically and enzymatically isolated from porcine small intestines. First, the small intestines were washed with cold PBS and then torn into muscle layers. The tissues were then cut into small fragments of 1–2 cm and digested with 1 mg/ml collagenase (Wako, Osaka, Japan) and 1 mg/ml hyaluronidase (Sigma, Saint Louis, MO) in PBS at 37°C for 20 min. After digestion, tissue was washed again with cold PBS and then redigested with 1 mg/ml pancreatin (Sigma) in 25 mM HEPES buffer at 37°C for 15 min. Isolated cells were washed with cold PBS and then purified using a 45% Percoll gradient (Amasham Pharmacia Biotech, Uppsala, Sweden). Following centrifugation, the upper layer of cells was collected and stained first with mouse IgG1 anti-porcine CD45 mAb (K252.1E4, Serotec Ltd., Oxford, UK) diluted 1:10 at 4°C for 30 min and then by microbead-conjugated rat anti-mouse IgG1 (Miltenyi Biotec, Bergisch Gladbach, Germany) diluted 1:5 at 4°C for 15 min to further remove contaminated leukocytes. Finally, IECs were negatively selected by auto-MACS (Miltenyi Biotec). Immunostaining with anti-cytokeratin mAb (Sigma) showed that the resulting IEC preparation was highly purified (>95%) [26].

Generation of mouse monoclonal antibodies

Purified porcine IECs were used for intraperitoneal immunization of BALB/c mice (2.0×10^6 cells/mouse). After 1 week, the mice received a booster intravenous injection of porcine IECs (1.0×10^6 cells/mouse). Four days after the booster, the mice were sacrificed so that splenic mononuclear cells could be harvested. Splenocytes

were fused with Sp2/0-Ag14 myeloma cells (ATCC, CRL-1581) in the presence of 50% (w/v) polyethylene glycol 1500 (Roche, Mannheim, Germany). Supernatants from the resulting hybridomas were screened with isolated cells from the small intestine by flow cytometry analysis. The isotype of the porcine IEC-reactive hybridoma was determined using a mouse monoclonal antibody-isotyping kit (Amersham Pharmacia Biotech). The mAbs, produced in the ascitic fluids of BALB/c mice by priming with pristane (Wako), were purified using Protein G sepharose (Amersham Pharmacia Biotech). Purified mAbs were biotinylated with EZ-Link Sulfo-NHS-LC-biotin (PIERCE, Rockford, IL). Among 10 mAbs generated, one clone of mAb (5-15-1: mouse IgG2b) was selected for the present study due to its specific and strong reactivity with the porcine intestinal epithelium. An optimal concentration of mAb (5-15-1) was determined for the different assay system used in this study, and those doses are indicated for the respective protocol or figure legend.

Immunohistochemistry

The porcine tissues were fixed in 4% paraformaldehyde (Wako), incubated overnight at 4°C, and then washed with 8% and 16% (w/v) sucrose solutions before being incubated overnight again at 4°C. The tissues were then embedded in Tissue-Tek OCT compound (SAKURA Finetechnical Company, Ltd., Tokyo, Japan). Tissue sections (5 μm) were incubated with 1.5% (v/v) normal goat serum (Vector, Burlingame, CA) for 20 min at room temperature (RT). They were then incubated overnight at 4°C with 10 μg/ml purified mAb (5-15-1, mouse IgG2b) and/or anti-porcine CD45 mAb (mouse IgG1, Serotec, Ltd.) diluted 1:10 or an isotype control (mouse IgG1 and/or mouse IgG2b, BD PharMingen, San Jose, CA). For single staining, they were incubated with FITC-conjugated goat anti-mouse IgG (IMMUNOTECH, Marseille, France) diluted 1:200 for 1 h at RT, and for double staining with FITC-conjugated anti-mouse IgG2b (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100 and rhodamine-conjugated anti-mouse IgG1 (Santa Cruz Biotechnology) diluted 1:100. Finally, the sections were counterstained with 1 μg/ml propidium iodate (Sigma) or 200 ng/ml DAPI (Sigma) for 30 min at RT and analyzed using a confocal laser scanning microscope (TCS SP2, Leica, Wetzlar, Germany).

Immunoprecipitation and Western blot analysis

The lysate of several tissues were washed with cold PBS and lysed in lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, and a protease inhibitor cocktail (Roche)]. After 1 h of incubation on ice followed by centrifugation, the lysate (5 mg/ml, 1 ml) was precleared with 40 μl protein G Sepharose (1:1 in PBS, Amersham Pharmacia Biotech) at 4°C for 1 h. After centrifugation, the lysate was incubated with mAb (5-15-1, 10 μg/ml) or an isotype control (mouse IgG2b, BD PharMingen) for 1 h at 4°C, before the addition of 40 μl Protein G Sepharose (1:1 in PBS) and incubation for 1 h at 4°C. Immune complexes were washed five times with cold PBS containing a protease inhibitor cocktail and eluted in Laemmli sample buffer with or without 2-ME. They were then subjected to an SDS-PAGE using a 12.5% polyacrylamide gel (Daiichi Pure Chemical, Tokyo, Japan) before being transferred to a polyvinylidene difluoride (PVDF) membrane (MILLIPORE, Billerica, MA) using a semidry transblot system (ATTO Instruments, Tokyo, Japan). The membranes were blocked in 1% BSA, 0.2% Tween-20/PBS overnight at 4°C, and incubated with biotinylated mAb (5-15-1, 10 μg/ml) at RT for 1 h and then with ABC-AP complex (Vector) at RT for 1 h. Finally, the reaction was detected with an Alkaline phosphatase-conjugated substrate kit (Bio-Rad, Hercules, CA). Carbohydrates were also stained with G.P. Sensor (HONEN, Yokohama, Japan) in accordance with the manufacturer's instructions.

Isolation of IELs, splenocytes, and PBMCs for flow cytometric analysis

Lymphocytes were isolated from small intestinal epithelium and spleen, as described previously with some modifications [26]. Briefly, in the case of IELs, the small intestinal epithelium was prepared and then stirred at 37°C in RPMI-1640 (Sigma) containing 1 mM EDTA for 20 min. Lymphocytes from the small intestinal epithelium and spleen were separated on a Percoll density gradient (Amersham Pharmacia Biotech). The cells layered between the 40% and 75% fractions were collected as IELs and splenocytes. PBMCs were separated on NycoPrep (AXIS-SHIELD PoC AS, Oslo, Norway) after blood was mixed with two volumes of PBS. IECs, IELs, splenocytes, and PBMCs were incubated with 1 μg/ml porcine IgG (Sigma) at 4°C for 20 min and then stained with 10 μg/ml purified mAb (5-15-1) or an isotype control (mouse IgG2b, BD PharMingen) at 4°C for 30 min, before being subjected to an FITC-conjugated anti-mouse IgG (IMMUNOTECH) and 10 μl/test VIAPROBE (BD Pharmingen) for 30 min at 4°C. Finally, the cells were analyzed by flow cytometry using FACSCalibur (Becton Dickinson, Franklin Lakes, NJ).

Purification and analysis of antigen by MALDI-TOF-MS and Tandem MS after triptic digestion

The antigen of mAb (5-15-1) was purified from the lysate of the small intestine by affinity chromatography using an Affi-Gel Hz Immunoaffinity Kit (Bio-Rad). Purified antigen was analyzed by SDS-PAGE with 12.5% polyacrylamide gel and stained by GelCode Blue Stain Reagent (PIERCE). The responding protein was digested with trypsin using a method previously described [27]. The resulting peptide samples were analyzed by time-of-flight mass spectrometry (Applied Biosystems, Foster, CA) after being spotted on a