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H. 知的財産権の出願・登録状況

①特許取得

該当事項無し

②実用新案登録

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③その他

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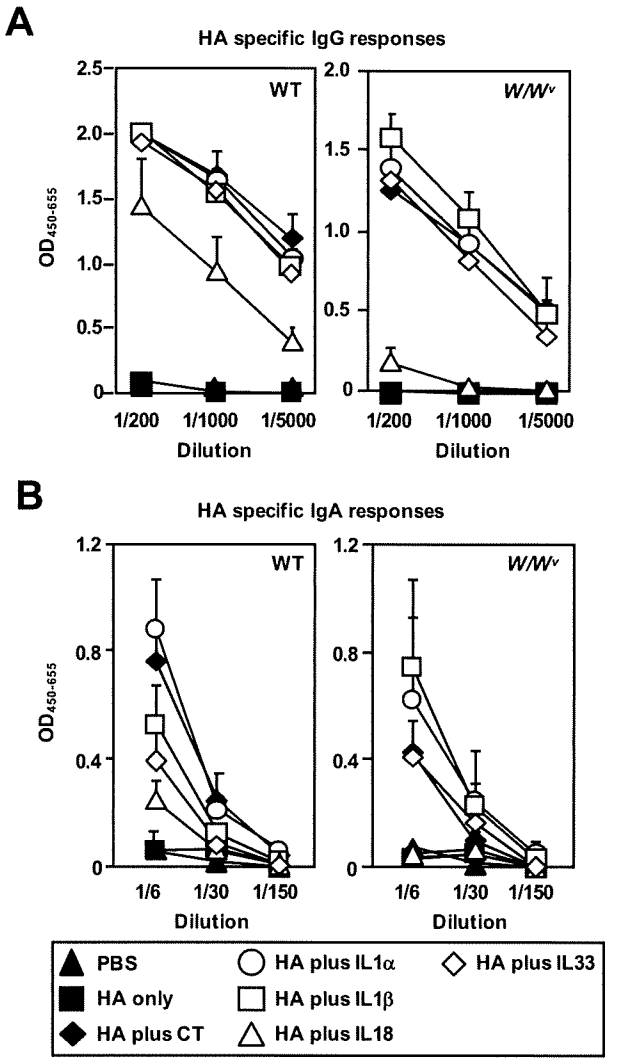

Figure 1. The dependency of MCs for the induction of rHA-specific immune responses by nasal immunization with rHA plus IL-1 family. WBB6F1 *W/W^v* and WT mice were intranasally immunized twice with rHA alone, rHA plus CT (1 μg/mouse) or rHA plus IL-1 family (1 μg/mouse) with an interval of four weeks between each immunization. Plasma and fecal extracts were collected 14 days after the final immunization and analyzed by ELISA for rHA-specific IgG responses in (A) plasma and rHA-specific SIgA responses in (B) fecal extracts.

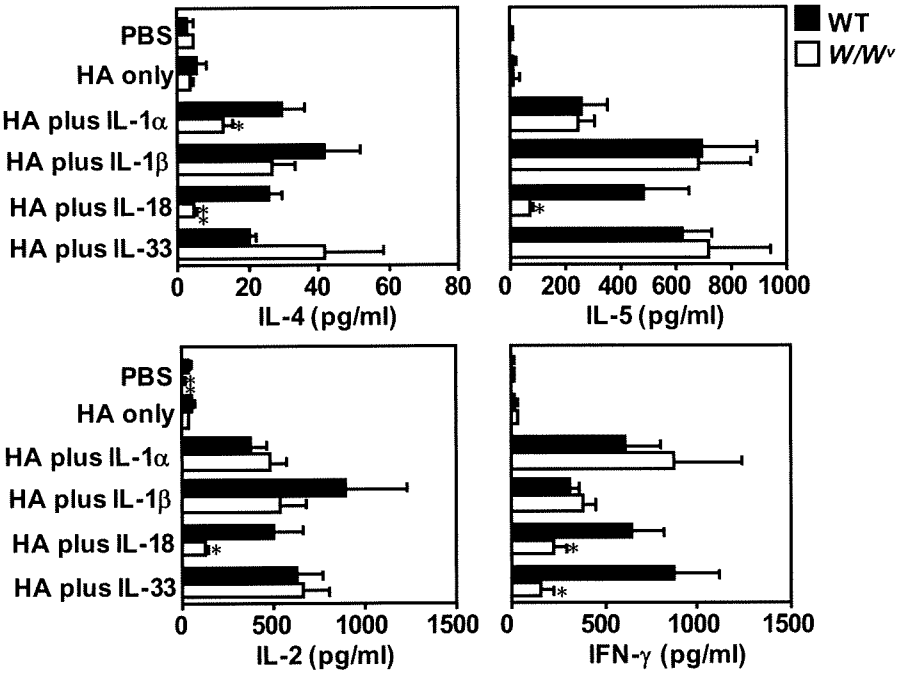

Figure 2. Cytokine responses induced by nasal immunization with HA plus adjuvants. WBB6F1 *W/W^v* and WT mice were intranasally immunized twice with rHA alone, rHA plus CT (1 μg/mouse) or rHA plus IL-1 family (1 μg/mouse) with an interval of four weeks between each immunization. Seven days after last immunization, splenocytes from each group of WBB6F1 *W/W^v* and WT mice were cultured with 10 μg/mL rHA. Culture supernatants were harvested after 3 days of incubation, and then (C) rHA-specific cytokine production in culture supernatant (IL-4, IL-5, IL-2, and IFN-γ) was analyzed using the Bio-Plex Multiplex Cytokine Assay. Data are presented as means ± SEM (n = 5; * $p < 0.05$, ** $p < 0.01$ versus value for rHA alone treated group by ANOVA).

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書 籍 名	出版社名	出版地	出版年	ページ
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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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TNF superfamily member, TL1A, is a potential mucosal vaccine adjuvant

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ABSTRACT

The identification of cytokine adjuvants capable of inducing an efficient mucosal immune response against viral pathogens has been long anticipated. Here, we attempted to identify the potential of tumor necrosis factor superfamily (TNFS) cytokines to function as mucosal vaccine adjuvants. Sixteen different TNFS cytokines were used to screen mucosal vaccine adjuvants, after which their immune responses were compared. Among the TNFS cytokines, intranasal immunization with OVA plus APRIL, TL1A, and TNF-α exhibited stronger immune response than those immunized with OVA alone. TL1A induced the strongest immune response and augmented OVA-specific IgG and IgA responses in serum and mucosal compartments, respectively. The OVA-specific immune response of TL1A was characterized by high levels of serum IgG1 and increased production of IL-4 and IL-5 from splenocytes of immunized mice, suggesting that TL1A might induce Th2-type responses. These findings indicate that TL1A has the most potential as a mucosal adjuvant among the TNFS cytokines.

Introduction

The majority of infectious pathogens, including HIV or influenza virus, enter through a mucosal surface. One important aspect of the immune response at mucosal surfaces is the production of secretory immunoglobulin (Ig) A antibodies, as well as the induction of cytotoxic T cells (CTLs) against epithelium-transmitted pathogens; therefore, the development of vaccines that induce effective immune responses at mucosal surfaces is important.

Mucosal vaccines administered either orally or nasally are highly warranted in order to combat these infectious diseases, as they would reduce the transmission of infectious pathogens more efficiently than parenteral administration by stimulating both mucosal and systemic immune responses [1,2]. The mucosal antigen-specific immune response, however, is weak because most protein antigens, such as non-living macromolecules or protein-subunit antigens, evoke only a weak or undetectable adaptive immune response when they are applied mucosally. Therefore, in order to develop mucosal vaccines, it is necessary to develop a mucosal vaccine adjuvant [3]. Both the cholera toxin and *Escherichia coli* heat-labile toxin are potent mucosal vaccine adjuvants, which have been used in experimental systems. Unfortunately, the watery diarrhea induced by these toxins makes their use as oral adjuvants clinically problematic to humans [4]. In addition, recent reports showed that a human vaccine containing inactivated influenza virus and the heat-labile toxin as an adjuvant resulted in a very high incidence of Bell's palsy [5]. Therefore, mucosal vaccine adjuvants with high efficacy and safety for the purpose of a clinical application are necessary.

Cytokines can trigger the innate and adaptive immune responses and also synergize with costimulatory molecules to improve the immune response. Therefore, cytokines are promising vaccine adjuvants that enhance the immune response against pathogens. The members of the tumor necrosis factor (TNF)/TNF receptor (TNFR) superfamily are critically involved in maintaining the homeostasis of the immune system [6–8]. Currently, more than 40 members of the TNF/TNFR superfamily have been identified and the majority is expressed by immune cells. The biological functions of this system encompass beneficial and protective effects in inflammation and host defense as well as a crucial role in immune organogenesis [7]. Among TNF superfamily cytokines, there are some candidates used as vaccine adjuvants to enhance the primary and memory immune responses against cancer and infectious

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disease. For example, 4-1BBL, CD27L, CD30L, GITRL, LIGHT, OX40L, and TNF-α, which have costimulatory functions for survival, expansion, and effecter function of T cells, enhance systemic immunity to co-administered antigens [8]. There is, however, no comparative study to investigate the potential of TNF superfamily cytokines as mucosal vaccine adjuvants.

In this study, to develop effective and safe cytokine-based mucosal vaccine adjuvants, we compared the potential of TNF superfamily cytokines. We showed that intranasal coadministration of APRIL, TL1A, or TNF-α along with antigen induced strong antigen-specific systemic IgG and mucosal IgA antibody responses. In particular, TL1A induced the strongest immune responses at mucosal sites among TNF superfamily cytokines. These data suggest that TL1A is an attractive prototype for a mucosal vaccine adjuvant. This study is the first report to clearly demonstrate the potential of TNF superfamily cytokines as mucosal adjuvants. To develop a mucosal vaccine adjuvant, a pool of such information would be of great benefit.

Materials and methods

Adjuvants. Cholera toxin B subunit (CTB) was purchased from List Biological Laboratories (Campbell, CA). Sixteen types of recombinant TNF superfamily cytokines included human APRIL (18.6 kDa), mouse BAFF (23.2 kDa), mouse 4-1BBL (25.7 kDa), mouse CD27L (19.2 kDa), mouse CD30L (22 kDa), mouse CD40L (25 kDa), mouse EDA (23 kDa), mouse GITRL (16 kDa), mouse LIGHT (21 kDa), mouse LT-α (18.5 kDa), mouse OX40L (18 kDa), mouse TL1A (20 kDa), mouse TNF-α (17.5 kDa), mouse TRIAL (20 kDa), mouse TRANCS (36 kDa), mouse TWEAK (17 kDa) were purchased from R&D Systems (Minneapolis, MN).

Mice and immunization protocols. Female BALB/c mice were purchased from SLC (Hamamatsu, Japan) and used at 6 weeks of age. All of the animal experimental procedures in this study were performed in accordance with the National Institute of Biomedical Innovation guidelines for the welfare of animals. Mice were intranasally immunized with 100 μg of ovalbumin (OVA; Sigma Chemical Co., St. Louis, MO) and 1 μg CTB or each TNF superfamily cytokine included human APRIL (5.4×10^{-11} M/mouse), mouse BAFF (4.3×10^{-11} M/mouse), mouse 4-1BBL (3.9×10^{-11} M/mouse), mouse CD27L (5.2×10^{-11} M/mouse), mouse CD30L (4.5×10^{-11} M/mouse), mouse CD40L (4.0×10^{-11} M/mouse), mouse EDA (4.3×10^{-11} M/mouse), mouse GITRL (6.3×10^{-11} M/mouse), mouse LIGHT (4.8×10^{-11} M/mouse), mouse LT-α (5.4×10^{-11} M/mouse), mouse OX40L (5.6×10^{-11} M/mouse), mouse TL1A (5.0×10^{-11} M/mouse), mouse TNF-α (5.7×10^{-11} M/mouse), mouse TRAIL (5.0×10^{-11} M/mouse), mouse TRANCE (2.8×10^{-11} M/mouse), mouse TWEAK (5.9×10^{-11} M/mouse) in a total volume of 20 μl per mouse (10 μl/nostril). Mice were intranasally treated with OVA plus each of the TNF superfamily cytokines three times at weekly intervals.

Sample collection. Seven days after the final immunization, plasma and mucosal secretions (nasal washes, vaginal washes, and fecal extracts) were collected to assess antigen-specific IgG and IgA responses. Nasal and vaginal washes were collected by gently flushing the nasal passage or vaginal canal with 200 or 100 μl of sterile PBS, respectively. Fecal pellets (100 mg) were suspended in 1 ml of PBS and then vortexed for 30 min. The samples were centrifuged at 15,000*g* for 20 min, and then the supernatants were collected as fecal extracts.

Detection of antigen-specific antibody responses by ELISA. Antigen-specific IgG and IgA levels in plasma, nasal washes, vaginal washes, and fecal extracts were determined by ELISA. ELISA plates (Maxisorp, type 96F; Nalge Nunc International, Tokyo, Japan) were coated with 10 μg/ml OVA in 0.1 M carbonate buffer, respectively, and incubated overnight at 4 °C. The plates were incubated with blocking solution (Block Ace; Dainippon Sumitomo Pharmaceuticals, Osaka, Japan) at 37 °C for 2 h, and serum (1/500) or mucosal secretion dilutions (1/50) were added to the OVA-coated plates. After incubation at 37 °C for 2 h, the coated plates were washed with PBS–Tween 20 and incubated with the horse horseradish peroxidase-conjugated goat anti-mouse IgG solution or a biotin-conjugated goat anti-mouse IgA detection antibody (Southern Biotechnology Associates, Birmingham, AL) solution at 37 °C for 2 h, respectively. For detection of IgA, the plates were washed with PBS–Tween 20 and then incubated with the horseradish peroxidase-coupled streptavidin (Zymed Laboratories South San Francisco) for 1 h at rt. After incubation, the color reaction was developed with tetramethylbenzidine (MOSS, Inc., Pasadena, MD), stopped with 2 N H_2SO_4, and measured by $OD_{450-655}$ on a micro plate reader.

Isolation of splenocytes. Spleens were aseptically removed and placed in RPMI 1640 (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% FBS, 50 μM 2-mercaptoethanol, and 1% antibiotic cocktail (Nacalai Tesque, Kyoto, Japan). Single-cell suspension of splenocytes was treated with ammonium chloride to lyse the red blood cells; they were then washed, counted, and suspended in RPMI supplemented with 10% FBS, 50 μM 2-mercaptoethanol, 1% antibiotic cocktail, non-essential amino acids solution, 1 mM sodium pyruvate, and 10 mM Hepes to a final concentration of 1×10^7 cells/ml.

Antigen-specific cytokine responses. Antigen-specific cytokine responses were evaluated using culturing splenocytes (5×10^6 cells/well) stimulated with OVA (1 mg/ml) *in vitro*. Cells were incubated at 37 °C for 72 h. Culture supernatants from *in vitro* unstimulated and OVA-stimulated cells were analyzed by the Bio-Plex Multiplex Cytokine Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The assay was read on a Luminex 100 (Austin, TX), and analyzed using the Bio-Plex Manager software. The mean concentration of cytokines in supernatants from OVA-stimulated cells over the unstimulated background was calculated.

Statistical analysis. All results are expressed as means ± SEM. Differences were compared using Bonferroni's method after analysis of variance (ANOVA).

Results and discussion

Development of novel mucosal vaccine adjuvants with high efficacy and safety has been expected. Among various adjuvant candidates, cytokines might be promising safe adjuvants because they are human-derived and able to enhance the primary and memory immune responses sufficiently to protect from various infections. In this study, we examined the potential of TNF superfamily cytokines as mucosal vaccine adjuvants.

In this study, we used 16 different TNF superfamily cytokines (APRIL, BAFF, 4-1BBL, CD27L, CD30L, CD40L, EDA, GITRL, LIGHT, LT-α, OX40L, TL1A, TNF-α, TRAIL, TRANCE, and TWEAK). To examine TNF superfamily cytokines' potential as mucosal adjuvants, the mice were intranasally immunized with 100 μg OVA plus each TNF superfamily cytokine (1 μg/mice) three times at weekly intervals. Seven days after the last immunization, we examined the level of anti-OVA IgG responses in serum by ELISA (Fig. 1A). As a result, intranasal immunization with OVA plus TL1A exhibited significantly higher OVA-specific IgG responses in serum than those immunized with OVA alone. OVA-specific IgG level of TL1A immunized mice was of similar magnitude to that induced by CTB, one of the most effective mucosal adjuvants. In addition, intranasal immunization with OVA plus APRIL or TNF-α also showed strong OVA-specific IgG responses in serum.

Murine serum IgG subclass responses have been used to assess the type of immune responses elicited by immunization with IgG1 indicative of Th2-type responses and IgG2a indicative of Th1-type responses. To assess the type of immune response induced by TNF superfamily cytokines, IgG subclass studies were also examined (Fig. 1B and C). As a result, OVA-specific IgG1 was significantly high in mice immunized with OVA plus TL1A and TNF-α, which are as strong as CTB. Mice immunized with OVA plus APRIL also tend to show strong OVA-specific IgG1 responses. On the other hand, APRIL showed the highest OVA-specific IgG2a responses as well as CTB. These results indicated that APRIL, TL1A, and TNF-α could induce strong immune responses via nasal administration and also induce an antigen-specific Th2-type immune response.

Next, in order to identify the characteristics of TNF superfamily cytokines as mucosal adjuvants, we examined the OVA-specific IgA responses in nasal washes from immunized mice as well as vaginal washes and fecal extracts, which are not administration sites (Fig. 2). In nasal washes, OVA-specific IgA level of TL1A immunized mice were significantly higher than OVA alone immunized mice, and this magnitude was similar to that induced by CTB (Fig. 2A). Mice immunized with OVA plus APRIL or TNF-α also tended to show strong OVA-specific IgA responses. In vaginal washes and fecal extracts, TL1A and TNF-α showed the highest OVA-specific IgA responses among TNF superfamily cytokines, while these magnitudes were lower than those immunized with OVA plus CTB (Fig. 2B and C). These results indicate that nasal immunization

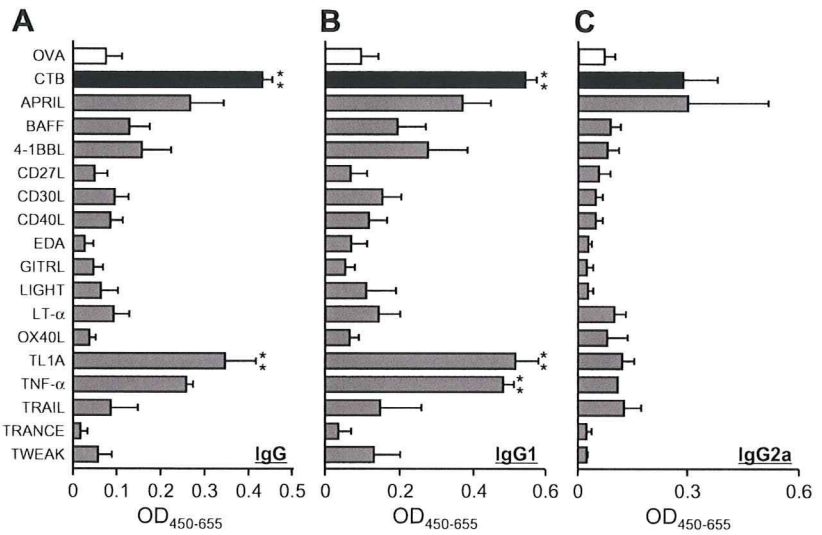

Fig. 1. Serum OVA-specific IgG responses by nasal immunization with OVA plus TNF superfamily cytokines. BALB/c mice were intranasally immunized with OVA alone, OVA plus CTB, or OVA plus each TNF superfamily cytokine three times at weekly intervals. Serum was collected 7 days after the last immunization and analyzed by ELISA for OVA-specific (A) total IgG, (B) IgG1, and (C) IgG2a responses at a 500-fold serum dilution. Data are presented as means ± SEM ($n = 5$; $^{**}P < 0.01$ versus value for OVA alone treated group by ANOVA).

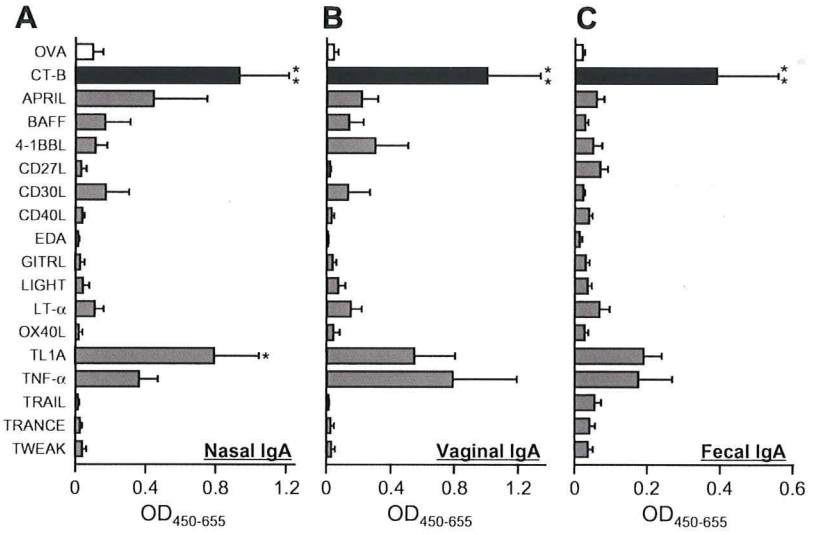

Fig. 2. OVA-specific mucosal IgA responses by nasal immunization with OVA plus TNF superfamily cytokines. BALB/c mice were intranasally immunized with OVA alone, OVA plus CTB, or OVA plus each TNF superfamily cytokines once a week for 3 weeks. Mucosal secretions were collected 7 days following the last immunization and then OVA-specific IgA responses in (A) nasal wash, (B) vaginal wash and (C) fecal extract were determined by ELISA. Data are presented as means ± SEM ($n = 5$; $^{*}P < 0.05$, $^{**}P < 0.01$ versus value for OVA alone treated group by ANOVA).

with TL1A effectively induced both antigen-specific systemic and mucosal immune responses, and indicated that TL1A might be a new candidate for mucosal vaccine adjuvants replacing toxin-based adjuvants. Among these cytokines, CD40–CD40L interaction is also known to play an important role in the generation of antigen-specific IgA positive B cells, and mount specific IgA responses to orally or parenterally administered antigens [9,10]. Indeed, Ninomiya et al. showed that intranasal administration of anti-CD40 antibody with antigens from the influenza virus induced protective immunity against influenza virus [11]. Our results showed that intranasal administration of CD40L could not enhance mucosal immunity against antigens, but the precise reason of these controversial results was unknown.

To clarify the mechanisms of immune response by each TNF superfamily cytokine, the release profiles of cytokines from splenocytes of immunized mice were analyzed. Culture supernatants from OVA-stimulated splenocytes collected from immunized mice were assessed for Th2-type cytokines IL-4 (Fig. 3A) and IL-5 (Fig. 3B) and Th1-type cytokines interferon-γ (IFNγ) (Fig. 3C) and TNF-α (Fig. 3D), using a multiplexed immunobeads assay. Splenocytes from mice immunized with OVA plus APRIL, TNF-α, or TL1A exhibited higher levels of Th2-type cytokines (IL-4 and IL-5) than those responses induced by OVA alone (Fig. 3A and B). In contrast, there was little difference in Th1-type cytokine (IFN-γ and TNF-α) secretion in all types of immunized mice (Fig. 3C and D). These results suggest that APRIL, TNF-α, and TL1A induced more strongly polarized Th2-type immune responses when used as mucosal vaccine adjuvants.

Our results indicate that TL1A induced the strongest mucosal immunity among the TNF superfamily cytokines. This is the first report to demonstrate TL1A mucosal adjuvant activity, induced antigen-specific systemic and mucosal immune responses in mice. The most recently identified member of the TNF superfamily, TL1A, is known to bind only to death receptor (DR) 3 expressed primarily on activated lymphocytes [12]. TL1A is also expressed on endothelial cells, normal lymphocytes, plasma cells, monocytes, and dendritic cells (DCs) [13,14]. TL1A can induce IFN-γ production of IL-12 and IL-18 primed T cells, whereas TL1A does not enhance IL-4 production from Th2 cells by systemic injection [15,16]. Therefore, it was believed that the TL1A/DR3 pathway played a dominant role in the Th1-type immune response by mucosal T cells in the inflamed mucosal site. On the other hand, a recent study showed that TL1A may act as soluble mediator or as cell-bound ligand to trigger DR3 on NKT and T cells, and this pair appears to provide an early signal for Th2-type cytokine production such as IL-4 and IL-13 [17]. We showed that the OVA-specific immune response of TL1A immunized mice was characterized by high levels of serum IgG1 and increased production of IL-4 and IL-5 from splenocytes of

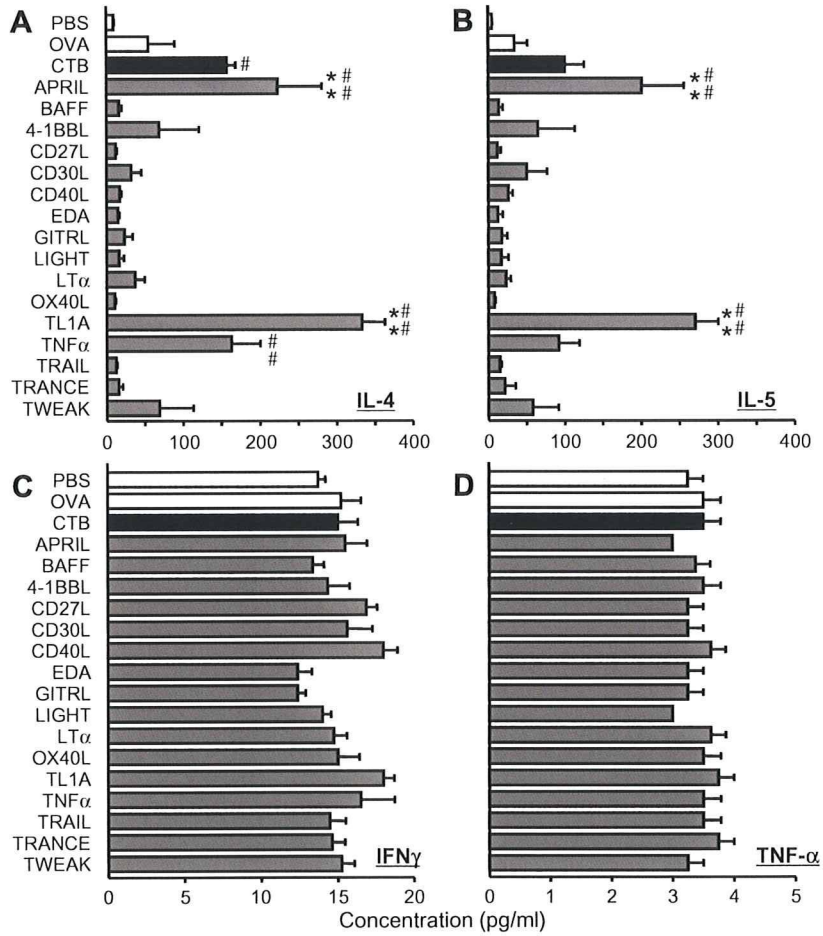

Fig. 3. Cytokine responses induced by nasal immunization with OVA plus adjuvants. BALB/c mice were intranasally immunized with OVA alone, OVA plus CTB, OVA plus each TNF superfamily cytokine three times at weekly intervals. Seven days after the last immunization, splenocytes from each group were cultured with 1 mg/ml OVA. Culture supernatants were harvested after 3 days of incubation, and then OVA-specific (A) IL-4, (B) IL-5, (C) IFNγ, and (D) TNF-α productions in culture supernatant were analyzed using the Bio-Plex Multiplex Cytokine Assay. Data are presented as means ± SEM ($n = 4$; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus value for PBS treated group by ANOVA; $^{**}P < 0.01$ versus value for OVA alone treated group by ANOVA).

immunized mice, suggesting a Th2-type immune response. The administration route of antigen immunization might be critical for influencing the type of immune response, although the precise reason for these contradictory results remains unclear. We are currently examining the mechanism of TL1A-induced Th2-type immune responses in more detail. In addition, the potential of TL1A as a mucosal vaccine adjuvant should be enhanced because the strength of immune responses by TL1A was lower than that of CTB. We have developed a novel technology to produce mutant cytokines using phage display [18–21]. We previously produced a bioactive mutant TNF-α with 6-fold stronger *in vitro* bioactivity and 13-fold stronger *in vivo* bioactivity compared with wild-type TNF-α [20]. We are now attempting to generate mutant TL1A with improved adjuvanticity.

We showed that APRIL and TNF-α also had the potential to function as a mucosal vaccine adjuvant, although the strength of immune responses in those immunized mice was lower than mice immunized with TL1A. The analysis of knockout mice suggested that APRIL would deliver B cell activation signals and induce IgA production against antigens from pathogenic microorganisms and viruses [22]. In addition, it was also reported that TNF-α played an important role in IgA production [23]. Future examination is needed to determine a more precise mechanism of IgA production by APRIL and TNF-α.

Regulation of Th1- and Th2-type immune responses is the major goal for the development of mucosal vaccines because these types of immune responses would control protective immunity against viral and bacterial infections by maximizing antigen-specific antibodies and CTL responses [1,3]. To induce both antigen-specific antibodies and CTL responses, combinatorial administration of TL1A with another mucosal adjuvant, which can induce Th1-type immune responses, is applicable. We have already screened the other cytokines and succeeded in finding candidates that can effectively induce CTL responses at the mucosal site. The combinatorial effect of the cytokines and TL1A as a mucosal vaccine adjuvant is now under examination.

In summary, our study showed that TL1A induced two layers of protective immunity when administered intranasally with a vaccine antigen. Our results indicate that TL1A is an effective mucosal adjuvant.

Acknowledgments

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The use of a mutant TNF-α as a vaccine adjuvant for the induction of mucosal immune responses

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ABSTRACT

Safe and potent adjuvants are required in order to establish effective mucosal vaccines. Cytokines are promising adjuvants because they are human-derived safe biomaterial and display immune-modulating functions. We have created a mutant tumor necrosis factor-α (TNF-α), mTNF-K90R, that exhibits high bioactivity and resistance to proteases. Here, we examined the potential of mTNF-K90R as a mucosal adjuvant. Initially, we showed that intranasal co-administration of mTNF-K90R with ovalbumin (OVA) potently produced OVA-specific Immunoglobulin (Ig) G antibodies (Abs) in serum and IgA Abs both at local and distal mucosal sites compared to co-administration with wild-type TNF-α. The OVA-specific immune response was characterized by high levels of serum IgG1 and increased production of interleukin-4 (IL-4), IL-5 and IL-10 from splenocytes of immunized mice, suggesting a Th2 response. Furthermore, intranasal immunization with an antigen from influenza virus plus mTNF-K90R exhibited mucosal adjuvant activity for induction of both systemic and mucosal immune responses. Importantly, histopathological examination of the nasal tissue of mTNF-K90R treated mice detected no signs of toxicity. These findings suggest that mTNF-K90R is safe and effective mucosal adjuvant and this system may have potential application as a universal mucosal adjuvant system for mucosal vaccines improving the immune response to a variety of viral antigens.

1. Introduction

Mucosal immunity forms the first line of defense against various infectious diseases. The majority of emerging and re-emerging pathogens, including *Vibrio cholerae*, pathogenic *Escherichia coli*, HIV or influenza virus, invade and infect *via* the mucosal surfaces of the host gastrointestinal, respiratory and/or genitourinary tracts [1,2]. An important aspect of the immune response at mucosal surfaces is the production of polymeric immunoglobulin (Ig) A antibodies (Abs), as well as their transport across the epithelium and release as secretory IgA [3]. Because this IgA response represents the major mechanism for defense against viral and bacterial infections, recent efforts have been focused on the development of vaccines that are capable of inducing IgA production as well as cytotoxic T cell activation efficiently in mucosal tissues.

Mucosal vaccines administered either orally or nasally have been shown to be effective in inducing antigen-specific immune responses at both systemic and mucosal compartments [4,5]. Because of this two-layered protective immunity, mucosal vaccines are thought to be an ideal strategy for combating both emerging and re-emerging infectious diseases. However the mucosal antigen-specific immune response is weak because most protein antigens, such as non-living macromolecules or protein-subunit antigens, can evoke only a weak or undetectable adaptive immune response when they are applied mucosally [6]. Therefore, one strategy to overcome the weakness of the immune response is a co-administration of mucosal adjuvant

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with the vaccine antigen [4]. Unfortunately, the development of safe and effective mucosal adjuvant has proved to be challenging. As a potent mucosal vaccine adjuvant, cholera toxin (CT) or heat liable toxin have been used in experimental studies. However, the watery diarrhea induced by the administration of these toxins precludes their use as oral adjuvants in humans [7]. In addition, recent reports show that a human vaccine containing inactivated influenza and heat liable toxin as a mucosal adjuvant results in a very high incidence of Bell's palsy [8]. Therefore, development of novel mucosal vaccine adjuvants with high efficacy and safety is urgently required for clinical applications.

Cytokines are promising candidate adjuvants because they are human-derived and able to enhance the primary and memory immune responses sufficiently for protection against various infections [9–11]. One of the most important cytokines of adaptive and innate immune response is tumor necrosis factor-α (TNF-α), a proinflammatory cytokine primarily produced by T cells and macrophages [12]. TNF-α has been reported to affect certain phases of the immune process, including innate immune activation, dendritic cells (DC) maturation/recruitment, T cell activation, or pathogen clearance [13]. Indeed many reports have shown that TNF-α exerts adjuvant activities against viral infection in various model systems [14–16]. Therefore the application of TNF-α in the development of a vaccine adjuvant has been anticipated for some time. However the application of TNF-α as a mucosal vaccine adjuvant has not been reported because TNF-α administered by mucosal routes is rapidly degraded at the mucosal surface. Therefore, the maximum adjuvant effects of TNF-α are quite limited in the mucosal environment.

Previously, we have produced a bioactive lysine-deficient mutant TNF-αs from a phage library expressing mutant TNF-αs in which all of the lysine residues that act as a site of trypsin-type protease recognition were replaced with other amino acids [17–19]. Lysine-deficient mutant TNF-αs were more resistant to proteolytic cleavage than wild-type TNF-α (wTNF-α) due to the lack of lysine residues. Furthermore we demonstrated that the mTNF-K90R, one of the lysine-deficient mutant TNF-αs, showed 6-fold stronger *in vitro* bioactivity and 13-fold stronger *in vivo* bioactivity compared with wTNF-α [18].

In this study, to develop effective and safe cytokine-based mucosal vaccine adjuvants, we examined the potential of mTNF-K90R as a nasal vaccine adjuvant. We demonstrate that intranasal administration of vaccine antigen with mTNF-K90R as an adjuvant induces a strong antigen-specific systemic IgG and mucosal IgA response. In addition, the safety of mTNF-K90R was confirmed by pathological examination. These results suggest that mTNF-K90R is an attractive mucosal vaccine adjuvant for clinical application.

2. Materials and methods

2.1. Recombinant TNF-αs

wTNF-α and mTNF-K90R were prepared in house as described previously [18]. Endotoxin level was quantified using a Limulus amebocyte lysate assay kit (QCL-1000, BioWhittaker, Walkersville, MD). The endotoxin content of purified TNF-α and its mutant was <0.02 EU μg $^{-1}$ protein.

2.2. Mice and immunization protocols

Female BALB/c mice were purchased from Nippon SLC (Kyoto, Japan) and used at 6–8 weeks of age. All of the animal experimental procedures were performed in accordance with the institutional ethical guidelines for animal experiments. Mice were intranasally immunized with a 20 μl aliquot (10 μl per nostril) containing 100 μg of ovalbumin (OVA; Sigma Chemical Co., St. Louis, MO) as antigen and 1 or 5 μg of wTNF-α or mTNF-K90R on days 0, 7 and 14. As positive control, mice were intranasally immunized with the same volume containing 100 μg of OVA and 1 μg cholera toxin B subunit (CTB; List Biological Laboratories, Campbell, CA) on days 0, 7 and 14. In the influenza virus studies, 1 μg baculovirus-expressed recombinant hemagglutinin (HA) derived from New Cal/99 virus (Protein Sciences, Meriden, CT), was immunized with 1 μg CTB or 5 μg mTNF-K90R on days 0, 7 and 14.

2.3. Sample collection

One week after the final immunization, plasma and mucosal secretions (nasal washes, saliva, vaginal washes and fecal extracts) were collected to assess antigen-specific Ab responses. Nasal and vaginal washes were collected by gentle flushing of the nasal passage or vaginal canal with 200 μl or 100 μl of sterile phosphate buffered saline (PBS), respectively. Fecal pellets (100 mg) were suspended in 1 ml of PBS and then vortexed for 30 min. The samples were centrifuged at 15 000g for 20 min and the supernatants were then collected as fecal extracts. Secreted saliva was collected from mice intraperitoneally injected with 0.2 mg of pilocarpine–HCl (Wako Pure Chemical Industries, Osaka, Japan).

2.4. Detection of antigen-specific Ab responses by enzyme-linked immunosorbent assay (ELISA)

Antigen-specific Ab levels in plasma, nasal washes, saliva, vaginal washes and fecal extracts were determined by ELISA. ELISA plates (Maxisorp, type 96F; Nalge Nunc International, Tokyo, Japan) were coated with 10 μg ml $^{-1}$ OVA or 2 μg ml $^{-1}$ HA in 0.1 M carbonate buffer and incubated overnight at 4 °C. The plates were incubated with blocking solution (Block Ace; Dainippon Sumitomo Pharmaceuticals, Osaka, Japan) at 37 °C for 2 h, and serum or mucosal secretion dilutions were added to the antigen-coated plates. After incubation at 37 °C for 2 h, the coated plates were washed with PBS–Tween 20 and incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG solution or a biotin-conjugated goat anti-mouse IgA detection Ab (Southern Biotechnology Associates, Birmingham, AL) solution at 37 °C for 2 h, respectively. For detection of IgA, the plates were washed with PBS–Tween 20 and then incubated with the horseradish peroxidase-coupled streptavidin (Zymed Laboratories, South San Francisco, CA) for 1 h at RT. After incubation, the color reaction was developed with tetramethylbenzidine (MOSS, Inc. Pasadena, MD), stopped with 2 N H_2SO_4, and measured by $OD_{450-655}$ on a microplate reader.

2.5. Isolation of splenocytes

Spleens were aseptically removed and placed in RPMI 1640 (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol and 1% antibiotic cocktail (Nacalai tesque, Kyoto, Japan). Single-cell suspension of splenocytes was treated with ammonium chloride to lyse the red blood cells, washed, counted, and suspended in RPMI supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 1% antibiotic cocktail, 10 ml L $^{-1}$ of a 100× nonessential amino acids solution (NEAA; Gibco-BRL), 1 mM sodium pyruvate, and 10 mM HEPES to a final concentration of 1×10^7 cells ml $^{-1}$.

2.6. Antigen-specific cytokine responses

Antigen-specific cytokine responses were evaluated by culturing the splenocytes (5×10^6 cells well $^{-1}$) stimulated with OVA (1 mg ml $^{-1}$) *in vitro*. Cells were incubated at 37 °C for 24 h (interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) assay), 48 h (IL-4 ELISPOT assay) or 72 h (multiplex cytokine assay).

2.7. Multiplex cytokine assay

Culture supernatants from *in vitro* unstimulated and OVA-stimulated cells were analyzed by the Bio-Plex Multiplex Cytokine Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The assay was read on a Luminex 100 (Austin, TX), and analyzed using Bio-Plex Manager software. The mean concentration of cytokines in supernatants from OVA-stimulated cells over the unstimulated background was then calculated.

2.8. Cytokine ELISPOT assay

An ELISPOT assay was performed to detect IFN-γ and IL-4 producing cells. After 24 h (IFN-γ) or 48 h (IL-4) incubation at 37 °C, the plate was washed, and the IFN-γ and IL-4 producing cells were measured by an ELISPOT assay kit (BD Biosciences), according to the manufacturer's instructions.

2.9. Fluorescence microscopy

BALB/c mice were administered intranasally with fluorescent isothiocyanate (FITC) labeled OVA (FITC-OVA; Molecular Probes–Invitrogen, Eugene, OR) at 50 μg mouse $^{-1}$ with or without mTNF-K90R (5 μg mouse $^{-1}$). After 15 min, the heads of the anesthetized mice were severed from the body. The heads were placed in fixative solution, and embedded in OCT compound (Sakura FineTek Japan Co. Ltd., Tokyo, Japan) and frozen tissue sections were prepared. FITC-OVA was observed under fluorescence microscopy (×20).

2.10. Histopathological analysis

For three times immunization protocol, BALB/c mice were immunized with OVA with or without mTNF-K90R at a dose of 1 μg, 5 μg or 25 μg on days 0, 7 and 14.

Seven days after the last immunization, heads of the mice were severed from the body and then placed in fixative solution (4% paraformaldehyde). Histopathological examination was performed by the Applied Medical Research Laboratory (Osaka, Japan). For single immunization protocol, BALB/c mice were immunized with OVA with or without mTNF-K90R at a dose of 5 μg. At 2 h after single immunization, histopathological examination was performed using the same protocol.

2.11. Statistical analysis

All results are expressed as mean ± SEM. Statistical significance in differences were evaluated by Newman–Keuls Multiple Comparison Test after analysis of variance (ANOVA).

3. Results

3.1. Mucosal adjuvant activity of mTNF-K90R

To examine the properties of mTNF-K90R as a mucosal vaccine adjuvant, mice were intranasally immunized with 100 μg OVA plus wTNF-α (1 μg mice^{-1}), mTNF-K90R (1 μg mice^{-1}, 5 μg mice^{-1}), or CTB (1 μg mice^{-1}) three times at weekly intervals. Seven days after the last immunization, we examined the level of anti-OVA Abs response in the serum by ELISA (Fig. 1A). Intranasal immunization with OVA plus 1 μg mTNF-K90R induced higher levels of anti-OVA IgG Ab response in serum than after immunization with OVA alone or OVA plus 1 μg wTNF-α. Furthermore, OVA-specific IgG Ab levels of mTNF-K90R immunized mice were of a similar magnitude to those immunized with CTB, a common laboratory mucosal adjuvant.

Serum IgG subclass responses have been used to assess the type of immune response elicited by immunization. For example, IgG1 is indicative of a Th2-type response whereas IgG2a is indicative of a Th1-type response. To assess the type of immune response induced by mTNF-K90R, serum OVA-specific IgG subclass responses were also examined (Fig. 1B). OVA-specific IgG1 Ab levels of 1 μg mTNF-K90R immunized mice were higher than those immunized with OVA alone or OVA plus 1 μg wTNF-α, and as well as in mice immunized with CTB. However, the level of OVA-specific IgG2a was low in all groups, indicating that mTNF-K90R may induce immune responses with an antigen-specific Th2 component.

Next, we examined the OVA-specific IgA secretion in nasal washes, vaginal washes, and fecal extracts from immunized mice (Fig. 2). As expected, immunization with OVA plus CTB induced strong anti-OVA IgA secretion in all mucosal tissues. Nasal immunization with OVA plus 1 μg of mTNF-K90R tended to induce high levels of OVA-specific IgA secretion in nasal tissue and fecal extract compared with after immunization with OVA plus 1 μg wTNF-α. The IgA level of 1 μg mTNF-K90R immunized mice in nasal tissue was of a similar magnitude to those immunized with CTB. In contrast, the IgA level in vaginal tissue and fecal extract was lower for the 1 μg mTNF-K90R treated mice compared with that of CTB treated mice. Nasal immunization with OVA plus 5 μg of mTNF-K90R induced high OVA-specific IgA secretion in multiple mucosal tissues compared to 1 μg of mTNF-K90R and comparable anti-OVA Abs responses to those induced with OVA plus CTB. These results indicate that mTNF-K90R is an attractive mucosal vaccine adjuvant for the induction of antigen-specific systemic IgG and mucosal IgA responses.

3.2. Antigen-specific cytokine responses of mTNF-K90R

To clarify the mechanism of immune response elicited by mTNF-K90R, the release profiles of cytokines from splenocytes of OVA-immunized mice were analyzed. Culture supernatants from

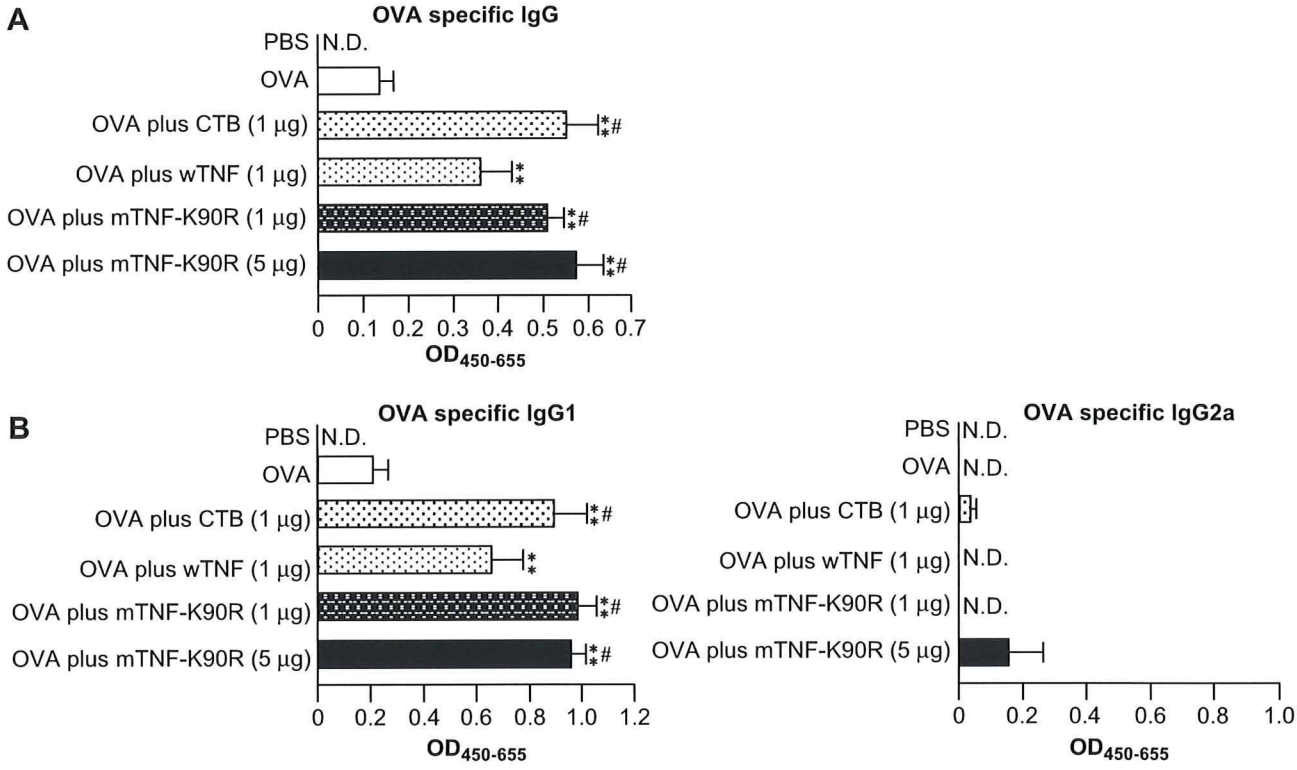

Fig. 1. Serum OVA-specific IgG Abs response after nasal immunization with OVA plus adjuvant. BALB/c mice were intranasally immunized with OVA alone, OVA plus 1 μg CTB, OVA plus 1 μg wTNF-α, OVA plus 1 μg mTNF-K90R or OVA plus 5 μg mTNF-K90R once a week for three consecutive weeks. Serum was collected 1 wk after the last immunization and analyzed by ELISA for OVA-specific IgG (A) and IgG subclass (B) at a 1:100 dilution of serum. Data represents the mean of absorbance 450 nm (reference wave, 655 nm). N.D; not detected. Data are presented as means ± SEM ($n = 7$; $^{**}P < 0.01$ *versus* value for OVA alone treated group by ANOVA; $^{\#}P < 0.05$ *versus* value for OVA plus wTNF-α treated group by ANOVA).

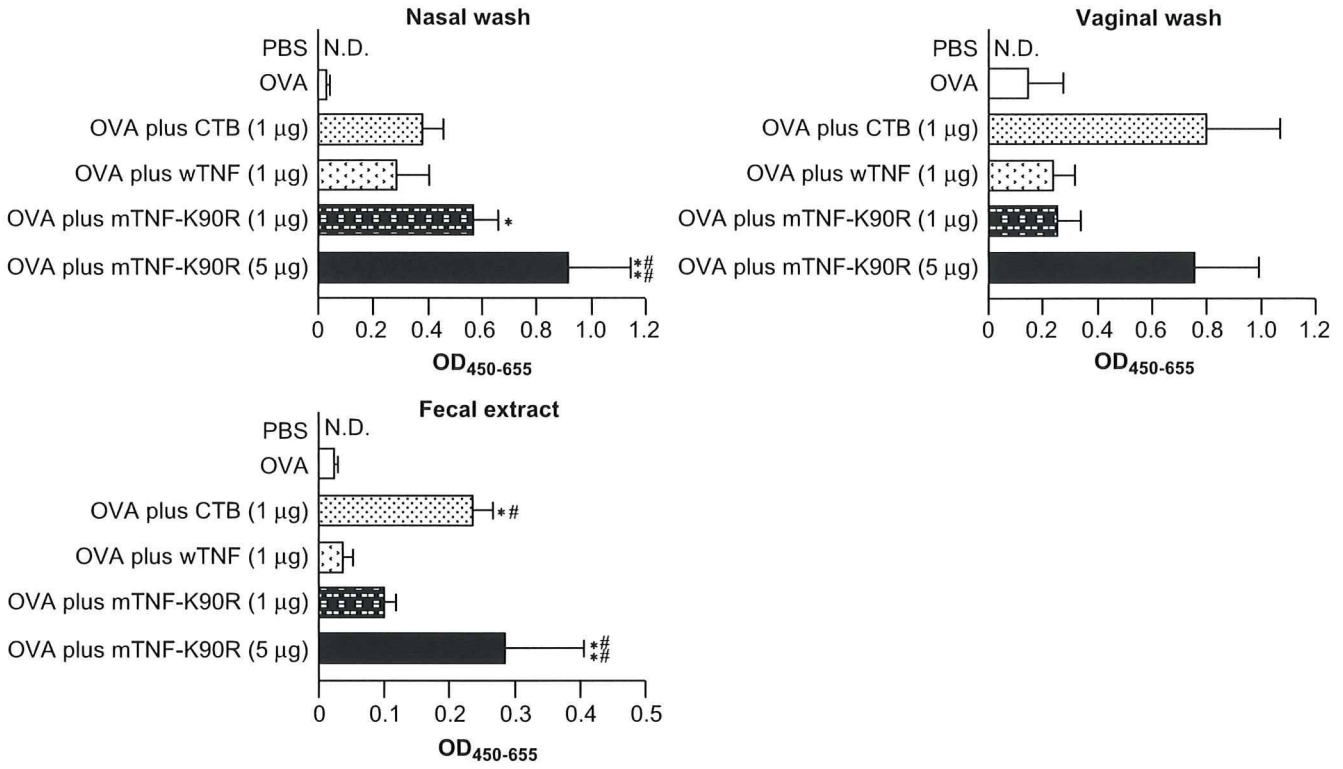

Fig. 2. OVA-specific mucosal IgA Abs response after nasal immunization with OVA plus adjuvant. BALB/c mice were intranasally immunized with OVA alone, OVA plus 1 μg CTB, OVA plus 1 μg wTNF-α, OVA plus 1 μg mTNF-K90R or OVA plus 5 μg mTNF-K90R once a week for three consecutive weeks. Mucosal secretions were collected 1 wk after the last immunization and OVA-specific IgA Abs responses in nasal wash, vaginal wash and fecal extract were determined by ELISA at a 1:8 dilution. Data represents the mean of absorbance 450 nm (reference wavelength, 655 nm). Data are presented as means ± SEM ($n = 7$; $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* value for OVA alone treated group by ANOVA; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* value for OVA plus wTNF-α treated group by ANOVA).

OVA-stimulated splenocytes collected from immunized mice were assessed for Th2-type cytokines IL-4, IL-5, IL-10, granulocyte macrophage colony-stimulating factor (GM-CSF) (Fig. 3A) and Th1-type cytokines IL-12 and IFN-γ (Fig. 3B), using a multiplexed immunobeads assay. Splenocytes from mice immunized with OVA plus 1 μg mTNF-K90R exhibited higher levels of Th2-type cytokines (IL-4, IL-5, IL-10 and GM-CSF) than those responses induced with OVA plus 1 μg wTNF-α or CTB (Fig. 3A). In contrast, there was hardly any difference in Th1-type cytokine (IL-12 and IFN-γ) secretion amongst all of the immunized mice (Fig. 3B). To further characterize the type of immune response, the level of OVA-specific IFN-γ and IL-4-secreting splenocytes from OVA-immunized mice with the various adjuvants was determined using a cytokine-specific ELISPOT assay (Fig. 4). The level of OVA-specific IL-4-secreting splenocytes from OVA-immunized mice with mTNF-K90R was greater than those responses observed after administration of wTNF-α or CTB. By contrast, the level of IFN-γ-secreting splenocytes in OVA-immunized mice with mTNF-K90R was no different from the levels observed for mice immunized with OVA alone. These results suggested that mTNF-K90R induced a more strongly polarized Th2-type immune response when mTNF-K90R was used as a mucosal vaccine adjuvant.

3.3. Mucosal adjuvant efficacy against influenza virus

We anticipated that a mucosal influenza virus-neutralizing antibody response would generate an ideal vaccine against these infectious diseases. To estimate the mucosal adjuvant efficacy of mTNF-K90R for influenza virus HA vaccine, the antibody response against HA was examined in mice intranasally immunized with mTNF-K90R (Fig. 5). Mice receiving HA plus 5 μg of mTNF-K90R induced a significantly greater anti-HA IgG or IgA response in serum, saliva or nasal wash compared with mice receiving HA alone or HA plus CTB. These results indicated that application of mTNF-K90R as a nasal vaccine adjuvant to viral infectious diseases might be an effective strategy.

3.4. Localization of antigens into nasopharyngeal-associated lymphoreticular tissue (NALT)

Because NALT plays an important role in the initial induction of the mucosal immune response, it was important to determine the tissue localization of the antigens. To characterize the mechanism of mTNF-K90R in the induction of OVA-specific mucosal and systemic immune responses, we examined the localization of OVA in NALT derived from mice that were immunized with OVA in the absence or presence of mTNF-K90R (Fig. 6). Fluorescence microscopic analysis revealed that FITC-OVA was mainly located beneath the nasal epithelium surrounding NALT in mice treated with FITC-OVA alone. By contrast, in addition to the subepithelial region, FITC-OVA was also observed within NALT after co-administration of mTNF-K90R. These results indicate that mTNF-K90R might induce the effective entry of antigens into NALT due to increasing nasal epithelial permeability. We believe that the observed response of mTNF-K90R in the nasal epithelial reflects one of the mechanisms for the induction of antigen-specific mucosal and systemic immune response in mice.

3.5. Safety examination of mTNF-K90R

While enterotoxin-based adjuvants display a strong mucosal response, they also induce severe central nervous system damage

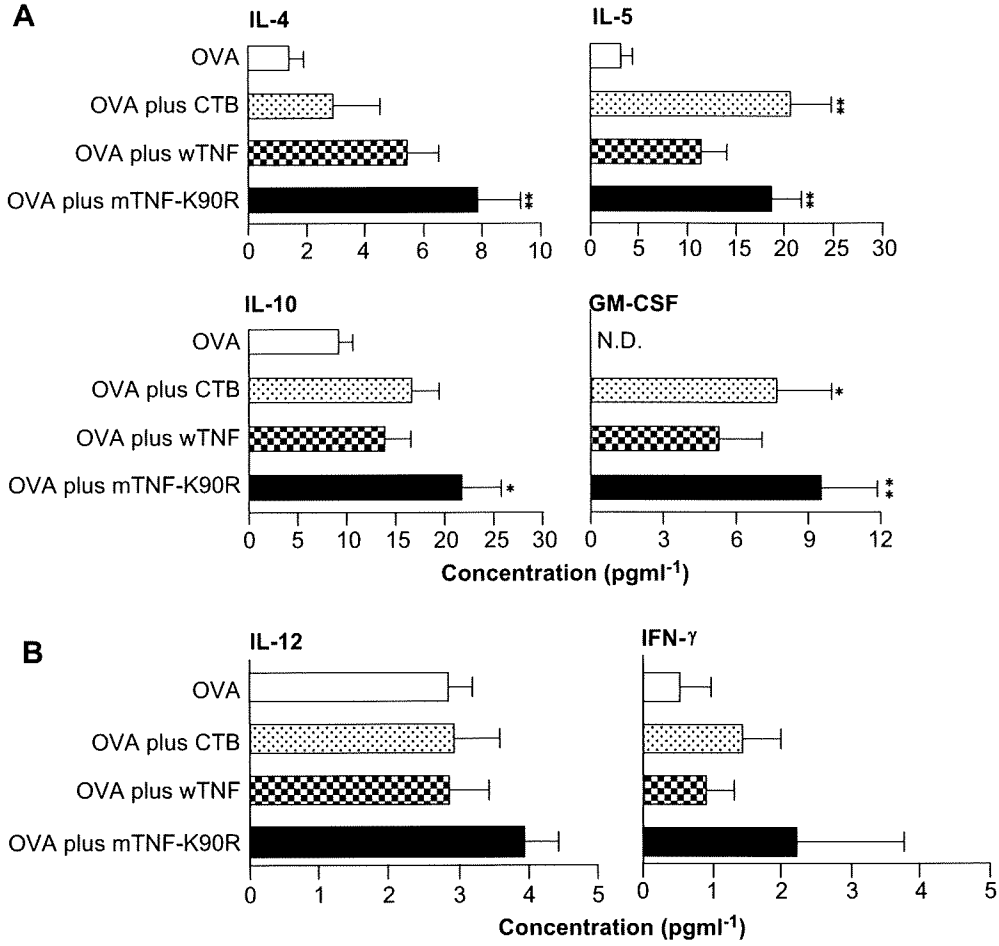

Fig. 3. Cytokine response induced after nasal immunization with OVA plus adjuvant. BALB/c mice were intranasally immunized with OVA alone, OVA plus 1 μg CTB, OVA plus 1 μg wTNF-α or OVA plus 1 μg mTNF-K90R once a week for three consecutive weeks. One week after the last immunization, splenocytes from each group were cultured with 1 mg ml^{-1} OVA. Culture supernatants were harvested following 3 days of incubation, and OVA-specific Th2-type (A) and Th1-type (B) cytokine productions in culture supernatant were analyzed by using the Bio-Plex Multiplex Cytokine Assay. Data are presented as means ± SEM ($n = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* value for OVA alone treated group by ANOVA).

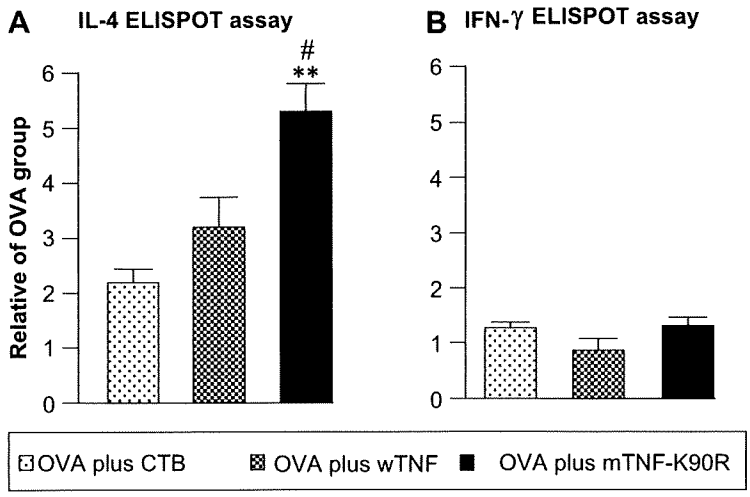

Fig. 4. Analysis of OVA-specific cytokine-secreting cells in mice nasally immunized with OVA plus adjuvant. BALB/c mice were intranasally immunized with OVA alone, OVA plus 1 μg CTB, OVA plus 1 μg wTNF-α or OVA plus 1 μg mTNF-K90R once a week for three consecutive weeks. One week after the last immunization, splenocytes from each group were cultured with 1 mg ml^{-1} OVA. The levels of OVA-specific IL-4 (a) and IFN-γ-producing cells (b) were examined by individual cytokine-specific ELISPOT assay. Data are presented as means ± SEM ($n = 3$; $^{**}P < 0.01$ *versus* value for OVA plus CTB treated group by ANOVA; $^{\#}P < 0.05$ *versus* value for OVA plus wTNF-α treated group by ANOVA).

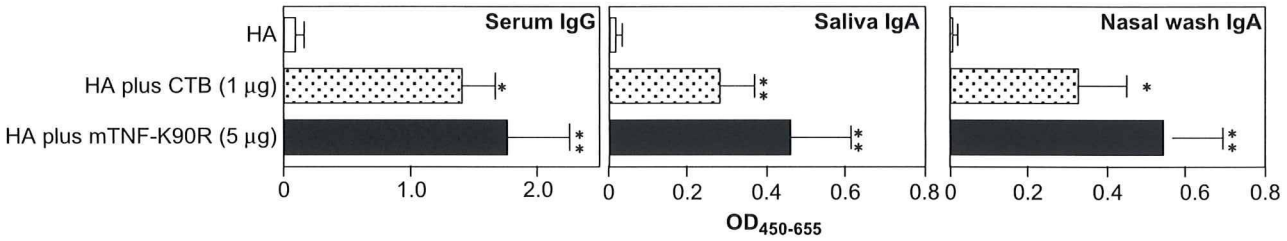

Fig. 5. mTNF-K90R induced mucosal IgA and IgG responses against influenza virus HA in mice. BALB/c mice were immunized intranasally with HA together with 1 μg CTB or 5 μg mTNF-K90R. One week after the last immunization, HA-specific IgG in serum at a 1:500 dilution and IgA in nasal or saliva at a 1:8 dilution were assessed by ELISA at a 1:8 dilution. Data represents the mean of absorbance 450 nm (reference wavelength, 655 nm). N.D; not detected. Data are presented as means ± SEM ($n = 4$–6; $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* value for HA alone treated group by ANOVA).

due to the presence of a specific receptor, GM1 ganglioside, which is highly expressed in neuronal tissue [20]. This neurotoxicity has severely restricted the use enterotoxins as adjuvants for mucosal vaccines in clinical practice. To evaluate the *in vivo* toxicity of mTNF-K90R, inflammatory response and tissue injury were assessed in the nasal tissue (Fig. 7). Tissue sections of the nasal cavity were prepared at various time points after immunization of OVA plus mTNF-K90R. However, no tissue injury could be detected in any of the sections. These observations indicate no membrane barrier disruption and/or inflammatory changes, not even after 2 h single immunization with 5 μg mTNF-K90R (Fig. 7A and B). Furthermore, increasing the dose of mTNF-K90R given intranasally from 1 μg to 25 μg did not seem to have an adverse effect on the mice after three immunizations (Fig. 7C and D). Thus, although further evaluation is required, the results of this initial study demonstrated that the toxicity of mTNF-K90R is likely to be relatively low.

4. Discussion

In this study, we examined the mucosal adjuvant activity of mTNF-K90R and showed that intranasal co-administration of mTNF-K90R with antigen strongly induced both antigen-specific IgG in serum and IgA at mucosal site (nasal cavity, oral cavity, vagina and intestine). The enhanced adjuvant effect of mTNF-K90R might be caused by improved bioactivity and protease resistance compared to wTNF-α. Although mTNF-K90R showed a potent adjuvant effect on mucosal immunity, it does not elicit excessive inflammatory symptoms, such as formation of edema or fibrosis. Therefore, we believe that mTNF-K90R is a potent mucosal adjuvant for vaccines against various infectious diseases.

The development of a safe and effective vaccine is critical in preventing the spread of influenza virus. It is generally thought that or anti-influenza virus-neutralizing antibody must be induced at mucosal surfaces in order to prevent influenza virus infection. Previous studies also reported that antigen-specific systemic IgG and mucosal IgA Abs have potentially important roles for protection against the influenza virus [21,22]. Therefore, our results suggest that mTNF-K90R might be a superior mucosal vaccine adjuvant against infectious diseases caused by influenza virus.

TNF-α is anticipated to be used as a therapeutic drug to treat cancer. TNF-α has been used clinically for the treatment of non-resectable high-grade sarcoma and melanoma by locoregional applications in combination with melphalan under the approval of the European Agency for the Evaluation of Medicinal Products because systemic administration of TNF-α at therapeutically effective doses is limited by its unacceptable toxic side effects [23,24]. Further, in a recent report, it has been suggested that TNF-α had the potentials of the genetic toxicity, because TNF-α caused DNA damage through its ability to induce reactive oxygen species [25]. Thus, it is important to examine the safety of mTNF-K90R in a protocol using a mucosal vaccine adjuvant. Previously, we reported that mTNF-K90R had 1.3-fold lower *in vivo* toxicity after systemic administration compared with that of wTNF-α because of a change in the pharmacokinetics. Similarly, no adverse effect on the nasal mucosa was observed in this study after intranasal administration of mTNF-K90R. Although detailed examinations are required, mTNF-K90R is expected to be a useful mucosal vaccine adjuvant. Furthermore, we have shown that conjugating cytokines with polyethylene glycol (PEG) improves their safety *in vivo* [17,18,26–28]. We have also developed a novel site-specific PEGylation process to overcome the

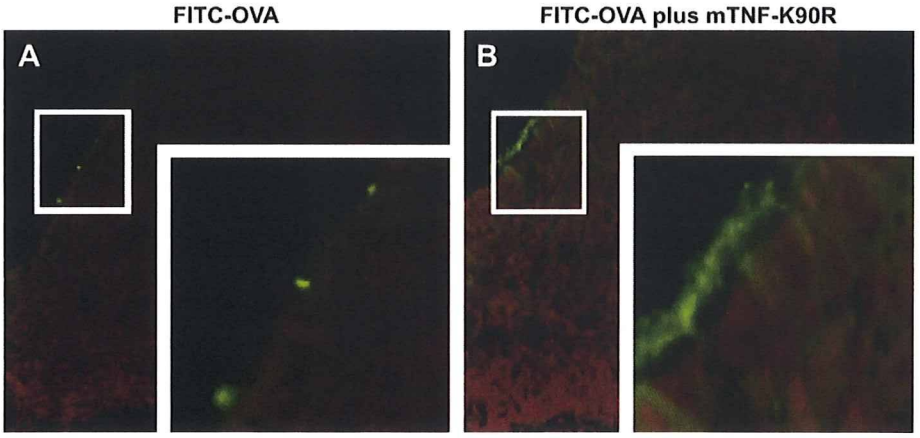

Fig. 6. Localization analysis of OVA in NALT. BALB/c mice were administered 50 μg FITC-OVA and a combination of 5 μg mTNF-K90R as a nasal vaccine adjuvant. Frozen sections of NALT resected from mice treated with FITC-OVA alone (A) and a combination of mTNF-K90R (B). The FITC-OVA (green) signals were detected by fluorescence microscopy. The nucleus was counterstained using PI (red). The original magnification of these photographs was 20×.

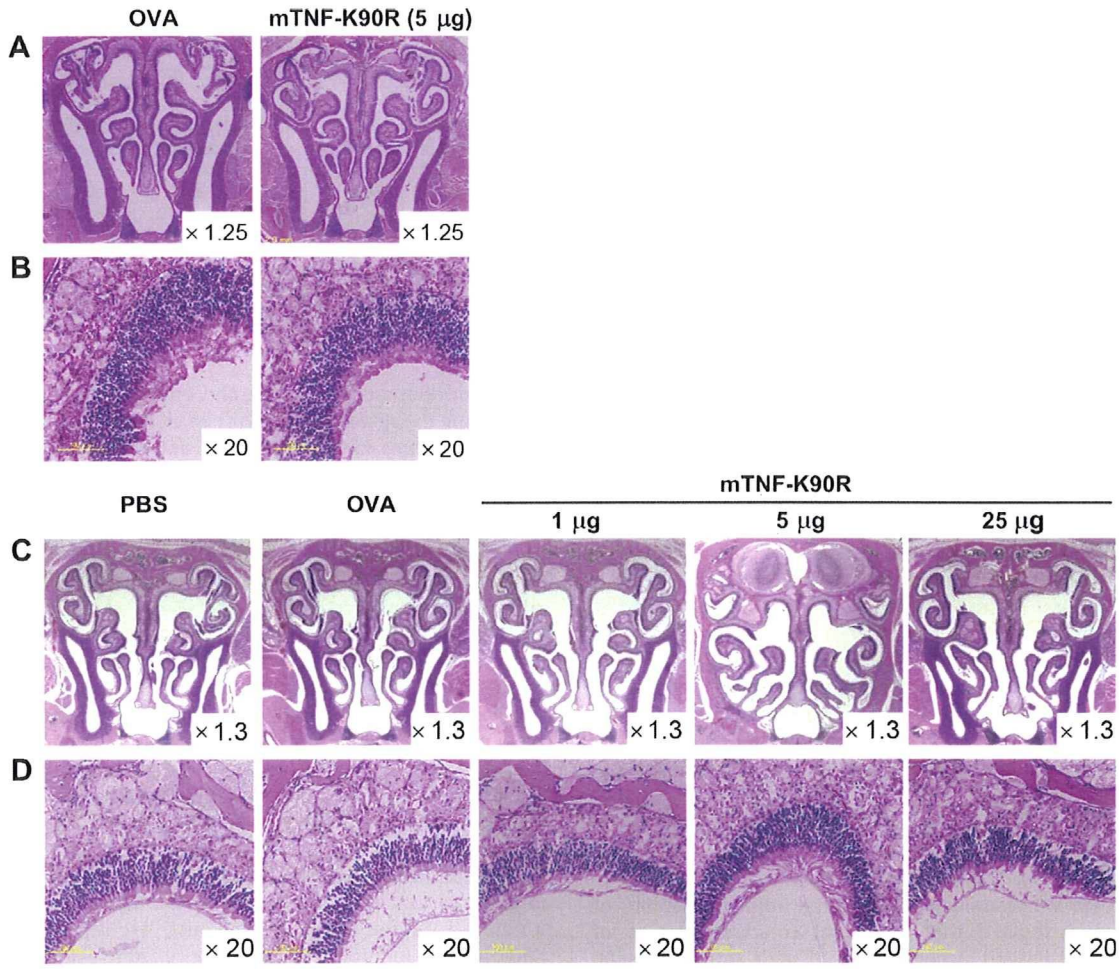

Fig. 7. Histopathological analysis of nasal cavity treated with mTNF-K90R. Frontal cross-sections of the nasal cavity from mice, taken 2 h after administration (A, B) or one week after three times administration of PBS, OVA alone, and OVA together with 1 μg, 5 μg or 25 μg mTNF-K90R (C, D). An overall view of the nasal passage is shown in (A) and (C). The region of nasal olfactory epithelia is shown in (B) and (D). Sections were prepared and the tissues were stained with H&E to assess the degree of tissue injury and local inflammation.

problems of PEGylation [17,18,26,28]. Previously we showed that the application of this technology to mTNF-K90R improved the safety and the anti-tumor effects of mTNF-K90R [18]. We are currently examining the safety and efficacy of site-specific PEGylated mTNF-K90R as a mucosal vaccine adjuvant.

The effects of mTNF-K90R at mucosal tissue was also analyzed. We reasoned that the adjuvant effect of mTNF-K90R may be related to its stimulation of antigen-presenting cells, such as DC. Indeed, DC plays a crucial role in eliciting T cell-dependent immunity. TNF-α is known to have profound effects on DC function and contributing to their activation, maturation and migration to, and accumulation within, draining lymph nodes [29,30]. Moreover, DC stimulated by TNF-α prior to anti-tumor vaccination or transfection with the TNF-α gene are reported to induce anti-tumor immunity [14,15]. However, we found that mTNF-K90R significantly enhanced the permeability of the nasal epithelial layer and diffusion of antigen into NALT. Consistent with our results, some reports have shown that TNF-α causes an increase in intestinal permeability [25,31]. Taken together, these results suggest that the strong mucosal adjuvant activity of mTNF-K90R is caused, at least in part, by increased epithelial permeability. In addition, TNF-α causes up-regulation of human polymeric Ig receptor on mucosal epithelial cells [32]. Polymeric Ig receptor transports polymeric IgA into external secretions as secretory IgA, which is critical for the defense of mucosal tissues [33]. We believe that multiple actions of mTNF-K90R contribute to its potent adjuvant activity. Currently, we are attempting to elucidate these various mechanisms.

Induction of both Th1- and Th2-type responses is the major goal for the development of mucosal vaccines because these responses would provide protective immunity against viral and bacterial infections by maximizing antigen-specific Ab and cytolytic T lymphocytes (CTL) responses. Although mTNF-K90R is not likely to induce CTLs, it could efficiently induce Abs responses. To induce both antigen-specific Ab and CTL responses, combinatorial administration of mTNF-K90R with another mucosal adjuvant, which can induce Th1-type immune responses, is applicable. We have already screened the TNF superfamily and other cytokines and succeeded in finding candidates that can effectively induce CTL at the mucosal site. The combinatorial effect of the cytokines and mTNF-K90R as a mucosal vaccine adjuvant is now under examination.

Recently, vaccine therapy has been attempted not only to combat cancer or viral infections but also for other diseases such as Alzheimer-type dementia. Schenk et al. demonstrated that intraperitoneal vaccination of β-amyloid peptide plus Freund's adjuvant to a murine Alzheimer's disease model resulted in a dramatic reduction of cerebral amyloidosis [34]. This therapeutic approach is clearly efficacious; however, the safety of this strategy is of paramount importance. In a clinical trial, approximately 6% of patients

administered a synthetic β-amyloid peptide plus adjuvant developed aseptic meningoencephalitis, most likely mediated by brain-infiltrating activated T cells [35,36]. This adverse effect seemed to be associated with the activation of Th1-type immunity by vaccination with β-amyloid peptide [37,38]. Nikolic et al. demonstrated that immunization capable of inducing Th2-type immunity predominantly constitutes an effective and potentially safe treatment strategy for Alzheimer's disease [39]. Therefore, our mTNF-K90R is a promising development in the establishment of an easy-to-use, efficacious, safe immunotherapy for Alzheimer's disease. However, further analyses are necessary in order to elucidate the Th2-dominant mechanism of mTNF-K90R.

5. Conclusions

In summary, our study showed that mTNF-K90R, engineered by using a phage display technique, induced two layers of protective immunity when administered intranasally with a vaccine antigen. Our results indicate that mTNF-K90R is a safe and effective mucosal adjuvant. Additionally, our technique of creating bioactive mutant cytokines might be an attractive generic approach for designing novel mucosal adjuvants.

Acknowledgements

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Appendix

Figures with essential color discrimination. Figs. 6 and 7 in this article may be difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.07.009.

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新規粘膜ワクチンアジュバントとしての機能性サイトカインの開発

特集　感染症対策に資する新規ワクチンシステムの開発

萱室裕之[*1,2]，角田慎一・堤　康央[*1~3]

Development of functional cytokines as novel mucosal vaccine adjuvants

Mucosal vaccines administered either orally or nasally have been shown to be effective in inducing antigen-specific immune responses at both systemic and mucosal compartments. Because of this two-layered protective immunity, mucosal vaccines are thought to be an ideal strategy for combating both emerging and re-emerging infectious diseases. There is, however, no mucosal vaccine to use in human due to the lack of mucosal adjuvant with high efficacy and safety. In this regard, we succeeded in augmentation of protective immunity to viral infection by applying a mutant tumor necrosis factor-α(TNF-α), mTNF-K90R, that exhibits high bioactivity and resistance to proteases as a mucosal vaccine adjuvant.

In this review, we describe about the feasibility of functional cytokines as mucosal vaccine adjuvants in vaccine therapy for infectious diseases.

粘膜ワクチンは，全身面と粘膜面の二重の防御免疫を誘導可能な唯一の方法であることから，新興・再興感染症に対する予防法として期待されている．しかし，生ワクチンを除いて，いまだ実用的な粘膜ワクチンは皆無であり，その実現に向けては，有効かつ安全なアジュバント開発が必要とされている．筆者らが開発を進めている機能性サイトカインは，防御免疫を効率よく誘導しうることから，粘膜アジュバントとしての可能性が大きく期待されるものである．

本稿では，粘膜ワクチンおよびアジュバント開発の現状と，筆者らの成果について概説する．

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key words : adjuvant, cytokines, mucosal vaccine, functional protein mutants, phage display library

腸管や呼吸器といった粘膜組織には，パイエル板や鼻咽頭関連リンパ組織(nasopharynx-associated lymphoid tissue：NALT)という粘膜固有の免疫誘導組織が存在し，粘膜免疫システムを発動させるための中枢として機能することが明らかとなってきた[1]．そして近年，この粘膜免疫システムを有効利用した経粘膜投与型ワクチン(粘膜ワクチン)が，インフルエンザやエイズといった新興再興感染症に対する次世代型ワクチンに適うものとして，大きく期待されている．

粘膜ワクチンは，全身レベルでの免疫のみならず，種々の病原体の侵入部位である粘膜面における抗原特異的免疫応答をも誘導することで，二段構えの予防免疫を誘導できるという，従来の注射型ワクチンでは達成できない効果を期待できる[2]．したがって昨今，世界的感染拡大を起こしている新型インフルエンザウイルスをはじめとするさまざまな病原体に対する感染予防策として，感染初発部位である粘膜局所での予防免疫を構築可能な粘膜ワクチンへの期待はますます高まっている(図1)．

粘膜ワクチンの有効性は，ポリオ生ワクチンによって実証されてきた．ポリオ生ワクチンを自然感染経路同様に経口接種すると，血清中におけるIgG抗体に加えて，腸管粘膜面においてIgA抗体産生が誘導され，すぐれた感染防止効果を発揮する[3]．このポリオ生ワクチンに倣い，経粘膜感染型ウイルスを標的とした粘膜ワクチンが，現在，盛んに研究されている[4]．

しかし，生ワクチンではなく，スプリットワクチンや成分ワクチンの場合には，ワクチン抗原を単独で経粘膜投与しても，抗体産生能に乏しく，感染防

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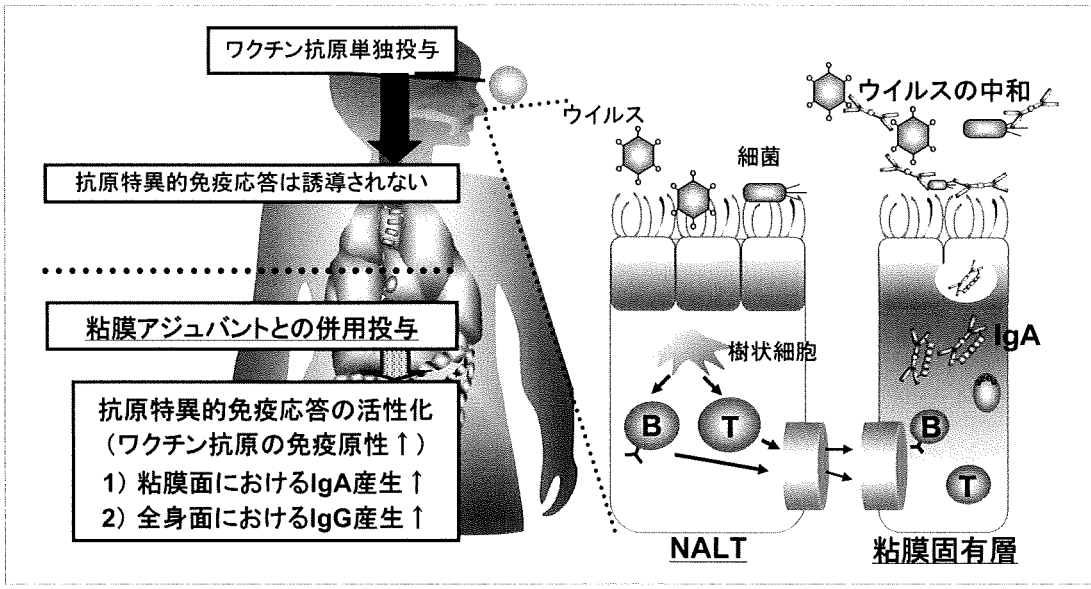

図1　粘膜ワクチン開発におけるアジュバントの必要性

御に充分な免疫を誘導できないことが明らかとなっている．したがって，粘膜ワクチン効果を最大限に発揮するためには，ワクチン抗原に対する抗原特異的な免疫応答を強化，活性化できる免疫増強剤（アジュバント）の併用が必須である[5]．

これまでに，粘膜アジュバントとして，コレラ毒素（cholera toxin：CT）や大腸菌易熱性毒素（heat labile enterotoxin：LT）が見いだされ，臨床応用も試みられてきた．しかし，これら細菌毒素由来のアジュバントは，粘膜面および全身面に効率よく免疫を誘導しうるものの，顔面神経麻痺（Bell's palsy）をはじめとする重篤な副作用を呈することが明らかとなり，臨床応用は断念されている[6]．

本観点から筆者らは，臨床応用可能な粘膜アジュバントを開発することを目的に，免疫調節因子であるサイトカインに着目し，新規粘膜アジュバントとしての有用性を検討してきた．サイトカインは，免疫応答の制御に必要不可欠な生体由来分子であり，おのおののサイトカインが抗原提示細胞の活性化作用や，T細胞・B細胞といった獲得免疫担当細胞の分化/増殖刺激作用などを担っている．したがって，サイトカインは，粘膜ワクチンアジュバントとして具備すべき，有効性と安全性の両者を兼ね備えているものと考えられた．

しかし，これまで粘膜免疫システムにおけるサイトカインの役割はほとんど解明されていないうえ，サイトカインを経粘膜投与した場合には，蛋白分解酵素やpH変化により速やかに失活・分解されることが予想された．

そこで筆者らは，これら問題点を克服し，サイトカインを粘膜アジュバントとして有効活用しうる方法論の確立を試みた．すなわち，サイトカインのなかでも特に強力な免疫活性化能を有することが知られる腫瘍壊死因子（TNF-α）に着目し，ファージ表面提示法を駆使した独自の機能性人工蛋白質創製技術により，活性が野生型TNF-α（wTNF）よりも飛躍的に向上し，かつ体内安定性にもすぐれた活性増強型TNF変異体（mTNF-K90R）を創製し，粘膜ワクチンアジュバントとしての応用を試みた[7]．

活性増強型TNF変異体の創出

上記観点から筆者らは，およそ20種類にも及ぶTNFスーパファミリーサイトカインについて，粘膜アジュバント活性の比較解析を実施した．その結果，TNF-αが特にすぐれた粘膜アジュバント効果を発揮すること，また重篤な副作用を伴うことなく粘膜免疫を誘導可能であることを世界に先駆けて見いだした[8]．

しかし，サイトカインは一般に，強力な免疫活性