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Mutant TNF-α, mTNF-K90R, is a novel candidate adjuvant for a mucosal vaccine against HIV

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The development of a safe and effective mucosal vaccine adjuvant is a crucial step for the development of vaccines against human immunodeficiency virus type-1 (HIV). We have previously reported that a mutant tumor necrosis factor-α (TNF-α), mTNF-K90R, possessed strong mucosal vaccine adjuvant activities in mice. Here, we evaluated the potential of mTNF-K90R as a mucosal vaccine adjuvant for the induction of systemic and mucosal immune responses against HIV. Nasal immunization of BALB/c mice with 5 μg of an HIV gp120 *env* protein immunogen together with mTNF-K90R induced higher serum anti-HIV gp120 protein immunoglobulin G (IgG) responses than gp120 alone. Furthermore, mTNF-K90R induced anti-gp120 IgA responses in nasal as well as vaginal washes from immunized mice, although these were not administration sites. Again, responses with mTNF-K90R were higher than with gp120 alone. These results indicate that mTNF-K90R may be applicable as a mucosal adjuvant for HIV vaccination to induce both systemic and mucosal immune responses.

1. Introduction

The development of highly active antiretroviral therapy has resulted in a reduced death rate from human immunodeficiency virus type-1 (HIV) infection in developed countries (Berzofsky et al. 2004). However, considering the high cost and potential toxicity of long-term highly active antiretroviral therapy (Kitahata et al. 2009), it is obvious that the development of vaccines against HIV is the most desirable option for the prevention of viral transmission and disease progression (Forthal et al. 2007). An effective vaccine against HIV will likely need to induce virus-specific neutralizing antibodies in both the systemic and mucosal compartments, as the latter is a target of HIV infection (Mazzoli et al. 1997). A variety of immunization protocols using HIV vaccines have been shown to induce the production of serum neutralizing antibodies after systemic vaccination, but immunization via this route poorly induces mucosal immune responses (Belyakov et al. 1998). Therefore, it is necessary to develop vaccines against HIV for augmentation of both systemic and mucosal immune responses (Belyakov and Berzofsky 2004).

Mucosal vaccines administered either orally or nasally have been shown to be effective in inducing antigen-specific immune responses at both systemic and mucosal level (Kunisawa et al. 2007). Because of this double protective immunity, mucosal vaccines are thought to be an ideal strategy for combating both emerging and re-emerging infectious diseases, such as HIV (Yuki et al. 2007). 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 (Ada 2001). One strategy to overcome the weakness of the immune response is co-administration of mucosal vaccine adjuvant with the vaccine antigen (Eriksson and Holmgren 2002). Both the cholera toxin and *E. coli* heat-labile toxin are potent mucosal vaccine adjuvants, which have been used in experimental systems. Unfortunately, recent reports showed that use of a human nasal vaccine containing the heat-labile toxin as an adjuvant resulted in a very high incidence of Bell's palsy (Mutsch et al. 2004). Therefore, mucosal vaccine adjuvants with high efficacy and safety for clinical application remain necessary.

Previously, we tested the potential of tumor necrosis factor (TNF) superfamily cytokines to function as mucosal vaccine adjuvants (Kayamuro et al. 2009a). We showed that TNF-α could significantly enhance antigen-specific antibody responses at both the systemic and mucosal level in mice. We have utilized a phage display system for creating modified functional peptides and proteins for testing as candidate adjuvants (Shibata et al. 2007, 2004). We developed a mutant form of TNF-α, mTNF-K90R, possessing a 6-fold higher *in vitro* bioactivity and a 13-fold higher *in vivo* bioactivity than wild type TNF-α

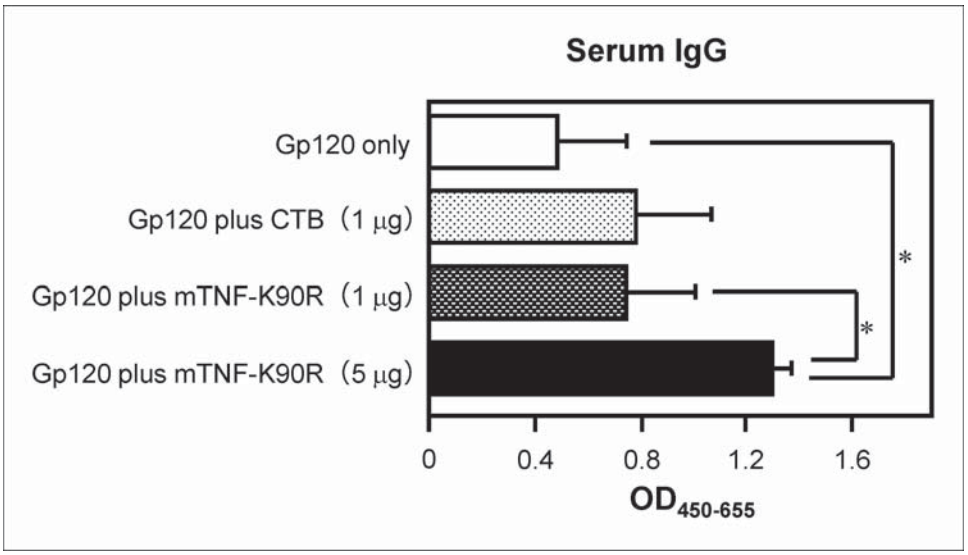

Fig. 1: Serum gp120-specific IgG responses following nasal immunization with gp120 plus mTNF-K90R. BALB/c mice were intranasally immunized with gp120 alone, gp120 plus CTB, or gp120 plus mTNF-K90R, four times at weekly intervals. Serum was collected 7 days after the last immunization and analyzed by ELISA for gp120-specific total IgG at a 100-fold serum dilution. Data are presented as mean ± SEM (n = 6; * $P < 0.05$)

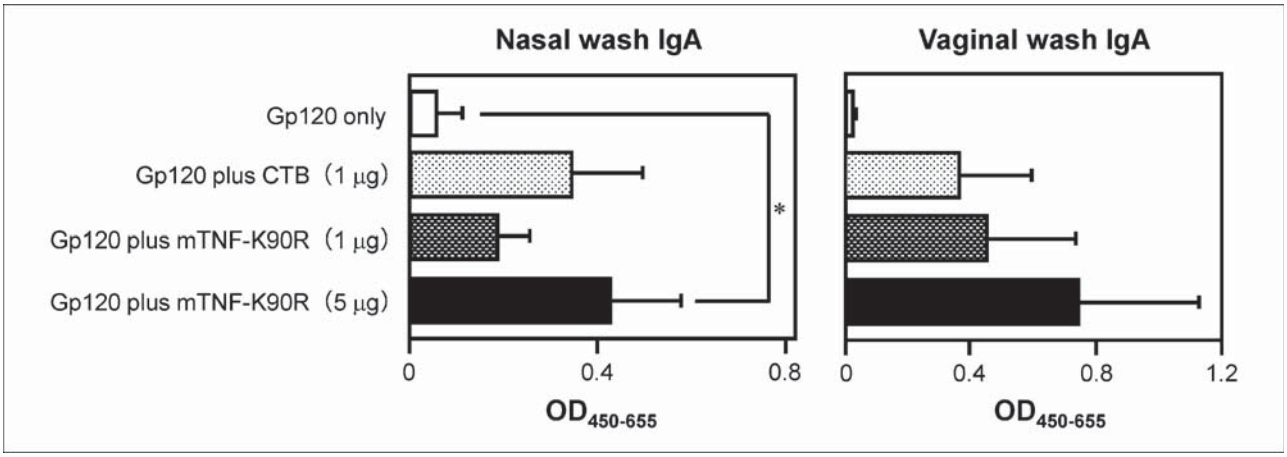

Fig. 2: gp120-specific mucosal IgA responses following nasal immunization with gp120 plus mTNF-K90R. BALB/c mice were intranasally immunized with gp120 alone, gp120 plus CTB, or gp120 plus mTNF-K90R once a week for four weeks. Mucosal secretions were collected 7 days after the last immunization and analyzed by ELISA for gp120-specific IgA in 8-fold diluted nasal wash or vaginal wash. Data are presented as mean ± SEM (n = 6; * $P < 0.05$)

(wTNF-α) (Shibata et al. 2004). In addition, we have previously demonstrated that intranasal administration of a model antigenic protein (OVA) together with mTNF-K90R induces a strong OVA-specific systemic IgG and also mucosal IgA response (Kayamuro et al. 2009b). These findings indicate that mTNF-K90R may be an attractive mucosal adjuvant for vaccination against HIV. Here, we evaluated mTNF-K90R as a mucosal vaccine adjuvant by assessing the production of IgA and IgG in mucosal tissue and blood.

2. Investigations, results and discussion

In this study, we evaluated the efficacy of mTNF-K90R as a mucosal adjuvant for vaccination against HIV. The HIV envelope protein, glycoprotein (gp) 120 is an important part of the spikes that decorate the surface of the virus and is a major target for neutralizing antibodies. One clinical study using gp120 protein for boosting is currently ongoing (Cleghorn et al. 2007). In the present study, BALB/c mice were nasally administered with HIV gp120 protein alone or together with cholera toxin B subunit (CTB), a conventional mucosal adjuvant, or mTNF-K90R, on days 0, 7, 14, and 21. Intranasal immunization with gp120 plus 1 μg of mTNF-K90R yielded higher antigen-specific IgG responses as measured in the serum than immunizing with gp120 alone. In fact, they were as strong as when using CTB (Fig. 1). In addition, use of 5 μg mTNF-K90R resulted in significantly higher IgG responses than 1 μg (Fig. 1). Next, to evaluate the induction of antibody responses in mucosal tissues, nasal and vaginal secretions were tested for the presence of gp120-specific IgA antibodies (Fig. 2). Immunization with gp120 plus CTB induced strong gp120-specific IgA secretion in all mucosal tissues. Nasal immunization with gp120 plus 1 μg of mTNF-K90R induced higher levels of gp120-specific IgA secretion than gp120 alone in nasal as well as vaginal washes from immunized mice, despite the fact that these were not administration sites. Again, use of 5 μg of mTNF-K90R induced higher gp120-specific IgA secretion in multiple mucosal tissues than 1 μg which were comparable to those induced with gp120 plus CTB. These results indicate that mTNF-K90R may be useful as a mucosal adjuvant for induction of gp120-specific serum IgG and mucosal IgA antibody responses when combined with HIV immunogens and administered via the nasal route.

The development of a safe and effective vaccine is critical in preventing the spread of HIV. However, the recent failure of the type 5 adenoviral HIV vaccine candidate developed by Merck in 2007 highlights the necessity for a new approach in developing a vaccine against HIV (Watkins et al. 2008). It is generally

thought that an anti-HIV neutralizing antibody must be induced at mucosal surfaces in order to prevent viral infection. Previous studies also reported that antigen-specific systemic IgG and mucosal IgA antibody may be important for protection against HIV (Veazey et al. 2003). Our results suggest that mTNF-K90R might be a superior mucosal vaccine adjuvant against infectious diseases caused by HIV and might be advantageous in this regard.

3. Experimental

3.1. Adjuvants

CTB was purchased from List Biological Laboratories (Campbell, CA). mTNF-K90R were prepared in-house as described previously (Shibata et al. 2004). The endotoxin level was quantified using a Limulus amebocyte lysate assay kit (QCL-1000, BioWhittaker, Walkersville, MD). The endotoxin content of purified mTNF-K90R was < 0.02 EU/μg protein.

3.2. Mice and immunization protocols

Female BALB/c mice were purchased from SLC (Hamamatsu, Japan) and used at 6 weeks of age. All of the experimental animal procedures in this study were performed in accordance with the National Institute of Biomedical Innovation guidelines for the welfare of animals. Mice were nasally immunized with 5 μg recombinant gp120 of HIV III_B strain (Immuno Diagnostics, Woburn, MA) together with 1 μg CTB, 1 or 5 μg mTNF-K90R on day 0, 7, 14 and 21.

3.3. Sample collection

Seven days after the final immunization, serum and mucosal secretions (nasal washes and vaginal washes) 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. The samples were centrifuged at 15000 × g for 20 min, and supernatants collected and stored at −80 °C.

3.4. Detection of gp120-specific antibody responses by ELISA

Gp120-specific IgG and IgA levels in serum, nasal washes, and vaginal washes were determined by ELISA. ELISA plates (Maxisorp, type 96F; Nunc, Roskilde, Denmark) were coated with 10 μg/ml gp120 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 (100 × dilution) or mucosal secretions (8 × dilution) were added to the gp120-coated plates. After incubation at 37 °C for 2 h, the coated plates were washed with PBS-Tween 20 and incubated with 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 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.

3.5. Statistical analysis

All results are expressed as mean ± SEM. Differences were compared using Student *t*-tests.

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