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# Cholesteryl Pullulan Nanoparticles-Encapsulated TNF- $\alpha$ : An Effective Mucosal Vaccine Adjuvant Against Influenza

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/64366>

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## Abstract

We encapsulated tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), a major proinflammatory cytokine, into cholesteryl pullulan (CHP) to prepare TNF/CHP nanoparticles. In this chapter, the immune response-enhancing capability of the nanoparticles to act as a vaccine adjuvant against influenza is described. TNF/CHP nanoparticles showed excellent storage stability, and they enhanced host immune responses to external immunogens. We applied the nanoparticles in a mouse model of influenza virus infection to investigate their adjuvant ability. Nasal administration of TNF/CHP nanoparticles combined with a conventional split vaccine was effective at inducing systemic IgG<sub>1</sub> as well as mucosal IgA, and it protected mice against a lethal challenge of A/PR/8/34 (H1N1) influenza virus. Mechanistic studies showed that the nanoparticles enhanced antigen uptake by dendritic cells (DCs) and moderately induced the expression of inflammation-related genes in nasal-associated lymphoid tissue (NALT), leading to the activation of both B and T cells. A preliminary safety study revealed no severe toxicity to TNF/CHP nanoparticles. Slight-to-moderate influences in nasal mucosa were observed only after repeated administration and they were reversible. Our data show that TNF/CHP nanoparticles effectively enhance both humoral and cellular immunity via nasal administration and could be a potential adjuvant for vaccines against infectious diseases like influenza.

**Keywords:** adjuvant, mucosal, nanoparticle, CHP, TNF- $\alpha$

## 1. Introduction

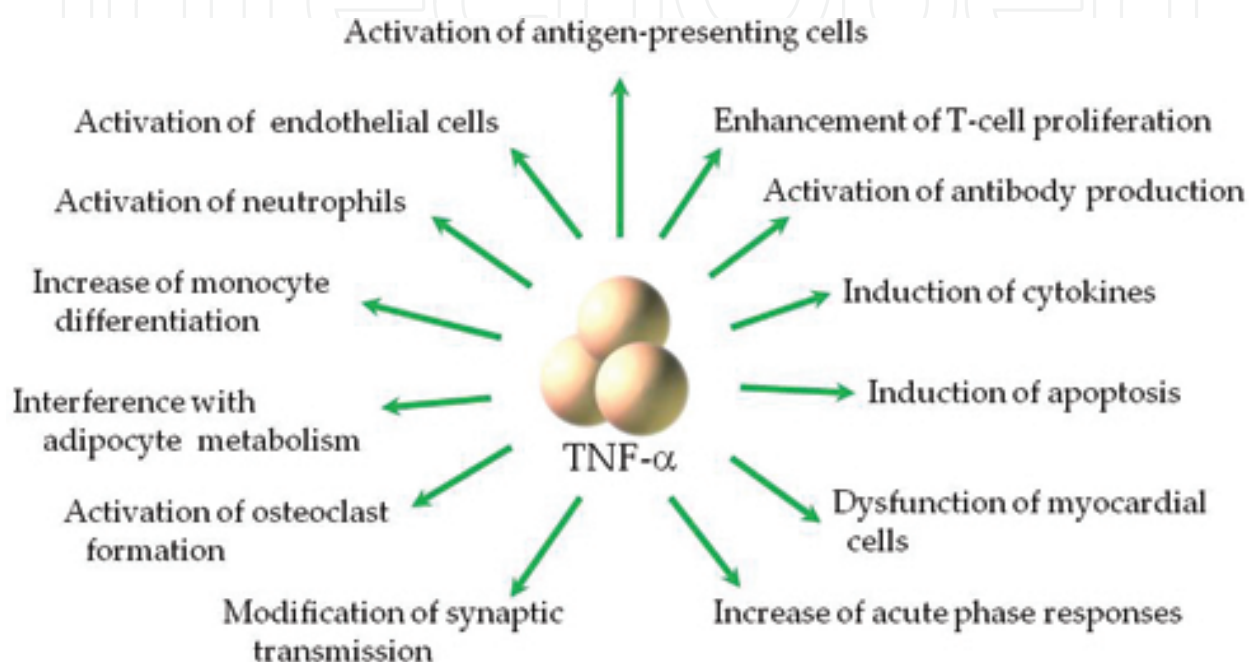
Vaccines are the most effective interventions against infectious diseases such as influenza. Many vaccines, however, are only effective at preventing onset and aggravation of symptoms, and less effective at preventing infection, particularly with respiratory infections. One reason for this is that the major administration routes of conventional vaccines, including subcutaneous (*s.c.*) and intramuscular (*i.m.*), induce neutralizing IgG antibody in blood but not mucosal IgA antibody, which is more effective at preventing infection. The efficacy of IgG antibody against variant or mutated viruses is very limited because it has highly restricted cross-protective capabilities. Conversely, IgA antibody on mucosa shows wide cross-protection and can block infection [1, 2]. When immunizations are delivered at the mucosa, IgA antibody is induced on mucosal surfaces throughout the body and IgG antibody is produced in the blood. Since mucosal vaccination induces immunity in both the systemic and mucosal compartments [3, 4], enhanced antigen-specific mucosal immunity is a clear goal for next-generation vaccines. Mucosal, especially nasal, vaccines are ideal because of their effectiveness in preventing infection via the respiratory tract. Nasal vaccines have the additional benefit of improved patient compliance and greater clinical convenience as well.

One significant drawback of mucosal vaccines is that they generally do not induce strong enough immune responses. The recent component-split vaccines, while avoiding many negative patient reactions, tend to be less immunogenic by themselves even in the case of intravenous (*i.v.*) or *i.m.* administration. Generally, children and the elderly tend to respond less to vaccinations, which may lower the preventive power of the population [5]. Therefore, adjuvants must be administered simultaneously with the vaccine in order to enhance vaccine-specific immune responses. Alum salts are the most commonly used adjuvant, but they are neither suitable for all vaccines nor always capable of eliciting the desired immune responses. Other types of adjuvants are being tested, such as liposomes, emulsions, and their combinations [6, 7]. The development of safe, effective, and suitable adjuvants is an important component in the future of mucosal vaccines.

Several groups have examined the use of cytokines (proteinaceous bioactive substances) as a new type of vaccine adjuvant due to their potent effects on the immune system [8–10]. Although some encouraging results have been reported, cytokines are not yet in the practical use as adjuvants. One of the important points to consider is the type of drug delivery system (DDS). Recently, our group tried to generate a new type of vaccine adjuvant by combining cytokines and biocompatible saccharide materials. This chapter describes the creation of human tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) encapsulated by cholesteryl pullulan (CHP) resulting in TNF/CHP nanoparticles. We investigated the potential of the nanoparticles as a nasal vaccine adjuvant by examining its ability to protect against lethal influenza infection in a mouse model and conducting further mechanistic analyses on innate and acquired immunity.

## 2. Tumor necrosis factor- $\alpha$ (TNF- $\alpha$ )

TNF- $\alpha$  is a major proinflammatory cytokine primarily produced by T cells and macrophages, and it is bioactive in a homotrimeric form [11]. It was first described as a potent anti-tumor factor, but it is now known to play an integral role in host defense. It activates innate and adaptive immunity by stimulating dendritic cell (DC) maturation and subsequent T cell activation as well as contributing to inflammatory responses [12, 13] (**Figure 1**).



**Figure 1.** Various actions of TNF- $\alpha$ .

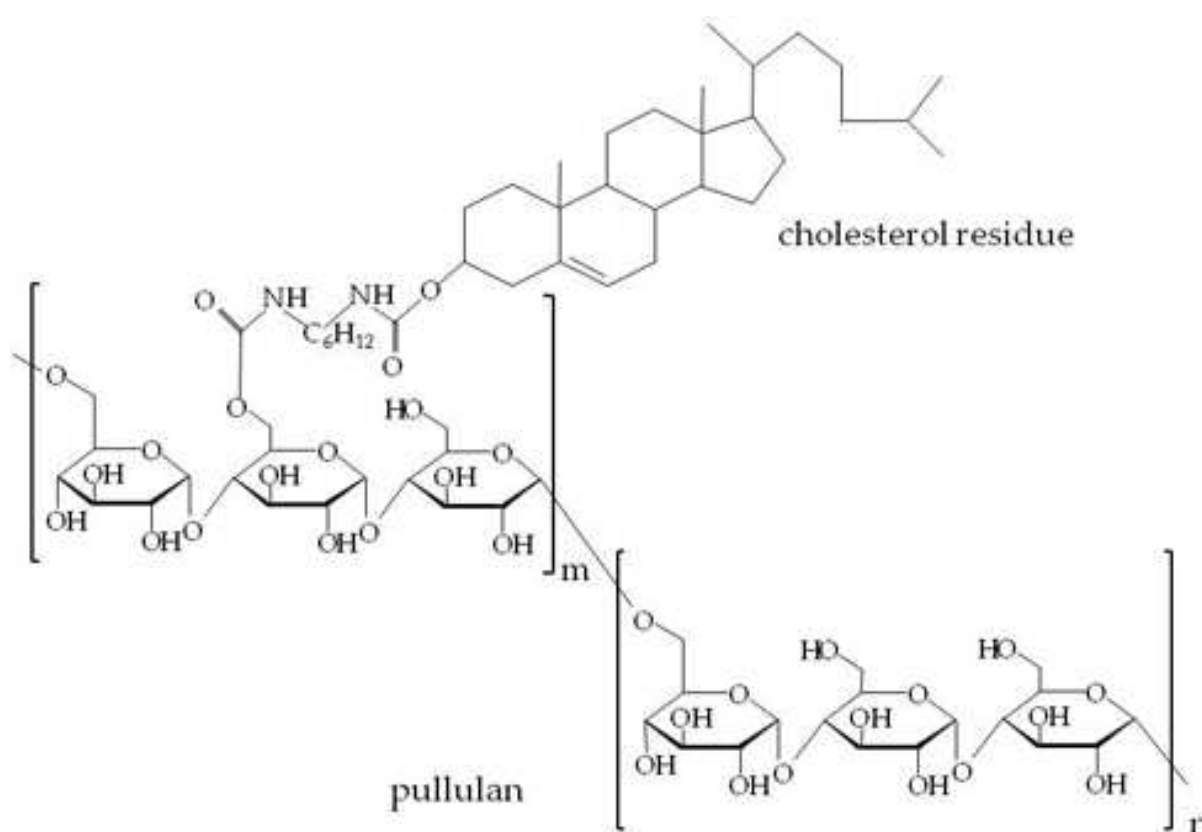
Interestingly, it was recently shown that TNF- $\alpha$  exerted adjuvant activities against pathogenic infections [14, 15]. Although there were some attempts to develop TNF- $\alpha$  (as well as other cytokines) as a vaccine adjuvant, successful practical results have not been reported. This is probably because TNF- $\alpha$  causes unfavorable biological reactions when administered systemically, and it is rapidly degraded when delivered at the mucosal surface. To overcome these obstacles, some investigators have attempted to generate protease-resistant mutant TNF- $\alpha$  molecules and have reported some potential as a vaccine adjuvant at the experimental level [16, 17].

## 3. Pullulan and cholesteryl pullulan (CHP)

One method of establishing a safer and more effective way to administer bioactive substances that is gaining popularity is a nanoparticle DDS. For nanoparticle materials, polysaccharides have been shown to possess several favorable characteristics in comparison with synthetic polymers currently used. Unlike synthetic polymers that could accumulate in the body to levels

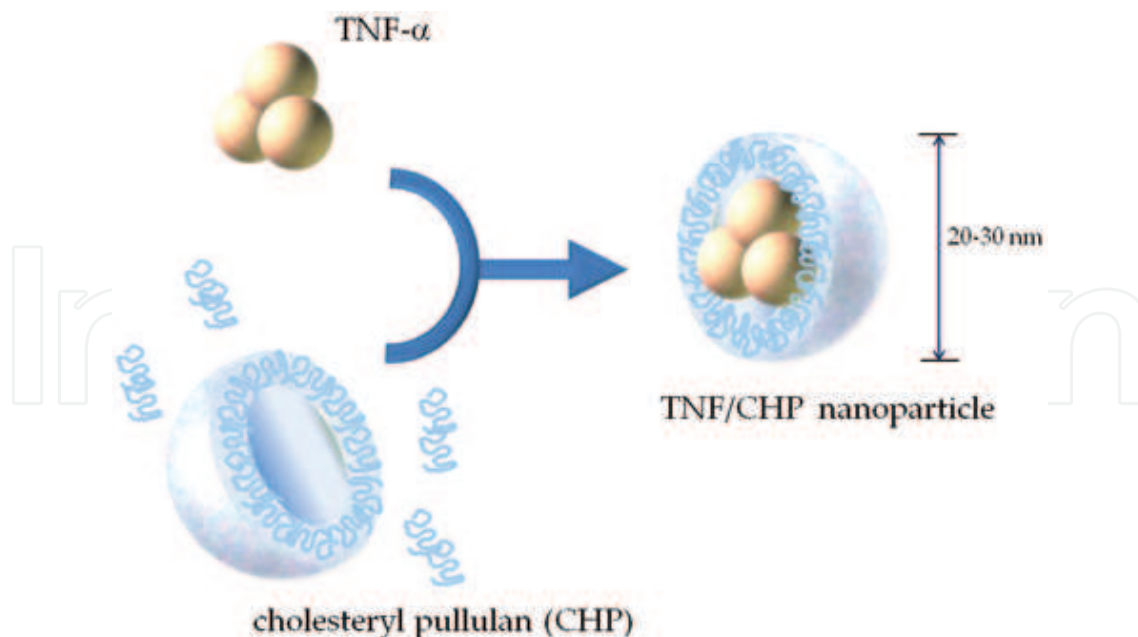
beyond the renal clearance, saccharides are biocompatible, meaning they are degraded by intrinsic enzymes inside the body [18, 19]. In this study, we used pullulan to create DDS nanoparticles.

Pullulan is a natural and chemically neutral homopolysaccharide consisting of  $\alpha$ -1, 6-linked maltotriose units (maltotriose is three glucose molecules linked with  $\alpha$ -1, 4 glycosidic bonds). It is produced primarily by fermentation of starch by strains of the fungus *Aureobasidium pullulans* [20]. By introducing hydrophobic moieties onto the hydrophilic pullulan molecule, amphiphilic copolymers can be generated. A representative is cholesteryl pullulan (CHP) [21, 22] (**Figure 2**).



**Figure 2.** Chemical structure of CHP.  $m, n$ ; integer values. In PUREBRIGHT CP-100T (NOF Co., Tokyo, Japan), 1–3% of glucose units are modified with cholesterol residues.

CHP self-assembles into nanoparticles in aqueous solution and entraps various molecules in its internal space through hydrophobic interactions. The hydrophilic shell serves as a stabilizing interface between the hydrophobic core and the external aqueous environment. It also protects the entrapped molecule from mechanical, chemical, or enzymatic attacks from outside the particle, and it acts as a superior carrier for delivery. It also allows slow release of the encapsulated materials [23–25]. Furthermore, the CHP nanoparticles showed prolonged circulation and thermodynamic stability in animal models [26].



**Figure 3.** Schematic model of self-assembling of TNF/CHP nanoparticles. TNF- $\alpha$  is shown in the trimeric, bioactive form. The diameter of the particle is approximately 20–30 nm.

Pullulan-based nanoparticles have been used for the delivery of proteins, anticancer drugs, imaging agents, and nucleotides. CHP nanoparticles are efficiently transferred to antigen-presenting cells such as macrophages and/or DCs, and they elicit strong immune responses [27, 28]. CHP is under vigorous investigation for establishing novel vaccine therapies against several types of cancers [29–31]. We created CHP nanoparticles containing TNF- $\alpha$ , which are described in the following sections (**Figure 3**).

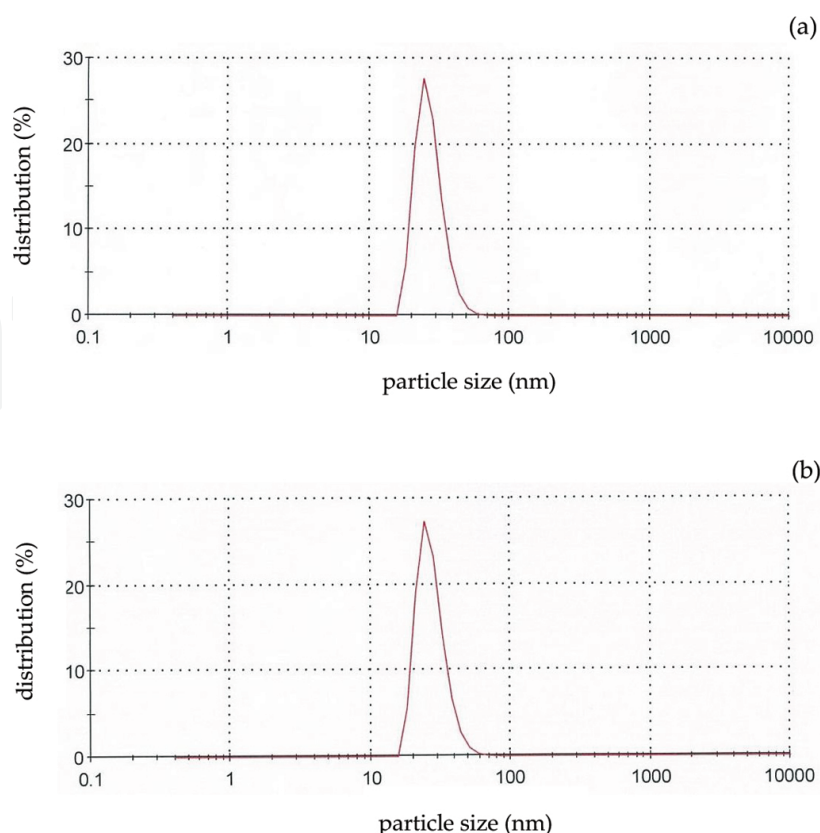
## 4. TNF/CHP nanoparticles

### 4.1. Preparation of TNF/CHP nanoparticles

In this study, we used TNF- $\alpha$  derived from a human lymphoblastoid cell line, BALL-1 [32], and CHP (PUREBRIGHT CP-100T) from NOF Corporation. CHP encapsulated active trimeric TNF- $\alpha$  to form stable nanoparticles as schematically shown in **Figure 3**. The encapsulating process was time- and temperature-dependent; at 37°C, more than 95% of the TNF- $\alpha$  was encapsulated into CHP complexes after 5 days of incubation; at 4°C, very few, if any, TNF/CHP nanoparticles were formed.

The resulting nanoparticles were relatively uniform. The mode and average sizes of the particles were 27.2 and 42.4 nm based on dynamic light scattering (DLS) results. This was not very different from the size of the blank CHP particles with a mode of 27.6 nm and an average of 42.8 nm (**Figure 4**). Stoichiometric analyses showed that a TNF/CHP nanoparticle consisted of a TNF- $\alpha$  active trimer (ca. 50 kDa) in a CHP tetrameric complex (ca. 400 kDa).





**Figure 4.** Size distribution of TNF/CHP nanoparticles. Two hundred fifty  $\mu\text{g/mL}$  TNF- $\alpha$  and 12 mg/mL CHP were mixed, sterilized by filtration, and incubated at 37°C for 5 days. CHP self-assembled with TNF- $\alpha$  molecules to form nanoparticles. The particle size was determined by DLS with a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). (a) TNF/CHP nanoparticles, (b) blank CHP particles. Reproduced with permission from Nagatomo D. et al., [33].

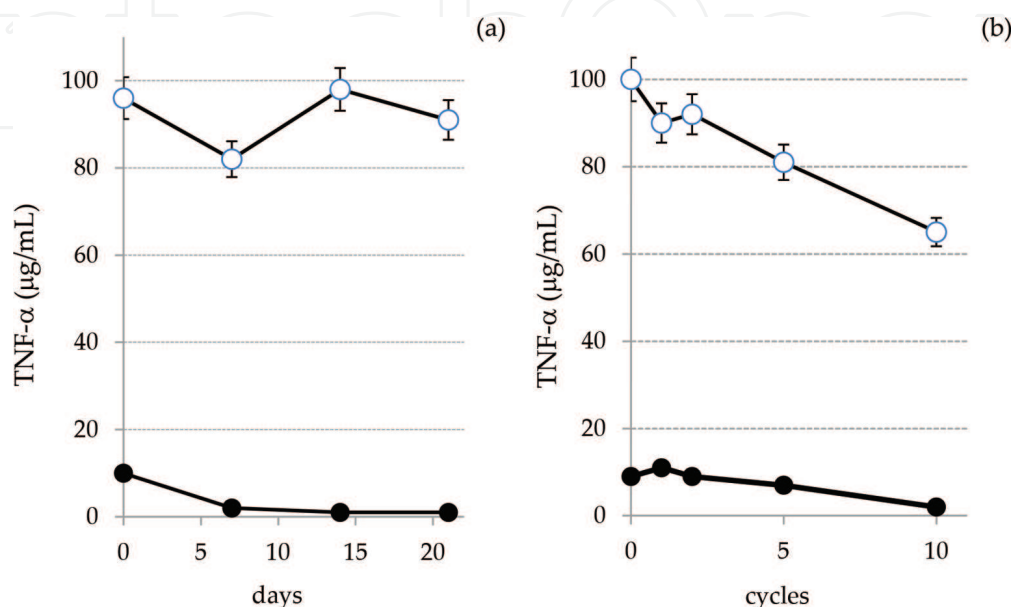
#### 4.2. Storage stability of TNF/CHP nanoparticles

Stability of the nanoparticles was evaluated after various treatments by measuring the level of TNF- $\alpha$ . To estimate the amount of encapsulated TNF- $\alpha$ , methyl- $\beta$ -cyclodextrin (Me- $\beta$ -CD) was used to disrupt the CHP complex and release the TNF- $\alpha$  as previously described [26]. The results showed that the nanoparticle retained its integrity and kept TNF- $\alpha$  molecules active inside the complex in aqueous solution at room temperature for at least 21 days (**Figure 5a**). Furthermore, even after five cycles of freezing and thawing, 80% of the particles remained intact (**Figure 5b**). These results show that the TNF/CHP nanoparticles have excellent storage stability. However, upon contact with high concentrations of dissolved proteins, such as serum albumin, the nanoparticles rapidly released the encapsulated TNF- $\alpha$  (data not shown), probably replaced by proteins from the external environment as reported [34]. This calls an attention to the usage of the nanoparticles, such as *i.v.* injection.

Me- $\beta$ -CD is known to interact with cholesteryl groups and disrupt CHP complexes to release the substance inside the particles [26]. The amount of Me- $\beta$ -CD required to disrupt the TNF/CHP nanoparticles was approximately 100 mg/mL, much higher than the 0.3 mg/mL reported for Interleukin-12 (IL-12)/CHP nanoparticles [35], suggesting that the affinity

between TNF- $\alpha$  and CHP was much stronger than that of IL-12 and CHP. The molecular interaction of TNF- $\alpha$  and CHP that creates this strength is an interesting area for further study.

Many medical formulations, especially biologicals, require storage at low temperatures or freezing. On the contrary, the TNF/CHP nanoparticles could be stored in solution and without refrigeration. Our formulation offers improved convenience of handling and transportation.



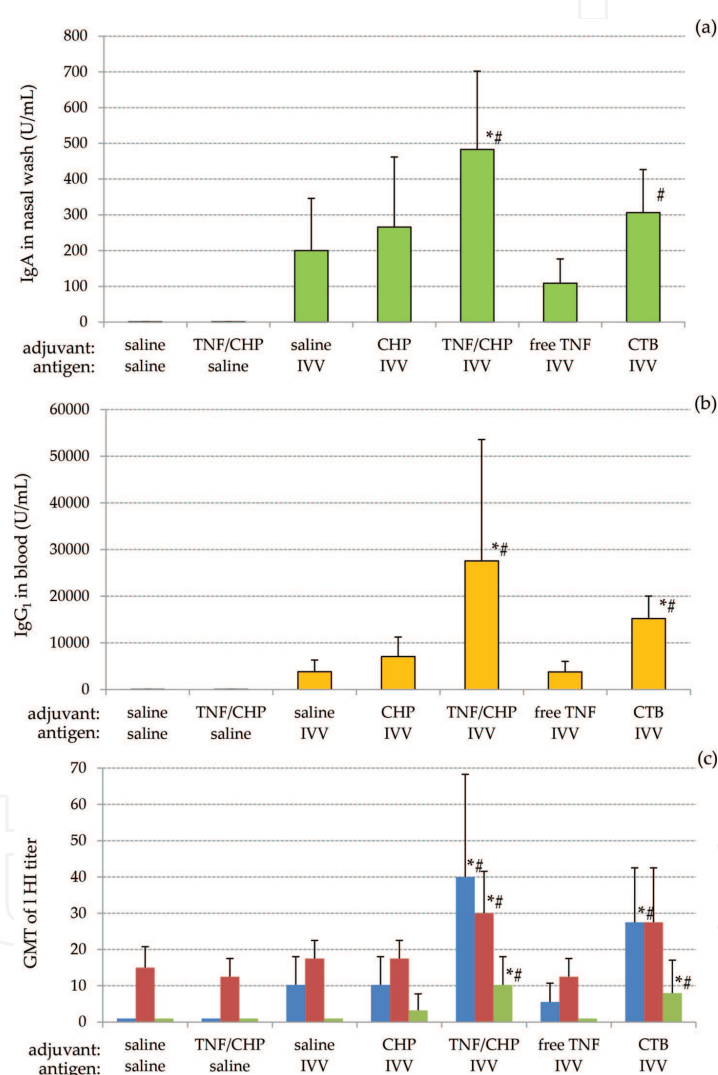
**Figure 5.** Stability of TNF/CHP nanoparticles *in vitro*. TNF/CHP nanoparticles were incubated in Dulbecco's phosphate buffer at 25°C or repeatedly freeze-thawed (−80°C/25°C). An aliquot was examined for the amount of active TNF- $\alpha$ . Samples were treated with 100 mg/mL Me- $\beta$ -CD at 37°C for 2 h to release TNF- $\alpha$  from the particles. The amount of TNF- $\alpha$  was determined by an enzyme-linked immunosorbent assay (ELISA) system. (a) storage stability at 25°C; (b) stability through freeze-thaw cycles. Open circle, treated with Me- $\beta$ -CD; closed circle, without Me- $\beta$ -CD. (mean  $\pm$  SD,  $n = 3$ ).

## 5. Immune responses induced by TNF/CHP nanoparticles administered nasally

Although TNF- $\alpha$  is known to have immune-enhancing activity [14], severe and unfavorable effects have hampered its practical use. Based on the stability described in the previous section, we hypothesized that delayed release of TNF- $\alpha$  from TNF/CHP nanoparticles would promote the beneficial effects of TNF- $\alpha$  while avoiding harmful events. We examined the adjuvant activity of the TNF/CHP nanoparticles, for example, enhanced induction of antigen-specific antibodies in mice, particularly in the case of nasal administration. We used a commercial influenza virus hemagglutinin vaccine (IVV), which is a component-split and trivalent vaccine for seasonal influenza. It consists of the inactivated hemagglutinin (HA) antigens from A/Brisbane/59/2007 (H1N1), A/Uruguay/716/2007 (H3N2), and B/Brisbane/60/2008. The nasally administered TNF/CHP nanoparticles combined with the IVV induced significant levels of

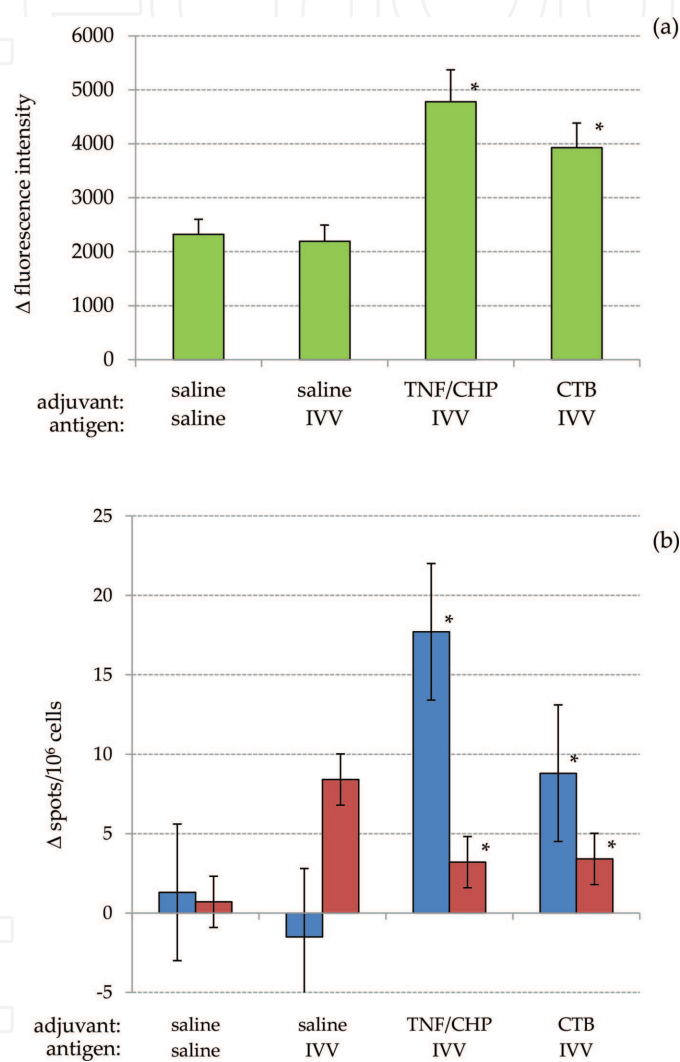


IgA in the nasal wash, as well as IgG<sub>1</sub> in blood plasma (**Figure 6a, b**). These are comparable to those of the positive control cholera toxin B subunit (CTB), the most powerful adjuvant in experimental settings [35]. Furthermore, IVV with CHP alone (without TNF- $\alpha$ ) or with free TNF- $\alpha$  failed to induce significant levels of antibodies when compared to IVV with no adjuvant. The TNF/CHP nanoparticles alone did not induce a measurable antibody response against IVV. To further examine antigen specificity, we performed hemagglutinin (HA)-specific hemagglutination inhibition (HI) assay for the different types of influenza virus included in the vaccine. The TNF/CHP nanoparticles with IVV induced significant HI activity against all types of HA used (A/H1N1, A/H3N2, and B) (**Figure 6c**).



**Figure 6.** Adjuvant effects of TNF/CHP nanoparticles administered nasally. BALB/c mice were nasally given IVV (SEIKEN, Denka Seiken Co., Ltd., Japan) (0.3  $\mu$ g/mouse) and TNF/CHP nanoparticles (5  $\mu$ g/mouse of TNF- $\alpha$ ) or CTB (0.8  $\mu$ g/mouse) once a week for 4 weeks. The nasal wash and blood plasma were prepared from the mice, and the levels of IVV-specific IgA and IgG<sub>1</sub> were determined by ELISA. (a) IgA levels in nasal wash, (b) IgG<sub>1</sub> levels in blood plasma, (c) HI titer in blood plasma against different HA types of influenza virus expressed in GMT (geometric mean titer). Blue column, type A/H1N1; red column, type A/H3N2; green column, type B. (mean  $\pm$  SEM, n = 8). \*,  $P < 0.05$  vs. saline/IVV; #,  $P < 0.05$  vs. free TNF/IVV. Adapted with permission from Nagatomo D. et al., [33].

These data indicate that TNF/CHP nanoparticles administered nasally can induce not only mucosal but also systemic immunity significantly and efficiently, comparable to the effects of CTB. In addition, the nasal vaccination covers a broad range of antigenicity as previous reports suggested [1, 2]. Also, just for reference, the induction of specific antibodies was seen for other antigens, such as Hepatitis virus type A vaccine, diphtheria toxoid, and cedar pollen allergen (data not shown). Those suggest that TNF/CHP nanoparticles have the potential as a vaccine adjuvant with a broad range of applications, as well as influenza.



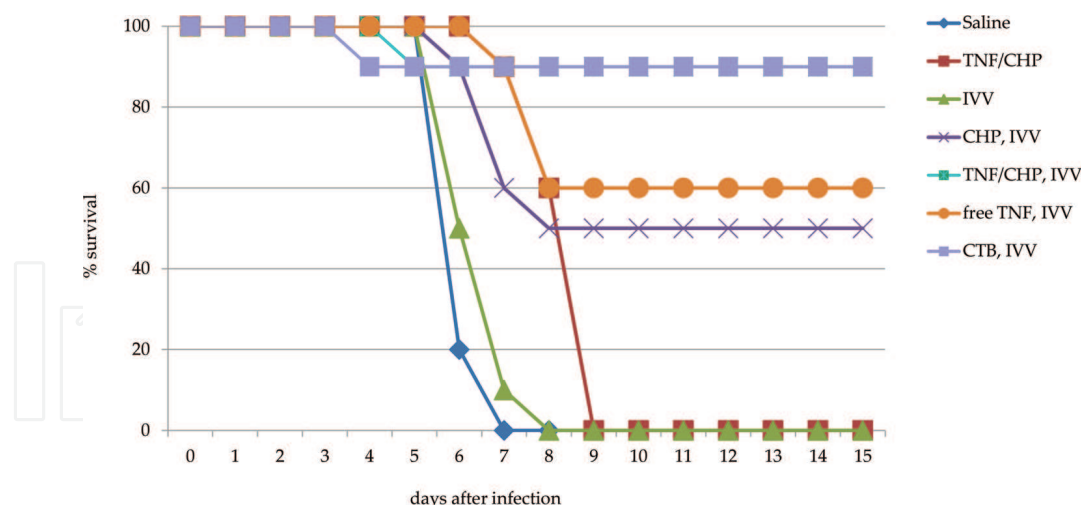
**Figure 7.** Proliferation and cytokine production by splenocytes from mice nasally administered TNF/CHP nanoparticles and IVV. BALB/c mice were given IVV (0.3  $\mu$ g/mouse) and TNF/CHP nanoparticles (5  $\mu$ g/mouse as TNF- $\alpha$ ) or CTB (0.8  $\mu$ g/mouse) by the nasal route as previously described. Splenocytes were prepared from the mice. Then, IVV-specific proliferation and IL-4/IFN- $\gamma$ -producing cells were examined with alamarBlue and ELISpot, respectively. The results represent the difference ( $\Delta$ ) between data from experiments with and without IVV antigen stimulation. (a) proliferation response. (b) cytokine production. Blue column, IL-4 production; red column, IFN- $\gamma$  production (mean  $\pm$  SEM,  $n = 8$ ). \*,  $P < 0.05$  vs. saline/IVV. Adapted with permission from Nagatomo D. et al. [33].

The antigen-specific T cell responses of the vaccinated animals were examined by challenging their isolated splenocytes with IVV. IVV-specific proliferation in the TNF/CHP nanoparticle

group was comparable to that of CTB (**Figure 7a**). We also measured cytokine production to understand what kind of T cell response occurred. IVV alone increased IFN- $\gamma$  production, while the addition of either adjuvant (TNF/CHP nanoparticle or CTB) suppressed IVV-induced Interferon- $\gamma$ (IFN- $\gamma$ ) production. The TNF/CHP nanoparticles combined with IVV also produced IL-4 cytokine to a higher level than IVV with CTB (**Figure 7b**). These experiments suggest that the nasally administered adjuvant shifted the Th1/Th2 balance to a Th2-dominant state, which confirms previous results obtained with a mutant TNF- $\alpha$  [17].

## 6. Protective effect of TNF/CHP nanoparticles in lethal challenge of influenza virus on mice

To directly address the stimulatory effect of the TNF/CHP nanoparticle adjuvant on protective immunity, we challenged immunized mice with a lethal dose of influenza virus. Mice were nasally immunized with IVV with or without TNF/CHP nanoparticles once a week for 3 weeks. Then, they were challenged with the antigenically distinct influenza virus A/Puerto Rico/8/34 strain at a lethal dose (**Figure 8**). The mice that received only IVV died by 8 days post challenge, which was comparable to mice without IVV immunization. The TNF/CHP nanoparticles without the IVV slightly delayed the time to death, but all of the animals eventually died. On the contrary, combined administration of IVV and TNF/CHP nanoparticles showed a highly protective effect with 90% of the mice surviving lethal challenge. The effect was comparable to that of the CTB adjuvant. Free TNF also showed a somewhat protective effect. Interestingly,



**Figure 8.** Protective effect of TNF/CHP nanoparticles adjuvant against lethal influenza virus challenge in mice. BALB/c mice were nasally administered with IVV with or without an adjuvant once a week for 3 weeks. Seven days after the final immunization, mice were challenged nasally with influenza virus (Puerto Rico/8/34, 10 LD<sub>50</sub>) and then monitored daily. Mice that were moribund or that had lost more than 20% of their body weight were considered to have reached an experimental endpoint and were humanely euthanized by anesthetization. Blue diamond, saline only; red square, TNF/CHP nanoparticles only (5  $\mu$ g/mouse of TNF- $\alpha$ ); green triangle, IVV only (0.3  $\mu$ g/mouse); purple cross, IVV with blank CHP nanoparticles (240  $\mu$ g/mouse); blue square, IVV with TNF/CHP nanoparticles (5  $\mu$ g/mouse of TNF- $\alpha$ ); orange circle, IVV with free TNF (5  $\mu$ g/mouse); purple dot, IVV with CTB (0.8  $\mu$ g/mouse) (n = 10). Reproduced with permission from Nagatomo D. et al. [33].

CHP only (without TNF- $\alpha$ ) provided a certain level of protection as an adjuvant, as we observed 50% survival. Importantly, the nasally administered TNF/CHP nanoparticles induced significant protective immunity in spite of the distinct antigenicities [36], suggesting that they have a potential for inducing broad cross-protection.

Antibodies became detectable after the second or third vaccination and reached plateau levels thereafter in mice vaccinated with TNF/CHP nanoparticles. The surviving animals immunized with IVV and TNF/CHP nanoparticles had immunological memory, including IgG<sub>1</sub> in plasma, and IgA in nasal/vaginal wash and feces. This memory was maintained at high levels for more than 90 days, and these mice responded to a boosting challenge of the IVV to further elevate the antibody levels (**Table 1**). These data indicate that the nanoparticles induced systemic immunity and long-lived memory, a critical feature for successful vaccine adjuvants. Overall, our data demonstrate that TNF/CHP nanoparticles are effective as a vaccine adjuvant for nasally delivered IVV.

TNF/CHP nanoparticles enhanced an IgA response not only at the site of application (e.g., in the nasal wash) but also at distant mucosal sites, such as the intestine (feces), vaginal, and salivary glands (data not shown). IgA antibody elicited at the mucosa is of vital importance as the natural route of infection for influenza is via the respiratory mucosa. Hence, local mucosal protection against pharyngeal carriage is likely to be decisive for preventing disease [37]. Conventional parenteral vaccines are not able to stimulate mucosal immune responses, thus restricting their efficacy in infections of mucosal surfaces such as the respiratory tract [1]. Our nasal vaccine/adjuvant formulation consisting of the IVV and TNF/CHP nanoparticles effectively induced both systemic and mucosal protective immunity.

		IgG <sub>1</sub> titer in plasma (U/mL)			IgA titer in feces extract (U/mL)			
Adjuvant	Unimmunized	None	TNF/CHP nanoparticles	CTB	Unimmunized	None	TNF/CHP nanoparticles	CTB
1 day before boost								
Day 90	n.d.	21,338 ± 2,438.9	16,917 ± 9,392.0	8,700 ± 5,510.3	n.d.	4.2 ± 3.5	14.4 ± 16.7	13.1 ± 10.8
12 days after boost								
Day 112	36.6 ± 43.2	19,764 ± 13,601.	53,769 ± 29,893.7	37,729 ± 29,281.	34.6 ± 33.3	19.8 ± 8.3	64.4 ± 63.4	58.2 ± 37.1
		2		3				

BALB/c mice were nasally administered with IVV (0.3  $\mu$ g/mouse), with or without TNF/CHP nanoparticles (5  $\mu$ g/mouse of TNF- $\alpha$ ) or CTB (0.8  $\mu$ g/mouse) once a week for 4 weeks. Ninety-one days after the immunization, the mice were boosted with IVV (0.9  $\mu$ g/mouse). Twenty-one days after the boosting challenge, the blood plasma was prepared and the feces were extracted with 10 volumes of water. The IgG<sub>1</sub> in blood plasma and IgA in feces extract were examined (mean  $\pm$  SEM,  $n$  = 8).  
n.d., not detected.

**Table 1.** Induction of immunological memory by TNF/CHP nanoparticles.

Muraoka et al. proposed that CHP-based nanoparticles preferentially deliver antigen to antigen-presenting cells in the lymph nodes, which potentiates effective immune responses [38]. This might be the reason why the TNF/CHP nanoparticles induced excellent protective immunity. They, however, reported that CHP itself did not show an adjuvant effect in the context of a tumor vaccine [31]. Interestingly, CHP only (without TNF- $\alpha$ ) showed a certain level of protective efficacy in our study. The reason for the discrepancy between their results and ours is not clear. It is unlikely, given the short time frame, that TNF- $\alpha$  was replaced *in vivo* by IVV antigens to form IVV/CHP nanoparticles that were then delivered to the lymph node. Another factor that is critical for adjuvant activity is particle size [39]; however, size is not an issue in our case since the DLS analyses showed no difference in particle size between the TNF/CHP nanoparticles and empty CHP particles (**Figure 4**). Most probably, the difference has to do with varying mechanisms to elicit protection against external pathogens, such as influenza virus, and internal antigens, such as tumor antigen.

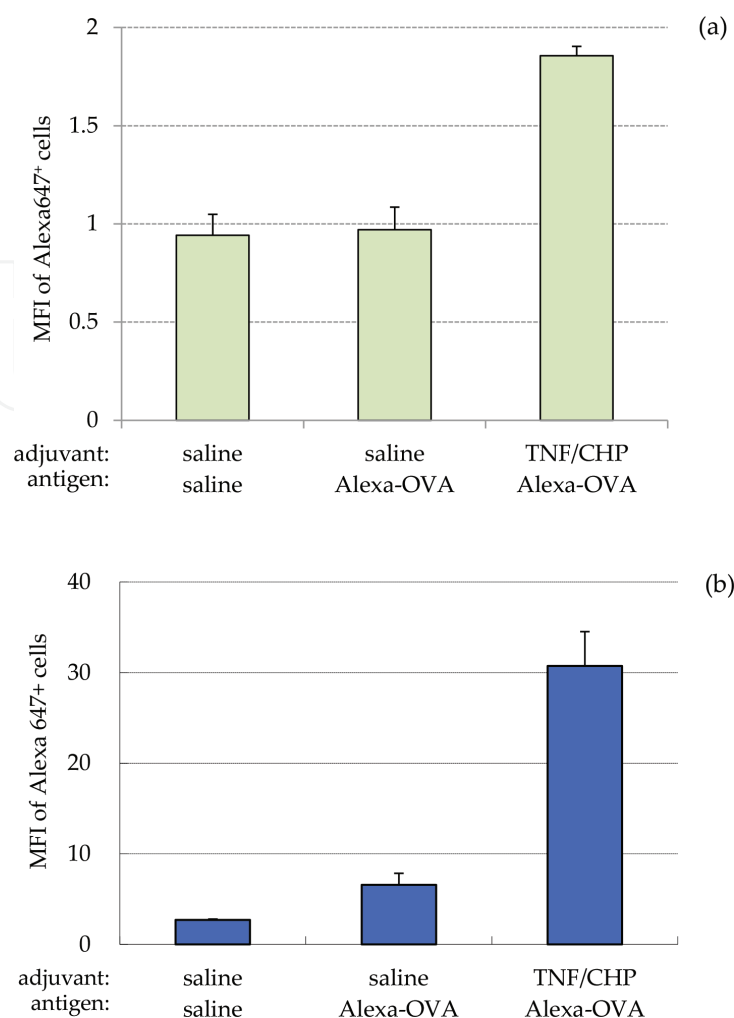
## 7. Mechanistic analyses of effects of TNF/CHP nanoparticles

### 7.1. Activation of immune cells in NALT

Being focused on the nasal route of vaccination, we examined immune cells in nasal tissues after immunization. The mucosal surfaces contain abundant B cells, T cells, and plasma (or DC) cells. After repeated immunization of animals for 3 weeks, cells from the nasal-associated lymphoid tissues (NALT) were prepared from mice, and expression of a surface marker for DCs (CD11c) and activation markers for B cells (CD80 and CD86) were examined by flow cytometry. The ratios of CD80<sup>+</sup>/CD11c<sup>+</sup> cells; CD86<sup>+</sup>/CD11c<sup>+</sup> cells were 0.20; 0.33, 0.30; 0.44, and 0.33; 0.50% for saline, IVV, and IVV with TNF/CHP nanoparticles, respectively (data not shown). Even though the degree was small, IVV vaccination with or without TNF/CHP nanoparticles activated DCs and B cells. Also, TNF/CHP nanoparticles did not have a prominent effect on DC or B cell activation.

### 7.2. Antigen uptake and activation of NALT and nasal passage cells

We next focused on the early immune response of nasal mucosal tissues. Uptake of antigen by the mucosal tissues is essential for the induction of immune responses [40]. Therefore, we examined antigen uptake by NALT-resident and nasal passage DCs, the inductive sites of the common mucosal immune system [41]. In these experiments, we used ovalbumin (OVA) as a model antigen and assessed antigen uptake by DCs in the NALT and nasal passage cells by flow cytometry at 6 h after immunization. Immunization of mice with OVA combined with TNF/CHP nanoparticle activated antigen uptake by both NALT and nasal passage DCs. TNF/CHP nanoparticles stimulated DCs most in the nasal passage mucosal immune tissue (**Figure 9**). We also found that TNF/CHP nanoparticles enhanced expression of DC and B cell activation markers (CD40, 80, and 86) in a bone marrow-derived immature DC preparation *in vitro* (data not shown).



**Figure 9.** Antigen uptake of NALT and nasal passage DCs after TNF/CHP nanoparticles administration. BALB/c mice were nasally immunized with 10  $\mu$ g of Alexa 647-labeled OVA antigen with or without TNF/CHP nanoparticles as an adjuvant. Six hours after the immunization the NALT and the nasal passage cells were prepared [42] and subjected to flow cytometric analysis. Antigen uptake was determined by detecting the Alexa 647 fluorescence intensity and the DC marker (CD11c<sup>+</sup> cells) in parallel, for example, ratio of Alexa 647<sup>+</sup>/CD11c<sup>+</sup>. (a) NALT DCs, (b) nasal passage DCs. MFI, mean fluorescence intensity (mean  $\pm$  SD, n = 4). Adapted with permission from Nagatomo D. et al. [33].

### 7.3. Expression of inflammatory signals in NALT

Vaccine adjuvants trigger the innate immune system allowing enhanced humoral and cellular responses against the co-administered vaccine antigens. To understand innate immune system activation caused by the TNF/CHP nanoparticles after immunization, we conducted gene expression profiling in NALT cells 2, 6, and 26 h after nasal administration of IVV antigen with or without the nanoparticles. By scattering analyses, the gene expression of inflammation- and immunity-related molecules was found to be significantly upregulated (data not shown). These included the triggering receptor expressed on myeloid cells 1, fibronectin 1, CD14, Toll-like receptor (TLR) 2, TLR3, IL-1 $\beta$ , IL-1 family 9, and IL-6. We confirmed the level of inflammation-related molecules in activated NALT cells by quantitative polymerase chain reaction (PCR) analysis. Expression of the inflammatory markers was enhanced when an adjuvant was included (TNF/CHP nanoparticles, free TNF, or CTB), while CHP itself did not show significant



enhancing activity. Among the molecules tested, significant increases in expression occurred for IFN- $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, CXCL2, IL-12 $\beta$ , CD14, and lipopolysaccharide (LPS)-binding protein (**Figure 10**). The degree of enhancement varied from gene to gene, but the greatest increase of expression was observed for IL-6 and IL-12 $\beta$ .

Overall, free exogenous TNF- $\alpha$  elicited the strongest increase in expression of inflammatory markers, and the enhancement tended to be highest at 2 h post-immunization. TNF/CHP nanoparticles elicited a moderate increase in gene expression by comparison, but the pattern over time was similar to that of free TNF- $\alpha$ . One reason for the discrepancy in gene expression between free TNF and the nanoparticles could be that the nanoparticles cause a slow release of TNF- $\alpha$ , prolonging the immune-stimulatory effect. The pattern of expression over time was very different for most genes when stimulated with CTB adjuvant. For example, although the expression of IL-12 $\beta$  was prominent at 6 h post-immunization when CTB was used as an adjuvant, the IL-12 $\beta$  response was much lower with TNF/CHP nanoparticles at the same time point.

Taken together, TNF/CHP nanoparticles delayed activation of innate immunity. By prolonging and dampening the stimulatory effect of TNF- $\alpha$  CHP seemed to minimize the unfavorable effects of TNF- $\alpha$  while promoting its beneficial activities. One important safety issue related to the development of nasal vaccines is the potential dissemination of vaccine antigens to the central nervous system (CNS). Past reports suggested that nasal administration of CTB allowed it to reach the CNS and accumulate in olfactory tissues. It caused Bell's Palsy in clinical studies, probably due to IL-12 $\beta$  production, and the use of CTB in humans was prohibited [43, 44]. In this context, the lower expression level of IL-12 $\beta$  seen in our experiments with TNF/CHP nanoparticles could be a beneficial safety feature.

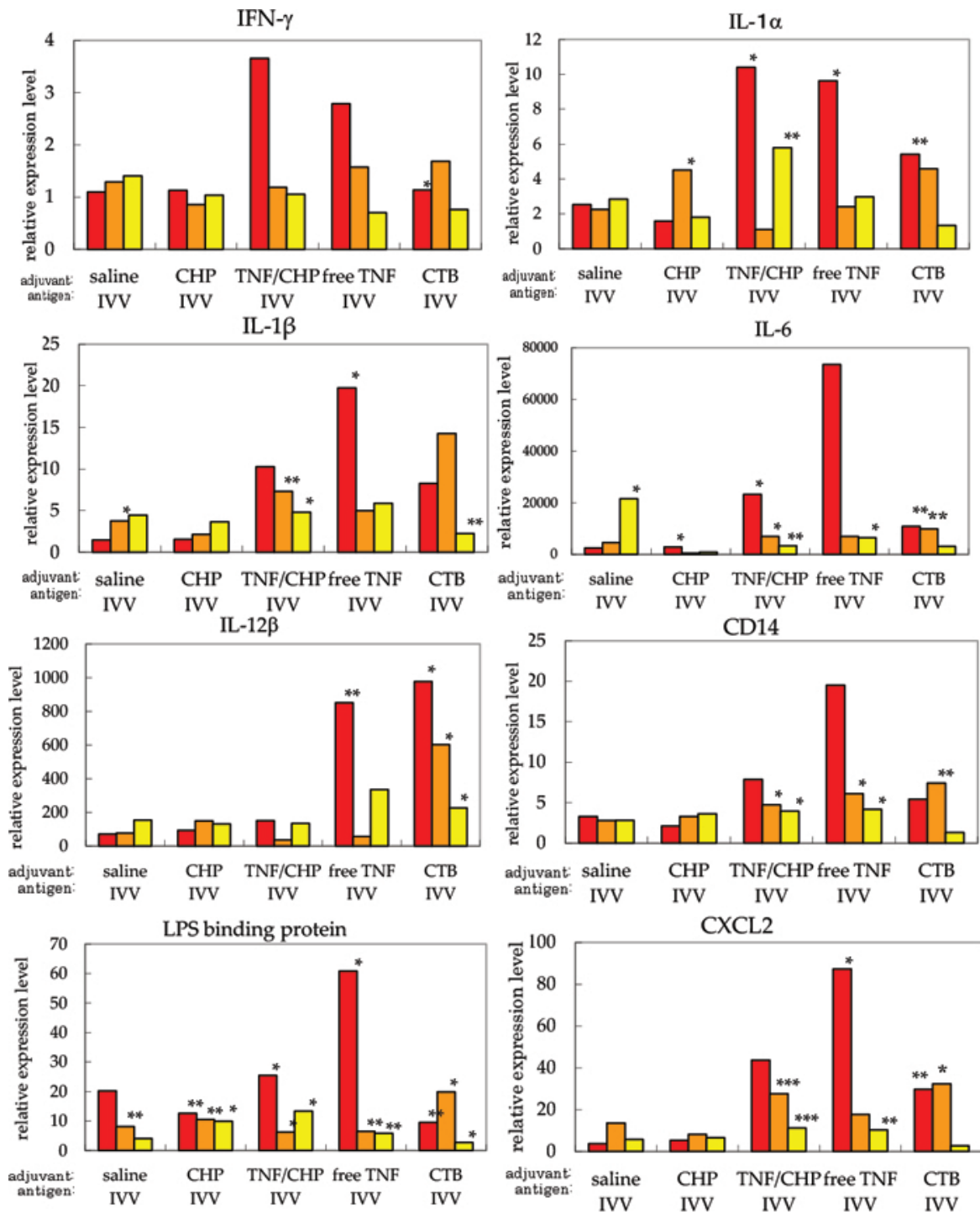
Regarding CHP itself, a shell of the nanoparticles did not show immune-enhancing activity, such as increasing IgG<sub>1</sub> and IgA or the expression of inflammation-related genes (**Figure 10**). However, CHP did confer a certain level of protection in a lethal influenza virus challenge (**Figure 8**). We cannot easily account for these conflicting observations; there are other pathways and mechanisms likely involved and waiting to be clarified.

## 8. Safety study of TNF/CHP nanoparticles

General safety was preliminarily examined according to the OECD guidelines for testing chemicals [45]. Mice nasally administered with a combination of TNF/CHP nanoparticles and IVV either once or four times were subjected to an acute or a repeated toxicity study, respectively.

### 8.1. General symptoms, ophthalmic examination, body weight, and body temperature

No general symptoms or behavioral anomalies in either male or female animals were correlated with the IVV with TNF/CHP nanoparticles treatment during the study period. In ophthalmic examinations, there were no test material-related ocular findings observed. Body



**Figure 10.** Gene expression in NALT after TNF/CHP nanoparticles administration. mRNA was prepared from NALT cells of BALB/c mice 2, 6, and 26 h after administration of TNF/CHP nanoparticles and IVV. Gene expression related to innate and adaptive immune responses was analyzed by quantitative PCR and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Data are shown as relative level vs. control (means of quadruple experiments). Red column, 2 h; orange column, 6 h; yellow column, 26 h post treatment. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  vs. saline control. Adapted with permission from Nagatomo D. et al. [33].

weight and body temperature were not statistically different among the treatment groups (data not shown).

## 8.2. Hematology, blood biochemistry, and urinalysis

Hematology evaluations were performed during and at the end of study. There were no differences in any of the tested parameters (white blood cell, red blood cell, hematocrit, lymphocyte, neutrophil, eosinophil, basophil, and monocyte) between controls and TNF/CHP nanoparticles with the IVV treatment. For blood biochemistry, we examined total protein, albumin, urea nitrogen, creatinine,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$ , inorganic phosphate, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), amylase (AMY),  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT), total cholesterol, triglyceride, high density lipoprotein (HDL)-cholesterol, total bilirubin, and glucose. Urobilinogen, bilirubin, ketone body, glucose, protein, pH, specific gravity, nitrite salt, and leucocytes were examined during the study period. All differences found during the study fell within historical control value ranges and were not considered test material-related (data not shown).

## 8.3. Pathology and major organ weights

Gross pathology of all the animals was examined at the end of the study. The major organs (brain, heart, lung, kidney, liver, ovary, testis, spleen, adrenal, and thymus) were weighed at the end of the study in all of the animals. No change was noted as test material-related (data not shown).

## 8.4. Histopathology

The histopathology of tissues from animals in each group was examined. As discussed in Section 7.3, there were some concerns of possible harmful effects on the CNS. However, no abnormalities in the brain, especially olfactory bulb, were noted in any animals after histological examination. There were some effects observed at the administration site (nasal mucosal tissue). While single administration showed no effect, repeated administration with IVV alone showed a slight infusion and TNF/CHP nanoparticles combined with IVV induced slight-to-moderate infusion and infiltration of inflammatory cells (lymphocytes, neutrophils, eosinophils, and mast cells). However, the response was reversed with time since the infusion diminished to trace proportions after the 2-week cessation period. No excessive inflammatory symptoms, such as formation of edema or fibrosis, were noted (data not shown).

Overall, no obvious immunotoxicity was detected. Although further evaluation is required, our results demonstrate that the toxicity of TNF/CHP nanoparticles is relatively low and safe as a nasal vaccine adjuvant against influenza.

Very recently, Onishi et al. reported that hydroxypropyl- $\beta$ -cyclodextrin (HP- $\beta$ -CD), another type of saccharide-based material that can form nanoparticles, exhibited adjuvant activity and elicited a strong protective effect against influenza virus in mice and cynomolgus macaques [46]. They suggested the involvement of follicular helper T cells via myeloid differentiation primary gene 88 (MyD88)- and TANK-binding kinase (TBK)-dependent pathways. Their findings may shed some light on additional mechanisms at play with nanoparticles as vaccine adjuvants. However, they mentioned the cytotoxicity of HP- $\beta$ -CD at more than 0.5% *in vitro*,

probably because of  $\beta$ -CD's ability to extract cholesterol out of cell membranes [47]. TNF/CHP nanoparticles might represent a preferable alternative.

A probable precaution for the practical use of TNF/CHP nanoparticles is avoiding contact with high concentrations of dissolved proteins as mentioned in Section 4.2. Unless, encapsulated TNF- $\alpha$  would be released from the nanoparticles and the adjuvant activity would be diminished. This means that the nanoparticles is not suitable for *i.v.* administration and premixed formulation with vaccine antigen. For the best performance, we recommend to administer the TNF/CHP nanoparticles on mucosa, such as nasal surface, after mixing with the vaccine antigen just before use.

## 9. Conclusions

The results of this study demonstrate that TNF/CHP nanoparticles are effective as a vaccine adjuvant against influenza when administered via the nasal mucosal route. Moreover, the ability of TNF/CHP nanoparticles to stimulate comparatively balanced systemic and mucosal immune responses makes them a potentially promising vaccine adjuvant for inducing immunity against infectious pathogens. In the short term, TNF/CHP nanoparticles may aid the development of new nasal influenza vaccines. Looking further ahead, we propose that combining TNF/CHP nanoparticles with next-generation vaccine platforms that do not rely on the cold chain will offer valuable alternatives for vaccination in a variety of settings.

## Acknowledgements

The author is very grateful to Dr. Ayato Takada of Research Center for Zoonosis Control, Hokkaido University for his expertise with influenza challenge of mice. The author is also thankful to the staff of R&D Center, Hayashibara Co., Ltd. for their excellent technical assistance, especially Mr. Daiki Nagatomo and Ms. Madoka Taniai for the mechanistic analyses of action, and Dr. Shigeyuki Arai for the histopathological examination.

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