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Prophylactic Vaccine Targeting TLR3 on Dendritic Cells Ameliorates Eosinophilic Pneumonia in a Mouse SARS-CoV Infection Model

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ABSTRACT
Putative subcomponent vaccines of severe acute respiratory syndrome coronavirus spike protein and ARNAX (TLR3-specific adjuvant for priming dendritic cells) were examined and compared with spike protein + Alum in a mouse BALB/c model. Survival, body weight, virus-neutralizing Ab titer in the blood, and viral titer in the lung were evaluated for prognosis markers. The infiltration degrees of eosinophils in the lung were histopathologically monitored at 10 d postinfection. The results were: (1) adjuvant was essential in vaccines to achieve a complete recovery from infection, (2) ARNAX displayed optimal body weight recovery compared with Alum, (3) ARNAX was optimal for the amelioration of eosinophilic pneumonia, and (4) the eosinophil infiltration score was not associated with the neutralizing Ab titer in the blood or viral titer in the lung. Although the pathological link between the TLR3 vaccine and lung eosinophil infiltration remains unclear, severe acute respiratory syndrome–mediated eosinophilic pneumonia can be blocked by the prior induction of dendritic cell priming by ARNAX. ImmunoHorizons, 2022, 6: 275–282.

INTRODUCTION
Severe acute respiratory syndrome (SARS) is a viral respiratory disease caused by SARS-coronavirus (CoV) infection (1, 2). SARS-CoV has been found to have originated from cave-dwelling horseshoe bats and led to the 2002–2004 SARS outbreak. Middle East respiratory syndrome CoV also induces severe pneumonia in humans (3). Although there has been no report of a worldwide SARS-CoV outbreak, SARS-CoV-2 caused the emergence of the coronavirus disease 2019 (COVID-19) pandemic. SARS-CoV shares ~80% sequence homology with SARS-CoV-2 (4), which emerged in late 2019 in Wuhan, China, and continues to cause outbreaks throughout the world. Vaccines against whole virus particles have been effective by promoting the generation of neutralizing Abs that recognize the SARS-CoV-2 S protein for protection (5). In addition, mRNA vaccines have been distributed throughout the world after short (<1 y) safety testing, and efficacy has been evaluated through mass vaccination. Although the mRNA vaccines have been favorably evaluated for their prophylactic ability, adverse events occur at
relatively high frequency, particularly in elderly individuals (6, 7). Until recently, no subcomponent vaccine consisting of both an Ag and adjuvant with a high safety profile has been developed for prevention of SARS infections (8), although subcomponent vaccine has a well-established safety profile.

Particularly challenging is the host response to viral infection being hindered by vaccination, which is rooted in the disruption of the ability of the human immune system to recognize Ag (e.g., S protein), presumably in dendritic cells (DCs). Typical antiviral responses are normally suppressed by infection with viruses, which possess a variety of immune-circumventing strategies that allow for viral spread. Moreover, vaccines should be designed to establish safe and effective protective immunity against SARS viruses. Recent understanding of the immune system suggests that both innate and acquired immune responses participate in the response to antiviral vaccines (9). The most problematic is the outcome of vaccination, which enhances inflammation and sometimes exacerbates viral diseases (10). Even if vaccines can prevent infection, they may cause a higher risk for adverse events through inducing inflammation, which can occasionally lead to life-threatening pneumonia. Lung pathology in patients exhibited an unexpected inflammatory response characterized by neutrophils and eosinophils, as well as immune-complex formation and complement activation in small airways.

Successive reports suggest that exceeding the innate response to viral infection occurs in vaccinated mice, resulting in cytokinemia (11). Vaccination can also occasionally induce vaccine-associated disease enhancement (VDE) (12). Although the mechanism of VDE is immunologically complex and remains etiologically undefined, antigenic sin and Th2 skewing in the acquired immune system appear to be involved in evoking VDE (13). The reason for the occurrence of eosinophilia in response to SARS-CoV postvaccination should also be investigated further. In addition, the relationship between the antiviral innate response and the Th2 skewing in SARS-CoV infection has been examined in postvaccinated BALB/c mice (14, 15). This mouse model employs a subcomponent vaccine, which does not always mimic human vaccination, and suggests that eosinophilia can be induced in S Ag-immunized BALB/c mice in response to SARS-CoV infection. This is because BALB/c mice possess a Th2 background, unlike black mice (e.g., C57BL/6).

In our mouse model of SARS-CoV, we demonstrate the importance of TLR agonists in vaccination for reverting the immune system back to a normal state (14). In this article, we further show that the TLR3 agonist ARNAX, which directly targets TLR3 in DCs, can sufficiently establish a Th1 shift and induce Ab production in response to s.c. vaccination with the SARS S protein. In addition, administration of the vaccine allowed the mice to recover from illness by fully inducing effective immunity against challenge with SARS-CoV infection.

**MATERIALS AND METHODS**

**Ethics statement**

All experiments involving recombinant DNA and pathogens were approved by the Committee for Experiments using Recombinant DNA and Pathogens at the National Institute of Infectious Diseases, Tokyo, Japan. The animal studies were carried out in strict accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. The animal experiments were conducted in strict compliance with animal husbandry and welfare regulations. All animals were housed in a Japan Health Sciences Foundation–certified facility. All animal experiments were approved by the Committee on Experimental Animals at the National Institute of Infectious Diseases in Japan (approval no. 120013), and all experimental animals were handled in biosafety level 3 animal facilities according to the guidelines of this committee (approval no. 20-03).

**Cells and viruses**

Vero E6 cells, derived from African green monkey kidney (ATCC No. CRL-1586; American Type Cell Collection, Manassas, VA), were cultured in Eagle’s MEM (Sigma-Aldrich Japan) containing 5% FBS (Sigma-Aldrich Japan), 50 IU/ml penicillin G, and 50 μg/ml streptomycin (5% FBS-MEM; Thermo Fisher Scientific). Stocks of a mouse-passaged Frankfurt 1 isolate of SARS-CoV, F-musX-VeroE6, were propagated twice and titrated on Vero E6 cells before cryopreservation at −80°C, as described previously. The infection dose of the virus was 3 × 10⁶ 50% tissue culture infectious dose (TCID₅₀) in 30 μl. Viral infectivity titers are expressed as the 50% TCID₅₀/ml on Vero E6 cells and were calculated according to the Behrens–Kärber method. All work with infectious SARS-CoV was performed under biosafety level 3 conditions.

**Immunogen (recombinant SARS S protein) and immunized mice**

Recombinant SARS-CoV spike (S) protein with tag (Strept-8-xHis) was used as immunogen to investigate the adjuvant effect of ARNAX120. The purified S protein was prepared using a baculovirus expression system as described previously (15, 16). The presence and size of the purified S protein were verified by western blotting as previously described (15). The predicted molecular mass of the recombinant S protein was 135 kDa. The immunogenicity of the purified S protein was assessed as previously described (15), and the dose used was 0.1 μg per mouse in this study.

To evaluate the adjuvant effects of ARNAX120 on the vaccine Ab response, we formulated the purified S protein with 3 or 10 μg of ARNAX120 in a total volume of 100 μl of PBS. BALB/c mice (female, 12–14 wk old [Japan SLC, Shizuoka, Japan]; n = 6–10; total, 16) were immunized s.c. twice at 2-wk intervals. Control mice were injected s.c. with PBS or without the S protein in 100 μl volume, and i.m. with the S protein with 1 mg of Alum (Thermo Fisher Scientific, MA) in
50 µl volume on the right thigh twice at 2-wk intervals (female, 12–14 wk old [Japan SLC]; n = 10; total, 30). Two weeks after each immunization, serum samples were collected from all mice for measurement of the Ab response.

**Virus infection of immunized mice**
Approximately 3 wk after the second immunization, the mice were anesthetized via i.p. injection of a mixture of 1.0 mg ketamine (Daichi Sankyo Company, Tokyo, Japan) and 0.02 mg xylazine (0.08 ml/10 g body weight [BW]; Byer Japan, Osaka, Japan). These mice were then inoculated intranasally with SARS-CoV (10⁶ TCID₅₀ in 30 µl of 2% FBS-MEM). The infected mice were then observed for clinical signs of infection, and their BW was measured daily for 10 d (n = 3–6 mice; total, 27 immunized mice). To analyze viral replication, we killed the animals at 3 d after inoculation (n = 3–4 mice per group; total, 19). The humane endpoint was defined as the appearance of clinically diagnostic signs of disease, including respiratory distress, ruffled fur, and weight loss of >25%. Animals were euthanized under anesthesia with an overdose of isoflurane if severe disease symptoms or weight loss was observed. Mice that survived until day 10 after challenge were euthanized for preparing lung tissue sections for histopathological examination.

**Virus titration**
Lung tissue homogenates (10%, w/v) were prepared in 2% FBS-MEM. The samples were clarified via centrifugation at 740 × g for 20 min, and the supernatant was inoculated onto Vero E6 cell cultures for virus titration.

**Neutralizing Ab test**
Serum samples were 2-fold diluted over a range of 1:4 to 1:256 in 2% FBS-MEM. Each sample was mixed with virus solution (F-musX-VeroE6 of 100 TCID₅₀ per well), and the mixtures were incubated for 1 h at 37°C for neutralization. After incubation, the mixtures were inoculated onto monolayers of VeroE6 cells in 96-well culture plates, followed by incubation at 37°C for 3 d. The cells were then examined for cytopathic effects. The sera titers of neutralizing Abs were calculated as the reciprocal of the highest dilution at which no cytopathic effects were observed. The method was originally reported in Saijo et al. (17).

**Histopathology and immunohistochemistry**
Mice were anesthetized and perfused with 2 ml of 10% phosphate-buffered formalin (Wako, Tokyo, Japan). The lungs were harvested, fixed, embedded in paraffin, sectioned, and stained with H&E. Eosinophils were identified via Astra Blue/Vital New Red staining, a combined eosinophil/mast cell stain (C.E.M. Stain Kit; DBS, Pleasanton, CA). Using the Astra Blue/Vital New Red-stained slides, the peribronchiolar area in five 147,000-µm² sections was assessed by light microscopy using a DP71 digital camera and cellSens software (Olympus, Tokyo, Japan), and the numbers of eosinophils counted in the lungs of each mouse were averaged as described previously.

**Statistical analysis**
Data are expressed as the mean and SEM. The statistical analyses were performed using Graph Pad Prism 9 software (GraphPad Software, La Jolla, CA). Virus titers, the neutralizing Ab titer assays, and eosinophil counts results were analyzed using nonparametric tests, that is, Dunn’s multiple comparisons test following the Kruskal–Wallis test. A p value <0.05 was considered statistically significant.

**RESULTS**
BALB/c female mice aged 11 wk were housed under the specific pathogen-free conditions and immunized with 0.1 µg of S protein ectodomain with or without adjuvant (15). The S protein ectodomain (without the transmembrane region) was cloned from SARS-CoV as described previously (14). We employed Alum (1 mg) or ARNAX (two doses, 3 and 10 µg). As an adjuvant, ARNAX has been defined as a TLR3-specific agonist and exclusively targets Ag-presenting DCs (CD8α⁺ or CD103⁺ DCs in mouse or CD141⁺ DCs in human) (16, 17). The Ag and adjuvant were mixed and simultaneously administered either s.c. or i.m. to the mice (Fig. 1). The mice were immunized twice at 2-week intervals. To verify the neutralizing Ab titers against the S protein, we collected blood three times from the tail vein of the mice as indicated in Fig. 1. Three weeks after the last immunization, the mice were challenged with SARS-CoV (3 × 10⁶ TCID₅₀/30 µl = 1000 LD₅₀). Ten days later, the

**FIGURE 1. The experimental protocol of this study.**
Numbers of mice in each group are shown in the inset table. Small-scale preliminary experiments were performed to determine the conditions of this study.
FIGURE 2. ARNAX adjuvant shows some advantages compared with Alum in vaccination. Adjuvant efficacy and lung histopathology in mice immunized with recombinant S protein with ARNAX120. Female BALB/c mice were vaccinated with each set of Ag/adjuvant. Mice immunized with 0.1 μg S protein with or without adjuvant were challenged with 10⁶ TCID₅₀ of mouse-adapted SARS-CoV (n = 6–10). (A) Serum neutralizing Ab titers after the second immunization. The line indicates the limit of detection (<4). (Continued)
mice were sacrificed to test the levels of eosinophil infiltration in the lung.

The neutralizing Ab titers were found to be increased in mice with S protein Ag + adjuvant, but not in those that received S protein Ag only (Fig. 2A). Thus, adjuvant was absolutely required for the induction of neutralizing Abs against S protein Ag, regardless of the source. Alum appeared to induce significantly higher titers of Abs than ARNAX (Fig. 2A). Ten micrograms of ARNAX almost works equivalent to 1 mg of Alum judging by the level of the neutralizing Ab.

The unvaccinated control mice all died within 5 d postinfection (Fig. 2B, 2C). One mouse in the Ag-only group died at 6 d postinfection. All mice in Ag + adjuvant groups survived >10 d postinfection. The BW of the mice in each group was monitored (Fig. 2B). The BW severely dropped around 3 d postinfection in all groups tested with Ag + adjuvant and gradually recovered. The BW recovery was rapid (within 6 d) in the Ag/ARNAX groups compared with those of the Ag/Alum group (Fig. 2B). All mice subjected to ARNAX sustained <25% decrease of BW during the course of infection.

Viral titers in the lung were measured on day 3 postinfection (Fig. 1). The titer was similar in the control and Ag-only mice, whereas the titer exhibited the highest reduction in the mice treated with Ag/Alum. The titer was moderately reduced in the mice treated with Ag/10 μg ARNAX, while no significant reduction was observed in the Ag/3 μg ARNAX group (Fig. 2D). These results suggest that the loss of BW does not always reflect the viral titers in lung or Ab titers in the blood.

Eosinophil infiltration into the lung was counted in mice sacrificed on day 10 postinfection. The degree of infiltration was scored according to a one-way ANOVA followed by Tukey’s test (Fig. 2E, 2F). High levels of eosinophil infiltration were observed in the Ag-only group and Ag/Alum group, compared with low levels in the Ag/ARNAX groups (Fig. 2E, 2F). Representative histopathological features are shown in Fig. 2E. The histology revealed that eosinophils appeared to be retained in the stroma around the pulmonary vein of the lung in the Ag/ARNAX group; however, they infiltrated into alveolar tissue in both the Ag/Alum and control groups (Fig. 2E).

In addition, some plasma cells were found in the stromal regions of mice treated with ARNAX. The 10 μg dose of ARNAX appeared to be more effective than that of 3 μg in the regimen. The histological features were confirmed with a high magnification in terms of the alveolar regions in the same specimens obtained from the mice treated with Alum (1 mg) or ARNAX (10 μg) (Fig. 3). Alveolar collapse was prominent in the Alum group compared with the ARNAX group (Fig. 3A). Alveolar infiltration of eosinophils was observed in the Alum group but barely in the ARNAX group (Fig. 3B). The results further suggest that the S protein/ARNAX subcomponent vaccine improves eosinophilic pneumonia irrespective of the neutralizing Ab titers in the blood or viral titers in the lung in vaccinated mice.

**DISCUSSION**

In this study, we demonstrated that vaccination with S protein and TLR3-specific adjuvant ARNAX could overcome the eosinophilic pneumonia resulting from SARS-CoV infection. In comparison with the results achieved with Alum, ARNAX was more responsible for amelioration of eosinophilic pneumonia in BALB/c mice. In this example, the adjuvant formulation in the vaccine governs the incidence of eosinophilia in the lung and is profoundly associated with the nature of the subcomponent adjuvant. Neither the relative levels of neutralizing Ab in the blood nor viral titers in the lung are involved in the lung pathogenesis in vaccinated mice. ARNAX is known to endow Th1 polarization to host in response to Ags (18). A rational explanation as to the mechanism by which vaccination affects eosinophilic SARS-CoV infection remains undetermined; however, the vaccine with a Th1 adjuvant helps BALB/c mice recover from severe SARS-CoV infection. Moreover, ARNAX targets TLR3 in DCs (18), which can be associated with vaccine-induced DC maturation with Th1 skewing. Although precise factors that assist in the onset of SARS-CoV eosinophilic pneumonia are intriguing, DC maturation via TLR3 is a factor that critically participates in the suppression of pulmonary eosinophil infiltration.

Eotaxin (CCL11) expedites the ability to attract eosinophils to the infected tissue. The levels of eotaxin appeared high in infected lungs when the S protein alone or S protein + Alum were administered as vaccines in mice (15). Yet, the levels of eotaxin were low in the lungs of mice administered with TLR agonists (15), suggesting that the TLR stimulation is involved in the regulation of eotaxin release. However, it remains unknown which TLR signal plays a part in circumventing eosinophilic pneumonia in SARS-infected mice. Our previous administration of TLR stimulants was composed of a mixture consisting of

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ARNAX VACCINE FOR SARS PREVENTION

Poly(I:C) can activate TLR3 in DCs and serves as an adjuvant similar to ARNAX, but induces robust cytokinemia (19, 21). This can be explained based on the fact that poly(I:C) stimulates the cytoplasmic RNA sensors, including RIG-I/MAVS, and RIG-I/MAVS in turn activates the MAVS pathway in whole-body cells similar to systemic virus infection (22, 23). ARNAX has a 5’-DNA cap that supports the effective incorporation of the ARNAX dsRNA into DC endosomes, where TLR3 is highly expressed (19). This is because poly(I:C), but not ARNAX, induces systemic activation of the MAVS pathway similar to those observed in virus infections (19–21). ARNAX activates only the TICAM-1 (Toll/IL-1R domain-containing adapter inducing IFN-β) pathway in DCs without significant increases of the levels of blood cytokines and IFNs (19–21, 23). Furthermore, TICAM-1 signaling, rather than MAVS signaling, can sufficiently upregulate DC cross-priming without inflammation (23). This may represent one reason explaining the difference in adverse events between poly(I:C) and ARNAX in the systemic response.

In the previous reports on cancer immunotherapy combined with ARNAX, this TLR3 agonist has been shown to specifically stimulate DC priming into mature DCs with the capacity for cross-presentation (19, 23). IFN-β and IL-12 derived from localized DCs participates in autocrine activation of the DCs and activation of T cells, respectively (23–25). In addition, Ag-specific CD8+ T cells proliferate in response to DC priming by ARNAX in tumor-bearing mice, resulting in tumor shrinkage (19, 23–25). This scenario rationally explains the previous reports on the importance of CTL in respiratory syncytial virus infection (26). This unanticipated event was first reported in the early 1960s during the trials with an inactivated respiratory syncytial virus vaccine (27). However, it has been further reported that the induction of Ag-specific CTLs is not mandatory in protection against SARS-CoV-2 infection (28). Furthermore, VDE may represent another reason why the vaccine provides poor protection against viral infection (28, 29). Immune aberrance caused by SARS is currently being addressed based on the clinical studies on COVID-19 (30, 31).

ARNAX-mediated Th1 polarization through DCs presumably induces CD4+ T cell help for Ab production in B cells. The neutralizing Ab titers of host were low in the ARNAX + Ag group compared with the Alum + Ag group in SARS-CoV–infected mice 3 d after immunization. Abs usually recognize the tertiary structures of Ag. Editing of the V region plays a role in antigenic sin and may cause VDE (4). This would be true in SARS-CoV-2–infected mice we recently reported using a lethal model (32).

Our previous results using an intranasal model suggested that prestimulation of innate signaling through TLR3/TICAM-1 in mucosal CD103+ DCs induced DC-dependent switch recombination for IgA production in C57BL/6 mice (21, 33); however, what happens for Ab production in BALB/c mice with s.c. ARNAX injection remained untested. Thus, the present results infer that ARNAX works well as adjuvant irrespective of the vaccination route. The results are consistent with the
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