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Enhanced potency of replicon vaccine using one vector to simultaneously co-express antigen and interleukin-4 molecular adjuvant

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We evaluated the utility of interleukin-4 (IL-4) as molecular adjuvant of replicon vaccines for botulinum neurotoxin serotype A (BoNT/A) in mouse model. In both Balb/c and C57/BL6 mice that received the plasmid DNA replicon vaccines derived from Semliki Forest virus (SFV) encoding the *Hc* gene of BoNT/A (*AHc*), the immunogenicity was significantly modulated and enhanced by co-delivery or co-express of the IL-4 molecular adjuvant. The enhanced potencies were also produced by co-delivery or co-expression of the IL-4 molecular adjuvant in mice immunized with the recombinant SFV replicon particles (VRP) vaccines. In particular, when AHc and IL-4 were co-expressed within the same replicon vaccine vector using dual-expression or bicistronic IRES, the anti-AHc antibody titers, serum neutralization titers and survival rates of immunized mice after challenged with BoNT/A were significantly increased. These results indicate IL-4 is an effective Th2-type adjuvant for the replicon vaccines in both strain mice, and the co-expression replicon vaccines described here may be an excellent candidate for further vaccine development in other animals or humans. Thus, we described a strategy to design and develop efficient vaccines against BoNT/A or other pathogens using one replicon vector to simultaneously co-express antigen and molecular adjuvant.

Introduction

The botulinum neurotoxins (BoNTs) are the most toxic proteins and can be classed into seven serotypes (A-G) which own similar structures but are antigenically distinct. Human botulisms are generally associated with botulinum neurotoxin serotypes A, B, E and F, of which serotype A is the most poisonous.[1,2] Botulisms can be effectively prevented by the presence of neutralizing antibodies against the BoNTs induced by immunization. The most commonly available vaccines for human are formalin-inactivated pentavalent toxoid (PBT). However, it has met with some drawbacks, including feasibility (neurotoxin yields from C. botulinum are relative low), danger associated with handling neurotoxins and secondary complications due to residual formaldehyde contamination.[3,4] To resolve the obstacles of the toxoid vaccines, improved vaccines designed to prevent botulism are urgently demanded. A feasible vaccine-design strategy based on the expression of non-toxic recombinant BoNT proteins in *Escherichia coli* or *Pichia pastoris* has currently been studied and developed.[4-7] DNA vaccines encoding the Hc domains of BoNTs have been developed as next generation botulinum candidate vaccines.[4,8-13] Candidate vaccines against BoNTs were also developed by using replicon vector.[13-15]

A number of studies have proved that DNA immunization can elicit antigen-specific antibody and cell-mediated responses to a variety of bacterial, viral, parasitic and tumor antigens and afford protective potency against these pathogens in various animal models.[16,17] And because DNA vaccines have a number of potential advantages such as simplicity of manufacture, high purity of product and ease of storage, DNA immunization has become an important direction of vaccine research and development. DNA vaccines against BoNTs induce protective humoral immune responses in mouse model, but when compared with conventional vaccines such as toxoid and subunit protein vaccines, DNA vaccines usually induce lower antibody level and protective efficacy and are still necessary to improve their potency for human use. A number of strategies have been investigated to increase the immunogenicity of DNA vaccines over the last few years, ranging from adjuvants, electroporation, cytokines, chemokines, CpG, viral replicon vector, liposomes to microparticles.[17-21]

In previous study, we indicated that plasmid DNA replicon vaccines encoding the Hc domains of BoNTs provide moderately efficient protection against BoNTs and more efficient potency than conventional plasmid DNA vaccines in mice.[11,12] In our recently continual efforts to further refine and enhance the

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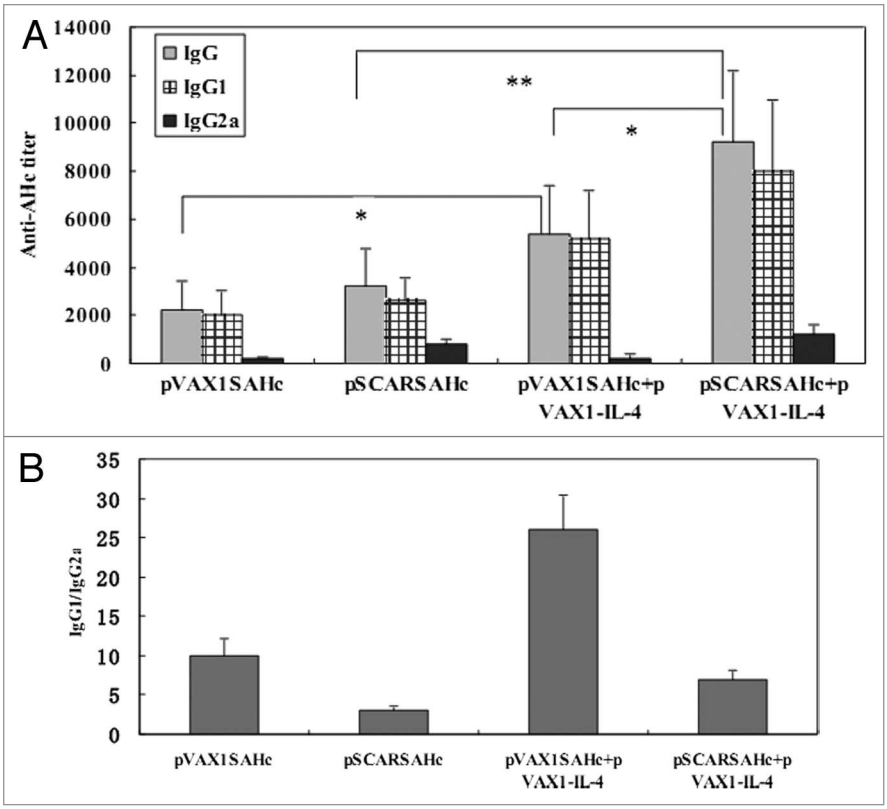

Figure 1. AHc-specific antibody responses in mice after i.m. vaccination with DNA vaccines. (**A**) Sera from each group of mice at 4 weeks after the last immunization were collected, and the specific anti-AHc total IgG titers and individual IgGs isotype (IgG1 and IgG2a) titers were analyzed by ELISA. Serum samples from individual mice were assayed and the group mean titer (GMT) was calculated for the group (n = 8). (**B**) The IgG1/IgG2 ratio of serum antibodies was determined to evaluate the type of response to immunization. Results for each group represent the average ratio ± SEM *p < 0.05; **p < 0.01.

Table 1. Survival and serum neutralization titers of mice after i.m. vaccination with DNA vaccines

Vaccine	Serum neutralization titer (IU/ml)	Number Alive[a]	
		10^3	10^4
pVAX1SAHc	< 0.16	4	0
pSCARSAHc	0.16	6[$]	0
pVAX1SAHc+pVAX1-IL-4	0.32	8*	4
pSCARSAHc+pVAX1-IL-4	0.64	8*	7[#]
pVAX1-IL-4	< 0.16	0	ND
pVAX1	< 0.16	0	ND

[a]Balb/c mice alive (8 mice/group) after i.p. challenge with the indicated dose (10^3 or 10^4 LD_{50}) of BoNT/A at 4 weeks after the last vaccination. *p = 0.00016 < 0.001 indicates a significant survival difference between the IL-4-codelivered groups that vaccinated with pVAX1SAHc+pVAX1-IL-4 or pSCARSAHc+pVAX1-IL-4 and the negative control groups (pVAX1-IL-4 or pVAX1). [#]p = 0.0014 < 0.01 indicates a significant survival difference between groups vaccinated with pSCARSAHc+pVAX1-IL-4 and pSCARSAHc or pVAX1SAHc. [$]p = 0.007 < 0.01 indicates a significant survival difference between the pSCARSAHc-vaccinated groups and the negative control groups.

protective immune response of this antigen, we have proved that formulation of the DNA vaccines with aluminum phosphate adjuvant can efficiently enhance antibody responses and protective efficacy against BoNTs and GM-CSF gene adjuvant can augment the immunogenicity of DNA replicon vaccine of BoNT/A.[22,23] In the present study, we also evaluated the potency of IL-4 as a molecular adjuvant of DNA vaccines to enhance antibody responses and protective efficacy against BoNT/A in both Balb/c and C57/BL6 mice.

Results

Co-delivery IL-4 molecular adjuvant induces stronger humoral and protective immune responses than DNA vaccine alone in mice. To evaluate whether the immunogenicity of DNA vaccines could be increased by IL-4 molecular adjuvant, the humoral immune responses and protective effects of pVAX1AHc or pSCARSAHc by co-delivery pVAX1-IL-4 DNA vector were compared with the same DNA vaccines alone. As shown in **Figure 1**, the mean antibody titers to AHc in the vaccinated Balb/c mice with IL-4 were higher than those obtained from the vaccinated mice without IL-4 ($p < 0.05$). The both DNA vaccines in the presence of IL-4 still predominantly induce Th2-type humoral immune responses as the both DNA vaccines alone, but with an IgG1 to IgG2a of ratio from approximately 10 to 26 for conventional pVAX1 DNA vector and 3 to 7 for replicon pSCAR DNA vector (**Fig. 1B**). These results suggest that stronger Th2-type humoral immune responses were modulated and elicited by the IL-4 molecular adjuvant.

Mice vaccinated with pVAX1AHc or pSCARSAHc co-delivered with pVAX1-IL-4 were completely protected against 1,000 50% lethal dose (LD_{50}) of BoNT/A and partly protected against 10,000 LD_{50} of BoNT/A, while pVAX1AHc or pSCARSAHc alone only provided part protection against 1,000 LD_{50} of BoNT/A and no protection against 10,000 LD_{50} of BoNT/A (**Table 1**). No protection was observed against 1,000 and 10,000 LD50 of BoNT/A in the negative control mice vaccinated with pVAX1-IL-4 or pVAX1. As shown in **Table 1**, the higher titers of neutralizing antibodies (0.32 or 0.64 IU/ml) were observed in the sera of mice vaccinated with the pVAX1AHc or pSCARSAHc in the presence of IL-4 and correlated well with group ELISA antibodies and protection levels.

In summary, IL-4 adjuvant significantly augmented the humoral immune responses and protective efficacy of the DNA vaccines against BoNT/A. Notably, the immunogenicity of pSCARSAHc + pVAX1-IL-4 is stronger than that of

pVAX1AHc + pVAX1-IL-4 ($p < 0.05$). Therefore, the effect of co-delivery or co-expression of IL-4 by the DNA replicon vector was investigated in next study.

Enhanced potency of plasmid DNA replicon vaccines against BoNT/A using one replicon vector to co-express the AHc antigen and IL-4 molecular adjuvant. Balb/c and C57/BL6 mice were immunized with various DNA replicon vaccines and the humoral and protective immune responses were assessed. As shown in **Figure 2**, the mean antibody levels of plasmid DNA replicon vaccines by co-delivery or co-expression the AHc antigen and IL-4 molecular adjuvant were significantly higher than that of the pSCARSAHc alone ($p < 0.05$). Antibody titers of co-expression the AHc and IL-4 were higher than that of co-delivery and there was statistically significant difference ($p < 0.05$), but not between dual-expression pSCARSAHc-26S-IL-4 and bicistronic IRES pSCARSAHc-IRES-IL-4 ($p > 0.05$). The higher titers of neutralizing antibodies against BoNT/A were also observed in the sera of mice immunized with the three adjuvanted-IL-4 DNA replicon vaccines (i.e., pSCARSAHc+pSCAR-IL-4, pSCARSAHc-26S-IL-4 and pSCARSAHc-IRES-IL-4). Mice immunized with the three adjuvanted-IL-4 DNA replicon vaccines were protected against 1,000 and 10,000 LD_{50} of BoNT/A, while pSCARSAHc alone provided part protection against 1,000 LD_{50} of BoNT/A and no protection against 10,000 LD_{50} of BoNT/A (**Table 2**). In particular, vaccination of co-expression the AHc and IL-4 provided complete protection against 10,000 LD_{50} of BoNT/A in both Balb/c and C57/BL6 mice. Results revealed that the immunogenicity and protective efficacy of co-delivery or co-expression the AHc antigen and IL-4 molecular adjuvant in Balb/c mice was comparable to that of C57/BL6 mice (**Fig. 2**; **Table 2**).

Therefore, co-delivery or co-expression of IL-4 by the DNA replicon vector was effective to induce stronger antibody responses and provide more effective protection against BoNT/A in both strain mice. More importantly, our results showed that co-expression of AHc antigen and IL-4 on the same plasmid DNA replicon vaccine by dual-expression (pSCARSAHc-26S-IL-4) or bicistronic IRES (pSCARSAHc-IRES-IL-4) well enhanced the generation of immune responses as co-delivery of separate plasmids expressing AHc antigen or IL-4 (pSCARSAHc+pSCAR-IL-4).

Enhanced potency of RVP vaccines against BoNT/A by co-expression the AHc antigen and IL-4 molecular adjuvant. The effect of RVP vaccines that co-expressed the AHc antigen and interleukin-4 molecular adjuvant were also assessed in Balb/c and C57/BL6 mice. As shown in **Figure 3**, the mean antibody levels of VRP vaccines by co-delivery or co-expression the AHc antigen and IL-4 molecular adjuvant were significantly higher than that of the VRP-AHc alone in both strain mice ($p < 0.01$). The higher titers of neutralizing antibodies against BoNT/A were also observed in the sera of mice immunized with the three adjuvanted-IL-4 RVP vaccines (i.e., RVP-AHc + RVP-IL-4, RVP-AHc-26S-IL-4 and RVP-AHc-IRES-IL-4). Mice immunized with the three adjuvanted-IL-4 DNA RVP were completely protected against 10,000 LD_{50} of BoNT/A, while alone RVP-AHc only provided part protection against 10,000 LD_{50} of BoNT/A (**Table 3**). Then, we rechallenged the mice of vaccination 5 d later with much higher neurotoxin doses (100,000 LD_{50}) and observed no animal deaths or symptoms of poisoning in the RVP-AHc-26S-IL-4 or RVP-AHc-IRES-IL-4-immunized mice ($p < 0.05$ compared with the VRP-AHc + VRP-IL-4-vaccinated group). However, the vaccination with RVP-AHc + RVP-IL-4 only provided part protection and the vaccination with RVP-AHc alone provided no protection. The immune protective effect was consistent with the serological reactivity results above.

In order to determine whether the IgG1 to IgG2a isotype ratio was affected by IL-4 molecular adjuvant encoded by replicon vector, isotypes of the IgG responses in both strain mice were also detected (**Fig. 2C and 3C**). Both types of replicon vaccines (DNA and RVP) in the presence of IL-4 still predominantly elicit Th2-type humoral immune responses as the both replicon vaccines alone, but with an IgG1 to IgG2a of ratio from approximately 3 to 10. These results suggest that stronger Th2-type humoral immune responses were also modulated and augmented by the IL-4 adjuvant in both Balb/c and C57/BL6 mice, which provided strong protection effect against BoNT/A.

Our findings revealed that co-delivery or co-expression of IL-4 by the RVP or DNA replicon vaccines was effective to induce stronger antibody responses and provide more effective protection against BoNT/A in mice of different genetic backgrounds. Notably, co-expression of AHc antigen and IL-4 on the same replicon vaccine by dual-expression or bicistronic IRES was more effective on the generation of immune responses and protective potency than co-delivery of two separate vectors expressing AHc antigen or IL-4.

Discussion

Naked DNA vaccines are effective in mice model, but their efficiency is restricted in larger animals or humans. Additionally, early DNA vaccines failed to afford sufficient immunogenicity in human clinical studies.[16] Thus, the potency of DNA vaccines must be improved to enable this technology to successfully apply to human clinical trial. It is clear from previous studies that exogenous cytokines are valuable adjuvants to vaccines aimed at stimulating and augmenting immune responses.[24,25] Moreover, adding an efficient adjuvant (such as IL-4) to DNA vaccines has also been demonstrated to be effective in modulating and enhancing the immune response to DNA vaccines.[26-30] In our study, we demonstrated that co-delivery or co-expression the AHc antigen and IL-4 molecular adjuvant can strongly potentiate antibody responses and protective efficacy of two types of DNA vaccines (i.e., conventional plasmid DNA vaccine and plasmid DNA replicon vaccine) against BoNT/A in both Balb/c and C57/BL6 mice. In agreement with previous studies,[23,26-30] IL-4 increases the immune response and protective efficacy induced by the DNA vaccines, which makes it a very good candidate for use as an adjuvant of vaccines against BoNT/A.

The humoral immune responses produced with naked DNA vaccines are relatively weak since intramuscular injection results in the production of relatively small amounts of antigen protein. Production of protein antigen alone was not sufficient to elicit a strong antibody response, and the need for an effective adjuvant

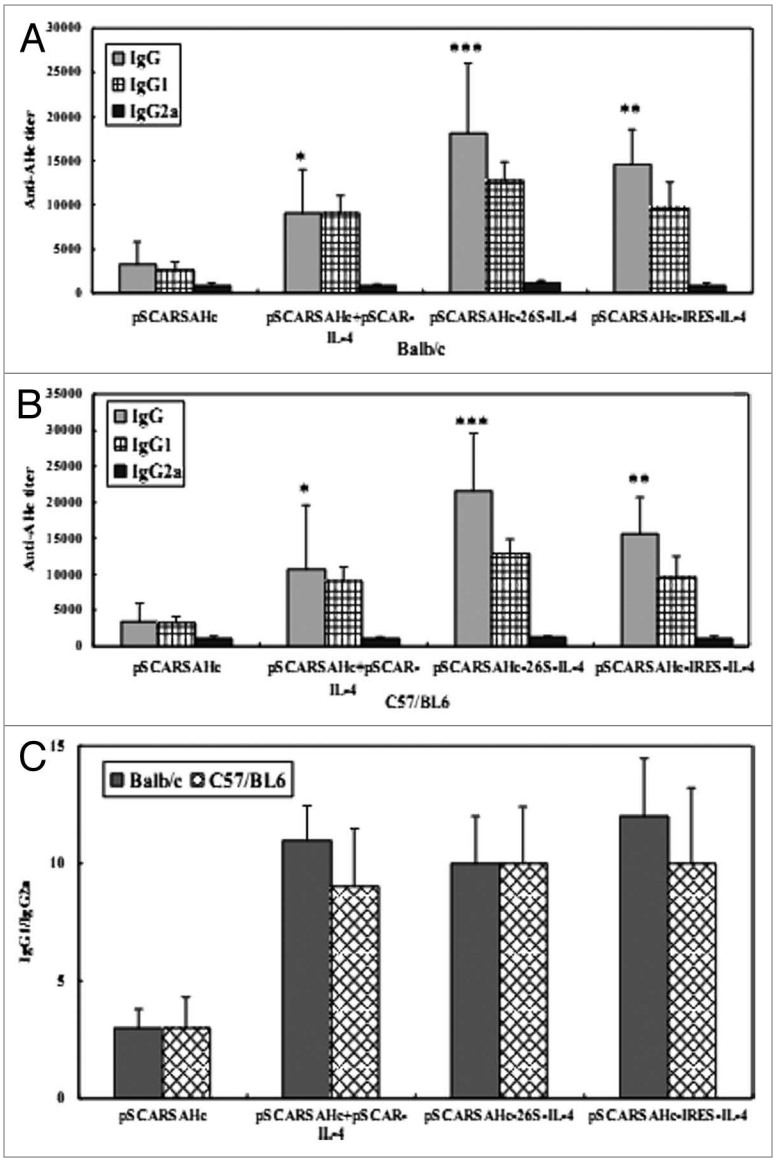

Figure 2. AHc-specific antibody responses in mice after i.m. vaccination with DNA replicon vaccines. Sera from each group of Balb/c (**A**) and C57/BL6 (**B**) mice immunized with different DNA replicon vaccines at 4 weeks after the last immunization were collected, and the specific anti-AHc total IgG titers and individual IgGs isotype (IgG1 and IgG2a) titers were analyzed by ELISA. Serum samples from individual mice were assayed and the group mean titer (GMT) was calculated for the group (n = 8). (**C**) The IgG1/IgG2a isotype ratio for each group represents the average ratio ± SEM *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with the pSCARSAHc-vaccinated group without IL-4 molecular adjuvant.

was clear.[25,31] IL-4 plays a role not only in the differentiation of Th2 cells but also as a key compensatory cytokine for all aspects of the Th2 responses.[32,33] IL-4 often modulates or introduces a Th2 bias into the immune responses against the antigen that they deliver. Similarly, DNA plasmids expressing IL-4 have also been used to preferentially augment B-cell mediated Th2 responses and Ig class switching.[24,27,33,34] Our results show that sera from the adjuvanted-IL-4 vaccine groups contain higher AHc-specific total IgG antibodies than the absence of IL-4 adjuvant. The plasmid DNA vaccines in the presence of IL-4 elicit much stronger Th2-type humoral immune responses with an IgG1 to IgG2a of ratio from approximately 10 to 26 for conventional DNA vector (pVAX1) and 3 to 7–10 for replicon DNA vector (pSCAR) in both strain mice as our previous studies on aluminum phosphate as adjuvant,[22,23] which is consistent with the potency of a Th2-type IL-4 adjuvant, but different to GM-CSF adjuvant with unconverted ratio of IgG1 to IgG2a.[23] Titers of neutralizing antibodies against BoNT/A were dramatically increased by 4–8-fold. As a result, protective efficacy against BoNT/A in mice model is stronger than the absence of IL-4 adjuvant. Further, stronger neutralizing antibodies and protection against BoNT/A were observed on the using of IL-4 than GM-CSF or aluminum phosphate as adjuvant in our laboratory.[22,23]

Another important purpose of the present study was to determine whether or not co-expression of IL-4 and antigen on the same plasmid DNA replicon vaccine well enhanced the generation of immune responses after DNA vaccination as co-delivery of separate plasmids expressing IL-4 or antigen. Therefore, in this study co-expression of IL-4 and AHc within same plasmid backbone were realized by dual-expression or bicistronic IRES using replicon vector. Our results showed that both types of co-expression strategies well-modulated and enhanced the antibody responses and protective efficacy as co-delivery in both strain mice. Moreover, the effect and feasibility of co-expressed IL-4 as molecular adjuvant were further performed on the recombinant SFV particles (VRP) vaccines. The enhanced potencies were produced by co-expression of the IL-4 molecular adjuvant in mice immunized with VRP vaccines as co-delivery.

More importantly, co-expression of AHc and IL-4 on the same replicon vaccine was more effective on the generation of humoral immune responses and protective potency than co-delivery of two separate vectors expressing AHc or IL-4. The immune responses were improved maybe because of the co-expression promised to target IL-4 to the suitable microenvironments to produce optimal adjuvant activity. Our results are consistent with previous studies demonstrating a bicistronic or two separate CMV promoter form of a vector expressing both proteins improved the immune response and co-expression was better than co-delivery of independent plasmids.[23,35,36] Although further studies are needed to prove these effects, it is clear that development of DNA vaccines using co-expression of antigen with molecular adjuvant (IL-4) may be an effective approach in modulating and enhancing immune responses against a variety of diseases. The co-expression of antigens of interest and molecular adjuvant from a vector may provide other advantages such as simplification of experimental immunization regimens and cost savings in manufacturing one product instead of two. Furthermore, the IL-4 is co-expressed and it acts at the site of antigen expression, thereby avoiding the toxicity of systemically administered cytokines.[25]

Table 2. Survival and serum neutralization titers of mice after i.m. vaccination with plasmid DNA replicon vaccines

Vaccine	Serum neutralization titer (IU/ml)		Number alive[a]			
	Balb/c	C57/BL6	10^3		10^4	
			Balb/c	C57/BL6	Balb/c	C57/BL6
pSCARSAHc	0.16	0.16	5	6	0	0
pSCARSAHc+pSCAR-IL-4	0.64	0.64	8	8	6*	7[$]
pSCARSAHc-26S-IL-4	1.28	2.56	8	8	8[#]	8[#]
pSCARSAHc-IRES-IL-4	1.28	2.56	8	8	8[#]	8[#]
pSCAR-IL-4	< 0.16	< 0.16	0	0	ND	ND
pSCAR	< 0.16	ND	0	ND	ND	ND

[a]Balb/c and C57/BL6 mice alive (8 mice/group) after i.p. challenge with the indicated dose (10^3 or 10^4 LD_{50}) of BoNT/A at 4 weeks after the last vaccination. *p = 0.007 < 0.01 indicates a significant survival difference between the IL-4-codelivered group that vaccinated with pSCARSAHc+pSCAR-IL-4 and the pSCARSAHc-vaccinated group without IL-4 molecular adjuvant in Balb/c mice. [#]p = 0.00016 < 0.001 indicates a significant survival difference between the IL-4-coexpressed groups that vaccinated with pSCARSAHc-26S-IL-4 or pSCARSAHc-IRES-IL-4 and the pSCARSAHc-vaccinated group. [$]p = 0.0014 < 0.01 indicates a significant survival difference between the IL-4-codelivered group that vaccinated with pSCARSAHc+pSCAR-IL-4 and the pSCARSAHc-vaccinated group in C57/BL6 mice. ND, not done.

In summary, our findings revealed that IL-4 is an effective adjuvant for the replicon vaccines, and the co-expression replicon vaccines described here may be an excellent candidate for further vaccine development. In future study, it is necessary to further prove the potency of co-expression of AHc antigen and IL-4 on the same replicon vaccine by dual-expression, or in particular bicistronic IRES, which can result in lower levels of expression of one or both of the genes, compared with the corresponding monocistronic constructs. The efficacy of this type of DNA vaccines against BoNT/A also needs to compare with that of the subunit AHc or toxoid vaccines for future clinical trial or clinical use.[37,38] To our knowledge, this is the first report to date demonstrating the potency of IL-4 as an adjuvant on replicon vaccines in both strain mice. Thus, we described a strategy to design and develop efficient vaccines against BoNT/A or other pathogens using one replicon vector to simultaneously co-express antigen and molecular adjuvant.

Materials and Methods

Construction of DNA plasmids. A viral DNA-based replicon vector pSCARSAHc (**Fig. 4A**) and a conventional DNA vector pVAX1SAHc (**Fig. 4E**) encoding the Hc domain of botulinum neurotoxin serotype A (AHc) were constructed by us and conserved in our lab.[12,22] Plasmid pCI-mIL-4 containing murine *IL-4* gene and pIRES containing the encephalomyocarditis virus (ECMV) internal ribosome entry site (IRES) from Clontech (Palo Alto, CA) were conserved by our lab.

To explore whether or not the immunogenicity to AHc antigen is augmented and modulated by the co-delivery or co-expression of the *IL-4* gene, a series of plasmid DNA vectors that expressed IL-4 were constructed by inserting the murine *IL-4* into pVAX1 or pSCAR (a viral DNA-based replicon expression vector derived from SFV).[11] Briefly, the *IL-4* gene amplified by PCR from pCI-mIL-4 was digested with *BamH* I and *Xho* I and inserted into pVAX1 to produce pVAX1-IL-4 (**Fig. 4F**). The *IL-4* gene was also digested with *BamH* I and *Nsi* I and inserted into pSCAR to produce pSCAR-IL-4 (**Fig. 4B**). For co-expression of IL-4 and antigen on the same plasmid DNA replicon vector pSCAR, the *IL-4* and *AHc* gene were cloned into the same plasmid DNA replicon vector backbone, but separate 26S promoters drove each gene. After pSCAR-IL-4 was digested by *Spe* I and *Msc* I, the 1300 bp DNA fragment containing 26S promoter and *IL-4* gene was inserted into pSCARSAHc which digested with *Spe* I and SmaI to produce pSCARSAHc-26S-IL-4 (**Fig. 4C**). Additionally, the IRES sequence from plasmid pIRES and *IL-4* were fusioned by PCR to produce IRES-IL-4 DNA fragment. The IRES-IL-4 was digested by *Nsi* I and inserted into the digested-Nsi I pSCARSAHc to produce pSCARSAHc-IRES-IL-4 which allow the simultaneous expression of AHc and IL-4 two proteins separately but from the same RNA transcript (**Fig. 4D**). All plasmids were prepared and purified using Endofree Mega-Q kits (Qiagen) for transfection and immunization. BHK-21 cells were transfected with pSCARSAHc, pSCARSAHc-26S-IL-4, pSCARSAHc-IRES-IL-4, pSCAR-IL-4, pVAX1SAHc or pVAX1-IL-4 and expression of AHc or IL-4 was analyzed as described previously (Data not shown).[12,22]

Preparation of DNA-based recombinant Semliki Forest virus replicon particles. Recombinant Semliki Forest virus replicon particles (VRP) were prepared as described previously.[13] Briefly, BHK cells were co-transfected with different DNA-based replicon expression vectors and helper vector (pSHCAR), and the VRP were harvested between 24 and 36 h post-transfection.

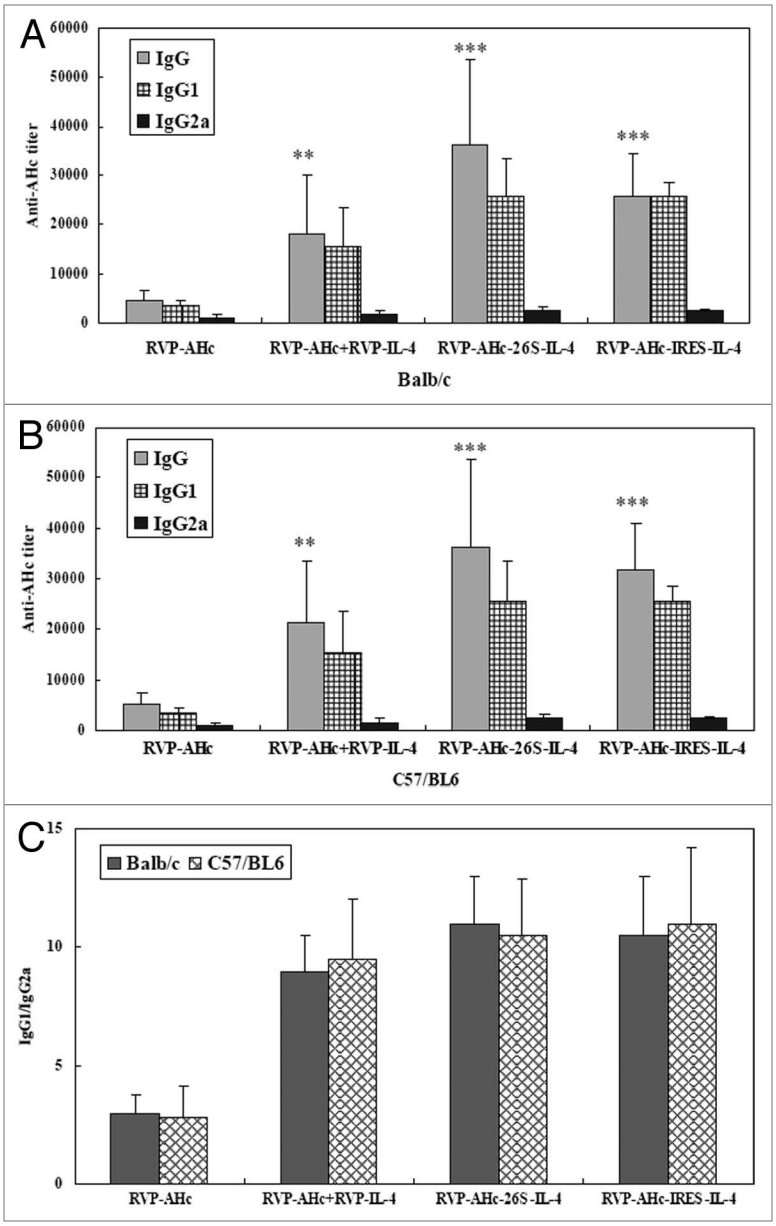

Figure 3. AHc-specific antibody responses in mice after s.c. vaccination with recombinant SFV particles (VRP) vaccines. Sera from each group of Balb/c (**A**) and C57/BL6 (**B**) mice immunized with different VRP vaccines at 4 weeks after the last immunization were collected, and the specific anti-AHc total IgG titers and individual IgGs isotype (IgG1 and IgG2a) titers were analyzed by ELISA. Serum samples from individual mice were assayed and the group mean titer (GMT) was calculated for the group (n = 8). (**C**) The IgG1/IgG2a isotype ratio for each group represents the average ratio ± SEM **p < 0.01 and ***p < 0.001 compared with the pSCARSAHc-vaccinated group without IL-4 molecular adjuvant.

VRP were concentrated and purified by centrifugation, and the virus pellets were resuspended in PBS buffer. Before use, VRP were activated in vitro with chymotrypsin, which renders them infectious by cleavage of the spike protein. For titer determination, BHK-21 cells were infected with serial dilutions of activated virus and monitored for expression of antigen by X-gal staining (VRP-lacZ) or immunofluorescence assay (IFA) (e.g., VRP-AHc, RVP-AHc-26S-IL-4, RVP-AHc-IRES-IL-4 or RVP-IL-4). This procedure typically yielded VRP titers of approximately 2×10^7 infectious particles/ml.

Vaccinations and challenge. Specific pathogen-free female Balb/c and C57/BL6 mice (purchased from Beijing Laboratory Animal Center) six weeks of age were randomly assigned to different treatment groups and vaccinated with DNA or VRP. DNA dosage used in the immunized groups was optimized by a series of preliminary experiments. For DNA vaccination, groups of eight mice were i.m. injected with 30 μg of each plasmid DNA replicon vaccines or conventional DNA vaccines in a total volume of 0.1 ml three times with 3-week intervals between each injection. As a negative control, mice were vaccinated with 30 μg of pSCAR or pVAX1 as above. For VRP vaccination, 10^6 infectious particles (one infectious unit = 1 IU) of each activated VRP (VRP-lacZ as negative control) in a total volume of 0.2 ml were s.c. inoculated three times with 3-week intervals in between. Blood from all groups was collected via the tail vein before each vaccination or neurotoxin challenge. Mice from all groups were challenged i.p. with different dosages of pure BoNT/A 4 weeks after the last vaccination. The mice were observed for 1 week after challenge, and survival was determined for each vaccination group. Repeated studies were performed and similar results were obtained in one additional experiment. The animal protocols in this study were approved by Institution Animal Care and Use Committee of our Institution.

Antibody titer measurements. Anti-AHc antibodies of sera from mice in the different groups were detected by ELISA as previously described.[12,23] Briefly, ELISA plates (Corning Inc.) were coated overnight at 4°C with 100 μl AHc (2 μg/ml). Serum samples were serially diluted at 1:2 increments beginning at 1: 100 and 100 μl was added to each well for 1 h at 37°C. After washing, 100 μl of a 1:2,000 dilution of goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Inc.) was added for 30 min at 37°C. Then, anti-AHc reactivity was visualized by adding 100 μl of citrate buffer (pH 5.0) containing 0.04% (w/v) of o–phenylenediamine and 0.02% (v/v) hydrogen peroxide for 5 min at 37°C. The reaction was stopped with 50 μl of 2 M H_2SO_4 and the absorbance was read at 492 nm using a Thermo Labsystems microplate reader. The individual isotype of IgG (IgG1 and IgG2a) titers were further determined using HRP-conjugated goat anti-mouse IgG1 and IgG2a antibodies (Santa Cruz Biotechnology) at a dilution of 1:2,000. Antibody titers were estimated as the reciprocal of the maximum dilution of serum. Serum samples from individual mice were detected and the group mean titer (GMT) was calculated for the group. Similar results were obtained in one additional experiment.

BoNT/A neutralization assay. Pooled sera from each group of mice above were diluted initially 1:8 and then 2-fold for serum neutralization titers as described previously.[37,38] Briefly, mixtures of serial dilutions of sera with 100 LD_{50} of BoNT/A (L+1/100)

Table 3. Survival and serum neutralization titers of mice after s.c. vaccination with recombinant SFV virus replicon particles (VRP) vaccines

Vaccine	Serum neutralization titer (IU/ml)		Number alive[a]			
	Balb/c	C57/BL6	10^3		10^4	
			Balb/c	C57/BL6	Balb/c	C57/BL6
VRP-AHc	0.32	0.32	8*	8*	2	3
VRP-AHc+VRP-IL-4	1.28	2.56	8*	8*	8#	8$
VRP-AHc-26S-IL-4	2.56	5.12	8*	8*	8#	8$
VRP-AHc-IRES-IL-4	2.56	2.56	8*	8*	8#	8$
VRP-IL-4	< 0.16	< 0.16	0	0	ND	ND
VRP (Control)	< 0.16	ND	0	ND	ND	ND

[a]Balb/c and C57/BL6 mice alive (8 mice/group) after i.p. challenge with the indicated dose (10^3 or 10^4 LD_{50}) of BoNT/A at 4 weeks after the last vaccination. *p = 0.00016 < 0.001 indicates a significant survival difference between the VRP-AHc-vaccinated groups and the negative control groups (VRP-IL-4 or VRP). #p = 0.007 < 0.01 indicates a significant survival difference between the IL-4-codelivered or co-expressed groups and the VRP-AHc-vaccinated group without IL-4 molecular adjuvant in Balb/c mice. $p = 0.026 < 0.05 indicates a significant survival difference between the IL-4-codelivered or co-expressed groups and the VRP-AHc-vaccinated group without IL-4 molecular adjuvant in C57/BL6 mice. ND, not done.

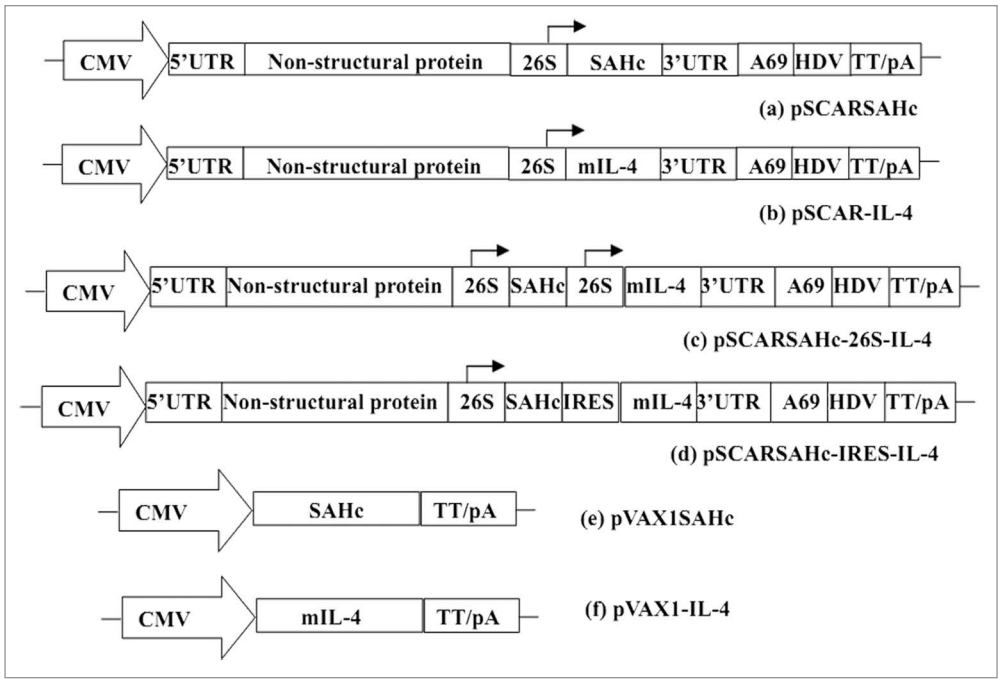

Figure 4. Schematic diagram of expression plasmids used in DNA immunization. CMV, cytomegalovirus immediate early (CMV IE) enhancer/promoter. TT/pA, BGH transcription termination and polyadenylation signal. HDV, HDV antigenomic ribozyme sequence. 26S, the subgenomic promoter of SFV. S, Igκ leader sequence. pSCARSAHc-26S-IL-4, a dual-expression plasmid DNA replicon vaccine that designed to co-express both AHc and IL-4 under the control of two separate 26s promoters. pSCARSAHc-IRES-IL-4, a plasmid DNA replicon vaccine that designed to expressed AHc and allow the simultaneous expression of IL-4 separately from the same RNA transcript using bicistronic IRES.

were incubated 0.5 h at room temperature and the mixtures were injected i.p. into mice (16–22 g) using a volume of 500 μl /mouse (four mice in each group). The concentration of neutralizing antibody in the sera was calculated relative to a World Health Organization botulinum neurotoxin serotype A and neutralizing antibody titers of sera were reported as international units per milliliter (IU/ml). Due to the limited amount of serum available, serum from each group of mice was pooled, and so only the average neutralization titer of the group could be assayed.

Statistical analysis. Differences in antibody titers were analyzed statistically using the Student's t-test or the paired t-test between group differences. Fisher's exact test was used to determine statistical differences in survival between the treatment groups. For all tests only data resulting in *P* values < 0.05 were regarded as statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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