No.	K22-3062	
研究課題名	Harnessing the group A streptococcus pilus to develop novel universal influenza vaccines	
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(Form 2-2)

Principal Investigator

IMSUT International Joint Usage/Research Center Project <International>

Joint Research Report (Annual/Project Completion)

Annual Report

Report

PilVax immunisation study

Background: PilVax is a novel peptide delivery platform (provisional patent obtained) using the group A streptococcus (GAS) pilus structure to display antigen on the surface of *Lactococcus lactis* vaccine vehicle. We proposed that new PilVax vaccines carrying the highly conserved M2e epitope can be an useful mucosal vaccination strategy against influenza A virus infection, which remains a challenge due to the genetic instability of the major antigens hemagglutinin and neuraminidase. In our pilot trial, M2e PilVax vaccines were prepared as heat-killed *Lactococcus* suspension produced in Auckland and shipped to Japan for the initial immunization and challenge study. However, no protective effect was observed from the vaccination and no IgA response was detected in the saliva samples collected at timepoints during the vaccination course. To follow up the results we confirmed the M2e antigen presentation on heat-killed *Lactococcus* by western blotting of the *Lactococcus* cell wall protein extract, and ruled out the possibility of antigen damage due to the heating process.

Progress: While the exact cause of reduced immune responses in this trial warrants further investigation, we suspect the loss of adjuvant activities in non-viable *Lactococcus* bacteria might explain the poor vaccination outcome. Therefore, a repeat of the immunization and challenge studies was planned, using frozen stock of live *Lactococcus* shipped from Auckland. Groups of BALB/c mice (female, 6-10 weeks, n=5) were nasally immunized with 1×10^8 CFU/10 mL PilVax-M2e, PilVax-M2e-M2e, PilM1 or WT *L. lactis* on 3 consecutive days, 2 weeks apart, for a total of 4 vaccinations (12 doses in total, see **Figure 1**). Mice vaccinated with synthetic M2e peptide mixed with poly(I:C) were included as positive controls. Another group of mice vaccinated with a PilVax strain containing MTP-PstS, a TB epitope, was also included as negative control.

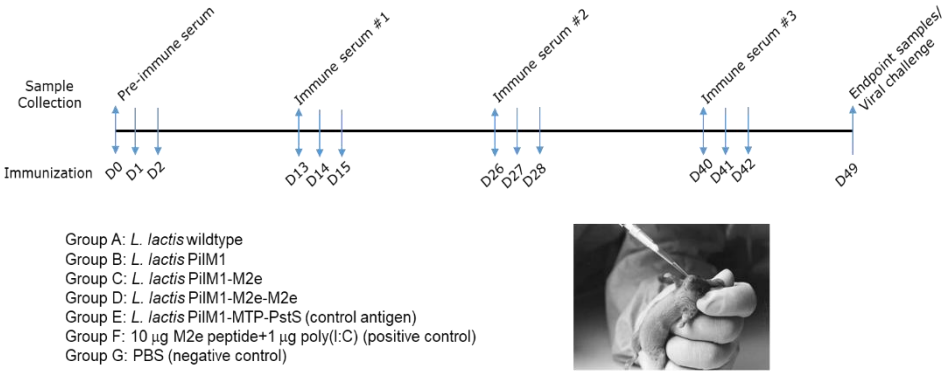

Figure 1. Immunization schedule and group designation.

The immunization procedures have been completed on 28 April. Serum, nasal wash and saliva samples were collected for antibody titre analysis before the animal were challenged by influenza A virus (A/California/7/2009 influenza virus at 5 x LD_{50}, 50 μL internally).

Pilus proteins as vaccine adjuvant

Background: We recently identified the GAS FCT-2 pilus as a toll-like receptor 2 (TLR-2) ligand, and a strong stimulant for the production of pro-inflammatory cytokines TNF and IL-8. Therefore, we hypothesize that the immune stimulating property of the pilus proteins can be exploited as vaccine adjuvant. To test if the pilin conjugation can increase immunogenicity of the M2e peptide, we generated 3 different recombinant proteins of M2e peptide fused to the soluble portions of Spy0128 and Spy0125 in Auckland. The antigen integrity of the fusion proteins was verified by a sandwich ELISA, and the ability of the proteins to stimulate TLR2 was examined by HEK-Blue hTLR2 reporter cell line.

Progress: For proof of concept, we shipped the recombinant proteins to Japan and immunized a

sentinel cohort of mice (n=5 per group) with different doses of Spy0125M2e recombinant protein. Results showed that the vaccination successfully stimulated antibody titers with a clear dose-dependent effect (**Figure 2A**). The highest dose of 50 μg was chosen for the following work, where groups of BALB/c mice (female, 6-10 weeks, n=5) were nasally immunized with recombinant protein, synthetic M2e peptide mixed with poly(I:C), or M2e alone. Two boosters were given on D7 and D21. On D27 serum and mucosal samples were collected for the analysis of antibody titers, and on D28 the animals were challenged with a lethal dose (5xLD_{50}) of H1N1 influenza A virus. Despite the elevated antibody titres observed (**Figure 2B**), no protection was seen in this immunization study.

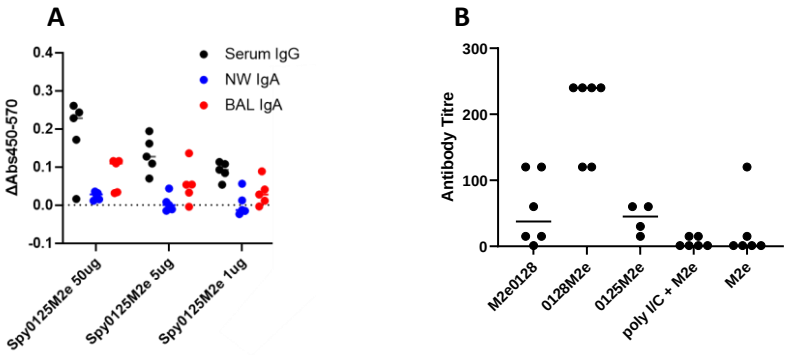

Figure 2. (A) Post-immunization anti-M2e antibody titres analysed by ELISAs. **(B)** Anti-M2e IgA titres in nasal wash analysed by ELISAs.

We decided the take a step back and investigate the adjuvant activity of the pilus proteins. We established the protocols of cell-based and flow cytometry assays, and repeated the experiments using murine macrophage cell line J774A.1 in Auckland. We first analyzed the stimulating effect of pilus proteins for macrophage proliferation. The results showed that at higher seeding densities ($5x10^4$ and $2.5x10^4$), proliferation of J774A.1 cells was seen in the presence of Spy0125 or Spy0128 (**Figure 3A**). In addition, in vitro activation of macrophages was investigated by determining the expression of CD80, CD86, F4/80 and MHCII by flow cytometry (**Figure 3B**). Both Spy0128 and Spy0125 exhibited stimulating activities for macrophage activation. These results suggest that the pilus proteins may also activate T cell response by upregulate the expression of surface co-stimulating molecules on APCs such as macrophages.

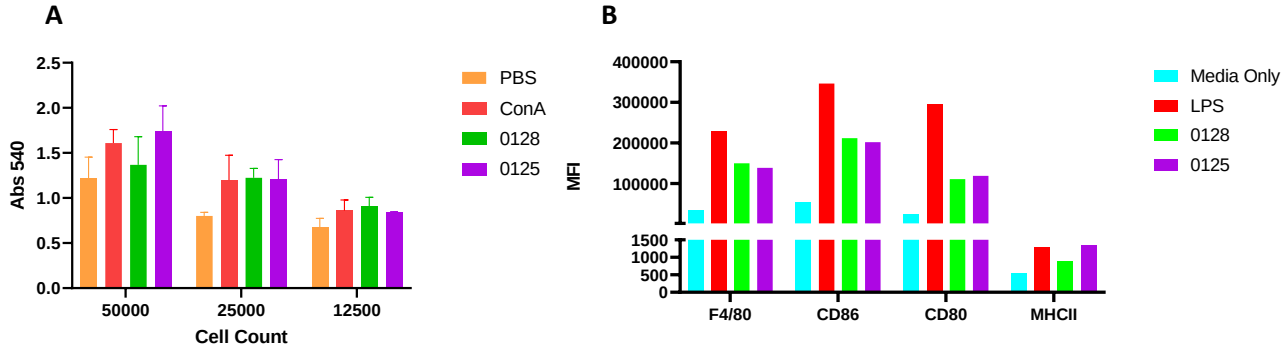

Figure 3. **(A)** Cell proliferation assay was performed using J774A.1 cells stimulated with Spy0128, Spy0125, ConA (positive control) or PBS (negative control). **(B)** Expression of surface markers by J774A.1 cells at 24h after treatment with Spy0125, Spy0128, or LPS (positive control).

Research output

[Conference presentations]

1. Webster Centre Infectious Disease Meeting during the 2022 Queenstown Research Week in Queenstown, New Zealand, 28 Augst-2 September, 2022 (oral presentation)
2. Lorne Infection and Immunity Meeting in Lorne, Australia, 15-17 February, 2023 (oral and poster presentations)
3. cSIMVa workshop and symposium in Chiba and Tokyo, Japan, 29 March – 1 April, 2023 (oral and poster presentations)

[Research publication]

1. Invited book chapter titled “Chapter 13. Mucosal Vaccine Delivery” in *Advanced Vaccination Technologies for Infectious and Chronic Diseases*, Elsevier (in press).
2. Review article titled “Mucosal vaccination: pros and cons” to be submitted to *Expert Review of Vaccines* for the article collection on the topic of *“The future of vaccines: new paradigms in vaccine and adjuvant technologies”* (in preparation).