Chapter 7

Whole-inactivated influenza virus as an adjuvant for influenza peptide antigens

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Abstract

Influenza peptide antigens have the potential to induce cross-protective influenza-specific T cells. However, short peptide antigens are poorly immunogenic and therefore need to be formulated with a potent adjuvant. In this study, it was investigated whether whole inactivated influenza virus (WIV) can act as an adjuvant for influenza peptide antigens. The immunogenicity of WIV mixed with the HLA-A*0201 restricted influenza peptide GILGFVFTL (GIL) was assessed in HLA-A2 transgenic mice by quantification of peptide-specific IFN-γ^+ $CD8^+$ T cells after vaccination. Subsequently, a Design of Experiments (DoE) approach was utilized to study the synergistic effect between WIV adjuvant and peptide antigen at different doses. Moreover, the roles of WIV fusogenicity, peptide-WIV association and co-localization on the adjuvanticity of WIV were investigated. To assess whether WIV could also act as an adjuvant for other peptides, a peptide pool with three wild type (WT) influenza peptides was adjuvanted with WIV. In addition, three chemically enhanced peptide ligands (CPLs) derived from the WT peptides, which possessed a higher binding affinity to the MHC molecules, were adjuvanted with WIV and screened for their immunogenicity compared to the WT peptides. WIV was found to be a potent adjuvant for the GIL peptide. The DoE study revealed that WIV was able to act as an adjuvant at even low concentrations. Co-localization of the peptide antigen and WIV adjuvant was important for the induction of a peptide-specific immune response, whereas peptide-WIV association and WIV fusogenicity were not. WIV was also able to act as an adjuvant for both WT and CPL peptide pools. This study shows the potential of WIV as an adjuvant for influenza peptides. The simple formulation process and the existing safety track record of WIV make this an attractive adjuvant, which could also be used for non-influenza antigens.

Introduction

Seasonal influenza vaccines mediate their protective effect mainly through the induction of virus-specific neutralizing antibodies. These antibodies are directed against the influenza surface proteins hemagglutinin (HA) and neuraminidase (NA). However, these proteins can undergo changes due to antigenic shifts and drifts. These antigenic changes impair the neutralizing ability of antibodies induced by vaccines, rendering these vaccines ineffective. Therefore, additional immune responses such as cellular responses against influenza need to be induced to increase vaccine effectiveness (1).

Cellular immune responses represent a potential alternative to antibody-mediated immune responses. Recently, Sridhar et al. found that cellular immune responses correlated with reduced morbidity in patients infected with pandemic influenza (2). Similarly, Wang et al. showed that patients with early influenza-specific $CD8^+$ T cell responses recovered faster from severe H7N9-induced disease (3). These studies confirmed that cellular responses against influenza can indeed be effective. Cellular immune responses such as cytotoxic T cells (CTLs) can effectively clear virus-infected host cells, thereby inhibiting viral replication and spread. Unlike most vaccine-induced antibodies, these CTLs recognize epitopes located on internal influenza proteins, which are conserved in many influenza strains. Owing to the conserved nature of these epitopes, cellular responses directed against these epitopes are potentially cross-reactive. Short linear peptides representing these epitopes are therefore attractive antigens for the development of cross-reactive influenza vaccines.

Peptide antigens as such, however, suffer from low immunogenicity due to inefficient delivery to antigen presenting cells (APCs) and the absence of pathogen-associated molecular patterns (PAMPs) or adjuvants to activate the APCs. Delivery of the peptide antigen to the cytoplasm of APCs is considered to be crucial for proper processing and subsequent presentation on MHC-I molecules, while activation of the APCs is important for licensing of naïve effector and memory $CD8^+$ T cells (4).

Formulation of peptide antigens with an appropriate adjuvant (which can be a delivery system or an immunopotentiator (5)) is thus crucial to induce a cellular immune response against the peptide antigen. Water-in-oil emulsions such as Incomplete Freund's Adjuvant (IFA) are commonly used and effective adjuvants for peptides, but are associated with severe adverse events such as lesion formation at the site of injection (6). Thus, alternative adjuvants for peptide antigens are highly sought after. Particulate adjuvant systems such as liposomes or virosomes formulated with influenza peptides derived from internal proteins have proven to be effective for the induction of peptide-specific CTLs (7, 8), especially in combination with Toll-like receptor (TLR) agonists (9, 10). However, the formulation of peptides into these delivery systems can be complicated and may result in low encapsulation rates. Adjuvants that can be directly admixed with peptide antigens would therefore be preferable.

Whole inactivated influenza virus (WIV) possesses an innate adjuvant capability in the form of viral single-stranded RNA (ssRNA). Previously it was shown that influenza ssRNA is a potent TLR7 agonist (11), that increases antibody responses and promotes cellular immune responses. Furthermore, WIV contains, aside from $CD8^+$ epitopes, $CD4^+$ epitopes, which provide invaluable T cell help that supports the induction of functional $CD8^+$ T cells (12). We therefore hypothesize that the addition of WIV to peptide antigens could promote the induction of peptide-specific T cell responses.

In addition to proper formulation of the peptide antigen, modification of the peptide could also improve the immunogenicity of the antigen. Previously, chemically enhanced altered peptide ligands (CPLs) derived from HLA-A2*0201-restricted epitopes were shown to possess a higher binding affinity to HLA-A2*0201, and to induce higher amounts of IFN-γ compared to wild type (WT) epitopes in an in vitro system (13). However, like other peptides, these CPLs are currently adjuvanted with IFA. Thus, we investigated whether WIV can act as an adjuvant for these modified peptides.

In the current study, we first investigated the adjuvanticity of WIV for the GILGFVFTL (GIL, $M1_{58-66}$) influenza peptide, an HLA-A2*0201-restricted $CD8^+$ T cell epitope, in a proof-of-principle study. Next, we performed a dose-finding study for the optimal WIV adjuvant and peptide antigen concentration to induce peptide-specific T cells by use of a Design of Experiments (DoE) approach. Furthermore, we studied the effect of WIV-peptide co-localization and WIV membrane fusion activity on the adjuvanticity of WIV. Finally, we tested the adjuvanticity of WIV with three WT T cell peptides and three CPL variants of the WT peptides.

Materials and methods

Formulation of vaccines

Influenza A/PR/8/34 virus was propagated on fertilized eggs and inactivated with β-propiolactone on a pilot scale as described before (14), which yielded PR8 WIV bulk vaccine. To study the effect of fusion activity on the immune response, WIV was fusion-inactivated by lowering the buffer pH to 4.5 with a pretitrated volume of 1 M HCl, and subsequently incubated at 37°C for 15 min. Afterwards, the sample was brought to physiological pH by dialyzing overnight against PBS pH 7.2. Membrane fusion capacity was subsequently determined by a hemolysis assay as described previously (10).

The Netherlands Cancer Institute kindly provided the HLA-A2*0201-restricted influenza GILGFVFTL (GIL, $M1_{58-66}$), FMYSDFHFI (FMY, PA_{46-54}), and NMLSTVLGV (NML, $PB1_{413-421}$) peptides, and CPLs [am-phg]ILGFVFTL (G1), [4-FPHE]MYSDFHF[2-AOC] (F5), and N[NLE]LSTVLGV (N53). Nonproteogenic amino acids introduced in the peptide sequences are shown in **Figure S1**. Influenza PR8 WIV and peptide antigens were formulated in PBS pH 7.2 (Life Technologies) at various concentrations. When mentioned, 50 μg of CpG ODN1826 (Invivogen) or 50% (v/v) Incomplete Freund's Adjuvant (Sigma-Aldrich) was added to the formulation.

Animal studies

Animal studies were conducted according to the guidelines provided by the Dutch Animal Protection Act, and were approved by the Committee for Animal Experimentation (DEC) of the National Institute for Public Health and the Environment (RIVM). Eight- to ten-week-old female HLA-A2 transgenic mice (Jackson Laboratory, maintained in-house) were used in all studies.

In the proof-of-principle study, mice (three per group) received immunizations subcutaneously (s.c.) in the flank at days 0 and 21 under isoflurane anesthesia, containing either PBS, 50 μg WIV, 1 μg GIL peptide adjuvanted with 50 μg WIV or 100 μg GIL adjuvanted with 50 μg CpG. For the dose finding study, a DoE approach was used (as described below). The selected formulations consisting of various doses of WIV and GIL peptide (shown in **Table SI**) were administered s.c. in the flank of mice (six per group) at day 0 and 21.

To study the effect of adjuvant co-localization, mice (six per group) were immunized at days 0 and 21 either s.c. in one flank with PBS or 100 μg GIL peptide adjuvanted with 25 μg WIV, or s.c. in separate flanks with 100 μg GIL peptide in one flank and 25 μg WIV adjuvant in the opposite flank. The effect of membrane fusion activity was assessed by immunizing mice (six per group) s.c. in the flank at day 0 and 21 with 100 μg GIL peptide adjuvanted with either 25 μg of fusion-active WIV or fusion-inactive WIV.

The adjuvant effect of WIV on a mix of multiple peptides was assessed with either a WT

peptide pool (GIL, FMY and NML; 100 µg each) or a modified peptide pool (G1, F5 and N53; 100 µg each). Mice (six per group) received an s.c. immunization in the flank at day 0 and 21 containing either PBS, WT peptide pool adjuvanted with 5 µg WIV or IFA, CPL peptide pool adjuvanted with 5 µg WIV or IFA, or only 5 µg WIV. In all studies, animals were sacrificed by cervical dislocation and bleeding under anesthesia at day 35.

Dose finding by design of experiments

An initial dose-response study was performed by a design of experiments approach in order to detect potential interactions and effects between the GIL peptide antigen dose and WIV adjuvant dose on the induction of GIL-specific T cell responses in vivo. A full factorial design was created using MODDE 10.0.0 (Umetrics AB). Results from both flow cytometry and ELISpot methods were selected as response parameters. The limits of the doses ranged from 1-100 µg GIL peptide and 1-25 µg WIV. This resulted in a design with seven formulations including three center points. To accommodate for the high variability in animal experiments, it was chosen to administer each formulation to six mice, resulting in a design as shown in **Table SI**. The models were fitted using partial least squares and subsequently optimized by deleting non-significant terms (15), until the model performance parameters goodness of fit (R^2), goodness of prediction (Q^2), validity, and reproducibility were at their highest.

Intracellular staining and flow cytometry

T cell populations were assessed by flow cytometry. In short, single-cell suspensions of spleens were plated at a concentration of $2*10^6$ cells in a 48-well plate in RPMI medium (Life Technologies) with 10% Hyclone fetal calf serum (FCS, Thermo Scientific), and stimulated overnight with either medium, 50 ng peptide or PR8 WIV. Cytokine transport was inhibited by incubating with Golgi-plug (BD Biosciences) for 4 hours. Cells were subsequently stained with anti-mouse CD8-FITC (BD Biosciences), anti-mouse CD4-PE (BD Biosciences) and Live-dead-Aqua (Invitrogen). Next, cells were fixated with fixation/permeabilization buffer (BD Biosciences) and washed with permeabilization wash buffer (BD Biosciences). Finally, cells were stained intracellular with anti-mouse IFN-γ-APC (BD Biosciences), and IFN-γ^+ $CD8^+$ T cells were quantified on a FACS Canto II flow cytometer (BD Biosciences). Acquired data was analyzed with FlowJo version 10 for Mac OSX (TreeStar Inc.).

Enzyme linked immunosorbent spot assay (ELISpot)

An ELISpot assay was used to determine IFN-γ spot-forming units in restimulated splenocytes. 96-wells Multiscreen PVDF filter plates (Millipore) were activated by adding 25 µL 70% ethanol for 2 min, and subsequently washed three times with PBS. Plates were coated overnight with anti-mouse IFN-γ antibodies (U-Cytech) at 4°C. Next, filter plates were washed three times and blocked with 5% Hyclone FCS (Thermo Scientific) for 1 hour at 37°C. Subsequently, $4*10^5$ isolated splenocytes resuspended in IMDM medium, 5% FCS were added to each well with or without 50 ng relevant peptide, and incubated overnight at 37°C. After overnight stimulation, filter plates were washed five times and IFN-γ was detected using biotinylated anti-mouse

antibodies (U-Cytech) and 100 µL BCIP/NBT reagent (Thermo Scientific) per well. Spots were allowed to develop for 15 min after which the plates were thoroughly washed with tap water. Spots were counted using an A.EL.VIS ELISpot reader (A.el.vis). The number of IFN-γ producing cells in antigen-stimulated splenocytes was obtained after background correction (subtracting the number of spots produced by splenocytes incubated with medium).

Determination of association between peptides and WIV

The association of peptides to WIV particles was studied by quantification of unassociated peptide in a mixture of peptides and WIV. Peptides were admixed with WIV in similar concentrations used in the animal studies. WIV particles were subsequently spun down by ultracentrifugation for 2 hours at 30,000 g. Supernatant was collected and analyzed for peptides by mass spectrometry. Percentage of unassociated peptide was calculated by comparing peptide content in supernatants of peptide mixed with WIV to peptide content in supernatants collected from solutions without WIV. No traces of WIV proteins were detected in supernatants, indicating that WIV was successfully separated from the free peptide.

Hemolysis assay

Virosome fusion activity was determined by using a hemolysis assay as described previously (16). Formulations were mixed with human blood erythrocytes and 0.1M 2-(Nmorpholino) ethanesulfonic acid (MES) buffer with pH's ranging from 4.5 to 5.5, and incubated at 37°C for 30 min. The released hemoglobin was quantified in the supernatant after centrifugation by reading absorbance at 540 nm using a Synergy Mx reader (Biotek). Hemoglobin release from erythrocytes mixed with water was set as maximal hemolysis (100%).

Statistics

Results were statistically analyzed with a one-way ANOVA followed by a Tukey-post test for multiple comparisons. All statistical analyses were performed using GraphPad Prism 6.04 for Windows (GraphPad Software Inc.).

Results and Discussion

Proof-of-principle of WIV as an adjuvant

The adjuvant effect of WIV for GIL peptide was assessed in HLA-A2 transgenic mice. Mice received two vaccinations of either peptide adjuvanted with CpG, peptide adjuvanted with WIV or WIV alone. Splenocytes restimulated with GIL peptide were analyzed for peptide-specific T cells by flow cytometry. The specificity of $CD8^+$ IFN-γ^+ T cells was determined by comparing peptide-stimulated splenocytes with mock-stimulated splenocytes (**Figure 1A**).

As expected, 100 µg GIL peptide adjuvanted solely with 50 µg CpG did not induce any peptide-specific CTL response in mice (**Figure 1B**). This can be attributed to a number of factors, such as the absence of $CD4^+$ helper epitopes, and the lack of delivery of antigen and adjuvant, both of which are crucial for the immunogenicity of short peptide antigens (6). In contrast, only 1 µg GIL peptide antigen adjuvanted with 50 µg WIV induced peptide-specific responses in mice. Mice that received only WIV also showed considerable T cell responses, which was attributed to the high dose of WIV as described earlier (17, 18). The GIL epitope is indeed present in PR8 WIV, which explains the induction of GIL-specific T cells by WIV at high concentrations. Furthermore, WIV might still act as an adjuvant for peptides at lower doses. Thus, in order to maximize peptide-specific T cell responses with minimal use of WIV, a dose-finding study was conducted.

Dose-finding and interaction study between GIL peptide and WIV using design of experiments

To investigate which concentrations of both WIV and peptide were still able to induce a peptide-specific T cell response a dose-finding study of both WIV and peptide was conducted by using a DOE approach. DOE approaches are commonly used for the optimization of (bio)

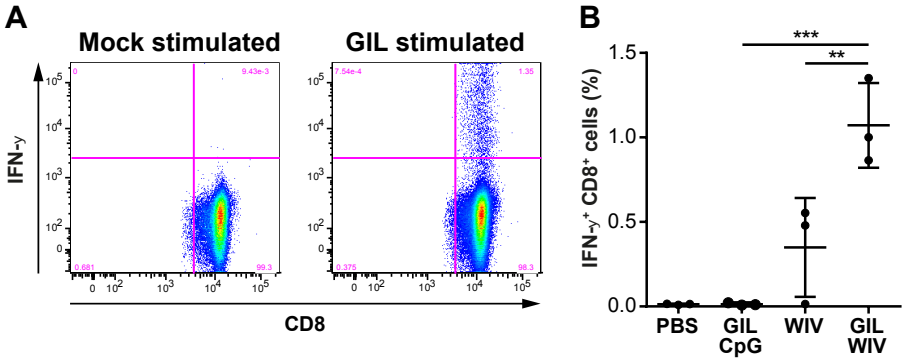

Figure 1. WIV acts as an adjuvant for peptide antigens.
Flow cytometry plot displaying specificity of IFN-γ^+ $CD8^+$ T cells in splenocytes from HLA-A2 transgenic mice immunized twice with 1 µg GIL peptide adjuvanted with 50 µg WIV (**A**). Splenocytes restimulated with GIL peptide were analyzed for IFN-γ^+ $CD8^+$ T cells with flow cytometry (**B**). Data is represented as mean ± SD, n=3; **p<0.01, ***p<0.001.

pharmaceutical formulations (19). However, there are currently no reports that utilized a DoE approach to assess the effect of formulation parameters on in vivo responses, such as cellular immune responses. A full factorial design was implemented by varying the peptide antigen dose from 1-100 µg, and the WIV adjuvant dose from 1-25 µg (**Table SI**).

The formulations, containing a variety of GIL peptide and WIV doses, were tested for their ability to induce GIL-specific T cell responses in HLA-A2 transgenic mice (**Figure 2A** and **2B**). A combination of 1 µg GIL peptide and 1 µg WIV was unable to induce CTL responses. However, when the GIL dose was increased to 100 µg, a significant increase of GIL-specific T cells was observed. This effect was also observed when the peptide dose was increased from 1 to 100 µg combined with a dose of 25 µg WIV. These results indicate that WIV is still able to boost the immune responses towards GIL peptide at concentrations as low as 1 µg WIV when combined with a high dose (100 µg) of peptide antigen.

Association between the GIL peptide and the WIV particles may be a contributing factor to the immunogenicity of peptide antigen. Thus, the association between the peptide and WIV was determined (**Table I**). Only low amounts of GIL peptide (1 µg) mixed with relatively high amounts of WIV (25 or 50 µg) showed some association. At higher peptide concentrations, association with WIV was negligible, which can be explained by the high molar abundance of GIL peptide compared to WIV or a low affinity between the two. In general, it can be concluded that association of the peptide to WIV did not have a significant influence on immunogenicity, contrarily to other delivery systems such as liposomes or virosomes (10, 20).

To assess the synergistic effect between the peptide antigen and the WIV adjuvant, a partial least squared (PLS) regression model was fitted for the T cell responses. Valid models were obtained for both flow cytometry (R^2=0.706, Q^2=0.633) and ELISpot (R^2=0.712, Q^2=0.629)

Table I. Association between GIL peptide and WIV at different concentrations.

Peptide (µg)	WIV (µg)	Unassociated peptide (%)
1	1	112 ± 10
100	1	111 ± 6
50	13	96 ± 5
1	25	77 ± 8
100	25	92 ± 20
1	50	87 ± 9

The fraction of unassociated peptide was determined in the supernatant by mass spectrometry. Data represents mean ± SD, n=3.

responses, and model prediction contour plots were generated (**Figure 2C** and **2D**). The contour plots illustrate that addition of WIV is essential for the peptide antigen to become immunogenic. Furthermore, the model indicates that theoretically the optimum of T cell responses has not been reached yet; however, the dose ranges used in this DoE model for both GIL peptide and WIV are at their maximum concerning peptide solubility and feasible WIV dose for human use, respectively.

The use of the DoE approach enabled us to illustrate the synergy between antigen and adjuvant, and to predict their effect on the cellular immune responses in vivo. The use of DoE in preclinical animal studies is difficult, due to the multiple factors, such as biological variability between animals and T cell assay variability, which can cause variability in each

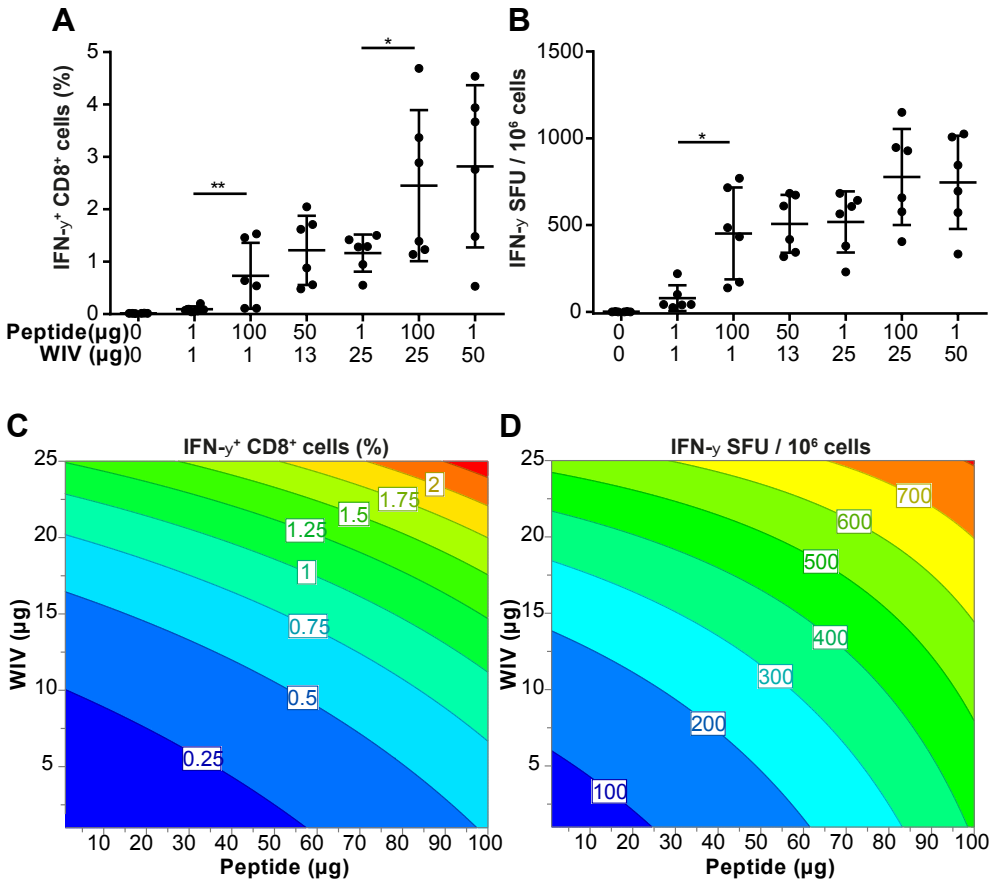

Figure 2. Dose-finding study of WIV and peptide by DoE.
HLA-A2 transgenic mice were vaccinated two times with different doses of GIL peptide and WIV. Splenocytes restimulated with GIL peptide were analyzed for peptide-specific $CD8^+$ T cells with flow cytometry (**A**) and ELISpot (**B**). Prediction contour plots obtained by DoE visualize the interaction between peptide antigen and WIV for the flow cytometry (**C**) and ELISpot (**D**) responses. The predicted responses are displayed in the white boxes. Data is presented as mean ± SD, n=6; *p<0.05, **p<0.01.

animal study. Nonetheless, the use of DoE provides valuable insight in the effect of antigen and adjuvant dose on the immune response in mice, and could be implemented in future vaccine development.

Involvement of WIV-peptide co-localization on immunogenicity

The viral ssRNA present in WIV is a TLR7 agonist, and likely contributes to the observed immunostimulating effect of WIV (11). For most adjuvants, including TLR ligands, co-localization with the antigen is necessary to provide local immunostimulatory signals.

In the model contour plots, it is predicted that a dose of 25 µg WIV combined with 100 µg GIL peptide is able to induce the highest peptide-specific T cell responses. Therefore, 25 µg WIV and 100 µg peptide was selected as the formulation to be used in mechanistic studies. To investigate the importance of co-localization, we administered 100 µg GIL peptide and 25 µg WIV s.c. at separate flanks, each draining to a different lymph node. When the peptide and WIV were administered separately at different sites, a significant decrease of the peptide-specific T cell response was observed (**Figure 3**). The observed response after separate vaccination is probably caused by the WIV only. It is likely that co-localization of WIV and GIL peptide in the endosomal compartment of APCs is required to benefit from the co-stimulatory adjuvant signal provided by the viral ssRNA (21). Moreover, a recent study suggested that particulate delivery of a TLR7 agonist can improve its immunostimulatory effect due to efficient delivery to the endosomal compartment (22), where TLR7 is located. WIV can deliver its own viral ssRNA in a similar manner, which might explain the immunostimulatory potential of WIV.

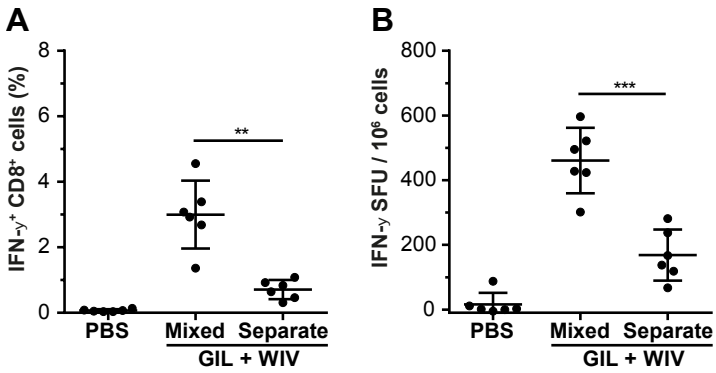

Figure 3. Effect of co-localization on WIV adjuvanticity.
HLA-A2 transgenic mice were vaccinated twice with 100 µg GIL peptide and 25 µg WIV either in one single flank (mixed) or separate flanks (separate). Splenocytes were restimulated with GIL peptide and analyzed with flow cytometry (**A**) or ELISpot (**B**). Data is presented as mean ± SD, n=6; **p<0.01, ***p<0.001.

Involvement of WIV membrane fusion activity on immunogenicity

Aside from co-localization, the role of membrane fusion activity of WIV was investigated. Fusion activity was shown to be important for the induction of cross-reactive T cell responses by WIV (23). Furthermore, other nearby molecules, such as the peptide antigen in our WIV-adjuvanted vaccine, can escape the endosomal compartment during membrane fusion of WIV with the endosomal membrane (24). Fusion activity might thus play a role in the adjuvanticity of WIV. Surprisingly, mice vaccinated with fusion-inactivated WIV mixed with GIL peptide still produced high amounts of peptide-specific T cells, comparable to those in mice receiving fusion-active WIV with peptide (**Figure 4A** and **5B**). A hemolysis assay confirmed the loss of pH-dependent fusion activity of WIV (**Figure 4C**). The current results indicate that fusion activity of WIV is not important for the induction of T cell responses against peptide antigens. The immunogenicity of antigens located inside the WIV particle itself might be compromised by fusion inactivation as shown before (23), but in the current study the admixed GIL peptide apparently was taken up and processed correctly by APCs regardless of WIV fusion activity.

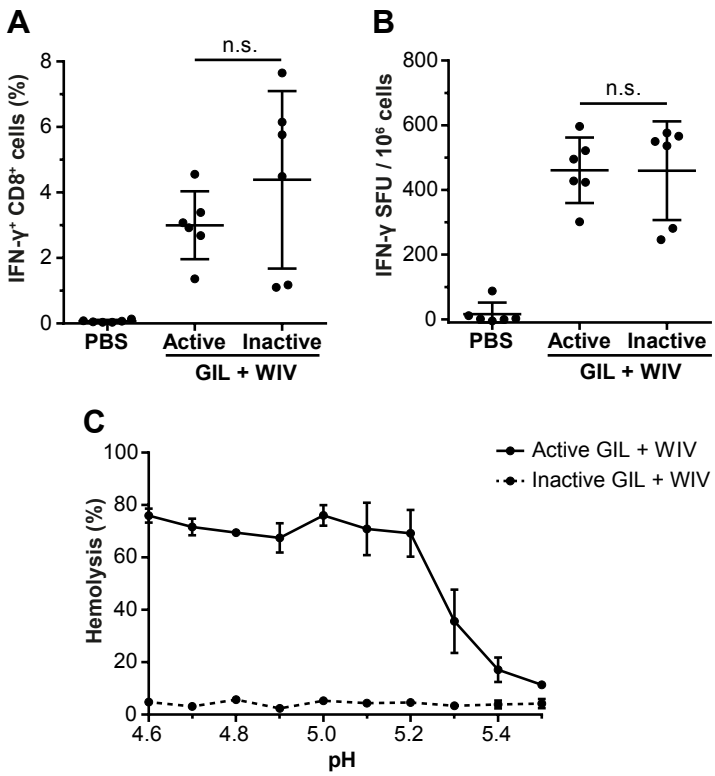

Figure 4. Effect of membrane fusion activity on WIV adjuvanticity.
HLA-A2.1 transgenic mice were vaccinated twice with 100 µg GIL peptide and 25 µg fusion-active (active) or fusion-inactive WIV (inactive). Splenocytes were restimulated with GIL peptide and analyzed by flow cytometry (**A**) or ELISpot (**B**). Fusion activity of active and inactive WIV-GIL formulations was determined by hemolysis assay (**C**). Immunogenicity data is presented as mean ± SD n=6; n.s.=not significant. Hemolysis data is presented as mean ± SD n=3.

This indicates that WIV is a robust adjuvant that retains its function even after loss of fusogenicity.

Adjuvation of multiple peptides by WIV

To investigate whether WIV also acts as an adjuvant for multiple peptides, a peptide pool of GIL and two additional human HLA-A*0201 restricted influenza epitopes, FMY and NML, was studied in combination with WIV. In addition, we selected three modified peptides to be combined with WIV, being G1, F5 and N53, which are CPLs derived from the three aforementioned WT peptide epitopes. Modification of WT peptides with non-proteogenic amino acids has previously shown to increase binding affinity with the MHC-I molecules, which might result in increased T cell responses (13). Since the selected epitopes are also present in WIV, a reduced WIV dose (5 μg) was chosen from the previously established prediction model. At this concentration, it was predicted that WIV still had an immunostimulating effect, while bringing the inherent T cell response generated by WIV itself to a minimum. Mice were vaccinated with either WT or modified peptide pools adjuvanted with WIV. As a control, peptide pools adjuvanted with IFA were included to compare the adjuvanticity of WIV to that of IFA.

The individual peptides in both WT and modified pools did not show significant association with the WIV particles, similar to the previous observations with the GIL peptide alone in this study (**Table SII**). Thus, it is unlikely that differences in induced immune responses by the peptide vaccines are caused by differences in association between peptide and WIV.

As seen previously in this study, the GIL peptide in the peptide pool was able to induce GIL-specific T cell responses after immunostimulation with WIV (**Figure 5A**). In contrast, IFA adjuvanted GIL peptide induced significantly lower T cell responses. The modified G1 peptide, however, was unable to induce potent GIL-specific responses, regardless of adjuvant. The G1 peptide adjuvanted with either WIV or IFA did induce a G1-specific T cell response, indicating that while the modified peptide was immunogenic in combination with an adjuvant, it failed to induce responses that reacted with the WT analog.

The WT FMY peptide was able to induce modest FMY-specific T cell responses in combination with either WIV or IFA (**Figure 5B**). Interestingly, the modified F5 peptide was able to induce significantly higher FMY-specific responses compared to the WT FMY peptide when adjuvanted with WIV. F5 peptide adjuvanted with IFA did not show such an increase, indicating that WIV is a more potent adjuvant than IFA for the F5 peptide. This difference was also observed with the F5-specific responses; F5 peptide induced significantly higher F5-specific T cell responses when adjuvanted with WIV than with IFA.

The subdominant NML peptide and the modified N53 were unable to induce any significant T cell responses, regardless of adjuvant (**Figure 5C**). IFA-adjuvanted peptides showed

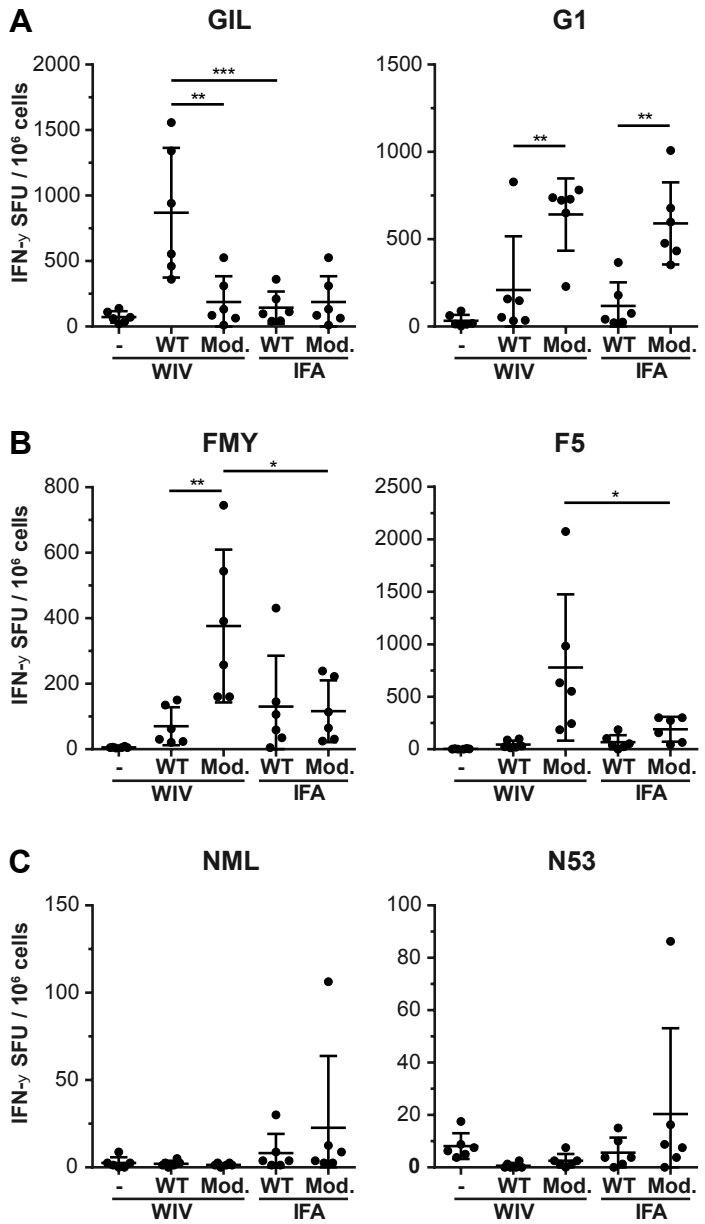

Figure 5. T cell responses against wild-type and modified peptides adjuvanted with WIV.
HLA-A2 transgenic mice were vaccinated twice with peptide pools containing 100 µg of wild type (WT) peptides (GIL, FMY and NML) or modified (mod.) peptides (G1, F5 and N53) adjuvanted with 5 µg WIV or 50% (v/v) IFA. Specific T cell responses induced by the peptide pools towards either GIL or G1 (**A**), FMY or F5 (**B**), NML or N53 (**C**) were determined for all groups. IFN-γ spot-forming units were determined with ELISpot. Data is presented as mean ± SD n=6; *p<0.05, **p<0.01, ***p<0.001.

incidental T cell responses in some animals, suggesting that IFA is a slightly better adjuvant than WIV for this specific peptide. It is unclear why WIV was not effective with NML and N53 peptides, while IFA-adjuvanted NML and N53 managed to induce a response in a few animals. It is possible that WIV contains epitopes which are more immunodominant than the NML epitope, decreasing the NML-specific T cell responses. However, since responses induced by IFA-adjuvanted NML and N53 peptides were not consistent in all animals, there was no significant difference between IFA- and WIV-adjuvanted groups.

These data indicate that WIV is a potent adjuvant for short peptides, both in WT or modified form. Other approaches such as peptide-lipid conjugates (25), liposomes (9), virosomes and nanoparticles have been used previously to increase the immunogenicity of short peptides (10, 26), but require multiple formulation steps and might not be suitable for every peptide due to differences in physicochemical attributes. In contrast, WIV can be readily mixed with peptide antigens, which is a simple process to scale up. Furthermore, WIV is already licensed and used for decades as an influenza vaccine, and recent studies show an excellent safety profile (27). With this prior knowledge on safety and tolerability, it should be feasible to include WIV in any prospective vaccine as an adjuvant.

Conclusion

While it is known that WIV possesses an innate adjuvant capacity, so far it has never been used as an adjuvant for peptide antigens. We showed that WIV is capable of effectively increasing the T cell response against GIL and FMY influenza peptides in HLA-A2 transgenic mice. Co-localization of antigen and adjuvant were necessary to induce a potent T cell response, but the membrane fusion capacity of WIV was not important for the immunogenicity of the formulation. Furthermore, we showed that WIV was also able to immunostimulate non-natural, modified peptides effectively. Due to the ease of production of WIV and its long time safety track record, it is an excellent candidate adjuvant for low-immunogenic antigens that induce cellular responses.

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Supplemental data

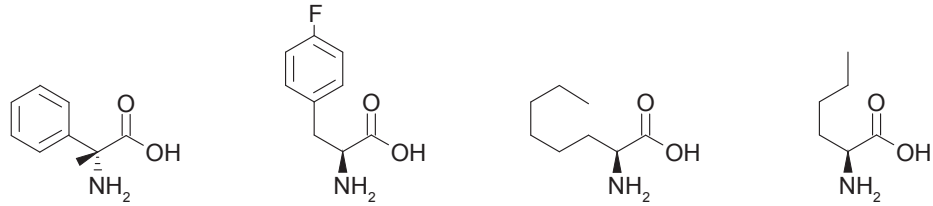

Figure S1. Nonproteogenic synthetic amino acids used for peptide modification.
The four nonproteogenic synthetic amino acids were introduced either in GILGFVFTL, FMYSDFHFI or NMLSTVLGV peptides, resulting in modified [am-phg]ILGFVFTL, [4-FPHE]MYSDFHF[2-AOC] and N[NLE] LSTVLGV peptides.

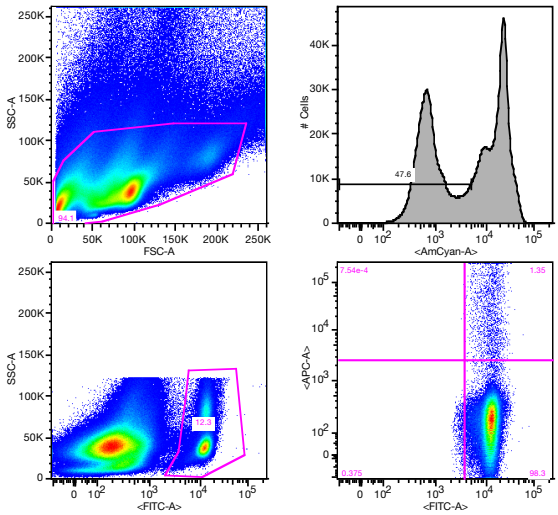

Figure S2. Gating strategy of CD8⁺ IFN-γ⁺ T cells in splenocytes.
An example of the gating strategy. The lymphocyte population was first gated (upper left). From this population, all live cells were selected (upper right). Subsequently, CD8⁺ cells were gated (lower left), after which a quadrant gate was created to select for CD8⁺ IFN-γ⁺ cells (lower right).

Table SI. Worksheet of full factorial design of dose-finding study.

All experimental points (initially seven) were duplicated and included six times to accommodate biological variation between the animals in the in vivo study.

No.	WIV	Peptide
1	1	1
2	1	1
3	1	1
4	1	1
5	1	1
6	1	1
7	25	1
8	25	1
9	25	1
10	25	1
11	25	1
12	25	1
13	13	50.5
14	13	50.5
15	13	50.5
16	13	50.5
17	13	50.5
18	13	50.5
19	1	100
20	1	100
21	1	100
22	1	100
23	1	100
24	1	100
25	25	100
26	25	100
27	25	100
28	25	100
29	25	100
30	25	100

Table SII. Association of peptides with WIV.

Peptides were admixed with WIV (in similar concentrations as used in vivo) and subsequently separated by ultracentrifugation. The fraction of unassociated peptide was determined in the supernatant by mass spectrometry. Data represents mean ± SD, n=3.

Peptide	Unassociated peptide (%)
GIL	87 ± 18
FMY	81 ± 22
NML	115 ± 21
G1	138 ± 34
F5	96 ± 8
N53	99 ± 12