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Adjuvants that stimulate TLR3 or NLRP3 pathways enhance the efficiency of influenza virus-like particle vaccines in aged mice

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Abstract

There is intense interest in the design and use of vaccine strategies against influenza to enhance protective immune responses in the elderly. To address the need for improved influenza vaccines for the aged, two inflammatory adjuvants, Imject® alum (a stimulator of the Nod-like receptor, Nalp3) and poly I:C (a toll-like receptor type 3 ligand), were used during vaccination with novel influenza virus-like particles (VLP). Adult (4 month old) or aged (24 month old) mice were vaccinated with VLPs alone or in combination with adjuvant. VLP-vaccinated adult mice were protected from a lethal influenza virus challenge without the use of either adjuvant. In contrast, only aged mice that were vaccinated with VLPs plus adjuvant survived challenge, whereas ~33% of the mice vaccinated with VLP only survived challenge. Mice vaccinated with adjuvant only did not survive challenge despite similar levels of activation of CD11b⁺/CD11c⁺ dendritic cells in the lungs. The protection was not associated with HAI titers or HA specific CD8⁺ T cells, since both adjuvants boosted the VLP-induced serum HAI titers and CD8⁺ responses in adult mice, but not aged mice. Influenza VLPs used in combination with two different inflammatory adjuvants during vaccination allow for the immune system to overcome the deficiency in the aged immune system to influenza virus infection.

Introduction

Influenza as a member of the *Orthomyxoviridae* family is an enveloped virus, and contains a segmented RNA genome (8 segments encoding for at least 11 proteins) of negative polarity. The segmented nature of the genome allows for the generation of viral diversity [1]. Influenza can be subdivided into three types (A, B and C). Influenza epidemics occur almost

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every winter around the globe, caused by circulating influenza A subtypes (H3N2 and H1N1) and influenza B. The annual influenza epidemics lead to approximately 300,000 hospitalizations and 36,000 deaths in the US alone. [2]. About 90% of all influenza related deaths occur among people older than 65 years of age [3]. Recently, highly pathogenic avian influenza (HPAI) of the H5N1 subtype has emerged as an extremely pathogenic virus in poultry and humans [4]. H5N1 has the potential to evolve into a virus that could cause a pandemic and therefore represents a serious threat for global health.

The immune control of influenza involves a combination of cellular and humoral responses [5, 6]. The viral antigens hemagglutinin (HA) and neuraminidase (NA) are the major surface glycoproteins of the virus and thus immune protective targets [7]. The goal of the influenza vaccine is to induce antigen-specific memory T and B cells. The memory B cells secrete high-affinity neutralizing antibodies to HA and NA [1]. The memory T-cell compartment, consisting of CD4+ and CD8+ T cells, can quickly respond by CD8+ T cells acquiring effector functions to kill infected cells and secrete pro-inflammatory cytokines that inhibit virus replication and CD4+ T helper cells in aiding B-cells and CD8+ T cells in their functions [8]. The efficacy of a vaccine is, like the control of an infection, dependent on the fitness of the immune system of the immunized individual. The immunization of immunocompromised people, very young children and especially of the aged is therefore less effective and more challenging.

Aging is associated with profound changes in the immune system, commonly termed immune senescence. The dysfunctions of the immune system in the aged occur within the innate and adaptive immune system [9, 10]. These defects include but are not limited to decreased numbers and functions of effector cells and lower levels of cytokines such as type I IFN. Vaccination against influenza is highly effective in preventing disease in healthy adults, but the aged population has a significantly lower response to seasonal influenza vaccines. A recent review of 31 influenza vaccine studies revealed that the antibody response in the aged compared to young adults is significantly lower [11]. Unfortunately, there is not much information available regarding influenza vaccine specific T-cell responses in the aged since the current inactivated virus vaccines elicit a poor cellular immune response, even though T cell-mediated immunity is crucial for the generation of high affinity neutralizing antibodies. The goal, therefore, is to develop new vaccine strategies to better protect aged individuals from infection with pandemic influenza by increasing the humoral and cellular immune responses to the vaccines.

One strategy to improve vaccine efficacy is by the addition of adjuvants. Adjuvants are believed to activate the innate arm of the immune system thereby enhancing the adaptive immune response to an antigen administered at the same time [12, 13]. There are only a few licensed adjuvants available for prophylactic vaccines in humans such as aluminum salts (commonly termed Alum), oil in water emulsions (MF59 and AS03) and the TLR4 ligand monophosphoryl lipid A (MPL) absorbed to Alum, known under the name AS04 [12, 14]. Several new adjuvants that are currently in clinical and pre-clinical trials target the toll-like receptor pathway, such as TLR3, TLR4, TLR5, TLR7/8 agonists and in the most advanced clinical studies, TLR9 agonists [12].

In this study, we assessed the pathology of H5N1 influenza infections and the efficacy of influenza H5N1 virus-like particles (VLP) as vaccine strategy in adult and aged mice. VLPs were comprised of the viral envelope proteins hemagglutinin (HA) and neuraminidase (NA) of H5N1 influenza and the Gag protein of HIV-1. Here, we demonstrate, that VLPs, produced in mammalian cells, provide protection against lethal H5N1 infection in adult and aged mice when used with either Imject® Alum, an analog of the approved aluminum salts or the investigational adjuvant, poly (I:C). Therefore, VLP vaccines can be very effective in aged animals via both the parenteral and mucosal routes.

Material and Methods

Viruses

Influenza reassortant H5N1 virus contained H5 HA and N1 NA derived from Influenza A/Indonesia/05/2005 (Indo/05/2005(H5N1)/PR8-IBCDC-RG2; termed Indo/05-PR8) or A/VN/1203/2004 (VN/04). The internal proteins were derived from the A/Puerto Rico/8/1934 (PR8) mouse adapted influenza A virus. The propagation of the H5N1-PR8 reassortant viruses was performed as previously described [15]. Female BALB/c mice were infected with 4×10^5 PFU (lethal dose) of the Indo/05-PR8 virus and 1×10^4 PFU (sub-lethal dose) of Indo/05-PR8. The viruses were administered intra-nasally in 50µl PBS to anesthetized BALB/c mice.

Sickness score

The sickness score included evaluation of ruffled fur, hunched back and activity as previously described [16]. The mice were evaluated for either the absence (score=0) or presence (score=1) in the case of ruffled fur and hunched back, or normal (score=0), reduced (score=1) and severely reduced (score=2) for the assessment of the activity level. Mice were evaluated daily and scored for the individual symptoms. The final score was the addition of each individual score. Therefore, the minimum score was 0 for a healthy mouse and 1-4 for a sick mouse.

Virus like particle vaccine

The H5N1 Indo/05 virus like particles (VLPs) were purified from HEK 293T cells which were transfected using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) with 5µg of each plasmid DNA expressing H5N1 A/Indonesia/05/2005 HA and H5N1 A/Thailand/1(KAN-1)/04 NA (codon optimized); and 10µg of plasmid DNA expressing HIV gag. Cells were incubated for 72 hours at 37C and supernatants containing VLPs were harvested. Supernatants were clarified by low speed centrifugation at 1000×g and sterile filtered using a 0.22µm filter. VLPs were purified by centrifuging clarified supernatant at 100,000×g through a 20% glycerol cushion and resuspended in PBS. Total protein was quantified via BCA protein assay (Pierce) and VLPs were aliquoted and stored at -80C.

Mice and vaccination

BALB/c mice (*Mus musculus*, females) were purchased from Harlan Sprague Dawley, (Indianapolis, IN, USA) and aged to 18-24 months of age. The adult BALB/c mice were 8-10 weeks of age. Mice, housed in microisolator units and allowed to free access to food

and water, were cared for under USDA guidelines for laboratory animals. For vaccination, mice were anesthetized with 0.03-0.04 ml of a mixture of 5ml Ketamine HCL (100mg/ml) and 1 ml xylazine (20mg/ml). Mice were vaccinated via the intramuscular or intranasal route with 10 µg of purified Indo/05 VLPs (in 50 µl volume) with or without 25µl Imject® Alum (Imject® Alum, Pierce, Rockford IL) or 50µg of the synthetic TLR3 ligand poly (I:C) (InvivoGen, San Diego, CA) as adjuvant and then boosted with the same dose at week 3 with or without 25µl Imject® Alum or 50µg of the synthetic TLR3 ligand poly (I:C). The mice were injected at day 0 and week 3 with the identical vaccine formulation. Imject® Alum contains an aqueous solution of aluminum hydroxide and magnesium hydroxide. The Imject® Alum -immunogen formulation was prepared according to the manufacturer instructions. Briefly, Imject® Alum was added dropwise with constant mixing to the VLPs so that the final volume ratio of Imject® Alum to immunogen is 1:1. The mixing is continued for 30 minutes after adding Imject® Alum.

Animals were monitored for survival and morbidity (weight loss, ruffling fur, hunched back, lethargy) weekly during the vaccination regimen and each day during viral challenge. Blood was collected from anesthetized mice via retro-orbital sinus. Blood was allowed to clot at room temperature. Tubes were centrifuged and sera was removed and frozen at -80 °C. All procedures were in accordance with the NRC guide for the Care and Use of Laboratory Animals and the Animal Welfare Act.

Isolation of cells from spleen and lung

Spleens and lungs were excised and placed in sterile PBS. For the isolation of lung cells, the lungs were placed in a petri dish and cut into small pieces with a razor blade. The lung pieces were incubated with 1µg/µl Collagenase D (Roche Diagnostics, Mannheim, Germany) and 0.01µg/µl DNase I (USB, Cleveland, OH, USA) for 30 minutes at 37 °C. A single cell suspension of lung and spleen cells was generated using a cell strainer (BD Biosciences, Bedford, MA, USA). Collected cells were centrifuged (1000×g for 5 min at 4 °C). The cells were resuspended in 2ml of RBC lysis buffer (eBioscience, San Diego, CA, USA) and incubated for 5 min at RT. PBS was added to each sample to neutralize the RBC lysis buffer and the cells were centrifuged. The supernatants were discarded, and the cells were then resuspended in RPMI medium with 10% fetal bovine serum (FBS) (cRPMI). Cell viability was determined by trypan blue exclusion staining.

Virus plaque assay

MDCK cells plated in 6-well tissue culture plates were inoculated with 0.2 ml of virus or lung cell supernatant serially diluted in Dubecco's modified Eagle medium (DMEM). Virus was adsorbed for 1h, with shaking every 15 min. Wells were overlaid with 1.6% w/v Bacto agar (DIFCO, BD Diagnostic Systems, Palo Alto, CA, USA) mixed 1:1 with L-15 media (Cambrex, East Rutherford, NJ, USA) containing antibiotics and fungizone, with 0.6mg/ml trypsin (Sigma, St. Louis, MO, USA). Plates were incubated for 2 days at 37 °C and plaques were visualized with crystal violet.

Serological assays

A quantitative ELISA was performed to assess anti-HA specific IgG in immune serum. Purified rHA (10 ng) was used to coat each well of a 96-well plate. Plates were blocked (25°C for 2 hr) with PBS containing Tween 20 (0.05%) and nonfat dry milk (5%) and then incubated with serial dilutions of each serum sample (25°C for 2 hr). Following thorough washing in PBS-Tween 20 (0.05%), samples were incubated (25°C for 1 hr) with horseradish peroxidase (HRP) rabbit anti-mouse IgG (1:5000) diluted in PBS-Tween 20 (0.05%) and nonfat dry milk (5%). The unbound antibody was removed, and the wells were washed. Samples were incubated with TMB substrate (1 hr), and the colorimetric change was measured as the optical density (O.D., 405 nm) by a spectrophotometer (BioTek, Winooski, VT, USA). The O.D. value of the age-matched naïve sera was subtracted from the samples using antisera from vaccinated mice. In order to measure the level of HA-specific IgG subtypes, biotinylated goat anti-mouse IgG1, IgG2a IgG2b, and IgG3 (1:100) antibodies were used to detect HA-antibody complexes.

IFN α levels in lung homogenates were quantified using a commercial mouse IFN α ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ) according to the manufacturer instructions. The IFN α ELISA detects IFN- α 1, - α 4, - α 5, - α 6, and - α 9. Colorimetric changes of enzyme substrates were detected at 450 nm wavelengths using a BioTek Power Wave microplate spectrophotometer (BioTek, Winooski, VT, USA).

Hemagglutination Inhibition activity

The hemagglutination inhibition (HAI) assay was used to assess functional antibodies to the HA able to inhibit agglutination of horse red blood cells (H5N1 viruses). The protocol was adapted from the CDC laboratory-based influenza surveillance manual and performed as previously described [15, 17].

Flow cytometry

A multi-parameter flow cytometry assay employing MHCI pentamer staining was used to detect influenza specific CD8⁺ T cells. The CD8⁺ T cell responses to nucleoprotein (NP) 147 (TYQRTRALV) are dominant followed by haemagglutinin (HA) 533 (IYSTVASSL) responses in influenza-virus-infected BALB/c mice. [18]. HA₅₃₃ was originally described for the haemagglutinin of A/PuertoRico/8/34 (H1N1). This epitope is conserved in H5N1 A/Indonesia/05/2005 and A/VN/1203/2004. The NP of the reassortant viruses is derived from A/PuertoRico/8/34. The cells were washed with FACS buffer (PBS, 1% FBS, 0.1% sodium azide) and were blocked with anti-CD16/CD32 mouse Fc receptor block (BD Biosciences, San Jose, CA, USA), followed by staining with a murine MHC-I encoded allele K^d-specific pentamer for the immunodominant HA₅₃₃ epitope or NP₁₄₇ epitope (ProImmune, Oxford, UK) conjugated with phycoerythrin (PE). Lymphocytes were then stained with anti-CD8 (Pacific Blue), anti-CD3 (PerCp), anti-CD19 (APC-Cy7), anti-CD44 (FITC) and anti-CD62L (PE-Cy7). The cells are then incubated with a viability dye (Molecular Probes, Invitrogen, Eugene, OR, USA) followed by fixation in 1% Formalin/PBS. For the analysis of innate cells, lung cells were stained with anti-CD11b (Pacific Blue), anti-CD11c (PE-Cy7), anti-CD40 (APC), anti-MHCII (Alexa Fluor 700) and anti-CD3, CD19, DX5 conjugated to FITC to eliminate lymphocytes and NK cells. The cells are then incubated with a viability

dye (Molecular Probes, Invitrogen, Eugene, OR, USA). Cells were fixed in 1% Formalin/PBS and the cells were acquired using a LSRII flow cytometer (BD Biosciences, San Jose, CA, USA). All antibodies were purchased from BD Biosciences, San Jose, CA or eBioscience, San Diego, CA. The data were analyzed with FlowJo software (Treestar, Ashland, OR, USA).

ELISPOT assays

The number of anti-HA, anti-HA₅₃₃ and anti-NP₁₄₇ specific murine splenocytes secreting IFN γ (mIFN γ) was determined by enzyme-linked immunospot (ELISPOT) assay (R & D Systems, Minneapolis, MN, USA). Briefly, pre-coated anti-IFN- γ plates were incubated (25 °C for 30 min) with cRPMI (200 μ l) and then were incubated with splenocytes (5×10^5 /well) isolated from infected mice 10 days post infection. Splenocytes were stimulated for 48 hours with overlapping HA peptide pools (BEI Resources; 1 μ g/ml per peptide) or peptides representing the immunodominant H2-K^d CD8⁺ T cell epitopes HA₅₃₃ and NP₁₂₇ or as a negative control the unspecific Ova₂₅₇ peptide (Pepscan Presto, Leystad, Netherlands). Additional wells were stimulated with PMA (50ng)/ionomycin (500ng) or were mock stimulated for 48 hours. In addition, IL-2 was added to all wells (10units/ml). Plates were washed 4 $\times$ with R&D wash buffer and were incubated (4°C, overnight) with biotinylated anti-mIFN γ . The plates were washed and then incubated (25°C for 2h) with streptavidin conjugated to alkaline phosphatase. Following extensive washing, cytokine/antibody complexes were incubated (25°C for 1h) with stable BCIP/NBT chromagen. The plates were rinsed with dH₂O and air dried (25°C for 2h) before the spots were counted by an ImmunoSpot ELISPOT reader (Cellular Technology Ltd., Cleveland, OH, USA).

Statistical analysis

Statistical analyses were performed using the Student's *t*-test and non-parametric data were analyzed employing the Mann-Whitney test or a two-way ANOVA. Significance was considered at a *p*-value <0.05.

Results

Morbidity and mortality after lethal and sub-lethal H5N1 infection in aged and adult mice

The goal of this study was to determine the efficacy of a H5N1 virus-like particle (VLP) based vaccine in aged and adult mice in combination with approved and novel adjuvants. Our research group previous analyzed the effects of seasonal influenza infections in aged and adult mice using a mouse-adapted influenza virus [16]. However, much less is known about HPAI infections in aged mice therefore, an influenza virus with the HA and NA from the influenza isolate, A/Indonesia/5/2005, pseudotyped onto the mouse adapted A/PR/8/1934 backbone virus was used to infect aged and adult mice.

Initially, aged (18-20 months) and adult (8-10 weeks) mice were infected with the Indo/05-PR8 virus (clade 2.1) and the mortality and morbidity was compared. Aged and adult mice infected with the lethal virus dose (4×10^5 PFU) rapidly lost 20% of their body weight over the course of the first 5 days post infection and required euthanasia by day 6 (Fig. 1A). Aged mice showed signs of sickness earlier and had enhanced morbidity compared to adult mice

as seen in the sickness score (Fig. 1B). Following a sub-lethal infection (1×10^4 PFU) with the Indo/05-PR8 virus, both adult and aged mice lost ~10-15% of their original weight by day 8-9 post-infection (Fig. 1C). However, aged mice had a statistically significant higher sickness score than adult mice and the clinical signs were sustained for 3 days longer than adult mice (Fig. 1D).

Innate and adaptive immune responses in aged and adult mice

In order to assess the efficacy of the immune system to control the virus, the lung virus load was determined in both age groups after sub-lethal infection. At day 3 and 6 post-infection, both aged and adult mice had similar viral lung titers ($\sim 1 \times 10^6$ to 1×10^7 pfu/g of lung tissue), but aged mice showed significantly higher virus titer at day 9 post infection compared to adult mice (Fig 2A).

At day 6 post-infection, IFN α peaked in the lungs of both age groups, but the IFN α levels in aged animals were significantly lower compared to the adult mice ($p < 0.05$) (Fig 2B). There were no significant differences in the percentage or total number of HA₅₃₃Pentamer⁺ specific CD8⁺ T cells in the lungs of aged and adult mice (Fig. 2C). In contrast, the percentage of NP₁₄₇Pentamer⁺ CD8⁺ T cells were significantly higher in adult (mean value: 6%) than aged (mean value: 3%) mice (Fig. 2C). At day 10, aged mice had higher numbers of lung CD8⁺ T cells ($\sim 2.1 \times 10^6$ CD8⁺ T cells/lung) than adult mice ($\sim 0.83 \times 10^6$ CD8⁺ T cells/lung) and therefore there was no difference in the number of NP₁₄₇⁺ CD8⁺ T cells present in the lungs of aged or adult mice (Fig. 2D). Analysis employing IFN γ -ELISPOT technology revealed that the aged mice had fewer influenza specific IFN γ producing T cells in their spleens compared to the adult mice (Fig. 2E).

VLP vaccine provides protection from lethal virus challenge in aged and adult mice

In order to assess the efficacy of influenza VLPs as vaccine candidates, aged and adult mice were immunized intramuscularly without the use of adjuvant. At week 5 following two immunizations of VLPs, all of the aged mice vaccinated with the Indo/05 VLPs had a 1:40 HAI titer against the homologous Indo/05-PR8 virus (Fig. 3A). Interestingly, only three of the seven (42%) adult mice had a HAI titer of 1:40 (Fig. 3A). Vaccinated mice were subsequently challenged intranasally with a lethal dose of Indo/05-PR8 virus. All immunized mice in both age groups were protected against a lethal challenge, whereas all non-vaccinated, naïve mice lost greater than 25% of their original body-weight by day 6 post-challenge and had to be euthanized (Fig. 3B and 3C). Even though aged mice were protected against death following challenge, these aged mice did have signs of disease including ~10% weight loss by day 6 post-infection (Fig. 3B). These mice recovered over the course of the next 16 days, but did not reach their original starting body weight. In contrast, the adult mice lost less than 5% of their original weight by day 6 and showed no overt signs of disease (Fig. 3B and 3C).

Alum adjuvant improves the efficacy of VLP vaccination in both age groups

In order to improve the efficacy of the VLP vaccines, both adult and aged mice were immunized via the intramuscular route with VLPs (10 μ g) in combination with Imject Alum®, which is similar to the widely used insoluble aluminum salts used as adjuvants in

human and animal vaccines [19]. At week 5, despite the addition of Imject® Alum, only 26% of the aged mice had HAI titers of 1:40 (GMT=20), whereas 85% of the adult mice had HAI titers 1:40 (GMT=58.56) (Fig 4A). Similarly, aged mice had a mean IgG endpoint dilution titer of 1:1455 and the adult mice of 1:12,800 (Fig. 4B).

Subsequent challenge of these immunized mice with a lethal dose of the Indo/05-PR8 virus protected 100% of the adult and aged mice from death. Additionally, the immunized aged mice lost ~5% of their initial body weight, which is comparable to the adult mice receiving VLP vaccine without the adjuvant (Fig. 3B and 4C). Therefore, despite no detectable increase in HAI titer in the aged animals receiving the VLP plus Imject® Alum adjuvant, the protective efficacy of the VLPs was increased. The adult mice immunized with the VLP vaccine plus Imject® Alum did not lose any body weight after virus challenge (Fig. 4C). In contrast, the non-immunized control mice in both age groups succumbed to the infection by day 6 (Fig. 4C). Next, we analyzed the frequency of the recalled influenza virus specific memory CD8⁺ T cells after challenge of VLP immunized and naïve aged and adult mice. At day 6 post infection, the CD8⁺ T cell response in the lungs of non-immunized mice in both age groups was low to undetectable (Fig. 4D). In contrast, adult and aged mice immunized with the VLPs plus Alum adjuvant demonstrated a slightly higher, albeit not statistically significant, low percentage of HA₅₃₃-pentamer⁺ CD8⁺ T cells (mean value adult: 0.3% and aged: 0.2%) specific for the HA₅₃₃ epitope in their lungs (Fig. 4D).

Dendritic cell activation by TLR3 ligand poly (I:C) in the lungs of adult and aged mice

In order to evaluate the efficacy of the VLP vaccine in inducing protective immunity in aged and adult mice via the mucosal route, a synthetic TLR3 ligand, poly (I:C), was successfully used. Mice were immunized intra-nasally with VLP alone, in conjunction with poly (I:C), or with poly (I:C) alone. After 24 hours, both adult and aged mice immunized with VLP alone had a significant increase in the number of cells per gram of lung tissue compared to non-immunized mice (Fig. 5A). Immunization with poly (I:C) alone significantly increased the number of cells in the adult mice. The same trend is visible in the aged mice, albeit not statistically significant (Fig. 5A). Curiously, the combination of VLP and poly (I:C) did not significantly increase the total number of lung cells compared to naïve mice ($p>0.05$) (Fig. 5A), however both adult and aged mice had a significant influx of conventional dendritic cells (CD11c⁺/CD11b⁺) (Fig. 5B); aged (naïve $4.4\% \pm 1.9$ versus VLP/poly (I:C) $10.29\% \pm 3.4$, $p<0.05$) and adult mice (naïve $3.2\% \pm 1.6$ versus VLP/poly (I:C) $12.7\% \pm 0.68$, $p<0.05$). In both age groups, the combination of VLP with poly (I:C) resulted in the largest increase in the percentage of conventional DCs in the lung compared to VLP or poly (I:C) alone (Fig 5B). Up-regulation of the activation markers MHC class II (Fig. 5C) and CD40 (Fig. 5D) on the lung DC surface 24 hours after immunization is shown by mean fluorescence intensity (MFI) flow cytometry analysis. Cell surface expression of MHC class II (MHCII) was significantly ($p<0.05$) up-regulated in adult mice after the stimulation with poly (I:C) compared to naïve mice, but there was no statistical significant increase over the naïve baseline observed after poly (I:C) treatment in the aged mice (albeit a trend in activation is detectable) (Fig. 5C). VLP immunization alone did not increase MHC class II up-regulation in either age group (Fig. 5C). Similarly, in adult mice, CD40 expression on the cell surface of lung DC was significantly up-regulated compared to naïve mice after poly (I:C)

immunization and the same trend is detectable after VLP/poly (I:C) combination immunization (Fig. 5D). Aged mice did require the combination of VLP plus poly (I:C) for statistically significant increase ($p<0.05$) in CD40 surface expression over the baseline in naïve mice (Fig. 5D). VLP immunization alone did not result in any measurable MHCII or CD40 increase over baseline expression (naïve mice) in adult and aged animals (Fig. 5C and D).

Mucosal immunization provides protection against lethal challenge

Having established that the H5N1 Indo/05 VLP vaccine in combination with poly (I:C) activates DC in adult and aged mice, groups of mice were immunized via the intranasal route with VLP vaccines with or without poly (I:C) as adjuvant. As a control, a group of mice received poly (I:C) alone. At week 5, the adult mice immunized intra-nasally with VLPs alone had anti-HA antibodies that recognized homologous recombinant HA with a mean geometric titer (GMT) of 1:1600 (Fig. 6A). In contrast, immunization with VLPs plus poly (I:C) increased the antibody titers in the adult mice to a GMT of 1:51,200. In comparison, aged mice vaccinated with VLPs alone had a GMT of 1:230, which was enhanced to a GMT of 1:1600 with the addition of poly (I:C) (Fig. 6A). Both aged and adult mice had a mixed IgG isotype profile, with IgG₁ and IgG_{2a} titers at equal ratios, as well as IgG_{2b} (data not shown). Approximately 60% of the adult mice had detectable HAI titers following vaccination with the VLPs alone, but all titers were $<1:40$ (GMT=11.5) (Fig. 6B). In contrast, 85% of the adult mice receiving the VLP plus poly (I:C) combination had detectable HAI titers of which 47% were $>1:40$ (GMT=37). Interestingly, none of the aged mice, regardless of the presence or absence of poly (I:C) as adjuvant had measurable HAI titers. As expected, immunization of aged and adult mice with poly (I:C) alone as a control, did not result in detectable HAI titers (Fig. 6B).

As previously noted, serum antibody responses and protection do not always correlate [20, 21]. Therefore, the mice were challenged with a lethal dose of Indo/05-PR8 virus to determine the protective efficacy of the intra-nasally administered VLP vaccine in both age groups. Aged mice receiving the VLP vaccine without adjuvant lost in average 15% of their initial body weight by day 6 and four out of six mice succumbed to the infection (Fig. 6C and Table 1). In contrast, the aged mice which received the VLP vaccine in combination with poly (I:C) did loose in average less than 5% of their initial body weight over the course of infection and 100% of the mice survived (Fig. 6C and Table 1). The adult mice receiving VLP with or without poly (I:C) all survived the challenge and there was no significant difference in weight loss between the groups (Fig. 6D and Table 1). The non-immunized control mice in both age groups lost greater than 20% of their body weight by day 6 and had to be euthanized (Fig. 6C and D). Additionally, HA-specific IgA, but not IgG, antibody secreting cells (ASC) were detected 6 days post infection in adult mice vaccinated without adjuvant (Fig. 6E). In contrast, HA-specific IgG and IgA antibody secreting cells were detected in the lungs of aged mice that received the VLP vaccine without adjuvant, but failed to provide a protective effect in these animals (Fig 6E).

Next, vaccine primed influenza specific memory CD8⁺ T cells following heterologous challenge were analyzed. Vaccinated mice were challenged with a lethal dose of the PR8

reassortant H5N1 A/VN/1203/2004 (VN/04-PR8) virus 6 days post infection. VLP vaccination alone resulted in a statistically significant recall of HA₅₃₃ specific CD8⁺ T cells in the aged mice after virus challenge compared to the primary response in non-immunized mice ($p < 0.05$) (Fig. 7). We observed the same trend in the VLP immunized adult animals (Fig. 7). Interestingly, poly I:C treatment alone resulted in a larger percentage of HA₅₃₃ CD8⁺ T cells in the lungs of the adult animals after challenge compared to non-adjuvanted mice (Fig. 7). This effect was also observed in the aged animals ($p < 0.05$). The combination of VLP plus poly I:C as adjuvant resulted in significant recall of influenza specific CD8⁺ T cells in both age groups after VN/04-PR8 challenge compared to the primary CD8⁺ T cell responses in non-immunized mice ($p < 0.05$) (Fig. 7).

Discussion

Influenza virus is a common respiratory virus causing disease in all age groups, but small children and the elderly are at highest risk for serious complications and the majority of influenza related deaths occurs among adults >65 years of age [22]. Immunization is the most effective weapon against infectious agents such as influenza virus. Unfortunately, due to the aging of the immune system, commonly termed immune senescence, vaccination efficiency and the ability to fight infections is decreased in the elderly, rendering this age group particularly vulnerable [23, 24]. New vaccination strategies specifically targeted at the elderly are therefore needed. In this study, we characterized the innate and adaptive immune responses elicited by a virus-like particle (VLP) based vaccine in combination with an approved or novel adjuvant in adult and aged mice.

The observed sustained weight loss and increased morbidity in the aged mice after sublethal infection suggests a less effective immune response in the aged compared to the adult animals. Interestingly, the aged and adult mice had similar lung virus titers early in infection, but the aged animals did have a ten times higher virus load in the lungs nine days post-infection, similar to previous observations where after A/PR8 infection virus was detectable in the lungs of aged mice for two additional days compared to adult mice [16].

Both innate and adaptive immune mechanisms are important for protection against influenza infection. The anti-viral cytokine, IFN α , is induced early after influenza infection, during the innate phase of the immune response [25]. The amount of IFN α expressed in the lungs was reduced six days post infection in the aged animals, which might be one reason for the observed delay in virus control in the aged animals (Fig. 2B). During infection with influenza A virus in mice the infected respiratory epithelium and plasmacytoid dendritic cells (pDC) are the major source of IFN α/β [26]. It has been found in aged mice that after TLR activation, aged murine pDC showed a defect in up-regulation of IFN-regulatory factor 7 during HSV-2 infection, leading to impaired IFN α expression [27]. One can speculate that this defect might play a role in the observed reduction in type I IFN induction after influenza infection in aged mice. Studies have been shown that in elderly humans TLR function is also impaired or dysregulated. (reviewed by [28]). Panda *et al.* observed reduced IFN α/β production after TLR7/9 activation by human pDC in individuals above 65 years of age [29].

The adaptive immune responses effective for virus clearance during a primary influenza infection consist mainly of virus specific CD8⁺ T cells eliminating virus infected epithelial cells and virus-specific IgM or IgA antibodies regulating free virus. A recent report indicates that serum IgA is increased in elderly humans [30, 31], however, these results may be misleading since these IgA molecules are predominantly monomeric and therefore cannot be transported across the mucosal surface as sIgA [32]. Serum IgA can initiate inflammatory reactions by binding to FcR on immune cell [33, 34], which might move to the lungs during the infection. However, IN VLP vaccinations of aged mice elicited both IgG and IgA antibody secreting cells (ASC), but these cells failed to prevent morbidity and mortality indicating that the local B cell responses are not predictive of protection in mice. The increased frequency of ASC in the cohort of vaccinated animals that displayed the most severe disease is likely due to increased viral burden and subsequent antigen-specific expansion of memory B cells. In contrast to previous reports [35, 36], we found relative IgG isotype frequency to be similar between adults and aged animals and as previously described, a decrease in innate function [16] could lead to decreases in non-neutralizing antibody function(s).

Influenza specific CD8⁺ T cells become detectable in the lung at day 5 post infection, with a peak response around day 9 post infection [37]. The lung cells were analyzed via MHC I pentamer flow cytometry recognizing CD8⁺ T cells specific for the H2-K^d immunodominant HA₅₃₃ and NP₁₄₇ epitopes. Aged and adult mice had the same number of virus specific HA₅₃₃ and NP₁₄₇ specific CD8⁺ T cells in their lungs at the peak of infection (Fig. 2 C and D). But, fewer IFN γ producing, influenza specific splenocytes, were detected in the spleen of aged mice after *ex vivo* peptide stimulation compared to the adult mice (Fig. 2E). These results indicate that a similar number of influenza specific CD8⁺ T cells are generated in the aged mice compared to adult mice, but the reduction in IFN γ secretion is indicative of a functional cell impairment, which is most likely a cause of the delay in virus clearance in the aged animals, leading to a longer recovery time.

These findings reflect the immune senescence phenotype that has been previously observed in aged mice [16, 38]. Influenza vaccination is efficient in young adults, but a high percentage of elderly people do not respond to the vaccination, which increases the chances of infection and disease complications in this population [39, 40]. Therefore, development of improved influenza vaccines for the elderly population is crucial. Virus-like particles are a new vaccine platform with several advantages over subunit or split vaccine approaches [41, 42]. One primary advantage of VLP vaccines is that the viral surface proteins are presented to the host in their correct three-dimensional structure, allowing them to maintain conformationally-dependent antigenic epitopes, as well as maintaining functional properties, without being able to replicate in the host. Additionally, the particulate nature of VLPs, promotes uptake by antigen-presenting cells, leading to enhanced activation of the innate and cellular arms of the immune response. Another successful strategy to improve vaccines is the addition of adjuvants [12, 43]. In this study, we merge both strategies by combining the VLP platform with the use of vaccine adjuvants.

Immunization with H5N1 Indo/05 VLPs without the addition of adjuvant protected both age groups from death after lethal H5N1 Indo/05-PR8 virus challenge, albeit resulting in some

weight loss in the aged animals (Fig. 3B and 3C). Baculovirus derived influenza VLPs have been shown to be successful in protecting aged mice from lethal H1N1 A/PR/8/34 infection when administered at a high dose [44]. Recently Sanofi-Pasteur licensed a high dose trivalent seasonal influenza vaccine for the use in persons over the age of 65 [45]. Unfortunately, the inactivated pandemic influenza H5N1 split vaccine is poorly immunogenic and requires already 12 times more antigen than the seasonal influenza vaccine in adults [46]. Further increasing the dose for the use in elderly would certainly create supply shortages in a pandemic situation. An alternative approach to antigen dose variation is the use of vaccine adjuvant, which has been shown to have dose-sparing effects [47]. Here we show, that the addition of Imject® Alum as adjuvant substantially reduced the observed weight loss in the aged animals after challenge when VLP vaccine was used at the same dose in both age groups, even though there was no measurable increase in hemagglutination inhibiting activity compared to VLP immunization alone (Fig. 4). Aluminum salts promote antigen uptake by antigen presenting cells and there are reports that aluminum salts activate innate immune cells via the NLRP3 inflammasome pathway [14]. Overall, the total HA specific antibody titer measured by ELISA and the HAI titer was lower in the aged animals compared to the adult animals, which has been previously reported [44, 48]. The reduction in weight loss in the aged mice when VLP was administered in combination with Imject® Alum might not be a result of increased quantity of hemagglutination inhibiting antibodies, but rather of an improved quality of the antibody response. VLPs administered with Imject® Alum elicited antibodies with higher affinity (measured via BIACORE® analysis) compared to VLP immunization alone (unpublished observations). Another reason for the reduction in morbidity in the aged mice could be the induction of influenza specific CD8+ memory T cells, which were recalled after virus challenge. A hallmark of immune senescence is the decline in T cell function, which might be overcome by the addition of adjuvant [22, 49, 50]. Adjuvant improves the uptake of the antigen by antigen presenting cells and/or activates the innate arm of the immune system, thereby enhancing the generation of the adaptive immune response [12]. In the elderly, both arms of the immune system, innate and adaptive, are impaired [16]. Enhanced activation of the innate immune system via the addition of adjuvant can therefore overcome the impairment of the adaptive immune response in the aged.

Influenza enters the host via the mucosal surfaces in the upper respiratory tract. Mucosal immune responses at the site of entry are therefore important to protect the host from infection with the pathogen. Administration of the vaccine via the mucosal route results in the induction of protective immune responses at the site of infection and induces enhanced heterotypic immunity than parenteral immunization [51, 52]. We therefore tested whether immunization via the mucosal route would improve immunity against influenza in aged mice. Since we already established that adjuvant improves the VLP vaccine when administered via the parenteral route (Figs. 3 and 4), we tested the efficacy of the synthetic TLR3 ligand poly (I:C) as mucosal adjuvant in aged mice. Poly (I:C) is an effective adjuvant for intranasal influenza vaccination in adult mice [47, 53]. TLR ligands activate the innate immune system through the up-regulation of co-stimulatory molecules on DCs and macrophages and the production of pro-inflammatory cytokines [54]. TLR3 is expressed on myeloid DCs and seems to play a key role in inducing the adaptive immune response after

viral infection [55]. Furthermore, TLR3 has an important role in cross-priming of CD8⁺ T cells [56]. Here, we found that poly (I:C) treatment alone and in combination with the VLP induced the influx of CD11b⁺/CD11c⁺ cells, also known as conventional DCs (cDC), into the lungs of aged and adult mice (Fig. 5B). DCs are professional antigen presenting cells (APCs) and among the first to encounter antigen. Interestingly there seems to be an increased influx of DCs when poly (I:C) and VLP are used in combination compared to treatment with the VLP or poly (I:C) alone (Fig. 5B). One can postulate that VLP, which are easily taken up by DCs due to their virus like structure activate the DCs to a small degree and in combination with the TLR3 ligand this becomes a synergistic effect. In order to present the antigen to CD4⁺ T cells the DCs up-regulate the expression of the MHC class II complex and increase the expression of co-stimulatory molecules such as CD40 [57]. Reduced antigen presenting activity of DCs has been shown in aged mice, resulting in impaired responses to antigen [58, 59]. Flow cytometric analysis of the infiltrating cDCs revealed that poly (I:C) treatment induced the up-regulation of MHCII and CD40 on the cDC in adult and aged mice. Intranasal immunization with the VLP in combination with poly (I:C) as adjuvant increased the induction of HA specific antibodies compared to VLP immunization alone in both age groups, but surprisingly there was no hemagglutination inhibiting activity detectable by HAI in the serum of aged mice (Fig. 6). In contrast, in the adult mice the addition of adjuvant improved the HAI titers in the serum. Despite the lack of measurable HAI titer in the aged mice, the intranasal VLP immunization alone protected 33% of the aged mice after virus challenge, whereas the addition of poly (I:C) substantially increased the efficacy of the VLP vaccine protecting 100% of the aged mice from death and weight loss after challenge. The adult mice did not require the addition of adjuvant for the VLP vaccine to protect all mice from death and weight loss. The addition of poly (I:C) most likely improved antigen presentation in the aged animal leading to an improved maturation of the adaptive immune response. The protective immune parameters seem to be independent of HAI responses, which has been previously shown in the ferret model, where VLP immunized ferrets, were protected from lethal H5N1 virus challenge despite the lack of measurable HAI titers [60]. The mice might be protected through other humoral responses such as the induction of mucosal IgA, antibodies with antibody-dependent cytotoxic lysis activity, or antibodies directed towards NA thereby blocking viral release of nascent influenza viruses and studies are underway to determine the nature of the protective agent(s). Alternatively, the induction of innate immune responses by poly I:C during VLP vaccination could have enhanced the induction of memory cytotoxic T-cells. Interestingly, the poly (I:C) immunization alone increases the presence of influenza specific CD8⁺ T cell responses in the adult and aged mice early after infection, maybe due to lasting activation of the innate immune system and/or altered cytokine milieu in the lung leading to an expedited response kinetic (Fig. 7). Even though administrating poly I:C only did increase the number and activation state of DCs (CD11b⁺/CD11c⁺) in the lungs of aged mice, it was only the combination of VLPs and poly I:C that elicited protective immune response in the aged animals (Fig. 5). It has recently been shown that intranasal administration of a stabilized chemical analog of double-stranded RNA (PIKA) prior to influenza infection reduces viral titers and protects the mice from death by changing the cellular composition and the cytokine milieu in the lungs [61].

For the first time, we show that mammalian cell-derived influenza virus-like particles in combination with adjuvant administered via the parenteral or mucosal routes constitute a successful vaccine strategy in both the adult and aged. Advantages of mammalian cell derived VLP in comparison to the baculovirus produced counterparts are the presence of mammalian cell derived post-translational modifications on the proteins and easier purification.

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Highlights

Need to improve influenza vaccines for aged

- > VLP-vaccinated adult mice survive challenge with or without an adjuvant
- > Aged mice need an TLR adjuvant administered intranasally with VLPs for survival
- > Protection is not associated with HAI or CD8+ T cell responses

Figure 1. Morbidity and Mortality after H5N1 Indo/05 challenge

Adult and aged mice (n=5) were challenged via the intranasal route with a lethal or sub-lethal dose of the Indo/05-PR8 virus. Mice were monitored for weight loss and signs of sickness (ruffled fur, hunched back and lethargy). Shown in (A) is the weight loss and in (B) the sickness score after lethal dose virus challenge (4×10^5 PFU). In figure (C) the weight loss and in figure (D) the sickness score after low dose virus challenge (1×10^4 PFU) is shown. Weight loss is plotted as percentage of the initial body weight (mean $\pm$ SEM). Mice that lost greater than 20% body weight were sacrificed. * indicates $p < 0.05$.

Figure 2. Innate and adaptive immune responses to virus challenge

Adult and aged mice (n=5) were challenged with a sub-lethal dose of the Indo/05-PR8 virus and (A) the virus titer in the lung and (B) the IFN α levels in the lungs at day 3, 6 and 9 post infection were determined (mean $\pm$ SEM). Additionally aged and adult mice were infected for 10 days and (C) the percentage of the HA₅₃₃ and NP₁₄₇ epitope specific CD8⁺ T cells was determined by pentamer staining and in (D) the number of HA₅₃₃-pentamer and NP₁₄₇-pentamer positive CD8⁺ T cells is shown. The whiskers in (C) and (D) designate the minimum and maximum values. The bar within the box designates the median. (E) IFN γ ELISPOT analysis with splenocytes collected 10 days post infection. * indicates p<0.05.

Figure 3. VLP vaccine provides protection in aged and adult mice from virus challenge

At week 5, adult and aged mice (n=7) vaccinated with H5N1 Indo/05 VLP vaccine via the intramuscular route were challenged intranasally with a lethal dose of Indo/05-PR8 virus and monitored daily for weight loss and mortality. (A) Prior to virus challenge serum was collected at week 5 and the serum HAI antibody responses were assessed (GMT $\pm$ SEM). Data were analyzed using a one-way AONOVA test. In (B) the percentage of original body weight and in (C) the percentage of survival following challenge with Indo/05-PR8 virus is shown. Mice that lost greater than 25% body weight were euthanized. Naïve mice were unvaccinated. Statistical analysis was performed by determining the p-value from comparison of percentage of initial body weight of aged vs. adult mice receiving the VLP and comparison of the percentage of initial body weight of the naïve aged vs. adult mice at the respective time points. * indicates $p < 0.05$.

Figure 4. Alum as adjuvant improves VLP vaccine in both age groups

Groups of mice (n=8-10) were immunized with H5N1 Indo/05 VLP vaccine in combination with Imject® Alum via the intra-muscular route. Antibodies elicited to the VLP vaccine with or without Imject® Alum were determined by (A) hemagglutination inhibition assay and (B) ELISA after the second immunization (week 5, ELISA compares VLP immunization in combination with Imject® Alum in both age groups) ($\pm$ SEM). (C) Mice were challenged with a lethal dose of Indo/05-PR8 virus and monitored daily for weight loss and mortality. The data are plotted as percentage of the initial body weight. Statistical analysis was performed by determining the p-value from comparison of percentage of initial body weight of aged vs. adult mice receiving the VLP + ImjectAlum and comparison of the percentage of initial body weight of the naïve aged vs. adult mice at the respective time points. (D) Separate groups of VLP+Imject® Alum immunized and naïve mice of both age groups were sacrificed on day 6 post infection and HA₅₃₃-pentamer staining was performed. The percentage of HA₅₃₃ pentamer + CD8⁺ T cells is shown for both age groups. The whiskers in (D) designate the minimum and maximum values. The bar within the box designates the median. * indicates p<0.05.

Figure 5. TLR agonist Poly (I:C) activates dendritic cells in aged and adult mice

Groups of aged and adult mice (n=3-5) were treated for 24 hours with H5N1 Indo/05 VLPs with or without 50µg of the synthetic TL3 ligand poly (I:C) via the intranasal route. (A) The total number of lung lymphocytes was determined in mice that received VLP, VLP plus poly (I:C) or poly (I:C) alone and compared to naive mice. The isolated lung cells were evaluated for viability by trypan blue exclusion. The number of cells was adjusted to the weight of the lung and reported as cells per g of tissue. (B) The percentage of CD11c⁺/CD11b⁺ cells (conventional DC, cDC) in both age groups was determined by flow cytometry. The activation of the cDCs was assayed via the expression of (C) MHC class II and (D) CD40. The mean fluorescence intensity (MFI) is shown. The whiskers designate the minimum and maximum values. The bar within the box designates the median. * indicates p<0.05.

Figure 6. Mucosal immunization with VLP requires adjuvant activity in aged mice

Mice (n=6-8) were immunized with H5N1 Indo/05 VLP vaccine alone or in combination with poly (I:C) via the intranasal route. As controls, mice were immunized with poly (I:C) alone or were non-immunized. (A) Antibodies elicited to the vaccine were determined by ELISA and (B) hemagglutination inhibition assay. In (C) aged and (D) adult mice were challenged with a lethal dose Indo/05-PR8 and monitored daily for weight loss and mortality. The data are plotted as percentage of the initial body weight. Statistical analysis was performed by comparing the percentage of initial body weight of the group receiving the VLP alone to the group receiving the VLP in combination with poly (I:C). (E) Mice immunized with the VLP vaccine alone were sacrificed 6 days post infection (DPI) and the number of HA-specific IgG and IgA antibody secreting cells in the lungs was determined. * indicates $p < 0.05$.

Figure 7. T cell recall responses

Mice were immunized with H5N1 Indo/05 VLP vaccine alone or in combination with poly (I:C) via the intranasal route. As controls, mice which were immunized with poly (I:C) alone and non-immunized mice were assayed (n=5-7). (A) The percentage of HA₅₃₃ pentamer⁺ CD8⁺ T cells in the lungs was determined 6 days post heterologous challenge with H5N1 Vietnam/04-PR8. The bar within the box designates the median. * indicates p<0.05

Table 1

Challenge Survivors.

	Poly I:C	VLP	VLP + Poly I:C	Naive
Aged	0/7 (0%)	2/6 (33%)	7/7 (100%)	0/5 (0%)
Adult	0/5 (0%)	5/5 (100%)	5/5 (100%)	0/5 (0%)

Percentage of mice surviving challenge. The number (percentage) of survivors are shown for the different age groups with each vaccine.