Tailored Vaccines Targeting the Elderly Using Whole Inactivated Influenza Vaccines Bearing Cytokine Immunomodulators

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Influenza and its complications disproportionately affect the elderly, leading to high morbidity and mortality in this ever-increasing population. Despite widespread vaccination efforts, the current influenza vaccines are less effective in the elderly; hence newer vaccine strategies are needed to improve their efficacy in this age group. We have previously shown that co-presentation of cytokines on the surface of inactivated influenza virus particles affords better protection from lethal homotypic viral challenge in young adult mice than conventional non-adjuvanted whole inactivated vaccine. Here, we determined the efficacy of these vaccine formulations in Balb/c mice “aged” to 17 months (“aged mice”) along with the addition of a membrane-bound interleukin-12 (IL-12) vaccine formulation. Our investigations found that a single low-dose intramuscular vaccination with inactivated whole influenza vaccine co-presenting IL-12 was sufficient to provide enhanced protection from subsequent influenza challenge as compared with non-adjuvanted whole inactivated vaccine. Our results indicate that incorporation of cytokines such as IL-12 in a membrane-bound formulation in whole inactivated vaccine may provide a means to lower the vaccine dose while eliciting enhanced protective responses in the elderly, an age group that responds poorly to current vaccination regimens.

Introduction

THE ELDERLY POPULATION, which encompasses people of age 60 years or older, is steadily increasing and is estimated to double from the current 11%–22% by the year 2050 worldwide (Nations 2009). Considering the significance of this segment of the population in the coming years and to meet the increasing demands of vaccines and drug production, new strategies should be developed to reduce the production time and costs of vaccines without compromising efficacy. Improving on existing viral vaccine platforms while maintaining broad efficacy across the adult lifespan of individuals would greatly reduce the costs associated with yearly vaccination regimens.

Influenza is a significant cause of morbidity and mortality in humans, typically resulting in 226,000 hospitalizations and an average of 36,000 deaths annually in the United States (Centers for Disease Control and Prevention 2009). Importantly, 90% of influenza-related deaths occur in the 65 years and above age group (Thompson and others 2003). This is also the risk group most likely to require hospitalizations due to influenza-associated secondary complications such as pneumonia that increased by 20% from 1988 to 2002 despite widespread vaccination efforts (Fry and others

2005) and represent almost 64% of the total influenza economic burden (Molinari and others 2007). This is mainly due to a weakened immune system or immunosenescence, age-associated frailty, and comorbidities, all of which are challenges to the development of effective influenza vaccine (McElhaney and others 2012).

Commercially available influenza Fluzone[®] High-Dose vaccine—approved in 2009—contains four times [60 µg hemagglutinin (HA) per subtype] the amount of antigen as compared with seasonal trivalent inactivated influenza vaccine (TIV; 15 µg HA/subtype) (Cate and others 2010). Despite superior induction of anti-viral antibody responses after Fluzone High-Dose vaccination, more adverse side effects were noted and it remains unclear whether it provides superior protection against influenza illness as compared with TIVs (Sullivan and others 2010). TIVs prevent 70%–90% of laboratory confirmed influenza illness during antigenic mismatch between vaccine and circulating viral strains (Jefferson and others 2007). In contrast to a greater than 90% vaccine efficacy in young adults, the efficacy in the adults over the age of 65 is low, ranging from 39%–69%, and varies significantly among studies (Smith and others 2006; Wilshut and others 2006; High 2007) due to different parameters used as correlates of protection (Gross and others 1995; Rivetti and

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others 2006). In the elderly population (over 64 years of age) with co-existing illnesses, successive influenza vaccination for at least three seasons was associated with reductions in hospitalizations for pneumonia and influenza by 48%–57%, chronic respiratory conditions (27%–39%) as well as for congestive heart failure (37%) (Nichol and others 1994).

The current available influenza vaccines in the elderly are designed to induce robust anti-viral antibody responses; however, vaccine-induced antibody responses are typically lower in the elderly when compared with younger individuals (Goodwin and others 2006), which was responsible for diminished efficacy of influenza vaccines in elderly (Sasaki and others 2011). There is only marginal information about the influenza vaccine-induced T cell-specific responses in older individuals, as the current vaccines elicit generally poor cellular immune responses. However, cell-mediated immunity is considered a more appropriate correlate of protection in the elderly, as cytotoxic T cells are involved in clearance of virus from the respiratory tract and provide clinical protection from disease (Ennis and others 1981). Hence, new vaccine strategies should include the activation of both humoral and cellular immune responses in the elderly, either through the use of more immunogenic vaccines or by using potent adjuvants and other immune modulators to enhance host immunity (Derhovanessian 2011).

Our laboratory has previously demonstrated that membrane-anchored immunomodulators co-presented on whole inactivated influenza virus particles (WIV) were able to serve as bioactive moieties stimulating humoral immune responses and providing superior protection against lethal challenge when used at low doses compared with traditional non-adjuvanted WIV in young adult mice (Herbert and others 2009). Our cytokine bearing influenza vaccine (CYT-IVAC) approach provides a novel vaccine platform to incorporate bioactive immunomodulators or cytokines on purified inactivated influenza virus produced by cell culture technology, eliminating the potential need of additional adjuvant substitutions. These vaccines may provide a means to significantly reduce the antigenic dose required to achieve protective immunity in the elderly. Here, we have tested the hypothesis that membrane-bound immunomodulators [interleukin-2 (IL-2), IL-4, and IL-12] presented in direct context with WIV will serve as adjuvants and elicit superior protective immune responses in “aged” mice, defined as >17 months of age.

Materials and Methods

Construction of expression plasmids and purification of CYT-IVACs

The murine IL-2 and -4 (mIL-2 and mIL-4) genes fused in frame to a short stalk, transmembrane, and cytoplasmic tail encoding domain derived from the HA gene of influenza A/WSN/33 has been previously described (Herbert and others 2009; Yang and others 2009). A murine single chain IL-12 p35p40 gene was amplified from pORF-mIL-12(p35p40) and fused in frame to the HA gene as described elsewhere (Khan 2012). The establishment of Madin–Darby Canine Kidney (MDCK) cell lines constitutively expressing the murine IL-12/HA fusion construct at the cell surface was established in a similar fashion as described for mIL-2/HA and mIL-4/HA (Herbert and others 2009; Yang and others 2009). Cell surface expression of the respective cytokines was verified by im-

munofluorescent surface staining using cytokine-specific antibodies. For CYT-IVAC production, MDCK cytokine-expressing cell lines were infected with Influenza A virus (A/PR/8/34) at a multiplicity of infection of 1 for 36–48 h at 37°C. Virus-containing supernatants were harvested, pre-cleared of cell debris by centrifugation at 1,500 rpm for 15 min at 4°C. Virions were banded and purified by ultracentrifugation through two sequential 10%–26% iodixanol continuous gradients (Optiprep™; Axis-Schield) at 18,000 rpm for 45 min at 4°C, and chemically inactivated by β -propiolactone (Acros Organics) for 30 min as previously described in detail (Herbert and others 2009).

Quantitation of membrane-bound IL-12 in viral particles

Quantitation of cytokine (IL-12) was performed using an IL-12/IL-23p40 specific bead assay as described by the manufacturer (eBioscience). Briefly, serial dilutions of virus particles were solubilized in the presence of 0.25% Triton X-100 before incubation with anti-IL-12 specific beads. Known quantities of recombinant murine IL-12 (eBioscience) were resuspended in 0.25% Triton X-100 to establish a standard curve for quantitation. In addition, as a negative control, CYT-IVAC formulations bearing IL-2 or IL-4 were evaluated for non-specificity of the IL-12 bead assay. In the present study, 23 pg (± 2 pg) of IL-12 was determined to be associated with 1 μ g of CYT-IVAC vaccine.

Vaccination and challenge studies

Seventeen-month-old female Balb/c mice (*Mus musculus*) were purchased from the National Institute of Aging, and all animal experiments were performed based on the guidelines of National Institutes of Health (NIH) and approval of Institutional Animal Care and Use Committee of Virginia Tech. For prime/boost vaccination, mice were administered 1 μ g (total viral protein) of whole inactivated virus vaccine (WIV, A/PR/8), CYT-IVAC^{mIL-2}, CYT-IVAC^{mIL-4}, and CYT-IVAC^{mIL-12} ($n = 22$ mice/group) intramuscularly (I.M.) in the right hindquarter. Phosphate buffered saline (PBS) served as a negative control ($n = 22$ mice/group). Animals were subsequently administered a booster dose of vaccine (0.3 μ g) I.M. in the left hindquarter at day 21. Blood was collected pre-booster vaccination and post-booster vaccination on days 16 and 28, respectively, via the retro-orbital sinus. At day 45 post-vaccination, lightly anesthetized animals ($n = 6$ mice/group) were challenged intranasally with mouse-adapted influenza virus A/PR/8/34 [1,000 (50% tissue culture infectious dose units) TCID₅₀/50 μ L]. All animals were euthanized at day 5 after challenge using Fatal-Plus (100 μ L intraperitoneal) and blood, spleen, and lung tissues were collected post-mortem. For the single-dose vaccination regimen, female Balb/c mice were vaccinated I.M. with 0.5 μ g of inactivated WIV, CYT-IVAC^{mIL-4}, and CYT-IVAC^{mIL-12} ($n = 23$ mice/group), and blood was collected on day 21. Animals were challenged on day 100 post-vaccination and monitored for a period of 16 days for reduction in weight loss and survival.

Enzyme-linked immunosorbent assay

Influenza virus-specific immunoglobulin G (IgG), IgG1, and IgG2a in sera were determined by enzyme-linked immunosorbent assay (ELISA) as previously described using

purified whole virus as an antigen (Herbert and others 2009). Data are presented as mean absorbance minus blank reduction. Specific anti-viral antibody was defined as ≥ 4 -fold (mean + 2 standard deviation) above the PBS group.

Neutralization assay

Vaccination-induced virus-neutralizing antibodies in serum collected at day 28 post vaccination were determined as previously described (Herbert and others 2009). Briefly, heat-inactivated sera were serially diluted 10-fold in PBS and incubated with an equal volume of PBS containing 100 hemagglutination units of influenza virus A/PR/8/34 for 1 h at room temperature. MDCK cell monolayers were subsequently infected with the sera-virus mixtures for 1 h at 37°C, washed, and incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-(tosylamido-2-phenyl) ethyl chloromethylketone (TPCK)-treated trypsin (2 µg/mL) for 72 h at 37°C. Neutralization titers were recorded as the reciprocal of the last serum dilution that completely blocked virus-induced cytopathic effect (CPE) (Weinberg and others 2010).

Enzyme-linked immunosorbent spot assay

Enzyme-linked immunosorbent spot (ELISPOT) plates (Millipore) were coated with capture antibodies for murine interferon gamma (mIFN γ) (BD Bioscience), mIL-2, mIL-4, and mIL-17A (eBioscience) overnight at 4°C according to the manufacturer's instructions. Briefly, freshly isolated splenocytes (4×10^6 cells/mL) were cultured with inactivated, purified influenza virus A/PR/8/34 antigen or inactivated vesicular stomatitis virus (VSV) (Indiana strain) antigen (10 µg/mL) as a negative control in triplicate and incubated at 37°C for 24–36 h. Media alone and concanavalin A/phorbol 12-myristate 13-acetate (PMA)/ionomycin-treated cells (20 µg/100 ng/1 µg per mL, respectively) served as negative and positive controls, respectively. After incubation with biotinylated detection antibody, plates were incubated with avidin-horseradish peroxidase and developed in the presence of 3-amino-9-ethylcarbazole substrate (BD Bioscience) for 60 min in the dark. Spots were counted using an AID ELISPOT plate reader (AID EliSpot).

Quantitation of viral loads in lung tissue

Viral burden (TCID₅₀/g lung tissue) was determined on *ex vivo* homogenized lung samples (Herbert and others 2009). Briefly, confluent MDCK cells in a 96-well tissue culture plate were incubated with serial 10-fold dilutions of lung homogenate prepared in PBS for 1 h at 37°C, rinsed, and cultured in DMEM supplemented with 1.5 µg/mL of TPCK-trypsin (Sigma-Aldrich) for 72 h at 37°C. Virus-induced CPE was recorded, and 50% tissue culture infectious dose units (TCID₅₀/mL) were determined by the Reed–Muench method (Reed and Muench 1938).

Intracellular cytokine staining of lung lymphocytes

Pooled lung samples ($n=6$ /group) were dissociated by incubation in glucose-potassium-sodium chloride buffer supplemented with 1.8 mg/mL Collagenase type 4 (Worthington Biochemical), 0.1 mg/mL deoxyribonuclease I (DNaseI) (Sigma-Aldrich), and 10% fetal bovine serum (FBS)

at 37°C for 30 min. After passage through 70 and 40 µm cell strainers, single-cell suspensions were subjected to erythrocyte lysis (155 mM ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM ethylenediaminetetraacetic acid) and washed extensively with PBS. Cells were cultured (3×10^6 cells/mL) with 50 ng/mL PMA and 10 µg/mL ionomycin in DMEM containing 10% FBS (Sigma-Aldrich) for 6 h in a 24-well plate in the presence of Brefeldin A (eBioscience) at 37°C. Cells were collected, washed in flow staining buffer (eBioscience), and stained with antibodies for lymphocyte surface markers such as CD3-APC, CD4-PECy7, and CD8-Pac blue (eBioscience) for 20 min in dark at 4°C. Cells were subsequently incubated in intracellular fixation and permeabilization buffer (eBioscience) and further incubated with phycoerythrin-conjugated antibodies directed against IFN γ (eBioscience) or granzyme B (GrB; Invitrogen) for 20 min in the dark. Cells were washed in PBS and analyzed using a BD FACS Aria.

Statistics

Statistical differences between vaccine and control groups were assessed using GraphPad Instat version 3.0a for Macintosh (GraphPad Software). One-way analysis of variance (ANOVA) using Bonferroni's multiple-comparisons post-test with PBS group as a control was used to analyze ELISA lung titers data sets. Microneutralization titers were analyzed by the Kruskal–Wallis test (nonparametric ANOVA) using Dunn's multiple comparison test. ELISPOT data sets were analyzed by one-way ANOVA followed by the Tukey–Kramer multiple comparison test or Bonferroni's multiple-comparison test.

Results

CYT-IVAC vaccine formulations are prepared from purified virions isolated from the supernatants of infected, membrane-bound cytokine-expressing MDCK cells. Importantly, both viral encoded, full-length HA, neuraminidase, and M2 proteins along with cytokine-linked HA-TM containing proteins are packaged into budding virions, although the latter are incorporated at much lower levels than full-length proteins (Herbert and others 2009). Previously, we evaluated our CYT-IVAC approach in young adult mice using the immunomodulators IL-2 and IL-4 (Herbert and others 2009). To overcome the weakened immune responses induced in the elderly with current vaccine formulations, we hypothesized that our CYT-IVAC formulations, particularly with

IL-12, may provide more robust protective responses in the elderly and potentially provide a means to lower the vaccine dose without compromising efficacy and reduce potential side effects due to high antigen dosing. Of note, using a murine IL-12 specific bead assay, the levels of membrane-bound IL-12 incorporated into our CYT-IVAC ~mIL-12 vaccine formulation was determined to be 23 pg (± 2 pg) per 1 µg of total viral protein.

Prime/boost vaccination with WIV induces high levels of anti-viral antibodies

Studies have suggested that protective immunity in “aged” animals may require high antigen dosage or the administration of several booster doses of vaccine (Falsey 2009).

FIG. 1. Comparison of antiviral serum IgG antibodies pre-boost and post-booster vaccination. Sera collected pre-boost (day 16 post vaccination) and post-boost (day 28 post vaccination) vaccination were analyzed for IgG titers by enzyme-linked immunosorbent assay. Serum IgG levels from animals vaccinated with PBS (A), WIV (B), CYT-IVAC IL2 (C), CYT-IVAC IL-12 (D) and CYT-IVAC IL4 (E). Data are displayed as blank reduced absorbance values (405 nm) for each group. The blank reduced absorbance values of each mice pre-boost and post-boost are plotted as a comparison and values joined together. Note the dashed line shows the threshold limit of seroconversion (≥ 4 -fold mean + standard deviation of PBS group). IgG, immunoglobulin G; PBS, phosphate-buffered saline.

Hence, our initial investigations into the efficacy of our CYT-IVAC formulations in the “aged mouse model” utilized higher doses of CYT-IVAC compared with our previous studies in young adult mice (Herbert and others 2009), and included a single booster dose of vaccine in animals aged to 17 months of age. Figure 1 depicts the influenza virus-specific serum IgG levels determined in each animal pre- and post-boost after vaccination. This dosing regimen led to high seroconversion rates preboost in all vaccinated animals compared with mock (PBS) (Fig. 1), although the IL-12 CYT-IVAC group exhibited the highest mean antibody levels. Post-boost antibody levels were only marginally enhanced and restricted to individual animals within each group. There was no direct indication as to why some animals responded better than others; however, these primary antibody responses were surprisingly high taking into consideration the age of the mice and reported weakened immune systems (Goodwin and others 2006). The overall trend in animals vaccinated with IL-12 CYT-IVAC showed an enhancement of serum antibodies after booster vaccination as compared with WIV-immunized animals that displayed only marginal increases or decreases in antibody levels post-boost (Fig. 1). Notably, there were a few non-responders in all vaccine groups (Fig. 1), and the levels of circulating IgG actually went down post-boost in some animals.

To further characterize the nature of the elicited antiviral antibody responses, we compared the influenza-specific IgG2a and IgG1 antibody levels post-boost (Fig. 2) as well as determined the levels of virus-neutralizing antibodies in serum (Fig. 3). Higher levels of IgG2a antibodies (Fig. 2B) as compared with IgG1 (Fig. 2A) were observed in all vaccine groups. The IL-12 CYT-IVAC group displayed slightly

higher mean IgG2a (Fig. 2B) and neutralizing antibody levels after vaccination compared with control WIV (Fig. 3).

Prime/boost vaccination reduces viral titers in lung tissue and induces enhanced splenic cellular responses after lethal challenge of aged mice

To assess the level of protection afforded by our CYT-IVAC formulations, vaccinated mice were challenged with a high lethal dose (100 LD₅₀) of mouse-adapted influenza A/PR/8/34 virus, and viral lung burden was assessed 5 days later as a measurement of protection. The IL-12 CYT-IVAC group was found to be most effective in reducing weight loss; the mock-vaccinated group lost ~20% of their original weight due to challenge (Fig. 4A). The viral lung burden was reduced in all immunized groups compared with control (sham) animals, suggesting that the low-dose prime/boost immunization regimen using whole inactivated vaccine is affording some degree of protection in “aged” animals (Fig. 4B). Importantly, despite the high degree of variation, 3 out of 6 animals in the CYT-IVAC~mIL-12 group and 1 animal in the CYT-IVAC~mIL-4 group had almost no detectable viral burden (10³ TCID₅₀/gm tissue). Of note, these animals also displayed the highest anti-viral antibody levels, including neutralizing antibody, suggesting a positive correlation between the level of anti-viral antibody and protection.

To further assess the nature of the immune response elicited by our vaccines, we assessed the virus-specific IFN γ (Th1 cytokine), IL-2 (T cell-stimulatory cytokine), IL-4 (Th2 cytokine), and IL-17A (Th17 cytokine) cellular responses by ELISPOT assay on day 45 post-vaccination. Interestingly,

FIG. 2. Whole inactivated influenza vaccines with or without immunomodulators enhance serum anti-viral IgG2a levels as compared with IgG1. Sera was collected from vaccinated animals ($N=9$) on day 28 post-vaccination and antibody titers for virus-specific IgG1 (**A**) and IgG2a (**B**) were determined by enzyme-linked immunosorbent assay. Data are displayed as antiviral IgG1 or IgG2a concentrations in ng/mL for each group. (** $p < 0.01$ compared with PBS I.M. group, one way one-way analysis of variance Bonferroni's multiple-comparison test).

there appeared to be a hierarchal influenza-specific $\text{IFN}\gamma$ cellular response elicited by our vaccines with the most robust response elicited by the $\text{CYT-IVAC}^{\sim\text{mIL-4}} > \text{CYT-IVAC}^{\sim\text{mIL-12}} = \text{CYT-IVAC}^{\sim\text{mIL-2}} > \text{non-adjuvanted WIV}$ (Fig. 5A). There were no significant differences in viral-specific splenic IL-4 responses among vaccinated groups (Fig. 5B), although there was a trend toward higher splenic IL-4 responses for WIV-vaccinated animals. Low levels of antiviral specific IL-2 splenic responses were observed in all groups, whereas IL-17A splenic responses were undetectable (data not shown).

To gain additional insights on the longevity and the nature of the protective cellular responses present within the lungs, we also performed a preliminary analysis of the type of lung T-cell responses inherent in vaccinated animals at day 65 post-vaccination. We theorized that the dominant recall responses present in the lungs at day 5 post-challenge would be indicative of the type of cellular protective response elicited by each vaccine formulation. Although this assay, which utilized pooled lung samples, is not quantitative, as such, it offers insights into the type of recall T-cell responses ($\text{IFN}\gamma$ and GrB) induced in vaccinated versus non-vaccinated animals on challenge. Notably, global stimulation increased the $\text{IFN}\gamma$ production capacity of inclined CD4^+ and CD8^+ T cells in all challenged animals. However, almost 75% of the CD4^+ and CD8^+ T lymphocytes in the IL-12 CYT-IVAC challenged mice group were induced to express $\text{IFN}\gamma$ on PMA/ionomycin stimulation, compared with 50%–56% in the IL-4 CYT-IVAC group and only 30%–40% in the control WIV and IL-2 CYT-IVAC groups. Interestingly, all vaccinated animals, regardless of the vaccine formulation, exhibited higher virus-induced levels of GrB-expressing CD8^+ T cells in the lungs compared with unvaccinated animals (PBS group). Almost 50% of the CD8^+ T cells in the lungs of the IL-12 and IL-2 CYT-IVAC animals exhibited GrB expression, compared with 26% observed in PBS-challenged animals, and 36% in the control WIV and IL-4 CYT-IVAC-

FIG. 3. IL-12 CYT-IVAC vaccination enhances virus-specific microneutralizing antibodies in serum post-booster vaccination. Sera collected on day 28 post-vaccination ($n=18$) was heat inactivated at 56°C for 30 min, serially 10-fold diluted, and incubated with 100 TCID_{50} of A/PR/8/34 for an hour at room temperature. Serum-virus mixtures were incubated with MDCK cells for 1 h and subsequently cultured for 3 days in DMEM supplemented with TPCK-treated trypsin ($1.5 \mu\text{g/mL}$). Microneutralization titer is presented as the reciprocal of sera dilution that was still able to neutralize and inhibit PR8 induced cytopathic effect. Note that a serum dilution of 1:5 is considered the lowest detectable dilution at which no neutralization (cytopathic effect) was observed (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with PBS I.M., Kruskal-Wallis test, Dunn's multiple-comparison test). IL-12, interleukin-12; CYT-IVAC, cytokine bearing influenza vaccine; TCID_{50} , 50% tissue culture infectious dose units; MDCK, Madin-Darby Canine Kidney; DMEM, Dulbecco's modified Eagle's medium; TPCK, L-(tosylamido-2-phenyl) ethyl chloromethylketone.

FIG. 4. Inactivated vaccines reduce viral loads in lung tissue at day 5 following lethal challenge. Aged mice vaccinated with wild-type inactivated vaccine (WIV) or with immunomodulators followed by booster on day 21 were challenged with a lethal dose (100 LD₅₀) of mouse-adapted A/PR/8/34 on day 45 post vaccination. **(A)** Body weights were monitored for 5 days after challenge and plotted as percent original weight. **(B)** Lung tissues were collected on day 5 post-challenge, homogenized and viral titers were determined by tissue culture infectious dose assay (calculated by Reed and Muench formula). Data represent TCID₅₀/g of lung tissue. Data not statistically significant.

vaccinated animals at time of sacrifice without any additional stimulation. On further stimulation with PMA/ionomycin, 10% and 12% increases in the GrB-expressing CD8⁺ populations were observed in the IL-12 and IL-4 CYT-IVAC groups, respectively. It is unclear why the lung IFN γ responses were not mirrored in the splenic responses as determined by ELISPOT (Fig. 5), but these could be reflective of the different time points that the samples were analyzed (day 45 versus day 65). In addition, the lung responses are likely elevated due to active recruitment of T cells (both non-specific and viral specific) as a consequence of challenge.

Single low dose of IL-12 CYT-IVAC provides superior protection against lethal influenza A/PR/8 challenge

To assess whether cytokine-bearing vaccines were more effective as single-dose formulations, we determined vaccine efficacy in aged animals after administration of a single dose of 0.5 μ g total viral protein. Due to the lack of enhanced efficacy of the IL-2 CYT-IVAC in the prime/boost study, we opted to focus on the efficacy of the IL-12 CYT-IVAC and the IL-4 CYT-IVAC. The latter resulted in significant protection

FIG. 5. Cellular immune response to CYT-IVAC vaccination. **(A)** IFN γ ELISPOT and **(B)** IL-4 ELISPOT assay on spleens from vaccinated mice challenged with A/PR/8 on day 45 post-vaccination and sacrificed 5 days after challenge. Data show average number of IFN γ spots or IL-4 spots from each group of mice ($N=6$) stimulated with VSV antigen or PR8 antigen. Data show average of reduced values of spots (PR8 Spots-VSV Spots). Error bars indicate standard errors **(A)** $*p < 0.05$ compared with WIV, $**p < 0.01$ compared with PBS, **(B)** $*p < 0.05$ compared with PBS one way one-way analysis of variance, (Bonferroni's multiple-comparison test). IFN γ , interferon gamma; ELISPOT, enzyme-linked immunosorbent spot assay; VSV, vesicular stomatitis virus.

FIG. 6. Single-dose immunization of inactivated influenza vaccines enhance serum anti-viral IgG1 and IgG2a antibody titers. Aged Balb/c mice (17 months old) were vaccinated intramuscularly (I.M.) with 0.5 μ g of WIV ($N=23$) or WIV-bearing membrane-bound mouse IL-4 ($N=23$) or IL-12 ($N=23$). PBS served as a negative control ($N=23$). Sera was collected on day 21 post-vaccination, and antibody titers for virus-specific IgG1 (**A**) and IgG2a (**B**) were determined by enzyme-linked immunosorbent assay. Data are displayed as blank reduced absorbance values (405 nm) for each group (* $p < 0.05$, *** $p < 0.001$ compared with PBS IM group by Bonferroni's multiple-comparison test).

when administered as a single low dose in young adult animals (Herbert and others 2009). Similar to the prime/boost regimen, most of the singly vaccinated animals seroconverted by d21 and had higher levels of anti-viral IgG2a compared with IgG1 (Fig. 6A, B). Notably, there were several non-responders in each vaccine group; this likely reflects age-associated non-responsiveness, as our previous studies in young adult animals generally resulted in 100% seroconversion rates (Herbert and others 2009).

To evaluate the long-term protective efficacy of single-dose vaccine formulations, animals were challenged on day 100 post-vaccination. 100% of the sham-vaccinated animals

(PBS) reached established end points by day 7 post-challenge and were euthanized (Fig. 7A). Vaccinated animals displayed varying clinical signs of disease depending on the vaccine group (eg, ruffled fur, weight loss, and inactivity) with recovery evident in some groups beginning at day 9 (Fig. 7A). Notably, 58% of the animals receiving the IL-12-bearing CYT-IVAC survived a high-dose lethal challenge, compared with only 23% of mice receiving the non-adjuvanted WIV (Fig. 7B). Interestingly, immunization with a single dose of the IL-4-bearing CYT-IVAC was not efficacious in providing long-term protection against lethal challenge despite the induction of high levels of anti-viral

FIG. 7. IL-12 CYT-IVAC vaccination protects mice from lethal homotypic viral challenge. Aged Balb/c mice vaccinated with wild-type inactivated vaccine or with immunomodulators were challenged with a lethal dose (100 LD₅₀) of mouse-adapted A/PR/8/34 on day 100 post-vaccination. Mice were monitored daily for percent reduction in body weights and survival after challenge. Data represent percent original/start weight monitored for 14 days after challenge (**A**) and percent survival after challenge (**B**). (**A**) * $p < 0.05$ compared with IL-4 PR8 IM on day 2 and *** $p < 0.001$ compared with PBS I.M. on days 3, 5, and 6 by one-way analysis of variance (Bonferroni's multiple-comparison test), (**B**) *** $p < 0.001$ compared with PBS I.M., logrank test.

FIG. 8. IgG1 and IgG2a antibodies are increased on homotypic lethal viral challenge. Animals ($N=12-13$) were challenged with 100 LD₅₀ of A/PR/8 on day 100 post-vaccination, and sera were collected over a period of 14 days as animals reached end points or from survived mice sacrificed at day 14 after challenge and evaluated for IgG1 (**A**) and IgG2a (**B**). Data represent difference in absorbance values (405 nm, blank). *** $p < 0.001$ compared with PBS, one-way analysis of variance, Bonferroni's multiple comparison.

antibody in some animals (see also Fig. 6). This is in contrast to our previous studies in young adult animals that responded positively to vaccination with CYT-IVAC^{~IL-4} (Herbert and others 2009). This suggests that immunomodulators have age-specific effects that may need to be “tailored” to a particular age group.

Sera from the challenged mice were also assessed for the scope and type of the antibody response (Fig. 8A, B). Both IgG1 and IgG2a antibody levels were higher post-challenge (Fig. 6), indicating expansion of memory responses as a consequence of challenge. Importantly, survivors exhibited higher pre-challenge anti-viral IgG antibody levels compared with other animals within the same group. Of note, all of the IL-12 CYT-IVAC-vaccinated animals displayed higher levels of IgG2a antibodies compared with other vaccine formulations. However, 5 of 12 mice in the IL-12 group succumbed to lethal challenge despite these high anti-viral antibody levels, suggesting that other protective factors were not sufficiently induced to confer complete protection. Together, these data suggest that the IL-12-bearing CYT-IVAC may hold promise of lowering the required antigen dose for the elderly while maintaining and improving on protective responses.

Discussion

We developed our CYT-IVAC approach as a means of providing additional immune-stimulatory signals during vaccination with WIV and as an alternative to overcome potential systemic cytotoxicity of soluble administered cytokines (Siegel and Puri 1991). WIVs are an attractive alternative to split/subunit vaccines, particularly as they are considered more immunogenic. Interestingly, recent studies suggest that the inactivating agent can have profound effects on the type and breadth of immune response elicited by WIV (Budimir and others 2010). Of note, β -propiolactone-inactivated WIV elicited potent antibody and cellular immune

responses to influenza in young adult mice, whereas formalin-inactivated WIV only led to antibody-mediated protection that was inferior to β -propiolactone-WIV (Budimir 2012). Our studies support and extend these observations to “aged” mice where β -propiolactone-inactivated WIV also results in the generation of robust humoral and cellular influenza-specific immune responses at low doses.

The elderly represent the most vulnerable population to influenza-associated illness and deaths (Thompson and others 2003). The increased vulnerability of this age group has largely been attributed to a deterioration or waning of immunity during the aging process evident by a decreased ability to mount effective innate and cellular immune responses to infections (McElhaney and Effros 2009). Vaccine efficacy in the elderly is also severely compromised, which necessitates the development of more immunogenic vaccines targeting this group (Shahid and others 2010). Initially, we tested whether the adjuvanticity of our CYT-IVAC formulations using a prime/boost immunization regimen could overcome age-associated vaccine non-responsiveness. Although this did not allow us to directly assess the impact of the co-presented cytokines, we were surprised that the dosing regimen employed (330 ng/100 ng of HA respectively based on total viral protein) was sufficient to reduce viral burden in lung tissues. Previous reports using formalin-inactivated influenza virus failed to provide protection at these doses without the addition of an adjuvant (Katz and others 2000). Of note, the animals displaying the lowest viral burden post-challenge also exhibited the highest levels of influenza-specific antiviral antibody, suggesting that in a prime/boost vaccination regimen, high anti-viral antibody levels positively correlate with reduced viral burden in aged animals.

In agreement with previous studies using inactivated WIV in young adult mice (Martin and others 2010), all vaccine groups exhibited influenza-specific splenocyte IFN γ and IL-4 responses; although the IL-2 splenic responses were only

marginally evident and did not reach statistical significance in the “aged” animals. The latter correlates well with diminished IL-2 clinical cellular responses to influenza vaccination observed in the elderly (McElhaney and others 1990). Interestingly, the IL-4 CYT-IVAC induced the highest splenic IFN γ cellular responses as compared with other vaccine groups, which is somewhat counter-intuitive, as IL-4 is known to drive the development of Th2-type responses and suppress Th1. However, there are reports that the presence of IL-4 during initial antigen priming, or when presented as a membrane-bound formulation, can lead to stimulation of IFN γ and IL-12 from antigen-presenting cells such as plasmacytoid dendritic cells, thus priming Th1 responses (Chakrabarti and others 2004; Yao and others 2005).

Due to the superior humoral and cellular responses elicited by the IL-4 and IL-12 CYT-IVAC formulations, we further evaluated whether the addition of these cytokine adjuvants were able to increase long-term protective efficacy using a single low dose formulation. All vaccine formulations administered as a single low dose (ca. 165 ng HA) with and without anchored cytokines were sufficient to induce type 1 humoral immune responses in aged animals evident by the dominant IgG2a type responses measured post-vaccination. Although the IL-4-bearing CYT-IVAC afforded superior protection in young adult mice (Herbert and others 2009), it failed to protect “aged” mice when administered as a single-dose formulation. In contrast to IL-4, the IL-12-bearing CYT-IVAC in a single-dose formulation afforded superior protection against lethal challenge compared with non-adjuvanted WIV. This suggests that a membrane-bound formulation of IL-12 directly co-presented on WIV has the potential to lower the protective dosage of vaccine in “aged” animals. This would represent a significant benefit if it could also mitigate the adverse side effects associated with high antigen dosage observed for the Fluzone High-Dose vaccine (Sullivan and others 2010).

IL-12 has multiple immunomodulatory functions, which serve to bridge both innate and adaptive immune responses. Importantly, IL-12 helps drive T-helper type 1 responses and can directly activate natural killer cells, enhancing their cytolytic activity as well as inducing the secretion of IFN γ and tumor necrosis factor α (Naume and others 1992; Robertson and others 1992). IL-12 is now well recognized as playing an important role in systemic and mucosal humoral responses, particularly impacting the IgG2a and IgG3 isotype responses in mice (Buchanan and others 1995; Germann and others 1995). Moreover, IL-12 can directly bind to B cells (Vogel and others 1996), potentially directly influencing their activation and induction of Th1-like differentiation (Durali and others 2003). The IgG1 and particularly the IgG2a levels were further increased on challenge in the CYT-IVAC^{~IL-12} group, supporting the role of IL-12 in modulating IgG2a protective responses. Therefore, there could be multiple potential target cells stimulated by the CYT-IVAC^{~IL-12} formulation *in vivo*. Ongoing studies to assess how the IL-12 CYT-IVAC directly impacts innate responses in antigen presenting cells such as dendritic cells and B-cells should help identify these target cells.

Our studies suggest that IL-12, when co-presented on the surface of WIV, serves to stimulate humoral and potentially cellular immune responses that are long lasting, as enhanced protection was observed 100 days post-vaccination in “aged” animals. The levels of neutralizing antibody were modest in all vaccine cohorts, although there was a trend

toward higher levels in the animals receiving the IL-12 bearing CYT-IVAC. This suggests that other cellular protective responses elicited by the IL-12-bearing CYT-IVAC were induced by the single-dose formulation. In a semi-quantitative analysis from pooled lung samples, we noted that the CYT-IVAC^{~IL-12}-vaccinated animals had higher levels of IFN γ and GrB expressing T cells in the lungs after challenge, suggesting that the cellular recall response was enhanced in these animals. In fact, almost 70% of the lung T cells (CD4⁺/CD8⁺) were inclined to express IFN γ and GrB compared with only 30% and 40%, respectively, in the lung T cells of non-adjuvanted WIV-vaccinated animals on challenge. Further studies incorporating influenza-specific tetramers will be required to fully assess the lung recall response after CYT-IVAC vaccination.

Recently, we tested the mucosal adjuvanticity of our CYT-IVAC formulations in young adult mice and were able to show that the IL-12-bearing CYT-IVAC was superior at eliciting robust and protective mucosal antiviral responses compared with non-adjuvanted WIV (Khan 2012). CYT-IVAC formulations bearing IL-12 administered by the intranasal route may prove to be a better alternative to deliver efficacious vaccines to the elderly and reduce potential parenteral-associated adverse side effects. Future studies evaluating mucosal responses of specific CYT-IVAC formulations in “aged” animals will help address this.

In conclusion, the CYT-IVAC approach offers several advantages compared with current egg-based vaccine production methodologies: It is cell culture based and can be “tailored” to elicit specific responses that are dependent on the cytokine used and the route of administration. In addition, the vaccine platform can easily be adapted to other vaccine-suitable cell lines such as the Vero cells. Our data also strongly suggest that WIV formulations need to be revisited for the elderly and along with suitable adjuvants, may allow for administration of lower-dosage vaccines with enhanced efficacy in this highly vulnerable age group.

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Author Disclosure Statement

No competing financial interests exist.

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