Protection by humoral elements independent of virus neutralization activity against an influenza virus challenge

Julia Sarkadi¹, David Kuti¹, Mate Jankovics¹, Eva Pallinger², Kinga Fodor¹, Zoltan Kis ¹, Eva Gonczol¹, Ildiko Visontai¹, Istvan Jankovics¹*

¹Division of Virology, National Center for Epidemiology, 2-6 Albert Flórian út, H-1097 Budapest, Hungary
²Department of Genetics, Cell- and Immunobiology, Semmelweis University, 4 Nagyvárad tér, H-1089 Budapest, Hungary

Running title: Protection of mice against a PR8 challenge is not HI, NI or VN dependent

SUMMARY
To investigate the protective efficacy of a seasonal trivalent inactivated whole virion influenza vaccine (TIV) adjuvanted with aluminum phosphate (Fluval AB, referred to here as TIV+Al), we immunized mice with the TIV+Al, and as controls, with TIV, TIV+Al combined with Freund adjuvant (TIV+Al+F), inactivated A/PR/8/34(H1N1) (PR8) strain or PBS, and challenged them with a lethal dose of a mouse-adapted PR8 virus. Serum pools from immunized mice were passively transferred to recipient mice that were then challenged similarly. All actively immunized mice survived the challenge. Of recipient mice receiving serum from mice actively immunized with TIV, TIV+Al or TIV+Al+F, 20%, 80%, and 100% survived, respectively. Rates of mortality and morbidity of recipient mice were inversely proportional to the hemagglutination inhibition (HI) antibody level to the vaccine virus in the absence of detectable PR8-specific HI, neuraminidase inhibition (NI) and virus neutralization (VN) antibodies. No cross-reactivity was observed between vaccine and PR8 strains in in vitro HI, NI or VN assays. In splenocytes from TIV+Al-immunized mice production of IFN-γ or granzyme-B protein and mRNA expression increased (p<0.05). Thus, antibodies play a major role in the protection against a mismatched challenge infection independent of HI, NI and VN activity, but cellular immune responses may contribute to full protection in actively immunized mice.

Key Words: Influenza vaccine, Protection, Serum transfer, Challenge infection, Cellular immunity.

* Corresponding author: Istvan Jankovics MD, T: +36.1.476.1100; FAX: +36.1.476.1226
e-mail: jankovics.istvan@oek.antsz.hu
INTRODUCTION

Vaccination is the most effective approach to prevent an influenza virus infection or disease. A seasonal influenza outbreak is associated with the annually occurring genetic drift in the hemagglutinin (HA) and neuraminidase (NA) genes of the virus, while influenza pandemics arise as a result of new combinations of HA and NA subtypes. Several types of vaccines for seasonal influenza are currently licensed, such as the inactivated vaccines, which may contain whole inactivated virus particles, or a virus disrupted by detergent (split vaccines), or purified HA and NA proteins (subunit vaccines), or the live attenuated influenza vaccine. Immune responses may differ with the type and dose of the influenza vaccine, the use and choice of an adjuvant and the route of immunization, and they may also be influenced by host factors such as age and host genetic characteristics. It is generally accepted that antibodies play a pivotal role against influenza virus infection. For protection against a homologous virus infection hemagglutination inhibition (HI) and neuraminidase inhibition (NI) antibodies are the most important components (Osterholm et al., 2012; van Els et al., 2014; Trombetta and Montomoli, 2016). It has also been long recognized that cell-mediated immune (CMI) responses are involved in protection against influenza, especially for the elderly (McElhaney et al., 2012). Influenza virus-specific CD8+ T cells can cross-react with various subtypes of influenza A virus, affording heterosubtypic immunity. CD4+ T cells are also considered responsible for the protection against an influenza virus infection, either via direct cytotoxic effects or indirectly by providing help to B cells and CD8+ T cells (Furuya et al., 2010; Budimir et al., 2012; Sridhar et al., 2013; Altenburg et al., 2015).

Fluval AB, a commercially available seasonal trivalent inactivated whole virion vaccine is a licensed influenza vaccine that is combined with aluminum adjuvant. Fluval AB has induced HI antibody responses in humans to all component strains in the vaccine in a titer that meets the requirements of the European Agency for the Evaluation of Medicinal Products for Interpandemic Influenza Vaccines, and more than 20 million doses of the vaccine have been used for annual seasonal immunization programs in Hungary since 1995 (European Committee for Proprietary Medicinal Products, 1997; Fiore et al., 2010; Vajo et al., 2008; 2010; 2012; van Els et al., 2014).

The CMI responses to and the protective efficacy of the Fluval AB had not been investigated earlier. Thus, the primary aim of our study was to find out whether a protective effect of Fluval AB vaccination could be determined in mice using active and passive immunization protocols and a lethal challenge with a mouse-adapted A/PR/8/34(H1N1) (PR8) influenza virus strain. To ensure that the observed results were not restricted to one mouse strain, two mouse strains, the outbred NMRI and the inbred Balb/c strains, were used in the experiments. For the identification of potentially cross-reactive antibodies in the protection, HI, NI and virus neutralization (VN) assays
were applied. For the role of CMI responses in the protection in the actively immunized mice, the expression of a major cytokine, IFN-γ, and of granzyme B (GrB), a mediator of T cell cytotoxicity, were measured. We ascertained that humoral components of the immune response independent of HI, NI and VN functions play a major role in the protection against a HI, NI and VN mismatched influenza virus challenge.

MATERIALS AND METHODS

Vaccine and the influenza virus strains
Fluval AB (2014/2015) is composed of the H1N1 A/California/07/2009 like NYMC X-179A reassortant strain (min. 15 µg HA), the H3N2 A/Texas/50/2012 like NYMC X-223A reassortant strain (min. 15 µg HA), and the B/Massachusetts/2/2012 wild-type strain (min. 15 µg HA) (Omininvest Ltd., Hungary), as recommended by the World Health Organization (World Health Organization, 2014). The A/California/7/2009 (H1N1)-like reassortant vaccine strain contains the HA, NA and PB1 genes donated from the A(H1N1) pandemic strain A/California/7/2009 (strain A(H1N1)pdm09), and the other internal genes donated from PR8 virus. The vaccine viruses are inactivated with formalin and adjuvanted with aluminum phosphate (National Institute of Pharmacy and Nutrition, Hungary, 2015). The Fluval AB vaccine is referred to here as TIV+Al. Additional immunogens used for immunization as controls were the TIV not adjuvanted, the TIV+Al combined with complete Freund adjuvant (TIV+Al+F), and the formalin-inactivated mouse-adapted PR8 strain (National Influenza Strain Collection, Hungary), or the inactivated PR8 strain combined with aluminum phosphate adjuvant (PR8+Al), or PBS. These viruses for the experiments were grown in eggs. The allantoic fluids were harvested 48 h after infection and assayed for the amount of virus by measuring the concentration of the HA (Klimov and Cox, 2003), and the infectivity was titered on Madin-Darby canine kidney (MDCK) cells, as described (Klimov et al., 2012).

Mice, immunization and challenge infection
Two mouse strains, the outbred NMRI (National Center for Epidemiology, Budapest) and the inbred Balb/c (Charles River Research Models and Services, Germany GmbH), were used for the experiments. For active immunization six groups of 6-8-week-old female NMRI mice (18 mice/group) were inoculated intramuscularly 3 times at 3-week intervals with the immunogens as follows: Group 1. TIV at a dose of 45 µg of HA (15 µg of each component), without adjuvant, Group 2. TIV combined with aluminum phosphate adjuvant (TIV+Al, i.e. Fluval AB ), Group 3. TIV+Al vaccine combined with complete Freund adjuvant (TIV+Al+F), Group 4. Formalin-inactivated PR8 strain at a dose of 15 µg, Group 5. Formalin-inactivated PR8 strain adjuvanted with aluminum phosphate (PR8+Al), Group 6. PBS. Blood samples were collected by heart
puncture from 5 mice in each group 2 weeks after the last immunization and before the administration of the challenge infection for serum transfer and for determination of HI, NI and VN titers. Thirteen mice/group were challenged by intranasal infection with 5 LD_{50} of the live PR8 strain in a volume of 50 µl under mild anesthesia by using a ketamine and xylazine mixture (Bayer Animal Health, Germany). Three mice/challenged groups were sacrificed on Day 6 after the challenge for lung virus titration. The mortality, daily body weight loss and clinical scores were monitored for 10 mice for 18 days after the challenge infection. Mice that lost 30% of their initial weight were euthanized by the ketamine-xylazine mixture and scored as dead. Body weight data are expressed as the change relative to the Day 0 measurement. Clinical scores were assigned like so: 0 = no clinical signs, 1 = rough fur, 2 = rough fur, passive during handling, 3 = rough fur, passive during handling, rolled up, 4 = rough fur, unresponsive, rolled up. The serum samples from the immunized mice were individually tested against the 3 components of the TIV by HI, then the samples in the same group were pooled and tested again in HI, NI, and VN assays against the A/California reassortant strain and the PR8 strain. Moreover, to measure IFN-γ and GrB responses, 20 mice were immunized in two groups. Ten mice were inoculated three times with TIV+Al at weeks 0, 4 and 8. Ten mice were immunized in a similar way, but the third dose of the vaccine was combined with equal volume of complete Freund adjuvant and served as positive controls. At Week 9 the mice of both groups were sacrificed and spleens were obtained for analysis of IFN-γ and GrB production in the splenocytes by flow cytometry. Six mice were inoculated three times with PBS and they served as negative controls.

In addition to NMRI mice, 6-7 week-old female Balb/c mice in 3 groups of 10 mice were immunized 3 times at 3-week intervals with TIV+Al, or as positive controls, with TIV+Al+F, or as negative controls, with PBS, with vaccine doses described for NMRI mice. Two weeks after the last immunization the mice were sacrificed, and blood samples and spleens were obtained. Serum pools were prepared for adoptive serum transfer and challenge experiments. The splenocytes were used for the determination of IFN-γ and GrB mRNA expression by qRT-PCR. All protocols were approved by the Laboratory Animal Care Committee at the National Center for Epidemiology.

Adoptive transfer of immunity with serum from actively immunized mice into naïve mice and challenge infection

From the serum pools collected from actively immunized NMRI or Balb/c mice, 300 µl (NMRI mice) or 100-200 µl (Balb/c mice) diluted with PBS to 500 µl were transferred to each mouse of the 5 to 6 recipient NMRI or Balb/c mice/group via intraperitoneal injection 24 hours prior to challenge with 5 LD_{50} of the PR8 strain. Serum samples from the recipient mice before challenge infection were obtained from the retroorbital plexus to compare HI titers in the donor and recipient mice.
Then the mortality, daily body weight loss and clinical scores in the passively immunized mice were monitored for 18 days after the challenge.

**Hemagglutination inhibition (HI) assay**
Serum antibody titers against the virus strains were measured in HI tests with chicken red blood cells following standard procedures (Klimov and Cox, 2003).

**Neuraminidase inhibition (NI) assay**
Serum neuraminidase inhibition titers against the virus strains were determined as described (Lambre et al., 1990).

**Virus neutralization (VN) assay**
Serum VN assays were carried out on MDCK cells using 100 TCID$_{50}$ of the virus strains/well in a 96 well plate, as described (World Health Organization, 2010), and the virus detection was performed by a HA assay using a chicken erythrocyte suspension (Klimov et al., 2012).

**Lung virus titers in NMRI mice**
Three mice/actively immunized groups were sacrificed on Day 6 after the challenge, and the lungs were removed and stored in -80°C until the determination of the infectious influenza virus titer. For the virus titration, the lungs were homogenized in a RPMI-1640 medium (Sigma) and cell debris was removed by centrifugation. Serial dilutions of the supernatants of lung homogenizes were inoculated on MDCK monolayers in a 96-well flat bottom culture plate using 4 parallels/dilution in the presence of a 2 µg TPCK trypsin (Sigma)/ml culture medium. After a 72-hour incubation period, the number of wells showing cytopathic effects was counted and the TCID$_{50}$ titer per gram of lung tissue was calculated (Sgarbanti et al., 2011; Reed and Muench, 1938).

**Flow cytometry**
Splenocytes were obtained from the NMRI mice on Day 7 after the third inoculation with the TIV+Al or with the TIV+Al+F vaccine or with PBS. Single-cell suspensions were prepared and 2x10$^6$ splenocytes were plated onto a 48-well flat-bottomed plate and stimulated for 24 h with the live A/California reassortant strain (MOI=2) or, as control, with culture medium. Brefeldin A (Beckton Dickinson, USA) was added at a final concentration of 1 µg/ml for the last 12 hours of the incubation. For the identification of certain subpopulations, the splenocyte preparations were stained with fluorochrome-conjugated monoclonal antibodies; namely, CD3-FITC, CD4-PerCP, CD8-PE, IFNγ-Alexa Fluor and GrB-eFluor (eBioscience, USA). To count the positive cells, 5000 to 10000 events were acquired by live gating on a FACS Calibur (Beckton Dickinson, USA) and analyzed by means of the CELLQuest pro software package.

**Quantitative real-time reverse transcription-PCR (qRT-PCR)**
In order to quantify the mRNA expression of IFN-γ and GrB, qRT-PCR assays were carried out as described for varicella-zoster vaccine-immunized guinea pigs (Sarkadi et al., 2015) with some modifications. Briefly, spleens were obtained from the Balb/c mice on Day 14 after the third inoculation with the TIV+Al or with the TIV+Al+F vaccine or with PBS. Single-cell suspensions were prepared and 2x10⁶ splenocytes/well were plated onto a 48-well flat-bottomed plate and stimulated for 24 hours with the live A/California reassortant strain (MOI=2) or, as control, with the culture medium. RNA was isolated from the in vitro stimulated splenocytes using the RNeasy Plus Kit (Qiagen, Germany) and transcribed by the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, UK). The primers and probe used for IFN-γ were the same as described earlier (sense primer, 5’-GAT ATC TGG AGG AAC TGG CAA AA-3’; antisense primer, 5’-CTT CAA AGA GTC GGT AGA AAG AGA TAA T-3’; probe, FAM-TGG TGA CAT GAA AAT CCT GCA GAG CCA-TAMRA) (Lin et al., 2005). The primers and probes for GrB (GenBank accession no. NM_013542.2) target genes were designed at the National Center for Epidemiology, GrB sense primer, 5’-CCA TCG TCC CTA GAG CTG AG-3’; antisense primer, 5’-GGA CAG AGC TGG TCC TTG TG-3’; probe, FAM-AGG ACT GCA AAG ACT GCC TTC ATG TCC-TAMRA. For the housekeeping gene peptidylprolyl isomerase A (PPIA) the primers and probe used were as described, sense primer: 5’- GGCCGATGACGAGCCC -3’;antisense primer: 5’- TGTCTTTGGAACTTTTGCTGCAA -3’; probe, FAM- TGGGCCGCGTCTCCTTCGA -TAMRA (RTPrimerDB, 2015). The product size was less than 150 bp for each amplicon. The qRT-PCRs were carried out in 10-µl final volumes. The PCR mixture (Roche, Germany) contained 2.5 µl of cDNA sample and 7.5 µl of a mastermix containing 0.4 µl (10 µM) each of the forward and reverse primers, 0.2 µl (20 µM) of probe, 5 µl of PCR mastermix and 1.5 µl of RNase-free distilled water. The PCR conditions were: 95 °C hot start for 15 min, followed by 50 amplification cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 10 s. Each measurement was performed in triplicate and the data values were analyzed via the LightCycler 480 II system (Roche, Germany). Expression levels were recorded as cycle threshold (C_T) values based upon the comparative C_T (2^(-ΔΔC_T)) method (Livak and Schmittgen, 2001). The relative expression was calculated as the ratio between the mean C_T values of the target gene and housekeeping gene in each sample, relative to a reference sample got from PBS-immunized animals.

Statistical analysis
All statistics were calculated using a Microsoft Excel 2007 software package. Statistical differences between different influenza vaccines relative to the negative control group were evaluated for survival proportion, change in body weight and clinical score. Body weight data are expressed as the change relative to the Day 0 measurement. Body weight and clinical scores were summarized as
a single outcome per animal using an Area Under the Curve (AUC) approach. Mice that died prior to the last day of the observation period were included in the analysis at their last recorded bodyweight and clinical score. Differences were determined by the Mann-Whitney test and p values <0.05 were considered statistically significant. Correlation coefficients were determined by applying the Pearson test.

RESULTS

The impact of immune responses in NMRI mice to TIV+Al on the protection against a challenge infection with a lethal dose of the mouse adapted PR8 strain

NMRI mice were immunized and challenged as described in the Materials and Methods for the active immunization protocol. All mice injected with PBS died by Day 10 after the intranasal administration of the challenge virus, while all mice immunized with TIV, adjuvanted or not, or immunized with the PR8 strain, adjuvanted or not, survived (Figure 1A). The body weight loss after the challenge infection indicated a low-level morbidity of the immunized mice, the mean body weight loss being within 7% in all mice during the observation period with no statistical difference between the immunized groups (p>0.05 for all variations). However, there was a strong statistical difference between PBS-inoculated mice and any of the immunogen-immunized groups (p<0.01 for all variations) (Figure 1B). The clinical score was significantly reduced, and defined as 0 in the groups immunized with TIV, adjuvanted or not, or with PR8, adjuvanted or not, as compared with PBS-inoculated mice, defined as 4 (p<0.01) (Figure 1C). Lungs were removed from three mice in each group on Day 6 after the challenge for titration of the PR8 challenge virus. The results obtained revealed that the virus titers in the lungs of mice immunized with the inactivated PR8 strain adjuvanted or not, were below the threshold of detectability. In mice in the three groups immunized with TIV or TIV+Al or TIV+Al+F, the lung virus titers were similar; between a mean of 3.7±0.4 to 4.4±0.2 log_{10}TCID_{50}/g, with no statistical difference (p>0.05) between the three groups. The virus titer in the lungs of mice inoculated with PBS had a mean of 5.1±0.3 log_{10}TCID_{50}/g, with a statistical difference of p<0.05 between this titer and titers in the lungs of TIV, or TIV+Al, or TIV+Al+F immunized animals.

The association of protection with serum antibodies in TIV+Al-immunized and PR8-challenged mice

To learn whether the observed protection against a lethal PR8 challenge after the immunization of mice with TIV, or TIV+Al, or TIV+Al+F, was associated with humoral response, we carried out passive immunization by transferring 300 µl (NMRI mice) or 100-200 µl (Balb/c mice) of serum pools from groups of actively immunized mice to each naive mouse/groups and then challenged the
recipient mice with the PR8 virus strain. In NMRI mice, immune serum obtained from donors immunized with inactivated PR8 virus, or the inactivated PR8+Al combination, or immunized with the TIV+Al+F, conferred protection against death in 100% of the recipient mice (p<0.01), while 80% of the recipients who received the serum from donors immunized with TIV+Al (p<0.05) survived compared with the set of mice that received serum from PBS inoculated donors. A non-statistical difference (p=0.174) for 20% survival of recipients that received serum from TIV-immunized vs PBS-inoculated mice, but a significant difference (p=0.047) for TIV vs TIV+Al-immunized mice was observed (Figure 2A). In Balb/c mice 200 µl or 100 µl serum obtained from TIV+Al+F-immunized donors, or 200 µl from the TIV+Al-immunized donors, conferred protection in 100% of the recipients (p<0.01), while 100 µl serum from TIV+Al-immunized donors conferred protection only in 50% of recipients (p=0.021), compared with the survival of recipients that received serum from PBS-inoculated mice. A significant difference was seen for the survival of the recipient mice obtaining 200 or 100 µl serum from TIV+Al-immunized mice (p=0.025) (Figure 2B). The body weight loss was significantly less in recipient NMRI mice receiving serum from PR8- or PR8+Al- or TIV+Al+F-immunized animals (p=0.009) or from TIV+Al-immunized mice (p=0.028), than in mice that received serum from PBS-inoculated donors (Figure 2C), indicating that the donor serum contained humoral components that protected the recipient mouse not only against death, but also against disease. However, a body-weight loss of recipient mice that received serum from mice immunized with TIV showed no statistical difference as compared with that of the mice who received serum from the PBS-inoculated donors (p=0.117), while it was significantly more severe than that of mice that received serum from TIV+Al-immunized mice (p=0.047). These results suggest that the combination of TIV with Al-adjvant was necessary to achieve a sufficiently high titer of humoral components for protection against disease (Figure 2C). The body-weight loss in the recipient Balb/c mice was significantly less in animals that received 100 µl or 200 µl serum from donors immunized with TIV+Al+F, or receiving 200µl serum from donors immunized with TIV+Al (p=0.0017), compared with recipients that received serum from PBS inoculated mice. Body-weight loss was more severe in recipients receiving 100 µl of serum as compared with 200 µl of serum from mice immunized with TIV+Al (p=0.035) (Figure 2D). During the observation period the clinical score for the recipient NMRI mice that received serum from TIV- or PBS-immunized donors was similarly high (score 4), but it was reduced for mice that received serum from TIV+Al+F-immunized mice (score 1-2), compared with a score of 0 in mice that received serum from PR8- or PR8+Al-immunized mice. The clinical scores were different (score 1-4) between the individual NMRI mice within the group of recipients that received serum from TIV+Al-immunized donors. The difference in the scores in recipient NMRI mice that received serum from donor mice
immunized with PR8, PR8+Al, TIV+Al, or TIV+Al+F, versus scores in recipient mice that received serum from PBS-inoculated mice, was significant (p=0.009). The scores for recipient mice that received serum from TIV-immunized donors showed a significant difference (p=0.028), compared with that of recipients who obtained serum from TIV+Al-immunized mice. (Figure 2E). Clinical scores for Balb/c recipients were similar to those for NMRI mice, but for the Balb/c recipients receiving only 100 µl of serum from TIV+Al-immunized donors the clinical scores remained 2 during the observation period, with big interquartile ranges (Figure 2F).

**Protection by serum components in mice is not dependent on HI, NI or VN antibodies**

To investigate the mechanisms of protection by the immune sera we evaluated the antibody response induced by the TIV against the components of the TIV and against the PR8 challenge virus by HI, NI and VN assays. First, the sera from NMRI mice of the 6 groups were tested individually in HI assays against the 3 strains of the TIV and the PR8 challenge strain. The results indicated that all mice immunized with TIV, adjuvanted or not, developed HI antibodies against the three components of TIV at a titer of ≥40, but no cross-reactive antibodies to the PR8 strain were detected (not shown). Similarly, all mice immunized with the PR8 strain, adjuvanted or not, developed HI antibodies against the PR8 strain at a titer of ≥1:40, but no cross-reactive antibodies to the TIV-strains were seen (not shown). Then serum pools were composed from the individual serum samples of the mice within the groups that were used for serum transfer experiments and were tested again against the immunizing A/California reassortant strain and the PR8 challenge strain in HI, NI and VN assays (Table 1). The serum pools contained high level of HI, NI and VN antibodies against the homologous immunizing strain. However, PR8-specific HI or NI or VN antibodies were not detectable in the sera obtained from mice immunized with the TIV vaccine, adjuvanted or not. Similarly, serum pools from PR8-immunized mice did not cross-react with the A/California reassortant strain. Thus humoral components other than the HI, NI or VN antibodies must have been involved in the protection. In order to evaluate the success of the serum transfer in NMRI mice, the HI antibody titers against the A/California reassortant strain in the serum samples got from the individual recipient mice before the challenge infection were also determined. Geometric mean titers (GMTs) of HI titers in the recipient NMRI mice, receiving 300 µl serum/mouse, were closely correlated with the HI titers in the corresponding donor pool, and were approximately 16-21 fold lower due to serum dilution in the recipient mice. In Balb/c mice, receiving 100 µl or 200 µl of serum/mouse, the HI titer in the recipient mice was 8-32 fold lower than that in the donor pool (Table 1).

**Correlation of protection against PR8 challenge infection with HI titer to A/California reassortant strain in the absence of PR8-cross-reacting HI antibodies**
High HI levels against the A/California reassortant vaccine strain (GMTs of 126-253 or 160-320 in the serum pool in recipient NMRI or Balb/c mice, respectively) (Table 1) were detected in the recipient mice that exhibited 80-100% protection against death by the PR8 challenge strain (Figures 2A and 2C, Table 1) and no cross-HI antibody reactivity was observed between the A/California reassortant vaccine strain and PR8 strain in HI, NI and VN assays (Table 1). Thus the bases for the protection may rely on the level of antibodies in the donor sera not related to HI, NI and VN activity. Assuming that antibodies to the various viral proteins in an inactivated whole virion vaccine develop proportionally after immunization, high-level HI antibodies indicate a high level of antibodies not related to HI. Our results told us that the correlation coefficient of the HI titers against the A/California reassortant vaccine strain in the combined groups of the NMRI and Balb/c mice and the survival proportion of the challenged recipient mice was 0.77, and the correlation coefficient for the HI titer and body weight change was 0.71. The positive correlation suggests that the high HI antibody titer specific to the homologous strain is an indication of some degree of protection against an influenza virus infection with no HI cross-reactivity.

**The production of IFN-γ and GrB in in vitro-stimulated splenocytes obtained from NMRI mice immunized with TIV+Al or, as controls, with TIV+Al+F, as measured by flow cytometry**

In order to ascertain the IFN-γ and GrB production in the splenocytes of TIV+Al-, or as controls, of TIV+Al+F-immunized NMRI mice, splenocytes were obtained from the mice, restimulated in vitro by a live_A/California virus preparation and investigated by flow cytometry. The percentage of cells harboring IFN-γ or GrB was determined in splenocyte subpopulations such as CD3+/CD4+ T helper cells, CD3+/CD8+ cytotoxic T lymphocytes and CD3-/CD8+ NK cells (Figure 3). TIV+Al immunization induced a significant increase (p<0.05) in the IFN-γ-producing CD3+/CD4+ and CD3+/CD8+ and CD3-/CD8+ cells (Panels A, B and C), and in the GrB-producing CD3+/CD4+ cells (Panel D), as compared with the splenocytes obtained from the PBS-inoculated mice. More IFN-γ or GrB-producing cells were present in all of the tested splenocyte subpopulations obtained from mice immunized with TIV+Al+F than that in splenocytes obtained from PBS-inoculated mice, with a statistical significance level of p=0.05 or p<0.005 (Panels A-F). Hence these results revealed a significant in vitro activation and expansion of some lymphocyte subpopulations producing IFN-γ and GrB, suggesting a CMI response after TIV+Al immunization.

**The mRNA expression of IFN-γ and GrB in the in vitro-stimulated splenocytes obtained from mice immunized with TIV+Al or, as controls, with TIV+Al+F, as measured by qRT-PCR**

Balb/c mice were immunized, splenocytes obtained and the qRT-PCR was carried out as described in the Materials and Methods Section. The relative quantification of the IFN-γ and GrB mRNA
expression in the in vitro stimulated splenocytes, obtained from the mice immunized with the TIV+Al or as controls, with TIV+Al+F, demonstrated a 20 to 30-times increase, as compared with splenocytes obtained from PBS-inoculated animals (p<0.05 for all combinations). The differences in the extent of mRNA expression in the splenocytes from TIV+Al or TIV+Al+F immunized mice were not significant in any combinations tested (p>0.05) (Figure 4). Thus, similar to the enhanced percentages of splenocytes containing IFN-γ or GrB, as determined by flow cytometry, the increased level of IFN-γ and GrB mRNA expression also indicated increased CMI responses after the TIV+Al immunization of mice.

DISCUSSION

For the first time, our challenge study tested the protective efficacy and some components of the protection of a trivalent inactivated whole virion influenza vaccine adjuvanted with aluminum (Fluval AB). We observed 100% protection against death by a lethal dose of PR8 strain in mice actively immunized with this vaccine. A 100%--80% protection was seen in recipient mice receiving a single 300µl-200µl of serum transfer from the immunized donor mice, the protection was attributed to humoral elements with no HI, no NI and no VN activity. Two components of TIV used in our study were reassortant strains containing the internal genes of the PR8 virus, and a mouse-adapted PR8 virus was used for the challenge infection of the TIV-immunized mice. Hence, functional antibodies directed to the internal proteins of the PR8 strain may have developed following TIV immunization and may be responsible for protection against a homologous PR8 challenge. The internal proteins of the influenza A viruses contain conserved epitopes, so the antibodies directed to the internal proteins may cross-react with mismatched influenza A strains providing protection against the mismatched strains (Vanderven et al., 2016). Similarly, antibodies induced by the conserved HA stalk epitopes present in the influenza A virus components of TIV and in the PR8 challenge virus may have contributed to the protection (Yassine et al., 2015; Klausberger et al., 2016; Zhong et al, 2016). The protective efficiency of a virosome-based trivalent subunit influenza vaccine (Inflexal V) against a heterosubtypic H5N1 challenge virus was tested earlier by passive transfer of sera obtained from the actively immunized mice, with the conclusion that a single transfer from donor mice did not, but a triple transfer did confer 80% survival rates in the recipient mice, and that non-neutralizing antibodies directed against conserved HA epitopes were responsible for the heterosubtypic protection (Roos et al., 2015). The immunization of mice with a modular capsomer presenting the ectodomain of matrix protein 2 (M2e) epitope adjuvanted with aluminum hydroxide, or recombinant M2e protein, induced high level M2e-specific antibodies that reduced disease severity and viral load in the lungs of challenged mice; the protection was attributed to the antibody-mediated immunity by antibody-dependent cell
mediated cytotoxicity (ADCC) or by complement-mediated phagocytosis (Wilbowo et al., 2014; Stepanova et al., 2015; Rappazzo et al., 2016). Cellular responses induced by the immunization of mice with influenza nucleoprotein (NP) were strongly associated with mouse survival after a lethal challenge with the PR8 strain (Wang et al., 2014), while other studies suggested that the non-neutralizing antibody response to NP may be an important protective mechanism (Carragher et al., 2008; LaMere et al., 2011). Furthermore, a heterosubtypic cross-protection in mice against the H1N1 PR8 challenge virus by a whole inactivated H5N1 virus vaccine was reported and cross-protective cytotoxic T lymphocytes were, but cross-reactive antibodies were not, suggested as responsible for the protection (Budimir et al., 2012). However, this study did not include the passive transfer of serum from actively immunized donor mice to recipient mice that were then challenged with a heterosubtypic virus strain.

Aluminum salts, the only adjuvants broadly licensed for human vaccines, have been widely used for more than eighty years. It is generally believed that aluminum adjuvants stimulate CD4+ cells to differentiate in the direction of T helper 2 (Th2) cells promoting predominantly antibody responses, but have little or no effect on the involvement of CD8+ cells in CMI responses (Bungener et al., 2008; Ehrlich et al., 2008; Aimanianda et al., 2009). Yet it has also been reported that aluminum-adjuvanted influenza proteins and a split A(H3N2) vaccine used for immunization can induce CD8+ and CD4+ T cells to proliferate, produce IFN-γ and protect mice from a lethal challenge with H1N1 and H2N2 viruses (Dillon et al., 1992; Mohr et al., 2010; Baz et al., 2012). It is not understood why some aluminum-adjuvanted influenza vaccines proved superior to non-adjuvanted ones in the induction of CMI responses, while others did not. It has been suggested that the viral RNA in the whole virion vaccines might act as a “built-in adjuvant” through TLR7 signaling (Tetsutani and Ishii, 2012), thereby influencing the results obtained in comparisons of immune responses following immunization with a whole virion vaccine administered with or without artificially added adjuvants. Seasonal trivalent inactivated influenza vaccines usually contain split or subunit viral elements and may contain various adjuvants, including MF59, ASO3 and ASO4 that have been shown to result in improved humoral and CMI responses in immunized subjects and experimental animals (van Els et al., 2014; Frey et al., 2014; Couch et al., 2014, Lee et al., 2014, Trombetta and Montomoli, 2016). However, regulatory and safety concerns have been raised in the case of some of these adjuvants (Miller et al., 2013). The CMI responses to the split or subunit vaccines are variable and do not seem to be optimal. As an illustration, it was observed that whole virus influenza vaccines activated dendritic cells and stimulated virus-specific CD8+ memory T cells more efficiently than did the split virus counterparts, and it was therefore concluded that vaccines against
seasonal and pandemic (-like) influenza strains that seek to stimulate cross-reacting CD8+ T cells should include whole virus formulations rather than split virus ones (Halbroth et al., 2014). Our results indicated that in mice, PR8-specific immunserum with an HI titer as low as 1:20 is sufficient for protection against a challenge infection with a homologous PR8 strain. Although the titer of the protective antibodies against the unidentified protein(s) in the challenge PR8 virus has not been determined, we observed that the HI titer to the TIV vaccine strains, used as an indicator for the level of unidentified protective antibodies, was high (in our experience HI titer of 1:126 or 1:160 or 1:253 or 1:320 to the A/California reassortant strain), and this was necessary for protection against a challenge infection with the PR8 virus. The low level protection in mice receiving immune serum from donor mice immunized with TIV or TIV+Al is most likely due to the relatively low antibody titer in the recipient animals (GMT of 1:50 or pool titer of 1:80 HI antibodies against the A/California reassortant strain). Correlates of protection, if protection is not based on HI, have not been defined, hence alternative assays should be developed to ascertain the protective antibody level that is not related to HI activity. Our study demonstrated that immunization with TIV+Al, vs. TIV not adjuvanted, was necessary to achieve a high serum antibody level sufficient for 80%-100% vs. 25% protection against mortality in the recipient mice, and a significant difference between body-weight loss in mice receiving serum from TIV+Al or TIV immunized donors (p<0.05) after the challenge infection. A further increase in antibody responses resulted in 100% protection against mortality and only a non-significant difference in body weight loss between mice receiving serum from mice actively immunized with TIV+Al+F or PR8 vaccines following the challenge infection. The morbidity rate of recipient mice as measured by the clinical scores gave similar results. The difference in the level of morbidity (change in body weight and clinical score) in mice actively immunized with TIV+Al vaccine (Figures 1B and C) and in mice passively immunized with immune serum obtained from these mice (Figures 2C and E) may indicate the contribution of CMI responses to the protection, or the higher antibody titers were responsible for the higher level of protection in the actively immunized mice (Table 1). We observed an increased IFN-γ and GrB expression in the in vitro-stimulated splenocytes obtained from TIV+Al-immunized mice (Figures 3 and 4). However, our preliminary results revealed that splenic lymphocytes obtained from Balb/c mice immunized with TIV+Al or TIV+Al+F then passively transferred to naive Balb/c mice by i.v. route did not protect the recipient mice against death or disease following the challenge infection with the PR8 strain (not shown). Clarification of the potential role of the TIV+Al-induced CMI in the protection against a PR8 challenge would be most useful and could be the subject of further research.
A systemic review and meta-analysis showed that in humans mismatched vaccines can reduce the risk of influenza infection by 54% for live attenuated influenza vaccine in children and by 52% for TIV in adults, but the protection rate was even higher when mismatched influenza A infections were calculated: namely, 75% for live attenuated influenza vaccine in children and 64% for TIV in adults, suggesting a beneficial role of vaccines in preventing influenza even when there is a mismatch between vaccine composition and the circulating strain (Tricco et al., 2013). It is difficult to compare the level of protection achieved in mice by the licensed influenza vaccines (e.g. Inflexal V) (Roos et al., 2015) or Fluval AB (our study) since immunizing doses of the vaccines, the amount of serum transferred, the antibody titers in the transferred serum and the nature and dose of the challenge viruses, which influenced the level of protection were different in these studies.

Our results indicate that the induction of immunity by a licensed, commercially available, aluminum-adjuvanted trivalent inactivated whole virion influenza vaccine should offer a reasonable level of serum-mediated protection against influenza A strains with no HI, NI or VN cross-reactivity. Before a universal influenza vaccine is developed, the Fluval AB, or other inactivated whole virion influenza vaccines containing efficient and safe adjuvants could be used not only for seasonal vaccination, but also for prevention at the beginning of the outbreaks of influenza infections caused by HA and NA mismatched influenza A viruses. After noting the strong booster effect of adjuvanted TIV inducing high antibody titers (Trombetta and Montomole, 2016), this approach might be especially helpful in populations with low-level or medium-level pre-existing immunity.

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REFERENCES


Table 1. Antibody titers in the pools of donor mice immunized with different vaccine compositions and GMs of HI titers in individual recipient mice after serum transfer

<table>
<thead>
<tr>
<th>Mice Immunogens</th>
<th>Titers against H1N1 A (California) reassortant</th>
<th>Titers against PR8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Donors</td>
<td>Recipients</td>
</tr>
<tr>
<td></td>
<td>HI</td>
<td>NI</td>
</tr>
<tr>
<td>NMRI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>&lt;10</td>
<td>&lt;40</td>
</tr>
<tr>
<td>TIV</td>
<td>1280</td>
<td>2560</td>
</tr>
<tr>
<td>TIV+Al</td>
<td>2560</td>
<td>&gt;5120</td>
</tr>
<tr>
<td>TIV+Al+F</td>
<td>5120</td>
<td>&gt;5120</td>
</tr>
<tr>
<td>PR8</td>
<td>&lt;10</td>
<td>&lt;40</td>
</tr>
<tr>
<td>PR8+Al</td>
<td>&lt;10</td>
<td>&lt;40</td>
</tr>
<tr>
<td>Balb/c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS (++)</td>
<td>&lt;10</td>
<td>ND</td>
</tr>
<tr>
<td>TIV+Al (++)</td>
<td>2560</td>
<td>ND</td>
</tr>
<tr>
<td>TIV+Al (+)</td>
<td>2560</td>
<td>ND</td>
</tr>
<tr>
<td>TIV+Al+F (++)</td>
<td>5120</td>
<td>ND</td>
</tr>
<tr>
<td>TIV+Al+F (+)</td>
<td>5120</td>
<td>ND</td>
</tr>
</tbody>
</table>

Serum pools collected from mice immunized actively with different vaccine compositions were tested in HI, NI and VN assays and transferred to recipient mice. NMRI recipients got 300 µl serum/mouse, Balb/c recipients got 200 µl serum/mouse (++) or 100 µl serum/mouse (+) from the donor pools. HI titers in individual sera of the recipient NMRI mice were tested and GM titers are shown. Serum pools of recipient Balb/c mice were tested. Protection: percentage survival rate after a lethal challenge with the PR8 strain.
Figure 1. Immunization of NMRI mice with TIV+Al protects against a A/PR/8/34 (H1N1) challenge infection.

Six groups of mice were immunized with TIV (▬), or TIV+Al (▬), or TIV+Al+F (▬Δ▬), or inactivated PR8 strain (▬), or PR8+Al (▬◊▬), or PBS (▬▬), and challenged with 5 LD50 of the PR8 strain as described in the Materials and Methods. Percent survival (Panel A), GM body weight change (Panel B) and median clinical score (Panel C) following challenge infection are shown. The asterisk indicates a statistically significant difference.

** p<0.01 for survival, body weight change and clinical score in all immunogen-immunized groups as compared with group inoculated with PBS.
Figure 2. TIV-specific immune serum confers protection against a challenge infection with PR8 virus in mice.

Serum transfer from immunized NMRI or Balb/c donor mice and the PR8 challenge infection of the recipient mice were carried out as described in the Materials and Methods. The survival rates of the recipient mice (Panels A and B), GM body weight change of recipient mice (Panel C and D), and median clinical score of the recipient mice (Panel E and F) following challenge infection are shown. Symbols for NMRI mice: mice receiving 300 µl of serum from TIV-immunized mice (▬◊▬); mice receiving 300 µl of serum from TIV+Al-immunized mice (▬Δ▬); mice receiving 300 µl of serum from TIV+Al+F-immunized mice (▬▲▬); mice receiving serum from PR8-immunized mice (▬х▬); mice receiving serum from PR8+Al-immunized mice (▬җ▬); mice receiving serum from PBS-immunized mice (▬▬▬). Symbols for Balb/c mice: mice receiving 200 µl of serum from TIV+Al-immunized mice (▬□▬); mice receiving 100 µl of serum from TIV+Al-immunized mice (▬○▬); mice receiving 200 µl of serum from TIV+Al+F-immunized mice (▬■▬); mice receiving 100 µl of serum from TIV+Al+F-immunized mice (▬●▬); mice receiving serum from PBS-immunized mice (▬▬▬).

The asterisk indicates a statistically significant difference as compared with mice receiving serum from PBS-inoculated mice. Error bars in panel E and F indicate interquartile range.

* indicates a p value of <0.05

** indicates a p value of <0.01
Figure 3. Percentages of IFN-γ+ or GrB+ cells in CD4+ or CD8+ T cell subpopulations or in NK cells in NMRI mice immunized with TIV+Al or TIV+Al+F or PBS.

NMRI mice were immunized, splenocytes were obtained and stimulated with live A(H1N1)pdm09 virus or culture medium, and the percentage of IFN-γ or GrB-producing cells in subpopulations of splenocytes was determined by flow cytometry, as described in the Materials and Methods. Symbols indicate individual mice. The percentages of IFN-γ+ or GrB+ cells in subpopulations of splenocytes stimulated with the virus in vitro after subtraction of the corresponding percentages in the culture medium-stimulated splenocytes are given. Horizontal lines are the mean percentages. *indicates a p value <0.05 and ** indicates a p value of <0.005, as compared with positive cells in the stimulated splenocytes of PBS-inoculated mice.
Figure 4. IFN-γ and GrB mRNA expression in virus-stimulated splenocytes taken from Balb/c mice immunized with TIV+Al, TIV+Al+F or PBS. The mRNA expression in virus-stimulated splenocytes is determined relative to that found in culture medium-stimulated splenocytes. * indicates a p value <0.05 as compared with PBS immunized mice.