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## Functional Immune Response to Influenza H1N1 in Children and Adults after Live Attenuated Influenza Virus Vaccination.

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### Abstract

Influenza virus is a major respiratory pathogen and vaccination is the main method of prophylaxis. In 2012, the trivalent live attenuated influenza vaccine (LAIV) was licensed in Europe for use in children. Vaccine-induced antibodies directed against the main viral surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA), play important roles in limiting virus infection. The objective of this study was to dissect the influenza-specific antibody responses in children and adults, and T cells responses in children induced after LAIV immunization to the A/H1N1 viruses. Blood samples were collected pre- and at 28 and 56 days post-vaccination from 20 children and 20 adults. No increase in microneutralization (MN) antibodies against A/H1N1 were observed after vaccination. A/H1N1 stalk specific neutralizing and NA-inhibiting (NI) antibodies were boosted in children after LAIV. Interferon  $\gamma$  producing T cells increased significantly in children, and antibody-dependent cellular-mediated cytotoxic (ADCC) cell activity increased slightly in children after vaccination, although this change was not significant. The results indicate that the NI assay is more sensitive to qualitative changes in serum antibodies after LAIV. There was a considerable difference in the immune response in children and adults after vaccination, which may be related to priming and previous influenza history. Our findings warrant further studies for evaluating LAIV vaccination immunogenicity.

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Conflicts of interest

The authors declare no conflict of interest.

## Keywords

Influenza A/H1N1; Children; Adults; Immune response; LAIV

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## Introduction

Influenza virus is a major respiratory pathogen causing annual epidemics leading globally to approximately 3–5 million severe infections, with 300,000–650,000 estimated deaths [1]. Influenza is a vaccine preventable disease and two main types of vaccines are available, the inactivated influenza vaccine (IIV) and the live attenuated influenza vaccine (LAIV). In 2012, the LAIV was licensed in Europe for use in children 2–17 years old, but not for adults. Trivalent live attenuated influenza vaccine (LAIV) has previously been shown to provide broader protection against influenza A infection [2–4]. LAIV mimics natural infection and both the innate and the adaptive immune responses are activated after LAIV [5–7].

The influenza virus has two major surface glycoproteins; the hemagglutinin (HA) and neuraminidase (NA), and antibodies directed towards both glycoproteins play an important role in protection against influenza virus. The haemagglutinin comprises an immunodominant head domain (composed of the majority of the HA1 subunit) and a stalk domain (mainly the HA2 subunit and the N- and C-terminal ends of HA1). Antibodies to the HA head inhibit viral attachment to the host cell receptors and can be measured by the hemagglutination inhibition (HI) assay. HI antibodies are measured as a surrogate correlate of protection [8–9] with an HI titer of 40 providing a 50% protective threshold in adults. The micro-neutralization assay also measures mainly HA head specific antibodies that can neutralize the influenza virus [11]. The HA head specific antibodies do not reflect the whole spectrum of protective antibodies and the HA stalk-specific antibodies can also confer protection [12]. The stalk domain is conserved and provides broad cross-protection by neutralizing antibody and through antibody-dependent cellular-mediated cytotoxicity (ADCC) by interaction of antibodies and Fc $\gamma$ R on natural killer cells [13–16]. The hemagglutinins of the influenza A viruses are divided into groups based on their amino acid sequence of mainly the head region, the more conserved stalk region may be a promising target for universal vaccine development. A recent animal model study identified broadly protective anti-HA stalk antibodies that were induced after LAIV vaccine expressing chimeric HA [17]. Furthermore, neuraminidase inhibition (NI) antibodies can also provide protection from influenza [18]. NA is an important viral surface glycoprotein with enzymatic activity cleaving sialic acid on the lumen of mucosal epithelial cells of the respiratory tract. NA is important in the infection process, clearing a path for infection, lowering the pH at the cell surface, and releasing of progeny virus from infected cells. Blocking NA activity is an effective way to inhibit infection and viral shedding [19,20].

During the 2014–15 season, the USA experienced unexpectedly low protection from the H1Npdm09 strain in the LAIV, which was not observed with IIV. This led the US Advisory Committee on Immunization Practices (ACIP) to withdraw its earlier preferential recommendation of LAIV in children. However, moderate protection was observed to the H1N1 strain in Europe, with 41.5% vaccine effectiveness in the UK and 47.9% in Finland,

although remaining lower than IIV [21,22]. A study in Senegal found that LAIV failed to protect against H1N1pdm09 in young children [23], whereas protection was found in a similar study in Bangladesh [24]. The reason for these differences is currently unknown but could be due to the vaccines were based on different backbones, or the population's exposure history [25,26]. The LAIV manufacturer reported that the A/H1N1 strain had a temperature sensitive mutation rendering it heat instable providing a possible explanation for the reduced protection observed, and has since updated the vaccine [27]. Importantly, exposure history to influenza will influence protection and differences in vaccine recommendations could possibly affect protection observed after LAIV. The USA recommends influenza vaccination for everybody from > 6 months, while the European vaccine recommendations primarily focus on risk groups.

In our previous study, we found no increase in HI antibodies to the H1N1 strain after LAIV, however most children had pre-existing antibodies [28]. However, a slight trend of rising H1N1 stalk specific antibodies was observed in children after LAIV, whereas adults had already higher levels of H1-stalk specific IgG antibodies pre-vaccination and no further increase was observed after vaccination. We suggested that this was due to preexisting antibodies present after vaccination or infection during the 2009 pandemic. The aim of this study was to further dissect the antibody responses, focusing on the functional immune response after LAIV immunization against H1N1.

## Materials and Methods

### Patients & Samples

Forty subjects (20 children (3–17 years old) and 20 adults (21–59 years old)) were intranasally immunized with 0.1 mL per nostril of the seasonal LAIV (Fluenz, Astra Zeneca, Liverpool, UK). LAIV (Fluenz) contained  $10^7$  fluorescent focus units (FFU) of A/California/7/2009(H1N1)pdm09-like and A/Victoria/361/2011(H3N2)-like strains in both 2012/13 and 2013/14 seasons, and either B/Wisconsin/1/209-like or B/Massachusetts/2/2012-like influenza B virus strains in the 2012/13 or 2013/14 seasons, respectively. The study was approved by the ethical and regulatory authorities (EUDRACT2012–002848-24, [www.clinicaltrials.gov](http://www.clinicaltrials.gov); ). Children received one (9 years old, n=6) or two doses (<9 years old, n=14) of LAIV in 2012 at a four-week interval, whereas adults received a single dose in 2013–14 season [5]. Blood samples were collected at day 0 (pre-vaccination), 28- and 56-days post-vaccination and plasma aliquoted and frozen for use in the serological assays, as previously described [28]. In children, cell preparation tubes (CPT, BD) were used to separate peripheral blood mononuclear cells (PBMCs) for the ELISpot assay.

### Viruses & Antigens

Viruses were propagated in the allantoic cavity of 10 day old embryonated hen's eggs. Allantoic fluid was harvested, clarified and frozen at  $-80^{\circ}\text{C}$  until used in the assays as described below. The reassortant A/California/7/2009(H1N1) virus (X-179A) was used for the micro-neutralization (MN) assay, the chimeric cH9/IN3 virus containing the HA stalk from A/California/7/2009(H1N1) and head from A/guinea fowl/Hong Kong/WF10/99 for the virus neutralization (VN) assay, and the reverse genetics H7N1 virus (NIBRG-127

containing the NA from A/California/7/2009(H1N1) and HA from the equine A/Prague/56 (H7N7) strain) for enzyme-linked lectin assay (ELLA). The wild type A/California/7/2009(H1N1) virus was used for the antibody dependent cellular cytotoxicity (ADCC) assay.

### Micro-neutralization assay (MN)

The microneutralization assay was conducted as previously described [28]. Briefly, plasma and control sheep serum samples (NIBSC, UK) were 2-fold serially diluted from 1:10 in flat-bottom 96-well cell culture plates (Nunclone Delta surface, USA) before incubation with one-hundred 50% tissue culture infectious doses (TCID<sub>50</sub>)/50µl/well of X-179A for 1 h at room temperature. Then, 1.5×10<sup>5</sup> MDCK (Mardin Darby Canine Kidney) cells/ml were added and incubated for 16–18 hours at 37°C. The propagation of influenza virus was detected using antibody to the nucleoprotein and TMB (3,3', 5,5'-Tetramethylbenzidine; Thermo Fisher Scientific, USA) before reading at 450nm and 620nm to obtain the final optical density (OD). The microneutralization titers (IC<sub>50</sub>) were calculated using the Reed and Muench method [29].

### Virus neutralization assay (VN)

The virus neutralization assay was conducted with the cH9/1 virus using a 3-day incubation period [30,31]. Briefly, cell culture plates (Flat-bottom 96-well Nunclone Delta surface, USA) were seeded with 1.5×10<sup>4</sup> MDCK cells/well and incubated at 37°C overnight. Next, heat-inactivated plasma samples were diluted to 1:10 and 2-fold serially diluted before incubation with cH9/1N3 (100 TCID<sub>50</sub>/50 µl) for 1h at 37°C. MDCK cells were washed with phosphate buffered saline (PBS), and plasma/virus dilutions were added and incubated for 1 h at 37°C. The mixture was removed, cells were washed with PBS, and 50 µl of serially diluted plasma plus 50 µl infection medium (DMEM medium containing 2.5µg/ml tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Biomedical, USA), PSA (100 IU/ml penicillin, 100mg/ml streptomycin and 0.25 µg fungizone; Lonza, Switzerland) and 0.14% bovine serum albumin (Sigma-Aldrich, USA) were added to each well before incubation at 37°C for 72 h. The virus neutralization titers were measured by haemagglutination assay using the supernatant (50 µl) and 50 µl of 0.7% human red blood cells and read after 30 min of incubation at room temperature. The highest dilution of plasma giving complete haemagglutination was read as the neutralizing antibody titer.

### Enzyme-linked lectin assay (ELLA)

The ELLA was used to measure antibodies inhibiting the ability of neuraminidase to cleave sialic acid as previously described [32–34] using the H7N1 virus containing the HA from an equine influenza virus strain and NA from A/California/07/09. Ninety-six well flat bottom Maxisorb plates (VWR, USA) were coated overnight at 4°C with 100µl coating solution containing 0.25µg fetuin per well (Sigma-Aldrich, USA; KPL; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Heat-inactivated (56°C for 45–60min) serially diluted serum and H7N1 virus were added and incubated at 37°C for 16–18 hours. The virus was diluted to a titer giving 90% neuraminidase activity. The plates were developed by adding 0.1µg/100µl/well horseradish peroxidase (HRP)- conjugated peanut agglutinin (PNA) (Sigma-Aldrich, USA) and incubation at room temperature for 2 hours, followed by adding o-

phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, USA) substrate (0.5mg/ml) in citrate buffer to all wells. After 10 min incubation at room temperature in the dark, the reaction was stopped with 100  $\mu$ l 1M sulphuric acid. The plates were read with a microplate reader by spectrophotometry at OD 490 nm. The anti-NA antibody titres (reported as 50% inhibition concentration, IC<sub>50</sub>) in the plasma samples were calculated as the reciprocal dilution of plasma which gave OD values equal to 50% of total OD (OD virus control + OD blank) in four-parameter non-linear regression analysis using GraphPad Prism.

### Antibody dependent cellular cytotoxicity (ADCC) assay

The ADCC Reporter Bioassay (Core Kit G7010/G7018, Promega, USA) was used to quantify pre- and post-vaccination ADCC antibodies [15]. MDCK cells as 'target cells' were seeded in 96 F-well white tissue culture plates (VWR, USA) at  $1.5 \times 10^4$  cells/well. Between 18–24 hours later, cells were infected with wild type A/California/7/2009(H1N1)pdm09 virus at a multiplicity of infection (MOI) of 3. On the day of assay, the medium was replaced with assay buffer (RPMI 1640 with 4% (vol/vol) low IgG fetal bovine serum (FBS) (Lonza, Switzerland) followed by the addition of 5-fold (starting at 1:10) serial dilutions of plasma. The infected cells and antibodies were incubated at 37 °C for 30 mins. The effector cells (Jurkat) at  $7.5 \times 10^4$  cells/well were added to the assay plates. After 6 hours incubation at 37°C, the Bio-Glo Luciferase Assay Reagent (Promega, USA) system was used to quantify using a plate reader with glow-type luminescence.

### ELISpot Assay

Antigen-specific interferon gamma (IFN- $\gamma$ ) cytokine-secreting T cells were quantified at the single-cell level by the ELISpot assay (Mabtech) [35]. Optimized libraries of peptides represented unique T cell epitopes from four of the initial H1N1pdm09 circulating strains for CD4<sup>+</sup> (originating from HA, NA, M1, NP, PB2) and CD8<sup>+</sup> (M1, NA, PA and NS2) responses (Table S1). Briefly, 400 000 PBMCs per well were stimulated with CD4<sup>+</sup> and CD8<sup>+</sup> conserved peptide pools (2  $\mu$ g/mL) anti-CD3 T-cell activator (positive control), or lymphocyte medium alone (negative control) [36]. Plates were incubated overnight at 37°C with 5% CO<sub>2</sub>. After developing the following day, the plates were read using an automated reader (Advanced Imaging Devices) and spot-forming units (SFUs) were counted. The background values were subtracted from the influenza virus-specific response.

### Statistics

The statistical tests and graphs were made using GraphPad Prism; v.7.0d for Mac (GraphPad Software, USA), where, P<0.05 was considered significant. Immune responses within each patient group, children or adults, were analysed using Friedman test (One-Way ANOVA, non-parametric, paired test). Comparison between children and adults were performed using Mann-Whitney test (t-Test, non-parametric, unpaired). Correlation analysis was performed using linear regression and Spearman correlation.

### Results

Twenty children (3–17 years old) and twenty adults (21–59 years old) were intranasally vaccinated with seasonal LAIV. The children received one ( 9 years old, n=6) or two doses

(<9 years old, n=14) of LAIV in 2012 at a four-week interval, whilst adults received one dose of vaccine. In this study, the objective was to analyze the functional HA and NA H1N1 specific responses in children and adults, and the T cellular immune response in children.

### **Presence of neutralizing serum antibodies by microneutralisation assay (MN)**

A MN titer of 80 is considered to protect 50% of individuals from disease [37]. Sixty percent of children had protective pre-vaccination MN titers (Figure 1A). Four children (20%) were boosted after 1<sup>st</sup> dose of vaccine and one of these children (5%) seroconverted after LAIV to a protective antibody titer. Only three children (15%) had no detectable MN antibodies both pre- and post-vaccination. Eleven adults (55%) had protective pre-vaccination MN titers of whom 25% increased post-vaccination (Figure 1A) and one (5%) adult sero-converted to protective antibody titers after LAIV. Of the adults with antibodies below the protective MN threshold before vaccination, 30% remained sero-negative post-vaccination. After vaccination MN antibody titers increased <2 fold in both children and adults at day 28 and decreased in adults at day 56 (Figure 1B). Children had higher MN titers than the adults both pre- and post -vaccination. As expected, both pre-and post-vaccination HI and MN titers correlated well (pre-vaccination: children- R=0.5546, adults- R=0.7514; post-vaccination: children- R=0.839, adults- R=0.3267; Figure S2). In summary, LAIV did not significantly boost micro-neutralizing antibody titers in adults or children against the H1N1 vaccine strain.

### **Assessing the stalk specific neutralizing serum antibodies by ELISA**

We have previously shown that only children, and not adults showed a slight trend of an increase in stalk specific antibodies measured by ELISA using chimeric HA proteins after LAIV vaccination [28]. We extended these observations by investigating the HA stalk specific virus neutralizing antibody responses using a chimeric cH9/1 virus. Nineteen (95%) children had high VN titer  $\geq$  80 pre-vaccination (geometric mean titer GMT: 114.2). H1N1 stalk-specific VN antibodies increased slightly after 1<sup>st</sup> dose of LAIV in 50% of children, (GMT=129.3) but decreased after the second dose (GMT=99.7) (Figure 1C). Fifteen adults (75%) had pre-vaccination stalk specific VN titers  $\geq$  80. No significant boost in stalk-specific VN antibodies was observed after LAIV, although 35% of adults had increases in VN antibodies (Figure 1C). In summary, neutralizing stalk antibodies showed a slight trend of an increase after LAIV vaccination in children after first 1<sup>st</sup> LAIV dose but were not maintained after the 2<sup>nd</sup> dose (Figure 1D). Of note, the method used for these assays is different than the standard MN assay used for wild type virus and is – in combination with the cH9/1N3 virus - more sensitive than standard MN assays, leading to a higher baseline as well.

### **Neuraminidase Inhibiting Assay (NI) showed increases titers in children and adults**

Neuraminidase inhibiting antibodies also play an important protective role in influenza virus infection. A neuraminidase inhibition (NI) titer of  $\geq$  40 reduced influenza illness in the human challenge model and has been recommended as protective titer [38,39]. Eight children had pre-vaccination NI titers above the protective titer, two children had low titers, and the remaining 10 children did not have detectable titers (<10) (Figure 2A). The median

NI titer increased significantly at 28 days post vaccination in children, and most children responded with higher NI titers, although not all responses were > 40.

In adults, thirty percent had protective NI antibody titers pre-vaccination, whilst 60 percent had low NI titers and ten percent had non-detectable NI titers (<10) before vaccination (Figure 2A). Vaccination boosted the NI titers in most of the adults, and the number of adults with protective level increased to forty percent. Overall the NI titers increased in most of the subjects, and very few had a decrease in titer (Figure 2B).

### **Children had stronger Antibody Dependent Cellular Cytotoxicity (ADCC) levels than adults after LAIV**

We examined the ability of antibodies to elicit ADCC activity in children and adults to the H1N1pdm09 virus after LAIV vaccination. Adults had higher ADCC antibody titers compared to children both pre- and post-vaccination. The mean ADCC titer was 95 pre-vaccination in children and increasing to 156 after 28 days, but this change was not significant (Figure 3A). The adults had a mean ADCC titer of 271 pre-vaccination and this dropped significantly to 147 after 28 days (Figure 3A). Significantly higher fold changes were found in children compared to adults after 1<sup>st</sup> dose of vaccine (Figure 3B).

### **LAIV elicited CD4<sup>+</sup>T cell activation in children but not CD8<sup>+</sup> cell activation**

As only children responded after LAIV we further extended this work by investigating the ability of LAIV vaccine to elicit IFN- $\gamma$  producing T-cells in children using H1N1pdm09 strain specific CD4 or CD8 peptides. These CD4<sup>+</sup> and CD8<sup>+</sup> peptides (Table S1) represented unique T cell epitopes from four of the initial H1N1pdm09 circulating strains. Previous work has shown that 100 spot forming units (SFUs)/10<sup>6</sup> PBMCs provided protection from laboratory confirmed influenza after LAIV immunization in children [40]. Although a recent population based study in the UK showed that T-cell responses to NP 20 SFU/10<sup>6</sup> PBMC were associated with protection in adults [41].

Most children had less than 100 IFN- $\gamma$  secreting CD4<sup>+</sup>100 SFUs per million PBMCs and only 1 child (5%) had above this level before vaccination. LAIV significantly boosted the CD4<sup>+</sup> IFN- $\gamma$  response (Figure 4A) with twenty percent of children had more than 100 IFN- $\gamma$  CD4<sup>+</sup> T-cells at 28 days post-vaccination. Two of these children (10%) maintained high IFN- $\gamma$  secreting CD4<sup>+</sup> T-cell after 2<sup>nd</sup> dose of LAIV. Two further children had increases in IFN- $\gamma$  to >100 SFUs/10<sup>6</sup> PBMCs after the 2<sup>nd</sup> dose of LAIV. However, we observed no changes after LAIV in IFN- $\gamma$  secreting H1N1 specific CD8<sup>+</sup> T-cells, except one (5%) child who showed an increase at day 56 (Figure 4B).

## **Discussion**

Earlier, the LAIV showed poor protective efficacy against the H1N1pdm09 strain and was therefore not recommended used by the ACIP, however in the 2018/19 season the vaccine was reinstated [42]. Several European studies showed other results and therefore the EU continued to recommend use of LAIV in children [43]. We have previously shown that LAIV immunization did not boost H1N1 HI titers in adults or children [28]. Although children had significantly higher H1N1 HI titers than adults prior to and up to 56 days after

vaccination, probably due to previous infection or the pandemic vaccination during the 2009 mass vaccination campaign. In this study, we evaluated the humoral (children and adults) and the cellular immune response (children only) after LAIV immunization to further understand the lower immune responses to H1N1pdm09. Our results demonstrate that the majority of children had relatively high pre-vaccination MN titers, perhaps due to either infection from circulating H1N1pdm09 or the previous pandemic vaccination. Although no boosting in MN antibodies was found after vaccination, in agreement with a previous report [44], the children had low but significant increases in neuraminidase specific antibody levels and CD4+ T cell responses, supporting findings that LAIV induces a broad immune response, which could have a protective effect.

### **No boost in H1N1 specific MN antibodies in children or adults after LAIV**

The immune response to LAIV is multifaceted and the HI titer underestimates protection achieved from LAIV [45]. Overall, no significant changes in MN antibodies were observed after LAIV, although a trend of an increase in MN titers was observed in both adults and children. Children have experienced less influenza virus exposure in their life-time compared to adults and therefore may have a stronger recall response to antigenically matched strains [46–48], perhaps explaining the higher MN antibody observed in the children (Figure 1C & 1D). Our study reveals that HI titers correlated well with the MN titers in both children and adults, pre- and 28 days post vaccination, with adults and children with HI titers > 40 also having higher MN titers. Previously the MN assay has been shown to have a higher sensitivity to H1N1pdm09 than the HI assay [37]. Interestingly, HI titers of 40 were associated with the geometric mean of MN titers over 160 in both children and adults in agreement with a previous study that suggested that MN titers of 160 are associated with protective HI titres [47], but this threshold may be different from study to study.

During the 2009 pandemic, Norway conducted a mass vaccination campaign with AS03 adjuvanted pandemic vaccine and nearly half of the population was vaccinated. In our study, half of the children and adults were previously vaccinated with adjuvanted pandemic vaccine and these vaccinated subjects had higher MN antibody titers compared to the unvaccinated group, in agreement with previous findings [50]. It has been shown that H1N1pdm09 specific HI antibodies persist after a single adjuvanted pandemic vaccination beyond the 2012 and 2013 seasons [51]. Interestingly, the adults vaccinated with the pandemic vaccine had significantly higher MN titres than unvaccinated adults, whereas this was not observed in the children many of whom may have had H1N1pdm09 as their first influenza virus infection which circulated in Norway in the 2009/10 and 2010/11 seasons (Figure S3). In children, influenza imprinting by early exposure to influenza virus influences subsequent influenza immunity [52,53], but whether the imprinting is specific to the first infection, or a result of the cumulative effect of repeated exposures and boosting, remains unclear. Although a child's first encounter with influenza virus (HA imprinting) has a large impact on future immune status [54,55].



### **An elevated H1N1 stalk specific neutralizing antibody titer were observed in children post LAIV vaccination**

MN antibodies prevent infection therefore are a direct measure of protection, although HA stalk-specific antibodies may also confer protection [12] through neutralization or by ADCC through Fc–Fc $\gamma$ R interactions [13]. We have previously reported a slight trend of an increase in group 1 HA stalk specific IgG in children after LAIV, but not in adults [28]. Stalk specific neutralizing antibodies also showed a slight boost after the first dose in children suggesting that LAIV could be used as a priming vaccine for inducing broadly cross-reactive stalk responses. However, no change in stalk neutralizing responses after LAIV vaccination was found in adults, irrespective of previous vaccination history. Furthermore, the stalk specific VN antibody response did not correlates with HI titer in either children or adults and confirm the different specificity of the assays (data not shown).

Overall children had more head specific neutralizing MN antibodies, whereas adults had higher stalk specific virus neutralizing antibodies (data not shown). Interestingly, we found both children and adults previously vaccinated with AS03 pandemic vaccination had elevated head specific MN antibodies, whereas elevated stalk specific VN antibody responses were observed in non-vaccinated children and adults (Figure S5). Infection do generally stimulate stronger antibody responses to stalk antigens than vaccination. This suggests induction of stalk specific neutralizing antibodies depends on previous exposure history but is not related age related [56,57].

### **LAIV boosts neuraminidase inhibition (NI) response in children**

NI antibodies can reduce release of new viruses from infected cells [58,59] and NA specific antibodies may be a correlate of protection [38,39] in man, independently of HA antibodies. Here, we found significant increases in NI antibodies after LAIV in children although titres often remained below the protection level (<40). In contrast, in the UK no increase in NI antibodies was found after LAIV4 perhaps because of the H1N1pdm09 having lowest replication of the four LAIV strains [44,60]. Here, LAIV boosted the NI response after the 1<sup>st</sup> dose in children which continued to increase after the 2<sup>nd</sup> dose in the previously vaccinated group, whereas, antibodies only increased after 1<sup>st</sup> dose in the previously unvaccinated group. Further analysis demonstrated that both children and adults with high HI or MN antibody titers (> 40) also had protective NI antibody titers, although these NI antibodies were only boosted significantly in children. Surprisingly, the NI antibody fold change after LAIV seemed to increase in children with increasing age, whilst decreasing in adults [18].

### **No boost in ADCC inducing antibodies**

We and others have previously shown that A(H1N1)pdm09 vaccination and infection induced HA-specific ADCC mediating antibodies, with the stalk-specific antibodies better at mediating ADCC than head-specific antibodies [30]. In the current study, we evaluated the ADCC activity to wild type H1N1pdm09 with the aim of detecting both H1 stalk and NA antibodies. Half of the children were vaccinated with AS03 pandemic vaccine in 2009 and these children had high stalk specific functional antibodies, as hypothesized previously [61–63]. But we also found high titers of stalk specific VN antibodies in the unvaccinated

children who may have experience natural infection with H1N1pdm 09 (Figure S4). Higher pre-existing ADCC inducing antibodies may limit replication of the LAIV strains and therefore limiting the ability of the vaccine to boost antibody responses [64]. Interestingly, our study illustrated that both children and adults had pre-existing ADCC antibodies, although the children had significantly lower antibody titers than adults, perhaps explaining the lower efficacy seen after LAIV in adults. Concurrently the adults had lower HA (HI and MN) and NA specific (NI) antibody titers than the children despite having higher ADCC titers pre and post vaccination. No significant changes in ADCC reporter activity were found after LAIV, in agreement with an earlier study [65].

### LAIV boosts CD4 T cells but not CD8 T cells in children

T-cells play an important role in coordinating the immune response and are also critical for the control of influenza infection. Early human studies showed an inverse correlation between influenza virus shedding and CD8<sup>+</sup> T cells in sero-negative adults [66,67]. The importance of CD8 cells was confirmed during the 2009 pandemic when pre-existing late-effector CD8<sup>+</sup> IFN- $\gamma$  T-cells with lung-homing and cytotoxic potential were associated with milder symptoms and less severe illness [68] in the absence of H1N1pdm09 specific antibody. In human challenge studies, pre-existing CD4<sup>+</sup> T-cells resulted in lower virus shedding and less severe influenza symptoms in sero-negative subjects [69]. We have previously shown that LAIV induces durable increases in IFN- $\gamma$  positive T cells to H1N1pdm09 in children and that pre-existing cross-reactive CD8 T cells are boosted in young children [70]. Here, we aimed to further understand the T cell response in children using optimized libraries of CD4 and CD8 peptides representing unique T cell epitopes from the H1N1pdm09 virus. In the children, we found that only H1N1pdm09 specific CD4 responses were boosted after LAIV and no change in CD8 responses was observed [71].

Our results suggest that the H3N2 LAIV strain may be responsible for our earlier observation of boosting of CD8 cross protective responses but will need to be confirmed using conserved CD8 peptides. In earlier LAIV studies we found that 25% had significant increases in virus specific T-cell responses after vaccination although no increase in serum responses was detected [5]. This study further extends these findings to show that although the children did not respond by traditional MN antibodies, they responded with increase in CD4 T-cells, which may provide clinical protection. LAIV has been shown to induce broader protection in ferrets [72] and in children when used in a school setting [73]. Furthermore, the inclusion of LAIV in the childhood vaccination programme in the UK has shown the benefits of herd immunity, which, are thought to be mediated by T-cells [74,75]. Therefore, the use of LAIV in children could be an important step in the protection against novel drifted or pandemic strains.

This study has focused on the functional systemic immune responses. The LAIV vaccine is intended to mimic a natural infection and stimulate the local immune response. We have in a previous study, shown that LAIV induces local influenza specific IgA production [71]. As there are some degree of compartmentalization of the immune system, and we cannot expect to observe the same degree of immune response systemically as with parenteral administered vaccines. Using established immune response thresholds validated for systemic vaccines

may have to be used with caution when evaluating LAIV. This warrant further work and development of improved evaluation tools for mucosal vaccines.

**In conclusion**, children and adults have different previous exposure histories to the influenza virus through infection and vaccination and this can impact upon the generation of immune responses after LAIV. The LAIV vaccine is administered mucosally requiring replication of the vaccine viruses, therefore pre-existing antibodies may limit replication and subsequent induction of immune responses as observed in the adults with relatively high pre-vaccination antibody levels. Although the children also had high MN antibodies they responded with significant increase in NI and CD4 T cells after vaccination. This indicate that the NI assay may be more sensitive to detect boosting in antibody responses after mucosal LAIV vaccination which needs further investigation. The multifaceted immune response after LAIV in children supports the continued recommendation of LAIV for use in children.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgement

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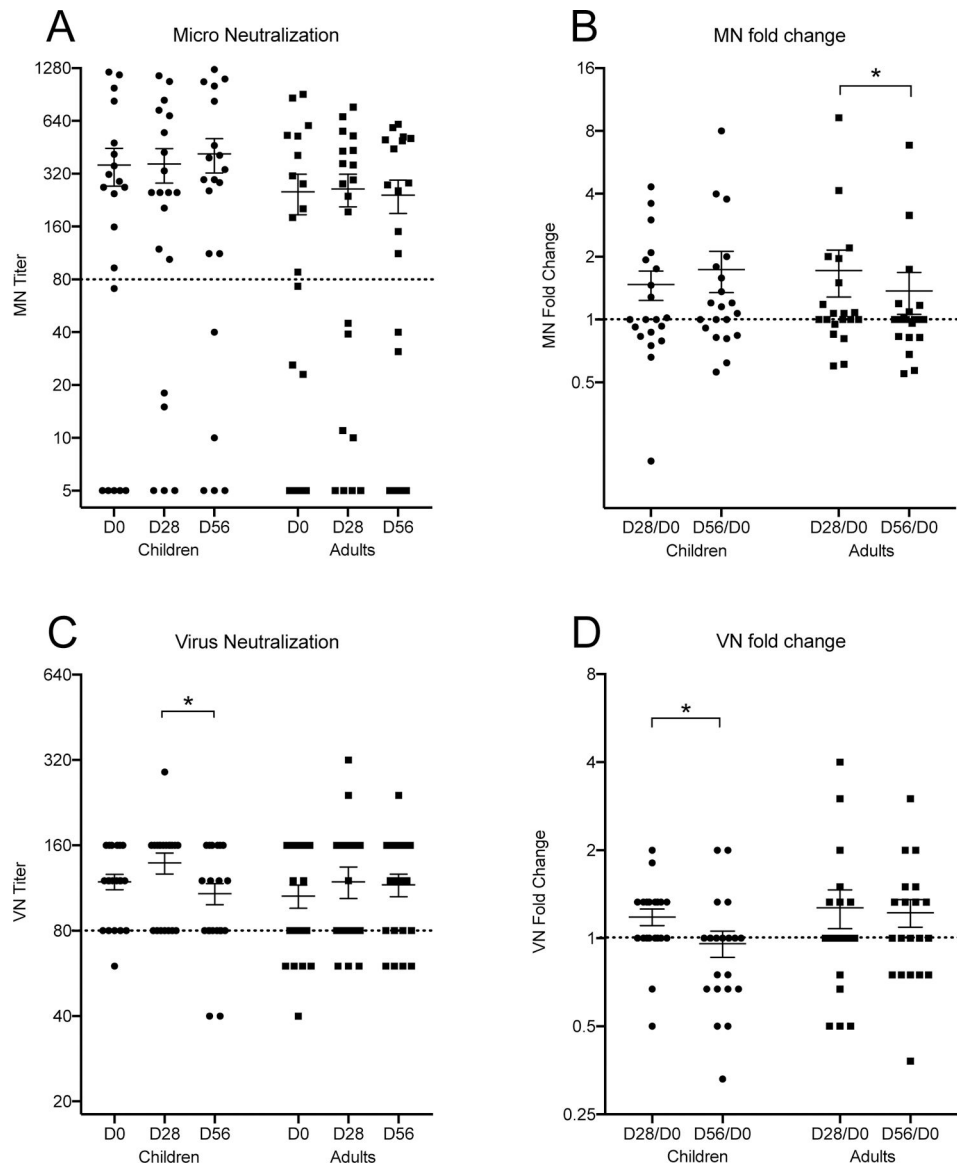
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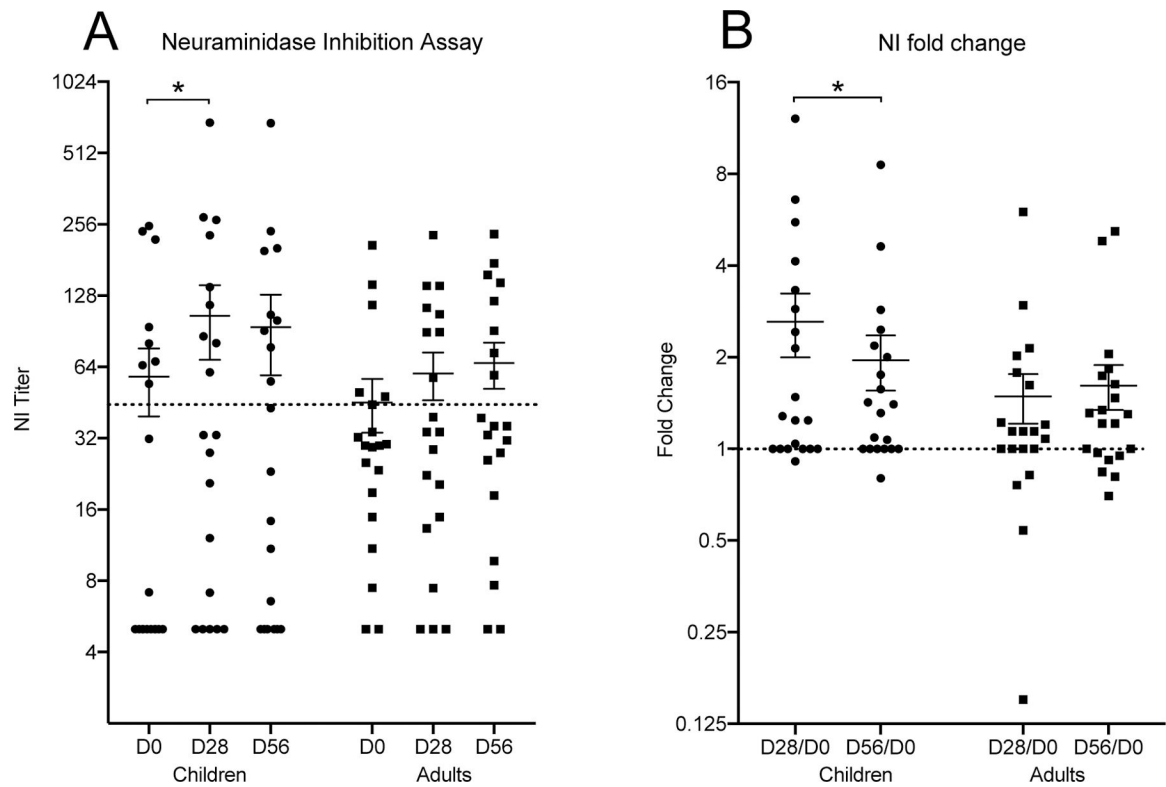
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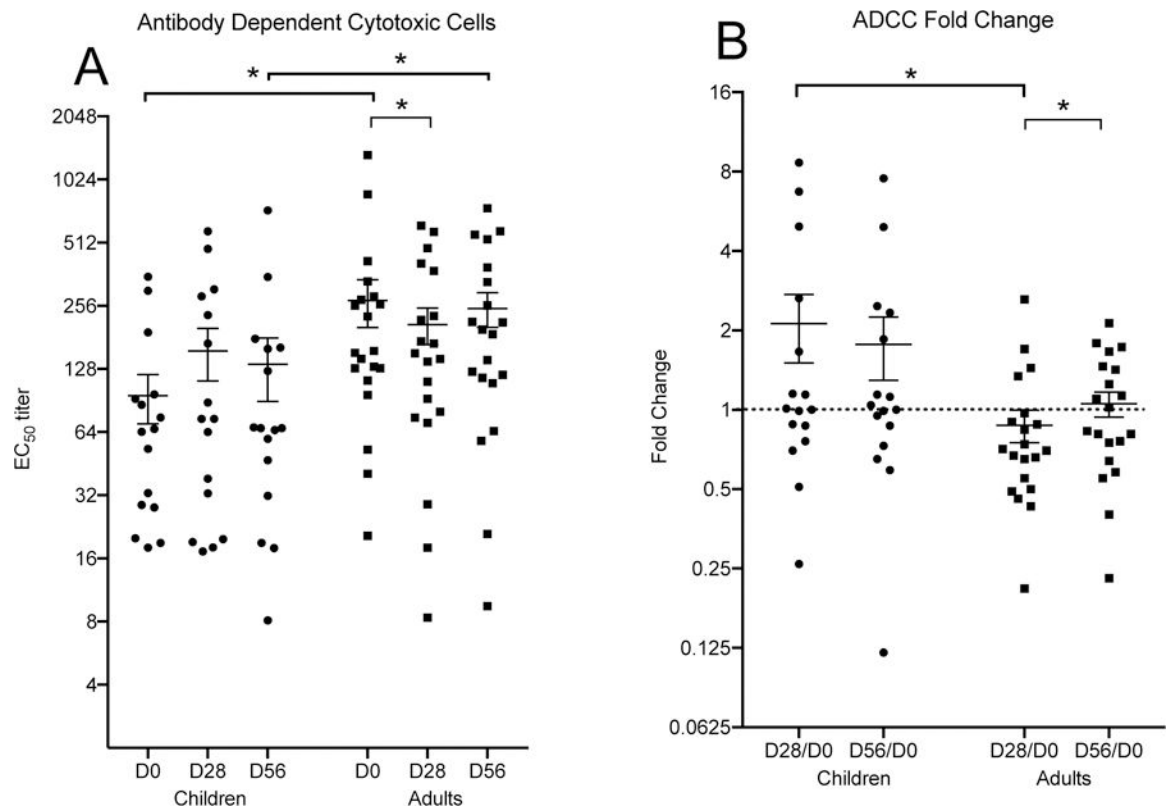


**Figure 1.** Dot plot of the microneutralization (MN, X-179A) and virus neutralization (VN, ch9/1N3) assays. Each dot represents data from one subject. The panels A to D are; MN, the fold change of MN, VN and fold change of VN respectively. The titers and fold change are indicated on the y-axis. The groups on the horizontal axis are children (circles) and adults (squares), sampled at day zero, and 28 and 56 after vaccination. The mean value  $\pm$  SEM of each group is indicated in all panels. In panels A and C, a horizontal dotted line indicates a protective threshold titer. A similar dotted line is indicating no titer change (fold change = 1). A significant difference in median values are indicated with asterisks above the group columns,  $p < 0.05$ .



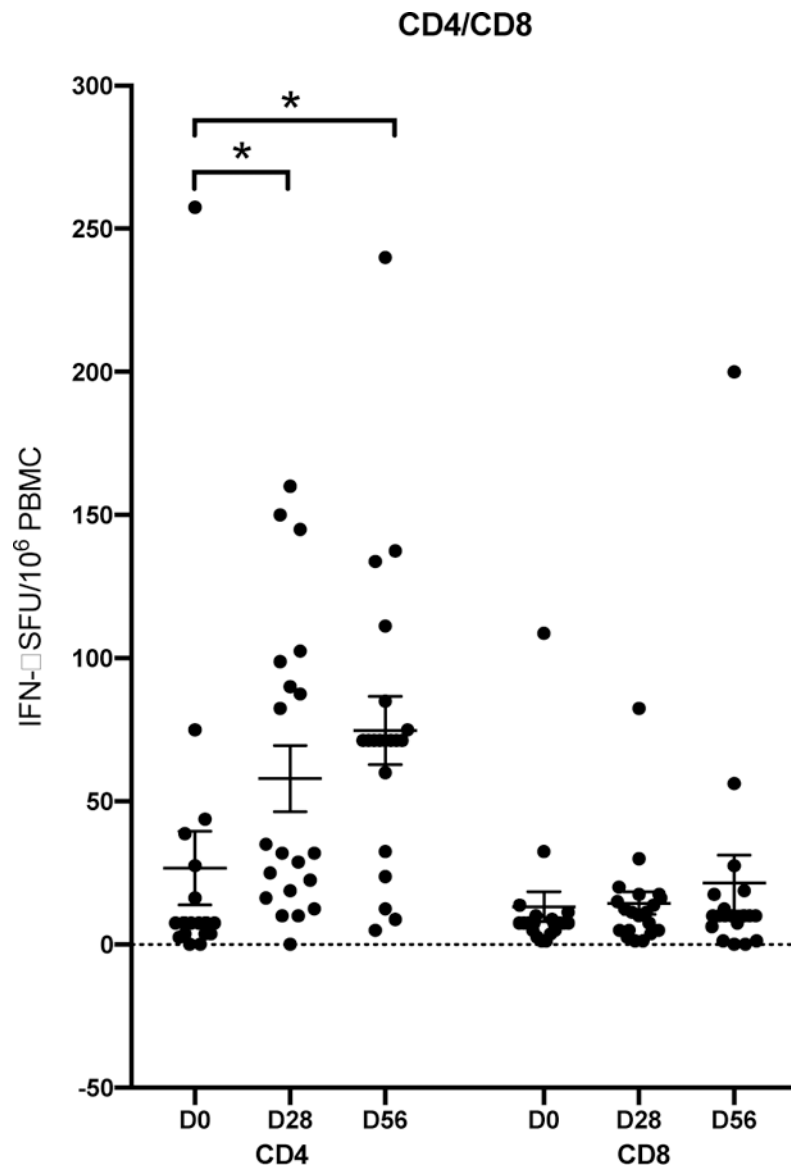
**Figure 2.**

Neuraminidase inhibition assay (NIBRG-127), tested at day zero and days 28 and 56 after LAIV vaccination in children (circles) and adults (squares). Each dot represents an individual test-point. The titer and fold change is indicated on the vertical axis and the groups on the horizontal axis. The mean value  $\pm$  SEM are shown for each group. A dotted line indicates the protective threshold in the left panel (A) and a no fold change in the right panel (B). A significant difference in median values are indicated with asterisks above the group columns,  $p < 0.05$ .



**Figure 3.**

The antibody-dependent cytotoxic cell (ADCC, A/California/7/2009(H1N1)) activity in children (circles) and adults (squares) after LAIV vaccination, each dot represents an individual test point. Panel A (left) shows the ADCC activity and panel B (right) shows the fold change in the ADCC activity. The groups; children and adults, and the time point after vaccination is shown on the horizontal axis. The ADCC titer and fold change are indicated on the vertical axis. The mean value and SEM is indicated for each group. A dotted horizontal line is shown in panel B, indicating a no fold change level. A significant change in median values are indicated with asterisks above the group columns,  $p < 0.05$ .



**Figure 4.** CD4<sup>+</sup> and CD8<sup>+</sup> IFN  $\gamma$  positive T-cell responses to influenza A H1N1 virus strains after live attenuated influenza vaccine (LAIV) in children. IFN  $\gamma$  <sup>+</sup> T-cell specific antibody response is shown in children per 10<sup>6</sup> peripheral blood mononuclear cells (PBMCs) after LAIV vaccination. The columns represent the CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses, and the timepoints after LAIV vaccination. Each symbol represents an individual child, and bars represent the means with standard errors of the mean. Significant change in median values are indicate with asterisks above the group columns,  $p < 0.05$ .