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**Author:** Rosendahl Huber, Sietske

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# Chapter 3

## Immunogenicity of influenza vaccines: evidence for differential effect of booster vaccination on humoral and cellular immunity

Sietske K. Rosendahl Huber <sup>1</sup>, Marion Hendriks <sup>1</sup>,  
Ronald H.J. Jacobi <sup>1</sup>, Jan van de Kasstele <sup>1</sup>, Jolanda C. Mandersloot-Oskam <sup>1</sup>,  
Renee A.J. van Boxtel <sup>1</sup>, Anne M.J. Wensing <sup>2</sup>, Nynke Y. Rots <sup>1</sup>,  
Willem Luytjes <sup>1</sup>, Josine van Beek <sup>1</sup>

<sup>1</sup>Centre for Infectious Disease Control, National Institute for Public Health and the Environment, Bilthoven, the Netherlands

<sup>2</sup>Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, the Netherlands

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## Abstract

While influenza vaccines are designed to induce neutralizing antibodies, little is known on T cell responses induced by these vaccines. In contrast, more data becomes available on the important role of cellular immune responses in limiting influenza disease. The 2009 pandemic provided us with the opportunity to evaluate the immune response to vaccination in a unique setting. We evaluated both antibody and T cell responses during two consecutive influenza seasons from 2009-2011 and compared the MF59-adjuvanted pandemic vaccine with the unadjuvanted seasonal vaccine. Antibody responses were determined by a hemagglutination inhibition assay in serum and vaccine-specific T cell responses were evaluated by detecting IFN- $\gamma$  producing peripheral blood mononuclear cells using whole influenza virus or vaccine-specific peptide pools as stimulating antigens. We show that one dose of the pandemic vaccine induced antibody responses sufficient for providing seroprotection and vaccine-specific T cell responses. A second dose further increased antibody responses but not T cell responses. Both responses could be boosted by the seasonal vaccine in the subsequent season. Furthermore, we show that the seasonal vaccine alone is capable of inducing vaccine-specific T cell responses, despite the fact that the vaccine did not contain an adjuvant. In addition, residual antibody levels remained detectable for over 15 months, while T cell levels had reduced back to baseline levels by that time. Hereby, we show that humoral and cellular immunity differ in their response to a second dose of the pandemic vaccine.

## Introduction

Influenza virus causes seasonal epidemics resulting in a major social and economic burden and 250,000-500,000 deaths each year, while pandemic outbreaks affect the population to an even greater extent (1-3). These outbreaks of influenza are the result of the variable nature of the surface proteins of influenza virus, hemagglutinin (HA) and neuraminidase (NA). Typically, antibodies directed to these proteins can provide neutralizing immunity. However, antigenic drifts can cause small changes in antibody binding sites that may render these antibodies ineffective. In addition, completely new subtypes can arise due to antigenic shifts, which occur when circulating viruses reassort with other viruses circulating in the human population or that of other species. During the emergence of such a new subtype, individuals depend even more on the activation of other arms of the immune system than the humoral response to the globular head of HA and NA. Although they cannot prevent infection, cross-reactive cytotoxic CD4<sup>+</sup> and CD8<sup>+</sup> T cells have been shown to provide an immunological advantage by limiting disease, improving recovery, and eventually clearing infection (4-6).

In 2009, A(H1N1)pdm09, a subtype from swine origin, was introduced into the human population. This was the first time in over 30 years that an influenza virus originating from an animal reservoir was able to transmit from human to human (7). As humans were expected to be naïve to this new subtype, an MF59-adjuvanted inactivated monovalent vaccine directed to the pandemic strain, was offered to classical Dutch risk groups, pregnant women, and health care workers in a two dose schedule (8, 9). MF59 is an oil in water emulsion that was shown to activate CD4<sup>+</sup> T cells, which play an important role in the induction of high affinity class switched antibodies (10-12). In the pandemic setting, MF59 was included to allow for a lower antigen dose, while still capable of inducing seroprotective antibody titers (13).

In this study, we analyzed the immunogenicity of the pandemic vaccine during the H1N1 pandemic in 2009, which allowed for evaluation of both the unusual two dose schedule and the effect of the addition of MF59 on humoral and cellular immunogenicity of the pandemic vaccine (14, 15). In addition, this study entailed the subsequent 2010-2011 season in which the A(H1N1)pdm09 strain was included in the unadjuvanted seasonal influenza vaccine, together with a new H3N2 strain (A/Perth/16/2009). This allowed for analysis of the booster effect of previous vaccination with the A(H1N1)pdm09 strain and comparison of immunogenicity of an adjuvanted pandemic vaccine versus an unadjuvanted seasonal vaccine. Analysis of immunogenicity was performed by measuring the standard correlate of protection for influenza vaccines, i.e., antibody responses. Furthermore, vaccine-specific T cell responses were investigated since little is known on the induction of T cells by vaccination, while more evidence is being published on their important role during influenza infections. T cell responses directed against epitopes of the influenza virus surface proteins HA and NA may serve the development of specific antibodies or mediate cytotoxic effects on their own (16).

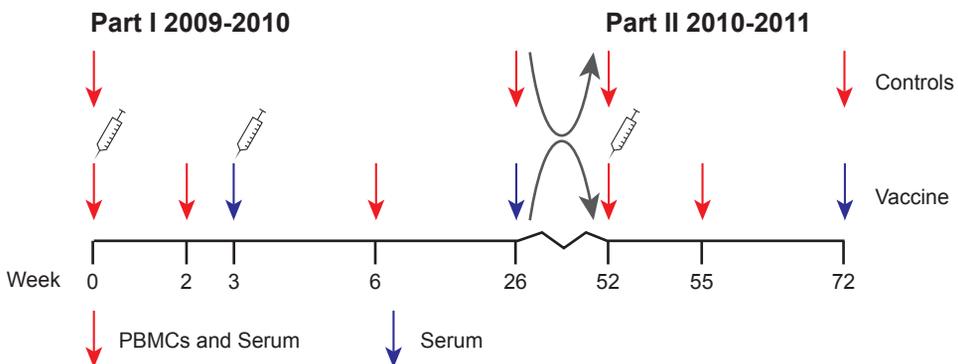
In this study, we evaluated the vaccine-specific antibody and T cell-mediated immune response during two consecutive influenza seasons from 2009 to 2011. During the first season, the additive value of a second dose of the pandemic vaccine was evaluated. In addition, a comparison of adjuvanted and unadjuvanted influenza vaccines is made. We show that one dose of the pandemic vaccine was sufficient to induce antibodies and T cell responses and that a second dose solely boosted antibody responses. The seasonal vaccine boosted both the humoral and cellular response and even induced T cell responses in individuals not vaccinated in the previous season. Antibody levels remained detectable until the end of the study, while T cell responses had reduced to baseline levels. Hereby, this study contributes to knowledge on the humoral and cellular immunity in response to influenza vaccination.

## Materials and Methods

### Experimental design

A non-randomized, multicenter, open-label controlled trial was conducted in the Utrecht area, during two consecutive influenza seasons between October 2009 and May 2011. The main objective was to evaluate whether a second dose with the pandemic adjuvanted vaccine was necessary for obtaining seroprotective antibody titers and whether the antibody response could be boosted in the second season with a seasonal unadjuvanted vaccine. In addition, humoral and cellular immune profiles after vaccination with pandemic and seasonal H1N1 containing vaccines were evaluated.

Healthy individuals aged between 18 and 52 years were recruited among workers of public health institutions in the Utrecht area. Exclusion criteria were: previous diagnosis with A(H1N1)pdm09, any history with serious allergic reaction to vaccine components, factors that might interfere with blood collection, and factors that might interfere with immunological analysis, including immune deficiencies, hematological disorders, bleeding disorders, usage of anticoagulants, corticosteroids, NSAIDs and/or statins, diabetes mellitus or having had an infectious disease with fever within the last two weeks before the start of the study. Study participants had the choice to be vaccinated or not in both seasons independently of their choice in the previous season, resulting in a vaccine and control group in the first season (2009-2010) and vaccine-vaccine (VV), vaccine-control (VC), control-control (CC) and control-vaccine (CV) groups in the second season (2010-2011).



**Figure 1: Design of the clinical study**

The study was performed during two consecutive influenza seasons. During the first season (2009-2010) individuals were vaccinated at the start of the study and three weeks later with the MF59-adjuvanted A(H1N1)pdm09 subunit vaccine. Three weeks before the start of the study or at week six, an optional seasonal 2009-2010 vaccination was allowed. However, this was not part of the study regime. Grey arrows depict reallocation in control and vaccine groups. During the second season (2010-2011), individuals in the vaccine group received the unadjuvanted seasonal 2010-2011 subunit vaccine (including A(H1N1)pdm09) at week 52.

The protocol was approved by the medical ethical reviewing committee (Central Committee on Research Involving Human Subjects (CCMO)) of the Netherlands and the study was conducted in accordance with Good Clinical Practice and the principles of the Declaration of Helsinki. The study was registered in the Netherlands Trial Register (NTR2070) and written informed consent was obtained from each participant.

### Vaccines

During the first season, individuals in the vaccine group received two pandemic influenza vaccine doses with a three-week interval (**Figure 1**). Vaccination with two doses of the A(H1N1)pdm09 vaccine was recommended by the Dutch Health Council, based on experience during outbreaks of H5N1 avian influenza, a subtype for which little to none pre-existing immunity was present in humans. The monovalent pandemic subunit vaccine (Focetria, Novartis) is a MF59-adjuvanted influenza vaccine containing A(H1N1)pdm09. MF59 already proved to be safe and immunogenic in combination with an inactivated seasonal influenza vaccine for elderly and has been registered in Europe since 1997 (17). Seasonal influenza vaccination was not part of the study regime in the first season, but was optional and had to take place at least three weeks prior to the study or at week 6 (**Table IA**), if so, individuals received the subunit vaccine Influxac 2009-2010 (Solvay, The Netherlands). This seasonal subunit vaccine contained A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2), and B/Brisbane/60/2008. During the second season, subjects in the vaccine group were vaccinated once with Influxac 2010-2011, containing vaccine strains A(H1N1)pdm09, A/Perth/16/2009 (H3N2), and B/Brisbane/60/2008 (Solvay, the Netherlands).

### Virus strains

The following virus strains were used for HI assays and virus ELISpots: A/California/07/09 (H1N1) was kindly provided by Institute Pasteur (Paris, France) and A/Perth/16/09 (H3N2) was obtained from the National Institute for Biological Standards and Control (NIBSC). Viruses were grown on Madin-Darby Canine Kidney (MDCK) cells. Sequences of hemagglutinin (HA) and neuraminidase (NA) proteins of these strains were obtained from GenBank and can be found under protein accession numbers ACP44189 (HA California), ACQ63272 (NA California), ACP44189 (HA Perth), and ACQ63272 (NA Perth).

### Blood collection

Blood was collected before vaccination, two weeks, and three weeks after the first dose, three weeks after the second dose and at the end of the influenza season, which was approximately five months after the second dose (**Figure 1**). During the second season, blood was drawn before and three weeks after vaccination and at the end of the influenza season. At most time points, blood was collected for PBMC isolation and serum, however three weeks after the first dose of the pandemic vaccine and at the end of both seasons, only serum was collected. Blood of individuals in the control group was collected for serum and PBMC isolation at the start and the end of both seasons. Serum was stored at -20°C until

analysis. PBMCs were isolated by Ficoll (Lymphoprep, Axis-Shield, Norway) density gradient centrifugation and stored at  $-135^{\circ}\text{C}$ .

### Hemagglutination-inhibition (HI) assay

HI assays against MDCK cell-grown A(H1N1)pdm09 wild type virus was performed in duplicate according to standard methods of the World Health Organization (WHO) at Viroclinics (Rotterdam, the Netherlands) (Luytjes et al., 2012). In short, a dilution series of cholera filtrate-treated serum samples was incubated with four Hemagglutinin Units (HAU) influenza virus for 20 minutes and 0.25% (v/v) turkey erythrocytes for 30 minutes at  $4^{\circ}\text{C}$  and scored for agglutination.

### EMA guidelines

EMA guidelines for influenza vaccines include criteria related to vaccine efficacy, which have to be met to obtain registration in the European Union (EU). First, the percentage of subjects who reach seroprotection, which is defined as an HI titer  $\geq 40$ , should increase by 70%. Second, the mean geometric increase of antibodies should be  $>2.5$ . Third, the percentage of individuals who reach seroconversion, which is defined as seroprotection with at least a fourfold increase in antibody levels should be  $>40\%$  (15). For a pandemic vaccine, all three criteria have to be met, while for a seasonal vaccine at least one in three is required. Antibody responses should be measured three weeks after vaccination.

### Enzyme-linked immunospot (ELISpot) assays

PVDF-membrane plates (Millipore Corporation, USA) were ethanol-activated, coated with  $5\ \mu\text{g/ml}$  1-D1K anti-IFN- $\gamma$  antibody (Mabtech Ab, Sweden) and incubated O/N at  $4^{\circ}\text{C}$ . Plates were blocked with AIM-V medium (Thermo Scientific, The Netherlands) containing 2% human AB serum (Sigma, MO, USA). For analysis of responses to the vaccine strains,  $2 \times 10^5$  PBMCs per well were incubated in AIM-V medium (Thermo Scientific, The Netherlands) containing 2% human AB serum (Sigma, MO, USA) and stimulated with influenza virus at a multiplicity of infection (MOI) of 4, mock (cell supernatant) or  $1\ \mu\text{g/ml}$  Staphylococcus Enterotoxin B (SEB) (Sigma). Analysis of the vaccine-specific antigens was performed by stimulation of  $4 \times 10^5$  cells per well with  $1\ \mu\text{g/mL}$  of a peptide pool spanning the entire HA or neuraminidase NA protein of A(H1N1)pdm09 or A/Perth/16/2009. Per protein, 15-mer peptides with 11 overlap (JPT peptide Technologies, Germany) were pooled and dissolved in DMSO. In the negative control wells, DMSO was added to the medium. After an incubation period of 18 hours, plates were washed with phosphate buffered saline (PBS) 0.2% triton-x100 to inactivate the virus and detection IFN- $\gamma$  antibody Biotin labeled antibody 7-B6-1 (Mabtech Ab, Sweden) in PBS 0.5% FCS (HyClone Thermo Scientific, USA) was added at  $1\ \mu\text{g/ml}$  for 2 hours at room temperature (RT). Plates were washed and incubated with streptavidin-alkaline phosphatase in PBS 0.5% FCS for 1 hour at RT. After washing the plates,  $100\ \mu\text{l}$  NBT/BCIP solution (Sigma, MO, USA) was added. Color reaction was stopped by washing the plates with tap water. Plates were dried O/N at RT and spots were counted with A.EL.VIS reader (A.el.vis, Germany).

### Statistical analysis

Mann Whitney U and Pearson Chi Square tests were applied to analyze the characteristics of the cohort, as indicated in the Results section. Statistical significance was defined as a p-value  $\leq 0.05$  and statistical analysis was performed with the SPSS 19.0 statistical software program for Windows. Data from peptide ELISpots were log-transformed and tested for significance with a two-tailed student's t-test, using GraphPad Prism 6.04 software.

Results from HI assays and ELISpot assays with virus-stimulated PBMCs, were analyzed by a mixed effects Negative Binomial regression model to quantify differences in immune responses between vaccinated and unvaccinated groups (18, 19). The Negative Binomial distribution was used to describe the number of spots, while the underlying spot rates were modelled by the regression model. SEB counts were included in the regression model as denominator in the so-called offset term, i.e., if the spot rate is constant, higher SEB spot counts will automatically result in higher virus specific spot counts. Possible confounders such as sex, vaccination history, and earlier influenza infections were taken into account as categorical variables and age was entered in the model as a natural cubic spline curve. A log-link function was used to relate the response rate with these fixed effects. To account for variation between participants, a random intercept was included in the model (20). Differences between groups are therefore presented as relative rates, including 95% confidence intervals and p-values. The Holm adjustment is applied to correct for multiple testing. All statistical analyses were performed in R using the R-INLA package (21, 22).

## Results

### Clinical trial design

In this study, 348 individuals were included of whom 288 chose to be vaccinated (vaccine group) and 60 chose not to be vaccinated (control group) (Figure 2). At the start of the second season individuals again had the choice to participate and to be vaccinated or not, independent of their choice in the previous season. This resulted in four different groups: 135 individuals remaining in the vaccine group (VV), 29 individuals switching to the control group (VC), 31 individuals remaining in the control group (CC) and 7 individuals switching to the vaccine group (CV) (Figures 1 and 2). Baseline characteristics of the study participants are described for season one (Table IA) and season two (Table IB). Vaccination history of all participants was recorded, which shows that the number of frequent vaccinees was higher in the vaccination groups (Table IA and IB).

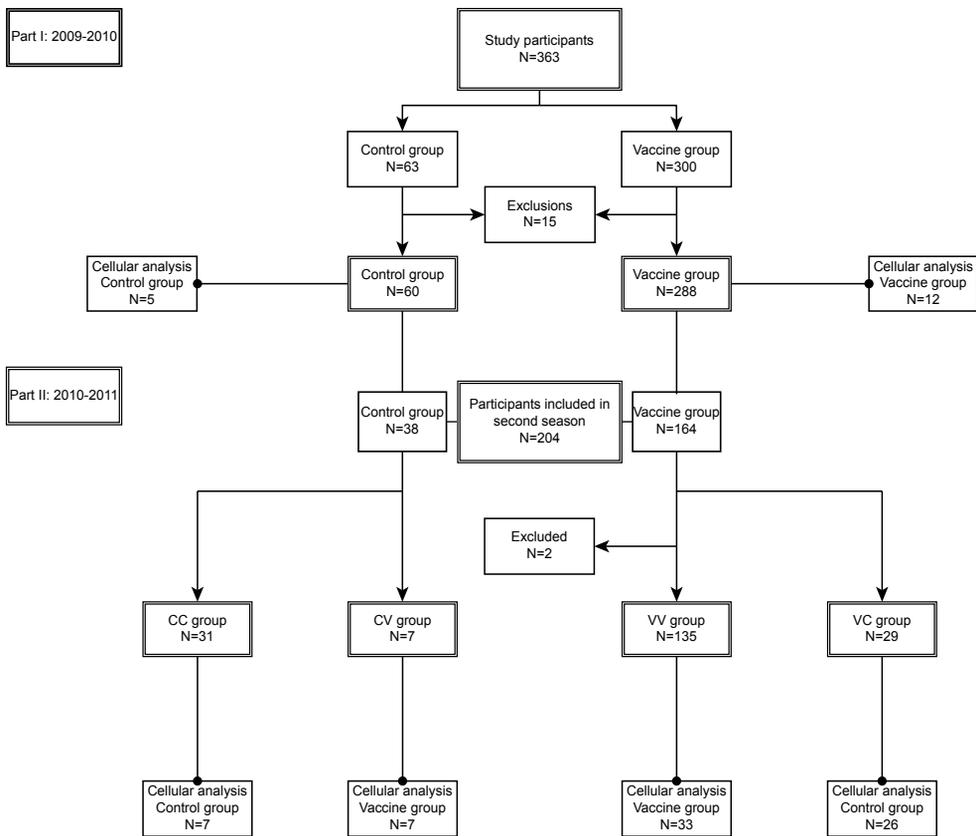
**Table IA: Baseline characteristics 2009-2010, season one**

	Control group (n=60)	Pandemic vaccine group (n=288)	p-value	
Mean age (range)	39.1 (25-52 yrs)	39 (19-52 yrs)	ns	
Gender (%)	Male	28.3	43.4	0.03
	Female	71.7	56.6	
Pregnant (%)	0	2.5	-	
Any previous influenza vaccination (%)	20	56.6	0.001	
Seasonal vaccination 2009-2010 before trial (%)	8.3	24.3	0.006	
Seasonal vaccination 2009-2010 at week 6 (%)	5	35.8	ns	

**Table IB: Baseline characteristics 2010-2011, season two**

	CC group (n= 31)	CV group (n=7)	VV group (n=135)	VC group (n=29)	p-value	
Mean age (range)	39.71 (27-52)	39.57 (25-52)	41.01 (19-52)	39.14 (23-52)	ns	
Gender (%)	Male	32.3	14.3	48.9	31	0.06
	Female	67.7	85.7	51.1	69	
Pregnant (%)	0	0	1.4	5	-	
Seasonal vaccination 2009-2010 (%)	76.3	85.7	76.3	20.7	0.0001	
Any previous influenza vaccination (%) 2009	3.2	100	63	27.6	0.0001	

To analyze vaccine immunogenicity, antibody responses of all participants were analyzed by HI assays. Furthermore, cellular responses were determined by IFN- $\gamma$  ELISpots in a subset of participants to investigate the presence of vaccine-induced T cell responses (Figure 2). To enable comparison of induction and duration of immune responses following vaccination, all responses were categorized. As hypothesized in Figure 3, baseline responses of participants are placed in category I, representing the variable background response of subjects (Table II). Responses of participants that are not vaccinated during the study are considered not to change considerably and therefore individuals will remain in category I, unless they do receive a vaccination during this study. Based on these rules, individuals can be placed in 11 different categories (Figure 3 and Table II). To account for individual variation and other confounding factors, results were analyzed statistically using the mixed effects negative binomial regression model. Differences between groups are expressed as relative rates (RR).

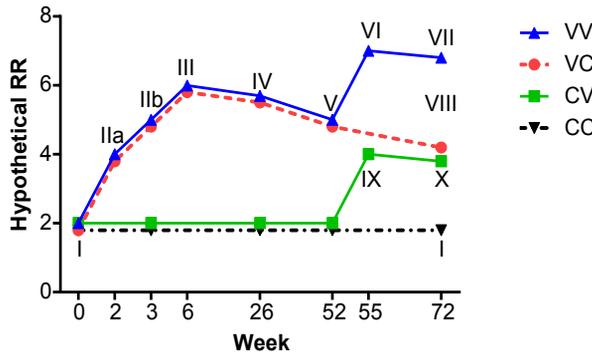


**Figure 2: Study disposition:**

Excluded in 2009-2010: Eight subjects were lost to follow up, two received occupational vaccination while in the control group, four only received first vaccination and, one was too old.

Excluded in 2010-2011: One withdrew consent, one due to use of corticosteroids

CC= Control group during both seasons, CV= Control group during first season, switch to vaccine group at the start of the second season, VV= Vaccine group during both seasons, VC= Vaccine group during the first season, switch to control group at the start of the second season.



**Figure 3: Hypothesis of Negative Binominal model of the immune responses**

Responses of individuals can be classified by 11 different categories corresponding with both time points at which samples were collected and the vaccination status of the individual at that time point (categories I-X). For example: Category I are baseline responses of individuals that were not vaccinated at the moment of sampling. Individuals receiving their first dose were placed in category II and a booster vaccination placed individuals in category III. Categories IIa and IIb are the cellular and antibody responses, respectively, after one dose.

CC= Control group during both seasons, CV= Control group during first season, switch to vaccine group at the start of the second season, VV= Vaccine group during both seasons, VC= Vaccine group during the first season, switch to control group at the start of the second season.

**Table II: Construction of categories of responses**

Category	Week	Serology	ELISpot	Name
I	0-72	Yes	Yes	Baseline
IIa	2	No	Yes	First dose (Cellular)
IIb	3	Yes	No	First dose (Serology)
III	6	Yes	Yes	Second dose
IV	26	Yes	No	Contraction phase V <sup>1</sup>
V	52	Yes	Yes	Maintenance phase V <sup>1</sup>
VI	55	Yes	Yes	Secondary seasonal 2010-2011 vaccination VV <sup>2</sup>
VII	72	Yes	No	Contraction phase VV <sup>2</sup>
VIII	72	Yes	Yes	Residual level VC <sup>3</sup>
IX	55	Yes	Yes	Primary seasonal 2010-2011 vaccination CV <sup>4</sup>
X	72	Yes	No	Contraction phase CV <sup>4</sup>

<sup>1</sup>V=Vaccine group, first season

<sup>2</sup>VV=Vaccine vaccine group

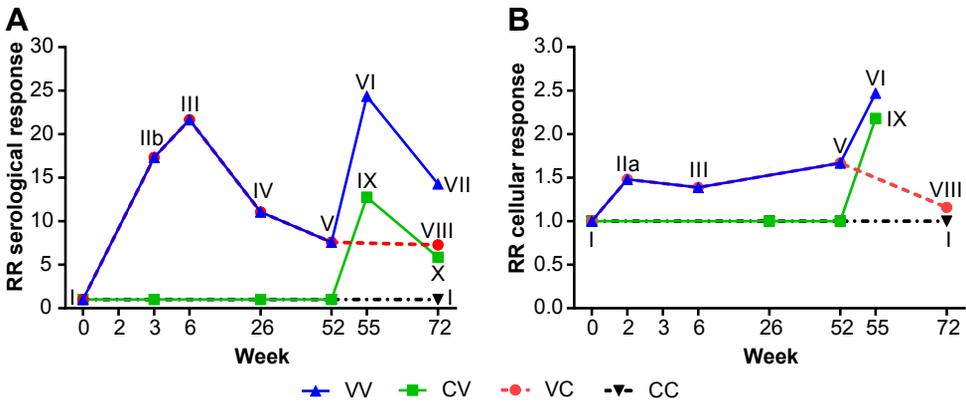
<sup>3</sup>VC=Vaccine control group

<sup>4</sup>CV=Control vaccine group



**One dose of the MF59-adjuvanted vaccine induced adequate antibody responses**

In **Figure 4A**, relative antibody responses to A(H1N1)pdm09 are depicted for all groups during both seasons. The first dose of the adjuvanted pandemic vaccine increased the RR of the antibody level 17.3 fold compared to baseline (IIb versus I;  $p < 0.001$ ) (**Table SIA**). The second dose induced a further relative increase of 1.3 compared to primary vaccination (III versus IIb;  $p < 0.001$ ), showing that there is a rapid induction of antibody responses after a first dose with the pandemic vaccine and that these responses increase after a second dose. To evaluate vaccine efficacy, standard analysis of HI titers was performed according to the EMA guidelines for pandemic vaccines. One dose of vaccine induced an 18-fold increase of the GMT, seroprotection in 87.7% and seroconversion in 78% of the vaccinated, which was sufficient to meet all three EMA criteria, while the second dose induced a further increase in antibody levels (**Table III**). After vaccination, antibody levels wane quickly, however, at week 26 antibody levels were significantly higher than baseline (RR: 11.1; IV versus I;  $p < 0.001$ ) (**Table SIA**).



**Figure 4: Relative response rates**

Profile of serological (A) and cellular (B) responses. X-axis depict sampling weeks and Y-axis depicts relative response.

**Residual antibody levels were boosted by seasonal vaccine**

At the start of the second season the RR of antibody levels in vaccinated individuals had declined further, but still remained higher compared to control individuals (RR: 7.6; V versus I;  $p < 0.001$ ) (**Figure 4A**). Seasonal vaccination resulted in a significant increase in RR of individuals in the VV group with a RR of 24.4 compared to primary baseline (VI versus I;  $p < 0.001$ ) (**Table SIB**). Titers of individuals that were vaccinated for the first time in the second season (CV) significantly increased 12.8 fold compared to baseline (IX versus I;  $p < 0.001$ ). This implies a booster effect of the seasonal vaccine on the antibody levels induced by the pandemic vaccine in the previous year since the RR of VV individuals was 2-fold higher compared to CV individuals (VI versus IX;  $p = 0.028$ ) (**Table SIC**). However, no significant difference was found between antibody levels of individuals that had received the first dose

of the adjuvanted pandemic vaccine and individuals vaccinated only in the second year with the unadjuvanted seasonal vaccine (IIb versus IX;  $p=0.599$ ). These results imply that 7.5  $\mu\text{g}$  HA antigen adjuvanted with MF59 is as efficient at inducing antibodies as a regular unadjuvanted antigen dose of 15  $\mu\text{g}$  HA.

One year after vaccination no further reduction in antibodies levels was observed in individuals that switched to the control group at the start of the second season, showing a duration of the antibody response for over 15 months (VIII versus V;  $p=0.699$ , RR:7.28; VIII versus I;  $p<0.001$ ) (**Table SIB**). Similar as the vaccine-induced antibody response in the first season, antibody levels of individuals vaccinated in the second season (groups VV and CV) significantly reduced between week 52 and the end of the study (VI versus VII  $p<0.001$  and IX versus X;  $p=0.032$ ) (**Table SIB**). At week 72, individuals in the VV group did end up with a larger residual antibody level compared to CV and VC individuals (VII versus X;  $p<0.001$  and VII versus VIII;  $p<0.001$ ), while no significant difference was observed between VC individuals and CV individuals at week 72 (VIII versus X;  $p=0.699$ ) (**Table SIC**). These results indicate an advantage of annual vaccination with the same vaccine strain on the height of the antibody levels.

### First dose of the MF59-adjuvanted vaccine induced cellular responses

Since the MF59-adjuvanted pandemic vaccine has been proposed to induce T cell responses, also cellular immune responses to the virus strains were analyzed in a subset of participants of the vaccinated and control groups. All subjects of the CV group were analyzed due to the small size of this group ( $n=7$ ). **Figure 4B** depicts relative rates of T cell responses to the A(H1N1)pdm09 strain. A significant increase with a RR of 1.5 was observed two weeks after the first dose of the pandemic vaccine (IIa versus I;  $p<0.001$ ) (**Table SIIA**). Three weeks after the second dose, the RR was 1.4 compared to the baseline level (III versus I;  $p<0.001$ ). The difference in T cell response ratio between weeks 2 and 6 was not significant, therefore we conclude that, contrary to antibody responses, a second dose did not boost T cell responses (RR: 0.9; IIa versus III;  $p=0.8$ ). Strikingly, no significant reduction in T cells, RR of 1.2, was observed between the level obtained after pandemic vaccination and the start of the second season (III versus V;  $p=0.11$ ) (**Table SIIB**).

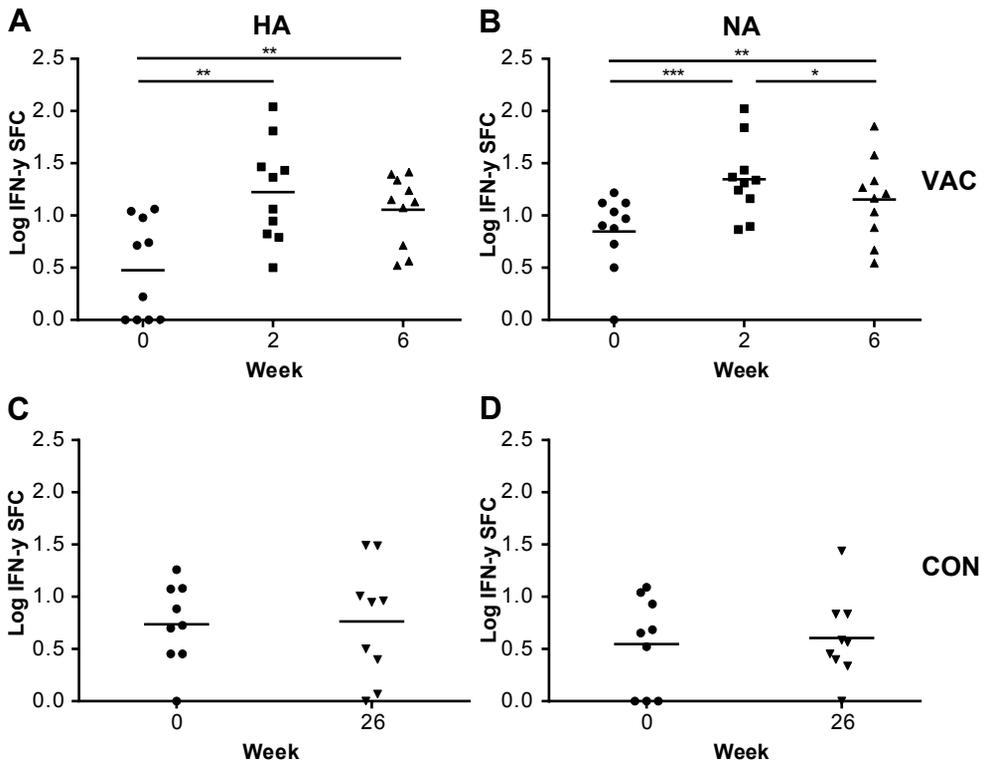
### Seasonal vaccine is capable of inducing T cell responses

Similar to antibody responses, at the start of the second season, a significantly higher level of T cells with a RR of 1.7 was observed in vaccinated individuals compared to non-vaccinated individuals (**Figure 4B**; V versus I;  $p<0.001$ ). Moreover, in VV individuals, a 2.5 increase in RR was observed after seasonal vaccination compared to the primary baseline (VI versus I;  $p<0.001$ ). In individuals not vaccinated in the previous year (CV) a significant induction of T cells was observed with a RR of 2.2 (IX versus I;  $p<0.001$ ). In addition, T cell responses to the new seasonal vaccine strain A/Perth/16/2009(H3N2) showed an increase in RR of 1.9, strengthening data on T cell induction by the seasonal vaccine (**Table SIII**). T cell levels to

A(H1N1)pdm09 obtained after singular vaccination (CV) and re-vaccination (VV) were similar (VI versus IX;  $p=0.819$ ). Therefore, previous pandemic vaccination does not appear to be an advantage for VV individuals compared to T cell responses of CV individuals. By week 72, the T cell response of individuals that switched to the control group in the second year (VC) had decreased to primary baseline level (VIII versus I; RR 1.2;  $p=0.544$ ), implicating a duration of the T cell response of approximately 15 months (Table SIIC).

**Pandemic and seasonal vaccine induce HA and NA-specific responses**

All cellular responses described above were analyzed by stimulation of PBMCs with live virus. As the vaccines only contained HA and NA from influenza virus, we postulate that vaccine-induced responses described after virus stimulation were mostly directed to the HA and NA proteins. To confirm this hypothesis, responses specific for the vaccine strains were further analyzed in an IFN- $\gamma$  ELISpot by stimulation of PBMCs with peptide pools spanning the entire HA or NA protein of A(H1N1)pdm09. In Figure 5, responses to the HA- and NA-peptide pools of A(H1N1)pdm09 are depicted for the first season. After one dose, there was a significant increase in T cell responses to HA, which was not boosted by the second dose (Figure 5A).

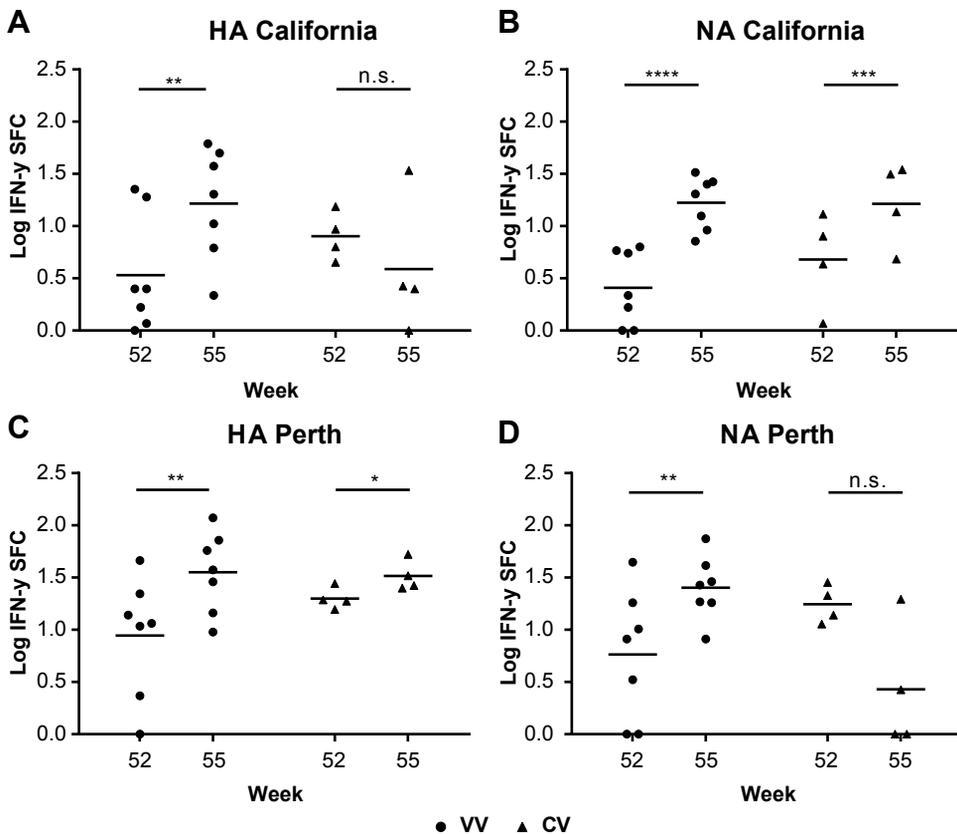


**Figure 5: Vaccine-specific T cell responses in the first season**

Responses against HA (A) and NA (B) peptide pools were measured with an IFN- $\gamma$  ELISpot in individuals of the vaccine group on samples taken at weeks 0, 2 and 6. Responses against HA (C) and NA (D) were measured on samples drawn at weeks 0 and 26. Number of spot forming cells (SFC) are corrected for non-stimulated controls. \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\* $p<0.001$

Similar observations were made for NA protein (**Figure 5B**). Responses of individuals in the control group remained similar during the first season (**Figures 5C and 5D**). Hereby, we show that the pandemic vaccine is indeed capable of inducing HA and NA-specific T cell responses.

Likewise, vaccine-specific T cell responses were observed during the second season. Three weeks post seasonal vaccination, PBMCs of individuals in the VV and CV groups were isolated and stimulated with HA or NA of both A(H1N1)pdm09 and A/Perth/16/2009(H3N2). VV individuals showed increased T cell responses to all peptide pools (**Figure 6**). Individuals in the CV-group had a significant induction of T cell responses after stimulation with NA derived from A(H1N1)pdm09 and HA of A/Perth/16/2009(H3N2) (**Figures 6B and 6C**). When comparing virus-stimulation and peptide-stimulation, individuals in the CV group had a significant increase in responses after only one vaccination as measured by virus stimulation which was confirmed by the NA of A(H1N1)pdm09 and HA of A/Perth/16/2009 (H3N2) peptide pool



**Figure 6: Vaccine-specific T cell responses in the second season**

Responses against A(H1N1)pdm09 HA (**A**) and NA (**B**) peptide pools were measured with an IFN- $\gamma$  ELISpot right before and three weeks after vaccination. Responses against A/Perth/16/2009(H3N2) HA (**C**) and NA (**D**) peptide pools were measured with an IFN- $\gamma$  ELISpot right before and three weeks after vaccination. Number of spot forming cells (SFC) are corrected for non-stimulated controls. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$

stimulations (**Figures 4B, 6B and C**). These results indicate an advantageous effect of 2010-2011 influenza vaccination.

### **Correlation of humoral and cellular immune response**

**Figure 4** summarizes the relative rates of antibody and T cell responses during both seasons, enabling a comparison of vaccine-specific antibody and T cell responses. The first dose of the pandemic vaccine resulted in a significant induction of both antibody and T cell responses, while a second dose only improved antibody responses. Individuals vaccinated in the first season had residual antibody and T cell responses and thus appear to have an advantage at the start of the second season. This advantage is reflected by antibody induction, but not T cell responses, as a single seasonal vaccination (CV) induces lower antibody titers but similar levels of T cell responses compared to VV individuals. At week 72, T cell responses were only measured for individuals in the control groups (CC and VC group), showing that responses of VC individuals had decreased to baseline level 15 months after their last vaccination. In contrast, antibody levels were measured for all groups and showed that residual levels of all groups that received at least one vaccination, remained significantly higher than baseline. Therefore, we can conclude that vaccine-induced antibody responses are detectable in the blood for a longer period than T cell responses measured in this study.

## Discussion

In this study, the antibody and T cell mediated immune response following influenza vaccination was evaluated during two consecutive influenza seasons from 2009 to 2011. The emergence of A(H1N1)pdm09 provided us with the opportunity to evaluate influenza vaccine immunogenicity in a unique setting. The Dutch Health Council recommended vaccination with two doses of a MF59-adjuvanted monovalent A(H1N1)pdm09 vaccine, which allowed us to evaluate both the unusual two dose schedule and the effect of MF59 adjuvation on immunogenicity of the pandemic vaccine. One dose of the pandemic vaccine induced antibody responses sufficient for providing seroprotection and, in addition, induced vaccine-specific T cell responses. A second dose further increased antibody responses but not T cell responses.

Furthermore, in the subsequent influenza season, the trivalent seasonal vaccine contained the pandemic strain of the previous season, A(H1N1)pdm09, and a new H3N2 strain, A/Perth/16/2009(H3N2), allowing for analysis of booster effect of previous vaccination with the A(H1N1)pdm09 strain. Both antibody and T cell responses could be boosted by the seasonal vaccine. In addition, a comparison could be made of an adjuvanted and unadjuvanted influenza vaccine. Immunogenicity of the influenza vaccines was evaluated by measuring both vaccine-specific antibody and T cell responses during both influenza seasons. Furthermore, we show that the seasonal vaccine alone is capable of inducing vaccine-specific T cell responses, despite the fact that the vaccine did not contain an adjuvant.

In addition, residual antibody levels remained detectable for over 15 months, while T cell levels had reduced back to baseline levels by that time. We conclude that vaccine-induced antibody responses are detectable in the blood for a longer period than T cell responses measured in this study. However, this does not necessarily indicate that vaccine-specific T cells are no longer present. Memory T cells might reside in (lymphoid) tissues instead of in circulation, which is not reflected by measuring PBMC-specific T cell responses in the blood (23-25).

During the first season, immunogenicity of the MF59-adjuvanted monovalent A(H1N1)pdm09 vaccine was evaluated. Adjuvants, such as MF59, have been shown to reduce the dose of antigen needed and to induce a longer lasting antibody-mediated immune response (8). To assure seroprotection, the Dutch Health Council chose to advise a two-dose schedule as recommended by the manufacturer. The choice of administering two doses was based on studies on avian influenza vaccination where two doses were needed to obtain sufficient antibody responses (26). These studies with H5 influenza vaccines showed that two adjuvanted vaccine doses were required to obtain antibody levels that correlate with protection according to EMA criteria and furthermore they induced memory B cells (27-30). In this study, we observed in a cohort of healthy individuals that one dose induced

antibody responses sufficient to conform to EMA guidelines for the registration of pandemic vaccines. In concordance, others have shown that one dose also induced adequate levels of seroprotection in other target groups of 2009 pandemic vaccination, i.e., infants, elderly and immunocompromised individuals (31-33). In addition, data on H9N2 vaccines indicate that one dose of an adjuvanted vaccine is sufficient for protection against H9N2 subtypes (34).

Efficacy of vaccines for newly emerging subtypes appear to be affected by cross-reactive immunity. For individuals that do not have pre-existing immunity, one or even two doses might not be sufficient to provide seroprotective antibodies as shown by a study with H5 subtypes (27). In contrast, a study on H9N2 vaccines showed that individuals who had cross-reactive H2 antibodies available, responded better to one dose of an H9N2 subunit vaccine than individuals that did not have cross-reactive antibodies available. This cross-reactivity has been proposed to be due to structure similarity of H2 and H9 (35). However, there is also literature available on neutralizing antibodies that are directed to the conserved stalk domain of HA (36-38). Therefore, cross-reactive immunity may provide partial protection that can be boosted by vaccination. Thus, when an influenza subtype crosses over to the human population for the first time, the presence of cross-reactive immunity could determine whether one or two doses are needed to provide seroprotection.

Although antibodies provide primary protection against influenza virus infection, T cells are needed to clear infection when these antibodies fail to induce neutralizing protection. The importance of T cells is especially clear in situations where low cross-protective neutralizing antibodies are observed, and shows the additive value of inducing T cell responses by vaccination (39-41). The MF59-adjuvanted vaccine has been shown to induce follicular helper CD4<sup>+</sup> T cells and presence of these cells predict antibody responses (42). Furthermore, MF59 recruits immune cells, such as macrophages and monocytes, to the site of infection, and was shown to induce differentiation of monocytes to DCs, which in turn can also prime CD8<sup>+</sup> T cell responses (43). Therefore, the MF59-adjuvanted vaccine is expected to induce T cell responses in addition to antibody responses.

Analysis of vaccine-induced T cell responses was performed by stimulation of PBMCs with whole influenza virus or HA or NA-specific peptide pools using an IFN- $\gamma$  ELISpot. In most individuals, we observe a background level of T cell responses before vaccination, which are more prominent in the whole virus stimulation assays. Background levels of these responses are the consequence of activation of T cells induced by natural infection or previous vaccination and will include the response to internal viral proteins. In the model, we correct for these background levels by studying an additional induction. Peptide pools solely containing vaccine antigens enabled us to make assumptions about vaccine-induced T cells alone. However, future studies are required to analyze the full cytokine profile of these responses, dissecting the nature of adjuvanted and unadjuvanted vaccine-induced T cells.

In the peptide ELISpot assay, we observed an induction of T cells already after vaccination with one dose of the adjuvanted vaccine. However, after the second dose, T cell responses remained similar to responses measured after one dose. McElhany et al. even found a negative correlation between antibody levels and cytokine ratios in elderly and proposed that a second dose might skew T cell responses to the production of IL-10, which limits CTL induction but is advantageous for antibody responses (44). We only evaluated T cell responses by IFN- $\gamma$  production and are therefore currently not able to support this notion. Others reported an inverse correlation between pre-vaccination IFN- $\gamma$  production and the magnitude of responses post-vaccination (45, 46). As described by Bodewes et al., annual vaccination with a seasonal vaccine hampers the development of influenza-specific CD8<sup>+</sup> T cells in children, indicating that vaccination history also affects the development of T cell responses (47). To conclude, both a second dose as well as previous vaccination and exposure to influenza might affect T cell responses induced by vaccination.

The number of doses and the quantity of antigen that are needed to induce sufficient protection during a pandemic might be related to the presence of cross-reactive antibody and T cell immunity. It is therefore important to obtain knowledge on pre-existing immunity to the virus, since this can be an indication whether a second dose is necessary. During the 2009 H1N1 pandemic, data became available that individuals had some cross-reactive T cells available that provided partial protection (48). In addition, antibodies cross-reacting to the pandemic strain were observed in older adults, which corresponds with the lower number of affected individuals in this age group (49). Although in this study individuals born during the previous H1N1 era, from 1917-1956, were excluded to limit the effects of cross-reactive immunity on the measurement of vaccine efficacy, there may still have been cross-reactive immunity present in younger individuals, which may in part explain why one dose of the pandemic vaccine already induced sufficient protection.

Therefore, it is also of significance what type of immune response is induced by regular seasonal vaccines, especially if administration of a second dose or annual vaccination might have a negative effect on the T cell response that is induced. In this study, we showed that both the adjuvanted pandemic vaccine containing 7.5  $\mu\text{g}$  HA and the unadjuvanted seasonal vaccine containing 15  $\mu\text{g}$  of HA were capable of inducing T cell responses. Others have shown that T cell responses can be induced by unadjuvanted split seasonal influenza vaccination in children, but focused only on internal influenza proteins (50). To date, not much data is available on the induction of T cells by the trivalent inactivated influenza vaccine containing HA and NA as viral antigens. However, we show that even an unadjuvanted subunit vaccine is capable of inducing T cell responses.

This study has some limitations. During this study, individuals were monitored for influenza-like illness (ILI) and ILI cases were laboratory confirmed for the presence of influenza within 72 hours of onset of symptoms. However, both the pandemic and consecutive year were

very mild influenza seasons in the Netherlands and only sporadic infections were observed in individuals in this study. Therefore, we could correct in our model for influenza infections during the study period. In addition, individuals with a laboratory-confirmed A(H1N1)pdm09 influenza infection before the start of the study were excluded. However, we cannot exclude the possibility that subclinical infections have occurred. Another confounding factor is the limited number of individuals that were enrolled in the CV group, which may have affected results of the HA and NA ELISpot assay, specifically. In addition, in this study, IFN- $\gamma$  was used as the only read-out for T cell responses, while other cytokines or assays may provide with a more complete picture of the T cell response. For example, it would be interesting to elucidate whether the T cell responses measured in this study can be contributed to CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells or both and the additional cytokines secreted by the activated T cells.

Summarizing, we showed that one dose of the MF59-adjuvanted pandemic vaccine induced seroprotective levels of antibodies, which were boosted after administration of a second dose. This second dose did not boost the number of vaccine-induced T cell responses. At the start of the second season, a residual level of antibody and T cell levels was detectable in individuals vaccinated in the previous season. Administration of the 2010-2011 seasonal vaccine boosted both antibody and T cell levels. Comparison of the adjuvanted and unadjuvanted vaccine showed that the adjuvanted vaccine induced significantly higher antibody levels, while T cell levels induced after pandemic or seasonal vaccination were similar. Furthermore, we show that antibody levels were still detectable after 15 months, whereas T cell levels had decreased back to baseline.

These findings have key implications for influenza vaccination strategies, especially during pandemic situations. When cross-protective immunity is available, in the form of conserved antibody or T cell responses, one vaccination dose might be sufficient to provide protection. Since repeated influenza vaccination may not be favorable for the induction of T cell responses, it is important to have knowledge on cross-reactive immunity available. Therefore, studies describing the immune response following influenza vaccination should not only focus on the humoral immune response, but should also include analysis of cellular responses.

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## Supplemental data

Table SIA: Serological responses 2009-2010

Vaccination response 2009-2010		RR (2.5-97.5% CI)	p-value
First dose (IIb)	Primary Baseline (I)	17.3 (15.5-19.4)	<0.001
Second dose (III)	Primary Baseline (I)	21.7 (19.3-24.2)	<0.001
Contraction V (IV)	Primary Baseline (I)	11.1 (9.8-12.4)	<0.001
Second dose (III)	First dose (IIb)	1.3 (1.1-1.4)	0.001
Second dose (III)	Contraction V (IV)	0.5 (0.5-0.6)	<0.001
Contraction V (IV)	Maintenance V (V)	1.5 (1.3-1.7)	<0.001

Table SIB: Serological responses 2010-2011

Vaccination response 2010-2011		RR (2.5-97.5% CI)	p-value
Maintenance V (V)	Primary Baseline (I)	7.6 (6.8-8.5)	<0.001
Secondary seasonal 2010-2011 VV (VI)	Primary Baseline CC (I)	24.4 (21.6-27.5)	<0.001
Contraction VV (VII)	Primary Baseline (I)	24.4 (21.6-27.4)	<0.001
Residual VC (VIII)	Primary Baseline (I)	7.3 (5.8-9.0)	<0.001
Primary seasonal 2010-2011 CV (IX)	Primary Baseline (I)	12.8 (8.0-19.3)	<0.001
Contraction CV (X)	Primary Baseline (I)	5.9 (3.7-8.9)	<0.001
Maintenance V (V)	Secondary seasonal 2010- 2011 VV (VI)	3.2 (2.8-3.6)	<0.001
Secondary seasonal 2010-2011 VV (VI)	Contraction VV (VII)	0.6 (0.5-0.7)	<0.001
Maintenance V (V)	Residual VC (VIII)	1.0 (0.8-1.2)	0.699
Primary seasonal 2010-2011 CV (IX)	Contraction CV (X)	0.5 (0.3-0.8)	0.032

**Table SIC: Comparison groups**

Comparison groups		RR (2.5-97.5% CI)	p-value
Secondary seasonal 2010-2011 VV (VI)	Primary seasonal 2010-2011 CV (IX)	2.0 (1.2-3.1)	0.028
Contraction VV (VII)	Contraction CV (X)	0.5 (0.4-0.6)	<0.001
Contraction VV (VII)	Residual VC (VIII)	2.0 (1.7-2.6)	<0.001
Residual VC (VIII)	Contraction CV (X)	1.3 (0.8-2.1)	0.699

**Table SID: Comparison time points**

Comparison time points		RR (2.5-97.5% CI)	p-value
First dose (IIb)	Primary seasonal 2010-2011 CV (IX)	0.7 (0.5-1.1)	0.599
Second dose (III)	Secondary seasonal 2010-2011 VV (VI)	1.1 (1.0-1.3)	0.328
Maintenance V (V)	Contraction CV (X)	0.7 (0.5-1.2)	0.674

**Table SIIA: Influenza A(H1N1)pdm09 specific T cell responses 2009-2010**

Vaccination response 2009-2010		RR (2.5-97.5% CI)	p-value
First dose (IIa)	Primary Baseline (I)	1.5 (1.3-1.7)	>0.001
Second dose (III)	Primary Baseline (I)	1.4 (1.2-1.6)	>0.001
Maintenance V (V)	Primary Baseline (I)	1.7 (1.4-1.4)	>0.001
First dose (IIa)	Second dose (III)	0.9 (0.8-1.1)	0.819
Second dose (III)	Maintenance V (V)	1.2 (1.0-1.4)	0.11

**Table SII B: Influenza A(H1N1)pdm09 specific T cell responses 2010-2011**

Vaccination response 2010-2011		RR (2.5-97.5% CI)	p-value
Secondary seasonal 2010-2011 VV (VI)	Primary Baseline (I)	2.5 (2.0-3.0)	<0.001
Primary seasonal 2010-2011 CV (IX)	Primary Baseline (I)	2.2 (1.5-3.1)	<0.001
Residual VC (VIII)	Primary Baseline (I)	1.2 (0.9-1.4)	0.544
Maintenance V (V)	Secondary seasonal 2010-2011 VV (VI)	1.5 (1.2-1.8)	0.001
Maintenance V (V)	Residual VC (VIII)	0.7 (0.6-0.9)	0.005

**Table SII C: Comparison groups influenza A(H1N1)pdm09 specific T cell responses**

Comparison groups		RR (2.5-97.5% CI)	p-value
Secondary seasonal 2010-2011 VV (VI)	Primary seasonal 2010-2011 CV (IX)	1.2 (0.8-1.7)	0.819
Secondary seasonal 2010-2011 VV (VI)	Residual VC (VIII)	2.1 (1.7-2.7)	>0.001
Primary seasonal 2010-2011 CV (IX)	Residual VC (VIII)	1.9 (1.2-2.8)	0.021

**Table SIII: Influenza A/Perth/16/2009(H3N2) specific T cell responses**

		RR (2.5-97.5% CI)	p-value
Vaccination	Baseline	1.9 (1.6-2.2)	<0.001