Dissecting the Immune Response to MF59-adjuvanted and Nonadjuvanted Seasonal Influenza Vaccines in Children Less Than Three Years of Age

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Introduction: Annual seasonal influenza epidemics are particularly dangerous for the very young, the elderly and chronically ill individuals, in whom infection can cause severe morbidity, hospitalization and death. Existing, nonadjuvanted influenza vaccines exhibit a suboptimal immunogenicity and efficacy in immunologically naive subjects such as young children.

Methods: This phase II, randomized clinical trial was conducted to evaluate the antibody and cell-mediated responses to a trivalent influenza vaccine administered without adjuvant (TIV) or adjuvanted with MF59 (ATIV) in previously nonvaccinated children less than 3 years of age.

Results: The MF59-adjuvanted vaccine was well tolerated, and induced higher titers of hemagglutination inhibition antibodies able to recognize strains different from the one used in the vaccine (heterovariant) than TIV. The presence of the adjuvant MF59 induced a larger expansion of vaccine-specific $CD4^+$ T cells. Interestingly, the adjuvant MF59 did not modify the cytokine profile of the elicited T cells, characterized by the production of IL-2 and TNF-α, and did not bias the response toward either Th1 or Th2. The advantage of ATIV over TIV was more pronounced for the virus strains that had not circulated in the years that preceded this study and for the heterovariant strains.

Conclusion: These data highlight the relevant role played by the oil-in-water adjuvant MF59 in enhancing the immunogenicity of inactivated influenza vaccines in immunologically naive individuals.

Key Words: influenza, vaccination, infant, MF59, $CD4^+$ T cells

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Influenza A and B viruses cause epidemic disease in humans and, in an average year, 20% of children are infected with influenza viruses. This attack rate is substantially higher during epidemic years and among restricted populations, such as children in day care. Furthermore, children shed large quantities of virus for longer periods of time, thus contributing to the spread of influenza within the community and household.[1] Some countries (including the United States) and the World Health Organization recommend vaccination of children;[2] however, inactivated influenza vaccines, administered without adjuvant, are less immunogenic in unprimed individuals, especially those who are of very young age,[3] and this inadequate immunogenicity translates into a suboptimal efficacy.[4]

The limited efficacy of trivalent inactivated vaccines (TIV) can potentially be overcome by vaccines formulated with an adjuvant (ATIV). Potent adjuvants for influenza are the oil-in-water emulsions like MF59.[5] MF59-adjuvanted influenza seasonal vaccines have been licensed in various countries since 1997 for immunization of elderly subjects (65 years and older) and have shown an optimal safety profile in different age groups[5–7] and a higher effectiveness against influenza hospitalization as compared with nonadjuvanted TIV.[8,9] The higher immunogenicity of MF59-adjuvanted vaccines is particularly evident in naive individuals. Vaccination of children with ATIV induces higher and more sustained antibody responses, as measured by hemagglutination inhibition (HI) and by microneutralization,[4,10] and reduces the attack rates of polymerase chain reaction-confirmed influenza-like illness.[4] We have previously shown that in naive adults the early expansion of vaccine-specific $CD4^+$ T lymphocytes associates in a predictive manner with the induction of high and sustained neutralizing antibody titers to avian and seasonal influenza viruses.[11,12] A question not addressed yet is how the addition of MF59 to TIV influences either the magnitude or the cytokine profile of the elicited $CD4^+$ T-cell response in children. The present phase II study was conducted to address this question and to compare the magnitude as well as the cytokine profile of vaccine-specific $CD4^+$ T lymphocytes in children, who were administered the nonadjuvanted (TIV) versus the MF59-adjuvanted subunit trivalent influenza vaccine (ATIV).

MATERIALS AND METHODS

Study Design

This study was a phase II, randomized, multicenter clinical trial (ClinicalTrials.gov NCT01342796) designed to evaluate both the antibody and the cell-mediated responses elicited by adjuvanted and nonadjuvanted seasonal influenza vaccines in children. The protocol and the informed consent form were reviewed and approved by Institutional Review Board/Independent Ethics Committee/Research Ethics Boards before study start. The study was conducted at 2 centers in Belgium: Sint Vincentius Ziekenhuis, Antwerp and Kinderartsenassociatie, Hasselt. Recruitment began in May and July 2011 and was reinitiated between October and December 2011 to achieve the target sample size.

Study Population and Procedures

Eligible participants were previously nonvaccinated healthy children aged 6 to <36 months of either gender whose parent(s) or legal guardian(s) had given written informed consent.

Exclusion criteria included any known or suspected impairment of the immune system, any serious medical condition or recent infectious disease, immunization with licensed vaccines within 2 weeks (for inactivated vaccines) or 4 weeks (for live vaccines) or

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any other investigational agent within 30 days prior to enrollment, or any history of hypersensitivity to any component of the study vaccine.

Demographic and baseline characteristics of subjects during the 2 phases of enrollment were similar across treatment groups: the mean age (21.4 months in the TIV group and 20.2 months in the ATIV group) and the distribution of subjects below or above 24 months of age were comparable, most of them were Caucasian (TIV 76% vs. ATIV 79%), and the mean weight, height and body mass index were similar. There were, however, 66% males enrolled in the TIV group and 53% in the ATIV group.

A total of 84 children (6 to <36 months of age) were enrolled. Each participant was randomized to receive 2 intramuscular doses (0.25 mL each) spaced 4 weeks apart (days 1 and 29) of a trivalent seasonal influenza vaccine either nonadjuvanted (TIV; n = 41) or adjuvanted (oil-in-water MF59 emulsion, ATIV; n = 43).

Overall, there were 11 subjects (27%) vaccinated with TIV and 18 subjects (42%) vaccinated with ATIV for which a major protocol deviation was reported; these were excluded from the immunogenicity analysis in a Per Protocol Set (PPS) (TIV; n = 30 for serology, 31 for cell mediated immunity and ATIV; n = 25) but retained in the Safety Set for as long as they participated in the study.

The antigen content of TIV and ATIV (both produced at Novartis Vaccines, Siena, Italy) was identical, each vaccine dose containing ~7.5 μg of hemagglutinin surface antigen from each of the 3 strains, A/California/7/2009 (H1N1), A/Perth/16/2009 (H3N2) and B/Brisbane/60/2008. Five milliliters of blood were taken before vaccination (day 1) and 3 weeks after administration of the second dose (day 50) for immunogenicity analysis.

Safety

All enrolled subjects were analyzed for safety and reactogenicity throughout the study. This corresponded to 100% subjects in each group for the first vaccination and 93% to 95% across vaccine groups after the second vaccination (Safety Set). Three subjects from the ATIV group and 2 subjects from the TIV group did not receive the second vaccination at day 29 and were therefore excluded from the safety analysis for the second vaccination.

Solicited local and systemic adverse events (AEs) were recorded for 7 days after each vaccination. Unsolicited AEs were recorded throughout the entire study period (days 1–50).

HI Assay

HI antibodies were measured as previously described. Antibody titers were measured to the influenza strains homologous (A/H1N1/California/07/2009, A/H3N2/Victoria/210/2009 and B/Brisbane/60/2008) and heterologous (A/H3N2/Uruguay/716/2007 and B/Malaysia/2506/2004) to those present in the study vaccines. Results were evaluated as seroconversion (defined as a change in HI titer of <1:10 on day 1 to an HI titer ≥1:40 on day 50) or a significant increase in HI titers (defined as a 4-fold or greater increase in titer in a subject with a day 1 titer ≥1:10); geometric mean ratio of HI geometric mean titer on postvaccination/prevaccination; and seroprotection (defined as the proportion of subjects achieving an HI titer ≥1:40 postvaccination). When the results were evaluated using the criteria of the Committee for Medicinal Products for Human Use (CHMP), the adult criteria were used since no CHMP criteria exist for children: (1) the proportion of subjects achieving seroconversion or a significant increase in HI titer should be >40%; (2) the geometric mean ratio should be >2.5; (3) the proportion of subjects achieving an HI titer ≥1:40 postvaccination should be >70%. In addition, seroprotection rates were also analyzed using higher antibody thresholds,[13] based on the data previously reported.[4]

Analysis was performed on both the Full Analysis Set (FAS) and the PPS. No difference was found between the 2 data sets.

Measurement of T-cell Responses by Intracellular Cytokine Staining

$CD4^+$ T-cell responses were measured by intracellular staining and flow cytometry at baseline and at day 50.[11] Purified peripheral blood mononuclear cells (PBMCs) were stimulated overnight, in vitro, with the subunit antigens (1 μg/mL) from the following homologous (A/H1N1/California/07/2009, A/H3N2/Victoria/210/2009 and B/Brisbane/60/2008) and heterologous strains (B/Florida/4/2006) in the presence of anti-human aCD28/CD49d antibodies (1 μg/mL; BD Bioscience, Sparks, MD) and brefeldin A (5 μg/mL; Sigma, St. Louis, MO). After overnight stimulation, PBMCs were stained with Live Dead (Invitrogen, Carlsbad, CA), then fixed, permeabilized and stained with anti-human antibodies to CD3 (clone SK7; BD Bioscience), CD4 (clone RPA-T4; BD Bioscience); IL-2 (clone 5344.111; BD Bioscience), IFN-γ (clone 4S.B3; BD Bioscience) and TNF-α (clone MAb11; BD Bioscience). Samples were acquired on a fluorescence-activated cell sorter BD LSR II analyzer. Results were analyzed blind using FlowJo software (Tree Star Software, Ashland, OR). The frequency and cytokine profile of antigen-specific $CD4^+$ T cells was calculated by summing the frequency of $CD4^+$ T cells producing all nonoverlapping permutations of the cytokines tested (Boolean and logical gates with FlowJo). Data are expressed as number of antigen-specific $CD4^+$ T cells per million of total $CD4^+$ T cells. Analysis was performed on both the FAS and the PPS. No difference was found between the 2 data sets.

Statistics

Distributions of HI titers are typically skewed to the right. However, log-transformed immunogenicity values are usually approximately normally distributed and so this transformation (base 10) was applied to all titers.

$CD4^+$ T-cell response variables were statistically analyzed in a similar way as the logarithmic transformed titers.

Values below the limit of detection were set to half that. Means and differences (ie, within vaccine group between visits and between vaccine groups across visits, respectively) and pertaining 95% confidence intervals were calculated using an analysis of covariance with vaccine group as a qualitative factor and while adjusting for age cohort and changes in baseline values.

Statistical inferences between vaccination groups were determined using the JMP software. Pairwise differences were evaluated by the Tukey–Kramer test on log normalized values, as described above. The level of significance for the statistical analysis will be set at 5% ($P \leq 0.05$).

All statistical-related analyses results were transformed back to be finally presented in the same unit as measured.

RESULTS

Safety

Consistent with previous studies,[6,7,14] most of the reported local and systemic AEs were mild to moderate in intensity and resolved within a few days of vaccination. The proportion of subjects reporting solicited AEs was higher in the group that received ATIV (see Table, Supplemental Digital Content 1, http://links.lww.com/INF/B945) while the incidence of unsolicited AEs was comparable in the 2 vaccine groups (see Table, Supplemental Digital Content 2, http://links.lww.com/INF/B946). The most commonly reported local AE after the first vaccine dose was tenderness at the

site of injection. There were no reports of severe local reactions in response to TIV or ATIV.

Antibody Responses

Both TIV and ATIV induced a significant increase of HI antibodies against the vaccine strains (A/H1N1California, A/H3N2Victoria and B/Brisbane). ATIV induced a significant increase of HI antibodies also against strains heterologous to the ones contained in the vaccine (A/Uruguay and B/Malaysia). HI antibody titers induced by ATIV were significantly higher than those induced by TIV for A/H3N2Victoria, B/Brisbane and for the heterologous B/Malaysia. HI titers for A/H1N1California were higher after ATIV as compared with TIV, but the difference between the 2 vaccines was not statistically significant (see Fig. 1 and Table, Supplemental Digital Content 3–4, http://links.lww.com/INF/B947 and http://links.lww.com/INF/B948). It is important to note that prevaccination HI titers were higher for A/H1N1California compared with A/H3N2Victoria and B/Brisbane ($P < 0.0001$ by the Tukey–Kramer HSD test). These results support a previous exposure of vaccinees to A/H1N1California, which extensively circulated since the 2009 pandemic, but not to the other strains analyzed in this study.

The serologic results were then evaluated using the criteria of the CHMP. Since there are no CHMP licensure criteria for influenza vaccines in pediatric age, we applied the ones used in adults (see Materials and Methods).

All 3 CHMP criteria were met for the influenza A strains by both TIV and ATIV. For the B strain all 3 criteria were met for ATIV while in the TIV group seroprotection was not met, with only 57% of children achieving an HI titer ≥1:40 (see Table 1 and Table, Supplemental Digital Content 3, http://links.lww.com/INF/B947). Recently it has been proposed that the adult correlate of seroprotection (HI titer ≥1:40) may not be appropriate in children since HI titers of 1:40, 1:110 and 1:330 predict 22%, 50% and 80% of clinical protection, respectively, in this age group.[13] Applying more stringent criteria of seroprotection, the difference between ATIV and TIV becomes more obvious with 80% of children in the ATIV group versus 53% in the TIV group reaching an HI ≥1:330 for A/H1N1California. Similarly, 92% of children in the ATIV group versus 23% in the TIV group reached an HI ≥1:330 for A/H3N2Victoria and 40% in ATIV versus 10% in TIV for B/Brisbane (see Fig. 2 and Table, Supplemental Digital Content 4, http://links.lww.com/INF/B948).

CD4$^+$ T-cell Responses to Vaccination

We then analyzed the impact of vaccination on the frequency and cytokine profile of vaccine-specific CD4$^+$ T cells. ATIV induced the expansion of CD4$^+$ T cells specific for both homologous (A/H1N1California, A/H3N2Victoria and B/Brisbane) and heterologous (B/Florida) influenza antigens. Conversely, TIV induced a significant expansion of CD4$^+$ T cells only for A/H1N1California and A/H3N2Victoria. The expansion of CD4$^+$ T cells specific for A/H1N1California was not significantly different after vaccination with TIV versus ATIV, whereas the expansion of CD4$^+$ T cells specific for A/H3N2Victoria or B/Brisbane was significantly higher after ATIV as compared with TIV ($P \leq 0.0001$) (Fig. 3).

The cytokine profile of influenza-specific CD4$^+$ T cells was comparable after vaccination with TIV and ATIV and was dominated by the production of IL-2 and TNF-α (Fig. 4). Only a small percentage of influenza-specific CD4$^+$ T cells produced IFN-γ and/or IL-13, and their frequency did not increase after vaccination and was comparable after vaccination with TIV and ATIV (Fig. 4). These data in children confirm our previous findings in adults and the elderly vaccinated with MF59-adjuvanted avian H5N1 or seasonal vaccines on the ability of influenza vaccination to expand vaccine-specific CD4$^+$ T cells, especially against those strains to which the populations have not been exposed and against heterovariant strains.[11,15] They also confirm the ability of MF59 to induce an increased expansion of vaccine-specific CD4$^+$ T cells without modifying their cytokine profile.

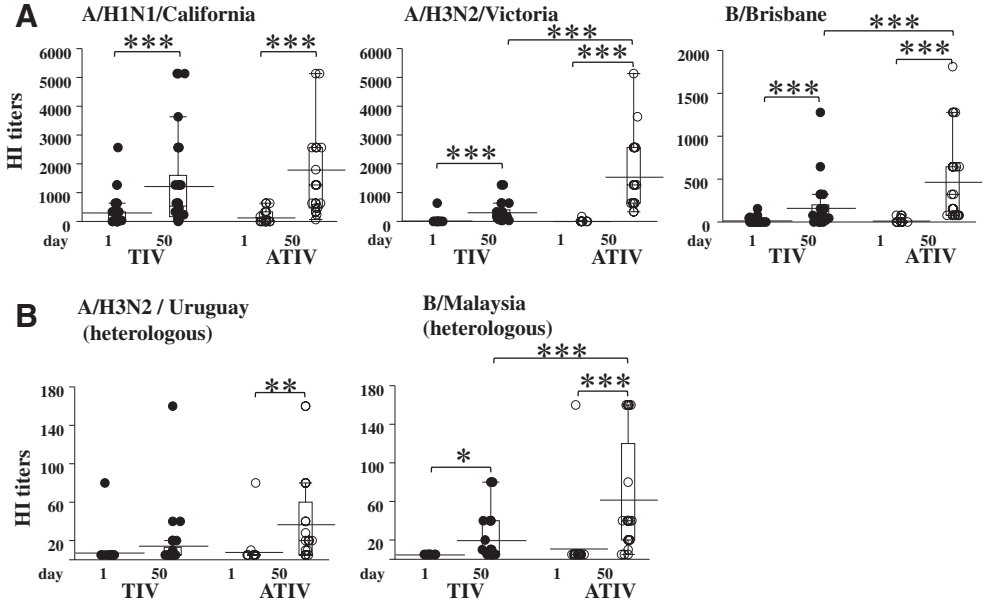

FIGURE 1. HI response to vaccination. HI antibody titers to influenza strains A) homologous and B) heterologous to the strains present in the vaccine were measured at baseline (day 1) and 3 weeks after the second dose (day 50) of TIV or ATIV. The lines crossing the box plots represent the mean titer of each group. The Tukey–Kramer HSD (honestly significant difference) test was used to determine statistical significance ($P \leq 0.05$) among groups (***$P < 0.0001$; **$P = 0.0007$; *$P = 0.0011$). All analyses were done using the PPS.

TABLE 1. Hemagglutination Inhibition Response to Homologous and Heterologous Strains

	Vaccine Group	TIV (n = 30)	ATIV (n = 25)
Homologous strain			
A/H1N1/California/07/2009	Day 1	47 (22–100)	29 (12–67)
	Day 50	387 (268–559)	1245 (831–1866)
	Day 50/day 1	9.99 (5.87–17)	41 (23–74)
A/H3N2/Victoria/210/2009	Day 1	5.87 (4.34–7.93)	6.05 (4.33–8.45)
	Day 50	206 (149–283)	1197 (838–1709)
	Day 50/day 1	35 (24–51)	199 (130–303)
B/Brisbane/60/2008	Day 1	9.66 (6.59–14)	8.65 (5.66–13)
	Day 50	50 (34–74)	300 (195–463)
	Day 50/day 1	5.63 (3.83–8.29)	34 (22–52)
Heterologous strain			
A/H3N2/Uruguay/07	Day 1	5.51 (4.52–6.72)	5.81 (4.66–7.23)
	Day 50	7.64 (5.21–11)	19 (13–30)
	Day 50/day 1	1.38 (0.91–2.09)	3.35 (2.11–5.3)
B/Malaysia/04	Day 1	5.06 (4.26–6)	5.88 (4.87–7.11)
	Day 50	11 (7.57–16)	41 (26–63)
	Day 50/day 1	2.28 (1.42–3.66)	6.49 (3.83–11)

(1.42-3.66)Geometric mean titers and 95% CI (at day 1 and at day 50) and geometric mean ratios (day 50/day 1). The CHMP criterion is GMR >2.5. All analyses were done using the PPS.

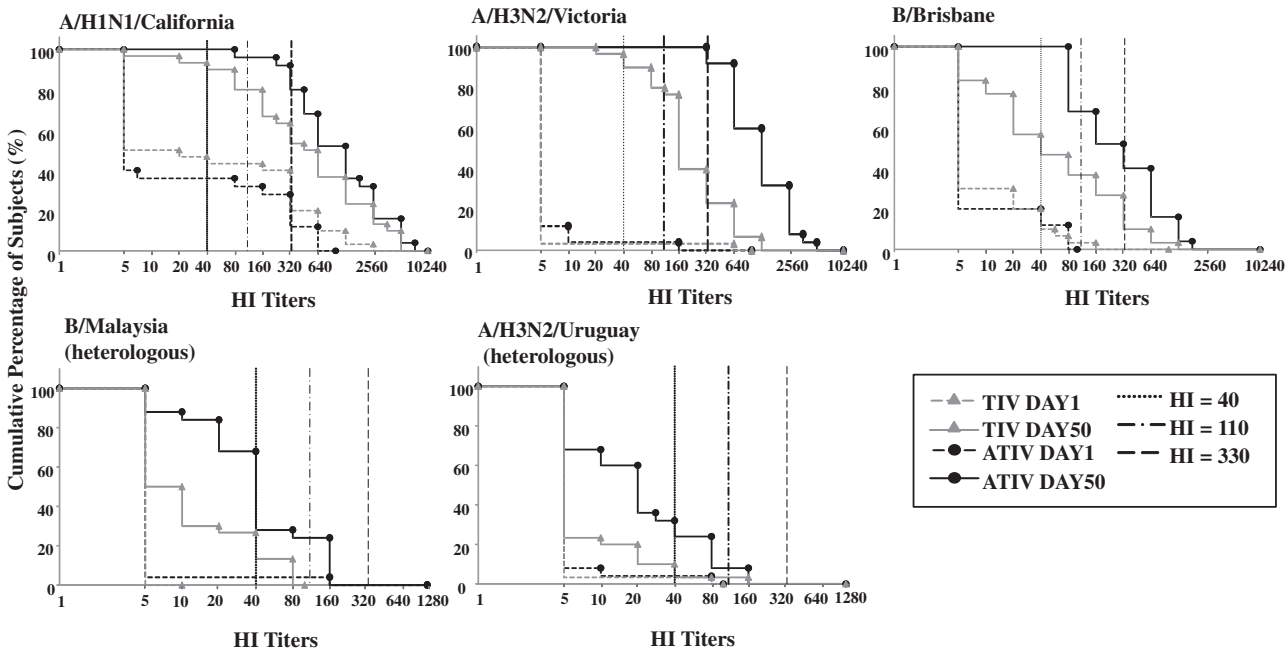

FIGURE 2. HI response to vaccination. Reverse cumulative distribution of HI titers after TIV (gray lines) and ATIV (black lines) at day 1 (dotted) and day 50 (solid). The vertical lines crossing the plots indicate HI titers of 1:40, 1:110 and 1:330. All analyses were done using the PPS.

DISCUSSION

Conventional, nonadjuvanted influenza vaccines have a long track record of use and success. Despite this, they fail to induce high and persistent immune responses in immunologically naive individuals. Formulation with adjuvants, especially oil-in-water emulsions like MF59, enhances the immunogenicity of influenza vaccines in naive individuals like children vaccinated against seasonal influenza[10,16] or adults vaccinated against avian H5N1, an antigen the population has not been exposed to.[11,12,17] In children, MF59-adjuvanted seasonal influenza vaccines induce stronger and more sustained HI and neutralizing antibody responses than the conventional nonadjuvanted vaccines and reduce the risk of infections.[4]

Despite the central role of HA-specific antibodies in protection from influenza infection, several reports point at a critical function for T lymphocytes in the direct killing of infected targets and also in orchestrating the optimal activation of B lymphocytes.[11,12] We have previously shown that in adults the administration of an MF59-adjuvanted A/H5N1 vaccine induces a rapid expansion of vaccine-specific $CD4^+$ T cells that predicts induction of high antibody responses and immune memory, thus supporting a central role for helper $CD4^+$ T cells in the optimal response to vaccination.[11,12]

In this study we show that, in previously unvaccinated children aged 6 to <36 months, the MF59-adjuvanted ATIV induces a strong immune response, as assessed by the increase in HI antibody

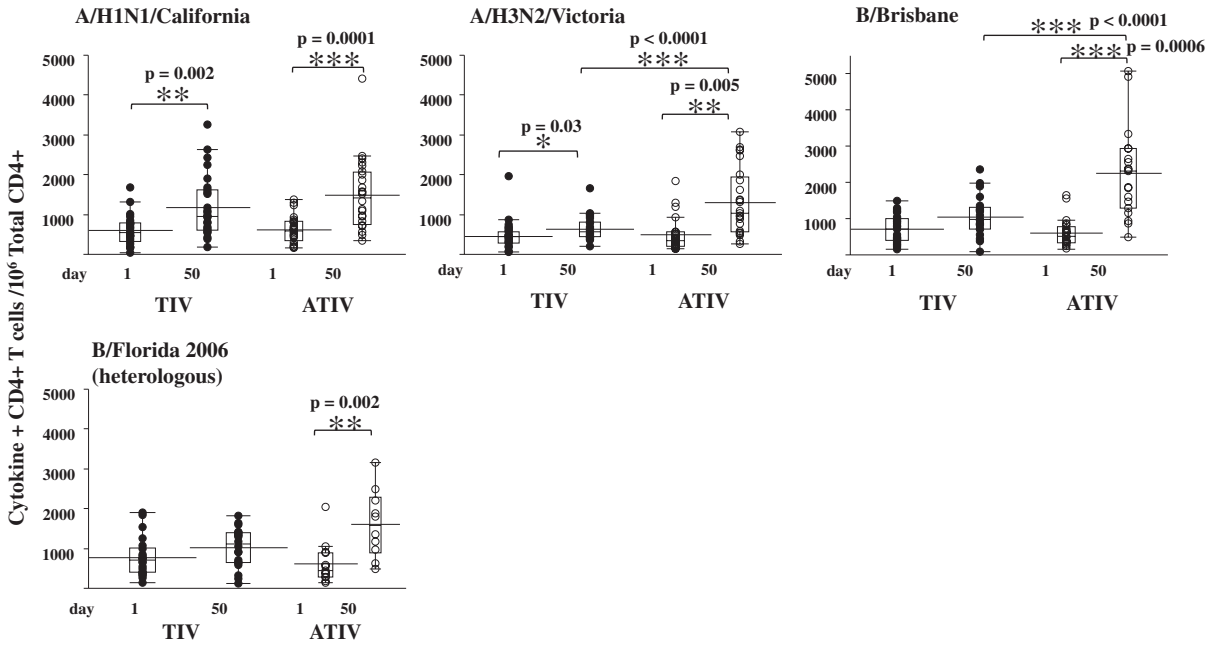

FIGURE 3. Influenza-specific CD4⁺ T cells before (day 1) and 3 weeks after the second vaccination (day 50) with TIV or ATIV – PPS. Number of cytokine⁺ CD4⁺ T lymphocytes after overnight, in vitro, stimulation of PBMCs with subunit antigen homologous and heterologous to vaccine strains. Data are expressed as number of cytokine⁺ CD4⁺ T lymphocytes/10^6 total CD4⁺ T lymphocytes. The lines crossing the box plots represent the mean of the group (***$P \leq 0.0005$; **$P \leq 0.005$; *$P \leq 0.05$ by the Tukey–Kramer HSD test). All analyses were done using the PPS.

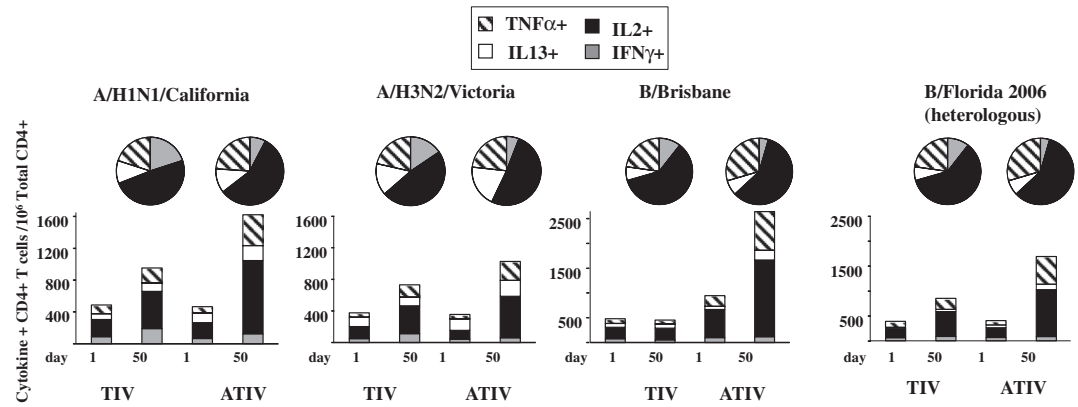

FIGURE 4. Cytokine profile of CD4⁺ T cells induced by vaccination with TIV and ATIV. Frequency of IFN-γ⁺ (gray), IL-2⁺ (black), IL-13⁺ (white) and TNF-α⁺ (striped) CD4⁺ T lymphocytes after overnight, in vitro, stimulation of PBMCs with influenza antigens homologous and heterologous to the vaccine. The bars represent the number of antigen-specific CD4⁺ T lymphocytes/10^6 total CD4⁺ T lymphocytes at day 1 and day 50. The pies represent the relative proportion of each cytokine producing CD4⁺ T lymphocytes at day 50. All analyses were done using the PPS.

titers and the expansion of vaccine-specific CD4⁺ T lymphocytes. The cytokine profile of influenza-specific CD4⁺ T cells was comparable after TIV and ATIV vaccination, and dominated by IL-2 and TNF-α-producing CD4⁺ T cells. The proportion of CD4⁺ T cells producing IFN-γ, a classic Th1 cytokine, or IL-13, a classic Th2-type cytokine, was limited and did not increase following vaccination with either TIV or ATIV. In children, influenza vaccination does expand antigen-specific CD4⁺ T cells with a Th0-like cytokine profile and the adjuvant MF59 does not bias the immune response toward either Th1 or Th2. This confirms in children the results obtained in other age groups on the ability of MF59 to increase the magnitude of both the antibody and the CD4⁺ T-cell response, without modifying the cytokine profile of vaccine-specific CD4⁺ T cells.[4,7,10,11] However, the observed absence of a Th2 bias is in contradiction with data reporting a strong production of Th2 cytokines in children, of the same age group, vaccinated with a split influenza vaccine.[18] Differences in the vaccine composition, the use of seasonal versus avian strains, the age of the children, previous exposure to the antigen as well as the experimental approaches used to detect cytokines can influence the quality of the immune responses.[18,19]

It is noteworthy that MF59-adjuvanted influenza (ATIV) induced stronger responses than the nonadjuvanted (TIV) vaccines against viruses both homologous (A/H3N2/Victoria and

B/Brisbane) and heterologous (A/H3N2/Uruguay, B/Florida and B/Malaysia) to the vaccine strains. The immune response to A/H1N1/California was not statistically different between TIV and ATIV. We believe that this difference can be explained by previous exposure of the children to A/H1N1/California but not to A/H3N2 or to B strains. The clinical study was, in fact, performed between April and December 2011. The pandemic strain A/H1N1/California spread to Europe in the summer of 2009 and circulated widely until the spring of 2010. During this time, the circulation of A/H3N2 strains was minimal; these virus strains did not reemerge until the beginning of 2012 when this study had already been concluded.[20] These epidemiologic data, together with the higher prevaccination HI titers against A/H1N1/California, suggest that most of the children enrolled in this trial had been exposed to A/H1N1/California and to a lower extent to the other 2 vaccine strains.

A limitation of this study is represented by the small sample size which was not powered to detect rare events following immunization. Despite this, the data obtained provide mechanistic insight into the adaptive immune response of adjuvant added to influenza vaccines used to vaccinate children and correlate with the safety profile of MF59-adjuvanted influenza vaccine in children already reported in larger studies.[4,6,7]

Taken together, the data presented here strongly support the advantage of using the MF59-adjuvanted ATIV over the non-adjuvanted TIV in priming an immune response in immunologically naive subjects, like young children, and to widen the breadth of the response to heterologous virus strains. We conclude that MF59-adjuvanted influenza vaccines are safe and immunogenic in children and support its use in this age group to confer higher and broader protection in unprimed children.

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