

# Parenteral Influenza Vaccination Influences Mucosal and Systemic T Cell–Mediated Immunity in Healthy Adults

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We sought to determine whether palatine tonsils (PTs) harbor naturally acquired influenza-specific T cell immunity and whether routine parenteral immunization with influenza vaccine influences mucosal and systemic T cell reactivity. We demonstrate that tonsillar and peripheral blood mononuclear cells (PBMCs) proliferate strongly to influenza antigens, suggesting that naturally acquired immunity exists within both the mucosal and systemic compartments. Influenza vaccination induced significantly stronger T cell responses in both PTs and blood, in addition to increasing titers of antiinfluenza antibodies in serum and saliva. More-rapid proliferative responses of PTs after vaccination were associated with a shift from a response involving both CD45RA<sup>+</sup> and CD45RO<sup>+</sup> T cells to an entirely CD45RO<sup>+</sup>-dependent response. Interestingly, the ratio of interferon- $\gamma$  to interleukin-5 was dramatically higher in cultures of PT T cells responding to influenza than in PBMCs. Our data indicate that parenteral influenza vaccination influences both mucosal and systemic naturally acquired T cell immunity.

Despite the availability of inactive split-virion and subunit vaccines, influenza-associated disease remains an important cause of respiratory-tract infection, hospitalization, morbidity, and mortality, which results in a significant economic burden throughout the world [1]. The results of previous studies have demonstrated that many healthy, unvaccinated adults show clear peripheral blood T cell responsiveness to influenza, presumably as a result of natural infection by the virus [2, 3]. Novak et al. [4] have reported that the influenza-driven proliferation of peripheral blood mononuclear cells (PBMCs) is largely attributable to CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>CD8<sup>-</sup> cells, and Bercovici et al. [5] have shown that peripheral blood CD8<sup>+</sup> lymphocytes

also proliferate when they are exposed to influenza antigens. Thus, natural systemic cell-mediated immunity to influenza is composed of both T helper and cytotoxic T cell responses. Given that infection with influenza occurs within the respiratory tract, it is also highly likely that naturally acquired immunity exists within the mucosal compartment.

In humans, the nasal-associated lymphoid tissue is composed of a ringlike structure known as Waldeyer's ring, which contains an adenoidal tonsil, a lingual tonsil, 2 tubal tonsils, and 2 palatine tonsils (PTs) [6, 7]. Studies of unvaccinated adults have demonstrated influenza-specific antibody-forming cells (AFCs) in nasal, PT, and adenoidal mucosal tissue [8, 9], which suggests that natural infection also elicits a humoral response in the mucosal compartment. Clearly, in any attempt to enhance immunity to influenza through vaccination, it would be advantageous to enhance local and systemic immunity.

To date, most routine influenza vaccinations have been administered by intramuscular (im) immunization [10]. Although it has been well documented that parenteral immunization of the naive host is not an effective means of stimulating mucosal immunity, it is

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noteworthy that, in the case of influenza vaccination, most adult recipients will harbor natural immunity from previous infection [10]. Thus, it is possible that parenteral vaccination after infection could restimulate both systemic and mucosal responses toward influenza [11]. In this regard, Brokstad et al. [12] have demonstrated that routine influenza vaccination modulates the humoral immunity observed in PT but does not induce a response in the human nasal mucosa [13]. This suggests that im delivery of influenza antigens can influence humoral immunity in the mucosal compartment but that this influence may be site dependent. Data regarding the effect of routine influenza vaccination on mucosal T cell immunity, which is important both as a regulator and an effector, are scarce.

In the present study, we show that systemic and mucosal T cells (derived from PTs) proliferate strongly in the presence of influenza antigens and that systemic—and, to a lesser extent, mucosal—T cell proliferative responses are enhanced after vaccination. We demonstrate clear changes in the nature of the T cell response in the PTs after vaccination.

## SUBJECTS, MATERIALS, AND METHODS

**Subjects.** Ten otherwise healthy adults (median age, 28 years 8 months [range, 16 years 11 months–32 years 2 months]; 5 female) undergoing tonsillectomy for recurring tonsillitis or airway obstruction were enrolled for vaccination. Because it was not possible to obtain clinical samples of PTs before and after vaccination, 10 control, unvaccinated healthy adults (median age, 22 years 1 month [range, 17 years 11 months–35 years 10 months]; 5 female) and 5 unvaccinated children (median age, 3 years 7 months [range, 3 years 2 months–3 years 9 months]; 2 female) were also enrolled. The study was conducted during a year in which influenza was not epidemic in the United Kingdom. Subjects with an influenza-like illness or previous immunization with influenza vaccine within 2 years before recruitment were excluded. The work was performed in accordance with the Helsinki Declaration, and written consent was obtained from all subjects or parent(s)/guardian(s) before the study. The collection and use of clinical material complied with relevant guidelines and institutional practices (United Bristol Healthcare Trust Local Ethics Committee application E5453).

**Immunization and sample collection.** Two weeks before tonsillectomy, subjects in the vaccine group were given a single im, 0.5-mL dose of an inactive trivalent split-virion influenza vaccine into the upper arm (Fluarix 2002/2003 vaccine; gift from GlaxoSmithKline). Each dose contained 15  $\mu\text{g}$  of hemagglutinin (HA) of each of the following strains: A/Moscow/10/99 (H3N2)-like strain, A/New Caledonia/20/99 (H1N1)-like strain, and B/Hong Kong/330/2001-like strain, adsorbed to aluminium hydroxide. Samples of saliva, serum, and citrated blood were obtained immediately before both vaccination and tonsillectomy. Tonsils were deposited into RPMI 1640 medium

supplemented with 1000 U/mL penicillin and 1 mg/mL streptomycin for transport to the laboratory. Saliva samples were obtained by use of sterile sponge swabs (Malvern Medical Developments) and were harvested by squeezing swabs into a 5-mL sterile plastic syringe; samples were then stored at  $-80^{\circ}\text{C}$ . Serum samples were stored at  $-80^{\circ}\text{C}$  until use. Blood, saliva, and PT samples were obtained from the subjects in the unvaccinated group at the time of operation.

**Preparation of mononuclear cells (MNCs).** Tonsil MNCs (TMNCs) and PBMCs were prepared as described elsewhere [14, 15]. Tonsils were dissected into 2-mm<sup>3</sup> pieces and dispersed into Hanks' balanced salt solution (HBSS) by use of steel gauze (Potter and Son). TMNCs were obtained by density-gradient centrifugation (Histopaque; Sigma). TMNCs were harvested, washed 3 times in HBSS (at 400 g for 10 min), and resuspended in complete RPMI (RPMI 1640 without glutamine supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, 4 mmol/L L-glutamine, and 10 mmol/L HEPES buffer). TMNCs were counted by use of 0.2% (wt/vol) trypan blue; diluted with complete RPMI to  $1.5 \times 10^6$ ,  $1.0 \times 10^6$ , and  $0.6 \times 10^6$  cells/mL; and plated out as 2-mL cultures in 24-well tissue culture clusters (Nunc) with a final concentration of 1% (vol/vol) heat-inactivated human AB serum (National Blood Services). PBMCs were isolated from citrated peripheral blood by density-gradient centrifugation, harvested, washed 3 times, and resuspended in complete RPMI. PBMCs were counted, diluted to  $1 \times 10^6$  cells/mL, and plated out as 2-mL cultures. Antigens were used within the following ranges: influenza vaccine, 0.2–1.8  $\mu\text{g}/\text{mL}$ , and tetanus toxoid vaccine (Aventis Pasteur), 50–100 ng/mL. Negative controls consisted of cells incubated with complete RPMI and human serum only. Cultures were incubated for up to 10 days at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ .

**Depletion of CD45RA<sup>+</sup> and CD45RO<sup>+</sup> populations.** TMNC preparations were depleted of CD45RA<sup>+</sup> and CD45RO<sup>+</sup> populations by magnetic bead-associated cell sorting, as described elsewhere [15]. Briefly,  $2 \times 10^8$  TMNCs were incubated in 1600  $\mu\text{L}$  of cold buffer (calcium magnesium-free PBS supplemented with 2 mmol/L EDTA and 0.5% [vol/vol] human AB serum) with 400  $\mu\text{L}$  of either CD45RA<sup>+</sup> or CD45RO<sup>+</sup> microbeads (Miltenyi Biotec) for 15 min. Washed cells were separated on an LS<sup>+</sup> column (Miltenyi Biotec) by use of a magnetic cell separator. Depleted cells were washed in ice-cold buffer, pelleted, resuspended in complete RPMI with 1% human AB serum (vol/vol), and plated out and stimulated as described above for undepleted TMNCs. The efficiency of cellular depletions was assayed by flow cytometry and was found to be >90% in all experiments (data not shown).

**Proliferation assays.** Tritiated-thymidine incorporation assays were used to monitor the proliferation of MNCs derived from tonsil and blood, as described elsewhere [15–17]. On days 3–9 after stimulation, cultures were mixed, and triplicates of

100  $\mu$ L were transferred to 96-well round-bottomed plates (Nunc). Triplicates were pulsed with 0.4 mCi of  $^3$ H thymidine (Amersham Pharmacia) and were incubated for 20 h at 37°C in 5% CO<sub>2</sub>. After incubation, MNCs were harvested onto glass-fiber filter mats, covered with melt-on scintillation strips, and counted by use of a scintillation counter (1450 Microbeta model; Wallac). Data were expressed as corrected counts per minute with backgrounds (derived from pulsed unstimulated cultures) subtracted (denoted as  $\Delta$ CCPM).

**Cytometric bead array (CBA).** Analysis of interleukin (IL)-2, IL-4, IL-5, IL-10, tumor necrosis factor (TNF)- $\alpha$ , and interferon (IFN)- $\gamma$  in culture supernatants was performed by use of a human-specific Th1/Th2 CBA (BD Pharmingen) [18], in conjunction with a FACScalibur flow cytometer, according to the manufacturer's instructions.

Samples were removed from cultures of TMNCs and PBMCs on days 3–9 after stimulation and stored at  $-80^{\circ}\text{C}$  until use. Samples were thawed, and cells were pelleted by centrifugation at 400 g for 10 min. Cytokine-specific antibody-coated beads were used to capture soluble cytokines, and an anti-human phycoerythrin detector antibody was used to quantify levels of bound cytokine in standards and samples. Standard curves were used to convert mean fluorescence intensities into concentrations of cytokine by use of CBA software (version 1.1; BD Biosciences). Time-course experiments permitted the resolution of peak cytokine responses: day 7 after stimulation for IFN- $\gamma$ , IL-5, and IL-10, and day 3 after stimulation for TNF- $\alpha$  (data not shown). Results are expressed as concentrations (pg/mL) of cytokine in individual samples on the appropriate day of peak cytokine production minus the background (cytokine in unstimulated cultures).

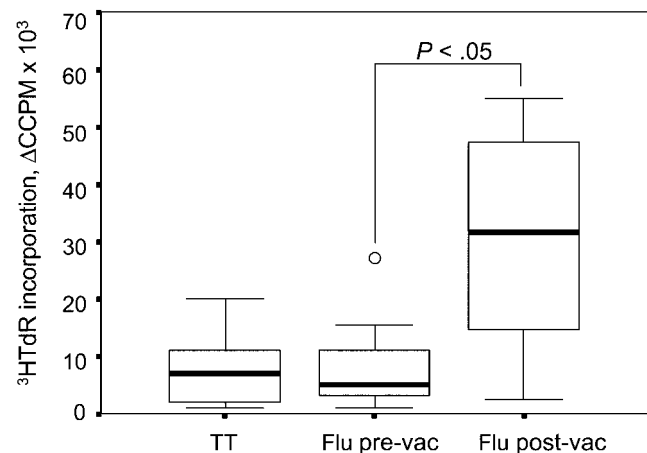
**ELISAs.** For ELISAs, 96-well microtiter plates (Maxisorp; Nunc) were coated with influenza antigens (Fluarix vaccine) at 5  $\mu$ g/mL (HA content), in 0.05 mol/L carbonate buffer (pH 9.6), and were incubated overnight at 4°C. Between each step, plates were washed 4 times with PBS that contained 0.05% Tween 20. Plates were blocked for 1 h at room temperature with 1% (wt/vol) bovine serum albumin (BSA) in PBS. Saliva and serum samples were diluted to 1:2 and 1:100 in BSA and PBS, respectively, and were titrated in duplicate as 2-fold serial dilutions and incubated for 2 h at room temperature. On each plate, internal standards were used that consisted of a sample from a 4-year-old child (serum only) and a previously vaccinated adult (serum and saliva). Secondary antibodies, goat anti-human alkaline phosphatase conjugates (Southern Biotech Associates), were used at 1:1000 dilution, and plates were incubated for 1 h at room temperature. As substrate, *p*-nitrophenyl phosphate, dissolved at 1 mg/mL in carbonate buffer with 0.5 mmol/L MgCl<sub>2</sub> (pH 8.0), was used. Plates were incubated with substrate for 30 min at room temperature, and reactions were stopped with 3 N sodium hydroxide. Plates were

read at 405 nm. End-point titers were calculated by regression analysis by use of an OD of 0.5 (equivalent to >5 times background) as a cutoff value.

**Statistical analysis.** Triplicates derived from proliferation assays yielded arithmetic means with SEs of <5%, which were used for subsequent statistical analysis. The Wilcoxon signed-rank test was used to compare PBMC proliferative responses, cytokine responses, and antibody titers of individuals before and after vaccination. Because it was not possible to obtain clinical samples of PTs before and after vaccination, the Mann-Whitney *U* test was used to compare tonsillar proliferative and cytokine responses in cohorts of vaccinated and nonvaccinated individuals. Statistical tests were performed by use of SPSS for Windows (version 11.0; Lead Technologies). *P* < .05 was considered to be statistically significant.

## RESULTS

**PBMC proliferative responses to influenza antigens.** To verify the efficiency of vaccination in our studies, PBMCs were assayed for proliferation by thymidine-incorporation before vaccination (day 0) and 2 weeks after vaccination (day 14). Peak proliferative responses toward influenza vaccine were significantly higher after vaccination (*n* = 10) than before (*n* = 10) (median, 5130 [range, 940–27,160] vs. 31,570 [range, 2360–54,890] CCPM above background, respectively; *P* < .05) (figure 1). Tetanus toxoid (TT), a positive control and recall antigen,



**Figure 1.** Peak proliferative responses (above background) of mononuclear cells derived from peripheral blood toward influenza vaccine and tetanus toxoid (TT). Peripheral blood mononuclear cells were isolated from individuals immediately before vaccination (flu pre-vac; *n* = 10) and 2 weeks after vaccination (flu post-vac; *n* = 10) and were stimulated with antigen. Proliferation was assayed by tritiated-thymidine ( $^3$ HTdR) incorporation over a period of 9 days of culture after stimulation. Results are expressed as the median no. of corrected counts per minute above background ( $\Delta$ CCPM) (thick black line) with interquartile range (box) and data range (thin black lines). *P* values were derived by use of the Wilcoxon signed-rank test.

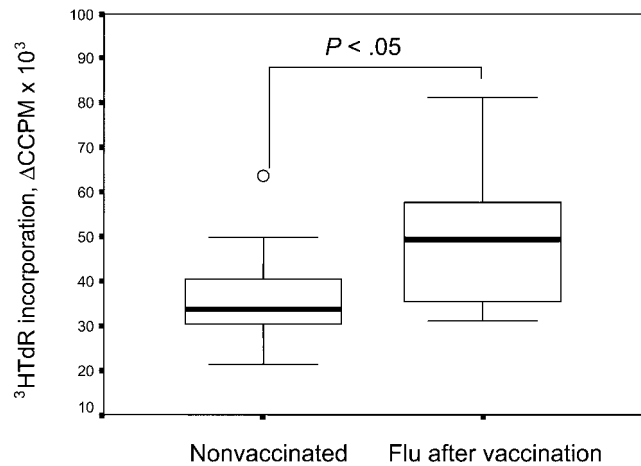
also elicited detectable proliferative responses in PBMC cultures. The median response was similar to that seen to influenza antigens before vaccination but was significantly lower than the median postvaccination response to influenza vaccine ( $P < .05$ ).

**TMNC proliferative responses toward influenza antigens.**

Tonsils obtained from subjects 2 weeks after vaccination and from nonvaccinated individuals were assayed for ex vivo proliferative responses toward influenza vaccine. Peak proliferative responses in influenza-stimulated TMNC cultures were significantly higher in the vaccinated cohort, compared with nonvaccinated individuals (median, 49,505 [range, 31,052–81,291] vs. 33,740 [range, 21,287–49,943] CCPM above background, respectively;  $P < .05$ ) (figure 2). TMNC cultures were also stimulated with TT, but detectable responses were not observed in either cohort (data not shown). TMNCs derived from nonvaccinated children <5 years old ( $n = 5$ ) were also assayed for proliferative responsiveness toward influenza vaccine. Children's TMNCs were found to have significantly lower proliferative responses (median, 21,336 [range, 849–25,563] CCPM above background), compared with those of nonvaccinated and vaccinated adults ( $P < .01$ ).

**Depletion of CD45RA<sup>+</sup>/RO<sup>+</sup> populations.** Cellular depletions were performed on TMNCs derived from vaccinated ( $n = 2$ ) and nonvaccinated ( $n = 2$ ) individuals, to determine whether proliferative responses observed toward influenza were dependent on the presence of memory (CD45RO<sup>+</sup>) and/or naive (CD45RA<sup>+</sup>) T cells. TMNCs derived from nonvaccinated individuals demonstrated proliferative responses toward influenza whether they were depleted of either CD45RA<sup>+</sup> or CD45RO<sup>+</sup> populations (figure 3). The peak of the response was early (day 6) when CD45RO<sup>+</sup> cells were the responders, whereas the peak was much later (day 9) in the culture where this population had been depleted. The response in undepleted cultures showed biphasic kinetics, with separate peaks occurring on both days 5 and 8. After vaccination, a single peak of proliferation was seen in the undepleted cultures, and this occurred early (day 4). The depletion of CD45RO<sup>+</sup> cells from vaccinee-derived TMNCs resulted in the abolishment of the proliferative response to influenza, whereas cultures depleted of CD45RA<sup>+</sup> cells readily proliferated, showing kinetics similar to those of the response of undepleted cells. Similar patterns of responsiveness were observed in the separate individuals tested.

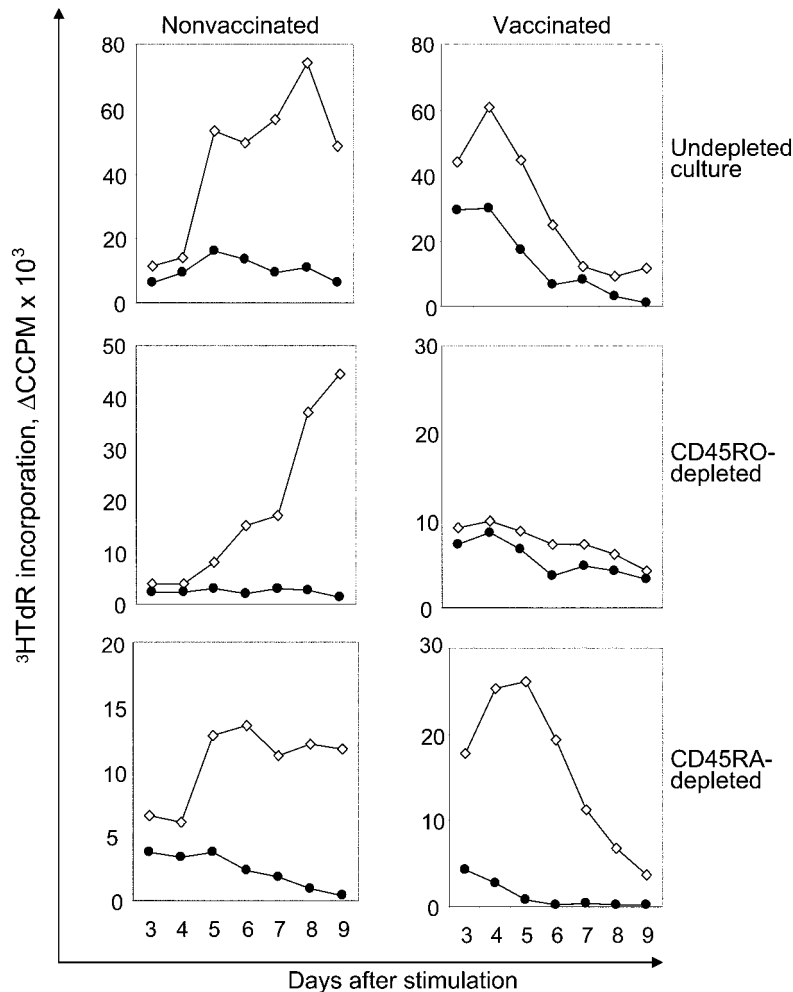
**CBA analysis of cytokine response.** CBAs were used to obtain the cytokine profile of the supernatants of stimulated cultures of TMNCs and PBMCs on days 3–9 after stimulation. CBA analysis revealed that levels of IFN- $\gamma$  and IL-5 secreted in PBMC supernatants were increased 2 weeks after vaccination, compared with those before vaccination, in 4 of 5 samples tested (figure 4A). In contrast, only modest increases in IL-10 and TNF- $\alpha$  titers were evident and were not consistent between individuals after vaccination.



**Figure 2.** Peak proliferative responses (above background) of tonsillar mononuclear cells derived from vaccinated ( $n = 10$ ) and nonvaccinated ( $n = 10$ ) individuals toward influenza vaccine. Proliferation was assayed by tritiated-thymidine (<sup>3</sup>HTdR) incorporation over a period of 9 days of culture after stimulation. Results are expressed as the median no. of corrected counts per minute above background ( $\Delta$ CCPM) (thick black line) with interquartile range (box) and data range (thin black lines).  $P$  values were derived by use of the Mann-Whitney  $U$  test.

Supernatants taken from stimulated cultures of TMNCs derived from vaccinated ( $n = 4$ ) and nonvaccinated ( $n = 5$ ) individuals were also analyzed. IFN- $\gamma$ , IL-5, and TNF- $\alpha$  were consistently detected in TMNC supernatants from both cohorts (figure 4B). It is noteworthy that, although the levels of IFN- $\gamma$  observed in the PT cultures were considerably higher than had been noted in PBMC cultures, the levels of IL-5 and TNF- $\alpha$  were much lower, indicating a different balance in the type of response in each compartment. Vaccination did not result in a marked change in levels of IFN- $\gamma$ , IL-5, or TNF- $\alpha$  in TMNC cultures, compared with those observed for nonvaccinated individuals. Titers of IL-10 were considerably lower in TMNC cultures derived from vaccinated individuals than in those from nonvaccinated individuals; however, this was not statistically significant. Neither IL-2 nor IL-4 was detected consistently in supernatants of PBMC or TMNC cultures when this assay was used (data not shown).

**Antiinfluenza antibodies in serum and saliva.** Serum and saliva samples taken before and 2 weeks after vaccination were assayed for influenza-specific antibodies by use of ELISA. Titers of total numbers of antiinfluenza antibodies were significantly increased in serum ( $P < .05$ ) and saliva ( $P < .01$ ) 2 weeks after vaccination, compared with prevaccination titers (figure 5). Antiinfluenza IgG antibodies elicited after vaccination were found to be significantly higher in serum ( $P < .05$ ) and saliva ( $P < .05$ ) than in prevaccination titers. Vaccination elicited increased IgA titers in serum ( $P < .05$ ) but did not boost IgA titers in saliva.



**Figure 3.** Representative data showing proliferative kinetics of tonsil mononuclear cells toward influenza antigens in undepleted cultures and cultures depleted of CD45RA<sup>+</sup> and CD45RO<sup>+</sup> cells. Data represent 2 separate experiments in either case. Proliferation was assayed by tritiated-thymidine (<sup>3</sup>HTdR) incorporation over a period of 9 days of culture after stimulation with influenza vaccine. Results are expressed as the median no. of corrected counts per minute ( $\Delta$ CCPM) for influenza-stimulated cultures (*hollow diamonds*) and unstimulated cultures (*filled circles*).

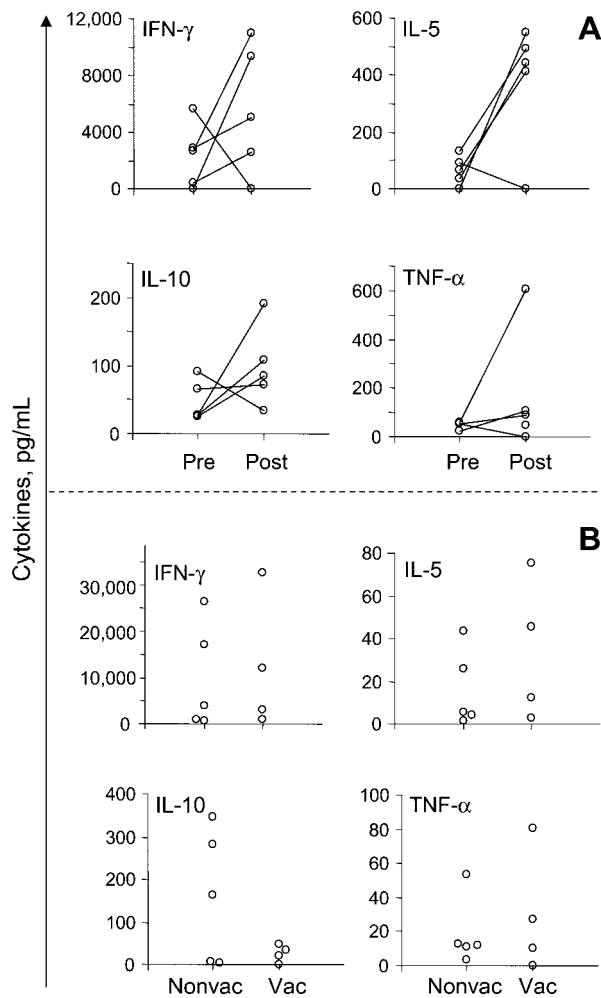
## DISCUSSION

Local immunity toward influenza is triggered after infection of the upper airway epithelium and is thought to involve several lymphoid tissues, including PTs and the mediastinal and cervical lymph nodes [19]. At present, routine vaccination against influenza is composed of a split-virion preparation representative of prevalent strains and is administered im. Given the compartmentalization of immune responses—in particular the perceived failure of parenteral immunization to trigger mucosal immunity [10, 13]—we investigated the effects of im vaccination on mucosal immune reactivity. An understanding of the cellular components of the mucosal response has been hampered by limitations in the techniques available, so, in our studies, we used assays that were optimized to investigate T cell immunity in human tonsils.

All of the adults enrolled in our study demonstrated marked

systemic responsiveness to influenza antigens before vaccination. These responses were evident even though none of the individuals had knowingly had an influenza-like illness within 2 years of the start of the study and were similar in magnitude and kinetics to the response exhibited toward TT. Although it is conceivable that some of the responses observed were those of naive T cells, our findings suggest, as has been reported elsewhere [2–5], that long-term memory to virus can be re-stimulated in the systemic compartment.

Investigations of TMNC responses to influenza antigens in the absence of vaccination revealed that the organized mucosal lymphoid tissues harbored influenza-specific T cells in all of the individuals tested. Although this was expected, given that influenza is a mucosal pathogen and that all of the individuals had demonstrable PBMC reactivity, this finding contrasts with that of a previous report, in which only 20% of adults were



**Figure 4.** Cytometric bead array profiling of cytokines produced by cultures of influenza-stimulated peripheral blood mononuclear cells (*A*) and tonsillar mononuclear cells (*B*). Cells were stimulated at day 0 with influenza vaccine and were assayed as described in Subjects, Materials, and Methods. Data are expressed as the level of cytokine produced (pg/mL) above background (nonstimulated cultures; data not shown). IFN, interferon; IL, interleukin; nonvac, not vaccinated; post, after vaccination; pre, before vaccination; TNF, tumor necrosis factor; vac, vaccinated.

shown to respond to influenza [20]. The discrepancy between our results probably reflects the fact that our assay system was optimized to detect relatively low-level responses to a variety of antigens [15]. We also found detectable, but significantly lower, influenza-driven proliferative T cell responses in PTs from children <5 years old, compared with PT responses from nonvaccinated adults (data not shown). These lower responses reflect that children <5 years old have not been previously exposed to influenza [21]. Our culture system supports the proliferation of naive T cells to antigens not previously encountered [15]; thus, many of the responses observed in this age group may have been of naive T cells. Collectively, these data suggest that PTs of nonvaccinated adults have been primed

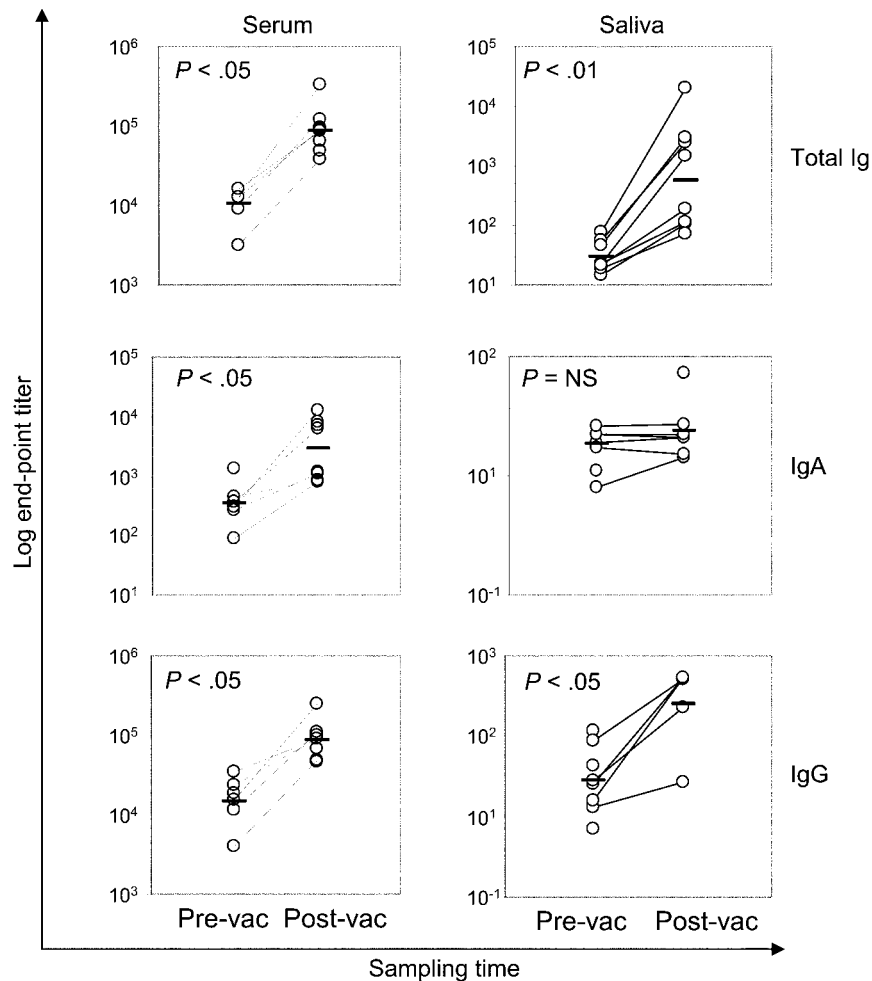
by previous exposure to influenza and harbor natural T cell-mediated immunological memory.

Conventional im vaccination unmistakably enhanced the proliferative responses of PBMCs toward influenza and was associated with a sharp increase in the levels of serum antibodies, in accordance with previous observations [2]. Importantly, im vaccination was also associated with a significant ( $P < .05$ ) increase in the levels of influenza-specific T cell proliferation in PTs, compared with those seen in unvaccinated subjects. This establishes that T cell immunity in the mucosal compartment can be affected by parenteral immunization with influenza.

The increased magnitude of the TMNC response to influenza antigens after vaccination was associated with a shift in the kinetics of the response. A biphasic response peaking on days 8–9 of culture was replaced with a more rapid response peaking on days 4–5. This shift reflected a dramatic change in the nature of the T cells proliferating to influenza after vaccination. Naive T cells express high levels of CD45RA and are CD45RO<sup>-</sup> [22, 23]. On activation, levels of CD45RA are down-regulated, and CD45RO becomes expressed. Therefore, CD45RO expression is widely used as a marker for memory T cells, although it is noteworthy that evidence indicates that some resting long-term memory cells may revert to the expression of CD45RA [24–26]. In unvaccinated individuals, strong proliferation was observed in TMNC cultures after the depletion of either CD45RA<sup>+</sup> or CD45RO<sup>+</sup> cells. This establishes the presence of influenza-specific CD45RO<sup>+</sup> T cell memory within PTs. The dividing CD45RA<sup>+</sup> cells could represent long-term memory cells, responding naive cells, or a mixture of both. Because CD45RA<sup>+</sup> T cell responses with similar characteristics have been reported by us when we used KLH [15], an antigen to which individuals would not have been previously exposed, we suggest that such naive T cells formed at least part of the response observed.

After vaccination, the depletion of CD45RO<sup>+</sup> cells reduced the levels of influenza-driven proliferation to near background levels, whereas reactivity in the CD45RA<sup>+</sup>-depleted fraction remained strong. The loss of reactivity in the CD45RA<sup>+</sup> pool indicates that the naive and/or long-term influenza-specific memory cells have been lost from PTs as a result of vaccination. In the case of naive T cells, this may result from the local presentation of antigen driving the differentiation of CD45RA<sup>+</sup> cells into CD45RO<sup>+</sup> effector cells. Alternatively, the absence of a response in the CD45RO<sup>+</sup>-depleted population may have resulted from the recruitment of naive T cells to the lymph node, draining the vaccination site and thereby reducing the numbers of naive precursors available to circulate through PTs. If the CD45RA<sup>+</sup> fraction also contains influenza-specific long-term memory cells [27], we speculate that these also may have been preferentially recruited to the draining lymph nodes.

Cytokine analysis of culture supernatants indicated that par-



**Figure 5.** Influenza-specific antibodies present in serum and saliva before and 2 weeks after vaccination. Levels of antiinfluenza total immunoglobulins, IgA, and IgG were assayed by ELISA by use of trivalent influenza vaccine as the coating antigen. Individual end-point titers and geometric mean end-point titers are represented by circles and thick black lines, respectively. Prevacination (pre-vac) and postvaccination (post-vac) end-point titers were compared by use of the Wilcoxon signed-rank test. NS, not significant.

entral vaccination influenced the effector populations in blood and PTs. Peripheral-blood T cell responses were characterized by increased postvaccination production of the Th1 cytokine IFN- $\gamma$  and the Th2 cytokine IL-5 in 4 of 5 individuals tested (figure 4A); however, these increases were not statistically different from cytokine titers observed before vaccination. These findings are in accordance with those of Skowronski et al. [28], who demonstrated that increased titers of IFN- $\gamma$  can be detected peripherally up to 6 months after vaccination, which suggests that systemic Th1 responses are not short lived. The increased production of both IFN- $\gamma$  and IL-5 in PBMC cultures after vaccination indicates that systemic immunological memory is associated with both Th1 and Th2 cells. In contrast, maximal levels of IFN- $\gamma$  produced in TMNC cultures in response to influenza antigens were  $\sim$ 3-fold higher than those in PBMC cultures, despite similar overall levels of proliferation in both compartments. Furthermore, IL-5 showed the opposite

trend—maximal levels were  $\sim$ 7-fold higher in PBMC than in TMNC cultures. These differences were evident in both vaccinated and unvaccinated individuals, indicating the compartmentalization of antiinfluenza immunity. There is a general perception that immune responses at mucosal sites are primarily not proinflammatory but are associated with help for IgA secretion [29]. Why the mucosal response toward influenza should be associated with a higher IFN- $\gamma$ :IL-5 ratio, suggestive of a more polarized Th1 response, is unclear, although the fact that IFN- $\gamma$  levels in vaccinated PT cultures were not significantly different from those observed in nonvaccinated PT cultures suggests that vaccination did not cause a marked shift in established natural immunity. Additionally, the observation that levels of IL-10 in TMNC cultures were lower in the vaccinated group, compared with the unvaccinated group, may provide indication that there was a reduction in the regulatory signals controlling the mucosal T cell response after vaccination. Great-

er numbers of individuals would be needed to determine whether this was a consistent difference, and it would be of interest to determine whether reduced IL-10 production was associated with the loss of CD45RA<sup>+</sup> influenza-responsive cells in the PT.

Studies of serum and salivary antibodies were used to determine whether the humoral immunity elicited by vaccination is also compartmentalized. Our data show that levels of anti-influenza antibodies were significantly increased in both serum ( $P < .01$ ) and saliva ( $P < .01$ ) after vaccination. The finding that the major increase in saliva was due to IgG rather than to IgA has several possible explanations. First, influenza-specific IgA has been reported to peak in saliva 9 days after vaccination [30]; hence, assaying at day 14 may have been too late to reveal vaccine-induced IgA levels. Second, our observation that IFN- $\gamma$  dominated over Th2 cytokines in the mucosal immune response may indicate a predominance of IgG production—IFN- $\gamma$  is known to have a potent effect on switching immunoglobulin isotype production to IgG. Third, we cannot exclude the possibility that the increase in salivary IgG is the result of the transudation of serum antibodies rather than increased local IgG production, although previous studies have demonstrated that PTs harbor influenza-specific AFCs and that these increase in number after influenza vaccination [8, 12, 13, 31]. Although tonsils harbor influenza-specific humoral immunity, there is considerable debate as to whether tonsillar AFCs directly contribute to the antibody content of saliva. We are currently engaged in in situ studies of PT antibody production that may bring clarity to this issue.

Vaccine strategies against mucosal pathogens aim to induce long-term memory at the appropriate immunological sites. The present results demonstrate, for the first time, the effects of routine influenza vaccination on human mucosal T cell-mediated immunity. It is evident that immunization against influenza influences both systemic and mucosal T cell responses and alters the natural immune state established by prior exposure to influenza through infection. Although the immunization delivery of influenza vaccine can effectively augment systemic T cell responses, it may not be optimal for eliciting mucosal responses. The distinct nature of the mucosal T cell response and how this is modified by repeated annual systemic vaccination needs to be considered for improving the design of current and future strategies to more effectively boost local T cell memory and enhance the antibody response to the virus.

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