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Ki-67 expression reveals strong, transient influenza specific CD4 T cell responses after adult vaccination

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Abstract

Although previous studies have found minimal changes in CD4 T cell responses after vaccination of adults with trivalent inactivated influenza vaccine, daily sampling and monitoring of the proliferation marker Ki-67 have now been used to reveal that a substantial fraction of influenza-specific CD4 T cells respond to vaccination. At 4–6 days after vaccination, there is a sharp rise in the numbers of Ki-67-expressing PBMC that produce IFN γ , IL-2 and/or TNF α in vitro in response to influenza vaccine or peptide. Ki-67+ cell numbers then decline rapidly, and ten days after vaccination, both Ki-67+ and overall influenza-specific cell numbers are similar to pre-vaccination levels. These results provide a tool for assessing the quality and quantity of CD4 T cell responses to different influenza vaccines, and raise the possibility that the anti-influenza T cell memory response may be substantially reshaped by vaccination, even if the overall memory cell numbers do not change significantly.

Keywords

Ki-67; influenza vaccine; CD4 T cell

Introduction

The human respiratory pathogen influenza virus is a significant threat for all age groups (1). The risk of influenza infection and the associated morbidity and mortality can be reduced by vaccination with partially purified subunit vaccines (TIV) or live attenuated viruses (LAIV) containing the predicted seasonal strains of A/H3N2, A/H1N1 and B viruses.

When matched, antibodies are unquestionably effective against influenza infection (2). However, CD4 and CD8 T cells assist resolution of infection in mice, and may play a similar role in humans (3–5). CD4 T cells provide help for B cell antibody responses and may also contribute to local inflammatory responses.

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In adults, TIV or LAIV vaccination does not significantly alter CD4 or CD8 T cell cytokine responses (6, 7). Almost all adults have detectable memory T cell responses to influenza due to repeated exposure to infection and/or vaccination. Thus preexisting memory to cross-reactive epitopes may mask elevated responses from immunization (8). Nevertheless, vaccination may reshape the repertoire or effector functions (9, 10) of the influenza response.

We have now measured the kinetics of expression of three cytokines (IL-2, IFN γ , TNF α) by T cells responding to TIV vaccination. Simultaneous measurement of Ki-67, a nuclear antigen expressed during and recently after proliferation (11), revealed an unexpectedly vigorous but transient T cell response to TIV, suggesting that vaccination may substantially reshape the CD4 T cell response, and providing a method for evaluating the quality of T cell vaccine responses.

Results

The lack of significant adult memory T cell responses to TIV (6–8) could be due to balanced expansion and contraction phases. To explore this possibility, we tested in vitro cytokine responses in daily samples to detect potential transient responses, and measured Ki-67 expression to determine whether a subset of influenza-specific T cells proliferated after vaccination.

CD4⁺CD8[−]CD45RA^{low} memory/effector cells expressing CD69, cytokines and Ki-67 were identified by ICS (Figure 1a). In two representative subjects (Figure 1b) at day 0, influenza antigens stimulated significant numbers of memory CD4 T cells to express cytokines in vitro. After vaccination, the responses initially declined on days 1–3 in some subjects (e.g. #6), but not others (e.g. #16). Circulating influenza-responsive CD4 T cells then increased on days 4–6, and returned to base levels around days 9–10. This pattern is consistent with initial sequestration of influenza-responsive cells in draining lymph nodes, followed by return of proliferated cells to the circulation.

Ki-67 is expressed selectively by proliferating T cells, but not induced in vitro within 10-hour stimulations (Figure 1b and (12)). Thus Ki-67 expression should identify cells that recently proliferated in vivo. In agreement with this, Ki-67 expression was low among influenza-responsive T cells before vaccination, increased markedly at days 4–6 and returned to lower levels by days 9–10 (Fig. 1b).

In the overall cytokine response of multiple subjects to influenza vaccine or peptide antigens, cytokine expression increased modestly during the expansion phase (days 4–6), and returned to pre-immunization values after ten days, although these changes did not reach statistical significance (Figure 2). TIV induced higher responses than H1N1 peptides, possibly due to the presence of both influenza A and B strains in TIV.

More striking changes occurred in influenza-specific Ki-67⁺ cytokine-expressing cells, which increased in all subjects for which day 0 and day 4–6 data were available (Figure 2). The number of Ki-67⁺ cells producing IFN γ , IL-2 or TNF α did not significantly increase after vaccination.

At the peak of the response (day 4–6) Ki-67⁺ cells comprised an average of 19% (95% confidence interval 13–25%) of circulating influenza responsive CD4 T cells expressing IFN γ , IL-2 or TNF α , while the influenza-specific Ki-67⁺ cells represented 6.1 $\pm$ 3.8% of the total Ki-67⁺ cells. As expected, vaccination did not change the low levels of Ki-67 expression in SEB-responsive CD4 T cells (Figure 3) or the total CD45RA^{low} CD4 T cell

population (not shown). Thus influenza TIV vaccination selectively induced Ki-67 expression in the influenza-specific but not bulk CD4 memory T cell populations.

Discussion

Although previous reports (6, 7) and our current data suggest that adult TIV vaccination does not significantly alter the overall number of influenza-reactive CD4 memory T cells, we have now shown a striking increase in Ki-67+ cells during the response, in some subjects reaching more than 30% of the cytokine-expressing cells at 4–6 days after vaccination. These apparently paradoxical results can be reconciled if it is assumed that the vaccine actually induces a vigorous memory cell proliferative response, but that this response is balanced by a rapid contraction phase, resulting in little change in overall numbers. This is supported by selective Ki-67 expression in the influenza-responsive but not total or SEB-stimulated populations.

Ki-67 expression before vaccination is low, consistent with the low rate of homeostatic renewal of influenza-specific resting memory CD4 T cells (13), and does not change after short term ex vivo stimulation ((12) and Fig 1). Thus Ki-67 expression is a measure of in vivo T cell proliferation, even when combined with short-term in vitro stimulation. The percentage of proliferating T cells is probably underestimated by the transience of Ki-67 expression (14), thus some circulating CD4+Ki-67- T cells may have previously expressed Ki-67. Ki-67 expression is also increased in antigen-responsive cells after tetanus toxoid vaccination (15). The Ki-67 response did not show a strong correlation with either pre-existing or boosted antibody titers (results not shown).

The unexpectedly large proliferative and contraction responses to TIV implied by our results could correlate with substantial reshaping of immune memory responses by influenza vaccination, because the final population could be derived from: minority populations in the original memory; further differentiation of original populations; or naïve responses to new epitopes. CD4 T cell specificities and effector functions could change substantially. Although influenza memory comprises predominantly Th1 cells, responses to new epitopes (J.M. Weaver, F.E. Lee and T.R. Mosmann, unpublished) may be biased towards the Thpp (uncommitted IL-2+ TNF α + IFN γ -CD4 T cells) responses induced by protein vaccines (16). As the selective expression of multiple Th1 cytokines can correlate with different protective outcomes (17, 18) such effector function changes could significantly influence the effectiveness of the vaccine response.

Materials and Methods

Vaccination

Fourteen healthy subjects, who had not received influenza vaccination for the current year were immunized during winter/spring 2010–2011 with seasonal TIV vaccine (FluLaval, GlaxoSmithKline). Blood was sampled prior to immunization and daily thereafter for 10 days. All protocols and procedures were approved by the Research Subjects Review Board at the University of Rochester Medical Center.

Antibodies

Antibodies used were: CD3-QDot605 (UCHT1), CD4-PE-TexRed (S3.5), CD8-QDot705 (3B5), CD14-QDot800 (TUK4), CD45RA-QDot655 (MEM-56) (Invitrogen); CD69-APC-Cy7 (FN50), IL-2-Pacific blue (MQ1-17H12), TNF α -PerCP-Cy5.5 (MAH1) (Biolegend); IFN γ -PE-Cy7 (B27), Ki-67-Alex700 (B56) (BD Pharmingen).

In vitro stimulation

PBMC were isolated from heparinized blood by Ficoll (Lonza) gradient centrifugation and incubated with or without antigens for 2 hours in RPMI (Mediatech, Manassas, VA) containing 10% FBS (Sigma-Aldrich, St. Louis, MO), then 2 μ M Monensin and Golgiplug (BD Pharmingen) were added for an additional 8 hours. H1N1 peptides (15mers incremented by 5), from all proteins of influenza virus A/California/04/2009 (H1N1), were obtained from Mimotopes (Clayton Victoria, Australia), pooled, and used at 20ng/ml/peptide. TIV influenza vaccine (Fluzone, A/California/7/2009 (H1N1), A/Victoria/210/2009 (H3N2) and B/Brisbane/60/2008) from Sanofi (Swiftwater, PA) was used at 1.25 μ g/ml HA. Negative controls contained 0.1% DMSO. Staphylococcal exotoxin B (SEB) (Sigma-Aldrich) was used at 1 μ g/ml.

Intracellular cytokine staining (ICS)

Cells were washed with ice cold HBSS containing 1%FBS and stained on ice in the same buffer with fluorochrome-conjugated antibodies specific for surface antigens. Stained cells were then fixed, permeabilized, stained with fluorochrome-conjugated cytokine, CD3, CD69 and Ki-67 antibodies using the ICS kit (BD Pharmingen), fixed with 1% formaldehyde and analyzed on an LSR-II Flow Cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR). Background values were subtracted after normalization to total CD4 T cell numbers in each sample.

Statistical analysis

To deal with repeated measures with missing observations, the linear mixed-effects (LME) model (19, 20) together with the AR(1) model were used. Data were normalized and log-transformed before background subtraction, and then used in the LME fitting. Based on the LME fitting results, the Tukey's all-pair comparison was used to test the difference between the baseline (day 0) and the peak window (pooled data from days 4–6) only, or to test the differences among the baseline, the peak window and the late stage (pooled data from days 9–10) simultaneously. To test the differences in the proportions of Ki-67 expression under different stimulations, the Wilcoxon signed-rank test was applied to data without background subtraction at each time point.

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Figure 1. Identification of influenza-specific CD4 T cells by flow cytometry PBMC from TIV immunized subjects were analyzed by in vitro antigen stimulation and ICS. A) Gating strategy to identify cytokine and Ki-67 expressing cells. B) Kinetics of cytokine expression in two subjects.

Figure 2. Transient increase in Ki-67+ cytokine-expressing T cells after TIV immunization
Cytokine expression was analyzed as in Figure 1, and the numbers of cytokine+ Ki-67+ T cells responding to H1N1 peptides or TIV vaccine were averaged for each subject for days 4–6 and 9–10. Solid line: significance by multiple comparison; dotted line, significance by direct comparison of 0 and 4–6 day values (* P<0.05 ** P<0.01).

Figure 3. Increased proportion of Ki-67 expression in influenza- but not SEB-responsive cells after vaccination

The percentage of Ki-67+ cells in each cytokine-expressing population was calculated for each subject in response to TIV vaccine or SEB (* $P < 0.05$, ** $P < 0.01$).