MAJOR ARTICLE

$CD4^+$ T-Cell Expansion Predicts Neutralizing Antibody Responses to Monovalent, Inactivated 2009 Pandemic Influenza A(H1N1) Virus Subtype H1N1 Vaccine

Jennifer L. Nayak,[1] Theresa F. Fitzgerald,[2] Katherine A. Richards,[3] Hongmei Yang,[4] John J. Treanor,[2] and Andrea J. Sant[3]

[1]Department of Pediatrics, [2]Department of Medicine, Division of Infectious Diseases, [3]David H. Smith Center for Vaccine Biology and Immunology, Department of Microbiology and Immunology, and [4]Department of Biostatistics and Computational Biology, University of Rochester Medical Center, New York

Background. The ability of influenza vaccines to elicit $CD4^+$ T cells and the relationship between induction of $CD4^+$ T cells and vaccine-induced neutralizing antibody responses has been controversial. The emergence of swine-origin 2009 pandemic influenza A virus subtype H1N1 (A[H1N1]pdm09) provided a unique opportunity to examine responses to an influenza vaccine composed of both novel and previously encountered antigens and to probe the relationship between B-cell and T-cell responses to vaccination.

Methods. We tracked $CD4^+$ T-cell and antibody responses of human subjects vaccinated with monovalent subunit A(H1N1)pdm09 vaccine. The specificity and magnitude of the $CD4^+$ T-cell response was evaluated using cytokine enzyme-linked immunosorbent spot assays in conjugation with peptide pools representing distinct influenza virus proteins.

Results. Our studies revealed that vaccination induced readily detectable $CD4^+$ T cells specific for conserved portions of hemagglutinin (HA) and the internal viral proteins. Interestingly, expansion of HA-specific $CD4^+$ T cells was most tightly correlated with the antibody response.

Conclusions. These results indicate that $CD4^+$ T-cell expansion may be a limiting factor in development of neutralizing antibody responses to pandemic influenza vaccines and suggest that approaches to facilitate $CD4^+$ T-cell recruitment may increase the neutralizing antibody produced in response to vaccines against novel influenza strains.

Keywords. influenza vaccines; pandemic H1N1 influenza; influenza virus; $CD4^+$ T cells; cellular immune response; immunodominance; epitopes.

Influenza A viruses can evade protective immune responses through both gradual antigenic drift of viral surface proteins and sporadic reassortment that can result in antigenic shift. Such reassortment occurred in 2009, when the novel, swine-origin pandemic influenza A virus subtype H1N1 (A[H1N1]pdm09) emerged and spread globally, resulting in the first influenza pandemic of the 21st century [1–4]. Characterization of immunity to this virus revealed little antigenic seroreactivity with contemporary seasonal influenza A virus subtype H1N1 (A[H1N1]) [5], relatively few conserved B-cell epitopes within the hemagglutinin (HA) protein [6], and little or no preexisting neutralizing antibody in unexposed children or adults aged <60 years [7–9]. In contrast, preexisting memory $CD4^+$ T cells, including cells directed against epitopes within the HA protein, were detected in peripheral blood mononuclear cells (PBMCs) of subjects not previously exposed to A(H1N1)pdm09 [6, 10–13]. Thus,

Received 29 June 2012; accepted 23 August 2012; electronically published 12 November 2012.

Presented in part: 6th Annual NIAID Centers for Excellence in Influenza Research and Surveillance Meeting, New York, New York, July 2012.

Correspondence: Jennifer L. Nayak, MD, Department of Pediatrics, Division of Infectious Diseases, University of Rochester Medical Center, 601 Elmwood Ave, Box 690 Rochester, NY 14642 (jennifer_nayak@urmc.rochester.edu).

The Journal of Infectious Diseases 2013;207:297–305

DOI: 10.1093/infdis/jis684

this pandemic offered a unique opportunity to study $CD4^+$ T-cell responses with an influenza virus strain that would simultaneously induce naive and memory $CD4^+$ T cells without large amounts of coexisting B-cell–mediated immunity.

Individuals are repeatedly exposed to influenza virus antigens through both vaccination and infection, resulting in competition between memory and naive lymphocytes over time. How this competition will affect the specificity of $CD4^+$ T-cell responses is unknown. While the typical seasonal trivalent inactivated vaccine (TIV) is enriched for HA, it also contains the more conserved internal viral proteins, such as nucleoprotein (NP) and matrix protein (M1) [14, 15]. Thus, both repeated infections and vaccinations may lead to a dominance of T cells specific for peptide epitopes conserved among virus strains. While recent studies examining the T-cell repertoire in humans have shown broad reactivity to diverse viral proteins [10, 16, 17], few studies have examined the distribution of reactivity among conserved and novel epitopes.

Neutralizing antibody is considered the major correlate of protection following influenza vaccination [18, 19], while the protective role of $CD4^+$ T cells remains more poorly understood. Whether $CD4^+$ T-cell responses even develop after vaccination with TIV remains controversial [12, 20–28]. Lack of consensus may be due to inadequate subject numbers, variable levels of baseline anti-influenza immunity in study populations, or the use of recall antigens that do not elicit the full repertoire of influenza-reactive cells. Knowledge of the relationship between $CD4^+$ T cells and the development of a neutralizing antibody response following administration of TIV is even more limited, with the few studies addressing this question failing to find a correlation between these parameters [12, 20], except when an adjuvanted influenza A virus subtype H5N1 vaccine was used [29].

In this study, we used an experimental approach designed to maximally detect antigen-specific $CD4^+$ T cells by using cytokine enzyme-linked immunosorbent spot (ELISPOT) assays with pools of overlapping synthetic peptides as recall antigens to quantify responses to conserved and novel epitopes following vaccination of adults with monovalent inactivated A (H1N1)pdm09 vaccine. We also examined the relationship between $CD4^+$ T-cell responses and neutralizing antibody titers. These analyses revealed a readily detectable increase in numbers of $CD4^+$ T cells directed against conserved portions of HA and the NP and M1 proteins. Further, $CD4^+$ T-cell expansion was correlated with the development of a neutralizing antibody response against this pandemic virus.

MATERIALS AND METHODS

Study Population and Procedures

Forty-nine healthy subjects were enrolled in 2 age groups (18–32 years and ≥60 years) between March and October 2010. Subjects with a history of previous laboratory-documented infection or vaccination with A(H1N1)pdm09, vaccination with A/New Jersey/76, egg allergy, immunosuppression, or active neoplastic disease were excluded. Younger subjects were excluded if they had a baseline A/California/07/09 hemagglutinin inhibition (HAI) titer of >10, but this was not an exclusion criterion for older adults because of greater levels of expected preexisting immunity. Blood was obtained before and at days 7, 14, and 28 after administration of inactivated subunit A/California/07/09 monovalent vaccine (Novartis, East Hanover, NJ). PBMCs were purified using Accuspin tubes with Histopaque-1077 cell separation media (Sigma-Aldrich, St. Louis, MO) and were frozen at a controlled rate in fetal bovine serum (FBS) containing 10% DMSO.

Microneutralization Assay

All sera were tested using the microtiter technique for neutralization of an egg-grown virus derived from the 2009 monovalent live attenuated influenza vaccine (lot 500914P; MedImmune, Gaithersburg, MD). Sera were heat inactivated prior to testing, starting at a 1:10 dilution. Viral growth was determined by enzyme-linked immunosorbent assay following fixation with acetone, using NP-specific monoclonal antibodies (WHO reagent kit). The antibody titer was defined as the reciprocal of the highest dilution that resulted in 50% inhibition of signal as compared to control wells. An end point titer was determined on all sera with an initial neutralizing antibody titer of ≥1280. Sera without detectable neutralization activity were assigned a titer of 5, and values >40 000 were assigned a titer of 40 000 for calculation purposes.

HAI Assay

HAI testing was performed on all sera in microtiter format, using turkey red blood cells with 4 HA units of egg-grown virus derived from the 2009 monovalent A(H1N1)pdm09 live attenuated influenza vaccine (lot 500914P; MedImmune, Gaithersburg, MD) as antigen. Sera were treated with receptor-destroying enzyme (Denka Seiken, Tokyo, Japan) and heat inactivated prior to testing, starting at a 1:10 dilution. The antibody titer was defined as the reciprocal of the highest dilution that resulted in inhibition of hemagglutination.

Quantification of $CD4^+$ T-Cell Responses

Cryopreserved PBMCs were thawed and then rested overnight at 37°C and 5% CO_2 in Roswell Park Memorial Institute 1640 medium containing 10% FBS and gentamicin (Life Technologies, Carlsbad, CA), with a typical yield of >85% viable cells after thawing. After rest, PBMCs were depleted of $CD8^+$ and $CD56^+$ cells, using MACS positive selection with LD separation columns as per manufacturer's instructions (Miltenyi Biotec, Auburn, CA). ELISPOT assays were performed as previously described [10], with 400 000 or 200 000 CD8- and

CD56-depleted PBMCs cocultured with either peptide pool or tetanus toxoid (Calbiochem, Billerica, MA). Plates were analyzed using an Immunospot reader series 2A with Immunospot software, version 3.2 (Cellular Technology, Shaker Heights, OH). Results were normalized to peptide-specific spots per 10^6 cells after averaging values for duplicate wells and subtracting background.

Synthetic Peptides

Peptides unique to A/California/07/09 were synthesized in our facility by using an Apex 396 system (AAPPTec, Louisville, KY) as described previously [30]. Other peptide sets were obtained through the National Institutes of Health Biodefense and Emerging Infections Research Resources Repository (Bethesda, MD) and included the A/California/04/09 HA peptides (NR-15433), the A/New York/384/2005(H3N2) HA (NR-2603) and neuraminidase (NA; NR-2608) peptides, and the A/New York/348/2003(H1N1) NP (NR-2611) and M1 (NR-2613) peptides.

Peptide Pools

A/California/07/09 HA and NA peptides containing <6 contiguous conserved amino acids as compared to recently circulating A(H1N1) strains were pooled into a HA/NA "unique" pool (38 peptides; Supplementary Table 1). A "HA-conserved" pool containing ≤1 divergent amino acid as compared to recently circulating A(H1N1) strains was also used (35 peptides; Supplementary Table 2). Because the A(H1N1)pdm09 vaccine was on an A/Puerto Rico/8/34 backbone that was largely conserved as compared to A/New York/348/2003, all NP and M1 peptides were combined into a "NP/M1-conserved" pool (123 peptides). As a control, divergent influenza A virus subtype H3N2 (A[H3N2]) peptides with <6 contiguous conserved amino acids as compared to A/California/07/09 (132 peptides) were pooled. The "total" influenza-specific response was calculated as the sum of the HA/NA unique, HA-conserved, and NP/M1-specific $CD4^+$ T-cell responses.

Statistical Analysis

T-cell responses against A(H1N1)pdm09 vaccine were compared between prevaccination and postvaccination time points, using the Wilcoxon signed rank test. The Mann–Whitney *U* test was used to compare unpaired groups. Correlations between groups were examined using Spearman rank correlation. To further investigate whether $CD4^+$ T-cell help was specificity dependent, 2 regression models were used to examine the joint effects of the NP/M1- and HA-conserved pools on the microneutralization titer; details of this analysis are in the Supplementary Materials. Statistical analyses were performed using SAS, version 9.2, or GraphPad Prism 5. All *P* values < .05 were considered statistically significant.

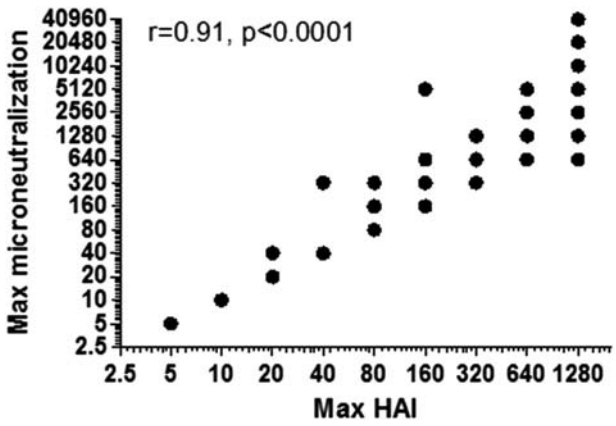

Figure 1. Comparison of the maximum hemagglutinin inhibition (HAI) and microneutralization (MN) titers in study subjects. HAI and MN titers were determined in all subjects. An end point MN titer was determined for all subjects with a neutralizing antibody titer of ≥1280. *r* and *P* values were determined using the Spearman rank correlation test.

Ethics Statement

The University of Rochester Research Subjects Review Board approved this study protocol, and human experimentation guidelines of the US Department of Health and Human Services and the University of Rochester were followed. Study procedures were in accordance with the ethical standards of the Declaration of Helsinki. All subjects provided written informed consent prior to study participation.

RESULTS

Study Subjects

Forty-nine healthy adults received the inactivated A/California/07/09 monovalent vaccine between March and October 2010. Seventeen subjects (35%) were aged 18–32 years (median age, 25 years), and 32 subjects (65%) were aged ≥60 years (median age, 68 years [range, 60–82 years]). Twenty-five subjects (51%) were female. Younger subjects were excluded if they had a prevaccination HAI titer of >10. Twelve older subjects had a baseline HAI titer of >10; 9 older subjects had a baseline HAI titer of ≥40.

Humoral Immune Responses

HAI and microneutralization titers were determined at all visits. Good correlation was seen between the maximum antibody titer as determined by HAI and microneutralization assay (Figure 1; $r = 0.91$, $P < .0001$). However, as neutralizing antibodies were fully titrated only with the microneutralization assay, these data are reported here. The maximum microneutralization titer ranged between <10 and >40 000, with a geometric mean of 482. Seroresponse (defined as a ≥4-fold increase in microneutralization titer) occurred in 41 subjects (84%).

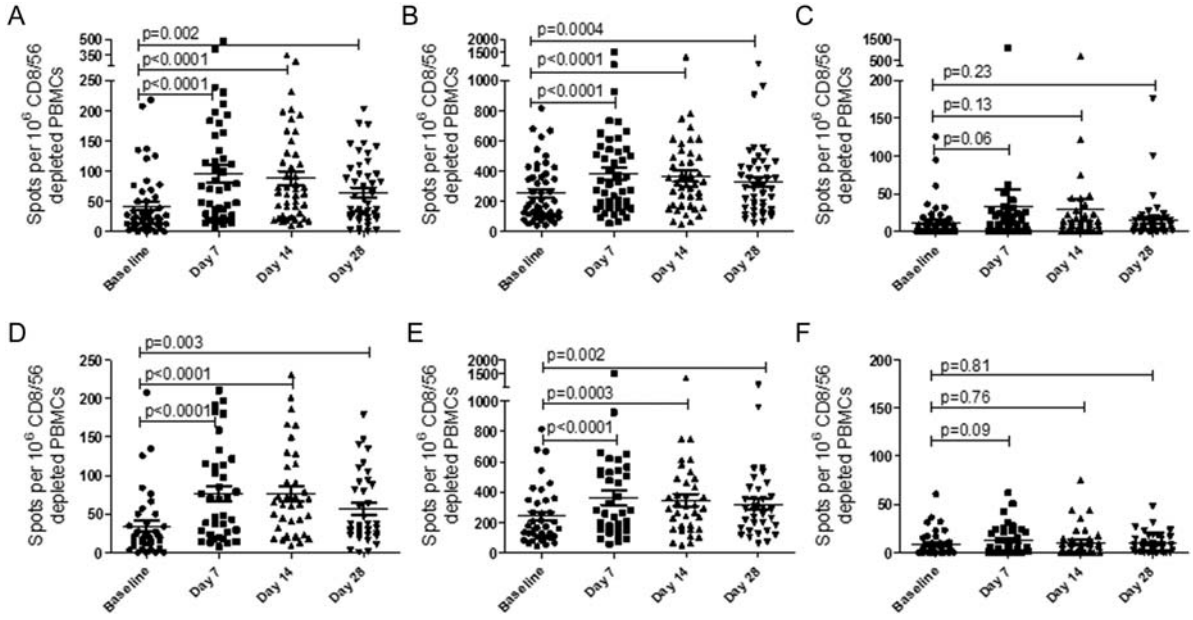

Figure 2. Expansion of $CD4^+$ T cells directed against conserved peptides is seen following monovalent 2009 pandemic influenza A virus subtype H1N1 (A[H1N1]pdm09) vaccination. Subjects received monovalent inactivated A/California/07/09 vaccine, and $CD4^+$ T-cell responses against conserved and nonconserved peptide pools were examined at baseline and at approximately days 7, 14, and 28 following vaccination, using interferon γ (IFN-γ) enzyme-linked immunosorbent spot assays. *A–C*, $CD4^+$ T-cell response of all subjects following vaccination. *D–F*, $CD4^+$ T-cell response of subjects with a baseline microneutralization titer of <40. *A* and *D*, Response to A/California/04/09 HA peptides expected to be conserved with recently circulating seasonal influenza A viruses. *B* and *E*, Response to a conserved nucleoprotein (NP)/matrix protein (M1) peptide pool. *C* and *F*, $CD4^+$ T-cell response to A (H1N1)pdm09 hemagglutinin (HA) and neuraminidase (NA) peptides expected to be nonconserved compared with seasonal influenza viruses. The horizontal line represents the mean of the $CD4^+$ T-cell response, with the error bars depicting the standard error of the mean. *P* values were determined by the Wilcoxon signed rank test. Abbreviation: PBMC, peripheral blood mononuclear cell.

$CD4^+$ T-Cell Responses

We initially addressed whether we could detect $CD4^+$ T-cell reactivity following A(H1N1)pdm09 vaccination. $CD4^+$ T-cell responses at all time points were quantified following restimulation with HA-conserved and NP/M1 peptide pools. A pool containing A(H3N2) peptides not present in the vaccine and tetanus toxoid were included as controls. Figure 2 shows the number of interferon γ (IFN-γ)–producing $CD4^+$ T cells per 10^6 CD8- and CD56-depleted PBMCs in both the cohort including all subjects (A–C) and only those subjects with preexisting microneutralization titers of <40 (D–F). Reactivity to the HA-conserved pool (Figure 2*A* and 2*D*) and NP/M1 pool (Figure 2*B* and 2*E*) was clearly detected following vaccination. When postvaccination responses were compared to baseline using the Wilcoxon signed rank test, these responses were statistically significant at all time points. The readily detectable responses to NP and M1 are likely the result of contaminating internal viral proteins within the vaccine, as has been demonstrated in previous studies [14, 15] and as we have confirmed in this vaccine, using a Western blot for NP (data not shown). No significant increases in responses to tetanus toxoid or the A(H3N2) divergent pool were observed (Supplementary Figure 1). These results provide strong evidence that subunit vaccines elicit $CD4^+$ T cells specific for influenza virus epitopes. In this study cohort, there was no effect of age or body mass index on either the maximum microneutralization titer achieved (Supplementary Figure 2*A* and 2*B*) or the overall expansion of $CD4^+$ T cells (Supplementary Figure 2*C* and 2*D*), although the preexisting immunity of older adults may have biased the results of the age analysis.

The second issue addressed was whether responses to previously unencountered peptide epitopes would develop in the face of existing memory $CD4^+$ T cells specific for conserved epitopes. To evaluate this, $CD4^+$ T cells specific for HA and NA peptides that were not conserved as compared to seasonal vaccines ("unique") were quantified before and after vaccination. We found a trend toward greater numbers of $CD4^+$ T cells specific for this unique pool, but the increase did not reach statistical significance (Figure 2*C*). When subjects with evidence of preexisting immunity (microneutralization titer, ≥40) were excluded, this trend became much less apparent (Figure 2*F*).

Because of the potential usefulness of preexisting $CD4^+$ T cells as a predictive biomarker [31–33], we next examined

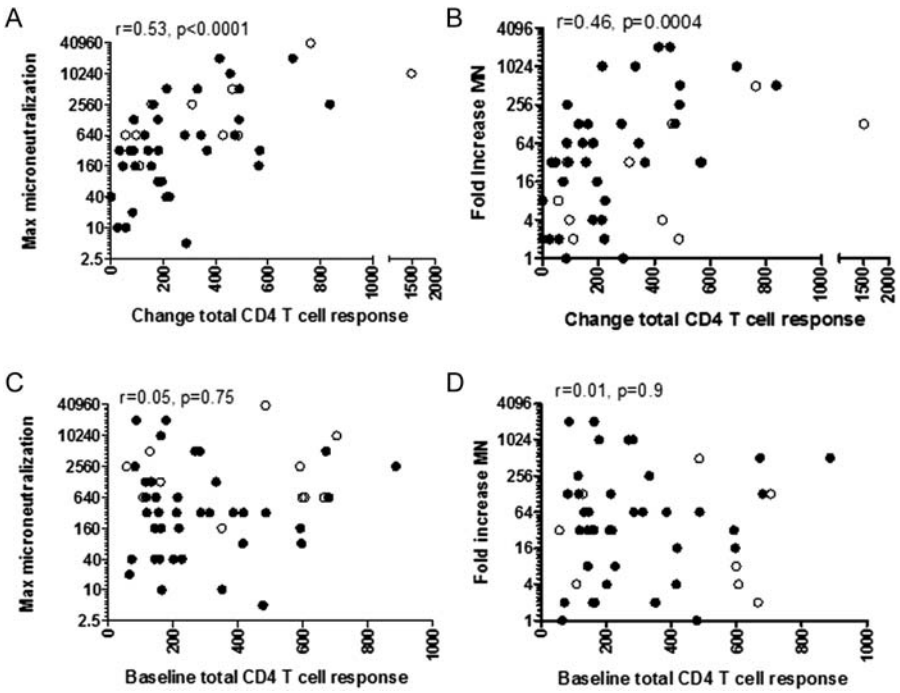

Figure 3. There is a relationship between the development of a neutralizing antibody response and expansion of but not the baseline number of $CD4^+$ T cells. The prevaccination number of $CD4^+$ T cells and the maximum expansion of $CD4^+$ T cells [maximum response – baseline response] were determined by interferon γ (IFN-γ) enzyme-linked immunosorbent spot assays, and the titer of neutralizing antibodies was determined using microneutralization (MN) assays. There is a statistically significant correlation between the maximum (*A*) or fold-increase (*B*) in MN titer and $CD4^+$ T-cell expansion. However, there is no correlation between the baseline number of $CD4^+$ T cells and either the maximum (*C*) or the fold-increase (*D*) in MN titer. Subjects with a prevaccination MN titer of ≥40 are indicated with an open circle. *r* and *P* values were determined by the Spearman rank correlation test.

whether prevaccination influenza virus–specific $CD4^+$ T cells correlated with postvaccination $CD4^+$ T-cell responses and, thus, potentially with the help available for antibody responses. We were not able to find a predictive relationship between total prevaccination influenza virus–reactive $CD4^+$ T cells and postvaccination responses when calculated as a change from baseline ([peak value – baseline value]; $r = 0.11$, $P = .46$). However, when the response magnitude was quantified as a fold-change [peak response/baseline value], an inverse correlation between these parameters was observed ($r = -0.5$, $P = .0002$), as has been previously reported [20, 25, 34]. If not all influenza virus–reactive $CD4^+$ T cells are recruited into the vaccine response, the inclusion of unstimulated cells in the denominator could artificially lessen the response estimate for subjects starting with higher baseline levels of immunity when calculating "fold-change." To avoid this potential pitfall, we chose to present the $CD4^+$ T-cell response as a "change from baseline."

Relationship Between the $CD4^+$ T-Cell and Neutralizing Antibody Responses

$CD4^+$ T-cell help is critical for production of high-affinity antibody responses, but it is not clear whether the $CD4^+$ T-cell response magnitude in any way correlates with or limits the magnitude of the antibody response. To evaluate this, the maximal change in the "total" $CD4^+$ response (sum of the HA/NA unique, HA-conserved, and NP/M1-specific $CD4^+$ T-cell responses) was determined for each subject and plotted against the maximum or fold-increase in neutralizing antibody titer. As demonstrated in Figure 3*A* and 3*B*, there was a highly significant correlation between $CD4^+$ T-cell expansion and both the maximum ($r = 0.53$, $P < .0001$) and fold-increase in ($r = 0.46$, $P = .0004$) microneutralization titer. The above correlations were also statistically significant when the maximal fold-increase in $CD4^+$ T cells was quantified and compared to the neutralizing antibody titer, when the antibody titer was quantified using HAI, and when all subjects with an HAI of ≥10 at baseline were excluded from the analysis (data not shown). In contrast, there was no detectable correlation between gains in neutralizing antibody titers and the number of influenza virus–reactive $CD4^+$ T cells present before vaccination (Figure 3*C* and 3*D*). We conclude from this that expansion of $CD4^+$ T cells rather than the baseline number of influenza virus–reactive cells correlates with and predicts the development of a neutralizing antibody response following A

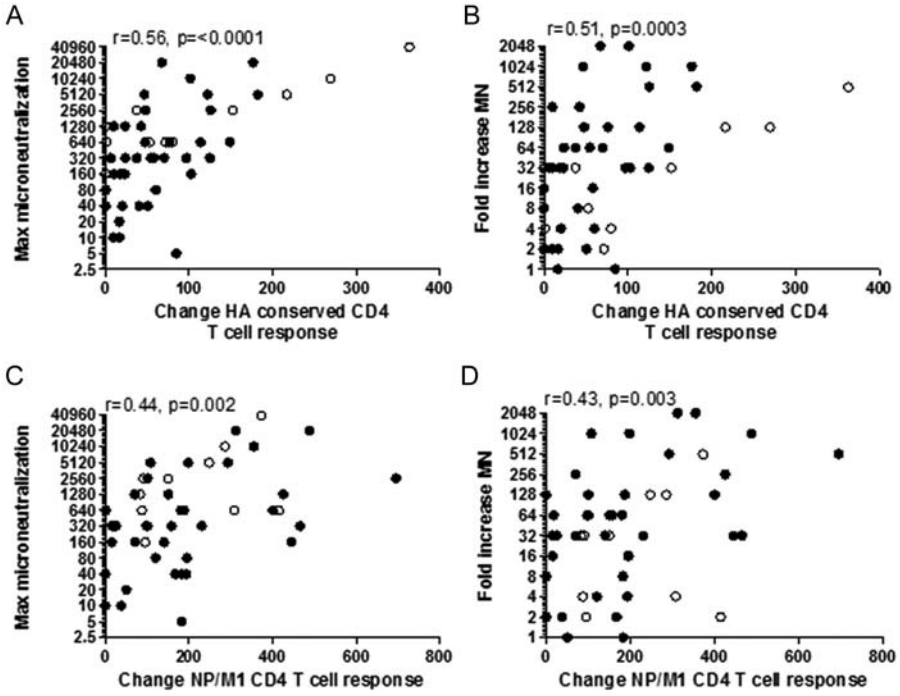

Figure 4. Both changes in the CD4$^+$ T-cell response directed against hemagglutinin (HA)–conserved and nucleoprotein (NP)/matrix protein (M1) peptides show a statistically significant correlation with the microneutralization (MN) titer, with the strongest correlation seen when HA reactivity is examined. *A* and *B*, Correlation between changes in the response directed against HA-conserved peptides and the maximum (*A*) or fold-increase (*B*) in MN titer. *C* and *D*, Correlation between expansion of NP/M1 specific CD4$^+$ T cells and the maximum (*C*) or fold-increase (*D*) in MN titer. Subjects with a prevaccination titer of ≥40 are indicated with an open circle. *r* and *P* values were determined by the Spearman rank correlation test.

(H1N1)pdm09 vaccination, a result that is consistent with the idea that CD4$^+$ T-cell help may limit the antibody response to pandemic influenza vaccines.

Effects of CD4$^+$ T-Cell Specificity

Under physiologic conditions, antigen-specific B cells primarily internalize antigen via the immunoglobulin receptor and recruit CD4$^+$ T cells via the major histocompatibility complex class II restricted display of peptides from this immunoglobulin-mediated event [35, 36]. If influenza virus proteins within the vaccine are not aggregated, other viral proteins will not be internalized by HA-specific B cells. This will lead to the selective presentation of HA-derived epitopes by HA-specific B cells. In this scenario, B cells producing neutralizing antibody will only recruit help from HA-specific CD4$^+$ T cells [37]. Clearly, the sampling limitations in humans preclude direct examination of B-cell antigen presentation within the draining lymph node following intramuscular vaccination. Thus, to evaluate the role of CD4$^+$ T-cell specificity in neutralizing antibody production, we examined correlations between the neutralizing antibody response and expansion of IFN-γ–producing CD4$^+$ T cells specific for peptides within either the HA-conserved or NP/M1 pools. Figure 4 demonstrates that there was a significant correlation between neutralizing antibody response and expansion of both HA-specific (Figure 4A and 4B) and NP/M1-specific (Figure 4C and 4D) CD4$^+$ T cells, with the strongest correlation seen when HA reactivity was examined. Figure 5 represents the subjects grouped by degree of CD4$^+$ T-cell expansion. Vaccine recipients with the largest CD4$^+$ T-cell response increases consistently had the greatest gains in neutralizing antibody production. Interestingly, subjects with a >100 spot increase in CD4$^+$ T cells specific for peptides within the conserved HA pool had a higher geometric mean titer of and fold-increase in neutralizing antibody production (Figure 5*A* and 5*C*) when compared to the similar NP/M1 group (Figure 5*B* and 5*D*), suggesting that HA-specific B cells may display a limited repertoire of peptides derived primarily from HA and thus may preferentially recruit cognate help from HA-specific CD4$^+$ T cells. However, even in this scenario, CD4$^+$ T cells specific for proteins such as NP and M1 that are stimulated in the lymph node may be able to promote antibody responses through noncognate interactions via the provision of cytokines [38].

To further distinguish the contribution of HA-specific and NP/M1-specific CD4$^+$ T cells to the neutralizing antibody response, multiple regression models were used. Modeling indicated that the majority of the effect of NP/M1-specifc CD4$^+$ T-cell expansion on the neutralizing antibody response could

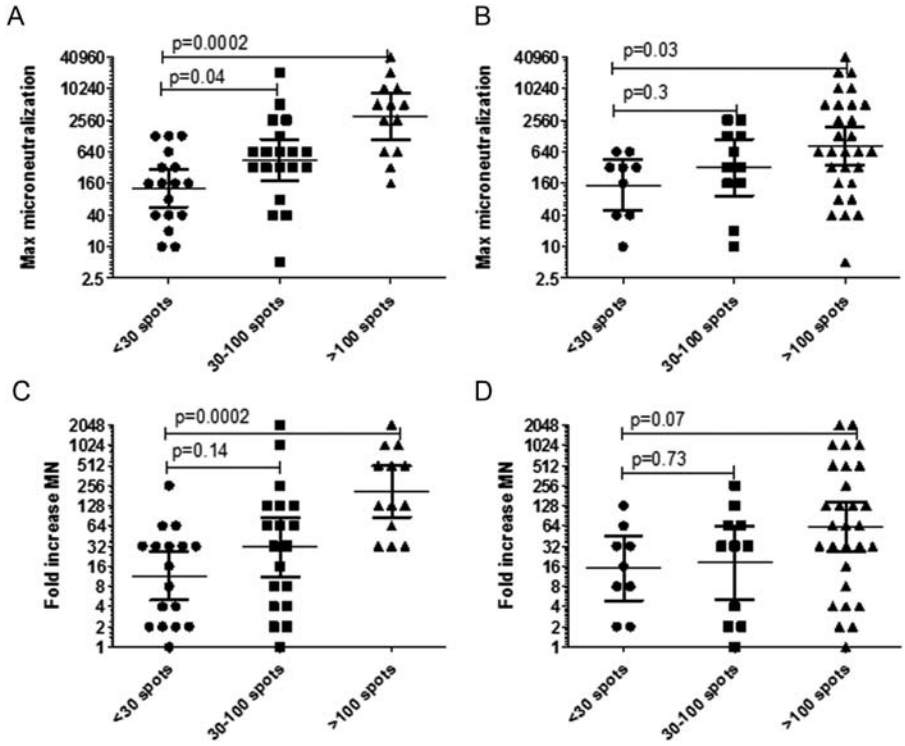

Figure 5. Degree of $CD4^+$ T-cell expansion is predictive of a robust neutralizing antibody response. When the $CD4^+$ T-cell response is subgrouped on the basis of the degree of expansion (negative, <30 spots per 10^6 $CD4^+$ T-cell increase; small, 30–100-spot increase; and large, >100-spot increase), subjects with the largest increase in $CD4^+$ T-cell count have greatest geometric mean microneutralization (MN) titer, with the highest geometric mean titer or fold-increase seen in subjects with a >100-spot increase in $CD4^+$ T cells specific for epitopes within the conserved hemagglutinin (HA) pool. *A* and *B*, Maximum MN titer achieved based on the degree of expansion of HA-conserved (*A*) or nucleoprotein (NP)/matrix protein (M1)–specific (*B*) $CD4^+$ T cells. *C* and *D*, Fold-increase in MN titer based on the degree of HA-conserved (*C*) or NP/M1 (*D*) $CD4^+$ T-cell expansion. The line represents the geometric mean, and the error bars depict the 95% confidence interval. Statistical testing was performed using the Mann–Whitney *U* test.

be accounted for by simultaneous increases in the HA-conserved response. Furthermore, increases in $CD4^+$ T cells specific for peptides within the HA-conserved pool predicted the antibody titer better than NP/M1 responses. However, this difference in prediction power did not reach statistical significance.

DISCUSSION

The experiments presented here demonstrate that the monovalent inactivated A(H1N1)pdm09 influenza vaccine elicits readily detectable $CD4^+$ T-cell responses and that the magnitude of these responses correlates with gains in neutralizing antibody. This positive relationship between $CD4^+$ T cells and antibody responses was most apparent when looking at $CD4^+$ T-cell responses directed against conserved peptide epitopes within the HA protein of the A/California/07/09 virus. On the basis of these results, we postulate that activation of $CD4^+$ T cells following vaccination may be one of the limiting factors for neutralizing antibody production following pandemic influenza vaccination.

As there is great interest in defining biomarkers that will predict success in vaccination, our studies evaluated whether prevaccination levels of influenza virus–specific $CD4^+$ T-cell reactivity predicted the magnitude of the neutralizing antibody response. We did not observe any correlation between these parameters but instead found a correlation between $CD4^+$ T-cell expansion and the titer of neutralizing antibody produced. This suggests that, following vaccination, only a subset of influenza virus–reactive cells are able to be recruited into the draining lymph nodes and expand, leading the cells that ultimately reenter the circulating pool to be most indicative of the help available for the antibody response. The effect this has on $CD4^+$ T-cell memory will be important to address in future studies. Our results contrast with what was recently reported in the study by Wilkinson et al [39], in which greater baseline circulating numbers of influenza virus–specific $CD4^+$ T cells were protective against development of severe disease in an influenza challenge model. One potential reason for this difference is that the study by Wilkinson et al predominantly examined the role of effector $CD4^+$ T cells that exerted their function prior to either viral clearance or the production of

neutralizing antibody, possibly via cytolytic activity. This contrasts with our study, which considered the neutralizing antibody produced in response to vaccine challenge. Additionally, more $CD4^+$ T cells may be able to be recruited into the immune response following infection, because of a greater abundance and diversity of epitopes displayed by antigen-presenting cells. Our ability to use peripheral blood $CD4^+$ T-cell reactivity to predict future vaccine-induced B-cell responses is likely to require a more refined definition of epitopes recruited by vaccination and a better understanding of the subsets of $CD4^+$ T cells that can participate in extrafollicular and germinal center responses to vaccine components. Further, it is possible that the observed relationship between $CD4^+$ T-cell and neutralizing antibody responses may be the result of an overall more robust vaccine response in some individuals. Future efforts to selectively boost $CD4^+$ T-cell responses will help to confirm the causal relationship between these parameters.

An important issue we sought to evaluate in these studies was whether the influence of $CD4^+$ T cells on neutralizing antibody responses to vaccination was related to antigen specificity. It is interesting that the strongest correlation observed was between expansion of HA-specific $CD4^+$ T cells and the neutralizing antibody response, although the distinction between NP/M1- and HA-specific expansion and the antibody response that we detected was modest. Both concurrent expansion of HA-reactive $CD4^+$ T cells and cells specific for the NP/M1 pool and the inclusion of M1 peptides within the NP pool, as M1 associates with the viral surface glycoproteins [40] and may be taken up with HA by B cells, may have lessened our ability to detect a potential linkage between B-cell and T-cell specificities. If HA-specific B cells do have preferential access to limited viral proteins, vaccine development efforts that focus the $CD4^+$ T-cell response on HA-derived epitopes may improve the neutralizing antibody response following vaccination.

One of the challenges to vaccination against pandemic influenza is that the viral protein composition of the next pandemic strain cannot be predicted [41, 42]. The failure to detect $CD4^+$ T-cell responses to novel epitopes in the current study suggests either that these epitopes are poorly immunogenic or that naive cells fail to successfully compete with the more abundant and rapidly recruited memory cells. Further studies involving individuals who are now primed with A(H1N1) pdm09 will help clarify the overall immunogenicity of these peptides and the effect of competing memory T cells on naive $CD4^+$ T-cell expansion and response kinetics. For antibody responses to A(H1N1)pdm09, there may have been enough conserved HA epitopes to promote antibody responses, but for more distant viruses conserved HA epitopes may be quite limited, possibly resulting in a correspondingly low neutralizing antibody response. This could explain the disparity between the robust responses to a single dose of A(H1N1) pdm09 vaccine [43, 44] as compared to A(H5N1) vaccine, to which responses are modest [45, 46]. If future studies substantiate the link between HA-specific $CD4^+$ T cells and anti-influenza virus neutralizing antibody production, efforts to enrich the $CD4^+$ memory population with T cells specific for potentially cross-reactive HA epitopes by prepriming with peptide-based vaccines or novel HA constructs [47] may increase the recruitment of HA-specific $CD4^+$ T cells on challenge with divergent HA proteins. Such a strategy could promote a more broadly cross-reactive and rapid response to novel strains of influenza virus, increasing pandemic preparedness by providing stand-alone protection while allowing dose-sparing efforts to facilitate rapid deployment of limited vaccine stocks to the population in the event of a pandemic.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Financial support. This work was supported by the National Institutes of Health (contract HHSN266200700008C and grant 1K12HD068373-01).

Potential conflict of interest. J. J. T. is on the scientific advisory board of Novartis, Immune Targeting Systems, and Visterra and has received grant support from Sanofi, Pfiser, GlaxoSmithKline, Vaxinnate, Protein Sciences, and Ligocyte. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Peiris JS, Poon LL, Guan Y. Emergence of a novel swine-origin influenza A virus (S-OIV) H1N1 virus in humans. J Clin Virol **2009**; 45:169–73.
2. Dawood FS, Jain S, Finelli L, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med **2009**; 360: 2605–15.
3. Brockwell-Staats C, Webster RG, Webby RJ. Diversity of influenza viruses in swine and the emergence of a novel human pandemic influenza A (H1N1). Influenza Other Respi Viruses **2009**; 3:207–13.
4. Neumann G, Kawaoka Y. The first influenza pandemic of the new millennium. Influenza Other Respi Viruses **2011**; 5:157–66.
5. Garten RJ, Davis CT, Russell CA, et al. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science **2009**; 325:197–201.
6. Greenbaum JA, Kotturi MF, Kim Y, et al. Pre-existing immunity against swine-origin H1N1 influenza viruses in the general human population. Proc Natl Acad Sci U S A **2009**; 106:20365–70.
7. Serum cross-reactive antibody response to a novel influenza A (H1N1) virus after vaccination with seasonal influenza vaccine. MMWR Morb Mortal Wkly Rep **2009**; 58:521–4.
8. Itoh Y, Shinya K, Kiso M, et al. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature **2009**; 460: 1021–5.

9. Hancock K, Veguilla V, Lu X, et al. Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. N Engl J Med **2009**; 361:1945–52.
10. Richards KA, Topham D, Chaves FA, Sant AJ. Cutting edge: CD4 T cells generated from encounter with seasonal influenza viruses and vaccines have broad protein specificity and can directly recognize naturally generated epitopes derived from the live pandemic H1N1 virus. J Immunol **2010**; 185:4998–5002.
11. Ge X, Tan V, Bollyky PL, Standifer NE, James EA, Kwok WW. Assessment of seasonal influenza A virus-specific CD4 T-cell responses to 2009 pandemic H1N1 swine-origin influenza A virus. J Virol **2010**; 84:3312–9.
12. Subbramanian RA, Basha S, Shata MT, Brady RC, Bernstein DI. Pandemic and seasonal H1N1 influenza hemagglutinin-specific T cell responses elicited by seasonal influenza vaccination. Vaccine **2010**; 28:8258–67.
13. Subbramanian RA, Basha S, Brady RC, Hazenfeld S, Shata MT, Bernstein DI. Age-related changes in magnitude and diversity of cross-reactive CD4+ T-cell responses to the novel pandemic H1N1 influenza hemagglutinin. Hum Immunol **2010**; 71:957–63.
14. Co MD, Orphin L, Cruz J, et al. In vitro evidence that commercial influenza vaccines are not similar in their ability to activate human T cell responses. Vaccine **2009**; 27:319–27.
15. Garcia-Canas V, Lorbetskie B, Bertrand D, Cyr TD, Girard M. Selective and quantitative detection of influenza virus proteins in commercial vaccines using two-dimensional high-performance liquid chromatography and fluorescence detection. Anal Chem **2007**; 79: 3164–72.
16. Babon JA, Cruz J, Orphin L, et al. Genome-wide screening of human T-cell epitopes in influenza A virus reveals a broad spectrum of CD4(+) T-cell responses to internal proteins, hemagglutinins, and neuraminidases. Hum Immunol **2009**; 70:711–21.
17. Assarsson E, Bui HH, Sidney J, et al. Immunomic analysis of the repertoire of T-cell specificities for influenza A virus in humans. J Virol **2008**; 82:12241–51.
18. Treanor J, Wright PF. Immune correlates of protection against influenza in the human challenge model. Dev Biol (Basel) **2003**; 115: 97–104.
19. de Jong JC, Palache AM, Beyer WE, Rimmelzwaan GF, Boon AC, Osterhaus AD. Haemagglutination-inhibiting antibody to influenza virus. Dev Biol (Basel) **2003**; 115:63–73.
20. Co MD, Orphin L, Cruz J, et al. Discordance between antibody and T cell responses in recipients of trivalent inactivated influenza vaccine. Vaccine **2008**; 26:1990–8.
21. Danke NA, Kwok WW. HLA class II-restricted CD4+ T cell responses directed against influenza viral antigens postinfluenza vaccination. J Immunol **2003**; 171:3163–9.
22. Gelder CM, Lamb JR, Askonas BA. Human CD4+ T-cell recognition of influenza A virus hemagglutinin after subunit vaccination. J Virol **1996**; 70:4787–90.
23. Guthrie T, Hobbs CG, Davenport V, Horton RE, Heyderman RS, Williams NA. Parenteral influenza vaccination influences mucosal and systemic T cell-mediated immunity in healthy adults. J Infect Dis **2004**; 190:1927–35.
24. Air GM, Feng J, Chen T, Joachims ML, James JA, Thompson LF. Individual antibody and T cell responses to vaccination and infection with the 2009 pandemic swine-origin H1N1 influenza virus. J Clin Immunol **2011**; 31:900–12.
25. He XS, Holmes TH, Zhang C, et al. Cellular immune responses in children and adults receiving inactivated or live attenuated influenza vaccines. J Virol **2006**; 80:11756–66.
26. Hoft DF, Babusis E, Worku S, et al. Live and inactivated influenza vaccines induce similar humoral responses, but only live vaccines induce diverse T-cell responses in young children. J Infect Dis **2011**; 204:845–53.
27. Zeman AM, Holmes TH, Stamatis S, et al. Humoral and cellular immune responses in children given annual immunization with trivalent inactivated influenza vaccine. Pediatr Infect Dis J **2007**; 26:107–15.
28. Li X, Miao H, Henn A, et al. Ki-67 expression reveals strong, transient influenza specific CD4 T cell responses after adult vaccination. Vaccine **2012**; 30:4581–4.
29. Galli G, Medini D, Borgogni E, et al. Adjuvanted H5N1 vaccine induces early CD4+ T cell response that predicts long-term persistence of protective antibody levels. Proc Natl Acad Sci U S A **2009**; 106:3877–82.
30. Alam S, Sant AJ. Infection with seasonal influenza virus elicits CD4 T cells specific for genetically conserved epitopes that can be rapidly mobilized for protective immunity to pandemic H1N1 influenza virus. J Virol **2011**; 85:13310–21.
31. Bucasas KL, Franco LM, Shaw CA, et al. Early patterns of gene expression correlate with the humoral immune response to influenza vaccination in humans. J Infect Dis **2011**; 203:921–9.
32. Nakaya HI, Wrammert J, Lee EK, et al. Systems biology of vaccination for seasonal influenza in humans. Nat Immunol **2011**; 12:786–95.
33. Frasca D, Diaz A, Romero M, et al. Unique biomarkers for B-cell function predict the serum response to pandemic H1N1 influenza vaccine. Int Immunol **2012**; 24:175–82.
34. He XS, Holmes TH, Sasaki S, et al. Baseline levels of influenza-specific CD4 memory T-cells affect T-cell responses to influenza vaccines. PLoS One **2008**; 3:e2574.
35. Lanzavecchia A. Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. Annu Rev Immunol **1990**; 8:773–93.
36. Rodriguez-Pinto D. B cells as antigen presenting cells. Cell Immunol **2005**; 238:67–75.
37. Sette A, Moutaftsi M, Moyron-Quiroz J, et al. Selective CD4+ T cell help for antibody responses to a large viral pathogen: deterministic linkage of specificities. Immunity **2008**; 28:847–58.
38. Perona-Wright G, Mohrs K, Mohrs M. Sustained signaling by canonical helper T cell cytokines throughout the reactive lymph node. Nat Immunol **2010**; 11:520–6.
39. Wilkinson TM, Li CK, Chui CS, et al. Preexisting influenza-specific CD4(+) T cells correlate with disease protection against influenza challenge in humans. Nat Med **2012**; 18:274–80.
40. Ali A, Avalos RT, Ponimaskin E, Nayak DP. Influenza virus assembly: effect of influenza virus glycoproteins on the membrane association of M1 protein. J Virol **2000**; 74:8709–19.
41. Webby RJ, Webster RG. Are we ready for pandemic influenza? Science **2003**; 302:1519–22.
42. Yen HL, Webster RG. Pandemic influenza as a current threat. Curr Top Microbiol Immunol **2009**; 333:3–24.
43. Plennevaux E, Sheldon E, Blatter M, Reeves-Hoche MK, Denis M. Immune response after a single vaccination against 2009 influenza A H1N1 in USA: a preliminary report of two randomised controlled phase 2 trials. Lancet **2010**; 375:41–8.
44. Greenberg ME, Lai MH, Hartel GF, et al. Response to a monovalent 2009 influenza A (H1N1) vaccine. N Engl J Med **2009**; 361:2405–13.
45. Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. N Engl J Med **2006**; 354:1343–51.
46. Manzoli L, Salanti G, De Vito C, Boccia A, Ioannidis JP, Villari P. Immunogenicity and adverse events of avian influenza A H5N1 vaccine in healthy adults: multiple-treatments meta-analysis. Lancet Infect Dis **2009**; 9:482–92.
47. Steel J, Lowen AC, Wang TT, et al. Influenza virus vaccine based on the conserved hemagglutinin stalk domain. MBio **2010**; 1:: e00018–10.