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

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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 conjunction 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.
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% CO2 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.

### 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 ≥1280. r and P values were determined using the Spearman rank correlation test.

#### 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%).

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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.
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 10⁶ 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 2A and 2D) and NP/M1 pool (Figure 2B and 2E) 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 2A and 2B) or the overall expansion of CD4+ T cells (Supplementary Figure 2C and 2D), 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 influenza viruses (“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 2C). When subjects with evidence of preexisting immunity (microneutralization titer, ≥40) were excluded, this trend became much less apparent (Figure 2F).

Because of the potential usefulness of preexisting CD4+ T cells as a predictive biomarker [31–33], we next examined
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 3A and 3B, there was a highly significant correlation between CD4⁺ T-cell expansion and both the maximum (\( r = 0.53, P < .0001 \)) and fold-increase (\( r = 0.46, P = .0004 \)) in MN 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 \( \geq 10 \) 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 3C and 3D). 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

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**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 \( \geq 40 \) are indicated with an open circle. \( r \) and \( P \) values were determined by the Spearman rank correlation test.
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 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 5A and 5C) when compared to the similar NP/M1 group (Figure 5B and 5D), 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-specific CD4\(^+\) T-cell expansion on the neutralizing antibody response could
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

**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.
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, Pfizer, GlaxoSmitKline, 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.

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