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## Rituximab-Treated Patients Have a Poor Response to Influenza Vaccination\*

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### Abstract

The efficacy of influenza vaccination in patients treated with rituximab is a clinically important question. Rheumatology clinics are populated with patients receiving rituximab for a broad array of disorders. Although several studies have explored the efficacy of other vaccines in rituximab-treated populations, results have been conflicting. We wished to define influenza vaccine efficacy in a rituximab-treated cohort. We examined 17 evaluable subjects treated with rituximab for rheumatologic conditions. T cell subsets, B cells subsets, T cell function, and B cell function were evaluated at specific time points along with hemagglutination inhibition titers after receiving the standard inactivated influenza vaccine. T cell subset counts were significantly different than controls but did not change with rituximab. B cells depleted in all patients but were in various stages of recovery at the time of vaccination. Influenza vaccine responsiveness was poor overall, with only 16% of subjects having a four-fold increase in titer. Pre-existing titers were retained throughout the study, however. The ability to respond to the influenza vaccine appeared to be related to the degree of B cell recovery at the time of vaccination. This study emphasizes that antibody responses to vaccine are impaired in subjects treated with rituximab and supports the concept that B cell recovery influences influenza vaccine responsiveness.

### Keywords

Rituximab; influenza; antibody; HAI titer; B cell

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Conflict of Interest

The authors declare that have no conflict of interest.

## Introduction

In the United States, influenza infections cause an excess of 225,000 hospitalizations a year and 36,000 deaths per year [1,2]. Mortality and hospitalization are not evenly distributed across all ages. Infants and the elderly are two recognized high risk populations while chronic illness and pregnancy represent additional high risk populations [3-12]. As a public health measure, influenza vaccination is widely recommended for patients with chronic illness and for those who are immunologically vulnerable [13]. The vaccine changes yearly to match the prevailing circulating strains and induces a protective antibody response. The critical antibodies are thought to be directed to the hemagglutinin molecule and to interfere with viral entry [14-16]. Seroprotection is usually defined as a hemagglutination inhibition (HAI) titer of  $\geq 1:40$ . This is based on data of young healthy vaccine recipients and it is clear that a titer of 1:40 is not similarly protective in the elderly and other immune compromised populations [17-24]. Without larger studies to define HAI titers providing clinical protection in specific populations, most studies continue to use 1:40 as the threshold for seroprotection. The goal of vaccination is to provide clinical protection and most studies utilize antibody responses as the relevant surrogate marker. Nevertheless, T cell responses occur and have been demonstrated to offer modest protection in murine models.

In patients with chronic autoimmune diseases, influenza and other infections represent a major source of morbidity and mortality [25-30]. Both the disease itself as well as immunosuppressive therapies can contribute to increased morbidity associated with infection. One of the cornerstones of prevention is influenza vaccination in immune compromised individuals. In general, studies of influenza vaccination in cohorts of patients with autoimmune diseases have suggested reduced but detectable vaccine responses [31-35]. Both medications and the autoimmune disease itself can contribute to globally poor vaccine responses [36,33,31,34,35]. As the use of rituximab has increased in most rheumatology clinics, the question of vaccine efficacy in rituximab-treated populations has become more important. There are limited data on the influenza vaccine specifically. Several studies have attempted to define effects on vaccine responses with conflicting results [33,37-40,36,41]. The variable response rates in these studies may have been due to different schedules of vaccination after rituximab. Repopulation of the B cell compartment is a dynamic and variable process [42]. Vaccination of patients with lymphoma treated with rituximab has likewise had limited efficacy [43,44]. We undertook a prospective study to investigate whether laboratory predictors of influenza responsiveness could be identified in a cohort of rheumatology patients treated with rituximab. We found that those patients who had undetectable circulating B cells were much less likely to respond, while there were modest responses to the inactivated influenza vaccine among patients who had any level of detectable B cells. We also found that pre-existing HAI titers were stable in individual patients over time.

## Methods

### Subjects

Twenty-five subjects who were on active rituximab therapy for autoimmune disease were enrolled in this prospective study of influenza vaccine effectiveness. We assessed baseline immunologic parameters, within four weeks of rituximab administration, on the day of vaccination (7-9 months after rituximab) and at 2 months and six months after vaccination. Subject received the inactivated influenza vaccine as part of their routine care. This study was approved by the Institutional Review Board. Demographic characteristics are listed in Table 1. An adult control cohort from the same city, which has in part been previously reported, had parallel studies performed on the day of vaccination and 4-8 weeks afterward

[45]. We age-matched the controls to the study population. Demographic characteristics are listed in Table 1.

## Vaccines

Four seasonal vaccines were used: For 2006-2007, A/New Caledonia/20/99 (H1N1)-like virus; A/Wisconsin/67/2005 (H3N2)-like virus; B/Malaysia/2506/2004-like virus. For 2007-2008, A/Solomon Islands/3/2006 (H1N1)-like virus; A/Wisconsin/67/2005 (H3N2)-like virus; B/Malaysia/2506/2004-like virus. For 2008-2009, A/Brisbane/59/2007 (H1N1)-like virus; A/Brisbane/10/2007, (H3N2)-like virus; B/Florida/4/2006-like virus. For 2009-2010, A/Brisbane/59/2007 (H1N1)-like virus; A/Brisbane/10/2007 (H3N2)-like virus; B/Brisbane/60/2008-like virus.

## Immunologic Assessments

A standard hemagglutination inhibition (HAI) assay optimized for the vaccine administered to each patient was used to define antibody responses to the vaccine [46]. Flow cytometry for T cell and B cell subsets was performed. Fixed stained T cells were run on an LSR II (BD Biosciences) and analyzed using FlowJo software (TreeStar). Approximately 200,000 to 500,000 total events were collected per assay. CD4 Naïve cells were defined as CD45RA+CD31+. CD4 Central Memory T cells were defined as CD27+CD45RO+CCR7+. CD4 Effector Memory T cells were defined as CD45RO+/CD27-/CCR7-. CD4 Reverted Memory T cells were defined as CD45RA+/CD31-/CCR7+. CD8 Naïve cells were defined as CD45RA+CD31+. CD8 Central Memory T cells were defined as CD27+CD45RO+CCR7+. CD8 Effector Memory T cells were defined as CD45RO+/CD27-/CCR7-. CD8 Reverted Memory T cells were defined as CD45RA+/CD31-/CCR7+.

Flow cytometry of B cell subsets was performed using fresh venous whole blood anti-coagulated with EDTA as described previously [42]. B cells were defined as CD19+ lymphocytes. Analyses were performed on a FACSCalibur with CellQuest software (Version 5.2.1, Becton Dickinson, San Jose, CA). CD19+ lymphocytes were analyzed for CD27 and IgM. The absolute B cell count was obtained by multiplying the absolute lymphocyte count by the CD19+ fraction. A minimum of 150 $\mu$ L of blood was analyzed. The limit of detection was <0.67 B cells/ $\mu$ L. Our criterion for complete B cell depletion at week 4 after rituximab was an absolute B cell count of  $\leq$  B cells/ $\mu$ L [47].

To examine functional responses to the vaccine, T cell ELISPOTs and B cell ELISPOTs were performed. A cocktail of influenza proteins (Protein Sciences, Meriden, CT) matched to the vaccine year was used as specific antigen (at 5  $\mu$ g/mL) in a standard  $\gamma$ -interferon T cell ELISPOT assay<sup>58</sup>. PMA and ionomycin (combined at 5  $\mu$ g/mL each) were used as a positive control. This assay examined a range of epitopes, and was HLA-dependent. Foreign antigen sources were avoided to minimize background. The B cell ELISPOT defined the frequency of memory B cells activated by influenza to produce antibody<sup>59,60</sup>. PBMC were stimulated for 6 days with pokeweed mitogen at 1:100,000, SAC at 1:10,000 and CpG-2006 at 6mg/ml (Sigma Aldrich St. Louis, MO). Following the stimulation period, cells were treated for six hours with the influenza protein cocktail described above (at 0.5  $\mu$ g/mL). Finally, IgG production assessed by quantification of effector cells using ImmunoSpot (CTL, version 4) software. Proliferation assays utilized carboxyfluorescein succinimidyl ester (CFSE)-loaded cells stimulated for five days with season-specific influenza virus. Flow cytometry was used to define the divided cells.

## Statistical analyses

The  $\chi^2$  test was used for the comparisons of responder frequency and seroconversion. Descriptive statistics were performed using the Mann-Whitney method. Two-tailed p values are reported. Significance was set at  $p \leq 0.05$ .

## Results

### Patient Characteristics

Twenty-five subjects were originally enrolled in this study to define predictors of influenza vaccine serologic responses. The demographic characteristics of the study cohort are given in Table 1. Patient diagnoses and B cell counts on the day of vaccination are provided in Table 2. As anticipated, the majority of the subjects were female (96%) and Caucasian (60%). The mean age was 49 years and the underlying diseases were diverse, with rheumatoid arthritis being the most common diagnosis (40%). Five subjects withdrew from the study: three were unwilling to receive the vaccine and two had increasing illness. Three subjects were withdrawn by the overseer due to failure to complete the essential time points, leaving 17 subjects for analysis. A subset of 12 patients with synchronized studies were used to define vaccine responder status. Enrollment into this study required only that the subject was expected to start rituximab specifically for their autoimmune disease. All subjects received the inactivated year-specific influenza vaccine as part of their routine care. Adult age-matched controls had the same laboratory studies performed at baseline and at 4-8 weeks after vaccination. Immunologic evaluations were obtained at the time of enrollment, at four weeks after a course of rituximab, at the time of vaccination (7-9 months after rituximab), two months and four months after vaccination. The immunologic assessments included year-specific HAI titers to the influenza vaccine, T cell subsets, B cell subsets, T cell proliferation, T cell cytokine production, B cell IgG production, and T cell repertoire analysis by spectratyping.

### B cell reconstitution

Three patients had received rituximab prior to study entry. The remainder were naïve to rituximab. We first analyzed B cell subsets by flow cytometry to characterize the pattern of B cell reconstitution. Depletion after rituximab was complete in these subjects (Figure 1) and no patient failed to deplete. At 7-9 months after rituximab, some subjects had reconstitution of their naïve B cells but switched memory (CD19+CD27+, IgM-) and non-switched memory (CD19+CD27+IgM+) B cells remained less than 10% of their starting values throughout the entire time frame (Figure 2).

### T cells in Subjects

All patients were on additional immunologically-modifying interventions in addition to rituximab (Table 2). On the day of vaccination, four patients were on low dose prednisone, four were on hydroxychloroquine, two were on leflunomide, one was on azathioprine and one was on methotrexate. To examine this cohort for evidence of T cell compromise, we initially examined T cell subsets over the same time points. We compared the T cells to the control group (Figure 3). Naïve and effector CD4 and CD8 T cells were lower in patients than controls at baseline. We could not discern a direct effect of the Rituximab. To assess T cell function, we analyzed CD4 and CD8 responses to influenza using purified cells and a  $\gamma$ -interferon ELISPOT. Responses were comparable to controls at baseline but there were no significant increases after vaccination (data not shown). Similarly, we measured proliferation after PHA and influenza stimuli and we did not detect a response after vaccination in the rituximab-treated cohort (data not shown). There were no changes in the T cell repertoire as detected by spectratyping over the time points (data not shown). Globally,

the T cell compartment was less altered in the patients compared to controls than the B cell compartment, as expected.

### Influenza Vaccine Responses

To define the rate of vaccine response, the rate of seroconversion was used. This represents a four-fold increase in titer after vaccination. Only two of 12 patients had a four-fold response and it was to a single serotype in both cases. These two patients had rheumatoid arthritis and polymyositis. In contrast, 10/15 of the adult controls had seroconversion after vaccination ( $p=0.009$ ) and in most cases it was to multiple serotypes. In spite of the failure to respond to the vaccine, pre-existing aggregate titers (defined as the sum of titers to the three serotypes) were retained over a long time frame (Figure 4)

To understand the determinants of the response to the inactivated influenza vaccination, we first defined B cell function. Overall, there were low numbers of antibody-secreting cells detected in a B cell ELISPOT assay (Figure 4). To examine correlates of the vaccine response, we identified five patients who had any detectable response to the vaccine. We defined responders as those who had a two-fold increase to at least one serotype at the two-month time point after vaccination. These five patients had rheumatoid arthritis (X2), polymyositis and Sjögren's syndrome (X2). We compared the T cell subsets defined in Figure 3 between responders and non-responders. There were no significant differences in T cell counts between the two groups (data not shown). However, the absolute B cell numbers were higher in responders compared to non-responders ( $p=0.004$ ) (Figure 5). This was not restricted to a single B cell subset (Figure 5). Responders had higher numbers of IgM and switched memory B cells compared to non-responders.

**Discussion**—The protection from influenza infection relies on innate defenses, humoral immunity and cytotoxic T cell responses. In murine models, T cell responses are more likely to be cross-protective, but they offer modest protection from severe disease [48-53]. In contrast, humoral immunity offers limited cross-protection against other serotypes but is strongly protective against infection [54-57]. Therefore, we focused on antibody responses.

This study was designed to determine whether there was a threshold B cell count that could predict serologic influenza vaccine responsiveness. In fact, patients with B cell counts of  $>1$  cell/ $\text{mm}^3$  did have some response to the vaccine (two-fold rise in titer to one serotype), while those subjects who had B cell counts  $<1$  cell/ $\text{mm}^3$  did not respond. This was a small study, however, and many of the B-cell counts clustered right around the threshold of one. Additional studies to test the predictive value of this threshold will be required. Recent progress has been made in identifying predictors of vaccine responses in healthy donors [58]. Specifically for influenza, the ability to stratify immunologically vulnerable subjects to maximize the effective use of the vaccine in resource-limited situations would be desirable. Repletion of the B cell compartment after rituximab is highly variable and influenza vaccine is available seasonally, making it difficult to develop standard protocols useful for all patients. Therefore identification of a laboratory marker would be extremely valuable.

Previous studies have identified diminished responses to various vaccines after rituximab treatment. Some studies have found an association of vaccine responses with the degree of B cell depletion [47,43,37] while others have not found such an association [36,38,39]. Our study is consistent with an association of B cell depletion and compromised vaccine responses. Differences between study results may be related to the specific vaccine utilized, the degree of priming, and the extent to which T cells provide help.

Based on this study, the uniform use of the inactivated influenza vaccine in patients treated with rituximab must be considered of limited serologic efficacy. Clinical efficacy due to T

cell responses is possible although in this cohort, vaccine-specific T cell responses were minimal [59,60]. In subjects with detectable peripheral blood B cells, the vaccine may induce a serologic response, however, patients should be cautioned that protection may not be complete. Since pre-existing titers to influenza were not affected, consideration should be given to vaccinating patients before treatment with rituximab.

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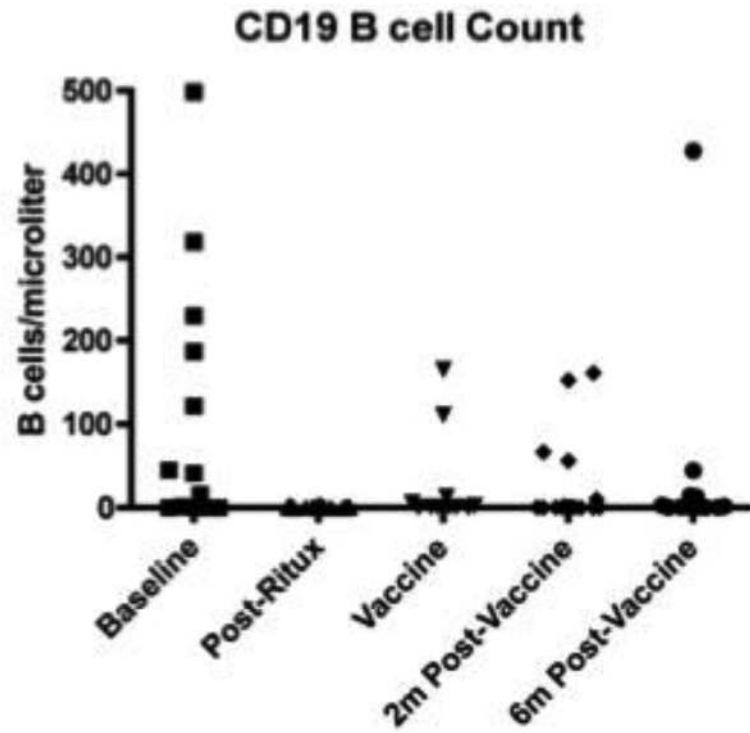
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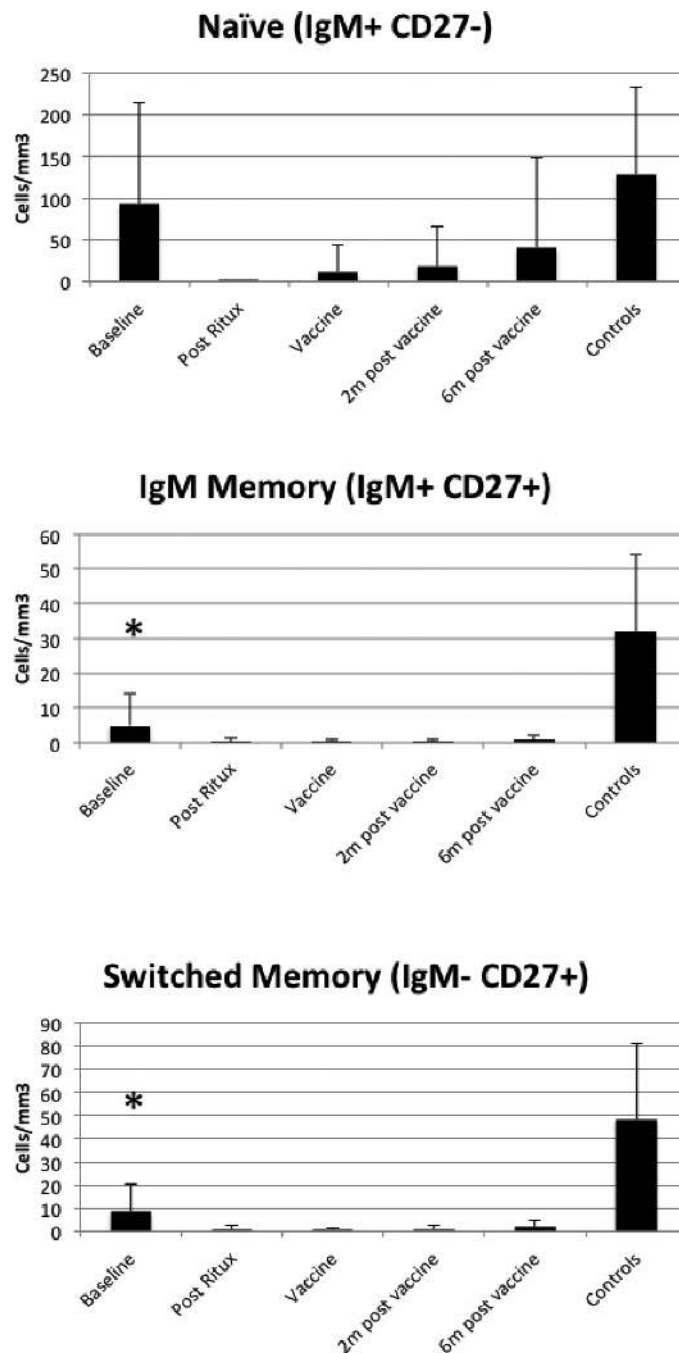
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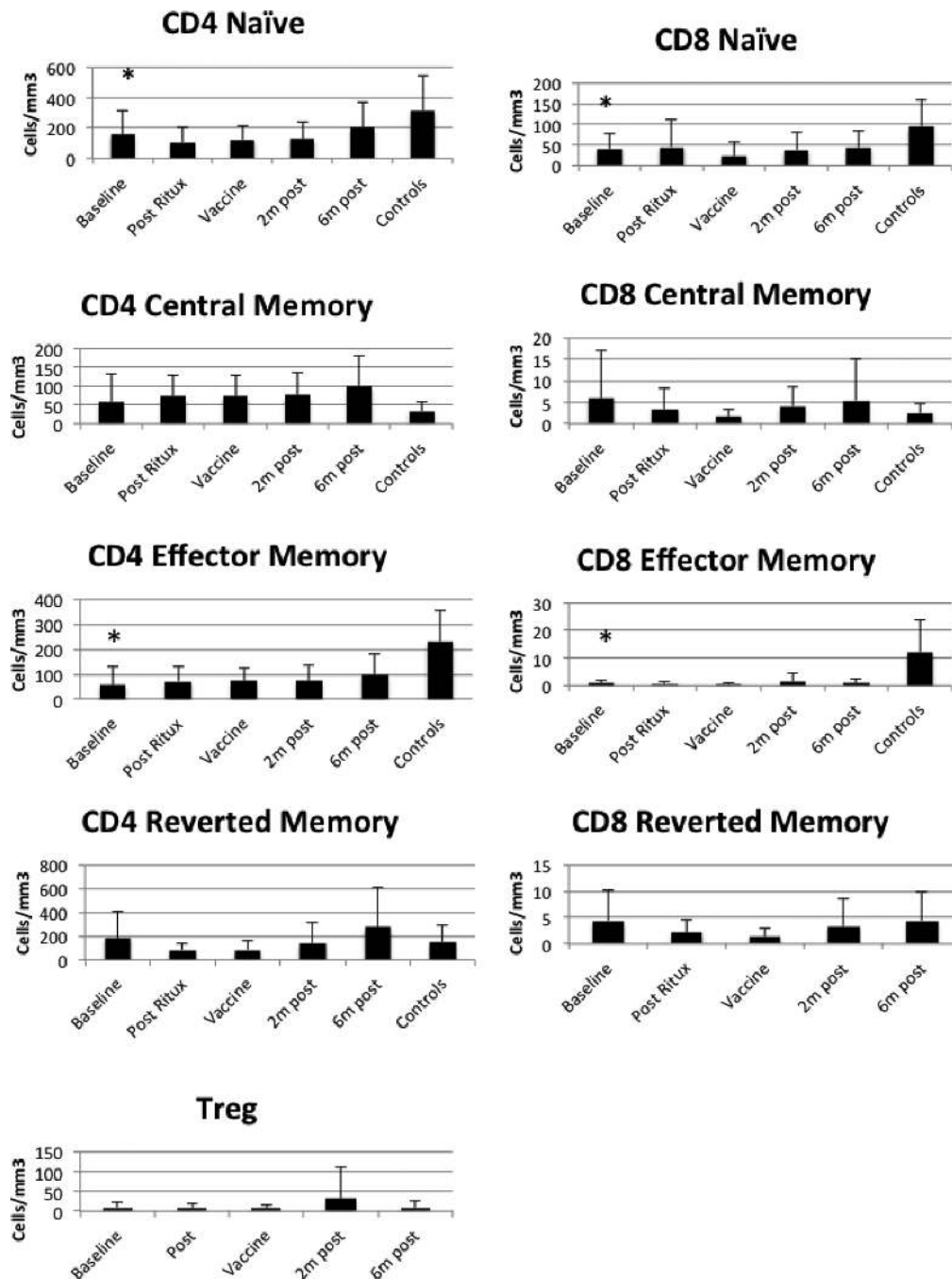
**Figure 1. B cell depletion kinetics**

Total B cells (CD19+) were measured at baseline, 4 weeks after rituximab, the day of vaccine administration (7-9 months after rituximab administration), 2 months after vaccination and 6 months after vaccination. B cells depleted well in all patients but recovery was variable.



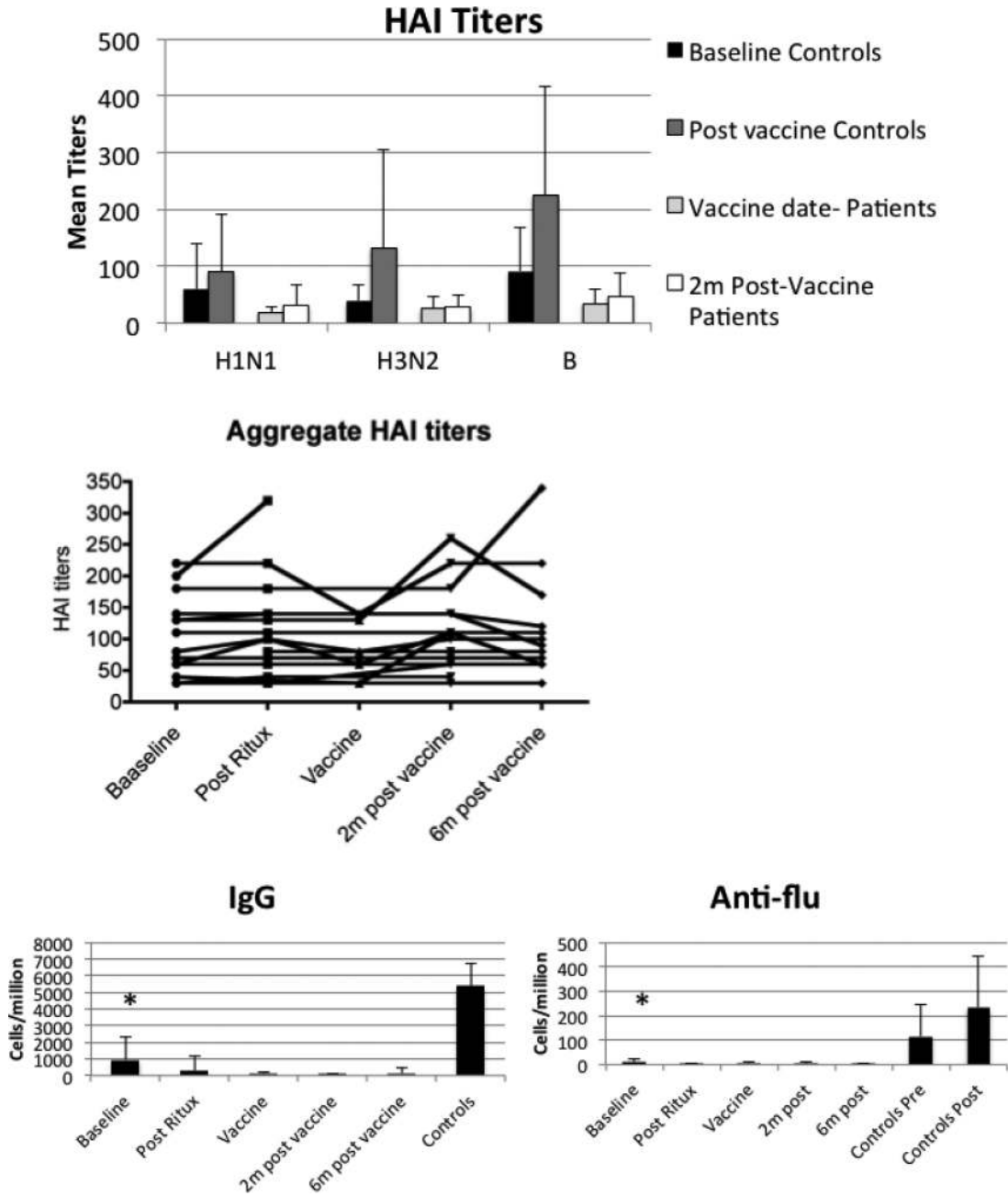
**Figure 2. B cell subsets**

B cell subsets were defined using flow cytometry. The mean and standard deviation are shown and the baseline evaluations for the controls are shown at the right of each graph. Baseline levels were compared between patients and controls. The IgM memory and switched memory subsets were different from controls at baseline ( $p < 0.001$  for both). On the day of vaccination, memory subset counts were significantly lower in patients than controls with  $p < 0.001$ .



**Figure 3. T cell subsets**

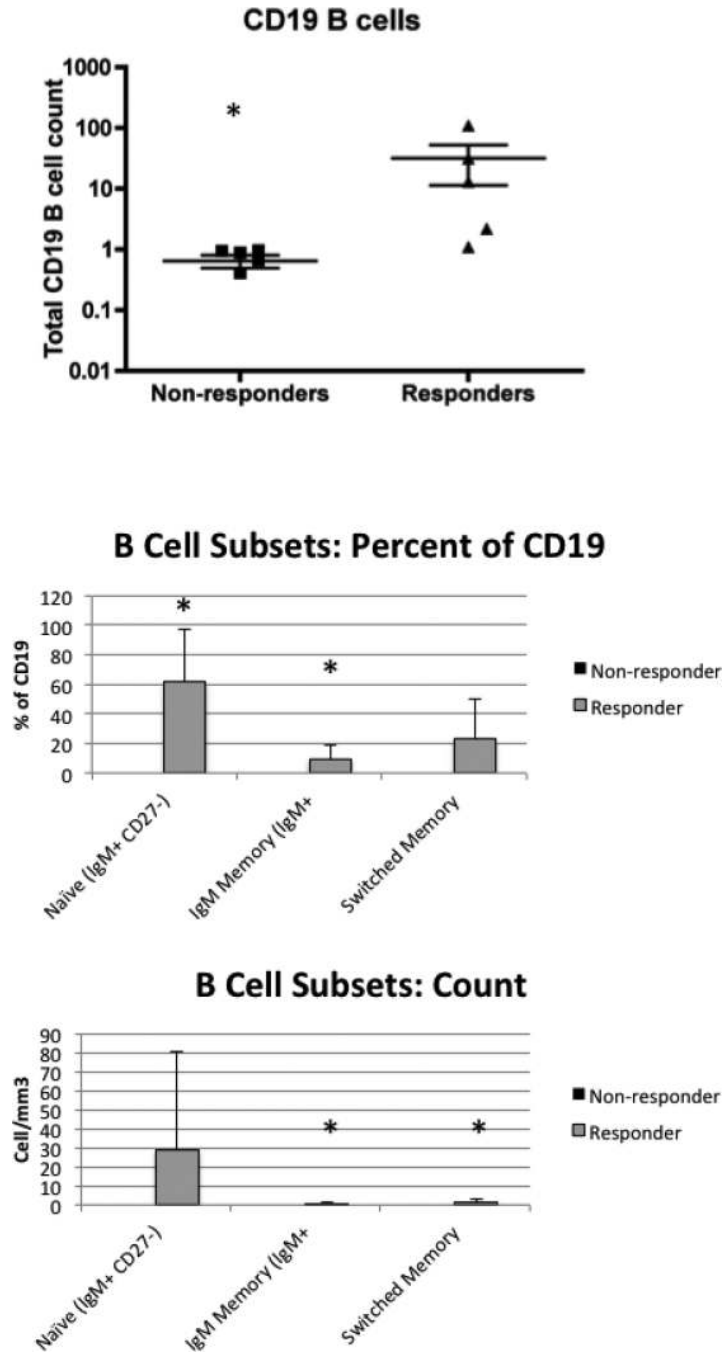
T cell subsets were defined using flow cytometry. The mean and standard deviation are shown at each time point and the baseline evaluations for the controls are shown on the far right of each graph. Baseline levels were compared between patients and controls and the asterisks indicate results statistically different from controls. The p values for CD4 naïve and effector subsets were  $p=0.05$  and  $p<0.01$  respectively. For CD8 naïve and effector subsets, the p values were  $p=0.01$  and  $p<0.01$  respectively.



**Figure 4. Antibody production**

Antibody responses to the vaccine were measured using the hemagglutination inhibition assay matched for each year of the vaccine (top panel). Mean titers and standard deviation are shown in the graph. Post vaccine responses in the patients were significantly lower than controls with  $p < 0.001$  for all three serotypes. In addition, the difference between the pre- and post-vaccine titers in the patients were non-significant. The existing HAI titers in the patients varied little over the course of the study. The second panel uses a sum of the individual HAI serotypes in the graph. Each line represents an individual study patient. We also assessed global IgG production and anti-influenza responses using a B cell ELISPOT assay. Although baseline levels are lower in patients than controls, there is detectable

antibody production in this assay. Nevertheless, all time points exhibited significantly less total IgG and influenza-specific responses than controls.



**Figure 5. Characteristics of the responder group**

Within the cohort, we compared B cell subsets on the day of vaccination between responders and nonresponders. The mean and standard error are shown. The non-responders are shown in dark grey but the values are so low, they appear superimposed on the X axis. The naïve B cell and IgM memory percentages were lower in nonresponders than responders with  $p < 0.01$  and  $p < 0.05$  respectively. The absolute counts were lower in non-responders than responders for IgM memory and switched memory B cell subsets with  $p < 0.05$  for IgM memory and  $P < 0.01$  for switched memory B cells. Although the responders had variable B cell counts, the non-responders uniformly had  $< 1$  B cell/mm<sup>3</sup>. Asterisks indicate significant differences.



**Table 1**

Demographic characteristics of the study population

<b>Patients</b>	<b>Number (n=17)</b>	<b>Percent</b>
Female	16	94%
Caucasian	11	65%
African American	4	24%
Asian	2	12%
Rheumatoid arthritis *	8	47%
Sjögren's syndrome *	6	35%
SLE *	2	12%
Polymyositis	2	12%
Wegener's vasculitis	1	6%
<b>Controls</b>	<b>15</b>	
Female	8	53%
Caucasian	11	73%

\* Two subjects had two concurrent diagnoses (rheumatoid arthritis and SLE, rheumatoid arthritis and Sjögren's syndrome)

**Table 2**

## Clinical characteristics

Disease group	Additional medications on the day of vaccination	Mean B cell count (cells/ $\mu$ l of whole blood $\pm$ SD) on the day of vaccination
Rheumatoid arthritis	Leflunomide, etanercept, prednisone	1 $\pm$ 1
Sjögren's	Hydroxychloroquine, ibuprofen, leflunomide, prednisone, methotrexate	37.6 $\pm$ 63.5
SLE	Hydroxychloroquine, prednisone, methotrexate	1 $\pm$ 0
Polymyositis	Methotrexate, hydroxychloroquine prednisone	7 $\pm$ 8.5
Wegener's	None	1