

# Loss of Discrete Memory B Cell Subsets Is Associated with Impaired Immunization Responses in HIV-1 Infection and May Be a Risk Factor for Invasive Pneumococcal Disease<sup>1</sup>

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**Invasive pneumococcal infection is an important cause of morbidity and mortality in HIV-1-infected individuals. B cells play an important role in maintaining serologic memory after infection. IgM memory B cells are significantly reduced in HIV-1-infected patients and their frequency is similar to that observed in other patient groups (splenectomized individuals and patients with primary Ab deficiency) who are also known to have an increased risk of invasive pneumococcal infection. Antiretroviral therapy does not restore marginal zone B cell percentages. Immunization with the 23-valent polysaccharide pneumococcal vaccine shows that HIV-1-infected patients have impaired total IgM and IgG pneumococcal vaccines compared with healthy controls. Loss of switched memory B cells was associated with impaired tetanus toxoid IgG vaccine responses. Results of this study demonstrate that defects in B cell memory subsets are associated with impaired humoral immune responses in HIV-1 patients who are receiving antiretroviral therapy and may be a contributory factor to the increased risk of invasive pneumococcal infection observed in HIV-1 infection. *The Journal of Immunology*, 2007, 178: 8212–8220.**

**I**nvasive pneumococcal infection is an important cause of morbidity even in HIV patients on antiretroviral therapy (ART)<sup>3</sup> (1). Ab responses to pneumococcal vaccination are reduced in ART-naive HIV patients with CD4 T cell counts <500 cells/ $\mu$ l (2); however, there is conflicting data on the efficacy of pneumococcal immunization in HIV-1 patients receiving ART (3, 4). Serological memory after exposure to pathogens or vaccination is maintained by plasma cells and memory B cells (5, 6). Intrinsic defects in B cell function resulting in failure of this cell population to respond to accessory CD4 T cell help (7, 8) and inhibition of Ig isotype class switching by HIV viral proteins may play a role in the development of impaired humoral immune responses seen in HIV-1 infection (9). Memory B cell (CD19<sup>+</sup>CD27<sup>+</sup>) counts are reduced in HIV-1-infected individuals (10–12) and are associated with reduced measles and tetanus Ab concentrations (13). Plasma levels of IgM and IgG pneumococcal Ab are reduced in HIV-1 infection and the capacity of PBMC to produce IgG and IgM pneu-

mococcal Ab following in vitro B cell polyclonal stimulation is impaired (14). Loss of CD19<sup>+</sup>CD27<sup>+</sup> memory B cells may be also be an additional factor responsible for impaired humoral immunity and poor vaccine responses in HIV infection.

Ag-experienced CD19<sup>+</sup>CD27<sup>+</sup> memory B cells are divided into two main populations (15, 16). Switched CD19<sup>+</sup>CD27<sup>+</sup>IgM<sup>−</sup>IgD<sup>−</sup> B cells are involved in T cell-dependent immune responses, secrete IgG and IgA Abs, and maintain long-term serological memory. IgM memory CD19<sup>+</sup>CD27<sup>+</sup>IgM<sup>high</sup>IgD<sup>low</sup>CD21<sup>high</sup> B cells are important in T cell-independent immune responses and secrete high-affinity IgM in the early phase of infection (17) to inhibit microbial replication in blood. This cell population is believed to be the circulating counterparts of splenic marginal zone B cells and responds to the 23-valent polysaccharide pneumococcal vaccine (18). Unlike switched memory B cells, IgM memory B cells do not promote long-term protective humoral immune responses and it has been proposed that this B cell subset be regarded as natural effector B cells which bridge innate and adaptive immune responses (15). The ontogeny of IgM memory B cells in humans remains unclear; one suggestion is that this B cell subset may be derived from transitional B cells which are a naive B cell population that need the spleen to complete their development to mature B cells (19, 20). Increased numbers of transitional B cells are a characteristic feature of several inherited humoral immunodeficiency conditions and HIV infection (21, 22) and are associated with decreased memory B cells (21). Expansion of transitional B cells may reflect increased immune activation which has been linked with impaired T cell-dependent Ag vaccine responses in patients with HIV-1 infection (23, 24).

The aims of this study were to analyze the distribution of IgM and switched memory B cell memory subsets in HIV-1 patients and to determine whether there was any association with the percentage of circulating transitional and memory B cell subsets and immunization responses to Pneumovax II (T cell-independent Ag) and tetanus toxoid (T cell-dependent Ag) in HIV-1 patients on ART. Finally, we also determined whether there was a relationship between a marker of immune activation (expression of CD38 on

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<sup>3</sup> Abbreviations used in this paper: ART, antiretroviral therapy; HC, healthy control; CVID, common variable immune deficiency; BAFF, B cell-activating factor; APRIL, a proliferating-inducing ligand; MBO, memory B cell O; MZB, marginal zone B cell.

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Table I. Demographic and immune parameters of controls and patient groups<sup>a</sup>

	HC <sup>a</sup>	HIV ART <sup>-</sup>	HIV ART <sup>+</sup>	CVID	Splenectomy
Age (years)	31 (28–40)	38 (35–44)	46 (41–52) <sup>b</sup>	50 (33–61)	40 (33–56)
M/F	12/5	27/2	51/4	11/17	3/5
Viral load RNA copies/ml	NA	48,187 (7,993–155,233)	<50 (<50–<50)	NA	NA
Time on ART (days)	NA	NA	1,013 (547–1,760)	NA	NA
CD4 cells/ $\mu$ l	788 (553–918)	374 (188–472) <sup>c</sup>	409 (279–556)	659 (401–854)	1,435 (905–2,323)
CD8 cells/ $\mu$ l	388 (280–516)	925 (711–1280)	793 (624–1,296)	298 (174–694)	713 (500–904)
CD19 cells/ $\mu$ l	171 (116–219)	111 (59–167)	249 (167–311)	155 (75–274)	686 (462–1,053)
% CD19 <sup>+</sup> CD27	31.0 (22.8–42.5)	18.6 (11.2–30.1) <sup>d</sup>	16.9 (10.8–26.6) <sup>d</sup>	9.8 (5.5–27.1) <sup>d</sup>	10.6 (7.9–19.0) <sup>d</sup>

<sup>a</sup> Demographic and flow cytometry data were not available for occupational health donors. The results shown for HC are for laboratory staff only. Table shows median and interquartile range for HC, HIV-1 patients not on ART (HIV ART<sup>-</sup>), HIV patients receiving ART (HIV ART<sup>+</sup>), CVID patients and patients with a history of splenectomy (Splenectomy).

<sup>b</sup> HIV-1 patients on ART were significantly older than controls and ART-naive patients ( $p < 0.001$ ).

<sup>c</sup> CD4 T cell counts were significantly lower in ART-naive patients compared with controls, CVID, and HIV-1 patients receiving ART ( $p < 0.01$ ).

<sup>d</sup> Memory B cell (CD19<sup>+</sup>CD27<sup>-</sup>) percentages were also significantly reduced in both HIV patient groups, CVID, and splenectomized patients compared with controls ( $p < 0.01$ ).

CD8 T cells) and CD4<sup>+</sup>CD28<sup>+</sup> T cell counts and immunization responses as has been described in previous studies of tetanus immunization in HIV-1-infected individuals (23, 24).

## Materials and Methods

### Controls and patient groups

Blood samples were taken from healthy controls (HC) ( $n = 83$ ), 17 laboratory staff, and 56 individuals who had attended an occupational health screening clinic in London. We studied 84 patients with HIV-1 infection; 55 patients were on successful ART with a sustained reduction in viral load for at least 6 mo and 29 patients had no history of ART. Patients with common variable immunodeficiency (CVID), characterized by recurrent bacterial infections and a failure to make pneumococcal vaccination responses, were recruited as disease controls ( $n = 28$ ). All patients with CVID satisfied the Pan-American Group for Immunodeficiency and the European Society for Immunodeficiency diagnostic criteria for this condition (25, 26). Twenty-five CVID patients were receiving IgG replacement containing exogenous pneumococcal and tetanus Abs. Bronchiectasis as defined by high-resolution chest computerized tomography scan was present in 17 patients. Eight patients with a history of splenectomy were recruited to the study as controls for reduced IgM memory B cells. Immunization with either tetanus toxoid (Aventis Pasteur) or the 23-valent polysaccharide pneumococcal vaccine (Pneumovax II; Aventis Pasteur) was performed in 33 of 55 HIV-1 patients on successful ART (HIV viral load <50 copies/ml for >6 mo). Eleven HIV-1 patients on ART were randomized to receive no vaccine and the remaining patients did not fulfill the criteria for test immunization. HIV-1-infected patients receiving tetanus and pneumococcal immunization were matched with the unvaccinated patients for nadir CD4 T cell counts. Criteria for test immunization were: baseline tetanus toxoid IgG Ab levels below the limit of long-term protection (1.0 IU/ml) and baseline levels of IgG pneumococcal Abs <50th centile for controls (80 U/ml). Vaccine responses were assessed at 4 wk and at 3–6 mo postimmunization and were compared with those observed in healthy controls. Criteria for successful IgM Pneumovax II immunization were a 4-fold rise between baseline and 4-wk Ab levels with postimmunization IgM levels of at least 200 U/ml. Successful IgG vaccine responses were defined as a 4-fold rise in between baseline and 1 and 3–6 mo immunization responses. The minimum postvaccine pneumococcal IgG target was a value >75th centile for healthy controls (225 U/ml) and the threshold for a satisfactory tetanus response was set at the 50th centile concentration observed for controls. Prior receipt of a pneumococcal vaccine and tetanus immunization within the previous 5 years formed part of the exclusion criteria for the study. Recruitment of patients to this study was approved by the local institutional ethics board.

### Flow cytometry

Absolute CD4<sup>+</sup> and CD8<sup>+</sup> T cell counts and percentages were determined using 100  $\mu$ l of EDTA blood samples by four-color flow cytometric analysis using a Beckman Coulter Epics XL flow cytometer. The following mAbs were used: FITC-conjugated anti-CD45, PE-cyanin red 5.1 (PC5) conjugated anti-CD3, PE-Texas Red (ECD) conjugated anti-CD8, and rhodamine 1 (RD1) conjugated anti-CD4 (all purchased from Beckman Coulter).

Analysis of B cell and T cell subsets was performed in 120  $\mu$ l of EDTA blood. Whole blood samples were washed in twice in 10% FCS/PBS solution to remove circulating Ig for analysis of B cells. All blood samples

were incubated with mAbs for 20 min at room temperature. The mAbs used to define human B cell subsets were: PE-cyanin 7 (PC7) conjugated anti-CD19 (Coulter Immunotech), PE-conjugated anti-human IgD (Southern Biotechnology Associates), Cy5-conjugated anti-human IgM (The Jackson Laboratory), FITC-conjugated anti-CD27 (DakoCytometry) and PE-conjugated anti-CD21, FITC-conjugated anti-CD38 (from BD Pharmingen). The expression of CD38 and CD28 on T cell subsets was determined using FITC-conjugated anti-CD8/PE-conjugated anti-CD38 (Coulter Immunotech). Allophycocyanin-conjugated anti-CD3, PerCP-conjugated anti-CD4, FITC-conjugated anti-CD8, PE-conjugated anti-CD28 (all purchased from BD Biosciences). After incubation, blood samples were lysed using Beckman Coulter TQ Prep and then washed twice with PBS. Cells were assessed using four-color flow cytometry on a FACSCalibur (BD Biosciences) and data were analyzed using CellQuest Pro software (BD Biosciences). Viable lymphocytes were examined using forward- vs side-scatter gating and B cells were analyzed using side-scatter vs CD19 gating; T cells were evaluated using a CD3 gate. Five thousand events were acquired on the B cell gate and the results are expressed as a percentage of CD19 events. Ten thousand events were acquired on the T cell gate and the results are expressed as a percentage of CD3 events. IgM memory B cells were defined as CD19<sup>+</sup>CD27<sup>+</sup>IgM<sup>high</sup>IgD<sup>low</sup> or CD19<sup>+</sup>CD27<sup>+</sup>IgM<sup>high</sup>CD21<sup>high</sup> B cells. There was very high level of agreement between these two sets of markers (correlation coefficient = 0.90). Data for IgM memory B cells is represented by the CD19<sup>+</sup>CD27<sup>+</sup>IgM<sup>high</sup>IgD<sup>low</sup> panel to avoid repetition and to allow us to display the switched CD19<sup>+</sup>CD27<sup>+</sup>IgM<sup>high</sup>IgD<sup>low</sup> memory B cell data. Transitional CD19<sup>+</sup>CD21<sup>low</sup>IgM<sup>high</sup>CD38<sup>high</sup> B cells were defined according to the Freiburg protocol (26). The proportion of IgM memory B cells and switched memory B cells in controls, CVID, and HIV-1 patient groups was analyzed according a modified Piqueras classification (27) where both IgM memory B cells and switched memory B cells are calculated as a percentage of the total B cell population. The MB2 phenotype is equivalent to healthy controls; IgM memory B cells and switched memory B cells are each >8% of the total B cell population. The MB1 phenotype refers to a selective depletion of switched memory B cells which are <8% of total B cell population. The memory B cell O (MBO) phenotype is characterized by a reduction in both IgM memory B cells and switched memory B cells; each B cell subset is <8% of total B cell population. The splenectomized patients had a reduction in IgM memory B cells (<8% of total B cell population), which we have called the marginal zone B cell (MZB<sup>-</sup>) phenotype.

### Determination of specific microbial Abs by ELISA

Serum titers of IgM and IgG antipneumococcal were determined using ELISA technology. Briefly, 96-well plates (Maxisorp; Nunc; Fisher-Scientific) were coated with Pneumovax II 23-valent vaccine overnight at 4°C. Patient samples and reference standards were incubated with cell wall pneumococcal polysaccharide Ag (5  $\mu$ g/ml; Statens Serum Institut) in PBS/0.1% Tween 20/1% BSA solution for 1 h at room temperature to remove nonspecific Abs which do not play a role in mediating protective immune responses. The 96-well plates were then washed three times with PBS/Tween 20 solution. Patients' samples (duplicates at four different dilutions) were then added and incubated at room temperature for 1.5 h. Plates were washed and then the appropriate HRP-labeled IgG or IgM conjugate (Sigma-Aldrich) was added. After a second wash step, plates were developed using *o*-phenylenediamine substrate, with results calculated from a standard curve. Pneumococcal IgG standard, assigned a value

Table II. Percentage of memory and transitional B cell subsets and of T cell subsets in controls and patients<sup>a</sup>

%	HC	HIV ART <sup>-</sup>	HIV ART <sup>+</sup>	CVID	Splenectomy
IgM memory B cell	14.9 11.6–23.1	6.6 3.7–14	5.5 2.9–8.6	7.3 4.0–21.9	3.3 1.6–8.1
Class switched memory B cell	14.9 11.2–23.0	14.3 7.4–20.1	10.7 6.4–19.5	2.6 1.2–5.2	7.8 3.9–11.9
Transitional B cell	0.8 0.4–1.6	7.5 3.7–12.3	2.6 1.4–3.85	1.7 0.9–4.0	2.1 0.9–6.8
CD8 <sup>+</sup> CD28 <sup>-</sup> T cell	18.5 12.3–28.8	70.7 60.3–81.0	48.0 36.6–62.7	35.3 20–51.3	17.9 9.9–41.3
CD4 <sup>+</sup> CD28 <sup>-</sup> T cell	0.1 0.0–0.5	9.6 3.1–22.9	6.7 2.0–12.0	0.1 0.0–0.5	0.2 0.1–0.3
CD8 <sup>+</sup> CD38 <sup>+</sup> T cell	4.2 2.5–12.2	32.1 24.4–42.5	8.2 5.7–12.9	9.9 6.5–18.5	8.6 4.1–9.7
CD4 <sup>+</sup> CD38 <sup>+</sup> T cell	16.5 13.5–23.9	27.7 20.9–34.2	17.3 11.3–24.9	21.4 14.8–32.5	20.3 7.2–27.3

<sup>a</sup> The percentage of the above B and T cell subsets was determined using flow cytometry. Data are shown for median and interquartile range for HC, HIV-1 patients not on ART (HIV ART<sup>-</sup>), HIV patients receiving ART (HIV ART<sup>+</sup>), CVID patients, and patients with a history of splenectomy (Splenectomy).

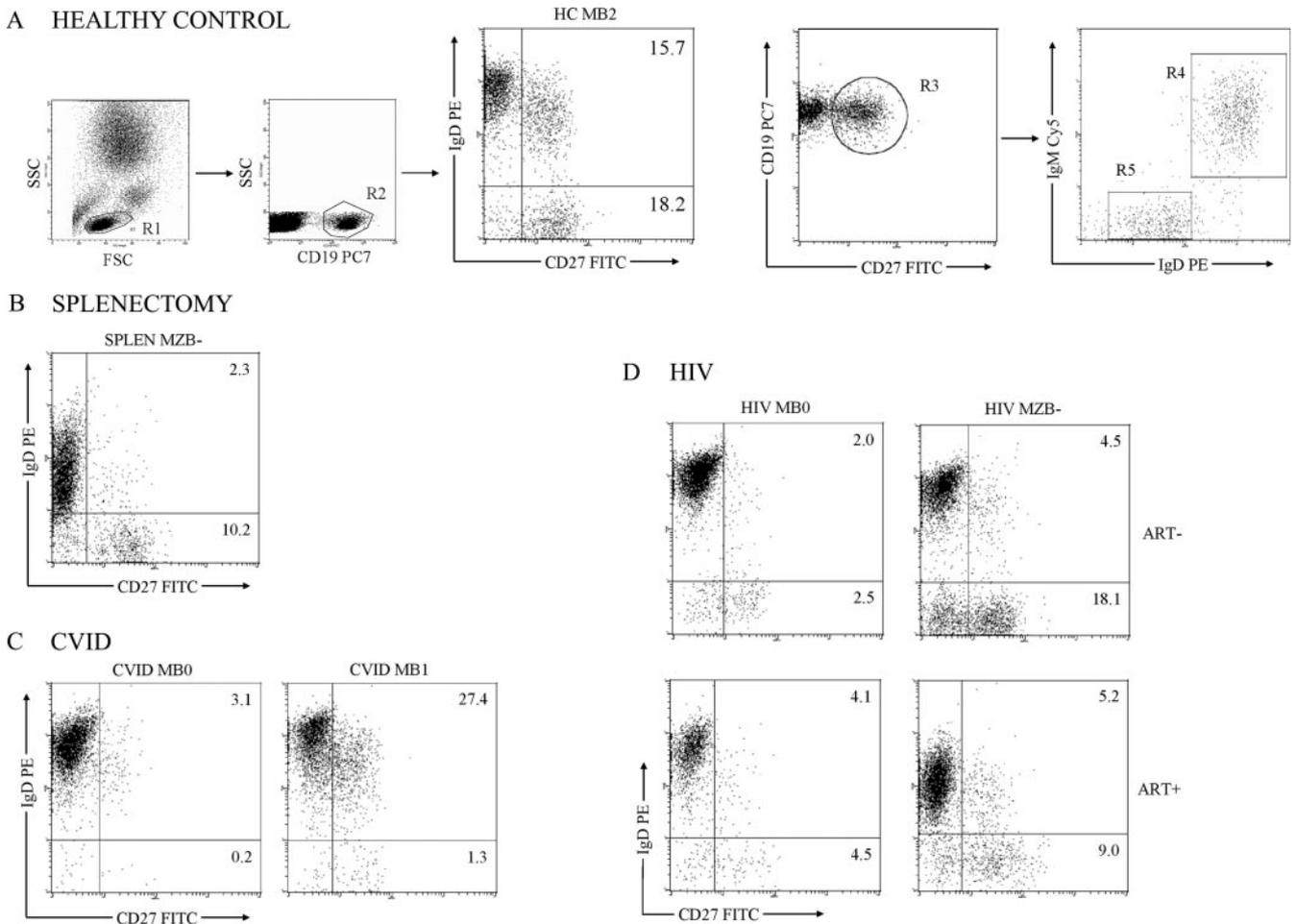
of 70 U (Protein Reference Unit, Sheffield, U.K.), was used together with an in-house standard for pneumococcal IgM levels to set up the standard curve.

The serum titer of IgG anti-tetanus toxoid was also measured. Ninety-six-well plates were coated with tetanus toxoid (National Institute for Biological Standards and Control, Potters Bar, U.K.) overnight at 4°C, washed in PBS/Tween 20 solution, and then a 5% milk/PBS/Tween 20 solution was added to individual wells for 1 h at 37°C. Patient samples and reference standards (National Institute for Biological Standards and Control, Potters Bar, U.K.) were then added for 1.5 h and samples were processed as outlined for the pneumococcal serology assays. For all ELISAs, all pre- and

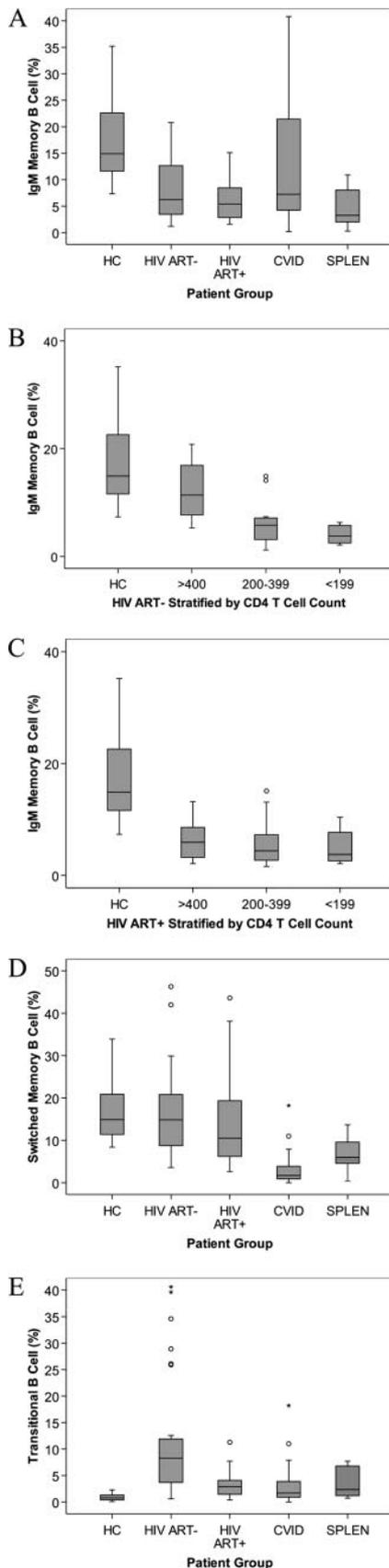
postvaccine sera were run on the same plate to minimize interassay variability. The concentration of IgG pneumococcal and tetanus Abs using this in-house method is similar to that seen with commercially available ELISAs or other in-house techniques in a U.K.-based external quality assurance scheme.

#### HIV viral load

Plasma viral load was routinely determined in all patients with HIV-1 infection using branched DNA amplification technology (HIV RNA 3.0 assay; Bayer Healthcare) with a lower limit of detection of 50 copies/ml.



**FIGURE 1.** FACS plots of memory B cell subsets in controls, splenectomized, CVID, and HIV-1 patients. *A*, Representative gating strategies used. Cells are gated on forward and side scatter (R1), then on a CD19 gate (R2). Expression of IgM and IgD shown on a representative HC, gated on CD27 (R3). All healthy controls satisfied the MB2 phenotype in the Piqueras classification for B cell differentiation. *B*, Reduced IgM memory B cells in patients with a history of splenectomy: MZB<sup>-</sup> phenotype. *C*, Memory B cell phenotype in patients with CVID: CVID patients with an MBO phenotype ( $n = 17$ ) and an MB1 phenotype ( $n = 9$ ). *D*, Memory B cell phenotype in HIV-1 patient groups who are not on ART (ART<sup>-</sup>) and who are receiving ART (ART<sup>+</sup>); HIV-1 (ART<sup>-</sup>) with an MBO phenotype ( $n = 16$ ) and an MZB<sup>-</sup> phenotype ( $n = 25$ ); HIV-1 (ART<sup>-</sup>) individuals with an MBO phenotype ( $n = 7$ ); HIV MZB<sup>-</sup> ( $n = 12$ ). Values (inset) correspond to the percentage of CD19<sup>+</sup> B cells within each quadrant. Data are representative of individuals within each subgroup analyzed.



**FIGURE 2.** B cell subset frequencies in controls, HIV-1 infection, CVID, and patients with a history of splenectomy. *A*, IgM memory B cell percentages in controls, HIV-1 patient groups, CVID, and splenectomized patients. *B*, IgM memory B cell percentage stratified by CD4 T cells counts in ART-naive HIV-1 patients. *C*, IgM memory B cell percentage stratified by CD4 T cells counts in HIV-1 patients on ART.

**Statistics**

The results for T and B cells subsets, HIV viral load, tetanus, and pneumococcal serology were expressed as median and interquartile ranges. Differences between patient groups were tested for statistical significance using the Mann-Whitney *U* test. Bonferroni correction analysis was performed to correct for multiple tests of statistical significance involving HIV patient groups and controls and the threshold for statistical significance was set at a *p* value of <0.01. Univariate correlations between different variables within a group were assessed using Spearman rank correlation tests. Partial regression analysis was also performed to correct for the influence of viral load on the relationship between CD4 T cell counts and different B cell subsets and on the relationship between CD4 T cells and B cell subsets. A  $\chi^2$  test with Yates' correction to correct for small sample size was used to compare the proportion of HIV-1 patients on ART who achieved IgM pneumococcal levels similar to healthy controls and to assess IgG pneumococcal vaccine responses. The Mann-Whitney *U* test was used to test our hypothesis that B cell memory proportions influenced postvaccine responses (IgM memory B cells and pneumococcal IgM levels: switched memory B cells and pneumococcal/tetanus IgG levels). All other secondary variables (CD4 T cell counts, markers of immune activation, viral load) which potentially could influence postvaccine Ab levels underwent Bonferroni analysis to correct for multiple statistical analysis. All statistical calculations were performed with SPSS version 14 software.

**Results**

HIV patients on ART had a significant increase in CD19<sup>+</sup> B cell counts compared with controls, ART-naive, and CVID patients (*p* = 0.009, Table I). Memory B cell (CD19<sup>+</sup>CD27<sup>+</sup>) percentages were reduced in all patient groups studied compared with controls (*p* < 0.001, Table I).

*IgM memory CD19<sup>+</sup>CD27<sup>+</sup>IgM<sup>high</sup>IgD<sup>low</sup>C21<sup>high</sup> B cells are reduced in HIV-1 infection and are not restored by ART*

The median percentage of IgM memory B cells was significantly reduced in both ART-naive and HIV-1 patients on ART compared with healthy controls (*p* < 0.001, Table II, Figs. 1C and 2A). Although absolute CD19 B cell counts were significantly elevated in the ART-treated HIV-1 patient group compared with healthy controls, median IgM memory B cells counts were still significantly lower (10.8 cells/ $\mu$ l vs 22.0 cell/ $\mu$ l *p* < 0.001). Patients with CVID had reduced circulating IgM memory B cells and, as expected, the most marked loss of IgM memory B cells was observed in individuals who had a history of splenectomy (Table II, Fig. 1B). Stratification of HIV patient groups by CD4 T cell counts showed that there was a significant decrease in median IgM memory B cell percentage in ART-naive HIV-1 patients with progressive infection (Fig. 2B). The percentage of median IgM memory B cells was 11.4% in the patient group with a CD4 T cell count of >400 cells/ $\mu$ l compared with 6.2% in patients with a CD4 T cell count of 200–399 cells/ $\mu$ l (*p* = 0.002). Partial regression analysis correcting for the influence of viral load showed a significant association between IgM memory B cell percentage and CD4 T cell count (*r* = 0.51, *p* = 0.01) in ART-naive patients. When ART-treated HIV patients were stratified by CD4 T cell count, the percentage of IgM memory B cells was significantly lower in all HIV patient groups, even in patients with CD4 T cells >400 cells/ $\mu$ l (Fig. 2C) compared with healthy controls (*p* < 0.001).

*Switched memory CD19<sup>+</sup>CD27<sup>+</sup>IgM<sup>-</sup>IgD<sup>-</sup> B cell percentage is markedly reduced in 25% of HIV-1-infected individuals*

There was no significant difference in the median percentage of switched memory B cells in both HIV patient groups compared

*D*, Class-switched memory B percentages in controls, HIV-1, CVID, and splenectomized patients. *E*, Transitional B cell percentages in controls, HIV-1, CVID, and splenectomized patients. HIV-1-infected patients not on ART (HIV ART<sup>-</sup>), HIV-1 patients receiving ART (HIV ART<sup>+</sup>), CVID patients.

Table III. Baseline pneumococcal and tetanus Ab levels in controls and HIV-1 patients<sup>a</sup>

	Pneumococcal IgM (U/ml)	Pneumococcal IgG (U/ml)	Tetanus IgG (IU/ml)
HC	30.0 (15–44.8)	88.0 (53.0–225.0)	2.7 (0.6–4.0)
HIV ART negative	21.0 (13.8–37.3)	43.0 (22.3–120.8)	0.4 (0.1–0.9)
HIV ART positive	17.0 (11.0–38.0)	29.0 (18.3–61.5)	0.4 (0.1–0.8)

<sup>a</sup> Data are shown for median and interquartile range for HC, HIV-1 patients not on ART (HIV ART negative), and HIV patients receiving ART (HIV ART positive). Almost all CVID (25 of 28) patients were on 3-weekly IgG replacement therapy (0.4 g/kg/mo), which contains exogenous tetanus and pneumococcal IgG Abs. Individuals with a history of splenectomy had already received pneumococcal vaccination as part of routine clinical care. Serology was performed as described in *Materials and Methods*.

with controls (Fig. 2D). Patients with CVID as expected had a significantly reduced median percentage of switched memory B cells compared with controls and with both HIV-1 patient groups. Classification of HIV patients on the basis of impaired B cell memory differentiation (27), showed that 15 of 55 (27%) HIV patients on ART and 6 of 29 (23%) ART-naive patients had marked reductions in switched B cell percentages (<8% of total B cell population) (Fig. 1C). Almost all patients with this B cell phenotype had a similar defect in IgM B cell percentage and would meet the MBO criteria as described by Piqueras et al. (27), which defines a subset of CVID patients (47% in this study) who have the greatest impairment in B cell differentiation. When the HIV patients on ART were stratified by CD4 T cell counts, a decline in switched memory B cell percentage was observed in HIV-1 patients with CD4 T cell counts <400 cells/ $\mu$ l compared with higher CD4 T cell numbers (8.9 vs 13.8%  $p = 0.002$ ) and controls (8.9 vs 14.9%  $p < 0.001$ ).

#### *HIV infection is associated with increased transitional B cell percentage*

The percentage of transitional B cell was significantly increased in all patient groups compared with healthy controls (Fig. 2E). Elevated transitional B cell counts were most striking in the ART-naive patient group and were of similar magnitude in patients with well-preserved CD4 T cell numbers as well as individuals with advanced disease (data not shown). Although there was an overall reduction in transitional B cell proportions in patients receiving ART, there was no significant difference between patients with good CD4 T cell responses to ART and individuals whose CD4 T cell counts had not reached 200 cells/ $\mu$ l. In contrast to the patient with CVID, we found no association between transitional B cell and CD27<sup>+</sup> memory B cell percentage in HIV-1-infected individuals (data not shown).

#### *Pneumococcal and tetanus Ab levels are reduced in HIV-1 patients*

Baseline pneumococcal IgG and tetanus toxoid Ab levels (Table III) were significantly reduced in both HIV patient groups compared with controls ( $p = 0.005$ ). Analysis of tetanus vaccination history for laboratory staff and ART-naive HIV-1 patient groups revealed no significant difference in time from previous tetanus immunization. Tetanus toxoid serum concentration was similar in both HIV-1 patient groups, which suggests that time since tetanus vaccination was unlikely to explain the reduction in tetanus Ab concentration observed in ART-exposed patients. Patients with CVID had a marked reduction in pneumococcal IgM levels compared with controls and HIV patient groups ( $p < 0.001$ ). There was no association seen with CD4 T cell numbers and pneumococcal (IgG and IgM) or tetanus toxoid (IgG) levels in controls or HIV patients. Comparative analysis of IgM memory B cell percentage and pneumococcal IgM Abs, which are markers of T cell-

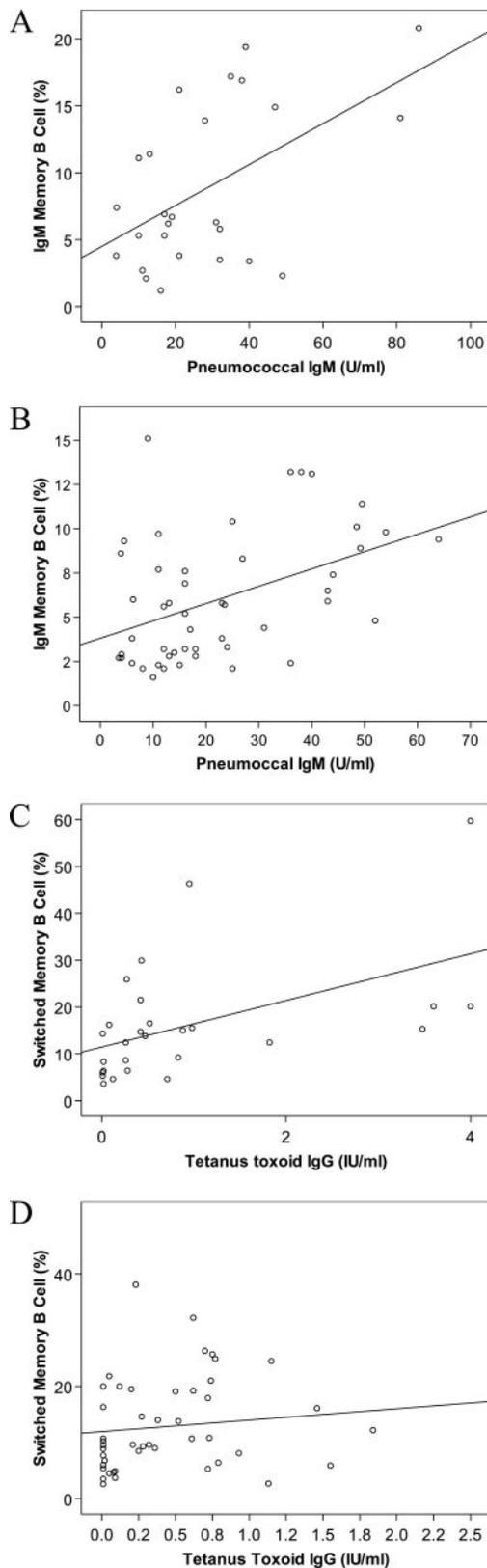
independent humoral immune responses, showed a significant association in ART-naive HIV-1 patients ( $r = 0.39$ ,  $p = 0.002$ ) (Fig. 3A) and in HIV-1 patients on ART ( $r = 0.36$ ,  $p = 0.011$ ) (Fig. 3B). We also found a significant association between class-switched memory B cell percentages and serum tetanus toxoid concentrations, which are indicators of T cell Ab responses, in ART-naive HIV-1 patients ( $r = 0.58$ ,  $p < 0.001$ ) (Fig. 3C) and in HIV-1 patients on ART ( $r = 0.30$ ,  $p < 0.034$ ) (Fig. 3D). No association between transitional B cells levels and tetanus and pneumococcal baseline serology was found in HIV-1 patients (data not shown).

#### *HIV-1-infected individuals on ART have impaired pneumococcal IgM vaccination responses that are associated with reduced IgM memory B cell percentages*

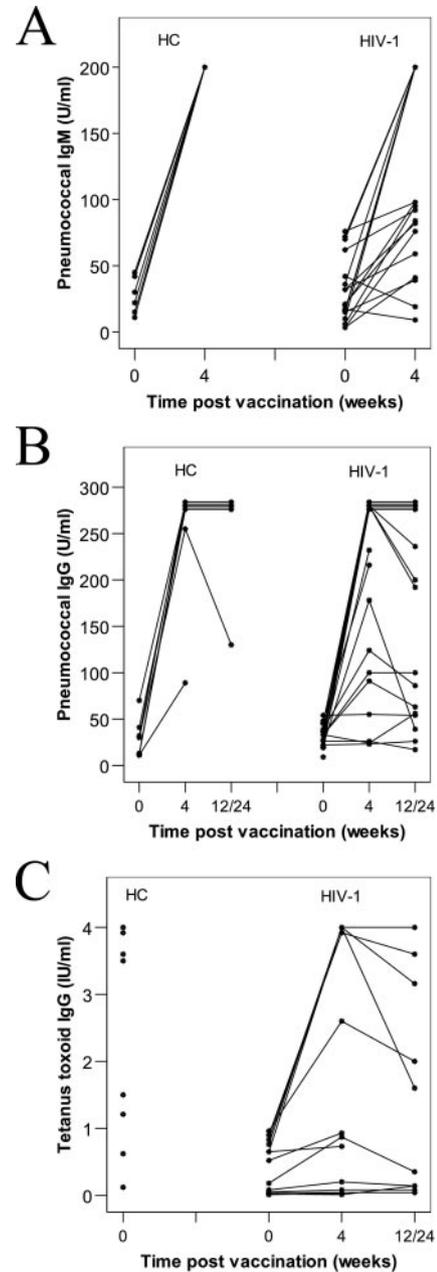
Vaccination responses to Pneumovax II were assessed in 19 patients on ART and in 8 controls without a history of respiratory disease. All controls achieved IgM pneumococcal Ab levels >200 U/ml at 4-wk postimmunization whereas only 7 of 19 patients with HIV infection mounted a similar vaccine response ( $p < 0.02$ , Fig. 4A). The percentage of IgM B cells was significantly higher in those HIV-1 patients who responded as well to Pneumovax II as healthy controls (Fig. 5A) compared with absent/impaired vaccine responders (7.6 vs 3.2%  $p = 0.003$ ). Baseline CD4 T cell, CD8<sup>+</sup>CD28<sup>-</sup> T cell, CD4<sup>+</sup>CD28<sup>-</sup> T cell, CD19 B cell counts, pneumococcal IgM Ab levels, transitional, switched B cell percentage, CD8<sup>+</sup>CD38<sup>+</sup> percentage did not influence postvaccine pneumococcal IgM Ab levels. Pneumococcal IgG vaccine concentrations at 4 wk were also reduced in HIV-1-positive patients compared with healthy controls; 7 of 18 HIV-1 patients on ART achieved a postimmunization concentration of 225 U/ml compared with 7 of 8 controls ( $p < 0.05$  Fig. 4B). Immunization responses 3–6 mo postvaccine showed that Ab levels had declined in two HIV-1-infected patients and in one healthy control, all of whom had achieved an initial postvaccine target pneumococcal Ab level. The median CD4 T cell count was slightly higher in HIV-1 patients who responded well to Pneumovax II 3–6 mo after immunization (696 cells/ $\mu$ l v 382 cells/ $\mu$ l); this difference was not significant after correction for multiple statistical analysis. No other significant correlation was found between any other baseline T cell, B cell immune parameter, or nadir CD4 T cell count with postvaccine pneumococcal IgG levels.

#### *Reduced switched B cell percentages are associated with absent/impaired tetanus vaccine responses in HIV-1 patients on ART*

The response to tetanus toxoid vaccination was also impaired in HIV-1 patients on ART compared with healthy controls who had a history of tetanus immunization in the United Kingdom (Fig. 4C). Four of 14 patients immunized with tetanus maintained a 4-fold rise in Ab response at 3–6 mo postimmunization. The most striking finding was that baseline tetanus serology strongly influenced postimmunization tetanus concentration: all patients with at

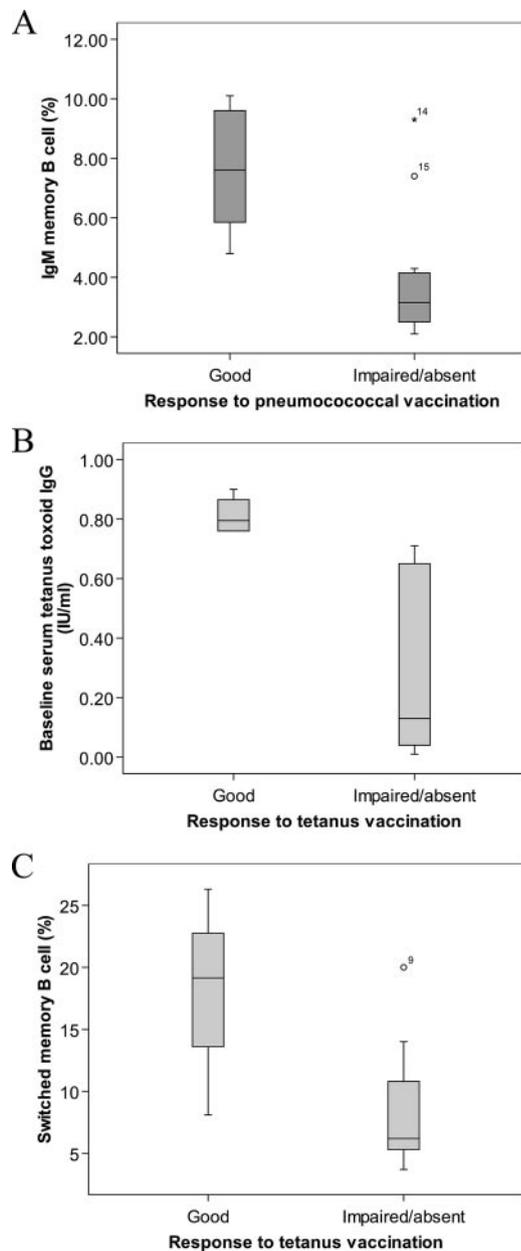


**FIGURE 3.** Scatter plots evaluating associations between pneumococcal IgM and tetanus IgG levels and B cell memory subsets in patients with HIV-1 infection. *A*, Association between pneumococcal IgM levels and IgM memory B cell percentages in ART-naive HIV-1 patients. *B*, Association between pneumococcal IgM levels and IgM memory B cell percentages in ART-treated HIV-1 patients. *C*, Association between class-switched memory B cell percentage and tetanus toxoid Ab concentration in ART-naive HIV-1 patients. *D*, Association between class-switched memory B cell percentage and tetanus toxoid Ab concentration in HIV-1 patients on ART.



**FIGURE 4.** Postimmunization pneumococcal IgM, IgG, and tetanus toxoid IgG vaccine responses in HIV-1 patients on ART. *A*, Postvaccine pneumococcal IgM levels in controls and HIV-1 patients. Data are shown for 19 patients and 8 controls at baseline and week 4. *B*, Postvaccine pneumococcal IgG levels in controls and HIV-1 patients. Data are shown for 19 patients at baseline and week 4 and 14 patients at 3–6 mo, eight controls at time 0, and four and six controls 3–6 mo postimmunization. *C*, Postvaccine tetanus toxoid IgG levels; data are presented for 14 HIV-1 patients at time 0, week 4, and 12 patients at 3–6 mo postimmunization. Serum tetanus toxoid concentration is shown for health care workers ( $n = 16$ ) with a known tetanus toxoid vaccination history. Although the inclusion criteria for tetanus vaccination was a serum toxoid concentration  $<1.0$  IU/ml almost all controls had serum toxoid levels greater than this value. The data shown illustrate the expected target range for post-tetanus vaccine responses in the HIV-1 patients receiving ART.

least a 4-fold rise in tetanus serology with long-term protection had tetanus toxoid Ab levels  $>0.5$  IU/ml (Figs. 4C and 5C). The median switched B cell proportion was higher in tetanus toxoid vaccine responders compared with patients who had impaired/absent immunization responses 3–6 mo postvaccination. (19.0 vs 6.2%,



**FIGURE 5.** Immune parameters which influence IgM pneumococcal and IgG tetanus toxoid Ab responses in HIV-1 patients on ART. *A*, IgM memory B cell percentage in HIV-1 patients with good and impaired/absent IgM pneumococcal Ab responses. *B*, Baseline serum tetanus toxoid Ab concentration in HIV-1 patients with good and impaired/absent IgG tetanus Ab responses. *C*, Class-switched memory B cell percentage in HIV-1 patients with good and impaired/absent IgG tetanus Ab responses. Criteria to define good and impaired/absent vaccine responses are described in *Materials and Methods*.

$p = 0.04$ ) (Fig. 5C). The difference in tetanus responses was not explained by current or nadir CD4 T cell counts.

## Discussion

HIV infection is associated with a wide range of B cell defects, including polyclonal hypergammaglobulinemia, enhanced expression of markers of B cell activation, decreased B cell survival (28–31), reduction of CD19<sup>+</sup>CD27<sup>+</sup> memory B cells (10–12), and impaired Ab responses to tetanus, influenza, and pneumococcal vaccination (7, 8, 32). In this study, analysis of two major B cell memory subsets showed that ART-naïve and -treated HIV

patients had a significant reduction in IgM memory B cells similar to that seen in patients with CVID. In 70% of all HIV patients studied, the percentage of IgM B cells was the same as that seen in individuals who had a history of splenectomy. A significant reduction or absence of IgM memory B cells is also found in other patient groups (the elderly, children under 2 years of age, or patients with congenital asplenia) who have an increased risk of invasive pneumococcal infection (33, 34). Further support for the importance of IgM memory B cells in host protection against pneumococcal infection comes from studies of CVID patients which show an association between recurrent chest infections and reduced concentration of pneumococcal IgM Abs and lower IgM memory B cell counts (33, 35). The median IgM memory B cell percentage in CVID patients with bronchiectasis in this study was lower than that in patients without bronchiectasis (5.1 vs 14.8), although the difference was not statistically significant. In this study, HIV patients with reduced IgM memory B cell counts were found to have impaired pneumococcal IgM Ab responses postvaccination compared with controls. A failure to mount IgM antipolysaccharide immune responses may account for the increased risk of invasive pneumococcal infection in HIV-1 patients. Further studies should elucidate the contribution of functional hyposplenism or impaired mucosal immune responses to the risk of invasive pneumococcal infection in HIV-1 infection. Some potent neutralizing HIV-1 Abs are generated by IgM memory B cells and it has been suggested that efforts to exploit the properties of this B cell subset may be a useful strategy to develop prophylactic vaccines against HIV (36). The reduction in numbers and function of IgM memory B cells suggests another potential obstacle for successful HIV vaccine development and may represent another immune evasion strategy used by the HIV-1 virus to subvert potent antiviral immune responses.

The efficacy of pneumococcal vaccination in HIV-1 infection is controversial; Ab responses are reduced in ART-naïve HIV-1 infected with CD4 T cell counts <500 cells/ $\mu$ l, resulting in a U.S. Public Health Service recommendation that pneumococcal vaccination should be offered early in HIV-1 infection as such patients will have well-preserved CD4 T cell counts (2, 37). Postvaccination pneumococcal IgG levels were also impaired in HIV patients on ART which agrees with previous data that the immune responses to polysaccharide pneumococcal vaccine are modest in this patient group (4).

Twenty-five percent of all HIV-1 patients in this study had a major reduction in switched B cell counts and in almost all cases this was associated with a significant loss of IgM memory B cells. Significant increases in serum toxoid Ab responses following vaccination were only seen in a minority of ART-treated HIV patients and were associated with higher pre-existing tetanus toxoid Ab levels and an increase in class-switched memory B cell percentage. Loss of IgM memory B cells was strongly associated with impairment of pneumococcal IgM vaccine responses; however, the relationship between loss of switched memory B cells and impaired/absent tetanus IgG vaccine responses was not as pronounced. The response to tetanus vaccination is CD40 dependent, which suggests that either intrinsic defects in the B cell response to CD4 T cell help or loss of CD4 T cells that directly promote CD40-dependent Ab responses may play an additional role in the impairment of T cell-dependent humoral immune responses in HIV-1 infection.

The ontogeny of IgM memory B cells in humans is uncertain; there is evidence that this cell population may be a distinct B cell lineage derived from precursor transitional B cells, or that they arise during GC B cell differentiation (19). Increased numbers of transitional B cells are a characteristic feature of several inherited

humoral immunodeficiency conditions and may also be seen in HIV infection (21, 22). Maturation of transitional B cells is dependent in part on the expression of receptors for TNF superfamily proteins, including B cell-activating factor (BAFF) and a proliferating-inducing ligand (APRIL) (16, 38–40). The HIV-1 gp120 protein enhances BAFF production by macrophages (41) and increased serum BAFF levels are associated with progressive HIV-1 disease (42). Increased expression of B cell maturation Ag (one of the receptors for APRIL) and reduced levels of BAFF receptor have been linked to decreased survival of B cells in HIV-1 infection (31). It has been suggested that BAFF-induced polyclonal activation could lead to functional B cell exhaustion and impairment of humoral immune responses in HIV-1 infection (41). Analysis of CVID patients has shown that abnormalities in the BAFF/APRIL/transmembrane activator and calcium modulator and cyclophilin ligand (TACI) receptor network are associated with disruption of B cell function (43–45) and further studies should investigate whether defects in the expression or function of TNF superfamily proteins accounts for the failure of B cell differentiation observed in this study.

In conclusion, the results of this study show that there are significant reductions in distinct memory B cell subsets in patients with HIV infection. Loss of discrete B cell memory is associated with diminished vaccination responses to T cell-dependent and -independent Ags in HIV-1-infected individuals. Data presented may account for the increased risk of invasive pneumococcal infection observed in HIV-1 infection. Intrinsic defects in B cell function may contribute to clinically significant complications of HIV-1 infection and be responsible for impaired humoral immune responses to vaccination observed in this condition.

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## Disclosures

The authors have no financial conflict of interest.

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