

## Impaired Naive and Memory B-Cell Responsiveness to TLR9 Stimulation in Human Immunodeficiency Virus Infection<sup>∇</sup>

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**Toll-like receptor 9 (TLR9) agonists such as unmethylated bacterial CpG DNAs activate B lymphocytes directly, potentially influencing their function and homeostasis. To assess B-cell responsiveness to TLR9 agonists in human immunodeficiency virus (HIV) disease, we examined the ability of naive and memory B cells to proliferation and to increase surface expression of CD80 in response to CpG oligonucleotides (ODN). CpG ODN induced expression of CD80 similarly in B cells from HIV-infected persons and from healthy controls. In contrast, proliferation responses to CpG ODN were markedly impaired in both naive and memory B-cell subsets from HIV-infected persons. Naive B-cell proliferation defects were related to plasma HIV RNA and, among memory B cells, to the frequencies of CD21-negative cells. Importantly, TLR9 mRNA levels were significantly diminished in freshly prepared naive B cells and especially so in memory B cells from HIV-positive viremic donors, suggesting a possible underlying mechanism for the observed functional impairments. Dose-response studies indicated that optimal induction of CD80 expression was achieved with much lower concentrations of CpG ODN than optimal induction of proliferation. We propose that the relatively low threshold of activation that is required for CD80 induction by CpG ODN might explain the preservation of this response in B cells from HIV-infected persons despite diminished TLR9 expression. Impaired responsiveness to TLR9 agonists may contribute to defects in humoral immunity in HIV infection.**

Human immunodeficiency virus (HIV) infection is associated with multiple defects in immune homeostasis and function. The most prominent pathogenic feature of HIV infection is the loss of CD4<sup>+</sup> T lymphocytes, which predicts an increased susceptibility to opportunistic infections. Nevertheless, there are marked perturbations in other cells that also may play an important role in progressive immunodeficiency. For example, B lymphocytes from HIV-positive (HIV<sup>+</sup>) donors display numerous abnormalities, including poor responses to mitogen stimulation *in vitro* (21, 25) and increased susceptibility to apoptosis and increased spontaneous secretion of immunoglobulins (Igs) (8, 21). These functional alterations may stem in part from sustained *in vivo* activation of B cells during HIV infection that results in increased frequencies of activated and terminally differentiated cells. B-cell dysfunction also may arise from lack of CD4 help. Regardless of the mechanism, disruption of normal B-cell function is one of the key immune deficiencies of HIV infection that may underlie the impaired antibody response to immunization seen in HIV disease (11).

Induction of humoral immunity is dependent on activation of the B-cell receptor (BCR) by cognate antigen as well as costimulation provided by helper T cells. BCR stimulation can be augmented by engagement of Toll-like receptors (TLRs), such as TLR9 (28). B cells stimulated through the BCR in the

presence of CpG oligonucleotides (ODN) that bind to TLR9, for example, expand vigorously and may undergo isotype switching even in the absence of CD4<sup>+</sup> T-cell help (28). In addition to playing a role in B-cell costimulation, TLR agonists also have been implicated as direct mediators of memory B-cell activation, supporting B-cell homeostasis and sustained Ig production (2). We and others have recently demonstrated that TLR9 agonists also can activate naive B cells in an antigen-independent manner, inducing proliferation and Ig production by these cells (14, 17). These responses may play an important role in the development of adaptive immunity during periods of microbial challenge.

Responsiveness to TLR agonists is an important consideration in HIV disease, where microbial translocation across the damaged gut mucosa may increase exposure to these agonists *in vivo* and may contribute to the chronic immune activation that is associated with disease progression (9; W. Jiang, M. Lederman, S. Sieg, K. Haley, B. Rodriguez, A. Asher, D. C. Douek, and J. Brechley, presented at the 15th Conference on Retroviruses and Opportunistic Infections, 2008). Although TLR9 ligation is thought to play an important role in B-cell function, the responses of B cells from HIV-infected persons to these agonists have not been fully characterized (7, 29, 32).

CpG ODN are synthetic DNA sequences with unmethylated CpG motifs that activate human plasmacytoid dendritic cells (pDCs) and B cells by interacting with TLR9 (20). Responsiveness to CpG ODN stimulation may be diminished in HIV disease in part due to numeric and functional deficiencies in pDCs (6, 10, 28). We hypothesized that B cells from HIV-infected persons also may be poorly responsive to TLR9 stim-

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TABLE 1. Clinical characteristics

Donor group and viral load	Total no.	No. female/male	Age (yr) <sup>a</sup>	CD4 <sup>+</sup> T-cell count (cells/mm <sup>3</sup> ) <sup>a</sup>	Plasma HIV RNA level (copies/ml) <sup>a</sup>	No. on HAART/treatment naive
HIV <sup>-</sup>	10	4/6	35 (23–50)			
HIV <sup>+</sup>						
≤400	21	4/17	44 (30–62)	611 (68–1,681)	≤400	20/1
>400	25	4/21	42 (23–65)	322 (0.8–1,041)	84,512 (503–303,000)	15/10

<sup>a</sup> Data are median (interquartile range) values.

ulation, since a number of other functional defects have been observed in these cells (22, 25, 32).

## MATERIALS AND METHODS

**Study subjects.** These studies were approved by the University Hospitals of Cleveland Institutional Review Board, and all human participants gave written informed consent. Peripheral blood samples from 46 HIV<sup>+</sup> donors and 12 HIV-negative (HIV<sup>-</sup>) donors were studied. Clinical characteristics of the subjects are shown in Table 1.

**Reagents.** Endotoxin-free CpG ODN 2006 (TCGTCGTTTGTGCGTTTGTGCGTTT) was provided by Coley Pharmaceutical Group Inc. (Wellesley, MA). Titration experiments using a range of concentrations from 0.01 to 24 μg/ml indicated that 6 μg/ml provided a minimum concentration with optimal effect.

**Cell preparation and culture.** Peripheral blood mononuclear cells (PBMCs) from HIV-infected donors and healthy subjects were isolated from whole blood by centrifugation using Ficoll-Hypaque density sedimentation. To measure induction of CD80 expression, PBMCs were suspended in RPMI 1640 supplemented with 10% human AB serum, plated at 2 million cells/well in 24-well culture dishes (BD Labware, Franklin Lakes, NJ), and incubated for 20 h with or without CpG ODN 2006 (6 μg/ml). To evaluate cell division, PBMCs or purified B-cell subsets obtained by negative magnetic bead selection (Miltenyi AutoMACS cell sorter) were labeled with carboxyfluorescein succinimidyl ester (CFSE) as previously described (17, 18) and incubated for 7 days. CFSE-labeled cells (2 × 10<sup>6</sup> cells/well) were cultured in 24-well plates with or without CpG ODN (6 μg/ml) for 7 days. Proliferation was quantified by measuring the percentage of cells that diluted tracking dye.

**Flow cytometry.** Cells were resuspended in phosphate-buffered saline-bovine serum albumin staining buffer and incubated at room temperature for 10 min with the fluorochrome-conjugated monoclonal antibodies anti-IgD-phycoerythrin (PE), CD27-PE or -allophycocyanin, CD20-peridinin chlorophyll protein, anti-CD19-allophycocyanin, anti-CD21-fluorescein isothiocyanate, and anti-CD80-PE or -PE-cyclin-5' and appropriate isotype control monoclonal antibodies (BD Pharmingen, San Jose, CA). Cells were washed twice prior to analyses on a FACS Calibur flow cytometer.

**Real-time PCR.** TLR9 mRNA expression in purified memory and naive B cells was measured by real-time PCR. Cells were purified by magnetic bead selection. Total B cells from human PBMCs were isolated using a cocktail of biotinylated anti-CD2, -CD14, -CD16, -CD36, -CD43, and -CD235a (glycophorin A) antibodies to deplete T cells, NK cells, monocytes, DCs, granulocytes, platelets, and erythroid cells. The purity of total B cells was always >98%. pDCs were further depleted from the purified total B cells with BDCA-4 microbeads, and pDC contamination was always <0.01%. Finally, purified naive B cells (CD27<sup>-</sup>; purity, >99%) and memory B cells (CD27<sup>+</sup>; purity, >95%) were obtained by use of CD27 microbeads. CD27 is a member of the tumor necrosis factor receptor family and is a marker of memory B cells (1, 12). Cells were lysed, and RNA was extracted using an RNA isolation kit (Qiagen, Hilden, Germany). An 8-μl aliquot of RNA was reverse transcribed with oligo(dT) primer (First Strand cDNA synthesis kit; Roche, Mannheim, Germany). The resulting cDNA was diluted 1:5 with water, and 5 μl was used for amplification. The real-time PCR was performed on an ABI 7700 instrument (Applied Biosystems, CA) using Sybr PCR mix (Applied Biosystems). To control for specificity of the amplification products, a melting curve analysis was performed. TLR9 mRNA levels were determined by quantitative real-time PCR and expressed as the number of transcripts per 10<sup>3</sup> copies of the glyceraldehyde-3-phosphate dehydrogenase (GADPH) housekeeping gene. The thermal cycler was programmed to perform an initial setup (95°C, 10 min) and 40 cycles of denaturation (95°C, 15 s) followed by annealing/extension (60°C, 1 min). The median mRNA copy number from three independent analyses was calculated for each sample. Primers were designed

using Primer3 software for TLR9 (forward, 5'-TGAAGACTTCAGGCCCAAC TG-3'; reverse, 5'-TGCACGGTCACCAGGTTGT-3') and for GADPH (forward, 5'-caatgaccctcattgacc-3'; reverse, 5'-gacaagctccggttctcag-3').

**Statistical methods.** Relationships between continuous variables were assessed by Spearman's test, and multivariate analyses were conducted by fitting multiple regression models using a backward stepwise strategy. The Wilcoxon rank test was used for comparison between two groups, and one-way analysis of variance (ANOVA) was used for comparisons among groups. All tests were two sided, and a *P* value of <0.05 was considered significant.

## RESULTS

**Impaired proliferation of naive and memory B cells from HIV-infected persons in response to CpG ODN.** Memory (2) and naive (17) B cells proliferate in direct response to CpG ODN. To ascertain whether B-cell proliferation in response to CpG ODN is impaired in HIV disease, we measured the expansion of naive (CD19<sup>+</sup> CD27<sup>-</sup>) and memory (CD19<sup>+</sup> CD27<sup>+</sup>) B cells following stimulation of PBMCs with an optimal concentration (6 μg/ml) of CpG ODN 2006 (Fig. 1). Both naive and memory B cells from HIV-infected persons demonstrated striking defects in cellular proliferation after stimulation with CpG ODN compared to the responses of B cells from healthy donors (Fig. 1A). These defects were particularly pronounced among individuals with detectable plasma HIV RNA levels (>400 copies/ml) (Fig. 1B) and were not different between viremic HIV<sup>+</sup> donors receiving highly active antiretroviral therapy (HAART) and viremic HIV<sup>+</sup> donors not on HAART (Fig. 1C). Importantly, we found little evidence of changes in CD27 expression following CpG ODN stimulation in purified naive and memory B-cell populations after stimulation with CpG ODN, ruling out the possibility that CpG ODN affected CD27 expression (data not shown).

To confirm these findings, in a smaller subset of subjects (*n* = 6 for HIV<sup>-</sup> donors, *n* = 4 for aviremic HIV<sup>+</sup> donors and *n* = 4 for viremic HIV<sup>+</sup> donors), we studied purified naive and memory B cells as the responder populations and also noted defects in cellular proliferation (median percentages of CFSE-low cells = 31.2, 20.2, and 1.2 for memory B cells and 22, 15.1, and 2.7 for naive B cells from HIV<sup>-</sup> donors, aviremic HIV<sup>+</sup> donors, and viremic HIV<sup>+</sup> donors, respectively). Thus, B cells from HIV-infected subjects have an intrinsic impairment in proliferation responses to TLR9 stimulation that is independent of any other cell types present in PBMC cultures.

**Induction of CD80 expression by CpG ODN in B cells from HIV<sup>+</sup> donors is related to CD4<sup>+</sup> T-cell counts.** Like other TLR agonists, CpG ODN can activate professional antigen-presenting cells to express costimulatory molecules. We assessed the expression of CD80 on naive and memory B cells in freshly isolated PBMCs (Fig. 2A and B) and on B cells in PBMC

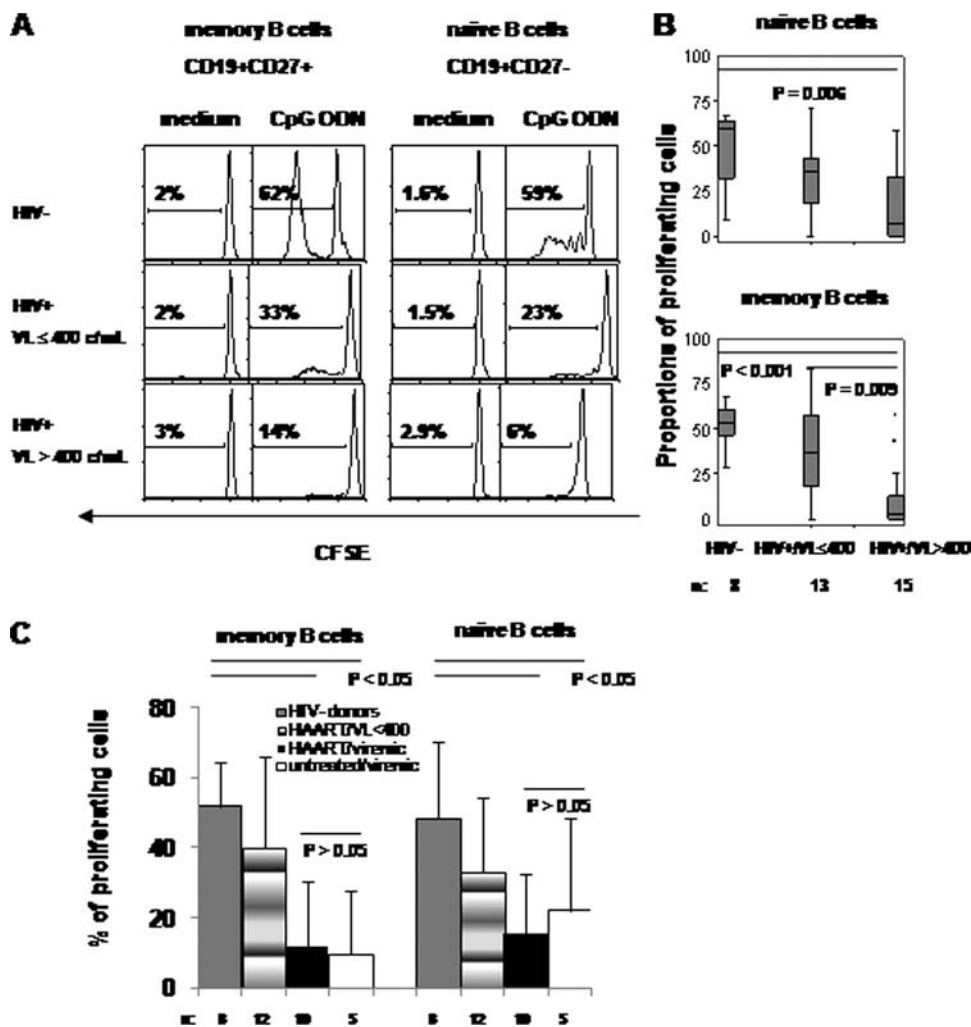


FIG. 1. Impaired expansion of naive and memory B cells in response to CpG ODN in viremic HIV-1 infection. PBMCs were labeled with CFSE and cultured with or without CpG ODN2006 at 6 μg/ml for 7 days. (A) Naive B-cell (CD19<sup>+</sup> CD27<sup>-</sup>) and memory B-cell (CD19<sup>+</sup> CD27<sup>+</sup>) expansion was evaluated by flow cytometric measurement of dye dilution. (B) Summary data (medians and interquartile ranges) of B-cell proliferation by CpG ODN for HIV-seronegative controls and for HIV-infected patients with undetectable (≤400 copies/ml) and detectable (>400 copies/ml) plasma HIV viremia. (C) Medians of B-cell proliferation for HIV-seronegative controls, HIV<sup>+</sup> aviremic patients receiving HAART, HIV<sup>+</sup> viremic patients receiving HAART, and untreated HIV<sup>+</sup> viremic patients. P values were calculated by one-way ANOVA tests.

cultures that had been incubated overnight in medium alone or in medium plus CpG ODN (Fig. 2C). The frequencies of CD80<sup>+</sup> B cells were increased among memory B cells in freshly isolated PBMCs from HIV<sup>+</sup> donors, especially among patients with plasma HIV RNA levels above 400 copies/ml (Fig. 2B). A similar trend to increase was observed among naive B cells, although the differences did not reach statistical significance (Fig. 2B). After overnight incubation in the presence of CpG ODN, CD80 expression on both memory and naive B cells was increased (Fig. 2C). CpG ODN exposure increased CD80 expression directly in purified naive (17) and memory (not shown) B cells, indicating that intermediary cells were not necessary for this effect. CD80 induction by CpG ODN was not significantly different between B cells from HIV<sup>-</sup> donors and B cells from HIV<sup>+</sup> donors (Fig. 2D). In HIV<sup>+</sup> viremic donors, there was a direct relationship between the magnitude of the induction of CD80 on memory B cells and CD4<sup>+</sup> T-cell counts (Fig. 2E) (Spearman's  $r = 0.62$ ,  $P = 0.006$ ), and a

similar trend was observed in naive B cells from these same donors, although the relationship did not reach statistical significance (Fig. 2E) (Spearman's  $r = 0.38$ ,  $P = 0.12$ ). With inclusion of all HIV<sup>+</sup> donors, the induction of CD80 on naive B cells was related to CD4 cell counts (Spearman's  $r = 0.32$ ,  $P = 0.05$ ) (data not shown). We found no evidence that the induction of CD80 expression was correlated with naive or memory B-cell proliferation in response to CpG ODN (Spearman's correlation test,  $P = 0.18$ ).

**Naive and memory B-cell proliferation defects are related to plasma HIV RNA levels and frequency of CD21-negative B cells.** Earlier studies (25) have established that circulating B cells from HIV-infected persons are enriched for activated cells lacking the complement receptor, CD21. Since CD21-negative B cells proliferate poorly in response to mitogens (25), we assessed the expression of CD21 on freshly isolated B cells from HIV<sup>+</sup> and HIV<sup>-</sup> donors. Striking differences in CD21 expression on naive and memory B cells from HIV<sup>+</sup> and

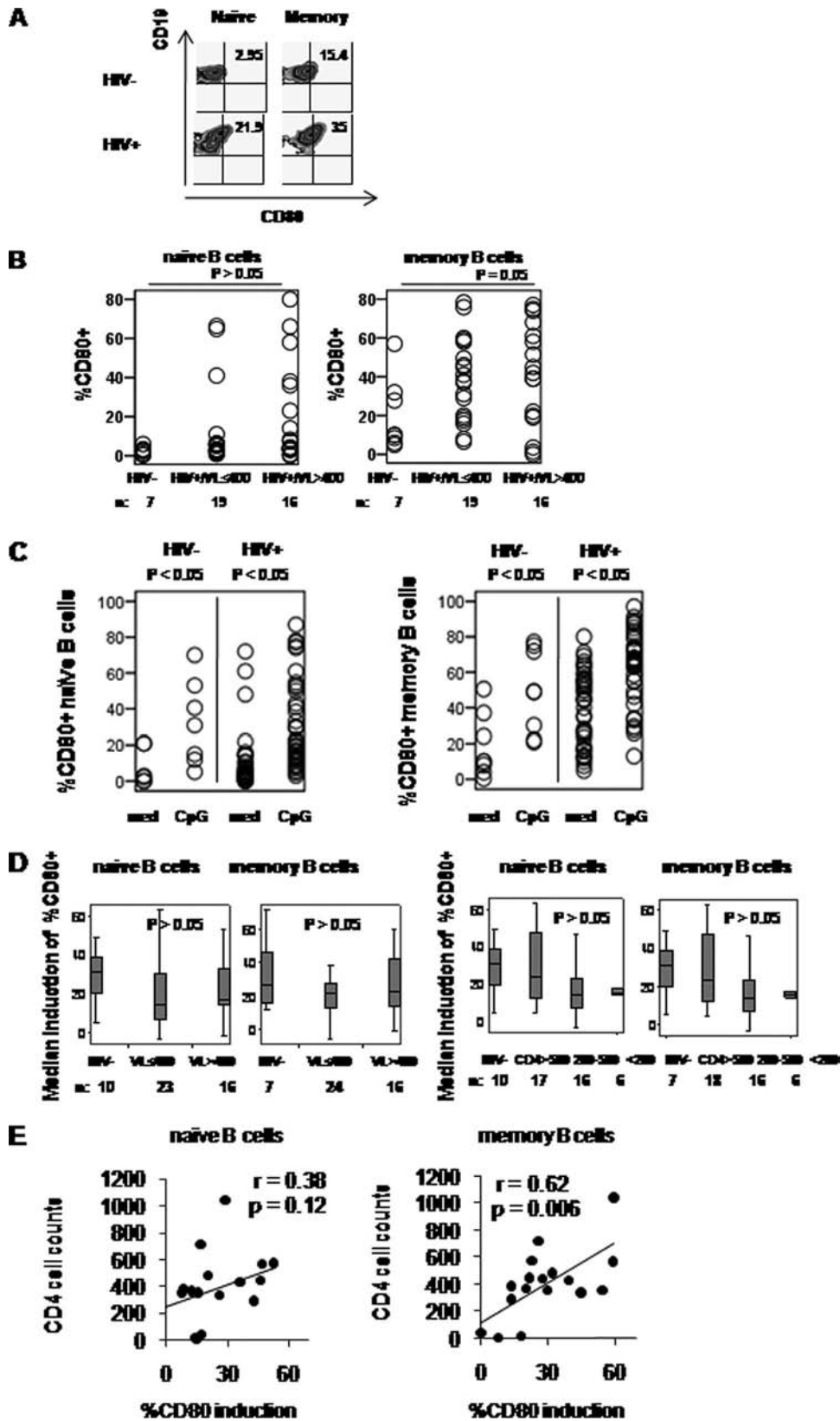


FIG. 2. Increased CD80 expression on memory B cells in HIV infection. PBMCs were analyzed by flow cytometry following staining with antibodies to CD19, CD27, and CD80. (A) Percentage of CD80 expression from one representative analysis among freshly isolated cells. (B) Percentages of CD80<sup>+</sup> naïve and memory B cells among freshly isolated cells. (C) Percentages of CD80<sup>+</sup> cells among naïve and memory B cells within unseparated PBMCs cultured overnight with or without CpG ODN. (D) Median percentage of cells that were induced to express CD80

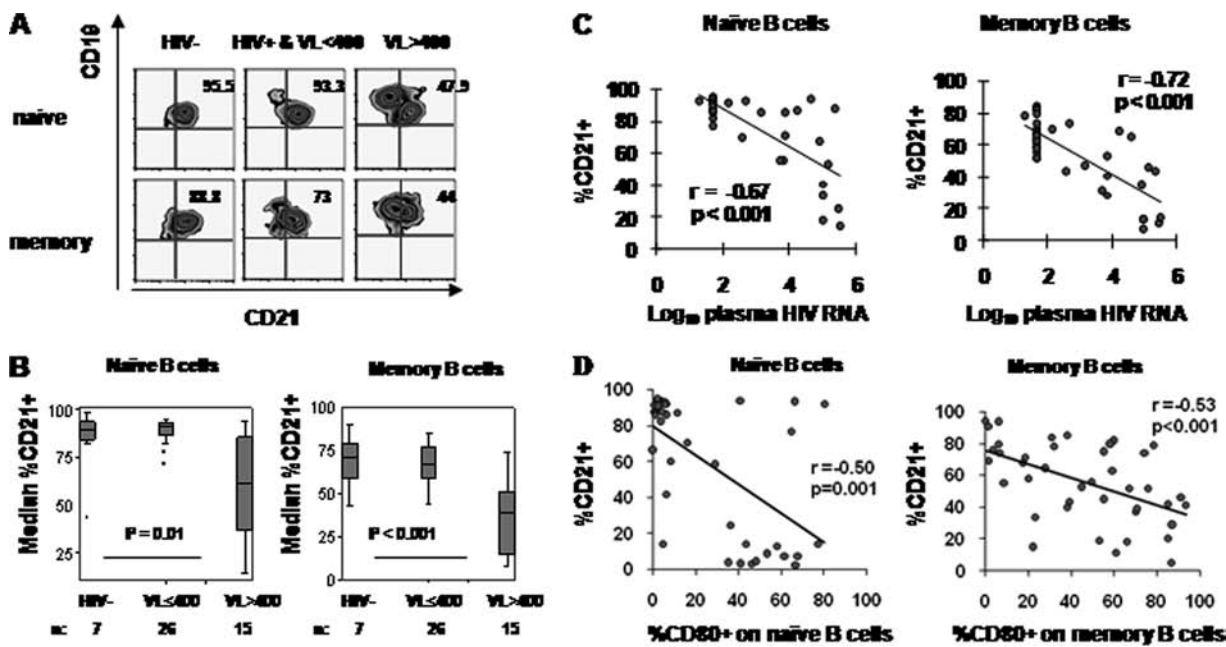


FIG. 3. CD21-positive B-cell frequencies in HIV infection are inversely related to plasma HIV RNA level and CD80 expression. (A) B-cell CD21 expression in one representative analysis among gated naive (CD27<sup>-</sup>) and memory (CD27<sup>+</sup>) B (CD19<sup>+</sup>) B cells. (B) Median percentages of CD21<sup>+</sup> naive and memory B cells in freshly isolated cells; comparisons were performed using one-way ANOVA and the Tukey test. (C) Among patients, plasma HIV RNA levels are inversely correlated with CD21 frequencies among freshly isolated naive and memory B cells ( $P < 0.001$ , Spearman's correlation). (D) In B-cell subsets from HIV-infected patients, the expression of CD80 was inversely related to the frequency of CD21<sup>+</sup> cells in both freshly isolated naive ( $P = 0.001$ ) and memory ( $P < 0.001$ ) B cells (Spearman's correlation).

HIV<sup>-</sup> donors were noted. CD21 expression was diminished among both the naive and memory B-cell subsets in HIV disease, and this was especially pronounced among individuals with plasma HIV RNA levels of >400 copies/ml (Fig. 3A and B). We found an inverse relationship between the frequency of CD21<sup>+</sup> naive and memory B cells and both plasma HIV RNA levels (Fig. 3C) and the frequencies of CD80 expression (Fig. 3D) among freshly isolated cells by Spearman's correlation test. Thus, viral replication and B-cell activation (as indicated by CD80 expression) may result in decreased frequencies of CD21<sup>+</sup> B cells in HIV disease.

We next examined the relationship between B-cell proliferation defects and CD80 induction by CpG ODN, frequencies of CD21<sup>+</sup> or CD80<sup>+</sup> B cells in freshly isolated samples, and clinical characteristics (plasma HIV RNA level, CD4<sup>+</sup> T-cell count, and age). CD4<sup>+</sup> T-cell counts were positively correlated with naive B-cell proliferation and HIV type 1 RNA levels were negatively correlated with naive B-cell proliferation in response to CpG ODN (Fig. 4A), and CD21 expression and CD4<sup>+</sup> T-cell counts positively correlated with memory B-cell expansion in response to CpG ODN (Fig. 4A). Multivariable regression analyses (Fig. 4B) indicated that CD21 expression was independently related to memory B-cell expansion and that the plasma HIV RNA level was independently related to

impaired naive B-cell expansion in HIV disease. Since plasma HIV RNA also was tightly related to CD21 expression among memory B cells (Fig. 3C), it is possible that viremia could contribute to memory B-cell defects by causing an accumulation of CD21<sup>-</sup> B cells in HIV disease. In contrast, HIV viremia, but not the frequency of CD21 expression on naive B cells, predicted impaired naive B-cell proliferation responses to CpG ODN. Taking the results together, it seems likely that HIV replication contributes to defects in the proliferation responses of both B-cell subsets in response to TLR9 stimulation.

**Reduced TLR9 mRNA expression among memory and naive B cells from viremic HIV<sup>+</sup> patients.** To determine whether altered TLR9 expression in HIV infection might contribute to the defects in TLR9 responsiveness, we measured ex vivo TLR9 mRNA levels among purified naive and memory B cells from HIV<sup>-</sup> and HIV<sup>+</sup> donors by real-time PCR (Fig. 5A). Importantly, these purifications included an additional depletion of CD123<sup>+</sup> pDCs, since these cells also may express TLR9 mRNA (20). As anticipated, memory B cells expressed higher levels of TLR9 mRNA than naive B cells (3). Notably, TLR9 mRNA was significantly lower in memory and naive B cells from viremic HIV-infected persons than in cells obtained from healthy controls, with the most pronounced deficiencies in the

by CpG ODN 2006 after 20 h of cultivation. The comparison among three groups was performed as noted above, and the comparison in the last panel was among HIV<sup>-</sup> donors, HIV<sup>+</sup> patients with CD4 levels of >500/ $\mu$ l, HIV<sup>+</sup> patients with CD4 levels of 200 to 500/ $\mu$ l, and HIV<sup>+</sup> patients with CD4 levels of <200/ $\mu$ l. One-way ANOVA and the Tukey test were used. (E) CD4 cell counts in relation to CpG-induced CD80 expression on memory B cells among HIV<sup>+</sup> viremic donors. Data were analyzed using Spearman's correlation.

**A Spearman's correlation analyses between B cell proliferation and various factors**

	naïve B cell proliferation		memory B cell proliferation	
	spearman's r	p value	spearman's r	p value
CD4 counts	0.413	0.026	0.394	0.038
plasma HIV RNA levels	-0.567	0.004	-0.28	0.13
age	-0.22	0.28	-0.04	0.87
%CD80+	-0.09	0.71	0.05	0.82
%CD80 induction by CpG	0.08	0.69	0.32	0.11
%CD21+	0.38	0.08	0.726	0.003

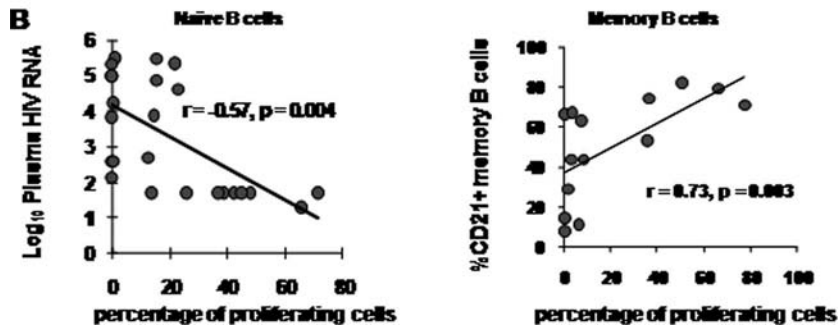


FIG. 4. CD21 expression predicts memory B-cell proliferation failure, and plasma HIV RNA level predicts naïve B-cell proliferation failure, in response to CpG ODN in HIV infection. (A) Age, CD4<sup>+</sup> T-cell number, plasma HIV RNA level, CD21 and CD80 expression among freshly isolated naïve and memory B cells, and induction of CD80 by CpG ODN in overnight cultures were analyzed for relationships to CpG-induced B-cell proliferation by Spearman's correlation. (B) Multiple regression analysis indicates that plasma HIV RNA level was the only independent predictor of naïve B-cell proliferation responses and that CD21 level was the only independent predictor of memory B-cell proliferation in response to CpG ODN in HIV infection.

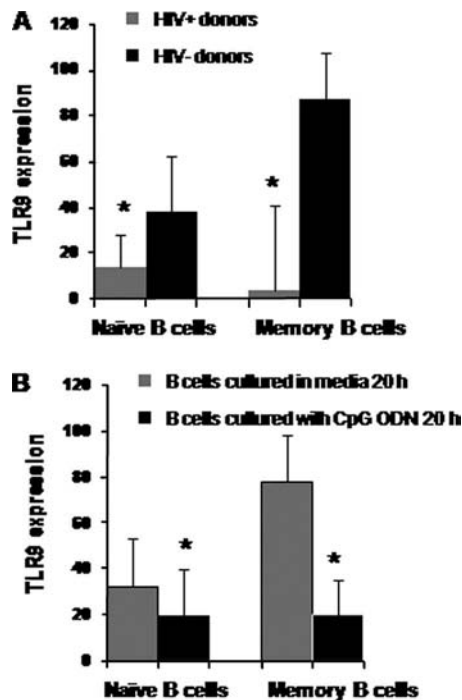


FIG. 5. Diminished TLR9 mRNA levels in both memory and naïve B cells from viremic HIV<sup>+</sup> patients. TLR9 mRNA levels were assessed by real-time PCR in purified memory and naïve B cells obtained ex vivo (A) and in purified memory and naïve B cells cultured with or without CpG ODN for 20 h (B) from five HIV<sup>-</sup> donors and six HIV<sup>+</sup> viremic donors (plasma HIV RNA levels of >400 copies/ml). Medians  $\pm$  standard errors of the means of TLR9 mRNA copies/1,000 GADPH are compared between HIV<sup>-</sup> and HIV<sup>+</sup> viremic donors by the Wilcoxon rank test (\*,  $P < 0.05$ ).

memory B-cell subset. Thus, diminished TLR9 expression could contribute to the functional defects of B cells in HIV disease.

Interestingly, we also found that exposure of purified B cells to TLR9 agonists in vitro resulted in decreased detection of TLR9 mRNA (Fig. 5B), raising the possibility that increased exposure to TLR9 agonists in vivo during the course of HIV disease could potentially underlie the TLR9 mRNA deficiency that we have observed in B cells from HIV-infected persons. Potential sources of TLR9 agonist activity in HIV disease include the virus itself (23), as well as potential exposure to bacterial products as a consequence of increased gut permeability and microbial translocation (9; Jiang et al., presented at the 15th Conference on Retroviruses and Opportunistic Infections).

Different concentrations of CpG ODN are required for optimal induction of CD80 expression and for optimal induction of proliferation in B lymphocytes. Although TLR9 mRNA expression and proliferation responses to CpG ODN were diminished in B-cell subsets from HIV-infected persons, the ability of these cells to increase surface expression of CD80 in response to CpG ODN was relatively preserved. To explain this apparent discrepancy, we considered the possibility that different thresholds of activation may be needed for triggering induction of CD80 and for initiation of cellular proliferation. Therefore, we examined CD80 expression and B-cell proliferation in response to a broad range of CpG ODN concentrations. As shown in Fig. 6, optimal CD80 induction was achieved by CpG ODN at a concentration of 0.5  $\mu$ g/ml. In contrast, a >10-fold-greater concentration of CpG ODN (6

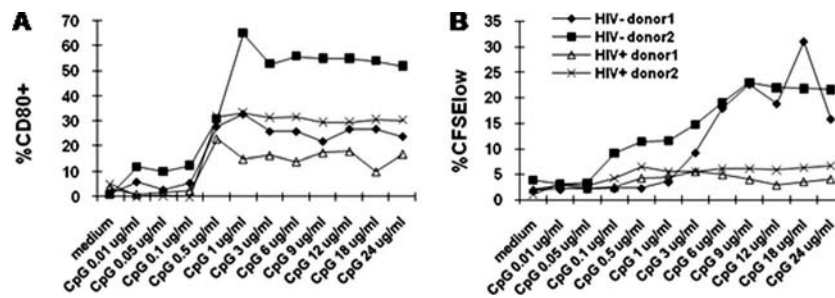


FIG. 6. Different thresholds for optimal CD80 induction and proliferation among B cells in response to CpG ODN. PBMCs from two healthy donors and two HIV<sup>+</sup> donors were cultured with increasing concentrations of CpG ODN 2006 (0.01 to 24  $\mu$ g/ml). The percentage of B cells (CD19<sup>+</sup>) induced to express CD80 was evaluated after 20 h of cultivation (A), and proliferation responses were measured by dilution of CFSE dye after 7 days (B).

$\mu$ g/ml) was needed for optimal proliferation responses. Thus, the activation threshold for inducing optimal CD80 expression appears to be substantially lower than the threshold for induction of proliferation in B cells responding to CpG ODN. Importantly, B cells from viremic patients did not achieve proliferation responses equal to the responses of cells from healthy controls even with high concentrations of CpG ODN (Fig. 6).

## DISCUSSION

TLR9 agonists can be used as potent adjuvants to enhance both humoral and cell-mediated immune responses (20). In addition to inducing alpha interferon production from pDCs and enhancing antigen-presenting cell function, these microbial products also regulate memory B-cell homeostasis and antibody production (3, 28, 29). Studies in humans and in nonhuman primate models indicate that CpG ODN administration can enhance vaccine responsiveness (30, 31, 33).

The potential utility of TLR agonists to enhance B-cell function in the setting of HIV infection is uncertain but may be impaired, since HIV infection is associated with a broad range of B-cell perturbations that include hypergammaglobulinemia (8, 9, 15, 19, 21, 24, 27), memory cells depletion (9, 32), and diminished responsiveness to BCR agonists, to CD40L, and to mitogens (21, 25). Previous studies have demonstrated the capacity of B cells from simian immunodeficiency virus-infected monkeys to proliferate in response to type C CpG ODN (29); however, there were limited numbers of animals to compare the magnitude of these responses between healthy and simian immunodeficiency virus-infected monkeys. Here, we provide evidence that both memory and naive B cells from HIV-infected persons proliferate poorly in response to TLR9 stimulation.

One mechanism that could explain poor proliferation function in B cells from HIV-infected persons centers on the accumulation of CD21-low/negative B cells in these persons. These cells appear to be terminally differentiated and consequently proliferate poorly in response to anti-Ig and other mitogens (25). CD21-low/negative cells are found at increased frequencies in peripheral blood of HIV<sup>+</sup> individuals, thereby diminishing the proliferative potential of the B-cell pool (25). Consistent with this understanding, our observations indicate that in HIV-infected persons, the frequency of CD21<sup>+</sup> memory B cells is directly related to the ability of these cells to

proliferate in response to CpG ODN stimulation. Furthermore, reduced proportions of CD21<sup>+</sup> memory B cells are tightly related to plasma HIV RNA levels, suggesting an important role for viral replication in the accumulation of these cells. Thus, our observations are consistent with a role for CD21-low/negative cells in memory B-cell dysfunction in HIV disease.

We also observed increased frequencies of CD21-negative naive B cells in peripheral blood samples from HIV-infected persons. CD21-negative naive B cells are not well defined functionally, but they may represent a population of transitional cells that are enriched in peripheral blood of HIV<sup>+</sup> donors with advanced disease (26). Notably, the proliferation response to CpG ODN among naive B cells from HIV-infected persons was not related to the frequencies of CD21 expression on naive B cells. However, our results indicated that naive B-cell proliferation in response to CpG ODN was inversely related to plasma HIV RNA levels, further indicating a role for viremia in B-cell dysfunction in HIV disease.

Although we found marked defects in the ability of B cells from HIV-infected persons to proliferate in response to CpG ODN, we found only modest, insignificant differences in the capacity of B cells from HIV-infected persons to increase CD80 expression after CpG ODN stimulation. Our dose-response studies indicate that higher concentrations of CpG ODN are required to induce optimal B-cell proliferation responses than to induce optimal CD80 expression suggesting the possibility that the signaling requirement for optimal induction of CD80 expression is less stringent than the signaling requirement for optimal induction of proliferation among B lymphocytes. Thus, we propose that diminished signaling via TLR9 (perhaps as a consequence of diminished TLR9 mRNA expression) in B cells from HIV-infected persons could result in defective proliferation responses without necessarily resulting in impairments of other responses such as induction of CD80 expression after activation of these cells with CpG ODN. Alternatively, the discrepancy in the ability of B cells from HIV-infected patients to express increased levels of CD80 and to proliferate after CpG ODN stimulation could result from differences in the durations of the respective assays. Thus, whereas induction of costimulatory molecules can be examined after short-term overnight cultures, proliferation requires several days to measure and consequently reflects both the ability of cells to divide and the ability of cells to survive in long-term

culture. Further studies that elucidate the mechanisms of B-cell proliferation failure in response to CpG ODN may help discern between these and other possibilities.

Our results indicate that B cells from HIV-infected persons express reduced levels of TLR9 mRNA, and this defect is especially pronounced among memory B cells. Memory B cells have longer half lives than naive B cells (days to weeks) (16), and they also can migrate to tissue sites, allowing them more opportunities to interact with microbial products, including TLR agonists. Translocation of bacterial lipopolysaccharide across the gut mucosa is elevated in HIV infection (5), and we have recently also found increased levels of bacterial DNA in plasmas from HIV-infected persons (Jiang et al., presented at the 15th Conference on Retroviruses and Opportunistic Infections). Thus, there is increased in vivo exposure to bacterial TLR agonists in chronic HIV infection, and our results indicate that exposure to TLR9 agonists could result in diminished TLR9 mRNA expression in B-cell subsets. The data on modulation of TLR9 expression by CpG ODN contradict previous studies (3, 4, 13); nevertheless, downregulation of TLR9 mRNA was observed in response to BCR ligation and to CpG ODN (28). In the present study, downregulation of TLR9 mRNA was confirmed by 3 h (modest downregulation) (data not shown) and 20 h of stimulation of CpG ODN in B cells from HIV<sup>-</sup> donors. Therefore, we propose that increased exposure to TLR9 agonists in vivo could result in reduced responsiveness to TLR9 agonists ex vivo, as seems to be the case for TLR4 ligand (5), although it is not yet clear if the latter is mediated by transcriptional regulation of the receptor.

TLR agonists are promising vaccine adjuvants and therapeutic agents that enhance both cellular and humoral immune responses. Previous studies have clearly demonstrated that TLR9 stimulation is sufficient to directly induce both naive and memory B-cell proliferation (2, 176, 8). Activation of memory B cells by TLR9 or other TLR agonists may play an important role in memory B-cell homeostasis and the sustained production of antibody after antigen is cleared (2). The impairment in the TLR9 responsiveness of memory B cells from HIV-infected persons could, therefore, contribute to the decline in memory B-cell numbers (9) and the shorter duration of antibody responses after immunization (32) that occur in the course of HIV infection. The impaired proliferation responses of B cells to CpG ODN in cells from HIV<sup>+</sup> donors was not fully restored in HAART-treated aviremic subjects (Fig. 1C), which suggests that there are other determinants of B-cell expansion defects besides viremia or that these defects, once induced, are persistent. As TLR agonists are being explored for their therapeutic and adjuvant properties, our data suggest that defects in naive B-cell responsiveness to CpG ODN in HIV infection could reduce the effectiveness of CpG ODN administration, particularly in circumstances where viral replication is poorly controlled. Ultimately, the mechanism that leads to this impairment will be important to discern, since the implications for immune homeostasis and humoral immunity in HIV disease are substantial.

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