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Mohamed Elrefaei; ... et. al

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Central Memory CD4$^+$ T Cell Responses in Chronic HIV Infection Are Not Restored by Antiretroviral Therapy[1]

Mohamed Elrefaei,[2]* Michael D. McElroy,* Christopher P. Preas,* Rebecca Hoh,† Steven Deeks,† Jeffrey Martin,‡ and Huyen Cao*

A strong CD4$^+$ T cell response has been correlated with better control of HIV infection. However, the effect of HIV on the maintenance of Ag-specific memory CD4$^+$ T cells is not fully understood. We characterized the function and phenotype of memory CD4$^+$ T cells generated by mumps and influenza A or B viruses in HIV-infected individuals receiving highly active antiretroviral therapy ($n = 21$), HIV-infected long-term nonprogressors ($n = 10$), and HIV-seronegative volunteers ($n = 10$). We observed significantly decreased proliferation of the Ag-specific central memory CD4$^+$ T cell population (CD28$^+$/CCR7$^+$/CD45RA$^-$) in the antiretroviral treated HIV-infected individuals compared with the seronegative controls. Restored CD4$^+$ T cell count and decreased HIV viral load while on highly active antiretroviral therapy did not result in increased proliferation, whereas nadir CD4$^+$ T cell count predicted the presence of Ag-specific proliferation. Our results indicate that HIV infection leads to impaired maintenance of virus-induced or vaccine-generated central memory CD4$^+$ T cells that is not restored by HAART. ***The Journal of Immunology,*** **2004, 173: 2184–2189.**

Protection following viral clearance or successful immunization requires the generation and maintenance of long-lived Ag-specific CD4$^+$ Th cells, defined as central memory CD4$^+$ T cells. Memory T cells have distinct phenotypes (reviewed in Ref. 1). Central memory CD4$^+$ T cells express CCR7, a homing molecule to the lymph nodes, and CD28, a costimulatory molecule that provides signals for specific CD4$^+$ T cell activation. Central memory CD4$^+$ T cells produce IL-2, undergo rapid expansion in response to Ag restimulation (2, 3), and are critical for the development of CD8$^+$ T cell memory (4–6).

HIV infection results in a progressive decline in CD4$^+$ T cell number and function, resulting in an increased risk of opportunistic infections (7–10). The introduction of highly active antiretroviral therapy (HAART)[3] has significantly reduced HIV-related morbidity and mortality (11–14). These clinical benefits have been attributed to decreased HIV replication and increased CD4$^+$ T cell number (reviewed in Ref. 15). The full effect of HAART on T cell immunity is still debated. HAART initiation has been associated with the detection of proliferative and Ab responses following immunization with influenza vaccine (16), tetanus toxoid, and inactivated hepatitis A vaccine (17), although these responses are often incomplete (18–21). We report in this work the consequence of chronic HIV infection on the maintenance of central memory CD4$^+$ T populations. We characterized the function and phenotype of memory CD4$^+$ T cells specific for mumps and influenza A or B (A/B) viruses in HIV-infected individuals. We demonstrated evidence of impaired maintenance of virus-induced or vaccine-generated central memory CD4$^+$ T cells in HIV-infected individuals that was not restored by HAART.

Materials and Methods

Study subjects and samples

HIV-positive volunteers ($n = 31$) were recruited from the Study of the Consequences of the Protease Inhibitor Era (SCOPE) cohort at San Francisco General Hospital (San Francisco, CA) (22). SCOPE is an ongoing cohort of 500 HIV-1 chronically infected adults. Subjects are evaluated every 4 mo. Prestudy treatment history and treatment responses (plasma HIV RNA, CD4$^+$ T cell count) were obtained via standardized patient interviews and medical chart review. A summary of the patient clinical data is shown in Table I. HIV RNA in the plasma (viral load) was quantified by the branched chain-DNA amplification test (Chiron, Emeryville, CA), and the lower limit of detection was 75 copies/ml. The study protocol was approved by the University of California Committee on Human Research, and all participants provided informed written consent. HIV-positive volunteers were divided into two groups: 1) 10 long-term nonprogressors (LTNP) having stable CD4$^+$ T cell counts for >10 years and no history of any antiretroviral treatment; 2) 21 HIV-positive volunteers currently receiving HAART who had achieved either complete (HIV RNA <75 copies/ml) or partial virologic response. Ten age-matched, HIV-seronegative control volunteers were also included in the study. None of the study participants had received a mumps vaccine booster for at least 10 years, and all had Abs against mumps, influenza A, and influenza B in the plasma, as determined by the standard diagnostic test, enzyme immunoassay (California Department of Health Services, Richmond, CA) (23, 24). PBMC were separated and cryopreserved in liquid nitrogen until assay time.

Antigens

Mumps virus Ag was prepared from the Enders strain (Microbix Biosystems, Toronto, Canada). Influenza A/B virus vaccine (Fluzone; Aventis Pasteur, Swiftwater, PA) was produced for the 2002–2003 influenza season, and was formulated to contain the prototype strains of influenza A and B viruses. The vaccine was dialyzed before use to remove all traces of the preservative thimerosal.

Proliferation of memory CD4$^+$ T cells

PBMC (2×10^6) were labeled with 4 μm CFSE (Molecular Probes, Eugene, OR) in PBS, then quenched with 100% FCS (Sigma-Aldrich, St. Louis, MO). Cells were resuspended in RPMI 1640 (Sigma-Aldrich) with

*California Department of Health Services, Richmond, CA 94804; and Departments of †Medicine and ‡Epidemiology and Biostatistics, University of California, San Francisco, CA 94110

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[2] Address correspondence and reprint requests to Dr. Mohamed Elrefaei, California Department of Health Services, 850 Marina Bay Parkway, Viral and Rickettsial Disease Lab, Richmond, CA 94804. E-mail address: melrefae@dhs.ca.gov

[3] Abbreviations used in this paper: HAART, highly active antiretroviral therapy; LTNP, long-term nonprogressor.

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Table I. *Characteristics of HIV-positive volunteers*[a]

HIV Positive	ID No.	Age (years)[b]	Year Diagnosed	HIV RNA[b]	Current CD4 Count	Nadir CD4 Count[c]
LTNP[d] ($n = 10$)	1027	46	1987	32,763	419	200
	1068	55	1984	105	748	351
	1086	53	1987	1,994	1,069	818
	1501	32	1995	16,690	505	309
	1504	41	1989	157	729	476
	1506	43	1989	13,374	446	400
	1516	47	1987	97	728	424
	1518	43	1992	<75	1,151	665
	4006	50	1998	3,303	1,099	737
	4007	48	1991	7,586	676	400
On HAART ($n = 21$)	1092	33	1992	<75	210	24
	2015	42	1988	<75	507	80
	2070	51	1999	<75	318	96
	2074	48	1990	<75	395	170
	2001	50	1982	<75	868	40
	2009	43	1986	<75	897	11
	2013	55	1986	<75	885	7
	3002	46	1986	364	290	11
	3019	55	1987	27,180	74	39
	3040	41	1987	8,856	243	147
	3042	42	1984	183	232	69
	3077	47	1985	2,423	239	176
	3085	55	1987	3,055	436	200
	3102	47	1994	17,507	290	24
	3135	56	1988	1,720	496	227
	3151	53	1978	37,999	119	72
	3167	52	1990	2,062	519	247
	3174	56	1984	257,849	89	87
	3502	66	1987	85,690	205	118
	3152	53	1985	8,442	125	43
	3158	39	2000	32,700	601	218

[a] Age, HIV RNA (copies per milliliter), and $CD4^+$ T cell count at the time the CFSE proliferation assay was performed.
[b] No statistically significant difference in age or HIV RNA between HIV positive on HAART and LTNP ($p = 0.27$; $p = 0.89$, respectively). HIV-seronegative volunteers consisted of 10, age-matched, HIV-seronegative control volunteers (data not shown).
[c] Documented lowest $CD4^+$ T cell count.
[d] No history of HAART.

10% FCS after washing with PBS. Cells were cultured in the presence of Ag (10 μg/ml) for 5 days at 37°C in 5% CO_2 and analyzed for the expression of surface markers. Media alone and PHA (10 μg/ml; Sigma-Aldrich) were used as negative and positive controls. The following Abs were used in different combinations: CD45RA PE, CD4 PerCP Cy5.5, CCR7 PE CY7, CD8 APC CY7, CD28 APC, and CD45RO APC (BD Pharmingen, San Diego, CA). Forty thousand lymphocytes per sample were acquired using an LSR II (BD Biosciences, Mountain View, CA), and analysis was performed by FLOWJO software (TreeStar, San Carlos, CA). Significant $CD4^+$ T cell proliferation, as measured by the extent of CFSE dilution, was defined as ≥1% after subtraction of background activity. All study participants demonstrated significant proliferation following PHA stimulation. Proliferation of $CD4^+$ T cells without Ag stimulation (negative controls) was <0.5%.

Flow-based intracellular cytokine staining

PBMC (0.5×10^6) were incubated for 2 h with Ag (10 μg/ml) at 37°C in 5% CO_2 in the presence of costimulatory anti-CD49d (1 μg/ml; BD Biosciences), then brefeldin A was added for 4–6 h. Cells were washed with PBS containing 0.1% FCS (wash buffer), and fixed by resuspending the cells in lysing solution (BD Biosciences) for 10 min at room temperature. Cells were washed once with wash buffer, then permeabilized by resuspension in 0.5 ml of permeabilizing solution 2 (BD Biosciences), and incubated for 10 min at room temperature. Cells were subsequently washed with wash buffer and stained with anti-IL-2 PE, anti-CD3 PerCP Cy5.5, and CD4 APC CY7 (BD Pharmingen). Forty thousand lymphocytes per sample were acquired using an LSR II (BD Biosciences), and analysis was performed by FLOWJO software (TreeStar). Percentage of cytokine-producing $CD3^+/CD4^+$ T cells without Ag stimulation was <0.05% (negative control). Results were expressed as: net percentage of IL-2-positive $CD4^+$ T cells (net percentage = percentage of Ag-specific − percentage of negative control).

Statistical analysis

Statistical analysis and comparisons were performed with PRISM software version 2.0 (GraphPad, San Diego, CA). Analysis was determined by Mann-Whitney *U* test and linear regression test. Statistical significance was defined as $p < 0.05$.

Results

Characteristics of study participants

The clinical characteristics of all HIV-positive volunteers are shown in Table I. There was no significant difference in age between the HIV-positive LTNP (aged 32–55; median = 46.5), or those receiving HAART (aged 33–66; median = 50) volunteers, and the HIV-seronegative (aged 26–61; median = 45.5) volunteers ($p = 0.9$ and $p = 0.4$, respectively). All HIV-positive and -seronegative study participants had Abs against mumps, influenza A, and influenza B virus Ags (as determined by enzyme immunoassay). HAART resulted in a significant increase in the mean $CD4^+$ T cell count (nadir = 100; current = 382; $p < 0.0001$) with nine volunteers having a restored $CD4^+$ T cell count >350 cells/mm^3 (ID 2015, 2074, 2001, 2009, 2013, 3085, 3135, 3167, 3158), while seven volunteers achieved a complete virologic response (HIV RNA <75 copies/ml; ID 1092, 2015, 2070, 2074, 2001, 2009, 2013).

Phenotypic analysis of central memory $CD4^+$ T cell subset

We first examined the phenotypes and proliferative ability of $CD4^+$ T cells following stimulation with mumps or influenza A/B virus Ags. Representative plots of the phenotype of the proliferating mumps- and influenza A/B-specific $CD4^+$ T cells are shown in Fig. 1, *A* and *B*, respectively. Most of the proliferating mumps-specific $CD4^+$ T cells expressed the $CD28^+/CCR7^+/CD45RA^-$

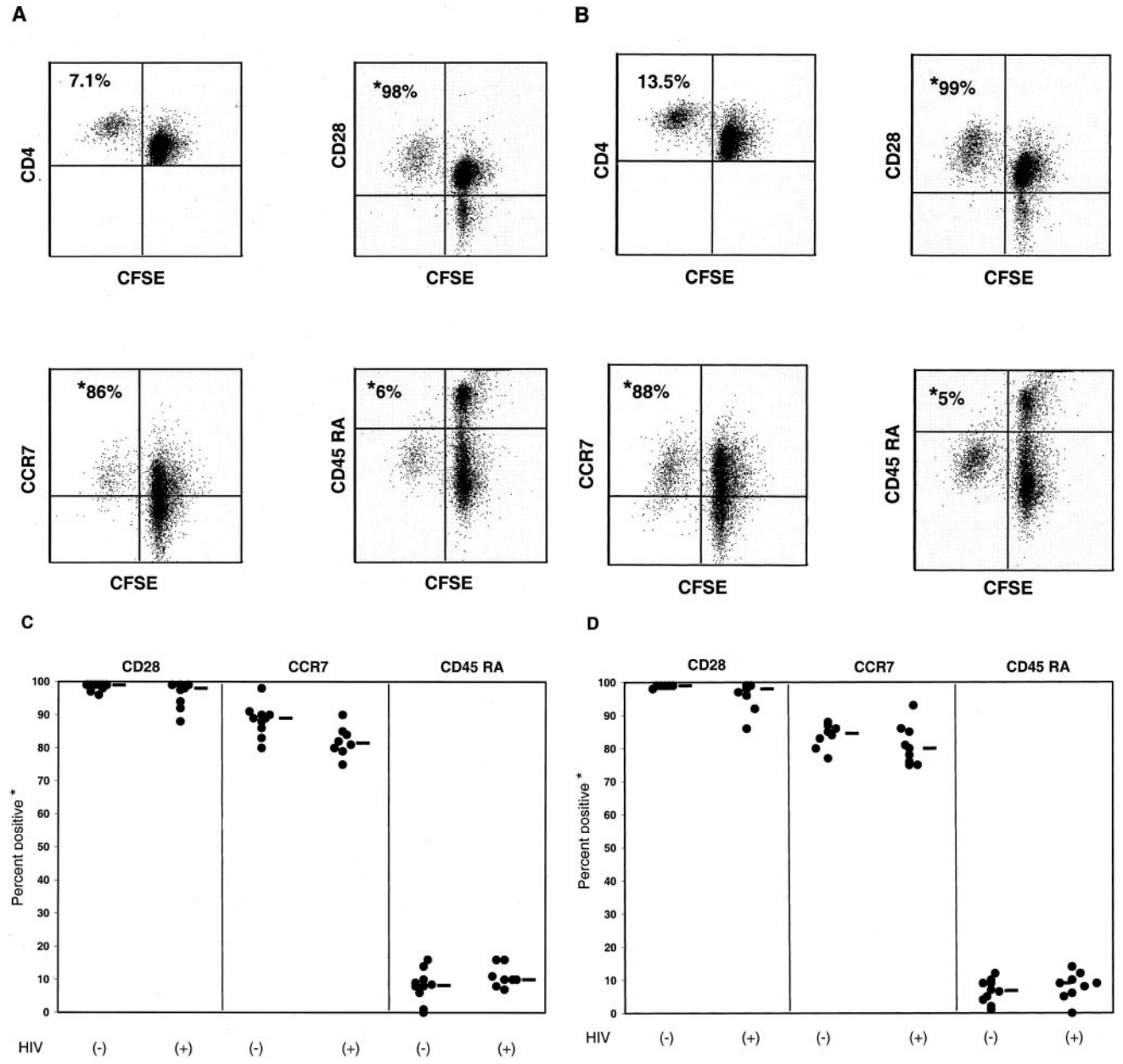

FIGURE 1. Phenotypic analysis of mumps- and influenza-specific central memory $CD4^+$ T cells. Representative plots of the phenotype of the proliferating mumps (*A*)- and influenza A/B (*B*)-specific $CD4^+$ T cells. CFSE-labeled PBMC were stimulated with either mumps or influenza A/B virus Ag for 5 days, then stained for various memory surface markers and assessed for proliferation by flow cytometry. Samples were first gated on the $CD4^+$ lymphocyte population, and percentage of proliferating $CD4^+$ T cells, as measured by the extent of CFSE dilution, was determined. The extent of CD28, CCR7, and CD45RA expression by the proliferating $CD4^+$ T cells was examined. *, Percentage values represent the fraction of proliferating mumps (*C*)- and influenza A/B (*D*)-specific $CD4^+$ T cells that express CD28, CCR7, or CD45RA over the total number of proliferating $CD4^+$ T cells (equivalent to 100%). Bars represent median values. Differences between HIV-positive and HIV-seronegative volunteers in *C* and *D* were not statistically significant ($p > 0.05$).

phenotype in both HIV-negative ($n = 10$) and HIV-positive volunteers ($n = 8$; Fig. 1*C*). The proliferating influenza A/B-specific $CD4^+$ T cells in both HIV-seronegative ($n = 10$) and HIV-positive ($n = 9$) volunteers also expressed similar phenotype (Fig. 1*D*). All proliferating mumps- and influenza A/B-specific $CD4^+$ T cells expressed the memory marker CD45RO (data not shown). This is consistent with the phenotypic criteria defining $CD28^+/CCR7^+/CD45RA^-/CD45RO^+$ as central memory $CD4^+$ T cells (2, 3). In addition, proliferation was associated with IL-2 production by the $CD4^+$ T cells in both HIV-seronegative and HIV-positive volunteers in response to mumps and influenza A/B virus Ags (Fig. 2), and differences between the two groups were not statistically significant ($p = 0.96$ and $p = 0.24$ for mumps and influenza A/B, respectively).

Effect of HIV infection on maintenance of mumps- and influenza-specific central memory $CD4^+$ T cell responses

We next examined the effect of chronic HIV infection on the maintenance of memory $CD4^+$ T cells specific for mumps and influenza A/B virus Ags. All HIV-seronegative volunteers ($n = 10$) demonstrated significant proliferative responses to both mumps (median = 2.05%; Fig. 3*A*) and influenza A/B (median = 4.75%; Fig. 3*B*) virus Ags. Similarly, 8 of 10 and 9 of 10 HIV-positive LTNP volunteers displayed significant proliferative responses to mumps

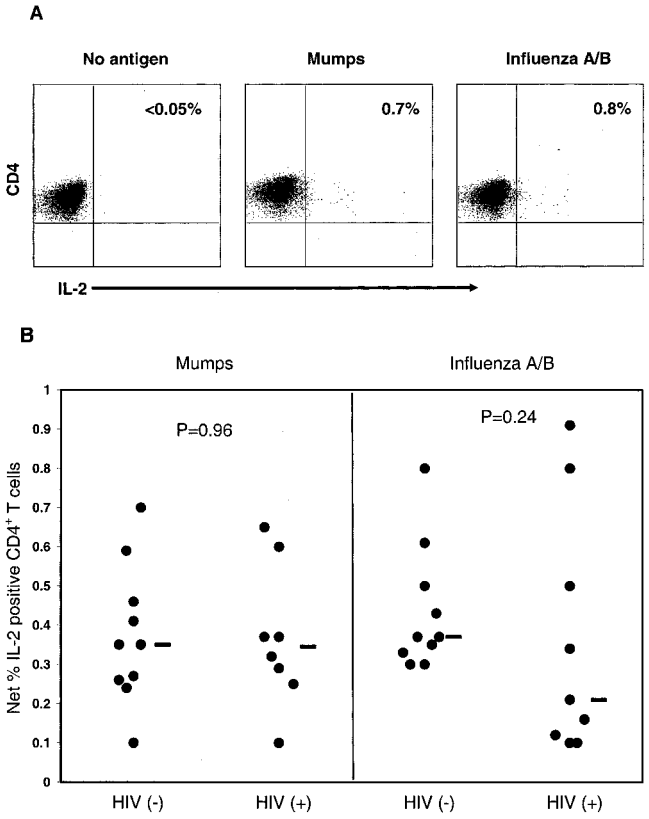

FIGURE 2. Frequencies of mumps- and influenza A/B virus-specific IL-2-positive $CD4^+$ T cells. PBMC were stimulated with mumps or influenza A/B virus Ag, then stained with anti-IL-2 PE, anti-CD3 PerCP Cy5.5, and anti-CD4 APC CY7, and analyzed by flow cytometry. Samples were first gated on the $CD3^+/CD4^+$ lymphocyte population, then the percentage of IL-2-positive cells was determined. *A*, Representative plots of the percentage of the mumps- and influenza A/B-specific $CD4^+$ T cells that produce IL-2. *B*, Results were expressed as net percentage of mumps- or influenza A/B-specific IL-2-positive $CD4^+$ T cells. Bars represent median values. Data are from individuals with significant mumps- or influenza A/B-specific proliferation only. Differences between HIV-positive and HIV-seronegative volunteers in *B* were not statistically significant ($p > 0.05$).

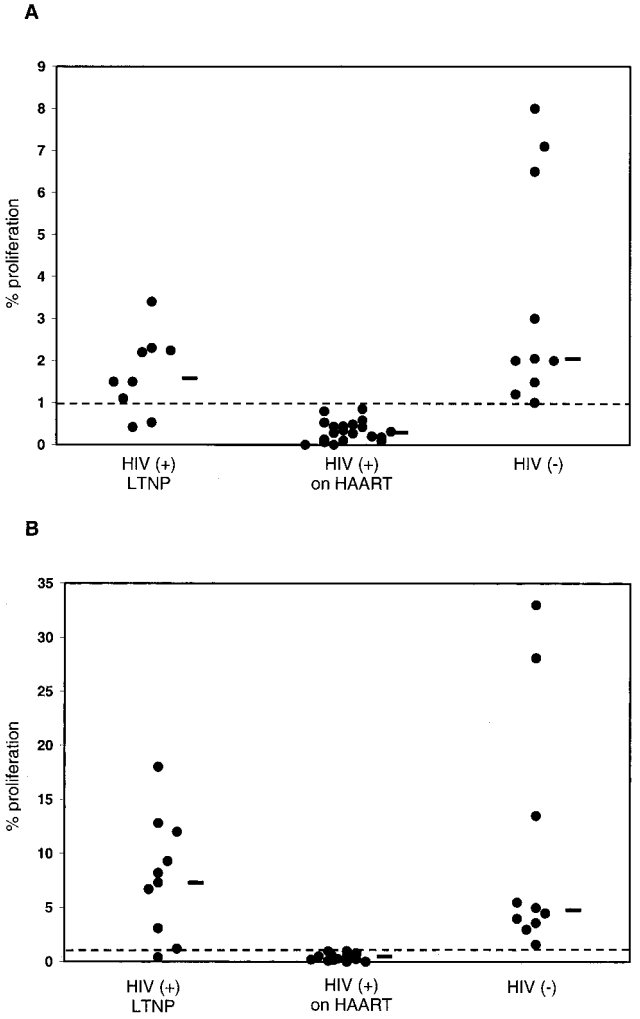

FIGURE 3. Magnitude of mumps- and influenza A/B-specific central memory $CD4^+$ T cell proliferation in HIV-positive individuals. CFSE-labeled PBMC were stimulated with mumps (*A*) or influenza A/B virus (*B*) Ag for 5 days, then assessed for proliferation by flow cytometry. Results are percentage of proliferating $CD4^+$ T cells as measured by the extent of CFSE dilution. Bars represent median values. Dashed line represents the cutoff for significant proliferation. Differences in *A* and *B* between HIV-positive volunteers receiving HAART compared with HIV-positive LTNP or HIV-seronegative volunteers were statistically significant ($p < 0.0001$ and $p < 0.0001$, respectively). No statistically significant differences were found between HIV-positive LTNP and HIV-seronegative volunteers ($p > 0.05$) in either *A* or *B*.

(median = 1.6%) and influenza A/B (median = 7.75%) virus Ags, respectively. The responses in HIV-seronegative and HIV-positive LTNP volunteers had similar magnitude that was not statistically significant ($p = 0.2$ and $p = 0.9$, for mumps and influenza A/B, respectively). In contrast, none of the HIV-positive volunteers receiving HAART ($n = 21$) generated proliferative responses to mumps or influenza A/B virus Ags (Fig. 3, respectively). These results were observed in volunteers who demonstrated restored $CD4^+$ T cell count, as well as in volunteers who achieved a complete virologic response. Differences in responses between HIV-positive volunteers receiving HAART compared with HIV LTNP or HIV-seronegative volunteers were significant for mumps ($p < 0.0001$ and $p < 0.0001$, respectively) and influenza A/B ($p < 0.0001$ and $p < 0.0001$, respectively) virus Ags. This lack of Ag-specific proliferation suggests that chronic HIV infection is associated with impaired maintenance of central memory $CD4^+$ T cell responses to mumps, influenza A, and influenza B viruses.

Effect of HAART on maintenance of central memory $CD4^+$ T cell responses

None of the HIV-positive volunteers receiving HAART who had a restored $CD4^+$ T cell count or had achieved a complete virologic response demonstrated significant central memory $CD4^+$ T cell responses to either mumps (Fig. 4*A*) or influenza A/B (Fig. 4*B*) virus Ags. Surprisingly, the two HIV-positive LTNP, who had a nadir $CD4^+$ T cell count ≤ 350 cells/mm^3, also demonstrated no significant central memory $CD4^+$ T cell responses to mumps (Fig. 4*A*), and only one of two was able to maintain significant central memory $CD4^+$ T cell responses to influenza A/B (Fig. 4*B*). In contrast, all of the HIV-positive LTNP, who had a nadir $CD4^+$ T cell count >350 cells/mm^3 ($n = 8$), were able to maintain significant central memory $CD4^+$ T cell responses to mumps (Fig. 4*A*) and influenza A/B (Fig. 4*B*) virus Ags.

Previous studies have demonstrated that HIV viremia correlated with diminished HIV-specific $CD4^+$ T cell proliferation in chronic HIV infection (25, 26). We determined the relationship between HIV viral load and the magnitude of central memory $CD4^+$ T cell responses and found no correlation between the viral load and responses to mumps (Fig. 5*A*) or influenza A/B (Fig. 5*B*) virus Ags.

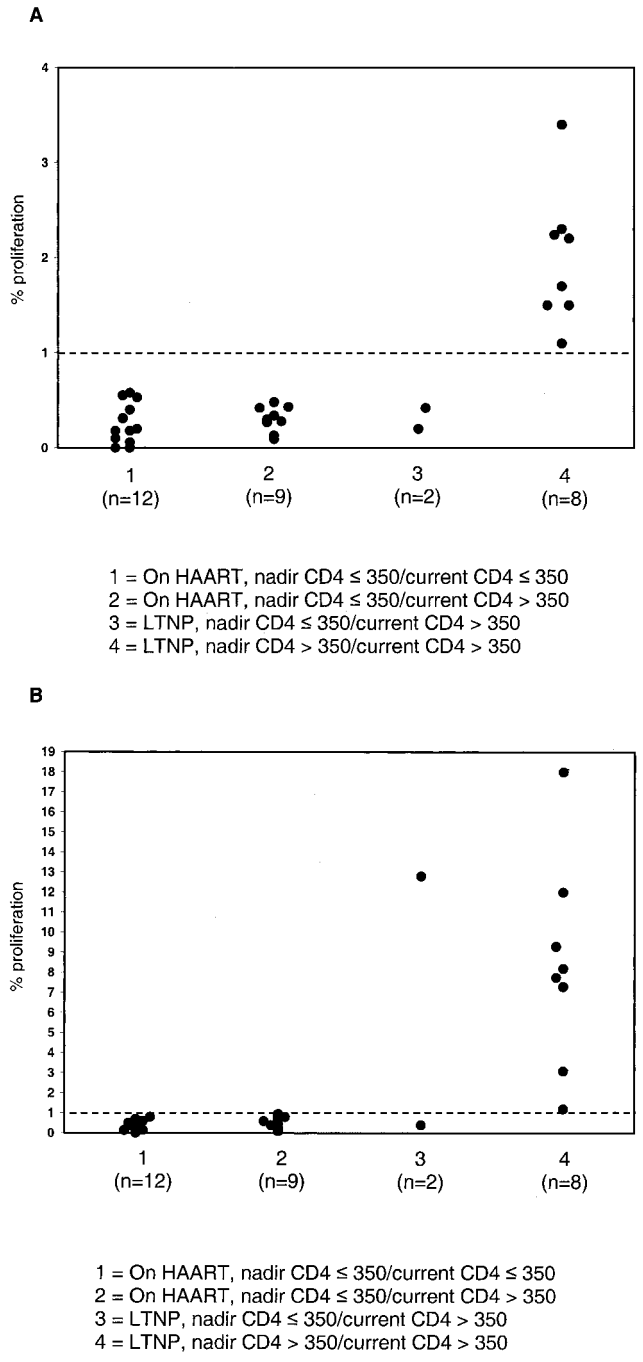

FIGURE 4. Relation of mumps- and influenza A/B-specific central memory $CD4^+$ T cell proliferation to the nadir $CD4^+$ T cell count in HIV-positive individuals. CFSE-labeled PBMC were stimulated with mumps (*A*) or influenza A/B virus (*B*) Ag for 5 days, then assessed for proliferation by flow cytometry. Results are percentage of proliferating $CD4^+$ T cells as measured by the extent of CFSE dilution. Dashed line represents the cutoff for significant proliferation. Differences in *A* between 4 vs 1, 2, or 3 were statistically significant ($p = 0.0003$, $p < 0.0001$, and $p = 0.04$, respectively). Differences in *B* between 4 vs 1 or 2 were statistically significant ($p = 0.0003$ and $p < 0.0001$, respectively). Differences between 4 vs 3 in *B*, between 1 vs 2 or 3 and between 2 vs 3 in *A* and *B* were not statistically significant ($p > 0.05$).

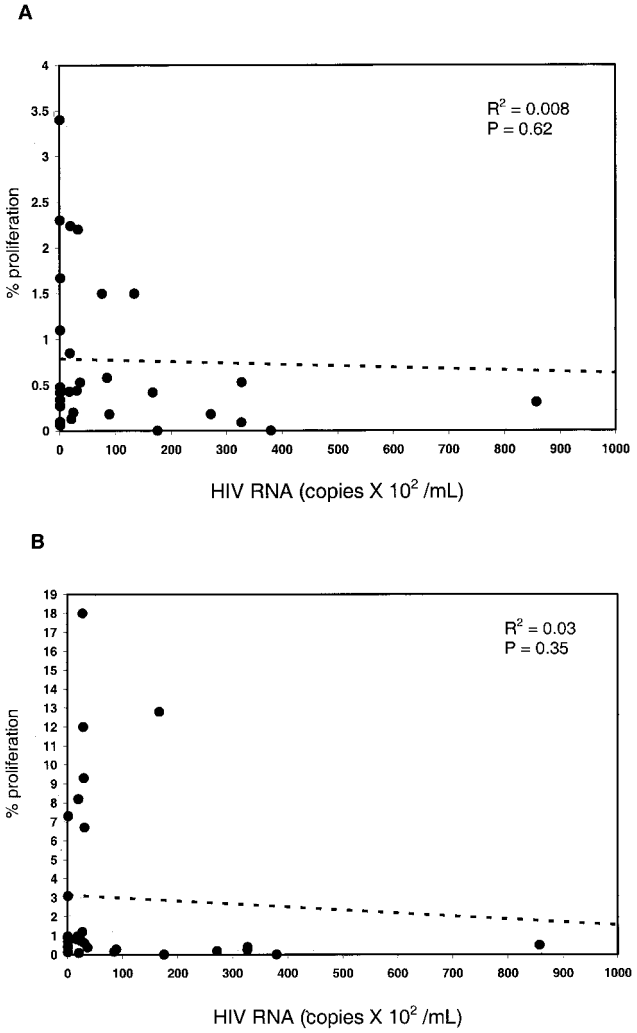

FIGURE 5. Relation of mumps- and influenza A/B-specific central memory $CD4^+$ T cell proliferation to HIV RNA titer. The $CD4^+$ T cell proliferative response to mumps (*A*) and influenza A/B virus (*B*) Ag, as measured by flow cytometry, is plotted against the HIV RNA titers (copies $\times 10^2$/ml). No correlation between the two parameters was observed in *A*, $R^2 = 0.008$, $p = 0.62$ or *B*, $R^2 = 0.03$, $p = 0.35$.

Discussion

The hallmark of immune protection is the long-term maintenance of memory T cell responses. $CD4^+$ T cell help is required for the control of infection and for the generation of functional memory $CD8^+$ T cells (27, 28). A strong $CD4^+$ T cell response has been correlated with better control of HIV infection (29). However, the mechanism by which HIV affects the homeostasis and the proliferative ability of memory $CD4^+$ T cells remains unresolved (reviewed in Ref. 30).

We first characterized the central memory $CD4^+$ T cells specific for mumps and influenza A/B virus Ags in a cohort of chronic HIV-infected volunteers. HIV-positive LTNP maintained significant proliferative responses to mumps and influenza A/B virus Ags comparable to that observed in HIV-seronegative volunteers. Detection of mumps-specific responses >10 years after natural infection or immunization suggests that central memory $CD4^+$ T cells are long-lived in the absence of Ag re-exposure. These results also suggest that the ability to generate or maintain effective central memory $CD4^+$ T cell responses is preserved in nonprogressive HIV disease with low-level HIV replication.

In contrast, none of the antiretroviral treated individuals had detectable proliferative responses to either mumps or influenza A/B virus Ags. This lack of responses persisted even in individuals with clear evidence of a treatment-mediated increase in $CD4^+$ T cell count

and low or undetectable viremia. These results suggest that progressive HIV disease may cause global impaired maintenance of central memory $CD4^+$ T cell responses, and that reconstitution of pre-existing immunity remains defective despite viral suppression and normalization of $CD4^+$ T cell count in response to HAART.

Data from several cohort studies support initiating treatment of HIV-positive individuals when $CD4^+$ T cell count reaches 200 cells/mm^3 (31–33). Introduction of HAART has significantly reduced HIV-related morbidity and mortality (11–14). However, the optimal time to initiate HAART in asymptomatic HIV-positive individuals with $CD4^+$ T cell count of <350 cells/mm^3 is still debated (reviewed in Ref. 34). In our study, indicators of clinical responses to HAART including restored current $CD4^+$ T cell count or suppression of HIV replication had no impact on maintaining effective virus-induced or vaccine-generated central memory $CD4^+$ T cell responses. In contrast, low nadir $CD4^+$ T cell count (≤ 350 cells/mm^3) predicted failure to preserve these responses. The ability to maintain central memory $CD4^+$ T cell responses in our HIV-positive study participants may reflect on immunologic characteristics specific to HIV-positive LTNP. However, the two LTNP that had a low nadir $CD4^+$ T cell count (≤ 350 cells/mm^3) were also unable to maintain mumps-specific central memory $CD4^+$ T cell responses. This interesting finding leads us to postulate that a low nadir $CD4^+$ T cell count results in a permanent loss of the long-lived Ag-specific central memory $CD4^+$ T cells. Our findings should contribute to decisions toward initiation of antiretroviral therapy and strategies of vaccination targeted to the HIV-infected population.

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