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CD4⁺CD8⁺ T Cells Represent a Significant Portion of the Anti-HIV T Cell Response to Acute HIV Infection

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Previous studies have revealed that HIV-infected individuals possess circulating CD4⁺CD8⁺ double-positive (DP) T cells specific for HIV Ags. In the present study, we analyzed the proliferation and functional profile of circulating DP T cells from 30 acutely HIV-infected individuals and 10 chronically HIV-infected viral controllers. The acutely infected group had DP T cells that showed more proliferative capability and multifunctionality than did both their CD4⁺ and CD8⁺ T cells. DP T cells were found to exhibit greater proliferation and higher multifunctionality compared with CD4 T cells in the viral controller group. The DP T cell response represented 16% of the total anti-HIV proliferative response and >70% of the anti-HIV multifunctional response in the acutely infected subjects. Proliferating DP T cells of the acutely infected subjects responded to all HIV Ag pools with equal magnitude. Conversely, the multifunctional response was focused on the pool representing Nef, Rev, Tat, VPR, and VPU. Meanwhile, the controllers' DP T cells focused on Gag and the Nef, Rev, Tat, VPR, and VPU pool for both their proliferative and multifunctional responses. Finally, we show that the presence of proliferating DP T cells following all HIV Ag stimulations is well correlated with proliferating CD4 T cells whereas multifunctionality appears to be largely independent of multifunctionality in other T cell compartments. Therefore, DP T cells represent a highly reactive cell population during acute HIV infection, which responds independently from the traditional T cell compartments. *The Journal of Immunology*, 2012, 188: 4289–4296.

Immature T cells express both CD4 and CD8 while undergoing thymic development (1). Traditionally it was thought that expression of either CD4 or CD8 was permanently lost once T cells transitioned to naive T cell status and exited the thymus. Despite this, it has been observed that both healthy and diseased humans, chickens, monkeys, mice, rats, and pigs exhibit a circulating pool of CD4⁺CD8⁺ double-positive (DP) T cells (2–7). The DP population generally represents ~3% of circulating T cells but shows considerable variability across individuals (3). In patients suffering from various neoplastic and infectious diseases, as well as in some ostensibly healthy individuals, circulating DPs can represent up to 43% of circulating T cells (8, 9).

Circulating DPs have often been thought to arise from thymic leakage of immature T cells (2, 10). Nonetheless, sporadic studies have examined the origins and functional abilities of extrathymic

DPs. DPs have been shown to localize to the sites of inflammatory processes in a variety of autoimmune disorders as well as in infectious diseases, such as hepatitis C virus (9, 11, 12). It has been reported that DPs with a highly activated memory phenotype and expressing both HIV coreceptors CCR5 and CXCR4 are located in the intestine, where HIV replication preferentially occurs during acute infection (13, 14). Kitchen et al. (15) and others have also shown that HIV is capable of infecting DP cells both in vitro and in vivo (16–19). Because DP cells are located at the sites of active HIV infection, express the appropriate coreceptors, and are capable of being infected, DP cells may be important targets of acute HIV infection.

The ability of the immune system to quickly respond during acute HIV infection and thereby decrease viral loads to low levels is thought to be an important determinant of long-term prognosis (20). Interestingly, Howe et al. (21) have shown that HIV-infected patients possess circulating HIV-specific DP T cells during the acute phase. These HIV-specific DP cells expressed IFN- γ and were either coexpressing IL-2 or a marker for degranulation (CD107a), and patients were more likely to have bifunctional DP cells than either bifunctional CD4 or CD8 T cells. Simultaneous expression of three or more functions at a time within HIV- and SIV-specific CD4 and CD8 T cells has previously been associated with improved disease outcomes (22–26). CD4 and CD8 T cell proliferation in response to HIV Ags has also been associated with better HIV disease status (27, 28). Therefore, in addition to the possibility of being a target of acute HIV infection, DP cells may represent an important component of the HIV-specific cellular immune response. At present, little is known about the breadth and functional profile of the DP response to HIV. As a result, we sought to determine the ability of circulating DP cells to respond to HIV Ags with a wide range of functionalities during the acute phase of HIV infection. Finally, we went on to compare these

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Abbreviations used in this article: DP, double-positive; EIA, enzyme immunoassay; ICS, intracellular cytokine staining; NAT, nucleic acid amplification test; VVNRT, VPR, VPU, Nef, Rev, and Tat.

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acute responses with those observed in a group of HIV-1 controllers.

Materials and Methods

Subjects

Persons with acute HIV infection were identified based on clinical presentation or by screening conducted by the state of North Carolina's Screening and Tracing Active Transmission Program that has identified individuals with acute HIV infections since 2002. Subjects are identified as being acutely infected through a combination of reported symptoms and serology (29). Acutely infected patients were then referred for further evaluation at either Duke University or the University of North Carolina-Chapel Hill. Following the provision of written informed consent, the referred patients were enrolled in either studies of antiretroviral treatment or an untreated longitudinal study (depending on patient choice) if they were 1) enzyme immunoassay (EIA)-negative and nucleic acid amplification test (NAT)-positive; 2) EIA-positive, NAT-positive, Western blot-negative/indeterminate; or 3) EIA-positive, NAT-positive, Western blot-positive, and documented EIA-negative within 45 d. Thirty patients who had been infected a median of 43 d (range, 22–105 d) before entry were studied (Table I) (29). Because of the study location, all 30 patients were presumed to have been infected with HIV-1 clade B viruses. At study entry, blood samples were acquired from these patients and PBMCs were isolated using standard density gradient centrifugation. Isolated PBMCs were cryopreserved in FCS supplemented with 10% DMSO and stored in vapor phase liquid nitrogen within 8 h from collection. Viral load and CD4 counts were also obtained at study entry. This acute patient cohort had a median viral load of 259,789.5 (range, 688–1,1503,872) copies/ml and a median CD4 count of 476.5 (range, 6–1,175) cells/mm³.

Additionally, nine virus controllers were separately recruited from the Duke Adult Infectious Diseases Clinic with informed consent under Duke University Medical Center Institutional Review Board approval. Virus controllers were required to have been diagnosed as HIV-positive for >1 y, be antiretroviral therapy naive, have CD4 counts >600 cells/mm³ blood, and have been controlling virus replication to <2700 viral RNA copies/ml blood (Table II) (30–32).

Table I. Acute patient data

Patient ID No.	Days after Infection	Viral Load (copies/ml)	CD4 Count (cells/mm ³)	DP Cells (% CD3)	FIEBIG
PHI02-70	54	6,281	1,175	4.97	5/6
Z023	24	255,507	392	2.04	3
Z026	43	1,338,235	558	4.62	4
Z029	36	2,027,400	316	10.05	3
Z030	34	1,408,114	703	11.48	1/2
Z032	57	559,145	N/A	4.33	5
Z035	26	2,251,415	291	8.21	1/2
Z042	38	572,904	N/A	6.48	5/6
Z055	56	21,488	311	3.62	5/6
Z059	45	565,421	538	5.55	5/6
Z064	84	11,503,872	6	28	5/6
Z065	49	3,071,359	342	4.87	5/6
Z067	22	562,000	521	5.08	1/2
Z068	40	750,001	180	5.55	3
Z069	33	100,001	250	4.37	3
Z072	46	25,788	558	2.44	5/6
Z073	33	6,732,441	673	3.29	1/2
Z075	38	170,826	241	4.21	5/6
Z078	58	750,001	760	9.99	5/6
Z080	58	264,072	508	5.26	5/6
Z085	67	189,036	422	5.48	5/6
Z091	43	37,706	541	4.98	5/6
Z095	26	37,706	808	7.52	5/6
Z098	43	59,603	1,106	6.73	5/6
Z099	26	394,649	377	5.13	1/2
Z100	31	33,959	806	5.44	5/6
Z106	105	688	574	6.84	5/6
Z109	74	46,470	438	7.23	5/6
Z112	51	189,930	445	4.11	5/6
Z117	48	193,306	200	8.43	3

Table II. Controller patient data

Patient ID No.	Viral Load (copies/ml)	CD4 Count (cells/mm ³)	DP Cells (% CD3)
VC10	2670	902	3.72
VC11	2020	924	4.00
VC16	519	628	2.06
VC18	<48	1514	1.98
VC20	601	627	2.65
VC23	424	806	4.36
VC24	894	643	2.24
VC26	118	668	2.43
VC27	2690	816	3.33

Peptides

Fifteen amino acid peptides overlapping by 11 aa residues representing the HIV-1 clade B consensus sequences of ENV (no. 9480), Gag (no. 8117), Nef (no. 5189), Pol (no. 6208), Rev (no. 6445), Tat (no. 5138), VPR (no. 6447), and VPU (no. 6444) were obtained from the National Institutes of AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health). Individual peptides were resuspended in DMSO and pooled, at a final concentration of 500 µg/ml, into a total of six peptide pools representing gp120, gp41, Gag, Pol peptides 5461–5585, Pol peptides 5586–5709, and a combination of VPR, VPU, Nef, Rev, and Tat (VVNRT).

Proliferation assays

Cryopreserved PBMCs were thawed and washed twice with RPMI 1640 containing 10% FCS and 1% penicillin/streptomycin (R10) and enumerated using a Guava Count system (Millipore). Following cell counting, the PBMCs were washed twice with PBS and subsequently resuspended at 20 × 10⁶ cells/ml. The cells were stained with CFSE for 8 min mixing once at 4 min (33). CFSE staining was quenched with 100% human serum for 2 min, and after washing with PBS, the cells were resuspended in R10 containing human serum. CFSE-stained cells were then plated at 1 × 10⁶ cells/ml and stimulated with peptide pools representing HIV gp120, gp41, Gag, Pol pool 1, Pol pool 2, VVNRT (1 µg/ml) or anti-CD3 (eBioscience) and anti-CD28 (BD Biosciences) Abs. Stimulated cells were then incubated at 36°C with 5% CO₂ for 6 d. Following 6 d stimulation, the cultures were washed twice with PBS and stained with a violet vital dye (Invitrogen) for 20 min at room temperature in the dark. Cells were again washed twice with PBS and then stained with anti-CD3 allophycocyanin-Cy7 (clone SK7; BD Biosciences), anti-CD4 PE-Cy5.5 (OKT4; eBioscience), and anti-CD8 Qdot605 (3B5; Invitrogen) Abs for 20 min at room temperature in the dark. Following Ab staining, the cells were washed thrice with PBS and fixed with 1% paraformaldehyde. Stained and fixed cells were then refrigerated in the dark until acquisition.

Intracellular cytokine staining assays

Cryopreserved PBMCs were thawed, resuspended at 2 × 10⁶ cells/ml in R10, and rested overnight at 36°C with 5% CO₂. After the overnight rest, the PBMCs were counted and resuspended at 1 × 10⁶ cells/ml in R10. Cells were then stimulated for 6 h at 36°C with 5% CO₂ with peptide pools representing CMV pp65, HIV gp120, gp41, Gag, Pol pool 1, Pol pool 2, or VVNRT (1 µg/ml). Stimulations were performed in the presence of 1 µg/ml each of anti-CD28 (L295; BD Biosciences) and CD49d (L25; BD Biosciences) Abs, anti-CD107a PE-Cy5 (H4A3; eBioscience), 5 µg/ml brefeldin A (Sigma-Aldrich), and 1 µg/ml monensin (BD Biosciences). Following the stimulation, the cells were washed with PBS containing 1% FCS and surface stained with aqua blue vital dye (Invitrogen), anti-CD4 PE-Cy5.5 (OKT4; eBioscience), anti-CD8 Qdot605 (3B5; Invitrogen), anti-CD27 PE-Cy7 (M-T271; BD Biosciences), anti-CD57 Qdot565 (TB01; AbD Serotec), and anti-CD45RO PE-TR (UCHL1; Beckman Coulter) for 20 min at room temperature in the dark. Following surface staining, the cells were washed again with PBS containing 1% FCS and then subsequently fixed and permeabilized with Cytofix/Cytoperm and Perm/Wash buffer (BD Biosciences) for 20 min and washed twice. Following the Fix/Perm step, cells were stained intracellularly with anti-CD3 Qdot655 (S4.1; Invitrogen), anti-IFN-γ Alexa 700 (B27; BD Biosciences), anti-IL-2 allophycocyanin (MQ1-17H12; BD Biosciences), anti-MIP-1β FITC (24006; R&D Systems), and anti-perforin PE (B-D48; Cell Sciences) for 20 min at room temperature in the dark. Subsequent to intracellular staining, cells were washed with Perm/Wash buffer and then fixed with 1%

paraformaldehyde. Following fixation, cells were refrigerated in the dark until acquisition.

Flow cytometry acquisition and analysis

Within 18 h of staining, fully stained cells from the proliferation and intracellular cytokine staining (ICS) assays were acquired on a custom LSRII (BD Biosciences) using FACSDiva. Following acquisition, flow data were analyzed using FlowJo software v.9.3.2 (Tree Star). For all assays, gates were set to include singlet events, live CD3⁺ cells, lymphocytes, and CD4⁺/CD8⁺/CD4⁺CD8⁺ subsets. For the proliferation assays, CFSE^{low} populations were then identified from each lymphocyte subset (Supplemental Fig. 1A). For the ICS assays, the naive population (CD27⁺CD45RO⁻) was identified and excluded from each lymphocyte subset. Within the memory population, cellular function-positive populations were identified individually for all cellular functions except perforin, which was only defined as positive if both perforin⁺ and IFN- γ ⁺ (Supplemental Fig. 1B). Using a Boolean gating strategy, the 32 combinations of the five cellular functions were identified. Based on these frequencies, we also calculated the total frequency of families of subsets expressing the same number of functions.

Statistical analysis

For the proliferation assays, relative proliferation values were obtained by subtracting the average CFSE^{low} population frequency of a patient samples' two unstimulated wells from the CFSE^{low} population frequency following stimulation, and then dividing the resulting value by the average CFSE^{low} population frequency of a patient samples' two unstimulated wells. For the intracellular cytokine staining assays, Pestle was used for background subtractions and Prism (GraphPad Software) and SPICE were used for frequency analysis. Pestle and SPICE were provided by Dr. M. Roederer (Vaccine Research Center, National Institutes of Health, Bethesda, MD).

DP response ratios were calculated by first multiplying each patient's frequency of HIV-specific DP, CD4, and CD8 T cells by the mean cell count in the DP, CD4, and CD8 compartments for each stimulation condition to obtain a normalized HIV-specific DP, CD4, and CD8 cell count. The normalized HIV-specific DP cell count was then divided by the sum of the normalized HIV-specific DP, CD4, and CD8 cell counts to give the DP response ratio.

Comparisons of responses within patient groups were performed using a Wilcoxon matched pairs test (Prism). Comparison across patient groups

used a Mann–Whitney *U* test (Prism). Regression analyses were performed using a linear regression (Prism). No adjustments for multiple comparisons were performed and *p* values should be interpreted with this in mind.

Results

HIV-specific proliferation of DP cells

Previous studies reported a correlation between the magnitude of HIV-specific proliferation of T cells and improved disease outcomes. Nonetheless, HIV-specific proliferation of DP cells has not been previously examined. Therefore, PBMCs from HIV⁺ patients were analyzed for their proliferative ability in response to stimulation with peptides representing the HIV clade B consensus peptide sequence. Following 6 d stimulation, CD4⁺CD8⁻ (CD4), CD4⁺CD8⁺ (DP), and CD4⁻CD8⁺ (CD8) populations were identified by flow cytometry (Supplemental Fig. 1A). The loss of CFSE staining was used to indicate cells within these populations that had undergone proliferation during the stimulation (33). As expected, we observed proliferative responses to the HIV proteome within both the CD4 and CD8 compartments of the acutely infected subjects. The relative proliferation within the CD8 compartment was greater than that seen in the CD4 compartment for both the acute (median [range], 1.78 [-4.93–25.5] versus 0.93 [-3.86–31.22]) and controller patients (3.28 [-0.19–29.69] versus 0.55 [-0.32–20.89]), although this difference was only significant in the controller cohort (Fig. 1A). In both acutely infected (median [range], 3.78 [-3.42–36.36]) and controller patients (6.61 [-0.13–98.13]), we observed significantly greater relative proliferation within the DP compartment following HIV Ag stimulation than within the CD4 compartment. DP proliferative responses were also higher than those observed in the CD8 compartments of both cohorts, but reached statistical difference only in the acute cohort (*p* = 0.01). Next, we determined the portion of the total T cell proliferative response to HIV that was

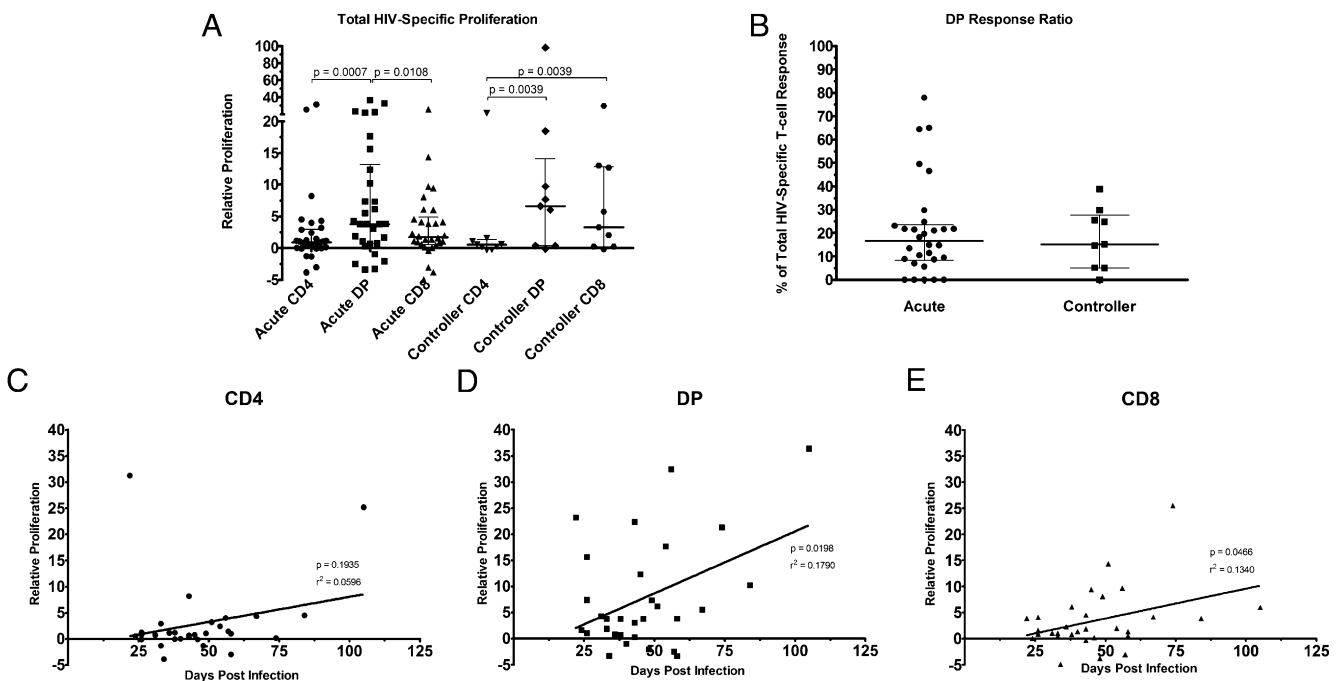


FIGURE 1. Total HIV-specific proliferation. (A) Cells were stained with CFSE and stimulated for 6 d with peptide pools representing the HIV proteome. The HIV-specific relative proliferation was calculated for each T cell subtype and plotted for each patient in the acute and chronic cohorts. (B) The percentage of the total anti-HIV proliferative response coming from the DP compartment was calculated for each patient in the acute and chronic cohorts. (C–E) The days after infection at which an acute blood sample was drawn was plotted against the HIV-specific relative proliferation in the (C) CD4 compartment, (D) DP compartment, and (E) CD8 compartment.

attributable to the DP compartment in the acute and controller cohorts, a calculation we call the DP response ratio. In both cohorts, the median proliferative DP response ratio was ~16% (range, 0–78; 0–39) (Fig. 1B). In 5 of 30 acute patients the DP response ratio was >40%. We also sought to determine whether there was a relationship between the time since infection and the magnitude of the proliferative response. There was no correlation between the CD4 proliferative response and time following infection (Fig. 1C). However, the HIV-specific proliferative response within the DP and CD8 compartments increases with time since infection ($p = 0.0198$, 0.0406), suggesting that both the DP and CD8 proliferative responses gain strength as the immune system partially controls viral replication (Fig. 1D, 1E). Meanwhile, the magnitude of the HIV-specific proliferative response within the controller cohort did not correlate with viral loads within these patients ($p = 0.6312$).

Polyfunctionality of HIV-specific DP cells

The polyfunctionality of HIV-specific cells was analyzed by staining for the expression of CD107a, IFN- γ , IL-2, MIP-1 β , and perforin after a 6-h stimulation. HIV-specific functionality was determined according to the gating strategy displayed in Supplemental Fig. 1B. Following the identification of each individual functional population, Boolean gating was applied to identify cells expressing all possible combinations of the five functions. We observed a significantly higher frequency of CD8 T cells expressing three or more functions in the acutely infected group than we observed within the CD4 compartment (Fig. 2A). HIV-specific multifunctional cells within the DP compartment (median [range], 20 [–0.19–100]) were significantly more frequent than in the CD8 compartment (0.34 [0.01–24.84]) in the acute infection group ($p = 0.0002$). Of interest, 100% of patient Z68's DP cells were specific for HIV and multifunctional at study entry.

Using the multifunctional response, we again compared the DP response ratios across cohorts to determine the fraction of all HIV-specific multifunctional T cells that reside within the DP compartment (Fig. 2B). The acute cohort had a median multifunctional DP response ratio of 73% (0–92%) whereas the controllers exhibited a median of 2% (0–100%). It is notable that the controllers exhibited an extremely variable DP response ratio with most well below the acute cohorts' median and two patients presenting a DP ratio of 100%. Due to this high variability, the response ratios of the two groups only trended toward a significant difference ($p = 0.1677$).

To understand the differentiation stage of the whole and HIV-1-specific multifunctional DP cell subsets, we also examined their CD27, CD45RO, and CD57 expression. Within the general mem-

ory population of the CD4, DP, and CD8 memory compartments (Supplemental Fig. 2A–F), we observed that the CD4 memory compartment was predominantly CD27⁺CD45RO⁺CD57[–]; the CD8 memory compartment was evenly distributed between CD27 and CD45RO expression status with the frequency of cells lacking CD57 expression being ~2-fold higher than those displaying CD57 expression. The DP compartment was often intermediate to the frequencies displayed in either the CD4 or CD8 compartments but more closely resembled the CD8 compartment. When the HIV-1-specific subsets were analyzed, we found that in both the acute and controller cohorts, a median of 92% (33–99%; 50–100%) of multifunctional DP cells lacked CD57 expression and therefore were not terminally differentiated (Fig. 2C). Within the time span covered by our acute cohort, there were no significant correlations between the frequencies of CD4, DP, or CD8 multifunctional cells and time postinfection (Supplemental Fig. 3A–C). Conversely, the frequency of HIV-specific multifunctional DP cells within the controller cohort correlated with the viral loads within these patients ($p = 0.0282$).

Ag-specific contribution to HIV-specific responses

Next, we sought to identify the antigenic regions responsible for evoking the proliferation and multifunctionality observed within the DP compartment. Within the acute HIV infection cohort, we observed no significant Ag-specific differences in proliferative capacity of the DP cells (Fig. 3A). In contrast, the controllers' DP cells focused on all non-Env peptide pools (Fig. 3B). In fact, the DP proliferative response to Gag was significantly higher within the controller cohort than within the acute cohort ($p = 0.0437$). All other Ag-specific responses were not significantly different between the patient cohorts (p values summarized in Supplemental Table I).

In contrast to their proliferative response, the acute patients' multifunctional response was most pronounced to VVNRT, although responses were robust to most of the peptide pools (Fig. 3C). Meanwhile, the controllers' multifunctional response did not focus on an individual HIV Ag pool (Fig. 3D). We compared the multifunctional responses to each Ag between patient cohorts and observed that they were significantly higher among the acute patients than the controllers for all peptide pools except the Pol pool 1, which displayed a trend toward significance (p values summarized in Supplemental Table I).

We also compared the proliferative and functional responses across assays for the two peptide pools (Gag and VVNRT) that generated the strongest functional and proliferative responses. This analysis revealed no correlation between the level of proliferating DP cells and the frequency of multifunctional DP cells (Fig. 4A, 4B). This was observed in both the acute and controller cohorts.

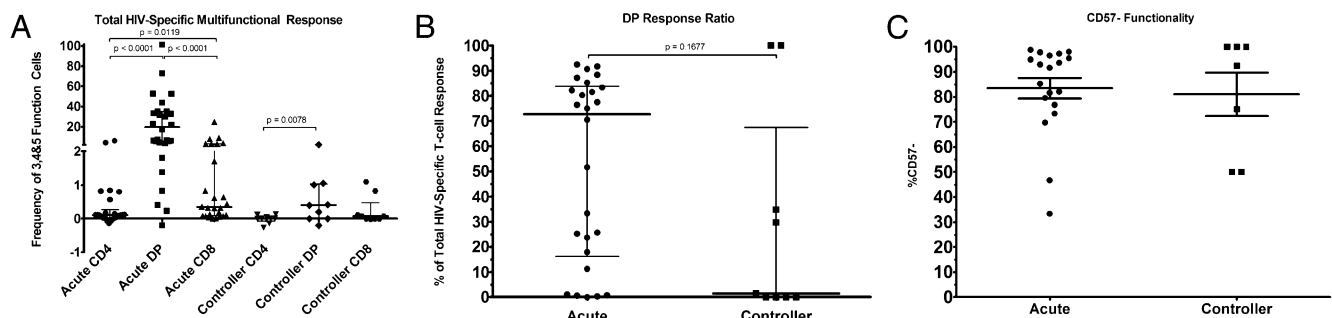
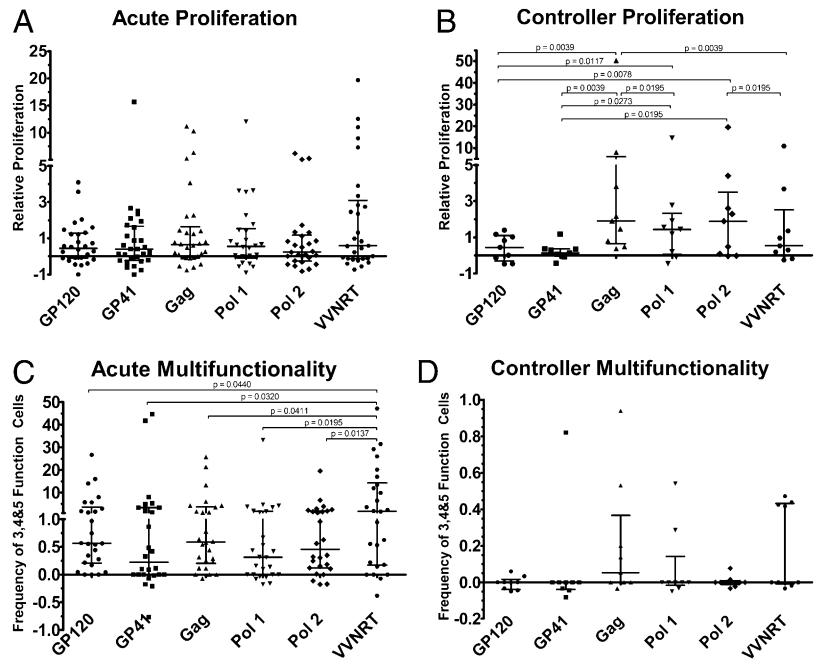


FIGURE 2. Total HIV-specific multifunctionality. (A) Cells were stimulated for 6 h with peptide pools representing the HIV proteome and then stained for expression of CD107a, IFN- γ , IL-2, MIP-1 β , and perforin. Using Boolean gating expression of all possible combinations of these functions was determined. The HIV-specific frequency of cells expressing three, four, or five of these functions within each T cell subtype was plotted for each patient. (B) The percentage of the total three-, four-, or five-function response coming from the DP compartment was calculated for each patient in the acute and chronic cohorts. (C) The percentage of multifunctional DP cells lacking expression of CD57.

FIGURE 3. HIV Ag-specific responses. **(A)** The relative proliferation of the DP compartment against each HIV Ag pool for patients in the acute cohort and **(B)** within the controller cohort. **(C)** The frequency of Ag-specific three-, four-, or five-function memory T cells responding to each HIV Ag pool in the acute cohort and **(D)** within the controller cohort.



Relationship between responses within the DP subset and the CD4⁺ and CD8⁺ T cell subsets

It is not clear whether DP cells leave the thymus as DP cells or whether they are derived in the periphery from the CD4 and/or CD8 compartments. To gain some insight into the origins of the DP response, we also determined the degree to which the magnitude of HIV-1-specific DP responses resembled those found within either the CD4 or CD8 compartments. Within the acute cohort, the level of proliferated DP cells was directly related to the proliferation observed within the CD4 compartment following stimulation with each HIV Ag (Fig. 5A and not shown). Furthermore, the magnitude of DP proliferation following stimulations with Gag, both Pol pools, and VVNRT also correlated with the proliferation observed in the CD8 compartment (Fig. 5A and not shown). Similarly, the controller DP proliferative response correlated with the CD4 response following stimulations with Gag and both Pol pools (Fig. 5B and not shown). However, the DP anti-Gag and VVNRT proliferative response correlated with the observed CD8 proliferation. Conversely, there were no significant correlations between the acute DP multifunctional response and the CD4 response. The magnitude of the acute anti-gp120 multifunctional DP response did correlate with the multifunctional frequencies observed in the CD8 compartment (Fig. 5C and not shown). Meanwhile, the controller DP multifunctional responses did not correlate with any CD4 or CD8 multifunctional responses (Fig. 5D and not shown).

The p values obtained from each of these comparisons are summarized in Supplemental Table I.

Discussion

Relative to the extensive analysis of the CD4⁺ and CD8⁺ T cell compartments, the role of circulating DP cells in immune responses to pathogens and cancer has rarely been studied. As a result, their immune function is poorly understood. In this study, to our knowledge we present the first evidence of Ag-specific DP cells simultaneously producing three or more functions.

Previously, a cohort of patients recently infected with HIV-1 was shown to contain DP cells that produced IFN- γ alone or in combination with either IL-2 or expression of CD107a in response to HIV (21). This work was performed at the level of the entire HIV proteome, leaving the Ag-specific breadth of DP cells unknown. Additionally, to our knowledge we present the first examination of HIV-specific DP cells within patients who naturally control HIV-1 infection. In this study, we sought to determine the Ag specificity of DP cells and whether DP cells were capable of mounting a wide variety of response modalities (CD107a, IFN- γ , IL-2, MIP-1 β , and/or perforin as well as proliferation) or were restricted to a narrow range of response types. This was done using peptide pools representing most of the HIV genome to stimulate cells isolated from acutely infected HIV patients. This study has shown that within these patients, DP cells represent an

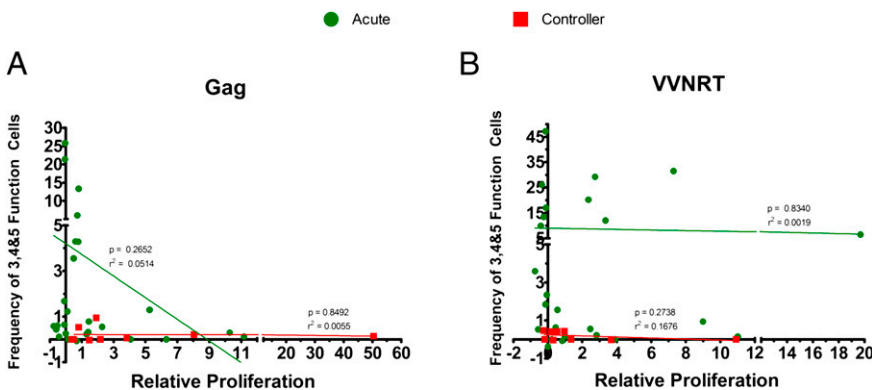


FIGURE 4. Relationship between multifunctionality and proliferation. **(A)** The frequency of Ag-specific three-, four-, or five-function DP cells is plotted against the relative proliferation of DP cells in response to gag stimulation and **(B)** in response to VVNRT stimulation.

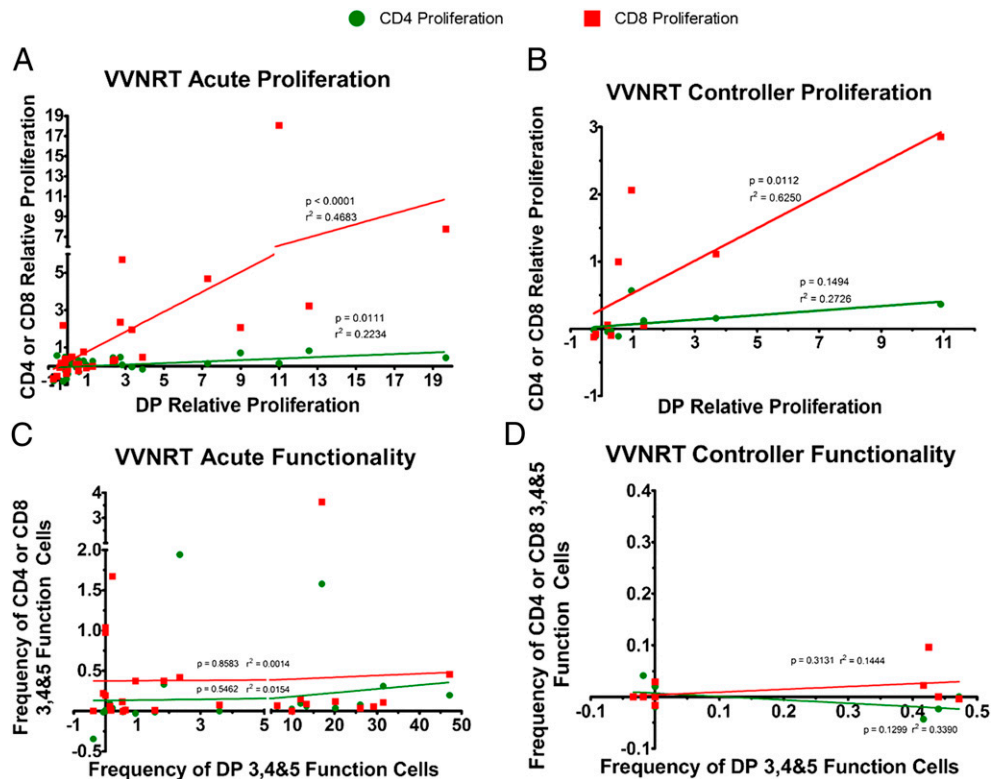


FIGURE 5. CD4 or CD8 responses versus DP responses. **(A)** The Ag-specific relative proliferation in the CD4 or CD8 compartment for each patient is plotted against that patient's Ag-specific relative proliferation in the DP compartment for patients in the acute cohort and **(B)** for patients in the controller cohort. **(C)** The Ag-specific frequency of three-, four-, or five-function CD4 or CD8 cells is plotted against that patient's Ag-specific frequency of three-, four-, or five-function DP cells for patients in the acute cohort and **(D)** for patients in the controller cohort.

immune subset that is capable of proliferation and mounting a multifunctional response. To our knowledge, we show for the first time that Ag-specific DP cells are capable of expressing three or more functions at a time. In fact, within the acute cohort studied, anti-HIV T cells expressing all five functions were almost exclusively DP cells (data not shown). Moreover, the multifunctional DP cells from the acute cohort were primarily focused on the VVVRT peptide pool, whereas the controller multifunctional DP cells were less focused in their Ag specificity.

The presence of multifunctional cells within the CD8 compartment during chronic infection has previously been shown to correlate with improved disease outcomes (22). Therefore, it will be important to track the DP response in treatment naive patients to determine whether multifunctional DP cells are as protective as multifunctional CD8 cells. Interestingly, the presence of multifunctionality in the DP population did not correlate with multifunctionality in the CD8 compartment. Our previous findings have shown that it may take as long as 55 wk to develop significant multifunctional CD8 responses (34). As a result, it is unclear whether there is truly a lack of a relationship between DP and CD8 multifunctionality or whether multifunctionality within the DP compartment may precede development of CD8 multifunctionality. Nonetheless, within our limited cohort of controllers there was a correlation between the frequency of multifunctional DP cells and lower viral loads at the time of sample acquisition. This suggests that multifunctional DP cells may have an impact on the successful control of virus replication.

We have shown that HIV-specific DP cells display both proliferative and multifunctional capabilities. These two qualitative aspects of the anti-HIV immune response have previously been correlated with improved disease outcomes when examined within other T cell compartments. Importantly, the samples in this study

were obtained while the patients' immune response would be expected to establish a partial control of viral replication toward the viral set point. Therefore, the DP responses described in this study are temporally associated with an expected decline in viral load and consequently may be important mediators of the temporary viral control experienced late in acute infection. For that reason, it will be important to isolate HIV-specific DP cells to test their ability to inhibit HIV *in vitro* either through direct inhibition of viral replication, by cytotoxic killing of HIV infected cells, and/or stimulation of Ab-dependent cellular cytotoxicity. Additionally, recent improvements in humanized mouse models may allow the selective addition and subtraction of DP cells to determine the role DP cells may play in the initial decline in viral loads following acute infection (35–38). If DP cells are able to not just respond to HIV Ags but in fact inhibit HIV, then it is important to note that a therapeutic vaccine trial has shown the ability to significantly increase HIV-specific IFN- γ production by DP cells in chronically infected patients with a single i.m. injection (39). This vaccine trial was able to demonstrate that not only are HIV-specific DP cells present in chronic infection, but that memory T cell responses result in the expansion of HIV-specific DP cells in addition to traditional CD4 and CD8 expansion. Therefore, DP cells may need to be considered in the development of therapeutic HIV vaccines. Additionally, Pahar et al. (13) have shown that DP cells are highly enriched in the gut, which is an important target site for protective HIV vaccine development. Our study revealed that the circulating HIV-specific multifunctional DP population generally lacks expression of CD57 and is therefore not terminally differentiated. Thus, it may be possible to generate a long-lasting protective DP memory pool. Unfortunately, Brenchley et al. (18) have shown that CD57⁻ memory T cells preferentially harbor HIV. Therefore, the DP cells we may want to elicit for protective im-

munity may also be the subset of DP cells HIV prefers to infect. As a result, extensive examination of dynamics between DP-mediated anti-HIV responses and infectability may be necessary.

Given previous work showing that DP cells are generally highly differentiated, our observation that they show evidence of proliferation following 6 d raises important questions about DP cell origins, at least in the HIV-1 infection model. Because a portion of DP cells resemble central memory T cells or even naive T cells, it is possible that the DP population is a self-sustaining population (21). Alternatively, a portion of single-positive CD4 and/or CD8 T cells could transition to DP status following antigenic activation and differentiation. In fact, previous work has shown that heavy stimulation (e.g., CD3/28, staphylococcal enterotoxin B) of CD8 T cells sustained over multiple days causes dim expression of CD4 (16, 40, 41). We observed CD4^{bright} in addition to CD4^{dim} DP cells within both stimulated and unstimulated conditions, and therefore sustained strong stimulation of CD8 cells is unlikely to explain the origin of the DP cells described in this study. Additionally, DP cells were also present and highly active in the ICS assay for which stimulations only lasted 6 h rather than multiple days as in the above studies. If the HIV-specific DP cells we observe are originating from a single-positive population, then the strong correlation between proliferated CD4 and DP cells means that it is likely that the CD4 compartment would be the primary source. Similarly, the work of Colombatti et al. (42) showing that DP cells show greater clonal similarity with CD4 cells than CD8 cells further supports this hypothesis.

In summary, we have demonstrated that DP cells are capable of mounting a large and highly diversified response to HIV Ags. Additionally, the DP response is maintained in viral controllers and similar responses within the CD4 and CD8 compartments have previously been correlated with improved disease outcomes. Therefore, it is important that this study forms the basis of further work delineating the effect this response has on viral replication and long-term disease outcomes. Furthermore, these DP cells may be an important vaccine target, which means we must work to more fully understand DP cell origins and development. In total, we have established DP cells as a major responding population to acute HIV infection and established a basis for extensive exploration of their origins and role in successfully combating HIV and other pathogens.

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Disclosures

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