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Human Immunodeficiency Virus Type 1 Controllers but Not Noncontrollers Maintain CD4 T Cells Coexpressing Three Cytokines▽

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Here, we evaluate the cytokine coexpression profiles of human immunodeficiency virus (HIV)-specific CD4 T cells for the expression of the cytokines gamma interferon (IFN-γ), interleukin-2, and tumor necrosis factor alpha. In controllers, CD4 T cells producing three or two cytokines (triple producers and double producers, respectively) represented >50% of the total response. In contrast, in noncontrollers ~75% of responding cells produced only one cytokine (single producers), mostly IFN-γ. Cells producing three cytokines were functionally superior to those producing single cytokines and showed an inverse correlation ($P < 0.001$) with viral load. These results demonstrate a strong association between the maintenance of highly functional CD4 T cells producing three cytokines and control of HIV-1.

Antiviral CD4 T cells play a vital role in the control of many viral infections. CD4 T-cell help is critical for the generation (3, 16, 23, 26) and maintenance (27) of functional CD8 responses. Similarly, CD4 T cells are crucial for the formation of germinal center reactions and the affinity maturation of B cells, which facilitates the generation of neutralizing antibody and formation of memory B cells (7, 20). Characteristics that are important for T-cell function in the control of viral infections include production of cytokines that mediate effector functions and support the expansion of the protective immune response (11, 24, 30), expression of CD40L for provision of costimulatory signals to responding B cells (19) and CD8 T cells (1, 5), and the ability to proliferate in response to stimulation by cognate antigen (14, 21). Recent studies have demonstrated a strong association between the maintenance of polyfunctional human immunodeficiency virus (HIV)-specific CD8 T cells capable of expressing multiple cytokines and control of HIV-1 (2). Here, we evaluate the cytokine coexpression profiles of virus-specific CD4 T cells from HIV-infected individuals for the expression of the cytokines gamma interferon (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor alpha (TNF-α) to better understand the relationship between the functional quality of antigen-specific CD4 T cells and viral control.

This study enrolled 37 HIV-1-infected adults (Table 1). These individuals included controllers (antiretroviral therapy-naïve subjects with plasma viremia of <1,000 HIV-1 RNA copies/ml for at least 1 year of follow-up [$n = 14$]), noncontrollers (antiretroviral therapy-naïve subjects with plasma viremia of >7,000 HIV-1 RNA copies/ml [$n = 8$]), and highly

TABLE 1. Clinical characteristics of study participants

Patient	CD4 count (no. of cells/μl of blood)	Viral load (no. of copies/ml of plasma)	HIV duration (yr)
Controllers			
201	493	640	16
202	841	680	18
341	720	400	14
353	999	440	16
357	534	82	2
358	1,005	120	5
360	470	280	4
364	596	628	2
365	447	990	3
366	500	50	1
367	1,423	50	2
368	1,051	50	2
369	764	712	1
370	613	251	1
Noncontrollers			
215	289	13,430	8
217	115	750,000	8
233	251	72,410	9
209	203	750,000	11
118	296	7,600	0.8
113	890	7,230	0.7
114	459	16,280	Unknown
126	491	84,580	0.2
HAART recipients			
203	725	50	17
205	494	120	8
206	310	50	8
212	281	540	3
214	518	50	2
219	575	50	20
221	172	620	3
222	273	70	10
229	410	1,220	23
230	139	1,400	9
235	524	660	14
236	453	50	6
343	523	200	1
348	313	360	19
351	182	50	6

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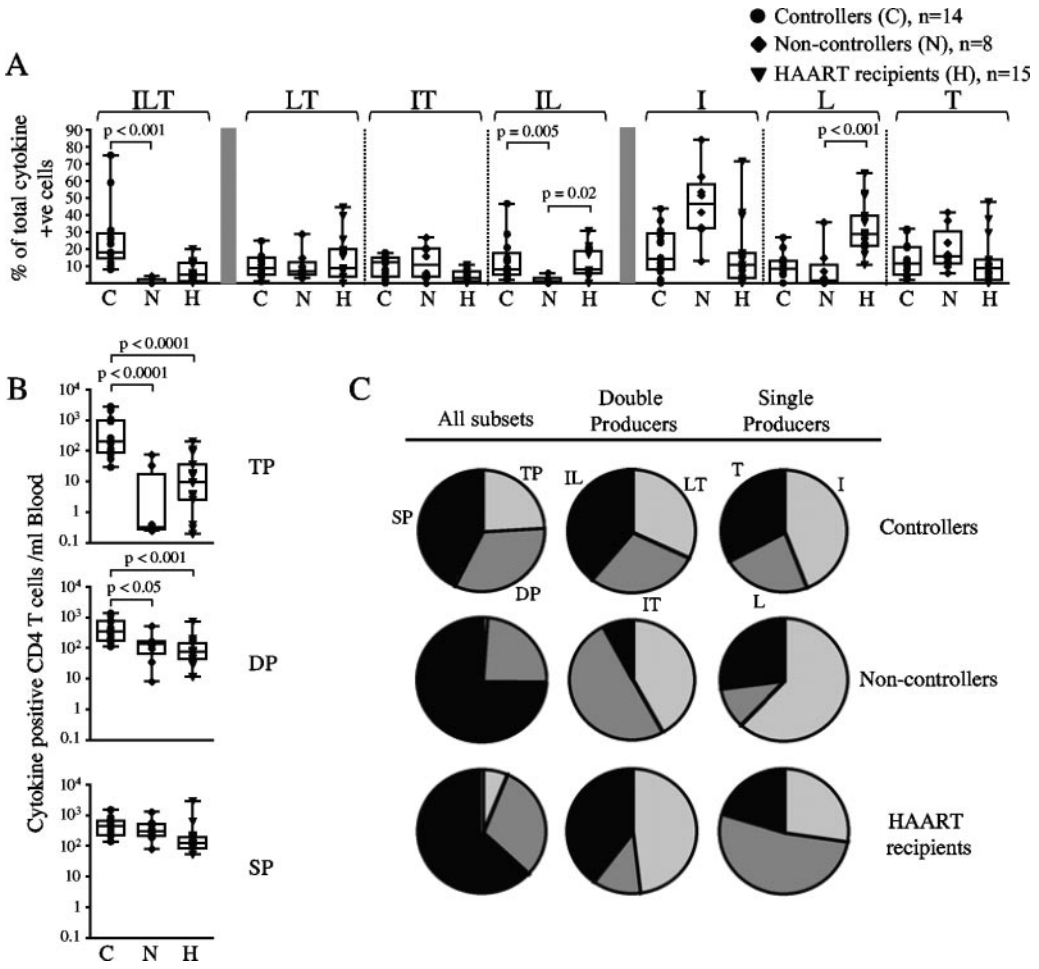

FIG. 1. Cytokine coexpression profile of HIV Gag-specific CD4 T cells in controllers, noncontrollers, and HAART-treated HIV-infected individuals. (A) The quality of response (cytokine subsets as a percentage of total cytokine-positive CD4 T cells). PBMC were stimulated with a pool of HIV-1 clade B consensus Gag-specific peptides (15-mers overlapping by 11; NIH AIDS Research and Reference Reagent Program catalog no. 8117). The ICS assay was performed as described previously (17). Approximately 500,000 lymphocytes were acquired on the LSRII (BD Immunocytometry Systems) and analyzed using FlowJo software (Treestar, Inc., San Carlos, CA). Lymphocytes were identified based on their scatter pattern, and $CD3^+$, $CD8^-$, and $CD4^+$ cells were all considered as CD4 T cells. These CD4 T cells were then gated for respective cytokine-positive cells. Boolean combination gating was then performed (see http://www.flowjo.com/v8/html/boolcomb.html for an example) to calculate the frequencies of the seven different combinations of cytokines using the Flowjo software. After subtracting the background, these seven different subsets were expressed as a percentage of total cytokine-positive cells and plotted for each antigen per individual. Responses that were greater than 0.07% of total CD4 T cells were considered for analysis. This criterion was defined based on the fact that we were dividing the total response into seven subsets and our detection limit was 0.01%, as described before (17). The percentage of cytokine subsets as a percentage of total cytokine-positive CD4 T cells was then plotted using Graphpad Prism. (B) Magnitude of response (absolute number of cytokine-positive cells per ml of blood). (C) Pie charts representing the quality of response. Mean frequencies of the indicated cytokine subsets are shown. TP, triple producers; DP, double producers; SP, single producers; I, IFN-γ; L, IL-2; and T, TNF-α.

active antiretroviral therapy (HAART) recipients (subjects who were on an effective HAART regimen for at least 1 year prior to enrollment [$n = 15$]) (9). For the study groups, the median CD4 T-cell counts were 667, 293, and 410 cells/μl of blood and the geometric mean viral loads were 246, 50,363, and 173 copies/ml of plasma for HIV-1 controllers, noncontrollers, and HAART recipients, respectively (Table 1). All of the HIV-infected individuals were recruited at the Emory Center for AIDS Research Clinical Research Core or at the Adult AIDS Clinic at the University of Alabama at Birmingham (UAB) in Institutional Review Board-approved studies. In the studies that follow, Wilcoxon's rank sum test was used to compare the magnitude of cytokine coexpression subsets, Wilcoxon's signed-rank test was used to compare cytokine mean fluorescent intensity (MFI) and frequency of CD40L-positive cells between triple producers and single producers, and Spearman's rank correlation test was used to assess the relationships between viral load and percentage of cytokine-positive CD4 T-cell subsets.

HIV-1 controllers but not noncontrollers maintained CD4 T cells coproducing IL-2, IFN-γ, and TNF-α. Cytokine coproduction profiles were assayed by intracellular cytokine staining (ICS) following stimulation of peripheral blood mononuclear cells (PBMC) with pools of overlapping clade B peptides (17). Antigen-specific CD4 T cells were categorized into seven different subsets consisting of triple producers, double producers,

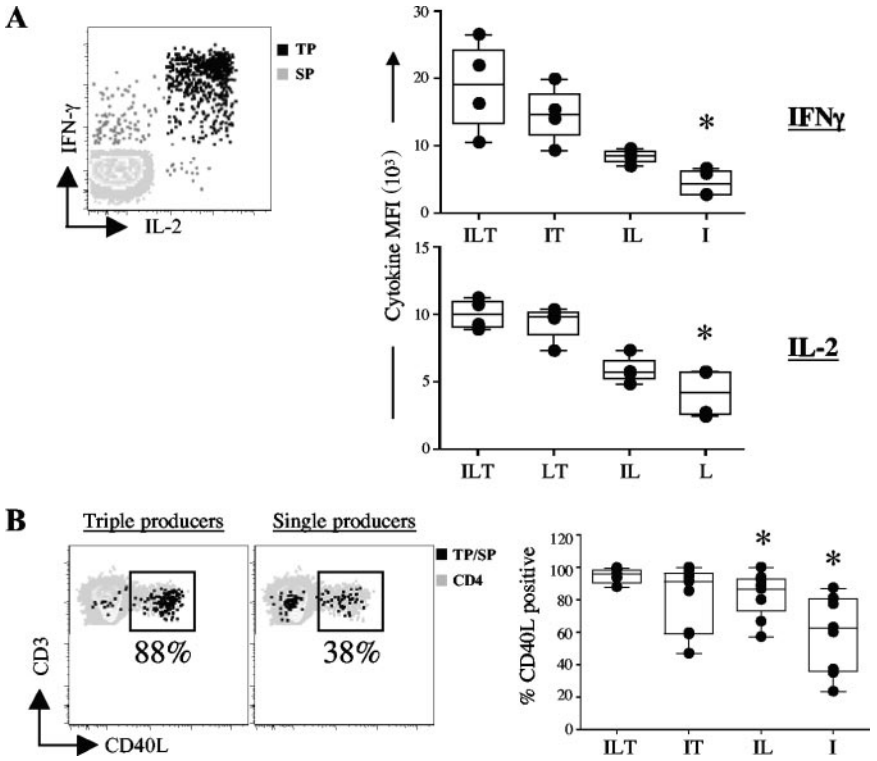

FIG. 2. Triple producers express higher levels of cytokines and CD40L than single producers. (A) Shown are a representative fluorescence-activated cell sorting plot giving the fluorescence intensity for IFN-γ and IL-2 for triple and single producers and a summary of MFI data for HIV Gag-specific CD4 response in four HIV-1 controllers. PBMC were stimulated with HIV-1 Gag peptide pools, and virus-specific CD4 T-cell responses were measured using an ICS assay. (See Fig. 1 for analysis.) The MFI of each cytokine for different cytokine coexpression subsets was analyzed. TP, triple producers; SP, single producers; I, IFN-γ; L, IL-2; and T, TNF-α. (B) Costimulatory potential of HIV-specific cytokine coexpression subsets. Shown are a representative plot giving the CD40L expression for triple and single producers and a summary of the HIV Gag-specific CD4 responses in four HIV-1 controllers, three noncontrollers, and two HAART recipients. Cytokine-coexpressing subsets were defined as described in the legend to Fig. 1. These subsets were then analyzed for expression of CD40L (CD154). Representative flow charts show the overlay of HIV-specific triple producers or IFN-γ single producers (black) on total CD4 T cells (gray). Numbers on the graphs represent the frequency of CD40L-positive cells as a percentage of the respective cytokine coexpression subset. *, significantly lower than triple producers ($P < 0.05$).

and single producers based on their coexpression of IFN-γ, IL-2, and TNF-α (Fig. 1). Individuals with CD4 responses that were greater than 0.07% of total CD4 T cells were considered for analysis. This criterion was defined based on the fact that we were dividing the total response into seven subsets and our detection limit was 0.01%.

The HIV-specific CD4 T cells from controllers consisted of all seven different subsets. Of the total response, 24% consisted of triple producers, 34% of double producers, and 42% of single producers (Fig. 1A and C). In contrast, CD4 T cells from noncontrollers consisted predominantly of single producers (mean of 75%), suggesting a preferential loss of triple producers in uncontrolled HIV infections ($P < 0.001$). Among the double producers, the Gag-specific response in controllers was equally distributed throughout the three subsets of double producers. In contrast, noncontrollers had similar levels of cells coproducing IL-2 and TNF-α and TNF-α and IFN-γ but lower levels of cells coproducing IFN-γ and IL-2 compared to controllers ($P = 0.005$). Among the single producers, the Gag-specific response consisted predominantly of IFN-γ-producing cells, which represented 19% of the response in the controllers and 47% of the response in the noncontrollers. HAART recipients had low levels of triple producers (6%) that were higher than in noncontrollers (1%; $P = 0.01$) but lower than that observed in controllers (24%; $P < 0.001$). They also had higher levels of IFN-γ-producing cells that coproduced IL-2 ($P = 0.02$) and IL-2 single producers ($P < 0.001$) than the noncontrollers. Consistent with the quality of the response, the magnitude of triple producers was also higher in controllers than noncontrollers (Fig. 1B).

Triple producers produce more cytokine per cell and possess better costimulatory potential than single producers. We next investigated whether triple producers are functionally superior to single producers. We studied the level of cytokine expression per cell as well as the coexpression of CD40L (Fig. 2). The amount of cytokine per cell was determined based on the MFI for each cytokine per subset. The triple and double producers produced higher levels of cytokines per cell than single producers ($P < 0.01$) (Fig. 2A). This was true for all three cytokines studied here (data not shown for TNF-α). The triple producers also produced higher levels of cytokines per cell compared to IFN-γ plus IL-2 double producers, with these differences being less pronounced than those of single producers. Tests for costimulation potential as measured by coexpres-

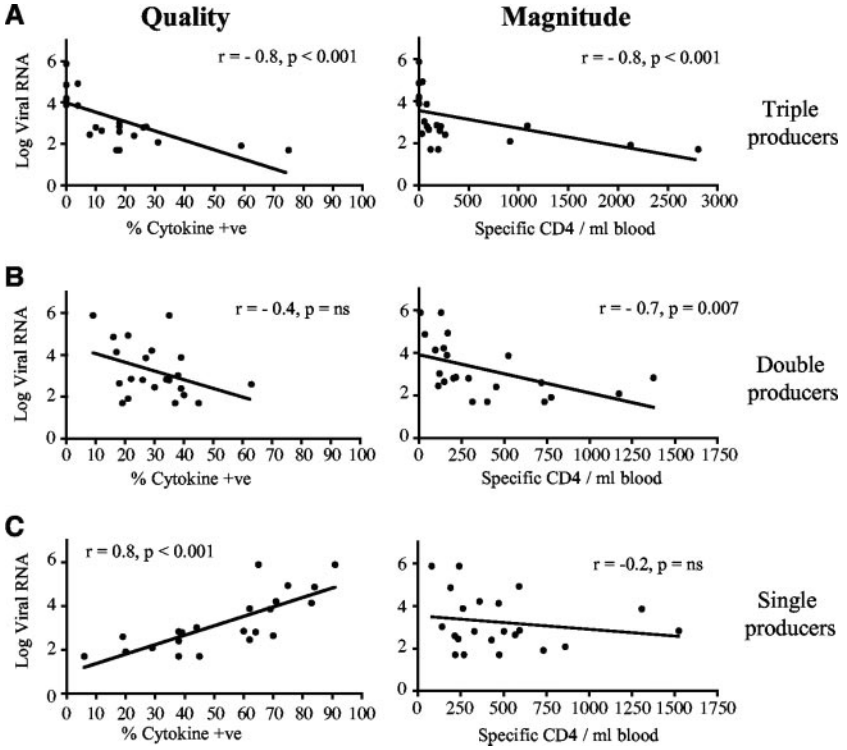

FIG. 3. Correlation between plasma viral load and cytokine coexpression subsets. Correlation between the quality (proportion of cytokine coexpression subset as a percentage of total cytokine-positive cells [left panel]) or magnitude (absolute number of cytokine-positive cells per ml of blood [right panel]) of cytokine-coexpressing cell subsets and plasma viremia for triple producers (A), double producers (B), and single producers (C) in untreated HIV-1-infected individuals.

sion of CD40L also revealed higher proportions of CD4 cells producing three and two cytokines than cells producing one cytokine coexpressing CD40L following stimulation (Fig. 2B). On average, 95% of triple producers expressed CD40L following stimulation, whereas only 61% of IFN-γ single producers expressed CD40L. These results show that HIV-specific triple producers are functionally more active in cytokine production and costimulation than single producers. These results are consistent with the results that we observed for triple and single producers for vaccinia virus, flu virus, and cytomegalovirus-specific CD4 T cells in humans (17).

Correlation between cytokine subset and plasma viral load. Correlations were made between the percentages of different cytokine coexpression subsets and levels of plasma viral RNA to better understand the relationship between the quality of the HIV-specific CD4 response and viral control (Fig. 3). These analyses revealed a strong inverse correlation between the triple producers and plasma viral RNA ($r = -0.8$; $P < 0.001$) (Fig. 3A) and a strong direct correlation between the single producers and plasma viral RNA ($r = +0.8$; $P < 0.001$) (Fig. 3C). A direct correlation was also observed between the percentage of IFN-γ single producers and viral load ($r = +0.6$, $P = 0.03$). There was no correlation between the percentage of double producers and viral load (Fig. 3B). No association was observed between IL-2 or TNF-α single producers and viral load (data not shown).

Further correlations tested how the magnitude of the response represented by the absolute number of different cytokine-coexpressing cells per ml of blood correlated with viral load. These correlations revealed strong inverse relationships between both triple producers ($r = -0.8$; $P < 0.001$) (Fig. 3A) and double producers ($r = -0.7$; $P = 0.007$) (Fig. 3B) and plasma viral RNA and no correlation between single producers and plasma viral RNA ($r = -0.2$; P, not significant) (Fig. 3C). These results demonstrate a strong association between low viremia and maintenance of high levels of HIV-specific CD4 T cells producing three cytokines in terms of both quality and magnitude.

The loss of highly functional CD4 T cells producing three cytokines in untreated HIV infections with high viral load could result from preferential killing, skewed maturation, exhaustion of T cells, or a combination of all three mechanisms. We do not consider preferential killing by direct viral replication alone as a mechanism because a similar phenomenon has been reported in mice for lymphocytic choriomeningitis virus infections, which do not kill CD4 T cells (4). We favor the hypotheses that persisting high levels of viral antigen induce a skewed maturation or exhaustion of HIV-specific CD4 T cells, phenomena that have been shown for CD8 T cells specific for HIV (2, 6) and lymphocytic choriomeningitis virus (29) and for CD4 T cells specific for HIV (10).

Consistent with previous reports (10, 12, 15, 18, 31), a higher proportion of HIV-specific CD4 T cells from HAART recipients produced IL-2 than HAART-naïve noncontrollers. However, comparison of the cytokine coexpression profiles revealed that the majority of IL-2 production in HAART

recipients is restricted to IL-2 single producers, whereas in controllers it is distributed throughout all three subsets (triple, double, and single producers). Thus, these results indicate that HAART generates a different quality of CD4 T-cell response than that seen in controllers with respect to their ability to help CD8 T-cell function and may explain the failure of HAART to control rebound viremia following treatment interruption (8, 13, 15).

Both IL-2 and CD40L have been shown to restore the function of HIV-specific CD8 T cells in vitro (22). Our results clearly demonstrate that triple producers produce more IL-2 per cell and preferentially express CD40L compared to single producers. This preferential expression of CD40L by triple producers and the loss of triple producers in HIV-1 noncontrollers is consistent with the previously reported impairment in the capacity of CD4 T cells to up-regulate CD40L during progressive HIV infection (25, 28). Collectively, our results suggest a critical role for the maintenance of CD4 T cells producing three cytokines for preservation of functional HIV-specific CD8 T cells during chronic HIV infection.

In conclusion, our results studying the coexpression profile of cytokines IFN-γ, IL-2 and TNF-α demonstrate a strong association between the maintenance of antiviral CD4 T cells capable of coexpressing two or more cytokines and viral control. In addition, they demonstrate that triple producers are functionally superior to single producers not only for the number but also for the level of produced cytokines per cell and for costimulatory potential. Our study also demonstrates that HAART restores a different quality of CD4 T cells comprised predominantly of IL-2 single producers and strongly suggests that therapeutic vaccination strategies for HIV should aim to elicit CD4 T cells that coproduce more than one cytokine.

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