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Distinct Transcriptional Profiles in Ex Vivo CD4$^+$ and CD8$^+$ T Cells Are Established Early in Human Immunodeficiency Virus Type 1 Infection and Are Characterized by a Chronic Interferon Response as Well as Extensive Transcriptional Changes in CD8$^+$ T Cells$^{\nabla}$†

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Changes in T-cell function are a hallmark of human immunodeficiency virus type 1 (HIV-1) infection, but the pathogenic mechanisms leading to these changes are unclear. We examined the gene expression profiles in ex vivo human CD4$^+$ and CD8$^+$ T cells from untreated HIV-1-infected individuals at different clinical stages and rates of disease progression. Profiles of pure CD4$^+$ and CD8$^+$ T-cell subsets from HIV-1-infected nonprogressors with controlled viremia were indistinguishable from those of individuals not infected with HIV-1. Similarly, no gene clusters could distinguish T cells from individuals with early infection from those seen in chronic progressive HIV-1 infection, whereas differences were observed between uninfected individuals or nonprogressors versus early or chronic progressors. In early and chronic HIV-1 infection, three characteristic gene expression signatures were observed. (i) CD4$^+$ and CD8$^+$ T cells showed increased expression of interferon-stimulated genes (ISGs). However, some ISGs, including CXCL9, CXCL10, and CXCL11, and the interleukin-15 alpha receptor were not upregulated. (ii) CD4$^+$ and CD8$^+$ T cells showed a cluster similar to that observed in thymocytes. (iii) More genes were differentially regulated in CD8$^+$ T cells than in CD4$^+$ T cells, including a cluster of genes downregulated exclusively in CD8$^+$ T cells. In conclusion, HIV-1 infection induces a persistent T-cell transcriptional profile, early in infection, characterized by a dramatic but potentially aberrant interferon response and a profile suggesting an active thymic output. These findings highlight the complexity of the host-virus relationship in HIV-1 infection.

The pathogenesis of human immunodeficiency virus type 1 (HIV-1) infection remains ambiguous despite intense research efforts in the past two decades. The infection is characterized by changes in T-cell function and homeostasis resulting in inexorable CD4$^+$ T-cell depletion and AIDS at a mean of 8 years of infection. Defects in T-cell function are observed at all stages of infection (9, 15). Specifically, CD4$^+$ T cells fail to produce interleukin-2 (IL-2) after antigenic stimulation (24); their levels of IL-2 receptor and CD28 decrease (7), their proliferation is decreased, and apoptosis is increased (17, 24, 34). This state, often referred to as anergy, has been linked to a general state of immune activation observed in HIV-1 infection (21), yet its mechanisms remain unclear. The changes occur in uninfected CD4$^+$ T cells as well as in CD8$^+$ T cells (7, 19, 34), which suggests a systemic, indirect effect of the infection. Chronic, systemic immune stimulation as a result of viral replication has been proposed as the cause of these defects (2). Others have suggested specific proteins, such as HIV-1 Tat or alpha IFN (IFN-α), as factors inducing the observed immune changes (42).

Various groups have monitored gene expression in cell lines and primary T cells infected with HIV-1 in vitro (1, 16, 40, 41). While informative, these studies ignore the fact that the HIV-1-induced T-cell abnormalities are systemic and affect all cells and not just the minor fraction of HIV-1-infected cells. Thus, studies examining the effect of direct HIV-1 infection on gene expression of infected cells in vitro are not representative of the systemic effects, which are observed in vivo. Other groups have monitored gene expression in peripheral blood mononuclear cells (PBMCs) in primate models of infection (4) and in human patients (31) as well as in unfractionated cells from lymph nodes (22, 31), in B cells from peripheral blood (26), and in latently infected T cells (8). Profiling of the response of human peripheral CD4$^+$ and CD8$^+$ T-cell subsets to in vivo HIV-1 infection, however, has not yet been reported. We hypothesized that direct examination of CD4$^+$ and CD8$^+$ T cells would yield further insights into why the majority of infected individuals are unable to control viral replication and to identify why CD4$^+$ and not CD8$^+$ T cells undergo progressive depletion.

In the current study, we compared global expression profiles of pure CD4$^+$ and CD8$^+$ T cells obtained ex vivo from males not infected with HIV-1 and from HIV-1-infected males at various clinical stages of HIV-1 infection, including early

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HIV-1 infection, chronic progressive infection, and nonprogression.

MATERIALS AND METHODS

Study participants. Clinical data from 5 HIV-1 male volunteers not infected with HIV-1 (participants N1 to N5) and 15 untreated HIV-1-infected individuals who were recruited for this study are listed in Table 1. Five individuals with early infection (participants A1 to A5) were infected by HIV-1 within 6 months of study (diagnosed by evolving Western blot analysis to examine HIV-1 levels) and were asymptomatic at time at which blood was drawn. Another five individuals (participants C1 to C5) had chronic progressive HIV-1 infection defined as documented HIV-1 infection for at least 1 year, with evident $CD4^+$ T-cell decline to <50 cells/μl and a viral load of >10,000 copies/ml (branched DNA). All chronic progressors were asymptomatic. The last five participants (participants L1 to L5) were nonprogressors as defined by chronic infection for at least 3 years, no evidence of $CD4^+$ T-cell decline, and a viral load of <500 copies/ml (branched DNA) without any therapy. None of the long-term nonprogressors carried the 32-bp deletion in the CCR5 gene, as determined by PCR using SP4.760 and PM 6.946 primers as detailed by Liu et al. (23). None of the individuals in this study were treated with antiretroviral medications at the time of or prior to study. Informed consent was obtained from participants in accordance with the guidelines for conduct of clinical research at the University of Toronto and St. Michael's Hospital, Toronto, Ontario, Canada. All investigational protocols were approval by the University of Toronto and St. Michael's Hospital institutional review boards. Samples from additional donors in all four groups were collected as well for use in confirmation experiments.

Isolation of peripheral blood T cells. PBMCs were obtained from 20 volunteer male donors through leukopheresis (Spectra apheresis system; Gambro BCT) between 9 a.m. and 3 p.m. Buffy coats were collected using Ficoll-Paque Plus (Amersham Biosciences) following the manufacturer's instructions. Pure $CD4^+$ and $CD8^+$ T cells were obtained by depletion using StemSep human $CD4^+$ T-cell enrichment cocktail (catalog no. 14052; StemCell) and human $CD8^+$ T-cell enrichment cocktail (catalog no. 14053; StemCell), respectively, following the manufacturer's instructions. PBMCs (2×10^8) were used as starting material for depletions from uninfected donors, while 50% more cells were used for depletions from HIV-1-infected donors. Purity levels of 94% to 98% were observed (data not shown). For the in vitro interferon treatment experiments PBMCs of other healthy donors were collected and T cells were isolated as described above.

RNA processing. Purified T cells were lysed, and RNA was extracted using an RNeasy Mini kit (QIAGEN). The viscosity of the lysates was reduced using a Qiashreder column (QIAGEN) per the manufacturer's instructions. Leftover genomic DNA was digested by DNase I treatment (DNA-free kit, catalog no. 1906; Ambion). Total RNA (5 to 6 μg) was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen), and second-strand synthesis was performed using a SuperScript double-stranded cDNA synthesis kit (catalog no. 11917-010; Invitrogen) per the manufacturer's instructions. cDNA was purified using the cDNA cleanup columns of a GeneChip sample cleanup module (catalog no. 900371; Affymetrix). A total of 10% of the double-stranded cDNA sample was saved for further analyses, while another 50% was used for in vitro transcription of biotin-labeled cRNA with a BioArray HighYield RNA transcript labeling kit (catalog no. 900182; Enzo Life Sciences) following the manufacturer's instructions. cRNA was purified using the IVT cleanup columns of a GeneChip sample cleanup module (catalog no. 900371; Affymetrix). Between 30 and 70 μg of cRNA was obtained. cRNA (0.5 μg or 0.1 μg) was reverse transcribed back to cDNA using random hexamers for further quantitative PCR (qPCR) analysis. Total RNA was also isolated from 26 PBMC samples as well as from an additional 7 cryopreserved PBMC samples. A 2.5 μg volume of each sample was reverse transcribed as described above and used in further qPCR analyses.

RNA quality control. Quality of total RNA and unfragmented cRNA was monitored using a BioAnalyzer 2100 microfluidics station and an RNA 6000 Nano LabChip kit (Agilent Technologies). For total RNA, samples showing degradation or an rRNA ratio (28S/18S) lower than 1.5 were reprocessed; for unfragmented cRNA the distribution of the fragments was monitored.

Microarray hybridizations and processing. All microarray procedures followed Affymetrix protocols (Affymetrix). Briefly, 20 μg of biotin-labeled cRNA was fragmented following GeneChip sample cleanup module protocols (catalog no. 900371; Affymetrix). Fragmented cRNA was hybridized to Hu133A microarrays (Affymetrix) for 16 h in a GeneChip 640 hybridization oven (Affymetrix) in hybridization cocktail. Washing, staining, and scanning were performed on site using a Fluidics 400 station (Affymetrix) for washes and a GeneArray scanner (Agilent) for scans. Initial image processing was performed using GCOS version 1.2 software (Affymetrix).

TABLE 1. Profiles of the participants[a]

Participant	Class	Age at donation (yr)	Viral load/ml	CD4 count/mm^3	Estimated duration of infection
A1	Early infection	25	8,459	710	2 mo
A2	Early infection	49	>500,000	440	3 mo
A3	Early infection	26	140,000	740	5 mo
A4	Early infection	27	325792	400	1 mo
A5	Early infection	32	459,640	520	4 mo
Avg		31.8		562.0	
C1	Chronic, no treatment	36	288,959	230	>1 yr
C2	Chronic, no treatment	40	63,106	490	7 yr
C3	Chronic, no treatment	32	182,741	240	>2 yr
C4	Chronic, no treatment	33	36,813	420	>3 yr
C5	Chronic, no treatment	39	17,498	390	2 yr
Avg		36.0		354.0	
L1	Long-term nonprogressor	59	<50	800	15 yr
L2	Long-term nonprogressor	39	<50	1,000	11 yr
L3	Long-term nonprogressor	34	124	670	9 yr
L4	Long-term nonprogressor	29	269	1,180	3.5 yr
L5	Long-term nonprogressor	44	<50	670	12 yr
Avg		41.0		880.0	
N1	Negative control	42	NA[b]	NA	NA
N2	Negative control	27	NA	NA	NA
N3	Negative control	34	NA	NA	NA
N4	Negative control	43	NA	NA	NA
N5	Negative control	32	NA	NA	NA
Avg		35.6			

[a] All participants were male.
[b] NA, not applicable.

Microarray data analyses. GCOS version 1.2 software (Affymetrix) was used with the MAS5 statistical algorithm to obtain the expression levels from microarrays along with those of the present and absent calls. The following settings were used: alpha1, 0.05; alpha2, 0.065; tau, 0.015; gamma1H, 0.0045; gamma1L, 0.0045; gamma2H, 0.006; gamma2L, 0.006; target, 500; NF, 1. Scaling factors ranged from 1.2 to 4.8 (mean, 3.0; standard deviation, 0.86). For further analyses data were $\log_2$ transformed, and genes evaluated by GCOS as present in fewer than three samples were eliminated. Significance of Microarray Analysis version 1.21 software (39), an Excel (Microsoft) add-in, was used to identify genes differentially expressed between groups based on a delta setting, resulting in false discovery rates (FDR) closest to 5% (two-class unpaired test, with 300 permutations, using 10-Nearest-Neighbors Imputer). Hierarchical clustering (by complete linkage) was performed with Genesis software (Graz University of Technology, Austria). Gene symbols and gene identification numbers used in this text were consistent with the Entrez Gene database as of June 2006. In later analyses, data from A and C samples as well as from N and L samples were combined for increased power.

RT qPCR. Primary cDNA samples were quantified using 10 ng of DNA, whereas cDNA samples obtained from cRNA were quantified using 1 ng of DNA. Specific primers <100 bp long designed for this study using Primer Express version 2.0 software (Applied Biosystems) are listed in Table S1 in the supplemental material. Real-time (RT) qPCR analyses were performed using an ABI Prism 7900HT sequence detection system and SDS version 2.1 software (Applied Biosystems). SYBR green (Applied Biosystems) detection was employed. Each reaction was run in triplicate; 10 μl reaction mixes contained 3 mM

$MgCl_2$, 0.2 μl ROX dye, 0.3 μl SYBR green dye, 0.2 μl of 0.10 mM deoxynucleoside triphosphate mix, 0.025 μl of Platinum *Taq* DNA polymerase, and 1 μM of each primer. Quantification was achieved using a known quantity of genomic DNA obtained from the salivary gland of a healthy female. Serial dilution of the genomic DNA was used to obtain a standard curve. It was assumed that each gene tested had two copies per genome; otherwise, appropriate corrections were applied to the copy estimates. After the elimination of outliers, the remaining replicates were used to estimate the original copy number of each message and the results were averaged.

Interferon treatments. Primary $CD4^+$ or $CD8^+$ T cells were plated in 6-well polystyrene plates (catalog no. 3516; Corning) at 1×10^6 cells/ml in 5 ml of complete RPMI 1640 medium (10% [vol/vol] fetal bovine serum, 1% [vol/vol] GlutaMax-1) (catalog no. 35050-061; Gibco-Invitrogen), 100 U/ml penicillin G, 100 μg/ml of streptomycin (catalog no. 15140-122; Gibco-Invitrogen), and 10 mM HEPES (catalog no. 35050-061, Gibco-Invitrogen). After overnight incubation, at time 0, IFN-α2b (Intron-A; Shering-Plough) was added for a final concentration of 1,000 units/ml to the 24-h treatment wells and an equivalent volume of medium was added to control wells. An identical IFN-α2b dose was added 16 h later to the 6-h treatment wells and after 22 h to the 2-h treatment wells. At 24 h after time 0, cells in all wells were collected, washed twice in phosphate-buffered saline, and lysed. RNA was isolated and reverse transcribed as described above under "RNA processing." Each treatment was run in duplicate using cells obtained from a single leukopheresis procedure as well as once more using cells from an independent donor (both donors were HIV-1 negative). Additional IFN-α2b treatments were performed on PBMCs from a frozen sample bank. Samples were thawed by quick immersion in a 37°C water bath and transferred to prewarmed RPMI 1640 medium (as described above) to be incubated overnight. The next day, any dead cells were washed away by two phosphate-buffered saline washes and the remaining PBMCs were resuspended in fresh medium in 6-well polystyrene plates as described above. IFN-α2b (Intron-A; Sherring-Plough) was then added to the treatment wells for a final concentration of 1,000 units/ml, and an equivalent volume of medium was added to control wells. PBMCs were collected after 4.5 h, washed, and lysed. RNA isolation and analysis were then performed as described above.

Microarray data accession number. Raw data will be made available through the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under accession no. GSE 6740.

RESULTS

Gene expression profiles for HIV-1-infected and uninfected individuals. We studied a cohort of HIV-1-infected individuals with various clinical stages of HIV-1 infection and healthy uninfected volunteers as a control group (Table 1). We included five individuals with early HIV-1 infection, five with chronic progressive HIV-1 infection, five individuals with nonprogressive HIV-1 infection with low or undetectable viral loads, and five individuals who were not infected with HIV-1. The HIV-1-infected individuals had never been on therapy prior to entering the study. One donor (participant L5) was subjected to leukopheresis again after 1 year to assess the variability of gene expression over time. No significant differences were observed in the expression levels of seven genes tested by qPCR (data not shown). None of the nonprogressors carried the 32-bp CCR5 deletion associated with resistance to infection (data not shown).

RNA from ex vivo $CD4^+$ and $CD8^+$ T-cell subsets (>94% purity by fluorescence-activated cell sorter analysis; data not shown) was used to obtain gene expression data from Affymetrix Hu133A microarrays, which contained 22,500 probe sets corresponding to approximately 16,500 genes. We employed SAM 1.21 software to identify differentially expressed genes in two-way comparisons between the four groups. SAM was selected over other methods of analysis because it avoids setting artificial fold-induction cutoffs and designating any particular samples as baseline. It is a nonparametric method of determining statistically significant gene expression patterns that also allows for estimating FDR.

At a setting of a 5% false discovery rate no differences were observed between N and L samples (Fig. 1a), and only four genes were differentially regulated between A and C samples (Fig. 1b). In contrast, many differentially expressed genes were identified in comparisons of data obtained from N versus C, N versus A, L versus A, and L versus C samples in both $CD4^+$ and $CD8^+$ T-cell gene expression profiles (Fig. 2a). Lack of discernible differences between profiles from L and N samples and A and C samples allowed us to combine these groups in order to increase the power of SAM to detect significant differences between these two patterns (Fig. 1c). In fact, more genes were identified as differentially expressed when using the combined classes (Fig. 2b). In this way, two distinct patterns of gene expression emerged, one that was typical in uninfected and long-term nonprogressors and one that was common to both early and chronic progressive HIV-1 infections. The genes differentially regulated in the comparisons of N plus L data versus A plus C data are listed in Table S2 in the supplemental material. As well, $CD8^+$ T cells contained strikingly more differentially regulated genes than $CD4^+$ T cells. Only $CD8^+$ T cells showed downregulated genes in A and C samples in comparison to N and L samples (Fig. 2b and d).

To confirm the microarray results we selected 77 genes and compared their transcript levels in the same samples by RT qPCR. The selected genes and their specific primer sets are listed in Table S1 in the supplemental material. A total of 63 genes had RT qPCR profiles matching those observed on microarrays (data not shown). Another six genes showed only partial similarity in that at least one sample showed a marked difference in the transcript abundance (relative to the other samples) from that of the microarray profile. Finally, eight (10%) genes did not show concordance between RT qPCR and microarray profiles (see Table S1 in the supplemental material). This result is slightly higher than the 5% FDR used to obtain our results, and it may reflect the fact that some Affymetrix probe sets are not fully specific and can cross-hybridize with nontarget sequences.

IFN-stimulated genes are upregulated in viremic HIV-1. Inspection of the genes upregulated in the A and C groups revealed many known type I IFN-stimulated genes (ISGs). We therefore compared our observed gene profiles obtained from SAM analysis to published IFN-α stimulation profiles (5, 18, 28, 37), as well as to profiles generated in house, and found a significant overlap, ranging from 15% to 51% in $CD4^+$ samples and from 24% to 51% in $CD8^+$ samples. Significantly, almost all the ISGs were found in the group of genes upregulated in A and C samples and not in the downregulated cluster observed in $CD8^+$ cells. Since ISG profiling has not been reported previously for primary $CD4^+$ and $CD8^+$ T cells, we considered the possibility that all of the upregulated genes we observed could be interferon regulated. To test whether this was the case, we tested for the IFN-α2b inducibility of those of the RT qPCR-confirmed genes that did not appear in any published ISG list but were upregulated in A and C samples in either $CD4^+$ or $CD8^+$ cells or both (15 genes). As a positive control we also tested for induction of six reported ISGs (DNAPTP6, HERC6, IFI44, IFI44L, OAS2, and SOCS1). We obtained freshly isolated ex vivo $CD4^+$ and $CD8^+$ T cells from

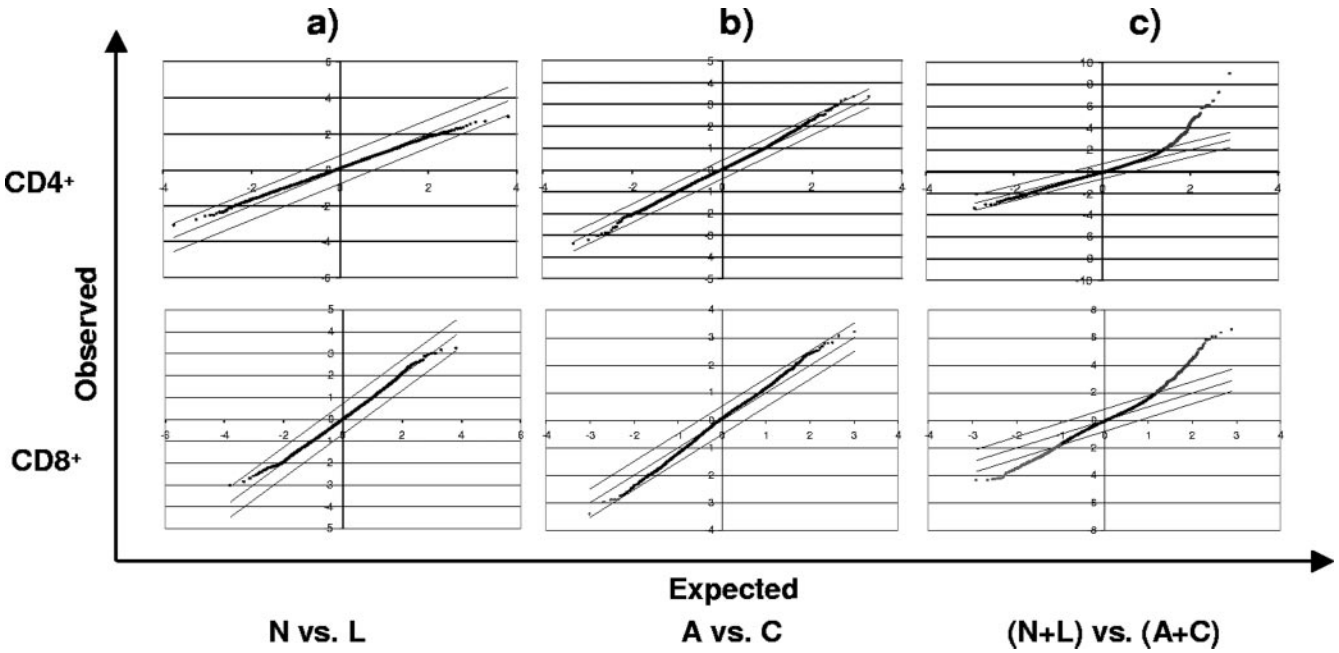

FIG. 1. SAM analysis of 20 $CD4^+$ and 20 $CD8^+$ samples across four clinical groups. Two-class SAM analysis was performed with 300 permutations. False discovery rate brackets of 5% were selected. The points outside the broken lines represent significantly upregulated genes (above the upper line) and downregulated genes (below the lower line). $CD4^+$ and $CD8^+$ T-cell transcriptional profiles revealed no significant differences between long-term nonprogressors (L) and uninfected individuals (N) (a) and only four genes differentiated between early (A) and chronic (C) infection (b); however, when combined cohorts were compared, large differences between L and N samples and A and C samples were observed (c).

two uninfected healthy volunteers and treated the cells with 1,000 U/ml of IFN-α2b for 0, 2, 6, and 24 h and then tested for changes in transcription of the selected genes by RT qPCR. Transcripts of all 6 known ISGs increased at either 2 or 6 h after addition of IFN-α2b compared to only 1 out of the 15 genes not known to be ISGs (the single new ISG is the transmembrane protein 15 gene TMEM156; GeneID 80008) (data not shown). This result suggests that IFN stimulation could not account for all the changes observed between A and C samples versus N and L samples.

We next asked whether ISGs alone could be used to cluster the samples in an unsupervised manner into distinct clinical subtypes. Using a set of well-established ISGs (see Table S3 in the supplemental material) we ran hierarchical clustering of both $CD4^+$ and $CD8^+$ samples. We found that an ISG data set alone was sufficient to separate A and C samples from N and L samples in both T-cell types, whereas a control set composed of a similar number of randomly selected non-ISG genes could not (Fig. 3a to c). Nevertheless, confirming observations from SAM analysis, analysis using ISGs was not sufficient to delineate between samples from patients infected early (A) and those infected chronically (C) or between uninfected (N) and nonprogressing (L) donors. When ISGs were removed from the full data set, the remaining genes failed to fully separate the samples, although the $CD8^+$ samples still showed partial separation (Fig. 3d).

In summary, based on the upregulation of ISGs in early and chronic infection, $CD4^+$ and $CD8^+$ T cells from early and chronic HIV-1 infection have global gene expression profiles that can be clearly distinguished from those of long-term nonprogressive infection (viral controllers) and of uninfected patients. Global T-cell gene expression profiles of nonprogressors are indistinguishable from those that have no HIV-1 infection.

We next focused on reported ISGs we did not find differentially regulated in our analyses. These could represent (i) false negatives, (ii) genes not expressed in T cells, or (iii) genes expressed but unchanged in T cells. We selected six ISGs for further analysis: CCL2, CCL8, CXCL9, CXCL10, CXCL11, and IL-15 alpha receptor (IL-15Rα). The first three, CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC), are chemokines known to act on T cells and monocytes through the receptor CXCR3, itself not an ISG (29). CCL2 and CCL8 are also chemokines for mononuclear cells (14). IFN-α induction of all five chemokines has been reported previously in PBMCs (37) but not specifically in T cells. Consistent with this, microarray results showed no expression of these five chemokines in $CD4^+$ and $CD8^+$ samples in any clinical group. However, when ex vivo $CD4^+$ and $CD8^+$ T cells freshly isolated from an uninfected donor were treated with IFN-α2b the expression of all five cytokines was strongly induced at 2 and 6 h in $CD4^+$ cells and more weakly in $CD8^+$ cells (Fig. 4).

The sixth gene we examined, IL-15Rα, was shown to be expressed at a low level in microarray data and by RT qPCR, but its expression was not raised in A and C patient samples in either T cells or PBMCs (not shown). However, the gene was observed to be IFN-α2b inducible at 2 and 6 h in purified $CD4^+$ and $CD8^+$ T cells (Fig. 4). Taken together, the data show that a number of chemokines and the IL-15Rα, which can be induced by type I IFN in vitro in T cells, are not

a)

CD4	N	L	A	C	
N	-	0	73	52	upregulated
L	0	-	51	21	
A	1	0	-	4	
C	5	0	0	-	
downregulated					

CD8	N	L	A	C	
N	-	0	771	86	upregulated
L	0	-	320	19	
A	798	138	-	0	
C	152	0	0	-	
downregulated					

b)

CD4	N + L	A + C
N + L	-	162
A + C	0	-

CD8	N + L	A + C
N + L	-	505
A + C	844	-

c)

d)

FIG. 2. Summary of SAM analysis. Numbers of genes found significant by SAM in various two-class, unpaired comparisons are shown. The groups represented by the letters in the upper horizontal bars in panels a and b were analyzed with respect to the groups represented in the vertical bars. Upregulated genes are shown in dark gray and downregulated genes in light gray (e.g., 52 genes were found upregulated in $CD4^+$ T cells of chronic progressors [C] compared to those of uninfected donors [N]). (a) Each of the four clinical groups was subjected to pairwise analysis in comparisons to each other group. (b) N and L groups were combined and analyzed in comparison to a combined A and C group. (c and d) Breakdown of genes upregulated (c) and downregulated (d) in A and C samples compared to N and L samples into those occurring in $CD4^+$ and $CD8^+$ T-cell subsets.

upregulated in early or chronic HIV-1 infection in vivo as are other ISGs.

The finding of ISGs upregulated in the T cells from patients infected with HIV-1 from 2 months to 7 years suggested that the type I IFN response is induced chronically in HIV-1 infection. This led us to investigate whether such a chronic IFN response limits further inducibility of ISGs in response to fresh IFN-α stimulation which could potentially prevent beneficial antiviral effects of interferon from affecting HIV-1 replication. To investigate this, we subjected PBMCs from a subset of 16 patients to IFN-α2b stimulation for 4.5 h and monitored levels of selected ISGs by RT qPCR. The fold increases of the RNAs for IFI16, IFI44, IFI44L, and CD38 in the IFN-α-treated samples were no lower in A or C samples than in N or L samples. In fact, in some A and C samples they were higher (Fig. 5). This indicates that the transcriptional response to type I interferon is not downregulated in T cells of HIV-1-infected individuals despite the chronic IFN response.

T-cell differentiation profile. Since not all genes identified by SAM analyses could be attributed to an IFN-like stimulus, we searched for other informative patterns in the $CD4^+$ and $CD8^+$ gene sets (20). In a study of transcriptional profiles of human T cells at various stages of differentiation, Lee et al. identified a panel of 180 genes that were differentially expressed between thymocyte subsets and peripheral T cells and which included genes involved with DNA replication, cell cycle regulation, cellular signaling, transcriptional regulation, and apoptosis (listed in Table S3 in the supplemental material). We identified similarities between this thymocyte-specific profile and that of genes differentially regulated in A and C samples. Specifically, a cluster of genes found to be enriched in single-positive thymocytes was also upregulated in A and C samples. When the select panel of genes identified by Lee et al. as immaturity markers (see Table S3 in the supplemental material) was used for the hierarchical clustering of samples from both studies we observed that while the intrathymic precursors, dual-positive thymocytes, and thymic stromal cells clustered

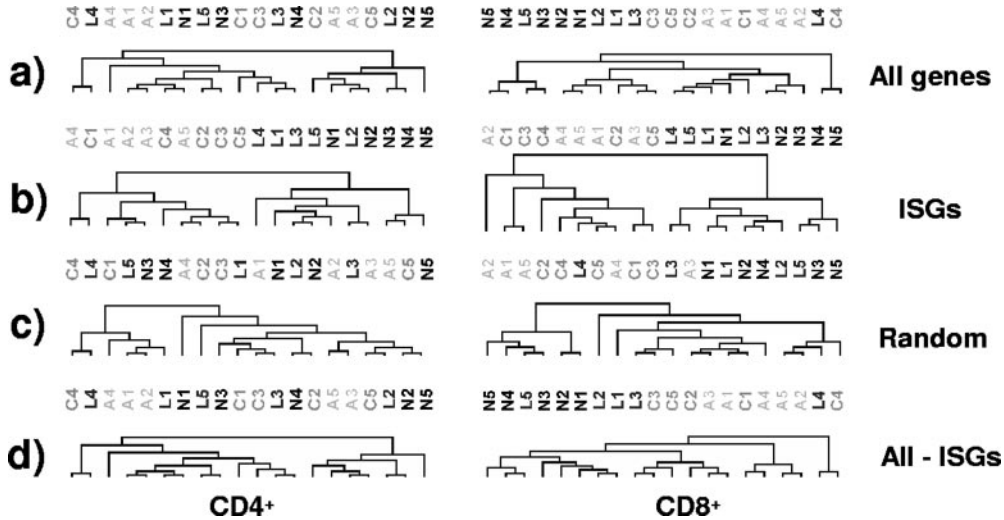

FIG. 3. Hierarchical clustering of gene expression profiles from $CD4^+$ and $CD8^+$ T-cell samples. The following sets of genes were used in clustering: (a) all genes; (b) only ISGs; (c) a random sample of genes equivalent in number to but excluding the genes represented in panel b; (d) all genes except ISGs. Samples: N, uninfected; L, long-term nonprogressors; A, early infection; C, chronic infection.

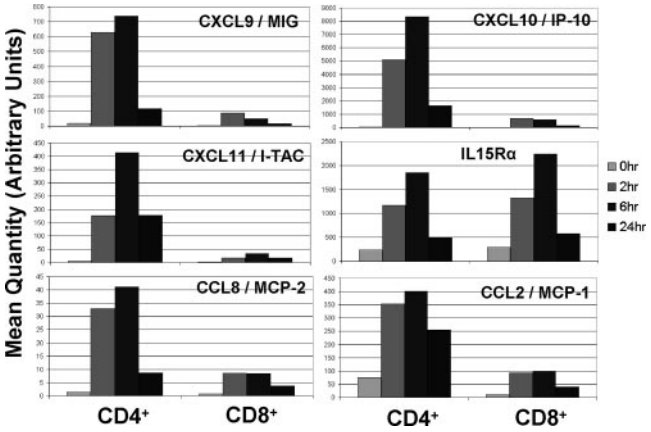

FIG. 4. IFN-α2b-mediated induction of selected genes in primary $CD4^+$ and $CD8^+$ T cells. Purified $CD4^+$ and $CD8^+$ T cells, obtained from two uninfected donors, were treated in vitro with IFN-α2b for 0, 2, 6, and 24 h. The mRNA levels of CXCL9, CXCL10, CXCL11, CCL2, CCL8, and IL-15Rα were determined by RT qPCR and quantified relative to the expression of a housekeeping gene (TATA-binding protein).

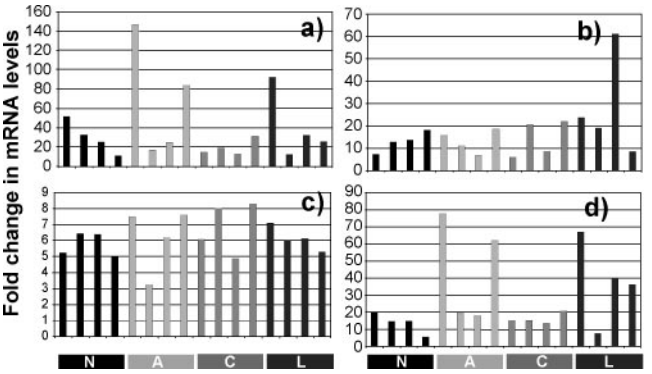

FIG. 5. IFN response in donor samples. PBMC samples from 16 donors were treated with IFN-α2b for 4.5 h, and the induction of interferon response was monitored by measuring the changes in the amount of mRNAs of the four selected ISGs by RT qPCR. Each bar represents the fold increase of a given mRNA in one clinical sample. Samples: N, uninfected; L, long-term nonprogressor; A, early infection; C, chronic infection. Genes: (a) IFI44; (b) CD38; (c) IFI16; (d) IFI44L.

separately, the single-positive thymocytes clustered with the majority of our samples from acutely and chronically infected HIV-1 patients while the cord blood and adult blood T-cell profiles from the Lee study clustered with our profiles from uninfected subjects and long-term nonprogressors (Fig. 6a). When the same set of genes was used for the hierarchical clustering of only the samples in this study (Fig. 6b) we observed that the $CD4^+$ T-cell samples were well classified into A and C subsets and N and L subsets (Fig. 6b, row 2) compared to the whole gene set (Fig. 6b, row 1) or a random gene set with the same number of genes (Fig. 6b, row 3). The classification was not perfect, as two $CD4^+$ C samples clustered with L and N samples. $CD8^+$ T-cell samples clustered by the same set of genes were divided into three clusters, one of which included 8 of 10 L and N samples (Fig. 6b).

Taken together, these observations suggest that the gene expression of $CD4^+$ and $CD8^+$ T-cell subsets from early and chronically infected patients shows a cluster that is characteristic of single-positive thymocytes whereas in long-term nonprogressors these genes have expression profiles resembling those of circulating peripheral T cells. The majority of genes which segregate the samples are involved in DNA replication, cell cycle regulation, and apoptosis.

$CD8^+$-specific cluster. When we removed both ISGs and the T-cell differentiation markers (DMs) from the data set and used the remaining genes in unsupervised clustering, the clusters of $CD8^+$ samples still preserved partial separation of A and C samples from N and L samples, in contrast to $CD4^+$ samples, which failed to separate to any degree (data not shown). This suggested that the remaining $CD8^+$ data contained more genes differentiating the clinical groups. In fact, while the ISGs and DMs account for the majority of the 162 differentially regulated genes found in $CD4^+$ samples, they constitute only a minority of 1,349 such genes in $CD8^+$ samples. The large difference in the number of genes differentially expressed (162 versus 1,349) in $CD4^+$ and $CD8^+$ samples and the fact that only $CD8^+$ cells showed downregulated genes are two unexpected findings, considering that it is the $CD4^+$ cells that undergo progressive depletion. Since it was possible that this imbalance was an artifact of our methods of microarray analysis, we have used RT qPCR to compare the levels of gene expression of 32 selected genes for which microarray data indicated differential expression in only one T-cell subset. For 29 of these genes the patterns were confirmed by RT qPCR (data not shown). Another possible reason for the difference is that the $CD8^+$ samples were more uniform in their gene expression and therefore presented a less noisy gene set, allowing for detection of larger number of small changes. To compare variability of the genes in $CD4^+$ and $CD8^+$ samples, we have compared the standard deviations of the expression values for all the genes in both T-cell subsets and found no skew that could point to standard deviations being significantly larger in either subset (data not shown). Taken together, these observations suggest that the microarray results reflect real differences in the extent of changes occurring in $CD8^+$ versus $CD4^+$ cells in HIV-1 infection.

Using L2L Microarray Analysis Tool software (30) we have compared the $CD8^+$-specific non-ISG, non-DM genes to over 800 gene sets previously reported (as of June 2006) in published microarray-based experiments, and we were unable to match our results to those previously obtained in any of those experiments. We were also unable to match the gene set to any specific pathway or cell state. This group of genes (see Table S2 in the supplemental material) contains many known immune regulatory genes such as SOCS2, SOCS3, SOCS5, TOB1, LEF1, TCF, and others, most of which were downregulated in A and C samples.

Among the many genes downregulated in $CD8^+$ T cells in A and C samples we noticed a group of known transforming growth factor beta (TGF-β)-inducible genes, including SMAD7. Since SMAD7 is also a known negative regulator of the TGF-β response (27), we therefore asked whether TGF-β-inducible genes are upregulated in these samples. Since we could not find any publications reporting profiles of TGF-β response in T cells, we treated $CD4^+$ and $CD8^+$ T cells from a single uninfected donor with TGF-β for 1 h and 3 h and profiled the response using Affymetrix U133A arrays. We observed that TGF-β induced expression of about 200 genes; compared to our patient profile

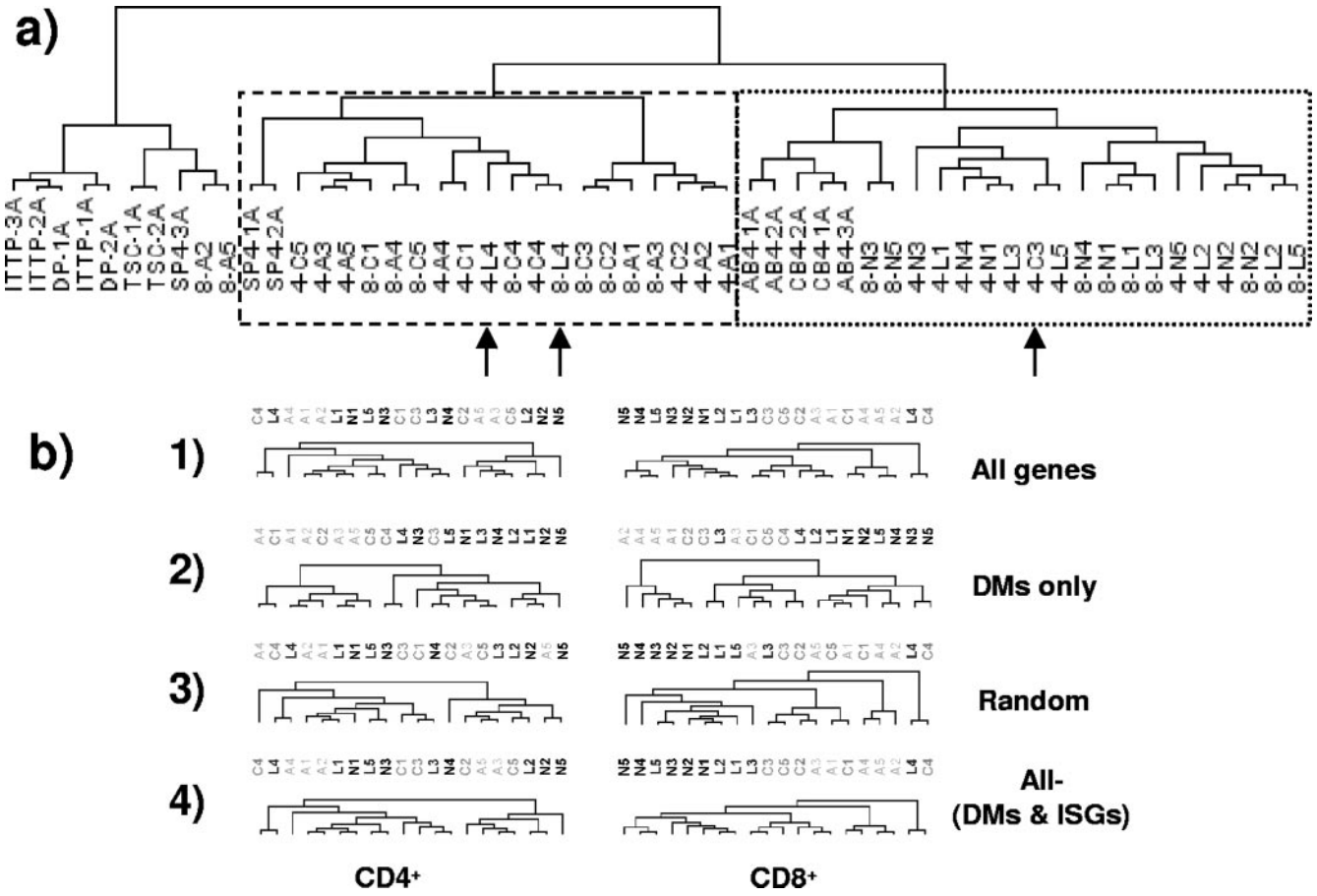

FIG. 6. Thymocyte development markers in $CD4^+$ and $CD8^+$ T-cell profiles. (a) Hierarchical clustering of the gene expression profiles from our study with those from a study by Lee et al. (20) of various thymocyte and T-cell populations by use of the cluster of immaturity markers identified in Lee et al. Abbreviations from Lee study: ITTP, intrathymic precursors; DP, dual-positive thymocytes ($CD4^+$ $CD8^+$); SP4, single-positive $CD4^+$ thymocytes; CD4, cord blood CD4s; AB4, adult blood CD4s; TSC, thymic stroma cells. Abbreviations from our study: 8, $CD8^+$ T-cell sample; 4, $CD4^+$ T-cell sample. Samples from participant L4 and the $CD4^+$ sample from participant C3 did not cluster with the majority of their respective clinical groups (arrows). (b) Hierarchical clustering of gene expression profiles from $CD4^+$ and $CD8^+$ T-cell samples using all genes (row 1); only DMs (row 2); a random sample of genes equivalent in number to but excluding those in row 2 (row 3); and all genes except DMs and ISGs (row 4). Samples: N, uninfected; L, long-term nonprogressor; A, early infection; C, chronic infection.

results, the transcription levels of the majority (~85%) of these genes did not change in HIV-1 infection, suggesting that the TGF-β response was unaffected.

In order to confirm that HIV-1 infection does not affect TGF-β hyperresponsiveness due to downregulated SMAD7, we treated PBMCs from 16 subjects employed in this study with TGF-β for 1.5 h and monitored the fold increase of selected TGF-β-responsive mRNAs by RT qPCR. We observed no significant differences in upregulation of any of the five genes tested (SMAD7, TGIF, TIEG, TIEG2, and SKIL) among any clinical groups (data not shown). Taken together these results indicate that TGF-β transcriptional response is not differentially regulated in HIV-1 infection.

Immune activation genes. Although HIV-1 infection has been described as a disease of immune activation, the nature and mechanisms of the immune activation of T cells in vivo have not been fully characterized. We asked whether gene expression patterns in the T cells reflected those found after signaling through the T-cell receptor and CD28. We compared our gene set results with those reported by Diehn et al. (12), who profiled T cells that were stimulated with αCD28/αCD3 antibodies. For $CD8^+$ T cells, only 16% of genes reported as upregulated twofold in T cells after 48 h of αCD28/αCD3 treatment were upregulated in A and C samples. Similarly, only 13% were codownregulated. For $CD4^+$ T cells, there was even less similarity. Thus, the gene expression profile of T cells from early or progressive HIV-1 infection is not typical of acute immune activation through the T-cell receptor.

DISCUSSION

We show that HIV-1 infection induces characteristic patterns of gene expression in $CD4^+$ and $CD8^+$ T cells and that these patterns can distinguish progression from nonprogression. First, we saw little difference in expression profiles of T cells from early infection (infected for under 6 months) and chronic progressors (infected >1 year), indicating that a stereotypical pattern of gene expression within T cells as a result of HIV-1 infection is already established early on in the infection and persists for years thereafter. Second, T-cell gene ex-

pression of HIV-1-infected nonprogressors is similar to that seen with uninfected individuals. Long-term nonprogressors form a unique group of HIV-1-infected individuals who have excellent virologic control and no overt disease (6, 32), while their T-cell functions are preserved and the lymph node architecture shows little evidence of immune activation despite the persistence of low-level viral replication (25). We found no evidence of gene clusters which were differently regulated that correlated with nonprogression compared to the results seen with persons who were not infected with HIV-1. That is, we found no transcriptional evidence that $CD4^+$ or $CD8^+$ T cells in these individuals were actively responsible for virological control, which was surprising given that $CD8^+$ T cells are classically viewed as important for viral clearance. We cannot rule out, however, the possibility that minor populations of $CD4^+$ and/or $CD8^+$ T cells transcriptionally programmed to control virus were diluted out in the total T-cell populations examined. Third, in early and chronic HIV-1 infection we find a greater perturbation of gene expression in $CD8^+$ versus $CD4^+$ T cells: although both $CD4^+$ and $CD8^+$ T cells had upregulated genes, only $CD8^+$ T cells had downregulated genes. This highlights the fact that HIV-1 infection has disparate effects on $CD8^+$ and $CD4^+$ T-cell populations. Finally, as only a small percentage of T cells are infected by HIV-1 in vivo, the changes in T cells during viremia likely represent bystander effects rather than effects in infected cells.

T cells in early and chronically HIV-1-infected individuals display a transcriptional signature of being chronically exposed to type I IFN-like signal; this response occurs early in HIV-1 infection. Type I interferons (IFN-α and IFN-β) are produced in response to microbial infections as part of the innate immune response (33). These cytokines induce a transcriptional program that can lead to antimicrobial and antiproliferative states in target cells, although the antiviral effects of IFNs have been characterized previously in most detail (35, 36). Additionally, recent studies show that type I IFNs have potent immunomodulatory functions, such as the ability to mature dendritic cells and modulate lymphopoiesis (3, 38). Among the hundreds of ISGs listed to date (10, 11), the best-characterized ones remain those involved directly in interference with viral replication, such as those of the OAS family, Mx1 and Mx2 and PKR (35). However, the sheer scale of the response suggests that the IFNs exert multiple and some as-yet-uncharacterized effects on cell function.

Our finding of IFN response signatures in T cells agrees with several recent studies that also reported type I IFN responses in HIV-1 infection. In a parallel study, Moir et al. have shown ISG induction in B cells of viremic patients compared to that in aviremic or uninfected donors (26). This observation, together with our finding that ISG upregulation is seen both in PBMCs and in pure $CD4^+$ and $CD8^+$ T cells, suggests that the stimulus responsible for ISG induction is acting broadly on all circulating leukocytes. The study by Li et al. that examined changes in lymph node biopsy samples pre- and post-highly active antiretroviral treatment showed that a large group of ISGs were downregulated in the post-highly active antiretroviral therapy samples, which is consistent with the model of these genes being upregulated prior to the therapy (22). This indicates that the effect of the IFN-like stimulus is also present in T cells residing in the lymph nodes and not just circulating T cells, and it may be that the IFN-like signature we observed in the peripheral T cells originates in the lymph nodes. Finally, in a study looking at simian-human immunodeficiency virus infection in cynomolgus macaques, Bosinger et al. observed ISGs to be induced in blood within 2 weeks postinfection (4), which supports our finding that the IFN-like gene transcription profile is induced early and may be sustained throughout the infection.

Based on the available transcriptional data and our in vitro studies, we find that a number of ISGs important for immune trafficking (CXCL9, CXCL10, CXCL11, and CCL8) and cytokine signaling (IL-15Rα) are not expressed in T cells in HIV-1 infection despite the fact that we can induce their expression with IFN-α2b in T cells from uninfected individuals. The lack of induction of these genes may affect the overall antiviral effects of the IFN response and allow viral replication to persist. It is also possible that some ISGs observed in acute IFN responses are repressed in chronic IFN responses as a result of immune regulation. We failed to detect significant differences in the ISG patterns between early and chronic HIV-1 infection, however, and so if such downregulation of ISGs takes place, it must occur earlier in infection than the time periods we examined. Interestingly, in a recently published study of gene expression changes in ex vivo T cells acutely infected with HIV-1 (24 h) (1), an ISG set similar to that seen in our study was identified; the results included demonstration of a lack of chemokine and IL-15Rα induction. That suggests that transcriptional induction of these ISGs is prevented in HIV-1 infection. Further work to elucidate the mechanisms for the atypical ISG expression is clearly warranted.

The second gene expression signature in $CD4^+$ and $CD8^+$ T cells in early and chronic progressors closely resembled that found in single-positive thymocytes. A number of possibilities could explain these findings. First, in contrast to peripheral T cells, thymocytes throughout their differentiation are actively undergoing proliferation and apoptosis. Indeed, a proportion of genes upregulated in this cluster are associated with cell cycle function. Thus, these findings are consistent with a more rapid turnover of $CD4^+$ and $CD8^+$ T cells in progressive HIV-1 infection. A second explanation would be that this transcriptional pattern reflects an enhanced release of recent thymic emigrants into the peripheral blood in order to compensate for peripheral T-cell destruction and maintain homeostasis, as has recently been demonstrated by Dion et al. (13). In addition, IL-7 has been shown to drive T-cell replenishment in HIV-1 infection. Further studies will be required to determine whether the transcriptional pattern we observed here reflects IL-7 signaling.

We also observed a large cluster of genes specific to $CD8^+$ samples of early and chronically infected patients that failed to match any published transcriptional profile. Future studies will determine whether this profile is unique to HIV-1 or is found in other virus infections. In addition, it is worth pointing out that the transcriptional patterns we observed in $CD4^+$ or $CD8^+$ T cells may be a result of enrichment for certain small subsets of T cells with large transcriptional changes as opposed to uniform changes occurring in all T cells. This is especially likely, since T cells from viremic individuals are enriched for T-cell subsets such as activated memory and effector cells, all of which have different gene expression profiles.

In summary, gene expression profiling of T cells in HIV-1 infection reveals a complex interplay between host and virus adaptation. Characteristic gene expression is established early during HIV-1 infection and persists during progression. It will be important to determine whether the interferon response which is induced during infection is aberrant and whether it can subsequently be manipulated to better control viral replication and limit T-cell dysfunction.

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