JOURNAL OF VIROLOGY, Dec. 2006, p. 11486–11497 Vol. 80, No. 23
0022-538X/06/$08.00+0 doi:10.1128/JVI.00324-06

Diminished Production of Monocyte Proinflammatory Cytokines during Human Immunodeficiency Virus Viremia Is Mediated by Type I Interferons▽†

John C. Tilton,[1] Alison J. Johnson,[1] Marlise R. Luskin,[1] Maura M. Manion,[1] Jun Yang,[2] Joseph W. Adelsberger,[2] Richard A. Lempicki,[2] Claire W. Hallahan,[1] Mary McLaughlin,[1] JoAnn M. Mican,[1] Julia A. Metcalf,[1] Christiana Iyasere,[1] and Mark Connors[1]*

Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland,[1] and Clinical Services Program, SAIC-Frederick Inc., Frederick, Maryland[2]

Received 14 February 2006/Accepted 29 August 2006

The effect of human immunodeficiency virus (HIV) infection and high-level HIV replication on the function of monocytes was investigated. HIV-positive patients had elevated levels of spontaneous production of some or all of the monocyte proinflammatory cytokines measured (interleukin-1β [IL-1β], IL-6, and tumor necrosis factor alpha [TNF-α]) compared to uninfected controls. In patients on therapy with high frequencies of monocytes producing proinflammatory cytokines, this frequency was diminished in the context of viremia during an interruption of therapy. Diminished production of proinflammatory cytokines during viremia was restored by culture with autologous CD4$^+$ T cells or monocytes from an on-therapy time point or lipopolysaccharide (LPS). Microarray analysis demonstrated that diminished monocyte production of proinflammatory cytokines was correlated with elevated type I interferon-stimulated gene transcripts. The addition of exogenous alpha 2A interferon diminished the spontaneous production of IL-1β, IL-6, and TNF-α but did not affect responses to LPS, recapitulating the changes observed for HIV-viremic patients. These results suggest that monocyte function is diminished during high-level HIV viremia and that this effect is mediated by chronic stimulation by type I interferons. This effect on monocytes during viremia may play a role in diminished innate or adaptive immune system functions in HIV-infected patients. In addition, the restoration of these functions may also play a role in some immune reconstitution syndromes observed during initiation of therapy.

One of the hallmarks of human immunodeficiency virus type 1 (HIV-1) infection is generalized immune system activation, although the mechanisms by which this is induced remain incompletely understood (reviewed in references 2 and 22). In HIV-infected patients, increased immune system activation is typically characterized by increased turnover of CD4$^+$ and CD8$^+$ T cells (24, 29, 40, 46) and increased expression of activation markers, such as CD38 (16, 31, 39) and HLA-DR (15, 26, 47), on immune system cells. The consequences of chronic activation on the function of cells of the immune system are not well defined and are likely important in the pathogenesis of HIV-1, the opportunistic infections and malignancies associated with HIV disease, and HIV-specific immunity.

Over the past several years, an increasingly detailed picture of the effects of HIV viremia on the immune system has emerged. It has become clear that HIV viremia has effects on a wide variety of cells involved in innate or adaptive immune responses and mediates these effects through a number of different direct or indirect mechanisms. HIV viremia causes an expansion of HIV-specific CD4$^+$ and CD8$^+$ T cells (17, 37, 38, 43, 49, 56). Other direct effects have also been described, including binding of viral products to non-antigen-specific receptors, such as envelope protein binding to CCR5 (55) or HIV RNA binding to toll-like receptor (TLR) 7 or 8 (5, 23). Effects of HIV viremia on immune system cells, such as those mediated by proinflammatory cytokines (reviewed in references 8 and 10) or cell-contact-dependent effects on natural killer (NK) cells (1, 36), might also be indirect. Although the details of many of these effects remain to be further defined, it appears clear that viremia has some direct or indirect effect on nearly every immune system cell type.

In the present study, we report an effect of HIV infection and high-level HIV viremia on the function of monocytes. HIV-positive patients (on or off therapy) had elevated levels of spontaneous production of some or all of the monocyte proinflammatory cytokines measured (interleukin-1β [IL-1β], IL-6, and tumor necrosis factor alpha [TNF-α]) compared to uninfected controls. In patients on therapy with high frequencies of monocytes producing proinflammatory cytokines, this frequency was diminished in the context of viremia during an interruption of therapy. Unexpectedly, HIV-1-infected patients on antiretroviral therapy with effective control of viral replication had markedly elevated levels of monocytes producing these proinflammatory cytokines, even compared with uninfected controls. Diminished frequencies of monocytes producing proinflammatory cytokines during active HIV-1 replication were tightly correlated with increased type I interferon (IFN)-stimulated gene expression and monocyte HLA-DR expression. Monocyte cytokine production was partially restored by cell-cell contact with autologous monocytes or CD4$^+$ T cells

* Corresponding author. Mailing address: LIR, NIAID, NIH, Bldg 10, Rm 11B-09, 10 Center Dr., MSC 1876, Bethesda, MD 20892-1876. Phone: (301) 496-8057. Fax: (301) 402-0070. E-mail: mconnors@niaid.nih.gov.

† Supplemental material for this article may be found at http://jvi.asm.org/.

▽ Published ahead of print on 27 September 2006.

from time points when the patients were on therapy. Treatment of peripheral blood mononuclear cells (PBMC) with recombinant IFN-α2A in vitro caused diminished production of monocyte proinflammatory cytokines, recapitulating the observations in patients off therapy. These findings suggest a potent effect of in vivo production of type I IFN on monocyte function. In addition, these findings likely have important implications for the pathogenesis of HIV-1, for opportunistic infections, and in reconstitution syndromes associated with improved immunologic function following control of HIV-1 replication.

MATERIALS AND METHODS

Study population. HIV-1 infection in study participants was documented by HIV-1/2 immunoassay. All HIV-1-infected subjects signed informed consent and participated in protocols approved by a National Institute of Allergy and Infectious Diseases (NIAID) investigational review board. An additional 22 HIV-1-seronegative, healthy volunteers from the National Institutes of Health donor apheresis clinic were recruited into a control cohort.

Storage of samples. PBMC were freshly isolated from peripheral blood or apheresis donor packs by sodium diatrizoate-Ficoll density centrifugation (with lymphocyte separation medium [ICN Biomedicals, Aurora, OH]). PBMC were cryopreserved in cell culture freezing medium-dimethyl sulfoxide (Gibco, Grand Island, NY) by using a Cryomed controlled-rate freezer (ThermoForma, Waltham, MA) and were stored at −140°C.

Multiplex supernatant cytokine assay (Luminex). Cytokine concentrations of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, granulocyte-macrophage colony-stimulating factor, TNF-α, and IFN-γ were measured in the supernatants of stimulated and unstimulated PBMC by using the human cytokine LINCOplex multiplex bead array (Linco Research, St. Charles, MO). Briefly, incubations and washes were performed in 1.2-μm filter membrane 96-well plates (Millipore, Bedford, MA). After the final wash, beads in the 96-well plate were resuspended in 150 μl of Luminex sheath fluid and were loaded onto the Luminex 200 system. Raw data (mean fluorescence intensities) from the beads were analyzed with the Statistical Ligand Immunoassay Analysis (StatLIA) software (Brendan Technologies, Grosse Pointe Farms, MI) in order to obtain concentration values. All samples were run in duplicate, and results were obtained two or three times.

Intracellular cytokine staining. Cryopreserved samples were thawed and aliquoted at 2×10^6 to 4×10^6 cells per stimulation tube under the following conditions: medium alone, 5 μg/ml aldrithiol-2-inactivated HIV-1_{ADA-M}, HIV-1_{JR-FL}, or HIV-1_{MN} (provided by Jeffrey Lifson of the AIDS Vaccine Program, SAIC, Frederick, MD), 100 ng/ml *Escherichia coli* lipopolysaccharide (LPS) (InvivoGen, San Diego, CA), or 100 U/ml IFN-α2A (PBL Biomedical Laboratories, Piscataway, NJ). Cell stimulation, fixation, permeabilization, staining, and flow cytometry were conducted as previously described (14). Briefly, cells were placed in an incubator at 37°C and 5% CO_2, and after 2 h, brefeldin A (Sigma Aldrich, St. Louis, MO) was added to the medium at a final concentration of 10 μg/ml to inhibit cytokine secretion. Following an additional 8-h incubation, cells were labeled with anti-CD14 phycoerythrin (PE) antibody and viability stain (Viaprobe Violet; Molecular Probes, Eugene, OR) and were then fixed in 2% paraformaldehyde (Sigma Aldrich). The cells were permeabilized and blocked overnight in a buffer containing saponin (Sigma Aldrich) and 5% milk. Intracellular staining was performed using anti-CD3 fluorescein isothiocyanate (FITC) and anti-CD4 peridinin chlorophyll protein and anti-IL-1β, anti-IL-6, or anti-TNF-α allophycocyanin (Becton Dickinson, San Jose, CA). Data were collected with a FACSAria three-laser cytometer. Between 25,000 and 250,000 $CD14^+$ events were collected and analyzed using FlowJo software (TreeStar Inc., Ashland, OR).

Mixing experiments, in which autologous PBMC of patients on therapy and off therapy were cultured together, were performed as follows: cryopreserved samples were prepared and the cells from one time point were labeled for 8 min in 0.625 μM CFSE [5 (and 6)-carboxyfluorescein diacetate succinimidyl ester] (Molecular Probes). Labeled PBMC were then washed and mixed with unlabeled PBMC at a 1:1 ratio. Purified $CD4^+$ T cells and monocytes were obtained by use of the $CD4^+$ T-Cell Isolation Kit II and the Monocyte Isolation Kit, respectively (Miltenyi Biotec, Bergish Gladbach, Germany), with >90% purity. These cells were subsequently labeled with CFSE as described above and cocultured with autologous PBMC of patients off therapy at various concentrations. Transwell experiments were performed by coculturing autologous PBMC of patients on and off therapy in 24-well plates, separated by a 0.4-μm transwell insert (Costar; Corning Inc., Corning, NY). For all mixing and transwell experiments, the incubation, fixation, staining, and data collection were performed as described above.

Microarray analysis. Peripheral blood $CD14^+$ cells were isolated by fluorescence-activated cell sorting, using a FACSVantage SE flow cytometer (Becton Dickinson). Frozen peripheral blood mononuclear cells were thawed and lymphocytes were stained according to the manufacturer's instructions with the monoclonal antibodies CD3 FITC (Becton Dickinson), CD4 PC5 (Beckman Coulter, Miami, FL), and CD14 PE (Becton Dickinson). Sorting for $CD14^+$ monocytes was performed using a quadrant gate on CD3 $FITC^-$ CD14 PE^+ cells. $CD14^+$ monocyte purity was determined by flow cytometry to be >95%. One-half to two micrograms of total RNA was isolated from 1 million to 2 million $CD14^+$ sorted cells. Target RNA was labeled as described in the Affymetrix 2003 technical note "GeneChip Eukaryotic Small Sample Target Labeling Assay Version II" and hybridized to Affymetrix U133A GeneChips following the manufacturer's recommended protocol. Chip-to-chip normalization and gene expression index were performed using Affymetrix MAS5.0 algorithms.

Expression data for $CD14^+$ cells from patients on therapy and off therapy were obtained from eight patients (16 samples total). A two-way analysis of variance was performed using PartekPro software with therapy (on therapy or off therapy) as the main effect and patients as a random effect. Differentially expressed genes were selected based on the following criteria: a P value of ≤0.05, an absolute mean expression level difference between groups greater than 30, and an absolute mean expression change (n-fold) between groups greater than 1.5. A total of 784 genes were selected. In a separate analysis, 1,382 genes with expression levels correlating to the fraction of monocytes staining positive for at least one of the proinflammatory cytokines (IL-1β, IL-6, or TNF-α) were also identified (r, ≥0.62 or ≤−0.62; P, <0.01; and maximum to minimum expression level difference, >40 or ratio >2 for a given gene). Functional annotation and biological category enrichment were performed with the DAVID knowledge base (11).

Statistical analysis. Because the data were not normally distributed, the percentages of cytokine-producing monocytes for independent groups were compared by the Wilcoxon two-sample test. In cases in which longitudinal data were analyzed, paired data were compared by the Wilcoxon signed-rank test. Medians are reported. Significance of correlations was determined by the Spearman rank method. The Bonferroni method was used to adjust P values for multiple testing.

Microarray data accession number. Microarray results have been deposited in the Gene Expression Omnibus database under accession number GSE5220.

RESULTS

Frequencies of monocytes producing proinflammatory cytokines are elevated in patients on antiretroviral therapy and are diminished during treatment interruption. The consequences of active HIV infection on cytokine production were examined by measuring supernatant cytokine concentrations in unstimulated PBMC cultures from patients undergoing antiretroviral treatment interruptions. Using a multiplex cytokine assay that simultaneously measured IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, granulocyte-monocyte colony-stimulating factor, TNF-α, and IFN-γ, we found that three of five patients examined showed marked elevations in the proinflammatory cytokines IL-1β, IL-6, and TNF-α in the PBMC cultures from the on-therapy time point compared with the corresponding off-therapy time point (data not shown). In these patients with elevated proinflammatory cytokines, flow-cytometric intracellular cytokine staining determined that >90% of the cells producing IL-1β, IL-6, and TNF-α were $CD14^+$ monocytes and revealed that there were marked reductions in the percentages of monocytes that produced these cytokines during treatment interruption (Fig. 1A).

Spontaneous monocyte proinflammatory cytokine production was subsequently analyzed by intracellular cytokine staining in patients undergoing scheduled treatment interruption (Fig. 1B). The clinical data for these patients are shown in Table 1. In agreement with the supernatant cytokine data, the intracellular cytokine staining revealed that a majority of

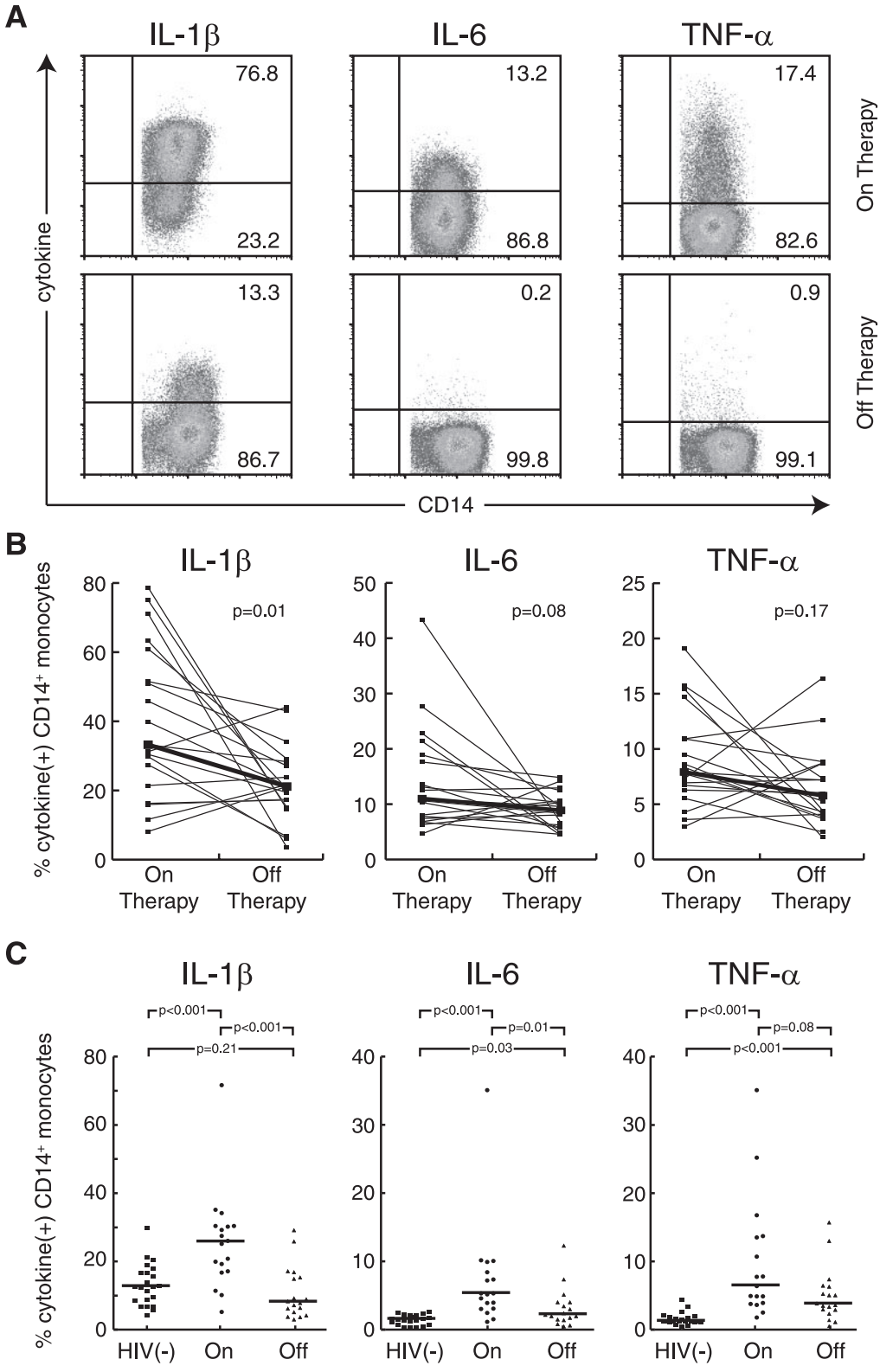

FIG. 1. HIV viremia is associated with diminished monocyte cytokine production [% cytokine(+) $CD14^+$ monocytes]. A. $CD14^+$ monocytes from a patient undergoing treatment interruption show a profound drop in levels of spontaneous IL-1β, IL-6, and TNF-α production. B. Monocytes from patients undergoing treatment interruption have a reduction in the frequencies of monocytes producing proinflammatory cytokines while off therapy (median values are indicated by bold lines). C. Cross-sectional analysis of IL-1β, IL-6, and TNF-α production in uninfected controls [HIV(−)] and HIV-1-infected patients either on or off therapy.

TABLE 1. Clinical characteristics of patients

Patient no.	Time on therapy (days)	Peripheral blood $CD4^+$ T-cell count (cells/μl)[a]		Time off therapy (days)	Plasma HIV RNA (copy eq/ml)[a]		Therapy[b]
		On therapy	Off therapy		On therapy	Off therapy	
203	>365	1,193	824	35	72	6,584	3TC/d4T/SQV
208	>365	666	896	37	<50	112	ZDV/3TC/IND
219	>365	677	700	180	<50	876	ZDV/3TC/ddI/EFV
221	>365	449	428	554	<50	60,582	ZDV/3TC/NVP
222	>365	718	496	41	<50	44,839	d4T/3TC/EFV
224	>365	752	652	52	<50	31,413	IND/d4T/3TC
225	>365	493	389	31	<50	11,515	d4T/IND/ddC
229	>365	1,230	791	252	<50	2,185	d4T/3TC/NEL
230	>365	822	469	97	<50	7,515	ZDV/3TC/IND
231	>365	516	329	400	<50	72,448	ZDV/3TC/TDF/NVP
232	>365	997	576	1,048	<50	35,114	ZDV/3TC/NEL
233	>365	487	258	43	<50	335,500	3TC/TDF/IND/RTV
234	>365	701	450	447	<50	7,491	3TC/ddI/IND
235	>365	494	162	1,652	<50	20,220	3TC/ABC/EFV
236	>365	928	684	247	<50	23,637	D4T/ddI/NVP
237	>365	1,433	1,077	56	<50	139,874	ZDV/3TC/EFV
238	>365	690	928	55	65	613	ZDV/3TC/IND
239	>365	759	579	63	<50	136,192	TDF/3TC/EFV
240	>365	745	362	55	<50	16,638	ZDV/3TC/ABC/TDF/LPVr
241	>365	720	609	56	<50	9,738	3TC/FTC/TDF/EFV
242	>365	934	562	62	<50	87,489	3TC/d4T/IND

[a] Listed values were obtained at the time of apheresis.
[b] ZDV, zidovudine; d4T, stavudine; ddC, zalcitabine; NEL, nelfinavir; 3TC, lamivudine; IND, indinavir; LPVr, lopinavir/ritonavir; NVP, nevirapine; RTV, ritonavir; SQV, saquinavir; EFV, efavirenz; ddI, didanosine; FTC, emtricitabine; TDF, tenofovir; ABC, abacavir.

patients undergoing treatment interruption had reduced frequencies of monocytes producing IL-1β, IL-6, and TNF-α. Overall, the patients studied longitudinally had diminished median frequencies of monocytes producing IL-1β ($P = 0.01$) when off therapy, although changes in IL-6 ($P = 0.08$) and TNF-α ($P = 0.17$) did not reach statistical significance. Decreases in proinflammatory cytokine production were not correlated with peak viral load, the number of days off therapy, or activation of $CD8^+$ T cells based upon CD38 expression (Spearman's rank correlation-adjusted P values of >0.5 for each comparison). This cohort included a number of patients with a relatively low production of these cytokines at both time points. For these patients, we did not observe large decreases given the low baseline cytokine production. High frequencies of monocytes producing cytokines in patients on therapy were strongly correlated with greater decreases in IL-1β ($r = -0.84$, $P < 0.001$), IL-6 ($r = -0.91$, $P < 0.001$), and TNF-α ($r = -0.89$, $P < 0.001$) when antiretroviral therapy was stopped. This suggested that the effect of viremia was more readily detected in patients with high frequencies of cells producing these cytokines at the on-therapy time point. In addition, there were strong correlations among the percentages of monocytes producing IL-1β, IL-6, and TNF-α (P was ≤0.001 and the Spearman rank correlation was >0.81 for all comparisons), and only one patient showed discordant changes in cytokine production during therapy interruption. This suggested that, in patients for whom a drop in proinflammatory cytokine production was observed, these changes were not isolated to one cytokine but reflected a more global effect of viremia on monocyte function.

To further define the effect of viremia on monocyte cytokine production in larger numbers of patients, cross-sectional groups of healthy controls, HIV-infected patients on therapy and patients off therapy were also studied (Fig. 1C). Surprisingly, compared with uninfected controls, patients who were on therapy demonstrated markedly increased frequencies of monocytes producing IL-1β, IL-6, and TNF-α (IL-1β, median 26.0% in patients on therapy versus 12.9% in controls, $P < 0.001$; IL-6, 5.5% versus 1.7%, $P < 0.001$; TNF-α, 6.6% versus 1.4%, $P < 0.001$). When 61 samples from patients on various combinations of antiretroviral therapy were analyzed, changes in monocyte cytokine production were not attributable to any of the following antiretrovirals: abacavir, didanosine, efavirenz, indinavir, lamivudine, lopinavir, nelfinavir, nevirapine, ritonavir, stavudine, tenofovir, or zidovudine. HIV-infected patients who were off therapy also had elevations in frequencies of monocytes producing IL-6 (2.4%, $P = 0.03$) and TNF-α (3.9%, $P < 0.001$), but not IL-1β (8.4%, $P = 0.21$), compared with controls. In the cross-sectional analyses, patients on therapy had elevated monocyte production of IL-1β ($P < 0.001$) and IL-6 ($P < 0.01$) compared with patients off therapy, but changes in TNF-α production did not reach statistical significance ($P = 0.08$).

The effect of prolonged suppression of viral replication or prolonged low-level viremia on spontaneous monocyte cytokine production was also examined. Results from six patients from whom more prolonged follow-up longitudinal samples were available are shown in Fig. 2. Interruption of therapy resulted in an initial decline in production of monocyte proinflammatory cytokines in each case. Reinstitution of therapy resulted in a rebound and subsequent decline in cytokine production. In general, this kinetic is similar to one recently described following initiation of therapy (7).

One possibility for the observed changes in monocyte func-

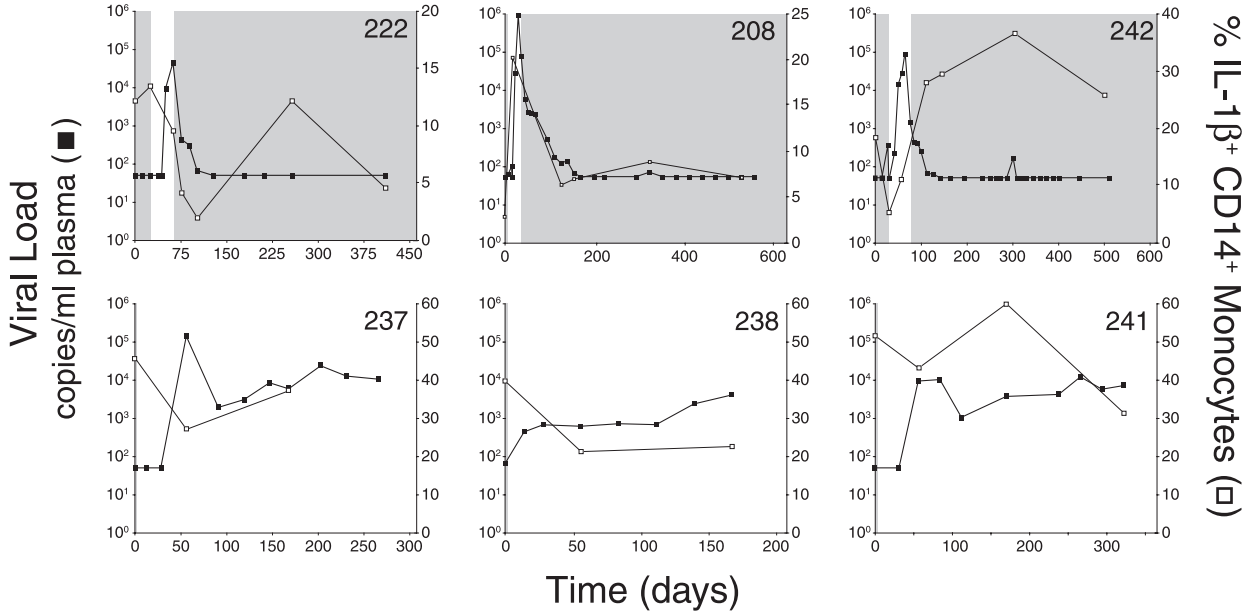

FIG. 2. Spontaneous in vitro IL-1β over time during an interruption of antiretroviral therapy. Patient numbers are indicated in the upper right corner of each graph. Shaded areas indicate periods of antiretroviral therapy. Day 0 for patients 237, 238, and 241 was the day of interruption of therapy.

tion during high-level viremia was that they were mediated by direct binding of HIV-1 virions (55). To address this possibility, peripheral blood monocytes from patients on or off antiretroviral therapy were stained with antibodies directed against Gag and Env proteins. HIV-infected patients who were off therapy demonstrated a slight increase in $CD14^+$ monocytes that were positive for HIV-1 viral products compared to the number at the on-therapy time point, although the percentages of positively stained cells ($<0.1\%$ of $CD14^+$ cells) were far too low to account for the large differences in the percentage of monocytes producing proinflammatory cytokines (data not shown). Furthermore, the addition of 2-aldothriol-inactivated HIV-1 viral particles did not diminish or augment monocyte proinflammatory cytokines. The same stimulation was sufficient to stimulate HIV-specific $CD4^+$ and $CD8^+$ T-cell responses and IFN-α production by plasmacytoid dendritic cells (data not shown). Thus, under the current experimental conditions, we were unable to demonstrate a direct effect of HIV virions on monocyte cytokine production directly ex vivo or in vitro.

Together, these data suggest that HIV-infected patients in general have significantly higher levels of spontaneous monocyte production of proinflammatory cytokines than uninfected controls. They also strongly suggest that high-level HIV viremia results in diminished production of these cytokines. The mechanisms by which active HIV replication impairs monocyte proinflammatory cytokine production were then further investigated.

Monocytes from off-therapy time points retain the ability to produce proinflammatory cytokines in response to LPS stimulation. Possible explanations for diminished levels of proinflammatory cytokine production by monocytes during viremia might include apoptosis of activated monocytes in vitro or trafficking of activated monocytes from the peripheral blood. However, viability dyes demonstrated that apoptosis during viremia was largely contained within T-cell populations. $CD14^+$ monocytes maintained a high level of viability regardless of the level of viremia (median viability, $97.7 \pm 3.3\%$ of $CD14^+$ cells). The ability of monocytes to respond to stimulation was also investigated. LPS, which is known to act via a TLR-4/CD14/MD-2-dependent mechanism, was used to stimulate monocytes in uninfected controls and HIV-infected patients on and off therapy. LPS induced significant increases in monocyte proinflammatory cytokine production levels in healthy controls and in HIV-infected patients either on or off therapy (Fig. 3). There were no differences between the three groups following LPS stimulation, suggesting that monocytes from off-therapy time points capable of producing proinflammatory cytokines remain in the peripheral blood. These results also suggest that decreased proinflammatory cytokine production was unlikely due to activation induced nonresponsiveness me-

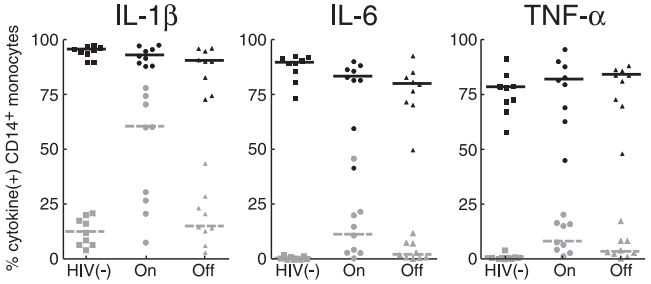

FIG. 3. $CD14^+$ monocytes from HIV-infected patients either on or off therapy retain the ability to produce proinflammatory cytokines in response to stimulation with LPS. There were no significant differences between the three groups. Baseline monocyte proinflammatory cytokine expression frequencies [% cytokine(+) $CD14^+$ monocytes] are indicated by gray symbols. The median values for each group are indicated by bars. HIV(−), uninfected group.

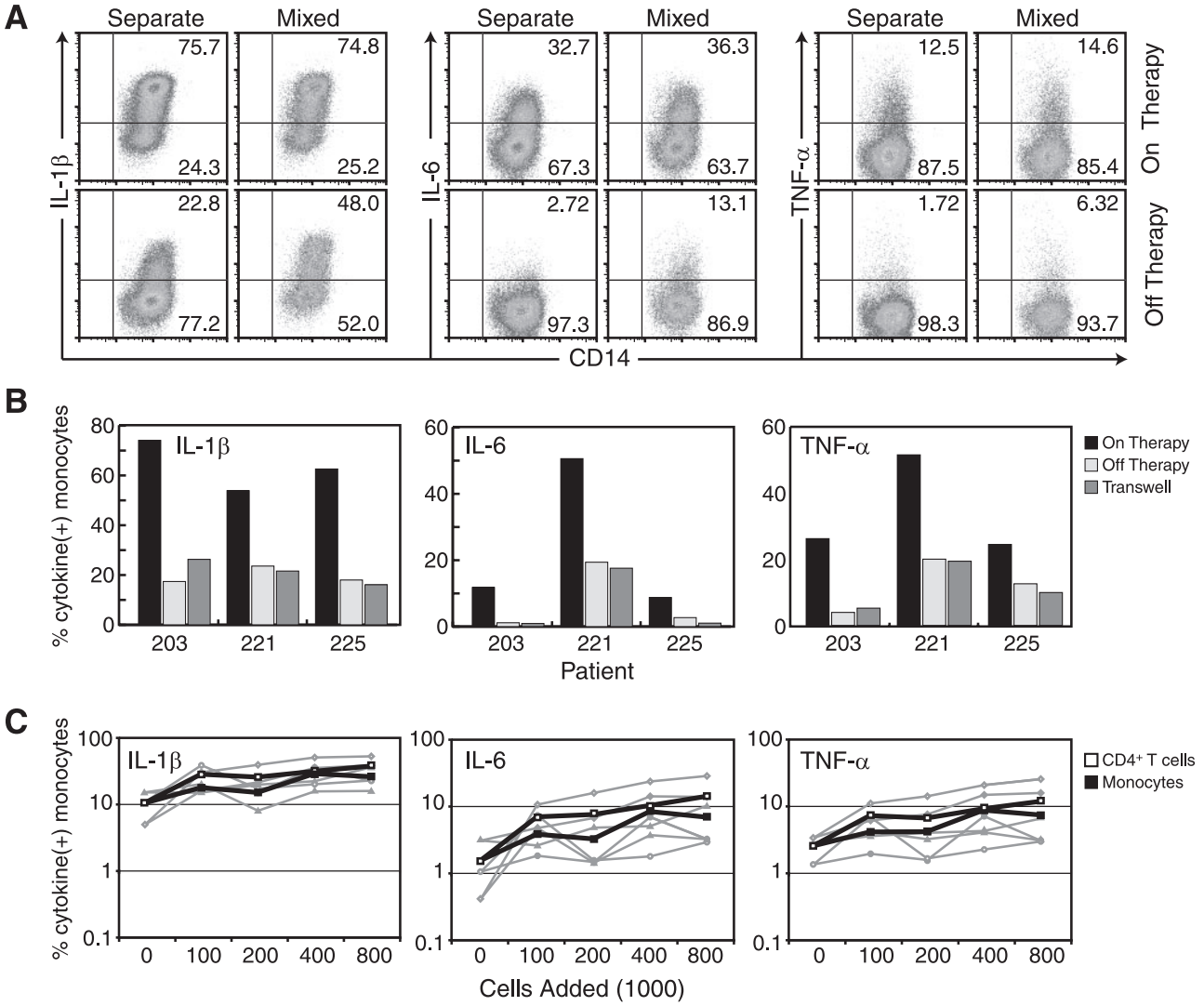

FIG. 4. Diminished monocyte proinflammatory cytokine production [% cytokine(+) $CD14^+$ monocytes] from HIV-1-infected patients off antiretroviral therapy can be augmented in a cell-cell-contact-dependent manner by autologous $CD4^+$ T cells or $CD14^+$ monocytes from on-therapy time points. A. (Top row) Frequencies of cytokine-producing $CD14^+$ monocytes from patients on antiretroviral therapy were similar when the cells were cultured alone (Separate) or with autologous PBMC from the off-therapy time point (Mixed). (Bottom row) In contrast, the frequencies of cytokine-producing $CD14^+$ monocytes from patients off antiretroviral therapy were augmented when cocultured with PBMC from the on-therapy time point (Mixed) compared to when they were cultured alone (Separate). Results from an experiment with cells from patient 203 are shown. B. In separate experiments with three patients, the frequencies of monocytes producing IL-1β, IL-6, and TNF-α in patients off therapy were not enhanced by the addition of autologous PBMC of patients on therapy when the populations were separated by a transwell insert. C. Coculture with autologous purified $CD4^+$ T cells and $CD14^+$ monocytes from on-therapy time points augmented the frequencies of cytokine-producing monocytes from off-therapy time points. Average values are indicated by bold lines and symbols.

diated by TLR-4/CD14 stimulation in vivo, since production was readily overcome by LPS stimulation in vitro.

Diminished monocyte cytokine production levels of patients off therapy are partially restored by cell-cell contact with autologous $CD4^+$ T cells and monocytes of patients on therapy. To determine whether the diminished frequencies of cytokine-producing monocytes observed for patients who were off therapy were due to the absence of stimulatory factors or due to the presence of inhibitory factors, we performed mixing experiments using autologous PBMC from patients undergoing treatment interruptions. Cells from patients' on-therapy time points were labeled with CFSE and cocultured with PBMC from the off-therapy time points. In all five patients tested, proinflammatory cytokine production by monocytes from patients off therapy was augmented by coculture with autologous PBMC of patients on therapy. Conversely, coculture of the two populations of cells did not diminish the percentages of monocytes that produced IL-1β, IL-6, and TNF-α in patients on therapy (results for a representative patient are shown in Fig. 4A).

Subsequently, transwell experiments were performed to determine whether a soluble factor or a cell-contact-dependent mechanism was responsible for the increase in monocyte proinflammatory cytokine production levels of patients off

therapy following mixing with autologous PBMC from patients on therapy. For all three patients tested, preventing cell-cell contact blocked the increased frequencies of monocytes producing proinflammatory cytokines observed in the mixing cultures (Fig. 4B).

The cells responsible for the increase in the frequencies of monocytes producing proinflammatory cytokines were also examined by magnetic bead purification and coculture experiments. Purified autologous $CD14^+$ monocytes and $CD4^+$ T cells of patients on therapy were both able to augment proinflammatory cytokine production by monocytes of patients off therapy (Fig. 4C). In each experiment, $CD4^+$ T cells were better able to recover monocyte cytokine production over a range of dilutions. Although these were highly enriched populations (purity, >90%), we cannot formally rule out the possibility that the effect of the addition of monocytes may be due to very small numbers of contaminating $CD4^+$ T cells. Taken together, these data suggest that recovery of cytokine production by monocytes from viremic patients can be mediated by autologous $CD4^+$ T cells or monocytes through a cell-contact-dependent mechanism.

Alterations in proinflammatory cytokine production by monocytes were inversely associated with type I IFN-stimulated gene regulation. Gene expression profiling using microarrays was performed on purified $CD14^+$ monocytes isolated from on-therapy and off-therapy time points in eight patients undergoing treatment interruption. This cohort included patients with a wide range of monocyte proinflammatory cytokine responses to treatment interruption, including several patients with large decreases, as well as several patients with no changes or slight increases, in cytokine production. Differentially expressed genes were selected based on an absolute mean expression level difference between the groups greater than 30, a P value of ≤ 0.05, and an absolute mean expression change (n-fold) between groups greater than 1.5, resulting in 784 differentially expressed genes (see Table S1 in the supplemental material). A group of interferon-induced genes were found to be highly enriched ($P = 8.4 \times 10^{-8}$) in the list of genes up-regulated during plasma viremia following treatment interruption (Fig. 5A). These data indicate that unrestricted viral replication is strongly correlated with type I IFN-stimulated gene activation in $CD14^+$ monocytes.

The microarray data were subsequently analyzed to determine whether individual genes that correlated with changes in the frequencies of monocytes producing proinflammatory cytokines in patients on or off antiretroviral therapy could be identified. A functional analysis of the 1,382 genes correlating with monocyte cytokine levels (correlated with at least one monocyte cytokine with an r value of ≥ 0.62 or ≤ -0.62 and a P value of ≤ 0.01; see Table S2 in the supplemental material) indicated that many of these genes were involved in type I interferon responses, NF-κB, mitogen-activated protein kinase, and Jun signaling pathways, general immune activation, immune down-regulation, protein degradation, protein secretion, and apoptosis (Fig. 5B). These data indicate that interruption of antiretroviral therapy is associated with alterations in monocyte gene expression across multiple functional categories, suggesting that diminished proinflammatory cytokine expression is part of a global effect of HIV viremia upon monocytes.

The elevations of type I interferon-stimulated gene expression in $CD14^+$ monocytes from patients off antiretroviral therapy suggest that these cells may be activated in vivo and that activation of monocytes may be linked to the diminished ability of these cells to produce proinflammatory cytokines. $CD14^+$ monocytes from 10 patients undergoing treatment interruption were assayed for surface expression of the activation marker HLA-DR, and increased HLA-DR expression was strongly correlated with decreased IL-1β ($r = -0.94$, $P < 0.001$), IL-6 ($r = -0.87$, $P = 0.004$), and TNF-α ($r = -0.94$, $P < 0.001$) production levels (data not shown). The inverse correlations of both interferon-stimulated gene expression and HLA-DR surface expression with the production of monocyte proinflammatory cytokines strongly suggests that the diminished cytokine production is tightly linked to the activation of monocytes in the context of HIV viremia.

The mRNA expression levels of IL-1β, IL-6, and TNF-α in the eight patients undergoing treatment interruption were also examined by microarrays in order to determine whether the reduced protein expression at the off-therapy time point correlated with reduced cytokine mRNA levels. The microarray analysis revealed that IL-1β and TNF-α mRNA levels were not different between patients who were on or off therapy. IL-6 mRNA levels were actually higher in patients when they were off therapy at a time when protein levels were diminished. The expression levels of the mRNAs for IL-1β, IL-6, and TNF-α all suggest that the diminished cytokine protein levels of patients off therapy are not due to decreases in mRNA levels but rather that a posttranscriptional regulatory mechanism, most likely operating at the level of mRNA translation or protein stability, is responsible for the changes in spontaneous monocyte cytokine production. This possibility is potentially supported by the inverse correlation between monocyte proinflammatory cytokine protein levels and the increased expression of genes involved in ubiquitination and proteasomal degradation pathways (see Table S2 in the supplemental material).

Treatment of $CD14^+$ monocytes with exogenous IFN-α2A recapitulates the changes in proinflammatory cytokines observed in HIV-infected patients off therapy. The inverse relationship between type I interferon-stimulated gene expression and monocyte proinflammatory cytokine expression raised the possibility that exposure to type I interferons in vivo might directly influence the cytokine production observed ex vivo. Exposure of monocytes to type I interferons induces maturation into dendritic cells over 24 to 48 h; however, its effects on baseline and LPS-induced proinflammatory cytokine production are not well characterized. $CD14^+$ monocytes from healthy patients were incubated with recombinant IFN-α2A and cultured with or without LPS stimulation. The frequencies of monocytes producing detectable baseline levels of IL-1β, IL-6, and TNF-α were significantly reduced by treatment with IFN-α2A ($P = 0.003$ for all three cytokines) (Fig. 6). However, IFN-α2A did not reduce the percentages of monocytes producing IL-1β, IL-6, and TNF-α in response to LPS stimulation (P values of >0.30 for all cytokines). This pattern of reduced baseline proinflammatory cytokine production with retained responsiveness to LPS stimulation following treatment with IFN-α2A was similar to that observed for HIV-infected patients off antiretroviral therapy. These results indicate that exposure to type I interferons can reduce the frequency of

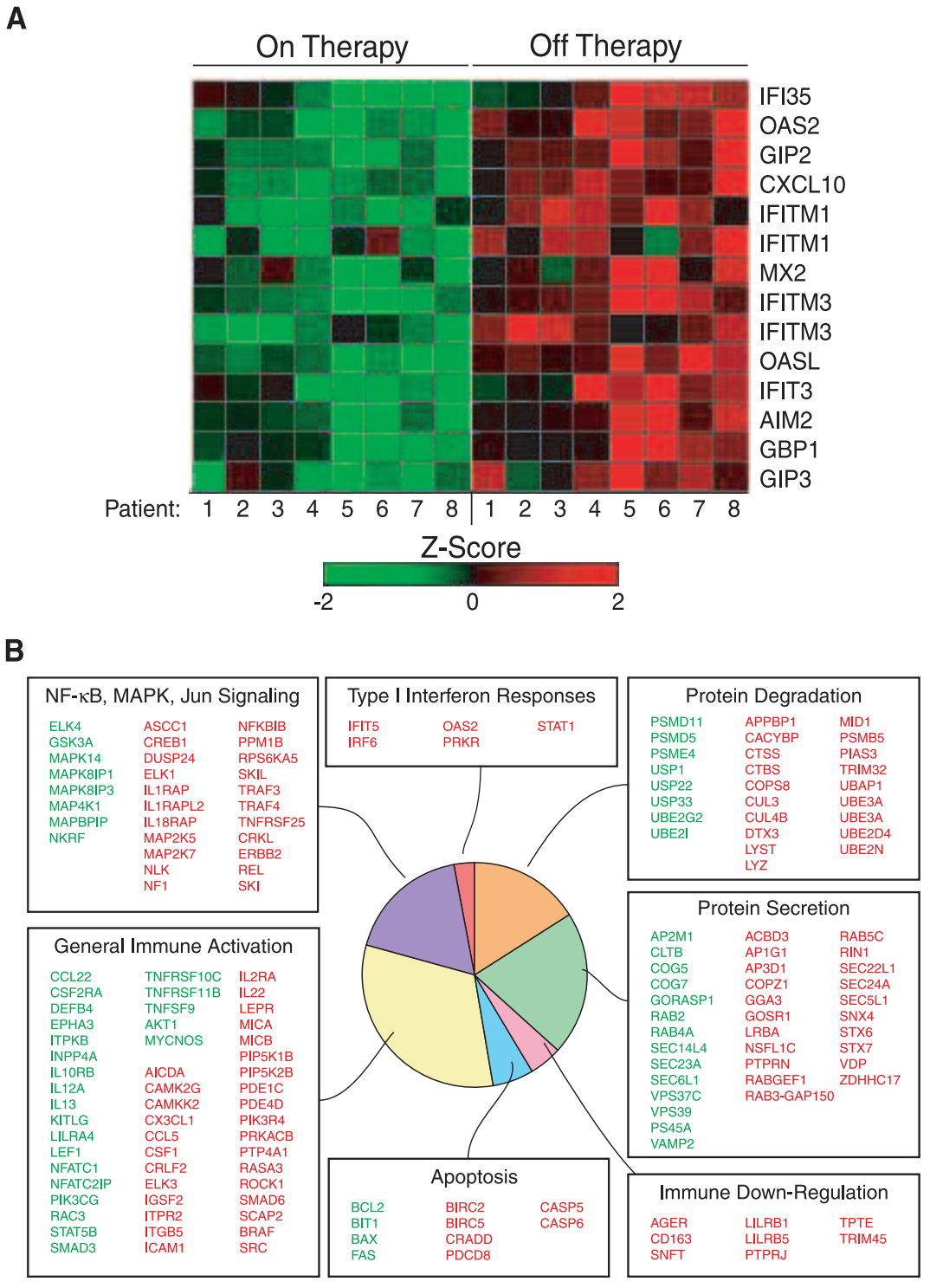

FIG. 5. Interferon-stimulated gene transcription is up-regulated in patients off antiretroviral therapy and is inversely correlated with monocyte proinflammatory cytokine production. A. Microarray analysis of eight patients undergoing treatment interruption revealed that genes significantly up-regulated in patients off therapy were enriched in interferon-stimulated genes. Relative expression levels (*z* score) of interferon-stimulated genes are indicated in the heat map, with green representing lower expression levels, black representing average expression, and red representing higher expression levels. Duplicate names represent distinct microarray probes for the same gene. B. Functional categories of genes significantly correlated with changes of at least one proinflammatory cytokine in patients undergoing treatment interruption. Genes involved in type I interferon responses, NF-κB, mitogen-activated protein kinase (MAPK), and Jun signaling pathways, general immune activation, immune down-regulation, protein degradation, protein secretion, and apoptosis were all associated with changes in monocyte-produced cytokines. Genes with expression values that inversely correlate with monocyte cytokine production are indicated in green, and genes with positive correlations are indicated in red.

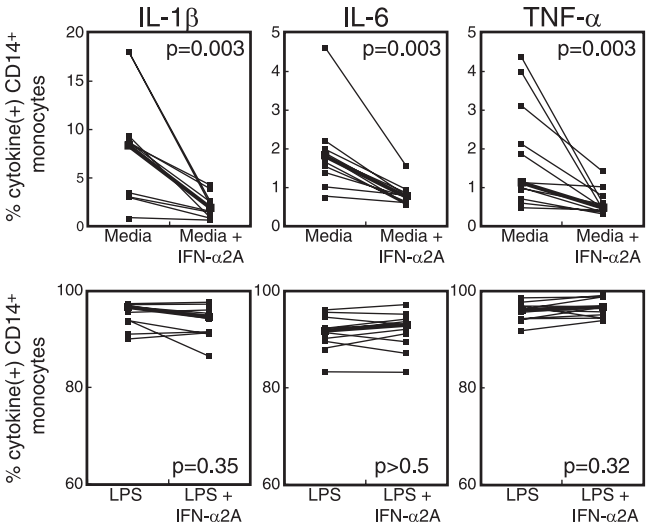

FIG. 6. Exogenous IFN-α2A inhibits spontaneous monocyte proinflammatory cytokine production [% cytokine(+) $CD14^+$ monocytes] but does not affect responsiveness to LPS. Frequencies of $CD14^+$ peripheral blood monocytes from healthy patients that spontaneously produced IL-1β, IL-6, and TNF-α were all significantly reduced by the addition of 100 U of recombinant IFN-α2A. In contrast, the frequencies of $CD14^+$ monocytes producing IL-1β, IL-6, and TNF-α in response to treatment with LPS were not inhibited by the addition of recombinant IFN-α2A.

monocytes producing detectable levels of proinflammatory cytokines and likely represent the mechanism underlying the observed changes in monocyte cytokine production in patients with unrestricted viral replication.

DISCUSSION

Unrestricted replication of HIV-1 in patients who are off antiretroviral therapy has been associated with alterations in the function of several types of immune cells. HIV-specific $CD4^+$ T cells in patients who are off therapy have diminished IL-2 production and reduced proliferation in response to HIV-1 antigens (21, 27, 44, 57). Viremic patients also demonstrate an expansion of a $CD56^-CD16^+$ NK cell subset impaired in cytotoxic function and cytokine secretion, as well as alterations in B-cell survival and costimulatory function (1, 34–36, 42). In comparison, relatively little has been published regarding the effects of HIV-1 viremia on the functions of monocytes. In the present study, we report that HIV-1-infected patients have marked elevations in the percentages of $CD14^+$ monocytes spontaneously producing some or all of the proinflammatory cytokines IL-1β, IL-6, and TNF-α compared with uninfected controls. In patients with high frequencies of monocytes producing proinflammatory cytokines while on therapy and undergoing treatment interruptions, active HIV-1 replication was associated with a decrease in the percentages of cytokine-producing monocytes. This diminished cytokine production could be restored by stimulating the cells with LPS, suggesting that the monocytes capable of producing these cytokines remained in the peripheral blood. This also suggests that activation and diminished function are unlikely secondary to stimulation by the microbial products acting through CD14 and TLR-4 in vivo. Diminished monocyte cytokine production could also be restored by the addition of $CD4^+$ T cells or monocytes in a cell-contact-dependent manner. The reduction of proinflammatory cytokine production in patients with active HIV-1 replication was associated with activation of these cells and correlated with increases in the expression of type I interferon-stimulated genes and activation markers on the monocytes. Treatment of monocytes with recombinant human IFN-α2A in vitro decreased spontaneous IL-1β, IL-6, and TNF-α production but did not affect responsiveness to LPS, recapitulating the pattern observed for viremic patients. Together, these data suggest that unrestricted replication of HIV-1 indirectly inhibits monocyte production of proinflammatory cytokines via the elevated production of type I interferon and transcription of type I interferon-stimulated genes.

There are mounting data to suggest that in vivo alpha interferon production may not simply be a consequence of ongoing viral replication but may play an important role in the pathogenesis of HIV. The major source of IFN-α is thought to be $CD11c^-$ $CD123^+$ plasmacytoid dendritic cells (50; reviewed in reference 9). IFN-α is produced by these cells following binding of viral RNA or DNA to TLRs 7/9 within the lysosomal compartment (5, 23, 30, 50). Alterations in the production levels of type I interferons were among the earliest reported laboratory abnormalities in AIDS patients, with descriptions of elevated serum levels of an unusual acid-labile IFN-α in patients with Kaposi's sarcoma and hemophilia (12, 13). Serum IFN-α has been detected in higher levels in subsets of patients with more advanced HIV infection and has been found to correlate with p24 antigenemia (54). More recently, HIV-induced activation of the type I interferon system has been demonstrated by microarray analysis of PBMC (R. Lempicki, personal communication) as well as with purified B cells (41) and, in the present study, with monocytes. In addition, up-regulation of type I IFN-regulated genes has been observed in gut mucosal (48) and lymph node (32) tissues in HIV-1-infected patients with active viral replication. Additional evidence of an important role for type I IFN in lentiviral infection has come from experimental simian immunodeficiency virus (SIV) infection of monkeys. SIV infection of sooty mangabeys is characterized by a lack of $CD4^+$ T-cell depletion and immune system activation, despite high-level viremia. Interestingly, recent preliminary reports have indicated that sooty mangabeys do not respond to SIV particles with the production of IFN-α, and PBMC from SIV-infected sooty mangabeys do not have elevations in type I interferon-stimulated genes (S. Klucking, A. P. Barry, R. Chavan, K. A. Dalbey, G. Silvestri, S. Staprans, and M. Feinberg, Keystone Symposia on HIV Pathogenesis, abstr. 22 and 244, 2005). These findings, in marked contrast to the response of humans and rhesus macaques infected with HIV and SIV, respectively, raise the possibility that activation of the type I IFN system may play a critical role in the pathogenesis of HIV-1 infection.

The results of the current study demonstrate that signaling via type I IFN in patients with unrestricted HIV-1 replication can diminish the production of proinflammatory cytokines by monocytes. The reasons the drop in production of these cytokines was restored by contact with $CD4^+$ T cells or monocytes from the on-therapy time point, as well as the molecular mechanisms of this drop and restoration, are not yet clear and are

the subject of ongoing work. Such an effect is potentially mediated by a number of cell surface molecules known to regulate monocyte cytokine production (19). It should be noted that IFN-α has been shown to inhibit production of IL-1β, TNF-α, and IL-6 in vitro in at least one report in tumor cell lines (25). In addition, IFN-α has been used to diminish inflammation associated with Behcet's syndrome (20). However, the consequences of prolonged stimulation of the type I interferon system in chronic viral infections in vivo, such as HIV, are not well characterized. In acute infections and in vitro assays, signaling via IFN-α/β has been implicated in the establishment of antiviral states within target cells, activation of innate immune cells, and modulation of adaptive immune responses. Persistent overactivation of the type I interferon pathway may affect all of these broad groups of antiviral immune defenses. In primary human cells, the findings of the present study are consistent with, and extend, observations in one recent report demonstrating that monocytes from patients with active HIV-1 replication have diminished up-regulation of costimulatory molecules and maturation into dendritic cells, suggesting that activation of the type I IFN system in vivo may have detrimental consequences on monocyte function (28). It is possible that these alterations in the innate immune system negatively impact adaptive immune responses and diminish the ability of the immune system to control HIV-1 replication and opportunistic infections associated with HIV disease.

Conversely, the results of the present study may also be interpreted in the context of a number of clinical observations suggesting improved monocyte function in HIV-infected patients during antiretroviral therapy. Following the initiation of antiretroviral therapy, some patients experience an immune reconstitution and inflammatory syndrome (IRIS), a condition in which some patients starting antiretroviral therapy experience severe clinical symptoms resulting from an inflammatory responses to non-HIV pathogens (33, 52). Of these pathogens, the vast majority are caused by pathogens for which monocytes are thought to play a critical role in containment, such as *Mycobacterium tuberculosis*, *Mycobacterium avium*, or some fungi. In this setting, the median time to onset of IRISs is 3 weeks, well before there were large increases in $CD4^+$ T cells in the peripheral blood in most patients. In approximately 10% of patients, these syndromes occur in the absence of increases in $CD4^+$ T-cell numbers in the peripheral blood. IRIS has also been associated with elevations of serum (51, 53) or spontaneous in vitro production of proinflammatory cytokines (7), suggesting that improvements in monocyte and macrophage function following control of HIV replication may play a role in the development of this syndrome. Although it is speculative, it is reasonable to suggest that a reversal of HIV-induced inhibition of monocyte function may underlie some forms of IRISs. If so, it is possible that therapies that modulate the effects of monocyte proinflammatory cytokines or their production, such as treatments with the TNF-α antagonist etanercept or alpha interferon, may have a role in treatment of these syndromes.

Taken in the context of prior work, the results of the present study also suggest that the interrelationship of HIV viremia and proinflammatory cytokine production is more complex than previously appreciated. Increases in serum levels of these cytokines have only intermittently been observed in the earliest stages of HIV infection, similar to the case for patients in the present study compared to uninfected controls. However, increases have been better documented for patients with advanced disease (3, 4, 18, 54). These data are, in general, consistent with those of the present study. It should be noted that monocytes are not the only source of TNF-α. The majority of HIV-specific $CD8^+$ T cells produce TNF-α and would be expected to be present at high levels during viremia and rapidly decline during effective therapy (6, 17). However, the effects of antiretroviral therapy on serum levels of these cytokines have been less well studied. Increases in serum levels of some proinflammatory cytokines following therapy of patients with IRIS have been documented, as noted above.

In the present study, it was somewhat surprising that even in the absence of disease, patients on antiretroviral therapy had the highest frequency of monocytes producing proinflammatory cytokines. A relatively wide range of frequencies of monocytes producing these cytokines was observed. This variation might be attributable to viral or host genetics of the response in monocytes or plasmacytoid dendritic cells and efficacy of or compliance with the antiretroviral regimen. Typically, in patients on antiretroviral therapy, nearly all immunological responses measured are equal to or approximate uninfected controls. However, it should be noted that even in the context of effective antiretroviral therapy with <50 copies of HIV RNA per ml of plasma, there is evidence of ongoing viral replication. When highly sensitive, real-time PCR methods are used, patients are observed to typically maintain a mode of 10 copies of HIV RNA per ml of plasma (45). In addition, there is evidence of ongoing immune system activation in such patients. Based upon expression array experiments, there is evidence of ongoing immune system activation in treated patients with <50 copies of HIV RNA per ml of plasma compared to uninfected controls (R. Lempicki, personal communication). It is most likely that the increases in proinflammatory monocyte cytokines observed for treated patients in the current study are related to HIV replication and not due to stimulation byproducts of opportunistic pathogens, given that this is a healthy cohort with relatively high $CD4^+$ T-cell counts. The observations from the present study, taken in the context of prior work, are most consistent with the view that during therapy, there is ongoing, most likely indirect activation of monocytes resulting from low-level HIV replication. In patients off antiretroviral therapy, there is ongoing monocyte activation but diminished function in the context of high-level viremia at least in part mediated by the chronic effects of high levels of type I IFNs. These functions may be rapidly restored in the context of initiation of antiretroviral therapy and may play a role in immune reconstitution syndromes.

Over the past several years, an increasingly detailed picture of the widespread nature of HIV-induced immune system activation has emerged. Improved tools for measuring immune responses in a more quantitative and reproducible manner, coupled with the ability to alter levels of HIV replication through antiretroviral therapy, are permitting a greater analysis of the cells involved, as well as primary and secondary effects of viral products on the immune system and the mechanisms involved, than was previously available. Further definition of these mechanisms may provide information that is

important for modulation of responses to non-HIV pathogens, adaptive responses to HIV, or pathogenesis of HIV disease.

ACKNOWLEDGMENTS

This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract NO1-CO-12400.

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

REFERENCES

1. **Alter, G., N. Teigen, B. T. Davis, M. M. Addo, T. J. Suscovich, M. T. Waring, H. Streeck, M. N. Johnston, K. D. Staller, M. T. Zaman, X. G. Yu, M. Lichterfeld, N. Basgoz, E. S. Rosenberg, and M. Altfeld.** 2005. Sequential deregulation of NK cell subset distribution and function starting in acute HIV-1 infection. Blood **106:**3366–3369.
2. **Appay, V., F. Boutboul, and B. Autran.** 2005. The HIV infection and immune activation: "to fight and burn". Curr. Infect. Dis. Rep. **7:**473–479.
3. **Aziz, N., P. Nishanian, and J. L. Fahey.** 1998. Levels of cytokines and immune activation markers in plasma in human immunodeficiency virus infection: quality control procedures. Clin. Diagn. Lab. Immunol. **5:**755–761.
4. **Bedini, J. L., F. Garcia, J. M. Miro, E. Aznar, J. Serrano, L. Lozano, J. Mallolas, X. Filella, J. Vidal, X. Latorre, J. M. Gatell, A. M. Ballesta, and E. Soriano.** 1998. Serum levels of beta2-microglobulin, neopterin, TNF-alpha and soluble receptors of TNF-alpha and interleukin-2 in intravenous drug abusers according to HIV-1 status and stage of the HIV-1 infection. Clin. Microbiol. Infect. **4:**4–10.
5. **Beignon, A. S., K. McKenna, M. Skoberne, O. Manches, I. DaSilva, D. G. Kavanagh, M. Larsson, R. J. Gorelick, J. D. Lifson, and N. Bhardwaj.** 2005. Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor-viral RNA interactions. J. Clin. Investig. **115:**3265–3275.
6. **Betts, M. R., M. C. Nason, S. M. West, S. C. De Rosa, S. A. Migueles, J. Abraham, M. M. Lederman, J. M. Benito, P. A. Goepfert, M. Connors, M. Roederer, and R. A. Koup.** 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. Blood **107:**4781–4789.
7. **Bourgarit, A., G. Carcelain, V. Martinez, C. Lascoux, V. Delcey, B. Gicquel, E. Vicaut, P. H. Lagrange, D. Sereni, and B. Autran.** 2006. Explosion of tuberculin-specific Th1-responses induces immune restoration syndrome in tuberculosis and HIV co-infected patients. AIDS **20:**F1–F7.
8. **Cohen, O. J., A. Kinter, and A. S. Fauci.** 1997. Host factors in the pathogenesis of HIV disease. Immunol. Rev. **159:**31–48.
9. **Colonna, M., G. Trinchieri, and Y. J. Liu.** 2004. Plasmacytoid dendritic cells in immunity. Nat. Immunol. **5:**1219–1226.
10. **Connolly, N. C., S. A. Riddler, and C. R. Rinaldo.** 2005. Proinflammatory cytokines in HIV disease—a review and rationale for new therapeutic approaches. AIDS Rev. **7:**168–180.
11. **Dennis, G., Jr., B. T. Sherman, D. A. Hosack, J. Yang, W. Gao, H. C. Lane, and R. A. Lempicki.** 2003. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol. **4:**P3.
12. **DeStefano, E., R. M. Friedman, A. E. Friedman-Kien, J. J. Goedert, D. Henriksen, O. T. Preble, J. A. Sonnabend, and J. Vilcek.** 1982. Acid-labile human leukocyte interferon in homosexual men with Kaposi's sarcoma and lymphadenopathy. J. Infect. Dis. **146:**451–459.
13. **Eyster, M. E., J. J. Goedert, M. C. Poon, and O. T. Preble.** 1983. Acid-labile alpha interferon. A possible preclinical marker for the acquired immunodeficiency syndrome in hemophilia. N. Engl. J. Med. **309:**583–586.
14. **Gea-Banacloche, J. C., S. A. Migueles, L. Martino, W. L. Shupert, A. C. McNeil, M. S. Sabbaghian, L. Ehler, C. Prussin, R. Stevens, L. Lambert, J. Altman, C. W. Hallahan, J. C. Lopez Bernaldo de Quiros, and M. Connors.** 2000. Maintenance of large numbers of virus specific CD8+ T cells in HIV infected progressors and long term nonprogressors. J. Immunol. **165:**1082–1092.
15. **Giorgi, J. V., and R. Detels.** 1989. T-cell subset alterations in HIV-infected homosexual men: NIAID multicenter AIDS cohort study. Clin. Immunol. Immunopathol. **52:**10–18.
16. **Giorgi, J. V., H. N. Ho, K. Hirji, C. C. Chou, L. E. Hultin, S. O'Rourke, L. Park, J. B. Margolick, J. Ferbas, J. P. Phair, et al.** 1994. CD8+ lymphocyte activation at human immunodeficiency virus type 1 seroconversion: development of HLA-DR+ CD38- CD8+ cells is associated with subsequent stable CD4+ cell levels. J. Infect. Dis. **170:**775–781.
17. **Gray, C. M., J. Lawrence, J. M. Schapiro, J. D. Altman, M. A. Winters, M. Crompton, M. Loi, S. K. Kundu, M. M. Davis, and T. C. Merigan.** 1999. Frequency of class I HLA-restricted anti-HIV CD8+ T cells in individuals receiving highly active antiretroviral therapy (HAART). J. Immunol. **162:**1780–1788.
18. **Gurram, M., N. Chirmule, X. P. Wang, N. Ponugoti, and S. Pahwa.** 1994. Increased spontaneous secretion of interleukin 6 and tumor necrosis factor alpha by peripheral blood lymphocytes of human immunodeficiency virus-infected children. Pediatr. Infect. Dis. J. **13:**496–501.
19. **Hamerman, J. A., and L. L. Lanier.** 2006. Inhibition of immune responses by ITAM-bearing receptors. Sci. STKE **2006:**re1. [Online.]
20. **Hamuryudan, V., F. Moral, S. Yurdakul, C. Mat, Y. Tuzun, Y. Ozyazgan, H. Direskeneli, T. Akoglu, and H. Yazici.** 1994. Systemic interferon alpha 2b treatment in Behcet's syndrome. J. Rheumatol. **21:**1098–1100.
21. **Harari, A., S. Petitpierre, F. Vallelian, and G. Pantaleo.** 2004. Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy. Blood **103:**966–972.
22. **Hazenberg, M. D., S. A. Otto, B. H. van Benthem, M. T. Roos, R. A. Coutinho, J. M. Lange, D. Hamann, M. Prins, and F. Miedema.** 2003. Persistent immune activation in HIV-1 infection is associated with progression to AIDS. AIDS **17:**1881–1888.
23. **Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer.** 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science **303:**1526–1529.
24. **Hellerstein, M. K., R. A. Hoh, M. B. Hanley, D. Cesar, D. Lee, R. A. Neese, and J. M. McCune.** 2003. Subpopulations of long-lived and short-lived T cells in advanced HIV-1 infection. J. Clin. Investig. **112:**956–966.
25. **Heslop, H. E., A. C. Bianchi, F. T. Cordingley, M. Turner, W. Chandima, C. P. De Mel, A. V. Hoffbrand, and M. K. Brenner.** 1990. Effects of interferon alpha on autocrine growth factor loops in B lymphoproliferative disorders. J. Exp. Med. **172:**1729–1734.
26. **Ho, H. N., L. E. Hultin, R. T. Mitsuyasu, J. L. Matud, M. A. Hausner, D. Bockstoce, C. C. Chou, S. O'Rourke, J. M. Taylor, and J. V. Giorgi.** 1993. Circulating HIV-specific CD8+ cytotoxic T cells express CD38 and HLA-DR antigens. J. Immunol. **150:**3070–3079.
27. **Iyasere, C., J. C. Tilton, A. J. Johnson, S. Younes, B. Yassine-Diab, R. P. Sekaly, W. W. Kwok, S. A. Migueles, A. C. Laborico, W. L. Shupert, C. W. Hallahan, R. T. Davey, Jr., M. Dybul, S. Vogel, J. Metcalf, and M. Connors.** 2003. Diminished proliferation of human immunodeficiency virus-specific $CD4^+$ T cells is associated with diminished interleukin-2 (IL-2) production and is recovered by exogenous IL-2. J. Virol. **77:**10900–10909.
28. **Jiang, W., M. M. Lederman, J. R. Salkowitz, B. Rodriguez, C. V. Harding, and S. F. Sieg.** 2005. Impaired monocyte maturation in response to CpG oligodeoxynucleotide is related to viral RNA levels in human immunodeficiency virus disease and is at least partially mediated by deficiencies in alpha/beta interferon responsiveness and production. J. Virol. **79:**4109–4119.
29. **Kovacs, J. A., R. A. Lempicki, I. A. Sidorov, J. W. Adelsberger, B. Herpin, J. A. Metcalf, I. Sereti, M. A. Polis, R. T. Davey, J. Tavel, J. Falloon, R. Stevens, L. Lambert, R. Dewar, D. J. Schwartzentruber, M. R. Anver, M. W. Baseler, H. Masur, D. S. Dimitrov, and H. C. Lane.** 2001. Identification of dynamically distinct subpopulations of T lymphocytes that are differentially affected by HIV. J. Exp. Med. **194:**1731–1741.
30. **Latz, E., A. Schoenemeyer, A. Visintin, K. A. Fitzgerald, B. G. Monks, C. F. Knetter, E. Lien, N. J. Nilsen, T. Espevik, and D. T. Golenbock.** 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat. Immunol. **5:**190–198.
31. **Lenkei, R., G. Bratt, V. Holmberg, K. Muirhead, and E. Sandstrom.** 1998. Indicators of T-cell activation: correlation between quantitative CD38 expression and soluble CD8 levels in asymptomatic HIV+ individuals and healthy controls. Cytometry **33:**115–122.
32. **Li, Q., T. Schacker, J. Carlis, G. Beilman, P. Nguyen, and A. T. Haase.** 2004. Functional genomic analysis of the response of HIV-1-infected lymphatic tissue to antiretroviral therapy. J. Infect. Dis. **189:**572–582.
33. **Lipman, M., and R. Breen.** 2006. Immune reconstitution inflammatory syndrome in HIV. Curr. Opin. Infect. Dis. **19:**20–25.
34. **Malaspina, A., S. Moir, S. Kottilil, C. W. Hallahan, L. A. Ehler, S. Liu, M. A. Planta, T. W. Chun, and A. S. Fauci.** 2003. Deleterious effect of HIV-1 plasma viremia on B cell costimulatory function. J. Immunol. **170:**5965–5972.
35. **Malaspina, A., S. Moir, S. M. Orsega, J. Vasquez, N. J. Miller, E. T. Donoghue, S. Kottilil, M. Gezmu, D. Follmann, G. M. Vodeiko, R. A. Levandowski, J. M. Mican, and A. S. Fauci.** 2005. Compromised B cell responses to influenza vaccination in HIV-infected individuals. J. Infect. Dis. **191:**1442–1450.
36. **Mavilio, D., G. Lombardo, J. Benjamin, D. Kim, D. Follman, E. Marcenaro, M. A. O'Shea, A. Kinter, C. Kovacs, A. Moretta, and A. S. Fauci.** 2005. Characterization of CD56-/CD16+ natural killer (NK) cells: a highly dysfunctional NK subset expanded in HIV-infected viremic individuals. Proc. Natl. Acad. Sci. USA **102:**2886–2891.
37. **McNeil, A. C., W. L. Shupert, C. A. Iyasere, C. W. Hallahan, J. Mican, R. T. Davey, Jr., and M. Connors.** 2001. High-level HIV-1 viremia suppresses viral antigen-specific CD4+ T cell proliferation. Proc. Natl. Acad. Sci. USA **98:**13878–13883.
38. **Migueles, S. A., A. C. Laborico, W. L. Shupert, M. S. Sabbaghian, R. Rabin, C. W. Hallahan, D. Van Baarle, S. Kostense, F. Miedema, M. McLaughlin, L. Ehler, J. Metcalf, S. Liu, and M. Connors.** 2002. HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. Nat. Immunol. **3:**1061–1068.
39. **Mocroft, A., M. Bofill, M. Lipman, E. Medina, N. Borthwick, A. Timms, L.**

Batista, M. Winter, C. A. Sabin, M. Johnson, C. A. Lee, A. Phillips, and G. Janossy. 1997. CD8+,CD38+ lymphocyte percent: a useful immunological marker for monitoring HIV-1-infected patients. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **14:**158–162.

40. **Mohri, H., A. S. Perelson, K. Tung, R. M. Ribeiro, B. Ramratnam, M. Markowitz, R. Kost, A. Hurley, L. Weinberger, D. Cesar, M. K. Hellerstein, and D. D. Ho.** 2001. Increased turnover of T lymphocytes in HIV-1 infection and its reduction by antiretroviral therapy. J. Exp. Med. **194:**1277–1287.
41. **Moir, S., A. Malaspina, O. K. Pickeral, E. T. Donoghue, J. Vasquez, N. J. Miller, S. R. Krishnan, M. A. Planta, J. F. Turney, J. S. Justement, S. Kottilil, M. Dybul, J. M. Mican, C. Kovacs, T. W. Chun, C. E. Birse, and A. S. Fauci.** 2004. Decreased survival of B cells of HIV-viremic patients mediated by altered expression of receptors of the TNF superfamily. J. Exp. Med. **200:**587–599.
42. **Moir, S., K. M. Ogwaro, A. Malaspina, J. Vasquez, E. T. Donoghue, C. W. Hallahan, S. Liu, L. A. Ehler, M. A. Planta, S. Kottilil, T. W. Chun, and A. S. Fauci.** 2003. Perturbations in B cell responsiveness to CD4+ T cell help in HIV-infected individuals. Proc. Natl. Acad. Sci. USA **100:**6057–6062.
43. **Palmer, B. E., E. Boritz, N. Blyveis, and C. C. Wilson.** 2002. Discordance between frequency of human immunodeficiency virus type 1 (HIV-1)-specific gamma interferon-producing $CD4^+$ T cells and HIV-1-specific lymphoproliferation in HIV-1-infected subjects with active viral replication. J. Virol. **76:**5925–5936.
44. **Palmer, B. E., E. Boritz, and C. C. Wilson.** 2004. Effects of sustained HIV-1 plasma viremia on HIV-1 Gag-specific CD4+ T cell maturation and function. J. Immunol. **172:**3337–3347.
45. **Palmer, S., A. P. Wiegand, F. Maldarelli, H. Bazmi, J. M. Mican, M. Polis, R. L. Dewar, A. Planta, S. Liu, J. A. Metcalf, J. W. Mellors, and J. M. Coffin.** 2003. New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma. J. Clin. Microbiol. **41:**4531–4536.
46. **Sachsenberg, N., A. S. Perelson, S. Yerly, G. A. Schockmel, D. Leduc, B. Hirschel, and L. Perrin.** 1998. Turnover of CD4+ and CD8+ T lymphocytes in HIV-1 infection as measured by Ki-67 antigen. J. Exp. Med. **187:**1295–1303.
47. **Salazar-Gonzalez, J. F., D. J. Moody, J. V. Giorgi, O. Martinez-Maza, R. T. Mitsuyasu, and J. L. Fahey.** 1985. Reduced ecto-5′-nucleotidase activity and enhanced OKT10 and HLA-DR expression on CD8 (T suppressor/cytotoxic) lymphocytes in the acquired immune deficiency syndrome: evidence of CD8 cell immaturity. J. Immunol. **135:**1778–1785.
48. **Sankaran, S., M. Guadalupe, E. Reay, M. D. George, J. Flamm, T. Prindiville, and S. Dandekar.** 2005. Gut mucosal T cell responses and gene expression correlate with protection against disease in long-term HIV-1-infected nonprogressors. Proc. Natl. Acad. Sci. USA **102:**9860–9865.
49. **Scott, Z. A., C. M. Beaumier, M. Sharkey, M. Stevenson, and K. Luzuriaga.** 2003. HIV-1 replication increases HIV-specific CD4(+) T cell frequencies but limits proliferative capacity in chronically infected children. J. Immunol. **170:**5786–5792.
50. **Siegal, F. P., N. Kadowaki, M. Shodell, P. A. Fitzgerald-Bocarsly, K. Shah, S. Ho, S. Antonenko, and Y. J. Liu.** 1999. The nature of the principal type 1 interferon-producing cells in human blood. Science **284:**1835–1837.
51. **Stone, S. F., P. Price, J. Brochier, and M. A. French.** 2001. Plasma bioavailable interleukin-6 is elevated in human immunodeficiency virus-infected patients who experience herpesvirus-associated immune restoration disease after start of highly active antiretroviral therapy. J. Infect. Dis. **184:**1073–1077.
52. **Stone, S. F., P. Price, and M. A. French.** 2004. Immune restoration disease: a consequence of dysregulated immune responses after HAART. Curr. HIV Res. **2:**235–242.
53. **Stone, S. F., P. Price, N. M. Keane, R. J. Murray, and M. A. French.** 2002. Levels of IL-6 and soluble IL-6 receptor are increased in HIV patients with a history of immune restoration disease after HAART. HIV Med. **3:**21–27.
54. **von Sydow, M., A. Sonnerborg, H. Gaines, and O. Strannegard.** 1991. Interferon-alpha and tumor necrosis factor-alpha in serum of patients in various stages of HIV-1 infection. AIDS Res. Hum. Retrovir. **7:**375–380.
55. **Weissman, D., R. L. Rabin, J. Arthos, A. Rubbert, M. Dybul, R. Swofford, S. Venkatesan, J. M. Farber, and A. S. Fauci.** 1997. Macrophage-tropic HIV and SIV envelope proteins induce a signal through the CCR5 chemokine receptor. Nature **389:**981–985.
56. **Wilson, J. D., N. Imami, A. Watkins, J. Gill, P. Hay, B. Gazzard, M. Westby, and F. M. Gotch.** 2000. Loss of CD4+ T cell proliferative ability but not loss of human immunodeficiency virus type 1 specificity equates with progression to disease. J. Infect. Dis. **182:**792–798.
57. **Younes, S. A., B. Yassine-Diab, A. R. Dumont, M. R. Boulassel, Z. Grossman, J. P. Routy, and R. P. Sekaly.** 2003. HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity. J. Exp. Med. **198:**1909–1922.