Cytokine Interactions in Human Immunodeficiency Virus–infected Individuals: Roles of Interleukin (IL)-2, IL-12, and IL-15

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Summary

Cytokines have been shown to be powerful regulators of the immune response. In this study, we analyze the effect that the newly recognized cytokine interleukin (IL)-15 has on proliferation and cytokine induction using peripheral blood mononuclear cells (PBMCs) and purified $CD4^+$ T cells from patients infected with human immunodeficiency virus (HIV) who are at various stages in their disease. We observed that IL-15 enhances the proliferative response in a dose-dependent manner from PBMCs of HIV-infected individuals when stimulated by polyclonal mitogen, tetanus toxoid, or HIV-specific antigen. The effects of exogenous IL-15 are substantially diminished by adding a neutralizing antibody to the β chain of the IL-2 receptor. Moreover, the ability of IL-15 to increase proliferation is enhanced by the presence of endogenous IL-2 produced in the cultures. The effect that exogenous IL-15 had on IL-2, IL-4, and interferon (IFN)-γ induction from PBMC's or $CD4^+$ T cells in response to mitogen or tetanus toxoid was also examined. This was compared to the effect that exogenous IL-2 and IL-12 had under the same conditions. Addition of IL-2 or IL-15 to short-term in vitro cultures of either PBMCs or $CD4^+$ T cells had little effect on IL-2, IL-4, or IFN-γ production. By contrast, IL-12 caused substantial enhancement of both IL-2 and IFN-γ production from these cultures. The role that endogenous cytokines have on IFN-γ induction was also studied. Addition of a neutralizing antibody to the α chain of the IL-2 receptor or IL-12 to antigen stimulated cultures caused a striking decrease in IFN-γ production. Neutralization of endogenous IL-15 also resulted in diminished IFN-γ production from cultures stimulated with mitogen. IL-4 and IFN-γ protein production by PBMCs and $CD4^+$ T cells stimulated with mitogen was assessed to see if we could detect a specific bias of cytokine production. Small amounts of IL-4 were detected from $CD4^+$ T cells but not PBMCs from most individuals tested. IFN-γ and IL-2, however, were also produced from these same cultures. These results further elucidate the mechanism of cytokine regulation in HIV- infected individuals, and they provide evidence that IL-15 may be a useful immune modulator.

During the past 10 years, there has been an explosion in the number of new cytokines isolated and intense interest in using them as therapeutics. IL-2, one of the first cytokines to be isolated (1), is a potent T cell growth factor that has been used successfully as a therapeutic in certain types of cancer (2). More recently, it has been shown to be effective in increasing the $CD4^+$ T cell count in patients infected with HIV (3). Another cytokine, IL-12, has also been shown to exert a powerful regulatory influence of the cellular immune response (4). These responses are most striking in several murine models of intracellular infection (4). There is also evidence to suggest that IL-12 may have a critical function in the progression of HIV disease (5, 6). These studies have been a prelude to clinical trials using IL-12 therapy in both cancer and AIDS. While these cytokines are important in mediating immune responses, it is clear that redundancy in function exists among various cytokines, leaving the door open for new growth factors to be isolated and studied, which might ultimately provide therapeutic benefit.

Recently, a new cytokine was cloned on the basis of its ability to cause proliferation of an IL-2–dependent cell line (7). It was given the designation IL-15. IL-15 appears to have many functions similar to those of IL-2. For example,

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IL-15 uses components of the IL-2 receptor for binding and signal transduction (8). Moreover, IL-15 is a potent growth factor for activated T cells and it enhances cytolytic function of both NK and $CD8^+$ T cells (7–9). In striking contrast to IL-2, IL-15 is not produced from T cells, but it is abundantly expressed by a wide variety of tissues and by activated monocyte/macrophages (7). Herein, using cells from both HIV^+ and HIV^- individuals, we study the effect that IL-15 has on both the proliferation and cytokine production. We also compare its effects to those of IL-2 and IL-12 on modulating these reponses. Moreover, we examine the role that endogenous IL-2, IL-12, and IL-15 have on IFN-γ production.

In addition to studying the function that exogenous cytokines have on the immune response, there has also been interest in defining the endogenous response from HIV-infected patients by the particular types of cytokines they produce (i.e., Th1 vs. Th2) (10–13). Most of the studies to characterize the immune response were done using PBMC as responder cells (10, 12). This population of cells contains multiple cell types that can produce the same cytokines, making it difficult to assess the production from $CD4^+$ T cells. In the present study, using both purified $CD4^+$ T cells and PBMCs from the same individual, we assessed the IL-2, IL-4, and IFN-γ protein content in response to various antigens. By using purified $CD4^+$ T cells, we were able to specifically identify whether a Th1 or Th2 type pattern was expressed from the same number of responding cells.

The following studies highlight the prominent role that both endogenous and exogenous IL-2, IL-12, and IL-15 exert on the immune response from patients infected with HIV.

Materials and Methods

Reagents. Complete media consisting of RPMI 1640 supplemented with 10% heat-inactivated human AB serum from Sigma Chemical Co. (St. Louis, MO), penicillin (100 U/ml), streptomycin (100 U/ml), and L-glutamine (2 mM) were used for all stimulations. PHA was purchased from Grand Island Biological Co. (Grand Island, NY). Tetanus toxoid was purchased from Connaught Labs (Swiftwater, PA). Recombinant gp 120 was a generous gift from Genentech (San Francisco, CA). A group of five synthetic peptides corresponding to the *env* of HIV-1 (T1, T2, Th4.1, P18IIIB, and p18MN) have been previously described (14, 15). Mouse anti–human magnetic beads were purchased from Advanced Magnetic Inc. (Cambridge, MA). Ficoll-Hypaque was purchased from Pharmacia Fine Chemicals (Piscataway, NJ).

Subjects. Whole blood was obtained by venipuncture from healthy HIV volunteer donors and HIV-1–infected individuals. In some experiments, PBMCs were obtained from HIV-1–infected individuals by apharesis. All specimens were processed and plated into the appropriate cultures immediately or within 16 h of the time the blood was collected.

Recombinant Lymphokines. Human rIL-2 with a specific activity of 6.5×10^6 BRMP U/mg was purchased from Genzyme Corp. (Boston, MA). Human rIL-12 with a specific activity of 5.26×10^6 U/mg was purchased from R & D Systems, Inc. (Minneapolis, MN). Simian IL-15 with a specific activity of 5.8×10^5 U/μg was provided by Immunex Corp. (Seattle, WA).

Antibodies. Humanized antibodies against the α chain (anti-TAC)[1] and β chain (anti–IL-2Rβ) of the IL-2 receptor were a generous gift of Dr. John Hakimi (Roche Laboratories, Nutley, NJ). Goat anti–human IL-12 antibody was purchased from R & D Systems Inc. Mouse anti–human antibodies to CD3, CD8, CD14, CD16, CD19, and CD56 were gift from Dr. Thomas Nutman (NIAID, NIH, Bethesda, MD). Antibody against human IL-15 was provided by Immunex (Seattle, WA).

Preparation of Cells. PBMCs, $CD4^+$ T cells, and APCs were prepared from the same starting population in the following manner: PBMCs were isolated from freshly drawn heparinized venous blood or buffy coat fractions by Ficoll-Hypaque density gradient centrifugation. T cell (E-rosette$^+$) and APC (E-rosette$^-$) populations were isolated by rosetting PBMCs with neuraminidase-treated SRBC. For $CD4^+$ T cells purification, the E-rosette$^+$ cells were resuspended at 2×10^7 cells per ml and mAbs (5 μg/ml) to CD8, CD14, CD16, CD19, and CD56 were added for 30 min at 4°C. For APC purification, the E-rosette$^-$ cells were incubated under similar conditions with antibodies to CD3, CD8, CD16, and CD56. At the end of the incubation, both E-rosette$^+$ and E- rosette$^-$ cells were washed twice, and they were mixed with mouse anti–human magnetic beads for 30 min at 4°. Positively staining cells were depleted by two 20-min cycles of exposure to a magnetic field. The remaining cells were washed twice in culture medium and used for studies. After this negative selection procedure, the percentage of $CD4^+$ T cells ranged between 80 and 90%, while >90% of the cells in the APC population stained for CD14 and CD19. In general, <1% of the cells in either population stained positively for CD8 or CD16. At least one different HIV^- control was used in each separate experiment.

Measurement of Proliferation in Response to Stimulation. PBMCs of HIV^+ or HIV^- individuals were suspended at a concentration of 2.5×10^6 cells per ml in complete media. A total of 2.5×10^5 cells were added to round-bottom 96 well plates (Nunc, Roskilde, Denmark) and stimulated in vitro for 5–6 d with PHA (final concentration of 1/500), a group of five peptides from the Env of HIV-1 (final concentration of 2.5 μM/ml), tetanus toxoid (final concentration of 1/3,000), or gp 120 (final concentration of 30 μg/ml). Various concentrations of simian IL-15 alone or in combination with TAC and IL-2β chain (MikB-1) at concentrations of 10 μg/ml were added to the cultures. In another group of experiments, PBMCs were stimulated with tetanus toxoid or HIV *env* peptides at the same concentrations as listed above in the presence of media alone, rIL-2 (1 ng/ml), rIL-12 (20 U/ml), or simian IL-15 (5 ng/ml). After 5 d, cultures were pulsed with 1 μCi of [^{3}H]thymidine and were harvested after 18 h. Results represent the mean of triplicate wells. The SEM was <10% in all experiments.

Induction of Cytokine Production by PBMCs and CD4 T Cells. To induce cytokine production, PBMCs (2.5×10^5 per well) or highly enriched $CD4^+$ T cells (1×10^5 per well) plus APC (1×10^4 cells per well) were added to round bottom 96-well plates and stimulated with PHA or tetanus toxoid in the presence of various cytokines and/or anticytokines. Supernatants were collected after 4–5 d of culture and stored at −70°C until used. It was determined from several time course experiments that optimal production of IL-2, IFN-γ and IL-4 could be detected 4–5 d after stimulation with mitogen or specific antigen.

Measurement of Cytokine Production. IL-2 content was assessed using an IL-2–specific ELISA kit (Endogen, Cambridge, MA).

[1]*Abbreviations used in this paper:* anti-TAC, humanized antibodies against the α chain of the IL-2 receptor; anti-IL-2Rβ, humanized antibodies against the β chain of the IL-2 receptor.

Table 1. *IL-15 Increases Proliferation of PBMCs from Both HIV$^+$ and HIV$^-$ Individuals in Response to Various Antigens*

Stimulus	HIV$^+$	HIV$^-$
Experiment 1		
PHA(1/500)		
–	25,908	79,770
Anti–IL-2RB	35,673	61,550
Anti–IL-15	11,222	36,778
IL-15 (10 ng/ml)	240,457	259,321
IL-15 + anti–IL-2RB	45,497	54,438
IL-15 + anti–IL-15	16,146	63,309
HIV pooled peptides		
–	2,151	762
IL-15 (10 ng/ml)	79,809	2,099
gp120		
–	14,864	333
IL-15 (10 ng/ml)	44,614	100
Experiment 2		
PHA (1/500)		
–	11,700	2,000
Anti–IL-2RB	16,241	1,413
Anti–TAC	5,213	1,361
IL-15 (5 ng/ml)	45,000	3,900
IL-15 (1 ng/ml)	17,300	2,700
IL-15 (5 ng/ml) + anti–IL-2RB	20,000	3,500
IL-15 (5 ng/ml) + anti–TAC	22,809	4,000
Tetanus		
–	9,467	12,220
Anti–IL-2RB	9,696	9,983
Anti–IL-15	7,483	11,173
Anti–TAC	1,448	951
IL-15 (5 ng/ml)	30,400	23,100
IL-15 (1 ng/ml)	17,300	14,900
IL-15 (5 ng/ml) + anti–IL-2RB	14,905	13,493
IL-15 (5 ng/ml) + anti–IL-15	11,336	8,824
IL-15 (5 ng/ml) + anti-TAC	9,849	4,616
HIV pooled peptides		
–	200	100
IL-15 (5 ng/ml)	8,900	800
IL-15 (1 ng/ml)	1,300	200
IL-15 (5 ng/ml) + anti–Il-2RB	800	300
IL-15 (5 ng/ml) + anti-TAC	3,300	100

The effect of IL-15 on proliferation of PBMCs from HIV$^+$ and HIV$^-$ individuals in response to various antigens. Fresh PBMCs were added (2.5×10^5/200 μl) to 96-well plates (round bottom) in the presence of the following antigens; PHA (1/500 final dilution), pooled HIV *env* peptides (2.5 μM final concentration), gp 120 (30 μg/ml), or tetanus (1/3,000 dilution). IL-15 was added at the concentrations indicated. Antibodies to IL-15, αTAC, and IL-2RB were added at a concentration of 10 μg/ml. In the absence of antigen, cpm were <1,000 from PBMCs of both HIV$^+$ and HIV$^-$ individuals in both experiments. In Experiment 1, cpm for cells cultured in the presence of IL-15 alone (10 ng/ml) were 1,500 and 6,000 for HIV$^+$ and HIV$^-$ individuals, respectively. In Experiment 2, cpm were 8,300 and 5,500, respectively, for HIV$^+$ and HIV$^-$ individuals. Background values for IL-15 alone were subtracted from values obtained in the presence of antigen plus IL-15. The data are combined from two separate experiments.

IL-15 did not show any activity in this assay. The lower limit of detection was 42 pg/ml. IL-4 was also assessed using an IL-4–specific ELISA kit (Endogen). The lower limit of sensitivity was found to be 3–10 pg/ml, which was similar to the sensitivity obtained using hCT.4S in a bioassay. Moreover, we noted that IL-15 caused significant proliferation of the hCT.4S cells, obviating their use in situations where IL-15 is endogenously produced or added. IFN-γ production was measured by ELISA as previously described (6). The lower limit of sensitivity was between 3 and 10 U/ml. Results for all cytokines represent the mean of triplicate wells. The SEM was <10% in all experiments.

Results

IL-15 Increases the Proliferative Response to Polyclonal Mitogen, Recall Antigen, and HIV-specific Antigen. We studied whether IL-15 could enhance the proliferative response of PBMCs from both HIV$^-$ and HIV$^+$ individuals in response to various antigens. PBMCs were isolated and stimulated with a "suboptimal" concentration of PHA for 4 d. This allowed a window in which to study the function that various cytokines can have on the proliferative response to PHA, since at an optimal dose of PHA, the proliferative response is maximal and exogenous cytokines have little effect. In Experiment 1 (Table 1), the HIV$^+$ individual had a ninefold increase in PHA-induced proliferation in the presence of IL-15, compared to a fourfold increase in the HIV$^-$ control, bringing the response of the HIV$^+$ donor to a level comparable to that of the HIV$^-$ control. Cells stimulated in the presence of both IL-15 and an antibody to IL-2Rβ or IL-15 showed similar values to the response obtained in the absence of exogenous IL-15, proving that the effect of IL-15 was specific. Moreover, anti–IL-2Rβ or anti–IL-15 did not appreciably diminish the baseline response. In the same experiment, the moderate baseline proliferative response to two different HIV-specific antigens (gp120 and pooled peptides) is also shown to be strikingly increased in the HIV-infected individual when IL-15 is present, whereas the HIV$^-$ individual had essentially no response in the presence or absence of IL-15. Experiment 2 (Table 1) shows additional data using tetanus toxoid as a recall antigen to stimulate the cells. Both the HIV$^+$ and HIV$^-$ individuals generated a baseline response to tetanus alone. Proliferation was increased approximately threefold in the HIV$^+$ individual and twofold in the HIV$^-$ individual in the presence of IL-15. We also tested whether anti-TAC influenced the IL-15–induced response. Anti-TAC caused substantial inhibition of the baseline proliferative response to tetanus, whereas anti–IL-2Rβ had no effect. These results are compatible with previous work demonstrating anti-

TAC to be better at inhibiting an IL-2– mediated proliferative response than anti–IL-2Rβ (8, 16). When IL-15 was added in the presence of anti-TAC, there was a threefold decrease in the response compared to IL-15 alone, but a sevenfold increase compared to when anti-TAC alone was added. This suggests the baseline proliferative response to tetanus is mediated in large part by IL-2, and the increase in proliferation seen with IL-15 is augmented by endogenous IL-2 production. PBMCs from both HIV^+ and HIV^- individuals showed essentially no baseline proliferation (cmp <1000) in response to HIV pooled peptides. Cells from HIV^+ individuals, however, when stimulated in the presence of IL-15, had a striking increase in proliferation that was completely abrogated when anti–IL-2Rβ was added in the cultures. Interestingly, anti-TAC inhibited the IL-15–mediated increase threefold. These data also supported the notion that IL-15 is enhancing a response in which small amounts of endogenous IL-2 are produced. It should be noted that although no proliferation was seen in the absence of exogenous IL-15, it is clear from earlier work using the same HIV peptides that IL-2 can be detected from cultures that do not show a proliferative response. Thus, although no proliferation is seen, small amounts of IL-2 may be present in these cultures (14, 15).

IL-2, IL-12, and IL-15 Increase the Proliferative Response to Recall Antigens and HIV-specific Antigen. It was recently demonstrated that IL-12 strikingly enhanced proliferation of PBMCs from HIV^+ individuals in response to recall or HIV-specific antigens (6). We compared the effect of exogenous IL-2, IL-12, and IL-15 on proliferation of PBMCs

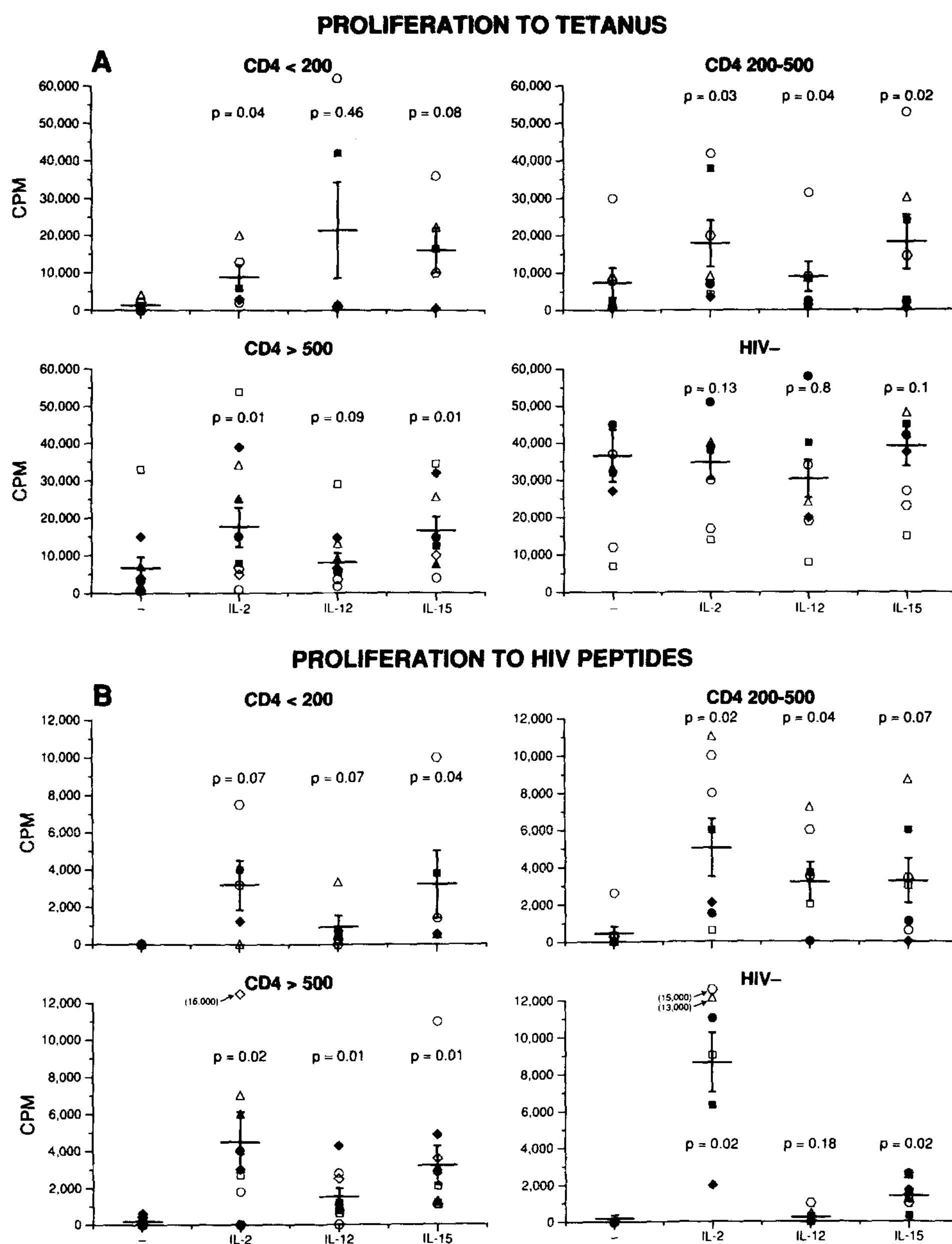

Figure 1. Proliferation of PBMCs from HIV^+ and HIV^- individuals stimulated with tetanus toxoid and HIV pooled peptides in the presence of IL-2, IL-12, and IL-15. Fresh PBMCs were added (2.5 × 10^5/200 μl) to 96-well plates (*round bottom*) and stimulated by a group of pooled HIV Env peptides (2.5 μM final concentration) or tetanus toxoid (1/3,000 dilution) in the presence or absence of IL-2 (1 ng/ml), IL-12 (20 U/ml), or IL-15 (5 ng/ml) for 5 d and then pulsed with 1 μCi of [^{3}H] thymidine, and harvested 18 h later. Results are reported as cpm. As a control, PBMCs were cultured for the same period in the presence of the various cytokines but without any antigen stimulation. In each individual, the background value for cells cultured in cytokine alone was subtracted from the value obtained when cells were cultured with antigen and cytokine. The data are pooled from more than eight experiments. At least one HIV^- control was included in each separate experiment. The *P* values shown were calculated by a Wilcoxon signed rank test by comparing the cpm when cells are stimulated with antigen alone vs. antigen in the presence of IL-2, IL-12, or IL-15.

Table 2. *IL-12 Increases IL-2 Production from PBMCs Stimulated with PHA*

CD4 count	Stimulus	PBMC			
		–	IL-15	αTAC	αTAC + IL-15
Experiment 1					
1,100	PHA	0	0	596	612
	TET	0	0	0	0
420	PHA	0	0	2,100	2,100
	TET	0	0	0	0
80	PHA	0	0	750	750
	TET	0	0	0	0
HIV$^-$	PHA	0	0	1,500	1,200
	TET	0	0	120	122
		PBMC			
Experiment 2		–	IL-12	αTAC	αTAC + IL-12
240	PHA	0	800	780	4,200
	TET	0	18	0	100
600	PHA	0	1,600	2,700	5,700
	TET	0	0	0	0
<200	PHA	0	109	681	1,051
	TET	0	0	0	0
1,100	PHA	0	0	234	910
	TET	0	0	0	0
HIV$^-$	PHA	0	105	1,200	7,000
	TET	0	0	73	59
HIV$^-$	PHA	0	0	573	783
	TET	0	0	444	263
		CD4$^+$ T cells			
240	PHA	1,900	3,000	1,800	4,500
	TET	0	0	0	0
600	PHA	2,700	2,100	2,100	4,000
	TET	0	0	0	0
<200	PHA	1,536	1,719	1,634	1,923
	TET	0	0	0	0
1,100	PHA	1,507	2,096	1,677	2,281
	TET	0	0	0	0
HIV$^-$	PHA	190	340	900	1,500
	TET	85	0	235	171
HIV$^-$	PHA	500	800	1,000	2,100
	TET	64	0	80	75

Effects of IL-12 and IL-15 on IL-2 production from PBMCs and CD4$^+$ T cells in response to PHA and tetanus (*TET*). PBMCs (2.5 × 10^5/200 µl) or highly enriched CD4$^+$ T cells (105/200 µl) plus APC (10^4 cells) were added to 96-well plates in the presence of PHA (1/100 final concentration) or tetanus (1/3,000 final concentration). In Experiment 1, IL-15 (5 ng/ml), anti-TAC (10 µg/ml), or both were added to cultures. In Experiment 2, IL-12 (20 U/ml), anti-TAC, or both were added to cultures containing PBMCs or CD4$^+$ T cells. Supernatants were collected after 4–5 d and IL-2 content was measured by ELISA (Endogen, Cambridge, MA). The sensitivity of the IL-2 ELISA was 42 pg/ml. Values shown are in picograms per milliliter. Total CD4 counts of the HIV$^-$ infected patients are listed in the left-hand column. Most HIV$^-$ controls had CD4 counts >1,000. The data are combined from two separate experiments.

from HIV$^+$ and HIV$^-$ individuals in response to tetanus toxoid or a group of HIV peptides. HIV$^+$ individuals were segregated into three groups based on their total CD4 count (<200, 200–500, >500/µl) and compared to a group of HIV$^-$ individuals with CD4 counts of generally >1,000/µl. HIV$^-$ individuals demonstrated a good proliferative response to tetanus toxoid that was not enhanced by IL-2, IL-12, or IL-15 (Fig. 1 *A*). By contrast, HIV$^+$ individuals with CD4 counts <200/µl showed no proliferation to tetanus toxoid in the absence of exogenous cytokines, but some enhancement of the response was noted in the presence of all three cytokines. Patients with CD4 counts between 200 and 500 and >500/µl showed increased baseline proliferation from the <200/µl group, and this was enhanced approximately twofold by IL-2 and IL-15, but not by IL-12. Thus, it appears that IL-12 had its most dramatic effect in two of the patients with CD4 counts <200 per µl, while IL-2 and IL-15 had effects in all groups tested. To characterize the HIV-specific responses, a group of five synthetic peptides corresponding to regions of the HIV-1 envelope used previously to characterize HIV-specific proliferative and cytokine responses were tried (6, 14, 15). Proliferation of PBMCs in response to these peptides from HIV$^-$ individuals in most instances was very low (cpm<1,000), although when stimulated in the presence of IL-2, there was significant proliferation (Fig. 1 *B*). Addition of IL-15 caused only a modest increase in proliferation (1,000 cpm above background), while IL-12 did not cause any increase in proliferation. These results make it difficult to assess the ability of IL-2 to "restore" a deficient response in the HIV$^+$ individuals. In this regard, IL-12 and IL-15 would provide more specific data. HIV$^+$ individuals in all groups had little proliferative response to the peptides in the absence of exogenous cytokines (Fig. 1 *B*). In patients with CD4 counts of 200–500 or >500/µl, IL-12 and IL-15 caused a modest increase in proliferation. The failure to detect a substantial baseline proliferative response from the HIV$^+$ individuals contrasts with the data obtained by Clerici et al. (6). This might be explained by the patient population and at which stage of disease they were tested. The ability of IL-12 to enhance the proliferative response is consistent with their data (6), although their responses were substantially greater. A major caveat to these studies, as in most studies on cells from HIV$^+$ patients, is that the patient groups are relatively small and variable in disease states, and the SE ranges are substantial.

IL-12 Significantly Increases IL-2 Production from PHA-stimulated PBMCs. Since IL-15 affected T cell proliferation, which is usually IL-2–dependent, it was of interest whether IL-15 might affect IL-2 production. PBMCs from patients with varying CD4$^+$ T cell counts were stimulated by PHA or tetanus in the presence of IL-15, anti-TAC, or IL-15 +anti-TAC. The use of anti-TAC prevents IL-2

consumption, and it allows measurement of the IL-2 that has accumulated. Table 2 (Experiment 1) shows that IL-15 does not enhance the IL-2 detected in response to PHA or tetanus compared to anti-TAC alone. We also examined the effect of IL-12 on IL-2 production. Previous work has shown that IL-12 increased proliferation and IL-2 production from PBMCs in response to HIV-specific antigen (6). Table 2 (Experiment 2) shows combined results from two separate experiments. In these experiments, the presence of IL-12 alone was able to enhance IL-2 production, even in the absence of anti-TAC, demonstrating the striking effect it has on IL-2 production. When both anti-TAC and IL-12 were added to cultures, IL-2 production was strikingly increased from PBMCs of both HIV^+ and HIV^- individuals. By contrast, IL-12 had only a modest effect on increasing IL-2 from highly enriched $CD4^+$ T cells, particularly in individuals with low $CD4^+$ T cell counts. Moreover, IL-2 production induced by a CD4/class II–restricted antigen such as tetanus was not enhanced using PBMCs or $CD4^+$ T cells. These results and other data not shown suggest that IL-12 may be enhancing IL-2 production from $CD8^+$ T cells, or that enhancement depends on the presence of other cells removed when the $CD4^+$ T cells are purified.

Early experiments studying proliferation or IL-2 production from cells of HIV-infected patients stimulated with PHA or tetanus raised the issue of whether a qualitative defect of IL-2 production existed in these patients (17–24). The data in Table 2 (Experiment 2), show that IL-2 pro-

Table 3. *Addition of IL-12 Enhances IFN-γ Production from PBMCs or $CD4^+$ T Cells Stimulated with Antigen*

CD4 count		PBMC –	PBMC IL-2	PBMC IL-12	PBMC IL-15
Experiment 1					
1,100	PHA	540	525	600	500
	TET	0	0	40	0
420	PHA	285	500	800	270
	TET	7	100	210	40
80	PHA	40	50	560	35
	TET	0	0	0	0
HIV^-	PHA	220	270	1,800	90
	TET	12	27	375	24

Experiment 2		PBMC –	IL-2	IL-12	IL-15	αTAC	α12	αTAC + 12	αIL-15
240	PHA	305	520	1,050	425	0	110	570	100
	TET	0	70	180	12	0	0	140	ND
600	PHA	600	500	515	600	250	175	410	400
	TET	0	30	120	7	0	0	70	ND
HIV^-	PHA	275	375	635	500	10	60	470	200
	TET	22	70	280	45	0	10	220	ND

		$CD4^+$ T cells –	IL-2	IL-12	IL-15	αTAC	α12	aTAC + 12
240	PHA	165	165	350	20	20	85	320
	TET	0	0	36	0	0	0	14
600	PHA	185	235	810	200	0	65	420
	TET	0	0	0	0	0	0	0
HIV^-	PHA	25	50	220	40	0	0	185
	TET	0	5	60	0	0	0	50

Effects of IL-2, IL-12, and IL-15 on IFN-γ production from PBMCs and $CD4^+$ T cells in response to PHA and tetanus. PBMCs (2.5×10^5/200 μl) or highly enriched $CD4^+$ T cells (10^5/200 μl) plus APC (10^4 cells) were added to 96-well plates and stimulated with PHA or tetanus in the presence of IL-2 (1 ng/ml), IL-12 (20 U/ml), or IL-15 (5 ng/ml). In Experiment 2, anti-TAC, anti–IL-12 (10 μg/ml), anti-TAC + IL-12, or anti–IL-15 (10 μg/ml) were added to the cultures. Supernatants were collected after 4 d and IFN-γ content was measured by ELISA. The sensitivity of the assay was between 3 and 10 U/ml. Values shown are in units per milliliter. The data represent two separate experiments.

duction was often higher from PBMCs and $CD4^+$ T cells in response to PHA in HIV^+ individuals compared to HIV^- controls. This could be explained if a greater number of the responding cells from the HIV^+ individuals were previously activated. Although PHA may not be a "physiologic" stimulus, these results suggest that under maximal stimulation conditions, there is not a deficit of IL-2 production when controlling for an equal number of $CD4^+$ responder cells from both HIV^+ and HIV^- individuals.

IL-12 Strikingly Enhances IFN-γ Production in Response to Antigen Compared to IL-2 or IL-15. IL-15 was compared to IL-2 and IL-12 in its ability to alter IFN-γ production by PMBC or $CD4^+$ T cells in response to PHA or tetanus toxoid. The presence of exogenous IL-2 and IL-15 had little effect on IFN-γ production (Tables 3 and 4). By contrast, cells stimulated in the presence of IL-12 show a marked increase in IFN-γ production from both the HIV^+ and HIV^- individuals in response to both stimuli. IFN-γ measured from PBMCs stimulated with HIV peptides was often below the limit of detection and was not enhanced by the presence of IL-15, although in selected patients, there was a response when IL-12 was added to the cultures (data not shown). These results suggest that IL-15 (5 ng/ml), at a dose sufficient to elicit maximal proliferation of T cell blasts (7), does not augment IFN-γ production. Interestingly, when the dose of IL-15 was increased to 100 ng/ml, there was an increase in spontaneous IFN-γ production from PBMCs. Whether this "high" dose of IL-15 elicits IFN-γ from NK cells in a similar manner to IL-2 is being examined. Moreover, at this dose of IL-15, IFN-γ was also increased in response to tetanus toxoid and HIV-specific antigen (data not shown).

Neutralization of Endogenous IL-2, IL-12, or IL-15 Reduces IFN-γ Production in Response to PHA. We analyzed the effect of inhibiting endogenous IL-12, IL-15, or binding of IL-2 to various subunits of its receptor on the in vitro production of IFN-γ. Neutralization of either IL-2Rα (anti-TAC) or IL-12 resulted in substantial inhibition of IFN-γ (Table 4). When the IL-2Rα was blocked in the presence of IL-12, however, there was partial restoration of the response. These results demonstrate the importance of both of these cytokines for optimal IFN-γ production. Neutralization of IL-15 also resulted in a two- to threefold decrease in IFN-γ production. This effect seemed more pronounced in the HIV-infected patients than in the HIV^- individuals (Table 4). Moreover, the presence of anti–IL-2Rβ chain antibody to PHA-stimulated cultures resulted in a similar degree of inhibition as did the anti–IL-15 (Table 4, Experiment 1), consistent with data showing that IL-15 acts through this subunit of the IL-2 receptor (7–9).

IL-2, IL-12, and IL-15 Do Not Alter IL-4 Production from PBMCs or PHA-stimulated $CD4^+$ T Cells. There has been

Table 4. *Antibodies against IL-12, IL-15, and Receptors for IL-2 Decrease IFN-γ Production from PBMCs and $CT4^+$ T Cells Stimulated with PHA*

	CD4 count	–	IL-2	IL-12	IL-15	aTAC	α12	αTAC+12	α15	αIL-2Rβ
			PBMC							
Experiment 1	491	750	570	900	650	55	ND	ND	275	265
	613	1,000	920	950	900	500	ND	ND	530	600
	HIV^-	600	500	500	500	200	ND	ND	320	310
			PBMC							
	CD4 count	–	IL-2	IL-12	IL-15	aTAC	α12	αTAC+12	α15	αIL-2Rβ
Experiment 2	155	1,900	1,400	2,000	1,000	225	600	1,050	600	ND
	1,129	180	200	850	300	0	75	370	80	ND
	HIV^-	400	400	950	500	0	150	700	500	ND
			$CD4^+$ T cells							
	155	140	150	1,650	100	0	55	950	0	ND
	1,129	150	200	1,100	275	60	110	630	100	ND
	HIV^-	550	750	2,500	575	250	250	2,500	200	ND

Effects of anticytokines and anti–cytokine receptor antibodies on IFN-γ production from PBMCs and $CD4^+$ T cells in response to PHA. PBMCs (2.5×10^5/200 μl) or highly enriched $CD4^+$ T cells (10^5/200 μl) plus APC (10^4 cells) were added to 96-well plates and stimulated with PHA of IL-2 (1ng/ml), IL-12 (20U/ml), or IL-15 (5 ng/ml. Anti-TAC (10μg/ml), anti–IL-12 (10 μg/ml), and anti–15 (10μg/ml), or anti–IL-2Rβ (10μg/ml) antibody were added to the cultures as indicated in the table. Supernatants were collected after 4 d and IFN-γ content was measured with ELISA. The sensitivity of the assay was between 3 and 10 U/ml. Values shown are in units per milliliter. The data are combined from two separate experiments.

considerable interest in quantitating IL-4 and IFN-γ production from cells of HIV+ individuals to determine if there is a bias toward a Th1- or Th2-type cytokine response (10–13). Previous studies measuring IL-4 protein used PBMCs as responder cells in the culture to various mitogens (10). Since multiple cell types can produce IL-4, and the Th1/Th2 paradigm is based on CD4-produced cytokines, we assessed IL-4 protein content from both PBMC and CD4+ T cells in response to PHA by using a sensitive IL-4–specific ELISA. PBMCs produced little or no IL-4 from both HIV+ and HIV− individuals. When IL-4 was measured from highly enriched CD4+ T cells, however, small amounts were detected from both groups (Table 5). IL-2 and IFN-γ were also measured from the same supernatants to determine if there was a predilection toward Th1 or Th2 cytokine production. In all cases, both IL-2 and IFN-γ were detected in both HIV+ and HIV− individuals. Moreover, production of IL-4 and IFN-γ were not substantially different among the two populations studied. Interestingly, the highest amount of IL-4 was detected from an HIV+ patient with a CD4 count of 1,100.

Lastly, the effects of exogenous IL-2, IL-12, or IL-15 were examined for their ability to alter IL-4 production from PHA-stimulated cells. These cytokines appear to exert no demonstrable influence on IL-4 production in these short-term in vitro cultures.

Table 5. *IL-4 Production from PBMCs and CD4+ T Cells Stimulated with PHA Is Not Affected by the Addition of IL-2, IL-12, or IL-15 to Cultures*

		PBMC					
CD4 count	Stimulus	–	IL-2	IL-12	IL-15	IL-2	IFN-γ
<200	PHA	0	0	0	0	681	1,900
240	"	0	0	0	0	780	305
600	"	0	0	0	0	2,700	600
1,100	"	15	17	13	17	234	180
HIV−	"	13	9	15	8	1,200	275
HIV−	"	0	0	0	0	573	40
		CD4+ T cells					
		–	IL-2	IL-12	IL-15	IL-2	IFN-γ
<200	PHA	61	51	39	76	1,634	140
240	"	7	10	11	13	1,800	165
600	"	5	5	6	8	2,100	185
1,100	"	155	137	21	147	1,677	150
HIV−	"	36	30	24	30	1,000	25
HIV−	"	65	45	40	53	900	550

Effects of IL-2, IL-12, and IL-15 on IL-4 production from PBMCs and CD4+ T cells in response to PHA. Cells were cultured under similar conditions outlined in Tables 2 and 3. Culture supernatants were collected after 4–5 d and IL-4 content was measured by ELISA (Endogen). The sensitivity of the IL-4 ELISA was 3 pg/ml. Values shown for the IL-4 are in pg/ml. IL-2 and IFN-γ content were measured as described in Tables 2 and 3. The data are combined from two separate experiments.

Discussion

These studies were designed to characterize the effects of a newly recognized cytokine, IL-15, and compare it to IL-2 and IL-12 in its ability to alter cytokine production and proliferation of both PBMC and CD4+ T cells in response to a panel of antigens. We were especially interested in whether IL-15 could effect an HIV-specific response as well as to recall antigen. The quantitation and specificity of the HIV-specific response was complex. Initially, envelope proteins, gp160 derived from baculovirus and gp41 derived from *Escherichia coli*, were used as antigens. In several experiments, they consistently induced proliferative responses from HIV− individuals and often elicited no responses from HIV+ individuals, suggesting a high false positive response, and making their usefulness as an HIV-specific antigen limited for these types of studies. We then used a group of *env*-derived peptides that have been well characterized and studied for these purposes (6, 14, 15). These peptides elicited essentially no proliferation from HIV− individuals, making them useful for studying HIV-specific responses. It should be noted that the proliferative responses detected from HIV+ patients at several stages of disease was also very low, similar to the HIV− group. Although the proliferative response to the HIV peptides was low, addition of IL-15 strikingly enhanced the response to the peptides as well as to tetanus toxoid from the HIV-infected individual (Table 1). The effects of IL-15 were abrogated by addition of either antibody to IL-2Rβ or IL-15 to the cultures, demonstrating IL-15 specificity. Moreover, the enhanced proliferation seen with IL-15 appeared to be synergistic with the endogenous IL-2 produced.

The second question we studied was the effect that IL-15 had on cytokine induction after short-term in vitro stimulation. At doses that have been shown to induce maximal proliferation on activated T cells (1–5 ng/ml) (7), IL-15 had no appreciable effect on altering the in vitro production of IL-2, IL-4, or IFN-γ in response to any of the antigens tested from either HIV+ or HIV− individuals (Tables 2–5). It is possible that higher doses of IL-15 may effect cytokine induction. Several experiments using 10 ng/ml of IL-15 gave similar results. However, when 100 ng/ml of IL-15 was used, there was an increase in IFN-γ production from cells cultured in the absence of antigen. Moreover, PBMCs stimulated with tetanus toxoid or HIV− peptides in the presence of 100 ng/ml of IL-15 had a significant increase in IFN-γ production above that seen when cells were cultured with IL-15 in the absence of antigen. Interestingly, IL-15 at 100 ng/ml did not increase IFN-γ production by PHA-stimulated PBMCs (data not shown). It is possible that IL-15 at a "high" dose may cause IFN-γ production from previously activated T cells or NK

cells. Alternatively, this dose may be required in situations in which IL-2 production is submaximal, as would be the situation when PBMCs from HIV-infected individuals are stimulated with tetanus or HIV^- peptides. Additional evidence that high dose IL-15 can increase IFN-γ production is supported by data obtained in a murine model of T cell priming. The presence of IL-15 at 300 ng/ml caused an increase an IFN-γ production from highly purified naive murine $CD4^+$ T cells from TCR transgenic mice that had been primed in vitro and restimulated, supporting its role as a Th1-inducing cytokine (Seder, R., manuscript submitted for publication). To study the role that endogenous IL-15 plays in IFN-γ induction, we added a neutralizing antibody against IL-15 to PHA-stimulated PBMCs. There appeared to be a two- to threefold decrease in IFN-γ in cultures from HIV-infected patients, but less inhibition in cultures from the HIV^- controls (Table 4). This interesting result may reflect the capacity of various infectious agents to induce IL-15 in HIV-infected individuals. Recent work in the murine system has shown that LPS, mycobacteria, and various parasites were capable of eliciting mRNA for IL-15 from macrophages (Doherty, M., personal communication).

Previous studies have shown IL-12 to be a potent regulator of T cell differentiation toward an IFN-γ–dominated response (26–28). Clerici et al. demonstrated that IL-12 could "restore" a deficient proliferative response using PBMCs from HIV-infected individuals stimulated with an HIV-specific antigen (6). They also noted that exogenous IL-12 caused a significant increase in IFN-γ production from these patients, but not the HIV^- controls. In our studies, IL-12 also had several striking effects. First, IL-12 caused a 2–10-fold increase in IFN-γ from both PBMCs and highly enriched $CD4^+$ T cells of HIV^+ and HIV^- individuals in response to PHA or tetanus toxoid (Table 3). Moreover, IL-12 was able to elicit IFN-γ production in several instances in which there was no IFN-γ produced with antigen alone. The role that endogenous IL-12 and IL-2 play in IFN-γ production was assessed by using neutralizing antibody to IL-12 or anti-TAC, respectively (Tables 3 and 4). Cultures in which anti-TAC was added had essentially complete inhibition of IFN-γ production, while the presence of anti–IL-12 resulted in a two- to fivefold decrease in IFN-γ production. Interestingly, when IL-12 was added in the presence of anti-TAC, it could partially restore the response. These data underscore the critical need for at least some IL-2 to optimally generate IFN-γ production. As we noted above, neutralization of IL-15 also caused a decrease in IFN-γ production, especially in HIV-infected patients (Table 4). Since there is marked redundancy in the functions of cytokines, it is interesting to speculate that IL-15 could act as a compensatory mechanism to IL-2 in situations in which IL-2 is diminished, as could occur in HIV infection. We are currently studying whether IL-15 can restore IFN-γ production in cultures where IL-2 is neutralized.

IL-12 also showed significant effects on increasing IL-2 production from PHA-stimulated PBMCs. This effect was not as dramatic when highly enriched $CD4^+$ T cells were used as responders. Moreover, responses to tetanus were not significantly effected by IL-12. This brings up the question of whether IL-12 is having a more beneficial response on $CD8^+$ T cells to produce IL-2. Preliminary data using highly enriched $CD8^+$ T cells suggests that IL-12 is able to enhance IL-2 production from this cell population. Our data are similar to those obtained by Clerici et al. in their demonstration that IL-12 increased proliferation and IL-2 production in HIV-infected patients in response to PHA. Additionally, they showed exogenous IL-12 increased IL-2 production by PBMCs stimulated with HIV-specific peptides or recall antigen (6). It is possible that the low or absent response to tetanus toxoid in our experiments may reflect the delayed kinetics compared to the PHA response, since Clerici et al. measured IL-2 after 7 d rather than 4–5 d in which we harvest our supernatant from. In experiments to address this issue, we compared IL-2 production from supernatants after 5 and 7 d of culture in response to tetanus toxoid or HIV^- peptides. IL-2 production was not enhanced when IL-12 was added to PBMCs stimulated for 5 or 7 d with tetanus toxoid or HIV pooled peptides, whereas IL-12 did cause a two- to fivefold increase from PHA-stimulated cells (data not shown).

A final issue addressed in this paper was whether PBMC or purified $CD4^+$ T cells taken from HIV-infected individuals at various stages of their disease exhibited a specific Th1 or Th2 pattern of cytokine production. Our results from a relatively small number of patients do not demonstrate a distinctive pattern of cytokine production related to the stage of HIV disease. Moreover, we found that both HIV^+ and HIV^- individuals produced both IFN-γ and IL-4 (Table 5), and that the HIV^+ individuals produced as much if not more of these cytokines in response to PHA. These results appear to be consistent with recent data published by Graziosi et al. showing that anti-CD3 stimulated cells from patients at various stages of disease had mRNA for all of these cytokines (13). By contrast, a recent report by Maggie et al. showed a reduction of both IL-4 and IFN-γ from HIV^+ PBMCs in response to anti-CD3 plus PMA (12). It is possible that the patient populations or the type of stimulus could explain these results. Differences that might explain why our data are different from experiments showing a deviation to a specific T helper response may again result from differing patient populations or the specific way in which subgroups of patients who produce IL-4 are defined. In conclusion, although our data do not show a distinctive pattern of cytokine production, it does not preclude a role of distinct types of cytokine production occurring in a certain group of patients. To definitively address the issue, large numbers of patients could be monitored throughout the course of their disease to determine if and when these specific T phenotypes occur.

The goal of these studies is to examine the mechanism of cytokine interaction to devise treatment strategies for disease. Immune mechanisms that could be useful in treating HIV disease would be to augment cytolytic activity, enhance cellular immunity, or generate neutralizing antibody. As noted earlier, IL-2 has been shown to be a useful thera-

peutic agent in many instances although it has been associated with significant toxicity. Since IL-15 is similar to IL-2, in its ability to enhance cytolytic function of $CD8^+$ T and NK cells, it might provide therapeutic benefit in those situations in which IL-2 is effective. Moreover, if IL-15 is able to mimic the effects of IL-2 with less toxicity, it might be a useful treatment in diseases such as cancer or AIDS. Toxicity studies in mice have shown IL-15 to have a sixfold reduction in capillary leak and pulmonary edema compared to IL-2 (28a). IL-15 could be combined with other cytokines (i.e., IL-12) to augment immune function and minimize toxicity. We have preliminary data in both human and murine systems which suggest that IL-12 and IL-15 can have additive effects on proliferation. This is consistent with recent data by Carson et al. showing that IL-12 and IL-15 have a synergistic effect in augmenting NK cytolytic activity and IFN-γ production (9). In these studies, as in others, IL-12 appears to be a good candidate for a therapeutic application based on its ability to augment cytolytic activity and enhance a cell-mediated response. For example, if a cellular immune response characterized by production of IFN-γ and IL-2 is desirable, the rationale for using IL-12 as it relates to its effects on increasing IFN-γ from $CD4^+$ T cells could be twofold. The first would be to change a Th2 to Th1 response. The second reason would be to restore a "deficient" cytokine response or enhance a relatively normal response above a critical threshold that would be needed to protect the host. In examining the first rationale, even if distinctive Th1 or Th2 patterns exist, there is little evidence that those $CD4^+$ T cells that are already immunologically committed can be functionally altered by cytokines (28). Moreover, in situations in which $CD4^+$ T cells have differentiated into a Th2 phenotype, IL-12, through its ability to act as a T cell growth factor, could enhance this response (29, 30). It is more likely that IL-12 would exert an influence toward a Th1 response on a naive T cell that had not yet encountered antigen or a cell that has been activated by antigen but is not fully committed, such as a Th0 cell. Thus, if cytokine therapy is to be useful, it might best be used in patients who have a relatively intact T cell repertoire containing cells that have not entirely committed to a fully differentiated Th2 phenotype, as well as a sufficient number of naive $CD4^+$ T cells to influence. The rationale to support the use of IL-12 to restore a deficient response is supported by the work of Chemini et al., who demonstrated impaired IL-12 production from PBMCs of HIV-infected patients (5). With regard to enhancing an existing response above a critical threshold, it has been demonstrated that IL-12 treatment protected mice infected with *Histoplasma capsulatum* protects from lethal infection by enhancing an already existing response characterized by endogenous production of IL-12 and IFN-γ (31).

In conclusion, the ability of IL-12 to enhance CD8 T cell and NK cytolytic activity, as well as the properties discussed above, make it an interesting candidate for treatment of HIV infection in combination with antiretroviral therapy. Moreover, in cases where IL-2 production is limiting because of a qualitative or quantitative T cell defect, combination therapy with low (nontoxic) doses of IL-2 or possibly IL-15 might enhance production of IFN-γ. One final caveat is that while IL-12 treatment has been shown in several animal models to be important in regulating infections caused by intracellular microorganisms, its potential effect on HIV viral replication must be considered as well.

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