

Thalidomide Stimulates T Cell Responses and Interleukin 12 Production in HIV-Infected Patients*

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ABSTRACT

We performed a placebo-controlled study to evaluate the effects of immunomodulatory treatment with thalidomide on HIV levels, TNF- α levels, and immune status of 31 HIV-infected individuals, after temporary suppression of viral replication with antiretroviral drugs. Treatment with a combination of zidovudine and lamivudine (ZDV/LMV) for 14 days resulted in a median decline in plasma viremia of 1.94 log₁₀ RNA equivalents/ml. After discontinuation of ZDV/LMV, thalidomide therapy (200 mg/day for 4 weeks) did not retard the prompt return of HIV titers to the pretreatment levels, and had no effect on plasma levels of TNF- α . In contrast, thalidomide treatment resulted in significant immune stimulation. We observed increased levels of plasma soluble IL-2 receptor, soluble CD8 antigen, and IL-12 ($p < 0.01$ for all parameters), as well as increased cutaneous delayed-type hypersensitivity reactions to recall antigens ($p < 0.01$) in thalidomide-treated patients. These changes were associated with a median increase in HIV titer of 0.2 log₁₀ RNA equivalents/ml in the thalidomide-treated group ($p < 0.05$), which resolved after stopping the drug. Further studies were performed *in vitro* to elucidate the mechanism of thalidomide-induced immune stimulation. When purified T cells from HIV-infected individuals were stimulated by immobilized anti-CD3 in the presence of thalidomide, a costimulatory effect of the drug was observed, resulting in increased production of IL-2 and IFN- γ , and increased T cell-proliferative responses. Further experiments showed that thalidomide increased IL-12 production by antigen-presenting cells in a T cell-dependent manner. Our findings suggest a potential application for thalidomide as a novel immune adjuvant in HIV disease.

INTRODUCTION

IN HIV-INFECTED INDIVIDUALS, viral burden is regulated by host factors that may promote or suppress viral turnover. Antiretroviral treatment of these patients directly suppresses viral replication and reduces plasma levels of virus. However, if treatment is discontinued, there is a prompt return of the plasma viral titer to the pretreatment level for a given HIV-infected individual.¹ This quasistable viral "set point" may be considered to reflect the net regulatory effect of the host environment on

viral replication. Immune activation caused by the administration of recombinant interleukin 2 (IL-2),² immunization^{3,4} or concomitant infection⁵ can cause increases in viral load in the blood of infected patients. Similarly, *in vitro*, the stimulation of HIV-infected CD4⁺ T cells with antigens, mitogens, or cytokines such as IL-2 stimulates viral replication⁶ and increases the susceptibility of CD4⁺ T cells to HIV infection.⁷ Viral production by HIV-infected macrophages can be increased by exposure to stimuli such as lipopolysaccharide (LPS) or proinflammatory cytokines such as tumor necrosis factor α

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(TNF- α).^{8,9} These observations suggest that modulation of certain host immune responses might modify viral levels in patients.

Since there is a correlation between levels of circulating HIV and TNF- α ¹⁰ or TNF- α receptor¹¹ in the plasma of infected patients, and since this cytokine induces viral replication via a shared NF- κ B-dependent transcriptional control mechanism,⁹ inhibition of TNF- α may reduce viral replication, resulting in a lowering of the viral set point. We have shown that the immunomodulatory drug thalidomide can inhibit the production of TNF- α *in vitro*¹² and *in vivo*,¹³ and can inhibit HIV replication in cultures of infected monocytes and peripheral blood mononuclear cells (PBMCs).^{14,15} These observations provided the rationale for exploring the potential of thalidomide to inhibit TNF- α , and thereby reduce viral replication in HIV-infected patients.

More recently, studies in our laboratory have revealed an additional and unexpected property of thalidomide in acting as a costimulator of T cells *in vitro*. Thalidomide treatment of T cells simultaneously receiving a primary stimulus through the T cell receptor results in enhancement of IL-2 and interferon γ (IFN- γ) production.¹⁶ Thus, *in vitro*, thalidomide has at least two immune-modulatory properties: inhibition of monocyte TNF- α production and T cell costimulation.

In the present study we examined whether thalidomide treatment of HIV-infected individuals reduced TNF- α and HIV levels. Previous studies have not shown a consistent effect of thalidomide treatment in reducing HIV levels.^{10,17} Therefore, we designed a different approach in which HIV replication was initially suppressed with a 14-day course of antiretroviral drugs, following which patients were randomly allocated to receive treatment with thalidomide or placebo for 4 weeks. Thus, the clinical trial was designed primarily to ask whether inhibition of TNF- α with thalidomide treatment could reduce the set point to which plasma viral levels would return after cessation of antiretroviral therapy.

Subsequently, in light of our recent findings *in vitro*, we also investigated the effects of thalidomide on parameters of immune activation in stored plasma samples from these patients. Finally, to extend our findings in the patient study, we investigated the effects of the drug on PBMCs and purified T cells from HIV-infected subjects stimulated *ex vivo* with immobilized anti-CD3 antibodies.

MATERIALS AND METHODS

Clinical study

Study subjects. Clinically stable HIV-infected male volunteers who were antiretroviral drug naive were recruited from an outpatient population in Chiang-Mai, Thailand. Patients were free of active opportunistic infections and had Karnofsky scores of at least 70. These studies were approved by the ethics committees of Chiang Mai University and the Rockefeller University. All patients gave written informed consent to participate.

Study design. After clinical evaluation, all patients were treated with an oral regimen of the antiretroviral drugs zidovudine (250 mg) and lamivudine (150 mg) twice daily (ZDV/LMV) (purchased from Glaxo-Wellcome, Research Tri-

angle Park, NC) from day 0 to day 14 of study. On study day 12 patients were randomly allocated to receive study drug: either thalidomide (Celgene, Warren, NJ), 200 mg by mouth once daily, or an identical placebo. A greater number of dropouts in the thalidomide-treated arm of the study was anticipated, because of transient drug-induced rashes that may occur in up to one-third of HIV-infected patients treated with thalidomide, as reported previously.^{10,18} Therefore the randomization was unbalanced, with a ratio of thalidomide to placebo recipients of 3:2. There was a 2-day overlap between the end of the course of antiretroviral therapy and the start of the study drug treatment phase to achieve maximal serum thalidomide levels before withdrawing antiretroviral drugs.¹⁹ Study subjects then continued on study drug for 28 days beyond the withdrawal of antiretroviral therapy (until study day 42), followed by a 2-week "washout" phase (no drugs), with final evaluation on study day 56.

Thalidomide and placebo were kindly provided by Celgene.

Virologic measurements. Blood samples were collected at the following time points: 2 weeks before starting the study (screening), and subsequently on study days 0 (baseline), 12 (randomization), 28, 42, and 56. Plasma was anticoagulated with EDTA and stored at -70°C until batch assay at the end of the study. HIV titers were measured by branched-chain DNA (bdNA) signal amplification (Chiron, Emeryville, CA). For the purpose of data analysis, undetectable virus was defined as equal to the lower limit of detection of the assay (500 HIV RNA equivalents/ml). Data from the first two assays for each individual were averaged to give the (geometric) mean baseline titer. Viral titers were expressed as \log_{10} RNA units per milliliter. Viral RNA from plasma collected on day 56 was evaluated with the line probe assay (Innogenetics, Ghent, Belgium) as described,²⁰ in accordance with the manufacturer instructions. This assay detects drug-resistance mutations in the HIV reverse transcriptase gene commonly induced by nucleoside analog therapy, including the codon 184 mutation, which causes a switch from methionine to valine (Val¹⁸⁴) and confers lamivudine resistance.

Immunologic assessments. Blood samples collected in the same way and at the same time points as described above were used for these studies. The following cytokines and soluble markers of T cell activation were measured in plasma by enzyme-linked immunosorbent assay (ELISA), in accordance with each manufacturer's instructions: TNF- α (Medgenix, Fleurus, Belgium; this assay measures both free and receptor-complexed TNF- α); IFN- γ (Genzyme, Cambridge, MA); interleukin 12 (IL-12) (Endogen, Woburn, MA; this assay measures both p40 and p70 subunits of IL-12); soluble interleukin 2 receptor (sIL-2R) (Genzyme); soluble CD4 antigen (sCD4) and soluble CD8 antigen (sCD8) (T Cell Diagnostics, Woburn, MA). The two initial values for each parameter were averaged to provide a mean starting value for the analysis.

T lymphocyte subsets (CD3⁺CD4⁺ and CD3⁺CD8⁺) were enumerated by flow cytometry (FACScalibur; Becton Dickinson, San Jose, CA), at baseline (day 0) and on study days 12 and 42.

Skin tests for delayed-type hypersensitivity (DTH) were conducted on study days 0, 42, and 56. Purified protein derivative of tuberculin (PPD), 5 tuberculin units (Connaught Laboratories, Willowdale, Ontario, Canada); *Candida* antigen, 0.1 ml of

undiluted reagent (ALK Laboratories, Berkely, CA); and *Tri-chophyton*, 0.1 ml of undiluted reagent (ALK Laboratories), were injected intradermally into the volar aspect of the forearms, and induration in two perpendicular diameters was measured 48 hr later. A positive response was defined as a mean diameter of induration greater than or equal to 5 mm.

Clinical evaluations. A full clinical evaluation, including medical history and physical examination, was performed at study baseline. Subsequently, patients were evaluated at weekly intervals while receiving study medications. Routine clinical investigations included chest radiography at baseline, and measurement of serum chemistries and complete blood counts on days 0, 12, 28, 42, and 56.

In vitro study

Study subjects. For *in vitro* studies, blood was drawn from clinically stable HIV-infected patients recruited through the outpatient clinic of the Rockefeller University Hospital.

Preparation of cells. PBMCs were freshly isolated from whole blood by Ficoll density gradient separation. CD4⁺ and CD8⁺ T cell subsets were purified by positive selection with magnetic beads coated with the appropriate monoclonal antibodies, in accordance with the manufacturer instructions (Dyna AS, Oslo, Norway). Selected cells were subsequently separated from the beads by incubation with a second antibody that competes for the binding site on the primary antibody (Detachabead; Dynal AS). This procedure routinely recovered cells that were >99% pure CD3⁺CD4⁺ or CD3⁺CD8⁺. Cells were suspended in RPMI medium (GIBCO, Grand Island, NY) supplemented with 10% pooled human AB serum and penicillin (50 U/ml)–streptomycin (0.05 µg/ml). Cultures were set up in a humidified 37°C incubator with a 5% CO₂ atmosphere.

Stimulation assays. T cells were stimulated by cross-linking CD3, a component of the T cell receptor (TCR) complex. Forty-eight- or 96-well flat-bottomed tissue culture plates were coated with mouse anti-human CD3 monoclonal antibody (a kind gift of R. Zivin, Ortho Biotech, Raritan, NJ) at a coating concentration of 10 µg of IgG/ml as previously described.¹⁶ For each experimental condition, duplicate cultures were set up at 10⁶ cells (either purified T cells or PBMCs) in 1 ml/well in the 48-well plates, and triplicates of 10⁵ cells in 0.2 ml/well in the 96-well plates. In further experiments, PBMCs in 48-well plates were stimulated with LPS obtained from *Salmonella minnesota* R595 (List Biological Laboratories, Campbell, CA) at a working concentration of 10 ng/ml, or baculovirus-derived recombinant human CD40 ligand–CD8 fusion protein at a working concentration of 1% of baculovirus culture supernatant (a kind gift of R.J. Noelle, Dartmouth Medical School, NH). Supernatant culture medium from the 48-well plates was harvested at the time points indicated and stored at –70°C until assayed by ELISA for IL-2, IFN-γ, IL-12 (p40 and p70) (Endogen), and IL-12 (p70 subunit only) (R&D Systems, Minneapolis, MN).

To estimate lymphocyte proliferation, DNA synthesis was assayed in the 96-well plates by measuring the incorporation of [³H]thymidine over the last 12 hr of 120-hr cultures as described previously.¹⁶ Proliferation data are expressed as “stimulation ratios” of thalidomide-treated cultures relative to dimethyl sulfoxide, (DMSO)-treated controls (cpm_{thalidomide}/cpm_{control}).

In some experiments, CD40 ligand (CD40L) on T cells was

blocked with mouse monoclonal anti-human CD40L IgG (clone TRAP1; PharMingen, San Diego, CA). Surface expression of CD40L was assessed on CD3⁺CD8⁺ and CD3⁺CD4⁺ lymphocyte subsets by three-color flow cytometry (anti-CD40L, PharMingen; anti-CD3, anti-CD4, and anti-CD8, Becton Dickinson).

Thalidomide. Thalidomide (α-phthalimido-glutarimide) (Celgene) was dissolved in DMSO to achieve stock concentrations of 20 mg/ml. Thalidomide at various concentrations or DMSO (control) was added at culture initiation so that the concentration of DMSO was identical under all culture conditions. Culture media were refreshed daily with 50% volume exchanges containing fresh thalidomide/DMSO or medium/DMSO.

Statistical analysis. The primary end point of the clinical trial was a reduction in plasma HIV titer in the group of patients on thalidomide treatment. For plasma virologic and immunologic parameters in the clinical study, changes from baseline were calculated for each patient at each study time point. SPSS (Chicago, IL) computer software was used for the analysis. Because of the small sample size, nonparametric methods were employed to describe and display the clinical data, and to test for significant differences. For display, box plots show the median, 25th, and 75th percentile values. Significance was set at a *p* value of ≤0.05. χ^2 analysis was used for comparing proportions, and simple linear regression analysis for evaluating relationships between continuous variables.

RESULTS

Clinical study

Tolerability of study drugs

ZDV/LMV: Thirty-six patients started treatment with ZDV/LMV. Five of these dropped out of the study before or at day 12 for the following reasons: opportunistic infection with *Penicillium marneffei* (*n* = 2, on days 6 and 12, respectively), acute respiratory tract infection (*n* = 1, on day 12), drug rash (*n* = 1, on day 7), nausea and vomiting (*n* = 1, on day 7). The data from these patients are not included in the analysis.

Thalidomide/placebo: The 31 patients who completed 12 days of antiretroviral treatment were randomized to receive thalidomide (*n* = 18) or placebo (*n* = 13). All subjects completed at least 2 weeks on thalidomide or placebo (up to study day 28). Five patients in the thalidomide treatment arm subsequently discontinued study drug: four because of skin rash, and one lost to follow-up. Data for these individuals are included up to the time of study drug discontinuation. Study drugs were otherwise well tolerated. In particular, no signs or symptoms of peripheral neuropathy, a known toxicity of thalidomide treatment, were detected.

Randomization. Study subjects subsequently randomized to thalidomide or placebo were well matched on study day 0 for demographic, clinical, and virologic characteristics (Table 1). Satisfactory matching was ascertained on study day 12 for immunologic parameters, since that was the day of randomization (Table 1). The analysis of individual changes from study day 0 for virologic and immunologic parameters also revealed satisfactory matching of the treatment groups at the time of ran-

TABLE 1. STUDY RANDOMIZATION DATA^a

Parameter (unit)	Placebo	Thalidomide	p value ^b
Number of patients	13	18	
Age (years)	32 (29–34)	31 (28–36)	0.96
Weight (lb)	117 (100–129)	117 (107–135)	0.38
Karnofsky score	100 (90–100)	100 (80–100)	0.54
HIV (log ₁₀ RNA, U/ml)	5.03 (4.56–5.33)	4.76 (4.47–4.96)	0.26
CD4 ⁺ T cell count (cells/ μ l)	195 (107–269)	156 (61–527)	0.72
CD8 ⁺ T cell count (cells/ μ l)	983 (716–1228)	1148 (743–1390)	0.79
TNF- α (pg/ml)	42.3 (13.4–49.8)	33.7 (23.6–49.9)	0.65
sIL-2R (pg/ml)	2298 (1722–3667)	2740 (2111–4324)	0.47
sCD8 (U/ml)	609 (478–635)	695 (494–891)	0.57
IL-12 (pg/ml)	199 (142–236)	295 (193–442)	0.16

^aClinical and virologic data are shown for study baseline. Immunologic data (CD4⁺ and CD8⁺ T cell counts, plasma TNF- α , sIL-2R, sCD8, and IL-12) are shown for study day 12, when the patients were randomized to thalidomide or placebo. Data are expressed as median values (interquartile range).

^bData for treatment groups are compared by Mann–Whitney *U* test.

domization (Figs. 1 and 2: day 12), indicating that the effect of ZDV/LMV on these parameters was evenly distributed.

Effect of ZDV/LMV and thalidomide on plasma HIV levels and LMV resistance mutation. At baseline, the overall median plasma HIV titer was 4.89 (4.53–5.15) log₁₀ RNA equivalents/ml (Table 2). After 12 days of ZDV/LMV, 19 of 31 subjects (61%) had plasma HIV titers less than 500 copies/ml, with a median decline in viremia of 1.94 log₁₀ RNA equivalents/ml (Table 2). After randomization to study drug and withdrawal

of antiviral treatment, patients in both treatment groups exhibited a similar return to baseline viral titers by study day 28 (i.e., 14 days after stopping antiviral drugs) (Fig. 1). However, by study day 42 (after 28 days of thalidomide treatment), HIV titers were increased by a median of 0.20 (0.10–0.37) log₁₀ RNA equivalents/ml over baseline values, whereas no such increase was observed in the placebo group ($p < 0.05$, Fig. 1). After withdrawal of study medications, viral titers returned to baseline by day 56, with a remarkable overall correlation between the baseline and end of study titers ($R = 0.95$, $p < 5 \times 10^{-12}$), supporting the notion of a robust viral set point in HIV disease. Thus, at the completion of the study, the viral load status of the patients was unchanged from the time of recruitment.

To test whether the 14-day course of ZDV/LMV treatment had induced genotypic LMV resistance, the line probe assay to detect drug resistance mutations in the HIV reverse transcriptase gene was performed on plasma samples of the 24 study subjects (11 treated with placebo, 13 with thalidomide) who completed the final, day 56 assessment. HIV cDNA was recovered from 20 of the 24 patient plasma samples, and the Val¹⁸⁴ mutation detected in one sample. Assay of the pretreatment specimen from this individual, who was subsequently randomized to receive thalidomide, revealed that the mutation was not present at study baseline.

Effect of ZDV/LMV and thalidomide on plasma immunologic markers. Plasma levels of TNF- α were not remarkably elevated at the start of the study (Table 2). In the whole group, there was a slight, but significant ($p < 0.01$) decline in circulating TNF- α levels in response to ZDV/LMV (Table 2). After withdrawal of ZDV/LMV and randomization to thalidomide or placebo, TNF- α levels returned to baseline levels by day 28. There was no difference in the changes in TNF- α levels throughout the study between the thalidomide- and placebo-treated patients (Fig. 2A).

Plasma levels of sIL-2R were not significantly affected by ZDV/LMV (Table 2), while levels of sCD8 were slightly, but consistently ($p < 0.05$) reduced by the antiretroviral treatment (Table 2). The effect of thalidomide on these parameters was striking, however: sIL-2R and sCD8 levels in plasma increased significantly by day 28 in the thalidomide treatment group com-

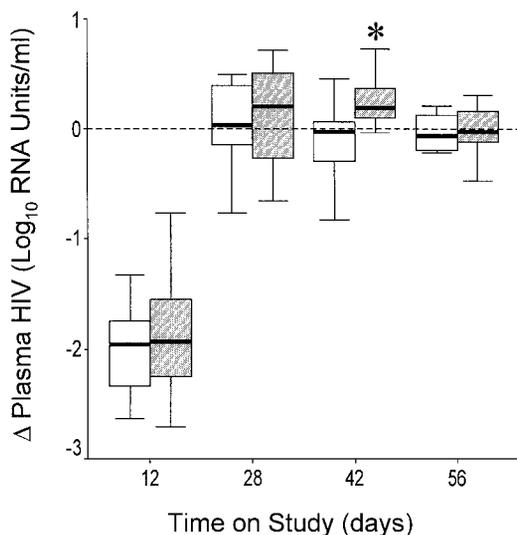


FIG. 1. Box plot displaying the effect of thalidomide and placebo on changes in plasma HIV levels from study day 0. HIV titers were measured by bDNA assay. All patients were treated with ZDV/LMV from study days 0 to 14. From study days 12 to 42, patients were randomly assigned to receive either thalidomide at 200 mg/day, or an identical placebo. Shaded boxes: patients treated with thalidomide; open boxes: patients treated with placebo. Boxes represent the 25th and 75th percentiles, with the median bar between. * p value < 0.05 when values for the treatment groups were compared by Mann–Whitney *U* test.

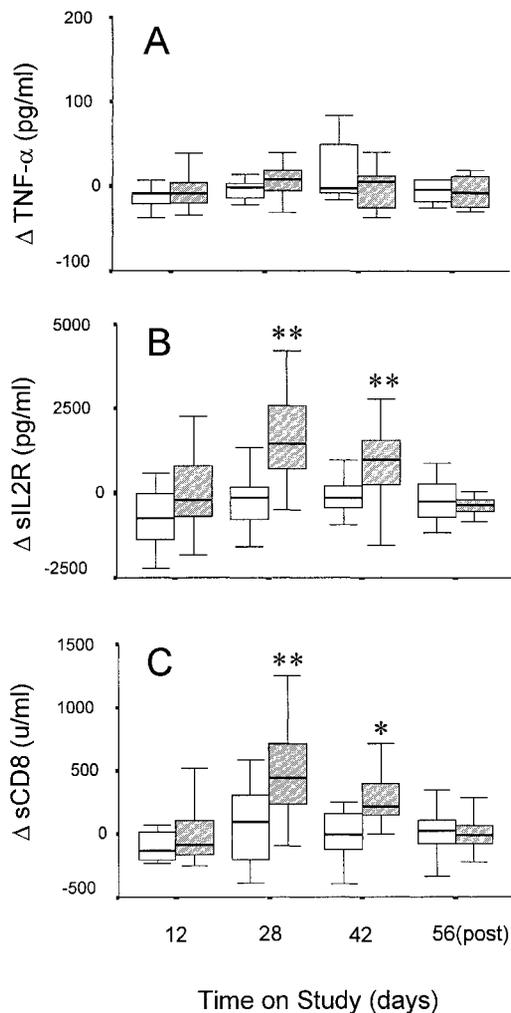


FIG. 2. Box plots displaying the effect of thalidomide and placebo on changes in plasma immunologic markers from study day 0. (A) Plasma TNF- α levels. (B) Plasma soluble sIL-2R levels. (C) Plasma sCD8 levels. All patients were treated with ZDV/LMV from study days 0 to 14. From study days 12 to 42, patients were randomly assigned to receive either thalidomide at 200 mg/day, or an identical placebo. Shaded boxes: patients treated with thalidomide; open boxes: patients treated with placebo. Boxes represent the 25th and 75th percentiles, with the median bar between. * p value < 0.05; ** p value < 0.01, when values for the treatment groups were compared by Mann-Whitney U test.

pared with the placebo group, and remained elevated until withdrawal of thalidomide on day 42 (Fig. 2B and C). Subsequently, sIL-2R and sCD8 levels returned to baseline levels (Fig. 2B and C). Levels of plasma sCD4 did not change in response to ZDV/LMV or thalidomide treatment (data not shown). IFN- γ was undetectable in the plasma of the majority of subjects and remained extremely low throughout the study (maximum level, 1.44 pg/ml), with no measurable effect due to thalidomide treatment (data not shown).

A modest decline in circulating IL-12 was observed in the whole group in response to antiretroviral therapy, almost achieving significance ($p = 0.06$) (Table 2). After randomiza-

tion, thalidomide treatment induced a highly consistent increase in circulating levels of IL-12, while in general, no change occurred in placebo recipients (Fig. 3, $p < 0.01$ on study days 28 and 42). Figure 3 shows an outlying observation from a single placebo recipient who exhibited an unexplained large increase in plasma IL-12 in the absence of any associated clinical findings. In general, plasma IL-12 levels peaked on day 42, subsequent to the day 28 thalidomide-induced peaks in the T cell activation markers sIL-2R and sCD8. This temporal relationship suggested that the increase in IL-12 was secondary to T cell activation. There was a prompt return of IL-12 to baseline levels after cessation of thalidomide treatment (not shown).

Thus, of the soluble immunologic parameters assayed, short-term antiretroviral therapy caused modest but consistent declines in TNF- α , sCD8, and IL-12. In contrast, subsequent treatment with thalidomide induced marked increases in sCD8, sIL-2R, and IL-12 levels, while having no effect on TNF- α or sCD4 levels.

Delayed-type hypersensitivity. The results of DTH skin testing are summarized in Table 3. At baseline, only 1 of a possible maximum of 36 positive tests (2.8%) was observed in the group subsequently randomized to placebo. Baseline results for the thalidomide group were 3 of 45 (6.7%) positive skin tests. After study drug treatment, there was a significant increase in skin test responses in the thalidomide treatment group, with 10 of 39 (25.6%) positives compared with 1 of 33 (3.0%) in the placebo recipients ($p < 0.01$). One individual exhibited a positive response to PPD at baseline and then had a blistering response after 4 weeks (study day 42) of thalidomide treatment, and so was not subsequently retested with this antigen. However, for the purpose of analysis, this individual was assumed to have a positive PPD response on day 56. At the final time point, after withdrawal of study drug (day 56), the proportions of responders were equivalent in the thalidomide and placebo groups (17.9 versus 15.4%, respectively, $p = 0.83$). Of note, at this time point all six of the responses in the placebo group and five of seven of the responses in the thalidomide group were to *Candida* antigen, suggesting possible sensitization by serial testing with this antigen.

T cell subsets. For the whole cohort completing 12 days of antiretroviral therapy, the median absolute peripheral blood CD4⁺ T cell counts increased modestly [from 84 (48–331) cells/ μ l on day 0 to 183 (65–325) cells/ μ l on day 12, $p = 0.02$], while CD8⁺ T cell counts did not change significantly (Table 2). There were no significant differences in these parameters between the groups randomized to placebo or thalidomide (Table 1). After withdrawal of antiretroviral therapy and treatment with thalidomide or placebo, CD4⁺ T cell counts returned to baseline levels and thalidomide treatment had no significant effect on either CD4⁺ or CD8⁺ T cell counts (data not shown).

The results from this patient study suggested that thalidomide exerted an immunostimulatory effect *in vivo* in this cohort of HIV-infected patients, reflected not only in increases in soluble immunologic markers, but also in the increased DTH responses.

In vitro experiments

Lymphocyte proliferation assays. To investigate the cellular mechanisms underlying the immune stimulation observed in

TABLE 2. EFFECT OF 12 DAYS OF ZDV/LMV THERAPY ON VIROLOGIC AND IMMUNOLOGIC PARAMETERS IN THE 31 PATIENTS SUBSEQUENTLY RANDOMIZED TO RECEIVE THALIDOMIDE OR PLACEBO^a

Parameter (unit)	Day 0	Day 12	p value ^b
HIV (log ₁₀ RNA, U/ml)	4.89 (4.53–5.15)	2.69 (2.69–2.86)	<0.0001
CD4 ⁺ T cell count (cells/ μ l)	84 (48–331)	183 (65–325)	0.02
CD8 ⁺ T cell count (cells/ μ l)	947 (765–1619)	1045 (714–1390)	0.57
TNF- α (pg/ml)	45.3 (37.3–60.1)	34.7 (21.1–50.3)	0.01
sIL-2R (pg/ml)	3212 (2572–3799)	2408 (1745–3992)	0.23
sCD8 (U/ml)	654 (592–839)	631 (483–846)	0.03
IL-12 (pg/ml)	298 (228–415)	236 (172–389)	0.06

^aData are expressed as median values (interquartile range).

^bPaired data for days 0 and 12 are compared by Wilcoxon signed rank sum test.

HIV-infected patients treated with thalidomide, we studied the *ex vivo* stimulatory effects of thalidomide on T cell and PBMC responses from HIV-infected patients. Highly purified CD4⁺ and CD8⁺ T cells from HIV-infected individuals were stimulated by cross-linking the TCR with immobilized anti-CD3 monoclonal antibodies (MAbs) in the presence of varying concentrations of thalidomide. Figure 4A shows the mean data from five experiments. Thalidomide induced a concentration-dependent increase in proliferation of CD4⁺ and CD8⁺ T cells. For some individuals, the thalidomide-induced augmentation of CD4⁺ T cell proliferative responses was identical to that of the CD8⁺ T cells, but on average, the CD8⁺ response was greater. In contrast, thalidomide caused modest increases in proliferation of anti-CD3-stimulated, unsorted PBMCs (Fig. 4A).

Cytokine production. We next examined the effects of thalidomide on cytokine production by T cells and PBMCs from

HIV-infected donors. Thalidomide consistently induced concentration-dependent increases in IL-2 and IFN- γ production by PBMCs and by purified CD4⁺ and CD8⁺ T cells stimulated with immobilized anti-CD3 antibodies. Figure 4B and C shows the mean results of five experiments. In contrast to the proliferative responses, the proportional increases in IL-2 and IFN- γ production induced by thalidomide were similar in PBMCs and purified T cells. These findings indicated that thalidomide can costimulate primary T cells from HIV-infected individuals *in vitro*.

As shown above, in thalidomide-treated HIV-infected patients, we observed increases in plasma IL-12, a cytokine that is produced by antigen-presenting cells (APCs) and not by T cells.²¹ We hypothesized that the drug-induced increase in IL-12 levels observed *in vivo* was secondary to T cell costimulation by thalidomide. Therefore, to study the effect of thalidomide on IL-12 production *in vitro*, in a system where the primary stimulus is delivered to the T cell, we stimulated PBMCs from HIV-infected donors with immobilized anti-CD3. The production of IL-12 and IFN- γ , as well as the expression of CD40L on T cells, were evaluated by ELISA and flow cytometry, respectively. Preliminary experiments revealed that maximum levels of IL-12, IFN- γ , and CD40L expression occurred 48 hr after stimulation with anti-CD3. Thalidomide-induced increases in IL-12 (p40 and p70) production were observed at 48 hr in seven of nine subjects. There was a significant positive correlation between changes in IL-12 secretion and increases in IFN- γ production in response to thalidomide at 1 μ g/ml ($R = 0.83$, $p = 0.006$) (Fig. 5) and at 10 μ g/ml ($R = 0.74$, $p = 0.02$) (data not shown). The two subjects who did not show increased IL-12 secretion in response to thalidomide were those with the smallest increments in IFN- γ secretion (Fig. 5). Interestingly, the latter subjects were two of the three female donors in the group.

In the same experiments, we observed modest thalidomide-induced increases in CD40L expression on T cells, which were significant only when total CD3⁺ or CD3⁺CD4⁺ T cell responses to 10 μ g of thalidomide per milliliter were analyzed (Fig. 6A). There was therefore an association between thalidomide-induced increases in T cell activation (indicated by increased IFN- γ production and CD40L expression) and IL-12 production. No IL-12 was detected in thalidomide-treated or control cultures in the absence of anti-CD3 (data not shown).

We next studied the effects of antibody blockade of CD40L on the production of IL-12 by PBMCs from HIV-infected sub-

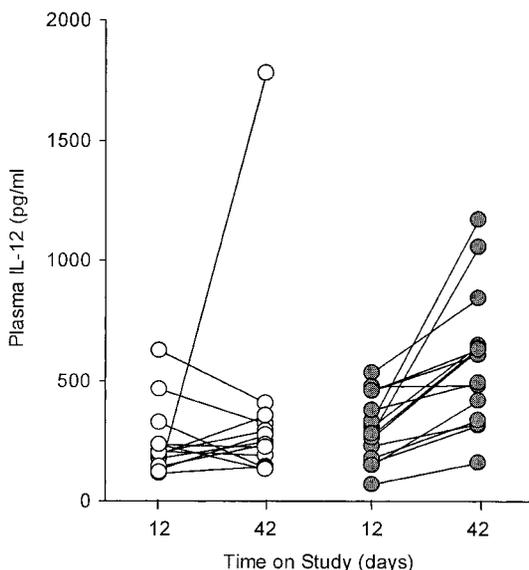


FIG. 3. Individual plasma IL-12 levels on study day 12 (day of randomization to thalidomide or placebo) and day 42 (end of study treatment). Open symbols: patients treated with placebo; shaded symbols: patients treated with thalidomide. The increases in plasma levels of IL-12 were maximal on day 42 in thalidomide-treated patients ($p < 0.01$ compared with the placebo group). Levels of IL-12 had returned to baseline within 14 days of stopping thalidomide (study day 56, data not shown).

TABLE 3. PATIENT DELAYED-TYPE HYPERSENSITIVITY RESPONSES

Treatment assignment	Skin test antigen	Total number of tests ≥ 5 mm in diameter		
		Day 0	Day 42	Day 56
Placebo	PPD	0	0	0
	<i>Trichophyton</i>	0	0	0
	<i>Candida</i>	1	1	6
		(n = 12) ^a	(n = 11)	(n = 10)
Thalidomide	PPD	1	4	2
	<i>Trichophyton</i>	0	1	0
	<i>Candida</i>	2	5	5
		(n = 15)	(n = 13)	(n = 13)
	Response index ^b	3/45	10/39	7/39
	p Value ^c	0.42	0.0079	0.83

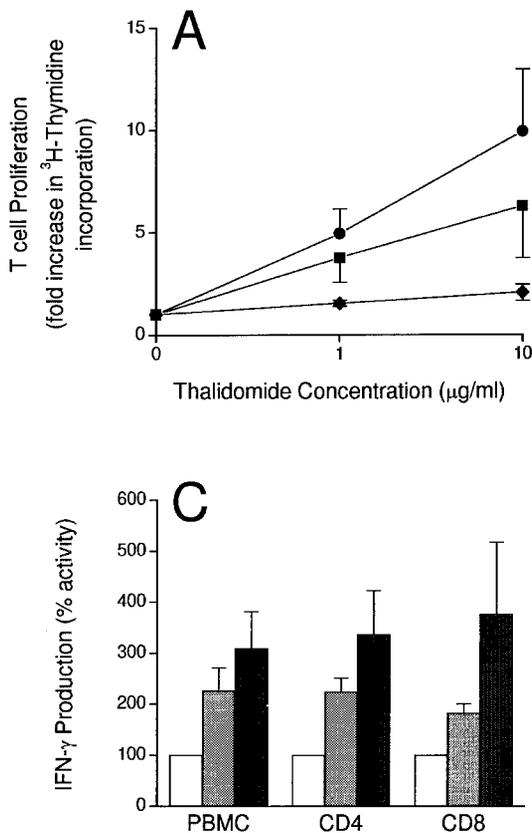
^an, Number of patients in group.

^bResponse index, number of positive tests per total number of possible positive tests (read at 48 hr).

^cResponse indices at each time point for thalidomide and placebo treatment groups, compared by χ^2 analysis.

jects treated *ex vivo* with thalidomide (Fig. 6B and C). Blockade of the CD40L-CD40 pathway resulted in inhibition of IL-12 production, and abrogated thalidomide-induced increases of this cytokine. Similar results were observed whether we measured total IL-12 (p40 and p70) (Fig. 6B), or the biologically active IL-12 (p70) heterodimeric form only (Fig. 6C).

Finally, to exclude the possibility that thalidomide was increasing IL-12 production by increasing the response of APCs to CD40L, PBMCs were stimulated with a CD40 ligand-CD8 fusion protein, thereby mimicking this T cell stimulus for IL-12 production, but in the absence of T cell activation. In addition, PBMCs were stimulated with LPS, a known T cell-inde-



PBMCs, 100.7 (± 42) pg/ml; CD4⁺ T cells, 45 (± 13.6) pg/ml; CD8⁺ T cells, 37 (± 4.7) pg/ml. (C) IFN- γ secreted into culture supernatants assayed at 72 hr. Data are expressed as the increase relative to DMSO control (normalized to 100%). Key as in (B). Mean (\pm SEM) IFN- γ in control cultures: PBMCs, 660.3 (± 314) pg/ml; CD4⁺ T cells, 125.0 (± 49.5) pg/ml; CD8⁺ T cells, 239.2 (± 71.6) pg/ml. All data represent means of five independent experiments.

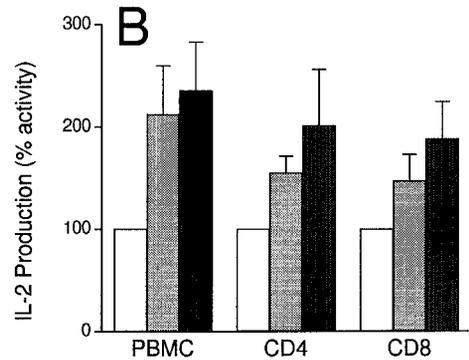


FIG. 4. Responses to thalidomide (1 and 10 μ g/ml) of PBMCs and purified CD4⁺ and CD8⁺ T cells from HIV-infected subjects stimulated *in vitro* with immobilized anti-CD3 antibody. (A) Proliferative responses after 5 days in culture. On day 5, 10⁵ cells/well in triplicate cultures were pulsed with 1 μ Ci of [³H]thymidine for 6 hr, and incorporation of [³H]thymidine into DNA was measured by counting β emission. Data are expressed as the ratio of counts per minute (cpm) of thalidomide-treated cultures to DMSO (control)-treated cultures. Circles: CD8⁺ T cells; squares: CD4⁺ T cells; diamonds: PBMCs. Mean (\pm SEM) cpm in control cultures: PBMCs, 98,104 ($\pm 29,019$); CD4⁺ T cells, 49,560 ($\pm 16,476$); CD8⁺ T cells, 3737 (± 826). (B) IL-2 secreted into culture supernatants assayed at 24 hr. Data are expressed as the increase relative to DMSO control (normalized to 100%). Open columns, DMSO control; gray columns, thalidomide, 1 μ g/ml; solid columns, thalidomide, 10 μ g/ml. Mean (\pm SEM) IL-2 in control cultures: PBMCs, 100.7 (± 42) pg/ml; CD4⁺ T cells, 45 (± 13.6) pg/ml; CD8⁺ T cells, 37 (± 4.7) pg/ml. (C) IFN- γ secreted into culture supernatants assayed at 72 hr. Data are expressed as the increase relative to DMSO control (normalized to 100%). Key as in (B). Mean (\pm SEM) IFN- γ in control cultures: PBMCs, 660.3 (± 314) pg/ml; CD4⁺ T cells, 125.0 (± 49.5) pg/ml; CD8⁺ T cells, 239.2 (± 71.6) pg/ml. All data represent means of five independent experiments.

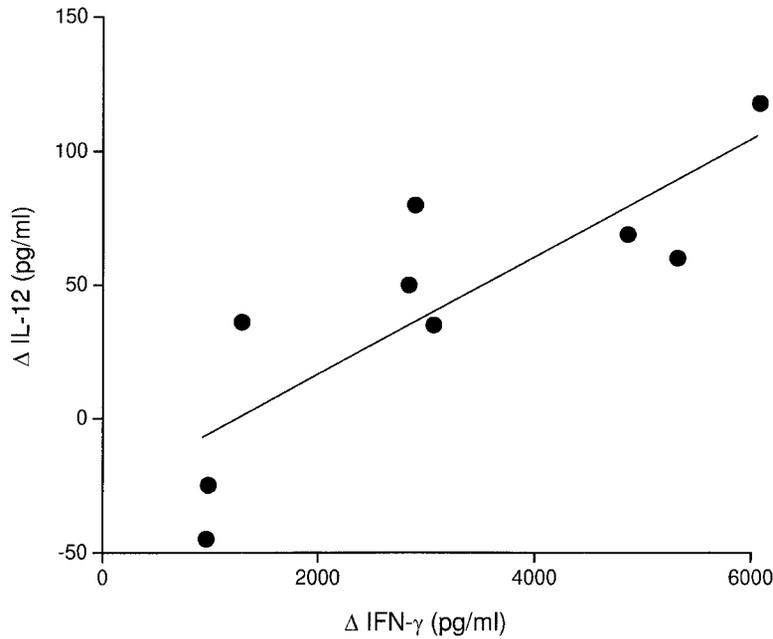


FIG. 5. Correlation of thalidomide ($1 \mu\text{g/ml}$)-induced increases (relative to DMSO control) in IFN- γ production and thalidomide-induced changes in IL-12 secretion by anti-CD3-stimulated PBMCs obtained from nine HIV-infected individuals. $R = 0.83$, $p = 0.009$ (by linear regression analysis). Supernatants for ELISAs were harvested at 48 hr (time of peak IL-12 production established in preliminary experiments).

pendent stimulus of IL-12 production. We observed that thalidomide inhibited IL-12 production in response to either stimulus (Fig. 7).

Together, these results show that thalidomide alone has no direct effect in increasing IL-12 production by PBMCs. How-

ever, when T cells were stimulated via the TCR, and the CD40-CD40L pathway is intact, thalidomide can stimulate production of IL-12 by APCs. On the other hand, when APCs are stimulated to produce IL-12 in the absence of activated T cells, the effect of thalidomide is inhibitory. Thus, these *in vitro* ex-

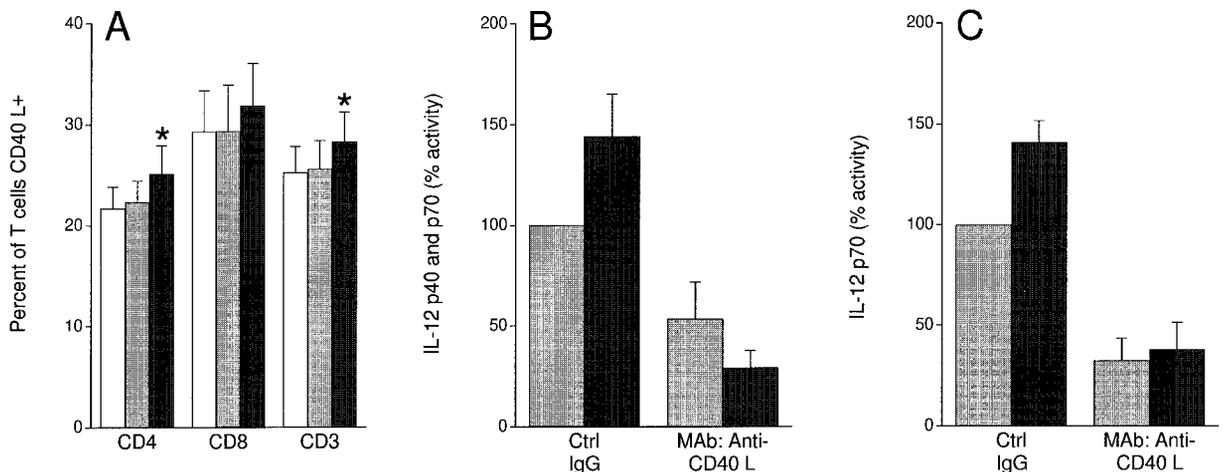


FIG. 6. (A) Effect of thalidomide on CD40L expression by CD4 $^+$, CD8 $^+$, and total CD3 $^+$ T cells in PBMCs stimulated by anti-CD3. Data are mean results from seven HIV-infected donors. Error bars represent SEMs. Cells were harvested for three-color flow cytometric analysis at 48 hr. Open columns, DMSO control; gray columns, thalidomide, $1 \mu\text{g/ml}$; solid columns, thalidomide, $10 \mu\text{g/ml}$. Significant ($p < 0.05$) increases relative to DMSO control are indicated by an asterisk. (B) Effect of antibody blockade of CD40L ($10 \mu\text{g/ml}$) on IL-12 (p40 and p70) production by anti-CD3-stimulated PBMCs from HIV-infected subjects in the presence or absence of thalidomide ($10 \mu\text{g/ml}$). Supernatants were harvested for assay at 48 hr. Gray columns, DMSO control; solid columns, thalidomide, $10 \mu\text{g/ml}$. Data are normalized to results for the DMSO-treated controls exposed to control mouse IgG. Mean of experiments performed with PBMCs from four HIV-infected individuals. (C) As in (B), but supernatants assayed for the IL-12 p70 heterodimer only. Key as in (B). Data shown are means (\pm SEM) of experiments with PBMCs from two HIV-infected individuals.

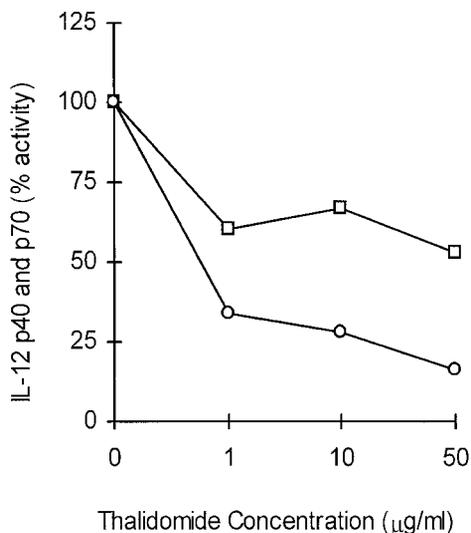


FIG. 7. Effect of thalidomide on IL-12 production by PBMCs stimulated with LPS (squares) or CD40L-CD8 fusion protein (circles). PBMCs (5×10^5) were stimulated with LPS or CD40L-CD8 in duplicate cultures in 48-well plates with the indicated concentrations of thalidomide. Supernatants were harvested at 24 or 48 hr for assay of IL-12 induced by LPS or CD40L-CD8, respectively. Data are expressed relative to DMSO control (normalized to 100%). In DMSO-treated control cultures, LPS and CD40L-CD8 induced 6710 and 647 pg of IL-12 per milliliter, respectively.

periments support our interpretation of the clinical data, suggesting that thalidomide exerts a primary effect in activating (costimulating) T cells, leading to secondary increases in IL-12 production by interacting APCs.

DISCUSSION

Thalidomide is a potent immunomodulatory drug that is likely to be used increasingly in the management of several complications of HIV disease, including the treatment of idiopathic aphthous oral ulceration¹⁷ and HIV disease-associated weight loss,¹⁰ as well as in a broad range of other chronic inflammatory and malignant diseases. There is therefore a need to advance our current, incomplete understanding of the mechanism of action of the drug. Here, we set out to test the hypothesis that specific inhibition of TNF- α by treatment of HIV-infected patients with thalidomide would reduce HIV replication. We hypothesized that any effect of the drug on HIV levels would be most readily detected during the rebound phase following temporary suppression of viremia. The rationale behind employing a ZDV/LMV pretreatment phase was twofold: (1) to quench some of the immune activation associated with uncontrolled viral replication at baseline and (2) to try and impose some uniformity on the viral response (i.e. during the rebound phase), in an attempt to optimize the power of a small, short-term study. We found no effect of thalidomide in retarding the return of viral titers to baseline levels, evaluated 14 days after cessation of antiretroviral treatment. However, 28 days of thalidomide treatment was associated with a modest "overshoot" in the rebound of viremia, which vanished after stop-

ping the drug. We did not observe an effect of thalidomide on plasma TNF- α levels. Levels of circulating TNF- α were not remarkably elevated at study baseline, but fell modestly during exposure to antiretroviral drugs. Similarly to viremia, TNF- α levels returned to baseline within 14 days of withdrawal of antiretrovirals, independently of the presence of thalidomide or placebo. We did, however, observe a correspondence between changes in HIV levels and TNF- α levels in patient plasma in this study, supporting the notion of a biologic relationship between this proinflammatory cytokine and HIV replication.^{10,11}

We considered that a 14-day course of LMV/ZDV therapy in this population was unlikely to induce drug resistance mutations. At the time we initiated our study, it was known that LMV monotherapy of HIV-infected, drug-naive patients could induce the Val¹⁸⁴ LMV resistance mutation in the HIV reverse transcriptase gene within 2 weeks,²² while ZDV monotherapy induced resistance only after several months of treatment.²³ However, it had been shown that when the two drugs were administered in combination to such drug-naive patients, the Val¹⁸⁴ mutation was not detectable before 4 weeks of treatment.²⁴ To address directly the question of whether the 14-day LMV/ZDV course of treatment induced this mutation in our patient cohort, we assayed circulating viral RNA for the Val¹⁸⁴ mutation at the end of the study. We found the mutation in 1 of 20 patient samples from which HIV cDNA was recoverable, and confirmed that the mutation was not present at baseline in this subject. Since short-term ZDV/LMV regimens are being evaluated to prevent mother-child transmission of HIV in resource-poor settings,²⁵ these data may have broader implications.

In contrast with the lack of effect on HIV and TNF- α plasma levels, we observed a remarkable immunostimulatory effect of thalidomide in HIV-infected patients. This was reflected in increases in DTH responses and plasma sIL-2R, sCD8, and IL-12 levels. These observations are consistent with preliminary observations made in two prior studies in HIV- and/or tuberculosis-infected patients, where thalidomide treatment appeared to induce increases in sIL-2R and CD8⁺ T cell numbers²⁶ and increased plasma levels of IFN- γ .²⁷ We now suggest that thalidomide directly stimulates T cells *in vivo*, corresponding to the *in vitro* T cell costimulatory properties of the drug observed in T cells from normal donors¹⁶ as well as in the T cells of HIV-infected patients (Fig. 4).

Optimal activation of T cells requires the delivery of a costimulus to the T cell in addition to primary antigenic stimulation via the TCR. Physiologically, such costimulation is afforded by the interaction of specific pairs of ligands on the APC and the T cell.^{28,29} Since antigenic stimulation in the absence of costimulation results in T cell anergy or apoptosis, the presence or absence of costimulation is critically important in the induction and regulation of cellular immunity.²⁹ We have shown that when highly purified primary T cells from healthy HIV-uninfected donors are stimulated via the TCR complex in the absence of physiologic costimulation by APCs, thalidomide can act as a costimulator, resulting in increased T cell proliferation and increased secretion of the helper T cell type 1 (Th1) cytokines IL-2 and IFN- γ .¹⁶ In the present study, we show that thalidomide acts as a costimulator for both CD8⁺ and CD4⁺ T cells from HIV-infected patients, when the cells are stimulated *ex vivo* with anti-CD3 antibody.

In this cohort of HIV-infected patients, the highly consistent increases in plasma IL-12 induced *in vivo* by thalidomide lagged

behind the increases in T cell activation markers, suggesting that IL-12 was augmented as a consequence of primary drug-induced T cell activation. IL-12 is a pivotal cytokine in the regulation of cellular immunity, steering primary T cell responses toward a Th1 cytokine (IL-2 and IFN- γ)-secreting profile, and away from a Th2 cytokine (IL-4 and IL-5)-secreting profile.²¹ This cytokine is produced primarily by antigen-presenting dendritic cells and monocyte-macrophages, and is regulated by both T cell-independent and T cell-dependent pathways.³⁰ The T cell-dependent pathway involves the two-way interaction of the CD40 molecule on APCs and CD40L on activated T cells. In addition, IFN- γ , a product of activated T cells, promotes IL-12 production by PBMCs from normal hosts,³¹ as well as those from individuals infected with *Mycobacterium leprae*³² or HIV.³³ Thus, a positive feedback loop exists to increase IL-12 and IFN- γ production by antigen-presenting cells and T cells, respectively.

In this study, we observed that *in vitro*, PBMCs from seven of nine HIV-infected subjects stimulated by immobilized anti-CD3 produced increased IL-12 in response to thalidomide, which correlated with increases in production of IFN- γ . In this system, IL-12 production was T cell dependent since blockade of the CD40L on activated T cells abrogated these responses. However, if the T cells were not activated, but the T cell stimulus to the APCs was mimicked with a CD40L-CD8 fusion protein, thalidomide exerted the opposite, inhibitory effect on IL-12 production. Furthermore, we confirmed the findings of Moller *et al.*, that thalidomide inhibits IL-12 production in a T cell-independent system *in vitro*, where bacterial products such as LPS are employed as direct stimuli of IL-12 production by APCs.³⁴ Together, these results strongly suggest that the present finding of thalidomide-induced increases in IL-12 levels *in vivo* is due to an indirect effect on APCs, mediated by thalidomide-costimulated T cells that express more CD40L. Thus, in a T cell-dependent system, thalidomide increases IL-12 production, while in a purely T cell-independent system, the drug can inhibit production of this cytokine. It follows that the immune-modulating effects of thalidomide in the complex *in vivo* situation may be dictated by the relative importance of T cell and macrophage activation in different immunopathologic states. Indeed, we can extend this argument to explain the dichotomous effect of thalidomide as an *in vivo* inhibitor of TNF- α in erythema nodosum leprosum (where the cellular target of the drug may be *M. leprae*-stimulated macrophages),¹³ while in the present study the drug did not inhibit TNF- α production, but was primarily an activator of T cells.

It is of interest that in the present study, marked immune stimulation by thalidomide was associated with only modest increases in HIV levels after 4 weeks of treatment. Importantly, HIV levels returned to the baseline set point after withdrawal of thalidomide. Kinter *et al.* have shown that *in vitro*, a differential effect of IL-2 in stimulating anti-HIV responses by CD8⁺ T cells outweighs the effect of this cytokine in promoting HIV replication in infected CD4⁺ T cells.³⁵ These authors suggest that such a net antiviral effect of T cell stimulation may break down in advanced HIV disease, as CD8⁺ T cell function declines. This may explain the exaggerated increases in HIV levels that follow IL-2 therapy of HIV-infected patients with advanced disease compared with those in the earlier stages.² Similarly, we suggest that in HIV-infected patients who are not receiving effective antiviral therapy, those with more advanced disease may be at greater risk of thalidomide-induced increases

in viral replication. Indeed, in a clinical trial by Jacobson *et al.*, thalidomide treatment of oral aphthous ulcers in extremely advanced HIV disease resulted in augmented viral levels.¹⁷ However, the advent of highly effective antiretroviral therapy presents the possibility of directly controlling viral replication where immunostimulatory therapies are being contemplated in advanced HIV disease.

In conclusion, the present findings may have twofold significance for possible future therapeutic applications of thalidomide in HIV disease: (1) direct pharmacologic costimulation of T cells by thalidomide may help to circumvent the documented defects in T cell costimulation that have been suggested to contribute to immune dysfunction in HIV disease^{36,37}; and (2) defective IL-12 responses have also been suggested to play an important role in the progressive immune deficiency of HIV disease.³⁸ It has been shown that deficient IL-12 responses in HIV-infected patients can be restored *in vitro* by the same T cell-dependent stimuli that thalidomide induces, namely CD40 ligand and IFN- γ .³⁹ Thus thalidomide therapy may achieve a restoration of IL-12 production in HIV-infected patients *in vivo*. We are currently pursuing studies to test the hypothesis that thalidomide treatment will have immune adjuvant effects in HIV disease, resulting in increases in primary immune responses to antigenic challenge.

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