

Thalidomide and a Thalidomide Analogue Drug Costimulate Virus-Specific CD8⁺ T Cells In Vitro

Patrick A. J. Haslett,^{1,2} Willem A. Hanekom,^{1,2} George Muller,³ and Gilla Kaplan¹

¹The Rockefeller University, New York, New York; ²University of Miami, Miami, Florida; ³Celgene Corporation, Warren, New Jersey

CD8⁺ T cell immunity is critical for protection from viral disease, such as that caused by the human immunodeficiency virus (HIV) or cytomegalovirus (CMV). It is therefore important to identify therapies that can boost antiviral immunity. The recent finding that thalidomide acts as a T cell costimulator suggested that this drug may boost antiviral CD8⁺ T cell responses. In this in vitro study, in a human autologous CD8⁺ T cell/dendritic cell (DC) coculture system, thalidomide and a potent thalidomide analogue were shown to enhance virus-specific CD8⁺ T cell cytokine production and cytotoxic activity. The drug-enhanced antiviral activity was noted in cells from both healthy donors and persons chronically coinfecting with HIV and CMV. This stimulatory effect was directed at CD8⁺ T cells, and not DCs. These results suggest an application for thalidomide and the thalidomide analogue as a novel immune-adjuvant therapy in chronic viral infections.

CD8⁺ effector T cells play a crucial role in the host immune response to virally infected cells [1–3]. In progressive HIV infection, however, cellular immunity is insufficient to control the infection. Even in the presence of highly active antiretroviral therapy (HAART) in chronically human immunodeficiency virus (HIV)-infected persons, immunologic abnormalities are present [4]. In particular, persistent defects in HIV-specific T cell responses may allow the continuous low-level replication of virus observed in the face of HAART [5]. Other viruses, such as cytomegalovirus (CMV) or Epstein-Barr

virus, that are normally controlled by an intact immune response can cause disease when immunity is depressed, whether pharmacologically or by coincident HIV infection. The enhancement of virus-specific immunity is of clinical importance in these groups of patients and has therefore been attempted in various ways, including the administration of therapeutic vaccines [6, 7], adoptive transfer of ex vivo-expanded virus-specific CD8⁺ T cells [8, 9], or immune-adjuvant therapy with recombinant interleukin (IL)-2 [10, 11].

A possible pharmacologic approach to immune enhancement is suggested by recent findings with the immune-modulatory drug thalidomide. Thalidomide's clinical anti-inflammatory activity was originally noted in erythema nodosum leprosum, a complication of leprosy [12]. The drug has since been shown to ameliorate the ulceration and inflammatory symptoms associated with Behçet's syndrome [13]. Similarly, thalidomide is also effective in reducing the aphthous ulceration associated with HIV disease [14]. Several activities of thalidomide have been described that may contribute to its clinical efficacy, including inhibition of the proinflammatory cytokine tumor necrosis factor- α (TNF- α) [15, 16], inhibition of new blood vessel formation [17], modulation of T cell function [18, 19], and stimulation of natural killer cell responses [20]. However, the precise mecha-

Received 26 December 2001; accepted 3 December 2002; electronically published 6 March 2003.

Presented in part: 8th Conference on Retroviruses and Opportunistic Infections, Chicago, 4–8 February 2001.

Informed consent was obtained from all participants in the studies described herein. The study protocol was approved by the Institutional Review Board of the Rockefeller University.

Financial support: National Institutes of Health (AI-47742 to P.A.J.H. and AI-22616 to G.K.); "Direct Effect" AIDS research organization.

G.M. is an employee of, and G.K. is on the Board of Directors of the Celgene Corporation, which provided the immunomodulatory drugs used in this work.

Reprints or correspondence: Dr. Patrick A. J. Haslett, University of Miami, Dept. of Microbiology and Immunology, 1600 Northwest 10th Ave., RMSB 3045-A, Miami, FL 33136 (phaslett@med.miami.edu).

The Journal of Infectious Diseases 2003;187:946–55

© 2003 by the Infectious Diseases Society of America. All rights reserved.
0022-1899/2003/18706-0009\$15.00

nism of action of the drug that accounts for its potency in various clinical situations remains unknown and may be multifactorial. Our recent work has focused on the effects of thalidomide on T cell responses. We demonstrated recently that thalidomide can act as a costimulator of T cell responses in vitro [18]. This costimulatory effect was manifested in a non-antigen-specific system by increased T cell proliferation and elevated cytokine production, exhibited by both CD8⁺ and CD4⁺ subsets. The subsequent findings that thalidomide treatment of patients with HIV infection [21, 22], tuberculosis [23], and scleroderma [24] is associated with immune activation, together with the observation that type 1 cytokine secretion is increased in healthy volunteers following a single dose of thalidomide [19], suggests that the drug has the potential to stimulate T cell immunity in vivo. The latter activity may be desirable in diseases in which effector CD8⁺ T cell responses are of critical importance, for example, in chronic infections with viruses such as HIV and CMV.

The well-known teratogenic and neurotoxic potential of thalidomide imposes serious limitations on the use of this agent. For this reason, there has been intense interest in developing analogues of thalidomide with an improved therapeutic ratio. Two classes of thalidomide analogue have emerged, originally selected for their increased potency as inhibitors of TNF- α production and their lack of teratogenicity in preliminary screening [25]. One of these classes comprises compounds that are inhibitors of phosphodiesterase IV [26] and that do not share the T cell-costimulating activity of the parent compound. Members of the other class are characterized by amino substitution of the phthalimide moiety of the parent molecule, are potent T cell costimulators, and, like thalidomide, lack phosphodiesterase IV inhibitory activity [27]. Herein we present the results of experiments done with thalidomide and one of the latter analogues (for molecular structure, see [27], compound "8a"), called CC-5013. Of importance, the latter drug is presently in phase 1/2 human trials [28].

To date, studies of the immune-modulatory activity of these agents have focused on their effect on non-antigen-specific immune responses. Here, with use of an in vitro dendritic cell (DC)/CD8⁺ T cell coculture system, we tested whether thalidomide and CC-5013 have the potential to enhance virus-specific CD8⁺ T cell effector function.

SUBJECTS, MATERIALS, AND METHODS

Study subjects. Healthy adults and asymptomatic chronically HIV-infected patients who were receiving HAART were eligible for enrollment in the study.

Cell preparation. Peripheral blood mononuclear cells (PBMCs) were isolated from freshly drawn heparinized blood by ficoll density gradient separation.

Immature DCs were prepared from CD14⁺ monocyte pre-

cursors by culture in recombinant human IL-4 (30 U/mL; Endogen) and recombinant human granulocyte-macrophage colony-stimulating factor (100 U/mL; Immunex), as described previously [29]. To achieve maturation of the DCs, 2×10^5 irradiated (3000 Rad) CD40 ligand-transfected fibroblasts (gift of J. Banchereau, Baylor Institute for Immunology Research, Dallas) were added on day 6 or 7 of culture per 3×10^6 input CD14⁺ precursor cells. On day 9 or 10, nonadherent mature DCs were harvested. These cells exhibited the characteristic stellate morphology of mature DCs, with the phenotype of mature DCs (CD14⁻, CD83⁺, CD86⁺, CD80⁺, HLA-DR^{bright}), verified by flow cytometry.

CD8⁺ T cells were negatively selected from PBMCs by means of immunomagnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. This technique routinely yielded populations that were >95% pure.

DC/T cell coculture. Mature DCs were resuspended at 10^7 cells/mL in serum-free medium and infected with recombinant vaccinia viruses (provided by Virogenetics) expressing the following genes: HIV-1 *gag* (vacGag), HIV-1 *pol* (vacPol), HIV-1 *env* (vacEnv), HIV-1 *nef* (vacNef), or CMV *pp65* (vacpp65), which codes for the CMV tegument protein pp65. Wild-type vaccinia virus (vacWT) was used as a negative control. Infections were done at a multiplicity of infection of 5 for 1 h at 37°C. Subsequently, DCs were washed twice and cultured at 37°C overnight to allow full expression of viral genes and antigen presentation. In experiments with HLA-A*0201-positive donors, mature DCs were pulsed with the HLA-A*0201-restricted influenza virus matrix protein CD8⁺ T cell epitope, GILGFVFTL, for 1 h, followed by washing and coculture with autologous CD8⁺ T cells. In some experiments, virus-infected DCs were fixed with 0.05% glutaraldehyde, followed by quenching with 0.2 M L-lysine, as described [18]. Purified T cells or PBMCs were preincubated with 0.04% dimethyl sulfoxide (DMSO; diluent vehicle: negative control), thalidomide (4–40 μ M), or CC-5013 (1 μ M). These concentrations of the test compounds were found in previous studies to be optimal for costimulating T cell responses in non-antigen-specific systems [18, 25]. Cultures were set up in 48-well tissue culture plates at 2×10^6 T cells or PBMCs per well in 1 mL of RPMI 1640 medium supplemented with 10% human AB serum, penicillin, and streptomycin (R10). The concentration of DMSO was the same in all conditions (0.04%). After 1 h of preincubation, antigen-loaded DCs were added at a ratio of 1 DC per 100 T cells (or PBMCs).

DC/T cell cocultures were incubated for 6 days. During incubation, fresh DMSO, thalidomide, or analogue was added on alternate days. On day 6 of coculture, cells were harvested to assess T cell function.

In some experiments, cocultures were treated with a polyclonal rabbit anti-human IL-2 antibody, 10 μ g/mL (Endogen), or control rabbit IgG, added on alternate days.

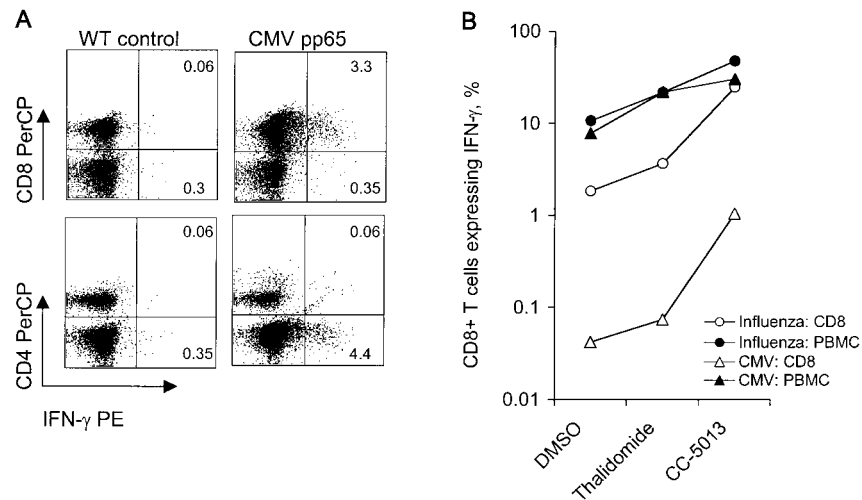


Figure 1. A, Dot plot showing cytofluorimetric detection of interferon- γ (IFN- γ) responses by CD8⁺, not CD4⁺, T cells from cytomegalovirus (CMV)-seropositive healthy donor, following stimulation by autologous dendritic cells (DCs) infected with vaccinia virus–CMV pp65 construct. Mature DCs were infected with vaccinia virus, either wild-type (WT control) or expressing the CMV tegument gene, pp65, and were cocultured with autologous peripheral blood mononuclear cells (PBMCs). After 6 days in culture, PBMCs were restimulated with the same antigens for 5 h, and intracellular IFN- γ was detected in either CD3⁺CD4⁺ or CD3⁺CD8⁺ T cells. Electronic gating was done on CD3⁺ lymphocytes. Numbers in dot plots indicate percentage of CD3⁺ cells in respective quadrants. Result shown is from 3 independent experiments. PE, phycoerythrin; PerCP, peridinin chlorophyll protein. B, Treatment with thalidomide and CC-5013 and increase of the number of CD8⁺ IFN- γ -positive T cells stimulated during 6-day cocultures with autologous DCs either infected with vaccinia virus–CMV pp65 (triangles) or pulsed with influenza virus matrix peptide (circles). The effect of immune-modulatory drugs on CD8⁺ T cell responses is similar in presence (PBMC [black symbols]) or absence (CD8 [white symbols]) of CD4⁺ T cells. Results shown are from 2 of 4 independent experiments with similar findings. DMSO, dimethyl sulfoxide.

Analysis of intracellular cytokines by fluorescence-activated cell sorting (IC/FACS).

Cells were collected from the DC/T cell cocultures, pelleted, and resuspended in 1 mL of culture medium. Aliquots of 2×10^5 cells were transferred to a 96-well V-bottomed tissue culture plate, and T cells were restimulated by the addition of autologous, virus-infected or peptide-pulsed DCs (DC to T cell ratio of 1:10–20). Brefeldin A (Sigma), 10 μ g/mL, was added to block cytokine secretion, followed by incubation at 37°C for 5 h. Subsequently, cells were fixed with 4% paraformaldehyde and permeabilized by resuspension and washing in permeabilization buffer (PBS containing 0.1% saponin and 0.1% bovine serum albumin). Cells were stained in 50 μ L of permeabilization buffer with combinations of 5 μ L each of the following fluorescence-conjugated antibodies: anti-CD8, anti-interferon- γ (IFN- γ), anti-TNF- α , or anti-IL-2. All antibodies were obtained from Becton Dickinson. Subsequently, cells were washed twice in permeabilization buffer, resuspended in PBS, and transferred to plastic tubes for analysis with a FACScan flow cytometer (Becton Dickinson).

ELISpot assay. In some experiments, single-cell CD8⁺ T cell IFN- γ production was evaluated by the ELISpot assay, as described previously [30]. On day 6 of DC/CD8 coculture, 10^5 cells were harvested and placed in duplicate in a 96-well ELISpot plate (Millititer; Millipore) coated with anti-IFN- γ monoclonal antibody (clone 1-DIK; MabTech), and restimulated with fresh DCs that had been infected with homologous vaccinia virus recom-

binants, at a DC to T cell ratio of 1:20. Cultures were incubated overnight. After incubation, cells were lysed and washed out, and spots of captured IFN- γ were revealed with a secondary IFN- γ monoclonal antibody (clone 7-B6-1; MabTech) conjugated to a peroxidase indicator system (Vector Laboratories). Resulting brown spots were counted with a dissecting microscope, each spot representing IFN- γ production by a single cell.

Chromium-release assay. Standard 5-h ⁵¹Cr-release assays were used to measure cytotoxic function. Effector cells were obtained from day 6 DC/CD8 cocultures and were resuspended in 1 mL of R10, as described above. Triplicate 100- μ L aliquots of effectors were placed in 96-well U-bottomed tissue culture plates, and serial 3-fold dilutions were prepared. Results are therefore expressed per dilution of effectors, ranging from 1 (undiluted) to 27 (1/27 dilution). As target cells, 10^6 autologous DCs, infected in the same way as DCs used in the primary cocultures, were suspended in 100 μ L of culture medium and incubated with 100 μ Ci of Na⁵¹CrO₄ for 1 h at 37°C. After 3 washes, radiolabeled DCs were resuspended in R10, and 5×10^3 targets were added to each well of the effector cells. After 5 h of incubation at 37°C, 50 μ L of supernatant medium/well was harvested for measurement of γ emission by means of a liquid scintillation counter (1450 “Trilux”; Wallac). For each effector population, parallel assays were set up with targets infected with the same virus used in the primary coculture and with control targets that were uninfected or infected with wild-

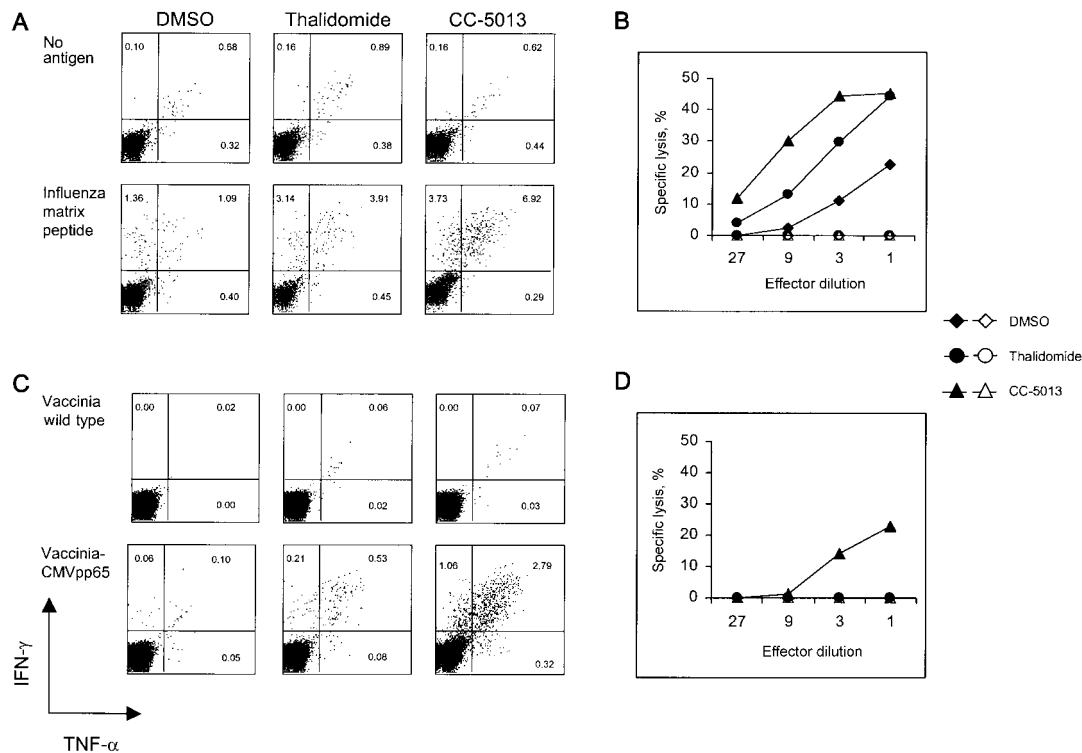


Figure 2. Enhancement, by thalidomide and CC-5013, of virus-specific CD8⁺ T cell responses. HLA-A*0201 dendritic cells (DCs) were pulsed with influenza virus matrix peptide GILGFVFTL and then were cocultured with autologous purified CD8⁺ T cells in presence or absence of either thalidomide (40 μ M) or CC-5013 (1 μ M). *A*, Dot plot showing cytofluorimetric detection of interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) responses by CD8⁺ T cells after restimulation, on day 6 of coculture, by autologous DCs. Electronic gating was done on CD8⁺ lymphocytes. Numbers in dot plots indicate percentage of CD8⁺ cells in respective quadrants. *B*, Cocultures, in same experiment, that were harvested and used as effectors versus ⁵¹Cr-labeled DC targets. Black symbols represent lysis of antigen-pulsed targets by antigen-stimulated effectors; white symbols represent lysis of unpulsed control targets. CD8⁺ T cells cocultured with unpulsed DCs did not lyse either pulsed or unpulsed targets (not shown). DMSO, dimethyl sulfoxide. *C* and *D*, Experiments performed as in *A* and *B* but with different healthy donor and with vaccinia virus–cytomegalovirus (CMV) to introduce CMV pp65 antigen. Vaccinia wild-type virus served as negative control. CMV-specific target lysis was observed only in culture treated with CC-5013. Results are from 4 independent experiments.

type vaccinia virus. Spontaneous and total release were assessed for each target population after treatment with culture medium and 1% SDS, respectively. Spontaneous release was calculated as $(\text{cpm}_{\text{spontaneous}}/\text{cpm}_{\text{total}}) \times 100$ and was < 20% for all targets. Specific lysis was calculated as $[(\text{cpm}_{\text{experimental}} - \text{cpm}_{\text{spontaneous}})/(\text{cpm}_{\text{experimental}} - \text{cpm}_{\text{spontaneous}})] \times 100$.

T cell proliferation–assays. Proliferative responses were assayed by measuring [³H]-thymidine (NEN Products) incorporation over 6 h by triplicate 50- μ L aliquots of resuspended day 6 cocultures, after transfer to 96-well U-bottomed tissue culture plates. DNA was harvested onto fiber mats with an automatic cell harvester (Skatron), and β emission was measured with a liquid scintillation counter (1450 “Trilux”; Wallac).

Evaluation of DC function. DC cultures were treated with DMSO, thalidomide (0.4–40 μ M), or CC-5013 (1 μ M), at the time that CD40L-transfected fibroblasts were added. Supernatants were collected at 48 h for assay of total IL-12 by ELISA (Endogen), according to the manufacturer’s instructions. At 72 h, DCs were evaluated phenotypically by flow cytometry to

assess expression of CD83, CD80, CD86, HLA-DR, and CD14, or by their capacity to stimulate allogeneic T cell proliferation: serial triplicate dilutions of DCs were made in 96-well U-bottomed plates to which allogeneic purified T cells (10^5 /well) were added. Tritiated thymidine incorporation during the last 6 h of 120-h cultures was assayed.

RESULTS

Thalidomide and CC-5013 costimulate virus-specific CD8⁺ T cell effector responses in DC/PBMC and DC/CD8 cocultures prepared from healthy donors. Monocytes prepared from the PBMCs of CMV-seropositive healthy adult volunteers were cultured in vitro to obtain mature DCs. The DCs were infected with the vaccinia virus constructs vacWT or vacpp65 and cocultured with autologous PBMCs at a DC to PBMC ratio of 1:100. On day 6, the cocultures were restimulated with DCs infected with the same vaccinia virus constructs for 5 h in the presence of brefeldin A. Subsequent IC/FACS analysis revealed CMV-spe-

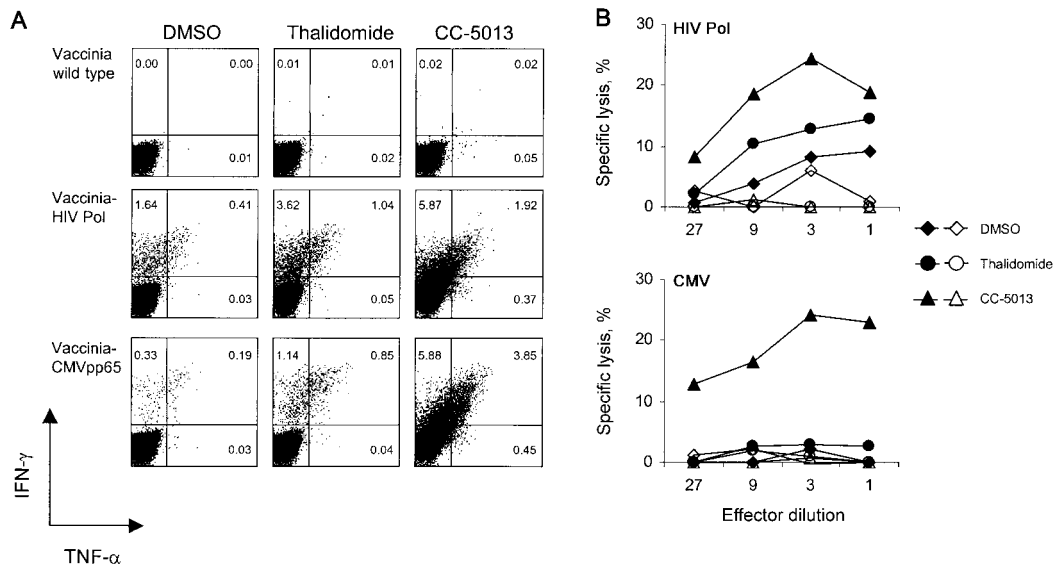


Figure 3. A, Dot plots showing enhancement by thalidomide (40 μ M) and CC-5013 (1 μ M) of human immunodeficiency virus (HIV) and cytomegalovirus (CMV)-specific interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) responses by CD8⁺ T cells from patient with chronic HIV disease. Experiment was set up as in figure 2C. B, ⁵¹Chromium-release assay showing enhancement by thalidomide and CC-5013 of HIV Pol-specific target lysis. CMV-specific killing was observed after treatment of cocultures with CC-5013, but not with either dimethyl sulfoxide (DMSO control) or thalidomide. Assays were set up as in figure 2D. Black symbols represent lysis of antigen-pulsed targets by antigen-stimulated effectors; white symbols represent lysis of unpulsed control targets. CD8⁺ T cells cocultured with unpulsed dendritic cells did not lyse either pulsed or unpulsed targets (not shown). Results are from >8 (intracellular cytokine staining) and 4 (⁵¹Cr-release assays) independent experiments.

cific intracellular IFN- γ expression by CD8⁺, but not by CD4⁺ T cells (figure 1A), confirming our previous observation that CD8⁺ T cell depletion abrogated the IFN- γ response to recombinant vaccinia virus/HIV constructs [30]. Restimulation of primary vacpp65-stimulated cocultures with the control vacWT or restimulation of primary vacWT-stimulated cocultures with vacpp65 elicited only background IFN- γ expression (not shown).

The lack of CD4⁺ T cell “help” in progressive HIV disease is

thought to be a factor that contributes to the functional failure of HIV-specific CD8⁺ T cell effector responses. It was therefore of interest to know whether the immune-modulatory effects of thalidomide and CC-5013 on virus-specific CD8⁺ T cell responses were evident in the presence or absence of CD4⁺ T cells. To test this, we compared the effects of these drugs on virus-specific CD8⁺ T cell IFN- γ production in DC/PBMC cocultures (CD4⁺ T cells present) versus DC/CD8 cocultures

Table 1. Enhancing effect of thalidomide and CC-5013 on virus-specific activities of CD8⁺ T cells from chronically human immunodeficiency virus (HIV)-infected patients.

Patient	Duration of HIV infection, years	HIV antigen	Baseline frequency of HIV-specific IFN- γ -positive T cells, %	Increase in virus-specific CD8 ⁺ IFN- γ -positive cells, compared with DMSO control, <i>n</i> -fold			
				HIV		CMV pp65	
				Thalidomide	CC-5013	Thalidomide	CC-5013
3	14	Pol	0.17	4.1	ND	ND	ND
11	14	Gag	0.19	1.2	1.7	2.2	1.6
30	8	Env	0.04	0.6	1.9	4.9	9.3
14	10	Pol	0.09	4.5	21.5	13.0	153.0
12	12	Pol	0.03	2.3	3.8	4.7	23.2
28	13	Nef	0.05	2.0	5.1	0.8	2.0
				2.0 ^{a,b}	3.8 ^{a,b}	4.7 ^{a,c}	9.3 ^{a,c}

NOTE. CMV, cytomegalovirus; DMSO, dimethyl sulfoxide; IFN- γ , interferon- γ ; ND, not done.

^a Median *n*-fold increase in virus-specific CD8⁺ IFN- γ -positive cells.

^b *P* < .01 (Friedman test).

^c *P* < .05 (Friedman test).

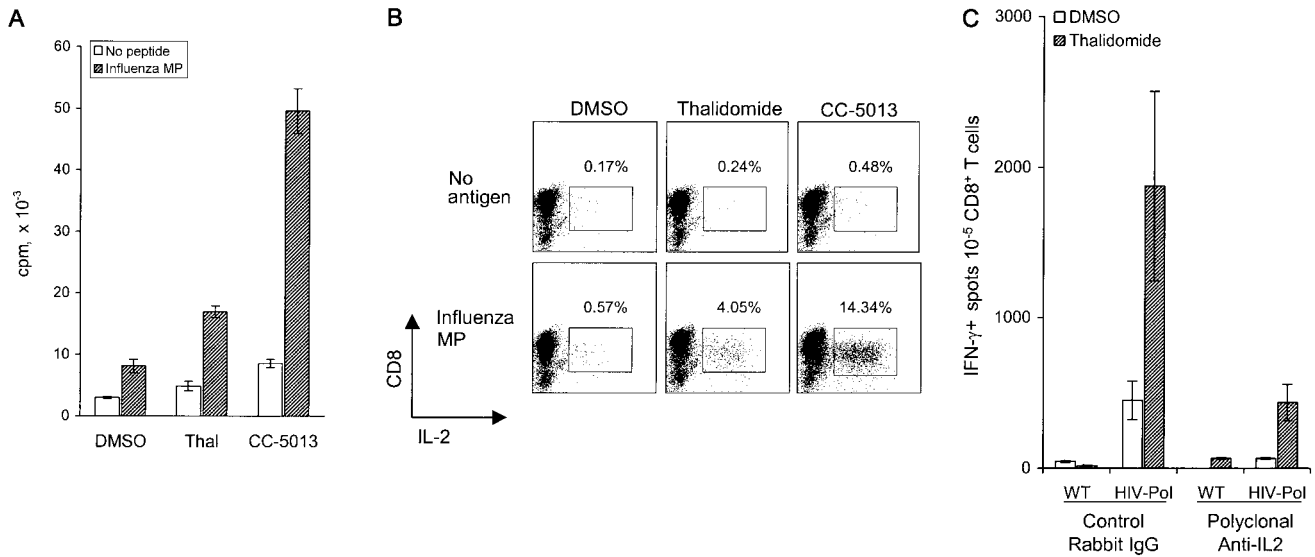


Figure 4. A, [³H]-thymidine incorporation by CD8⁺T cells cocultured with autologous influenza virus matrix peptide (MP)-pulsed or -unpulsed dendritic cells (DCs) in presence or absence of either thalidomide (40 μ M) or CC-5013 (1 μ M). On day 6, equal volumes of cocultures were transferred to 96-well plates in triplicate and were pulsed for 6 h with [³H]-thymidine. Results are from 3 experiments. B, Dot plots showing increased numbers of influenza virus-specific interleukin-2 (IL-2)-containing CD8⁺ T cells after treatment of DC/CD8 cocultures with thalidomide (40 μ M) or CC-5013 (1 μ M). Results are from 3 experiments. C, Results of ELISpot assay showing that enhancement by thalidomide of human immunodeficiency virus (HIV) Pol-specific expansion of CD8⁺ T cells is IL-2-dependent. DCs were infected with vaccinia virus-HIV Pol constructs or wild-type control vaccinia virus (WT) and were cocultured with autologous purified CD8⁺ T cells in presence of thalidomide (40 μ M) or dimethyl sulfoxide (DMSO control) and in presence of polyclonal neutralizing antibody to IL-2 or rabbit immunoglobulin G (isotype control). On day 6 of coculture, CD8⁺ T cells were transferred to ELISpot plates (10⁵ cells per duplicate well) and were restimulated in situ with autologous DCs. Each interferon- γ (IFN- γ) spot represents single IFN- γ -producing cell. Results are from 3 experiments.

(CD4⁺ T cells absent). PBMCs and purified CD8⁺ T cells prepared from healthy donors were cultured with autologous mature DCs that had been pulsed with influenza virus matrix peptide or with no antigen (HLA-A*0201-positive donor), or infected with vacWT or vacpp65 (CMV-seropositive donor). The cocultures were set up in the presence of 0.04% DMSO (control), 40 μ M thalidomide, or 1 μ M CC-5013. After restimulation on day 6, FACS analysis was performed to assess intracellular IFN- γ expression, with data collected on 50,000 CD8⁺ T cells. In these experiments, day 6 responses represented variable expansions of antigen-specific CD8⁺ T cells over day 0 responses (day 0 response = IFN- γ expression after the first 5 h of coculture in the presence of brefeldin A), ranging from 7- to >150-fold increases in the percentage of CD8⁺ T cells that were IFN- γ positive (data not shown). Moreover, neither thalidomide nor CC-5013 increased the numbers of IFN- γ -positive cells over the first 5 h. We therefore focused on the capacity of the immunomodulatory drugs to expand antigen-specific CD8⁺ T cells over 6 days of culture. Figure 1B shows results from 2 representative experiments, in which thalidomide and, to a greater extent, CC-5013 enhanced CMV and influenza virus-specific CD8⁺ T cell IFN- γ responses in both DC/PBMC and DC/CD8 cocultures. The degree of enhancement of virus-specific IFN- γ expression by thalidomide and CC-5013 was

similar in both systems, although the absolute number of IFN- γ -positive CD8⁺ T cells was less in DC/CD8 than in DC/PBMC cocultures. Hence, the presence of CD4⁺ T cells did not influence the enhancing effect of thalidomide or CC-5013 on CD8⁺ T cell responses. Subsequent experiments were set up with the "minimal" system of cocultured DCs and purified CD8⁺ T cells.

To study CD8⁺ T cell effector responses in more detail, we next evaluated the effect of thalidomide and CC-5013 on simultaneous IFN- γ and TNF- α production, and cytotoxic activity of virus-specific CD8⁺ T cells, by IC/FACS and ⁵¹Cr-release assays, respectively.

DC/CD8 cocultures were prepared as described above, and FACS analysis was done after restimulation of the cultures on day 6. Both influenza virus matrix peptide and CMV pp65 stimulated cytokine responses that were enhanced by thalidomide and further increased by CC-5013 (figure 2A and 2C). Of note, the majority of cytokine-positive cells coexpressed both IFN- γ and TNF- α .

In parallel, effector cells were harvested on day 6 of the DC/CD8 coculture, and their capacity to lyse antigen-pulsed ⁵¹Cr-labeled autologous DC targets was evaluated. Thalidomide and, to a greater extent, CC-5013 enhanced the lysis of influenza virus matrix peptide-pulsed DC targets (figure 2B), whereas only CC-5013 stimulated a detectable CMV-specific lytic re-

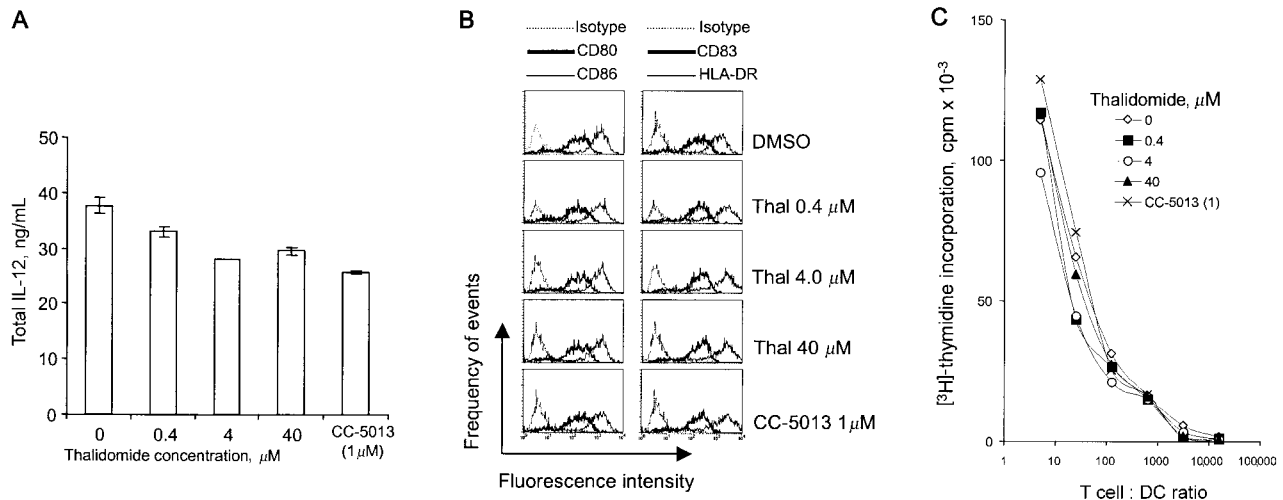


Figure 5. A, CD40L-stimulated interleukin-12 (IL-12) production by monocyte-derived dendritic cells (DCs). Immature DCs were stimulated by CD40L-expressing fibroblasts in presence or absence of either thalidomide or CC-5013, at indicated concentrations. At 48 h, supernatant culture medium was assayed for total IL-12, by ELISA. B, Histograms showing expression of T cell costimulatory molecules CD80 and CD86, DC maturation marker CD83, and antigen-presenting molecule HLA-DR, by CD40L-stimulated (i.e., matured) DCs cultured in presence of thalidomide or CC-5013. C, [³H]-thymidine incorporation by allogeneic CD4⁺ T cells that had been stimulated for 4 days by DCs matured in presence of indicated concentrations of thalidomide or CC-5013. Results are from 2 experiments.

sponse (figure 2D). The target cell lysis was antigen specific, because the same effectors did not lyse control targets that had not been pulsed with antigen (figure 2B) or that were infected with vacWT (figure 2D). Similarly, no lysis of target cells was observed in experiments in which non-antigen-pulsed or vacWT-infected DCs were used in the initial 6-day coculture, demonstrating that an antigenic stimulus is necessary for the drugs to exert their costimulatory effect and that priming or enhancement of vaccinia virus-specific lytic responses was not occurring to a measurable extent during the 6-day coculture. From these experiments, we conclude that, in healthy donors, thalidomide and CC-5013 can costimulate CD8⁺ T cell effector functions against influenza virus and CMV, two viruses of clinical importance, in the absence of CD4⁺ T cell help.

Thalidomide and CC-5013 promote HIV- and CMV-specific responses in CD8⁺ T cells from chronically HIV- and CMV-coinfected persons. Next, we used the same autologous culture system to evaluate the potential of thalidomide and CC-5013 to enhance the virus-specific activities of CD8⁺ T cells isolated from chronically HIV-infected patients who were undergoing treatment with HAART. DCs prepared from PBMCs obtained from these patients were infected with vacWT, vacGag, vacPol, vacEnv, vacNef, or vacCMV. The presence of baseline CD8⁺ T cell responses to the individual HIV gene products by these patients had been determined previously in ELISpot assays [30]. In the present experiments, we observed that thalidomide and CC-5013 enhanced HIV- and CMV-specific IFN- γ and TNF- α expression by CD8⁺ T cells, as well as virus-specific lytic activity (figure 3). Table 1 shows the IFN- γ data from 6

independent experiments done with cells isolated from 6 HIV-infected patients. In general, the immune-modulatory compounds enhanced HIV- and CMV-specific responses ($P < .01$ and $P < .05$, respectively, by Freidman's nonparametric analysis of variance by ranks). Thus, thalidomide and CC-5013 can stimulate HIV- and CMV-specific immunity in CD8⁺ T cells from chronically HIV-infected patients currently undergoing treatment with HAART.

Thalidomide enhances virus-specific responses by stimulating autocrine IL-2-driven expansion of effector CD8⁺ T cells. In direct ex vivo assays in which DC/CD8 cocultures were immediately treated with brefeldin A and IC/FACS analysis was done after 5 h, thalidomide had little or no enhancing effect on virus-specific IFN- γ responses (data not shown). In contrast, after 5–7 days of in vitro expansion of CD8⁺ cells by coculture with DCs, the enhancing effect of thalidomide and CC-5013 was apparent, suggesting that the drugs were enhancing clonal expansion of antigen-specific memory-effector CD8⁺ T cells. In support of this, [³H]-thymidine incorporation was increased when DC/CD8 cocultures were set up in the presence of thalidomide or CC-5013 (figure 4A). Parallel evaluation of intracellular IL-2 expression in restimulated day 6 cocultures revealed consistent increases in numbers of IL-2-positive CD8⁺T cells (figure 4B). When cultures were treated with a neutralizing antibody against IL-2, the thalidomide-induced expansion of antigen-specific CD8⁺ T cells was largely reversed (figure 4C). Control experiments confirmed that the IL-2-neutralizing antibody did not cross-react with IFN- γ (not shown). These results strongly suggest that, in our experimen-

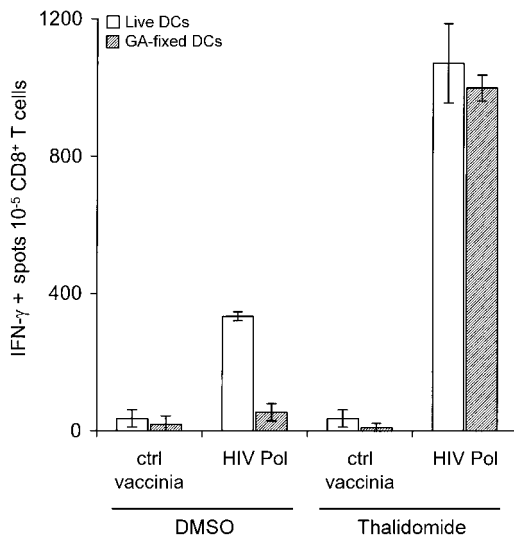


Figure 6. ELISPOT data showing that glutaraldehyde fixation of antigen-expressing dendritic cells (GA-fixed DCs) does not affect enhancement of virus-specific CD8 $^+$ T cell responses by thalidomide (40 μ M). Mature monocyte-derived DCs were prepared from chronically human immunodeficiency virus (HIV)-infected patients and infected with vaccinia virus-expressing HIV Pol or wild-type vaccinia virus control (ctrl vaccinia). DCs were GA-fixed or left unfixed and cocultured with autologous purified CD8 $^+$ T cells. On day 6 of coculture, cells were restimulated in duplicate wells of ELISPOT plates with autologous DCs. Each interferon- γ (IFN- γ) spot represents single IFN- γ -producing cell. Results are from 3 experiments.

tal system, the enhancement of virus-specific CD8 $^+$ T cell responses by thalidomide is a consequence of pharmacologic costimulation of CD8 $^+$ T cell IL-2 production.

Thalidomide and CC-5013 exert a direct costimulatory effect on CD8 $^+$ T cells and do not enhance costimulation by DCs. In the experiments described thus far, both DCs and T cells were exposed to the experimental immune-modulating compounds. It was therefore possible that the augmented T cell responses observed were due to pharmacologic enhancement of DC function and/or direct effects on T cells. To address this, we performed experiments to evaluate the effect of thalidomide on key DC functions that could, in turn, increase T cell responsiveness: that is, IL-12 production [31], expression of surface molecules involved in T cell activation, and the capacity of DCs to prime an allospecific T cell response.

Immature DCs, obtained from healthy HIV-uninfected persons, were incubated with thalidomide or CC-5013 during CD40L-induced DC maturation. CD40L is a potent stimulus of IL-12 production. This cytokine is maximally produced as the DCs mature to become highly efficient antigen-presenting cells [32]. IL-12 was assayed in culture supernatants at 48 h. At this time, DCs were harvested and assayed by FACS analysis for surface molecule expression or were combined with HLA-mismatched purified T cells to assess their capacity to prime an allospecific lymphoproliferative response. We observed that

neither thalidomide nor CC-5013 enhanced IL-12 production by DCs, but rather both drugs exerted a modest inhibitory effect on the secretion of this cytokine by these cells (figure 5A). On analysis of surface marker expression by FACS, we observed no effect of the drugs on the expression of the DC maturation marker CD83, the costimulatory molecules CD80 or CD86, or the antigen-presenting molecule HLA-DR (figure 5B). This lack of effect was confirmed functionally, because the drugs had no effect on the allostimulatory capacity of DCs (figure 5C). These experiments suggested that thalidomide and CC-5013 were unlikely to exert their T cell-costimulatory effect via direct augmentation of DC function.

Finally, we performed experiments in which mature DCs that had been infected with vaccinia virus constructs were fixed with glutaraldehyde before coculture with autologous CD8 $^+$ T cells, so that any biologic effect of the experimental agents in this antigen-specific system would have to be directed at the T cell. After 6 days, the CD8 $^+$ T cells were restimulated with infected (unfixed) DCs in ELISPOT plates. The thalidomide-induced boosting of T cell responses was evident regardless of whether the DCs used for the initial stimulation had been fixed (figure 6). Indeed, the effect of thalidomide relative to DMSO control-treated cultures was even more striking after DC fixation, because fixation reduced the T cell response to antigen in the absence of thalidomide (figure 6).

We conclude from these experiments that thalidomide and CC-5013 exert their immune-stimulatory effect directly on CD8 $^+$ T cells, that they can do so in the absence of CD4 $^+$ T cells, and that this effect is not by increasing the efficiency of antigen presentation.

DISCUSSION

Herein, we show that, in vitro, thalidomide and a selected thalidomide analogue enhance antigen-specific CD8 $^+$ T cell responses against HIV, CMV, and influenza virus—all viruses of clinical importance. Our data indicate that the stimulatory effect of the drugs is directed at the T cell and is not accounted for by direct augmentation of antigen-presenting cell function. These studies support the hypothesis that thalidomide has an immune-adjunct effect and, thus, may be of clinical utility for diseases in which enhancement of antigen-specific CD8 $^+$ T cell immunity is desirable, such as in chronic viral infections or cancer.

In previous clinical studies, we have shown that thalidomide treatment of patients with HIV infection [21, 22], tuberculosis and HIV coinfection [23], scleroderma [24], and sarcoidosis [33] is associated with immune activation. One feature of this is consistently increased IL-12 production. This has also been demonstrated recently in healthy volunteers treated with thalidomide [19]. However, IL-12 is produced by antigen-pre-

senting cells, not T cells, whereas the data presented here suggest that the immune-stimulatory effects of thalidomide are directed at the T cell rather than the antigen-presenting cell. To explain this apparent contradiction, the physiologic regulation of IL-12 production must be considered. IL-12 production by antigen-presenting cells (DCs and monocytes) is stimulated by activated T cells that express CD40L (T cell-dependent pathway—mimicked in the present work by CD40L-expressing fibroblasts) or by pathogen-associated molecules, such as lipopolysaccharide (T cell-independent pathway). In the present report, in contrast to the consistent and direct costimulatory effect of thalidomide and CC-5013 on CD8⁺T cell function, we show that the direct effect of these drugs on CD40 ligand-induced IL-12 production by DCs was modestly inhibitory. This is consistent with the findings of Moller et al. [34], which demonstrate that thalidomide directly inhibits T cell-independent monocyte production of IL-12 in a dose-dependent manner. To reconcile the different effects of thalidomide on IL-12 production, we suggest that, in the clinical settings cited above, costimulation of T cells by thalidomide results in the up-regulation of T cell signals (i.e., CD40 ligand and IFN- γ) for antigen-presenting cells to produce IL-12 [22, 25], outweighing the direct inhibitory effects of the drug on the latter cells. However, it is possible that, in some clinical situations, the effect of the drug on IL-12 levels in vivo will be inhibitory if the predominant stimulus for IL-12 production is microbial (i.e., T cell independent). Indeed, in vitro experiments have revealed this dichotomy: when peripheral blood mononuclear cells are stimulated by T cell receptor activation, thalidomide enhances IL-12 production, but, when PBMCs are stimulated by lipopolysaccharide, thalidomide inhibits the production of this cytokine [25].

Analogues of thalidomide that possess enhanced potency as T cell costimulators have been described recently [25]. Here we show that CC-5013, an analogue compound that is currently in phase 1/2 clinical trials [28], also displayed increased potency, relative to thalidomide, in enhancing antigen-specific CD8⁺T cell responses. Further support for the potential importance of an immune-enhancing or adjuvant effect of this class of compounds was provided in a recent publication by Dredge et al. [35], which showed that another T cell-costimulating thalidomide analogue, CC-4047, potently enhanced protective anti-tumor immunity when administered to mice during the priming phase of tumor cell immunization. The identification of the molecular target(s) of thalidomide and its analogues that mediate this immune-adjuvant activity will be of crucial importance in refining analogue development and, more generally, in identifying potentially new cellular targets for immune-modulatory therapy.

A key unanswered question is how, or whether, the putative immune-adjuvant effects of thalidomide are related to the clin-

ical effects observed during therapy with this drug. “Paradoxical” exacerbations of some diseases have been reported following thalidomide treatment, in graft-versus-host disease [36], toxic epidermal necrolysis [37], and tuberculosis meningitis [38], which are readily explained by pharmacologic enhancement of T cell activity. However, in those conditions in which there is controlled evidence of clinical benefit—erythema nodosum leprosum [12] and HIV- or Behçet’s syndrome-associated mucosal ulceration [13, 14]—the apparent effect of the drug is anti-inflammatory. The TNF- α -inhibitory activity of thalidomide is an appealing and widely accepted explanation for the latter responses. However, it is possible that the T cell-directed immune-adjuvant effect of thalidomide may promote active immune regulation. This may occur via stimulation of regulatory T cell subsets [39] or by the induction of IL-2-dependent control of memory T cell populations [40], resulting in resolution of inflammation. Recent clinical studies by our group suggest that thalidomide promotes an active healing process when used in the treatment of cutaneous sarcoidosis, in which therapy is associated with maturation, followed by involution of sarcoid granulomata [33]. The hypothesis that thalidomide stimulates host immune regulation will be tested in further, detailed clinical-immunologic studies. This area of investigation may lead to the development of alternative approaches to treating inflammatory diseases, approaches that harness the host’s active immune-regulatory potential.

In summary, we show here that thalidomide and CC-5013, a thalidomide analogue currently in clinical trials, are costimulators of virus-specific CD8⁺T cell effector functions. These data contribute to a growing body of evidence that thalidomide has immune-potentiating effects. Further studies will elucidate the relevance of this activity of the drug to the known clinical benefits of thalidomide and will permit prospective testing of the hypothesis that useful stimulation of antiviral immunity can be achieved with these agents in the clinic.

References

1. Koup RA, Safrit JT, Cao Y, et al. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* **1994**;68:4650–5.
2. Moskophidis D, Kioussis D. Contribution of virus-specific CD8⁺ cytotoxic T cells to virus clearance or pathologic manifestations of influenza virus infection in a T cell receptor transgenic mouse model. *J Exp Med* **1998**;188:223–32.
3. Rickinson AB, Lee SP, Steven NM. Cytotoxic T lymphocyte responses to Epstein-Barr virus. *Curr Opin Immunol* **1996**;8:492–7.
4. Autran B, Carcelain G, Li TS, et al. Positive effects of combined antiretroviral therapy on CD4⁺ T cell homeostasis and function in advanced HIV disease. *Science* **1997**;277:112–6.
5. Furtado MR, Callaway DS, Phair JP, et al. Persistence of HIV-1 transcription in peripheral-blood mononuclear cells in patients receiving potent antiretroviral therapy. *N Engl J Med* **1999**;340:1614–22.
6. Birx DL, Redfield RR. HIV vaccine therapy. *Int J Immunopharmacol* **1991**;13:129–32.

7. Valentine FT, Kundu S, Haslett PA, et al. A randomized, placebo-controlled study of the immunogenicity of human immunodeficiency virus (HIV) rgp160 vaccine in HIV-infected subjects with ≥ 400 CD4 T lymphocytes/mm³ (AIDS Clinical Trials Group Protocol 137). *J Infect Dis* **1996**; 173:1336–46.
8. Riddell SR, Walter BA, Gilbert MJ, Greenberg PD. Selective reconstitution of CD8⁺ cytotoxic T lymphocyte responses in immunodeficient bone marrow transplant recipients by the adoptive transfer of T cell clones. *Bone Marrow Transplant* **1994**; 14:S78–84.
9. Tan R, Xu X, Ogg GS, et al. Rapid death of adoptively transferred T cells in acquired immunodeficiency syndrome. *Blood* **1999**; 93:1506–10.
10. Kovacs JA, Vogel S, Albert JM, et al. Controlled trial of interleukin-2 infusions in patients infected with the human immunodeficiency virus. *N Engl J Med* **1996**; 335:1350–6.
11. Smith KA. Low-dose daily interleukin-2 immunotherapy: accelerating immune restoration and expanding HIV-specific T-cell immunity without toxicity. *AIDS* **2001**; 15(suppl 2):S28–35.
12. Waters MF. An internally controlled double blind trial of thalidomide in severe erythema nodosum leprosum. *Lepr Rev* **1971**; 42:26–42.
13. Hamuryudan V, Mat C, Saip S, et al. Thalidomide in the treatment of the mucocutaneous lesions of the Behcet syndrome: a randomized, double-blind, placebo-controlled trial. *Ann Intern Med* **1998**; 128:443–50.
14. Jacobson JM, Greenspan JS, Spritzler J, et al. Thalidomide for the treatment of oral aphthous ulcers in patients with human immunodeficiency virus infection. National Institute of Allergy and Infectious Diseases AIDS Clinical Trials Group. *N Engl J Med* **1997**; 336:1487–93.
15. Sampaio EP, Sarno EN, Galilly R, Cohn ZA, Kaplan G. Thalidomide selectively inhibits tumor necrosis factor alpha production by stimulated human monocytes. *J Exp Med* **1991**; 173:699–703.
16. Sampaio EP, Kaplan G, Miranda A, et al. The influence of thalidomide on the clinical and immunologic manifestation of erythema nodosum leprosum. *J Infect Dis* **1993**; 168:408–14.
17. D'Amato RJ, Loughnan MS, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. *Proc Natl Acad Sci USA* **1994**; 91:4082–5.
18. Haslett PA, Corral LG, Albert M, Kaplan G. Thalidomide costimulates primary human T lymphocytes, preferentially inducing proliferation, cytokine production, and cytotoxic responses in the CD8⁺ subset. *J Exp Med* **1998**; 187:1885–92.
19. Verbon A, Juffermans NP, Speelman P, et al. A single oral dose of thalidomide enhances the capacity of lymphocytes to secrete gamma interferon in healthy humans. *Antimicrob Agents Chemother* **2000**; 44:2286–90.
20. Davies FE, Raje N, Hideshima T, et al. Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. *Blood* **2001**; 98:210–6.
21. Hanekom WA, Hughes J, Haslett PA, et al. The immunomodulatory effects of thalidomide on human immunodeficiency virus-infected children. *J Infect Dis* **2001**; 184:1192–6.
22. Haslett PA, Klausner JD, Makonkawkeyoon S, et al. Thalidomide stimulates T cell responses and interleukin 12 production in HIV-infected patients. *AIDS Res Hum Retroviruses* **1999**; 15:1169–79.
23. Bekker LG, Haslett P, Maartens G, Steyn L, Kaplan G. Thalidomide-induced antigen-specific immune stimulation in patients with human immunodeficiency virus type 1 and tuberculosis. *J Infect Dis* **2000**; 181:954–65.
24. Oliver SJ, Moreira A, Kaplan G. Immune stimulation in scleroderma patients treated with thalidomide. *Clin Immunol* **2000**; 97:109–20.
25. Corral LG, Haslett PA, Muller GW, et al. Differential cytokine modulation and T cell activation by two distinct classes of thalidomide analogues that are potent inhibitors of TNF-alpha. *J Immunol* **1999**; 163:380–6.
26. Muller GW, Shire MG, Wong LM, et al. Thalidomide analogs and PDE4 inhibition. *Bioorg Med Chem Lett* **1998**; 8:2669–74.
27. Muller GW, Chen R, Huang SY, et al. Amino-substituted thalidomide analogs: potent inhibitors of TNF-alpha production. *Bioorg Med Chem Lett* **1999**; 9:1625–30.
28. Richardson PG, Schlossman RL, Weller E, et al. Immunomodulatory drug CC-5013 overcomes drug resistance and is well tolerated in patients with relapsed multiple myeloma. *Blood* **2002**; 100:3063–7.
29. Granelli-Piperno A, Zhong L, Haslett P, Jacobson J, Steinman RM. Dendritic cells, infected with vesicular stomatitis virus–pseudotyped HIV-1, present viral antigens to CD4⁺ and CD8⁺ T cells from HIV-1–infected individuals. *J Immunol* **2000**; 165:6620–6.
30. Haslett PA, Nixon DF, Shen Z, et al. Strong human immunodeficiency virus (HIV)–specific CD4⁺ T cell responses in a cohort of chronically infected patients are associated with interruptions in anti-HIV chemotherapy. *J Infect Dis* **2000**; 181:1264–72.
31. Bhardwaj N, Seder RA, Reddy A, Feldman MV. IL-12 in conjunction with dendritic cells enhances antiviral CD8⁺ CTL responses in vitro. *J Clin Invest* **1996**; 98:715–22.
32. Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A, Alber G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T–T help via APC activation. *J Exp Med* **1996**; 184:747–52.
33. Oliver SJ, Kukuchi T, Krueger JG, Kaplan G. Thalidomide induces granuloma differentiation in sarcoid lesions associated with disease improvement. *Clin Immunol* **2002**; 102:225–36.
34. Moller DR, Wysocka M, Greenlee BM, et al. Inhibition of IL-12 production by thalidomide. *J Immunol* **1997**; 159:5157–61.
35. Dredge K, Marriott JB, Todryk SM, et al. Protective antitumor immunity induced by a costimulatory thalidomide analog in conjunction with whole tumor cell vaccination is mediated by increased Th1-type immunity. *J Immunol* **2002**; 168:4914–9.
36. Chao NJ, Parker PM, Niland JC, et al. Paradoxical effect of thalidomide prophylaxis on chronic graft-vs.-host disease. *Biol Blood Marrow Transplant* **1996**; 2:86–92.
37. Wolkenstein P, Latarjet J, Roujeau JC, et al. Randomised comparison of thalidomide versus placebo in toxic epidermal necrolysis. *Lancet* **1998**; 352:1586–9.
38. Schoeman JF. Thalidomide therapy in childhood tuberculous meningitis. *J Child Neurol* **2000**; 15:838.
39. Shevach EM, McHugh RS, Thornton AM, Piccirillo C, Natarajan K, Margulies DH. Control of autoimmunity by regulatory T cells. *Adv Exp Med Biol* **2001**; 490:21–32.
40. Ku CC, Murakami M, Sakamoto A, Kappler J, Marrack P. Control of homeostasis of CD8⁺ memory T cells by opposing cytokines. *Science* **2000**; 288:675–8.