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## CONCISE COMMUNICATION

## Thalidomide Suppresses Up-Regulation of Human Immunodeficiency Virus Coreceptors CXCR4 and CCR5 on CD4<sup>+</sup> T Cells in Humans

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Concurrent infection in patients with human immunodeficiency virus (HIV) infection increases the expression of HIV coreceptors CXCR4 and CCR5. Thalidomide has beneficial effects in a number of HIV-associated diseases. The effect of thalidomide on CXCR4 and CCR5 expression on CD4<sup>+</sup> T cells was determined. Thalidomide produced a dose-dependent inhibition of lipopolysaccharide (LPS)-induced up-regulation of CXCR4 and CCR5 *in vitro*. Antibody to tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) also attenuated the LPS-induced HIV coreceptor up-regulation, which was not further reduced by thalidomide. Thalidomide (400 mg) was orally administered to 6 men, and their blood was stimulated *ex vivo* with LPS, staphylococcal or mycobacterial antigens, or antibody to CD3 or CD28 cells. All stimuli induced up-regulation of HIV coreceptors, which was reduced after ingestion of thalidomide. Thalidomide may be beneficial in the treatment of intercurrent infections during HIV infection by reducing the up-regulation of CXCR4 and CCR5 expression on CD4<sup>+</sup> T cells induced by bacterial and mycobacterial antigens, by a mechanism that involves inhibition of TNF- $\alpha$ .

Concurrent infections in patients infected with human immunodeficiency virus (HIV) induce an increase in HIV replication, resulting in a higher plasma virus load and progression of HIV disease. The chemokine receptors CXCR4 and CCR5 can act as HIV coreceptors and are essential for entry of virus into cells [1]. An increase in CXCR4 and CCR5 expression is associated with an enhanced entry of HIV into cells of the immune system [1]. In previous experiments, we found an increased expression of CXCR4 and CCR5 on circulating CD4<sup>+</sup> T cells after intravenous injection of lipopolysaccharide (LPS) into healthy humans and after *in vitro* stimulation of whole blood with various bacterial and mycobacterial antigens, suggesting that intercurrent infections during HIV infection may induce HIV replication by up-regulation of HIV coreceptors [2].

Thalidomide has been rediscovered as a beneficial agent in

a number of diseases with different causes and pathophysiologic features. It has been successfully used in the treatment of patients with refractory tuberculosis (TB) [3] or with an infection due to *Mycobacterium avium* [4]. Thalidomide was reported to stimulate a Th1-type immune response in HIV patients [5]. In particular, patients with concomitant HIV and TB benefit from thalidomide treatment, and the benefit is greater than that among patients with HIV infection alone [3]. *In vitro*, HIV expression in macrophages stimulated with lipoarabinomannan (LAM, a highly immunogenic cell-wall component of *Mycobacterium tuberculosis*) was inhibited by thalidomide, further confirming the beneficial effect of thalidomide in HIV-positive TB patients [6].

Studies of the mechanism of action of thalidomide report the selective degradation of mRNA of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) by thalidomide, resulting in reduced production of TNF- $\alpha$  protein [7]. TNF- $\alpha$  can enhance HIV replication *in vitro* [8]. Furthermore, other reports suggest that thalidomide reduces HIV replication, at least in part, by inhibiting TNF- $\alpha$  production [9, 10]. In the present study, we show that thalidomide inhibits HIV coreceptor expression on CD4<sup>+</sup> T cells induced by bacterial and mycobacterial antigens. This may be a mode of action of thalidomide in HIV patients with concomitant disease.

### Material and Methods

*In vitro studies.* For each experiment, blood was collected from 6 healthy donors by using a sterile collecting system consisting of

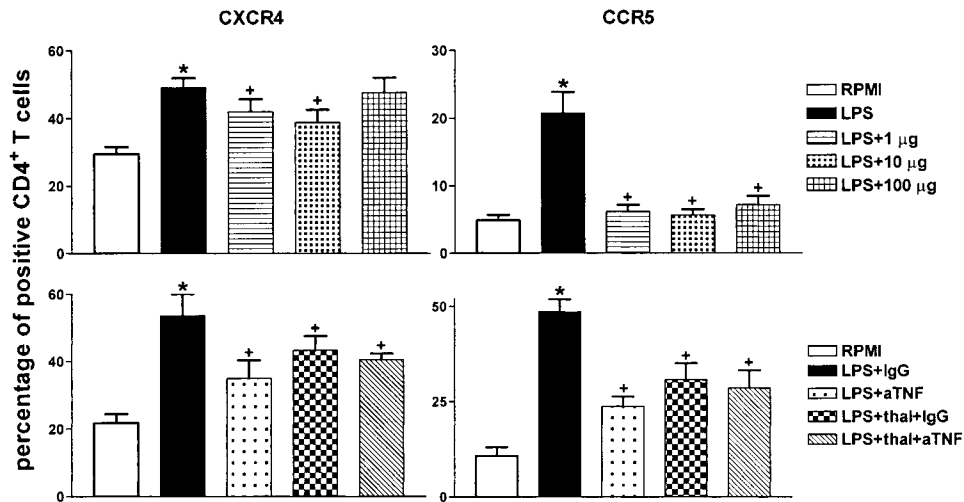
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The studies presented were approved by the institutional scientific and ethics committees of the Academic Medical Center, and written, informed consent was obtained from all volunteers.

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**Figure 1.** Upper panels, Percentage of CD4<sup>+</sup> T cells expressing CXCR4 and CCR5 after stimulation of whole blood with lipopolysaccharide (LPS, 10 ng/mL) in the presence of different concentrations of thalidomide. Lower panels, Percentage of CD4<sup>+</sup> T cells expressing CXCR4 and CCR5 after stimulation of whole blood with LPS in the presence of thalidomide or antibody to tumor necrosis factor- $\alpha$  (aTNF). Whole blood was incubated for 8 h at 37°C. Data are mean  $\pm$  SE of 6 donors. \* $P < .05$  vs. RPMI; + $P < .05$  vs. LPS.

a butterfly needle connected to a syringe (Becton Dickinson, Mountain View, CA). Anticoagulation was obtained by using heparin (Leo Pharmaceutical Products, Weesp, The Netherlands; 10 U/mL of blood). Whole blood was diluted 1 : 1 with RPMI 1640 (BioWhittaker, Verviers, Belgium) to which LPS (10 ng/mL, from *Escherichia coli*, serotype 0111 B4; Sigma, St. Louis) and/or different doses of thalidomide (racemic mixture; Grünenthal, GmbH, Stolberg, Germany), dissolved in dimethyl sulfoxide (DMSO; Merck, Munich) and further diluted in RPMI, were added. Control specimens were incubated with RPMI containing the same amount of DMSO in which the thalidomide was dissolved. Also, blood was stimulated with LPS in the presence or absence of thalidomide (10  $\mu$ g/mL) or a neutralizing mouse anti-human TNF- $\alpha$  monoclonal antibody (MAK 195F; Knoll, Ludwigshafen, Germany) or both [11] or an isotype-matched mouse IgG (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service [CLB], Amsterdam) (both 10  $\mu$ g/mL). Blood was stimulated at 37°C for 8 h, after which fluorescence-activated cell sorter (FACS) analysis was performed, as described below.

**Ex vivo study.** Six healthy men with a median age of 38 years (range, 33–44 years) ingested 400 mg of thalidomide orally. Blood was obtained directly before ingestion of thalidomide and 3, 6, and 24 h thereafter, and one of the following reagents was added: LPS (10 ng/mL), LAM (mannose-capped, isolated, and prepared from *M. tuberculosis* strain H37Rv, which was provided by J. T. Belisle, Colorado State University, Fort Collins, under National Institutes of Health contract N01-A1-75320), 1  $\mu$ g/mL lipoteichoic acid from *Staphylococcus aureus* (LTA; Sigma), staphylococcal enterotoxin B (SEB; 1  $\mu$ g/mL; Sigma), or anti-CD3/CD28 (mouse anti-human CD3, clone SPVT3b, 1 : 500; mouse anti-human CD28, 1 : 1000 [CLB, Amsterdam]). Blood was stimulated at 37°C for 8 h, after which FACS analysis was performed.

**Flow cytometry.** In preparation for FACS analysis, erythrocytes were lysed with bicarbonate-buffered ammonium chloride

solution (pH 7.4). Leukocytes were recovered and counted after centrifugation at 400  $g$  for 5 min, and  $1 \times 10^6$  cells were resuspended in PBS containing EDTA at a concentration of 100 mM, 0.1% sodium azide, and 5% bovine serum albumin (complete PBS) and were placed on ice. Triple staining was obtained by incubation for 1 h with the following directly labeled antibodies: anti-CD3-PE, anti-CD4-Cy (both from Coulter Immunotech, Marseille, France), and either antibody to CXCR4-fluorescein isothiocyanate (FITC) or antibody to CCR5-FITC (R&D Systems, Abingdon, UK). Nonspecific staining was controlled for by incubating cells with FITC-labeled mouse IgG2 (Coulter Immunotech). Cells were washed twice in complete PBS and resuspended for flow cytometric analysis (Calibrite; Becton Dickinson Immunocytometry Systems, San Jose, CA), and  $\geq 8,000$  lymphocytes were counted. Data on the number of CD4<sup>+</sup> T cells expressing either CXCR4 or CCR5 were obtained by setting a quadrant marker for nonspecific staining.

**Statistical analysis.** All values are given as mean  $\pm$  SE. Data on in vitro stimulations were analyzed using the Wilcoxon test for matched samples. Ex vivo data were analyzed using 1-way analysis of variance.  $P < .05$  was considered statistically significant.

## Results

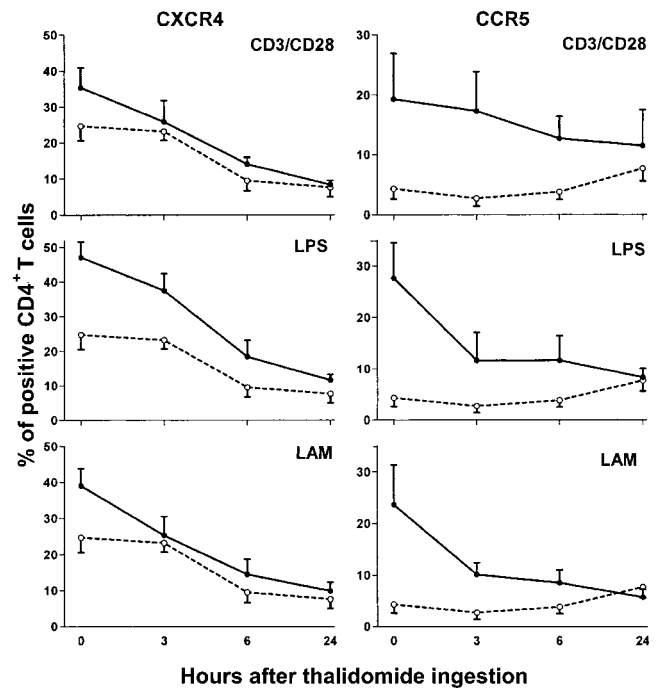
**Thalidomide produces dose-dependent inhibition of LPS-induced HIV coreceptor expression in vitro.** To determine the effect of thalidomide on HIV coreceptor expression, we stimulated blood from healthy donors with LPS in the presence of different concentrations of thalidomide (figure 1). LPS induced an up-regulation of both CXCR4 and CCR5 on CD4<sup>+</sup> T cells, compared with control ( $P < .05$ ). Addition of thalidomide caused a dose-dependent inhibition of this LPS effect. A dose

of 1  $\mu\text{g}/\text{mL}$  inhibited CXCR4 by  $15.3\% \pm 3.2\%$ , and 10  $\mu\text{g}/\text{mL}$  resulted in a  $21.2\% \pm 4.7\%$  inhibition, compared with CXCR4 expression after incubation with LPS alone. CCR5 was inhibited by  $53.9\% \pm 11.4\%$  (1  $\mu\text{g}/\text{mL}$ ) and  $56.8\% \pm 10.3\%$  (10  $\mu\text{g}/\text{mL}$ ). Interestingly, the effect of thalidomide on HIV coreceptor expression appeared biphasic, with 100  $\mu\text{g}/\text{mL}$  having less influence than 10  $\mu\text{g}/\text{mL}$ .

*Thalidomide does not influence HIV coreceptor expression in the presence of antibody to TNF- $\alpha$ .* Thalidomide inhibits TNF- $\alpha$  production by mononuclear cells [7, 12]. To determine whether thalidomide inhibited HIV coreceptor expression via inhibition of TNF- $\alpha$ , we stimulated whole blood with LPS in the presence of thalidomide or a neutralizing antibody to TNF- $\alpha$  or both (figure 1). Both thalidomide and antibody to TNF- $\alpha$  partially blocked LPS-induced up-regulation of CXCR4 and CCR5 on the fraction of CD4<sup>+</sup> T cells, compared with incubation with an irrelevant antibody ( $P < .05$ ). Simultaneous addition of antibody to TNF- $\alpha$  and thalidomide did not further reduce CXCR4 or CCR5 expression, compared with the effects of either antibody to TNF- $\alpha$  or thalidomide alone.

*Reduced HIV coreceptor expression on CD4<sup>+</sup> T cells after ingestion of thalidomide.* Having established that thalidomide can inhibit LPS-induced up-regulation of HIV coreceptors on CD4<sup>+</sup> T cells in vitro, we next determined the effect of a 400-mg oral dose of thalidomide on CXCR4 and CCR5 expression after stimulation of whole blood ex vivo. Aside from drowsiness, volunteers experienced no side effects. Ingestion of thalidomide did not result in a change in leukocyte counts or differentiation. In addition, CD4<sup>+</sup> and CD8<sup>+</sup> counts did not change after ingestion of thalidomide. In unstimulated blood (blood immediately processed for FACS analysis), neither the number of CD4<sup>+</sup> T cells nor the fraction of CD4<sup>+</sup> T cells expressing CXCR4 and CCR5 changed after ingestion of thalidomide (data not shown).

Concurrent infections in HIV-infected patients can be caused by gram-negative, gram-positive, or mycobacterial organisms. Therefore, the effect of an oral dose of thalidomide on HIV coreceptor expression on CD4<sup>+</sup> T cells in humans was determined after ex vivo stimulation with LPS (a cell-wall component of gram-negative bacteria), LAM (a cell-wall lipoglycan of *M. tuberculosis*), LTA (a cell-wall component of *S. aureus*), and SEB (a superantigen from *S. aureus*). In addition, the effect of thalidomide on HIV coreceptor expression induced by anti-CD3/CD28, a specific T-cell stimulus, was determined. Each stimulus induced an increase in CXCR4 and CCR5 expression on CD4<sup>+</sup> T cells vs. incubation with RPMI ( $P < .05$ ). Ingestion of thalidomide inhibited bacterial- and mycobacterial-induced up-regulation of CXCR4 and CCR5 ( $P < .05$  vs.  $t = 0$  for LPS, LAM, and CD3/CD28; figure 2), an effect that was evident after 3 h and peaked after 24 h (thereby ruling out a circadian effect). In addition, thalidomide tended to inhibit LTA- and SEB-induced CXCR4 up-regulation (LTA increasing from  $43.1\% \pm 4.4\%$  to  $9.3\% \pm 2.0\%$  and SEB increasing from



**Figure 2.** Percentage of CD4<sup>+</sup> T cells expressing CXCR4 and CCR5 in blood from 6 volunteers after ingestion of thalidomide. Blood was obtained before and at various time points after oral ingestion of thalidomide (400 mg), and it was subsequently incubated for 8 h without stimulus or with anti-CD3/CD28, lipopolysaccharide (LPS, 10 ng/mL), or lipoarabinomannan (LAM, 10  $\mu\text{g}/\text{mL}$ ). Solid lines represent incubation with the indicated stimuli; dashed lines represent control incubation (without stimulus) with RPMI alone. Data are mean  $\pm$  SE.

$40.0\% \pm 5.3\%$  to  $9.3\% \pm 1.8\%$  positive CD4<sup>+</sup> T cells after 24 h), but this effect did not reach statistical significance. There was no effect of thalidomide on CCR5 expression. Interestingly, thalidomide also reduced CXCR4 expression on unstimulated CD4<sup>+</sup> T cells, probably because CXCR4 expression increases during incubation at 37°C [13].

## Discussion

Concurrent infections in patients with HIV are associated with an increase in HIV replication. The chemokine receptors CXCR4 and CCR5 serve as coreceptors for HIV entry in CD4<sup>+</sup> T cells [1]. Enhanced expression of HIV coreceptors CXCR4 and CCR5 is correlated with an increase in HIV load [1]. We had previously found an up-regulation of CXCR4 and CCR5 expression on CD4<sup>+</sup> T cells in humans injected with endotoxin and after in vitro stimulation with bacterial and mycobacterial antigens [2]. The results of the present study, taken together with those earlier observations, suggest that pathogens commonly found in HIV-infected patients may increase the virus burden in blood by up-regulating HIV coreceptors. In this study, reduced expression of CXCR4 and CCR5 on CD4<sup>+</sup> T

cells was found in blood from volunteers after ingestion of thalidomide and ex vivo stimulation with antigens derived from *M. tuberculosis* and gram-positive and gram-negative bacteria. We hypothesize that, in HIV patients with concurrent disease, a mechanism of action of thalidomide may be the inhibition of HIV coreceptor expression.

Thalidomide reduces symptoms in patients with mycobacterial disease, presumably by inhibiting production of TNF- $\alpha$ . Indeed, in patients with HIV and TB, thalidomide induced a reduction in TNF- $\alpha$  levels, which was associated with weight gain [3]. Most studies on the mechanism of action of thalidomide have concentrated on monocytic cell lines, in which thalidomide selectively inhibits TNF- $\alpha$  production [7]. TNF- $\alpha$  induces HIV replication [9], and antibody to TNF- $\alpha$  blocks HIV replication [6]. Thalidomide can inhibit HIV replication in monocytes stimulated with LPS or LAM [6, 9]. Thalidomide may reduce the HIV load via inhibition of TNF- $\alpha$  synthesis. We previously found that antibody to TNF- $\alpha$  inhibits the expression of CXCR4 and CCR5, whereas recombinant TNF- $\alpha$  increases it [2]. In the present study, both thalidomide and antibody to TNF- $\alpha$  inhibited HIV coreceptor expression on CD4<sup>+</sup> T cells. When thalidomide and antibody to TNF- $\alpha$  were added simultaneously, no further inhibition was seen. As has been shown by studies of the mechanism of action of thalidomide in monocytes [7], thalidomide seems to influence HIV coreceptor expression on CD4<sup>+</sup> T cells, at least in part, by inhibiting TNF- $\alpha$  production.

Previous in vitro experiments provided no evidence that thalidomide has an effect on purified CD4<sup>+</sup> T cells. In addition, concentrations of thalidomide  $\leq 50$   $\mu\text{g/mL}$  were not toxic to CD4<sup>+</sup> T cells [14]. In the present study, thalidomide reduced HIV coreceptor expression on CD4<sup>+</sup> T cells in whole blood stimulated with bacterial and mycobacterial antigens as well as with T cell-activating anti-CD3/CD28. An explanation may be that the effect of thalidomide on CD4<sup>+</sup> T cells requires an environment in which all blood cells are present. Thalidomide treatment in a murine model of pulmonary TB reduced lung mRNA expression not only of TNF- $\alpha$  but also of interleukin (IL)-6 and IL-10 [15]. Therefore, part of the beneficial effect of thalidomide may be produced by modulation of cytokines other than TNF- $\alpha$ .

In summary, ingestion of thalidomide reduced the expression of CXCR4 and CCR5 on CD4<sup>+</sup> T cells in human whole blood stimulated ex vivo with bacterial and mycobacterial antigens, in part via inhibition of TNF- $\alpha$  production. The beneficial effects of thalidomide in HIV-infected patients with intercurrent

infections may be produced by the drug's ability to inhibit HIV coreceptor expression.

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