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Differential Cytokine Modulation and T Cell Activation by Two Distinct Classes of Thalidomide Analogues That Are Potent Inhibitors of TNF-α[1]

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Differential Cytokine Modulation and T Cell Activation by Two Distinct Classes of Thalidomide Analogues That Are Potent Inhibitors of TNF-α[1]

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TNF-α mediates both protective and detrimental manifestations of the host immune response. Our previous work has shown thalidomide to be a relatively selective inhibitor of TNF-α production in vivo and in vitro. Additionally, we have recently reported that thalidomide exerts a costimulatory effect on T cell responses. To develop thalidomide analogues with increased anti-TNF-α activity and reduced or absent toxicities, novel TNF-α inhibitors were designed and synthesized. When a selected group of these compounds was examined for their immunomodulatory activities, different patterns of cytokine modulation were revealed. The tested compounds segregated into two distinct classes: one class of compounds, shown to be potent phosphodiesterase 4 inhibitors, inhibited TNF-α production, increased IL-10 production by LPS-induced PBMC, and had little effect on T cell activation; the other class of compounds, similar to thalidomide, were not phosphodiesterase 4 inhibitors and markedly stimulated T cell proliferation and IL-2 and IFN-γ production. These compounds inhibited TNF-α, IL-1β, and IL-6 and greatly increased IL-10 production by LPS-induced PBMC. Similar to thalidomide, the effect of these agents on IL-12 production was dichotomous; IL-12 was inhibited when PBMC were stimulated with LPS but increased when cells were stimulated by cross-linking the TCR. The latter effect was associated with increased T cell CD40 ligand expression. The distinct immunomodulatory activities of these classes of thalidomide analogues may potentially allow them to be used in the clinic for the treatment of different immunopathological disorders. ***The Journal of Immunology,*** **1999, 163: 380–386.**

Tumor necrosis factor α, a highly pleiotropic cytokine produced primarily by monocytes and macrophages, plays a central role in the host protective immune response to bacterial and viral infections. For example, TNF-α is essential for granuloma formation and the control of bacterial dissemination in experimental tuberculosis in mice (1, 2). In addition, TNF-α added to infected cells in vitro inhibits the replication of both DNA and RNA viruses (3, 4). However, the cytokine may also play a role in the pathogenesis of disease. Perhaps the best evidence for this is the dramatic reduction in disease activity observed in rheumatoid arthritis and inflammatory bowel disease after treatment of patients with neutralizing anti-TNF-α Abs (5, 6). Additionally, elevated levels of TNF-α have been associated with the fevers, malaise, and weight loss that accompany chronic infections (7), and reductions in TNF-α levels have been linked with an amelioration of clinical symptoms in a number of disease states (8–11).

Our previous work has shown thalidomide to be a relatively selective inhibitor of TNF-α production by human monocytes in vivo and in vitro. Leprosy patients with erythema nodosum leprosum treated with thalidomide, experience a reduction of serum TNF-α levels with a concomitant abrogation of clinical symptoms (9). In patients with tuberculosis, with or without HIV infection, thalidomide lowers plasma TNF-α protein levels and leukocyte TNF-α mRNA levels in association with an accelerated weight gain (8). In vitro, thalidomide has been shown to selectively partially (50–70%) inhibit TNF-α produced by monocytes and macrophages stimulated with LPS (12).

Recently, we have reported the ability of thalidomide to costimulate T cells in vitro (13). Thus, in addition to its monocyte cytokine-inhibitory activity, thalidomide exerts a costimulatory or adjuvant effect on T cell responses that includes increased production of IL-2 and IFN-γ. This effect may contribute to the immunemodulating effects of the drug.

To obtain drugs that are more efficient TNF-α inhibitors than thalidomide, structural analogues of the parent molecule have been synthesized and examined for inhibition of TNF-α production. Recently, some of these thalidomide analogues have been described (12, 14, 15, 47). On a molar basis, these reported compounds are up to 50,000-fold more potent than thalidomide at inhibiting TNF-α production by PBMC in vitro. In this study, we have selected six of these compounds and evaluated them for their effects on the production of other monocyte cytokines, as well as their immunomodulatory effects on T cells.

Materials and Methods

Preparation of cells

PBMC were isolated from the blood of healthy volunteers by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density centrifugation as previously described (12). T lymphocytes were purified from PBMC by rosetting with neuraminidase-treated sheep erythrocytes and subsequent incubation of erythrocyte-rosetting cells on a nylon wool column. Nonadherent cells eluted from the column were >93% CD3 Ag positive by flow cytometry (FACStar, Becton Dickinson, San Jose, CA). Leukocytes were cultured in RPMI medium (Life Technologies, Grand Island, NY) supplemented with

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10% AB^+ human serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies).

Thalidomide and analogues

Thalidomide and analogues (Celgene, Warren, NJ) were dissolved in DMSO (Sigma, St. Louis, MO); further dilutions were made in culture medium immediately before use. The final DMSO concentration in all assays was 0.25%. The following structural analogues were used: CII-A is compound 3a (14) and CC-1069 (12); CII-B is compound CC-3052 (16) with the carboxymethyl group replaced by an amide moiety; CII-C is an amino-substituted analogue of compound 4b (14); compounds CI-A, CI-B, and CI-C are amino-substituted analogues of thalidomide. CI-A is 5a, CI-B is 8a and CI-C is 14 (47).

PBMC stimulation by LPS

PBMC (2×10^5 cells) incubated in 96-well flat-bottom polystyrene Costar tissue culture plates (Corning, Corning, NY) were stimulated by 1 μg/ml LPS from *Salmonella* minnesota R595 (List Biological Labs, Campbell, CA) for the induction of TNF-α , IL-1β, IL-6, IL-8, IL-10, and IL-12 (12). Cells were incubated with or without thalidomide or analogues for 20 h, and supernatants were collected for the determination of cytokine levels by ELISA.

PBMC stimulation by anti-CD3 Ab

PBMC (1×10^6 cells) were stimulated by cross-linking of the TCR by immobilized monoclonal mouse anti-human CD3 (Orthoclone OKT3, a kind gift of Dr. R. Zivin, Orthobiotech, Raritan, NJ) as previously described (13). The anti-CD3 Ab was diluted to 10 μg/ml in 100 μl PBS and coated onto 48-well flat-bottom polystyrene Falcon tissue culture plates (Becton Dickinson, Franklyn Lakes, NJ) by overnight incubation at 4°C. Appropriate dilutions of thalidomide and analogues were added at the start of the cell cultures. Supernatants were collected at 24, 48, and 72 h and assayed for IL-10, IL-12, and TNF-α levels. Cells were collected at 48 h for evaluation of CD40 ligand (CD40L)[3] and CD3 surface expression by two-color flow cytometry (anti-CD40L, PharMingen, San Diego, CA; anti-CD3, Becton Dickinson, San Jose, Ca).

T cell stimulation and proliferation assays

Purified T cells (2×10^5 cells/well) in 96-well Costar tissue culture plates (Corning) previously coated with anti-CD3 mAb (as above) were treated daily with thalidomide or analogues for up to 120 h. Supernatants were harvested for IFN-γ assay at 72 h. T cell-proliferative responses were assayed by measuring [^{3}H]thymidine (NEN Products, Boston, MA) incorporation during the last 18 h of 120-h cultures. DNA was harvested onto fiber mats with an automatic cell harvester (Skatron, Stirling, VA), and [^{3}H]thymidine incorporation was measured with a LKB 1205 Betaplate liquid scintillation counter (Wallac, Gaithersburg, MD)

Phosphodiesterase 4 (PDE4) inhibition assay

PDE4 inhibition was evaluated in purified extracts of promonocytic U937 cells using a modified method of Hill and Mitchell (17) as previously described (14). Cells (1×10^9) were washed in PBS and lysed in cold homogenization buffer (20 mM Tris-HCl, pH 7.1; 3 mM 2-ME; 1 mM MgCl; 0.1 mM EGTA, 1 μM PMSF, 1 μg/ml leupeptin). After homogenization with a Dounce homogenizer, the supernatant was collected by centrifugation and loaded onto a Sephacryl S-200 column equilibrated with homogenization buffer. PDE4 was eluted in homogenization buffer, and enzyme activity was determined in 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, and 1 μM cAMP (of which 1% was [^{3}H]cAMP) as described in detail by Thompson et al. (18). Reactions were performed at 30°C for 30 min and terminated by boiling for 2 min. Briefly, cyclic 3′,5′-[^{3}H]AMP was converted to 5′-[^{3}H]AMP by phosphodiesterase. The separation of 5′-[^{3}H]AMP from 3′,5′-[^{3}H]AMP was achieved by enzymatically converting 5′-[^{3}H]AMP to [^{3}H]adenosine with nucleotidase present in snake venom (Sigma, V-0376), 1 mg/ml at 30°C for 15 min. Adenosine was separated from the unreacted cyclic substrate by addition of 200 μl of AG1-X8 resin (Bio-Rad, Hercules, CA) that absorbs cyclic 3′,5′-[^{3}H]AMP. Samples were then spun at 3000 rpm for 5 min, and 50 μl of the aqueous phase were taken for counting of adenosine radioactivity by liquid scintillation techniques. Enzyme activity was determined in the presence of varying concentrations of compounds. IC_{50} values were determined from dose-responses curves derived from at least three independent experiments done in duplicate. IC_{50} values were calculated by nonlinear regression analysis (variable slope) using Prism by GraphPad Software (San Diego, CA).

Cytokine assays

Culture supernatants were harvested at indicated times and frozen immediately at −70°C until assayed in triplicate or duplicate. TNF-α , IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12 (p40 and p70), and IFN-γ levels were measured by ELISA (Endogen, Cambridge, MA) as described by the manufacturer.

Statistical analysis

Data were evaluated by the Friedman test, a nonparametric ANOVA, in view of the small sample size. The SPSS computer program was used. Significance was set at $p < 0.05$.

Results

Effect of thalidomide analogues on LPS-induced cytokine production

A group of thalidomide analogues were selected for their capacity to inhibit TNF-α production by LPS-stimulated PBMC. Their IC_{50} values for TNF-α (the concentration at which each compound was able to inhibit TNF-α levels by 50%) were established when screening these agents (Table I). Although all compounds were efficient TNF-α inhibitors, their dose-response curves were not identical (data not shown). For some compounds, dose-response curves were the classical sigmoidal curves seen for pharmacological antagonists (class II compounds, see below) (12). Other compounds, however, showed a flatter, thalidomide–like dose response (class I compounds, see below) (12). Subsequent experiments were conducted with the compounds at three concentrations: their approximate TNF-α IC_{50}; 3 times the TNF-α IC_{50}; and 10 times the TNF-α IC_{50}. Rolipram, a known TNF-α inhibitor (19), was used as a control. The effect of these drugs on other LPS-induced cytokines was also investigated. Compounds were added at the mentioned concentrations to LPS-stimulated human PBMC, and cytokine secretion into the culture supernatant was evaluated. Fig. 1 shows the effect of the drugs on the production of TNF-α , IL-1β, IL-6, IL-8, IL-10, and IL-12. Compounds clearly segregated into two different classes according to their effects on LPS-induced IL-1β, IL-6, IL-10, and IL-12 cytokines. One class of compounds (class I) showed significant inhibition of IL-1β, at their TNF-α IC_{50}, and almost complete inhibition at higher concentrations, whereas compounds from class II had a more modest inhibitory effect, albeit significant at the higher concentrations (Fig. 1). Similarly, class I compounds significantly inhibited IL-6 levels, whereas class II compounds did not affect IL-6 production (Fig. 1). IL-8 levels were not significantly affected by either class of compounds, although class I showed a very minor trend toward inhibition of IL-8 production (Fig. 1).

Table I. TNF-α and PDE4 inhibition

Compound	IC_{50} Values (μM)[a]	
	TNF-α	PDE4
Thalidomide	194	>500
CI-A	0.01	>100
CI-B	0.10	>100
CI-C	0.04	>100
CII-A	12.6	9.4
CII-B	20.6	15.0
CII-C	0.21	0.04
Rolipram	0.15	0.40

[a] TNF-α IC_{50} values were determined in LPS-stimulated human PBMC from dose-response curves derived from four independent experiments with different donors. PDE4 IC_{50} values were determined in U937-purified enzyme from dose-response curves derived from three independent experiments. IC_{50} values were calculated by nonlinear regression analysis (variable slope) using Prism by GraphPad Software.

[3] Abbreviations used in this paper: CD40L, CD40 ligand; PDE4, phosphodiesterase 4.

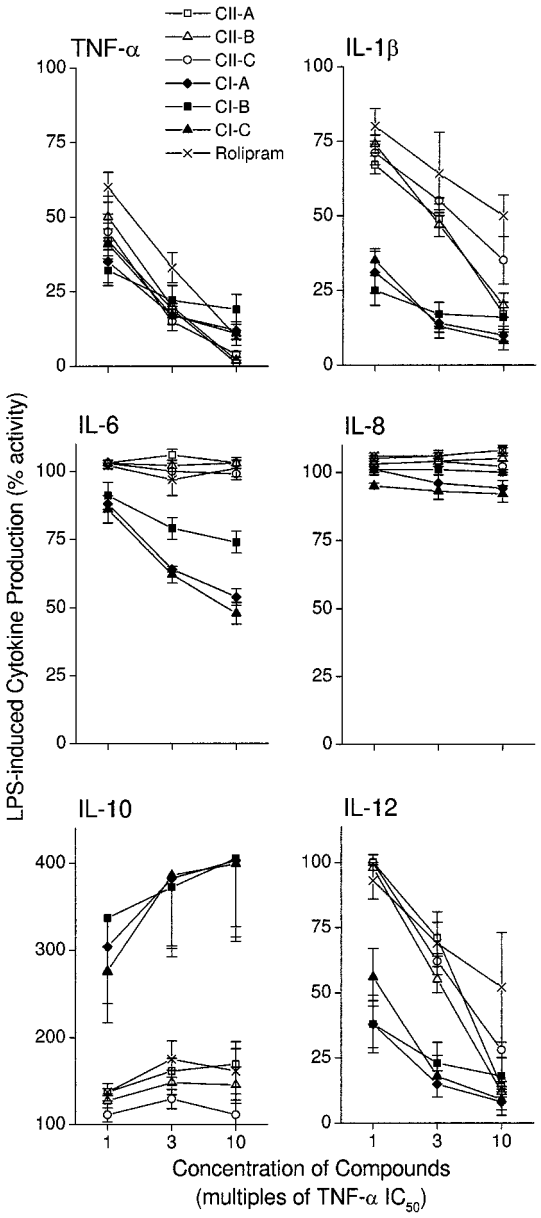

FIGURE 1. Effect of thalidomide analogues on LPS-induced cytokines. PBMC were cultured in triplicate and stimulated with LPS in the absence or presence of the indicated concentrations of thalidomide analogues. Supernatants were collected at 20 h after incubation and tested for cytokine levels by ELISA. Results, expressed as percentage of activity, are averages ± SEM of three to five independent experiments with different donors. Drugs were tested at their TNF-α IC_{50}, 3-fold TNF-α IC_{50}, and 10-fold TNF-α IC_{50}. Class I compounds (closed symbols) inhibited IL-1β and IL-12 and stimulated IL-10 levels significantly ($p < 0.001$) at all concentrations. IL-6 levels were significantly inhibited ($p < 0.001$) at concentrations above the TNF-α IC_{50} values for these drugs. Class II compounds and rolipram (open symbols) inhibited IL-1β and IL-12 significantly only at 3-fold their TNF-α IC_{50} or higher ($p < 0.05$). These compounds did not inhibit IL-6 or IL-8. CII-A, CII-B, and rolipram significantly ($p < 0.05$) increased IL-10 levels at the two higher concentrations.

The effect of these compounds on the levels of the antiinflammatory cytokine IL-10 was also tested. All compounds, except for CII-C, significantly increased IL-10 production. However, IL-10 stimulation by class I compounds was clearly more extensive at all concentrations (Fig. 1).

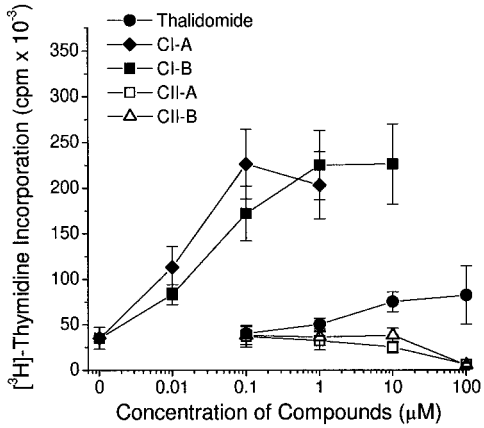

FIGURE 2. Effect of thalidomide and analogues on the proliferative responses of T cells. Purified (>97% $CD3^+$) T cells were cultured in triplicate and stimulated by 10 μg/ml immobilized anti-CD3 in the absence or presence of the indicated concentrations of thalidomide and analogues. [^{3}H]Thymidine incorporation was measured for the last 18 h of 120-h cultures. Results, expressed as cpm, are averages ± SEM of five independent experiments with different donors. Thalidomide and class I compounds increased cell proliferation significantly ($p < 0.005$). Cell proliferation was significantly inhibited by CII-B ($p < 0.05$).

LPS-induced IL-12 levels were significantly inhibited by both classes of compounds at the higher concentrations, but class I compounds were more potent (Fig. 1). Thus, in summary, compounds from class I caused a more pronounced inhibition of LPS-induced IL-1β and IL-12 in addition to the inhibition of IL-6 and a much greater stimulation of IL-10. Class II compounds showed significant inhibitory activities against LPS-induced IL-1β and IL-12 but only at concentrations above their TNF-α IC_{50} values. A modest but consistent stimulation of IL-10 was observed for class II compounds CII-A and CII-B. Rolipram, used as a control, showed cytokine-modulatory profiles comparable with those of class II compounds (Fig. 1).

Effect of class I and class II compounds on T cell-proliferative responses to immobilized anti-CD3 mAb

Optimal T cell activation requires two types of signals (20). Signal 1 is delivered by clustering of the T cell Ag receptor-CD3 complex through engagement of specific foreign peptides bound to MHC molecules on the surface of an APC. Signal 1 can be mimicked by cross-linking the TCR complexes with anti-CD3 mAb. Signal 2 (or costimulation) is Ag independent and may be provided by cytokines or by surface ligands on the APC that interact with their receptors on the T cell. Costimulatory signals are essential to induce maximal T cell proliferation and secretion of cytokines including IL-2 which ultimately drives T cell clonal expansion (20).

Thalidomide was recently reported to provide a costimulatory signal to T cells receiving primary stimulation via the TCR, resulting in increased cytokine production and proliferation (13). We now examined the effect of the two classes of thalidomide analogues on the proliferative responses of purified T cells stimulated by anti-CD3 mAb. Two compounds from each class were tested in these assays. Again, the two classes of compounds showed differential activities. Compounds from class II exerted a modest inhibition of T cell proliferation in response to immobilized anti-CD3, significant for only one of the two compounds (Fig. 2). Class I compounds, however, were potent costimulators of T cells and increased cell proliferation significantly in a dose-dependent manner. As expected, thalidomide was also significantly costimulatory in this assay but not as potent. There was no proliferative response

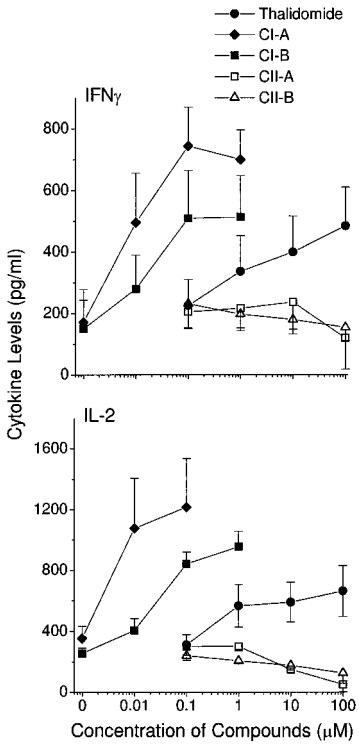

FIGURE 3. Effects of thalidomide and analogues on T cell cytokine production. Purified T cells (for IFN-γ determination) or PBMC (for IL-2 determination) were cultured in triplicate and stimulated with immobilized anti-CD3 in the absence or presence of the indicated concentrations of thalidomide and analogues. IFN-γ levels and IL-2 levels were determined by ELISA. Results, expressed as picograms per milliliter of cytokine, are averages ± SEM of three independent experiments with different donors. Thalidomide and class I compounds increased IL-2 and IFN-γ production significantly ($p < 0.05$ and $p < 0.01$, respectively).

to these drugs in the absence of anti-CD3, indicating that these drugs are not mitogenic per se but provide a secondary, costimulatory signal (data not shown).

Thus, whereas class I compounds had a thalidomide-like costimulatory effect on the proliferative responses of T cells, compounds from class II were modest inhibitors. Rolipram, used as a control, modestly inhibited the proliferation of anti-CD3-stimulated T cells, similarly to class II compounds (data not shown).

Effect of class I and class II compounds on T cell cytokine production

We next evaluated the effect of the two classes of compounds on production of the T cell cytokines IL-2 and IFN-γ. Class I compounds induced significant concentration-dependent increases in IFN-γ at 72 h [peak of the production of this cytokine in this system (13)] (Fig. 3). Class II compounds, on the other hand, either had no effect or slightly inhibited IFN-γ production at higher drug concentrations. As reported previously, thalidomide significantly stimulated IFN-γ production, although it required higher dosages (2–3 logs of magnitude) for activity.

Similarly, IL-2 production was significantly increased in anti-CD3-stimulated PBMC by class I compounds, whereas class II compounds showed no effect (Fig. 3). Thalidomide stimulated IL-2 production significantly but only at higher doses.

Thus, class I compounds were found to be efficient T cell costimulators leading to the augmented production of the T cell cytokines IL-2 and IFN-γ.

Effect of class I and class II compounds on PDE4 activity

PDE4 is one of the major phosphodiesterase isoenzymes found in human myeloid and lymphoid lineage cells. The enzyme plays a crucial role in regulating cellular activity by degrading the ubiquitous second messenger cAMP and maintaining it at low intracellular levels (21). Inhibition of PDE4 and the consequent increased cAMP levels result in the modulation of LPS-induced cytokines including inhibition of TNF-α. As previously reported, class II compounds, similarly to rolipram, are potent PDE4 inhibitors (14). Therefore, we examined the effect of class I compounds on PDE4 activity in purified fractions of the monocytic cell line U937. These compounds did not show significant PDE4 inhibitory activity at up to 100 μM (Table I). These results strongly suggest that the molecular target of the class I compounds is not PDE4. Thus, class I compounds constitute a new class of immunomodulators. These compounds are efficient TNF-α inhibitors but do not act as PDE4 inhibitors. Unlike PDE4 inhibitors, which usually decrease T cell activity, class I compounds are potent stimulators of T cell proliferation and IFN-γ and IL-2 production.

Differential effects of class I compounds on T cell-dependent and T cell-independent cytokine production

IL-12 is produced primarily by APC (monocytes/macrophages and dendritic cells) and is regulated by both T cell-dependent and T cell-independent pathways. LPS induction of IL-12 is an example of the T cell-independent pathway. In the T cell-dependent pathway, on the other hand, the production of IL-12 is induced primarily by the interaction of CD40L on activated T cells with CD40 on IL-12-producing APC (22, 23). To study the effect of thalidomide and class I compounds on cytokine production in a T cell-dependent system, PBMC were stimulated through the TCR with immobilized anti-CD3 mAb, and IL-12, TNF-α, and IL-10 were measured. In this system, both thalidomide and the class I compound CI-A induced significant increases in IL-12 production (Fig. 4). However, thalidomide did not affect the production of TNF-α and IL-10 by anti-CD3. On the other hand, the class I drug CI-A slightly stimulated TNF-α production but significantly inhibited IL-10 production in this system.

We next examined the effect of thalidomide and two class I compounds on the expression of CD40L on T cells stimulated by anti-CD3. Thalidomide and class I compounds induced a dose-dependent and significant increase in CD40L expression that paralleled the increases in IL-12 production induced by anti-CD3 (Fig. 5).

We also tested the effect of the drugs on T-cell independent IL-12 production. PBMC were stimulated with LPS in the presence and absence of the drugs, and IL-12, TNF-α, and IL-10 levels were determined. LPS-induced IL-12 and TNF-α levels were significantly inhibited by thalidomide and by the class I drug CI-A, whereas IL-10 was significantly stimulated (Fig. 4). Similar results were obtained with another class I compound, CI-B (data not shown). Thus, class I compounds modulate IL-12, TNF-α, and IL-10 production differently in cells from the same donor depending on whether the stimulus is directed at the monocytes/macrophages (LPS) or T cells (anti-CD3).

Discussion

To develop analogues of thalidomide with increased anti-TNF-α potency and reduced or absent teratogenic potential, a program to identify improved TNF-α inhibitors was initiated. Here we report that when a selected group of these TNF-α-inhibitory compounds was further characterized, a dichotomous pattern in cytokine modulation activities was revealed. Although all tested compounds

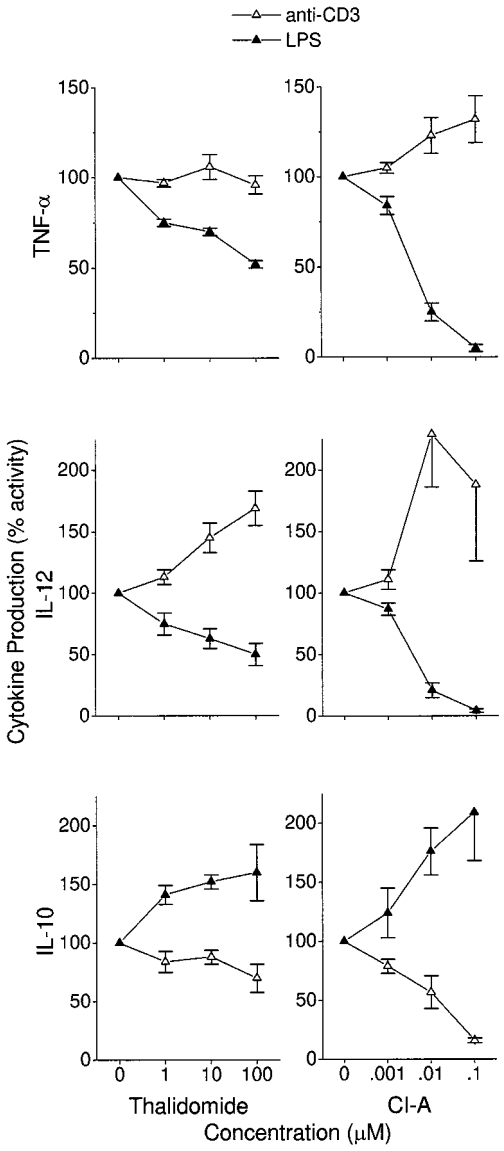

FIGURE 4. Effect of thalidomide and class I compound CI-A on anti-CD3 and LPS-induced TNF-α, IL-12, and IL-10 levels. PBMC were stimulated with anti-CD3 or LPS and treated with thalidomide and Class I-A at the indicated concentrations. LPS-induced cytokines were determined at 24 h after stimulation. Anti-CD3-induced TNF-α was determined at 24 h; anti-CD3 induced IL-12 and IL-10 levels were determined at 72 h after stimulation. Results, expressed as percentage of activity, are averages $\pm$ SEM of three to six independent experiments with different donors. Thalidomide and CI-A increased anti-CD3-induced IL-12 production significantly ($p < 0.05$), whereas LPS-induced IL-12 and TNF-α levels were significantly inhibited by the drugs ($p < 0.01$ and $p < 0.05$, respectively). LPS-induced IL-10 levels were increased by thalidomide and CI-A ($p < 0.05$) whereas anti-CD3-induced IL-10 was significantly inhibited by CI-A only ($p < 0.05$). No IL-12, IL-10, or TNF-α was detected in cultures with or without added compounds in the absence of LPS or anti-CD3 (data not shown).

were much more potent TNF-α inhibitors than the parent drug thalidomide, they differed in the slope of their dose-response curves as well as in the modulation of other monocyte and lymphocyte cytokines. Members of one class of compounds, referred to here as class I, were broad inhibitors of the LPS-induced proinflammatory monocyte cytokines TNF-α , IL-1β, IL-6, and IL-12 while potently augmenting the secretion of the antiinflammatory cytokine IL-10. Class II compounds, on the other hand, inhibited

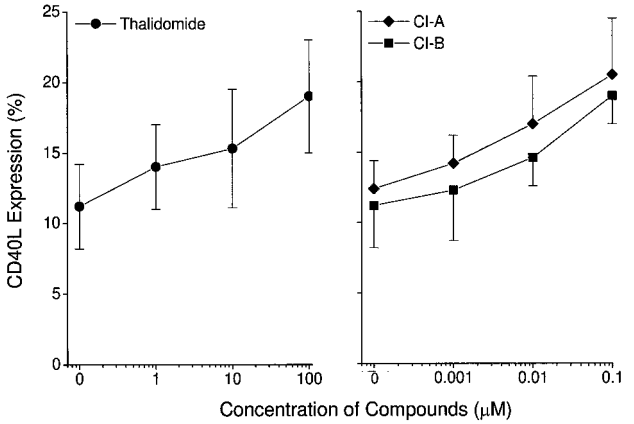

FIGURE 5. Effect of thalidomide and two class I compounds on CD40L expression by $CD3^+$ cells in PBMC stimulated by anti-CD3. Cells were treated with thalidomide and analogues and harvested for two-color cytometric analysis at 48 h. Results, expressed as percentage of cells staining for CD40L, are averages $\pm$ SD from four independent experiments with different donors. All three drugs increased CD40L expression significantly ($p < 0.05$).

both TNF-α and IL-12 in LPS-stimulated PBMC but had little effect on the production of other LPS-induced monocyte cytokines such as IL-1β, IL-6, or IL-8. The latter drugs also produced a modest stimulation of LPS-induced IL-10 levels. In all of these effects, class II compounds closely resemble thalidomide (12).

Recently, we reported that thalidomide provides a costimulatory signal to T cells, resulting in increased T cell proliferation and augmented IL-2 and IFN-γ production (13). Resting T cells require a costimulus in addition to the primary signal mediated by the T cell Ag receptor to achieve optimal activation (20). Such a costimulus alone will not activate the T cell. Similarly to thalidomide, class I compounds also exhibited T cell costimulatory properties but were far more potent than the parent molecule in this respect. Thus, these compounds caused marked increases in proliferation and secretion of IL-2 and IFN-γ by anti-CD3-stimulated T cells. In the absence of the TCR-mediated stimulus, however, the drugs had no activating effect. Costimulation by class I compounds also resulted in increased CD40L expression on T cells, associated with enhanced T cell-dependent IL-12 production. These findings show that in addition to their strong antiinflammatory properties, class I compounds efficiently costimulate T cells, achieving both effects with 100 to 1000 times the potency of thalidomide.

The different cytokine-modulatory profiles of the two classes of compounds are likely to be related to their molecular targets. Class II compounds are potent inhibitors of PDE4 (14). PDE4 inhibition leads to increases in intracellular cAMP levels resulting in the suppression of TNF-α and IL-12 production and increased production of the antiinflammatory cytokine IL-10 (24, 25). IL-6 and IL-8, on the other hand, are not directly regulated by cAMP (25, 26). IL-1β is only partially affected by inhibition of PDE4 (26, 27). Thus, PDE4 inhibitors appear to have a selective antiinflammatory action. It is also well established that raising cAMP levels in T cells during the early phase of mitogen or Ag activation results in a decrease in proliferative potential (28, 29). Indeed, class II compounds modestly but consistently inhibited T cell proliferation and T cell cytokine production, in accord with rolipram and other known PDE4 inhibitors (29, 30). In addition, class II compounds either inhibited or had no effect on CD40L expression on T cells. Although there are no reports on the effects of PDE4 inhibitors on CD40L expression, other cAMP-elevating agents have been shown to be unable to induce CD40L expression on T lymphocytes (31).

All these results are consistent with the notion that the class II compounds described here act via PDE4 inhibition.

In contrast, thalidomide and class I compounds do not have activity against PDE4 at concentrations below 100 μM. The possibility that metabolites or degradation products of these drugs may weakly inhibit PDE4 has not been ruled out. However, because PDE4 inhibition is not associated with increased T cell activation, thalidomide and class I compounds must have another molecular target. The molecular mechanism of action of thalidomide and class I compounds is currently under investigation.

Thalidomide has been reported to inhibit IL-12 production by LPS-stimulated monocytes (32), and here we have confirmed this effect. In vivo, however, thalidomide treatment has been found to increase IL-12 levels in the plasma of tuberculosis and HIV-infected patients (Ref. 48 and our manuscript in preparation). The dual and opposite effects of thalidomide on IL-12 production may be explained by the present findings. Whereas thalidomide inhibits LPS-induced monocyte IL-12 production, the drug stimulates the production of this cytokine in T cell-dependent systems. Thus, the effect of the drug on IL-12 varies according to the nature of the stimulus and the cell type being stimulated. Indeed, this Janus-faced attribute of thalidomide can explain the coincidence of seemingly opposite effects in some clinical situations. For example, in the management of the Behçet syndrome, thalidomide is very effective in healing the debilitating orogenital ulceration (antiinflammatory effect), while simultaneously inducing or exacerbating erythema nodosum (possible T cell-costimulatory effect) in these patients (33).

The analogues studied here appear to have "inherited" different properties from the parent drug. The distinct immunomodulatory activities of two classes of thalidomide analogues suggest that they may have differing applications in different immunopathologic disorders. Therapeutic elevation of intracellular cAMP levels by PDE4 inhibitors has antiinflammatory effects, with consequent benefits in a variety of diseases such as asthma (34), rheumatoid arthritis (35), and atopic dermatitis (36). However, in general, the efficacy of PDE4 inhibitors in clinical trials has been disappointing because of dose-limiting side effects, most prominently nausea and vomiting (37). Preliminary results with thalidomide-derived PDE4 inhibitors in animals have not shown any evidence of emesis.

In addition to their potential use as antiinflammatory drugs, class I compounds could also be useful in clinical settings where there is a defect in T cell function or IL-12 responses, as in HIV disease (38). IL-12 has been shown to restore HIV-specific cell-mediated immunity in vitro (39). In addition, IL-12 has been shown to exhibit potent antitumor activity in murine tumor models through various mechanisms including the stimulation of NK cells (40), $CD8^+$ cytotoxic T cells (41), and IFN-γ-mediated antiangiogenesis (42). Our recent finding of the preferential costimulation of $CD8^+$ T cells by thalidomide (13) and the observation that thalidomide and class I compounds induce T cell-dependent IL-12 production suggest possible applications of these drugs in the control of viral infections (43, 44) or in boosting antitumor immunity (45, 46).

In conclusion, through the design and selection of analogues more active than thalidomide in inhibiting LPS-induced TNF-α production, we have identified two different classes of immunomodulators that share with thalidomide its anti-TNF-α activity. However, the two classes of compounds possess different properties. Preliminary results indicate that at least some of these new compounds are nontoxic and nonteratogenic (Ref. 16 and unpublished observations). These compounds with distinct immunomodulatory effects will be used as investigational tools in animal disease models to define mechanisms of pathogenesis and to continue to elucidate the mechanism of action of thalidomide.

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