Immunomodulation by thalidomide and thalidomide analogues

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Tumour necrosis factor α (TNFα), a key cytokine involved in the host immune response, also contributes to the pathogenesis of both infectious and autoimmune diseases. To ameliorate the pathology resulting from TNFα in these clinical settings, strategies for the inhibition of this cytokine have been developed. Our previous work has shown that the drug thalidomide is a partial inhibitor of TNFα production in vivo. For example, when leprosy patients suffering from erythema nodosum leprosum (ENL) are treated with thalidomide, the increased serum TNFα concentrations characteristic of this syndrome are reduced, with a concomitant improvement in clinical symptoms. Similarly, we have found that in patients with tuberculosis, with or without HIV infection, short-term thalidomide treatment reduces plasma TNFα levels in association with an accelerated weight gain. In vitro, we have also shown that thalidomide partially inhibits TNFα produced by human peripheral blood mononuclear cells (PBMC) responding to stimulation with lipopolysaccharide (LPS). Recently, we found that thalidomide can also act as a costimulatory signal for T cell activation in vitro resulting in increased production of interleukin 2 (IL2) and interferon γ (IFNγ). We also observed a bidirectional effect on IL12 production: IL12 production is inhibited by thalidomide when PBMC are stimulated with LPS, however, IL12 production is increased in the presence of the drug when cells are stimulated via the T cell receptor. The latter effect is associated with upregulation of T cell CD40 ligand (CD40L) expression. Thus, in addition to its monocyte inhibitory activity, thalidomide exerts a costimulatory or adjuvant effect on T cell responses. This combination of effects may contribute to the immunomodulating properties of the drug.

To obtain drugs with increased anti-TNFα activity that have reduced or absent toxicities, novel TNFα inhibitors were designed using thalidomide as template. These thalidomide analogues were found to be up to 50 000 times more active than thalidomide. The compounds comprise two different types of TNFα inhibitors. One class of compounds, shown to be potent phosphodiesterase 4 (PDE4) inhibitors, are selective TNFα inhibitors in LPS stimulated PBMC and have either no effect or a suppressive effect on T cell activation. The other class of compounds also inhibit TNFα production, but do not inhibit PDE4 enzyme. These compounds are also potent inhibitors of several LPS induced monocyte inflammatory cytokines. Also, the latter compounds markedly stimulate the anti-inflammatory cytokine IL10. Similarly to thalidomide, these drugs that do not inhibit PDE4 act as costimulators of T cells but are much more potent than the parent drug. The distinct immunomodulatory activity of these new TNFα inhibitors may potentially allow them to be used in the clinic for the treatment of a wide variety of immunopathological disorders of different aetiologies.

TNFα is a key player in the immune response

TNFα is a pleiotropic cytokine produced primarily by monocytes and macrophages, but also by lymphocytes and NK cells. TNFα plays a central part in the host immune response to viral, parasitic, fungal and bacterial infections. The importance of TNFα and TNFα signalling through its receptors in the host immune response to disease has become clearer as a result of a number of seminal studies. For example, mice genetically deficient in TNFα have a significantly reduced humoral immune response to adenovirus infection.1 In Leishmania major infection, TNFα signalling is important for protection as mice lacking TNFα p55 receptor (TNFR-p55) show delayed elimination of the parasites compared with controls and the lesions formed failed to resolve.2 Mice deficient in TNF-R-p55 are also significantly impaired in their ability to clear infection with Candida albicans and readily succumb to the infection. TNFα signalling is also crucial in resisting Streptococcus pneumoniae infections in mice.3 In addition, TNFα is essential for protection against murine tuberculosis. TNF-R-p55 deficient mice have been shown to be more susceptible to tuberculosis infection. When TNFα was neutralised in vivo by monoclonal antibodies impaired protection against mycobacterial infection was observed.4 The data from both models also established that TNFα and the TNF-R p55 are essential for production of reactive nitrogen intermediates by macrophages early in infection.

TNFα contributes to disease pathogenesis

Although TNFα is crucial to the protective immune response, it also plays a part in the pathogenesis of both infectious and autoimmune diseases. Increased concentrations of TNFα have been shown to trigger the lethal effects of septic shock syndrome.5 TNFα has also been implicated in the development of cachexia, the state of malnutrition that complicates the course of chronic infections and many cancers.5 In rheumatoid arthritis, TNFα is a critical mediator of joint inflammation and therefore an important therapeutic target.
Recently, it has been shown that treatment of patients with neutralising anti-TNFα antibodies produces a dramatic reduction in disease activity in this condition.9 Similarly, it has been shown that in inflammatory bowel disease, neutralisation of TNFα results in a profound amelioration of clinical symptoms.9 10 Reductions in TNFα levels have also been linked with a significant reduction of clinical symptoms in leprosy patients with ENL, including fever, malaise, and arthritic and neuritic pain.11 In tuberculosis patients, reduction of TNFα levels was associated with accelerated weight gain.12

**Thalidomide inhibits TNFα production by monocytes**

The pathology associated with TNFα production is profound and in many diseases leads to significant morbidity and mortality. This has led to a concerted effort to discover drugs that will down regulate the production of this cytokine. Agents conventionally used in these diseases may inhibit TNFα production, but are also often broadly immunosuppressive (for example, cyclosporin A and corticosteroids) and therefore associated with extensive side effects.12 Drugs that are potentially more specific in inhibiting TNFα are under active investigation and development. Our previous work has shown that the drug thalidomide (α-N-phthalimidiglutaramide) is a relatively selective inhibitor of TNFα production by human monocytes in vivo. This property of thalidomide was first described in leprosy patients with ENL, an acute inflammatory condition in leprosy. In addition, thalidomide inhibited TNFα production by human monocytes in vitro in association with enhanced degradation of TNFα mRNA.13 It also inhibits the activation of the nuclear factor κB (NfκB),14 20 a promoter for the transcription of TNFα as well as transcription of HIV-1.21 22

**Thalidomide has T cell costimulatory properties**

Recently, we reported that thalidomide also has a hitherto unappreciated immunomodulatory effect: the drug was shown to costimulate human T cells in vitro, synergising with stimulation via the T cell receptor complex to increase IL2 mediated T cell proliferation and T cell IFNγ production.23 Optimal T cell activation requires two signals.24 The first signal or signal 1 is delivered by clustering of the T cell antigen-receptor-CD3 complex through engagement of specific foreign peptides bound to MHC molecules on the surface of an antigen presenting cell (APC). Signal 1 can be mimicked by crosslinking the T cell receptor (TCR) complexes with anti-CD3 antibodies. Signal 2 (or costimulation) is antigen 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 IL2, which ultimately drive T cell clonal expansion. As antigenic stimulation in the absence of costimulatory signals leads to T cell anergy or apoptosis, costimulation is critically important in the induction and regulation of cellular immunity.

Thalidomide appears to act as a costimulator to T cells that have received signal 1 via the TCR.25 In our experiments in vitro, stimulation of purified T cells with anti-CD3 antibodies, in the absence of signal 2, induced only minimal T cell proliferation. However, the addition of thalidomide to this cell culture system resulted in an increase in T cell proliferation and secretion of cytokines, including IL2, which ultimately drive T cell clonal expansion. As antigenic stimulation in the absence of costimulatory signals leads to T cell anergy or apoptosis, costimulation is critically important in the induction and regulation of cellular immunity.24 The thalidomide mediated costimulation of T cell proliferation was accompanied by increases in IL2 and IFNγ production. It is noteworthy that in the absence of anti-CD3, there was no T cell proliferative response to thalidomide, indicating that the drug is not mitogenic in itself. It is also interesting to note that in these experiments, thalidomide did not inhibit TNFα production by purified T cells stimulated by anti-CD3 antibodies. This is in contrast with the effects of the drug on TNFα produced by monocytes. As already described above, thalidomide inhibits monocyte TNFα production. The costimulatory effect of thalidomide was greater on the CD8+ T cells than on the CD4+ T cell subset.

In addition to its effects on T cell proliferation and T cell cytokine production, we observed that thalidomide induced the up-regulation of CD40L expression on activated T cells.25 26 CD40L/CD40 interaction occurs early in the sequence of signalling events between T cells and antigen presenting cells (APC). Signalling through CD40 has been shown to activate APC and to induce expression of costimulatory molecules such as B7, as well as stimulating production of IL12.27 28 Thus, CD40 signalling results in a stimulatory feedback mechanism in which the activated APC amplifies the T cell response.29 It has also been suggested that CD40L function is essen-
tial for the survival of CD8+ T cells and that in its absence these cells die or become anergic.30

These studies show that in addition to its inhibitory effect on the production of mono-
cyte cytokines, thalidomide exerts a costimulatory or adjuvant effect on T cell responses. The
immune modulating effects of the drug in patients may thus be attributable to a balance be-
tween the inhibition of production of mono-
cyte cytokines, including TNFα, and the costimulation of T cell activity. The effects of
thalidomide in vivo in HIV infected patients seem to reflect the costimulatory activity of the
drug.25 In a placebo controlled study to eval-
uate the effects of in vivo immunomodulation with thalidomide, the drug was administered
for four weeks to HIV infected patients. Thalidomide treatment did not affect TNFα
levels in these patients. In contrast, thalidomide
treatment resulted in significant immune stimulation. This was reflected by increases in
dTH responses and increased plasma levels of T cell activation markers such as soluble IL2
receptor (sIL2R) and soluble CD8 antigen. An ear-
erly study of tuberculosis patients treated
with thalidomide showed increased plasma
levels of IFNγ suggesting an immunostimula-
tory effect of the drug.12 Recently, patients suf-
fering from sarcoidosis have shown consistent
increases in sIL2R plasma levels after thalido-
mide treatment (Oliver et al, manuscript in
preparation). In the same study, thalidomide
treatment increased the proliferation of sarco-
aid patient T cells in response to concanavalin A in
vitro. These results strongly suggest that
thalidomide directly stimulates T cells in vivo
in patients, corresponding to the T cell
costimulatory properties of the drug observed
in vitro in T cells from normal donors23, 25 as
well as in the T cells of HIV infected patients.26

Thalidomide analogues are improved
TNFα inhibitors
In addition to being the drug of choice for the
treatment of ENL, thalidomide has been
shown to be useful in a number of clinical situ-
ations including rheumatoid arthritis, HIV
associated aphthous ulcers and chronic graft
versus host disease.31–34 However, thalidomide
is a potent teratogen and ingestion of the drug
by a pregnant woman can lead to catastrophic
birth defects.35 In addition, thalidomide treat-
ment is often accompanied by a number of side
effects, including peripheral neuropathy.16
Therefore, the use of thalidomide requires
strict monitoring of all patients.37 Thus, there is
a pressing need to develop drugs with increased
TNFα inhibitory activity and reduced or
absent toxicities. Towards this end, structural
analogues of thalidomide have been designed
and synthesised at Celgene Corporation (War-
ren, New Jersey) and screened for inhibition of
TNFα production. A large number of potent
novel TNFα inhibitors were thus identified.
Recently, some of these compounds were
described.20–38 On a molar basis, the more
potent of these thalidomide analogues were
found to be up to 50 000-fold more potent
than thalidomide at inhibiting TNFα produc-
tion by human PBMC stimulated by LPS in
vitro. Furthermore, we have shown that some
of these compounds retain high activity in LPS
stimulated human whole blood.40 In vivo,
several of these new compounds showed
improved activity in reducing LPS induced
TNFα levels in mice39 and in inhibiting the
development of adjuvant arthritis in rats.40

Thalidomide analogues comprise two
distinct classes of molecules
A group of thalidomide analogues, selected for
their capacity to potently inhibit TNFα pro-
duction by LPS stimulated PBMC, was further
investigated (fig 1). When tested for their effect
in vitro on LPS induced cytokines, different
patterns of cytokine modulation were shown.25
One class of compounds, class I or IMiDs
(Immunomodulatory Imide Drugs) showed not
only potent inhibition of TNFα but also
marked inhibition of LPS induced monocyte
IL1β and IL12 production. LPS induced IL6
was also inhibited by these drugs, albeit
partially. These drugs were potent stimulat-
or of LPS induced IL10, increasing IL10 levels by
200–300%. In contrast, the other class of com-
ponents, class II or SelCiDs (Selective Cytokine
Inhibitory Drugs), while still potently inhib-
iting TNFα production, had a more modest
inhibitory effect on LPS induced IL1β and
IL12, and did not inhibit IL6 even at high drug
centrations. In addition, SelCiDs produced a
more modest IL10 stimulation (20–50%
increases). In all of these characteristics, SelCi-
Ds were more similar to thalidomide than IMiDs.16, 17

Further characterisation of the SelCiDs
showed that they are potent PDE4 inhibitors.10
PDE4 is one of the major phosphodiesterase
isoenzymes found in human myeloid and lym-
phoid lineage cells.41 The enzyme plays a
crucial part in regulating cellular activity by
degradation the ubiquitous second messenger
cAMP and maintaining it at low intracellular
levels. Inhibition of PDE4 results in increased
cAMP levels leading to the modulation of LPS
induced cytokines including inhibition of
TNFα.42 Increasing intracellular cAMP levels
have been shown to inhibit TNFα production
in monocytes as well as in lymphocytes.41, 42
although it is not clear how this inhibition is

Figure 1 Chemical structures of thalidomide and selected thalidomide analogues.
Thalidomide and IMiDs modulate cytokines differently according to cell type and stimulation pathway

As described above, thalidomide has been shown to inhibit IL12 production by LPS stimulated monocytes in vitro. In vivo, however, thalidomide treatment of HIV infected and M. tuberculosis infected patients induced increases in plasma IL12 levels (Bekker et al, submitted data). Thalidomide treatment also resulted in increases in plasma IL12 levels in patients with scleroderma and sarcoidosis (Oliver et al, manuscripts in preparation). These dual and opposite effects of thalidomide may be explained by the differential modulation of cytokines according to target cell type and specific pathways of cellular stimulation.

IL12 is produced primarily by APC (monocytes/macrophages and dendritic cells) and is regulated by both T cell dependent and T cell independent pathways. LPS directly induces T cell independent IL12 production by APC, which is inhibited by thalidomide. In the T cell dependent pathway, on the other hand, the production of IL12 by the APC is induced primarily by the interaction of CD40 on the surface of the APC with CD40L on the surface of activated T cells. When T cells were stimulated by anti-CD3, thalidomide and IMiDs treatment caused a significant stimulation of IL12 production. Thalidomide and IMiDs also induced an up-regulation of CD40L on the surface of T cells. Blockade of this pathway inhibits the production of IL12 and abolishes the stimulatory effect of thalidomide. Interestingly, in HIV infected patients, the consistent increases in plasma IL12 levels induced by thalidomide treatment lagged behind the increases in T cell activation markers. This observation suggested that IL12 production was augmented as a consequence of drug induced T cell activation.

The dichotomous nature of thalidomide cytokine modulation may explain the seemingly opposite effects observed in different clinical situations. When patients with Behçet’s syndrome are treated with thalidomide, healing of inflammatory aphthous ulcers occurs, but is sometimes accompanied by exacerbation of erythema nodosum. Similarly, the paradoxical worsening of graft versus host disease and toxic epidermal necrolysis reported in clinical trials of thalidomide may be a manifestation of the unsuspected immune stimulatory effect of this drug.

Potential clinical applications of thalidomide and thalidomide analogues

The thalidomide analogues discussed here seem to have retained different properties of the parent drug (table 1). The distinct immunomodulatory activities of these two classes of drugs suggest they may have applications in different immunopathological disorders. SelCIDs, which inhibit PDE4, may be used in clinical situations in which PDE4 inhibition and selective TNFα inhibition are beneficial. Therapeutic increase of intracellular cAMP levels by PDE4 inhibitors has anti-inflammatory effects, which may afford consequent benefits in a variety of diseases such as asthma, atopic dermatitis and rheumatoid arthritis. Indeed, in an animal model of adjuvant arthritis, thalidomide derived PDE4 inhibitors have shown efficacy in suppressing the development of disease as measured by ankle swelling, hind limb radiographic changes and weight gain. The suppression of arthritis was accompanied by a reduction in TNFα and IL2 mRNA levels in the ankle joints of treated rats.

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Table 1 Immunomodulatory profiles of thalidomide and thalidomide analogues
other known selective PDE4 inhibitors, such as rolipram, have been reported to have dose limiting side effects, such as nausea and vomiting, which limit the therapeutic use of these drugs.\(^5\) \(^4\) These side effects may be produced by the lack of specificity of these drugs—that is, the compounds inhibit one or more PDE isoenzymes in non-target tissues. For example, it is probable that the emetic activity of PDE4 inhibitors is attributable to an action of the drugs in the CNS.\(^9\) \(^9\) Intensive effort is being directed towards identifying compounds with improved therapeutic ratios. Preliminary results with thalidomide derived PDE inhibitors indicate that these novel drugs are selective inhibitors of PDE4 and may be better tolerated than other PDE4 inhibitors, as they have not shown evidence of emesis in animals. One of these drugs has been recently shown to be well tolerated in a small human safety trial in the United Kingdom (D Stirling, personal communication).

The IMiDs, as thalidomide, are anti-inflammatory drugs that do not target PDE4. These compounds, in addition to their potential use to decrease inflammation, could also be useful in clinical settings where there is a defect in T cell function, as in HIV disease. HIV infection is accompanied by deficiencies in the production of IL12 and in the up-regulation of CD40L.\(^5\) \(^6\) IL12 has been shown to restore HIV specific cell mediated immunity in vitro\(^5\) and to increase HIV specific CTL responses in vitro\(^9\) and in vivo.\(^5\) Also, deficient IL12 responses in HIV infected patients can be restored in vitro by CD40L and IFN\(\gamma\),\(^5\) the same costimulatory factors induced by thalidomide and IMiDs. Thus, these drugs may eventually be used to restore or stimulate IL12 production in immune deficient patients.

IL12 has also been shown to exhibit potent anti-tumour activity in murine tumour models through various mechanisms including the stimulation of natural killer cell activity,\(^7\) activation of CD8+ cytotoxic T cells\(^5\) and increased IFN\(\gamma\) mediated anti-angiogenesis.\(^4\) Thalidomide has also recently been reported to exhibit anti-tumour activity through the inhibition of angiogenesis in vivo.\(^6\)\(^5\) However, this anti-angiogenic effect does not seem to be mediated by TNF\(\alpha\) inhibition. Although these studies did not determine the mechanism of thalidomide's anti-angiogenic activity, it is conceivable that stimulation of IFN\(\gamma\)/IL12 levels may be at least partly responsible. One report indicates that thalidomide may have anti-angiogenic activity in multiple myeloma in humans.\(^8\)

In summary, our recent findings that thalidomide and IMiDs preferentially costimulate CD8+ T cells and induce T cell dependent IL12 production suggest possible applications of these drugs in the control of viral infections\(^5\) or in boosting anti-tumour immunity.\(^7\)\(^7\) Also, there are anecdotal reports of the efficacy of thalidomide in treating refractory inflammatory bowel disease.\(^7\) \(^4\) Recently, preliminary findings were announced from a pilot study with patients with Crohn's disease refractory to standard treatments (Annual Digestive Disease Meeting, May 1999, Orlando, FL). In this study, two third of the patients experienced a significant improvement in their condition. This therapeutic effect may be due to the combination of TNF\(\alpha\) inhibition and CD8+ T cell stimulation.\(^7\)\(^7\)

Conclusions
In several disease conditions such as septic shock, chronic infections and cancer, overproduction of TNF\(\alpha\) is accompanied by severe toxicities. Thalidomide inhibits TNF\(\alpha\) production in different diseases without causing the immunosuppression often associated with standard agents such as glucocorticoids and cyclosporin A. Our results indicate that the immunomodulating effects of thalidomide may occur via the inhibition of TNF\(\alpha\) production and/or the stimulation of T cell responses, without the suppression of host immunity.

Recent efforts have concentrated on developing TNF\(\alpha\) inhibitors that are efficient, safe and specific. The collaboration between Rockefeller University and Celgene Corporation scientists has led to the discovery of two different classes of immunomodulators derived from thalidomide and selected for their potent anti-TNF\(\alpha\) inhibitory activity. Preliminary results indicate that at least some of these new compounds are non-toxic and non-teratogenic.\(^29\) The two classes of thalidomide analogues, however, possess distinct properties. IMiDs are potent inhibitors of monocyte inflammatory cytokine production and also are strong costimulators of T cell activity. SelCiDs, on the other hand, are potent PDE4 inhibitors and thus, more selective inhibitors of TNF\(\alpha\). Unlike IMiDs, these compounds do not costimulate T cells but inhibit T cell activity. Thus, the two classes of compounds may prove to be useful in different clinical settings according to their immunomodulatory properties. The thalidomide analogues are being used as investigational tools in animal disease models to define mechanisms of pathogenesis and to continue to elucidate the mechanisms of drug action.

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