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Immunomodulatory effects of thalidomide analogs on LPS-induced plasma and hepatic cytokines in the rat

Eduardo Fernández-Martínez[a], Martha S. Morales-Ríos[b], Víctor Pérez-Álvarez[a], Pablo Muriel[a,*]

[a]*Section of Pharmacology, CINVESTAV-I.P.N., Apdo. Postal 14-740, México 07000, DF*
[b]*Department of Chemistry, CINVESTAV-I.P.N., Apdo. Postal 14-740, México 07000, DF*

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Abstract

Thalidomide has shown to inhibit, selectively and mainly the cytokine tumor necrosis factor-α (TNF-α), thus, thalidomide has inhibitory consequences on other cytokines; this is ascribed as an immunomodulatory effect. Novel thalidomide analogs are reported with immunomodulatory activity. The aim of this work was to synthesize some of these analogs and to assess them as immunomodulatory agents in an acute model of LPS-induced septic challenge in rat. Animal groups received orally twice a day vehicle carboxymethylcellulose (0.9%), or thalidomide in suspension (100 mg/kg), or analogs in an equimolar dose. Two hours after last dose, rats were injected with saline (NaCl, 0.9%, i.p.) or LPS (5 mg/kg, i.p.). Groups were sacrificed 2 h after injection and samples of blood and liver were obtained. TNF-α, interleukin-6, -1β, and -10 (IL-6, IL-1β, IL-10) were quantified by enzyme linked immunosorbent assay (ELISA) and studied in plasma and liver. After 2 h of LPS-induction, different patterns of measured cytokines were observed with thalidomide analogs administration evidencing their immunomodulatory effects. Interestingly, some analogs decreased significantly plasma and hepatic levels of LPS-induced proinflammatory TNF-α and others increased plasma concentration of anti-inflammatory IL-10. Thalidomide analogs also showed slight effects on the remaining proinflammatory cytokines. Differences among immunomodulatory effects of analogs can be related to potency, mechanism of action, and half lives. Thalidomide analogs could be used as a pharmacological tool and in therapeutics in the future.

Keywords: Cytokines; Thalidomide analogs; Endotoxin; Immunomodulation; Liver; Serum

1. Introduction

Cytokines are soluble hormone-like protein mediators produced by diverse cell types in response to various stimuli including other cytokines. Although they play important roles in the normal physiology of cells and organs, cytokines are most distinguished for their activities associated with immune response, inflammation, tissue injury or repair, and organ dysfunction [1,2]. The liver is an important organ in the metabolism of cytokines with the capacity of both to produce and to remove them. All cells normally resident in the liver are capable of producing cytokines, which by stimulating surrounding cells (paracrine effect) or themselves (autocrine effect) lead to further cytokine production and amplification of an inflammatory response. While some cytokines are released by resting cells, the concentrations and variety of cytokines released are considerably increased following stimulation by a variety of inducers [2].

Gram-negative bacterial endotoxin or lipopolysaccharide (LPS), present in the wall of these bacteria, is associated with tissue injury and fatal outcome in septic shock.

Abbreviations: LPS, endotoxin or lipopolysaccharide; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-1β, interleukin-1β; IL-10, interleukin-10; NF-κB, nuclear factor-κB; PBMC, peripheral blood mononuclear cells; V, vehicle; S, saline; T, thalidomide; PDA, 3-phthalimido-3-(3,4-dimethoxyphenyl)-propanoic acid; PDP, 3-phthalimido-3-(3,4-dimethoxyphenyl)-propanamide; PDPMe, methyl 3-phthalimido-3-(3,4-dimethoxyphenyl)-propanoate; $4NO_2$PDPMe, methyl 3-(4-nitrophthalimido)-3-(3,4-dimethoxyphenyl)-propanoate; 4APDPMe, methyl 3-(4-aminophthalimido)-3-(3,4-dimethoxyphenyl)-propanoate; TFPDPMe, methyl 3-tetrafluorophthalimido-3-(3,4-dimethoxyphenyl)-propanoate; PEMN, 3-phthalimido-3-(3-ethoxy, 4-methoxyphenyl)-propanitrile; PDE4, phosphodiesterase-4; AGP, α_1-acid glycoprotein.

* Corresponding author. Tel.: +52 55 50 61 33 03; fax: +52 55 57 47 70 95.
E-mail address: pamuriel@mail.cinvestav.mx (P. Muriel).

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Proinflammatory cytokines, including interleukin-1 and -6 (IL-1, IL-6) and tumor necrosis factor-α (TNF-α), are produced in response to LPS and are thought to mediate many of these effects. Cytokines such as TNF-α and IL-1 are produced in large quantities during systemic inflammation and have been implicated in many of the pathophysiological responses that accompany endotoxemia [3–5].

During bacterial sepsis, as opposed to focal infections or local inflammatory responses, the main organ responsive to LPS is the liver [3,6]. The development of liver failure after sepsis or septic shock remains a difficult clinical problem that is refractory to therapeutic intervention [4,7]. Since this important organ is an endotoxin target, different levels of hepatic damage are present after LPS administration or in pathological states complicated with systemic sepsis or endotoxemia. Concerning this, various experimental models (human and animal) have been performed, and these have shown that cytokines are strongly implicated because increased levels of these proteins have been found. For example, rat intoxication with the hepatotoxic agent aflatoxin B_1 is enhanced by LPS and such mechanism depends on TNF-α [8]; also there is evidence of increased sensitivity to endotoxemia in cirrhotic rats wherein TNF-α is involved [9].

The molecular mechanism involved in septic shock is a complex network of events. After the injection or exposure to LPS/endotoxin, liver and systemic LPS binds to CD14, a protein produced by liver, and other cell surface molecules on the Kupffer cell, macrophages, neutrophils and endothelial cells, activating and triggering these cells to produce nitric oxide (NO) and several proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, IFN-γ. These cytokines primarily stimulate Kupffer cells, macrophages and non-macrophage-type cells, resulting in the activation of nuclear transcription factors, e.g., nuclear factor-κB (NF-κB), among other mediators, besides the stimulation of caspases downstream. Some of these mediators are considered to be involved in the pathogenesis of liver injury and in other organs during endotoxemia. Furthermore, anti-inflammatory mediators, such as IL-10 are also induced and contribute to the modulation of inflammatory liver or systemic responses [2,5,9–12].

Thalidomide (α-N-phthalimidoglutarimide) is an immunomodulatory and anti-inflammatory drug that was originally used as a sedative, although it is now widely associated with its teratogenic and neurotoxic properties [13]. Thalidomide selectively inhibits TNF-α production by LPS-stimulated human monocytes [14]. Its inhibitory action on TNF-α is exerted by enhancing messenger RNA degradation. This inhibition is selective, and other cytokines are almost unaffected [15]. Currently, there is an emerging interest on the potential clinical use of this drug. Present applications include treatment of leprosy, particularly erythema nodosum leprosum (ENL); wasting disease associated with HIV/AIDS; a variety of dermatologic conditions; and some immunologic diseases, as well as abnormal angiogenesis [16,17]. Novel structural thalidomide analogs, which possess improved immunomodulatory effects, increased stability, and besides minor adverse effects, have been prepared and are being assessed in laboratory studies prior to clinical trials [13,17,18].

All above-mentioned data prompted us to synthesize some of these analogs and to assess them as immunomodulatory agents in an acute model of LPS-induced septic challenge in rat, regarding special attention on hepatic and plasma cytokines implicated in processes leading to liver or systemic damage.

2. Materials and methods

2.1. Chemistry

All organic and inorganic reagents were purchased from Aldrich Chemical Company. Solvents were purchased from Merck Germany and Alyt Mexico, and were used without further purification.

Synthetic routes to obtain thalidomide analogs were performed as described by Muller et al. [19,20]. The reaction conditions were modified and developed in our laboratory, based on previously reported methods [21–23]. The thalidomide analogs synthesized were:

- 3-Phthalimido-3-(3,4-dimethoxyphenyl)-propanoic acid (PDA)
- 3-Phthalimido-3-(3,4-dimethoxyphenyl)-propanamide (PDP)
- Methyl 3-phthalimido-3-(3,4-dimethoxyphenyl)-propanoate (PDPMe)
- Methyl 3-(4-nitrophthalimido)-3-(3,4-dimethoxyphenyl)-propanoate ($4NO_2$PDPMe)
- Methyl 3-(4-aminophthalimido)-3-(3,4-dimethoxyphenyl)-propanoate (4APDPMe)
- Methyl 3-tetrafluorophthalimido-3-(3,4-dimethoxyphenyl)-propanoate (TFPDPMe)
- 3-Phthalimido-3-(3-ethoxy, 4-methoxyphenyl)-propanitrile (PEMN)

2.2. Animal treatment

Male Wistar rats weighing around 200–250 g were used. The animals had free access to food (standard Purina chow diet; Purina, USA) and water ad lib. Animal groups were as follows ($n = 6$, each group): the group V + S (control) was administered orally twice with an interval of 12 h in between with the vehicle carboxymethylcellulose (CMC, 0.9% w/v); then, 2 h after last dose of vehicle, rats were injected intraperitoneally with saline (NaCl, 0.9% w/v). Group V + LPS (damaged control) also received vehicle orally twice, and 2 h after last dose of vehicle, rats were injected intraperitoneally with LPS (5 mg/kg, solved in

saline) from *Escherichia coli* serotype 026:B6 (Sigma Chemical Co., USA). Group T + LPS was administered orally twice with thalidomide (100 mg/kg) suspended in CMC 0.9%, and 2 h after last dose of thalidomide, rats were injected intraperitoneally with LPS. The remaining groups underwent the same schedule as thalidomide group; analogs were suspended in vehicle and thus received orally twice as the equimolar dose of 100 mg/kg of thalidomide, again 2 h after last dose of drugs rats were injected intraperitoneally with LPS. Two hours after saline or LPS injection, rats were sacrificed under light ether anesthesia. Blood was collected by cardiac puncture using a syringe containing sodium heparin as anticoagulant. Liver was rapidly removed and rinsed in saline. All samples were kept on ice.

It is important to mention that animals, treated with thalidomide or its analogs, did not show any sign of sickness behavior or side effects elicited by drugs administration during the dosage period previous to LPS challenge; in addition, LPS (5 mg/kg) did not produce an evident alteration in animals' health.

All animals received human care according to the institution's guidelines and the Mexican Official Norm (NOM-062-ZOO-1999) regarding technical specifications for production, care, and use of laboratory animals.

2.3. Cytokine determinations

Blood samples were immediately centrifuged at 3000 rpm for 10 min. Sera were collected, frozen, and kept at −70 °C. In order to obtain liver extracts, pieces of 1 g of liver were homogenized on ice in 5 mL of cold phosphate buffered saline (PBS), pH 7.4, containing a protease inhibitors cocktail (Tablets Complete Roche, Germany). The homogenates were centrifuged at 15,000 rpm (17,147 × *g*) for 15 min at 4 °C. Supernatants were filtered through a 0.45 μm filter (Millex-HA, Millipore, France) and again centrifuged at 15,000 rpm for 15 min at 4 °C. Liver extracts were removed and kept at −70 °C, until cytokine analysis were performed. Plasma and hepatic TNF-α, IFN-γ, IL-6, IL-1β, and IL-10 levels were quantified. Cytokine concentrations were determined by enzyme linked immunosorbent assay (ELISA), using commercial kits that are selective for rat cytokines (Biosource International, USA). Manufacturer's directions were followed. The minimum detectable concentration of TNF-α is 0.7 pg/mL, and 3–8 pg/mL for the rest of cytokines; hepatic amounts of cytokines were calculated per 1 g of wet tissue in 5 mL of PBS. Plasma cytokine concentrations are expressed as picogram per milliliter and hepatic cytokine amounts are expressed as picogram per milligram of tissue.

2.4. Statistics

For statistical analysis, ANOVA with the Student–Newman–Keuls test were used to compare groups [24]. Resulting data are expressed as means ± S.E.M. of six rats in each group, and analyzed using SigmaStat version 1.0 software (Jandel Corporation, EUA). In all cases, a difference was considered significant when $P < 0.05$.

3. Results

3.1. Plasma cytokines

Groups administered with thalidomide analogs plus LPS were only named as analog abbreviations, for simplification in all shown figures.

Plasma TNF-α was undetectable in control group, V + S (Fig. 1A), but it increased dramatically after 2 h of LPS injection (V + LPS). Interestingly, some thalidomide analogs decreased plasma concentration of proinflammatory TNF-α in statistically significant way, such analogs are $4NO_2$PDPMe and PEMN, this last one showed the best inhibition on TNF-α levels, lowering almost by half plasma concentration when compared with V + LPS.

In plasma, IL-6 was not detected in control group (V + S) as can be seen in Fig. 1B, but LPS-induced stimulation elevated the cytokine concentration in plasma in all groups. Although some LPS-stimulated groups seem to undergo immunomodulatory effects, neither thalidomide nor analogs were capable of down-modulating significantly IL-6. These experimental groups had large SEM, thus, differences among them did not reach statistical significance.

Plasma IL-1β was not increased despite the administration of LPS (Fig. 1C), then, it was undetectable in the majority of groups. Nevertheless, very modest concentrations of this cytokine were quantified in some groups where thalidomide and its analogs were assessed. The plasma concentration of IL-1β in $4NO_2$PDPMe + LPS group rose up and it was the unique statistically significant effect.

Fig. 1D shows the plasma levels of the anti-inflammatory IL-10. This interleukin was not detectable in control group (V + S), but the cytokine-inductor LPS caused a marked increase on plasma IL-10 levels. Two thalidomide analogs, enhanced significantly even more IL-10 concentration, these were PDP and PDPMe. The rest of the groups showed similar values compared to damaged control (V + LPS), thus, thalidomide and other analogs were unable to modify this plasma cytokine.

3.2. Hepatic cytokines

Hepatic TNF-α was quantified in all groups (Fig. 2A). LPS induced an almost twofold increase in this cytokine in damaged control (V + LPS), as in most of the groups. Nevertheless, thalidomide analogs $4NO_2$PDPMe, 4APDPMe, and PEMN inhibited partially but significantly the elevation of hepatic TNF-α induced by LPS administration. It is worth noting that the groups' behavior has a very similar pattern as the LPS-induced plasma profile of

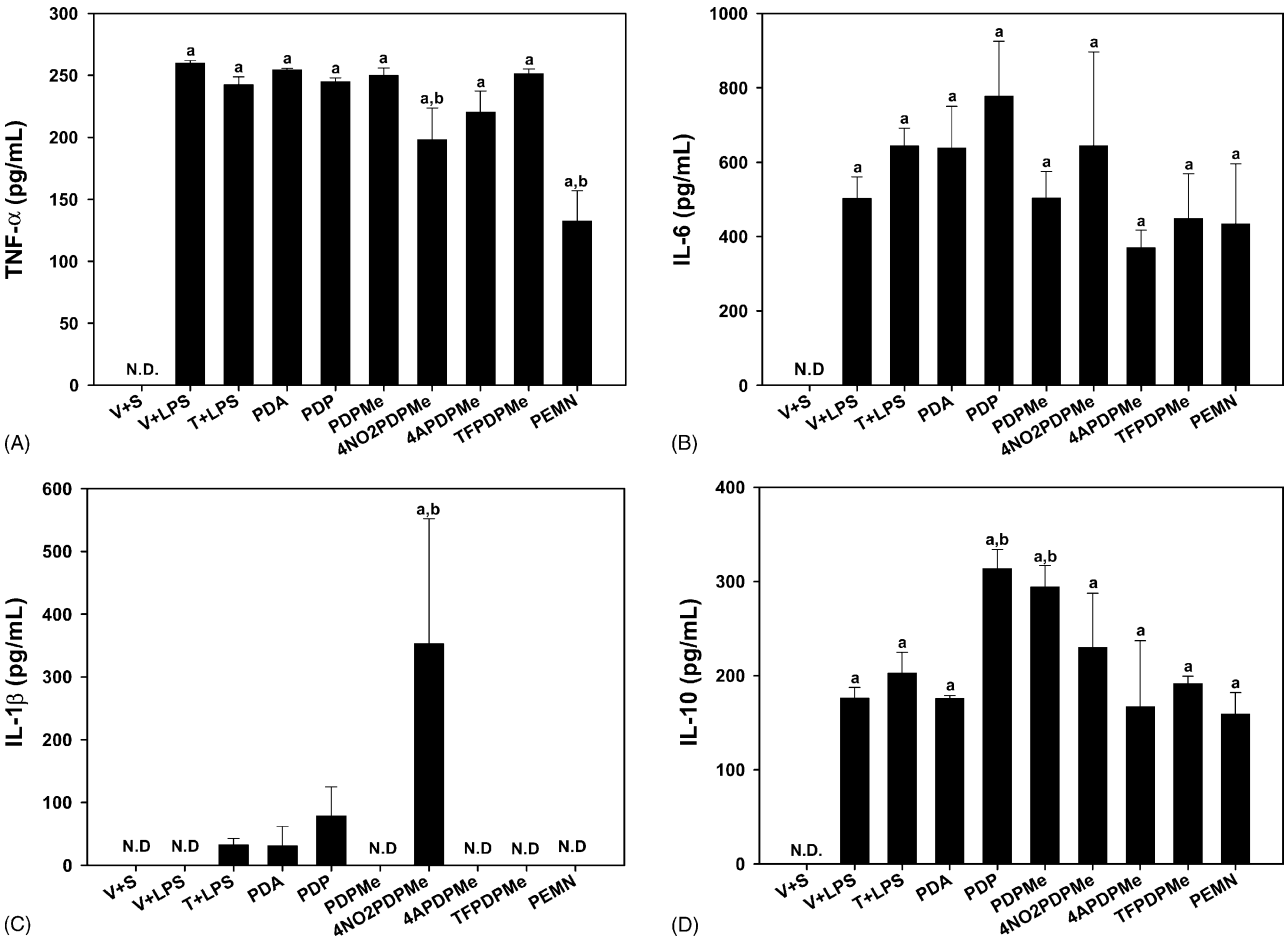

Fig. 1. Plasma concentration of (A) TNF-α, (B) IL-6, (C) IL-1β, and (D) IL-10 determined by ELISA from rats treated twice a day with vehicle (V), thalidomide (T, 100 mg/kg), or its analogs in equimolar dose. Two hours after last dose of drugs, rats were injected with saline (S) or LPS (5 mg/kg). Groups administered with thalidomide analogs plus LPS were only named as analog abbreviations for simplification. Two hours after injections, rats were sacrificed and blood samples were taken. Each bar represents the mean value of experiments performed in duplicate assays with samples from five to six animals ± S.E.M. (a) indicates significant difference from control group (V + S); (b) indicates significant difference from damaged control group (V + LPS). ANOVA Student–Newman–Keuls' Test: $P < 0.05$.

TNF-α, suggesting that plasma and hepatic TNF-α are interrelated and probably synchronized.

Fig. 2B shows that IL-6 amount in livers from control group maintained a very similar value to damaged control group (V + LPS). However, four thalidomide analogs were able to diminish in significant mode, although modest, IL-6 concentration in tissue. Such thalidomide analogs were PDPMe, 4NO_2PDPMe, 4APDPMe, and TFPDPMe. Hepatic concentration profile is not identical to plasma pattern, but it seems to have some resemblance in its behavior.

LPS caused an increment in hepatic concentration of IL-1β when it was compared with control group (V + S) (Fig. 2C). This increment was sustained in the rest of challenged groups and only the analog PEMN decreased the amount in liver of that cytokine significantly; however, this inhibition was weak. It is important to notice that this concentrations profile showed a similar pattern like hepatic TNF-α (Fig. 2A).

After 2 h of LPS injection, the cytokine IL-10 was unaffected in the liver (Fig. 2D). Values of IL-10 hepatic concentration in V + S and V + LPS groups were quite similar. However, thalidomide and its analogs decreased in growing manner, most of them significantly, this cytokine in the hepatic tissue. Interestingly, hepatic IL-10 profile did not resemble its counterpart in plasma (Fig. 1D).

4. Discussion

Cytokines are central mediators of pathological processes and they are involved in necrosis, inflammation, apoptosis and fibrosis. Endotoxin is a potent systemic cytokine inductor. In liver, cytokines play an important role in pathological states, included LPS-induced shock. Thus, the aim of this work was to synthesize some thalidomide analogs and to assess them as immunomodulatory agents in a preventive acute model in rat.

The thalidomide dose of 100 mg/kg was chosen because it is a reasonable middle dose from a broad range, and it is in agreement with various studies wherein authors used

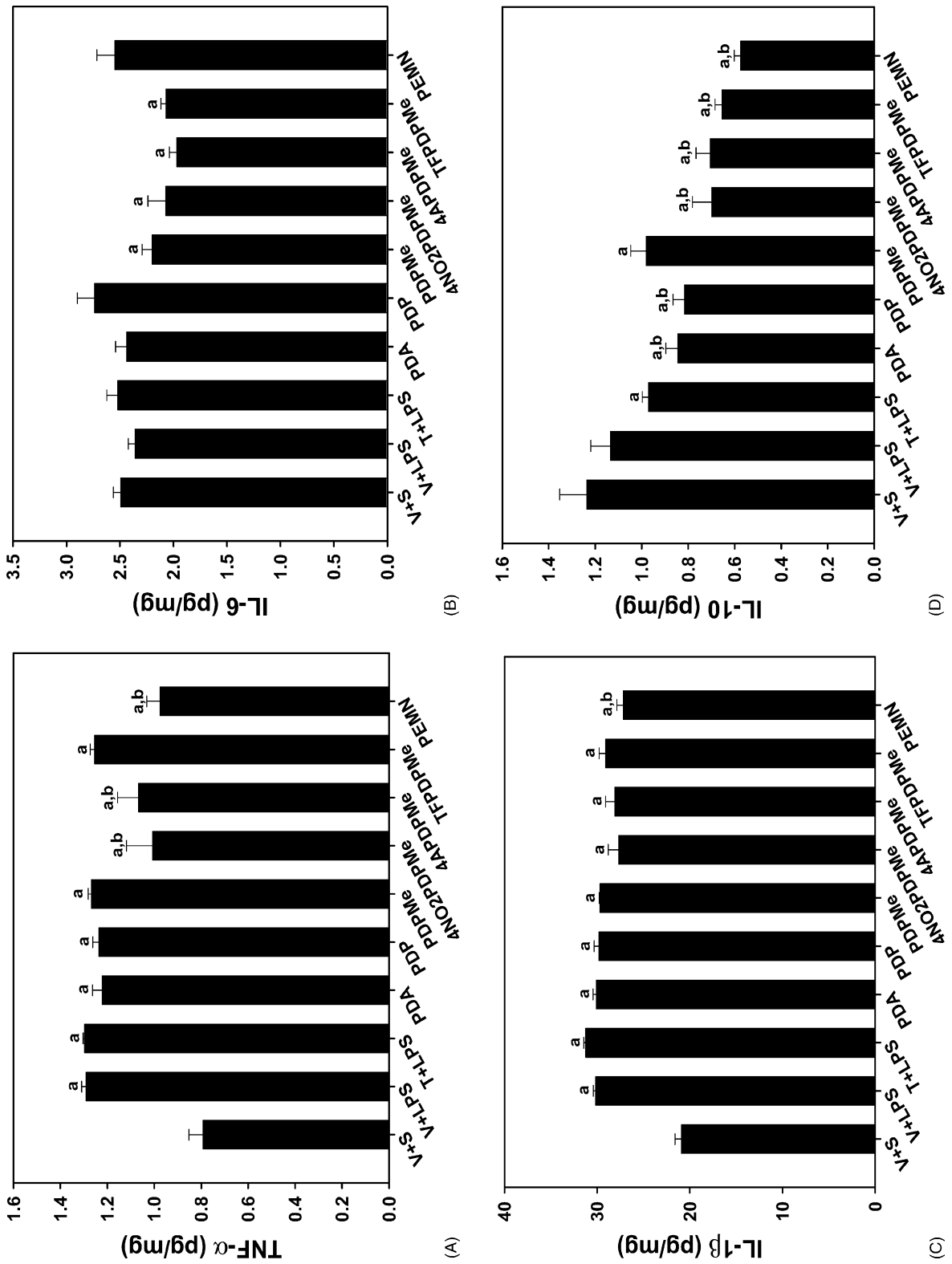

Fig. 2. Hepatic amount of (A) TNF-α, (B) IL-6, (C) IL-1β, and (D) IL-10 determined by ELISA from rats treated twice a day with vehicle (V), thalidomide (T, 100 mg/kg) or its analogs in equimolar dose. Two hours after last dose of drugs, rats were injected with saline (S) or LPS (5 mg/kg). Groups administered with thalidomide analogs plus LPS were only named as analog abbreviations for simplification. Two hours after injections, rats were sacrificed and liver samples were taken. Each bar represents the mean value of experiments performed in duplicate assays with samples from five to six animals ± S.E.M. (a) indicates significant difference from control group (V + S); (b) indicates significant difference from damaged control group (V + LPS). ANOVA Student–Newman–Keuls' Test: $P < 0.05$.

approximately or just 100 mg/kg in rodent species to elicit a biological response in diverse in vivo models [25–27]. In our preventive whole animal model, we used equimolar doses of analogs related to 100 mg/kg of thalidomide with no regard on their intrinsic potency. In our work, we selected 5 mg/kg intraperitoneal dose of the cytokine inducer LPS. This is a sufficient dose to reach, in short time, a high concentration of plasma and hepatic cytokines involved during endotoxic shock in rat and mouse [5,8,9,11,28]. We chose to sacrifice the animals 2 h after LPS challenge because plasma cytokine concentrations have been reported to peak within 2 h of endotoxin injection, after which they rapidly decline [29,30]; in fact, TNF-α, the most important proinflammatory cytokine, is not longer detected 3–4 h after LPS exposure [3,31]. Our results evidenced that, in plasma and in liver, in 2 h cytokine concentrations increased after intraperitoneal LPS injection in majority of treated groups.

TNF-α is a peptide mediator released by monocytes and macrophages in response to various stimuli including bacterial LPS [32]. It has been hypothesized to be the principal mediator of deleterious effects of endotoxin [4]. TNF-α, which is secreted from Kupffer cells by LPS, causes hepatic injury and hepatocyte apoptosis [10]. Overproduction of TNF-α is associated with a wide range of pathologic conditions and has therefore led to much recent effort to find ways to down-regulate its production or inhibit its effects in vivo [13]. In our study, 2 h after LPS induction, TNF-α rose to higher plasma and hepatic levels when compared with control group values, making it evident that such time is enough to observe endotoxin-induced cytokine response in rat.

Immunomodulatory effects of thalidomide analogs on TNF-α were clearly shown, keeping a similar pattern at both levels, systemic and hepatic. Three analogs of thalidomide, $4NO_2$PDPMe, 4APDPMe, and PEMN, out of the eight compounds tested, had significant inhibitory effects on TNF-α, while the rest, including thalidomide, showed no significant effect. In agreement, $4NO_2$PDPMe, 4APDPMe, and PEMN are analogs reported with enhanced potency as TNF-α inhibitors tried in LPS-stimulated peripheral blood mononuclear cells (PBMC); in fact, PEMN has been reported as the most potent thalidomide analog from a series of compounds structurally related [20]. Corral et al. [28] have described that in LPS-stimulated cells, inhibition of TNF-α production by thalidomide and its analogs could be documented as early as 2 h after LPS stimulation. They also found in vitro that thalidomide as well as PDPMe and mainly PDP, significantly reduced TNF-α mRNA accumulation in cells. Furthermore, their study showed that in vivo when mice were challenged with LPS, a single 100 mg/kg dose of analog PDP, administered intraperitoneally 2 h before to challenge, inhibited circulating TNF-α almost 90%. Nevertheless, in our study, we observed only very mild effects of the same compound on this cytokine in rat, administered orally at an equimolar dose with respect to 100 mg/kg of thalidomide and under our schedule of treatment and challenge. Different immunomodulatory effects of analog may be a consequence of dissimilar animal metabolisms and administration ways.

IL-6 is an important proinflammatory cytokine produced by monocytes/macrophages, and in the liver mainly by Kupffer cells. In this study, we observed a remarkable increment of plasma IL-6 due to LPS stimulation after 2 h. Nevertheless, in some groups, the final immunomodulation by thalidomide analogs was not clear. That event may be related to the slight immunomodulatory effects of thalidomide analogs on LPS-induced IL-6. It has been reported that, in cultured human monocytes stimulated by LPS, the analogs PDP, PDPMe, and a very resembled analog to 4APDPMe, had poor or no significant reduction on the elevated IL-6 [13,18,28]. On the other hand, Mathiak et al. [33] have reported that LPS-induced IL-6 has the highest plasma concentration peak around 4–6 h; thus, the lack of a stable pharmacological effect on this cytokine in plasma may be due to the fact that we measured it 2 h after LPS administration.

In the case of liver IL-6, references point out that the intraperitoneal injection of LPS results in a significant elevation of IL-6 mRNA levels within 30 min post-LPS, and are still observed after 3 h [11]. In liver samples cultured with LPS, it is reported a progressive increment of IL-6 during a 5–6 h period [3]. Regardless these studies mentioned previously, after 2 h of LPS-induction, we did not observe a significant dissimilarity between hepatic IL-6 amount of control group and the most of LPS-challenged groups. It is necessary to consider the different measuring techniques for cytokines, when is quantified either gene expression or protein. However, four compounds showed some but significant down-regulating capacity on hepatic IL-6; these were PDPMe, $4NO_2$PDPMe, 4APDPMe, and TFPDPMe.

IL-1β is a proinflammatory cytokine that has been implicated as a mediator of LPS toxicity in vivo and in vitro. The biological properties of IL-1β are remarkably similar to those of TNF-α, and synergism between effects of these two molecules is evident in several models. The highest LPS-induced IL-1β concentration in serum has been found around of 4–6 h [33]. In plasma, we observed that IL-1β was not detectable, either in control group or in damaged control, this last in spite of the previous 2 h LPS-induction, but thalidomide and some of its analogs ($4NO_2$PDPMe, PDA, and PDP) showed slight up-regulating effects, although only $4NO_2$PDPMe achieved statistical significance. In contrast, some authors reported that in PBMC cultures, stimulated by LPS and IL-1β, secretion was inhibited modest, but significantly by PDP and PDPMe [13,18,28]. Opposite immunomodulatory effects can be related to dissimilar models, and to the optimal time of maximum plasma IL-1β concentration after LPS stimulation.

In the liver, activated Kupffer cells are capable of releasing IL-1β [2]. Sang et al. [11] described that intraperitoneal injection of LPS (4 mg/kg) into rats resulted in a significant elevation in the liver levels of IL-1β within 30 min post-LPS. These reports are in agreement with our results of liver IL-1β. LPS injection induced a significant increment of hepatic IL-1β, in addition, the hepatic LPS-induced profile obtained after 2 h was quite dissimilar when compared with plasma profile. In fact, it is possible to observe an immunomodulatory tendency of thalidomide analogs similar to hepatic TNF-α pattern; however, this effect was not large enough to reach statistical significance in most of groups; nevertheless, PEMN prevented significantly the increment of hepatic IL-1β. We suggest that similitude between hepatic TNF-α and IL-1β results from likeness of biological properties of these two cytokines, as it was mentioned in above paragraphs, and therefore immunomodulatory effects of analogs are then also similar in liver.

IL-10 is a potent and pleiotropic anti-inflammatory cytokine produced by lymphocytes and macrophages [34]. It inhibits the synthesis of proinflammatory cytokines, such as IL-6 and TNF-α, by T helper type 1 cells, mono/macrophages, and polymorphonuclear cells, and reduces T-cell activation in vitro and in vivo [11,35]. We observed a notable difference between control group and LPS-stimulated control group. Two analogs induced a significant up-regulation of IL-10, these were PDP and PDPMe. Our results are in agreement with several reports where it was found a modest but consistent stimulation of IL-10 due to these compounds, either in LPS-stimulated PBMC cultures or in mice challenged with LPS [13,18,28].

The liver is a major source of IL-10. The potential cellular sources of IL-10 in the liver include macrophages, Kupffer cells, T and B lymphocytes, and hepatocytes [35]. Specifically, in rodents IL-10 down-regulates proinflammatory cytokine synthesis including the LPS-induced synthesis of the major proinflammatory cytokine TNF-α in Kupffer cells, and also IL-6 [2,34]. In this work, it was found that hepatic IL-10 amount of control group was almost equal to the amount of damaged control group at 2 h of LPS stimulation. Surprisingly, most of the groups administered with thalidomide analogs underwent an important and significant inhibition of IL-10. These findings can be explained perhaps because after 2 h of LPS-challenge the liver spills, as a main source of IL-10, its major part of this cytokine to the rest of economy through blood torrent. Furthermore, it is necessary to mention that there are other organs that can produce IL-10, increasing the systemic plasma IL-10 concentration after endotoxin-induction [36].

It is very important to notice that the kind of structural thalidomide analogs, which we synthesized and tried, possess besides the TNF-α inhibitory activity, phosphodiesterase-4 (PDE4) inhibitory effects [18]. It is well documented that elevated levels of cAMP inhibit TNF-α production in activated monocytes and in PBMC [20]. Thus, inhibition of PDE4 has been shown to be an effective method for inhibition of TNF-α production due to these compounds, especially for perfluorinated analogs such as TFPDPMe [37]. Thalidomide is not a PDE4 inhibitor [20] and has different mechanisms of action, likely this is one of the reasons why this compound was not able to decrease or modulate TNF-α. There are two possible immunomodulatory mechanisms of action for thalidomide, the first is the best known and more cited, concerning to selective inhibition of TNF-α by enhancing mRNA degradation [14,15]; while its analogs do not affect the stability of TNF-α mRNA [13]. The second proposed mechanism is related to the fact that thalidomide binds to α_1-acid glycoprotein (AGP) with high specificity, this implies that AGP may mediate the immunomodulatory effect of the drug due to biological properties of AGP; however, thalidomide analogs do not compete for AGP thalidomide binding site and therefore they do not share this mechanism of action [37–39].

Other possible explanations for the poor inhibitory effects of thalidomide on TNF-α, observed in our study, are that the effect of thalidomide depends both on the cell type under study and the cytokine inducer, in the case of TNF-α, the result may be production or inhibition [40–42]. Shannon and coworkers suggested that the production-inhibition thalidomide effect may be due to variations in experimental dosages and related to hydrolysis of thalidomide [43,44]. Thalidomide suffers hydrolysis on both rings, especially at physiological pH, but the glutarimide ring appears to be more stable than the phthalimide ring, it leads to a short half-life around of 5 h [45]. In addition, several studies have reported that thalidomide also possesses regulatory properties depending on the enantiomer, R or S, employed [46–48]; we used a racemic mixture in our study. Thus, differences of effectiveness between thalidomide and its analogs in our work seem to be due to variations in metabolism, pharmacokinetics, bioavailability, or a bidirectional cytokine regulation; as well as the use of a middle dose that given orally was likely not enough to reach a significant inhibitory effect.

In order to explain the differences among immunomodulatory effects of thalidomide analogs that we observed not only on TNF-α but on all remaining cytokines studied here, it is necessary to comment that causes of these differences may be found in many factors, as follows: (a) thalidomide analogs have quite dissimilar half-lives reported in human plasma at 37 °C [28]; for example, in the case of PDP its half-life is around 8 h, for PDPMe approximately 3 h, and an analog which resembles to 4APDPMe has a half-life of about 4 h. Differences in half-lives may reflect the solubility and stability of each compound in organism and therefore their bioavailability; then, this is a very important factor because some analogs could be

metabolized or eliminated from organism at different rates without reaching a significant immunomodulatory effect. (b) Potency is another plausible reason to explain why analogs yielded variable immunomodulatory effects; their potencies as both TNF-α and PDE4 inhibitors have been reported [20], for instance, PDP inhibitory concentration-50 (IC_{50}) are 13 and 9.4 μM, respectively; PDPMe is a more potent inhibitor of TNF-α and PDE4 with IC_{50} of 2.9 and 2.5 μM, respectively; TFPDPMe is more potent as TNF-α inhibitor with 0.26 μM than as PDE4 inhibitor with IC_{50} = 4.7 μM, and PEMN the most potent of this class of thalidomide analogs, as either TNF-α or PDE4 inhibitor, with 0.12 and 0.13 μM, respectively. It is important to notice that the given potencies were obtained by in vitro experiments and different efficacy may be expected in whole animal models, such as in our study [20]. (c) Dose, via of administration and schedule of treatment are other striking factors, which are capable of leading to variation of immunomodulation, besides to the previous points, some of these analogs may be not bioavailable enough to achieve significant effects within 2 h after LPS-induction. Although further studies with different doses, animal species, cytokine inducers, and time courses for cytokine determination are required to clarify and optimize immunomodulatory effects, we propose that the resultant effects of these compounds on cytokines, at the hepatic and systemic level, open a wide perspective to use them as immunomodulatory agents.

Finally, thalidomide and its analogs have quite dissimilar immunomodulatory properties and inclusive different mechanism of action, though both share the final inhibition on TNF-α. The explanation for dissimilarities in the immunomodulatory effects of thalidomide analogs seems to be found in different half-lives, stability of each compound, solubility, and potency [13,28]. There are various important factors to take into account to evaluate the immunomodulatory effects of this kind of thalidomide analogs, among these factors are the administration of a racemic mixture, experimental model, sampling and time courses, cellular types, the inducer agent, doses or concentrations, animal species, and pathological conditions [23].

In conclusion, we observed immunomodulatory effects of thalidomide analogs on LPS-induced plasma and hepatic cytokines in rat. To explain these findings is difficult, because there is a complicated network of regulatory mechanisms for cytokines, such as one modulating itself and each other. If it is possible down-regulating proinflammatory cytokines and up-regulating anti-inflammatory cytokines by immunomodulatory drugs, then it could be possible to control biological responses during pathological processes. Further studies of dose-effect, dissimilar cytokine inducers, and time courses are required; nevertheless, we suggest that thalidomide analogs offer a new opportunity to explore immunological therapeutics acting as useful pharmacological tools in the future.

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