not clear whether these multiple alleles represent separate translocation events or aneusomy of a previously translocated chromosome. The latter possibility is favored, given that: (1) the partner status (7q32.3 or other) was identical in the allele pairs from each case; (2) two of the three cases demonstrated aneuploidy; and (3) 6p25.3 translocations appear to be an early event during lymphomagenesis, based on our constant finding of this translocation in initial biopsies from patients with multiple tumor specimens over time (AL Feldman, unpublished observation). In addition to the lack of a normal copy of the 6p25.3 allele, multiple copies of the translocations could have implications on the partner locus. For example, we previously reported overexpression of microRNAs (especially *MIR29B1)* that reside near the 7q32.3 breakpoint in ALK-negative ALCLs with t(6;7)(p25.3;q32.3) (Feldman *et al*., 2009).

In summary, we present three cases of pcALCL with biallelic rearrangements of 6p25.3 and no intact copies of the *DUSP22–IRF4* locus. These cases indicate one mechanism by which both copies of the *DUSP22* gene can be disrupted or deleted in T-cell lymphomas, and provide further evidence that *DUSP22* may represent a tumor-suppressor gene.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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# Lysophosphatidic Acid Mediates the Release of Cytokines and Chemokines by Human Fibroblasts Treated with *Loxosceles* Spider Venom

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## TO THE EDITOR

*Loxosceles* spiders are a genus of arachnids, whose bites cause necrotizing skin lesions. They are distributed worldwide in temperate and tropical regions. In Brazil, approximately 10,000 cases of *Loxosceles* spider bites are reported annually. *L. intermedia*, *L. gaucho*, and *L. laeta* are prevalent in most of the southern states of Brazil, whereas *L. similis* has been described mainly in the state of Minas Gerais. *L. reclusa* and *L. deserta* cause the majority of accidents in North America. The venoms of these species all have similar biochemical and pharmacological profiles (Barbaro *et al.*, 2005; Silvestre *et al.*, 2005; Chatzaki *et al.*, 2012). The envenomation, described as loxoscelism, is characterized by pain, local edema, and erythema, followed by dermonecrosis that require weeks to heal. The genesis of loxoscelism is attributed to a family of sphingomyelinase D enzymes, also known as

*Abbreviations: LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LsV, L. similis venom; PLD, phospholipase D; recLiD1, recombinant L. intermedia dermonecrotic protein 1*

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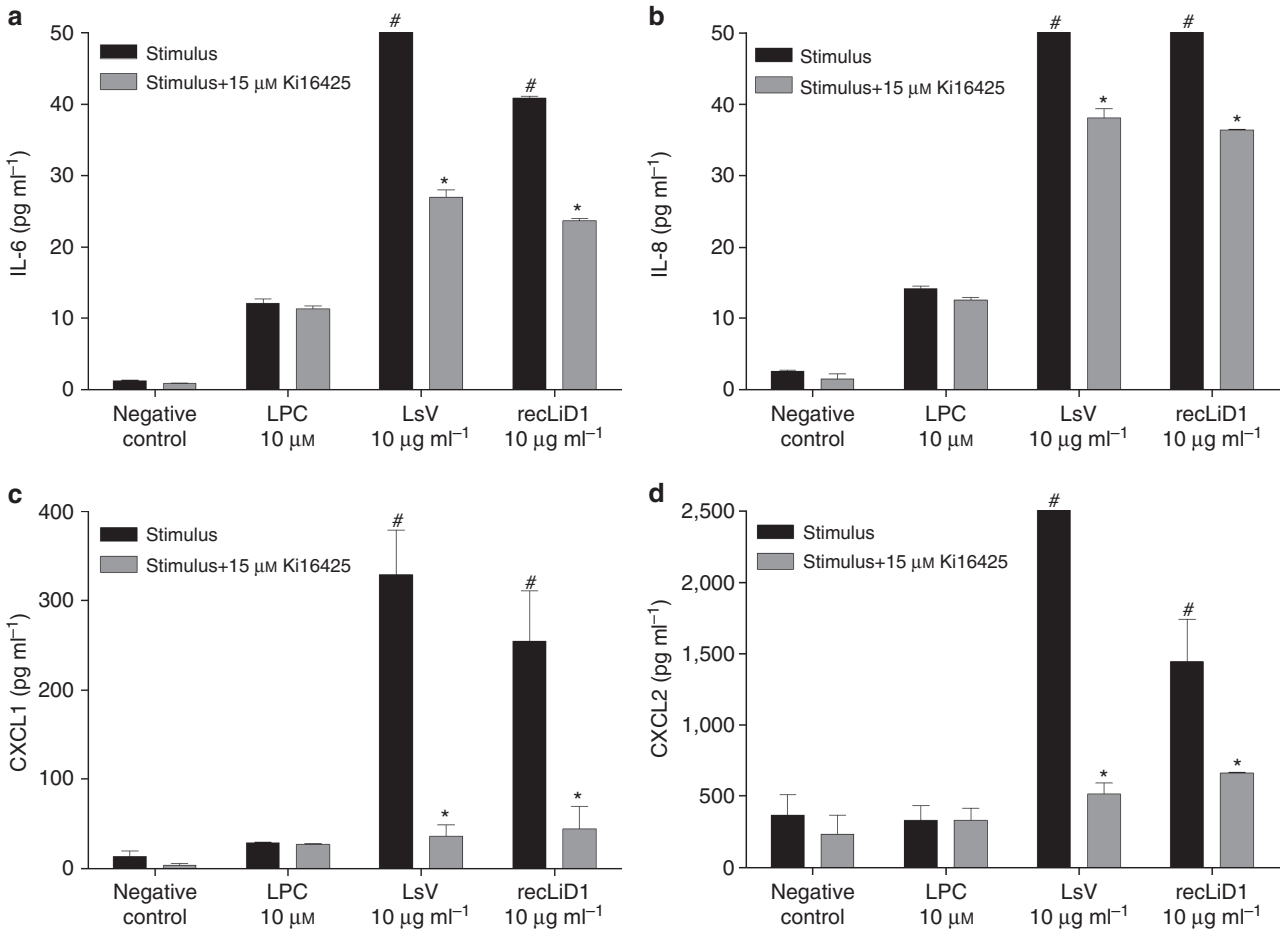


**Figure 1. The lysophosphatidic acid (LPA) receptor antagonist Ki16425 decreases the release of cytokines/chemokines induced by stimulation of HFF-1 cells with *L. similis* venom (LsV) or recombinant *L. intermedia* dermonecrotic protein 1 (recLiD1).** LsV and recLiD1 were pre-incubated for 1 hour at 37 °C with 10 μM 1-oleoyl-lysophosphatidylcholine (LPC; complexed to 5 mg ml$^{-1}$ fatty acid-free albumin). HFF-1 cells were then incubated for 12 hours with 10 μM LPC, 10 μg ml$^{-1}$ LsV or recLiD1, in the absence or presence of 15 μM Ki16425. Untreated cells served as negative controls. Graphs show the levels (pg ml$^{-1}$) of IL-6 (**a**), IL-8 (**b**), CXCL1 (**c**), and CXCL2 (**d**) in conditioned medium from cell cultures. Values represent the mean ± SEM of triplicate determinations from three independent experiments. $^{\#}P<0.05$ compared with LPC control group; $^{*}P<0.05$ compared with experimental LsV/recLiD1 groups (two-way analysis of variance, Bonferroni *post-hoc* test).

Loxtox proteins (Kalapothakis *et al.*, 2007). Since the discovery that these enzymes have several phospholipid substrates besides sphingomyelin, they have been named phospholipase D (PLD; Van Meeteren *et al.*, 2004; Lee and Lynch, 2005; Chaim *et al.*, 2011). Furthermore, the complex pathophysiological mechanisms underlying cutaneous loxoscelism have been discussed. Dragulev *et al.* (2007) showed that *L. reclusa* PLD upregulated the expression of proinflammatory cytokines/chemokines in human fibroblasts. The authors proposed that ceramide-1-phosphate formed by the hydrolysis of plasma membrane sphingomyelin is responsible for this effect. This speculation was challenged by Van Meeteren *et al.* (2007), who hypothesized that lysophosphatidic acid (LPA), a product of lysophosphatidylcholine (LPC) hydrolysis, is the likely trigger for the observed response, rather than ceramide-1-phosphate. LPC is an abundant plasma component (approximately 150 μM) and serves as a genuine physiological substrate for PLD (Van Meeteren *et al.*, 2004).

On the basis of this intriguing discussion, we investigated the role of LPA receptors in the release of cytokines/chemokines and in cell death caused by treatment of fibroblasts with *L. similis* venom (LsV). For comparison, we also used recombinant *L. intermedia* dermonecrotic protein 1 (recLiD1), a well-described toxin (Kalapothakis *et al.*, 2002; Felicori *et al.*, 2006). PLD activity was detected for LsV and recLiD1 (data not shown).

HFF-1 human fibroblast cells (BCRJ/UFRJ, Rio de Janeiro, Brazil) were maintained in DMEM with 15% serum. Before treatment, cells were maintained in serum-free DMEM for 4 hours. Subsequently, the medium was changed, and the cells were treated for 12 hours with 10 μg ml$^{-1}$ LsV, 10 μg ml$^{-1}$ recLiD1 (both pre-incubated for 1 hour at 37 °C with 10 μM LPC), 15 μM LPA, 10 μM LPC, or 10 ng ml$^{-1}$ tumor necrosis factor-α in serum-free DMEM. Untreated cells served as negative controls. Treatments were performed in the absence or presence of 15 μM Ki16425, an LPA receptor ($LPA_1/LPA_3$) antagonist, which was

added 30 minutes before the addition of each agent. After treatment, aliquots of conditioned medium from cell cultures were collected for analysis of cytokine/chemokine levels using ELISA kits for human IL-6, IL-8, IL-1β, RANTES (Life Technologies, Rockville, MD), CXCL1 (Abnova, Taipei, Taiwan), and CXCL2 (Immuno-Biological, Gunma, Japan). AlamarBlue assay (Life Technologies) was performed to confirm cell viability after stimulation, with 10 μg ml$^{-1}$ LsV or recLiD1 for 12 hours (data not shown).

Exposure of HFF-1 to LsV or recLiD1 stimulated the release of IL-6, IL-8, CXCL1, and CXCL2. Cells produced lower levels of mediators after LPC stimulation (Figure 1). *Loxosceles* venoms have been shown to promote the release of inflammatory mediators in different experimental models (Barbaro *et al.*, 2010). However, the participation of LPA receptors in this venom inflammatory response has not yet been investigated. In our assays, Ki16425 significantly inhibited the production of cytokines/chemokines by HFF-1 after treatment with LsV and recLiD1 (Figure 1). It is known that HFF-1 cells express $LPA_1$, $LPA_2$, and $LPA_3$ receptors (Zhang *et al.*, 1999). Control cells treated with LPA exhibited results similar to those of cells treated with LsV or recLiD1, and tumor necrosis factor-α induced secretion of the mediators tested (data not shown).

These data indicate that LPA, formed by PLD activity of LsV and recLiD1, induces liberation of cytokines/chemokines via LPA receptor-mediated pathways. LPA is a bioactive phospholipid that is involved in many cellular functions, including cytokine/chemokine secretion (Fang *et al.*, 2004). As *Loxosceles* PLD has several phospholipid targets, it is necessary to acknowledge that other mediators may also participate in these inflammatory effects.

We next investigated whether LPA receptors were involved in cell death induced by longer incubation with LsV. Previous results from AlamarBlue assays revealed that cell survival decreased after 24–48 hours of incubation with LsV (data not shown). Therefore, HFF-1 monolayers were stimulated with 10 μg ml$^{-1}$ LsV, 10 μg ml$^{-1}$ recLiD1 (both pre-incubated with 10 μM LPC), 15 μM LPA, or 10 μM LPC in 15% serum DMEM for 48 hours. Ki16425 was added as previously described. Untreated cells served as negative controls. After incubation, cells were trypsinized and washed with phosphate-buffered saline. The cells

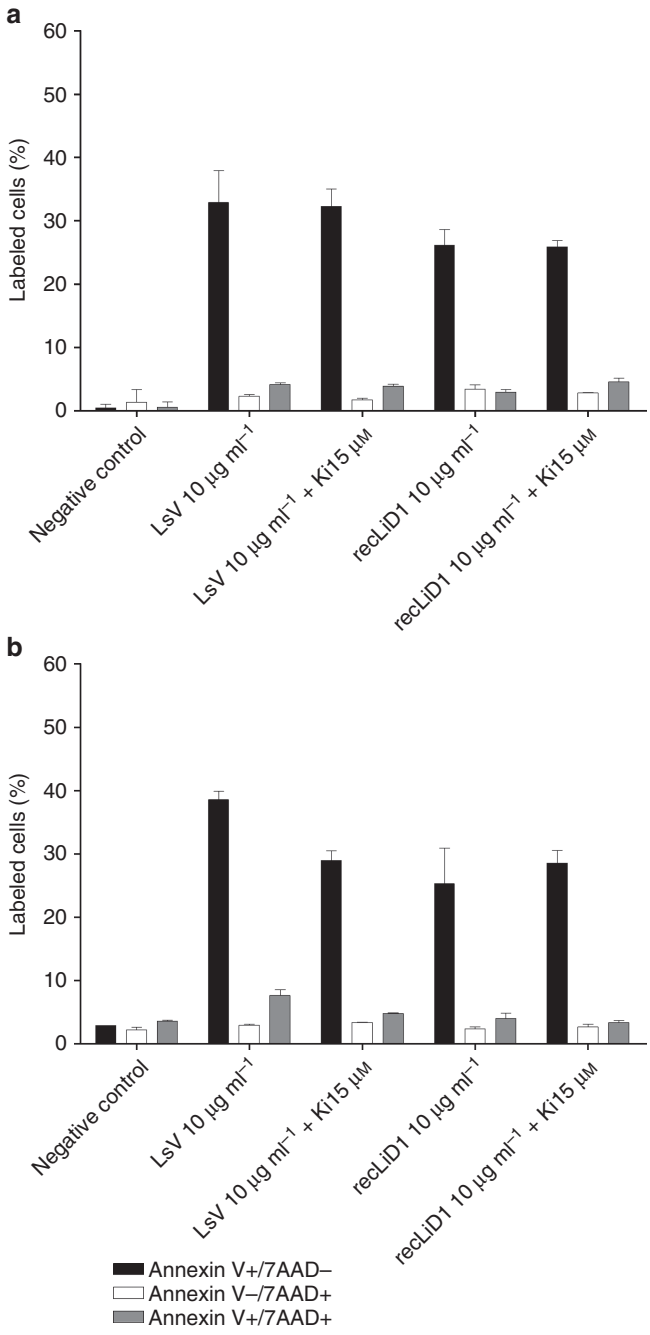


**Figure 2. Lysophosphatidic acid (LPA) receptors are not involved in apoptotic cell death of human fibroblast and endothelial cells treated with *L. similis* venom (LsV) or recombinant *L. intermedia* dermonecrotic protein 1 (recLiD1).** LsV and recLiD1 were pre-incubated for 1 hour at 37 °C with 10 μM 1-oleoyl-lysophosphatidylcholine (LPC; complexed to 5 mg ml$^{-1}$ fatty acid-free albumin). Human umbilical vein endothelial cells (HUVECs) and HFF-1 cells were then incubated for 48 hours with 10 μg ml$^{-1}$ LsV or recLiD1. Untreated cells served as negative controls. Treatments were performed in the absence or presence of 15 μM Ki16425. Graphs show the percentage of HFF-1 cells (**a**) and HUVECs (**b**) labeled with Annexin V-Cy3 and/or 7-aminoactinomycin d (7-AAD). Values represent the mean ± SEM of triplicate determinations from three independent experiments. Note: cells in early apoptosis are Annexin V-Cy3-positive (black bars).

were then incubated in Apoptosis/Necrosis Detection Kit buffer containing 1% v/v 7-AAD and Annexin V-Cy3 (Enzo LifeSciences, Farmingdale, NY), and subjected to flow cytometry.

Treatment with LsV or recLiD1 caused HFF-1 cell death after 48 hours, primarily due to apoptosis, which was not inhibited by Ki16425 (Figure 2a). The lack of inhibition was expected, as treatment with LPA did not cause cell death. Control LPC did not injure cells either (data not shown). As LPA can act as a cell survival or apoptotic factor, dependent upon the cell type (Ye *et al.*, 2002), we performed the same procedures using human umbilical vein endothelial cells, which yielded similar results (Figure 2b).

Thus, we did not find that LPA participates in apoptosis induced by LsV or recLiD1 in fibroblast and endothelial cells *in vitro*. These cells are known sites of *Loxosceles* venom interaction. Our results support the idea that PLD initiates the effects of *Loxosceles* venom through stimulation of acute inflammatory responses.

In conclusion, LPA receptors are involved in the release of proinflammatory cytokines/chemokines provoked by LsV and recLiD1. The present work contributes to the open discussion regarding the participation of LPA in the pathophysiology of cutaneous loxoscelism and paves the way for investigation of new therapeutic strategies.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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# Mutations in ERBB4 May Have a Minor Role in Melanoma Pathogenesis

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## TO THE EDITOR

In the recent past, an increasing amount of evidence has indicated that several alterations to the complex molecular machinery that provides checks and balances in normal melanocytes may be involved in the pathogenesis of melanoma (Palmieri *et al.*, 2009; Tsao *et al.*, 2012). The different molecular pathways associated with melanomagenesis seem to correspond to specific subsets of melanoma patients, with distinguished biological and clinical behavior of the disease. In the era, just begun, of innovative targeted therapies for the treatment of melanoma (Ascierto *et al.*, 2012; Flaherty *et al.*, 2012), identification of such different subsets of melanoma patients is becoming mandatory.

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