Lysophosphatidic acid enhances interleukin-13 gene expression and promoter activity in T cells

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Rubenfeld, Joshua, Jia Guo, Nitat Sookrung, Rongbing Chen, Wanpen Chaicumpa, Vincenzo Casolaro, Yutong Zhao, Viswanathan Natarajan, and Steve Georas. Lysophosphatidic acid enhances interleukin-13 gene expression and promoter activity in T cells. Am J Physiol Lung Cell Mol Physiol 290: L66–L74, 2006. First published September 30, 2005; doi:10.1152/ajplung.00473.2004.—Lysophosphatidic acid (LPA) is a lysophospholipid with wide-ranging effects on multiple lung cells including airway epithelial and smooth muscle cells. LPA can augment migration and cytokine synthesis in lymphocytes, but its potential effects on Th2 cytokines have not been well studied. We examined the effects of physiological concentrations of LPA on IL-13 gene expression in human T cells. The Jurkat T cell line and human peripheral blood lymphocytes were transfected with LPA alone or with IL-13 promoters containing a sequence sufficient for tissue-specific IL-13 expression. The effects of LPA were determined using transcriptional reporter assays. LPA increased transcriptional activation of the IL-13 promoter via regulatory elements contained within proximal 312 bp. The effects of LPA on IL-13 mRNA stability were examined using actinomycin D to halt ongoing transcription. Expression of mRNA encoding LPA2 and LPP-1 increased with T cell activation. LPA augmented IL-13 secretion and responsiveness to both Th1 and Th2 cytokines. LPA significantly enhanced transcriptional activation of the IL-13 promoter under conditions of submaximal T cell activation.

T lymphocytes; transcription factors; inflammation; lysophospholipids; transcriptional regulation

LYSOPHOSPHATIDIC ACID (LPA) is a lysophospholipid with far-reaching effects on different cell types ranging from platelet activation to enhancing cell proliferation. LPA binds to several G protein-coupled receptors including LPA1, LPA2, and LPA3 (formerly known as endothelial differentiation gene or Edg receptor-2, -4, and -7, respectively). A fourth LPA receptor related to the purinergic receptor family was recently identified (37). LPA is detectable in normal serum at a concentration in the micromolar range, where it is bound by both albumin and gelsolin (33). Major sources of LPA include platelets, epithelial cells, and certain tumor cells (24).

LPA is generated by the action of both secretory phospholipase A2 (via hydrolysis of phosphatidic acid) and lysophospholipase D (via cleavage of extracellular lysophosphatidylcholine) on membrane phospholipids, with the latter pathway representing a potential pathway of cell activation in cancer (14, 34).

Although most studies to date have examined the effects of LPA on structural tissue cells such as endothelium and epithelium, emerging data suggest that LPA and related lysosphingolipids [e.g., sphingosine-1-phosphate (S1P)] activate circulating leukocytes including lymphocytes (19). Pioneering work by Goetzl and colleagues (17–19, 53) established that human T cells express LPA1 and LPA2 mRNA and that LPA could induce the chemotaxis of Jurkat T cells through Matrigel membranes. Zheng et al. (54) found that LPA receptor expression and responsiveness were altered by mitogen activation and that LPA enhanced IL-2 secretion in phytosagglutinin (PHA)-primed CD4 T+ cells. This body of work was the first to demonstrate the ability of LPA to regulate CD4+ T cell cytokine gene expression, although the molecular mechanisms by which LPA acts in this regard are not well understood. Additionally, recent data by Panther and associates (40) established that LPA could augment the differentiation of myeloid dendritic cells. Together, these studies suggest that LPA directly or indirectly can affect T cell-driven immune responses.

IL-13 is a 112-amino acid T cell-derived Th2 cytokine that has been shown to play a crucial role in the pathogenesis of asthma (49). It has been implicated in the induction of airway hyperresponsiveness and mucus hypersecretion, both of which are inhibited by IL-13 blockade (21, 29, 50). The IL-13 gene is located on chromosome 5q in a cluster of other Th2 cytokine genes, and the molecular factors that regulate IL-13 gene expression are beginning to be understood. Kishikawa et al. (26) recently reported that the proximal IL-13 promoter was faithfully expressed in T cells (but not non-T cells) in transient transfection assays, arguing that the promoter contains sequences sufficient for tissue-specific IL-13 expression. The IL-13 promoter contains binding sites for the transcription factors nuclear factor of activated T cells (NFAT) and GATA-3 (10, 26), and the signal transduction pathways initiated by ligation of the T cell receptor (TCR) and coreceptors that lead to IL-13 expression are beginning to be understood. In different experimental models, activation of calcium-, PKC-, MAPK-, and cAMP-dependent pathways have been implicated in IL-13 gene regulation (5, 10, 38, 46). Furthermore, agonists that signal through G protein-coupled receptors can also en-
hance IL-13 gene expression [e.g., histamine (13), adenosine (2), and prostaglandin D2 (45)].

We wondered whether LPA could affect the secretion of other T cell cytokines besides IL-2. Here we report that LPA augments the secretion of IL-13 in Jurkat and CD4+ T cells. LPA by itself did not induce cytokine secretion but synergized with other signals, especially under conditions of submaximal activation. We investigated the mechanism by which LPA augments IL-13 gene expression and demonstrate that LPA acts in part at the level of transcriptional activation by regulatory elements located in the IL-13 promoter.

MATERIALS AND METHODS

Reagents and ELISA. Cells were maintained in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% FCS (Hyclone) and Glutamax (GIBCO). LPA (Biomol) was diluted in ethanol to a stock concentration of 10 mM and stored at −20°C. Aliquots of LPA were evaporated under N2 and sonicated for 30 s in 1 ml of medium with 0.1% BSA (Sigma) as carrier immediately before use. T cells (0.5–1 x 10⁶/condition) were stimulated for 8–18 h with pharmacological agonists including the calcium ionophore A-23187 (0.1 and 0.5 μM), the phorbol ester PMA (5 and 50 ng/ml), and the cAMP analog 8-bromo adenosine 3′,5′-cyclic monophosphate (8-BrcAMP; 1 mM, all from Calbiochem) as indicated. In some experiments, actinomycin D (3 μg/ml, Sigma) was added to halt ongoing transcription followed by cell harvesting at different time points (see Supplementary Fig. 1). Supplemental data for this article may be found at http://ajplung.physiology.org/cgi/content/full/00473.2004/DC1).

Twenty-four well plates were incubated overnight with different concentrations of an anti-CD3 antibody in coating buffer (0.1–1 μg/ml as indicated, clone 145.2C11, Pharmingen). After being washed, 0.5 × 10⁶ CD4+ T cells were added to each well in duplicate with or without LPA or an antibody directed against CD28 (5 μg/ml, clone 37.51, Pharmingen) and incubated for 18 h followed by harvesting of the cell supernatant for ELISA and cell pellet for RNA or protein extraction. Commercially available ELISAs for IL-4 and IL-13 were performed according to instructions provided by the manufacturer (range: 2.5–300 pg/ml, Beckman-Coulter). All samples were diluted as needed to fall within the standard curve.

Cells. Jurkat T cells (a kind gift of Dr. J. Strominger, Harvard Univ.) were maintained in supplemented RPMI medium and incubated at 37°C in 5% CO2 and 95% air. Three hours before treatment with submaximal concentrations of pharmacological agonists the cells were cultured in reduced-serum RPMI containing 1% FBS, followed by stimulation with various combinations of the pharmacological agonists with and without LPA as described above. Peripheral blood was obtained from normal volunteer donors using a protocol approved by the Johns Hopkins Medicine Institutional Review Board-5. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using standard techniques. CD4+ T cells were purified from the PBMC fraction via negative selection using the RosetteSep technique according to the manufacturer’s instructions (Stem Cell Technologies, Victoria, BC, Canada). Purity of CD4+ cells was >95% as assessed by immunofluorescence and flow cytometry (data not shown). RT-PCR. Total RNA was isolated from Jurkat T cells or human CD4+ T cells using RNeasy spin columns (Qiagen) according to the manufacturer’s specifications, followed by incubation with RNase-free DNase (Ambion) for 1 h at 37°C. Integrity and purity of RNA were assessed by spectrophotometry and agarose gel electrophoresis. One microgram of total RNA was reverse transcribed using a GeneAmp protocol (Perkin-Elmer). PCR was performed to assess expression of the LPA1, LPA3, and LPA4 genes using primers described by Wang et al. (48). The following primers specific for LPA1, IL-13, the lipid phosphate phosphatases (LPP)1–3, and GAPDH genes were synthesized: hLPA4: (forward)-GCACCAAAATCCAGTGACATAC, (reverse)-CACGTTACATGCACAGCA-TTTG; hIL-13: (forward)-AAGGCTCCGTCTCAGATGG, (reverse)-GGGCCACCTGTATT- TTCGGT; hLPP1: (forward)-CTTTCAACCCAGATAGAAGGAG, (reverse)-CTTGGTAGATTGCCTGGATAGTG; hLPP2: (forward)-CA-TCTCAGACTTTCCTCAAAGCCG, (reverse)-CAGCACTACTCT- GTACTCTCG; hLPP3: (forward)-CCCCATTATAGGAGACC- AGCTGC, (reverse)-CTTTGATTTTGTACGCGCTT; hGAPDH (forward)-GGAGCCAAAAGGGTCATCATCTC, (reverse)-AGTG- GGTGTCGTGGTGTGAATG.

For PCR, each 50-μl reaction mixture contained 1 μl DNTPs (200 mM), 1.5 μl MgCl2, 1 μl each primer, 5 μl 10X reaction buffer, 0.4 μl Taq polymerase, 2 μl cDNA, and 38.1 μl double distilled H2O. PCR was performed as follows: 94°C for 1 min, 57°C for 30 s, 72°C for 90 s repeated for 35 cycles. The reaction was then extended for 10 min at 72°C after the final cycle. PCR products were resolved on a 1% agarose gel stained with ethidium bromide.

Transfections and plasmids. Promoter fragments containing 312, 151, and 123 bp of the human IL-13 promoter (numbered upstream from the transcription start site according to Ref. 32) were amplified from human genomic DNA by PCR and ligated into the pKml and Sau3A sites of the luciferase-based reporter construct pGL3 (Promega). Reporter constructs in a pGL3 backbone containing 2,666, 1,157, and 771 bp of the human IL-13 promoter were kindly provided by Dr. Donata Vercelli (Univ. of Arizona). Multimerized reporter constructs containing consensus AP-1, NF-κB, and NFAT sequences were purchased (PathDetect System, Promega). Transfection of Jurkat T cells (10 × 10⁶/condition) was performed in quadruplicate by electroporation (290 V, 960 μF) with 8 μg of promoter reporter constructs in 300 μl of serum-free medium. Transfected cells were either left untreated or stimulated with various combinations of A-23187, PMA, 8-BrcAMP, and LPA as indicated. Eighteen hours after transfection, cells were analyzed by luminometry using a Monolight 3010 Luminometer and using an assay kit according to the manufacturer’s instructions (Promega). Transfection efficiency was controlled for by cotransfecting cells with pSEAP2-Control (1 μg, Clontech) and normalizing firefly luciferase activity to secreted alkaline phosphatase measured by chemiluminescence (Great EscApE SEAP kit, Clontech).

In some experiments, cells were cotransfected with sense or antisense GATA-3 expression vectors (kindly provided by Dr. Anuradha Ray, Univ. of Pittsburgh).

Western blot. Whole cell lysates of T cells (40 μg/lane) were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked overnight with 6% milk in Tween PBS and then incubated with rabbit polyclonal antibodies directed against LPA1 and LPA2 (1:1,000 dilutions, courtesy of Dr. K. Belmonte, Glaxo Smith Kline) or mouse monoclonal antibodies directed against GATA-3 (1 μg/ml, Santa Cruz). After being incubated for 4–8 h, the membranes were washed and incubated with 1:300 dilution of horseradish peroxidase-conjugated secondary antibody for 30–60 min at room temperature and developed using enhanced chemiluminescence according to the manufacturer’s instructions (Amersham).

RNA stability experiments. Jurkat T cells (5 × 10⁶) were stimulated with submaximal concentrations of pharmacological agonists (0.1 μM A-23187, 5 ng/ml PMA, and 1 mM 8-BrcAMP) alone or with LPA (1 μM) for 6 h, followed by the addition of actinomycin D (3 μg/ml) to halt ongoing transcription. After 1, 2, and 3 h, cells were pelleted and total RNA was extracted using TRizol (GIBCO) according to the manufacturer’s instructions. RNA was cleaned of contaminating genomic DNA using an RNA clean-up kit (Ambion). RNA was quantitated by spectrophotometry and agarose gel electrophoresis, and 0.5 μg were subjected to end point RT-PCR using the following primers specific for IL-13, β-actin, and peptidyl prolyl isomerase A (PPIA) as controls: IL-13 (forward)-AAGGCTCCGGTCCTCGCA- GG, IL-13 (reverse)-GGGCCACCTTGATTTGTTGT; GAPDH (forward)-GGAGCCTAAAGGGTCATCATCTC, GAPDH (reverse)-
Fig. 1. A: RT-PCR was performed using RNA isolated from resting Jurkat T cells and primers for the 3 lysophosphatic acid (LPA) receptors (LPAR), LPA1, LPA2, and LPA3. The PCR was performed in duplicate with and without reverse transcriptase (RT) as indicated. Jurkat cells constitutively express mRNA for LPA1 and LPA2, but not LPA3, and no bands were detected in the absence of RT. B: RT-PCR was performed using RNA from resting Jurkat (Jkt) T cells, primary bronchial epithelial cells (Epi), and human CD4+ cells using primers specific for LPA3. A PCR product of the expected size was observed using only the bronchial epithelial cell mRNA, but not T cell mRNA. C: Western blotting of whole cell lysates from resting Jurkat T cells was performed using antibodies directed against LPA1 and LPA2 (see MATERIALS AND METHODS), demonstrating that Jurkat T cells constitutively expressed LPA1 and LPA2 protein.

AGTGGGTTGTTCGTTGGAAGTC; PPIA (forward)-GCCGCGTGCT-CCTTCGAGCTG, PPIA (reverse)-TGCTTGCCATCCGACGGCTCA. PCR was performed for 29 cycles with an annealing temperature of 55°C. PCR products were resolved on a 1% agarose gel stained with ethidium bromide and imaged using a Bio-Rad gel documentation system. IL-13, β-actin, and PPIA band intensities were analyzed by densitometry using NIH Image software (version 1.62). β-Actin and PPIA were essentially unchanged under all conditions. The expression of IL-13 mRNA was then analyzed using a linear fit model of R² ≥ 0.97 for all samples, and the half-life was calculated accordingly.

STAT6 phosphorylation in primary human bronchial epithelial cells. Primary human bronchial epithelial cells were isolated from normal human lung obtained from anonymous lung transplant donors (exempt from RB review) as described (51). P1 passage cells were incubated at 37°C in 5% CO₂ and 95% air to ~80% confluence in basal essential growth medium (supplied by Clonetics, BioWhittaker, Walkersville, MD) in 12-well, collagen-coated dishes. Supernatant from CD4+ T cells activated with and without LPA were added to cells for 15 min in a blinded fashion. Human bronchial epithelial cells were rinsed twice with ice-cold PBS and lysed in 1 ml of lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 5 mM β-glycerophosphate, 1 mM MgCl₂, 1% Triton X-100, 1 mM sodium orthovanadate, 10 μg/ml protease inhibitors, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. Western blotting was performed by using anti-phospho-STAT6 antibody and anti-STAT6 antibody (BD Science, Bedford, MA).

Statistical analysis. Data are presented as means ± SE, and differences among groups were analyzed using the Wilcoxon signed rank test (StatView 5.0). A P value < 0.05 was considered statistically significant.

RESULTS

LPA₁ and LPA₂ are constitutively expressed by Jurkat T cells. To better characterize the ability of LPA to regulate effector T cell cytokine secretion, we wanted to establish a model of LPA-dependent T cell activation. We first used Jurkat T cells because these cells have been reported to express LPA receptors and respond to LPA and can also be transfected with high efficiency (22, 53). We used RT-PCR and Western blot analysis to examine the expression of LPA receptors in Jurkat cells. Consistent with prior reports, these cells constitutively express mRNA for LPA₁ and LPA₂ (Fig. 1A). We did not detect expression of LPA₃ nor of the recently described receptor LPA₄ (Figs. 1B and 2). By Western blot analysis using antibodies directed against LPA₁ and LPA₂, we found constitutive expression of immunoreactive LPA₁ and LPA₂ consistent with results using RT-PCR (Fig. 1C). Emerging data suggest that the expression of lysophospholipid receptors depends on the state of T cell activation (6). Therefore, we next examined LPA receptor gene expression using RT-PCR and RNA isolated from cells at different time points after activation. Figure 2 shows that LPA₂ gene expression gradually increased during cell activation, whereas LPA₁ expression was relatively constant. In these experiments, we confirmed the observation of Chu et al. (6) that expression of mRNA encoding S1P₁ (or Edg-1, a receptor for S1P) was downregulated in
activated T cells. The biological activity of LPA is regulated by hydrolysis by LPPs (51), but whether T cells express LPPs is currently unknown. Using PCR primer pairs specific for the LPP family members LPP1–4, we found that Jurkat cells constitutively express mRNA for LPP1 but not LPP2–4 (Fig. 2 and data not shown). Thus Jurkat T cells express predominantly LPA1 and LPA2, with LPA2 being dominant in activated cells.

**LPA increases expression of IL-13 in submaximally stimulated T cells.** To determine whether LPA altered the expression of IL-13, we stimulated Jurkat T cells with LPA alone and with varying combinations of the calcium ionophore A-23187 [to activate the calcineurin/NFAT pathway (41)], the phorbol ester PMA (to activate PKC and downstream effectors), and the cAMP analog 8-BrcAMP [to activate GATA-3 (5)]. We established maximal activation conditions and reasoned that LPA might be more effective under submaximal conditions given recent experience with T cells and the related lipid mediator lysophosphatidylcholine (see also DISCUSSION) (30). We used every possible combination of different agonists, and Fig. 3 shows those combinations yielding significant results. In the absence of LPA, expression of IL-13 was induced by costimulation of Ca\(^{2+}\) and PKC-dependent signaling pathways together. Coincubation with the cell-permeable cAMP analog 8-BrcAMP further augmented IL-13 secretion in cells stimulated with Ca\(^{2+}\) ionophore plus PMA (Fig. 3). Similar results were obtained when IL-13 gene expression was analyzed by RT-PCR (data not shown). Interestingly, coincubation with LPA increased IL-13 secretion approximately fivefold at submaximal concentrations of Ca\(^{2+}\) ionophore plus PMA, which by themselves induced negligible expression of this cytokine. Furthermore, under submaximal conditions, LPA strikingly synergized with cAMP-dependent signals to restore IL-13 secretion to maximal levels (∼10-fold induction). LPA alone slightly reduced constitutive IL-13 secretion, and coculture with LPA did not affect cell viability or proliferation as assessed by trypan blue staining (Fig. 3 and data not shown). Consistent with prior reports (4), we did not detect any secretion of the related Th2 cytokine IL-4 from Jurkat cells under any condition examined (data not shown). Thus LPA represents a novel activator of IL-13 secretion in Jurkat T cells.

**LPA augments IL-13 expression at the transcriptional level.** Although less well studied than the related Th2 cytokine IL-4, data are accumulating that the IL-13 promoter is sufficient for tissue-restricted and inducible IL-13 expression in T cells (26). To study possible effects of LPA on the transcriptional regulation of IL-13, we next transfected Jurkat T cells with plasmids encoding reporter constructs containing varying lengths of the IL-13 promoter and studied their activity in cells stimulated with and without LPA. Reporter constructs were synthesized to isolate elements with potential regulatory activity including a purine box (PuB, centered around −130 bp upstream from the transcription start site) and potential GATA-3 binding sites (centered ∼ −90 bp, see schematic in Fig. 4). Cells were stimulated under submaximal conditions as defined in Fig. 3 to uncover potential enhancing effects of LPA. As shown in Fig. 4, constructs containing 312 and 151 bp of the IL-13 promoter were readily inducible in Jurkat cells, but deletion of the PuB abrogated both basal promoter activity and inducibility by pharmacological agonists. Activity of the 151-bp construct was consistently higher than the 312-bp construct, suggesting that this region harbors a negative regulatory element (NRE). Interestingly, costimulation with LPA led to a significant approximate threefold increase in activity of the full-length 312-bp promoter construct, an effect that was eliminated with shorter promoter constructs (Fig. 4A). At least two possibilities can explain this observation. First, the region between 312 and 151 bp upstream of the IL-13 transcription start site might contain a binding site for an LPA-activated transcription factor. Second, LPA could act by displacing a putative repressor factor that interacts with the NRE. Future experiments will be needed to distinguish between these and other possibilities. Sequence inspection of this region of the promoter did not reveal any obvious consensus motifs for transcription factors previously implicated in LPA-induced gene activation (including NF-κB, AP-1, or serum response factor, data not shown).

Transcriptional regulation of the IL-13 gene has been linked to the calcium-dependent factor NFAT, which can bind the PuB in gel-shift assays (10). NFAT frequently cooperates with members of the AP-1 family to induce transcriptional activity. To investigate the potential for LPA to regulate these transcription factor pathways, we studied the effects of LPA on transcription driven by canonical NFAT and AP-1 multimers. We also examined the potential role of NF-κB, given that LPA has been shown to activate this factor in other cells (43). After transfection, cells were stimulated with and without LPA followed by analysis of reporter gene expression using luminometry. Cell stimulation induced varying degrees of AP-1, NF-κB, and NFAT-dependent transcription (Fig. 4B). Interestingly, incubation with LPA alone or coincubation with other agonists did not further enhance the transcriptional activity of any pathway (Fig. 4B and data not shown).

Because GATA-3 has been shown to activate the mouse IL-13 promoter in EL-4 T cells (26), we wondered whether LPA was enhancing GATA-3-dependent transcriptional activation. We first confirmed that GATA-3 enhanced human IL-13 promoter activity in Jurkat cells by cotransfecting a GATA-3 expression vector together with the 312-bp promoter.
LPA ENHANCES IL-13 EXPRESSION

Fig. 4. LPA augments IL-13 promoter activity. A: Jurkat T cells were transiently transfected with 1 of 3 IL-13 luciferase (luc) reporter constructs containing 312, 151, and 123 bp of the promoter and an internal control (pSEAP control), followed by incubation with A-23187 (0.1 μM), PMA (5 ng/ml), + 8-BrcAMP (1 mM) with or without LPA as indicated. Eighteen hours later, reporter gene expression was analyzed by luminometry (see MATERIALS AND METHODS). A schematic of the promoter constructs is indicated (bottom) with the relative locations of a potential negative regulatory element (NRE), LPA response element (LPARE), the purine box (PuB), and potential GATA-3 sites. Data are means ± SE of n = 4 expressed relative to the activity of a control vector (pSEAP control, see MATERIALS AND METHODS). B: multimerized response elements containing canonical AP-1, nuclear factor of activated T cells (NFAT), and NF-kB binding sites driving luciferase expression were transfected by electroporation, followed by cell activation and analysis of reporter gene expression as in A. Data are means ± SE of n = 5 expressed relative to the baseline activity of each reporter in unstimulated cells, which was set equal to 1. *P < 0.05 for the effect of LPA using Wilcoxon’s signed rank test. RLU, relative light units; SEAP, secreted alkaline phosphatase.

Fig. 5. GATA-3 augments IL-13 promoter activity by a distinct mechanism from LPA. A: Jurkat T cells were transiently transfected with a 312-bp IL-13 luciferase reporter construct together with expression vectors containing sense or antisense GATA-3 (courtesy of Dr. A. Ray), followed by incubation with different pharmacological agonists as indicated. Eighteen hours later, reporter gene expression was analyzed by luminometry. Data are means ± SE RLU of n = 3–4 expressed relative to the activity of a control vector (pSEAP control, see MATERIALS AND METHODS). B: whole cell lysates from Jurkat cells stimulated with and without LPA and different pharmacological agonists as indicated were analyzed for GATA-3 expression by Western blot. A single band of the expected molecular weight was observed in all lanes. IB, immunoblot.

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Fig. 5A shows that overexpression of GATA-3 potently enhanced promoter activity, whereas antisense GATA-3 suppressed reporter gene activity to background levels. Interestingly, maximal promoter activation was observed in GATA-3 cotransfected cells after activation of calcium-, PKC-, and cAMP-signaling pathways (similar to results obtained when analyzing IL-13 protein secretion, Fig. 3). We then analyzed the promoter elements involved in GATA-3-driven IL-13 promoter activity by cotransfecting GATA-3 together with the three promoter deletion constructs. Figure 5B shows that GATA-3 potentiated transcription driven by the 312-bp construct as well as the 151-bp construct lacking the LPA response element (LPARE), suggesting that LPA and GATA-3 activate the IL-13 promoter through distinct mechanisms. Interestingly, deletion of the PuB (in construct 123 luc) abrogated the transacting ability of GATA-3, even though this construct contains the putative GATA binding sites identified by Kishikawa et al. (26). This suggests that GATA-3 cooperates with a factor that binds further upstream, an observation that was not pursued further in this report. In support of the idea that LPA and GATA-3 act independently, we found that stimulation with LPA did not affect GATA-3 protein expression as assessed by Western blot analysis (Fig. 5C), nor did stimulation with LPA further enhance activity of the promoter constructs in cells cotransfected with GATA-3 (data not shown).

The magnitude of activation of the 312-bp promoter construct by LPA was less than that observed for protein secretion (~3-fold vs. ~10-fold, compare Figs. 3 and 4). Several possibilities could explain this difference, including that LPA could be acting through additional regulatory elements located outside of the proximal promoter or by a posttranscriptional mechanism. To investigate these possibilities further, we first studied the effects of LPA on extended IL-13 promoter reporter constructs containing 2,666, 1,157, and 771 bp upstream of the IL-13 transcription start site (see MATERIALS AND METHODS). These constructs contain all of the elements known to regulate the IL-13 promoter, including a polymorphic NFAT site associated with human asthma (47). In additional transfection experiments, however, we found that LPA costimulation did not pursue further in this report. In support of the idea that LPA and GATA-3 act independently, we found that stimulation with LPA did not affect GATA-3 protein expression as assessed by Western blot analysis (Fig. 5C), nor did stimulation with LPA further enhance activity of the promoter constructs in cells cotransfected with GATA-3 (data not shown).

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not further enhance transcription driven by these constructs (data not shown). We next wondered whether LPA could be acting at the posttranscriptional level to enhance IL-13 mRNA stability. To address this possibility, we coincubated Jurkat cells under submaximal conditions with the transcription inhibitor actinomycin D and analyzed the decay of IL-13 mRNA expression at various time points. In these experiments, we found that although IL-13 mRNA stability was slightly enhanced in submaximally activated Jurkat cells (half-life of 7.9 vs. 6.6 h), this was only slightly increased by coincubation with LPA (half-life of 8.3 h, see Fig. 6). Together, these data suggest that the enhancing effect of LPA on IL-13 protein secretion is mediated at least in part at the level of the proximal promoter, but we cannot absolutely exclude effects of LPA on chromatin remodeling or translational regulation.

LPA enhances IL-13 expression in submaximally stimulated human CD4+ T cells. The studies using Jurkat cells showed that LPA augmented the expression of IL-13 under submaximal activation conditions. We next examined whether LPA would augment IL-13 expression using CD4+ T cells stimulated under conditions of submaximal TCR ligation. To test this possibility, the ability of LPA to augment CD4+ T cell IL-13 secretion was examined in cells activated by different concentrations of plate-bound anti-CD3 antibody (to mimic TCR signaling) together with a fixed concentration of anti-CD28 (to mimic B7-driven costimulation). Using CD4+ lymphocytes isolated from peripheral blood of healthy donors, we first confirmed that these cells constitutively expressed LPA1 and LPA2 as determined by RT-PCR and Western blot assay (data not shown). Interestingly, coincubation with LPA further enhanced IL-13 secretion but only under conditions of submaximal TCR ligation (Fig. 7, A and B). Thus, similar to Jurkat T cells stimulated with pharmacological agonists, LPA can augment IL-13 secretion in CD4+ cells, but only under submaximal activation conditions. Costimulation of cells with LPA and higher concentrations of plate-bound anti-CD3 (1–10 μg/ml) did further augment (and occasionally reduced) IL-13 secretion (Fig. 6 and data not shown). To determine whether the enhancing effects of LPA were specific for IL-13, we analyzed CD4+ T cell supernatants for other cytokines including IFN-γ and IL-4. Figure 7 shows that LPA did not enhance IFN-γ or IL-4 secretion in submaximally activated CD4+ T cells. Data are means ± SE of n = 4–5. *P < 0.05 for an effect of LPA using Wilcoxon’s signed rank test.

![Fig. 6. LPA coinoculation only slightly affects IL-13 mRNA stability. Jurkat T cells were treated under submaximal conditions with and without LPA as in Fig. 3, and actinomycin D was used to halt ongoing transcription (see MATERIALS AND METHODS). IL-13 mRNA was analyzed by end point PCR and densitometry after 1, 2, and 3 h, and mRNA half-life was calculated using linear regression.](image-url)
analysis of the activation of the IL-13-induced transcription factor STAT6 by Western blot. Figure 8 shows that supernatants from LPA-costimulated T cells induced substantially more STAT6 phosphorylation than from cells stimulated without LPA. There was no detectable production of IL-4 in these experiments, indicating that STAT6 phosphorylation was due to the effects of IL-13 (data not shown). STAT6 activates the transcription of many proinflammatory genes in epithelial cells, including chemokines such as eotaxin (31). Therefore, these data show that LPA-costimulated T cells could enhance airway inflammation by activating epithelial cells.

**DISCUSSION**

There is a growing appreciation that LPA and related lysosphingolipids can augment several aspects of T cell function including apoptosis, cell migration, and cytokine synthesis (17–19, 53, 54). In this report we show that LPA exerts an enhancing effect on IL-13 expression in T cells, especially under conditions of submaximal activation. Our observations are in keeping with the prior studies of Goetzl et al. (18, 54), who showed that LPA could regulate the synthesis of other T cell cytokines including IL-2. We extend these results and demonstrate that LPA selectively enhances IL-13 expression and does so at the level of transcriptional activation through a novel regulatory element located in the proximal IL-13 promoter.

We found that LPA alone had minimal effects on IL-13 gene expression as determined by ELISA or RT-PCR (Figs. 1 and 5, and data not shown). These results are similar to studies by Goetzl et al. (18) who found that LPA by itself did not affect IL-2 secretion in T cells but rather modulated the effects of CD3/CD28 signaling. This contrasts with effects of LPA on other cell types, where LPA alone can readily induce gene expression (12, 39). The biochemical basis for these differences between T cells and other cell types remains to be determined. In other cell types, LPA receptors can couple to Gα, Gβ, and G12/13, and in Jurkat cells LPA has been shown to increase intracellular calcium concentrations (20, 44). In future studies, it will be important to investigate how LPA receptors are coupled to different G proteins and downstream effector cascades in CD4 cells and how these pathways interact with signaling driven by the TCR and costimulatory molecules.

Our observation that in both Jurkat T cells and CD4+ lymphocytes LPA was most effective at conditions of submaximal activation is strikingly reminiscent of the findings of Le et al. (30), who studied T cells from mice lacking the lysosphingadlycholine (LPC) receptor G2A. G2A-deficient mice develop a late-onset autoimmune syndrome characterized by enhanced T cell proliferation, suggesting that LPC inhibits T cell expansion in vivo. Differences in proliferation rate in vitro, however, were only apparent under conditions of submaximal CD3 ligation (30). The physiological correlate of submaximal T cell activation likely reflects immune responses where the quantity or affinity of the antigen is limiting. Interestingly, T cell receptor signal strength is emerging as an important regulator of T cell development and differentiation (3, 8, 23, 25, 42). Together, these results suggest that lysosphospholipids may have previously underappreciated roles as immune modulators. Future experiments in which TCR signal strength and can be titrated in the absence or presence of lysosphospholipids (e.g., with transgenic mice) should help address this issue.

We found that Jurkat and CD4+ T cells express predominantly LPA1 (Edg-2) and LPA2 (Edg-4) mRNA and protein, but not LPA3 (Edg-7) or the recently described receptor LPA4 (or GPR23) (18, 37). Expression of LPA2 mRNA progressively increased in Jurkat cells stimulated with calcium ionophore and phorbol ester (Fig. 3), suggesting that LPA2 is preferentially expressed in activated T cells. Interestingly, Zheng et al. (54) found that mitogen priming with PHA induced a modest reduction in LPA2 and an increase in LPA1 mRNA expression in CD4+ T cells. Differences in cell types or stimuli may account for these apparent discrepancies. Whether differential LPA receptor expression translates into differential T cell responsiveness is currently unknown. Very little is also known about the expression and function of LPPs in lymphocytes. We found by RT-PCR that activated Jurkat cells express mRNA for LPP1, suggesting that this enzyme may limit the biological activity of LPA after T cell activation. We acknowledge that in some cells, LPA1 and possibly other receptors may be expressed intracellularly [e.g., in the nuclear compartment (16)] and that we have not conclusively demonstrated that LPA receptors are expressed on the T cell surface. Currently available antibodies do not allow this issue to be resolved with certainty. In preliminary experiments using purified nuclear fractions from resting and activated Jurkat and CD4+ T cells, however, we did not detect any evidence of nuclear LPA1 or LPA2 expression by Western blot analysis (data not shown). Although this suggests that these receptors are expressed mainly on the cell surface, future studies using antibodies directed against extracellular domains of the receptors will help resolve this issue.

In contrast to the related Th2 cytokine IL-4, relatively little is known about the transcriptional regulation of the IL-13 gene. Dolgovan et al. (10) identified a Puβ that bound NFAT proteins in gel-shift assays and was required for Ca2+ ionophore and PMA-induced promoter activity in Jurkat cells. Kishikawa et al. (26) recently identified two GATA-3 binding sites in the proximal murine IL-13 promoter and showed that GATA-3 could enhance promoter activity in EL-4 cells. Our deletion analysis confirms the critical importance of the Puβ in IL-13 transcriptional regulation. However, we found that LPA does not activate transcription driven by the Puβ-containing construct 151 luc, which suggests that LPA is not acting through the calcineurin-NFAT pathway. In support of this, we did not observe activation of the multimerized NFAT response element in LPA-stimulated cells. Instead, our promoter deletion...
analysis suggests that LPA acts through an LPARE located further upstream.

We found that the enhancing effects of LPA on IL-13 secretion were most pronounced in Jurkat cells coincubated with 8-BrcAMP, which also potentiated IL-13 secretion in the absence of LPA (Figs. 3 and 5). Stimulation of the adenylyl cyclase/cAMP cascade has long been known to induce Th2-type cytokines (and inhibit Th1 cytokines) in effector T cells (1, 5, 27, 36). Interestingly, Elliott et al. (13) found that cAMP-dependent signaling was required for histamine-induced IL-13 expression in Th2 cells. The precise mechanisms by which the cAMP cascade enhances Th2 cytokine gene expression are unknown. Chen et al. (5) recently showed that cAMP activated a p38 MAPK-dependent pathway resulting in GATA-3 phosphorylation and IL-13 secretion in D10 and in vitro differentiated Th2 cells. Therefore, one possibility is that transcription factors induced by LPA interact with DNA-bound, phosphorylated GATA-3 to drive IL-13 expression. However, we found no evidence that LPA was enhancing GATA-3 expression or its transactivating functions at the IL-13 promoter.

In summary, we have described a novel role for LPA in enhancing expression of the Th2 cytokine IL-13. Our studies add to the growing body of literature showing that lysosphospholipids regulate many aspects of T cell function. Excess generation of LPA may contribute to dysregulated IL-13 expression in inflammatory lung diseases such as allergic asthma.

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