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Lysophosphatidic Acid Receptor 1 Suppression Sensitizes Rheumatoid Fibroblast-like Synoviocytes to Tumor Necrosis Factor–Induced Apoptosis

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Objective. **To investigate the role of lysophosphatidic acid (LPA) receptors in the proliferation and apoptosis of fibroblast-like synoviocytes (FLS) from patients with rheumatoid arthritis (RA).**

Methods. **Expression of LPA receptors 1–3 was analyzed by real-time polymerase chain reaction (PCR). *LPAR1* and *LPAR2* were suppressed in RA FLS by small interfering RNA (siRNA) transfection. Proliferation of RA FLS after tumor necrosis factor (TNF) and LPA stimulation was determined with a luminescent cell viability assay. Apoptosis was analyzed by quantification of nucleosome release and measurement of activated caspase 3/7. Genes involved in the apoptotic response were identified with a human apoptosis PCR array and validated with Western blot assays. The requirement of these genes for apoptosis sensitization was assessed by siRNA transfection. Secretion of mediators of inflammation was analyzed by enzyme-linked immunosorbent assay.**

Results. **Only *LPAR1* and *LPAR2* were expressed by RA FLS, and their levels were higher than those in osteoarthritis (OA) FLS. Suppression of *LPAR1* abrogated TNF-induced proliferation and sensitized the RA FLS, but not the OA FLS, to TNF-induced apoptosis. These changes occurred despite an increased early inflammatory response to TNF. Sensitization to apoptosis was associated with changes in expression of multiple apoptosis-related genes. Three of the up-regulated proapoptotic genes were further studied to confirm their involvement. In contrast, suppression of *LPAR2* showed no effect in any of these analyses.**

Conclusion. **LPA_1 is an important receptor in RA FLS. Its suppression is accompanied by a global increase in the response to TNF that is ultimately dominated by sensitization to apoptosis.**

Rheumatoid arthritis (RA) is a chronic disease characterized by synovial hyperplasia and inflammation of peripheral joints, leading to progressive destruction of cartilage and bone. The relevance of mediators of inflammation to the pathogenesis of RA has been confirmed by the efficacy of the new treatments that block proinflammatory cytokines (1–3), yet 20–40% of RA patients do not respond adequately. Resident synoviocytes not targeted by available medications could be involved in treatment resistance because of their contribution to the production of cytokines, chemokines, and metalloproteases that results in RA damage.

In RA, dysregulated proliferation and apoptosis seem to be pivotal to the expansion of resident synoviocytes that have been demonstrated in situ in synovial tissues and in vitro in cultured fibroblast-like synoviocytes (FLS). The dysregulated apoptosis of RA FLS is characterized by resistance to apoptosis despite expression of the functional death receptors Fas/CD95, TRAILR-1, TRAILR-2, and tumor necrosis factor receptor (TNFR) (3–5). This cell phenotype is strongly influenced by the pleiotropic effect of tumor necrosis

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Drs. Orosa, González, Gómez-Reino, and Conde have filed a patent application concerning lysophosphatidic acid receptor targeting in rheumatoid arthritis.

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factor (TNF), which is able to induce cell proliferation and apoptosis, in addition to its well-known effect as a proinflammatory mediator. In RA, TNF-mediated FLS cell death is insignificant, whereas TNF-mediated FLS proliferation and inflammation are important (6,7).

Lysophosphatidic acid (LPA) is a phospholipid produced by cleavage of the choline group from lysophosphatidylcholine by lysophospholipase D, also called autotaxin. LPA is recognized by 6 G protein–coupled receptors (LPA_{1-6}). LPA_{1-3} are encoded by the genes *LPAR1* through *LPAR3* (formerly called *EDG2*, *EDG4*, and *EDG7*, respectively) and share sequence homology between them (~50–60% amino acid homology), whereas LPA_{4-6} sequences, which are encoded by *LPAR4* through *LPAR6*, are more dissimilar. These receptors interact with several G proteins, namely, $G_{i/o}$, $G_{q/11,}$ and $G_{12/13}$, depending on the cell type, and lead to activation of several intracellular pathways. For example, interaction with $G_{i/o}$ protein leads to a decrease in cAMP and activation of phosphatidylinositol 3-kinase/Akt and Ras/MAPK, whereas interaction with $G_{q/11}$ and $G_{12/13}$ proteins signals for activation of phospholipase C and RhoA, respectively (8–11).

Involvement of the LPA signaling pathway in RA has been suggested by studies showing expression of the LPA-producing enzyme autotaxin in FLS and synovial fluid samples from RA patients (12,13) as well as expression of LPA receptors in RA FLS (14,15). In addition, LPA has been shown to induce production of the inflammatory mediators interleukin-6 (IL-6), IL-8, and cyclooxygenase 2 in RA FLS (14,15).

In the present study, we confirmed that the main LPA receptor in RA FLS is LPA_1, and we showed that suppression of this LPA receptor critically regulates TNF signaling by increasing apoptosis and decreasing proliferation of FLS. We also identified 3 of the pro-apoptotic mediators involved in this response as being TRAIL, TRADD, and PYCARD.

MATERIALS AND METHODS

Preparation of FLS. FLS were derived from synovial tissue obtained from 10 patients with RA and 4 patients with osteoarthritis (OA) who were undergoing synovectomy or total joint replacement surgery. RA patients fulfilled the American College of Rheumatology (ACR) 1987 criteria for the classification of RA (16). OA was diagnosed according to clinical signs and symptoms. The study was performed according to the recommendations of the Declaration of Helsinki and was approved by the Comite Etico de Investigación Clínica de Galicia.

Synovial tissue was minced and incubated with 10 μg/ml of collagenase in serum-free Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen) for 3 hours at 37°C. After digestion, FLS were filtered through a nylon cell strainer (BD Falcon), washed extensively, and cultured in DMEM supplemented with 10% volume/volume fetal calf serum (FCS), 1% penicillin/streptomycin, and 1% L-glutamine (all reagents from PAA Laboratories) in a humidified atmosphere consisting of 5% CO_2. Adherent cells at 80–90% confluence were trypsinized and diluted at a split ratio of 1:3. FLS from a minimum of 6 patients and from passages 3 and 8 were used for all experiments. It is interesting to note that these cells were continuously exposed to LPA, as they were cultured in medium containing ≥1% FCS, and as reported by Nagasaki et al (17), 0.5–2% serum represents ~60–240 n*M* LPA in the culture medium.

Small interfering RNA (siRNA) transfection of FLS. We purchased siRNAs for *LPAR1*, *LPAR2*, *Apo2L/TRAIL*, *TRADD*, and *PYCARD/TMS1/ASC*, as well as nonsilencing siRNAs (control siRNAs), from Thermo Scientific Dharmacon. RA FLS (8.5×10^4 cells/well in 6-well plates) were transiently transfected with 20 n*M* *LPAR1*, 20 n*M* *LPAR2*, 50 n*M* *TRAIL*, 100 n*M* *TRADD*, or 100 n*M* *PYCARD* siRNAs or with nonsilencing control siRNAs in Opti-MEM I reduced serum medium (Gibco) using 1.25 mg/ml of Dharmafect 1 (Dharmacon). The degree of suppression was determined by Western blotting or by real-time quantitative polymerase chain reaction (qPCR). Experiments were performed 72–112 hours after siRNA transfection.

Proliferation assay. After siRNA transfections, RA and OA FLS (2×10^3 cells/well) were cultured in 96-well plates with DMEM and 1% FCS. Cells were treated with 10 ng/ml of TNF, 2.5 μ*M* LPA, or both for 24, 48, or 72 hours, and proliferation was determined using a CellTiter-Glo luminescent cell viability assay (Promega) according to the manufacturer's instructions.

Apoptosis assay. After siRNA transfections, RA and OA FLS (3×10^3 cells/well) were cultured in DMEM with 1% FCS in 96-well plates and treated with 10 ng/ml of TNF for 48 hours. Apoptosis was determined by quantifying mono- and oligonucleosomal DNA using a cell death detection enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics) as previously described (18). Apoptosis was confirmed by analysis of activated caspase 3/7 using a Caspase-Glo 3/7 assay (Promega). RA FLS (10^4 cells/well) were incubated in 96-well plates for 1 hour with reconstituted Caspase-Glo 3/7 reagent, and the luminescence signal generated after cleavage of DEVD–aminoluciferin substrate by caspase 3/7, was measured using a FluoStar Optima microplate reader (BMG Labtech).

Real-time qPCR. Total RNA was obtained using an RNeasy kit and an RNase-Free DNase set (Qiagen) according to the manufacturer's instructions. Real-time qPCR was performed in duplicate in an Mx3005P real-time qPCR system, using Brilliant SYBR Green Single-Step qRT-PCR Master Mix (both from Stratagene), according to the manufacturer's protocol. Relative levels of gene expression were normalized to the β-actin gene using the comparative C_t method, where C_t is the cycle at which the amplification is initially detected. The relative amount of mRNA was calculated according to the $2^{-\Delta\Delta C_t}$ method, where

$$\Delta C_t = C_{t\,\text{target}} - C_{t\,\beta\text{-actin}}$$

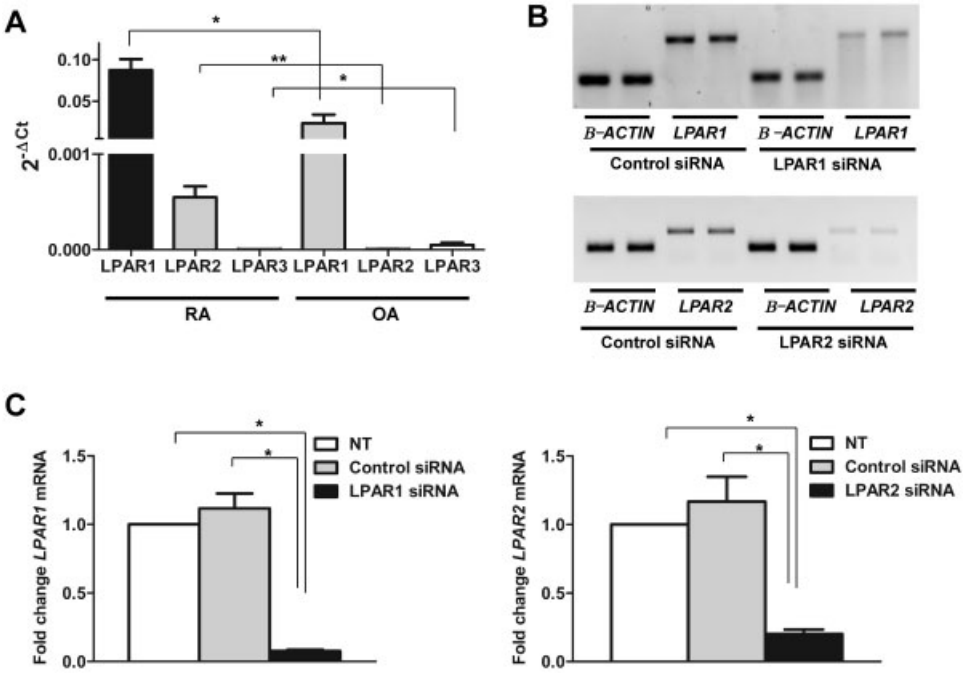

Figure 1. Expression of lysophosphatidic acid (LPA) receptors in rheumatoid arthritis (RA) and osteoarthritis (OA) fibroblast-like synoviocytes (FLS) and efficiency of *LPAR* silencing with small interfering RNA (siRNA). **A,** Levels of mRNA for LPA receptors relative to that for β-actin were determined. **B** and **C,** RA FLS were nontransfected (NT) or were transfected with *LPAR1*, *LPAR2*, or control siRNA. After transfection, total RNA was extracted and real-time quantitative polymerase chain reaction was performed. Shown are representative results from agarose gel electrophoresis (**B**) as well as the fold change in the expression of mRNA for *LPAR1* and *LPAR2* relative to nontransfected RA FLS (**C**). Values in **A** and **C** are the mean ± SEM of FLS from 6 RA patients and 4 OA patients. * = $P < 0.05$; ** = $P < 0.005$.

and

$$\Delta\Delta C_t = (C_{t\ target} - C_{t\ \beta\text{-actin}})_{control\ siRNA} - (C_{t\ target} - C_{t\ \beta\text{-actin}})_{experimental\ siRNA}$$

For the control siRNA, $\Delta\Delta C_t = 0$, and $2^0 = 1$. For the experimental siRNA, the value $2^{-\Delta C_t}$ indicates gene expression relative to β-actin and the value $2^{-\Delta\Delta C_t}$ indicates the fold change in gene expression relative to the control siRNA. Melting curves and agarose gel electrophoresis were used to establish the purity of the amplified PCR product.

The primer sets used were as follows: for *LPAR1*, 5′-TGTCTCGGCATAGTTCTGGA-3′ (forward) and 5′-CATTTCTTTGTCGCGGTAGG-3′ (reverse); for *LPAR2*, 5′-CATCATGCTTCCCGAGAACG-3′ (forward) and 5′-GGGCTTACCAAGGATACGCAG-3′ (reverse); for *LPAR3*, 5′-CTGCGGCAGTGATCAAAAACAGA-3′ (forward) and 5′-ATGGCCCAGACAAGCAAAATGAGC-3′ (reverse); for *IL6*, 5′-GTGGCTGCAGGACATGACAA-3′ (forward) and 5' TGAGGTGCCCATGCTACATTT-3′ (reverse); for *IL8*, 5' AAGAGCCAGGAAACCACC-3′ (forward) and 5′-GGAAAACGCTGTAGGTCAGAA-3′ (reverse); for *MCP1*, 5′-ACTCTCGCCTCCAGCATGAA-3′ (forward) and 5′-TTGATTGCATCTGGCTGAGC-3′ (reverse), and for β-actin, 5′-AGAAGGATTCCTATGTGGGCG-3′ (forward) and 5′-CATGTCGTCCCAGTTGGTGAC-3′ (reverse).

Human apoptosis PCR array. The relative expression of 84 apoptosis-related genes was assessed using the human apoptosis PCR array (SABiosciences). RA FLS (4×10^5 cells/well) were cultured for 12 hours with 10 ng/ml of TNF. Total RNA was isolated using an RNeasy Mini kit (Qiagen). The complementary DNAs (cDNAs) were reversed transcribed from 1 μg of RNA using a ReactionReady First-Strand cDNA Synthesis kit (SABiosciences). A total of 20 μl of cDNA from each sample was mixed with RT^2 Real-Time SYBR Green/ROX PCR Master Mix (SABiosciences) and qRT-PCR was performed. Relative levels of gene expression were normalized to the levels of 5 housekeeping genes using the comparative C_t method. The raw array data were processed and analyzed using PCR Array Data Analysis System software (online at http://sabiosciences.com/pcrarraydataanalysis.php), with a fold-change threshold set at 2.0.

Western blot analysis. RA FLS (8.5×10^4 cells/well in 6-well plates) were treated for 12 hours with 10 ng/ml of TNFα, washed twice with ice-cold phosphate buffered saline, and proteins were extracted using lysis buffer (50 m*M* Tris HCl [pH 7.5], 250 m*M* NaCl, 1% Triton X-100, 30 m*M* $Na_4P_2O_7$, 5 m*M* EDTA [pH 8.0], 100 m*M* NaF, 1 m*M* Na_3VO_4, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, and 1 m*M* phenylmethylsulfonyl fluoride). The protein concentration in the extracts was determined using a Qubit fluorometer (Invitrogen) according to the manufacturer's protocol. Whole cell lysates (10–30 μg of protein) were fractionated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to PVDF membrane (Hybond-P; Amersham Biosciences), and probed

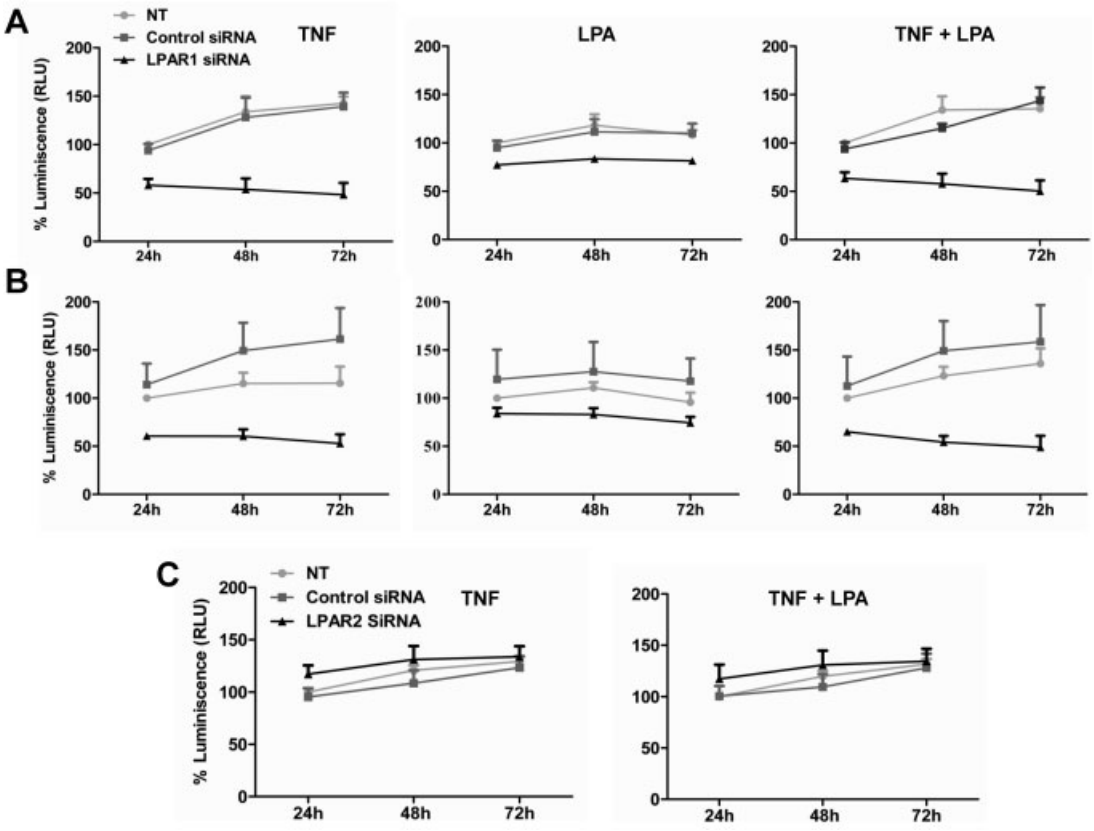

Figure 2. Effect of *LPAR1* or *LPAR2* suppression by silencing with small interfering RNA (siRNA) on the proliferation of fibroblast-like synoviocytes (FLS). **A** and **B,** Proliferation of control and *LPAR1*-suppressed rheumatoid arthritis (RA) FLS (**A**) or osteoarthritis (OA) FLS (**B**) after stimulation with 10 ng/ml of tumor necrosis factor (TNF), 2.5 μM lysophosphatidic acid (LPA), or both for 24, 48, or 72 hours. **C,** Proliferation of control and *LPAR2*-suppressed RA FLS stimulated with TNF or with TNF plus LPA for 24, 48, or 72 hours. Results are shown as the percentage of nontransfected (NT) control luminescence obtained at 24 hours, which is expressed in relative luminescence units (RLU). Values are the mean ± SEM of FLS from 6–7 RA patients and 4 OA patients.

with antibody to phospho-p38, p38, phospho-ERK, ERK, or β-actin (all from Sigma-Aldrich), phospho-JNK, JNK, or PYCARD (all from Santa Cruz Biotechnology), TRAIL (Cell Signaling Technology), or TRADD (BD PharMingen). Bound antibodies were revealed with horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology), and blots were developed using an ECL Plus detection system (Amersham Biosciences) or SuperSignal West Femto maximum sensitivity substrate (Thermo Fisher Scientific).

ELISA. RA FLS (1×10^4 cells/well) were stimulated for 12 hours with TNFα (10 ng/ml; Sigma-Aldrich). The supernatants were harvested at 12 hours, as indicated below, and assayed for IL-6, IL-8/CXCL8, and monocyte chemoattractant protein 1 (MCP-1)/CCL2 by ELISA according to the manufacturer's instructions (BD Biosciences).

Statistical analysis. Differences between experimental groups were assessed by Wilcoxon's matched-pairs test and by repeated-measures analysis of variance. *P* values less than 0.05 were considered significant.

RESULTS

Reduced TNF-induced proliferation of RA or OA FLS in the absence of LPA_1. Two previous studies have analyzed the expression of *LPAR1–3* in FLS from RA patients (14,15). LPA_1 expression was demonstrated in both studies, while messenger RNA (mRNA) for LPA_2 and LPA_3 was found only in the study by Zhao et al (14). We analyzed the mRNA expression of these LPA receptors in FLS from 10 RA and 4 OA patients by real-time qPCR. As shown in Figure 1A, LPA receptors were differentially expressed in RA and OA FLS. LPA_1 mRNA expression was higher in RA FLS than in OA FLS ($P = 0.019$), whereas LPA_2 mRNA was detected only in RA FLS. LPA_3 mRNA was not found in RA FLS, whereas weak expression was observed in OA FLS.

RA and OA FLS were transfected with *LPAR1* and *LPAR2* siRNA, and real-time qPCR was performed to verify the efficiency of silencing. The results from the RA FLS transfections are shown in Figures 1B and C. *LPA1* and *LPA2* siRNA produced nearly complete inhibition of *LPAR1* and *LPAR2* transcription, whereas expression in FLS transfected with control siRNA was similar to that in the nontransfected control FLS. Similar suppression efficiency was obtained for OA FLS (data not shown).

Next, we analyzed the effect of *LPAR1* and *LPAR2* knockdown on the proliferation of RA FLS induced by treatment with TNF, LPA, or both for up to 72 hours. As shown in Figure 2A, TNF-induced proliferation was significantly reduced in *LPAR1* siRNA–transfected FLS as compared to nontransfected FLS or control siRNA–transfected FLS ($P = 4 \times 10^{-4}$ and $P = 7 \times 10^{-4}$, respectively). LPA treatment did not induce proliferation, whereas TNF plus LPA treatment had a similar effect as TNF stimulation alone. Proliferation was significantly reduced by *LPAR1* knockdown ($P = 0.003$ and $P = 0.002$ versus nontransfected and control siRNA–transfected cells, respectively). Similar results were found with OA FLS (Figure 2B), with comparable levels of proliferation induced by TNF and by LPA plus TNF, a lack of proliferation induced by LPA, and reduced proliferation by *LPAR1* silencing ($P = 0.009$ versus control siRNA–transfected cells). *LPAR2* suppression did not modify the proliferation induced by TNF or by TNF plus LPA (Figure 2C).

***LPAR1* suppression and sensitization of RA FLS to apoptosis mediated by TNF.** The reduced proliferation and decreased cell number of *LPAR1* suppressed FLS, as was already observed with all treatments at 24 hours. This suggested the possibility of increased cell death. RA FLS were therefore stimulated with TNF for 48 hours, and apoptosis was determined by quantification of nucleosome release and caspase 3/7 activity. As shown in Figure 3A, a significant increase in nucleosome release and caspase 3/7 activity occurred in RA FLS lacking LPA_1, as compared with nontransfected or control siRNA–transfected FLS ($P = 0.031$ for nucleosome release; $P = 0.016$ for caspase 3/7 activity). This effect was specific to *LPAR1* knockdown, as suppression of *LPAR2* did not sensitize RA FLS to TNF-induced apoptosis (Figure 3B). Analysis of apoptosis in OA FLS showed similar resistance to that observed in RA FLS (Figure 3C), consistent with previous reports (19,20). Unlike in RA FLS, *LPAR1* silencing did not sensitize OA FLS to TNF-induced apoptosis. Therefore, increased apoptosis after *LPAR1* silencing was specific to RA FLS.

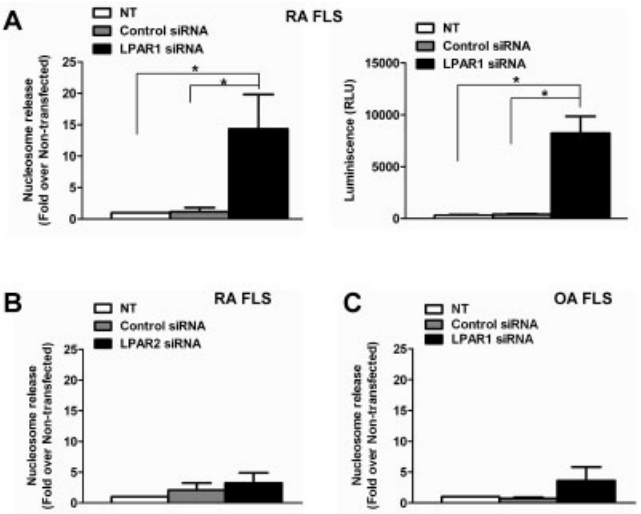

Figure 3. Effect of *LPAR1* or *LPAR2* suppression by silencing with small interfering RNA (siRNA) on apoptosis of fibroblast-like synoviocytes (FLS). Apoptosis of rheumatoid arthritis (RA) and osteoarthritis (OA) FLS following 48 hours of stimulation with 10 ng/ml of tumor necrosis factor (TNF) was quantified by nucleosomal release enzyme-linked immunosorbent assay and shown as relative to the nontransfected (NT) cell value (**A–C**), as well as by analysis of caspase 3/7 activity and shown as relative luminescence units (RLU) (**A**, right panel). Values are the mean ± SEM of FLS from 6–7 RA patients and 4 OA patients. $* = P < 0.05$.

Up-regulation of apoptosis-related genes by *LPAR1* deficiency. To explore the mechanisms underlying the sensitization to TNF-induced apoptosis in *LPAR1*-deficient RA FLS, we used a human apoptosis PCR array to analyze the expression of 84 apoptosis-related genes in FLS from 6 RA patients after the cells were exposed to 10 ng/ml of TNF for 12 hours. (Data on the differential expression of *LPAR1* siRNA– and control siRNA–transfected RA FLS genes are available upon request from the author.) The threshold used for change in expression as reported herein was ≥2.0-fold change at $P < 0.05$. Among the 17 up-regulated genes in RA FLS lacking LPA_1, 9 were proapoptotic genes (*BCL2L11*, *CASP1*, *CASP10*, *CASP3*, *CASP7*, *PYCARD*, *TNFRSF25*, *TNFSF10* or *TRAIL*, and *TRADD*), 4 were antiapoptotic genes *(BCL2*, *BIRC3*, *XIAP*, and *CFLAR*), and 4 genes had proapoptotic or antiapoptotic functions, depending on the stimulus and tissue studied (*CD40*, *RIPK2*, *TNF*, and *CD70*). Three genes with proapoptotic function (*TRAIL*, *TRADD*, and *PYCARD*) were chosen for confirmatory experiments, given their significant change in expression ($P \leq 0.01$) and their upstream localization in the apoptosis pathway. Their overexpression in RA FLS was confirmed by Western blot analysis.

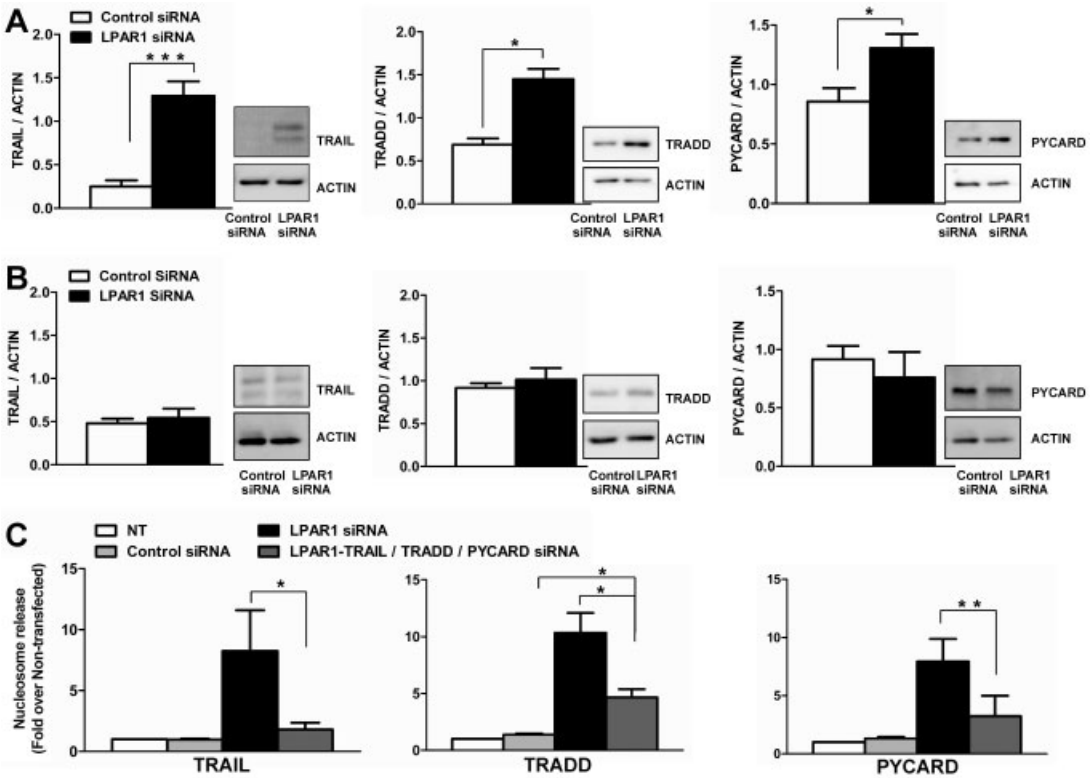

Figure 4. *LPAR1* suppression increases expression of TRAIL, TRADD, and PYCARD proteins in tumor necrosis factor (TNF)–stimulated rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS), and they are required for sensitization to apoptosis. **A** and **B,** *LPAR1* small interfering RNA (siRNA)–transfected or control siRNA–transfected RA FLS were treated for 12 hours with 10 ng/ml of TNF (**A**) or were left untreated (**B**), and total proteins were extracted for Western blot analysis. Representative blots are shown at the right. Densitometric quantification of band intensity normalized to actin control was then performed (left). Values are the mean ± SEM of FLS from 6–8 RA patients. $* = P < 0.05$; $*** = P < 0.001$. **C,** RA FLS were transfected with control, *LPAR1*, *LPAR1* + *TRAIL*, *LPAR1* + *TRADD*, or *LPAR1* + *PYCARD* siRNAs, and apoptosis was determined following 48 hours of stimulation with 10 ng/ml of TNF. Results are shown as the fold nucleosome release as compared with nontransfected (NT) cells, as determined by enzyme-linked immunosorbent assay. Values are the mean ± SEM of FLS from 6–8 RA patients. $* = P < 0.05$; $** = P < 0.01$.

As shown in Figure 4A, higher levels of TRAIL, TRADD, and PYCARD proteins were observed when *LPAR1* was suppressed by siRNA than when not suppressed ($P < 0.001$, $P < 0.015$, $P < 0.015$, respectively) (Figure 4A). We also analyzed whether *LPAR1* suppression regulates the constitutive (without TNF stimulation) expression of TRADD, TRAIL, and PYCARD in RA FLS. We found that similar levels of these proteins were observed in control and in *LPAR1*-suppressed RA FLS (Figure 4B).

Apoptosis sensitization by *LPAR1* suppression is dependent on TRAIL, TRADD, and PYCARD. To further analyze the role of TRAIL, TRADD, and PYCARD proteins in the sensitization to TNF-induced apoptosis of RA FLS lacking LPA_1, we used siRNA suppression. RA FLS were transfected with control siRNA or with *LPAR1* siRNA, either alone or in combination with *TRAIL*, *TRADD*, or *PYCARD* siRNAs. The efficiency of suppression was verified by Western blot analysis of total FLS proteins, which showed that expression of TRAIL, TRADD, and PYCARD proteins was suppressed by a mean ± SEM of 76.6 ± 0.06%, 95.2 ± 0.03% and 74.9 ± 0.09%, respectively.

The results of TNF-induced apoptosis in RA FLS with suppression of *LPAR1* and *TRAIL*, *TRADD*, or *PYCARD* are shown in Figure 4C. Suppression of any of the 3 proapoptotic genes reverted apoptosis sensitivity ($P = 0.03$, $P = 0.03$, and $P = 0.008$ for TRAIL, TRADD and PYCARD, respectively, versus *LPAR1* suppression alone). Apoptosis mediated by TNF was abrogated when *LPAR1* suppression was combined with *TRAIL* or *PYCARD* suppression, as the level of apoptosis was similar to that observed in nontransfected or control siRNA–transfected RA FLS. Reduction of apoptosis

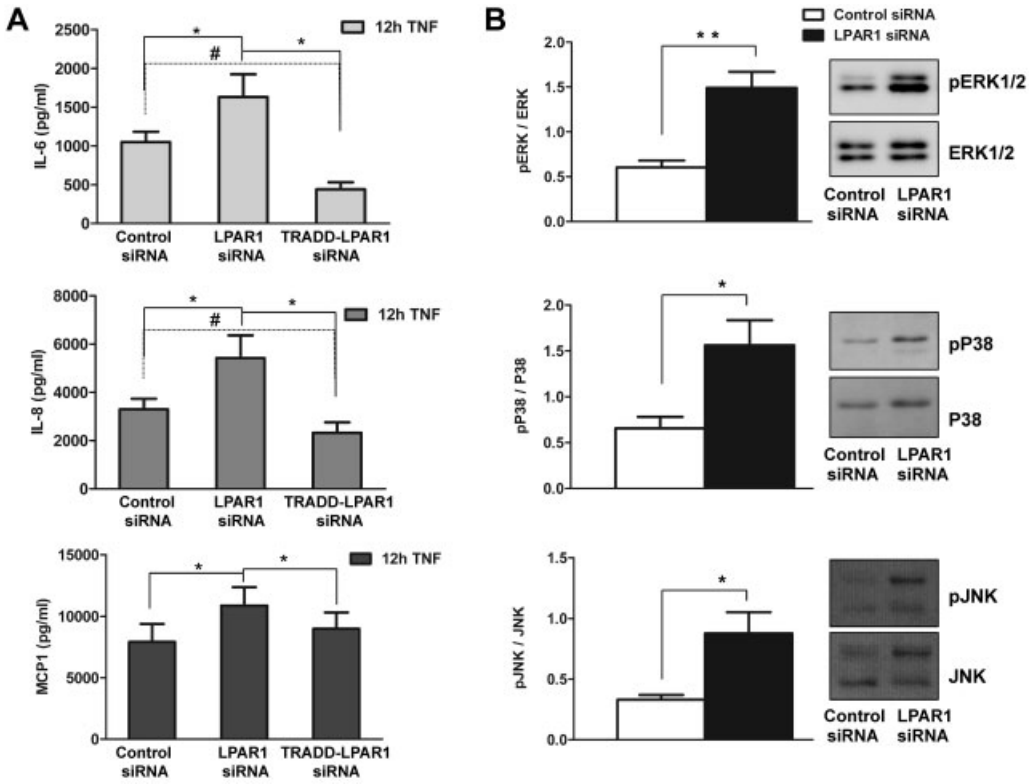

Figure 5. Enhanced tumor necrosis factor (TNF)–induced inflammatory response in *LPAR1*-suppressed rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS). Control, *LPAR1*, or *LPAR1* + *TRADD* small interfering RNA (siRNA)–transfected RA FLS were stimulated for 12 hours with 10 ng/ml of TNF. **A,** Secretion of interleukin-6 (IL-6), IL-8, and monocyte chemoattractant protein 1 (MCP-1) in culture supernatants was determined by enzyme-linked immunosorbent assay. **B,** MAPK activation was determined by Western blotting (representative blots at right) with densitometric quantification of band intensity (left). Values are the mean ± SEM of FLS from 6–9 RA patients. $* = P < 0.05$; $\# = P < 0.05$; $** = P < 0.005$.

was lower when *LPAR1* and *TRADD* were suppressed, but it was still significantly larger than that in the RA FLS transfected with only the control siRNA.

***LPAR1* suppression and increased response of inflammatory RA FLS to TNF.** To further evaluate the effects of *LPAR1* suppression on the response of RA FLS to TNF, we analyzed the production of inflammatory mediators and the activation of p38, JNK, and ERK downstream in the TNF signaling pathway. Transfected RA FLS were stimulated with TNF, and levels of IL-6, IL-8, and MCP-1 secretion in culture supernatants were determined by ELISA. As shown in Figure 5A, *LPAR1* suppression increased the TNF-induced production of the 3 inflammatory mediators. This higher inflammatory response to TNF was accompanied by increased MAPK activation, as shown by significantly higher levels of phospho-p38, phospho-ERK, and phospho-JNK in LPA_1-deficient RA FLS as compared to control siRNA–transfected FLS (Figure 5B).

Since TRAIL, TRADD, and PYCARD proteins have also been shown to be involved in inflammation, we analyzed the impact of their suppression on the increased TNF inflammatory response in LPA_1-deficient RA FLS. As shown in Figure 5A, the absence of TRADD in FLS lacking LPA_1 significantly reduced the increased inflammatory response to TNF. However, the absence of TRAIL or PYCARD did not modify the levels of IL-6, IL-8, or MCP-1 production in *LPAR1*-deficient RA FLS (data not shown).

DISCUSSION

LPA signaling has previously been shown to be involved in the inflammatory response of RA FLS (14,15), but its role in the proliferation or apoptosis of RA FLS, which is pivotal for the characteristic synovial hyperplasia of RA, had not heretofore been addressed. This study is the first to show that LPA_1 signaling is

important to the occurrence of hyperplasia, even if LPA did not induce increased FLS proliferation. In addition, when *LPAR1* was suppressed, TNF induced more apoptosis than proliferation, leading to a decrease in FLS cell numbers. These results suggest that blockage of LPA_1 will be useful as a treatment in RA.

The effect of LPA in the promotion of the proliferation of several cell types, especially cancer cells (21–24), is well known. However, LPA may have negative effects on cell growth, as in myeloma, or it may have no effect at all, as in astrocytes and Jurkat cells (25). Our results demonstrate that LPA neither induces proliferation in RA or OA FLS nor increases TNF-induced proliferation. This discrepancy between cell types reflects the fact that the effect of LPA is complex and is dependent on the tissue being analyzed.

Our study confirmed the expression of LPA_1 and LPA_2 in RA FLS, showed that LPA_1 is the major LPA receptor, and showed that its expression is increased in these cells compared to OA FLS, suggesting a role in the pathogenesis of RA. Furthermore, inhibition of LPA_2 had no effect on RA FLS proliferation, whereas LPA_1 abrogated the induced proliferation and sensitized the cells to apoptosis. Discrepancies in the functions of the LPA receptors have previously been noted. For example, LPA_1-null mice show important perinatal mortality and multiple development abnormalities in the surviving mice, whereas LPA_2-null mice are born normal and have no discernible phenotype (26). Thus, it is not surprising that we found a different response after the two LPA receptors were suppressed in these studies. This complexity is consistent with studies in colonic cancer cells. Proliferation of some cells depends on LPA_1, and in other cells, it depends on LPA_2 or LPA_3 (27), due to the diversity of mechanisms underlying the proliferative effects of LPA, which are mediated through multiple independent pathways.

The expected TNF-induced proliferation of FLS (28–31) was completely abrogated by *LPAR1* deficiency, both in RA FLS and in OA FLS. In contrast, *LPAR1* suppression sensitized RA FLS, but not OA FLS, to TNF-mediated apoptosis. This finding is very interesting because RA FLS are resistant to FasL-, TNF-, and TRAIL-induced apoptosis (3,32,33) despite their expression of death receptors. We tried to elucidate the mechanisms underlying the increased sensitivity of RA FLS lacking LPA_1 to TNF-induced apoptosis using an apoptosis PCR array analysis and analyzing other outcomes of TNF signaling in cytokine secretion and MAPK activation. These analyses identified 3 critical players in the sensitization to TNF-induced apoptosis and defined the fact that it is accompanied by increased signaling through the MAPK pathways and by increased secretion of the inflammatory cytokines IL-6, IL-8, and MCP-1. The variety of effects secondary to suppression of *LPAR1* due to interaction of LPA signaling with other signaling pathways (34) and the multiple levels of the TNF pathways at which they were found make it impossible to define a unique step or process that could be considered a determinant of the observed apoptosis sensitization.

The absence of *LPAR1* in TNF-stimulated RA FLS led to up-regulation of 17 of the genes analyzed, 9 of which were proapoptotic, 4 were antiapoptotic, and 4 were proapoptotic or antiapoptotic depending on the cell type or other factors. After selection of 3 proapoptotic genes (*TRAIL*, *TRADD*, and *PYCARD*), we confirmed their increased protein expression by Western blotting and showed that this regulation is dependent on TNF stimulation. The key role of these proteins was confirmed by showing that sensitization to TNF-induced apoptosis was reduced or abolished after knockdown of each of the genes. These results indicate the complexity and sensitivity of the mechanisms involved. In effect, apoptosis sensitization of RA FLS requires TRAIL, TRADD, and PYCARD molecules, which play a role in different stages of apoptosis signaling.

TRAIL is the most proximal of the 3 in the apoptosis pathways. It is a proapoptotic transmembrane glycoprotein that can be cleaved from the cell surface to form a soluble ligand. Similar to other extracellular proapoptotic ligands, such as TNF and FasL, it transmits signals through proapoptotic death receptors (DRs) to the caspase machinery. Binding of the homotrimeric TRAIL to TRAILR-1/DR-4 and TRAILR-2/DR-5 leads to assembly of the death-inducing signaling complex (DISC), which is formed by the oligomeric receptors, the adaptor protein FADD, caspase 8 and/or caspase 10, and depending on the circumstances, the cellular FLICE-inhibitory protein. DISC promotes the activation of caspases 8 and 10, which in turn, cleave and activate the effector caspases. TRAIL also promotes the formation of the so-called signaling complex II that contains TRADD. Signaling complex II is composed of FADD, TRADD, caspase 8, caspase 10, receptor-interacting protein 1 (RIP-1), TNFR-associated factor 2 (TRAF2), and IKKγ (NF-κB essential modulator), and it promotes the activation of IKK, JNK, p38 MAPK, and protein kinase B/Akt pathways (35–36). The central role of TRAIL is consistent with previous experiments showing that exogenous TRAIL induces apoptosis in a subset of RA FLS, depending on the relative expression of the

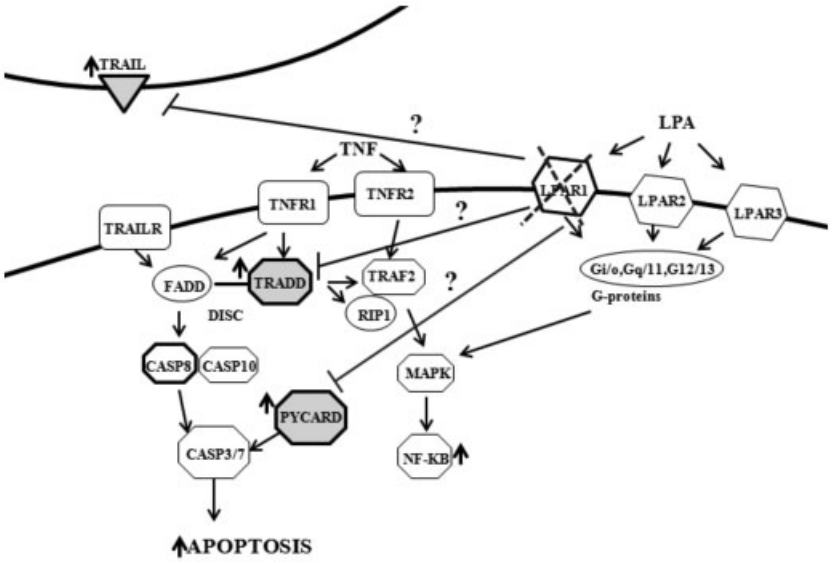

Figure 6. Schematic representation of the likely events leading from the *LPAR1*-induced suppression of tumor necrosis factor (TNF)–stimulated rheumatoid arthritis fibroblast-like synoviocytes to the sensitization to apoptosis through the up-regulation of TRAIL, TRADD, and PYCARD. LPA = lysophosphatidic acid; LPAR2 = LPA receptor 2; TNFRI = TNF receptor type I; DISC = death-inducing signaling complex; TRAF2 = TNFR-associated factor 2; RIP-1 = receptor-interacting protein 1; CASP8 = caspase 8.

death receptors TRAILR-1/DR-4 and TRAILR-2/DR-5 and the decoy receptors (DcR) TRAILR-3/DcR-1 and TRAILR-4/DcR-2 (37,38). Additional data from experimental arthritis models have demonstrated that intra-articular injection of TRAIL ameliorated the severity of arthritis (39,40).

TRADD is an adaptor protein that acts distal to TRAIL in the apoptosis pathways. TRADD is included in signaling complex II, which is formed after TRAIL signaling, and in the 2 complexes induced by TNF signaling through TNF receptor type I (TNFRI). TNFRI complex I is formed by TNFRI, TRADD, TRAF2, and RIP-1, and it mediates the activation of MAPKs and NF-κB. Subsequently, TRADD, RIP-1, and TRAF2 dissociate from TNFRI and interact with FADD to form complex II, which activates caspase 8 and leads to apoptosis (41–43). Participation of TRADD in TNFRI complex I could explain why its suppression in FLS was accompanied by a lack of increased cytokine secretion in our experiments, whereas no effect was observed with TRAIL or PYCARD suppression. Therefore, TRADD overexpression increases the early formation of complex I, which leads to activation of MAPK pathways and cytokine secretion and, subsequently, forms part of complex II, which along with TRAIL and PYCARD, leads to FLS apoptosis.

More distal is the participation of PYCARD (also known as target of methylation-induced silencing 1 [TMS-1] and as ASC), which is an adaptor protein that is up-regulated by TNF and TRAIL in some tissues. It forms large molecular complexes, and it is involved in the intrinsic and extrinsic pathways of apoptosis (44–46) and in the inflammasome, which is a large cytosolic protein complex, where PYCARD bridges the interaction between NLRP3 and caspase 1, leading to activation of the latter (47,48). Its involvement in the extrinsic apoptosis pathway seems to be related to activation of caspase 8 (49).

The roles of TRAIL, TRADD, and PYCARD in the different stages of apoptosis signaling in RA FLS may be described as follows. *LPAR1* suppression in RA FLS induces the up-regulation of TRADD, TRAIL, and PYCARD, which leads to an increase in the global response to TNF (Figure 6). Up-regulation of TRADD induces both inflammatory and survival responses, as well as sensitization to TNF-mediated apoptosis. The first effect is mediated by activation of MAPKs and NF-κB through complex I (TNFR, TRADD, TRAF2, and RIP-1). Apoptosis sensitization is mediated through complex II (TRADD, TRAF2, RIP-1, and FADD) leading to caspase activation. Up-regulation of TRAIL contributes to sensitization of apoptosis in RA FLS, through activation of DISC, which is also upstream of the caspase activation cascade. PYCARD is involved in

caspase activation as well. Therefore, sensitization to apoptosis, finally, dominates the inflammatory and survival responses.

The results described herein are of great interest, given the pivotal role of FLS apoptosis resistance in the pathogenesis of RA. Current treatments for RA are focused on targeting factors involved in inflammatory responses, but in many patients, they do not lead to complete remission. The insufficient response could be due to the contribution of other factors, such as the chronic hyperplasia of FLS. Data presented herein point to LPA_1 as a therapeutic target by which to block synovial hyperplasia by inducing apoptosis in RA FLS.

In conclusion, our study is the first to show that *LPAR1* suppression sensitizes RA FLS to TNF-induced apoptosis through the up-regulation of proapoptotic molecules, such as TRAIL, TRADD, and PYCARD, and points to LPA_1 as a therapeutic target.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Conde had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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