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Lpa2 Is a Negative Regulator of Both Dendritic Cell Activation and Murine Models of Allergic Lung Inflammation

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Negative regulation of innate immune responses is essential to prevent excess inflammation and tissue injury and promote homeostasis. Lysophosphatidic acid (LPA) is a pleiotropic lipid that regulates cell growth, migration, and activation and is constitutively produced at low levels in tissues and in serum. Extracellular LPA binds to specific G protein-coupled receptors, whose function in regulating innate or adaptive immune responses remains poorly understood. Of the classical LPA receptors belonging to the Edg family, *lpa2* (*edg4*) is expressed by dendritic cells (DC) and other innate immune cells. In this article, we show that DC from *lpa2*^{-/-} mice are hyperactive compared with their wild-type counterparts and are less susceptible to inhibition by different LPA species. In transient-transfection assays, we found that *lpa2* overexpression inhibits NF- κ B-driven gene transcription. Using an adoptive-transfer approach, we found that allergen-pulsed *lpa2*^{-/-} DC induced substantially more lung inflammation than did wild-type DC after inhaled allergen challenge. Finally, *lpa2*^{-/-} mice develop greater allergen-driven lung inflammation than do their wild-type counterparts in models of allergic asthma involving both systemic and mucosal sensitization. Taken together, these findings identify LPA acting via *lpa2* as a novel negative regulatory pathway that inhibits DC activation and allergic airway inflammation. *The Journal of Immunology*, 2012, 188: 000–000.

Originally described as an intermediate in intracellular lipid biosynthesis, lysophosphatidic acid (LPA; or monoacyl-*sn*-glycero-3-phosphate) is now recognized as a pluripotent extracellular mediator that regulates cell growth, migration, and activation (1). Extracellular LPA is generated primarily by hydrolysis of lysophosphatidylcholine by the enzyme autotaxin (or lysophospholipase D) (2, 3). A major advance in the field came from the molecular cloning of specific LPA receptors (4–6). There are at least five established LPA receptors, three of which belong to the Edg G protein-coupled receptor superfamily: LPA1 (Edg2), LPA2 (Edg4), and LPA3 (Edg7) (7). Signal transduction via these classical LPA receptors leads to activation of MAPKs, PI3K, and Rho kinases, which affect cell activation, survival, and migration (reviewed in Ref. 8). Activation of G α i and PI3K/Akt is emerging as particularly important for LPA-directed cell migration (9–13).

LPA has physiologic roles in wound repair and development (14–20) and emerging roles in disease states, including cancer, atherosclerosis, lung fibrosis, and asthma (21–31). LPA may play a broader role in regulating innate and adaptive immune responses (32). For example, constitutive expression of autotaxin in high endothelial venules contributes to lymphocyte homing to secondary lymphoid organs, presumably by inducing local LPA production and T cell emigration from the vasculature (33). Although LPA has proinflammatory effects (28), it can also inhibit inflammation in some contexts. For example, i.v.-injected LPA protected mice from LPS-induced peritonitis (34), and LPA attenuated cytokine secretion in human monocyte-derived dendritic cells (DC) (9, 35). However, the inhibitory mechanisms and receptor(s) by which LPA attenuates immune responses remain poorly defined.

In this article, we report that LPA2 (*edg4*) negatively regulates DC activation and allergic airway inflammation using different mouse models of asthma. Compared with wild-type controls, *lpa2*^{-/-} DC exhibit a hyperactive phenotype both in vitro in DC/T cell coculture, as well as following adoptive transfer into the mouse airway. Although LPA inhibits activation of wild-type DC in response to different pattern recognition receptor ligands, *lpa2*^{-/-} DC are resistant to LPA-dependent inhibition. In transfection studies, we show that expression of LPA2 inhibits LPS-induced NF- κ B activation. Finally, we studied allergic airway inflammation using OVA as a model allergen and protocols known to induce DC activation by either systemic immunization (OVA plus alum i.p.) or mucosal immunization (inhaled OVA plus low-dose LPS); *lpa2*^{-/-} mice developed greater allergen-driven lung inflammation than did their wild-type counterparts. Taken together, these studies uncover a novel anti-inflammatory role for *lpa2* and identify a new pathway involved in the suppression of innate immune responses.

Materials and Methods

Mice

Wild-type mice on the C57BL/6 background were from The Jackson Laboratory. LPA2-deficient (*lpa2*^{-/-}) mice were derived from frozen gene-

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Abbreviations used in this article: BAL, bronchoalveolar lavage; BM-DC, bone marrow-derived dendritic cell; DC, dendritic cell; GSK, GlaxoSmithKline; LPA, lysophosphatidic acid; PTX, pertussis toxin; RT, room temperature; VEGF, vascular endothelial growth factor.

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targeted embryos provided by Deltagen (San Mateo, CA), in collaboration with GlaxoSmithKline (GSK), and backcrossed for more than six generations onto the C57BL/6 background. Wild-type and gene-targeted mice were maintained at the University of Rochester, and age- and gender-matched littermate controls were used in all experiments. C57BL/6.PL OT-II TCR transgenic mice recognizing OVA peptide OVA₃₂₃₋₃₃₉ in the context of I^{ab} were a gift of Dr. David Topham (University of Rochester). Mouse protocols were reviewed by the University of Rochester Committee on Animal Resources and the GSK Institutional Animal Care and Use Committee and were conducted in accordance with institutional guidelines and the GSK Policy on the Care, Welfare, and Treatment of Laboratory Animals.

Bone marrow-derived DC

DC were derived from bone marrow precursors using modifications of previously published protocols (36). Briefly, wild-type and *lpa2*^{-/-} mice were euthanized and prepared aseptically to remove femurs and tibias for bone marrow harvest. On day 0, bone marrow cells were seeded at a density of 1×10^6 cells/ml in RPMI 1640 media supplemented with 10% heat-inactivated FBS (lot #103057; Tissue Culture Biologicals, Los Alamitos, CA), 1 M HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 20 mM 2-ME, and 50 mg/ml gentamicin using six-well plates (media and additives from Life Technologies, Carlsbad, CA), supplemented with 25 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ). The media were changed and supplemented with the same concentration of GM-CSF together with 10 ng/ml IL-4 (PeproTech) on days 2, 5, and 7. Bone marrow-derived DC (BM-DC) grown using this protocol typically express CD11c, high levels of MHC class II (Ia/Ie) and CD11b, and low levels of CD8 α and plasmacytoid dendritic cell Ag 1 (Ab panels available upon request). On day 8, DC were harvested and cocultured with allogeneic T cells at DC/T cell ratios of 1:1, 1:5, 1:25, 1:125, and 1:625. Cell proliferation was analyzed 72 h following coculture using a BrdU Cell Proliferation ELISA (Roche). Cell supernatants were analyzed by ELISA (IL-6 detection limit: 1.6 pg/ml; TNF- α detection limit: 1.8 pg/ml; vascular endothelial growth factor [VEGF] detection limit: 3 pg/ml; Quantikine kits, R&D Systems) or multiplex cytokine/chemokine bead array (IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, TNF- α , cotaxin, G-CSF, GM-CSF, IFN- γ , MCP-1, MIP-1 α , MIP-1 β , RANTES, and KC; Bio-Plex Pro Mouse Cytokine 23-plex Assay; Bio-Rad, Hercules, CA), according to the manufacturers' instructions.

Transfection of HEK293T cells with LPA2

HEK293T cells stably transfected with TLR4 and MD2 (a kind gift of Dr. Jian Dong Li, University of Rochester Medical Center, Rochester, NY) were maintained in DMEM (Life Technologies Invitrogen, Carlsbad, CA) containing heat-inactivated 10% FBS (Tissue Culture Biologicals), 100 μ g/ml streptomycin, 100 U/ml penicillin, and 0.5 mg/ml Geneticin (Invitrogen, Carlsbad, CA) at 37°C in 5% CO₂. Confluent HEK293-TLR4/MD2 cells were detached with 0.05% Trypsin-EDTA (Invitrogen) and subcultured every 2–3 d. A full-length *lpa2* cDNA expression vector was obtained from University of Missouri-Rolla cDNA Resource Center (Rolla, MO). HEK293-TLR4/MD2 cells were grown to ~80% confluency and transfected using jetPRIME transfection reagent (Polyplus Transfection, New York, NY), according to the manufacturer's protocol. Briefly, cells were seeded in 10% serum DMEM on poly-D-lysine-coated 24-well plates for 24 h. One microgram of plasmid DNA and 2 μ l jetPRIME transfection reagent were mixed with transfection buffer and incubated for 10 min at room temperature (RT), after which 25 μ l the mixture was added to each well. For NF- κ B reporter assay, cells were transfected with pNF κ B-luc (Stratagene) and pcDNA-3HA-LPA2 or pcDNA empty plasmid at a 1:1 ratio using jetPRIME transfection reagent, as described above. Twenty-four hours after transfection, cells were washed with PBS; the medium was replaced with 1% serum DMEM containing 0.5 mg/ml Geneticin, with or without LPS as indicated, and then incubated with reporter lysis buffer; and reporter gene activity measured by luminometry using a Monolight 3010 luminometer (Analytical Luminescence Laboratory, San Diego, CA), according to the manufacturer's protocol. Protein concentration was determined by Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL).

Western blot assays

HEK293T cells were collected and lysed with RIPA buffer supplemented with protease inhibitors. Protein concentrations were determined using Pierce BCA protein assay kit (Thermo Scientific). Protein samples were prepared by adding 6 $\times$ Laemmli sample buffer to a final concentration of $1 \times$ plus an equal amount of 8 M urea and incubated at RT for 20 min with

occasional mixing. The samples were then loaded on 10% SDS gel and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% milk/TBST for 1 h at RT. Anti-hemagglutinin (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted at 1:200 in 5% milk/TBST and incubated with the membrane overnight at 4°C. After washing with TBST, the membrane was incubated with 1:1000 anti mouse-HRP conjugate (Cell Signaling Technology, Danvers, MA) for 1 h at RT. The signal was developed using Amersham ECL Plus (GE Healthcare, Buckinghamshire, U.K.). As a loading control, the membrane was also probed for GAPDH (ab8245; 20 ng/ml final; Abcam, Cambridge, MA).

Cell stimulation and luciferase assay

Cell culture was carried out using medium supplemented with 10% FCS, unless otherwise indicated. The following reagents were used in cell culture experiments to stimulate cells. *Escherichia coli* LPS (Sigma-Aldrich, St. Louis, MO) was prepared in PBS. Pertussis toxin (PTX) was from Calbiochem (San Diego, CA). Different species of LPA, including 16:0 (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate), 18:1 (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate), and 20:4 (1-arachidonoyl-2-hydroxy-*sn*-glycero-3-phosphate; all from Avanti Polar Lipid, Alabaster, AL) were stored in methanol:H₂O. Working solutions were prepared fresh by evaporating methanol under N₂ gas and dissolving the residue in PBS containing 1% fatty acid free BSA. Ki16425 was kindly provided by Dr. Andrew Tager (Harvard University, Cambridge, MA), and wortmannin was purchased from Calbiochem.

DC adoptive transfer

Wild-type and *lpa2*^{-/-} BM-DC were generated, as described above. On day 8, DC were plated at 8×10^6 cells/condition and pulsed with 400 μ g/ml Grade V OVA (Sigma-Aldrich) or saline control. On day 9, 1×10^6 OVA and control wild-type and *lpa2*^{-/-} DC were intratracheally instilled by oropharyngeal aspiration (50 μ l) into wild-type recipients. The recipients were then aerosol challenged with Grade V OVA inhalation on days 19–22 (1%, 30 min) and sacrificed for analysis of airway inflammation.

OVA sensitization and challenge

Female wild-type and *lpa2*^{-/-} littermates were used at 6–8 wk of age. We used systemic and mucosal protocols to sensitize and challenge mice using OVA as a model allergen. In the systemic protocol, mice were immunized by i.p. injection of Grade II OVA (20 and 100 μ g) plus alum (1.3 and 6.5 mg; Thermo Scientific) on days 0 and 14, respectively, followed by aerosol challenge with Grade V OVA inhalation (1%, 30 min) on days 24, 26, and 28. Some mice were sacrificed on day 10 for analysis of OVA-specific serum IgE levels using a commercially available ELISA (MD Biosciences). To immunize mice via the airway, separate groups of mice were exposed to Grade V OVA (100 μ g, Sigma-Aldrich), purified by Endotoxin Removing Columns (Thermo Scientific), either alone or together with low-dose LPS (100 ng; Sigma-Aldrich) by oropharyngeal aspiration (50 μ l), followed by aerosol challenge with Grade V OVA inhalation on days 14–16 (1%, 30 min). Bronchoalveolar lavage (BAL) was collected for analysis of cell counts and differentials using standard techniques, as previously reported (37). BAL supernatants were analyzed for expression of IL-13 and VEGF using commercially available ELISA kits (eBioscience). Some lungs were inflated with 4% paraformaldehyde and embedded in paraffin, and 8- μ m sections were stained with H&E. Lung sections were scored by blinded observers using a semiquantitative scoring system, which takes into account extent and severity of inflammation, on a 0–4 scale.

Bone marrow chimeras

Female wild-type and *lpa2*^{-/-} littermates were exposed to 1000 rad delivered via a [¹³⁷Cs] source using whole-body radiation without shielding. The radiation was delivered in a split dose (500 rad) 4 h apart. Immediately following the second round of radiation, the mice were infused with 1×10^7 bone marrow cells from wild-type and *lpa2*^{-/-} donor mice via the tail vein. To permit complete chimerism, we allowed 6 wk of reconstitution before sensitizing and challenging wild-type and *lpa2*^{-/-} bone marrow chimeras using the mucosal immunization protocol described above.

Results

BM-DC from *lpa2*^{-/-} mice are hyperactive and not susceptible to inhibition by exogenous LPA

Using primers specific for the classical Edg family of LPA receptors, we confirmed that BM-DC constitutively express *lpa1*, *lpa2*, and *lpa3*, as previously reported for human monocyte-

derived DC (Fig. 1A) (35). The yield of cells and cell surface expression of MHC class II and costimulatory molecules, including CD40, CD80, and CD86, were similar when comparing DC derived from wild-type and *lpa2*^{-/-} littermates (data not shown). However, when comparing the ability of DC to stimulate allogeneic naive CD4⁺ T cells in coculture assays, we found that *lpa2*^{-/-} DC induced significantly more T cell proliferation than did their wild-type counterparts, especially at lower DC/T ratios (black bars, Fig. 1B). In coculture supernatants, we detected almost twice as much IL-13 secretion from T cells stimulated with *lpa2*^{-/-} DC compared with their wild-type counterparts (Fig. 1C). In contrast, DC obtained from *lpa1* gene-targeted mice behaved similarly to wild-type mice in these assays (gray bars, Fig. 1). Pretreating DC with the LPA1/3 antagonist Ki16425 had no effect on the ability of DC to induce T cell proliferation or activation (38), whereas the PI3K inhibitor wortmannin (0.1–10 μ M) inhibited the ability of both wild-type and *lpa2*^{-/-} DC to stimulate T cells equally well (data not shown, Supplemental Fig. 1). These data indicate that, in the absence of *lpa2*, DC acquire a hyperactive phenotype and suggest that LPA2 is an inhibitory receptor that attenuates DC activation.

We explored this possibility further using two approaches. First, we studied the secretion of inflammatory and immunoregulatory cytokines by wild-type and *lpa2*^{-/-} DC in response to LPS, which is known to activate DC in a TLR4/MyD88-dependent manner. We first confirmed that the expression of TLR4 was not affected by *lpa2* deficiency (data not shown). Compared with their wild-type counterparts, we found that when stimulated in the presence of 10% serum, *lpa2*^{-/-} DC secreted significantly more VEGF than did their wild-type counterparts, whereas secretion of other cytokines, including IL-6, IL-12p70, and TNF- α , was similar between genotypes (data not shown, Supplemental Fig. 2). One explanation for this finding is that LPA present in serum-

containing tissue culture medium is sufficient to suppress LPS-dependent DC activation in an *lpa2*-dependent manner. To test this possibility, we next stimulated wild-type and *lpa2*^{-/-} DC under reduced serum conditions with LPS (1 μ M), alone or together with 16:0, 18:1, or 20:4 LPA (1–10 μ M). If *lpa2* normally inhibits DC activation, then wild-type DC should be more susceptible to LPA-dependent inhibition than *lpa2*^{-/-} DC. Fig. 2 shows that exogenous LPA inhibited LPS-driven IL-6 secretion from wild-type (open bars) but not *lpa2*^{-/-} DC (closed bars); the data are expressed as relative inhibition compared with cells stimulated with LPS alone, and the dotted line indicates no inhibition. Table I shows that, under reduced serum conditions, LPS-stimulated *lpa2*^{-/-} DC secreted significantly less IL-10 and more TNF- α than did their wild-type counterparts, whereas IL-12p70 secretion was comparable between genotypes. Furthermore, three exogenous LPA species partially suppressed LPS-driven TNF- α secretion in wild-type DC, an effect that was lost using *lpa2*^{-/-} DC (Table I). Interestingly, both saturated and unsaturated LPA species inhibited LPS-driven IL-6 and TNF- α secretion, which contrasts with the preferential ability of unsaturated LPA species to induce DC migration in vitro (39) (see Discussion).

Expression of *lpa2* inhibits LPS-dependent NF- κ B activation

Signal transduction via the TLR4 receptor complex is known to induce cytokine secretion in an NF- κ B-dependent manner. To test the possibility that *lpa2* interferes with NF- κ B-dependent gene expression, we used HEK293T cells stably expressing TLR4 and MD2, which do not express LPA2 at baseline (data not shown). We first confirmed that, after cotransfection with a full-length expression vector, LPA2 is expressed in these cells and localizes to the cell membrane (data not shown, Supplemental Fig. 3). As expected, LPS induced transcriptional activation of an NF- κ B-driven reporter construct in cells cotransfected with an empty expression vector (Fig. 3). In contrast, LPS-dependent NF- κ B activation was significantly attenuated in LPA2-expressing cells. Levels of secreted IL-6 were at or below detection limits in these experiments (data not shown). Treatment with exogenous 16:0 LPA alone, or in combination with LPS, did not result in additional inhibition of reporter gene activity (data not shown). Interestingly, transient transfection of an LPA1 expression vector also attenuated LPS-dependent NF- κ B activation in HEK293T cells expressing TLR4/MD2 (N. Meednu, unpublished observations); the mechanisms and consequences of this effect are being pursued in a separate study. Taken together, these data support the idea that endogenous serum LPA inhibits LPS-induced NF- κ B-dependent gene expression, at least in part, in an *lpa2*-dependent manner.

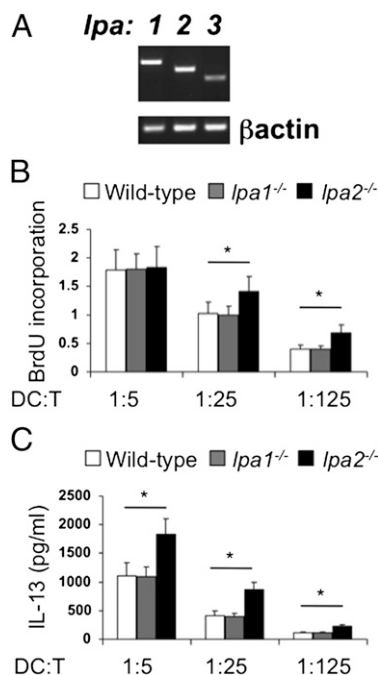


FIGURE 1. *lpa2*-deficient DC are hyperactive in vitro: T cell stimulation. (A) Expression of LPA1–3 by BM-DC using RT-PCR. Wild-type (white bars), *lpa1*-deficient (gray bars), and *lpa2*-deficient DC (black bars) on the C57BL/6 background were incubated with allogeneic (BALB/c) naive CD4⁺ T cells at the indicated DC:T ratios for 72 h, followed by analysis of T cell proliferation (B) and IL-13 production (C). Results are mean $\pm$ SEM ($n = 5$ –6 mice). * $p < 0.05$.

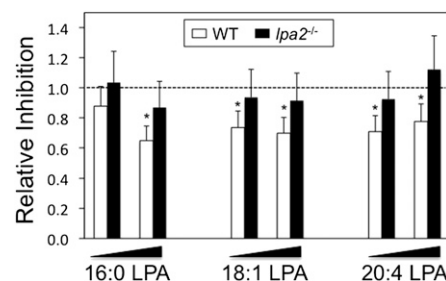


FIGURE 2. *lpa2*-deficient DC are refractory to inhibition by different LPA species. Wild-type and *lpa2*-deficient DC were stimulated under reduced serum conditions with LPS alone or together with increasing concentrations of the indicated LPA species (1–10 μ M) for 48 h, followed by analysis of IL-6 secretion by ELISA. Results are expressed relative to LPS-induced IL-6 production in the absence of LPA and are the mean $\pm$ SEM of $n = 3$ mice/group. * $p < 0.05$.

Table I. Cytokine production (pg/ml) by wild-type and LPA2^{-/-} BM-DC

Cytokine/Genotype	Unstimulated	LPS	LPS + 16:0 LPA	LPS + 18:1 LPA	LPS + 20:4 LPA
IL-10					
Wild-type	11 ± 1	428 ± 13	432 ± 67	450 ± 21	706 ± 67
lpa2 KO	23 ± 5	268 ± 5*	264 ± 15	264 ± 28*	298 ± 9*
IL-12p70					
Wild-type	42 ± 1	57 ± 1	51 ± 2	55 ± 1	57 ± 2
lpa2 KO	50 ± 3	95 ± 21	105 ± 21	101 ± 14*	104 ± 23
TNF-α					
Wild-type	12 ± 0	1038 ± 154	785 ± 34	769 ± 72	770 ± 111
lpa2 KO	14 ± 0	1765 ± 132*	2011 ± 19*	1795 ± 46*	1800 ± 80*

LPS was used at 1 μM, and LPA species were used at 10 μM. All data are mean ± SEM (n = 3/genotype).

*p < 0.05 for genotype-specific effect.

KO, Knockout.

PTX augments the ability of wild-type, but not lpa2^{-/-}, DC to stimulate T cell proliferation

Many of the effects of LPA2 are mediated by coupling with Gαi. If lpa2 were inhibiting DC activation in a Gαi-dependent manner, we reasoned that we should be able to augment the activation of wild-type DC more than lpa2^{-/-} DC using PTX. Fig. 4 shows that pretreatment with PTX significantly augmented the ability of wild-type DC to induce T cell proliferation in a dose-dependent manner. Similarly to Fig. 1, we found that untreated lpa2-deficient DC induced more proliferation in responding T cells than did their wild-type counterparts (white bars; p < 0.05 versus wild-type); in contrast to wild-type cells, preincubation with PTX had no significant effect on the T cell-stimulating capacity of lpa2-deficient DC. Because no exogenous LPA was supplied in this experiment, these data further support the idea that LPA present in serum transduces a tonic inhibitory signal that dampens DC activation via LPA2.

Lpa2-deficient DC are hyperactive and proallergic in vivo

Using in vitro assays, we found that lpa2-deficient DC induced more T cell proliferation and IL-13 production, as well as secreted more VEGF, than did their wild-type counterparts, suggesting that they may promote Th2-driven allergic immune responses in vivo (40, 41). To test this possibility, we used an adoptive-transfer model in which wild-type mice received allergen-pulsed wild-type or lpa2^{-/-} DC by intratracheal administration, followed by aerosol allergen challenge using OVA as a model allergen (modified from Refs. 42–44). Mice receiving control, saline-pulsed DC followed by OVA aerosol challenge developed little or no lung inflammation, as determined by BAL cell counts (>95% macro-

phages), whereas significant lung eosinophilia developed in mice that received OVA-pulsed wild-type DC followed by OVA aerosol challenge (Fig. 6B, data not shown). Interestingly, adoptive transfer of lpa2-deficient, OVA-pulsed DC resulted in substantially more lung inflammation than did transfer of wild-type DC following OVA challenge (Fig. 5). Taken together with the results shown in Figs. 1–4, these data indicate that lpa2-deficient DC are hyperactive in both in vitro and in vivo assays.

Greater allergen-driven airway inflammation in lpa2^{-/-} mice compared with controls

Finally, we compared wild-type mice with lpa2-deficient mice in two models of allergic airway inflammation known to involve DC activation. First, we used the well-established model of systemic immunization using i.p. injection of OVA plus alum. Second, we used a mucosal immunization approach and sensitized mice with inhaled endotoxin-free OVA using low-dose LPS (100 ng) as adjuvant (45). Using both systemic- and mucosal-immunization approaches, we found that lpa2-deficient mice developed greater allergic sensitization, airway inflammation, and airway hyper-responsiveness than did their wild-type counterparts (Figs. 6, 7). In the OVA-plus-alum model, lpa2-deficient mice developed more airway eosinophilia and higher BAL IL-13 and VEGF levels at 48 and 72 h following allergen challenge (Fig. 6A–C, data not shown), indicative of greater Th2-driven allergic airway inflammation. To determine whether lpa2 deficiency resulted in augmented allergen sensitization, we sacrificed a separate group of mice 10 d after OVA-plus-alum immunization and found that serum OVA-specific IgE levels were almost twice as high in lpa2-deficient mice compared with wild-type controls (Fig. 6D).

Mucosal immunization protocols result in less severe airway inflammation than does systemic immunization following recall

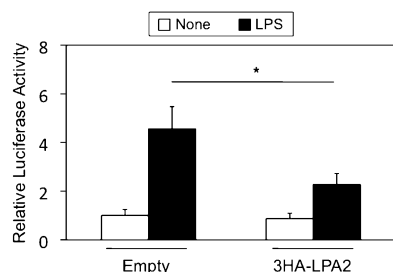


FIGURE 3. Expression of lpa2 inhibits NF-κB activation. TLR4/MD2-expressing HEK293T cells were cotransfected with an empty or LPA2 expression vector (3HA-LPA2) as indicated, together with an NF-κB-dependent luciferase reporter construct. Cells were then incubated in medium control (open bars) or with LPS (1 μM, closed bars) for 48 h, followed by analysis of reporter gene activity using luminometry. Data are expressed as luciferase activity relative to unstimulated cells and are the mean ± SEM of three independent experiments. *p < 0.05.

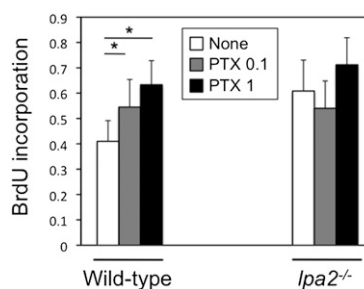


FIGURE 4. PTX enhances the ability of wild-type, but not lpa2-deficient, DC to stimulate T cells. DC from wild-type or lpa2^{-/-} mice were pretreated with 0.1 or 1 μg/ml PTX, washed, and then cocultured with allogeneic naive T cells for 48 h. T cell proliferation was measured using BrdU incorporation. Results are the mean ± SEM of three independent experiments. *p < 0.05.

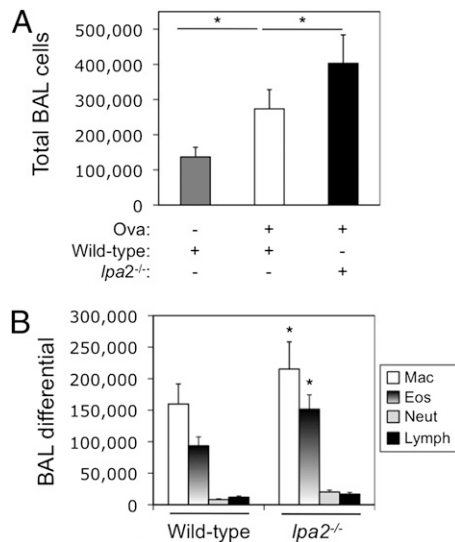


FIGURE 5. *lpa2*-deficient DC are hyperactive and proallergic in vivo. DC from wild-type or *lpa2*^{-/-} mice were pulsed with 400 μ g/ml OVA for 24 h, and adoptively transferred intratracheally into wild-type recipients. Control mice received unpulsed DC from wild-type mice. Ten days later, mice were challenged for four consecutive days with 1% OVA aerosol and sacrificed 24 h later for analysis of total BAL cell counts (**A**) and BAL cell differentials using cytospin (**B**). Results are the mean $\pm$ SEM of three independent experiments. * p < 0.05, recipients of wild-type versus *lpa2*-deficient DC.

allergen challenge, but they are a more physiological route of allergen encounter. Using the approach described by Eisenbarth et al. (45), with low-dose LPS as inhaled adjuvant, we found that *lpa2*-deficient mice developed a greater influx of inflammatory cells into BAL fluids than did their wild-type counterparts 48 h after allergen challenge (Fig. 7A). Interestingly, although the percentages of eosinophils and neutrophils were similar between the groups (eosinophils: 22 $\pm$ 2% versus 17 $\pm$ 2% and neu-

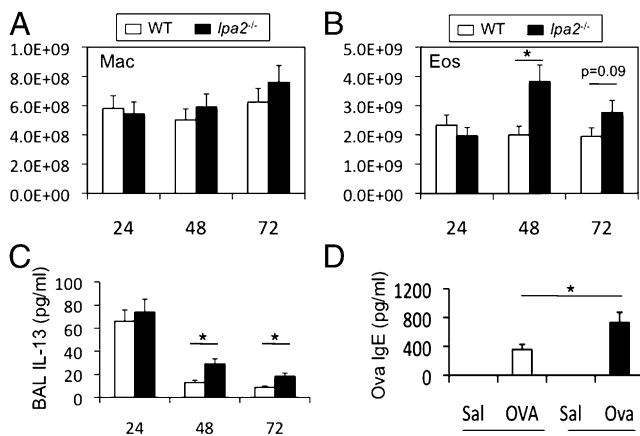


FIGURE 6. *lpa2*-deficient mice develop more eosinophilic airway inflammation than do wild-type littermates after systemic immunization. Wild-type and *lpa2*^{-/-} mice were sensitized with OVA plus alum by i.p. injection, followed by OVA aerosol challenge, and were sacrificed 48 h later for analysis of BAL cell counts and cytokines. Total numbers of macrophages (**A**) and BAL eosinophils (**B**) were significantly elevated in *lpa2*-deficient mice compared with wild-type mice. (**C**) BAL IL-13 levels were also increased in *lpa2*^{-/-} mice. (**D**) Wild-type and *lpa2*^{-/-} mice were sensitized with OVA plus alum by i.p. injection, and serum OVA-specific IgE was measured 10 d later. Results are the mean $\pm$ SEM of six mice/time point. * p < 0.05, wild-type versus gene-targeted littermates.

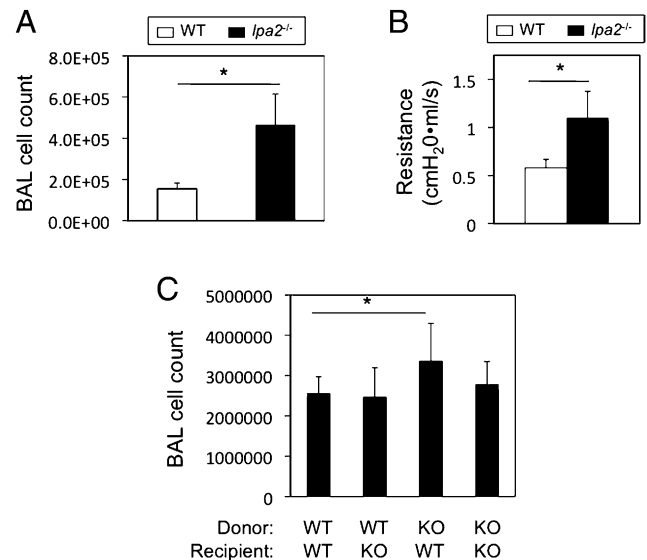


FIGURE 7. *lpa2*-deficient mice develop more airway inflammation and hyperactivity than do wild-type mice after mucosal immunization in a manner dependent on a radiosensitive hematopoietic cell. Wild-type (open bars) or *lpa2*^{-/-} (closed bars) mice were sensitized with endotoxin-free OVA together with 100 ng LPS as adjuvant by inhalation, followed by OVA aerosol challenge 2 wk later. Twenty-four hours after the last OVA challenge, total BAL cell counts were determined (**A**), and airway hyperactivity to methacholine (0.1 mg/ml) was measured in a separate group of sedated and paralyzed mice (**B**). (**C**) Reciprocal donor/recipient bone marrow chimeras were created, as indicated, and subjected to the mucosal-immunization protocol, demonstrating enhanced recovery of BAL cells from wild-type recipients of *lpa2*-deficient bone marrow. Results are the mean $\pm$ SEM of 5–12 mice/condition. * p < 0.05, ANOVA, followed by Wilcoxon signed-rank test.

trophils: 8 $\pm$ 2% versus 11 $\pm$ 3%, wild-type versus *lpa2* knockout, respectively, mean $\pm$ SEM of n = 9–11), airway hyperactivity measured in sedated and paralyzed mice was significantly greater in *lpa2*-deficient mice compared with wild-type controls (Fig. 7B). We used reciprocal bone marrow chimeras to investigate the requirement for LPA2 in hematopoietic and nonhematopoietic cells in suppressing allergic lung inflammation following mucosal immunization. We found that wild-type mice reconstituted with *lpa2*-deficient bone marrow developed significantly greater airway inflammation than did those reconstituted with wild-type bone marrow (Fig. 7C). Furthermore, *lpa2*-deficient recipients of either wild-type or *lpa2*-deficient bone marrow reacted similarly to wild-type recipients of wild-type bone marrow. These data indicate that *lpa2* expression by a radiosensitive bone marrow-derived cell(s) normally restrains allergic lung inflammation.

Discussion

Using complementary approaches, we uncovered a novel role for *lpa2* (Edg4) in suppressing DC activation and allergic immune responses. DC from *lpa2*-deficient mice were hyperactive using in vitro assays compared with their wild-type counterparts and induced greater allergic airway inflammation after adoptive transfer in vivo. Wild-type (but not LPA2-deficient) DC were susceptible to inhibition by different exogenous LPA species, and PTX enhanced the ability of wild-type DC to induce T cell proliferation, an effect not seen using LPA2-deficient DC. Collectively, these data support a model in which LPA, acting via LPA2 coupled to G α_i , acts to tonically inhibit DC activation. Thus, in addition to regulating cell recruitment and survival, our data establish a novel role for LPA as a negative regulator of innate immunity.

Negative regulation of innate immune responses is important to prevent excess inflammation and tissue injury (46). Negative regulatory mechanisms have been identified that suppress activation of innate immune cells and the dysfunction of which may be associated with disease states (47). Because LPA is constitutively present in serum and BAL fluids (48, 49), one possibility is that the extravasation of LPA-containing serum into tissues that occurs during inflammation may be sensed as an anti-inflammatory or proresolution signal (50). In support of this notion, LPA promotes epithelial barrier function, a key step in the restoration of normal tissue integrity following inflammatory insults (51). A corollary of this hypothesis is that dysfunction of the LPA/*lpa2* axis may contribute to persistent inflammation in chronic disease states. Taken together, these findings suggest that lysolipids may play a broader role in dampening immune responses than previously suspected.

Our data support a model in which LPA2 coupling to G α i suppresses NF- κ B-dependent DC activation. Precedence for the idea that PTX can augment DC activation is provided by the work of Ausiello et al. (52), and our data firmly implicate a role for LPA2 in this regard. The C-terminal tail of LPA2 contains unique sequences that support macromolecular complex formation (53), and it is attractive to speculate that this complex negatively regulates TLR4-dependent activation of NF- κ B. Future studies will be needed to explore this and other mechanistic possibilities.

We found that allergic lung inflammation was substantially greater in *lpa2*^{-/-} mice compared with wild-type littermates using both systemic- and mucosal-immunization strategies. Our data contrast with the observations of Zhao et al. (54), who reported that heterozygous *lpa2*^{+/-} mice were partially protected from lung inflammation following challenge with *Schistosoma* egg Ag. The reasons for this apparent discrepancy are not immediately obvious, but they may relate to the nature of the Ags used, immunization protocols, or genetic backgrounds. Using bone marrow chimeras, we uncovered a key role for *lpa2* expression by radiosensitive hematopoietic cells in suppressing allergic airway inflammation. Our results using adoptive-transfer experiments firmly implicate DC in this regard and are supported by the observation that OVA-specific IgE responses are enhanced in the absence of LPA2.

LPA is constitutively present in epithelial lining fluids of the human lung and is significantly enriched during the late-phase response following segmental allergen challenge (49). Based on our findings and previously published research, we can construct a working model in which LPA has both pro- and anti-inflammatory effects in asthma. Proinflammatory effects can result from the ability of LPA to promote cell recruitment or activation (9, 27–29), especially in response to submaximal stimuli (55). LPA can also augment airway hyperresponsiveness by direct effects on smooth muscle cells (30, 31). However, because LPA also restores epithelial barrier function (51), enhances IL-13R α 2 expression (56), inhibits epithelial RANTES production (57), and attenuates DC activation (this report), it has the potential to dampen airway inflammation. The observation that LPA is constitutively present in epithelial lining fluids supports its potential role in maintaining lung homeostasis. One intriguing possibility is that LPA contributes to airway remodeling in long-standing asthma. By promoting fibroblast recruitment (25) and smooth muscle mitogenesis (58), local generation of LPA during cycles of airway injury and repair could lead to airway fibrosis and smooth muscle hypertrophy. More research is needed to understand the mechanisms and timing of LPA generation in the airway, as well as the roles of different LPA receptors and target cells in airway inflammation and remodeling.

Taken together, our results establish a novel role for *lpa2* as an inhibitory receptor of a key innate immune cell type and uncover

a new pathway involved in tonic dampening of allergic immune responses. Future studies investigating the function of the inhibitory LPA/*lpa2* axis in inflammatory diseases should prove worthwhile. We also speculate that LPA2-specific agonists may have anti-inflammatory properties and therapeutic efficacy in allergic and inflammatory diseases.

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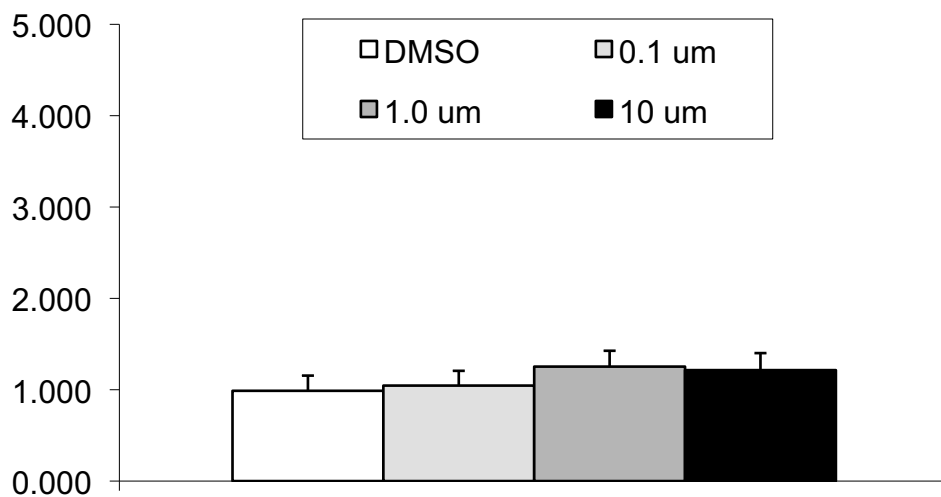
Disclosures

The authors have no financial conflicts of interest.

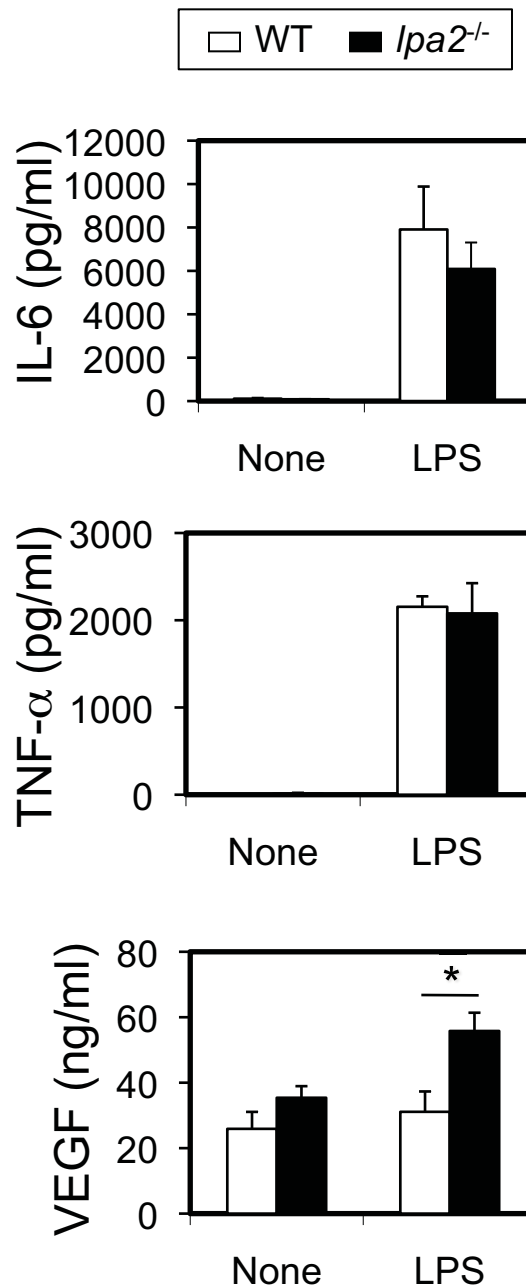
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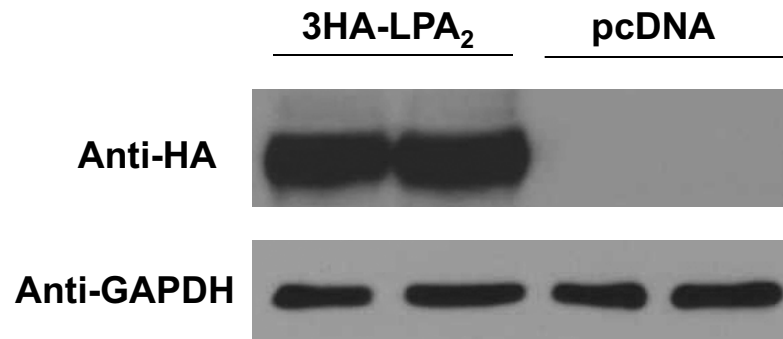
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Supplementary Figure 1. Dendritic cells were derived from bone marrow precursors as reported in the main text, and then exposed to DMSO control (open bars), or increasing concentrations of the LPA 1/3 antagonist Ki16425 (0.1 to 10 μ M, shaded bars, courtesy of Dr. Andrew Tager, Harvard University). Twenty-four hours later, DC were washed and co-cultured with allogeneic naïve T cells for 72 hours followed by analysis of T cell proliferation by measuring BrdU incorporation. Results are mean $\pm$ SEM of n=4 mice. There was no significant difference between groups as determined by ANOVA ($p>0.05$).



Supplementary Figure 2. *lpa2*-deficient DC are hyperactive in vitro: cytokine secretion. Wild-type and *lpa2*-deficient DC were stimulated with LPS for 48 hours, followed by analysis of cytokine secretion by ELISA. Results are mean±SEM of n=6 mice, *p<0.05.



Supplementary Figure 3. HEK293 cells stably expressing TLR4/MD2 were transfected as described with pcDNA-3HA-LPA2 or empty vector. Forty-eight hours later, whole cell lysates were analyzed by Western blot assays demonstrating expression of HA-tagged LPA2 (above). Parallel experiments using immunofluorescent microscopy of cell cytospin confirmed predominant membrane expression of the HA-LPA2 construct (not shown).