Research Article

Cancer Immunology Research

Lysophosphatidic Acid Inhibits CD8 T-cell Activation and Control of Tumor Progression

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Abstract

CD8 T lymphocytes are able to eliminate nascent tumor cells through a process referred to as immunosurveillance. However, multiple inhibitory mechanisms within the tumor microenvironment have been described that impede tumor rejection by CD8 T cells, including increased signaling by inhibitory receptors. Lysophosphatidic acid (LPA) is a bioactive lysophospholipid that has been shown repeatedly to promote diverse cellular processes benefiting tumorigenesis. Accordingly, the exaggerated expression of LPA and LPA receptors is a common feature of diverse tumor cell lineages and can result in elevated systemic LPA levels. LPA is recognized by at least six distinct G protein–coupled receptors, several of which are expressed by T cells, although the precise function of LPA signaling in CD8 T-cell activation and function has not been defined. Here, we show that LPA signaling via the LPA_5 receptor expressed by CD8 T cells suppresses antigen receptor signaling, cell activation, and proliferation *in vitro* and *in vivo*. Importantly, in a mouse melanoma model tumor-specific CD8 T cells that are LPA_5-deficient are able to control tumor growth significantly better than wild-type tumor-specific CD8 T cells. Together, these data suggest that the production of LPA by tumors serves not only in an autocrine manner to promote tumorigenesis, but also as a mechanism to suppress adaptive immunity and highlights a potential novel target for cancer treatment. *Cancer Immunol Res; 1(4); 245–55. ©2013 AACR.*

Introduction

The adaptive immune system is able to detect and eliminate nascent tumors through a process referred to as immune surveillance and mediated in large part by cytotoxic CD8 T cells. However, this immune response to tumor may also contribute to tumorigenesis by providing selective pressure to which tumors adapt and eventually evade eradication, a process coined cancer immunoediting (1, 2). Tumors that evade the initial T-cell response create an immunosuppressive microenvironment from which variants arise, escape immune control, and grow without restraint. The mechanisms used by tumors to either evade the initial CD8 T-cell response or promote the tumor immunosuppressive environment are not fully defined. However, the recent success of immunotherapies that interfere with tumor-derived immune suppression (3–5) has underscored the importance of identifying the mechanisms by which tumors suppress CD8 T cells to escape immune control (6, 7).

The activation of cytotoxic CD8 T cells by either foreign or tumor antigen is initiated via signals transmitted by the T-cell antigen receptor (TCR; ref. 8). TCR signaling and the subsequent function of mature T cells can be regulated in a positive or negative manner by different surface coreceptors (9). Multiple inhibitory mechanisms within the tumor microenvironment that impede tumor rejection by tumor-infiltrating lymphocytes (TIL) have been described (6, 10), including the increased signaling by CD8 T-cell inhibitory receptors, such as the well-characterized CTLA-4 molecule (11).

Lysophospholipids comprise a small family of structurally simple lipids that include sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA), and they induce diverse biologic and pathophysiologic effects by signaling through specific G protein–coupled receptors (GPCR; refs. 12, 13). The LPA lysophospholipid is recognized by six specific GPCR, LPA_{1-6} (14), and although T lymphocytes are known to express several LPA receptors (13, 15–17), the immune regulatory activities of LPA are not well understood. LPA concentration in the blood of healthy individuals has been reported to vary from high nanomolar to low micromolar levels (12, 18). *In vivo*, production of LPA results predominantly from the activity of autotaxin (ATX; ref. 19), an extracellular lysophospholipase D originally isolated and identified from a human melanoma as an autocrine motility factor (20). Since then LPA has been found aberrantly produced in a number of different malignant cell

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types (21–23), resulting in significantly increased systemic levels that can reach 60 μmol/L in malignant effusions (24–26). At these elevated levels, LPA has been shown to promote tumor progression by enhancing tumor migration, survival, metastasis, angiogenesis, and therapeutic resistance (27–31).

Previously LPA has been shown to modulate the activation of different cell types (17), and in this study, we investigated if LPA could influence CD8 T-cell activation. Here, we report that CD8 T cells express the LPA_5 receptor and signaling by this GPCR inhibits CD8 T-cell receptor signaling, activation, and proliferation. Furthermore, we show that tumor-specific CD8 T cells lacking LPA_5 can control the progression of established tumor progression more efficiently than the LPA_5-sufficient tumor-specific CD8 T cells. Thus, our findings reveal a novel role for lysophospholipid-mediated protection of tumor from adaptive immunity.

Materials and Methods

Mice

C57BL/6 (CD45.2) and CD45.1 (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ) were obtained from The Jackson Laboratory or bred in the Biological Resource Center (BRC) at National Jewish Health (NJH; Denver, CO). CD45.1 OT-I mice (a gift from Dr. Ross Kedl, University of Colorado, Denver, CO), *Lpar2*$^{-/-}$ mice (a gift from Dr. Jerold Chun, Scripps Research Institute, La Jolla, CA), *Lpar5*$^{-/-}$ mice, TCRα$^{-/-}$ mice (a gift from Dr. Philippa Marrack, NJH) were bred in the BRC at NJH. $LPA_5{}^{-/-}$ mice were generated as described in the Supplementary Data. All mice used were 8 to 12 weeks of age, housed under specific pathogen-free conditions, and were maintained in accordance with the regulations of the Institutional Animal Care and Use Committee.

Calcium mobilization

Erythrocyte-lysed cells isolated from spleen, pooled lymph nodes, or purified CD8$^+$ T cells were suspended at 20×10^6 cells/mL in RPMI-1640 medium supplemented with 2.5% fatty acid–free (faf) bovine serum albumin (BSA; Calbiochem) and loaded with Indo1-AM (Molecular Probes) as described in the Supplementary Data.

qPCR

CD8$^+$ T cells were isolated from the spleens and lymph nodes of wild-type mice and LPA receptor expression measured by real-time quantitative reverse transcriptase (RT-PCR) using Platinum SYBR Green quantitative PCR (qPCR) SuperMix-UDG (Invitrogen Life Technologies). The details of qPCR analysis including specific forward and reverse LPA receptor primers are provided in the Supplementary Data.

Cytometry

All antibodies were purchased from eBiosciences, BD Pharmingen, Biolegend, or were produced in our laboratories. Cells were stained in 2% BSA–PBS+0.1% sodium azide with blocking Fc receptor antibody (2.4G2) on ice for 20 to 30 minutes. For viability assessment, 7-amino actinomycin D (7-AAD) was added 10 minutes before data acquisition. All flow-cytometric analysis was conducted on the LSRII flow cytometer (BD) and analyzed with FlowJo v8 (TreeStar) and GraphPad Prism software (v 5.0).

Lipid preparation

LPA (C16 LPA; Avanti Polar Lipids) was solubilized to 5 mmol/L concentration in 0.1% BSA–PBS, aliquoted, and stored at −20°C. Aliquots were diluted to 1 mmol/L in RPMI-1640 medium supplemented with 2.5% faf-BSA (Calbiochem) before use. Octodecenyl thiophosphate (OTP) was generated as previously described (32), stored as a powder, and solubilized in 95% methanol to create aliquots. Virgin glass tubes and caps were sterilized by autoclave for aliquots that were stored at −20°C. The concentration was confirmed by phosphorus assay. OTP was solubilized for experimental use by sonication with FBS- or BSA-containing culture media or vehicle (2% propanediol and 1% ethanol in PBS) for *in vitro* or *in vivo* usage, respectively. For *in vitro* experiments, OTP was solubilized to 50 μmol/L and passed through a 0.2-μm filter for further sterilization. For *in vivo* experimentation, solubilized OTP was transferred to siliconized Eppendorf tubes and animals were dosed at 5 mg/kg every 8 hours.

Generation of bone marrow–derived dendritic cells

Congenic gender-matched bone marrow–derived dendritic cells (BMDC) were generated by flushing of femur and tibia and culture at 10^6 cells/mL in RPMI-1640 with 20 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF), 10% FBS (Omega Scientific), penicillin–streptomycin, and GlutaMAX (Invitrogen). Media was refreshed on days 3 and 5. On day 7, BMDCs were harvested from culture and stimulated with 1 ng/mL LPS for 90 minutes and pulsed with peptide for the last hour of LPS treatment. BMDCs were washed five times to remove LPS and unbound peptide before transfer.

In vitro T-cell activation and proliferation

To determine how LPA affected antigen-specific activation of CD8 T cells, OT-I splenocytes were isolated, erythrocyte lysed, and labeled with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen). For all CFSE labeling, cells were suspended at 15×10^6 cells/mL in PBS and CFSE was added to a final concentration of 2 μmol/L for 10 minutes and then washed in media. Splenocytes were pulsed with 1 μmol/L of the SIIGFEKL (G4; AnaSpec, Inc.) or SIINFEKL (a gift from Philippa Marrack) peptides for 4 hours or 90 minutes, respectively, in 5% faf-BSA RPMI, then washed. Cells were cultured in 96-well plates at 2.5×10^6 cells/mL in the presence or absence of 50 μmol/L OTP that was sterile-filtered before addition to culture. Cells were enumerated by flow cytometry, and the proportion of cells proliferated was calculated by FlowJo analysis. The mean fluorescence intensity (MFI) values of activation marker expression were normalized.

To assess *in vitro* cytokine production, OT-I effector T cells were generated by pulsing erythrocyte-lysed OT-I splenocytes with 1 μmol/L SIINFEKL and culture with interleukin-2 (IL-2) for 5 days. On day 5 of culture, target cells (EL4 cells) were pulsed with 1 μmol/L SIINFEKL and cultured at an effector to

target ratio of 0.625:1 with OT-I effector T cells for 4 hours in the presence of Brefeldin A, in the presence or absence of sterile-filtered 50 μmol/L OTP.

In vivo T-cell transfer and antigen-specific stimulation

BMDCs were generated as described earlier. One day before BMDC transfer, $CD8^+$ T cells were purified from OT-I spleen and lymph node cells with a $CD8^+$ T-cell enrichment kit (Miltenyi) to a purity of 95% or more, and 10^6 CFSE-labeled $CD8^+$ T cells were transferred to CD45 allotype-mismatched recipient C57BL/6 mice. SIINFEKL-BMDC (10^6) were suspended in PBS and transferred subcutaneously in the scruff of individual recipients. On day 3 postimmunization, animals were sacrificed and draining lymph nodes (dLN; axilary, brachial, and cervical), non-draining lymph nodes (ndLN; inguinal and mesenteric), and spleen were harvested. After erythrocyte lysis, cells were counted by Z2 Coulter Particle Count and Size Analyzer (Beckman-Coulter) and 10×10^6 cells were stained for flow cytometry. Cells were suspended in fluorescence-activated cell sorting (FACS) buffer and stained with 7-AAD for viability before analysis on the BD LSRII.

B16.cOVA tumor experiments

The OVA-transfected B16 tumor cell line (B16.cOVA) was kindly provided by Dr. Ross Kedl. Cells were maintained in RPMI-1640 (Cellgro), supplemented with 10% heat-inactivated FBS (Omega Scientific), GlutaMAX, penicillin–streptomycin, minimum essential medium (MEM) nonessential amino acids, sodium pyruvate (Invitrogen), and 0.75 mg/mL G418 sulfate selection (Cellgro).

To determine how $LPA_5^{-/-}$ OT-I T cells responded to tumor, 10^5 B16.cOVA cells were transferred subcutaneously into the hind leg of recipients, and 5 days later, 0.5×10^6 CFSE-labeled OT-I T cells were transferred via the tail vein. Recipients were sacrificed 5 days later and tumor and dLN were harvested. Lymphoid organs were mashed through a cell strainer (100 μm; BD), erythrocyte lysed, and counted. Tumors were digested in 0.5 mg/mL collagenase D (Fisher Scientific) and 60 U/mL DNase (Sigma-Aldrich) for 30 minutes with perturbation every 10 minutes, and then neutralized with 5 mmol/L EDTA for 5 minutes. Tumor cells were homogenized by pipette tituration and passed through a 100 μm filter, erythrocyte lysed, and counted. Tumor diameter was measured with calipers and body weight and overall appearance were assessed every 2 days. Mice were sacrificed when tumor diameter exceeded 10 mm or at 8 days after T-cell transfer, whichever came first.

Statistical analysis

All statistical analysis was conducted with GraphPad Prism software (version 5.0) using a two-tailed unpaired Student *t* test.

Results

LPA inhibits T-cell antigen receptor-mediated calcium mobilization via LPA_5

Both human and murine T cells express LPA receptors (15, 16), so we initially questioned whether LPA could influence TCR signaling. Specifically, we tested whether LPA could modulate TCR-induced intracellular calcium mobilization. These results revealed that 20 μmol/L of LPA potently inhibited intracellular calcium mobilization by $CD8^+$ T cells in response to TCR stimulation achieved by antibody-mediated cross-linking of the TCR (Supplementary Fig. S1). In contrast, LPA treatment in the absence of antigen receptor stimulation did not affect intracellular calcium levels. LPA inhibition of CD8 TCR signaling was further shown to be dose dependent with a reduction of intracellular calcium levels achieved with LPA concentrations as low as 1 μmol/L (Fig. 1A), approximating the levels reported in the blood of healthy individuals (12, 18).

There are currently six validated LPA receptors: LPA_{1-6} (14) and qPCR analyses revealed that purified CD8 T cells expressed LPA_2, LPA_5, and LPA_6 (Fig. 1B), consistent with previous

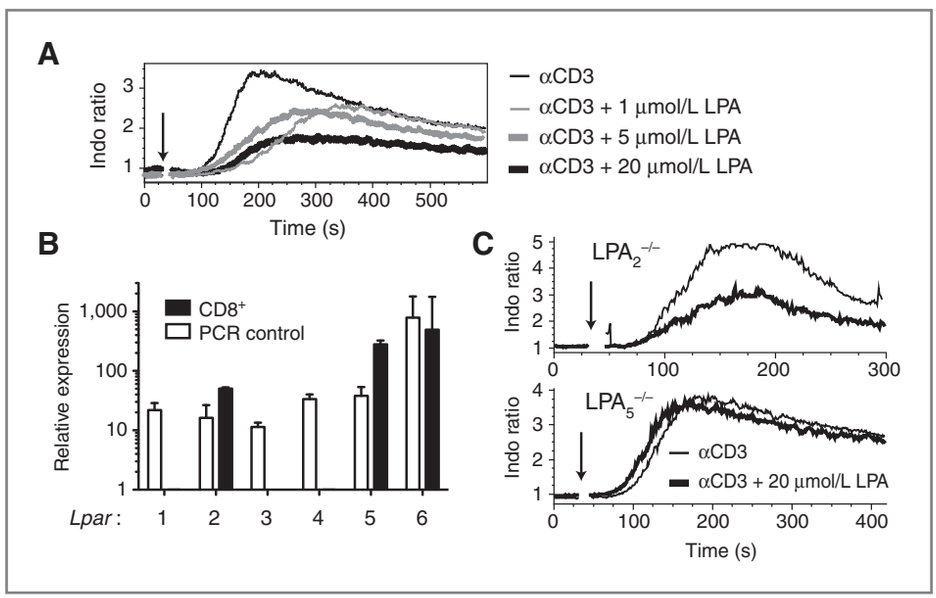

Figure 1. LPA signals via LPA_5 to inhibit $CD8^+$ T-cell TCR–mediated intracellular calcium release. A, intracellular calcium concentration was assessed in purified $CD8^+$ T cells stimulated with 10 μg/mL of anti-CD3 in the absence (black line) or presence of the indicated titrated concentrations of LPA. B, expression of *Lpar1-6* by isolated $CD8^+$ T cells as measured by qPCR. C, $LPA_2^{-/-}$ or $LPA_5^{-/-}$ $CD8^+$ T cells were isolated, and intracellular calcium mobilization was assessed in response to anti-CD3 stimulation in the presence (bold line) or absence (thin line) of 20 μmol/L LPA.

findings (15, 16). To determine which LPA receptor(s) was responsible for LPA inhibition, we compared calcium mobilization in TCR-stimulated $CD8^+$ T cells from $LPA_2^{-/-}$ and $LPA_5^{-/-}$ mice ($LPA_6^{-/-}$ mice were not commercially available). These results revealed that although LPA inhibition was intact in the LPA_2-deficient T cells, LPA inhibition was absent in LPA_5-deficient T cells (Fig. 1C). These data show that LPA_5 expression is required for the inhibition of TCR-mediated calcium mobilization.

LPA signaling inhibits proliferation and activation *in vitro*

Increased intracellular calcium concentration is an important early consequence of antigen receptor signaling that leads to the activation of distinct transcriptional programs important for T-cell activation and function (33). Thus, we next tested whether LPA suppression of TCR-induced calcium mobilization would lead to reduced CD8 T-cell activation *in vitro*. Initial experiments revealed that LPA was able to inhibit TCR-mediated activation of $CD8^+$ T cells, as determined by reduced CD62L downregulation (34), however, only when T cells were stimulated with suboptimal concentration of anti-CD3 (Supplementary Fig. S2). To investigate whether LPA could inhibit antigen-specific stimulation of CD8 T cells, we used the well-characterized OT-I TCR transgenic mouse model that features CD8 T cells that express a TCR specific for the chicken ovalbumin peptide, SIINFEKL, in the context of the MHC molecule, H-$2K^b$ (35). This system also allowed us to modulate T-cell activation with different stimulatory peptides (36). More specifically, the OT-I TCR displays relatively high affinity ($K_D \sim 5$ μmol/L) for the SIINFEKL ovalbumin peptide, but it also recognizes the altered peptide ligand, SIIGFEKL (G4), with relatively weaker affinity ($K_D \sim 10$ μmol/L; ref. 37). Thus with these peptides, we could compare the effects of LPA signaling after relatively high or low affinity TCR stimulation. Because LPA is degraded both *in vitro* and *in vivo* (38), we also used a metabolically stabilized LPA analog, OTP, to induce LPA signaling (39, 40). Of note, OTP is preferentially recognized by LPA_5 relative to other LPA receptors and displays a much lower EC_{50} for LPA_5 compared with other LPA receptors (41).

Using this system, we monitored CD8 T-cell proliferation and expression of activation markers after antigen-specific TCR stimulation *in vitro* in the absence or presence of LPA signaling achieved with OTP. Proliferation was monitored by CFSE dilution and showed that in the presence of OTP, CD8 T cells were acutely inhibited from proliferating in response to G4 peptide stimulation (Fig. 2A). Specifically, by day 3, control cells had typically started to divide with the majority having undergone at least two divisions by day 7, whereas the majority of OTP-treated cells remained undivided in the same period. OTP inhibition of CD8 T-cell activation was further evidenced by significantly reduced expression of both the CD25 and CD44 activation antigens after TCR stimulation (Fig. 2B). Likely as a consequence of inhibiting activation and proliferation, OTP treatment also resulted in a reduced accumulation of $CD8^+$ T cells (Fig. 2C) without any significant effect on viability as compared with unstimulated controls (Fig. 2D). Importantly, OTP treatment did not affect the ability of antigen-presenting cells to present peptide to OT-I T cells as OTP-treated and -untreated antigen-presenting cells were equally able to induce CD25 upregulation (Supplementary Fig. S3). Similar to our initial experiment with anti-CD3 stimulation, we found that the ability of LPA signaling to dampen CD8 T-cell proliferation and activation was less effective after the TCR was stimulated with the higher affinity SIINFEKL peptide (Fig. 2E). In addition, OTP treatment did not seem to significantly influence SIINFEKL-stimulated TNF-α or IFN-γ cytokine production by *in vitro*–generated effector $CD8^+$ T cells (Fig. 2F). Together, these findings show that LPA signaling potently suppresses TCR signaling, cell activation, and proliferation when T cells are stimulated by relatively weak-affinity antigens.

Increased LPA signaling dampens activation and proliferation *in vivo*

Our *in vitro* data show that LPA signaling via LPA_5 on CD8 T cells inhibits TCR signaling, activation, and proliferation. We next addressed whether this LPA regulatory pathway operated similarly *in vivo*. To accomplish this, we transferred purified, CFSE-labeled OT-I $CD8^+$ T cells into C57BL/6 recipients followed by antigen-specific stimulation achieved by the subsequent transfer of BMDC previously pulsed with TCR-specific peptide (Fig. 3A). Under these conditions, BMDC pulsed with the relatively weak-affinity G4 peptide were unable to stimulate OT-I $CD8^+$ T cells as determined by the absence of increased activation antigen expression or proliferation (data not shown). In contrast, when SIINFEKL peptide-pulsed BMDC (SIINFEKL-BMDC) were transferred, OT-I CD8 T-cell proliferation was observed clearly 3 days later in the dLN (axillary, brachial, and cervical) relative to the site of BMDC transfer (Fig. 3B).

To promote LPA signaling *in vivo*, we treated mice with OTP subcutaneously, which results in detectable levels in the blood after 1 hour with an approximate half-life of 5.5 hours (G. Tigyi; unpublished data). Mice were treated with 5 mg/kg OTP every 8 hours, with the first dose preceding SIINFEKL-BMDC transfer by 1 hour (Fig. 3A). Three days after antigen-specific stimulation, the OT-I CD8 T cells recovered from the dLN of OTP-treated animals had proliferated considerably less compared with those from the vehicle-treated animals (Fig. 3B). Furthermore, the number of OT-I $CD8^+$ T cells recovered in the dLN of OTP-treated mice was significantly reduced relative to those recovered from the vehicle-treated animals and consistent with an inhibition of proliferation (Fig. 3C). In addition, antigen-specific T-cell activation was diminished, as evidenced by the reduced expression of CD25 by the transferred OT-I CD8 T cells in OTP-treated mice compared with those of the vehicle-treated mice (Fig. 3D). Taken together, these findings show that increased LPA signaling inhibits antigen-specific CD8 T-cell proliferation and activation both *in vitro* and *in vivo*.

LPA_5-deficient CD8 T cells show enhanced proliferation in response to antigen-specific stimulation *in vivo*

To directly test whether LPA_5 signaling suppressed CD8 T cells *in vivo*, we measured antigen-driven proliferation of LPA_5-deficient and wild-type OT-I CD8 T cells after adoptive transfer. $LPA_5^{-/-}$ mice are phenotypically unremarkable

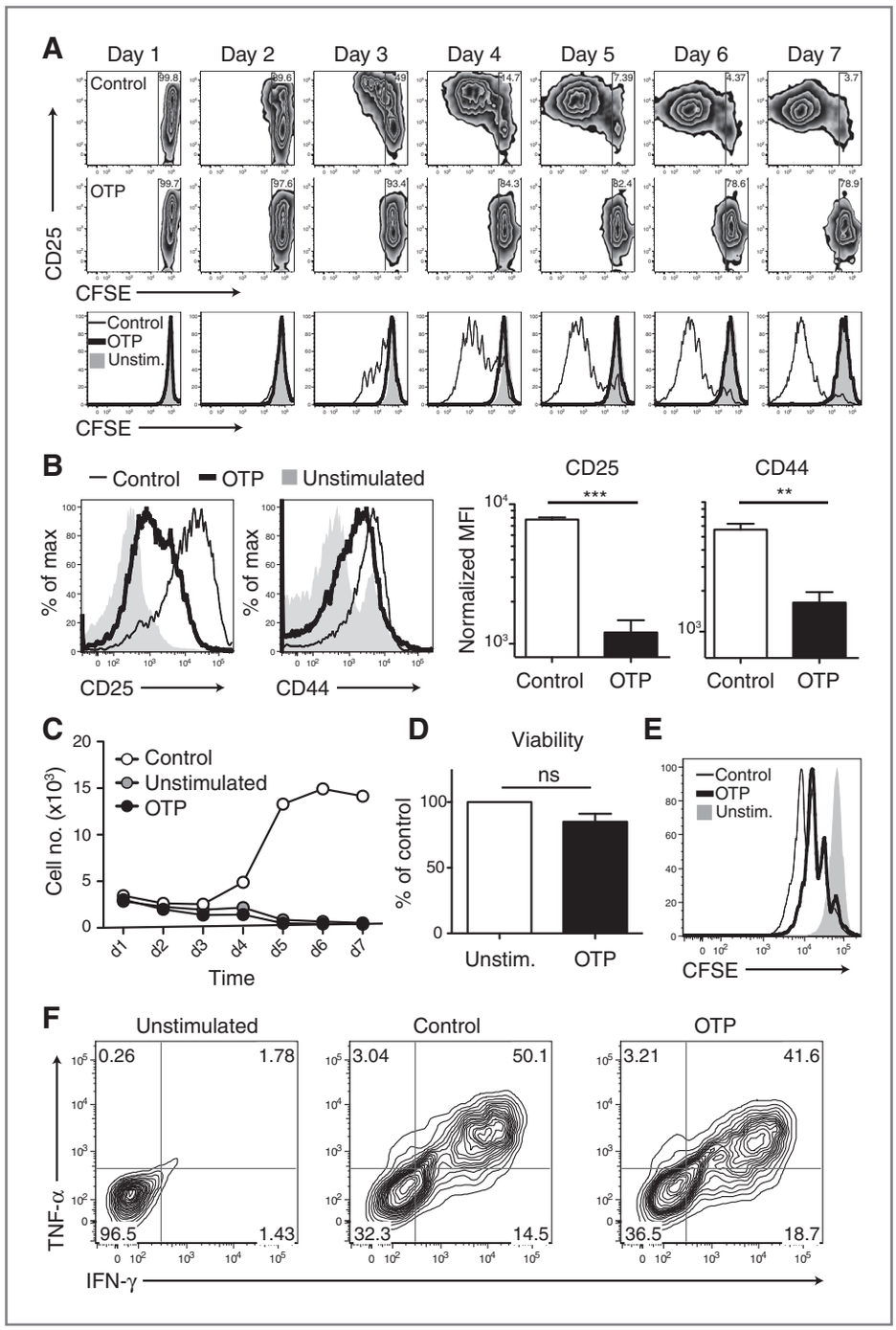

Figure 2. LPA signaling inhibits *in vitro* TCR-induced activation and proliferation. A, $CD8^+$ T cells were stimulated with specific antigen in the absence (top row) or presence (middle row) of OTP and analyzed for expression of the CD25 activation antigen and CFSE fluorescence at the indicated times. Overlay of CFSE histograms by day is shown in bottom row in the absence (thin line) or presence of OTP (bold line) or when left unstimulated (Unstim.; gray filled). Representative of three independent experiments. B, CD25 and CD44 expression on $CD8^+$ T cells is shown in histograms after 3 or 4 days, respectively, of *in vitro* culture in the absence of stimulation (gray filled) or after G4 peptide stimulation alone (thin line), or in the presence of OTP (bold line). Histograms are representative of three independent experiments, and normalized MFI values are summarized in bar chart (mean + SEM; **, $P < 0.01$; ***, $P < 0.0001$). C, enumeration of *in vitro*–cultured peptide-specific OT-I $CD8^+$ T cells at the indicated times in the absence of peptide (gray circles) or after peptide stimulation alone (open circles) or in the presence of 20 μmol/L OTP (black circles). D, cell viability was determined by 7-AAD staining at the peak of CD25 expression, and data are representative of three independent experiments (mean + SEM); ns, not statistically significant. E, splenocytes from OT-I transgenic mice were labeled with CFSE, stimulated with SIINFEKL peptide, and cultured for 3 days before analyses of CFSE fluorescence. F, effector OT-I $CD8^+$ T cells were left unstimulated (left) or restimulated with SIINFEKL in the absence (center) or presence (right) of OTP before analysis of TNF-α and IFN-γ expression. Data are representative of two independent experiments.

compared with wild-type littermates and display comparable numbers and frequencies of splenic $CD8^+$ and $CD4^+$ T-cell populations (Supplementary Fig. S4), similar to that reported for an independently generated LPA_5-deficient mouse strain (42). Furthermore, *in vitro* LPA_5-deficient CD8 T cells displayed similar viability as wild-type cells (data not shown). To address whether LPA inhibited antigen-specific CD8 T-cell responses under normal physiologic conditions, $LPA_5^{+/+}$ or $LPA_5^{-/-}$ OT-I $CD8^+$ T cells were isolated, CFSE-loaded, transferred into recipients, which were subsequently immunized with SIINFEKL-pulsed BMDC similar to those shown in Fig. 3A except neither group was treated with OTP. The results from these experiments show that 3 days after antigen-specific stimulation, a considerable proportion of wild-type OT-I CD8 T cells had undergone cell division. In contrast, similar stimulation of LPA_5-deficient OT-I CD8 T cells resulted in an increased percentage of LPA_5-deficient OT-I CD8 T cells that had undergone cell division and fewer transferred cells that

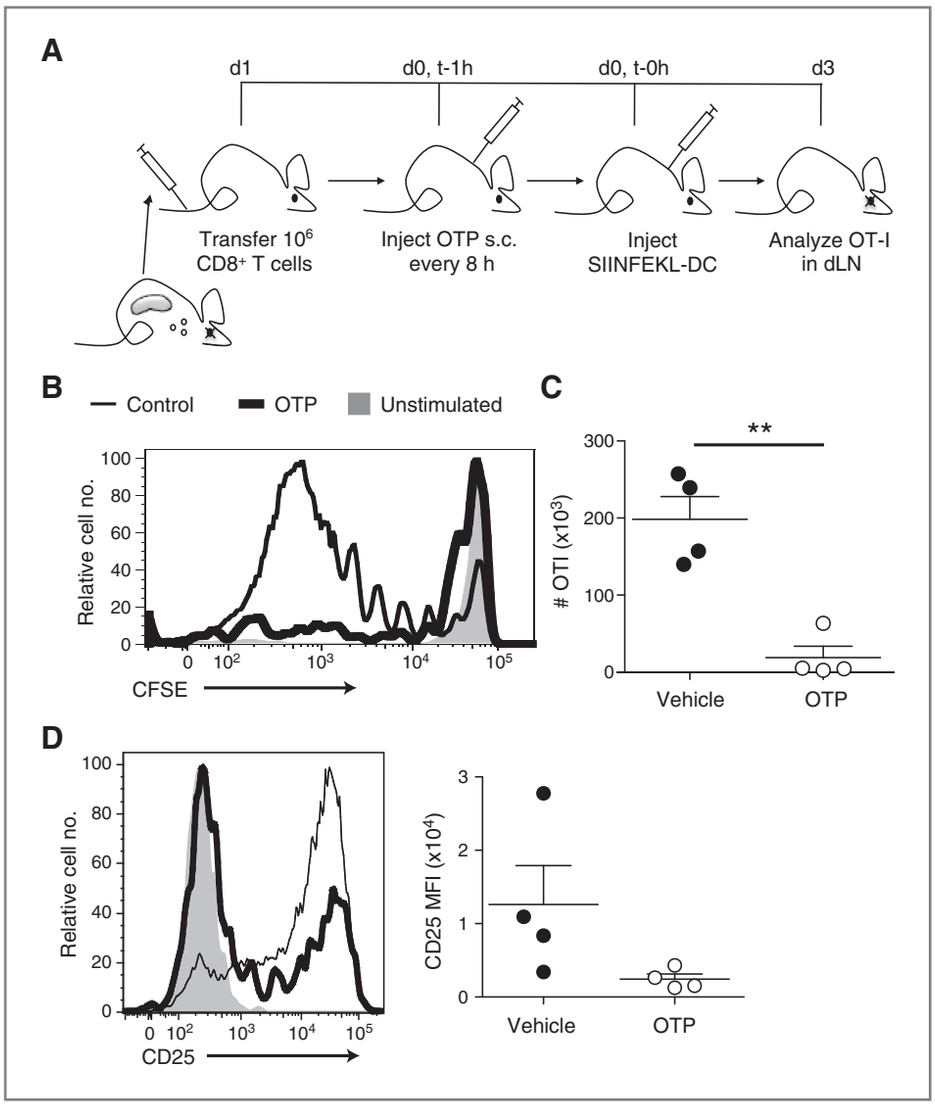

Figure 3. LPA signaling inhibits TCR-mediated CD8$^+$ T cell activation *in vivo*. A, schematic of adoptive transfer of OT-I CD8$^+$ T cells and *in vivo* OTP treatment. B, histograms of CFSE dilution in vehicle-treated control (thin line), OTP-treated (bold line), and unstimulated control (gray filled) OT-I CD8$^+$ T cells isolated from dLN. C, number of OT-I CD8$^+$ T cells in dLN. D, representative histograms of CD25 expression by OT-I CD8$^+$ T cells after vehicle-treatment (thin line), OTP-treatment (bold line), or unstimulated (gray shaded). Right, expression of CD25 as MFI by OT-I CD8$^+$ T cells in the dLN from individual mice after vehicle treatment (closed circles) or OTP treatment (open circles). Data in B, C, and D are representative of two independent experiments ($n = 4$ mice/group; mean + SEM; **, $P < 0.01$).

remained CFSE^{high} (Fig. 4A). Consistent with an increased frequency of LPA$_5^{-/-}$ OT-I CD8 T cells having proliferated, higher numbers of mutant OT-I CD8 T cells were recovered from the dLN compared with wild-type cells (Fig. 4B). Together, these data reveal that in the absence of LPA$_5$-mediated suppression, antigen-specific stimulation of OT-I CD8 T cells leads to a higher frequency of cells that proliferate and accumulate in the dLN.

Transfer of LPA$_5$-deficient tumor-specific CD8 T cells controls tumor progression

Our data thus far show that LPA signaling via LPA$_5$ expressed by CD8 T cells suppresses T-cell activation and proliferation. A number of different tumor cell types have been reported to produce elevated levels of LPA that promote survival, growth, and tumorigenesis (26, 43). Thus, we next compared the ability of LPA$_5$-deficient and -sufficient tumor-specific CD8 T cells to control progression of an established tumor.

To address this question, we used the B16 melanoma cell line, B16.cOVA, which stably expresses chicken ovalbumin (with the SIINFEKL peptide) and used OT-I T cells as tumor-specific CD8 T cells as previously reported (44). B16.cOVA cells were subcutaneously implanted in the rear leg of wild-type C57BL/6 mice, and 5 days later either naïve LPA$_5^{-/-}$ or wild-type OT-I CD8$^+$ T cells were transferred into these recipients and the tumor diameter measured every 2 days thereafter (Fig. 5A). The results from these experiments showed that tumors grew similarly in the presence or absence of wild-type OT-I CD8 T cell transfer (Fig. 5B and E), consistent with previous reports showing tumor progression in the presence of wild-type tumor-specific CD8 T cells (45, 46). In contrast, tumor growth was clearly abated in mice 6 and 8 days after receiving LPA$_5$-deficient OT-I CD8$^+$ T cells (Fig. 5B and E). Compared with the wild-type tumor-specific CD8 T cells, LPA$_5$-deficient tumor-specific CD8 T cells were also found typically at higher numbers within the tumor compared

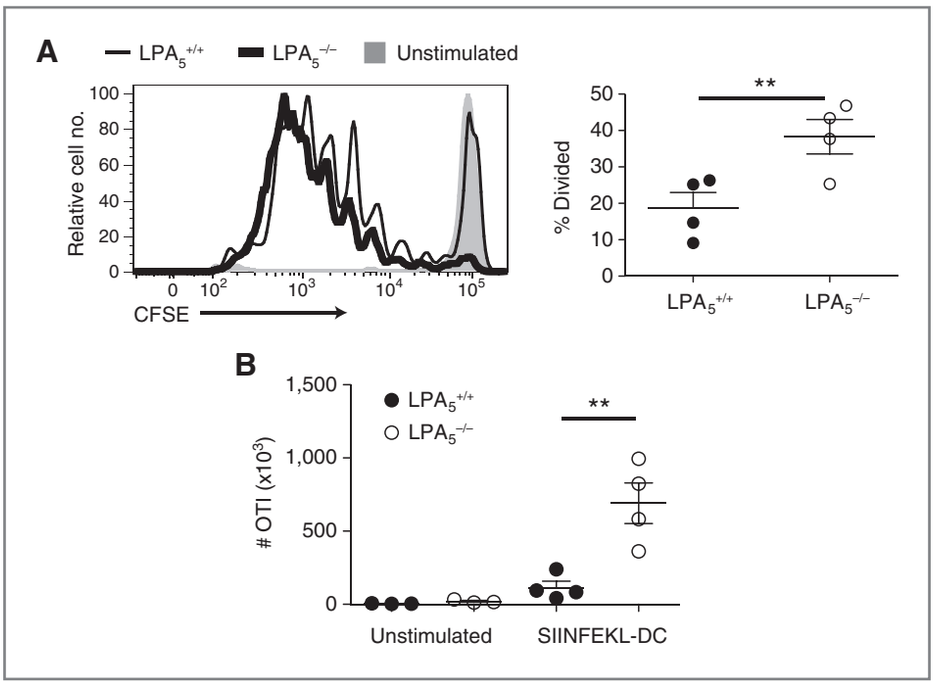

Figure 4. LPA_5-deficient $CD8^+$ T cells stimulated by peptide *in vivo* exhibit enhanced proliferation and accumulation. A and B, purified $LPA_5^{+/+}$ or $LPA_5^{-/-}$ OT-I $CD8^+$ T cells were CFSE labeled and transferred to C57BL/6 recipients, similar to Fig. 3A. Recipients were immunized 1 day later with SIINFEKL-pulsed BMDCs. On day 3 after immunization, mice were sacrificed and draining lymph nodes harvested for analysis. A, proliferation of $LPA_5^{+/+}$ (thin line), $LPA_5^{-/-}$ (bold line), or unimmunized (filled gray) OT-I $CD8^+$ T cells as indicated by CFSE dilution. Data are representative of two independent experiments. B, number of OT-I $CD8^+$ T cells harvested from the dLN of $LPA_5^{+/+}$ (closed circles; $n = 3$–4) and $LPA_5^{-/-}$ (open circles; $n = 3$–4) OT-I $CD8^+$ T-cell recipients in the absence of stimulation or 3 days after peptide stimulation (**, $P < 0.01$). Data are representative of two independent experiments.

with wild-type (Fig. 5C). However, compared with the wild-type CD8 T cells, these LPA_5-deficient tumor-infiltrating CD8 T cells seemed to express similar levels of IFN-γ, TNF-α, the inhibitory PD-1 receptor, and the CCR7 chemokine receptor 5 days after transfer (Fig. 5D and data not shown). Averaging tumor size across a treatment group showed that while the tumors were similar in size at the time of T-cell transfer, a significant reduction in tumor size was observed at 6 and 8 days post-transfer of $LPA_5^{-/-}$ tumor-specific CD8 T cells compared with the tumor size in mice transferred with wild-type CD8 T cells (Fig. 5B). Further evidence that tumor-specific $LPA_5^{-/-}$ CD8 T cells were better able to control tumor progression as compared with wild-type CD8 T cells was provided by the significantly smaller tumors that were harvested 8 days after T-cell transfer (Fig. 5E and F). These data show that tumor growth is inhibited to a greater degree when LPA_5 signaling is prevented in tumor-specific CD8 T cells.

Discussion

CD8 T cells are specialized cells of the adaptive immune system with the ability to recognize and eliminate nascent tumors. However, tumors are notorious for promoting an immunosuppressive environment through different mechanisms that thwart this adaptive immune response. Notably, the identification and targeting of some of these immunosuppressive mechanisms has led to relative success in the immunotherapeutic treatment of melanoma (3–5). Our data reveal a previously uncharacterized ability of the LPA lysophospholipid to suppress CD8 TCR signaling and *in vivo* activation, proliferation, and tumor control. As aberrant production of LPA is a common feature of diverse cancer cell types, these data further suggest that tumor cells can exploit a naturally occurring lipid to not only promote tumorigenesis but also to create an immunosuppressive environment.

Precedence for lysophospholipid regulation of adaptive immunity has been established previously in studies of S1P, which plays an important role in directing lymphocyte trafficking, localization, and development (47, 48). Similar to S1P, LPA is recognized by multiple GPCR that are differentially expressed by lymphocytes; in previous reports using primary human CD4 T cells and T-cell lines, LPA has been shown to regulate intracellular calcium mobilization (49) and cytokine production (16, 50). However, these *in vitro* studies did not identify the precise T-cell signaling pathway regulated by LPA, or which LPA receptor mediated these activities. In this report, we show that LPA engagement of LPA_5 suppresses $CD8^+$ T-cell receptor signaling, activation, and tumor immunity.

CD8 T cells express several LPA receptors and we have identified LPA_5 expression to be required for not only negative regulation of TCR-induced calcium mobilization but also for inhibiting *in vivo* antigen-mediated proliferation. TCR-mediated increase in cytosolic calcium is an early signaling event important for both proximal and distal CD8 T-cell activities (33). Indeed, the primary encounter between antigen-specific CD8 T cells and the specific antigen is known to program the subsequent proliferation and differentiation of antigen-specific cells *in vitro* and *in vivo* (51, 52). Analyses of TCR-mediated intracellular calcium mobilization unambiguously showed the ability of LPA to suppress TCR signaling at 1 μmol/L, the lowest concentration tested, which approximates the physiologic level of LPA in normal individuals (18). At higher concentrations of LPA (or OTP), antigen-mediated CD8 T-cell activation and proliferation *in vitro* were considerably suppressed. However, it is not yet clear whether the source of LPA that suppresses CD8 T-cell tumor immunity is derived from the

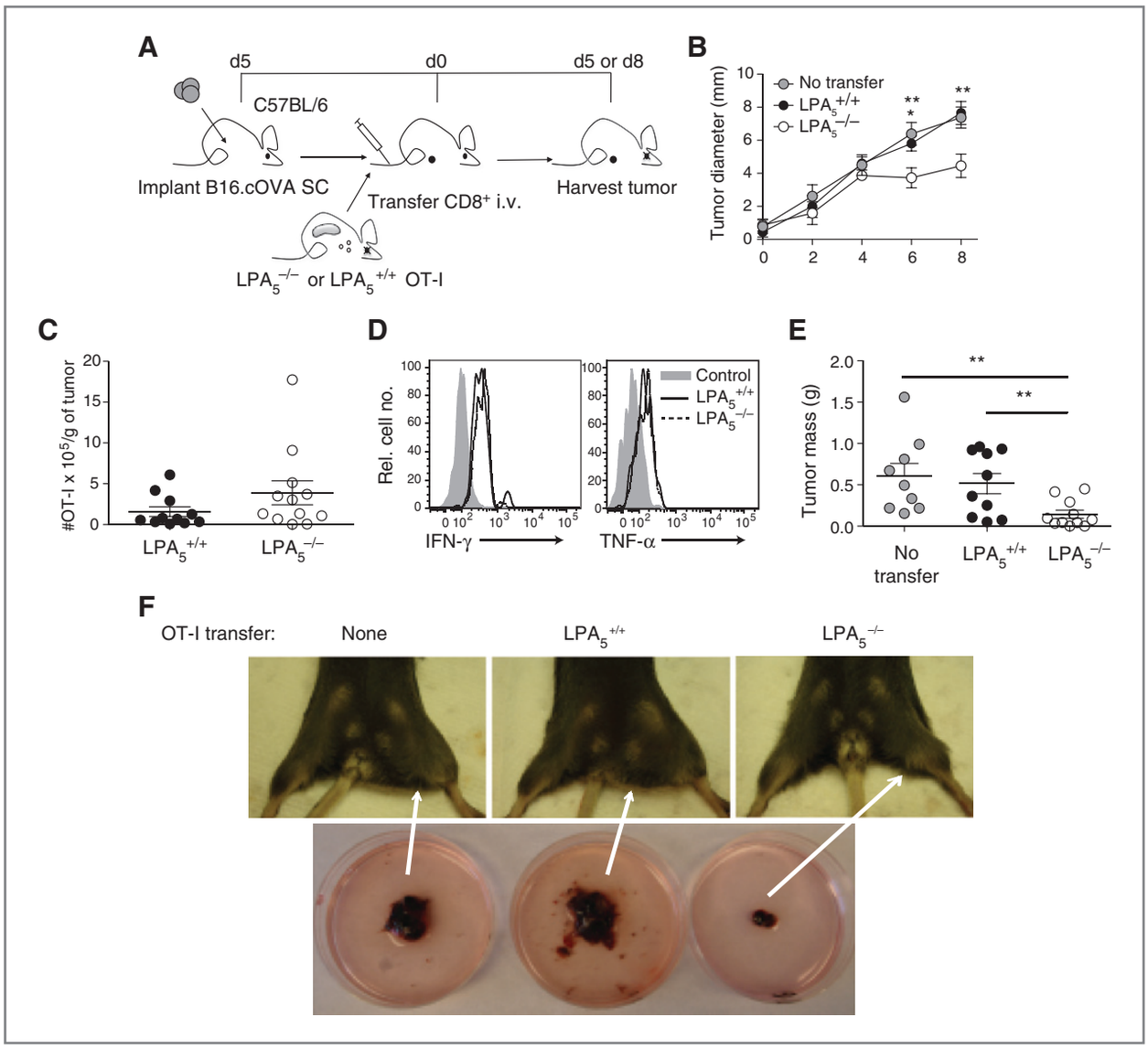

Figure 5. Enhanced control of tumor progression by LPA_5-deficient $CD8^+$ T cells. A, schematic of tumor establishment and subsequent OT-I $CD8^+$ T-cell adoptive transfer. C57BL/6 recipients were implanted subcutaneously in the leg with 10^5 B16.cOVA cells. Five days after implantation, 0.5×10^6 $LPA_5^{+/+}$ or $LPA_5^{-/-}$ OT-I $CD8^+$ T cells were transferred intravenously and tumor progression evaluated at the specified time points. B, average combined tumor burden at the indicated days in the absence of T-cell transfer (gray circles; $n = 9$) or after adoptive transfer of $LPA_5^{+/+}$ OT-I (black; $n = 10$), $LPA_5^{-/-}$ OT-I (white; $n = 11$), or untreated controls (gray; $n = 9$). Data are cumulative of three independent experiments (mean ± SEM; *, $P < 0.05$; **, $P < 0.01$). C, number of tumor-specific $CD8^+$ T cells per gram of tumor at day 5 after adoptive transfer of $LPA_5^{+/+}$ (black; $n = 11$) or $LPA_5^{-/-}$ (white; $n = 11$) OT-I $CD8^+$ T cells. Data are cumulative of three independent experiments (mean ± SEM; $P = 0.08$). D, tumors were harvested on day 5 after T-cell transfer and $LPA_5^{+/+}$ (black line) and $LPA_5^{-/-}$ (dotted line) OT-I CD8 T cells evaluated for IFN-γ and TNF-α expression and compared with tumor-free wild-type lymph node OT-I T cells (gray histogram). E, tumor mass 8 days after adoptive transfer of $LPA_5^{+/+}$ OT-I (black; $n = 10$), $LPA_5^{-/-}$ OT-I (white; $n = 11$), or untreated controls (gray; $n = 9$). Data are cumulative of three independent experiments (mean ± SEM; **, $P < 0.01$). F, representative tumors *in situ* and *ex vivo* from C57BL/6 mice in the absence of T-cell transfer or 8 days after adoptive transfer of $LPA_5^{+/+}$ or $LPA_5^{-/-}$ tumor-specific $CD8^+$ T cells.

tumor, the endogenous systemic levels, or from elsewhere, which is currently under investigation in our laboratory. It is important to note that the significance of the systemic levels of LPA in regard to LPA receptor signaling is not yet established. Current models suggest that biologically relevant LPA signaling results from localized and directed autocrine/paracrine LPA production and signaling (19, 53) where concentrations can reach considerably higher levels. In this model, secreted autotaxin has been proposed to associate with integrins on a cell surface where LPA is locally produced and engages nearby LPA receptors.

To assess the ability of LPA–LPA_5 signaling to suppress antigen-specific CD8 T-cell responses *in vitro* and *in vivo*, we used an established model system that allowed us to either increase LPA signaling using an LPA analogue, OTP, or prevent LPA inhibitory signaling using LPA_5-deficient CD8 T cells. Our

in vitro data suggest that LPA suppression of antigen-specific CD8 T cells operates more efficiently for relatively weak-affinity peptide antigens (Fig. 2 and Supplementary Fig. S2). However, in our studies neither wild-type nor $LPA_5^{-/-}$ OT-I CD8 T cells were stimulated *in vivo* with BMDC pulsed with the lower-affinity G4 peptide. Thus, whether LPA_5 suppression operates particularly for relatively weak affinity antigens could not be confirmed *in vivo*, but this is an area of ongoing investigation. As endogenous tumor-specific CD8 T cells normally express TCR with relatively low affinity for tumor antigens (54–56), this would suggest that tumor-derived LPA may be particularly effective at suppressing CD8 T-cell tumor immunity.

Our results clearly show that LPA signaling via LPA_5 inhibits CD8 T-cell TCR signaling, activation, and proliferation and that LPA_5-deficient tumor-specific CD8 T cells are better than the wild-type cells at controlling the growth of established tumor. However, analysis of tumor-infiltrating CD8 T cells 5 days after adoptive transfer did not reveal any apparent difference between tumor-specific $LPA_5^{-/-}$ and wild-type CD8 T cells with respect to the expression of IFN-γ, TNF-α, PD-1 inhibitory receptor, or the CCR7 chemokine receptor. Nevertheless, when stimulated with specific antigen *in vivo*, LPA_5-deficient CD8 T cells consistently proliferated to a greater extent and accumulated to higher numbers relative to wild-type CD8 T cells (Figs. 4 and 5C). Thus, together these data support a model in which $LPA_5^{-/-}$ tumor-specific CD8 T cells are more easily stimulated to proliferate in response to tumor antigen and the increased proliferation and accumulation equate, at least in part, to better tumor control. We note, however, that LPA has been shown to influence T-cell homing (57) and migration (58). Thus, it remains possible that $LPA_5^{-/-}$ tumor-specific CD8 T cells also display altered migration and trafficking, or they could influence CD8 T-cell function by other indirect mechanism(s) and, which is the focus of ongoing experiments.

As enhanced LPA production is a feature of many malignant cell types, our findings further suggest that in addition to the role of this lipid in enhancing tumorigenesis (27–29), LPA production by these cells also represents an additional inhibitory mechanism within the tumor microenvironment that serves to impede tumor rejection by CD8 T cells. The recent success of immunotherapies that antagonize inhibitory receptor signaling by T cells suggests that identification and characterization of additional signaling pathways used by tumors to suppress CD8 T-cell tumor immunity will only improve cancer immunotherapy (6, 7). Data presented here show that LPA_5 functions as an additional inhibitory receptor on CD8 T cells. Given the association of LPA with multiple cancers (26, 43, 59), blockade of LPA_5 signaling may be a promising additional strategy to promote host CD8 T-cell tumor immunity.

Disclosure of Potential Conflicts of Interest

T. Oravecz has ownership interest (including patents) in Lexicon Pharmaceuticals. G. Tigyi has ownership interest (including patents) in RxBio Inc. and is a consultant/advisory board member of the same. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.K. Oda, R.M. Torres
Writing, review, and/or revision of the manuscript: S.K. Oda, A. Al-Shami, T. Oravecz, G. Tigyi, R. Pelanda, R.M. Torres
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.K. Oda, P. Strauch, R.M. Torres
Study supervision: R. Pelanda, R.M. Torres

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