Inhibition of serine palmitoyltransferase delays the onset of radiation-induced pulmonary fibrosis through the negative regulation of sphingosine kinase-1 expression

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Abstract The enforcement of sphingosine-1-phosphate (S1P) signaling network protects from radiation-induced pneumonitis. We now demonstrate that, in contrast to early postirradiation period, late postirradiation sphingosine kinase-1 (SphK1) and sphingoid base-1-phosphates are associated with radiation-induced pulmonary fibrosis (RIF). Using the mouse model, we demonstrate that RIF is characterized by a marked upregulation of SIP and dihydrosphingosine-1-phosphate (DHS1P) levels in the lung tissue and in circulation accompanied by increased lung SphK1 expression and activity. Inhibition of sphingolipid de novo biosynthesis by targeting serine palmitoyltransferase (SPT) with myriocin reduced radiation-induced pulmonary inflammation and delayed the onset of RIF as evidenced by increased animal lifespan and decreased expression of markers of fibrogenesis, such as collagen and α-smooth muscle actin (α-SMA), in the lung. Long-term inhibition of SPT also decreased radiation-induced SphK activity in the lung and the levels of SIP-DHS1P in the lung tissue and in circulation. In vitro, inhibition or silencing of serine palmitoyltransferase attenuated transforming growth factor-β1 (TGF-β)-induced upregulation of α-SMA through the negative regulation of SphK1 expression in normal human lung fibroblasts. These data demonstrate a novel role for SPT in regulating TGF-β signaling and fibrogenesis that is linked to the regulation of SphK1 expression and SIP-DHS1P formation. —Gorshkova, I., T. Zhou, B. Mathew, J. R. Jacobson, D. Takekoshi, P. Bhattacharya, B. Smith, B. Aydogan, R. R. Weichselbaum, V. Natarajan, J. G. N. Garcia, and E. V. Berdyshev. Inhibition of serine palmitoyltransferase delays the onset of radiation-induced pulmonary fibrosis through the negative regulation of sphingosine kinase-1 expression. J. Lipid Res. 2012. 53: 1553–1568.

Supplementary key words sphingosine-1-phosphate • dihydrosphingosine-1-phosphate • myriocin

Radiation-induced lung injury (RILI), comprised of pneumonitis and fibrosis (RIF), is a serious and dose-limiting complication of thoracic radiotherapy (1). Commonly used radiotherapy regiments result in an incidence of radiation pneumonitis in up to 35% in lung cancer and breast cancer patients (2–4), and patients receiving total body irradiation before bone marrow transplant are also at risk (4). Although clinical symptoms of radiation pneumonitis, which usually manifests within a few months after radiotherapy, may subside, most patients having radiation pneumonitis develop gradual and progressive fibrosis within 6 to 24 months after radiotherapy, and in some cases, RIF develops without previous history of pneumonitis (3). RIF usually progresses over a period of 2 years and then remains stable but can result in severe respiratory compromise and core pulmonary when significant lung volumes are affected.

Substantial progress has been achieved in recent years in understanding signaling mechanisms and cell populations involved in initiation and progression of pulmonary fibrosis. However, there are no available therapies to stop and reverse its progression, including that of RIF. Being multifactorial in nature, pulmonary fibrosis requires nontrivial therapeutic approaches targeting multiple signaling and cellular elements involved in fibrogenesis at all stages of its progression. Even such a previously

Abbreviations: BAL, bronchoalveolar lavage; DHS1P, dihydrosphingosine-1-phosphate; DSHp, dihydrosphingosine; Gs, grey; nHLFB, normal human lung fibroblasts; RIF, radiation-induced lung fibrosis; RILI, radiation-induced lung injury; SIP, sphingosine-1-phosphate; SIP1, sphingosine-1-phosphate lyase; SIPR, sphingosine-1-phosphate receptor; SMA, smooth muscle actin; Sph, sphingosine; SphK, sphingosine kinase; SPT, serine palmitoyltransferase; TGF-β, transforming growth factor beta; TNFa, tumor necrosis factor alpha

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This online version of this article (available at http://www.jlr.org) contains supplementary data in the form of two tables and one figure.
nondisputed relationship between inflammation and fibrogenesis is questioned now because standard anti-inflammatory therapies (by corticosteroid or cytotoxic agents) have shown little efficacy in affecting fibrogenesis in idiopathic pulmonary fibrosis (5, 6) and lung cancer (7) patients. Furthermore, the same elements involved in the process of fibrogenesis may play opposite roles depending on the cell type involved and the stage of fibrogenesis. For example, macrophages involved in tissue inflammation and fibrogenesis play a distinct role in the inflammatory and remodeling phases of the process (8). Then, such a potent proinflammatory cytokine as TNF-α fulfills a profibrotic role by stimulating interleukin-1β and TGF-β signaling in multiple cell types, including macrophages and fibroblasts (6, 9, 10), but also blocks collagen synthesis in myofibroblasts (11). Therefore, it is important to better understand the role of each cellular and molecular element involved in fibrogenesis at different stages of the process and to find novel signaling mechanisms involved in inflammatory and proliferative responses characteristic for fibrogenesis.

We recently reported a beneficial role for sphingosine-1-phosphate (SIP) signaling system in ameliorating radiation pneumonitis evaluated at 6 weeks postirradiation in the mouse model of RILI (12). In the above studies, we demonstrated the ability of SIP receptor (S1PR) agonists SEW2871 and FTY720-PH to decrease radiation-induced subacute lung inflammation. In addition, mice with targeted deletion of sphingosine kinase-1 (SphK1) (SphK1<sup>-/-</sup>), SIP receptors 2 and 3 (S1PR2<sup>-/-</sup>, S1PR3<sup>-/-</sup>), or with the reduced expression of SIP receptor 1 (SIPR1<sup>+/--</sup>) showed a marked increase in RILI, thereby demonstrating a protective role for SIP and SIP signaling in response to radiation. We attributed the observed beneficial effect of S1PR ligands to the known potent endothelial cell barrier-enhancing properties of SIP-induced (mainly through the S1PR1) signaling events (13, 14). In the current study, our investigation focuses on the late, fibrotic phase of RILI development. We affected RIF by global targeting of sphingolipid and SIP metabolism through the inhibition of serine palmitoyltransferase (SPT), a key enzyme controlling biosynthesis of all sphingolipids (15), by myricin (Fig. 1). The inhibition of SPT decreased inflammation and fibrogenesis and normalized SIP homeostasis in the lung. Furthermore, the in vitro modeling using normal human lung fibroblasts and transforming growth factor-β1 (TGF-β1)-induced α-smooth muscle actin (α-SMA) expression as a marker of fibroblast transdifferentiation revealed the involvement of SPT in regulation of TGF-β1-induced α-SMA and SphK1 expression. Our data identified SPT as a novel protein involved in TGF-β signaling and a potential target for RIF management. Our data also suggest that, in contrast to its beneficial role during the development of early inflammation in response to irradiation, SphK1/S1P-dihydrosphingosine-1-phosphate (DHS1P) signaling axis is detrimental rather than protective at the late RIF phase of radiation-induced lung injury.
collagen content (per Biocolor instructions), and determining the expression of α-SMA (by Western blotting) in the lung. Before biochemical analyses, the harvested lung tissues were pulverized in liquid nitrogen to ensure sample homogeneity.

**Cell culture**

Normal human lung fibroblasts (nHLFB) and FBM-2 culture medium were purchased from Lonza (Walkersville, MD). Passages between 5 and 7 were used for experiments. Cells were grown at 37°C in a humidified atmosphere with 5% CO₂ and 95% air. All experiments were designed to terminate when cells reached 80% confluency.

**Immunoblotting**

Lung tissue samples were lysed with 100–300 μl cell lysis buffer (Cell Signaling Technology, Danvers, MA) containing Halt ThermoFisher protease inhibitor cocktail (Fisher Scientific, Pittsburgh, PA). Tissue lysates were cleared by centrifugation at 5,000 × g for 10 min and boiled with the Laemmli sample buffer for 5 min. Tissue lysates (20–30 μg protein) were separated on 10% or 4–20% NuPage precast gels (Life Technologies, Grand Island, NY), transferred to PVDF membranes, and blocked in TBST containing 5% BSA before incubation with primary antibodies (1:1,000 dilution) overnight. After blocking, washing, and incubation with appropriate secondary antibody, blots were developed using an ECL chemiluminescence kit. The bands of interest were quantified using a densitometry (1:1,000 dilution) overnight. After blocking, washing, and incubation with appropriate secondary antibody, blots were developed using an ECL chemiluminescence kit. The bands of interest were quantified using a densitometry.

**Lipid extraction and sample preparation for electrospray ionization-LC/MS/MS**

Tissue lipids were extracted by a modified Bligh and Dyer procedure (21) with the use of 0.1 N HCl for phase separation as described in (22). C17-SIP (40 pmol), C17-ceramide (30 pmol), and C17-sphingosine (C17-Sph) (30 pmol) were used as internal standards and were added during the initial step of lipid extraction. The extracted lipids were dissolved in methanol/chloroform (4:1, v/v), and aliquots were taken to determine total phospholipid content as described in (23). Samples were concentrated under a stream of nitrogen, redissolved in methanol, transferred to autosampler vials, and subjected to sphingolipid LC/MS/MS analysis. Plasma and BAL fluid samples were processed similarly except that lipid phosphorus was not determined and data were expressed per sample volume.

**Analysis of sphingoid base-1-phosphates, ceramides, and sphingoid bases**

Analyses of sphingoid base-1-phosphates, ceramides, and sphingoid bases were performed by electrospray ionization tandem mass spectrometry. The instrumentation used included an API4000 Qtrap and AB Sciences 3500 QTRAP hybrid triple quadrupole linear ion-trap mass spectrometers (Applied Biosystems, Foster City, CA) equipped with a turboionspray ionization source interfaced with an automated Agilent 1100 series or 1200 series liquid chromatograph and autosampler (Agilent Technologies, Wilmington, DE). S1P and DHS1P were analyzed as bis-acetylated derivatives with C17-S1P as the internal standard using reverse-phase HPLC separation, negative ion electrospray ionization, and multiple reaction monitoring analysis as described in (24). Ceramides and sphingoid bases were analyzed with C17-ceramide and C17-Sph as internal standards using reverse-phase HPLC separation, positive ion ESI, and multiple reaction monitoring analysis as described in (22).

**Sphingosine kinase, SIP lyase, and serine palmitoyltransferase enzymatic activity assays**

Lung tissues were pulsed in liquid nitrogen for homogeneity and lysed in the buffer consisting of 5 mM MOPS, 1 mM EDTA, 0.25 M sucrose, 1 mM PMSF, and 10% (v/v) glycerol (pH 7.4). Debris was removed by low-speed centrifugation (1,000 × g for 5 min), and the supernatant was used as a total tissue homogenate.

The serine palmitoyltransferase activity assay was performed essentially as described previously (25, 26) with 1 mM stable isotope-labeled [L-1-¹³C,¹⁵N]serine and 0.4 mM 16:0-CoA as substrates. The reaction was carried out with 20 μg of total lysate protein/reaction for 20 min at 37°C in a buffer consisting of 20 mM HEPES (pH 8.0) containing 5 mM EDTA, 10 mM dithiothreitol, and 50 μM pyridoxal-5’-phosphate. The reaction was stopped by lipid extraction with C17-Sph as the internal standard. The stable isotope-labeled [M+3] analog of 3-keto-DHSP was quantified by the LC/MS/MS by detecting a specific transition from m/z 303 to m/z 285, which corresponds to the M+3 isotope analogs of molecular and product ions of 3-keto-dihydrosphingosine (DHSPh). The standard curve of

**RNA isolation, real-time RT-PCR, and microarray analysis**

Total RNA was isolated from lung tissues using TRIzol® reagent according to the manufacturer’s instructions. RNA (1 μg) was reverse transcribed using a cDNA synthesis kit (Bio-Rad), and real-time PCR and quantitative PCR were performed to assess expression of the SphK1, SphK2, S1PL, SPT1, and SPT2 using primers designed for mouse mRNA sequences. iQ SYBR Green Supermix was used to perform the real-time measurements using iCycler by BioRad. Amplicon expression in each sample was normalized to 18S RNA content. Analysis of results and fold differences were determined using the comparative CT method. Fold change was calculated using a comparative quantification algorithm from the ΔΔCt values with the formula (2^(-ΔΔCt)), and data are presented as relative to the endogenous normalizer 18S mRNA expression. For the microarray analysis, RNA was isolated from total lungs using an RNAeasy Mini Kit (Qiagen) according to the manufacturer’s instructions, and gene expression was evaluated using GeneChip mouse chip 1.0ST (Affymetrix, Santa Clara, CA). Chips were scanned using a GeneChip Scanner 3000 (Affymetrix). Chip quality and present calls were determined by Affymetrix GCOS software. The chip data were analyzed using the Affymetrix Power Tools v.1.12.0 (http://www.affymetrix.com/analysis/netaffx/). Genes potentially dysregulated were identified by pairwise comparison using SAM (Significance Analysis of Microarrays) algorithm (17) using the criteria of false discovery rate <5% and fold change >2. We searched for any enriched KEGG (Kyoto Encyclopedia of Genes and Genomes) (18) physiological pathways among the dysregulated genes using the NIH/DAVID (19, 20).

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response of variable amounts of 3-keto-DHSPh versus fixed amounts of C17-Sph was created to perform a proper quantitative determination of the formed product.

Sphingosine kinase activity assay was carried out in a buffer consisting of 10 mM HEPES (pH 7.4) containing 100 mM MgCl₂, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na₃VO₄, and 10 mM MgCl₂ with 20 µg total lysate protein/reaction for 30 min at 37°C. Reaction was initiated by the addition of the substrate (5 µM sphingosine [Sph]) and ATP (200 µM) and stopped by lipid extraction as described above. C17-SIP (40 pmol) was added during initial phase of lipid extraction as the internal standard. Generated SIP was quantified as bis-acetylated derivative using the LC/MS/MS approach as described (24) with a background SIP level subtraction.

SIPL activity assay was performed as described (27). The SIPL reaction was initiated by mixing 0.025 ml of 0.4 mM SIP in 1% Triton X-100 in water, 0.175 ml of reaction buffer (35 mM potassium phosphate buffer [pH 7.4], 0.6 mM EDTA, 70 mM sucrose, 36 mM sodium fluoride, 0.57 mM pyridoxal-5′-phosphate), and 0.05 ml of protein preparation (20 µg protein) in lysis buffer. The reaction was performed for 20 min at 37°C and was stopped by the addition of 2 ml of methanol. All samples were processed for lipid extraction as described above. Internal standard ((2E)-t5-hexadecenal, 20 pmol) was added during the initial phase of lipid extraction. After lipid extraction, chloroform was evaporated by a stream of nitrogen, and aldehyde semiacetylation derivatives were prepared and analyzed by the LC/MS/MS as described (27).

Statistical analyses
Each animal group contained at least five animals; in vitro experiments were performed at least in triplicate. Two-way ANOVA was used to determine the interaction effect between two or more experimental variables. Post hoc Student’s t-test was used where appropriate. A GraphPad Prism 5.02 statistical package was used for statistical analyses. Differences between groups and significance of the interaction effect were considered statistically significant at \( P < 0.05 \). Results are expressed as means ± SEM.

RESULTS

Mouse model of thoracic radiation-induced pulmonary fibrosis
C57Bl/6 mice are susceptible to radiation and develop dose-dependent pulmonary inflammation (12) and fibrosis (28, 29). A dose of 20 Gy of thoracic irradiation results in pulmonary fibrosis within 12 to 15 weeks and death between 15 and 23 weeks postirradiation (28). In our hands, 20 Gy of thoracic irradiation resulted in the animal’s loss of body weight of up to 25% by week 2 postirradiation. Animals recovered their original weight within 4 weeks; however, 20 Gy caused the development of skin lesions in some animals, which were unresponsive to antibacterial/antifungal treatment, resulting in animal elimination from the studies. At 12 to 15 weeks postirradiation, animals began to lose weight, developed severe pulmonary inflammation (Fig. 2B,C) and fibrosis (Fig. 2D–F), and died between 15 and 19 weeks postirradiation (Fig. 2A). Fibrosis was confirmed by lung tissue staining with Sirius Red, which showed extensive tissue remodeling, collagen deposition, and loss of normal alveolar morphology (Fig. 2D) as well as by the in vitro assessment of total lung tissue collagen content (Fig. 2E) and expression of α-SMA, the marker of fibroblast transdifferentiation into myofibroblasts (Fig. 2F). In accordance with previous reports (28, 29), fibrogenesis in irradiated mice was accompanied by a secondary wave of inflammation marked by massive tissue infiltration by immune cells (Fig. 2C) and the increase in BAL fluid leukocyte and protein content (Fig. 2B).

Myriocin decreases radiation-induced pulmonary inflammation and fibrosis
Previously, we found thoracic irradiation to cause a significant dysregulation of pulmonary sphingolipid signaling system, which suggested its involvement in RILI (12). Because the biosynthesis of all sphingolipids is initiated by serine palmitoyltransferase (SPT) (Fig. 1), it can be affected through SPT inhibition. To investigate a potential link between radiation-induced dysregulation of sphingolipid metabolism and RIF, animals were treated with myriocin, the inhibitor of SPT (30). The chosen dose and regimen of myriocin administration was deducted from studies where myriocin did not show toxic but rather demonstrated positive effects in different experimental settings (31–33). Also, our preliminary studies demonstrated toxic effects of higher myriocin dose (0.5 mg/kg p.o.), with daily administration resulting in death of irradiated (but not nonirradiated) animals within 2 to 4 weeks. Myriocin treatment (0.375 mg/kg p.o. 3×/week) initiated 3 days before irradiation and continued throughout the experimental period delayed...
Fig. 3. Changes in mouse lung gene expression and the effect of myriocin on dysregulated gene expression developed at RIF phase. A: Dysregulated genes in RIF animals and animals receiving myriocin treatment were identified using a SAM algorithm and subjected to ontology analysis. The dysregulated pathways were aligned according to the power of dysregulation. Dotted line represents the threshold for statistical significance (false discovery rate <5% and fold change >2). The TGF-β signaling pathway is presented due to the known fundamental role of TGF-β signaling in fibrogenesis and the fact that TGF-β2 gene expression was statistically significantly upregulated (2.47-fold; q = 0.7) at RIF stage (see supplementary Table II). B: Hierarchical clustering of genes dysregulated at RIF phase is shown as identified by SAM. Blue, white, and red represent mRNA levels below, at, and above the average level of the corresponding gene, respectively.

the onset of the final body weight decline by about 2 weeks (data not shown); extended animal survival by about 2 to 3 weeks \( (P = 0.0172) \) (Fig. 2A); and decreased BAL protein level, neutrophil infiltration (Fig. 2B), and the interstitial infiltration of the lung tissue with immune cells in comparison to nontreated animals (Fig. 2C). Histological (Fig. 2D) and biochemical (Fig. 2E,F) analyses revealed the ability of myriocin to slow the development of RIF, as demonstrated by the decrease in collagen deposition and the expression of α-SMA in the lung tissue. In BAL, the level of a potent profibrotic TGF-β protein was substantially upregulated at RIF stage, and myriocin treatment had a tendency to decrease it (supplementary Fig. I). By itself, the long-term application of myriocin moderately increased tissue levels of acid-soluble collagen and α-SMA (Fig. 2E,F) but substantially decreased radiation-induced upregulation of their expression. Although the influence of myriocin treatment on radiation-induced inflammation at the early stages of RILI progression was not the focus of the current study, we evaluated the effect of SPT inhibition on BAL protein content and cell count at 6 weeks postirradiation. Myriocin treatment did not modulate protein level and total cell counts in BAL fluid (data not shown). This limits the potential importance of the early immunomodulatory consequences of SPT inhibition on the final outcome manifested by partial protection from RIF and associated pulmonary inflammation.

Myriocin alleviates the dysregulation in lung gene expression at RIF stage

To link the protective effect of myriocin in murine model of RIF to genomic influences of myriocin treatment, we performed genome-wide mRNA profiling from lung tissues from control, irradiated, and myriocin-treated animals at RIF time point (18 weeks postirradiation). Genes potentially differentially regulated were identified
we performed lung tissue, plasma, and BAL fluid analysis 6 weeks postirradiation. To study whether the imbalance in first week postirradiation but was markedly upregulated content in the lung was substantially reduced during the declined 6 to 12 weeks postirradiation. In contrast, ceramide levels in the lung tissue at RIF stage, whereas dihydroceramide levels were upregulated more than that of DHS1P at RIF stage (Fig. 4A). SIP-DHS1P levels in BAL were extremely low in comparison to their levels in plasma, but the low levels could not be explained by a possible blood leak during BAL collection as follows from BAL cell lineage attenuation. The detailed look at ceramide molecular species and sphingoid bases in the lung tissue, plasma, and BAL in RIF animals provided a better understanding of metabolic environment favoring DHS1P-S1P formation with RIF development (Fig. 4A). The opposite dynamics in changes of ceramides-Sph versus dihydroceramides-Sph in the lung tissue and in plasma also support the notion of separate metabolic routes involved in the final development. A heat-map view of their changes versus age-matched, solvent-treated controls indicated mostly negative shifts in ceramide levels in the plasma and in the lung tissue at RIF stage, whereas dihydroceramide levels were upregulated (Fig. 4B). Yet, the level of sphingolipid in lung tissue was increased, which supports increased S1P level in the lung (Fig. 4A). Similar changes were detected in plasma levels of SIP and DHS1P, whereas in BAL SIP levels were upregulated more than that of DHS1P at RIF stage (Fig. 4A). The analysis revealed a 3-fold increase in the level of DHS1P and a smaller increase in SIP level in the lung (Fig. 4A). S1P-DHS1P levels in BAL were extremely low in comparison to their levels in plasma, but the low levels could not be explained by a possible blood leak during BAL collection as follows from BAL ceramide analyses when comparing changes in individual ceramide molecular species (see below). We also found that myriocin counteracted radiation-induced upregulation of SIP and DHS1P levels in lung tissue and in circulation seen with RIF development (Fig. 4A,B).

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Fig. 4. Myriocin inhibits upregulation of S1P-DHS1P levels and modifies ceramide-sphingoid base levels in RIF animals. A: Inhibition of S1P-DHS1P levels in the lung tissue, plasma, and in BAL 18 weeks after a 20 Gy thoracic irradiation by myriocin (Lung DHS1P P = 0.0118; Plasma S1P P = 0.0457; Plasma DHS1P P = 0.0013). B: Heat-map presentation of the changes in the lung tissue, plasma, and BAL ceramide, dihydroceramide, sphingoid base, and sphingoid base-1-phosphate levels at RIF stage and the effect of myriocin in comparison to the age-matched, solvent-treated control animals. Blue, white, and red represent lipid levels below, at, and above the level of corresponding analyte in control animals, respectively, presented as a ratio to the level in nontreated controls. All changes in the levels of analytes below 0.9-fold and above 1.2-fold over control values were statistically different with P < 0.05 at minimum. C: Relative content of major ceramide molecular species in the lung tissue, plasma, and BAL from solvent-treated nonirradiated animals. ** P < 0.01, *** P < 0.001 versus nonirradiated control (n = 5). N.S., nonsignificant.
sustained through sphingolipid de novo biosynthesis (22) and S1P generation supported through a consecutive degradation of complex sphingolipids to ceramides and Sph (Fig. 1). Regarding the BAL, ceramides and dihydroceramides demonstrated a remarkable upregulation late postradiation (Fig. 4B). It is not understood what could be their origin and if this is a reflection of inflammatory and fibrotic processes in the lung or an indication of ceramide functional role in pulmonary fibrogenesis. Still, this finding may lead to the development of novel biomarkers of RIF development.

In accordance with previously published reports (31–33), the long-term application of myriocin substantially decreased circulatory ceramide and dihydroceramide levels along with sphingosine and S1P-DHS1P (Fig. 4B). The long-term animal treatment with myriocin did not decrease lung tissue levels of ceramides and sphingoid bases (Fig. 4B), whereas in short-term experiments (up to 2 weeks) such a treatment decreased their levels (data not shown). This suggests the development of a compensatory mechanism aimed at alleviating global inhibition of sphingolipid biosynthesis with a long-term but periodic (only three times per week) animal treatment. Yet, such a treatment reduced the expression of SPTLC2 subunit and the activity of SPT in the lung of RIF animals (see below). Interestingly, the superposition of myriocin treatment and a single 20-Gy dose of thoracic irradiation inversed the effect of myriocin on ceramide and dihydroceramide levels in plasma and in BAL seen in nonirradiated animals but only moderately reversed myriocin’s effect on the level of ceramides in the lung tissue at RIF stage. (Fig. 4B). There should be a link between RIF development and changes in the dynamic exchange of sphingolipids between tissues and circulation that complicates the interpretation of the observed modulations in sphingolipid levels and merits further investigation.

To define a link between myriocin-provided protection from RIF development and the upregulation of S1P and DHS1P levels late postirradiation (18 weeks), we explored the effect of myriocin on the expression and
Fig. 6. S1P lyase protein but not the enzyme activity is increased in the lung tissue at 18 weeks after a single dose (20 Gy) of thoracic irradiation. Irradiation upregulates S1P lyase protein level (A) and its mRNA level (B) but not its enzymatic activity (C). Myriocin treatment does not substantially affect S1P lyase background protein (A) and mRNA (B) levels but decreases radiation-induced changes in S1P lyase transcription (\(P_{\text{int}} = 0.0444\)) or translation (\(P_{\text{int}} = 0.0238\)) (A, B). Neither radiation nor myriocin affects S1P lyase enzymatic activity (C). S1PL activity is expressed as pmol \(\Delta^2\)-hexadecenal formed per minute per mg total cell lysate protein.* \(P < 0.05\), *** \(P < 0.001\) versus nonirradiated control (\(n = 5\)). N.S., nonsignificant.

Fig. 7. Serine palmitoyltransferase expression and activity are upregulated by a single dose (20 Gy) of thoracic irradiation at RIF stage. Mice received a single dose (20 Gy) of thoracic irradiation; the SPT1-SPT2 subunit mRNA levels (A), SPT2 subunit protein expression (B), and SPT activity (C) were evaluated at 18 weeks postirradiation. Myriocin decreased SPT2 subunits’ mRNA level (\(P_{\text{int}} = 0.0406\)) (A) and SPT activity (\(P_{\text{int}} = 0.0137\)) (C) in RIF animals. SPT activity is expressed as pmol 3-keto-dihydrosphingosine formed per minute per mg total cell lysate protein.* \(P < 0.05\), ** \(P < 0.01\), and *** \(P < 0.001\) versus nonirradiated control (\(n = 3–5\)). N.S., nonsignificant.
**Fig. 8.** Myriocin decreases TGF-β-induced upregulation of α-SMA and SphK1 in normal human lung fibroblasts through the inhibition of SPT. A: nHLFB cells were serum deprived for 3 h in 0.1% FBS-FBM and treated with myriocin (2 μM) for 24 h. TGF-β (5 ng/ml) was added to cells for the next 24 h. B: nHLFB cells were treated as above but with DHSph (1 μM) added twice during first 24 h then added once again concomitantly with TGF-β. The expression of SphK1 and α-SMA was evaluated by Western blotting and normalized to GAPDH expression. Cell treatment with DHSph reversed the inhibitory effect of myriocin. **P < 0.01, ***P < 0.001 versus nontreated control (n = 3).

presenting those data due the unknown nature of the Triton X-100 effects on SphK1/2 activities and to the fact that Triton X-100 differentially stimulated SphK1 activity in lung tissue lysates from nonirradiated animals (~10-fold over the activity without Triton X-100) and in lysates from irradiated animals (only ~5-fold over the activity without Triton X-100).

To test the possibility that the observed increase in S1PL and DHS1P levels may be determined in part by the modulation of S1PL expression and/or activity, we characterized these parameters in the lung tissues from control and irradiated animals treated with myriocin or not treated with myriocin. Thoracic irradiation increased S1PL protein expression and S1PL transcription at 18 weeks postirradiation (Fig. 6A,B); however, the S1PL protein increase was not accompanied by the increase in S1PL activity (Fig. 6C). Also, myriocin had no discernable effect on the background level of S1PL activity or on its activity in the lung tissues from irradiated animals at RIF stage (Fig. 6C). These results suggest inactivation and/or impaired processing of S1PL at the fibrotic stage that favors S1P-DHS1P accumulation in the tissue.

SphK1 uses de novo synthesized DHSph to synthesize DHS1P in cells (22). It is therefore likely that the observed increase in DHS1P formation is in part due to the increased expression or activity of serine palmitoyltransferase, which is the initial and rate-limiting enzyme in the biosynthesis of all sphingolipids (15). We therefore carried out a qRT-PCR analysis of SPTLC1 and SPTLC2 SPT subunits’ expression and determined their transcript levels in lung tissues at RIF stage (18 weeks postirradiation). We found that SPTLC2, but not SPTLC1 subunit expression, was slightly but statistically significantly upregulated at RIF stage (Fig. 7A); this effect was paralleled by about 2-fold increase in SPT2 protein expression (Fig. 7B) and by a slight increase in SPT activity (Fig. 7C). Long-term treatment with myriocin downregulated SPTLC2 mRNA levels and SPT activity in RIF animals but did not affect SPTLC1 mRNA level and only slightly affected SPT2 protein expression (Fig. 7). These data confirm that the observed radiation-induced upregulation of DHS1P levels at RIF stage is driven by a concert work of SphK1 and SPT, with the latter providing an increased supply of de novo synthesized DHSph for DHS1P formation.

**Myriocin interferes with TGF-β signaling by inhibiting SphK1 expression**

Long-term animal treatment with myriocin counteracted radiation-induced increase in the expression of collagen and α-SMA in the lung at fibrotic stage (Fig. 2E,F). Transforming growth factor-β (TGF-β) is one of the most potent known inducers of fibrogenesis and is intimately involved in radiation-induced fibrosis (35). In mice, TGF-β level in BAL and TGF-β2 mRNA expression in the lung tissue were substantially increased at RIF stage (supplementary Fig. 1; supplementary Table I), confirming the well-established link between TGF-β signaling and fibrogenesis in RIF. Moreover, myriocin treatment clearly downregulated TGF-β2 gene expression (supplementary Table I). To provide mechanistic insights into the in vivo antifibrotic action of myriocin, we tested myriocin interference with TGF-β-induced effects in vitro using normal human lung fibroblasts (nHLFB). First, we treated nHLFB with 2 μM myriocin for 24 h and then stimulated cells with TGF-β (5 ng/ml) for an additional 24 h and evaluated α-SMA expression by Western blotting. We found that 2 μM myriocin did not affect background α-SMA expression in nHLFB but decreased TGF-β-induced upregulation of α-SMA (Fig. 8A). Myriocin also decreased TGF-β-induced upregulation of SphK1 expression (Fig. 8A). To address the role of SPT and sphingolipid de novo biosynthesis in
the inhibition of TGF-β-induced upregulation of α-SMA and SphK1 expression by myriocin, we supplemented nHLFB culture medium with DSHsp concomitantly with the inhibition of SPT by myriocin to bypass the block of sphingolipid de novo biosynthesis (Fig. 1). Such a treatment counteracted myriocin-induced inhibition of TGF-β signaling and partially restored TGF-β-induced effects on α-SMA and SphK1 expression (Fig. 8B), confirming the role of sphingolipid de novo biosynthesis in supporting TGF-β signaling. We also found that myriocin at concentrations up to 10 µM did not affect SphK or S1PL activities in vitro, as confirmed by the LC/MS/MS quantitation of the products of SphK and S1PL reactions (data not shown). This excludes myriocin’s direct modulation of enzymes pivotal for controlling S1P homeostasis in cells and highlights transcriptional regulation of SphK1 expression by myriocin as part of its interference with profibrotic/TGF-β signaling.
To further support the role of SPT in TGF-β profibrotic signaling, we inhibited SPT2 expression by small interfering RNA and tested its effect on TGF-β-induced α-SMA and SphK1 expression. The downregulation of SPT2 expression significantly decreased TGF-β-induced α-SMA and SphK1 expression (Fig. 9). Moreover, this experiment revealed that TGF-β, in addition to the upregulation of SphK1 expression, increases the expression of SPT2 subunit of SPT (Fig. 9A). Next, and in concert with previous findings (36–38), SphK1 was confirmed to mediate TGF-β-induced signaling in nHLFB. In these cells, the downregulation of SphK1 by SphK1 siRNA substantially decreased TGF-β-induced α-SMA expression (Fig. 9B). These data demonstrate a novel regulatory link between SPT expression/activity and SphK1 expression that provides mechanistic explanation for myriocin-induced effects in the settings of TGF-β and radiation-induced fibrogenesis.

### DISCUSSION

There is no full understanding regarding the role of signaling sphingolipids in the initiation, progression, and culmination or resolution of RILI. Regardless of an overall acknowledgment of proapoptotic role for ceramides as mediator of radiation-induced injury, especially in microvasculature (39), limited information is available on the role of signaling sphingolipids in RILI. Recently, we highlighted an important beneficial role of S1P signaling in the protection from radiation-induced pulmonary inflammation at an early stage of RILI development (12). The focus of the current work is on the late fibrosis stage of RILI progression, and our findings suggest that at this stage the S1P signaling system may be associated with RIF progression, thus making the overall intricate story of S1P signaling in RILI complicated and provocative. RILI is a long, multistep process, and with a 20-Gy single dose of thoracic irradiation mice develop RIF and die between 15 and 20 weeks postirradiation. Pulmonary inflammation (pneumonitis) develops much earlier (around 3–6 weeks postirradiation), and it is not clear if RIF is a consequence of radiation pneumonitis or it develops at least in part, irrespective of early inflammation caused by radiation-induced tissue damage. However, the process of RILI progression is characterized by dynamic changes in tissue and circulatory levels of sphingoid base-1-phosphates and their signaling counterparts, ceramides (Fig. 4; supplementary Table I) (12). Our focus in previous and in current studies was on the elements of S1P signaling system, which include SphK1, a major sphingosine kinase regulating the formation of S1P and DHS1P, the endogenous analog of S1P. When analyzing lung gene expression at the pneumonitis stage, we found increased expression of SphK1 and considered it to be a compensatory mechanism to overcome drastically reduced tissue S1P-DHS1P levels detected at this time point (12). In support of such a hypothesis, we demonstrated (12) a significant protection against radiation pneumonitis provided by S1PR1 ligand SEW2871 and a novel S1PR agonist (S)-FTY720-phosphonate (41). In the current study, we also found SphK1 upregulation at the level of transcription, translation, and enzymatic activity (Fig. 5; Supplementary Table II), but this time lung tissue and circulatory S1P-DHS1P levels were substantially increased (Fig. 4; supplementary Table I) and concurred with the development of pulmonary fibrosis and RIF stage inflammation. This may appear to contradict our earlier findings; however, radiation pneumonitis and RIF are time-separate events with no active fibrogenesis going on at the pneumonitis stage of RILI. In fact, current findings are well supported by the known profibrotic (36–38) and proliferative (40, 42, 43) role for the SphK1-S1P signaling axis, reflecting the increased remodeling, the hallmark of fibrogenesis, in RIF lungs.

A more detailed look at sphingolipid changes in the lung tissue at RIF stage revealed that SphK1 upregulation is accompanied by a preferred increase in DHS1P rather than S1P levels (Fig. 4). Simultaneously, tissue levels of dihydroceramides and dihydrosphingosine but not ceramides are increased. This prompted us to investigate the involvement of serine palmitoyltransferase (SPT) in the observed shift in sphingolipid homeostasis. SPT catalyzes the first step in the chain of reactions leading to the formation of all complex sphingolipids (Fig. 1), determines the rate of de novo sphingolipid biosynthesis (15), and may provide increased sphingoid base supply affecting dihydrosphingosine-dihydroceramide levels. Importantly for the current findings, which highlight a profibrotic role of SphK1 upregulation, SPT directly provides dihydrosphingosine, one of the substrates for SphK1 (22). In fact, we found increased expression of the SPT2 regulatory subunit in the lung tissue at RIF stage as confirmed by the analysis of its mRNA and protein levels (Fig. 7; supplementary Table II), with a resulting total increase in SPT activity (Fig. 7).

To test if the modulation of SPT activity affects sphingolipid levels and RIF development, we pharmacologically inhibited SPT in mice. Knocking down SPT is embryonically lethal (44), but pharmacological inhibition of SPT is possible through the use of myriocin, the antifungal antibiotic isolated from Myriococcus albomyces (30, 45). Regardless of a substantial interest in myriocin as the SPT inhibitor, its ability to negatively regulate sphingolipid biosynthesis and the consequence of such inhibition on S1P signaling system and radiation-induced injury has not been investigated. Our recent data clearly demonstrate the ability of a long-term myriocin application to delay RIF development and the accompanying RIF pulmonary inflammation (Fig. 2). Interestingly, the link between the inhibition of SPT and the delay in RIF progression seems to converge at SphK1, the enzyme that is known to be induced by TGF-β and is critical for fibroblast differentiation and fibrogenesis (36–38). We show here a substantial upregulation of SphK1 expression and activity and elevated levels of DHS1P and S1P in the lung tissue and plasma in RIF mice, with myriocin being able to decrease the observed RIF-associated changes in all those parameters. Out data do not suggest any role for SphK2 in the observed
modulation in S1P-DHS1P homeostasis because SphK2 expression was decreased with the onset of pulmonary fibrosis (Fig. 5; supplementary Table II). As to the possible connection between fibrogenesis and SphK1 expression/activity, RIF in mice was characterized by increased BAL level and lung tissue expression of TGF-β (supplementary Fig. 1; supplementary Table II), which provided us sufficient grounds to further investigate TGF-β-SphK1-SPT cross-talk in the in vitro system.

The in vitro modeling of fibrogenesis using normal human lung fibroblasts and TGF-β-induced fibroblast transdifferentiation with the upregulation of α-SMA expression as a marker of profibrotic transformations further supported the proposed link between SPT expression and activity and the SphK1 accessory role in TGF-β signaling. Similar to its effect in vivo, myriocin decreased TGF-β-mediated upregulation of α-SMA and SphK1 expression in the in vitro model (Fig. 8A). Mechanistically, the interference of myriocin with TGF-β signaling in vitro was SPT dependent and converged once again at SphK1 as the bypass of the inhibition of sphingolipid de novo biosynthesis by myriocin through medium supplementation with DHSph annulled myriocin effects on TGF-β-stimulated expression of α-SMA and SphK1 (Fig. 8B). Importantly, the downregulation of SPT2 expression by small interfering RNA had the same effect as the inhibition of SPT by myriocin, resulting in the inhibition of TGF-β-induced α-SMA and SphK1 expression (Fig. 9A). This confirms that the effect of myriocin is SPT-mediated and not determined by its possible off-target effects or by its cellular metabolites. Finally, our novel observation regarding the ability of TGF-β to upregulate SPT2 expression provides additional support to the existence of a regulatory relationship between the expression of SPT and SphK1 and brings a novel element, SPT, to the known SphK1-S1P signaling network as an important contributor to TGF-β-driven profibrotic transformations.

Our work does not provide an answer regarding the best timing to initiate SPT inhibition to warrant maximum protection from RIF. Taking into account the fact that RIF develops several months after irradiation and is preceded by radiation pneumonitis, the extent of which can be diminished through the enforcement of S1PR1-mediated signaling (12), targeting SPT at a particular time period postirradiation may be more beneficial than the inhibition of the enzyme over the entire period of RILI progression. This notion of selective time targeting extends into a seemingly opposite (beneficial during early pneumonitis phase [12] and potentially profibrotic at RIF phase) role for SphK1 throughout RILI progression. Regarding the effects of myriocin in long-lasting in vivo experiments, we cannot exclude a potential role for myriocin metabolites generated by biotransformation in vivo in delaying or preventing RIF. Such a possibility can be inferred from the fact that synthetic α-mannosyl ceramide analogs with myriocin instead of sphingosine as the backbone unit were shown to stimulate a particular Vα19 subset of NKT cells (46). In view of the important role played by the immune system in the development of RIF and RILI (9, 10, 47–53), investigation into myriocin biotransformations and the ability of myriocin derivatives to modulate the immune response may be of interest. However, regardless of any additional to SPT inhibition modalities of myriocin action, our studies clearly suggest that at least part of the myriocin interference with RIF development is SPT and SphK1 dependent. At the end, the normalizing effect of myriocin treatment is the most pronounced among the signaling pathways involved in the regulation of cell-cell interactions and in extracellular matrix production (Fig. 3), the pathways that are most associated with active fibrogenesis.

RIF-associated changes in sphingolipid homeostasis cover a wide range of molecules that are not limited to the currently explored S1P/ceramide metabolic network. Moreover, (DH)SIP/(DH)Sph/(DH)ceramide levels undergo differentially directed changes depending on the biological matrix analyzed, as exemplified by comparison of lipid dynamics in the lung tissue, plasma, and BAL. This precludes us from drawing a definite conclusion regarding which sphingolipid metabolic pathway prevails at RIF. Our studies also do not provide a direct answer of whether DHS1P and S1P fulfill the same (profibrotic) or opposite role in radiation-induced pulmonary fibrogenesis. Answering all these questions will require separate and comprehensive studies. However, our microarray analysis, regardless of its limitation (n = 3 per group), does not indicate that major sphingolipid biosynthetic pathways are substantially modulated at RIF stage (supplementary Table II), leaving enzymes and receptors of the S1P signaling pathway (Sphk1, Sgppl2, Sgppl1, and S1pr2,4,5) being the most and statistically significantly dysregulated. Still, care should be taken when interpreting the results of microarray and even qRT-PCR and protein expression analyses, as was the case with S1P lyase. Thus, microarray (supplementary Table II) and qRT-PCR (Fig. 6A) analyses of mRNA levels as well as S1P lyase protein expression analysis (Fig. 6B) clearly demonstrated the upregulation of S1P lyase at RIF stage; yet, the enzyme activity was unaffected (Fig. 6C). Due to the extreme sensitivity and versatility of our LC/MS/MS-based S1P lyase activity assay (27), we believe that S1P lyase becomes partially inactive at the stage of developed RIF that supports the upregulation of tissue and circulatory S1P-DHS1P levels (Fig. 4; supplementary Table I). It is unclear if this inhibition is determined by impaired protein processing, by posttranslational modification(s) such as phosphorylation or nitration, or by negative regulation by yet to be identified signaling pathway(s). There is no understanding of how the activity of S1P lyase is endogenously regulated, and our data provide the first indication for the existence of endogenous mechanisms affecting S1P lyase activity.

In summary, we provide evidence for the association of dysregulated sphingolipid de novo biosynthesis and the development of pulmonary RIF. Furthermore, we show that SphK1 emerges as an important element associated with radiation-induced pulmonary fibrogenesis. Given the known protective role of S1P-mediated signaling in the model of total lung radiation-induced pneumonitis (12),...
it becomes critical to identify criteria for a timely switch from the enforcement of SphK1-S1P/DHSL1-S1PR signaling network at early stages of RILI progression to its inhibition in order to prevent the development of RIF. The ability of myricin to defer radiation-induced SphK1 activation and RIF progression and to counteract signaling network at early stages of RILI progression to its enforcement of SphK1-S1P/DHS1P-S1PR signaling in breast cancer patients. J. Natl. Cancer Inst. 96: 1676–1681.

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Figure 1. Sphingosine lipids in pulmonary fibrosis.
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