Temsirolimus induces surfactant lipid accumulation and lung inflammation in mice

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Washino S, Ando H, Ushijima K, Hosohata K, Kumazaki M, Mato N, Sugiyama Y, Kobayashi Y, Fujimura A, Morita T. Temsirolimus induces surfactant lipid accumulation and lung inflammation in mice. Am J Physiol Lung Cell Mol Physiol 306: L1117–L1128, 2014. First published May 2, 2014; doi:10.1152/ajplung.00251.2013.—Interstitial lung disease (ILD) is a well-known adverse effect of mammalian target of rapamycin (mTOR) inhibitors. However, it remains unknown how lung toxicities are induced by mTOR inhibitors. Here, we constructed a mouse model of mTOR inhibitor-induced ILD using temsirolimus and examined the pathogenesis of the disease. Male ICR mice were treated with an intraperitoneal injection of different doses of temsirolimus (3 or 30 mg·kg−1·wk−1) or vehicle. Temsirolimus treatment increased capillary-alveolar permeability and induced neutrophil infiltration and fibrinous exudate into the alveolar space, indicating alveolar epithelial and/or endothelial injury. It also induced macrophage depletion and the accumulation of excessive surfactant phospholipids and cholesterol. Alveolar macrophage depletion is thought to cause surfactant lipid accumulation. To further examine whether temsirolimus has cytotoxic and/or cytostatic effects on alveolar macrophages and alveolar epithelial cells, we performed in vitro experiments. Temsirolimus inhibited cell proliferation and viability in both alveolar macrophage and alveolar epithelial cells. Temsirolimus treatment caused some signs of pulmonary inflammation, including upregulated expression of several proinflammatory cytokines in both bronchoalveolar lavage cells and lung homogenates, and an increase in lymphocytes in the bronchoalveolar lavage fluid. These findings indicate that temsirolimus has the potential to induce alveolar epithelial injury and to deplete alveolar macrophages followed by surfactant lipid accumulation, resulting in pulmonary inflammation. This is the first study to focus on the pathogenesis of mTOR inhibitor-induced ILD using an animal model.

Temsirolimus; mammalian target of rapamycin inhibitor; interstitial lung disease; inflammation; surfactant lipids

RAPAMYCIN/SIROLIMUS and its derivatives are immunosuppressor macrolides that block mammalian target of rapamycin (mTOR), a central regulator of intracellular signaling pathways involved in cell growth and proliferation, cellular metabolism, and angiogenesis (53). Rapamycin and everolimus are a class of drugs used for the prevention of solid organ transplant rejection (19). Temsirolimus and everolimus have been recently used for the treatment of various tumors, including renal cell carcinoma, breast cancer, pancreatic neuroendocrine tumors, and subependymal giant-cell astrocytomas and angiomylipoma in tuberculous sclerosis (5, 6, 21, 27, 36, 57). Although these drugs are generally well tolerated, they have been associated with an 8–14% incidence of interstitial lung disease (ILD) in solid organ transplant recipients with a wide spectrum of disease severity (9, 35, 52). Similar findings have been reported for the use of temsirolimus and everolimus in the treatment of cancer, although a higher incidence (13.5–36%) of clinically identified ILD has been detected (5, 15).

Clinicopathological patterns of mTOR inhibitor-induced ILD include interstitial pneumonitis with or without fibrosis, bronchiolitis obliterans organizing pneumonia (BOOP) (9, 40, 52), lymphocytic interstitial pneumonia (52), granulomatous ILD (10), and alveolar hemorrhage (40). T helper cell lymphocytosis has also been reported in bronchoalveolar lavage (BAL) findings (20, 40). An immunological mechanism is speculated to be involved based on findings of lung biopsies and BAL as well as the observed clinical response to corticosteroids (53). However, the mechanism(s) involved in mTOR inhibitor-induced lung toxicities remains to be determined. To the best of our knowledge, few animal or in vitro studies have evaluated the pathogenesis of mTOR inhibitor-induced ILD.

In the present study, we constructed a mouse model of mTOR inhibitor-induced ILD using temsirolimus and evaluated a potential mechanism of the adverse effects. Mice treated with temsirolimus exhibited signs of alveolar epithelial injury, and temsirolimus treatment caused alveolar macrophage depletion, resulting in lipid accumulation in the lungs.

MATERIALS AND METHODS

Drug and its preparation. Temsirolimus was kindly provided by Pfizer (New York, NY) and was suspended in 100% ethanol to a concentration of 30 mg/ml and stored at −20°C until use. The stock solution of temsirolimus was diluted to a final concentration of 0.1 or 1 mg/ml using a diluent of 5% Tween 80 and 5% polyethylene glycol 400.

Animals. Male ICR mice were obtained from Japan SLC (Hamamatsu, Japan) at 6 wk of age and were maintained under specific pathogen-free conditions with controlled temperature and humidity. They were given a standard laboratory diet and water ad libitum. Mice were allowed a 7-day acclimation period before experiments. All animal procedures were approved by the Use and Care of Experimental Animals Committee of Jichi Medical University (Tochigi, Japan).

Treatment. Temsirolimus (1 and 10 mg·kg−1·day−1 for the low- and high-dose groups, respectively) or vehicle was administered 3 days/wk for 2, 4 or 8 wk. The doses and dosing interval of temsirolimus were chosen based on previous studies (28, 56) that evaluated tumor growth inhibition and hematological toxicities of temsirolimus at doses of 3–100 mg·kg−1·day−1 in mice and the following unpublished company data (Pfizer): a single intravenous dose of 1 mg/kg temsirolimus in mice resulted in areas under the plasma concentra-
tion-time curves of temsirolimus (1,460 ng·h⁻¹·ml⁻¹) and sirolimus (2,470 ng·h⁻¹·ml⁻¹), which were equivalent to those (1,349 and 3,793 ng·h⁻¹·ml⁻¹, respectively) in patients repeatedly treated with the recommended dose (25 mg) of the drug. At the end of the dosing period, animals were anesthetized with pentobarbital (120 mg/kg ip) and euthanized by exsanguination, and samples were obtained.

**BAL**. BAL was performed five times with PBS in 1-ml aliquots. Cell count in the BAL fluid (BALF) was determined using a hemocytometer with trypan blue staining. Differential cell counts were determined using Diff-Quick staining (Sysmex, Kobe, Japan) on cytospin preparations. To detect intracellular neutral lipids, cytospin BALF cells were also stained with oil red O. Differential cell count and oil red O positivity were quantified by counting at least 200 cells on each slide. The concentration of total protein in the BALF supernatant was measured using BCA protein assay kits (Thermo Scientific Pierce, Yokohama, Japan).

**Lung permeability assay.** Permeability changes were measured by Evans blue dye (EBD) leakage from blood into airways. EBD (20 mg/kg, Sigma-Aldrich, St. Louis, MO) was administered by tail vein injection (25). One hour later, mice were anesthetized and euthanized by exsanguination, and the pulmonary vasculature was flushed by a right ventricular puncture. Pulmonary vessels were perfused with 3 ml PBS to remove EBD from vascular spaces. Lungs were removed en bloc and snap frozen in liquid nitrogen. Frozen lungs were homogenized in 2 ml PBS. The homogenate was diluted with 2 volumes of formamide and incubated at 70°C for 24 h followed by centrifugation at 5,000 g for 30 min. The supernatant was collected, and absorbance was measured at 620 and 740 nm in a dual-wave spectrometer. The following formula was used to correct optical density (E) for contamination with heme pigments:

\[
\text{Absorbance at } 620 \text{ nm (corrected)} = \frac{\text{Absorbance at } 620 \text{ nm}}{1 - (1.426 \times E_{254} + 0.030)}
\]

**Histological analysis.** After an intratracheal injection of 1.5 ml of 10% (vol/vol) neutral buffered formalin, the lungs were excised, postfixed for 24 h, and embedded in paraffin. Sections were cut into slices (5 μm thick), which were stained with hematoxylin and eosin solution. Alternatively, these sections were used for immunohistochemical analysis. The protocol for immunohistochemical analysis was obtained using Serotec Worksheets (www.abdserotec.com). In brief, 5-μm sections were deparaffinized and the median was changed. Cells were incubated for two rounds for 60 s each. Slides were counterstained with proteinase K (20 μg/ml) for 3 min at room temperature. Sections were blocked for endogenous peroxidase activity with 0.3% H₂O₂ in 70% methanol-PBS, avidin/biotin blocking solutions, and serum-free protein block (DAKO Japan, Tokyo, Japan) before incubation with the primary F4/80 antibody (1:50, Serotec, Raleigh, NC) overnight at room temperature in a humidified chamber. On the next day, after being washed three times in PBS containing 0.1% Tween 20, sections were incubated with biotinylated rabbit anti-rat IgG, mouse adsorbed (1:100, Vector, Burlingame, CA), for 30 min at room temperature. Standard avidin-biotin immunoperoxidase methods with 3,3′- diaminobenzidine were used for detection. Methyl green (3%) was used for counterstaining.

**Lipid extraction and analysis.** Extraction of total lipids from BAL cells and BALF was performed as previously described (2). In brief, total lipids were extracted from BAL cells and fluid in chloroform-methanol-1 M NaCl [2:1:1.25 (vol/vol/vol)]. The organic phase was obtained by centrifugation at 1,500 rpm. Lipids were dried under a gentle stream of nitrogen gas. Enzymatic assays for total cholesterol, phospholipids, and triglycerides were performed using kits purchased from Sysmex (Kobe, Japan), Wako (Osaka, Japan), and Shimizu Medical (Tokyo, Japan), respectively. Lipid contents were expressed as milligrams of lipid per milligram of protein in BAL cells or milligrams of lipid per milliliter of BALF.

**RNA extraction and real-time quantitative PCR.** Isolation of total RNA was achieved using the RNasy Mini Kit according to the manufacturer’s instructions (Qiagen, Valencia, CA). Reverse transcription was performed using the PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). Real-time quantitative PCR was performed using the Applied Biosystems StepOnePlus Real-Time PCR system and TaqMan Gene Expression Assays (Life Technologies, Carlsbad, CA). To control the variation in gene expression levels, the expression of target sequences was normalized to the expression of an endogenous control, GAPDH (Gpdh) or β-actin (Actb). GenBank accession numbers and assay identifications were as follows: colony stimulating factor (CSF)-1, NM_001113529.1 and Mm00432686_m1; chemokine (C-C motif) ligand 2 (Ccl2), NM_011333.3 and Mm00441242_m1; TNF-α, NM_013693.2 and Mm00443258_m1; IL-6, NM_031168.1 and Mm00446190_m1; CSF-2, NM_009966.4 and Mm01290062_m1; IL-1β, NM_008361.3 and Mm01336189_m1; GAPDH, NM_008084.2 and Mm09999915_g1; and β-actin, NM_031491.1 and Mm00607939_s1. Data were analyzed using the comparative threshold cycle method.

**ELISA.** Protein extraction from lungs was performed using Cell,Lytic MT (Sigma-Aldrich)—containing protease inhibitor following the manufacturer’s protocol. The cytokine concentrations in lung homogenates were measured by ELISA using a kit (R&D Systems, Minneapolis, MN).

**Perfusion of alveolar macrophages.** Alveolar macrophages were obtained by BAL from ICR mice as previously described (58). The purity of the macrophage populations on Diff-Quik-stained cytospin slides was >95%, and the cell viability as determined by trypan blue dye exclusion was >98%.

**In vitro temsirolimus exposure to macrophages and alveolar epithelial cells.** For in vitro exposure to drugs, isolated macrophages were suspended in DMEM (Life Technologies) containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin and were seeded on 48-well plates (2 × 10⁵ cells/well) for the dead cell assay and 4-well culture slides (4 × 10⁵ cells/well, BD Japan, Tokyo, Japan) for the TUNEL assay. After 2 h for cell attachment, cells were washed with PBS twice and cultured in the same medium overnight before incubation in the same medium with or without drugs for 24 h at 37°C in a 5% CO₂ humidified atmosphere. Human pulmonary alveolar epithelial cells obtained from ScienCell (Carlsbad, CA) were seeded to poly-L-lysine coated 48-well plates (1 × 10⁶ cells/well) in alveolar epithelial cell medium (ScienCell) containing 2% FBS, epithelial cell growth supplement, penicillin, and streptomycin and cultured overnight. After the medium was changed, Cells were incubated for an additional 3 days until the culture was ~70% confluent before incubation in the same medium with or without temsirolimus for the indicated durations.

**Cell proliferation assay.** After incubation in 100 μl medium with vehicle or temsirolimus for the indicated durations, cell proliferation assays were performed using cell proliferation WST-1 reagent (Roche, Basel, Switzerland) following the manufacturer’s protocol. In brief, 10 μl WST-1 reagent was added to the plate and incubated for 1 h at 37°C in a 5% CO₂ humidified atmosphere before the absorbance of the samples was measured at 450 and 650 nm. Cell proliferation was calculated as follows: absorbance at 450 nm − absorbance at 650 nm. Absorbance is directly correlated with cell number.

**Dead cell assay by laser scanning confocal microscopy.** After incubation with or without drugs in 48-well plates for 24 h, cells were stained with 8.6 μM propidium iodide (PI; BD Japan) and 10 μg/ml Hoechst 33342 (Dojindo, Kumamoto, Japan). Nuclei were visualized using an IX71 phase-contrast confocal laser scanning microscope (Olympus, Tokyo, Japan) with a WU excitation filter in Hoechst 33342 and a WIG excitation filter in PI. Dead and viable cells were identified by Hoechst 33342 (blue), whereas dead cells were identified...
by PI (red). Dead cells (in %) were quantified as follows: PI-positive cells/Hoechst 33342-positive cells × 100, with at least 200 cells in each well.

_TUNEL assay_. The TUNEL assay was performed using a commercially available kit (Promega, Tokyo, Japan) following the manufacturer’s protocol. In brief, four-well culture slides were fixed in 10% (vol/vol) neutral buffered formalin and rinsed in PBS. Cells were permeabilized in PBS containing 0.2% Triton X-100 solution for 5 min. After preincubation with rTdT buffer, slides were incubated with rTdT buffer containing rTdT- and FITC-labeled dUTP in a humid atmosphere at 37°C for 60 min. EDTA (10 mM in distilled H2O) was added to the slides for 15 min to stop the reaction. Slides were washed in PBS and counterstained with 1 μg/ml PI before they were washed again with distilled H2O. Nuclei were visualized using an IX71 phase-contrast confocal laser scanning microscope with an enhanced green fluorescent protein excitation filter in FITC and a WIG excitation filter in PI. TUNEL-positive cells (in %) were quantified as follows: TUNEL-positive cells/PI-positive cells × 100, with at least 200 cells on each slide.

**RESULTS**

_Temsirolimus induces histological changes in lungs_. The histological findings in mice treated with temsirolimus for 2 or 4 wk were similar. Compared with control mice, mice treated with temsirolimus exhibited some histological changes, including infiltration of inflammatory cells in the alveolar space and wall and focal accumulation of foamy alveolar macrophages in the alveolar space (Fig. 1A). These histological changes seemed to be more pronounced and accompanied with the...
appearance of fibrinous exudate into the alveolar space at 8 wk of treatment. However, the lung architecture was not altered by temsirolimus.

Temsirolimus increases capillary-alveolar permeability and inflammatory cells. Next, we examined the total protein concentration in BALF and the amount of EBD in lungs to assess the capillary-alveolar permeability in the lungs. As shown in Fig. 1, B and C, the variables significantly increased in mice treated with temsirolimus in a dose-dependent manner. We further investigated whether temsirolimus induced recruitment of inflammatory cells into the alveolar space. Diff-Quick staining of BAL cells revealed that the number of inflammatory cells, including neutrophils, lymphocytes, monocytes, and eosinophils, were elevated by temsirolimus in a dose-dependent manner. Increases in neutrophils and monocytes were predominantly detected at 2 wk of treatment, whereas lymphocytes in BALF were increased in a treatment duration-dependent manner (Fig. 1, D–F).

Temsirolimus induces lipid accumulation in alveolar macrophages and alveolar spaces. Diff-Quick staining of BAL cells also revealed that, compared with control mice (Fig. 1D), foamy alveolar macrophages were frequently observed in the temsirolimus-treated group (Fig. 1E). Treatment with temsirolimus increased oil red O-positive cells in a dose-dependent manner (Fig. 2, A–C), indicating that neutral lipids accumulated in alveolar macrophages. Intracellular and extracellular compositions of the lipids in the lungs were determined by measuring the levels in BAL cells and BALF, respectively (Fig. 2, D and E). Compared with control mice, both cellular and extracellular contents of cholesterol significantly increased.
Temsirolimus increases proinflammatory cytokine expression in BAL cells as well as in lungs. We evaluated the mRNA expression of proinflammatory cytokines in the lungs. In lung homogenates, mRNA expression of IL-6, CCL2/monocyte chemotactic protein (MCP)-1, granulocyte (G)-macrophage (M)-CSF, TNF-α, and IL-1β were upregulated at 4 and/or 8 wk of treatment with temsirolimus (Fig. 3A). We further evaluated protein levels of MCP-1, IL-1β, and IL-6 in lung homogenates and mRNA expressions of these cytokines in BAL cells. As shown in Fig. 3B, protein levels of MCP-1 and IL-1β in lungs
Table 1. Lipid composition in serum

<table>
<thead>
<tr>
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<th>Cholesterol, mg/dl</th>
<th>Triglyceride, mg/dl</th>
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<tbody>
<tr>
<td></td>
<td>Total</td>
<td>HDL</td>
</tr>
<tr>
<td>Vehicle</td>
<td>89.4 ± 11.6</td>
<td>68.2 ± 8.1</td>
</tr>
<tr>
<td>Low-dose temsirolimus</td>
<td>126.0 ± 15.4</td>
<td>96.7 ± 12.4</td>
</tr>
<tr>
<td>High-dose temsirolimus</td>
<td>115.2 ± 7.0</td>
<td>90.6 ± 9.3</td>
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Values are means ± SE; n = 4 mice. Shown is the lipid composition in serum of mice treated with vehicle or temsirolimus for 2 wk. Total, high-density lipoprotein (HDL), and non-HDL cholesterol levels did not change significantly among the groups, nor did total, HDL, or non-HDL triglyceride levels.

DISCUSSION

Alveolar epithelial and/or endothelial injury after infiltration of inflammatory cells into the alveolar interstitium and space is the first event in the pathogenesis of ILD, including interstitial pneumonitis and BOOP (12, 13, 38, 49). It is believed that the increase in capillary-alveolar permeability, resulting in the leakage of plasma proteins followed by fibrin formation within the alveolar lumen, is a sign of alveolar epithelial and/or endothelial injury (49, 12). We found an increase of fibrin concentrations and amounts of EBD in the lungs in temsirolimus-treated mice, indicating an increase in capillary-alveolar permeability (Fig. 1, B and C). Furthermore, the infiltration of inflammatory cells as well as fibrosin exudate within alveolar spaces in temsirolimus-treated mice were demonstrated by BAL cells analyses and histological findings (Fig. 1, A and F). These findings indicate that alveolar epithelial and/or endothelial injury are induced by temsirolimus. To further examine whether temsirolimus has cytotoxic and/or cytocstatic effects on alveolar epithelial cells, we cultured human pulmonary alveolar epithelial cells and A549 cells, a human alveolar epithelial cell line. Temsirolimus inhibited cell viability (>1 μM) and cell proliferation (>0.1 μM; Fig. 5, F and G). Previous in vitro studies using human umbilical vein endothelial cells and human pulmonary microvascular endothelial cells have demonstrated that endothelial cell viability and proliferation are blocked by temsirolimus or rapamycin at lower drug concentrations (0.1–100 nM) (4, 8, 47, 58). In vivo studies have also shown that rapamycin treatment augments lung injury induced by BLM or lipopolysaccharide in mice with acute lung injury (17, 28). Clinically, serum Krebs von den Lungen 6, which is a biomarker of interstitial lung disease and is associated with epithelial cell injury and capillary-alveolar permeability changes, is elevated in patients with everolimus-induced ILD (24, 34). Based on these data, it is speculated that mTOR inhibitors have the potential to cause alveolar epithelial injury, which could, in turn, initiate and/or augment lung injury (as shown in the left pathway in Fig. 6).

Surfactant abnormalities are problematic in many lung diseases, including acute respiratory distress syndrome, sarcoidosis, asthma, and pulmonary alveolar proteinosis (2, 7, 48).

Homogenates were increased in mice treated with temsirolimus in a dose-dependent manner. However, IL-6 protein levels were not different among the groups. In BAL cells, mRNA expression of several proinflammatory cytokines, such as IL-6, CCL2, GM-CSF, and M-CSF, appeared to be upregulated in mice treated with temsirolimus (Fig. 3C).

Temsirolimus does not induce hyperlipidemia in mice. Because mTOR inhibitors are responsible for the development of hyperlipidemia in humans, it might be possible that temsirolimus-induced lipid accumulation in the lungs was a consequence of hyperlipidemia. To address this possibility, we examined whether temsirolimus affected lipid compositions in serum and found that concentrations of total and non-high-density lipoprotein-cholesterol and triglycerides in the serum were not significantly increased by the agent (Table 1). These data indicate that temsirolimus-induced lipid accumulation in lungs might not be caused by hyperlipidemia, at least in this model.

Temsirolimus depletes alveolar macrophage. Dichloromethylene diphosphonic acid-induced alveolar macrophage depletion has been reported to be associated with an increased surfactant pool in rats (18). Because mTOR inhibitors are also known to deplete the macrophage-monocyte lineage (51, 32, 55), we next investigated whether temsirolimus depletes alveolar macrophage. As shown in Fig. 1F, temsirolimus reduced alveolar macrophage numbers in BALF. F4/80 immunostaining of lungs confirmed alveolar macrophage depletion by temsirolimus (Fig. 4, A–C).

Temsirolimus inhibits cell viability and proliferation in alveolar macrophage as well as alveolar epithelial cells in vitro. As shown in Fig. 5A, temsirolimus inhibited the proliferation of RAW264.7 cells, a macrophage cell line, at lower concentrations of the agent. In addition, the drug induced the death of cultured alveolar macrophages in a concentration-dependent manner (Fig. 5, B and C). Therefore, we further studied whether apoptosis was involved in the mechanism of cell death induced by temsirolimus. In contrast to a positive control, bleomycin (BLM) treatment, temsirolimus did not induce an obvious increase in TUNEL-positive cells (Fig. 5D).

Temsirolimus also inhibited cell viability and proliferation in alveolar epithelial cells (Fig. 5, E–G).

Fig. 3. Temsirolimus increases proinflammatory cytokine expression in lungs as well as BAL cells. A: total RNA from lungs was analyzed by real-time PCR. CCL2, chemokine (C-C motif) ligand 2; GM-CSF, granulocytic (G)-macrophage (M)-colony-stimulating factor (CSF). Data are means ± SE; n = 4 individual mice. Data were analyzed by two-way ANOVA followed by a Bonferroni multiple post hoc comparison test in lungs. *P < 0.05 and **P < 0.01 vs. vehicle. B: expressions of monocyte chemotactic protein (MCP)-1, IL-1β, and IL-6 in lungs from mice treated with vehicle or temsirolimus for 8 wk were determined by ELISA (n = 7 individual mice). Data were analyzed using the Kruskal-Wallis test followed by a Dunn’s multiple post hoc comparison test. *P < 0.05 vs. vehicle. C: total RNA from BAL cells pooled from three mice treated with vehicle or temsirolimus for 8 wk was analyzed by real-time PCR. Data are shown as the mean of two sets of pooled BAL cells.
Surfactant is produced by type II pneumocytes; two pathways have been described in the clearance of surfactant. Type II pneumocytes endocytose surfactant lipids and complexes and recycle them into new surfactant. Alveolar macrophages phagocytose and degrade surfactant and are considered to be the primary cells involved in the clearance and catabolism of surfactant (50).

In the present study, we demonstrated that temsirolimus treatment induced an accumulation of surfactant lipids (Fig. 2, A–E), which is considered to be a functional defect of lipid clearance. Because alveolar macrophages are the primary cells involved in the clearance of surfactant, their depletion may lead to increased surfactant. Indeed, dichloromethylene diphosphonic acid-induced alveolar macrophage depletion is associated with an increased surfactant pool in rats (18). Similarly, in the present study, temsirolimus treatment depleted alveolar macrophages (Figs. 1F and 4, A–C), which might lead to an accumulation of surfactant lipids (Fig. 2, A–E). There potentially can be different additional mechanisms for surfactant lipid accumulation. One possibility is that alveolar macrophages have acquired a functional defect in lipid processing such as uptake or reverse transport. However, in the present study, gene expressions of lipid uptake (scavenger receptor cluster of differentiation 36 and scavenger receptor class A) and reverse transport (ATP-binding cassette subfamily A member 1, ATP-binding cassette subfamily G member 1, and apolipoprotein E) in BAL cells were generally upregulated or unchanged by temsirolimus (data not shown), although BAL cells contained not only alveolar macrophages but also other cells. The other possibility is impaired lipid uptake/degradation by type II pneumocytes. However, we speculate that temsirolimus has the potential to deplete macrophages and that alveolar macrophage depletion may contribute to the accumulation of pulmonary surfactant lipids (as shown in the right pathway in Fig. 6).

We found that temsirolimus treatment depleted alveolar macrophages in vivo and that temsirolimus inhibited macrophage proliferation at lower drug concentrations (>0.1 nM), whereas it induced cell death in primary cultured alveolar macrophages at higher concentrations (>3 μM) in vitro (Fig. 5, A–C). A previous study (54) has shown that temsirolimus treatment decreased the number of macrophages in alveolar epithelial neoplasia in K-ras^K14 mice. Several clinical case reports of everolimus-induced lung injury also present macrophage depletion in BAL cell analyses (43, 44). Furthermore, mTOR inhibitors have been shown to induce leukopenia in clinical and animal studies (21, 36, 56). Other in vitro studies have also demonstrated that rapamycin inhibits the proliferation of monocytes/macrophages and that mTOR inhibitors induce cell death through autophagy and apoptosis in the macrophage-monocyte lineage (11, 32, 51, 55); we obtained similar results. In the present study, temsirolimus induced cell death, whereas it induced only a slight increase in TUNEL-positive cells, in contrast to BLM (Fig. 5D), which is considered to be more compatible with necrosis rather than apoptosis.

Foamy, lipid-laden, alveolar macrophages are found in the lungs of patients with various lung diseases, such as BOOP, granulomatous ILD, and lipid pneumonia (31, 37, 46). Foamy macrophages are also detected in drug-induced ILD, including mTOR inhibitor-induced ILD (16, 23, 39). Although it is unclear whether foamy macrophage formation is involved in the pathogenesis of these lung diseases, there is strong evidence that dysregulation of lipid metabolism contributes to both foamy macrophage formation and pulmonary inflammatory responses in animal studies (3, 30, 45). Alveolar endothelial and/or epithelial injury also contribute to an inflammatory state in the lung (12). Because we found that temsirolimus caused not only foamy macrophage formation and lipid accumulation in lungs but also signs of epithelial injury, we evaluated the expression of proinflammatory cytokines in lungs and BAL cells. In both lungs and BAL cells, the expression of several proinflammatory cytokines was upregulated in mice treated with temsirolimus (Fig. 3, A–C). Furthermore, the number of lymphocytes in BALF increased in mice treated with temsirolimus (Fig. 1F). The lung inflammation seemed to be pronounced with long-term treatment (Figs. 1, A and F, and 3A). It is speculated that temsirolimus treatment induced pulmonary inflammation through excessive surfactant lipid accumulation and alveolar epithelial injury.

A previous study (42) has demonstrated that mTOR inhibitors have diverse effects on inflammatory response. mTOR controls cell progression from the G1 to S phase in cytokine-
stimulated T cells. Hence, mTOR inhibitors potently decrease the proliferation of CD4$^+$ T lymphocytes, which is an immunosuppressive property. In addition, mTOR inhibitors have been shown to decrease IL-6 and other cytokine/chemokine expressions and to inhibit cell migration stimulated by MCP-1 (1, 14). On the other hand, mTOR inhibitors also have proinflammatory properties. Inhibition of mTOR enhances IL-12 and IL-23 expressions in human and murine macrophages upon

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Fig. 5. Temsirolimus inhibits cell viability and proliferation in both alveolar macrophages and alveolar epithelial cells. A: results of the cell proliferation assay in RAW264.7 cells treated with different doses of temsirolimus or vehicle for 48 h. Data are mean percentages of control from two independent experiments; $n = 12$. Data were analyzed by one-way ANOVA with a Dunnett’s post hoc comparison test. *$P < 0.001$ vs. vehicle. B: representative images of alveolar macrophages from ICR mice were subjected to treatment with vehicle, temsirolimus, or 500 $\mu$g/ml bleomycin (BLM) for 24 h, stained with propidium iodide (PI) and Hoechst 33342 dye, and examined by confocal microscopy. Scale bars = 50 $\mu$m. C: data are mean percentages of PI-positive cells from three independent experiments; $n = 6$. D: alveolar macrophages were subjected to treatment with vehicle, 10 $\mu$M temsirolimus, or 500 $\mu$g/ml BLM for 24 h, analyzed using the TUNEL assay, and examined by confocal microscopy. Data are mean percentages of TUNEL-positive cells from three independent experiments; $n = 6$. E: representative images of human pulmonary alveolar epithelial cells subjected to treatment with vehicle, temsirolimus, or 100 $\mu$g/ml BLM, stained with PI and Hoechst 33342 dye, and examined by confocal microscopy. Scale bars = 100 $\mu$m. F: data are mean percentages of PI-positive cells from two independent experiments; $n = 6$. Data were analyzed using the Kruskal-Wallis test followed by a Dunn’s multiple post hoc comparison test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. vehicle. G: results of the cell proliferation assay in A549 cells treated with different doses of temsirolimus or vehicle for 24 h. Data are mean percentages of control from two independent experiments; $n = 12$. Data were analyzed by one-way ANOVA with a Dunnett’s post hoc comparison test. *$P < 0.01$ vs. vehicle.
stimulation with lipopolysaccharide and *Mycobacterium tuberculosis* (42). Moreover, in other studies, rapamycin potentiated thrombin-induced ICAM-1 expression by accelerating and stabilizing NF-κB activation in endothelial cells, which, in turn, induced leukocyte adherence followed by transendothelial migration (33). Based on these findings, temsirolimus might have complicated effects on the inflammatory response in the model of this study.

Alveolar macrophages play a central role in initiating and orchestrating the immediate pulmonary host defense against invading pathogens. However, evidence for the equally important regulatory role of alveolar macrophages in the resolution process has been derived from in vitro and in vivo studies (22, 26, 41). One such study (26) reported the consequences of inadequate alveolar macrophage function in a solution of experimental pneumococcal pneumonia. Compared with control mice, macrophage-depleted animals not only had higher concentrations of intrapulmonary cytokines and greater numbers of activated neutrophils but also greater numbers of apoptotic and dead neutrophils. The lungs of alveolar macrophage-depleted mice showed focal areas of parenchymal destruction, which were absent in the control group. Therefore, alveolar macrophage depletion and inadequate alveolar macrophage function may contribute not only to an increase in pulmonary surfactants but also to the resolution of inflammatory states in the lungs.

Our study has some limitations. First, in temsirolimus-treated mice, the histology did not exhibit organized pneumonia or granuloma, which is found in human patients with angiography.
mTOR inhibitor-induced ILD. In addition, inflammatory cell infiltration in temsirolimus-treated mice was less severe than that reported in patients with mTOR inhibitor-induced ILD. Thus, modest inflammation was induced by temsirolimus in mice compared with human patients. This might be due to the difference in species or the difference in basal inflammatory states between cancerous or transplant patients and normal mice. Next, the present study was done in one mouse strain, so it is possible that the temsirolimus phenotype is strain related. However, we did a preliminary study to evaluate lung histology in C57BL/6 mice treated with temsirolimus in the same treatment schedule for 8 wk, and the histological findings in C57BL/6 mice were similar as those in ICR mice (Fig. 7).

In conclusion, temsirolimus treatment caused signs of alveolar epithelial injury and macrophage depletion in mice. Alveolar epithelial injury resulted in increased capillary alveolar permeability and neutrophil infiltration, and alveolar macrophage depletion resulted in lipid accumulation in the lungs. Lung inflammation might be induced through mechanisms such as those shown in Fig. 6. Thus, the present study provides new insights into the pathogenesis of mTOR inhibitor-induced ILD. However, further studies are needed to confirm the usefulness of this animal model.

**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: S.W. conceived the project, performed experiments, analyzed data; S.W. drafted manuscript; H.A. and A.F. edited and revised manuscript, interpreted results of experiments; S.W. prepared figures; S.W. and A.F. interpreted results of experiments; S.W. wrote the paper.

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