Ablation of Pericyte-Like Cells in Lungs by Oropharyngeal Aspiration of Diphtheria Toxin

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Abstract

We demonstrated previously that FoxD1-derived cells in the lung are enriched in pericyte-like cells in mouse lung. These cells express the common pericyte markers and are located adjacent to endothelial cells. In this study, we demonstrate the feasibility of administering diphtheria toxin (DT) by oropharyngeal aspiration as an approach to ablating FoxD1-derived cells. We crossed mice expressing Cre-recombinase under the FoxD1 promoter to Rosa26-loxP-STOP-loxP-iDTR mice and generated a bitransgenic line (FoxD1-Cre; Rs26-iDTR) in which FoxD1-derived cells heritably express simian or human diphtheria toxin receptor and are sensitive to DT. We delivered low-dose (0.5 ng/g) and high-dose (1 ng/g $\times$ 2) to FoxD1-Cre;Rs26-iDTR mice and littermate control mice by oropharyngeal aspiration and evaluated ablation by flow cytometry and immunohistochemistry. FoxD1-Cre mice showed a 40–50% reduction in PDGFR β ⁺ cells by flow cytometry at Days 2 and 7 after DT administration, with a return of PDGFR β ⁺ cells at Day 28. Confocal microscopy revealed an observable reduction in pericyte markers. Bronchoalveolar lavage fluid analysis revealed no significant differences in total protein, bronchoalveolar

lavage fluid red blood cell, or white blood cell counts at low dose. However, at high-dose DT, there was a proinflammatory effect in the control mice and increased mortality associated with systemic toxicity in Cre⁺ mice. Low-dose DT reduced lung PDGFR β ⁺ stromal cells in the FoxD1-Cre;iDTR transgenic model without a differential effect on lung inflammation in DT-sensitive and DT-insensitive animals. Low-dose DT is a viable method for transient lineage-specific stromal cell ablation in the lung that minimizes systemic toxicity.

Keywords: pericytes; platelet-derived growth factor receptor β ; ablation; diphtheria toxin; FoxD1

Clinical Relevance

The ability to specifically ablate pericyte-like cells in the lungs will enable future scientific work that examines the function of pericyte-like cells in the lung, both in homeostasis as well as in response to injurious stimuli.

Pericytes are stromal cells of mesenchymal origin that surround endothelial cells in the microvasculature and share a common basement membrane with the endothelium (1, 2). During vasculogenesis, pericytes play a critical role in the maturation of developing vessels and the stabilization of the endothelium (3, 4). More recently, studies have revealed additional biological

roles of pericytes in leukocyte trafficking during acute inflammation and in wound healing (5–9), although their roles in different organs may be unique. There is scant literature on the biological roles of stromal cell subpopulations in the lung. Elegant genetic lineage tracing by Rock and colleagues demonstrated that multiple stromal populations contribute to fibrosis

after lung injury (10). In our own transgenic mouse model work, we found that cells originating from progenitors transiently expressing the forkhead transcription factor FoxD1 during embryonic development (FoxD1-derived) are enriched for markers associated with pericytes (PDGFR β , NG2, and CD146). These cells are adjacent to endothelial

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cells by histology, suggesting their role as pericyte-like cells (11). Importantly, they contribute to approximately one-half the population of α -smooth muscle actin-positive myofibroblasts in the bleomycin model of lung fibrosis (11). However, the functional biology of pericytes in lung homeostasis, injury, and healing remains unknown.

Recently, several studies tested the functional biology of pericytes in organ injury and healing by ablating pericytes after injurious stimuli (8, 9). The most common method of pericyte ablation involves the generation of transgenic mice with pericyte-restricted expression of simian or human diphtheria toxin receptor (iDTR), in which only cells expressing the receptor are sensitive to diphtheria toxin (DT) exposure. Conventionally, DT is administered systemically by intraperitoneal injections (9, 12, 13). However, this method of ablation, although effective, results in high mortality when used for pericyte ablation, suggesting that pericytes have important homeostatic functions (13). With the growing sophistication in our understanding of lung stromal subpopulations and their unique roles in lung injury and repair, refinement in methods for selective ablation of stromal subpopulations is desperately needed. Mammalian lungs interact continuously with the external environment and can be accessed with relative ease in mouse models. In this study, we leveraged this unique property of the lungs to test oropharyngeal administration of DT as a practical alternative to systemic DT delivery.

Materials and Methods

Mice and Reagents

Transgenic mice expressing Cre-recombinase under the control of FoxD1 promoter were crossed with Rosa26-loxP-STOP-loxP-iDTR mice (Rs26-iDTR) to generate transgenic mice (FoxD1-Cre;Rs26-iDTR) in which FoxD1-derived cells heritably express iDTR and are sensitive to DT. We also generated triple transgenic FoxD1-Cre;Rs26-iDTR;Rs26-tdTomato mice through breeding, in which FoxD1-derived cells coexpress iDTR and tdTomato reporter. Control mice were Rs26-iDTR littermates without Cre-recombinase transgene. Animal protocol was approved

by the Institutional Animal Care and Use Committee at the University of Washington, Seattle. The following antibodies were used: rat antimouse CD16/CD32 (Mouse BD Fc Block, BD Pharmingen 553141, San Jose, CA); rabbit anti-PDGFR β monoclonal antibody (Cell Signaling, C82A3; Danvers, MA); rabbit anti-NG2 (EMD Millipore AB5320, Billerica, MA); AlexaFluor 647-conjugated goat antirabbit IgG (H + L) (Jackson ImmunoResearch, 111-605-144; West Grove, PA), fluorescein isothiocyanate (FITC)-conjugated rat anti-CD45 (eBioscience clone 30-F11); FITC-conjugated rat anti-CD31 (BD Pharmingen clone MEC 13.3); and FITC-conjugated rat anti-CD326 (eBioscience clone G8.8, San Diego, CA). AbD Serotec (Raleigh, NC) Reagent A and B (Catalog No. BUF09) were used for fixation and permeabilization of lung single cells for flow cytometry experiments. DT (Sigma-Aldrich D0564, St. Louis, MO) was diluted to 0.25 ng/ μ l in sterile Dulbecco's phosphate-buffered saline for oropharyngeal administration and was delivered at a dose of 0.5 ng/g $\times$ 1 (low dose) or 1 ng/g $\times$ 2 (high dose).

Oropharyngeal Aspiration of DT

FoxD1-Cre;Rs26-iDTR mice and littermate control mice between the ages of 5 and 8 weeks were administered DT via oropharyngeal aspiration. Briefly, the mice were anesthetized under isoflurane and suspended by their front incisors on a vertical board. The tongues were gently retracted away with forceps, and DT (0.5 ng/g or 1 ng/g, volume ranging from 20 to 50 μ l) was pipetted into the oropharyngeal space. With spontaneous respirations, the anesthetized mice were allowed to aspirate the contents. The mice were returned to their cages before awakening from anesthesia. The mice were killed at 2, 7, or 28 days after DT administration.

Evaluation of Vascular Permeability

In some animal experiments, we administered Evans blue dye (EBD) (10 mg/kg body weight) to the mice by retroorbital injection 90 minutes before they were killed and the organs harvested. At harvest, we collected blood samples, followed by perfusion of the right and left ventricles with ice-cold phosphate-buffered saline (PBS). Solid organs including the brain, lungs, liver, and kidney were then collected, air dried at 60°C for 72 hours,

and weighed. EBD from dried organs was extracted in 500 μ l of formamide (Sigma) by incubating at 60°C for 24 hours and was measured against a standard curve by spectrophotometry (620-nm absorbance) in a 96-well microplate reader (Biotek, Winooski, VT). Total measured EBD (nanograms) was normalized to dry tissue weight (milligrams).

Sample Collection

Bronchoalveolar lavage fluid (BALF) was obtained by lavaging whole lungs with 1 ml of PBS 0.6 mM ethylenediaminetetraacetic acid. The right lungs were harvested for single-cell preparation as described previously (11), and the left lungs were inflated with 4% paraformaldehyde in PBS at 25 cm H₂O pressure at 4°C for 90 minutes, followed by immersion in 18% sucrose in PBS overnight at 4°C, and cryopreserved in TissueTek Optimum Cutting Temperature Compound (VWR, Radnor, PA). Total white blood cell (WBC) count, total red blood cell (RBC) count, and total protein were measured in the BALF collected. Total WBC count was measured using the Nexcelom Cellometer 2000. WBC differential on the BALF was counted manually on a cytospin preparation by counting 200 nucleated cells per sample. RBCs were counted manually with a hemocytometer.

Flow Cytometry

We demonstrated previously that the vast majority of FoxD1-derived stromal cells were PDGFR β ⁺. Thus, we used PDGFR β labeling as a surrogate marker to assess FoxD1-derived stromal cell depletion in the lungs. For flow cytometry, single-cell suspension from right lung digests was prepared as described (11). 1×10^6 cells were resuspended in 100 μ l of flow cytometry buffer (PBS without Ca²⁺ and Mg²⁺ containing 2 mM ethylenediaminetetraacetic acid and 0.5% body surface area). Samples were incubated with purified rat anti-mouse CD16/32 (BD Biosciences, San Jose, CA) (1 μ g per 1×10^6 cells) at 4°C for 15 minutes and then stained with phycoerythrin-conjugated rat anti-CD31/45/326 at 4°C for 30 minutes. After washing, the cells were fixed with AbD Serotec reagent A at room temperature for 15 minutes, followed by permeabilization using AbD Serotec reagent B at room temperature for 30 minutes. Samples were stained for

PDGFR β during the permeabilization step per manufacturer instructions, followed by washing and secondary antibody labeling with AlexaFluor 647-conjugated goat antirabbit IgG at room temperature for 30 minutes. Flow cytometry was performed using the BD FACS Canto II 6-color analyzer with 488-nm and 647-nm lasers at the Cell Analysis Core at the University of Washington. The PDGFR β ⁺ stromal population was defined as CD31⁺, CD45⁺, CD326⁺, PDGFR β ⁺ and was reported as a percentage of total stromal cells. Flow cytometry data were analyzed using FlowJo (v 8.7; Ashland, OR).

Immunofluorescent Microscopy

Cryopreserved lung sections were permeabilized using 0.1% TritonX-100 in PBS for 5 minutes at room temperature, followed by blocking with 5% goat serum in PBS for 30 minutes at room temperature. Sections were then incubated with anti-PDGFR β antibody in blocking solution (1:100) overnight at 4°C, followed by incubation with fluorophore-conjugated goat antirabbit IgG antibody in blocking serum (1:400) for 1 hour at room temperature. After being washed, the sections were mounted in Vectashield with 4',6-diamidino-2-phenylindole (DAPI). Immunostained sections were visualized and images were captured with the Nikon A1R confocal microscope at the Institute for Stem Cell and Regenerative Medicine at the University of Washington, Lynn and Mike Garvey Cell Imaging Lab.

Results

BALF Analysis at Days 2 and 7 Using Low-Dose DT

We initially attempted pericyte ablation through conventional intraperitoneal injection of DT (with doses as low as 5 ng/g) in our transgenic model. However, we observed neurologic sequelae and mortality in 100% of the FoxD1-Cre;Rs26-iDTR animals (abbreviated as Cre⁺ DT for simplicity) compared with their littermate control mice (abbreviated as WT DT for simplicity) 48–72 hours after DT administration (data not shown). Given the mortality associated with the conventional method using this transgenic model, we evaluated the efficacy of low-dose DT (0.5 ng/g) by oropharyngeal aspiration. At Day 2 after DT administration, Cre⁺ DT

Figure 1. (A) BALF collected at Day 2 after DT administration in WT DT and Cre⁺ DT animals. BALF WBC in the WT DT group was $7.9 \pm 1.6 \times 10^4/\text{ml}$ versus $13.1 \pm 2.4 \times 10^4/\text{ml}$ in the Cre⁺ DT group (mean $\pm$ SEM, $P=0.1$, $n \geq 8$). BALF RBC in the WT DT group was $3.9 \pm 1.7 \times 10^4/\text{ml}$ versus $55.4 \pm 59.9 \times 10^4/\text{ml}$ in the Cre⁺ DT group (mean $\pm$ SEM, $P=0.37$, $n \geq 8$). BALF total protein in the WT DT group was $173.8 \pm 10.1 \mu\text{g}/\text{ml}$ versus $193.4 \pm 36.9 \mu\text{g}/\text{ml}$ in the Cre⁺ DT group (mean $\pm$ SEM, $P=0.68$, $n \geq 6$). Cell differential of BALF showed predominantly macrophages ($80.4 \pm 3.4\%$ WT DT versus $78.3 \pm 6.2\%$ Cre⁺ DT) with slight neutrophilia ($14.8 \pm 3.9\%$ WT DT versus $20.7 \pm 6.3\%$) (mean $\pm$ SEM, $n \geq 6$). (B) At Day 7 after DT administration, BALF WBC in the WT DT group was $16 \pm 3.2 \times 10^4/\text{ml}$ versus $16 \pm 1.9 \times 10^4/\text{ml}$ in the Cre⁺ DT group (mean $\pm$ SEM, $P=0.998$, $n \geq 6$). BALF RBC in the WT DT group was $1.5 \pm 0.5 \times 10^4/\text{ml}$ versus $36.3 \pm 21.7 \times 10^4/\text{ml}$ in the Cre⁺ DT group (mean $\pm$ SEM, $P=0.08$, $n \geq 6$). No difference was observed in BALF total protein between the two groups ($352.3 \pm 55.1 \mu\text{g}/\text{ml}$ WT DT versus $401.8 \pm 33.8 \mu\text{g}/\text{ml}$ Cre⁺ DT, $P=0.49$, $n \geq 5$). Cell differential of BALF at Day 7 showed predominantly macrophages ($95.4 \pm 1.1\%$ WT DT versus $91.7 \pm 4.3\%$ Cre⁺ DT) without significant difference in cell differential (mean $\pm$ SEM, $n \geq 6$). BALF, bronchoalveolar lavage fluid; Cre⁺ DT, FoxD1-Cre;Rs26-iDTR; DT, diphtheria toxin; Lymph, lymphocytes; Mac, macrophages; PMN, polymorphonuclear cells; RBC, red blood cells; WBC, white blood cells; WT DT, control.

mice showed no significant differences in BALF WBC counts ($13.1 \pm 2.4 \times 10^4/\text{ml}$ versus $7.9 \pm 1.6 \times 10^4/\text{ml}$, $P=0.1$, $n \geq 8$), RBC counts ($55.4 \pm 59.9 \times 10^4/\text{ml}$ versus $3.9 \pm 1.7 \times 10^4/\text{ml}$, $P=0.37$, $n \geq 8$), total protein concentrations ($193.4 \pm 36.9 \mu\text{g}/\text{ml}$ versus $173.8 \pm 10.1 \mu\text{g}/\text{ml}$, $P=0.68$, $n \geq 6$), or cell differential, compared with WT DT (Figure 1A). At Day 7 after DT administration, Cre⁺ DT mice showed no significant difference in BALF WBC count ($16 \pm 1.9 \times 10^4/\text{ml}$ versus $16 \pm 3.2 \times 10^4/\text{ml}$, $P=0.998$, $n \geq 6$), BALF RBC counts ($36.3 \pm 21.7 \times 10^4/\text{ml}$ versus $1.5 \pm 0.5 \times 10^4/\text{ml}$, $P=0.08$, $n \geq 6$), total protein concentrations ($401.8 \pm 33.8 \mu\text{g}/\text{ml}$ versus $352.3 \pm 55.1 \mu\text{g}/\text{ml}$, $P=0.49$, $n \geq 5$), or cell differential, compared with WT DT (Figure 1B).

Assessment of Ablation at Days 2 and 7

At Day 2 after DT administration, the percentage of PDGFR β ⁺ stromal population in the Cre⁺ DT group was decreased compared with that in WT DT ($44 \pm 6\%$ versus $24 \pm 3\%$). At Day 7 after DT administration, there continued to be a decrease in PDGFR β ⁺ population in lung digests of Cre⁺ DT mice compared with WT DT ($36 \pm 2\%$ versus $22 \pm 3\%$) (Figures 2A and 2B). Histological examination of lung sections revealed patchy areas of decreased PDGFR β and NG2 immunostaining in Cre⁺ DT mice compared with control mice at Day 7 (Figure 2C). The heterogeneity observed in PDGFR β and NG2 immunostaining suggests that the aspiration method results in patchy DT exposure of sensitive FoxD1-lineage cells in the lung, an inherent weakness in this model.

High-Dose DT

Given the incomplete ablation in a single aspiration model, we escalated the dosing regimen by delivering 1 ng/g of DT on two consecutive days, aiming to decrease the patchy exposure of DT with a single aspiration. We harvested mice at 7 days and 28 days after the last DT exposure to assess BALF and the PDGFR β ⁺ stromal cell population. At Day 7, we observed a significant difference in BALF WBC and total protein between Cre⁺ DT and WT DT animals (Figure 3A). High-dose DT elicited an inflammatory BALF profile showing elevated WBC in WT DT versus Cre⁺

DT animals ($43.5 \pm 4 \times 10^4/\text{ml}$ versus $18.1 \pm 2 \times 10^4/\text{ml}$, respectively) and elevated total protein ($664.5 \pm 56.1 \mu\text{g}/\text{ml}$ versus $254.2 \pm 16.4 \mu\text{g}/\text{ml}$, respectively) (Figure 3A). We observed a similar decline in PDGFR β ⁺ stromal cells ($\sim 40\%$) using the high-dose regimen compared with the low-dose regimen ($0.5\text{ng}/\text{g} \times 1$) (Figure 3A). Importantly, we observed a 25% mortality in the Cre⁺ DT group, in which animals displayed neurologic effects including what appeared to be myoclonic jerks and seizures (Figure 3A). At 28 days, the inflammatory BALF profile observed previously at Day 7 in WT DT animals resolved, but Cre⁺ DT animals continued to experience mortality (Figure 3B). Interestingly, we observed no difference in PDGFR β ⁺ stromal cells at 28 days, suggesting that the depletion observed at Day 7 was transient and that the ablated cells had repopulated. Repopulation via self-renewal and/or differentiation from another progenitor population may be responsible for this observation.

Assessment of Vascular Permeability

Mice exposed to high-dose DT displayed mortality associated with clinical signs of seizures and myoclonic jerks. Because similar effects were also observed in 100% of FoxD1-Cre;Rs26-iDTR mice given DT by intraperitoneal injections, we speculated that the mortality might be a consequence of systemic absorption. Specifically, pericytes are important regulators of vascular permeability in the brain. Systemic exposure to DT in our transgenic mice might have altered vascular permeability in the brain. To test this, we examined vascular permeability by EBD extravasation in mice exposed to the high-dose DT scheme. We harvested the mice 48 hours after DT challenge to capture all mice before the onset of mortality. WT DT and Cre⁺ DT animals showed no significant differences in EBD extravasation in the lungs, liver, or kidneys (Figure 3C). However, there was a significant increase in EBD extravasation in the brain of Cre⁺ DT mice, lending support to our speculation that high-dose DT leads to systemic absorption and subsequent central nervous system side effects (Figure 3C).

Triple Transgenic Model

Expression of PDGFR β in the lungs may be dynamic in response to injury and repair. Thus, using PDGFR β as a surrogate marker

to assess ablation indirectly, while convenient, may not accurately reflect ablation of FoxD1-lineage cells. For a direct assessment, we generated the triple transgenic line FoxD1-Cre;Rs26-tdTomato;Rs26-iDTR (Figure 4), in which FoxD1-derived cells permanently and heritably coexpress the fluorescent reporter tdTomato and iDTR. Because we observed unacceptably high mortality with the high-dose DT scheme, we lowered the dosage to $0.5 \text{ ng}/\text{g} \times 2$ in this model. We assessed ablation at Day 7 by comparing the tdTomato⁺ stromal population in triple transgenic mice given DT and the triple transgenics given PBS. Consistent with our previous findings by PDGFR β ⁺ flow cytometry, we observed a 30–40% reduction in tdTomato⁺ stromal cells (Figure 4). Although we did not observe any mortality at this dosage, we did observe a modest increase in BALF WBC, as well as increased total protein in mice given DT compared with PBS, suggesting that the double dose at $0.5 \text{ ng}/\text{g}$ still leads to some degree of lung inflammation (Figure 4).

Discussion

As appreciation for stromal subpopulations in lung biology deepens, the methods used to examine their functional roles will be ever more important. One approach to detailing the functional biology of lung stromal populations is through lineage-specific ablation of a defined subpopulation of lung stromal cells in iDTR transgenic mice. Conventional approaches to DT ablation studies involve systemic administration of DT by intraperitoneal injection. However, the toxicity of systemic pericyte ablation limits the utility of this approach. Although conditional ablation using a lung-specific stromal cell promoter would be of great value in lung stromal cell biology, such a promoter has yet to be identified. Therefore, work on alternative methods to achieve lung-restricted ablation of stromal populations will be needed. In this study, we examined a method for FoxD1-lineage stromal cell ablation by oropharyngeal aspiration of DT and demonstrated ablation of FoxD1-lineage cells.

Exposure of DT at a $0.5 \text{ ng}/\text{g}$ dose resulted in $\sim 40\%$ decrease in PDGFR β ⁺ cells at Days 2 and 7. There was no significant difference in BAL RBC, WBC, or

Figure 2. (A) Gating of PDGFR β ⁺ stromal populations in lung single-cell preparations from WT DT and Cre⁺ DT groups harvested on Day 2. Graph represents PDGFR β ⁺ cells as a percentage of stromal cells in WT DT and Cre⁺ DT groups at Day 2 ($44 \pm 6\%$ versus $24 \pm 3\%$, respectively, mean $\pm$ SEM, $P=0.02$, $n \geq 4$). (B) Gating of PDGFR β ⁺ stromal population at Day 7 after DT administration. Graph represents PDGFR β ⁺ cells as a percentage of stromal cells in WT DT and Cre⁺ DT groups at Day 7 ($36 \pm 2\%$ versus $22 \pm 3\%$, respectively, mean $\pm$ SEM, $P=0.003$, $n \geq 5$). (C) Immunostaining for PDGFR β ⁺ cells (green) and NG2 (purple) in lung sections from WT DT (top two panels) and Cre⁺ DT (bottom two panels) mice at Day 7 (nuclei represented by blue 4',6-diamidino-2-phenylindole staining). Cre⁺ DT mice display patchy PDGFR β and NG2 immunostaining. (Inset) Magnified views of normal PDGFR β and NG2 staining in several alveoli from WT mice in contrast to areas with diminished immunostaining in Cre⁺ DT mice. NG2, neural/glial antigen 2; PDGFR β , platelet-derived growth factor receptor- β .

Figure 3. Characterization of high-dose DT. (A) BALF analysis at Day 7 after DT exposure. BALF WBC in the WT DT group was $18.2 \pm 3.8 \times 10^4$ /ml versus $12.6 \pm 1.4 \times 10^4$ /ml in the Cre⁺ DT group (mean $\pm$ SEM, $P = 0.3$, $n \geq 5$). BALF RBC in the WT DT group was $12.2 \pm 6 \times 10^4$ /ml versus $20.7 \pm 10.7 \times 10^4$ /ml in the Cre⁺ DT group (mean $\pm$ SEM, $P = 0.45$, $n \geq 5$). BALF total protein in the WT DT group was $664.5 \pm 56.1 \mu\text{g/ml}$ versus $254.2 \pm 16.4 \mu\text{g/ml}$ in the Cre⁺ DT group (mean $\pm$ SEM, $P = 0.0002$, $n \geq 5$). All mice in the WT DT group survived to Day 7, compared with only 75% of mice in the Cre⁺ DT group. (B) BALF analysis at Day 28 after DT exposure. BALF WBC in the WT DT group was $43.5 \pm 4 \times 10^4$ /ml versus $18.1 \pm 2.1 \times 10^4$ /ml in the Cre⁺ DT group (mean $\pm$ SEM, $P = 0.001$, $n \geq 5$). BALF RBC in the WT DT group was $8.2 \pm 3 \times 10^4$ /ml versus $4.1 \pm 1.2 \times 10^4$ /ml in the Cre⁺ DT group (mean $\pm$ SEM, $P = 0.37$, $n \geq 5$). BALF total protein in the WT DT group was $195.5 \pm 17.5 \mu\text{g/ml}$ versus $187.2 \pm 22.5 \mu\text{g/ml}$ in the Cre⁺ DT group (mean $\pm$ SEM, $P = 0.78$, $n \geq 5$). All mice in the WT DT group survived to Day 28, compared with only 63% of mice in the Cre⁺ DT group. (C) Evans blue dye (EBD) extravasation in four organs 48 hours after high-dose DT exposure. Graphs represent total EBD (nanograms) normalized to tissue dry weight (milligrams) (mean $\pm$ SEM). The brain showed significantly elevated EBD extravasation in the Cre⁺ DT group compared with WT DT (22.3 ± 1.7 ng/mg versus 9.4 ± 2.2 ng/mg, respectively, $P = 0.004$, $n \geq 3$).

total protein at Days 2 and 7. Similarly, neither FoxD1-Cre;iDTR nor control mice experienced significant weight loss, and both groups tolerated the aspiration approach well. One hundred percent of the FoxD1-Cre;iDTR mice survived to 30 days after DT exposure (data not shown), suggesting that this dosage has minimal complications that are usually associated with systemic pericyte ablation. Escalation to high-dose DT did not improve the ablation efficiency. However, phenotypic differences resulting from high-dose DT pose interesting questions about the function of FoxD1-lineage stromal cells in the lung.

First, we observed proinflammatory changes in the BALF from littermate control mice in the high-dose, but not in the low-dose, scheme. DT exposure has been shown to induce an inflammatory response in mice even in nonsensitive wild-type animals (14). Prior studies have used local administration of DT (by intranasal or intratracheal approaches) to ablate immune regulatory and effector cells in the lungs, including Foxp3⁺ Treg and CD11c⁺ dendritic cells (14–16). Several features delineate our model from these previous methodologies. First, DT used to deplete immune effector cells in these studies required much higher dosages, varying

from 50 to 100 ng per instillation. Second, the proinflammatory response observed in these previous studies might have been confounded by the ablation of immune effector cells, regulatory cells, and/or the immune stimuli used. Because stromal cell ablation in the lung has not been attempted to date, and because of the much lower dosage of DT used in our study, the inflammatory effect of DT in lung stromal cell ablation is not known. With the low-dose DT regimen, we observed a mild neutrophilic inflammatory response in the BALF with elevations in WBC and total protein in both control and DT-sensitive cohorts. The elevations were modest and,

Figure 4. (Top) Diagram of triple-transgenic animal in which FoxD1-Cre controls the expression of Rs26-iDTR and Rs26-TdTomato transgenes. (Bottom) Triple-transgenic mice were exposed to PBS (control group) or DT in triple-transgenic mice. Graphs represent mean ± SEM. BALF WBC in the PBS group was $9.9 \pm 1.4 \times 10^4/\text{ml}$ versus $19 \pm 3.3 \times 10^4/\text{ml}$ in the DT group ($P = 0.08$, $n \geq 3$). BALF RBC was $0.25 \times 10^4/\text{ml}$ in the PBS group and $0.9 \pm 0.3 \times 10^4/\text{ml}$ in the DT group ($P = 0.3$, $n \geq 3$). BALF total protein in the PBS group was $137 \pm 16 \mu\text{g}/\text{ml}$ versus $376.3 \pm 44.7 \mu\text{g}/\text{ml}$ in the DT group ($P = 0.007$, $n \geq 3$). By flow cytometry, $18.2 \pm 2.2\%$ of stromal cells were tdTomato⁺ (FoxD1-lineage) in the control group (PBS), whereas only $11.9 \pm 0.4\%$ of stromal cells were tdTomato⁺ in the DT group ($P = 0.02$, $n \geq 3$). iDTR, human diphtheria toxin receptor; TdT, tdTomato.

more importantly, they were not significantly different between the two groups. Consistent with other published findings, however, high-dose DT resulted in more significant elevations of BALF WBC and total protein at Day 7 in WT DT mice, confirming the proinflammatory effect DT elicits independent of iDTR expression. Interestingly, in Cre⁺ DT mice, in which FoxD1-derived cells are ablated, there is an attenuation of inflammatory markers in the BALF (WBC and total protein). FoxD1-derived cells may participate in the acute lung inflammation associated with DT exposure, and partial ablation diminishes the proinflammatory effects of DT. Alternatively, the presence of apoptotic cells (from DT ablation) may alter the inflammatory environment in the lung. These findings highlight the importance of using proper controls in lung stromal cell ablation studies, which include DT administration to DT-insensitive littermates as control animals. Furthermore, the phenotypic difference in inflammation observed using the high-dose approach suggests that pericyte-like cells may have functional relevance in acute inflammatory responses.

Second, the clinical manifestations and mortality observed with high-dose DT in Cre⁺ DT mice suggested systemic

absorption of DT via the lungs. Indeed, examination of the brain revealed evidence of increased EBD extravasation in Cre⁺ DT compared with WT DT mice, supporting the idea that pericyte-like cells play functional roles in maintaining the blood-brain barrier and in endothelial transcytosis (17). In contrast, we did not observe differences in EBD extravasation in the liver or kidney, suggesting that vascular permeability by FoxD1-derived cell ablation is unaffected in these organs. There was a nonsignificant decrease in EBD extravasation in the Cre⁺ DT lung, which is consistent with the attenuated inflammatory response observed in Cre⁺ DT mice with high-dose DT. Given the limited number of mice, the current study may be underpowered to detect a significant difference.

Third, FoxD1-lineage stromal cells that were ablated at Day 7 returned by Day 28, as measured by PDGFRβ expression. Whether the FoxD1-derived cells regenerate by self-renewal or by differentiation from a local progenitor cell of another lineage remains unanswered. We are actively studying this regenerative process using the triple transgenic mouse described in this work.

The oropharyngeal aspiration approach has several limitations. First, the delivery of DT by aspiration resulted in

patchy regions of ablation and is an inherent weakness of this approach. Second, although we did not observe increased vascular permeability in the lungs, our observations are limited to a model in which only 40% of FoxD1-derived cells were ablated. Furthermore, other potential homeostatic roles of stromal cells in the lung, including physiologic responses to hyperoxia and hypoxia, airway reactivity, or lung mechanics, were not tested in this study. Understanding the functional role of stromal cells with respect to these homeostatic functions is important, and refinements in this model may facilitate experiments that will address these questions. Third, the ablation is transient only. Although this method may facilitate studies examining acute responses to injury, infection, or physiologic perturbations in the lungs after stromal cell ablation, studies requiring longer time points such as fibrosis may require ablation of the cells using alternative DT schedules, such as delivery of DT during the recovery phase in a bleomycin lung injury model.

In conclusion, oropharyngeal aspiration of low-dose DT is a viable method for lung stromal cell ablation. Transgenic mice tolerated the procedure well, without evidence of morbidity or mortality, and were able to thrive

beyond the acute time frame after DT administration. High-dose DT did not lead to more efficient ablation in this model, but did lead to inflammatory changes in the lung. These observations highlight the need to understand and characterize the baseline phenotypic changes associated with DT exposure in

DT-sensitive and DT-insensitive transgenic animals and to consider these effects in experimental design. The ablation using this method is transient, and PDGFR β ⁺ stromal cells return at Day 28. This model of lung stromal cell ablation may be a valuable tool for researchers interested in the

functional biology of lung stromal subpopulations under normal lung homeostasis, acute responses to lung injury, and the biology of stromal cell regeneration. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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