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Protective Role of Macrophages in Noninflammatory Lung Injury Caused by Selective Ablation of Alveolar Epithelial Type II Cells

Yasunobu Miyake,* Hitomi Kaise,* Kyo-ichi Isono,† Haruhiko Koseki,‡ Kenji Kohno,§ and Masato Tanaka2,*

Macrophages have a wide variety of activities and it is largely unknown how the diverse phenotypes of macrophages contribute to pathological conditions in the different types of tissue injury in vivo. In this study we established a novel animal model of acute respiratory distress syndrome caused by the dysfunction of alveolar epithelial type II (AE2) cells and examined the roles of alveolar macrophages in the acute lung injury. The human diphtheria toxin (DT) receptor (DTR), heparin-binding epidermal growth factor-like growth factor (HB-EGF), was expressed under the control of the lysozyme M (LysM) gene promoter in the mice. When DT was administrated to the mice they suffered from acute lung injury and died within 4 days. Immunohistochemical examination revealed that AE2 cells as well as alveolar macrophages were deleted via apoptosis in the mice treated with DT. Consistent with the deletion of AE2 cells, the amount of surfactant proteins in bronchoalveolar lavage fluid was greatly reduced in the DT-treated transgenic mice. When bone marrow from wild-type mice was transplanted into irradiated LysM-DTR mice, the alveolar macrophages became resistant to DT but the mice still suffered from acute lung injury by DT administration. Compared with the mice in which both AE2 cells and macrophages were deleted by DT administration, the DT-treated LysM-DTR mice with DT-resistant macrophages showed less severe lung injury with a reduced amount of hepatocyte growth factor in bronchoalveolar lavage fluid. These results indicate that macrophages play a protective role in noninflammatory lung injury caused by the selective ablation of AE2 cells. The Journal of Immunology, 2007, 178: 5001–5009.

Macrophages exhibit a variety of activities including induction of inflammation, engulfment of microorganisms and dead cells, Ag presentation, and regulation of extracellular components (1–3). Consistent with these activities, macrophages play diverse roles at the site of tissue injury. At the site of bacterial infection, macrophages recognize invading microorganisms via a wide variety of surface receptors that bind the cellular components common to many bacterial surfaces, thereby engulfing them for elimination. Macrophages encountering bacteria also produce and secrete inflammatory cytokines and chemokines such as TNF-α, IL-6, and MIP-1α. These factors induce inflammation, and the inflammatory responses play a critical role in the effective clearance of invading bacteria and the induction of appropriate adaptive immunity. The inflammatory responses, however, can cause deleterious injury to local tissues.

In contrast, at the site of tissue injury macrophages also recognize cell debris and dead cells (4–6). Tissue injury and inflammation results in the death of resident stromal and parenchymal cells. In addition, infiltrating neutrophils are eliminated by apoptotic cell death during the late course of inflammation. Macrophages rapidly phagocytose these dead cell corpses by means of specific phagocytic receptors for dying cells. The clearance of dead cells prevents the release of potentially toxic or immunogenic intracellular materials from a dead cell corpse. Thus, the prompt elimination of dying neutrophils and damaged resident cells is required for the resolution of inflammation and normal tissue repair (7, 8). Apoptotic cell clearance by macrophages is associated with active immunosuppression by the production of such anti-inflammatory cytokines as TGF-β and IL-10 (9–11). This suppressive effect of macrophages in association with the clearance of dead cells may contribute to normal tissue repair. Thus, macrophages have both proinflammatory and anti-inflammatory phenotypes. However, these phenotypes have been defined predominantly in the in vivo culture of macrophages and it is largely unknown how these diverse phenotypes of macrophages contribute to pathological conditions in the different types of tissue injury in vivo.

To study the effects of loss of a specific cell type in animal models, several approaches have been attempted for inducible cell-specific ablation. Recently, Saito et al. (12) reported a method for conditional cell ablation called TRECK (toxin receptor-mediated cell knockout) that works by expressing the human diphtheria toxin (DT) receptor (DTR) in transgenic mice. Human heparin-binding epidermal growth factor-like growth factor (HB-EGF) acts

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3 Abbreviations used in this paper: DT, diphtheria toxin; AE1, alveolar epithelial type I; AE2, alveolar epithelial type II; ARDS, acute respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; DIG, digoxigenin; DTR, DT receptor; ES, embryonic stem; FasL, Fas ligand; HB-EGF, heparin-binding epidermal growth factor-like growth factor; HGF, hepatocyte growth factor; LysM, lysozyme M; SP, surfactant protein.
FIGURE 1. Targeting strategy for the introduction of human DTR into the LysM gene. A, Schematic diagram of the LysM-DTR targeting construct. Exons 1 through 4 of the LysM gene are indicated by solid boxes. In the targeting construct, human diptheria toxin receptor (hDTR) cDNA, the loxP-flanked neomycin resistance gene (neo) cassette, and the thymidine kinase (TK) cassette are indicated by boxes. The probe used for Southern blot analysis is indicated as a solid line together with the predicted hybridizing fragments. WT, wild type. B, Genomic Southern blot analysis of LysM-DTR mice using an EcoRI digest in combination with the indicated probe. WT, wild type. C, Surface expression of human DTR protein on peritoneal macrophages from LysM-DTR mice. Peritoneal cells were prepared from wild-type (+/+) or LysM-DTR (+/LysMOTR) mice. Macrophages and B cells were stained with anti-DTR mAb and analyzed with a flow cytometer. Black and green lines indicate no staining and anti-DTR staining, respectively. D, Sensitivity of macrophages to DT ex vivo. Thioglycollate-elicited peritoneal macrophages were prepared from wild-type (+/+) or LysM-DTR (+/LysMOTR) mice and cultured overnight. The cells were then treated with the indicated concentrations of DT for 20 h. Cell viability was measured using a colorimetric reagent, WST-8. The assay was performed in triplicate and average values are shown with the SD. E, Inducible ablation of peritoneal macrophages by DT administration to LysM-DTR mice. DT at 40 μg/kg was injected i.p. into wild-type (+/+) or LysM-DTR (+/LysMOTR) mice. Forty-eight hours later, peritoneal cells were prepared from these mice and stained with anti-F4/80 and anti-B220 Abs for macrophages and B cells, respectively.

as a DTR, whereas mouse HB-EGF possesses negligible affinity to DT. Thus, mouse cells are more resistant to DT than human cells (13–15). When the human HB-EGF gene is transduced into mice under the control of a cell-specific promoter, the target cells are transiently depleted by DT administration in vivo (12, 16). This technique allows us to achieve efficient inducible ablation of a specific cell type by the administration of DT to the mice.

In this study we have developed a line of transgenic mice expressing human DTR under the control of the lysozyme M (LysM) gene promoter. Our initial goal was to induce ablation of various macrophages in vivo. However, in addition to macrophages the alveolar epithelial type II (AE2) cells in the lung were killed by administration of DT, and the mice suffered from acute respiratory failure caused by the selective ablation of a specific cell type by the administration of DT to the mice.

Materials and Methods
Targeting vector construction and generation of LysM-DTR mice

A genomic clone containing the LysM gene was obtained from a BAC library of 129/Sv mouse genomic DNA (Research Genetics). To generate the targeting vector, the region from −1.6 to +6.6 kb relative to the transcriptional start site of the LysM gene was subcloned into pBluescript II SK+ vector (Stratagene). Then, a 115-bp fragment in exon 1 was replaced with human DTR (HB-EGF) cDNA with a polyadenylated tail by a recombinant PCR technique. To select for homologous recombinants, a loxP-flanked Neo cassette was cloned downstream of the human DTR gene.

The thymidine kinase gene was inserted downstream of the 3′ arm to select against random integrants. The final targeting vector was linearized with PvuII for transfection.

To generate LysM-DTR mice, R1 embryonic stem (ES) cells were transfected with the targeting vector by electroporation. G-418- and gancyclovir-resistant clones were screened for homologous recombination by Southern blot analysis. Germline chimeric mice were generated by aggregation methods (17). Chimeric mice with a high ES cell contribution were crossed with C57BL/6 mice to produce LysM-DTR mice. +/LysMOTR mice were backcrossed to C57BL/6 mice three to six times, and the wild-type (+/+) and heterozygous LysM-DTR (+/LysMOTR) littermates were used for analysis. All mice were housed in a specific pathogen-free facility. Experiments were performed according to institutional guidelines.

For Southern blot analysis, genomic DNA was prepared from a mouse tail biopsy as described previously (18). For Southern hybridization, genomic DNA was digested with EcoRI, separated by electrophoresis on a 0.8% agarose gel, and transferred to a Biodyne A membrane (Pall). Hybridization was conducted with a 200-bp digoxigenin-labeled DNA fragment located outside the targeting vector according to the recommended protocols (DIG System for Filter Hybridization; Roche).

Antibodies

Anti-human DTR mAb was prepared by immunizing Armenian hamsters with human DTR-expressing cells. In brief, a DNA fragment for the full-length coding sequence of human DTR from pMS7 vector (12) was subcloned into the pEF-BOS-EX vector (19). The expression plasmid was introduced into human 293T cells by the calcium phosphate precipitation method. Beginning 24 h later, human DTR-expressing cells (1.5 × 10^7 cells) were injected s.c. into hamsters four times with 1-wk intervals between injections. A final booster was performed by injecting 1 × 10^7 cells of human DTR-expressing cells into the footpads. Three days after the final boost, the hamster was sacrificed and lymphocytes were harvested from the lymph nodes and fused with NS0 cells (20). Hybridomas were tested by ELISA with human DTR-expressing cells and three clones for anti-human DTR Ab were obtained. The hybridomas were cultured in GIT medium (Nihon Seiyaku) and Ab was purified by protein A-Sepharose 4F beads (Amersham Biosciences). Fluorescent labeling of
Ab was performed using an Alexa Fluor 488 mAb labeling kit (Molecular Probes).

Anti-FcγRII/III receptor Ab (clone 2.4G2), PE-anti-CD11b Ab (clone M1/70), and PE-anti-B220 Ab (clone RA3-6B2) were obtained from BD Biosciences. Anti-SP-A, -B, and -D Abs were obtained from Chemicon International. Anti-TTF-1 Ab (clone SGT7G3/1) and HRP-labeled anti-mouse Ig and anti-rabbit Ig Abs were obtained from DakoCytomation. Anti-Tlr1 Ab (clone 8.1.1) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Anti-CD169 Ab (clone 3d6.112) was obtained from Serotec. Cy3-labeled anti-rat IgG Ab and HRP-labeled anti-hamster IgG Ab were obtained from Jackson ImmunoResearch Laboratories. Biotinylated anti-F4/80 Ab (clone CE-A3-1) was obtained from Caltag Laboratories, Anti-F4/80 Ab (clone 6-16A; M. Tanaka and S. Nagata, unpublished observations) was provided by Dr. S. Nagata (Osaka University, Osaka, Japan). Fluorescein-anti-DIG Ab was obtained from Roche.

Ex vivo cytotoxic assay

Thioglycollate-elicted peritoneal macrophages (1 × 10⁶ cells) were cultured in DMEM containing 10% FCS overnight. DT was added to macrophage culture and incubated for 20 h and then cell viability was measured by Cell Counting Kit-8 (Dojindo Laboratories).

FACS analysis

Mouse peritoneal elicited cells (1 × 10⁶ cells) were preincubated with anti-FcγRII/III receptor Ab and were stained with biotinylated anti-F4/80 Ab (clone 6-16A). Cells were then washed, incubated with PE-anti-B220 or PE-CD11b Abs and streptavidin-Alexa Fluor 488 (Molecular Probes), and analyzed by a FACSCalibur system with the CellQuest program (BD Biosciences). To detect the DTR expression, cells were stained with an Alexa Fluor 488-labeled anti-DTR mAb (KM018).

Histology and immunohistochemistry

In all of the experiments except those with H&E staining the lungs were inflated with 4% paraformaldehyde and 4% sucrose in 0.1 M phosphate buffer (pH 7.2) through the trachea. For paraffin sections the lungs were immersion fixed in 4% paraformaldehyde in PBS overnight at 4°C and embedded in paraffin. For cryosections, the lungs were immersion fixed with 4% paraformaldehyde and 4% sucrose in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature. Then, the lungs were washed with PBS, immersed in 10 and 20% sucrose in phosphate buffer, and embedded in Tissue-Tek OCT (Sakura).

Paraffin sections (5 μm) were mounted on coated slides (Matsunami adhesive slides; Matsunami Glass Industries) and stained with H&E. Apoptotic cells were detected by TUNEL staining using a peroxidase in situ apoptosis detection kit (Chemicon), followed by counterstaining with methyl green in paraffin sections.

For Tlα and TTF-1 staining, paraffin sections were deparaffinized in xylene and rehydrated through a graded series of ethanol and then the slides were autoclaved for Ag retrieval. The sections were then quenched with 3% hydrogen peroxide in PBS, and blocked with 1.5% normal goat serum and 2% Block Ace (Snow Bland Milk Products). Slides were subsequently immunostained with anti-Tlα Ab overnight at 4°C or an anti-TTF-1 Ab for 1 h at room temperature. Sections were washed and incubated for 1 h at room temperature with HRP-labeled anti-hamster IgG (for Tlα) or anti-mouse Ig (for TTF-1). Signals were detected with a liquid diaminobenzidine substrate kit (Zymed Laboratories).

For F4/80, SP-A, -B, and -D staining, cryosections (4 μm) were mounted on 3-aminopropyltriethoxy silane-coated slides. Sections were fixed in cold acetone and blocked with 1.5% normal goat serum and 2% Block Ace. Slides were incubated with biotinylated anti-F4/80 Ab (Caltag Laboratories) for 1 h at room temperature or with anti-SP-A, -B, or -D Abs (Chemicon International) overnight at 4°C. The sections were then incubated with peroxidase-labeled streptavidin (Roche) for F4/80 or HRP-labeled anti-rabbit Ig Abs for SP-A, -B, and -D Abs (Chemicon International) overnight at 4°C. The sections were then incubated with DIG-dUTP and blocked with 1.5% normal goat serum and 2% Block Ace. The sections were then incubated with anti-CD169 Ab followed by fluorescein-anti-DIG Ab and Cy3-anti-rat IgG Ab. The stained sections were mounted with FluorSave (Calbiochem) and observed by fluorescence microscopy (Olympus IX-71).

For transmission electron microscopy, the lungs were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), washed with 0.14 M sucrose and 0.1 M phosphate buffer, postfixed with 1% OsO₄, 0.21 M sucrose, and 0.05 M phosphate buffer, and embedded in Epon 812. Sections (800–1000 Å) were prepared with U-5 Ultratome (LKB-BROMMA), stained with uranyl acetate and lead citrate, and observed with a JEOL 100S electron microscope (JEOL).

Analysis of bronchoalveolar lavage fluid (BALF) and large aggregate

Mouse lungs were lavaged via tracheal cannula two times with 700-μl aliquots of PBS. Cell debris was removed by centrifugation at 200 g for 10 min. For SP-B analysis, the large aggregate was separated from the BALF sample by centrifugation at 20,000 × g for 60 min and the pellet was resuspended in PBS.

For Western blot analysis, BALF samples and large aggregates were electrophoresed on polyacrylamide gels and then transferred to an Immobilon-P membrane (Millipore). Membranes were blocked in 5% skim milk in TBST before incubation with anti-SP-A, -B, and -D Abs. After washing, membranes were incubated with HRP-labeled anti-rabbit Ig Abs. Signals were detected using SuperSignal West Pico substrate (Pierce).

For cytokine ELISA, BALF was obtained from bone marrow chimeric mice after 24 h of DT treatment. The concentration of albumin and hepatocyte growth factor (HGF) in the BALF was measured by ELISA (Bethyl Laboratories and Institute of Immunology, Tokyo, Japan, respectively) according to the manufacturer’s protocols.

FIGURE 2. Lethal effect of DT administration on Lysm-DTR mouse lung. A. The indicated amount of DT was i.p. injected into 6- to 8-wk-old wild-type (+/+) or Lysm-DTR (+/LysmDTR) mice. The number of mice in each group was 6–11 and the percentages of the mice that survived until the indicated time were plotted. B–D, DT at 40 μg/kg was i.p. injected into wild-type (+/+) or Lysm-DTR (+/Lysm-DTR) mice. Mice were sacrificed 24 or 48 h after DT administration. B, Macroscopic observation of the lung. C and D, Lungs were fixed with 4% paraformaldehyde and embedded in paraffin. The sections were stained with H&E (C) or TUNEL with methyl green staining (D). Scale bar, 100 μm.
Bone marrow transplantation

For each chimera, 1–1.5 × 10⁷ bone marrow cells from donor mice were transferred i.v. into recipient mice that had received 9 gray from an x-ray irradiation system, the Gammacell 40 Exactor (¹³⁷Cs; MDS Nordion) before transfer. The recipient mice were analyzed 6–10 wk after reconstitution. The recipient mice were analyzed 6–10 wk after reconstitution.

Results

Generation of LysM-DTR mice

To establish the inducible macrophage depletion mice, we generated LysM-DTR mice in which human DTR was expressed under the control of the LysM gene, which is specifically expressed in myelomonocytic cells such as macrophages and monocytes (21–23). Human DTR cDNA was introduced into the endogenous ATG start site within the first exon of the LysM gene (Fig. 1A). The linearized targeting construct was transfected into ES cells, and heterozygous ES cells carrying a human DTR knock-in allele were aggregated with blastocysts to generate LysM-DTR chimeric mice. The chimeric mice were then crossed with C57BL/6 wild-type mice to obtain LysM-DTR heterozygous animals. Homologous recombination of LysM-DTR mice was confirmed by Southern blot analysis (Fig. 1B).

We first examined the expression levels of human DTR in the cells from LysM-DTR mice. FACS analysis revealed that human DTR was expressed on the peritoneal macrophages from LysM-DTR mice (Fig. 1C). In contrast, the DTR expression was not detected on the surface of peritoneal B cells from LysM-DTR mice. Surface expression of human DTR was also detected on thioglycollate-elicited peritoneal macrophages and bone marrow-derived macrophages from LysM-DTR mice (data not shown).

Next, we examined whether the macrophages from LysM-DTR mice were sensitive to DT. When the thioglycollate-elicited macrophages from LysM-DTR mice were treated with DT, the cells were killed in a dose-dependent manner (Fig. 1D). In contrast, the macrophages from wild-type mice were completely resistant to DT. Furthermore, when DT was i.p. injected into LysM-DTR mice, peritoneal macrophages (F4/80⁺) but not B cells (B220⁺), were selectively killed (Fig. 1E). These results indicated that macrophages from knock-in mice expressed the functional human DTR on the cell surface.

DT administration causes severe lung injury in LysM-DTR mice

We next examined the effects of DT injection on LysM-DTR mice. Various amounts of DT were i.p. injected into LysM-DTR mice, and the injection of a high dose of DT killed the mice (Fig. 2A). When 40 μg/kg DT was injected into the transgenic mice, all mice showed hypomotility, hunched posture, and ruffled fur and died within 96 h. In the case of 10 μg/kg injection, half of the mice were killed within 1 wk after injection. In contrast, none of the wild-type mice were killed by the administration of 40 μg/kg DT.

Lactose dehydrogenase levels in the serum of LysM-DTR mice were increased by >7-fold following the administration of 40 μg/kg DT, suggesting severe damage in certain tissues (data not shown). Macroscopic observation revealed that the lungs

FIGURE 3. Effects of DT administration on alveolar macrophages and alveolar epithelial cells in lungs of LysM-DTR mice. Wild-type (+/+; a–c) or LysM-DTR (+/LysMDTR; d–f) mice were i.p. injected with 40 μg/kg DT. Lungs were obtained from these mice 48 h after DT administration. A, The lung sections were immunostained with F4/80 (a and d), T1α (b and e), and TTF-1 (c and f), which are specific markers for alveolar macrophages, AE1, cells and AE2 cells, respectively. Scale bar, 100 μm. B, Transmission electron microscopic observation of the lung sections of wild-type (a and c) and LysM-DTR (b and d) mice. The red arrow indicates AE2 cells. Scale bar, 10 μm.

FIGURE 4. Effect of DT administration on surfactant proteins in the lung. A, DT at 40 μg/kg was i.p. injected into wild-type mice (+/+; a) and LysM-DTR (+/LysMDTR) mice. BALF was harvested 24 or 48 h after DT administration and the amount of SPs was analyzed by Western blot analysis using anti-SP-A, anti-SP-B, and anti-SP-D Abs. These results are representative of three independent experiments. B, Lungs were obtained from wild-type (+/+; a–c) or DT-administered LysM-DTR (+/LysMDTR) mice (d–f) 48 h after DT administration and embedded in OCT compound. Cryosections were immunostained with anti-SP-A, anti-SP-B, and anti-SP-D Abs. Scale bar, 100 μm.
had a hemorrhagic appearance (Fig. 2B). Histological analysis of the lungs demonstrated that the alveolar walls became thick and the alveolar spaces were diminished (Fig. 2C). Moreover, RBC were notably expanded in the interstitial mesenchyme, indicating severe congestion in the lungs. We also microscopically observed other organs, including the heart, kidney, and liver. However, none of these organs showed any abnormalities except the liver that showed slight congestion (data not shown). These results indicated that the lungs were primarily injured in LysM-DTR mice by DT administration.

DT is integrated into cells through its receptor and induces apoptosis by ADP ribosylation of elongation factor 2 (15, 24, 25). We next performed TUNEL staining with the lung sections to detect apoptotic cells. In wild-type mice, no TUNEL-positive cells were observed in the lungs by DT administration (Fig. 2D). In contrast, a large number of cells became TUNEL-positive in the lungs of LysM-DTR mice 24 h after DT administration. TUNEL-positive cells were located in the epithelial layer of alveoli as well as inside the alveolar space, indicating that some epithelial cells as well as alveolar macrophages underwent apoptosis by DT. We also performed TUNEL staining with the sections of other tissues. Macrophages in several organs also showed TUNEL-positive staining. However, we could detect neither TUNEL-positive cardiomyocytes nor hepatocytes in the LysM-DTR mouse injected with DT (data not shown).

The alveolar epithelial type II cells are deleted by DT in LysM-DTR mice

The lung alveolar epithelial layer consists of alveolar epithelial type I (AE1) cells and AE2 cells. While AE1 cells cover up to 95% of the surface area of the lungs and are responsible for gas exchange, AE2 cells occupy only 5% of the surface area and produce and secrete the surface-active agents collectively known as surfactants. It has been reported that both alveolar macrophages and AE2 cells express the LysM gene (26, 27). Therefore, in the present study it was most likely that both alveolar macrophages and AE2 cells expressed human DTR and that they were killed by DT administration. To assess this speculation, immunohistochemical analysis for alveolar macrophages and AE2 cells was performed in the lung sections of DT-administered mice. F4/80, T1α/H9251, and TTF-1 were used as specific markers of alveolar macrophages, AE1 cells, and AE2 cells, respectively (28–31). The number of both alveolar macrophages and AE2 cells were notably decreased by DT administration to LysM-DTR mice, while that of AE1 cells was not affected (Fig. 3A). TTF-1 was also expressed in epithelial cells lining conducting airways (Fig. 3, Ac and Af, lower right area for both wild-type and LysM-DTR mice); however, DT administration had a negligible effect on the epithelial cells lining conducting airways. To further confirm the absence of AE2 cells in LysM-DTR mice injected with DT, the lung sections were analyzed by transmission electron microscopy. In wild-type mice, AE2 cells were located at the alveolar corners and displayed typical cuboidal features with lamellar bodies (Fig. 3B).
3, Ba and Bc, arrow). In contrast, intact AE2 cells were hardly observed in LysM-DTR mice treated with DT (Fig. 3Bb). Some apoptotic AE2 cells were found at the alveolar corners in these mice (Fig. 3Bb). These results indicated that alveolar macrophages and AE2 cells were ablated by DT administration in LysM-DTR mice.

One of the important roles of AE2 cells is the synthesis and secretion of bioactive SPs, which stabilize alveolar spaces by reducing the surface tension of alveoli (32, 33). Thus, it was expected that the ablation of AE2 cells led to the depletion of SPs in alveolar spaces. We next analyzed the effect of DT administration on SPs in BALF. Although the amount of SP-D was not altered, those of SP-A and SP-B were notably decreased by DT administration (Fig. 4A). Reduced production of SPs was also confirmed by immunohistochemistry with anti-SP Abs. As shown in Fig. 4B, cells producing SP-A, -B, and -D were detected in the lung sections of wild-type mice. In contrast, few SP-producing cells were detected in the lungs of LysM-DTR mice with DT injection. These results indicated that DT administration to LysM-DTR mice induced apoptosis in AE2 cells and caused respiratory failure due to the decreased amount of surfactants.

Contribution of macrophages to lung pathology in the LysM-DTR mice

In LysM-DTR mice, DT administration resulted in the ablation of AE2 cells as well as alveolar macrophages and caused severe respiratory failure possibly due to the decreased amount of SPs. To examine how the ablation of alveolar macrophages contributed to lung pathology in the mice, we performed transplantation of bone marrow cells from wild-type mice into LysM-DTR mice that had been lethally irradiated. Six weeks after the bone marrow transplantation, the sensitivity of peritoneal macrophages to DT was determined in these mice. As shown in Fig. 5A, peritoneal macrophages were completely resistant to DT in the LysM-DTR mice reconstituted with wild-type bone marrow cells (W-L mice), whereas these cells were deleted by DT administration in the LysM-DTR mice reconstituted with LysM-DTR bone marrow cells (L-L mice). Consistent with this, alveolar macrophages were not ablated by DT injection in the W-L mice (Fig. 5Bc). Some alveolar macrophages in the DT-treated W-L mice phagocytosed apoptotic cells (possibly apoptotic AE2 cells), indicating that DT-resistant, functional alveolar macrophages were successfully reconstituted in the W-L mice. (Fig. 5Cc, arrow) In contrast, AE2 cells both in the L-L and the W-L mice were almost completely deleted by DT administration (Fig. 5, Bj and Bk). We also examined the amount of SP-A in BALF, and found that it was greatly reduced both in the W-L and the L-L mice (Fig. 5D). These results indicated that the W-L mice showed selective deletion of AE2 cells with intact alveolar macrophages by DT administration.

We then compared the severity of lung injury induced by DT administration between W-L mice and L-L mice. Although both mice were killed by DT administration, macroscopic observation indicated that lung congestion seemed to be more severe in the L-L mice than in the W-L mice (Fig. 6A). Histological examination also gave the same results (Fig. 6B). The early phase of lung injury caused by LPS or bleomycin is often associated with an inflammatory reaction, including neutrophil recruitment. However, few neutrophils were observed in the injured lungs even in the presence of alveolar macrophages (W-L mice), indicating that the deletion of AE2 cells by DT administration to LysM-DTR mice did not induce any inflammatory reactions.

To further evaluate the severity of lung injury, we examined the leakage of albumin into alveolar spaces upon DT administration. As shown in Fig. 6C, albumin concentrations were much higher in BALF from L-L mice than in that from the W-L mice. No leakage of albumin was observed in WT mice reconstituted with LysM-DTR bone marrow cells (L-W mice) in which alveolar macrophages but not AE2 cells were deleted by DT. These results indicate that the injury of AE2 cells is a major contributor to respiratory failure in DT-treated LysM-DTR mice and that the presence of macrophages diminishes this noninflammatory lung injury caused by AE2 cell deletion.

Production of cytokines in DT-treated LysM-DTR mice

We next examined the concentrations of several cytokines in BALF from these mice. We could not detect the production of
either inflammatory cytokines such as TNF-α, or immunoregulatory cytokines such as IL-10 and TGF-β in BALF from any of these mice (data not shown). This is consistent with the histological findings of no inflammatory cells in the lungs of DT-treated mice (W-L and L-L). The HGF is considered to be a candidate growth factor for AE2 cells and is produced by bronchial epithelial cells as well as alveolar macrophages engulfing dying neutrophils in a mouse model of bacterial pneumonia (34). Therefore, we measured HGF concentrations in BALF from these mice. As shown in Fig. 6D, BALF from DT-treated L-L mice contained a larger amount of HGF than those from W-L mice. This result suggests that the amount of HGF is correlated with the severity of the lung injury caused by AE2 cell deletion and that alveolar macrophages do not contribute to HGF production in noninflammatory AE2 cell injury.

Discussion
To explore the pathophysiology of respiratory distress, several animal models have been used as acute lung injury models. The intratracheal instillation of bleomycin in rodents has been widely used as a model to study the mechanisms of lung injury and repair. In this model, the administration of bleomycin induces early inflammatory responses followed by fibrosis. Histological examination demonstrated that both vascular endothelial cells and AE1 cells, but not AE2 cells, were specifically injured in the lungs of mice treated with bleomycin (35). However, the pathogenesis of the injury and of the subsequent lung disease caused by bleomycin remains obscure. Intratracheal instillation of LPS also induces a massive infiltration of inflammatory cells, including neutrophils and macrophages, causing acute respiratory distress (36, 37). This model, at least in part, represents infection-induced acute respiratory distress syndrome (ARDS) in human. However, the precise mechanism of LPS-induced lung injury remains elusive.

In this study we established transgenic mice expressing human DTR in AE2 cells as well as macrophages. When DT was injected into the transgenic mice AE2 cells selectively underwent apoptosis and the mice showed lethal respiratory failure, possibly due to a rapid decrease in the amount of surfactants produced by AE2 cells. DT injection also killed alveolar macrophages in these mice. Bone marrow transplantation from wild-type mice could not rescue the lethal phenotype of the LysM-DTR mice, indicating that the ablation of AE2 cells is primarily responsible for the respiratory failure in the mice injected with DT. To our knowledge, this transgenic mouse line is the only mouse model of acute respiratory distress that is possible due to the selective injury of AE2 cells and the subsequently reduced production of surfactants. In addition to the unique mechanism of acute lung injury in these mice, lung injury was easily and reproducibly induced by i.p. injection of DT, whereas intratracheal instillation of the reagent is required for bleomycin- or LPS-induced acute lung injury in mice.

Injury or apoptosis of AE2 cells has been reported to play an important role in the pathogenesis of several lung diseases. Hypoxia, an important feature of acute lung injury, has been shown to induce apoptosis in AE2 cells in vitro (38). Chronic hypoxia has also been shown to cause morphological changes of AE2 cells including delamination in vivo (39), suggesting the functional failure of AE2 cells in hypoxia-induced lung injury or fibrosis. In other studies AE2 cells have been reported to express the Fas receptor, a transmembrane protein that transduces death signals upon stimulation (40, 41). When primary cultured AE2 cells were treated with the Fas ligand (FasL) the cells underwent apoptosis in vitro. Moreover, intratracheal administration of FasL or agonistic anti-Fas Ab induces AE2 cell death in vivo (40–42). A consider-

able amount of the soluble FasL, produced by shedding of the membrane-bound form (43, 44), was detected in BALF of ARDS patients (45), suggesting that AE2 cells could be damaged by FasL/Fas activation in ARDS. As a matter of fact, abnormal surfactant function and changes in the surfactant subfraction, including a decrease in the amount of SPs, were observed in ARDS patients (33, 46–49). Taking these observations into consideration, it is possible that in some ARDS cases AE2 cell death is at least partly responsible for the pathogenesis of ARDS. In this respect the pathology of respiratory failure in the LysM-DTR mouse partly resembles that in human ARDS and, thus, this system will provide a new animal model to investigate the pathophysiology of human ARDS.

In this study we found that the presence of macrophages diminished lung injury caused by AE2 cell deletion. Although it is likely that alveolar macrophages contribute to the protective effects, we cannot deny the possibility that lung interstitial macrophages or macrophages in other organs, such as Kupffer cells, may also contribute to some extent to diminishing lung damage in this model of noninflammatory lung injury. In the acute phase of tissue injury macrophages often perform injury-inducing roles. For instance, the depletion of macrophages in the early phase of carbon tetrachloride-induced liver injury resulted in reduced scarring and fewer myofibroblasts (50). In acute rejection of renal transplantation, infiltrating macrophages promoted tissue damage (51). In this study, by contrast, alveolar macrophages contributed to the reduction of lung injury and serum albumin leakage into alveolar spaces caused by AE2 cell deletion. This result suggests that macrophages play protective roles in noninflammatory tissue injury. In the DT-treated W-L mice, alveolar macrophages phagocytosed apoptotic AE2 cells. This clearance of dying cells by macrophages may be involved in the protective effects. During the late course of inflammation, prompt elimination of neutrophils by macrophages is required for the resolution of inflammation and normal tissue repair (7, 8). The hyaluronan receptor CD44 plays an important role in the phagocytosis of apoptotic neutrophils by macrophages. CD44-deficient mice have been shown to succumb to unremitting inflammation in association with impaired clearance of apoptotic neutrophils in bleomycin-induced acute lung injury (52, 53). Although the lung injury caused by AE2 cell deletion is not associated with the infiltration of neutrophils, the clearance of dying parenchymal cells by macrophages may be a critical factor determining the degree of tissue damages in such noninflammatory tissue injury.

HGF is considered to play an important role in the repair of the pulmonary epithelium in acute lung injury. In mouse bacterial pneumonia the expression of HGF in the lung showed a biphasic pattern (34); that is, bronchial epithelial cells produced the cytokine in the early phase of infection and alveolar macrophages engulfing dying neutrophils produced it in the late phase of infection. In the present study, HGF was produced in acute lung injury caused by the deletion of AE2 cells, and the expression levels in L-L mice were higher than those in W-L mice. These results suggest that bronchial epithelial cells, but not alveolar macrophages, are the major producers of HGF in the case of noninflammatory lung injury without any infiltration of neutrophils. The precise mechanisms of HGF production by bronchial epithelial cells are not known. Because the presence of alveolar macrophages that phagocytosed dying AE2 cells reduced the levels of HGF in lung injury in this study, cellular components released from dying AE2 cells may stimulate HGF production by bronchial epithelial cells.

LysM-DTR mice are also expected to serve as a useful model to study the regeneration of AE2 cells. AE2 cells are considered to be
stem cells of the adult alveolar epithelium (54). Primary cultured AE2 cells differentiate into AE1 cells under certain conditions in vitro (55, 56). After lung injury, AE2 cells divide and differentiate into AE1 cells in vivo (57, 58). In the present study most AE2 cells were killed in LysM-DTR mice by DT administration. We could not monitor the course of repair for AE2 cell injury by i.p. DT administration because of early death. However, if AE2 cells in a regional group of lung alveoli are ablated by intratracheal instillation of DT, it may be possible to observe the course of lung repair after AE2 cells are selectively injured.

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Disclosures
Y. Miyake and M. Tanaka, together with RIKEN, have a pending patent on the LysM-DTR mouse as a mouse model for acute respiratory distress syndrome.

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