Online Data Supplement

Streptococcus pneumoniae triggers progression of pulmonary fibrosis through pneumolysin

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Methods

Animals

Female C57BL/6N mice were purchased from Charles River (Sulzfeld, Germany). Transgenic SPC-DTR mice on a C57BL/6N background (> 10 backcrosses) were generated as described previously in detail [1], and were housed together with their respective wild-type littermates under conventional conditions with free access to food and water in the Central Animal Facility of Hannover Medical School. Transgenic SPC-DTR heterozygotes were mated with WT littermates followed by offspring genotyping of tail snip DNA by polymerase chain reaction. TLR4 KO mice on a C57BL6/J background (>5 backcrosses) were purchased from Jackson Laboratories. For experiments involving TLR4 KO mice, C57BL6/J wild-type controls purchased from Charles River were used. In all experiments, female mice were used at the age of 8 to 12 weeks. All animal experiments were approved by local government authorities.

Antibiotics

Clarithromycin (CLR) was purchased from Sigma-Aldrich (Deisenhofen, Germany). CLR was prepared at a stock concentration of 15 mg/ml in 95 % ethanol and phosphate-buffered saline (PBS, ratio of 1:10), and was then sonicated for 30 min (Ultrasonic Cleaner, VWR, Germany). Amoxicillin (Amox) obtained from Sigma-Aldrich was prepared at a stock concentration of 40 mg/ml resuspended in 0.1 M NaOH diluted in sterile water. Antibiotics were always freshly prepared prior to each experiment. Mice received a single application of either CLR (50 mg/kg body weight i.p.) or Amox (100 mg/kg body weight i.p.) at 24 h and 48 h post infection (corresponding to days 15 and 16 post AdTGFβ1 treatment).

Administration of adenoviral vectors and diphtheria toxin treatment of mice

Replication-deficient adenoviral vectors carrying the gene for active TGFβ1 (AdTGFβ1) and control vector without any gene inserted (AdDL70-3) were prepared as previously described in detail [2]. Vectors were diluted in PBS to a concentration of $1x10^8$ plaque-forming units (PFU)/50 μl PBS. Diphtheria toxin (DT; Merck, Darmstadt, Germany) was applied via orotracheal routes to the lungs of mice at a concentration of 10 ng DT/50 µl PBS once daily for seven consecutive days. Mock treated animals received o.t. instillations of 50 µl PBS once daily for seven consecutive days. Orotracheal instillations were performed according to previously described protocols [3, 4]. Briefly, mice were anesthetized with xylazine (5 mg/kg of body weight; Bayer, Leverkusen, Germany) and ketamine (75 mg/kg; Albrecht, Aulendorf, Germany) and were then orotracheally (o.t.) intubated with a 29-gauge Abbocath catheter (Abbott, Wiesbaden, Germany), which was inserted into the trachea under visual control with transillumination of the neck region. For repetitive DT applications, mice were anesthetized daily with desflurane (Baxter). Subsequently, mice were brought back to their cages with free access to food and water.

Culture and quantification of *Streptococcus pneumoniae* and infection of mice

For infection experiments, we used a Gram-positive capsular group 19 *Streptococcus pneumoniae* strain (EF3030), which is known to primarily cause focal pneumonia in mice. In addition, we used an isogenic pneumolysin-deficient *S. pneumoniae* (EF3030Δ*ply*), and Gram-negative serotype 2 *K. pneumoniae* (ATCC 43186), as previously described [5-7]. Pneumococci were grown in Todd-Hewitt broth (THB) (Oxoid, Wesel, Germany) supplemented with 20 % fetal calf serum (FCS) to mid-log phase, and *K. pneumoniae* was grown in nutrient broth [5]. Aliquots were snap-frozen

in liquid nitrogen and stored at -80°C until use. Spn and Kpn stocks were quantified by plating serial dilutions on sheep blood agar plates (BD Biosciences, Heidelberg, Germany), or nutrient agar plates (ATCC medium 3) respectively, followed by incubation of the plates at 37°C and 5 % CO_2 for 18 h and subsequent determination of colony-forming units (CFU).

Mice were infected o.t. with serotype 19 Spn (EF3030) or its pneumolysin-deficient, isogenic mutant EF3030Δply adjusted to ~1x10^7 CFU/50 µl PBS/mouse, or were infected o.t. with Kpn (ATCC 43186) adjusted to 5x10^6 CFU/50 µl PBS/mouse according to previously published protocols [7, 8].

Experimental groups

The following experimental groups were studied: (1) Mice received AdTGFβ1 or control vector to characterize AdTGFβ1 induced pulmonary fibrosis in mice by day 7-28 post vector treatment. (2) Mice received instillations of AdTGFβ1 or control vector for 14, 21, or 28 days, followed by infection of mice with Spn for 1 or 3 days to analyze the host defense capacity of fibrotic lungs post bacterial infection. (3) Mice received AdTGFβ1 for 14 days, and subsequently were either mock-infected or infected with Spn for 7 and 10 days to examine the effect of bacterial infection on pulmonary fibrosis. (4) In a TGFβ1-independent mouse model of pulmonary fibrosis, WT mice or transgenic SPC-DTR mice received DT daily o.t. for 7 days, and after 14 days (i.e., when fibrosis was established), mice received mock or Spn infection o.t. for 7 days to examine infection-induced exacerbation in a TGFβ1 gene transfer independent model of pulmonary fibrosis. (5) Mice were pretreated as described for experimental group 3, but received clarithromycin (CLR) or amoxicillin (Amox) applied at 24 h and 48 h after infection to examine the effect of antibiotic treatment on *S. pneumoniae*-induced fibrosis exacerbation. (6) Mice received AdTGFβ1 for 14

days, and then received either mock, or Spn, or SpnΔply o.t. for 7 days to investigate the effect of Ply on progression of pulmonary fibrosis. (7) WT mice or transgenic SPC-DTR mice received DT for 7 days, and at day 14 post DT treatment, mice were either mock infected, or infected with Spn or SpnΔply o.t. for 7 days to examine the effect of Ply on pulmonary fibrosis progression. (8) Mice received AdTGFβ1 for 14 days, and then received daily o.t. instillations for three consecutive days of either cytotoxic Ply (~40-50 hemolytic units (HU) per mouse, corresponding to approx. 10^7 CFU of Spn), or its non-cytotoxic derivative, PdB, or its non-cytotoxic, non-complement activating derivative, PdT to examine its effect on pulmonary fibrosis progression. (9) To investigate the role of TLR4 on Ply-induced pulmonary fibrosis progression, WT mice and TLR4 KO mice received AdTGFβ1 for 14 days, and then received daily o.t. instillations for three consecutive days of either cytotoxic Ply (~50 hemolytic units (HU) per mouse), or non-cytotoxic PdB, and lung hydroxyprolin levels were determined on day 21. (10) To assess the effect of previous immunization on Ply-induced fibrosis progression, mice were vaccinated on day -7 with i.p. injection of 10 µg PdB-alum, and on day +7 with i.p. injection of 20 µg PdB-alum. On days 14, 15, and 16 after AdTGFβ1 instillation, mice received o.t. instillations of PdB or Ply (~50 hemolytic units (HU) per mouse).

PdB precipitation in alum and determination of Ply-specific antibody titers

PdB precipitation was performed in aluminium potassium sulphate (PdB-alum) (Sigma, Deisenhofen, Germany), and the protein precipitate was neutralized with potassium hydroxide solution before the pellet was washed and resuspended in sterile PBS.

Determination of Ply-specific antibody titers in plasma of immunized mice was performed as described recently in detail [9, 10]. Briefly, plates were coated with highly

purified Ply (2,5 µg/ml) overnight at 4°C. After blocking with PBS/2 % (wt/vol) BSA (AppliChem, Darmstadt, Germany) for 1 hour, 100 µl of plasma samples were added and incubated for 2 h at room temperature. For detection of Ply-specific IgG1, plasma was diluted 1:3,000; for detection of IgA and IgG2a, plasma samples were diluted 1:30 in PBS/ 2 % BSA. Subsequently, biotinylated anti-IgG1 (clone A85-1) and anti-IgG2A (clone R19-15), or anti-IgA (clone C10-1) (all purchased from BD Biosciences, Heidelberg, Germany) was added to the wells for 1 h. After washing, streptavidin-horseradish peroxidase (Dako, Hamburg, Germany), diluted 1:100 in PBS/ 2 % BSA, was added to the wells and incubated for 1 h. Finally, ABTS (Roche, Mannheim, Germany, 100 µl per well) was used for detection and, after shaking at room temperature for 20 min, OD measurements were performed at 405 nm.

Determination of lung collagen content

Mice of the various experimental groups were euthanized with an overdose of isoflurane (Baxter) at various time points post treatment. Lung collagen content measurement was performed using the hydroxyproline dye binding assay as previously described [11].

Lung function testing

Pulmonary function was measured in isoflurane-anaesthetized orotracheally intubated mice. Lung resistance (R_L) and dynamic lung compliance (C_{dyn}) were calculated from measured transpulmonary pressure, tidal flow and volume signals over each complete breath cycle of the spontaneously breathing mice. The technique for lung function testing used in this study was previously described in detail [12-14].

Lung histopathology

Mice were euthanized with an overdose of isoflurane (Baxter) and non-lavaged lungs were inflated in situ with PBS-buffered formaldehyde solution (4,5 %, pH 7, Roth, Deisenhofen, Germany), then removed and immersed en bloc in formaldehyde solution for at least 24 h at room temperature. Paraffin-embedded lung sections (3 µm) were stained with hematoxylin/eosin (HE) and trichrome (Elastica-van-Gieson) and subsequently analyzed by an experienced lung pathologists (DJ) blinded to respective treatment regimens, using an Olympus microscope, model BX53 at a x 200 magnification. Pictures were taken with a Keyence BZ 9000 camera and were edited with Adobe Photoshop software. The degree of lung fibrosis was determined using Ashcroft scoring [15].

Bronchoalveolar lavage (BAL)

Mice were euthanized with an overdose of isoflurane (Baxter, Unterschleissheim, Germany) and bronchoalveolar lavage was performed as previously described in detail [8].

Determination of bacterial loads in BAL and lung parenchymal tissue

At indicated time points post bacterial infection, mice were euthanized with an overdose of isoflurane (Baxter) and bacterial loads were determined in BAL fluid (BALF) and lung homogenates of mice of the respective treatment groups. BALF aliquots (100 µl) were plated in 10-fold serial dilutions on sheep blood agar or nutrient agar plates followed by incubation of the plates at 37°C in 5 % CO_2. Subsequently, CFU were counted. For determination of bacterial loads in lung parenchymal tissue, lung tissue was dissected, homogenized in 2 ml Hanks' balanced salt solution without supplements and 10-fold serial dilutions were plated on sheep blood agar or nutrient agar plates followed by incubation of the plates at 37°C in 5 % CO_2 [16, 17].

Isolation of type II alveolar epithelial cells

Type II alveolar epithelial cells (AT II cells) were isolated from the lungs of mice subjected to AdTGFβ1 followed by treatment with either PdB or Ply for induction of fibrosis exacerbation, using previously published protocols [11]. Isolated type II epithelial cells were further subjected to high-speed cell sorting to achieve sufficient purities for subsequent FACS analysis of apoptosis induction in AT II cells. AT II cells were gated according to their FSC-A versus SSC-A characteristics, followed by hierarchical sub-gating on $CD45^{neg}$, $MHCII^{pos}$ cells. Flow sorting was carried out under sterile conditions at a constant temperature of 4° C. Post-sort analysis of sorted cells revealed purities of > 98%. Electron microscopic examination of sorted cells confirmed a typical morphology of type II alveolar epithelial cells, including the presence of lamellar bodies (data not shown).

ELISA

Quantification of biologically active TGFβ1 protein and TNF-α in BALF of mice was performed using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (detection limit, 31 pg/ml) (R&D Systems, Wiesbaden, Germany).

Statistical analysis

All data are presented as mean ± SD. Data were analyzed for normal distribution and differences between treatment groups were analyzed by a nonparametric Mann-Whitney U test, Student t test, one-way analysis of variance (ANOVA) followed by post hoc Bonferroni adjustment or two-way ANOVA using SPSS for Windows

software. P-values below 0.05 were considered to indicate statistically significant differences.

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