Online Data Supplement

Natural Killer T Cell-Derived IL-17 Mediates Lung Ischemia-Reperfusion Injury

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Materials and Methods

Animals. This study utilized 8-12 week old, male C57BL/6J (WT), Rag-1$^{-/-}$, IL-17A$^{-/-}$ and Jα18$^{-/-}$ mice which were randomly assigned to either a sham group (2 hr reperfusion) or IR group (1 hr left-lung ischemia followed by 2 hrs reperfusion). The WT and Rag-1$^{-/-}$ mice (on C57BL/6J background) were purchased from the Jackson Laboratory (Bar Harbor, ME). The IL-17A$^{-/-}$ mice have been backcrossed onto the C57BL/6J background for 10 generations (E1). Jα18$^{-/-}$ mice, which are exclusively deficient in invariant Valpha14(+) CD1d-restricted NKT cells, were provided by Dr. M. Taniguchi (RIKEN Research Center for Allergy and Immunology, Chiba University, Chiba, Japan) and have been backcrossed onto the C57BL/6J background for 10 generations (E2). For *in vivo* blocking of IL-17A, WT mice were treated, intravenously, 5 minutes before ischemia with 150 μg of either anti-IL-17 mAb (clone 50104, R&D Systems, Minneapolis, MN) or IgG2A isotype control (clone 20102, R&D Systems). Clone 50104 antibody has no cross-reactivity with recombinant mouse IL-17B, IL-17C, IL-17D, IL-17E, or IL-17F as described by the manufacturer. This study conformed to the "Guide for the Care and Use of Laboratory Animals" published by the National Institute of Health (NIH publication No. 85-23, revised 1985) and was conducted under protocols approved by the University of Virginia's Institutional Animal Care and Use Committee.

Lung IR model. An *in vivo* hilar clamp model of IR was used as previously described (E3). Mice undergoing IR were subjected to 1 hr lung ischemia (via left hilar occlusion) followed by 2 hrs of reperfusion. Briefly, mice were anesthetized with inhaled isoflurane, intubated with PE-60 tubing and connected to a pressure-controlled ventilator (Harvard Apparatus Co, South Natick, MA). Mechanical ventilation with room air was performed at 150 strokes/min, 1.0 cc stroke volume, and peak inspiratory pressure less than 20 cm H_2O. Heparin (20 U/kg) was given

immediately preceding the ischemic period via external jugular injection to minimize thrombosis in the pulmonary vasculature during ischemia. Left thoracotomy was performed by dividing the left 3rd rib, and the left hilum was exposed. A 6-0 prolene suture was passed around the left hilum facilitated by a tip-curved (22G) gavage needle. Both ends of the suture were threaded through a 5-mm long PE-50 tube. Hilar occlusion was achieved by synching down the hilar suture and securing the PE-50 tube with a small surgical clip to maintain consistent tension against the hilum. The thoracotomy was then suture-closed, and the mouse was extubated and allowed to recover during the entire 1 hr hilar occlusion period. Mice were extubated during the ischemic and reperfusion periods in order to minimize injury due to mechanical ventilation. The average time on the ventilator for each animal was 10 min. Five minutes before reperfusion was to begin, the mouse was re-anesthetized and re-intubated. Reperfusion was achieved by removing the clip, tube and hilar suture. Again, the chest was suture-closed. The mouse was extubated and placed back in the cage during the 2-hr reperfusion period. Sham animals received the same surgeries as described above but without hilar occlusion.

Measurement of pulmonary function. At the end of the 2-hr reperfusion period, pulmonary function was evaluated using an isolated, buffer-perfused mouse lung system (Hugo Sachs Elektronik, March-Huggstetten, Germany) as previously described (E3, E4). Once properly perfused and ventilated, the isolated lungs were maintained on the system for a 5-min equilibration period before data was recorded for an additional 5 min. Hemodynamic and pulmonary parameters were recorded by the PULMODYN data acquisition system (Hugo Sachs Elektronik). The values during the 5-min data acquisition period remained constant in all animals, and the pulmonary function recorded for each animal reflected the average of this 5-min period.

Bronchoalveolar Lavage (BAL). After pulmonary function measurements, left lungs were lavaged with 0.4 ml PBS, and the BAL fluid was stored at –80°C until further analysis.

Bronchoalveolar lavage. After pulmonary function measurements, left lungs were lavaged with 0.4 ml PBS. The BAL fluid was centrifuged at 4°C (500 *g*, 5 min), and the supernatant was collected and stored at –80°C until further analysis.

Cytokine measurements. Cytokine content in BAL fluid was quantified using the Bioplex Bead Array technique using a multiplex cytokine panel assay (Bio-Rad Laboratories, Hercules, CA). The final concentration was expressed as pg/ml.

Measurement of MPO. MPO levels were measured in BAL fluid using a commercially-available mouse MPO ELISA kit (Cell Sciences, Canton, MA).

Lung wet/dry weight ratio. Using separate groups of animals (n=5/group), fresh lungs were harvested, weighed, and then placed in a 54°C vacuum oven until a stable dry weight was achieved. The lung wet/dry weight ratio was then calculated as an indicator of edema.

Pulmonary microvascular permeability. Microvascular permeability in lungs was estimated using the Evans blue dye extravasation technique, which is an index of change in protein permeability, as previously described (E5). Using separate groups of animals, Evans blue (20 mg/kg, Sigma-Aldrich) was injected intravenously via tail vein 30 min before euthanasia. The pulmonary vasculature was then perfused for 10 min with PBS to remove intravascular dye. Lungs were then homogenized in PBS to extract the Evans blue and centrifuged. The absorption of Evans blue was measured in the supernatant at 620 nm and corrected for the presence of heme pigments: A_{620} (corrected) = A_{620} - (1.426 x A_{740} + 0.030). The concentration of Evans blue was determined according to a standard curve and expressed as μg/gram wet lung weight.

Immunohistochemistry and neutrophil counting. Lungs were inflation-fixed (under constant 20 cm H_2O pressure) with 4% paraformaldehyde at 4°C for 15 hours and then embedded in paraffin. Immunostaining of lung sections was performed with rat anti-mouse neutrophil antibody (GR1.1, Santa Cruz Biotechnology) using Vectastain ABC kit (Vector Laboratories, Burlingame, CA) as described previously (E3). Alkaline phosphatase–conjugated anti-rat immunoglobulin G (Sigma Aldrich, St. Louis, MO) was used as secondary antibody. The signals were detected with Fast-Red (Sigma Aldrich, St. Louis, MO). Purified normal rat immunoglobulin G (eBioscience Inc, San Diego, CA) was used as a negative control. The sections were counterstained lightly with hematoxylin. Lung sections (1 slide per mouse) were used for semi-quantitative cell counts in peripheral lung tissue. These cell counts did not distinguish among cells in various components of the lung (e.g. airspace, interstitial or marginated) but included all cells in peripheral (alveolar) lung tissue. For each lung, neutrophils were counted in five random fields at 40X magnification and averaged.

Purification and adoptive transfer of $CD4^+$ T cells. Splenocytes were isolated from mice and placed in RPMI 1640 containing 10% FCS. Cells were released by blunt dissection of spleen followed by incubation with collagenase D at 37°C (for 30 min), strained through a 40-µm nylon mesh (Collector Tissue Sieve; E-C Apparatus), and washed in PBS. Red blood cells were removed by hypertonic lysis buffer (Sigma Aldrich, St. Louis, MO) for 5 min at room temperature and three washes with 10% FCS-containing medium. To facilitate $CD4^+$ T cell separation, a commercially available magnetic bead-based cell isolation kit was utilized (Miltenyi Biotec, Germany). We routinely achieved a relative enrichment of $CD4^+$ T cells of >90% using this negative selection process. Purified $CD4^+$ T cells (2×10^7 cells/animal) were injected into recipient Rag-$1^{-/-}$ mice via tail vein 7 days before study. Efficient reconstitution of $CD4^+$ T cells

in both spleen and lungs was documented by flow cytometry after staining with PE anti-TCRβ (5 μg/ml, H57-597; BD Biosciences), APC anti-CD4 (5 μg/ml, RM4-5; BD Bioscience), and blocking of nonspecific Fc binding with anti-mouse CD16/CD32 (10 μg/ml; eBioscience). Subsequent flow cytometry data acquisition was performed on FACS-Canto (BD Biosciences), and data were analyzed by FlowJo software (Tree Star).

Purification and adoptive transfer of iNKT cells. Spleens were removed from mice, and splenocytes were passed through a 40-μm nylon cell strainer (BD Biosciences) and collected in PBS. After lysis of red blood cells, cells were suspended in FACS staining buffer containing anti-mouse CD16/CD32 mAb (eBioscience) to block non specific FcR binding. Cells were then incubated with CD1d tetramer-Alexa647 (NIH Tetramer Facility at Emory University) and enriched by positive magnetic bead selection using anti-Alexa647 microbeads (Miltenyi Biotec, Germany). The enriched cells were stained for 30 min with FITC-conjugated anti-CD19 and PE-conjugated anti-TCRβ (eBioscience) and sorted using a FACSVantage SE Turbo Sorter (Becton Dickinson) resulting in cell populations of >98% pure CD1d tetramer^{+} TCRβ^{+} iNKT cells. Purified iNKT cells (2.5 x 10^{5}) were then adoptively transferred into adult, male Jα$18^{-/-}$ mice via tail vein injection 4 days prior to lung IR.

Flow cytometry. Left lungs from mice were minced and incubated for 15 min at 37°C with collagenase type IA (Sigma) in Dulbecco's PBS buffer with 0.5% BSA and 2mM EDTA. The digested lung tissue suspension was then passed through a 40-μm cell strainer (BD Falcon) and centrifuged at 1000 rpm for 10 min. The cell pellet was washed with 0.5% BSA in PBS and resuspended in 1 ml FACS staining buffer (0.1% BSA, 0.01% sodium azide in PBS). Cells were blocked with anti-mouse CD16/CD32 (1 μg/ml; eBioscience) before surface labeling, and 7-aminoactinomycin (7-AAD; 2 μg/ml; Invitrogen) was added to the surface labeled samples 15

min before analyzing the samples to distinguish between live (7-AAD negative) and dead (7-AAD positive) cells. APC–Alexa Fluor 750–labeled CD45 (eBioscience), FITC labeled TCRβ or Gr-1 (Ly6G) (eBioscience), V450 labeled CD4 (BD Bioscience), V500 labeled B220 (BD Bioscience), and Alexa647 labeled CD1d tetramer loaded with PBS57 (1:500), an analog of α-galactosylceramide (αGalCer) (NIH Tetramer Facility at Emory University), were used for the surface staining. Intracellular IL-17A detection was performed by FACS as previously described by our lab (E6). AD- (actinomycin, 10μg/ml, Invitrogen) and PE-labeled anti-mouse IL-17A (TC11-18H10; BD Biosciences) were added to the samples following cell fixation and permeabilization using a cytofix/perm kit (BD Bioscience). Intracellular isotype controls were performed at the same time. Cells were washed, and data was analyzed by 8 color FACS (Cytek). FACS data was analyzed by using Flowjo software 8.8.

For cell counting by FACS, the lung cell suspension (50 μL) was mixed thoroughly with 120 μL FACS staining buffer and 30 μL of Caltag Counting Beads (994 beads/μL) before acquisition by 8 color FACS (Cytek). Caltag Counting Beads (Invitrogen Life Technologies) were used to normalize for differences in cell recovery among samples. At least 1000 bead events over 2 min were acquired to ensure the accuracy of the assay. $CD45^+$ cell absolute count (per lung) = events of $CD45^+$ cells counted/total number of beads counted (A+B) X input bead number. The leukocyte subset cell number (per lung) was multiplied by the $CD45^+$ cell number and by the percentage of the subset. For example, CD1d $tetramer^+$ cell number (per lung) = total $CD45^+$ cell number X percent of CD1d $tetramer^+$ gated on the $CD45^+$ $B220^-$ $TCR\beta^+$ population.

ELISPOT Assay. $CD4^+$ T cells and neutrophils were isolated and purified from WT lungs after sham or IR surgery (n=5/group) using magnetic bead-based separation protocols via $CD4^+$ T cell and Ly-6G microbead kits, respectively (Miltenyi Biotec, Germany). The purity of

these cells (>95%) was confirmed by FACS analysis. $CD4^+$ CD1d $tetramer^+$ iNKT cells were isolated from lungs via cell sorting using FACS turbo sorter (Becton Dickinson). Cells were then assessed for IL-17A production using a commercially available murine IL-17A ELISPOT assay (R&D Systems, Minneapolis, MN). In precoated wells, $CD4^+$ T cells (1×10^5), $CD4^+$ CD1d $tetramer^+$ iNKT cells (0.5×10^5), or neutrophils (1×10^5) were plated in duplicate wells with medium containing 50 ng/ml phorbol 12-myristate-13 acetate (PMA) and 0.5 μg/ml calcium ionomycin for 3 hrs at 37 ^oC. Neutrophils which were not stimulated with PMA/ionomycin were also included as controls. The plate was then processed according to the manufacturer's instructions, and the spots were manually counted under a dissection microscope. Results are presented as the average number of spot forming cells (SFC) per total number of cells plated and corrected for the background medium.

Statistics. PASW statistics18 software was used for all statistical analysis. Values are presented as the mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) with post-hoc Bonferroni's multiple comparisons, two-way ANOVA, or Student's t-test was used as appropriate to compare experimental groups. A P value of less than 0.05 was considered significant.

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