Online data supplement

Pulmonary Neutrophil Infiltration in Murine Sepsis: Role of Inducible Nitric Oxide Synthase

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Methods.

Reciprocally Bone-marrow (BM)-Transplanted (BMT) iNOS Chimeric Mice.

Reciprocally BMT iNOS chimeric mice were generated as previously described (E1,E2). At four weeks of age, recipient mice were BM-depleted by lethal irradiation (960 cGy via ^{60}Co). Within four hours, donor BM was infused intravenously, and BM reconstitution allowed to occur over four to six weeks. During this time, BMT mice were kept individually in sterile filter isolator cages, and administered neomycin (100 mg/L) and polymyxin B (10mg/L) in sterile drinking water.

Based on the iNOS genotype of the BM donor mice and recipient mice, two reciprocal BMT iNOS chimeric groups were generated: +to- and -to+. +to- indicates that iNOS+/+ BM cells were transplanted into iNOS-/- recipient mice, such that iNOS expression and activity are limited to donor BM-derived cells, including macrophages and neutrophils, with no iNOS in recipient tissue stromal (i.e. parenchymal) cells. In the reciprocal -to+ BMT chimeric mice, iNOS expression/activity are restricted to recipient stromal cells, whereas all donor BM-derived inflammatory cells are iNOS-/-. A third BMT group, +to+, was also generated, in which iNOS+/+ BM was infused into iNOS+/+ recipient mice, such that all cells are iNOS+/+, similar to wild-type mice. This important group controls for the lethal irradiation and BMT procedures.

Pulmonary Intravital Videomicroscopy (IVM).

Pulmonary microvascular neutrophil sequestration was quantified by pulmonary IVM using a modification of a technique we previously described for rats (E3). At various time points after sham or CLP procedures, mice were anesthetized (subcutaneous ketamine 200mg/kg, and xylazine 10mg/kg). The mice were then tracheotomized and subjected to mechanical ventilation

at the following settings: tidal volume 0.1 mL, rate 130 breaths/minute, PEEP 2 cm H2O, FiO2 1.0 (Harvard rodent respiration pump, model 683, Harvard Apparatus, South Natick, MA). A 10-mm diameter, transparent window was implanted in the right lateral thoracic wall and the mice were placed in the right lateral decubitus position on the stage of an inverted intravital videomicroscope (DIAPHOT-300/DIAPHOT-200 epi-fluorescence microscope, Nikon Inc. Melville NY) such that the thoracic window was apposed to an opening in the stage. In this position, respiratory motion of the dependent right lung is minimized, but not eliminated (Figures E1, E2). A working distance 32x10.4 objective was used. The field of view was visualized by digital videophotography (MTI-VE 1000 Video Camera, Dage-MTI, Michigan City IN), and was displayed on a video monitor (WV-BM 1400 Panasonic video monitor, Markertek Video Supply, Saugerties, NY), and captured on videotape.

Neutrophils were fluorescently-labelled *in vivo* by intravenous administration of dihydro-rhodamine-6G (9 μmol/kg, Sigma) and were visualized by fluorescence Leitz N2 filter block (excitation 530-560 nm, emission 580 nm). Fluorescent neutrophils that were stationary in the field for >10 seconds were counted. We have chosen this longer, more conservative time to define stationary neutrophils than previously reported in rats (2 seconds) and rabbits (5 seconds), in order to not overestimate the numbers of pulmonary microvascular sequestered neutrophils (E4,E5). Similar to our previous observations in rats (E3), sepsis in mice was not associated with increased pulmonary microvascular neutrophil rolling (data not shown).

In Vitro Trans-Endothelial Neutrophil Migration.

Mouse pulmonary microvascular endothelial cells (PMVEC) were isolated by the dual microbead technique (E6). Naïve iNOS+/+ and iNOS-/- mice were sacrificed with subcutaneous

pentobarbital, the pulmonary vasculature flushed with 10 mL phosphate buffered saline, and the lungs removed. Mouse lung tissue was finely minced into 2-3mm pieces, placed into a sterile Ca^{++}, Mg^{++}-free Hanks Balanced Salt Solution (HBSS, Sigma, St. Louis, MO) buffer containing 100U/ml penicillin and 100µg/ml streptomycin, and rinsed thoroughly. The lung tissue was digested (Ca^{++}, Mg^{++}-free HBSS containing 500 U/ml collagenase II, 0.6 U/ml Dispase, and 0.1% bovine serum albumin) for 40 min at 37° C under constant bidirectional agitation. The tissue was mechanically dissociated using a Dounce homogenizer, the resultant cell suspension filtered sequentially through 100µm and 30µm nylon mesh and washed twice in M199 cell culture medium. Cells and microvascular fragments were pelleted at 200xg at 4°C and resuspended in M199 medium containing lectin (Bandeiraea simplicifolia, BS-I)-coated microbeads (Dynal, Inc., Lake Success, NY) at a 4:1 bead to cell ratio. The cells with attached microbeads were magnetically captured (MPC magnet, Dynal Inc). PMVEC were seeded in gelatin-coated cell culture flasks and incubated in EGM medium with supplements for microvascular cells (Clonetics media, Cambrex Bio Science. Baltimore, MD) until confluent. This procedure yields cell populations consisting of 75-80% of microvascular endothelial cells as confirmed by the fluorescin-isothiocyanate-labeled (FITC) acetylated-low density lipoprotein (LDL) uptake assay.

To increase PMVEC homogeneity, confluent monolayers (passage 1) were trypsinized, washed in Ca^{++}, Mg^{++}-free HBSS buffer, and then resuspended and incubated for 30 minutes in M199 medium containing 20µg/ml monoclonal anti-platelet-endothelial-cell-adhesion-molecule (PECAM)-1 (CD-31) antibody under constant bi-directional agitation. Subsequently, the cells were washed twice, and were incubated in medium containing microbeads M-450 (Dynal Inc.) coated with goat anti-mouse monoclonal antibody at room temperature for 20 minutes under

constant bi-directional agitation. Microbead-coated cells were again captured magnetically. PMVEC were seeded in gelatin-coated cell culture flasks and incubated in EGM medium with endothelial cell growth supplements (Clonetics). The dual magnetic bead technique yields >90% homogeneity of PMVEC as confirmed by staining for von Willebrand factor, PECAM-1, and the uptake of FITC-labeled acetylated-LDL. 90% confluent PMVEC monolayers were trypsinized in 0.025% trypsin / 0.01% EDTA buffer, and subcultured at a ratio 1:3. PMVEC were used for experiments at passage 3-5.

iNOS+/+ and iNOS-/- mice were sacrificed with subcutaneous pentobarbital, tibias and femurs were aseptically removed, both ends of each bone transected, and each long bone was flushed with HBSS. Harvested BM tissue plugs were meticulously broken up by tituration, and single cell suspensions were separated by centrifugation (1060 x g, 30 mins, 4°C) on a three-step Percoll gradient (78%, 68%, 54%). The mature neutrophils, which sediment at the lower boundary, were carefully aspirated, washed with HBSS, and counted using a hemecytometer. Cytospin slides of these purified neutrophils revealed purity >98%, and trypan blue exclusion showed >99% viability.

References

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Figure Legends.

Figure E1. Representative video recording of fluorescent intravital videomicroscopic images of naive iNOS+/+ mouse lung, following intravenous infusion of fluorescently-labelled (rhodamine) neutrophils. Three neutrophils are stationary in the microvascular network. Note that the video recording is looped.

Figure E2. Representative video recording of fluorescent intravital videomicroscopic images of septic iNOS+/+ mouse lung, following intravenous infusion of fluorescently-labelled (rhodamine) neutrophils. A large number (approximately 20) of neutrophils are stationary, and two neutrophils transiently pass through the microvascular network. Note that the video recording is looped.