Surfactant Proteins A and D Suppress Alveolar Macrophage Phagocytosis via Interaction with SIRPa

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ONLINE DATA SUPPLEMENT

Supplemental Materials and Methods

Antibodies and Reagents

Anti-SIRPα P84 hybridoma and SIRPα constructs were a gift from Dr. Axel Ullrich (1). Anti CD-47 mIAP 301 was provided by Dr. Frederik Lindberg (Washington University) (2). Soluble CD-47-Fc ligand was a gift from Dr. E. J. Brown (University of California) (3). Rabbit anti-human SP-A IgG and rabbit anti-mouse SP-A IgG antibodies were obtained from Chemicon (Temecula, CA). Rat anti-mouse CD3 was purchased from BD Biosciences (Franklin Lakes, NJ). Sodium stibogluconate and Rho-kinase inhibitor Y-27632 were purchased from Calbiochem (San Diego, CA). Purified human C1q (10μg/ml) was obtained from Quidel Corp (San Diego CA). Lipopolysaccharide (*Escherichia coli* O55:B5) was obtained from List Biological Laboratories (Campbell, CA).

Animals

ICR mice (8-10 weeks old) were obtained from Taconic (Germantown, NY) and used in all murine experiments unless otherwise indicated. SP-D knockout (KO) mice were a kind gift from Dr. James Fisher (Denver, Colorado) (4). SP-D KO mice have no detectable SP-D protein in bronchoalveolar lavage fluid and SP-A levels are decreased by 25%(4). SP-D KO mice were outbred with five generations of National Institutes of Health Swiss Black mice. Age and gender-matched wild type Swiss Black controls were used in all experiments. SHP-1 -/- ($Ptpn6^{mev}/Ptpn6^{mev}$) mice and littermate controls were obtained from Jackson Laboratory (Bar Harbor, Maine) at 4 weeks of age. They were housed for one week under pathogen-free

conditions before use. Wild type C57BL/6 mice and green fluorescent protein (GFP)-expressing mice (C57BL/6-Tg(UBC-GFP)30Scha/J) were also obtained from Jackson Laboratory.

Generation of Chimeric GFP-Expressing Mice

Bone marrow cells were isolated under sterile conditions from the hindlimbs of 4 week-old GFP-expressing donor mice. Briefly, femurs and tibias were removed from donor mice and extra muscle and connective tissue were mechanically dissociated. Marrow cells were liberated by gently crushing the bones and then filtering cell isolates through a 70-μm mesh (BD Biosciences) in DMEM supplemented with 10% FCS. Cell suspensions were centrifuged for 10 minutes at 200*g* and decanted. Pellets were resuspended in sterile HBSS, filtered through a 40-μm mesh, and centrifuged at 200*g*. Following a third wash step, the cells were resuspended in HBSS at a concentration of $25x10^7$ cells/ml. Recipient C57BL/6 mice were sedated with Avertin and then exposed to 9 Gy of total body irradiation. To protect resident alveolar macrophages from radiation injury, lead strips (1 cm thick x 2 cm wide) were positioned directly over the thorax. A total of $5x10^7$ GFP-expressing cells were transplanted via lateral tail vein injections into recipient animals in a volume of 0.2 ml. Resulting chimeric mice were characterized by GFP-negative resident alveolar macrophages and GFP-positive hematopoietic cells. Chimeric mice were housed under specific pathogen-free (SPF) conditions with antibiotic-containing food. LPS (200 μg in 50 μl PBS) was administered via direct intratracheal injection 4 weeks after transplantation, a time at which over 90% of RAM were of host origin (GFP-negative) and greater than 95% of peripheral blood leukocytes were of donor origin (GFP-positive).

Isolation of Primary Cells

Human alveolar macrophages (AMs) were isolated by bronchoalveolar lavage (BAL) from normal volunteers (7). Human AMs were plated in 24-well tissue culture plates (BD Biosciences, Franklin Lakes NJ) at 1 x 10^6 AMs/well, allowed to adhere for 30 min, washed with X-vivo medium (Biowhittaker, Wakersville MA), and incubated for an additional 2 h before experimentation. Human neutrophils were obtained from healthy donors and isolated via density centrifugation as previously described (8).

Mouse AMs were isolated immediately after euthanasia of the animals with intraperitoneal pentobarbital (Abbott Laboratories, North Chicago, IL). BAL was performed ten times on each mouse using 1 ml aliquots of ice-cold PBS supplemented with 100μM EDTA. Cell suspensions were centrifuged at 200*g* for five minutes, excess fluid was decanted and AM were resuspended in culture media. For experiments in which AM were isolated from LPS-treated mice, purification was performed as previously described (9). Briefly, BAL samples were centrifuged, washed once in Hanks balanced salt solution (HBSS), layered over a Percoll gradient (78%, 66%, and 54%) and centrifuged at 1060*g* for 30 minutes. Cytospin samples of the 66%: 54% interface contained more than 90% macrophages.

Mouse peritoneal macrophages (PMs) were isolated from mice following euthanasia with inhaled CO_2. Peritoneal lavage was performed with 10ml of ice-cold HBSS. Lavage fluid was centrifuged and PMs were resuspended in DMEM supplemented with 10% fetal calf serum and plated overnight in culture.

Murine thymocytes were isolated from the thymuses of 4-week-old, female C57 BL/6 mice. Thymuses were removed freshly euthanized animals and then gently ground through a sterile 40-μm strainer (Fisher Scientific) to separate individual cells. The cells were washed twice in PBS before use.

Transient Cell Transfection with SIRPα

Active SIRPα constructs and mutant SIRPα constructs were obtained from Dr. Axel Ullrich (Martinsried, Germany). In the mutant constructs, all 4 tyrosines in the intracellular domain were replaced with asparagine to render the molecule inactive (12). Swiss 3T3 cells, which do not contain SIRPα de novo, were plated in 6-well plates (3.0×10^5 cells/ml) for 24 hrs. Transfection was carried out using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 48 hours, the cells were pre-treated with SP-A (10μg/ml) and CD47-Fc (10 μg/ml) for 20 minutes, washed once to remove unbound protein, and then incubated with Jurkat T-cells as above.

Induction of Apoptosis

Apoptosis was induced in Jurkat T cells, murine thymocytes, and human neutrophils by exposure to ultraviolet radiation (254 nm) for 10 minutes as described previously (13). Jurkat T cells or murine thymocytes were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 for 3 h before use. Apoptosis was verified by nuclear condensation using light microscopy on Wright-Giemsa stained samples. Flow cytometry confirmed that greater than 85% of cells treated in this fashion were annexin V positive. Approximately 25% were propidium iodide positive. Human neutrophils were cultured in RPMI 1640 containing 0.5% BSA at 37°C for 90 minutes. Apoptosis was then assessed by light microscopy on Wright-Giemsa stained samples every 20 minutes. Neutrophils were deemed ready for use when 80% of neutrophils exhibited nuclear condensation.

Flow Cytometry

BAL samples were fixed in 4% parformaldehyde immediately after BAL. Cells were then suspended in HBSS containing 2% FBS (Atlanta Biologicals, Atlanta, GA) at a concentration of 10^6 cells per 100µl. FcγR was blocked using mAb 2.4G2 (Becton Dickinson, Mountain View, CA). Cells were incubated with 1µg of primary antibody on ice for 30 minutes, washed twice, and then incubated with secondary antibody (1:50 dilution) on ice for 30 minutes. Flow cytometry was performed using a FACScan cytometer (Becton Dickinson). Data were collected using Cellquest software (Becton Dickinson) and analyzed with Flowjo software (Tree Star, Ashland, OR). Cell sorting was performed on fresh BAL specimens resuspended in DMEM with 10% FBS using a MoFlo cell sorter (Dako, Denmark). For cell sorting and flow cytometry experiments, resident AM and recruited mononuclear phagocytes were distinguished from each other and from neutrophils based on forward-scatter, side-scatter, and FL1 autofluorescence.

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