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Enhanced Susceptibility to Endotoxic Shock and Impaired STAT3 Signaling in CD31-Deficient Mice

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Platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31), an adhesion molecule expressed on hematopoietic and endothelial cells, mediates apoptosis, cell proliferation, and migration and maintains endothelial integrity in addition to its roles as a modulator of lymphocyte and platelet signaling and facilitator of neutrophil transmigration. Recent data suggest that CD31 functions as a scaffolding protein to regulate phosphorylation of the signal transducers and activators of transcription (STAT) family of signaling molecules, particularly STAT3 and STAT5. STAT3 regulates the acute phase response to innate immune stimuli such as lipopolysaccharide (LPS) and promotes recovery from LPS-induced septic shock. Here we demonstrate that CD31-deficient mice have reduced survival during endotoxic LPS-induced shock. As compared to wild-type controls, CD31-deficient mice showed enhanced vascular permeability; increased apoptotic cell death in liver, kidney, and spleen; and elevated levels of serum tumor necrosis factor α (TNF-α), interferon γ (IFNγ), MCP-1, MCP-5, sTNRF, and IL-6. In response to LPS *in vivo* and *in vitro*, splenocytes and endothelial cells from knockout mice had reduced levels of phosphorylated STAT3. These results suggest that CD31 is necessary for maintenance of endothelial integrity and prevention of apoptosis during septic shock and for STAT3-mediated acute phase responses that promote survival during septic shock. *(Am J Pathol 2005, 166:185–196)*

Platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31) belongs to the immunoglobulin family of cell adhesion molecules.[1–3] CD31 mediates homophilic and heterophilic binding in hematopoietic and endothelial cells and also modulates intracellular signaling.[4–10]

CD31-mediated adhesion enhances transendothelial migration of leukocytes to sites of acute inflammation.[11] Recent work also suggests that homophilic CD31 binding between endothelial cells maintains vascular integrity and prevents prolonged changes in permeability.[12] Cultured endothelial cells from CD31-deficient mice demonstrate enhanced transendothelial migration of T lymphocytes and prolonged permeability changes in response to histamine. In experimental autoimmune encephalomyelitis (EAE), the animal model of human multiple sclerosis, CD31-deficient mice develop earlier onset of disease due to enhanced migration of immune cells into the brain and spinal cord.

CD31 regulation of intracellular signaling occurs through recruitment of adapter and signaling molecules to an immunoreceptor tyrosine activation motif (ITAM) on the cytoplasmic domain of CD31.[6–10] By serving a scaffolding function, it has been hypothesized that CD31 mediates tyrosine phosphorylation of two members of the STAT (signal transducers and activators of transcription) family, STAT3 and STAT5.[8] Based on its multiple roles in cell adhesion, leukocyte migration, and cell signaling, we reasoned that CD31 would also regulate the acute phase response (APR). Either infection or tissue injury can trigger the APR.[13] In acute infection, binding of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) on gram-negative bacteria and lipotechoic acids on gram-positive bacteria to the Toll-like family of pattern recognition receptors initiates the APR.[14] Although this innate immune response is necessary to host survival during severe infection, impaired regulation of the APR can lead to septic shock.[15] For example, activation of Toll receptors on macrophages and other immune cells leads to local release of pro-

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inflammatory cytokines such as interleukin 6 (IL-6).[16] IL-6 and related cytokines signal through phosphorylation of the transcription factor, STAT3.[17,18] During the APR, phosphorylated STAT3 (pSTAT3) stimulates transcription of pro- and anti-inflammatory molecules. In cell-specific gene knockout models, STAT3 deficiency in hepatocytes impairs the APR to LPS, and, in mice with STAT3-deficient monocytes and neutrophils, there is reduced survival from endotoxic shock.[19,20]

We hypothesized that CD31 maintenance of endothelial integrity and regulation of phosphorylation of STAT3 enhance recovery from endotoxic shock. We here show that CD31-deficient mice are markedly more sensitive to LPS-induced shock as compared to wild-type (WT) mice. In response to LPS, these mice demonstrate reduced survival, increased vascular permeability and apoptosis in solid organs, elevated serum TNF-α, IFNγ, MCP-1, MCP-5, sTNFR1, and IL-6 and decreased levels of phosphorylated STAT3.

Materials and Methods

Mice

Female C57Bl/6CR mice (6 to 8 weeks of age) were obtained from Charles River Laboratories (Wilmington, MA). CD31-deficient mice on the C57Bl/6CR background were generated and analyzed as described previously.[12,21] They were bred in our facility at Yale University and have been backcrossed onto the C57Bl6 background for greater than 10 generations.[12]

LPS-Induced Endotoxic Shock

LPS (*E. coli* serotype 055:B5; Sigma Chemical Co., St. Louis, MO) was administered intraperitoneally (i.p.) at doses of 200 μg or 600 μg per mouse in 200 μl phosphate-buffered saline (PBS). Control mice received PBS. Clinical status was monitored twice daily for 7 days.

Vascular Permeability

One day after a 200-μg dose of LPS, Evans blue dye was injected intravenously. One hour later, mice were anesthetized with ketamine/xylazine, and intracardiac perfusion was performed with ice-cold PBS. Lung, liver, and kidney were isolated, and dye was extracted in formamide (5 μl/mg of tissue) for 3 days at room temperature. Absorbance at 650 nm was measured to determine dye concentration as described.[12]

Histology

Wild-type and CD31-deficient C57BL6 were injected with 200 μg of LPS i.p. One day later, mice were anesthetized with ketamine/xylazine, and intracardiac perfusion was performed with ice-cold PBS followed by paraformaldehyde-lysine-periodate (PLP) fixative. Lung, liver, and kidney were harvested and fixed in PLP overnight. Tissue was dehydrated through graded ethanol, cleared in xylene, and embedded in paraffin. Five-μm sections were stained with hematoxylin/eosin (H&E).

Cytokine Analysis

Mouse Th1/Th2 Cytokine CBA (BD Biosciences, San Jose, CA) was performed according to manufacturer's instructions (Manual No. 551287) on serum samples obtained by eye bleed from wild-type or CD31-deficient mice treated with 10 μg/g LPS for 24 hours.

ELISA for mouse TNF-α was obtained from Endogen (Endogen-Pierce mouse TNF α ELISA Minikit No. KMT-NFA) and performed according to manufacturer's instructions (Pierce Biotechnology, Inc., Rockford, IL).

Mouse Cytokine Antibody Array (Kit No. MA6060) (Panomics, Redwood City, CA) was performed on serum from mice injected with 10 μg/g LPS for 6, 12, or 24 hours, plus saline controls, according to manufacturer's instructions.

Splenocyte Cell Culture

Sex- and age-matched wild-type and CD31-deficient C57Bl6 mice were sacrificed by cervical dislocation and spleens removed under sterile conditions. Splenocytes were teased from the spleens by crushing between the frosted ends of microscope slides in sterile PBS. The resulting cell suspension was then run through a cell strainer (Falcon), spun out, and the red blood cells lysed in ACK buffer (0.15 mol/L NH_4Cl, 10 mmol/L $KHCO_3$, 0.1 mmol/L Na_2EDTA pH 7.2). Lymphocytes were then isolated by gradient centrifugation in LSM (ICN Biomedicals) and plated at 5.5×10^6 cells in 60-mm dishes. Lymphocytes were then exposed to 0, 10, and 100 ng/ml LPS (Sigma, *E. coli* serotype 055:B5) for 24 hours in Clicks Media (Irvine Scientific) supplemented with 10% fetal bovine serum (FBS), L-glutamine, pen/strep, and 2-mercaptoethanol. Cells were lysed in 20 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10 mmol/L $Na_4P_2O_7$, 1% Brij-35, Complete Protease Inhibitors (Roche), 5 mmol/L NaF, 250 μmol/L NaOV, and 1 mmol/L PMSF, and assayed for protein content by bicinchoninic acid (BCA) assay (Pierce Biotechnology, Inc., Rockford, IL). Twenty μg of each sample was loaded onto 8% SDS-PAGE for immunoblotting with anti-pY(705) STAT3 and anti-STAT3 (Cell Signaling, both at 1:1000 in 0.05% T-TBS plus 1% normal donkey serum, followed by anti-rabbit IgG-HRP conjugate. Signals were detected with Western Lightning Reagent (Perkin Elmer, Boston, MA) on Hyperfilm MP (Amersham/Pharmacia).

Endothelial Cell Culture

Immortalized CD31-reconstituted and CD31-deficient mouse lung endothelial cells were cultured in Dulbecco's modified Eagle's medium enriched with 10% FBS, L-glutamine, non-essential amino acids, sodium pyruvate, HEPES, and β-mercaptoethanol as described.[12] Puromy-