Paraplegia remains a significant complication of thoracoabdominal aortic interventions. In some high-risk populations, lower limb deficits can approach 15%. Although perioperative approaches have reduced overall paraplegia rates, the lack of mechanistic understanding of both ischemic insult and over-exuberant inflammatory response to reperfusion has limited the development of pharmacological agents to attenuate this injury.

Resident macrophages have been implicated in exacerbating tissue injury after ischemic insults in a variety of tissue beds. A reduction of ischemia–reperfusion (IR) injury after macrophage depletion has been shown in the kidney, liver, and lung. Microglia are the resident macrophages of the central nervous system and become rapidly activated after injury. Inhibition of microglial activation has demonstrated neuroprotective effects in multiple models in the central nervous system.

Microglial activation can occur through a toll-like receptor (TLR)-4-mediated pathway. The presence of functional TLR-4 has shown deleterious effects in neurodegenerative and stroke models and in some disease processes plays an integral part in microglial signaling. We hypothesize that delayed paraplegia after thoracic aortic occlusion results, in part, from TLR-4–mediated microglial activation in mice.

Methods

Animal Procedures

The Animal Care and Use Committee at the University of Colorado approved all experiments, and preoperative approaches have reduced overall paraplegia rates, the lack of mechanistic understanding of both ischemic insult and over-exuberant inflammatory response to reperfusion has limited the development of pharmacological agents to attenuate this injury.

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Microglial activation can occur through a toll-like receptor (TLR)-4-mediated pathway. The presence of functional TLR-4 has shown deleterious effects in neurodegenerative and stroke models and in some disease processes plays an integral part in microglial signaling. We hypothesize that delayed paraplegia after thoracic aortic occlusion results, in part, from TLR-4–mediated microglial activation in mice. To test this hypothesis, the effects of intact TLR-4 receptor will be tested with regard to motor function, neuronal viability, and cyto-architectural changes after thoracic aortic occlusion in a murine model of delayed paraplegia. Furthermore, to implicate the role of microglial TLR-4 signaling in the deleterious effects after ischemia, mutant and wild-type microglia will be isolated, subjected to oxygen and glucose deprivation (OGD), and analyzed for the expression of TLR-4 and proinflammatory cytokines.
this investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (www.nap.edu/catalog/5140.html). Male mice aged between 10 and 20 weeks were used for all experiments. C3H/HeJ mice with TLR-4 loss-of-function mutation were obtained from Jackson Laboratories. Wild-type C3H/HeNc/NcIbr mice with functional TLR-4 were obtained from Charles Rivers Laboratories.

**Aortic Cross-Clamping**
Mice were anesthetized using 2% isoflurane and placed in the supine position. Surgery was performed under normothermic conditions, with core body temperature maintained at 36.5±0.5°C using a rectal temperature probe and automatic temperature-adjusting bed (Vestavia Scientific, Birmingham, AL). The aortic arch was exposed using a cervicohoracic approach as previously described. Occclusion was achieved by placing vascular clamps on the aortic arch distal to the left common carotid artery and the subclavian artery for 5 minutes. To confirm ≥90% reduction in distal flow, a laser Doppler blood flow monitor (Moor Instruments, UK) was placed over the left femoral artery.

**Functional Scores**
The Basso Mouse Scale (BMS), which ranges from a score of 0 for complete paraplegia to a score of 9 for normal function, was used to quantify hind-limb function in mice after ischemia. Function was scored at 12, 24, 36, 48, and 60 hours after reperfusion.

**Histological Analysis and Neuronal Quantification**
After 60 hours of reperfusion, the animals were euthanized. The vertebral column was removed en bloc from T8-L3. The spinal cord was removed from the vertebral column by injection of PBS (pH 7.4) into the spinal column. Spinal cords were transferred to 4% formalin where they remained for 24 hours before paraffin embedding, sectioning, and hematoxylin and eosin staining. Sections were evaluated blindly for neuronal viability quantified as neurons per microscopic field. Neurons within the anterior horn that contained prominent nucleoli and loose chromatin were considered normal.

**Microglial Isolation**
Adult male C3H/HeJ and C3H/HeNc/NcIbr mice were euthanized. The vertebral columns were removed en bloc as above. After dissection of spinal cords, the tissue slices were digested in a solution of Hibernate A (InVitrogen, Grand Island, NY) and papain (Worthington Biochemical Corporation, Lakewood, NJ). The digested tissues were triturated several times and applied to OptiPrep gradient (Sigma Aldrich, St. Louis, MO) to separate cell lines by a density gradient. After centrifugation, microglia were harvested for further plating on -lysine–coated dishes (Sigma-Aldrich, St. Louis, MO) with media of DMEM with high glucose and glutagro, 10% fetal bovine serum, glutamax, and pen/strep media (Gibco, Grand Island, NY). After 2 hours. Confluent cell cultures were returned to DMEM containing 10% fetal bovine serum, glutamax, and pen/strep media (Gibco, Grand Island, NY) and returned to a 37°C incubator with atmospheric oxygen. Media and cell cultures were removed at 1- to 36-hour time points for protein analysis.

**Oxygen and Glucose Deprivation**
DMEM without glucose (Gibco, Grand Island, NY) was pretreated in a humidified hypoxic chamber (1% O2, 5% CO2, 94% N) at 37°C for 2 hours. Confluent cell cultures were transferred to hypoxic chamber and placed in pretreated media where they remained for 24 hours. After OGD, cell cultures were returned to DMEM containing 10% fetal bovine serum, glutamax, and pen/strep media (Gibco, Grand Island, NY) and returned to 37°C incubator with atmospheric oxygen. Media and cell cultures were removed at 1- to 36-hour time points for protein analysis.

**Immunoblotting**
Spinal cords were removed from wild-type (n=9) and mutant (n=9) mice that had no manipulation or at 6 and 36 hours after IR surgery. Galectin-3/mac-2 (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a marker of microglial activation at 1:500 dilutions. Microglia cells were lysed with M-PER mammalian protein extraction reagent (Fisher Scientific, Pittsburg, PA). Microglial lysates were assessed for TLR-4 expression with TLR-4 polyclonal antibody (1:1000; Cell Signaling, Danvers, MA) at 1 to 24 hours after OGD. Protein extracts were placed in 4x Laemmli sample buffer with β-mercaptoethanol and boiled for 10 minutes at 100°C. loaded into a 15-well 4% to 20% gradient ready gels (Bio-Rad, Hercules, CA), and run at 160 V for 450 minutes. The gels were transferred to nitrocellulose membranes at 100 V for 60 minutes and then cross-linked using an ultraviolet Stratalinker (Stratagene, La Jolla, CA). The membranes were blocked in 5% dry milk in 0.1% Tween in PBS (T-PBS) and rinsed 3x in 0.1% T-PBS. The blocked membranes were incubated with primary antibody overnight at 4°C (5% BSA in 0.1% T-PBS). The membranes were washed in 0.1% T-PBS 3x and incubated in appropriate horseradish peroxidase-conjugated secondary antibodies diluted to 1:5000 in 5% BSA in 0.1% T-PBS for 1 hour at room temperature. SuperSignal West Dura Chemiluminescent Substrate for horseradish peroxidase was used for 5 minutes, and membranes were visualized by Chemidoc Hi Sensitivity (BioRad, Hercules, CA) scanner. Mean density values of bands were quantified by QuantityOne software (BioRad, Hercules, CA).

**Cytokine Quantification**
Expression of tumor necrosis factor (TNF)-α and interleukin-6 (IL-6) was chosen as likely outputs of activated microglia. Based on previous studies from our laboratory, time points of 6 and 36 hours were chosen. Spinal cords were removed from wild-type and mutant mice that had no manipulation or at 6 and 36 hours after IR surgery as above. A comparison of 3 mice in each genotype was made at each time point, with a total of 18 mice included in the analysis. Microglial cultures from 3 separate cell lines had media removed at 6, 24, and 36 hours after OGD, and cytokines were quantified using ELISA (R & D systems, Minneapolis, MN) performed according to the manufacturer’s instructions. Absorbance of standards and samples was determined spectrophotometrically at 595 nm using a microplate reader (Bio-Rad, Hercules, CA). Results were plotted against the linear portion of the standard curve, and the protein concentration of each sample was expressed as pg/mL of sample.

**Statistical Analysis**
Statistical analysis was performed using repeated measures and the Kruskal–Wallis nonparametric ANOVA with Bonferroni/Dunn post hoc test using StatView (SAS Institute, Inc, Cary, NC). Data are presented as mean±SE. A P<0.05 was considered significant for all statistical comparisons.

**Results**

**Functional Outcomes With TLR-4 Mutation**
Sham mice (n=4), 2 of each strain, showed no functional deficits at any point after surgery. Wild-type mice with functional TLR-4 (n=9) had a mild initial function deficit at 12 hours after IR, which progressed to moderate paralysis at 60 hours. Mutant mice (n=9) had mild functional deficit at 12 hours after aortic occlusion, which was not significantly different from wild-type mice. Mutant mice, however, did not exhibit the same progressive functional decline that was observed in mice with functional TLR-4. Statistical significance was observed at 24 hours until the duration of the experiment (Figure 1).

**Histological Changes**
Wild-type mice that underwent IR had an increased amount of pyknotic nuclei compared with mutant and sham mice, suggesting a greater degree of cellular injury. In addition, these
mice had impressive inflammatory infiltrates in the gray matter surrounding the anterior horn motor neuron, with distortion of the gray–white junction (Figure 2).

Neuronal Viability
All sham mice had significantly more anterior horn neurons compared with both the mutant and wild-type mice undergoing IR. Mice with mutant TLR-4 receptor had significantly more viable anterior horn neurons compared with wild-type mice (Figure 3).

Microglial Activation
Spinal cord microglial activation was assessed by galectin-3/mac-2 at 6 (Figure 4A) and 36 hours (Figure 4B) postoperatively and compared with baseline values. At both postoperative time points, wild-type mice had increased microglial activation compared with mutant mice and baseline values.

Cytokine Expression
Spinal cord microglial activation paralleled the production of proinflammatory cytokines. The absence of functional TLR-4 on microglial cells inferred a significant reduction in the expression of proinflammatory cytokines TNF-α and IL-6 both in vivo and in vitro (Figures 5 and 6). Statistical significance (*P<0.05) was observed for both IL-6 and TNF-α at 6 hours postoperatively. A reduction to values near baseline was observed at 36 hours, at which point statistical significance was lost (Figure 5). Similarly, wild-type microglia had a significant increase in the expression of proinflammatory cytokines. This was seen in a time-dependent manner and was significant with IL-6 at 24 and 36 hours. Although the concentrations of TNF-α were much lower than expected, there was a significant reduction in TNF-α expression by mutant microglia at 36 hours (Figure 6).

Expression of TLR-4 on Microglia After OGD
Isolated microglial cultures demonstrated the presence of TLR-4 with increased expression after 24 hours of OGD. This expression continued to increase after OGD and was significant at 24 hours when there was nearly a 30% increase in the expression of TLR-4 when normalized to GAPDH (Figure 7).

Discussion
These data demonstrate the pathological role of TLR-4–dependent activation of microglia in the development of delayed paraplegia seen clinically after aortic surgery. The in vivo data suggest that, after thoracic aortic occlusion in mice, the absence of a functional TLR-4 is associated with preserved hind-limb function and neuronal viability and an attenuation of microglial activation and proinflammatory cytokine production. Furthermore, the in vitro data demonstrate that TLR-4 is upregulated in microglia after OGD and microglia with intact TLR-4 express a significantly higher amount of proinflammatory cytokines.

The insult sustained by the spinal cord during thoracoabdominal aortic intervention has classically been defined as ischemia–reperfusion. The injury, however, should be separated into 2 distinctly different insults. Clinically, this is seen in the bimodal distribution of paraplegia after thoracoabdominal interventions. Patients experience either immediate paralysis after interventions, which is rarely reversible, or subsequently develop paraplegia in the hours to days after surgery, which can commonly be reversible, with the delayed injury more prominent in contemporary series.1 To further clarify these clinical entities, we have developed a murine model of immediate and delayed paraplegia.11

Ischemia causes metabolic derangement, with wide variations in the degree of injury to motor neurons.12 If this ischemic injury is severe enough, the patient will experience immediate paraplegia secondary to neuronal apoptosis or necrosis in

**Figure 1.** Hind-limb functional scores in mice at 12-hour intervals after 5 minutes of aortic occlusion or sham surgery. There was a significant difference in all mice that underwent ischemia–reperfusion (IR) compared with sham mice (P<0.05). In addition, mice with functional toll-like receptor (TLR)-4 receptors had significantly worse hind-limb function at 36 hours after ischemia compared with nonfunctional mutant TLR-4 receptors (*P<0.05).

**Figure 2.** Hematoxylin and eosin stain of spinal cord at 60 hours after aortic occlusion or sham surgery with transverse (left) and sagittal sections (right). Mice with functional toll-like receptor (TLR4)-4 (wild type [wt]) that underwent ischemia–reperfusion (IR) had increased vacuolization and loss of gray–white matter differentiation compared with TLR-4 mutant (mut) or sham mice. Sagittal sections of spinal cords after IR or sham surgery demonstrated a preservation of viable anterior horn neurons in the mut mice compared with wt mice (magnification ×200; ×40 in box).
the motor neurons. Dense paraplegia from these irreversibly injured neurons will not recover. With reperfusion, the restoration of blood flow results in an amplified inflammatory response from both resident and systemic leukocytes, leading to further neuronal injury from production of reactive oxygen species and cytokines. The secondary injury attributed to reperfusion provides a window of intervention for the potential reversal or prevention of delayed injury.

To investigate the contribution of TLR-4 signaling to the development of delayed paraplegia, mice with TLR-4 loss-of-function mutation were subjected to lower body ischemia. The duration of ischemia was selected by titrating the ischemic duration until wild-type mice exhibited a delayed progressive function deficit. Mice with TLR-4 mutation had significantly preserved neurological function and neuronal viability compared with wild-type controls. Both groups subjected to IR had significantly worse functional outcomes compared with sham mice. The difference is likely related to the metabolic effects of initial ischemic insult. Ischemic spinal cord also demonstrated a significant increase in microglial activation with intact TLR-4 receptors. These data suggest the contribution of TLR-4 signaling to reperfusion injury in the spinal cord.

Microglia, the resident immune cells in central nervous system, serve a complicated role. Their presence is associated with a host of beneficial and detrimental effects. Although microglia are necessary for neuronal development and viability, dysregulation of these cells after physiological insults can result in neurotoxicity in a process known as reactive microgliosis. On activation, microglia undergo a dramatic phenotypic transformation and can produce a host of neurotoxic factors, such as TNF-α and nitric oxide. Others have shown that microglial inhibition significantly attenuates IR injury in the brain and spinal cord. The role of the microglia seems to be significant with regard to the reperfusion arm of this injury.

Microglia have a plethora of receptors that can be activated by a variety of stimuli. Exogenous stimuli, such as bacterial and viral pathogens, can cause activations of microglia. Recently, TLRs, in particular TLR-4, have gained attention for their role in various other models of IR. These receptors represent a family of proteins that regulate the innate immune response. Although multiple cell lines, such as astrocytes and neurons, have TLR-4 receptors, the TLR-4 concentration is significantly increased in microglial activation with intact TLR-4 receptors. These data suggest the contribution of TLR-4 signaling to reperfusion injury in the spinal cord.

**Figure 3.** All mice that underwent ischemia–reperfusion (IR) surgery had significantly less viable anterior horn neurons compared with sham mice (*P<0.05). Mice with intact toll-like receptor-4 (wild type [wt]) that underwent IR surgery had the lowest number of normal-appearing anterior horn neuron, which was significantly different from mutant mice (mut; #P<0.05).

**Figure 4.** Immunoblotting of galectin-3/mac-2 in spinal cord after ischemia–reperfusion (IR) surgery, with a comparison to baseline values. A, Galectin-3/mac-2 immunoblotting 6 hours after IR surgery with increased expression in wild-type (wt) mice after surgery compared with mutant mice or baseline values. B, Representation of galectin-3/mac-2 immunoblotting 36 hours after IR surgery. wt mice had an increased expression of microglial activation marker compared with mutant mice (mut) or baseline values.

**Figure 5.** Compared with baseline values, the expression of proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor (TNF)-α was significantly (*P<0.05) elevated at 6 hours after ischemia–reperfusion (IR) surgery in wild-type (wt) mice compared with mutant mice (mut). At 36 hours after IR surgery, values decrease and return to near baseline.

**Figure 6.** Expression of tumor necrosis factor (TNF)-α and interleukin-6 (IL-6) after 24 hours of oxygen and glucose deprivation (OGD). Mice with intact toll-like receptor-4 (wild type [wt]) had a significant increased expression of IL-6 at 24 and 36 hours and TNF-α at 36 hours (*P<0.05) compared with mutant mice (mut).
Microglial TLR-4 Expression Following OGD

Figure 7. Toll-like receptor (TLR)-4 expression in wild-type microglia after oxygen and glucose deprivation (OGD). The expression of TLR-4 increased in a time-dependent manner after OGD, and significant increases were noted at 12 and 24 hours (*P<0.05).

disease. To date, this had not been shown in the spinal cord, but this receptor could be a potentially powerful source of intervention to attenuate paraplegia.

Bacterial lipopolysaccharide, a ligand of TLR-4, has been associated with proinflammatory cytokine production. Similarly, ischemic cells release cell signalers, such as heat shock protein 60, resulting in TLR-4–mediated microglial activation. Stimulation of TLR-4 causes translocation of TLR-4 to the nucleus and production of proinflammatory cytokines and chemokines. The cell culture data demonstrate that TLR-4 is significantly upregulated after OGD. This upregulation of receptors, when it occurs, continues to increase for a period of time and likely sensitizes microglia to become activated after physiological insults. Upregulation of TLR-4 also paralleled proinflammatory cytokine production. Microglia with functional TLR-4 had a higher expression of proinflammatory cytokines TNF-α and IL-6 after OGD.

To summarize, mice lacking functional TLR-4 did not exhibit the progressive functional decline seen in wild-type mice after thoracic aortic occlusion. In addition, a reduction of microglial activation and proinflammatory cytokine production was observed in mutant mice compared with wild-type controls. The in vitro data presented demonstrate that TLR-4 upregulation occurs in microglia after OGD and is associated with the expression of proinflammatory cytokine production. In conclusion, TLR-4 activation of microglia in the spinal cord after IR injury is critical in the mechanism of injury progression, which could be important in future prevention of the clinical entity of delayed paraplegia after thoracoabdominal aortic intervention.

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Disclosures

None.

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Toll-Like Receptor 4–Dependent Microglial Activation Mediates Spinal Cord Ischemia–Reperfusion Injury

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