Myocyte TLR4 enhances enteric and systemic inflammation driving late murine endotoxic ileus

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Am J Physiol Gastrointest Liver Physiol 308: G852–G862, 2015. First published March 6, 2015; doi:10.1152/ajpgi.00211.2014.—Myocytes are nonhemopoietic in origin and functionally essential in generating gastrointestinal motility. In endotoxemia, a rapid-onset nonhemopoietic mechanism potently triggers early ileus in a Toll-like receptor 4 (TLR4)/myeloid differentiation primary response gene 88 (MyD88)-dependent manner. Moreover, synergistically with hemopoietic cells, nonhemopoietic cells escalate late ileus via an IL-6 receptor-dependent inflammation-driven pathway. We therefore specifically investigated the role of myocytes in TLR4-driven ileus. TLR4+/-, TLR4-/-, bmTLR4+/+TLR4-/-, chimera, SM22-Cre-/-/TLR4flox/flox, and selective myocyte TLR4-deficient (SM22-Cre+/-/TLR4flox/flox) mice were injected intraperitoneally with purified lipopolysaccharide. SM22-driven Cre recombinase activity was selectively detected in cardiac, gastrointestinal, skeletal, and vascular myocytes, of small-sized vessels in a two-color fluorescent Cre reporter mouse. In contrast to nonhemopoietic TLR4 deficiency, deletion of myocyte TLR4 signaling prevented neither endotoxin-induced suppression of spontaneous jejunal contractility in vitro nor early ileus in vivo at 6 h. Circulating plasma colony-stimulating factor 3 was greatly elevated during endotoxemia, independent of myocyte TLR4 signaling or time. TLR4 activation of myocytes contributed significantly to an early enteric IL-6 mRNA induction and systemic IL-6 release, as well as to a late increase in circulating chemokine (C-X-C motif) ligand 1 (CXCL1) and IL-17. Consequently, inhibition of myocyte TLR4 signaling allowed functional recovery of motility by preventing inflammation-driven late ileus at 24 h. Direct TLR4 activation of myocytes is not responsible for nonhemopoietic-mediated early ileus. However, myocytes are proinflammatory cells that potently drive enteric and systemic inflammation, subsequently fueling late mediator-triggered ileus. Specifically, the myocyte TLR4-dependent inflammatory signature of elevated plasma IL-6, CXCL1, and IL-17 is strongly associated with late rodent ileus.

gastrointestinal motility; lipopolysaccharide; Cre reporter IRG mouse

SEVERE SEPSIS HAS A MORTALITY RATE OF 20–60% AND KILLS >200,000 PEOPLE EVERY YEAR IN THE UNITED STATES ALONE, WHICH TRANSLATES TO 1 PERSON EVERY 2.5 MIN (2). UNFORTUNATELY, LITTLE PROGRESS HAS BEEN MADE IN DEVELOPING EFFECTIVE PHARMACOLOGICAL AGENTS TO IMPROVE SEPSIS SURVIVAL RATES AND THE MORBID COMPLICATIONS OF SEPSIS, SUCH AS ILEUS ACCOMPANIED BY BACTERIAL TRANSLLOCATION AND THE INABILITY TO TOLERATE AN ORAL DIET. GRAM-NEGATIVE BACTERIAL ENDOTOXIN SIGNALS VIA TOLL-LIKE RECEPTOR (TLR) 4 (20). WE HAVE SHOWN THAT TLR4 ACTIVATION TRIGGERS ILEUS VIA TWO INDEPENDENT MECHANISMS THAT DIFFER IN THE TIME OF ONSET. AT THE EARLY TIME POINT OF 6 H AFTER INTRAPERITONEAL ENDOTOXIN INJECTION, WE HAVE DEMONSTRATED THAT SIGNALING VIA THE TLR4/MYELOID DIFFERENTIATION PRIMARY RESPONSE GENE 88 (MyD88) PATHWAY IN CELLS OF NONHEMOPOIEtic ORIGIN POTENTLY DRIVES ILEUS (6). IN CONTRAST, ACTIVATION OF TLR4 SIGNALING SOLELY IN HEMOPoietic-DERIVED CELLS RESULTS IN A TIME-DELAYED ONSET OF GASTROINTESTINAL DYSMOTILITY AFTER 24 H OF ENDOTOXEMIA. THIS LATE ENDOTOXIN-INDUCED ILEUS IN MICE IS KNOWN TO BE DRIVEN BY INFAMmatory CYKINES, AND, IN PARTICULAR, INTERLEUKIN (IL)-6 PLAYS A CENTRAL ROLE (5). HEMOPoietic AND NONHEMOPoietic CELLS ARE MAJOR SOURCES OF IL-6, AS WELL AS NUMEROUS OTHER INFAMmATORY MEDIATORS. Thus dual activation of these two cell lineages can synergistically generate amplified inflammation, sustained ileus, and, ultimately, death. However, the nonhemopoietic cell subpopulation that mediates the early mechanism of TLR4-triggered ileus and/or late inflammatory synergy remains elusive.

Early TLR4-mediated ileus is characterized by diminished spontaneous contractility of the intestinal muscularis (6). Importantly, myocytes comprise the majority of the intestinal muscularis syncytium, and, functionally, these cells represent the effector arm of gut motility. It has been reported that direct exposure of cultured human colonic smooth muscle cells to endotoxin in vitro induced a time- and dose-dependent contractile dysfunction, and similar observations were made in murine cardiomyocytes (4, 21). Furthermore, TLR4 stimulation in vascular myocytes can cause a phenotypic modification from the contractile to the proliferative-inflammatory subtype. This switch hypothetically results in generation of inflammatory cytokines and loss of vascular tone, consequently aggravating endotoxic shock (10).

Accordingly, in the present study our objective was to delineate the contribution of myocytes to endotoxin-induced inflammation and ileus over time. Surprisingly, direct myocyte TLR4 signaling was not found to mediate nonhemopoietic-mediated early ileus. Still, TLR4-activated myocytes potentially trigger enteric and systemic inflammation, thereby secondarily driving an IL-6-dependent late ileus. Consistent with this finding, the abrogation of TLR4 signaling consequently permitted accelerated recovery of the digestive tract
from endotoxin-induced dysmotility, suggesting that sepsis-associated morbidity may be alleviated by silencing myocyte TLR4.

**MATERIALS AND METHODS**

Animals, genetic modification, and experimental groups. Adult male C57BL/6 (TLR4+/+) mice, 2–3 mo of age, were purchased from Jackson Laboratory (Bar Harbor, ME). Male TLR4−/− mice on a C57BL/6 background (22) were bred in homozygous colonies at the University of Pittsburgh. bmtTLR4+/+/?TLR4−/− (TLR4lox/lox/mice) were created by TLR4−/− bone marrow transplantation in lethally irradiated TLR4−/− mice, and chimeric animals convalesced for >3 mo to allow for engraftment and repopulation of TLR4-competent bone marrow and peripheral tissue immunocytes. Cre-lox technology was used to serially breed smooth muscle 22-kDa protein (SM22, or transgelin)-Cre mice to selectively delete in myocyte TLR4. Matched pairs of the genotyped SM22-Cre+/−; TLR4lox/lox (TLR4lox/lox/mice) and SM22-Cre−/−; TLR4lox/lox (TLR4lox/lox/mice) were used for experiments, and their genotype was reconfirmed after harvest.

SM22-Cre+/− IRG+/+ mice were generated by crossing SM22-Cre+/− mice with IRG−/− mice (Jackson Laboratory). The insulator/red (green) (IRG) mouse, a two-color fluorescence reporter mouse, was created for visual assessment of promoter-specific Cre-mediated gene recombination of loxp gene sequences (8). In these mice, loxP-flanked red fluorescent DsRed-Express is constitutively expressed in a tissue-specific manner. Upon Cre-driven gene recombination, DsRed-Express and a stop codon are floxed out, and the downstream enhanced green fluorescent protein (EGFP) sequence is transcribed selectively in Cre recombinase-expressing cells.

Animals were maintained in a laminar flow facility at the University of Pittsburgh, and the experiments were approved by the University of Pittsburgh Animal Care and Use Committee. Age-matched animals were intraperitoneally injected at 0 and 2 h with ultrapurified Escherichia coli strain 0111:B4, endotoxin activity 106 endotoxin units/mg, as determined with an amebocyte lysate assay (InvivoGen, San Diego, CA). Mice were fixed with 2% paraformaldehyde for 1 h at room temperature. All organs except the muscle strips was recorded using an isometric force transducer (catalog no. ML T0202, ADInstruments, Colorado Springs, CO). Digital recordings were analyzed with Chart 5 software (v5.5.6, ADInstruments), and values were normalized to weight and length of the muscle strips.

Quantitative PCR of intestinal muscularis. Messenger RNA was extracted from intestinal muscularis samples with the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Equal amounts of DNase-treated mRNA were used for cDNA synthesis (Invitrogen). mRNA expression of mouse GAPDH (5’-TTTCCACCATGGAGAGGC-3’ (forward) and 5’-GGCATGGACTGTTGTCATAGA-3’ (reverse)), mouse IL-6 (5’-TCAATTCGAAACCGCTATGA-3’ (forward) and 5’-CAGCCAGCAGCTGCAAGAA-3’ (reverse)), mouse IFN-γ, mouse TNF-α, mouse IL-12p40, mouse IL-12p70, IL-13, IL-17, leukemia inhibitory factor (LIF), monokine induced by IFN-γ (MIG), macrophage inflammatory protein-1α (MIP-1α), tumor necrosis factor-α (TNFα), and vascular endothelial growth factor (VEGF) (Millipore, Billerica, MA) was quantified in duplicate by SYBR Green two-step RT-PCR [supplemented with uracil N-glycosylase (UNG)] on a Prism 7000 detection system (Applied Biosystems, Foster City, CA) as described previously (6). TaqMan gene expression assay for mouse CCL2 (assay ID No. Mm00438334_m1) was performed using TaqMan gene expression master mix with UNG (all reagents from Applied Biosystems). The amount or treatment of the genes of interest was quantified relative to the murine housekeeping gene GAPDH. All comparative analyses for any strain or treatment were calculated relative to the sham TLR4+/+ group.

**RESULTS**

Animal characterization and intestinal TLR4 muscularis expression. In tissues of adult IRG−/− mice, strong native red fluorescence without EGFP expression was present in enteric neurons throughout the digestive tract and the central nervous system, gastrointestinal myocytes, striated muscle of the heart, and skeletal system (Fig. 1A, i–xii and xvii–xxi), as well as in the intestinal crypt stem cells and the pancreas, indicating constitutive expression of the intact IRG gene. Hepatocytes, alveolar epithelial cells, renal tubules (with the exception of glomeruli), resident macrophages, splenocytes, endothelial cells, intestinal epithelial cells, and, in particular, vascular myocytes lacked native DsRed protein expression (Fig. 1A, xiii–xvi and xxi–xxiv). Despite the tissue-specific absence of this internal control, a functional CCAG-loxP-DsRed-STOP-loxP-EGFP gene was detected in multiple cells, for example, in endothelial and hemopoietic cells in Tie2Cre+/−IRGfloxed−/− reporter mice. In these mice, skeletal muscle of the hindlimb

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and back, as well as the abdominal and thoracic wall, expressed green fluorescence macroscopically (Fig. 1B, iv). Microscopically, emergence of the green fluorescent signal over the red fluorescence in transgenic mice was detected selectively in smooth muscle cells of the entire gastrointestinal tract and in striated muscle cells of heart and skeletal muscle (Fig. 1C, i–xii). Additionally, scattered SM22-positive fibers in the central nervous system and intestinal submucosa, which most likely represent the muscular media of small-sized vessels, exhibited green fluorescence (Fig. 1C, xvii–xxiv). However, the fluorescent signal conversion was not found in vascular myocytes of medium- and large-sized arteries (Fig. 1C, xiii–xvi). Similarly, the pancreas, enteric neurons, and intestinal crypt stem cells, as well as all other organs examined, showed no visible Cre recombinase activity (Fig. 1B, iv; Fig. 1C, i–iv; Fig. 1C, xx–xiv; and images not shown).

Fig. 1. Smooth muscle 22-kDa protein (SM22)-driven Cre recombinase activity was selectively detected in myocytes and efficiently reduced Toll-like receptor 4 (TLR4) gene transcription within the intestinal muscularis externa. A–C: representative images from 3 independent experiments (n = 5 per group). A: DsRed is constitutively expressed in selected tissues of insulator/red/green (IRG)-heterozygous (IRG+/−) Cre reporter mice (i–xxiv). B: the pancreas (*) of adult IRG+/− mice turned macroscopically red, and SM22-driven Cre recombinase activity was macroscopically observed in the abdominal wall (#) and skeletal muscle (‡) in adult SM22+/− IRG+/− mice. C: cell-specific SM22-driven Cre recombinase activity is detected in gastrointestinal (i–iv), cardiac (v–viii), and skeletal (ix–xii) myocytes, but not in the aortic media (xiii–xvi). *, Esophagus; #, trachea; ‡, ascending aorta. Similar to the network of enteric neurons (i–iv, ‡), cerebellar neurons and intestinal epithelial cells did not exhibit SM22-driven Cre recombinase activity, but the SM22 promoter was functional in myocytes of small-sized vessels of the brain and the intestinal submucosa (xvii–xxiv). D: SM22-driven Cre activity effectively diminished intestinal muscularis TLR4 gene transcription. PELPS, purified Escherichia coli lipopolysaccharide. Values are group means ± SE of data pooled from 36 independent experiments (n = 5–7 mice per group). *P < 0.05 vs. all sham. #P < 0.05 vs. TLR4fl/fl sham.
To further characterize the myocyte TLR4-deficient mouse model, we assessed SM22-Cre-mediated altered TLR4 gene transcription within the intestinal muscularis externa. Primer specificity was confirmed, since TLR4 mRNA was not detectable in murine intestinal muscularis extracts of TLR4<sup>+/+</sup> mice (Fig. 1D). Insertion of the loxP sequences led to a decreased efficiency of TLR4 gene transcription in TLR4<sup>flx/flx</sup> mice with a level of only 60% of that found in genetically unmodified TLR4<sup>+/+</sup> mice. Myocyte-specific Cre recombinase activity further reduced intestinal muscularis TLR4 message to 18% (Fig. 1D). Insertion of the loxP sequences led to a decreased efficiency of TLR4 gene transcription in TLR4<sup>flx/flx</sup> mice with a level of only 60% of that found in genetically unmodified TLR4<sup>+/+</sup> mice. Myocyte-specific Cre recombinase activity further reduced intestinal muscularis TLR4 message to 18% (Fig. 1D). Insertion of the loxP sequences led to a decreased efficiency of TLR4 gene transcription in TLR4<sup>flx/flx</sup> mice with a level of only 60% of that found in genetically unmodified TLR4<sup>+/+</sup> mice. Myocyte-specific Cre recombinase activity further reduced intestinal muscularis TLR4 message to 18% (Fig. 1D). Insertion of the loxP sequences led to a decreased efficiency of TLR4 gene transcription in TLR4<sup>flx/flx</sup> mice with a level of only 60% of that found in genetically unmodified TLR4<sup>+/+</sup> mice. Myocyte-specific Cre recombinase activity further reduced intestinal muscularis TLR4 message to 18% (Fig. 1D). Insertion of the loxP sequences led to a decreased efficiency of TLR4 gene transcription in TLR4<sup>flx/flx</sup> mice with a level of only 60% of that found in genetically unmodified TLR4<sup>+/+</sup> mice. Myocyte-specific Cre recombinase activity further reduced intestinal muscularis TLR4 message to 18% (Fig. 1D). Insertion of the loxP sequences led to a decreased efficiency of TLR4 gene transcription in TLR4<sup>flx/flx</sup> mice with a level of only 60% of that found in genetically unmodified TLR4<sup>+/+</sup> mice. Myocyte-specific Cre recombinase activity further reduced intestinal muscularis TLR4 message to 18%.
of baseline levels in TLR4+/+ mice and 31% of muscularis TLR4 gene expression in TLR4flox/flox mice. With the assumption of 100% Cre recombination efficiency, myocyte TLR4 expression, therefore, comprised 69% of intestinal muscularis TLR4 message, with the remaining TLR4 mRNA presumably derived from other muscularis externa cells, such as neurons, glia, endothelial cells, and macrophages. Intestinal macrophages reside as a dense network of highly immunocompetent cells within the intestinal muscularis surrounded by other immunocytes, such as neutrophils, mast cells, monocytes, and lymphocytes (16). Replenishment of TLR4-expressing bone marrow into TLR4−/− mice restored intestinal muscularis TLR4 gene transcripts in TLR4−/− mice to 22% of the TLR4 levels observed in TLR4+/+ mice. The latter indicates that we can detect TLR4-competent passenger leukocytes and repopulated resident immunocytes in the chimeric mice.

Myocyte TLR4 signaling regulates enteric and systemic inflammation during early endotoxemia. Because TLR4 signals upstream of many inflammatory cascades, we assessed endotoxin-induced alterations in inflammatory mediators. In general, TLR4 signaling potently triggered the release of circulating cytokines and the cytokine subfamily of chemokines, but an overall reduced amount of inflammatory mediators in TLR4flox/flox mice was observed compared with nontransgenic TLR4+/+ mice. This is most likely explained by the diminished TLR4 gene expression in TLR4flox/flox mice after insertion of the loxP sites. Therefore, we concluded that TLR4flox/flox littermates were the appropriate control group for comparison with the experimental TLR4−myocyte mice.

We were intrigued to find that myocyte TLR4 signaling distinctively regulates systemic inflammation. The analyzed circulating mediators could be categorized according to the induction pattern into 1) TLR4-unresponsive cytokines, 2) TLR4-dependent, but myocyte-independent, cytokines, and 3) dual TLR4-dependent and myocyte-dependent cytokines (Fig. 2A). More specifically, the TLR4-unresponsive cytokines IFN-γ, CSF2, IL-12p70, and VEGF did not show significant regulation by TLR4 signaling. Moreover, the set of TLR4-

Fig. 2. Deletion of myocyte TLR4 signaling distinctively regulated early systemic inflammation. A: distinct patterns in endotoxin-mediated regulation of circulating mediators. These patterns were identified as 1) TLR4-unresponsive cytokines [colony-stimulating factor (CSF) 2, IFN-γ, IL12p70, IL-13, and VEGF], 2) TLR4-dependent, but myocyte-independent cytokines [CSF3, IL-12p40, leukemia inhibitory factor (LIF), monocyte induced by IFN-γ (MIG), macrophage inflammatory protein 1α (MIP1α), and regulated on activation normal T cell expressed and secreted (RANTES)], and 3) dual TLR4-dependent and myocyte-dependent cytokines, with the latter subdivided into early [chemokine (C-C motif) ligand 2 (CCL2), IL-1ß, IL-6, IL-10, and IFN-γ-induced protein 10 (IP10)] and late [chemokine (C-X-C motif) ligand 1 (CXCL1) and IL-17] myocyte dependence (in reference to Fig. 6). B–D: TLR4 signaling on myocytes significantly contributed to systemic IL-6, but not early CXCL1, release. Plasma CSF3 levels were mainly derived from hemopoietic cells during endotoxemia. Values are group means ± SE of data pooled from 36 independent experiments (n = 6 mice per group). *P < 0.05 vs. all sham. #P < 0.05 vs. TLR4flox/flox PELPS at 6 h.
responsive, but myocyte-independent, mediators (IL-12p40, IL-13, MIG, RANTES, TNFα, MIP1α, CSF3, and LIF) could be separated from TLR4 myocyte-dependent cytokines at 6 h after the onset of endotoxemia (IL-1β, IL-10, IP10, CCL2, and IL-6; Fig. 2A). In the latter group, myocyte TLR4 deficiency significantly diminished TLR4-triggered circulating plasma levels, as shown in more detail for IL-6 (Fig. 2B). In contrast, systemic CSF3 and CXCL1 were highly induced during early endotoxemia, despite deletion of myocyte TLR4 signaling (Fig. 2, C and D). In TLR4 chimeric mice, systemic IL-6 levels were dependent on the presence of nonhemopoietic TLR4, which also regulated systemic CXCL1-1 levels but not hemopoietic-derived circulating CSF-3 (Fig. 2, B–D). Importantly, this early reduction of endotoxin-induced plasma IL-6 levels in TLR4^Δmyocyte mice was similar to that in TLR4^Δnonhemopoietic mice, suggesting that intestinal myocytes were the main nonhemopoietic subpopulation contributing to TLR4-triggered systemic IL-6 release.

We next determined the molecular changes in the intestinal muscularis for a selected set of the above-analyzed inflammatory mediators, namely, IL-6, CXCL1, and CSF3, as representative cytokines for each of the categories. The TLR4-triggered induction pattern of IL-6 within the intestinal muscularis completely mirrored the inflammatory plasma response, with high gene expression in TLR4^{lox/lox} mice and a significantly diminished mRNA induction in selective myocyte TLR4-deficient and nonhemopoietic TLR4-deficient animals (Fig. 3A). In contrast, expression of intestinal muscularis CXCL1 was not significantly regulated by nonhemopoietic cells or by myocytes (Fig. 3B). The robust plasma response of the hemopoietic growth factor CSF3, which also functions as a modulator of inflammation (14), was not reflected by molecular alterations in the intestinal muscularis (Fig. 3C). This difference is most likely explained as follows. 1) The biological process of mRNA translation to a sequence of amino acids during protein synthesis is tightly regulated by the presence of RNA-stabilizing proteins and microRNAs, as well as phosphorylation status of the ribosomes. 2) Specific mRNA expression patterns in various tissues depend on its cellular composition, while the circulating CSF3 proteins are collectively secreted from all organs and from the highly active circulating immunocytes into the blood circulation. Similarly, the close contact of the endothelial lining with the blood circulation might account for the observed difference, with significant CXCL1 circulating protein alterations but insignificant intestinal muscularis CXCL1 mRNA regulation in the nonhemopoietic TLR4-deficient mice.

Fig. 3. Myocyte TLR4 triggers early intestinal muscularis IL-6 gene expression. A–C: TLR4-triggered intestinal muscularis IL-6 mRNA expression was dependent on myocyte TLR4 signaling. Muscularis CXCL1 and CSF3 transcription was highly induced by TLR4 stimulation but independent of myocyte TLR4. Values are group means ± SE of data pooled from 36 independent experiments (n = 5–7 mice per group). *P < 0.05 vs. all sham. #P < 0.05 vs. TLR4^{lox/lox} PELPS at 6 h.
Early endotoxin-induced ileus is not blocked by deletion of myocyte TLR4 signaling. Gastrointestinal motility was normal in all sham-treated strains (Fig. 4A). Consistent with previous results, mice lacking nonhemopoietic TLR4 signaling did not display early endotoxin-induced ileus, similar to TLR4-deficient mice (GC = 9.5 ± 0.5 and 8.7 ± 0.5, respectively). In contrast, endotoxemia potently shut down gastrointestinal motility in wild-type mice at 6 h after the first endotoxin injection (GC = 3.8 ± 0.5). Similar results were seen in TLR4<sup>−/−</sup> and TLR4<sup>Δmyocyte</sup> mice (GC = 5.5 ± 0.3 and 5.1 ± 0.6, respectively), with no significant impact of the above-described reduction in endotoxin-triggered inflammatory responses in these strains on early endotoxin-triggered ileus (Fig. 4B).

Myocyte TLR4 deficiency does not forestall endotoxin-induced loss of spontaneous jejunal contractility in vitro. Previously, we identified endotoxin-induced suppression of spontaneous myocyte contractility as one potential causative event in early TLR4-triggered gastrointestinal dysmotility (6). Recordings from sham-treated animals of all strains demonstrated regular spontaneous phasic contractile activity in jejunal circular muscle strips (Fig. 5A, i–v). Confirming our previous results, in vivo TLR4 activation caused a significant decrease in the spontaneous contractility in all mice with TLR4-competent signaling in nonhemopoietic cells (Fig. 5A, vi–x). Muscarinic-stimulated contractile forces tended to be diminished by low-dose endotoxin treatment but were not significantly suppressed at high doses of bethanechol in any strain studied, despite a second endotoxin injection (Fig. 5B).

Myocyte TLR4-triggered immune response enhances late mediator-driven ileus and is associated with the inflammatory mediators IL-6, CXCL1, and IL-17. Time-delayed ileus is driven by inflammatory mediators and is dependent on IL-6 receptor (5). To examine the role of myocyte TLR4 in this process, we next examined systemic inflammation and late ileus in TLR4<sup>Δmyocyte</sup> mice at 24 h after endotoxin treatment. Presumably as a result of dampened early inflammation, TLR4<sup>Δmyocyte</sup> mice recovered from ileus at 24 h, while TLR4<sup>−/−</sup> and TLR4<sup>Δmyocyte</sup> mice suffered from sustained ileus (Fig. 6A). The normalization of gastrointestinal motility in TLR4<sup>Δmyocyte</sup> mice occurred in parallel with a further decline in plasma IL-6 and a major reduction in plasma CXCL1 (Fig. 6B). Additionally, we observed a general improvement in convalescence of the TLR4<sup>Δmyocyte</sup> animals, with a disappearance in sickness behaviors, such as shivering, hunching, and lethargy. However, plasma CSF3 remained elevated. Interestingly, IL-17 emerged as a possible biomarker of ileus during late endotoxemia, as plasma levels increased over time in TLR4<sup>−/−</sup> mice but decreased to near baseline levels in myocyte TLR4-deficient animals (Fig. 6C). Therefore, IL-17 and CXCL1 were categorized as TLR4-dependent and late myocyte-dependent cytokines (Fig. 2A).

**DISCUSSION**

Infection-triggered morbidity and mortality remain major challenges in the modern era of critical care medicine. Thus, further insights into the mechanisms and, particularly, into cell-specific contributions during sepsis are needed to provide a rational basis for the development of new diagnostic and therapeutic modalities. We used the Cre-lox technology, which allows for precise, cell-specific DNA rearrangements, genetic switches, or gene deletion in living mice (18), to assess the contribution of myocyte TLR4 signaling to endotoxin-induced inflammation and ileus. Utilizing the two-color fluorescence Cre reporter IRG mouse, we demonstrated that the SM22 promoter provided excellent muscle-specific Cre recombinase activity. However, contrary to the original description of the SM22-Cre strain (26), floxed alleles not only were present in adult enteric smooth muscle cells, cardiac striated muscle cells, and vascular myocytes of small-sized vessels but also were intensely expressed in skeletal striated muscle cells. Moreover, SM22 is described as an established pan-smooth muscle cell marker (24), but, in our hands, vascular myocytes of intermediate- and large-sized vessels did not exhibit functional SM22 promoter activity in the IRG reporter strain. SM22 promoter activity in vascular myocytes may depend on cell differentiation or proliferation and other factors, such as environmental stimuli, but the exact reason for the specific pattern of differential SM22 expression remains unclear. Yet, importantly, SM22 expression was consistently restricted to myocytes and...
has not been found in any other nonhemopoietic cell population.

Endotoxin strongly induces inflammation and ileus via the TLR4/MyD88 pathway. Human colonic myocytes, a subpopulation of nonhemopoietic cells, respond to TLR4 stimulation, exhibiting a time- and dose-dependent contractile dysfunction in vitro (21). Yet direct TLR4 activation in myocytes did not trigger the early nonhemopoietic mechanism of endotoxin-triggered ileus in vivo in our studies. Additionally, functional preservation of the in vitro contractile responses to muscarinic...
stimulation indicated the absence of sustained myocyte dysfunction or injury. Nonetheless, we confirmed both disturbed in vivo gastrointestinal motility and in vitro spontaneous contractile activity of the intestinal myocytes in endotoxin-treated mice with intact nonhemopoietic TLR4 signaling. With the exclusion of myocytes, other candidates, such as interstitial cells of Cajal, as well as endothelial, epithelial, glial, and neuronal cells, have to be considered as the responsible nonhemopoietic subpopulation directing early TLR4-induced ileus. The enteric nervous system, as well as the central nervous system, control neuronal signals among distinct gut regions and coordinate propulsive digestive activity (12). Detailed immunohistochemical studies revealed the expression of TLR4 in the myenteric and submucosal neuronal plexus of murine intestine and human ileum (3), thereby, theoretically, allowing TLR4 responsiveness of enteric neurons during endotoxemia. However, we hypothesize that a direct activation of neuronal TLR4 would require local tissue infection or endotoxin extravasation. The latter might occur if the well-described endotoxin-induced vascular hyperpermeability is taken into consideration (9). Furthermore, in vitro application of the neural blocking agent tetrodotoxin to muscle strips from endotoxin-pretreated mice does not alter the endotoxin-induced suppression of jejunal circular muscle contractions (data not shown).

In contrast, vascular endothelial cells are directly exposed to endotoxin from drained infected organs or during bloodstream infections. We observed a substantial intestinal muscularis gene induction and systemic release of the chemokine CXCL1, a mouse functional homolog of human IL-8. CXCL1 can be induced by endotoxin, IL-1β, and TNFα controlled by NF-κB motifs (23). This chemokine is mainly derived from platelets, macrophages, and endothelial cells (15). Its functions include regulation of cell proliferation and leukocyte trafficking, which are important features of inflammation. In our study, 60% of systemically circulating CXCL1 during early endotoxemia was derived from nonhemopoietic cells; nevertheless, in accordance with the aforementioned literature, myocytes did not appear to be a direct source of CXCL1. These findings strongly point toward a significant early, TLR4-triggered activation of endothelial cells. Endothelial cells express TLR4 and endotoxin-induced CXCL1, and leukocyte rolling in cerebral microvessels was absent in transgenic mice lacking endothelial TLR4 (27). Moreover, the capacity of endothelial cells to

Fig. 6. Myocyte TLR4 drives late systemic inflammation fueling cytokine-driven ileus during late endotoxemia. A: deletion of myocyte TLR4 signaling allowed recovery from endotoxin-induced gastrointestinal dysmotility at 24 h. Values are group means ± SE of data pooled from 25 independent experiments (n = 4–6 mice per group). *P < 0.05 vs. all sham. ‡P < 0.05 vs. TLR4fl+/flox PELS at 24 h. †P < 0.05 vs. TLR4fl+/flox PELS at 6 h. B and C: 24 h after endotoxin treatment, plasma IL-6 levels declined further, dependent on myocyte TLR4 signaling, and highly elevated CXCL-1 and IL-17 plasma levels dropped significantly when myocyte TLR4 signaling was interrupted; however, circulating hemopoietic-derived CSF3 remained high. Values are group means ± SE of data pooled from 18 independent experiments (n = 6 mice per group). *P < 0.05 vs. TLR4+/+/ sham. #P < 0.05 vs. TLR4fl+/flox PELS at 24 h. †P < 0.05 vs. TLR4fl+/flox PELS at 6 h.
eradicate lethal systemic gram-negative bacteremia underlines the functional significance of endothelial TLR4 signaling in sepsis (1). Yet myocyte TLR4 signaling appeared to contribute to CXCL1 liberation over time indirectly, most likely via IL-1β and TNFα pathways. We were intrigued to find that the significantly reduced CXCL1 plasma levels in TLR4−/−mice during late endotoxemia correlated with improved outcome.

Another key observation in our study was that IL-17 was the only circulating mediator that further increased in wild-type mice at 24 h after endotoxin treatment, correlating with ileus severity and duration. IL-17 is a prototypical Th1 cytokine that is released by Th17 cells, an effecter CD4 T cell subset. In general, endotoxemia amplifies activation of adaptive immunity as measured by Th1 responses and generation of Th17 cells. Interestingly, antibody-mediated neutralization of IL-17A improved survival in sepsis triggered by cecal ligation and puncture, even when administration of anti-IL-17A was delayed for up to 12 h after cecal ligation and puncture (11). Furthermore, IL-17A was recently shown to promote hypocontractility of intestinal smooth muscle via inducible nitric oxide synthase induction in muscularis macrrophages (19).

Th17 cell differentiation is promoted by IL-6 derived from mature plasmacytoid dendritic cells. In contrast, neutralization of IL-6 stabilizes newly generated T regulatory cells, favoring the development of Th17 cells (13, 17). Our recent data suggest that IL-6, which is synergistically generated by hemopoietic and nonhemopoietic lineages, plays a central role in the cytokine-driven mechanism of late TLR4-triggered ileus. Mechanistically, IL-6 promotes intestinal barrier failure and bacterial translocation, as shown in a model of gut ischemia-reperfusion (25). Here, we report that myocytes are the main nonhemopoietic cell subpopulation that contributes to TLR4-triggered IL-6 expression in the inflamed gut, as well as IL-6 release into the systemic circulation. Thus, interruption of TLR4 signaling selectively on myocytes may be a promising approach by which to restrict IL-6 production and, thereby, indirectly restrain activation of hemopoietic inflammation and TLR4-mediated late ileus.

In conclusion, myocytes do not directly mediate the early nonhemopoietic mechanism of TLR4-triggered murine ileus but, rather, appear to be central in triggering inflammation-driven murine ileus during late endotoxemia. Specifically, a distinct pattern of TLR4 myocyte-dependent circulating inflammatory mediators consisting of continuous CXCL1 and IL-17 increases, as well as a temporary surge in IL-6, is strongly associated with experimental ileus. We hypothesize that IL-1β and IL-6 production by direct TLR4 activation on myocytes leads to prolonged endothelial CXCL1 release and increasing hemopoietically derived Th17 release, respectively. Consequently, diminution of IL-6 and IL-1β by specific deletion of myocyte TLR4 signaling may help prevent sepsis-associated ileus.

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