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Role of Interleukin-6 in Hemopoietic and Non-hemopoietic Synergy mediating *TLR4*-triggered Late Murine Ileus and Endotoxic Shock¹

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Abstract

Background—Early murine endotoxin-induced ileus at 6 hours is exclusively mediated by non-hemopoietic *TLR4*/MyD88 signaling despite molecular activation of hemopoietic cells which included a significant IL-6 mRNA induction. Our objective was to define the role of hemopoietic cells in LPS/*TLR4*-triggered ileus and inflammation over time, and identify mechanisms of ileus.

Methods—CSF-1^{−/−}, *TLR4* non-chimera and *TLR4* chimera mice were single-shot injected with intraperitoneal ultrapure lipopolysaccharide (UP-LPS) and studied up to 4 days. Subgroups of *TLR4*^{WT} mice were additionally intravenously injected with exogenous recombinant IL-6 (rmIL-6) or murine soluble IL-6 receptor blocking antibody (anti-sIL-6R mAB).

Key Results—Hemopoietic *TLR4* signaling independently mediated UP-LPS-induced ileus at 24 hours, but chemotactic muscularis neutrophil extravasation was not causatively involved and mice lacking CSF-1-dependent macrophages died prematurely. Synergy of hemopoietic and non-hemopoietic cells determined ileus severity and mortality which correlated with synergistic cell lineage specific transcription of inflammatory mediators like IL-6 within the intestinal muscularis. Circulating IL-6 levels were LPS-dose dependent, but exogenous rmIL-6 did not spark off a self-perpetuating inflammatory response triggering ileus. Sustained therapeutic inhibition of functional IL-6 signaling efficiently ameliorated late ileus while preemptive antibody-mediated IL-6R blockade was marginally effective in mitigating ileus. However, IL-6R blockade did not prevent endotoxin-associated mortality nor did it alter circulating IL-6 levels.

Conclusions and Inferences—A time-delayed bone marrow-driven mechanism of murine endotoxin-induced ileus exists, and hemopoietic cells synergize with non-hemopoietic cells

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disclosures

All authors declare no conflict of interest exists.

Authors' contribution: BB provided the working hypothesis, designed the experimental study, performed the research and data analysis, and wrote the manuscript. RC participated in the performance of the research and edited the manuscript. TB participated in study design and coordination, and edited the manuscript. AB provided the working hypothesis, contributed to the study design and wrote the manuscript. All authors read and approved the final manuscript.

thereby prolonging ileus and fueling intestinal inflammation. Importantly, IL-6 signaling via IL-6R/gp130 drives late ileus, yet it did not regulate mortality in endotoxic shock.

Keywords

lipopolysaccharide; inflammation; cell signaling; IL-6R blockade; gastrointestinal motility; endotoxin; sepsis; shock

Introduction

Nature has developed receptors for non-self recognition with highly conserved molecular motifs in an effort to minimize a pathogen's ability to escape detection by the immune system.¹ This is exemplified by the family of Toll-like receptors (*TLRs*), which are differentially expressed on immune and non-immune cells with each playing a key role in the innate immune response.² Although in principle, a competent activated immune system is essential for combating bacterial invasion, an overwhelming unrestrained inflammatory response can be severely detrimental to the host. Signaling through *TLR4*,³ gram-negative sepsis continues to result in substantial morbidity and mortality despite the availability of effective antimicrobial therapy and interdisciplinary critical care. In the US, sepsis gives rise to annual healthcare expenditures of \$16.7 billion and remains the 10th leading cause of death with a mortality rate of 30 %.^{4;5}

Gastrointestinal dysfunction plays a major role in the high morbidity of the septic patient,⁶ and the established model of endotoxin-induced rodent ileus has served well to investigate possible mechanisms involved.⁷ Recently, it has become evident that MyD88 dependent activation of non-hemopoietic cells is the primary event triggering early *TLR4*-mediated murine ileus (6 hours after endotoxin treatment).^{8;9} Notably, despite early molecular activation of bone marrow cells, the hemopoietic lineage is incapable of independently initiating early *TLR4*-mediated ileus.⁸ However, regardless of the initial pathogenesis, mechanisms of ileus might vary over time, and indeed compelling evidence points towards time-dependent pathways.^{10–13}

In any case, it is not well studied until now whether inflammation-triggered bioactive substances can compromise propulsive gastrointestinal function *in vivo*. Interestingly, the gut is a major cytokine generating organ during endotoxemia,^{14;15} thereby hypothetically poisoning itself via paracrine mechanisms. Interleukin-6 (IL-6), a predictive inflammatory marker of organ failure in human sepsis,¹⁶ can be produced by both hemopoietic and non-hemopoietic cells,⁸ and overexpression of the shared IL-6 cytokine family receptor subunit gp130 has been shown to render mice hypersensitive to LPS.¹⁷ Interestingly, exogenous high dose IL-6 supplementation by intraperitoneal injection caused anorexia and gastroparesis in rats.¹⁸ Moreover, in rodent intestinal transplantation, circulating endogenous IL-6 protein levels correlated with recipient gastric emptying rates.¹⁹

We, therefore, investigated first the cell lineage specific effects of selective *TLR4* activation and secondly the effect of antibody-mediated IL-6 receptor (IL-6R) blockade on gastrointestinal motility at the late time point of 24 hours after endotoxin challenge. In this study, we demonstrate that *TLR4*-triggered ileus occurs via two different lineage specific signaling pathways, specifically through (i) early onset, sustained nonhemopoietic signaling and (ii) a time-delayed hemopoietic driven mechanism that is independent of neutrophil muscularis infiltration. Synergistic activation of these dual pathways determines an overwhelming inflammatory response including the systemic release of IL-6 and endotoxin-triggered mortality over time. Therapeutic single-cytokine targeting by neutralization of IL-6 signaling efficiently ameliorated late *TLR4*-triggered ileus but, in contrast to a targeted

disruption of hemopoietic and non-hemopoietic synergy, could not prevent endotoxin-associated mortality.

Material and Methods

Animals

Adult male inbred *TLR4* competent (C3H/HeOuJ = *TLR4*^{WT}) and *TLR4* mutant (C3H/HeJ-Pde6brd1 = *TLR4*^{ΔPS-d}) mice aged 2–3 months were purchased from Jackson Laboratory (Maine, USA). Animals were maintained in a laminar flow facility at the University of Pittsburgh with water supplied ad libitum and the experimental designs were approved by the University IACUC. As previously described, four groups of *TLR4* chimera mice were generated by bone marrow transplantation in lethally irradiated mice (total body irradiation with 10 Gy, Cs¹³⁷ source): *TLR4* competent bone marrow in *TLR4* competent mice (bm *TLR4*^{WT}/*TLR4*^{WT}), *TLR4* competent bone marrow in *TLR4* mutant mice (bm *TLR4*^{WT}/*TLR4*^{ΔPS-d}), *TLR4* mutant bone marrow in *TLR4* competent mice (bm *TLR4*^{ΔPS-d}/*TLR4*^{WT}), and *TLR4* mutant bone marrow in *TLR4* mutant mice (bm *TLR4*^{ΔPS-d}/*TLR4*^{ΔPS-d}). Studies in chimera mice were performed 12–15 weeks after the adoptive transfer to allow complete bone marrow engraftment with establishment of tissue chimerism through repopulation of resident hemopoietic cells and natural depletion of radioresistant cells like macrophages and mast cells.^{8;10}

Genetically macrophage/microglia-depleted and monocytopenic osteopetrotic (B6C3Fe a/a-Csf1op/J, spontaneous CSF-1^{-/-}, synonymous op^{-/-}) mice were bred at the University of Pittsburgh and offsprings were genotyped using a custom made single nucleotide polymorphism genotyping assay (Applied Biosystems) as described previously.⁸

LPS treatment and organ harvest

Endotoxemia was induced by a single intraperitoneal injection of highly purified lipopolysaccharide from *Escherichia coli* (UP-LPS, 5 mg/kg i.p., strain O111:B4, endotoxin activity level as assessed by chromogenic limulus amoebocyte lysate (LAL) assay 2.575 10⁶ EU/mg and 6.75 10⁶ EU/mg, List Laboratories) under short isoflurane narcosis while sham treatment consisted of anesthesia and vehicle injection (0.9 % saline). Functional and molecular studies were conducted 24 hours after sham or UP-LPS injection unless otherwise stated.

Functional in vivo studies

In vivo gastrointestinal transit was measured by evaluating the aboral intestinal distribution of an orally fed non-absorbable liquid marker, fluorescein isothiocyanate (FITC)-labeled dextran (10 μl, 70,000 Da, Molecular Probes), after 75 minutes as described in the past.⁸ Additionally, either cumulative endotoxin-associated mortality or post-endotoxemic resolution of gastrointestinal dysmotility was investigated four days after UP-LPS administration.

Histochemistry and Immunohistochemistry

Micro-dissected mucosa-free mid-jejunal muscularis whole-mounts were fixed with 100 % ethanol for 10 minutes at room temperature and either histochemically stained for extravasated polymorphonuclear neutrophils with Hanker-Yates reagent (Polysciences, Warrington, Pennsylvania, USA) or immunostained for F4/80⁺ monocytes/macrophages (AbD Serotec, A488 conjugated, rat anti mouse, 1: 100).²⁰ Cellular recruitment was quantified in 5 representative high power fields at a magnification of 200× (fluorescent microscope LeicaDMRX, McHenry, Illinois, USA) and the cell counts were averaged.

In vitro mid-jejunal contractile activity

The effect of *in vivo* LPS administration on muscarinic mid-jejunal smooth muscle contractility was measured *in vitro*. Briefly, mucosa-free individual circular muscle strips of 0.5 mm width were mounted horizontally in organ bath chambers perfused with prewarmed (37 °C), pH adjusted and oxygenated modified Krebs solution. After equilibration, normalized muscarinic dose-dependent intestinal smooth muscle contractile responses (bethanechol, 0.1 µM to 300 µM) were assessed.

Relative mRNA quantification of muscularis externa extracts

Total mRNA was extracted from snap-frozen isolated intestinal muscularis externa of *TLR4* chimera mice. mRNA expression of the inflammatory target genes chemokine (C-X-C motif) ligand 10 (CXCL10), granulocyte-macrophage colony stimulating factor (GM-CSF), heme oxygenase-1 (HO-1), inter-cellular adhesion molecule-1 (ICAM-1, synonymous CD54), interleukin 6 (IL-6), interleukin-10 (IL-10), interleukin-12 (IL-12), and inducible nitric oxide synthase (iNOS) were quantified by standardized SYBR Green two-step real-time reverse transcription-PCR (RT-PCR) using the same standards as in our previous work.⁸ Sequences of the quantitative PCR (qPCR) primers are provided in table S I. Relative fold increase of the genes of interest was quantified over the murine house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All comparative statistical analyses in this study were performed with the appropriate *bm TLR4^{WT}/TLR4^{WT}* sham group.

Recombinant murine IL-6 treatment and antibody-mediated IL-6 receptor inhibition

Recombinant mouse IL-6 (rmIL-6, 10 ng/g, endotoxin level < 1.0 EU per µg protein, R & D systems, Minneapolis, USA) or volume-equal carrier solution PBS (5 µl/g) was given intravenously to *TLR4^{WT}* mice at 4 hours and 8 hours after a single intraperitoneal injection of saline or low-dose UP-LPS (1 mg/kg). The rmIL-6 dose was calculated based on reference IL-6 protein plasma levels in murine endotoxemia, and a 3-fold dose of total circulating IL-6 was chosen.

The murine soluble IL-6 receptor blocking antibody MR16-1 (anti-sIL-6R mAb, 10.3 mg/ml PBS, endotoxin activity level 0.01 EU/mg IgG) was a kind gift from Chugai Pharmaceutical Co. Ltd., Japan.²¹ The binding region of the anti-sIL-6R was mapped to the N-terminal half of the fibronectin domain II of mouse IL-6R (amino acids 214 – 285), and although the antibody was generated in mouse soluble IL-6R (sIL-6R)-immunized Wistar rats further studies suggested that the antibody is not only binding to sIL-6R thereby blocking IL-6 signal transduction but also capable to inhibit membrane-bound IL-6R function as it suppressed IL-6 induced proliferation of cultured lymphocytes. *TLR4^{WT}* mice were treated intravenously with a dose of 100 µg/g anti-sIL-6R mAb either simultaneously with intraperitoneal UP-LPS 5 mg/kg, or twice at 4 hours and 8 hours after UP-LPS injection. Intravenous volume-equal PBS (10 µl/g) and protein-equal isotype control antibody rat IgG₁ (IgG₁, 10.11 mg/ml, antigen horseradish peroxidase, endotoxin activity level < 1.4 EU/mg, BioXCell, West Lebanon, NH, USA) were used as sham and control treatment, respectively.

Assessment of plasma IL-6 protein levels

Heparinized blood was drawn from the caval vein at the time of harvest, centrifuged at 10,000 g for 10 minutes at 4 °C and plasma was stored at – 80 °C until further analysis. IL-6 protein levels were determined in duplicates utilizing enzyme-linked immunosorbent assay (ELISA, R & D systems, Minneapolis, USA) according to the manufacturer's instructions.

Solutions and data analysis

Modified Krebs solution constituents and drugs used were purchased from Sigma Chemical Company (St. Louis, Missouri, USA) unless otherwise stated. The results are expressed as mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed either by one-way analysis of variance (ANOVA) with Tukey posthoc test using InerSTAT-a v1.3 or EZAnalyze add-in for Microsoft Excel for F test and Bonferroni posthoc group comparisons where appropriate. A probability level of $p < 0.05$ was considered statistically significant.

Results

Endotoxin-induced ileus is independently mediated by lineage specific TLR4 signaling in a time-dependent fashion: time-delayed hemopoietic mechanism and sustained non-hemopoietic mechanism

Our previous publications demonstrated that UP-LPS caused a rapid molecular activation of hemopoietic derived cells within the muscularis externa, which however did not trigger ileus in the absence of non-hemopoietic *TLR4*/MyD88 signaling during the early phase of endotoxemia (6 hr).⁷⁻⁹ Nonetheless, we postulated that the functional significance of hemopoietic *TLR4* signaling might change over time. Therefore, we investigated UP-LPS induced alterations to *in vivo* gastrointestinal transit in *TLR4* bone marrow chimera mice 24 hours after endotoxin injection. As expected, UP-LPS administration to fully *TLR4* competent bm *TLR4*^{WT}/*TLR4*^{WT} chimera mice disrupted gastrointestinal propulsive motor activity yielding a delayed GC value of 3.4 ± 0.55 comparable to non-chimeric *TLR4* competent mice (Figure 1A and B) (N=5-6), while complete *TLR4* deficiency (bm *TLR4*^{LPS-d}/*TLR4*^{LPS-d}) protected from late endotoxin-induced ileus in the experimental chimera model (GC 10.6 ± 0.59) (Figure 1B) (N=5-7). Analogous to the observations made at the early time point of 6 hours after single hit endotoxemia,⁸ bm *TLR4*^{LPS-d}/*TLR4*^{WT} mice were severely affected with a significant suppression in gastrointestinal motility (GC 5.2 ± 0.68), which demonstrates the functional significance of a sustained *TLR4* mediated response by non-hemopoietic derived cells. Importantly, in sharp contrast to the early phase of endotoxemia,^{8,9} bm *TLR4*^{WT}/*TLR4*^{LPS-d} mice were no longer protected from endotoxin-induced ileus (GC of 7.0 ± 0.73) at 24 hours after UP-LPS administration. The gastrointestinal effect of functional hemopoietic signaling was still present 48 hours after UP-LPS treatment of bm *TLR4*^{WT}/*TLR4*^{LPS-d} mice (GC 8.6 ± 0.4 , N=4, data not shown). Hence, these investigations elucidate the existence of a time-delayed hemopoietic-driven mechanism operating independent of the non-hemopoietic *TLR4* cellular signaling machinery. Notably, however, the severity of UP-LPS-induced ileus in bm *TLR4*^{WT}/*TLR4*^{LPS-d} was diminished when compared to bm *TLR4*^{LPS-d}/*TLR4*^{WT}, and ileus induction in both hetero-chimera groups tended to be less marked compared to fully *TLR4* competent chimera mice.

Recruited muscularis polymorphonuclear leukocytes are not the causative hemopoietic subpopulation mediating late onset LPS-induced ileus while the role of CSF-1 dependent macrophages in ileus remains unclear

When LPS activates tissue macrophages, their chemotactic signaling properties guide circulating hemopoietic derived leukocytes to the site of inflammation and also to remote organs.^{22,23} Therefore, we quantified polymorphonuclear (PMN) and macrophage/monocyte cell influx into mid-jejunal muscularis whole-mounts. The intestinal muscularis externa of all sham vehicle injected mice (*TLR4*^{WT}, *TLR4*^{LPS-d}, and *TLR4* chimeras) showed no more than one neutrophil within each microscopic field of an individual whole-mount. As previously reported for wild-type rodents,^{7,24,25} and shown in Figure 1C (N=5 each), UP-LPS treatment induced a significant *TLR4*-dependent transmigration of neutrophils into the intestinal muscularis of both *TLR4*^{WT} (12.3 ± 2.15 cells/field) and bm *TLR4*^{WT}/*TLR4*^{WT}

chimera mice (12.3 ± 2.15 and 12.1 ± 0.9 cells/field, respectively). Interestingly, both hetero-chimera groups displayed markedly diminished numbers of extravasated neutrophils (bm $TLR4^{LPS-d}/TLR4^{WT}$ LPS 2.0 ± 0.48 and bm $TLR4^{WT}/TLR4^{LPS-d}$ LPS 2.0 ± 0.68) despite the presence of ileus.

Although a complete network of resident muscularis macrophages stained positive for F4/80 in all sham and UP-LPS treated groups, no monocytic infiltration was detected in any of the chimera or non-chimera mice 24 hours after UP-LPS (data not shown) (N=3–5). Intriguingly, LPS-treated CSF-1 deficient mice died prematurely with a mortality rate of 80 % (time range 14 hours to 22 hours after endotoxin treatment) (Figure 1 A) (N=5) prohibiting further investigations on the role of macrophages in late $TLR4$ -mediated ileus utilizing this transgenic approach, and implicating the necessity of a balanced macrophage-derived anti-inflammatory response restricting inflammation.

In vitro muscarinic jejunal smooth muscle contractility is not compromised during late endotoxin-induced ileus

To investigate a persistent effect of UP-LPS on jejunal smooth muscle function, we generated *in vitro* dose-response curves using the muscarinic agonist bethanechol ($0.1 \mu\text{M}$ to $300 \mu\text{M}$). Quantitatively, $100 \mu\text{M}$ bethanechol stimulation resulted in equivalent robust contractile responses, regardless of UP-LPS exposure. ($TLR4^{WT}$ Sham= 1.7 ± 0.18 gr/mm²/sec and $TLR4^{WT}$ LPS 1.8 ± 0.22 gr/mm²/sec). Similarly, as expected, UP-LPS had no significant effect on *in vitro* circular muscle contractions in the LPS deficient mice ($TLR4^{LPS-d}$ Sham= 2.0 ± 0.20 gr/mm²/sec and $TLR4^{LPS-d}$ LPS 1.97 ± 0.15 gr/mm²/sec at bethanechol $100 \mu\text{M}$) (Figure 2) (N=6). These results indicated the absence of sustained alterations of the smooth muscle contractile apparatus after *in vivo* UP-LPS administration at this dose.

Cell lineage synergism regulates the $TLR4$ induced intestinal muscularis inflammatory response

$TLR4$ mediated activation of its bifurcated downstream adapter protein pairs MyD88/MAL and TRIF/TRAM elicits the transcription of a broad variety of inflammatory genes.^{9;14;24;26} In line with molecular events during early endotoxemia,^{8;9} all inflammatory target genes (including: GM-CSF, iNOS, IL-6, IL-12, CXCL10, ICAM-1, and the anti-inflammatory cytokines IL-10 and HO-1) analyzed were significantly induced within the intestinal muscularis during late endotoxemia in a $TLR4$ -dependent manner (table S II) (N=6–7). In fully $TLR4$ competent (bm $TLR4^{WT}/TLR4^{WT}$) chimera mice, endotoxin activated all inflammatory genes even 3 to 10-fold higher relative to non-chimeric $TLR4^{WT}$ mice, while these inductions were virtually absent in bm $TLR4^{LPS-d}/TLR4^{LPS-d}$ LPS mice (Figure 3 A–D). A detailed description of the cell-lineage specific contributions to the individual inflammatory gene transcription is provided as supplemental data. In short, analysis of $TLR4$ -dependent cell lineage specific transcription within the intestinal muscularis revealed GM-CSF gene induction only in non-hemopoietic cells while increased CXCL-10, IL-12, iNOS and IL-10 gene transcription occurred predominantly in hemopoietic cells. In contrast, ICAM-1, HO-1 and IL-6 message was amplified in both cellular systems. These observations were similar to molecular events during early endotoxemia, indicating a distinct time-independent functional role for both intestinal cell lineages in inflammation (Figure 3 A–D) (N=4). Most interestingly, LPS-mediated muscularis gene induction in the selective cell lineage $TLR4$ responsive mice (bm $TLR4^{LPS-d}/TLR4^{WT}$ and bm $TLR4^{WT}/TLR4^{LPS-d}$) was always diminished compared to fully $TLR4$ competent chimera mice, and especially IL-6 mRNA was only marginally increased by UP-LPS in hetero-chimera mice.

Endotoxin-associated mortality is mediated by synergy of hemopoietic- and non-hemopoietic cell derived TLR4 signaling

We next examined endotoxemia associated mortality and resolution of ileus in mice with *TLR4* competence in both or individual hemopoietic- or non-hemopoietic cells. Intraperitoneal UP-LPS treatment resulted in a high mortality rate of 68% in age-matched *TLR4*^{WT} non-chimera mice within 3 days post-injection (Figure 6 E). A similar dose of UP-LPS injected into bm *TLR4*^{WT}/*TLR4*^{WT} mice was consistently lethal with a more rapid and higher mortality rate of 100% within 30 hours of injection (Figure 4 A) (N=4–6).

Correlating with lessened muscularis inflammation, *TLR4* deficiency in individual cell lineages - either hemopoietic or non-hemopoietic - was sufficient to completely protect mice from this lethal insult with a 100% survival rate on post-injection day 4. Additionally, as shown in Figure 4 B, both bm *TLR4*^{LPS-d}/*TLR4*^{WT} and bm *TLR4*^{WT}/*TLR4*^{LPS-d} mice completely recovered after four days from the earlier UP-LPS-mediated disruption in gastrointestinal motility displaying GC values of 10.4±0.22 and 10.1±0.85, respectively (N=4).

Exogenous IL-6 supplementation does not spark off a self-perpetuating inflammatory circuit

Prompted by the impressive synergistic induction of intestinal muscularis IL-6 message and its association with mortality, we investigated the effect of exogenous IL-6 (rmIL-6) on gastrointestinal motility either alone or combined with low dose LPS (1 mg/kg i.p.). As shown in Figure 5A–B, low dose LPS treatment did not significantly slow down liquid gastrointestinal transit in *TLR4*^{WT} mice in line with rather low circulating IL-6 protein levels. Although, by observation these mice were severely sick with anorexia, dehydration, lethargy and moderate amounts of optical white haze. But in contrast to lethal doses of LPS, they did not shiver or appear succumbing (N=5).

Mice intravenously injected with rmIL-6 alone showed neither sickness behavior nor significant alterations in gastrointestinal motility while systemic IL-6 protein levels were 7-fold higher compared to sham treated mice. Also, the combination of a sublethal dose of LPS and subsequent exogenous rmIL-6 injections did not significantly impact gastrointestinal motility, still single cytokine supplementation by rmIL-6 treatment further augmented IL-6 protein levels compared to low dose LPS alone (Figure 5A–B) (N=5).

Therapeutic blockade of IL-6R signaling prevents late ileus but not inflammation and mortality

Further exploring the role of functional IL-6, we sought to investigate whether neutralization of IL-6 signal transduction can ameliorate synergistically non-hemopoietic and hemopoietic mediated *TLR4*-triggered ileus and inflammation.

While sham treatment with intravenous PBS substitution did not amend *TLR4*-triggered ileus, control treatment with intravenous rat IgG₁ showed some improvement of LPS-induced gastrointestinal dysmotility which however was not statistically significant from LPS+PBS treated *TLR4*^{WT} mice (Figure 6A and C) (N=4–5). Intravenous anti-sIL-6 receptor antibody (anti-sIL-6R mAb, MR16-1) treatment simultaneously with intraperitoneal LPS injection slightly improved liquid gastrointestinal transit, however the increase in transit was not significantly different from *TLR4*-triggered ileus in mice additionally injected with saline or IgG₁, respectively (Figure 6A). Still, antibody mediated IL-6R blockade was remarkably efficient in reversing *TLR4*-mediated ileus when given in a therapeutic approach (4 hr and 8 hr after LPS treatment) yielding almost normal GC values, which were statistically significant from both LPS+PBS and LPS+IgG₁ groups (Figure 6C).

In the dose used, no toxicity of the murine antibody was observed. Although anti-sIL-6R mAb by itself induced systemic IL-6 protein levels 16-fold compared to sham treated mice, theoretically indicating a negative feedback loop by IL-6 signaling on IL-6 release, an unspecific immunoglobulin-mediated phenomenon might account for this observation (Figure 6B). But, similar to IgG₁, anti-sIL-6R mAb did not increase or decrease the elevated circulating IL-6 levels during endotoxemia (Figure 6B and D) (N=4–5). Consistent with an unchanged inflammatory response, mice showed severe sickness behavior despite IL-6R blockade and the mortality rate of these mice was similar to LPS alone in non-chimeric TLR4^{WT} mice (Figure 6E) (N=6–7). Survivor animals of all groups recovered displaying normal gastrointestinal transit at 4 days after treatment, and IL-6 serum levels declined to near baseline values (data not shown).

Discussion

The clinical importance of a disruption in gastrointestinal motor function is that it leads to feeding intolerance, malnutrition, aspiration, bacterial overgrowth and mucosal barrier breakdown accompanied by an ensuing gut-derived cytokine storm contributing to SIRS and the high incidence of death due to MOF. Without a doubt, microbial recognition is the crucial primary catalyst in all imaginable scenarios and the fundamental task is to elucidate discrete mechanisms involved in this pathophysiological process. Therefore, we have focused on the specific role of LPS/TLR4 signaling in gastrointestinal dysmotility and inflammation.

Interestingly, we have recently shown that LPS/TLR4 sensitive early ileus at 6 hours is exclusively dependent on MyD88 signaling on non-hemopoietic derived cells.^{8,9} The distinct lack of a role for hemopoietic TLR4 signaling in early endotoxin-induced ileus was unexpected, especially with regard to the observed rapid and potent transcriptional activity in leukocytes. Also, hemopoietic TLR4 signaling would have most likely been eradicated through evolution if not functional or needful to the host. But, in fact, the chimera data at 24 hours after endotoxin exposure clearly revealed the existence of a novel second wave TLR4/hemopoietic driven mechanism, which occurred concurrently with the sustained non-hemopoietic cell lineage driven mechanism. Interestingly, Binck et al. observed a similar dual lineage mechanism of endotoxic cardiac contractile dysfunction after 18 hours concluding that myocardial depression is caused by the cumulative responses of both the classical leukocytes as well as parenchymal tissues.¹⁰

At present, the specific bone marrow derived subpopulation(s) that are involved in the late hemopoietic component of endotoxin-induced ileus have not been identified. TLR4 mediated transmigration of the monocytic lineage into the intestinal muscularis externa did not occur, which however does not preclude a role for the dense network of tissue macrophages in the hemopoietic mechanism of ileus. Surprisingly, CSF-1 deficient mice that are macrophage depleted and monocytopenic reacted more sensitively to LPS than wild-type littermates. Most likely, a macrophage-derived anti-inflammatory response is lacking in these mice tilting the inflammatory balance and rendering them more prone to organ failure and subsequent death. Unfortunately, based on the paradoxical observation between inflammation-triggered mortality and ileus, the contribution of tissue macrophages to hemopoietic-driven ileus remains currently unclear.

However, the data demonstrate local chemotaxis via TLR4-dependent induction of GM-CSF, CXCL-10/IP-10 and ICAM-1 within the intestinal muscularis externa. Both adhesion molecule induction and neutrophil extravasation were contingent on full endotoxin responsiveness in both hemopoietic and non-hemopoietic systems (TLR4^{WT} and bmTLR4^{WT}/TLR4^{WT} mice). Yet, the neutrophil influx in hetero-chimera mice did not

correlate with the extent of ileus at 24 hours, as the hetero-chimeras displayed ileus in the absence of a significant neutrophil recruitment. In contrast, the degree of neutrophil infiltration paralleled sepsis induced mortality in the individual groups of chimera mice. Hence, the recruited leukocytes may be key features and indicators of sepsis induced organ injury^{27,28} but do not seem to play a causative role in producing endotoxic ileus.

Interestingly, IL-12, the hallmark cytokine of the adaptive immune system, was significantly induced within the gut muscularis in the fully *TLR4* competent mice, most likely derived from antigen-presenting cells such as M1 macrophages and dendritic cells^{29,30} which are both known to innately respond to LPS. Dendritic cells are recruited to the intestine upon intestinal manipulation and TLR4 ligation,^{31,32} and are critical in maintaining intestinal homeostasis and instructing T cells.^{33–35} However, T cells express functional *TLR4* themselves³⁶ and immunomodulation by these cells greatly impacts survival outcome in both rodents and humans, as rapidly occurring systemic apoptosis-induced lymphopenia reflecting a state of protracted immunosuppression is associated with a high rate of mortality. Hence, further work will be required to investigate the specific functions of individual hemopoietic subpopulations and their causative role in the hemopoietic mechanism of ileus.

Irrespective of the specific hemopoietic subpopulation(s) involved, whether the hemopoietic mechanism is mediated as part of a local leukocyte response and/or via systemic inflammatory mediators was yet unknown. Hypothetically, leukocyte-derived effector molecules could play a key role in the initiation and prolongation of sepsis-induced ileus, as in the past implicated for iNOS, IL-1 β , MCP-1, and IL-6.^{1,14} IL-6 is an established predictive biomarker in human sepsis,³⁷ and links the innate and the adaptive immune system.³⁸ This pleiotropic cytokine is known to be upregulated in numerous intestinal injury and disease states, including postoperative ileus,³⁹ ischemia-reperfusion injury¹⁹ and inflammatory bowel disease. Its many roles include immunoregulation and modulation of enteric neurotransmission.⁴⁰ Here, over time, crosstalk of hemopoietic and non-hemopoietic cells generated an escalating inflammatory response within the muscularis externa with IL-6 message rising to 3-fold of the levels reached during early endotoxemia.⁸ This snowballing phenomenon was associated with high mortality of fully *TLR4* competent mice in our study. Interestingly, muscularis IL-6 mRNA induction was significantly less in animals with partial *TLR4* deficiency. Indeed, compared to 6 hours after LPS treatment,⁸ IL-6 message in hetero-chimera mice was already declining to half of the values, which was accompanied by amelioration of ileus at 24 hours, significantly reduced endotoxin-triggered mortality and complete recovery from endotoxic ileus. Complementing this, anti-inflammatory strategies aiming at reducing ileus like lipid-rich nutrition and preconditioning protocols¹⁵ always demonstrated reduced IL-6 induction.

However, no attempts have been made so far to causatively link IL-6 to LPS-induced dysmotility. Circulating IL-6 protein levels were LPS dose dependent in our experimental model but exogenous intravenous IL-6 treatment did not initiate ileus neither when given alone nor when supplemented to a low dose LPS. *In vitro*, IL-6 alone had no direct effect on smooth muscle cells⁴¹ but healthy rodents developed gastroparesis 3 hours after intraperitoneal injections of high dose IL-6 (125 ng/g).¹⁸ These contradictory results might be caused not only by different timing but also by different doses and routes of administration used. As with all rodent experiments, efforts should be made to use relevant pathophysiological doses. We therefore had calculated the intravenous dose of IL-6 based on reference IL-6 plasma protein levels during early endotoxemia taking the total blood volume of the mouse into account but, admittedly, could not mimic continuous systemic IL-6 elevation.

Despite the failure of IL-6 to trigger ileus and self-amplifying inflammation in our model, we were interested whether single-cytokine targeting via antibody-mediated blockade of IL-6 signaling would be a valuable therapeutic option to prevent *TLR4*-induced ileus and inflammation. The tripartite IL-6 signaling complex consists of IL-6 protein, the ligand-binding IL-6 receptor (IL-6R), also known as CD126, and the transmembrane-spanning glycoprotein gp130 (CD130), which is the signal transducing component. Importantly, two different forms of the IL-6R exist, including a cell-surface bound IL-6R α chain with its expression mainly restricted to hepatocytes and hemopoietic subpopulations such as monocytes, neutrophils, T cells and B cells, and a second soluble form of IL-6R (sIL-6R) originating from either alternative splicing or metalloproteinase-mediated ectodomain cleavage of membrane-bound IL-6R.⁴²⁻⁴⁴ In direct contrast to other soluble cytokine receptors like secreted sIL-1RI and sIL-1RII,^{45;46} sIL-6R does not act as a decoy providing competitive inhibition of IL signal transduction but instead agonistically mediates IL-6 responsiveness in a broad range of cell types that are lacking IL-6R but express gp130 such as fibroblasts, vascular smooth muscle cells, endothelial cells and astrocytes.⁴⁷⁻⁵⁰ Importantly, anti-sIL-6R mAB was shown to competitively inhibit binding of IL-6 to IL-6R thereby mediating neutralization of IL-6 signal transduction.²¹

Preemptive treatment with MR16-1, a murine rat-derived anti-IL-6 receptor antibody directed against sIL-6R with neutralization capacity of both soluble and membrane-bound IL-6R,²¹ had a marginal benefit in ameliorating LPS-induced ileus at 24 hours. Yet, a more clinically relevant approach with repeated intravenous injection of anti-sIL-6R mAB at 4 hours and 8 hours after onset of endotoxemia, a time where animals become lethargic and high IL-6 levels are present, was remarkably beneficial in causing a near normalization of liquid gastrointestinal transit. Control treatment with rat IgG₁, which can potentially alter the immune system through both pro- and anti-inflammatory mechanisms,⁵¹ exerted only minor effects on LPS-induced gastrointestinal dysmotility, confirming that the significant prevention of *TLR4*-triggered ileus was specifically mediated by IL-6R blockade.

However, despite protection of gastrointestinal motor function, inhibition of functional IL-6 signaling did not modify sickness behavior and did not avert mortality implicating other bioactive substances as causative factors in organ failure. IL-6 levels demonstrated LPS-dose dependency, therefore serving well as biomarker of inflammation, but were not self-regulated by IL-6 receptor signaling. Interestingly, in parallel with our findings for muscularis IL-6 message, serum IL-6 levels late in endotoxemia were dependent on mice being *TLR4* competent in both the hemopoietic and the non-hemopoietic lineage,⁵² and excessive IL-6 plasma levels were indicative of rodent mortality within a four day period after cecal ligation and puncture.⁵³

The exact mechanism of MR16-1 in preventing *TLR4*-triggered ileus is currently unclear and warrants further investigation. One of the major gastrointestinal motility modulating cell population is the extrinsic and intrinsic neuronal network. Interestingly, neuro-immune interaction has been reported with increased neurotrophin expression and neuronal survival in response to IL-6 if sIL-6R is present.^{54;55} Furthermore, IL-6 combined with IL-1 β regulated norepinephrine release from the longitudinal muscle myenteric plexus and myenteric nerve varicosity preparations explaining altered neurotransmitter release in the inflamed intestine.⁵⁶ On the other hand, especially with regard to the hemopoietic contribution, it is known that IL-6 blockade recalibrates the balance of the adaptive immune system in graft-versus-host disease after bone marrow transplantation,⁵⁷ and that inhibition of IL-6 signaling was beneficial in murine collagen-induced arthritis by suppression of Th17 cell induction.⁵⁸

In conclusion, this study aids unraveling another puzzle piece of the complex picture of TLR4 signaling. LPS/*TLR4* induced ileus is the result of multi-dysfunctional elements which participate in disturbing motility, and we demonstrated that two independent pathways of late-endotoxin induced ileus coexist as illustrated in Figure 4. Dual activation of *TLR4*-dependent signaling on hemopoietic and non-hemopoietic cell lineages synergistically produce maximal expression of intestinal muscularis IL-6 message and endotoxin-associated lethality, while *TLR4* deficiency of one cell lineage permits resolution of LPS-induced ileus. Here, deletion of a single cytokine out of the complex LPS-triggered inflammatory repertoire by antibody-mediated IL-6R blockade efficiently reinstated gastrointestinal motor function, however it did not improve survival. Finally, it needs to be pointed out that the gut, as a major cytokine producing organ, can intoxicate itself over time via paracrine mechanisms, but a certain threshold of inflammation is needed to trigger ileus. Still, it appears that gastrointestinal dysmotility is not proportionally linked to endotoxin-triggered mortality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

anti-sIL-6R mAb	murine soluble interleukin-6 receptor blocking antibody
CXCL-10	chemokine (C-X-C motif) ligand 10
GM-CSF	granulo-macrophage colony stimulating factor, synonym CSF-2
HO-1	heme oxygenase-1
iNOS	inducible nitric oxide synthase
rmIL-6	recombinant mouse interleukin-6
TRIF	Toll-IL-1-resistance (TIR) domain-containing adaptor-inducing IFN- β
UP-LPS	ultra-pure® lipopolysaccharide

Reference List

1. Buchholz BM, Bauer AJ. Membrane TLR signaling mechanisms in the gastrointestinal tract during sepsis. *Neurogastroenterol Motil.* 2010 Mar; 22(3):232–245. [PubMed: 20377787]
2. Medzhitov R, Janeway J. The Toll receptor family and microbial recognition. *Trends in Microbiology.* 2000 Oct 1; 8(10):452–456. [PubMed: 11044679]
3. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol.* 2004 Jul; 4(7):499–511. [PubMed: 15229469]
4. Angus DCM, Linde-Zwirble WT, Lidicker JM, Clermont GM, Carcillo JM, Pinsky MRM. Epidemiology of severe sepsis in the United States: Analysis of incidence, outcome, and associated costs of care. *Critical Care Medicine.* 2001 Jul; 29(7):1303–1310. [PubMed: 11445675]
5. Melamed A, Sorvillo FJ. The burden of sepsis-associated mortality in the United States from 1999 to 2005: an analysis of multiple-cause-of-death data. *Critical Care (London, England).* 2009; 13(1):R28.
6. Deitch EA. Bacterial translocation or lymphatic drainage of toxic products from the gut: what is important in human beings? *Surgery.* 2002 Mar; 131(3):241–244. [PubMed: 11894026]
7. Eskandari MK, Kalff JC, Billiar TR, Lee KK, Bauer AJ. Lipopolysaccharide activates the muscularis macrophage network and suppresses circular smooth muscle activity. *Am J Physiol.* 1997 Sep; 273(3 Pt 1):G727–G734. [PubMed: 9316478]

8. Buchholz BM, Chanthaphavong RS, Bauer AJM. Nonhemopoietic cell TLR4 signaling is critical in causing early lipopolysaccharide-induced ileus. *J Immunol.* 2009 Nov 15; 183(10):6744–6753. [PubMed: 19846874]
9. Buchholz BM, Billiar TR, Bauer AJ. Dominant role of the MyD88-dependent signaling pathway in mediating early endotoxin-induced murine ileus. *Am J Physiol Gastrointest Liver Physiol.* 2010 Aug; 299(2):G531–G538. [PubMed: 20508155]
10. Binck BW, Tsen MF, Islas M, White DJ, Schultz RA, Willis MS, et al. Bone marrow-derived cells contribute to contractile dysfunction in endotoxic shock. *Am J Physiol Heart Circ Physiol.* 2005 Feb 1; 288(2):H577–H583. [PubMed: 15458952]
11. Mollen KP, Levy RM, Prince JM, Hoffman RA, Scott MJ, Kaczorowski DJ, et al. Systemic inflammation and end organ damage following trauma involves functional TLR4 signaling in both bone marrow-derived cells and parenchymal cells. *J Leukoc Biol.* 2008 Jan 1; 83(1):80–88. [PubMed: 17925504]
12. Chakravarty S, Herkenham M. Toll-Like Receptor 4 on Nonhematopoietic Cells Sustains CNS Inflammation during Endotoxemia, Independent of Systemic Cytokines. *J Neurosci.* 2005 Feb 16; 25(7):1788–1796. [PubMed: 15716415]
13. Hui W, Jinxiang Z, Heshui W, Zhuoya L, Qichang Z. Bone marrow and non-bone marrow TLR4 regulates hepatic ischemia/reperfusion injury. *Biochem Biophys Res Commun.* 2009 Nov 13; 389(2):328–332. [PubMed: 19723506]
14. Overhaus M, Togel S, Pezzone MA, Bauer AJ. Mechanisms of polymicrobial sepsis-induced ileus. *Am J Physiol Gastrointest Liver Physiol.* 2004 Sep; 287(3):G685–G694. [PubMed: 15331356]
15. Schwarz NT, Engel B, Eskandari MK, Kalff JC, Grandis JR, Bauer AJ. Lipopolysaccharide preconditioning and cross-tolerance: the induction of protective mechanisms for rat intestinal ileus. *Gastroenterology.* 2002 Aug; 123(2):586–598. [PubMed: 12145811]
16. Bozza FA, Salluh JI, Japiassu AM, Soares M, Assis EF, Gomes RN, et al. Cytokine profiles as markers of disease severity in sepsis: a multiplex analysis. *Crit Care.* 2007; 11(2):R49. [PubMed: 17448250]
17. Greenhill CJ, Rose-John S, Lissilaa R, Ferlin W, Ernst M, Hertzog PJ, et al. IL-6 trans-signaling modulates TLR4-dependent inflammatory responses via STAT3. *J Immunol.* 2011 Jan 15; 186(2):1199–1208. [PubMed: 21148800]
18. McCarthy DO. Tumor necrosis factor alpha and interleukin-6 have differential effects on food intake and gastric emptying in fasted rats. *Res Nurs Health.* 2000 Jun; 23(3):222–228. [PubMed: 10871537]
19. Buchholz BM, Masutani K, Kawamura T, Peng X, Toyoda Y, Billiar TR, et al. Hydrogen-enriched preservation protects the isogeneic intestinal graft and amends recipient gastric function during transplantation. *Transplantation.* 2011 Nov 15; 92(9):985–992. [PubMed: 21956195]
20. Schmidt J, Stoffels B, Moore BA, Chanthaphavong RS, Mazie AR, Buchholz BM, et al. Proinflammatory Role of Leukocyte-Derived Egr-1 in the Development of Murine Postoperative Ileus. *Gastroenterology.* 2008 Sep; 135(3):926–936. [PubMed: 18652830]
21. Okazaki M, Yamada Y, Nishimoto N, Yoshizaki K, Mihara M. Characterization of anti-mouse interleukin-6 receptor antibody. *Immunol Lett.* 2002 Dec 3; 84(3):231–240. [PubMed: 12413742]
22. Kalff JC, Schwarz NT, Walgenbach KJ, Schraut WH, Bauer AJ. Leukocytes of the intestinal muscularis: their phenotype and isolation. *J Leukoc Biol.* 1998 Jun; 63(6):683–691. [PubMed: 9620660]
23. Kalff JC, Carlos TM, Schraut WH, Billiar TR, Simmons RL, Bauer AJ. Surgically induced leukocytic infiltrates within the rat intestinal muscularis mediate postoperative ileus. *Gastroenterology.* 1999 Aug; 117(2):378–387. [PubMed: 10419919]
24. Eskandari MK, Kalff JC, Billiar TR, Lee KK, Bauer AJ. LPS-induced muscularis macrophage nitric oxide suppresses rat jejunal circular muscle activity. *Am J Physiol.* 1999 Aug; 277(2):G478–G486. [PubMed: 10444463]
25. Turler A, Schwarz NT, Turler E, Kalff JC, Bauer AJ. MCP-1 causes leukocyte recruitment and subsequently endotoxemic ileus in rat. *Am J Physiol Gastrointest Liver Physiol.* 2002 Jan; 282(1):G145–G155. [PubMed: 11751168]

26. Lodato RF, Khan AR, Zembowicz MJ, Weisbrodt NW, Pressley TA, Li YF, et al. Roles of IL-1 and TNF in the decreased ileal muscle contractility induced by lipopolysaccharide. *Am J Physiol.* 1999 Jun; 276(6 Pt 1):G1356–G1362. [PubMed: 10362638]
27. Xu H, Gonzalo JA, St PY, Williams IR, Kupper TS, Cotran RS, et al. Leukocytosis and resistance to septic shock in intercellular adhesion molecule 1-deficient mice. *Journal of Experimental Medicine.* 1994 Jul 1; 180(1):95–109. [PubMed: 7911822]
28. Andonegui G, Zhou H, Bullard D, Kelly MM, Mullaly SC, McDonald B, et al. Mice that exclusively express TLR4 on endothelial cells can efficiently clear a lethal systemic Gram-negative bacterial infection. *J Clin Invest.* 2009 Jul; 119(7):1921–1930. [PubMed: 19603547]
29. Pearce EJ, Kane CM, Sun J. Regulation of dendritic cell function by pathogen-derived molecules plays a key role in dictating the outcome of the adaptive immune response. *Chem Immunol Allergy.* 2006; 90:82–90. [PubMed: 16210904]
30. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Biosci.* 2008; 13:453–461. [PubMed: 17981560]
31. Koscielny A, Boerner T, Wehner S, Kurts C, Kalff JC. The Role of Dendritic Cells in the Gastrointestinal Field Effect. *Transplantation Proceedings.* 2007 Jul; 38(6):1815–1817. [PubMed: 16908290]
32. Engel DR, Koscielny A, Wehner S, Maurer J, Schiwon M, Franken L, et al. T helper type 1 memory cells disseminate postoperative ileus over the entire intestinal tract. *Nat Med.* 2010 Dec; 16(12):1407–1413. [PubMed: 21113155]
33. De TC, Pajak B, Brait M, Glaichenhaus N, Urbain J, Moser M, et al. TLR4 and Toll-IL-1 receptor domain-containing adapter-inducing IFN-beta, but not MyD88, regulate Escherichia coli-induced dendritic cell maturation and apoptosis in vivo. *J Immunol.* 2005 Jul 15; 175(2):839–846. [PubMed: 16002681]
34. Napolitani G, Rinaldi A, Berton F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol.* 2005 Aug; 6(8):769–776. [PubMed: 15995707]
35. Coombes JL, Powrie F. Dendritic cells in intestinal immune regulation. *Nat Rev Immunol.* 2008 Jun; 8(6):435–446. [PubMed: 18500229]
36. Zanin-Zhorov A, Tal-Lapidot G, Cahalon L, Cohen-Sfady M, Pevsner-Fischer M, Lider O, et al. Cutting Edge: T Cells Respond to Lipopolysaccharide Innately via TLR4 Signaling. *J Immunol.* 2007 Jul 1; 179(1):41–44. [PubMed: 17579019]
37. Song M, Kellum JA. Interleukin-6. *Crit Care Med.* 2005 Dec; 33(Suppl)(12):S463–S465. [PubMed: 16340422]
38. Fang C, Zhang X, Miwa T, Song WC. Complement promotes the development of inflammatory T-helper 17 cells through synergistic interaction with Toll-like receptor signaling and interleukin-6 production. *Blood.* 2009 Jul 30; 114(5):1005–1015. [PubMed: 19491392]
39. Wehner S, Schwarz NT, Hundsdoerfer R, Hierholzer C, Tweardy DJ, Billiar TR, et al. Induction of IL-6 within the rodent intestinal muscularis after intestinal surgical stress. *Surgery.* 2005 Apr; 137(4):436–446. [PubMed: 15800492]
40. Ruhl A, Hurst S, Collins SM. Synergism between interleukins 1 beta and 6 on noradrenergic nerves in rat myenteric plexus. *Gastroenterology.* 1994 Oct; 107(4):993–1001. [PubMed: 7926489]
41. Montuschi P, Preziosi P, Navarra P. Interleukin-1 α and tumor necrosis factor inhibit rat gastric fundus motility in vitro. *Eur J Pharmacol.* 1993; 233:303–304. [PubMed: 8467878]
42. Jones SA, Horiuchi S, Topley N, Yamamoto N, Fuller GM. The soluble interleukin 6 receptor: mechanisms of production and implications in disease. *FASEB J.* 2001 Jan; 15(1):43–58. [PubMed: 11149892]
43. Lust JA, Donovan KA, Kline MP, Greipp PR, Kyle RA, Maihle NJ. Isolation of an mRNA encoding a soluble form of the human interleukin-6 receptor. *Cytokine.* 1992 Mar; 4(2):96–100. [PubMed: 1633265]
44. Briso EM, Dienz O, Rincon M. Cutting edge: soluble IL-6R is produced by IL-6R ectodomain shedding in activated CD4 T cells. *J Immunol.* 2008 Jun 1; 180(11):7102–7106. [PubMed: 18490707]

45. Sha Y, Markovic-Plese S. A role of IL-1R1 signaling in the differentiation of Th17 cells and the development of autoimmune diseases. *Self Nonself*. 2011 Jan; 2(1):35–42. [PubMed: 21776333]
46. Francis J, MohanKumar PS, MohanKumar SM. Lipopolysaccharide stimulates norepinephrine efflux from the rat hypothalamus in vitro: blockade by soluble IL-1 receptor. *Neurosci Lett*. 2001 Aug 3; 308(2):71–74. [PubMed: 11457562]
47. Cichy J, Rose-John S, Pryjma J, Travis J. Effect of soluble interleukin-6 receptor on interleukin-6 synthesis in human skin fibroblasts. *Biochem Biophys Res Commun*. 1996 Oct 14; 227(2):318–321. [PubMed: 8878515]
48. Klouche M, Bhakdi S, Hemmes M, Rose-John S. Novel path to activation of vascular smooth muscle cells: up-regulation of gp130 creates an autocrine activation loop by IL-6 and its soluble receptor. *J Immunol*. 1999 Oct 15; 163(8):4583–4589. [PubMed: 10510402]
49. Van Wagoner NJ, Oh JW, Repovic P, Benveniste EN. Interleukin-6 (IL-6) production by astrocytes: autocrine regulation by IL-6 and the soluble IL-6 receptor. *J Neurosci*. 1999 Jul 1; 19(13):5236–5244. [PubMed: 10377335]
50. Modur V, Li Y, Zimmerman GA, Prescott SM, McIntyre TM. Retrograde inflammatory signaling from neutrophils to endothelial cells by soluble interleukin-6 receptor alpha. *J Clin Invest*. 1997 Dec 1; 100(11):2752–2756. [PubMed: 9389739]
51. Aschermann S, Lux A, Baerenwaldt A, Biburger M, Nimmerjahn F. The other side of immunoglobulin G: suppressor of inflammation. *Clin Exp Immunol*. 2009; 160(2):161–167. [PubMed: 20041883]
52. Juskewitch JE, Kudsen BE, Platt JL, Nath KA, Knutson KL, Brunn GJ, et al. LPS-induced murine systemic inflammation is driven by parenchymal cell activation and exclusively predicted by early MCP-1 plasma levels. *Am J Pathol*. 2012; 180(1)
53. Xiao H, Siddiqui J, Remick DG. Mechanisms of mortality in early and late sepsis. *Infect Immun*. 2006 Sep; 74(9):5227–5235. [PubMed: 16926416]
54. März P, Cheng J-G, Gadiant RA, Patterson PH, Stoyan T, Otten U, et al. Sympathetic neurons can produce and respond to interleukin-6. *Proc Natl Acad Sci USA*. 1998; 95(6):3251–3256. [PubMed: 9501249]
55. März P, Heese K, Dimitriades-Schmutz B, Rose-John S, Otten U. Role of interleukin-6 and soluble IL-6 receptor in region-specific induction of astrocyte differentiation and neurotrophin expression. *Glia*. 1999; 26(3):191–200. [PubMed: 10340760]
56. Ruhl A, Hurst A, Collins SM. Synergism between interleukins 1 beta and 6 on noradrenergic nerves in rat myenteric plexus. *Gastroenterology*. 1994; 107(4):993–1001. [PubMed: 7926489]
57. Chen X, Das R, Komorowski R, Beres A, Hessner MJ, Mihara M, et al. Blockade of interleukin-6 signaling augments regulatory T-cell reconstitution and attenuates the severity of graft-versus-host disease. *Blood*. 2009; 114(4):891–900. [PubMed: 19491393]
58. Yoshida H, Hashizume M, Mihara M. IL-6 blockade preferentially inhibits Th17 differentiation in collagen-induced arthritis. *Rheumatol Int*. 2011 Jan; 31(1):127–131. [PubMed: 20658238]
59. Bauer AJ. Mentation on the immunological modulation of gastrointestinal motility. *Neurogastroenterol Motil*. 2008 May; 20(Suppl 1):81–90. [PubMed: 18402645]
60. Overhaus M, Moore BA, Barbato JE, Behrendt FF, Doering JG, Bauer AJ. Biliverdin protects against polymicrobial sepsis by modulating inflammatory mediators. *Am J Physiol Gastrointest Liver Physiol*. 2006 Apr; 290(4):G695–G703. [PubMed: 16537973]
61. Moore BA, Otterbein LE, Turler A, Choi AM, Bauer AJ. Inhaled carbon monoxide suppresses the development of postoperative ileus in the murine small intestine. *Gastroenterology*. 2003 Feb; 124(2):377–391. [PubMed: 12557144]
62. Nakao A, Moore BA, Murase N, Liu F, Zuckerbraun BS, Bach FH, et al. Immunomodulatory effects of inhaled carbon monoxide on rat syngeneic small bowel graft motility. *Gut*. 2003 Sep; 52(9):1278–1285. [PubMed: 12912858]
63. Nakao A, Otterbein LE, Overhaus M, Sarady JK, Tsung A, Kimizuka K, et al. Biliverdin protects the functional integrity of a transplanted syngeneic small bowel. *Gastroenterology*. 2004 Aug; 127(2):595–606. [PubMed: 15300591]

B

Figure 1. Both hemopoietic and non-hemopoietic TLR4 signaling can independently mediate late endotoxin-induced ileus but neutrophils do not comprise the causative hemopoietic subpopulation

Single-shot endotoxemia triggered a long-lasting TLR4-dependent gastrointestinal dysmotility as indicated by the calculated geometric center for the gastrointestinal transit (A). Although less severe compared to bmTLR4^{WT}/TLR4^{WT} LPS mice, late endotoxin-mediated ileus occurred in both hetero-chimera groups (bmTLR4^{LPS-d}/TLR4^{WT} LPS and bmTLR4^{LPS-d}/TLR4^{WT} LPS) while bmTLR4^{LPS-d}/TLR4^{LPS-d} LPS were protected (B) (*=p<0.05 versus all sham groups, #=p<0.05 versus bmTLR4^{WT}/TLR4^{WT} LPS, N=5–7). Genetic macrophage-depletion resulted in premature death of LPS-treated CSF-1^{-/-} mice with a mortality rate of 80 % precluding further investigations on the role of macrophages in late LPS-induced gastrointestinal dysmotility (A). Late TLR4-induced ileus was associated with a polymorphonuclear leukocyte recruitment within the intestinal muscularis externa of fully TLR4 competent mice but neutrophil extravasation was significantly diminished in both TLR4 hetero-chimera mice (C) (*=p<0.05 for TLR4^{WT} and bmTLR4^{WT}/TLR4^{WT} LPS versus all sham and all other LPS-treated groups, N=5).

Figure 2. In vitro muscarinic jejunal contractile apparatus function is not significantly altered during late endotoxin-induced ileus

The dose-dependent increment of bethanechol stimulated contractile force of midjejunal muscle strips was preserved in UP-LPS-treated *TLR4* non-chimera mice indicating a lack of permanent smooth muscle cell damage ($p > 0.05$ for all groups at 100 μM bethanechol, $N=6$).

Figure 3. Both hemopoietic and non-hemopoietic TLR4 signaling contributes differentially to the maximal molecular inflammatory response within the muscularis externa

Inflammatory gene transcripts were lineage-specific upregulated within the intestinal muscularis externa in a *TLR4*-dependent manner (A–D). Non-hemopoietic cells are the exclusive source of GM-CSF (A) while CXCL10 mRNA was solely induced in hemopoietic cells (C). Note the vigorous induction of IL-6 mRNA in bm *TLR4*^{WT} / *TLR4*^{WT} LPS mice while *TLR4* deficiency of one compartment resulted in significantly decreased induction of these central inflammatory cytokines (B) (‡=p<0.01 versus all sham groups and indicated LPS groups, #=p<0.01 versus all sham groups and p<0.05 versus indicated LPS groups, N=4–6).

A

Figure 4. Synergy of non-hemopoietic and hemopoietic TLR4 signaling determines severity of ileus and endotoxin shock associated mortality

Deficiency of either hemopoietic or non-hemopoietic *TLR4* signaling completely protected from endotoxin-induced mortality observed in fully *TLR4* competent mice (N=4–6) (A) and allowed recovery from endotoxin-triggered ileus at 4 days after single UP-LPS treatment (N=4) (B). Single-time activated hemopoietic and non-hemopoietic pathways of TLR4-mediated ileus differ in time of onset and severity of ileus induction (..... Non-hemopoietic TLR4 signaling, ----- hemopoietic TLR4 signaling, — — — synergistic non-hemopoietic and hemopoietic TLR4 signaling, - - - - extrapolated synergistic non-hemopoietic and hemopoietic TLR4 signaling († symbolizes rodent death)) (B).

Figure 5. Exogenous supplementation of IL-6 does not significantly aggravate below threshold endotoxemia

Despite sickness behavior, gastrointestinal transit was not significantly slowed down in TLR4^{WT} mice by low-dose UP-LPS. Intravenous recombinant murine IL-6 did neither significantly induce ileus by itself nor when given in combination with low-dose UP-LPS (A) (N=5). Plasma IL-6 protein levels were raised significantly only by combined treatment with exogenous IL-6 and low-dose UP-LPS (B) (*= $p < 0.05$ versus sham, N=5).

Figure 6. Neutralization of IL-6 function by antibody-mediated receptor blockade ameliorates ileus but does not prevent endotoxin-associated mortality

Inhibition of IL-6 signaling was most effective in preventing TLR4-triggered gastrointestinal dysmotility when given as a posttreatment after the onset of endotoxemia while immunomodulatory IgG₁ only slightly improved gastrointestinal transit during endotoxemia (A,C) (*= $p < 0.05$ versus sham, N=4–5). However, LPS-triggered elevated systemic IL-6 protein levels were unchanged by anti-sIL-6R mAb or IgG₁ treatment (B,D) and survival could not be improved by blockade of IL-6 signal transduction (E) (N=6–7).