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Title: Effect of hemorrhagic shock on gut barrier function and expression of stress-related genes in normal and gnotobiotic mice

Authors: Runkuan Yang[1], David J. Gallo[2], Jeffrey J. Baust[2], Simon K. Watkins[3], Russell L. Delude[1,4], and Mitchell P. Fink[1,2]

Affiliations: Departments of Critical Care Medicine[1], Surgery[2], and Pathology[4] and the Center for Biologic Imaging[3]
University of Pittsburgh School of Medicine
Pittsburgh, PA 15260

Correspondence: Mitchell P. Fink, M.D.
Department of Critical Care Medicine
616 Scaife Hall
3550 Terrace Street
Pittsburgh, PA 15260
Tel: (412) 383-8905
Fax: (412) 648-4977
Email: finkmp@anes.upmc.edu

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Abstract

We sought to determine whether gut-derived microbial factors influence the hepatic or intestinal inflammatory response to hemorrhagic shock and resuscitation (HS/R). Conventional and gnotobiotic mice contaminated with a defined microbiota without Gram-negative bacteria were subjected to either a sham procedure or HS/R. Tissue samples were obtained 4 h later for assessing ileal mucosal permeability to fluorescein isothiocyanate dextran and hepatic and ileal mucosal steady-state IL-6, iNOS, COX-2 and TNF mRNA levels. Whereas HS/R significantly increased ileal mucosal permeability in conventional mice, this effect was not apparent in gnotobiotic animals. HS/R markedly increased hepatic mRNA levels for several pro-inflammatory genes in both conventional and gnotobiotic mice. HS/R increased ileal mucosal IL-6 and COX-2 mRNA expression in conventional but not gnotobiotic mice. If gnotobiotic mice were contaminated with *Escherichia coli* C25, HS/R increased ileal mucosal permeability and up-regulated expression of IL-6 and COX-2. These data support the view that the hepatic inflammatory response to HS/R is largely independent of the presence of potentially pathogenic Gram-negative bacteria colonizing the gut, whereas the local mucosal response to HS/R is profoundly influenced by the microbial ecology within the lumen during and shortly after the period of hemorrhage.

Key Words: translocation, bacterial; permeability, mucosal; tumor necrosis factor; cyclo-oxygenase-2; inducible nitric oxide synthase; IL-6

Short title: Hemorrhagic shock in gnotobiotic mice

Introduction

The pathological mechanisms underlying the development of multiple organ dysfunction in victims of trauma and hemorrhagic shock (HS) remain poorly understood. Nevertheless, a widely held view is that poorly controlled systemic activation of the inflammatory response somehow leads to cellular dysfunction and/or destruction in various organs, including the liver, lungs and kidneys. Although a large body of data supports the idea that the systemic inflammatory response syndrome (SIRS) and the multiple organ dysfunction syndrome (MODS) are causally related, the SIRS/MODS model is insufficient by itself to explain some common clinical observations. For example, some victims of major trauma and hemorrhagic shock and resuscitation (HS/R) are fortunate and recover relatively uneventfully. Others, however, suffer complications such as acute respiratory failure, azotemia, jaundice, and systemic infection. Of course, the likelihood of developing MODS depends, in part, on the magnitude of the initial insult (50). Nevertheless, because the magnitude of the initial stressful event fails to reliably predict the occurrence of MODS, it seems probable that one or more other, possibly host-related, factors is also involved. One of these additional factors may be the leakage of bacteria or microbial products, notably lipopolysaccharide (LPS), from the lumen of the gut into the systemic compartment, leading to the initiation or amplification of a deleterious inflammatory response. This view is supported by results from a number of clinical studies, which have documented increases in intestinal epithelial permeability in a variety of acute conditions, including trauma and HS/R (28;36;41). Moreover, in several recent studies, increased intestinal permeability has been shown to be associated with an increased risk of complications, MODS, or even mortality (2;13;15;40).

Despite these observations, only very limited data are available to support a direct link between gut-derived microbes or microbial products and the inflammatory response in distant organs following HS/R (6;19;64;67). One might presume that the most direct way to test the leaky gut hypothesis in the laboratory would be to compare the effects of HS/R in normal and germ-free animals, using markers of systemic inflammation and/or organ injury as the read-outs. Indeed, some previous studies have employed just this strategy (16;21;22;31;38;48;68). However, numerous structural and functional immunological abnormalities are present in animals that have been maintained under truly germ-free conditions (5;7;9;39). Accordingly, drawing inferences about alterations the inflammatory response after HS/R related to the absence of microbes in the intestine could be confounded by pre-existing immunological abnormalities.

One way to minimize some of the concerns regarding the use of germ-free animals is to employ gnotobiotic mice colonized with a defined microbiota called the altered Schaedler flora (51). These animals are born by Cesaerian delivery and raised in a germ-free environment, but are deliberately contaminated with eight strains of bacteria with low pathogenic potential. The eight strains consist of the following: two strains of Lactobacillus (ASF 361 and ASF 360); a strain closely related to the genus Porphyromonas (ASF 360); a spiral-shaped strain in the Flexistipes phylum (ASF 457); and four strains of extremely oxygen-sensitive fusiform Gram-positive bacteria (ASF 356, ASF 492, ASF 502 and ASF 500). Unlike germ-free animals, gnotobiotic mice colonized with various bacterial strains with low pathogenic potential manifest relatively normal resistance to opportunistic bacteria (20) and other aspects of immunological responsiveness, such as the number of IgA-secreting lymphocytes in the intestinal mucosa (26;35). Thus, studies using gnotobiotic mice carrying the Schaedler flora might provide a more clinically relevant model for testing the role of gut-derived pathogenic bacteria in the pathogenesis of organ system injury and inflammation following resuscitation from hemorrhagic shock.

Accordingly, in the present study, we subjected conventional and gnotobiotic (ASF-contaminated) Black Swiss mice to HS/R. Whereas HS/R significantly increased ileal mucosal permeability to a hydrophilic macromolecular tracer--fluorescein-labelled dextran with an average molecular weight of 4000 Da (FD4)--in conventional mice, this effect was not apparent in gnotobiotic animals. HS/R activated the pro-inflammatory transcription factor, NF-κB, in liver in both conventional and gnotobiotic mice. Similarly, HS/R markedly increased hepatic steady-state mRNA levels for several pro-inflammatory genes in both conventional and gnotobiotic mice. In contrast, HS/R increased ileal mucosal IL-6 and cyclooxygenase-2 (COX-2) mRNA expression in conventional but not gnotobiotic mice. Collectively, these data support the view that the hepatic inflammatory response to HS/R is largely independent of the presence of potentially pathogenic Gram-negative bacteria colonizing the gut, whereas the local mucosal response to HS/R is profoundly influenced by the microbial ecology within the lumen during the period of hemorrhage and resuscitation.

Materials And Methods

This research protocol complied with the regulations regarding animal care as published by the National Institutes of Health and was approved by the Institutional Animal Use and Care Committee of the University of Pittsburgh Medical School. Male black swiss wild type and gnotobiotic mice, age 4-8 weeks, were obtained from Taconic (Germantown, NY). The wild type animals were maintained at the University of Pittsburgh Animal Research Center with a 12-hour light-dark cycle and free access to standard laboratory feed and water. The gnotobiotic mice were kept in a sterilized facility with free access to germ-free food and water. Animals were not fasted prior to the experiments. All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless otherwise noted.

Experimental design for animal experiments. Pilot studies using 30 conventional mice were carried out to determine the optimal time after resuscitation to maximize the signal for the primary read-out of interest (ileal mucosal permeability) while minimizing the loss of animals due to mortality induced by the HS/R protocol. After completing these pilot experiments, four groups of mice (N=8 each) were studied for the main series of experiments. The two sham groups (conventional and gnotobiotic) were anesthetized but subjected neither to shock nor resuscitation. The two HS/R groups (conventional and gnotobiotic) were subjected to hemorrhagic shock and resuscitated with shed blood and Ringer's lactate solution (RLS). The shock model employed has been described in detail previously (65). Briefly, mice were anesthetized using intramuscular pentobarbital sodium (90 mg/kg). Both femoral arteries were surgically prepared and cannulated. The left artery was used for continuous blood pressure monitoring. The right artery was used for blood withdrawal and blood and fluid administration. The mice were subjected to hemorrhagic shock by withdrawal of blood (2.25 ml/100 g body wt) over 10 min to achieve a mean arterial pressure (MAP) of 30 mm Hg. MAP was maintained at 30 mm Hg for 2.0 h with continuous monitoring of blood pressure and withdrawal and return of blood as needed. Cannulas, syringes, and tubing were flushed with heparin sodium (1,000 U/ml) before all procedures. The animals were resuscitated to an initial MAP≥80 mm Hg by administration of all remaining shed blood plus intra-arterial injection of 2× shed blood volume of RLS over 30 min. Sham controls were subjected to the same anesthetic and cannulation procedures, but were not subjected to hemorrhagic shock. Four h after resuscitation (or sham shock), surviving mice were re-anesthetized with intramuscular sodium pentobarbital (90 mg/kg), and the following procedures were performed: a segment of ileum was harvested for determination of mucosal permeability; the mesenteric lymph node (MLN) complex was harvested to measure bacterial translocation; blood was aspirated from the heart to measure the plasma concentration of alanine aminotransferase (ALT); and portions of the liver and ileum were harvested for determination of NF-κB activation using electrophoretic mobility shift assay (EMSA) and/or expression of pro-inflammatory genes using semi-quantitative RT-PCR.

Because of the results obtained, a fifth experimental group was added to the protocol. This group consisted of gnotobiotic mice that were enterally contaminated with *Escherichia coli* C25. The initial innoculum was a gift from Dr. H. Ford (University of Pittsburgh School of Medicine). The bacteria were grown overnight in brain-heart infusion. The bacteria were washed twice in sterile saline, being pelleted by centrifugation (4,000 *g* for 10 min) between and after the washes. The bacteria were resuspended in normal saline at a final concentration of 5×10^{10} cfu/ml. The concentration of bacteria was estimated by measuring the absorbance of the suspension at 550 nM and assuming that an optical density of 1.0 corresponds to a concentration of 3×10^9 cfu/ml. Eighteen h before inducing hemorrhagic shock, the mice were anesthetized with ketamine HCl (20 mg/kg) and gavaged with 0.2 ml of the bacterial suspension.

Intestinal mucosal permeability. Intestinal mucosal permeability to the fluorescent tracer, fluoroscein isothiocyanate dextran with a molecular weight of 4,000 Da (FD4) was determined using an everted gut sac method, as previously described by Wattanasirichaigoon et al. (62) and modified by Yang et al. for use with mice (65). Everted gut sacs were prepared in ice-cold modified Krebs-Henseleit bicarbonate buffer (KHBB, pH 7.4). One end of the gut segment was ligated with a 4.0 silk. The segment was then everted onto a thin plastic rod, and the resulting gut sac was secured with a 4.0 silk suture to the grooved tip of a 3 ml plastic syringe containing KHBB. The sac was gently distended by injecting 1.5 ml of KHBB. The sac was suspended in a 50 ml beaker containing 40 ml of KHBB plus FD4 (40 mg/ml). The solution in the beaker was temperature jacketed at 37 °C and was continuously bubbled with a gas mixture containing 95% O_2 and 5% CO_2. A 1.0 ml sample was taken from the beaker prior to putting in the gut sac in order to determine the initial external (i.e., mucosal surface) FD4 concentration. The sac was incubated for 30 min in the KHBB solution containing FD4. The length of the gut sac was measured. Fluid from the inside of the sac was aspirated for the determination of FD4 concentration. The serosal and mucosal samples were centrifuged for 10 min at 1,0000*g*. 300 µL of the supernatant was diluted with phosphate buffer saline (2.7 ml). Fluorescence was measured using a Perkin-Elmer LS-50 fluorescence spectrophotometer (Palo Alto, CA) at an excitation wavelength of 492 nm (slit width = 2.5 nm) and an emission wavelength of 515 nm (slit width = 10 nm). Permeability was expressed as the mucosal-to-serosal clearance of FD4 as previously described (65).

Bacterial translocation. The skin was cleaned with 10% povidone-iodine. Using sterile technique, the abdominal cavity was opened and the viscera exposed. The MLN complex was removed, weighed, and placed in a grinding tube containing 0.5 ml of ice-cold phosphate buffer saline. The MLN were homogenized with glass grinders, and a 250 µL aliquot of the homogenate was plated onto brain-heart infusion and MacConkey's agar (Becton Dickinson, Sparks, MD). The plates were examined 24 h later after being aerobically incubated at 37 °C. The colonies were counted and results expressed as colony forming units (CFU) per g of tissue.

Serum ALT measurement. 200 µL of blood was obtained by cardiac puncture and placed in a 0.5 ml centrifugation tube on ice. The samples were then centrifuged at 5,000 *g* for 3 min. The serum was collected and assayed for ALT using an automated assay system.

Immunohistochemistry. Tissues were fixed in 2% paraformaldehyde for 1 h, then treated with 30% sucrose overnight. Fixed sections were washed three times in PBS containing 0.5% BSA and 0.15% glycine, pH 7.4 (Buffer A). The fixed and washed sections were incubated for 30 min with purified goat IgG (50 mg/ml) at 25 °C and then washed three more times with Buffer A. All the preceding steps were designed to ensure minimal non-specific reaction to the antibodies used. Sections were then incubated for 60 min with a primary antibody to iNOS (1μg/ml; Transduction Laboratories, Newcastle, England). This step was followed by three washes in Buffer A and a 60 min incubation with a fluorescently-labeled second antibody (Alexa 488; 1-2 mg/ml; Molecular Probes, Eugene OR) mixed with Buffer A. The sections were then washed six times (5 min/wash) in Buffer A, washed for 1 min in PBS containing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), a uv-excited DNA stain to delineate nuclei, and then mounted in gelvatol and coverslipped for light microscopy. Observation was with an Olympus Provis microscope equipped with a Cooled CCD camera (Mangnifer, Olympus US).

Assessment of NF-κB activation. DNA binding of NF-κB was assess using the electrophoretic mobility shift assay as previously described by our laboratory (65). To prepare nuclear extracts, murine tissue samples were homogenized with T-PER™ (Pierce, Rockford, IL), using a 1:20 ratio of tissue to the sample preparation reagent, as directed by the manufacturer's instructions. The samples were centrifuged at 10,000 *g* for 5 min to pellet tissue debris. The supernatant was collected and frozen at –80 °C. Nuclear protein concentration was determined using a commercially available Bradford assay (Bio-Rad, Hercules, CA).

The EMSA assay for NF-κB nuclear binding was performed using a duplex oligonucleotide probe based on the NF-κB binding site upstream of the murine iNOS promoter. The sequence of the double-stranded NF-κB oligonucleotide was as follows: sense: 5'-AGT TGA GGG GAC TTT CCC AGG C-3'; antisense: 3'-TCA ACT CCC CTG AAA GGG TCC G-5' (Promega; Madison, WI). The oligonucleotides were end-labeled with γ-^{32}P adenosine triphosphate (New England Nuclear; Boston, MA) using T4 polynucleotide kinase (Promega; Madison, WI). 6 μg of nuclear protein was incubated at room temperature with γ-^{32}P-labeled NF-κB probe in 4 μl of 5X binding buffer (65 mM HEPES, 325 mM NaCl, 5 mM DTT, 0.7 mM EDTA, 40% glycerol, pH=8.0) in the presence of 2 μg of polyDI-DC for 20 min, the total volume of the binding reaction mixture being 20 μL. The binding reaction mixture was electrophoresed on 4%

nondenaturing polyacrylamide electrophoresis (PAGE) gels. After electrophoresis, the gels were dried and exposed to Kodak (Rochester, NY) X-Omat film at -80 °C. The autoradiograms were quantified by scanning densitometry.

To determine the specificity of binding reactions, "cold competition" studies were carried out using a 100-fold molar excess of either unlabeled NF-κB duplex oligonucleotide (specific competition) or an irrelevant oligonucleotide probe. For the latter, we used a probe containing the hypoxia inducible factor (HIF)-1 binding sequence from the human erythropoeitin 3' enhancer. Supershift assays were performed by incubating nuclear extracts with 2 µL of anti-p65 and anti-p50 antibodies (Santa Cruz Biotechnology; Santa Cruz, CA) for 1 h prior to the addition of radiolabeled probe. The binding reaction mixture was electrophoresed on 4% PAGE gels. After electrophoresis, the gels were dried and exposed to XAR-5 film (Kodak; Rochester, NY) at –80 °C for overnight using an intensifying screen.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Steady-state levels of TNF, iNOS, COX-2, and IL-6 mRNA were estimated using semi-quantitative RT-PCR, using methods and primers that have been previously reported in detail by our group (65).

Statistical methods. In general, results are presented as means ± SEM. Results from studies to estimate bacterial translocation were not normally distributed and included a few outlying values. Accordingly, these data are presented as median values and the differences between groups were analyzed using Wilcoxen's U-test. Other continuous data were analyzed using Student's t-test or analysis of variance followed by Fisher's LSD test, as appropriate. P values < 0.05 were considered significant. Summary statistics are presented for densitometry results from studies using RT-PCR to estimate mRNA expression, but these results were not subjected to statistical analyses since the method employed was only semi-quantitative and the samples sizes (N=3-4) were small (65).

Results

Pilot studies using conventional mice. MAP was maintained at 30 mm Hg for 2.0 h in all animals prior to initiating resuscitation. Survival was 8/8 (100%) at 30 min after starting resuscitation, 9/12 (75%) at 4 h after resuscitation, and 5/10 (50%) at 24 h after resuscitation. Ileal mucosal permeability to FD4 was similarly increased at 4 h and 24 h after resuscitation. Accordingly, detailed studies were carried out using the 2 h hemorrhage plus 4 h resuscitation model.

Intestinal barrier dysfunction. Although ileal mucosal permeability to FD4 tended to be somewhat higher in sham-hemorrhaged gnotobiotic as compared to conventional mice, the difference was not consistent enough to achieve statistical significance (Figure 1). Subjecting conventional mice to HS/R significantly increased ileal mucosal permeability to FD4. In contrast, when gnotobiotic mice were subjected to HS/R, ileal mucosal permeability was not affected. As expected, bacterial translocation to MLN was minimal in sham-hemorrhaged animals, irrespective if whether they were maintained under conventional or gnotobiotic conditions; median values were 0 cfu per g of MLN tissue in both groups. The number of aerobic or facultative anaerobic colony-forming units in MLN increased significantly in conventional mice subjected to HS/R (median value = 714 cfu/g), but not in gnotobiotic mice subjected to the same insult (median value = 0 cfu/g; $p<0.05$ versus conventional group).

Hepatocellular damage. The mean plasma ALT concentration increased significantly following HS/R in both conventional and gnotobiotic mice (Figure 2). Although the circulating levels of ALT tended to be lower following HS/R in gnotobiotic as compared to conventional animals, the difference was not sufficiently reproducible to achieve statistical significance.

NF-κB activation. We used EMSA to detect DNA binding by the transcription factor, NF-κB, in nuclear extracts prepared from samples of liver and ileal tissue obtained from conventional and gnotobiotic mice 4 h after resuscitation from hemorrhagic shock or at a similar time after the sham procedure. As noted previously by our laboratory (65) and others (44), there was a high level of basal DNA binding of NF-κB in samples of ileal mucosa. Following resuscitation from HS/R, NF-κB DNA binding increased in liver tissue (Figure 3) but was unchanged in ileal mucosa. Hepatic post-HS/R NF-κB DNA binding was similar in conventional and gnotobiotic mice. As in a recent previous report from our group (65), we carried out studies to confirm the identity of the activated protein-DNA complex. Binding of the protein to the radioactive consensus NF-κB binding element was completely inhibited by a 100-fold excess of unlabeled NF-κB duplex oligonucleotide but not by a similar molar excess of the consensus binding sequence for hypoxia inducible factor-1, an irrelevant duplex oligonucleotide. Supershift assays were carried out using samples that were pre-incubated with specific antibodies directed against p50 and p65. As in a previous report from our laboratory (65), we failed to observe a supershift with the anti-p50 antibody, but observed decreased intensity of the main NF-κB band with the p65 antibody and evidence of a supershifted complex.

Expression of inflammatory genes. We used semi-quantitative RT-PCR to estimate steady-state mRNA levels for several inflammatory genes. 18S RNA were used as an internal control to document equal loading of RNA. Hepatic IL-6, iNOS, COX-2 and (to a lesser extent) TNF steady-state mRNA levels increased to a similar degree following HS/R in both conventional and gnotobiotic mice (Figure 4). In other words, the gnotobiotic state had little or no influence on the hepatic expression of several inflammatory genes after HS/R. In ileal mucosa, however, the gnotobiotic state had a clear effect on the post-HS/R expression of IL-6 and COX-2 and a lesser effect on the post-HS/R expression of iNOS and TNF (Figure 5). Specifically, whereas ileal mucosal expression of IL-6 and COX-2 increased substantially in conventional mice after HS/R, ileal mucosal steady state levels of IL-6 and COX-2 mRNA were very low in both sham-treated gnotobiotic mice and gnotobiotic mice subjected to HS/R. There was a relatively small increase in ileal mucosal iNOS and TNF expression in conventional mice after HS/R. However, in gnotobiotic mice, steady state iNOS and TNF mRNA levels were very low in mice subjected to either the sham procedure or HS/R.

Immunohistochemistry. In conventional mice, HS/R was associated with increased staining of hepatic parenchyma (Figure 6) and ileal mucosa (Figure 7) for iNOS. In gnotobiotic mice, HS/R was associated with increased staining of liver tissue for iNOS However, staining for iNOS in ileal mucosa was decreased in gnotobiotic animals, both after sham manipulation and, particularly, after HS/R.

Effect of HS/R in gnotobiotic mice contaminated with E. coli C25. We were impressed that HS/R in gnotobiotic mice caused minimal changes in ileal mucosal permeability or the expression of several stress-related genes in samples of ileal mucosa. We considered two alternative hypotheses to explain these observations. First, gnotobiotic animals might have an intrinsic alteration in their capacity to mount an innate immune response to a pro-inflammatory stimulus. Alternatively, alterations in the microflora within the lumen of the gut--such as the absence of Gram-negative bacteria--in gnotobiotic mice might prevent the development of mucosal inflammation following HS/R. To test this latter hypothesis, we carried out an additional series of experiments, wherein gnotobiotic mice were acutely contaminated with *E. coli* C25 18 h prior to being subjected to HS/R. When compared to uncontaminated gnotobiotic mice subjected to HS/R, *E. coli* C25-contaminated gnotobiotic mice manifested increased ileal mucosal permeability to FD4 (Figure 8) as well increased expression of several pro-inflammatory genes, most notably IL-6 and COX-2 (Figure 9) following hemorrhage and resuscitation.

Discussion

The notion that leakage of microbes or microbial toxins from the gut into the systemic compartment might contribute to systemic illness in HS/R is far from novel. Indeed, versions of this concept have been appearing in the biomedical literature for many years. As early as 1903, the Russian biologist, Metchnikoff, suggested that the systemic absorption of microorganisms or their products from the GI tract could lead to mortality (32). Subsequently, in 1923, the great Harvard physiologist, Walter B. Cannon, suggested that a toxic factor originating from the gut was responsible for the development of irreversibility in cases of prolonged, profound hemorrhagic shock (8). During the 1950s and 1960s, Jacob Fine promoted the idea that endotoxin derived from intraluminal Gram-negative bacteria was the gut-derived toxic factor responsible for irreversibility in hemorrhagic shock (17). In support of this concept, Fine and his co-workers amassed a huge corpus of experimental evidence. For example, these workers showed that using antibiotics to decontaminate the gastrointestinal tract could improve mortality in animals subjected to hemorrhagic shock (18;24) and documented the appearance of endotoxin in the circulation during shock (47). Fine's concept was further supported by results from an elegant, if complex, study performed by Lillehei, who showed that irreversibility was prevented in dogs subjected to hemorrhagic shock, when oxygenated blood from healthy unshocked animals was used to maintain normal intestinal (but not hepatic) perfusion during the period of hypotension in the animals subjected to bleeding (29).

The work by Fine and colleagues was repudiated by Zweifach et al. (38;68) and others (21;31), who showed that mortality rates after HS/R were similar in conventional animals and animals raised under germ-free conditions. Fine argued that these results were explainable because the food fed to the germ-free animals contained sufficient LPS to cause systemic contamination during hemorrhage (17), a view that was substantiated many years later by Rush and colleagues (48). Indeed, Fine's whole concept that gut-derived bacteria and/or toxins play a crucial role in the pathogenesis of HS/R-induced mortality or MODS was revived during the 1980s and 1990s by numerous investigators. The reawakening of interest in the "leaky gut" hypothesis was partly prompted by results from some pivotal studies (16;22;48), which contradicted the earlier data reported by

Zweifach and others (21;31;38;68) by showing that long-term survival after HS/R is significantly better in germ-free as compared to conventional rats.

A number of other fairly recent findings can be cited to support the notion that a gut-derived microbial factor—notably LPS—contributes to the development of distant inflammation, MODS and/or mortality after HS/R. For example, Guo et al. reported that post-HS/R plasma IL-6 and TNF levels are lower in rats whose gut flora has been decontaminated by pretreatment with oral antibiotics as compared to rats with normal gut flora (19). Similarly, Yamashita et al. showed that pretreatment of rats with recombinant human bactericidal/permeability-increasing protein (BPI), a compound that is known to bind and neutralize LPS, attenuates HS/R-induced increases in TNF, IL-6 and plasminogen activator inhibitor-1 mRNA levels (64). Along these same lines, survival after HS/R is improved when rats are pretreated with BPI (66) or a monoclonal antibody to LPS (4) or , an antibiotic known to bind and neutralize LPS, polymyxin B (67). Furthermore, circulating TNF levels are significantly higher and elevated blood lactate levels persist longer and mortality is significantly greater in endotoxin-sensitive C3H/HeN as compared to endotoxin-resistant C3H/HeJ mice subjected to HS/R (12;42).

In contrast to these observations, other recent data support the view that gut-derived microbial toxins are *not* important in the pathogenesis of MODS and/or mortality after HS/R. For example, Ayala et al. were unable to detect circulating LPS at any time points between 2 and 24 h after HS/R in mice (3). Similar findings have been reported from studies of patients admitted to the hospital with HS or major trauma (14;34). Moreover, HS/R was associated with profound alterations in myelopoiesis in both germ-free and conventional rats (30).

The data presented herein support the view that the presence of normal intestinal microflora is not a major factor contributing to the early hepatic (distant) inflammatory response to HS/R. Activation of the important pro-inflammatory transcription factor, NF-κB, was similar in both conventional and gnotobiotic mice. Furthermore, steady-state levels of several pro-inflammatory genes increased after HS/R to a similar extent in liver tissue in both conventional and gnotobiotic mice. Consistent with these findings, we found that the degree of early HS/R-induced hepatocellular injury (as assessed by leakage of the enzyme, ALT, into plasma) was also similar in conventional and gnotobiotic mice.

At least two explanations might account for these findings. First, injury and inflammation in the liver during and after hemorrhage is probably initiated, at least in part, by tissue ischemia and redox stress. Certainly, it is well established that HS/R is associated with the release of potent oxidants in the liver and other organs (25;33;37;52;57). Moreover, redox-mediated events have been implicated in the pathogenesis of the inflammatory response after HS/R (52;65), possibly as a result of activation of the important pro-inflammatory transcription factor, NF-κB (1;53-55;65). Reactive oxygen species (ROS) also have been implicated in activating other pro-inflammatory signaling pathways. For example, activation of activator protein (AP)-1 is at least partially redox-dependent (27;45;46). Modifying the microflora in the gut would not be expected to modulate redox-mediated activation of these important pro-inflammatory signaling pathways.

An alternative explanation for the similar degree of hepatic inflammation and injury observed reported here in gnotobiotic and conventional mice subjected to HS/R has already been alluded to above. Specifically, although the gnotobiotic animals used in this study were maintained under strict germ-free conditions and were fed sterilized chow, no efforts were made to remove LPS from the diet supplied to these animals. Accordingly, the presence of LPS in the lumen of the gut, rather than the presence or absence of normal intestinal microflora, may be the key determinant of hepatocellular injury or activation of hepatic pro-inflammatory genes after HS/R.

Although colonization of mice with the altered Schaedler flora rather than the range of microbes normally found in the lumen of gut failed to modulate hepatocellular injury or hepatic expression of pro-inflammatory genes after HS/R, the gnotobiotic condition was associated with a marked alteration in the intestinal mucosal (local) response to this stressful perturbation. We observed a four-fold increase in ileal mucosal permeability to the hydrophilic macromolecule FD4 when gut sacs were harvested from conventional mice 4 h after HS/R. This finding is entirely consistent with previous observations reported by our laboratory (61;63) and other groups (49). In contrast, when gnotobiotic mice were subjected to HS/R, intestinal permeability FD4 did not increase at all. When we analyzed ileal mucosal samples using semi-quantitative RT-PCR, we observed consistent increases following HS/R in steady-state mRNA levels for several pro-inflammatory genes. This HS/R-induced up-regulation of pro-inflammatory genes was not observed in similar samples obtained from gnotobiotic animals.

Our observations regarding the effect of the gnotobiotic state on the intestinal mucosal response to HS/R are consistent with (at least) two possible mechanisms. First, it is well-known that the immune system in germ-free mice is markedly abnormal (9) (5;7;39)and that the release of a number of cytokines is impaired in mice born and maintained under perfectly sterile conditions (23). Even gnotobiotic mice (i.e., mice colonized with a defined but abnormal microflora) such as the ones used for the present study are not entirely normal immunologically (35). Accordingly, the failure of HS/R to alter either gut mucosal barrier function or the expression of several pro-inflammatory genes in gnotobiotic mice could have been due to defects in the immune system in these animals. Alternatively, alterations in the microflora within the lumen of the gut--such as the absence of Gram-negative bacteria--in gnotobiotic mice might have directly interfered prevent the development of mucosal inflammation following HS/R. To test this latter hypothesis, we carried out an additional series of experiments, wherein gnotobiotic mice were contaminated with *E. coli* C25 18 h prior to being subjected to the HS/R protocol. When compared to uncontaminated gnotobiotic mice, *E. coli* C25-contaminated gnotobiotic mice manifested both increased ileal mucosal permeability to FD4 as well increased expression of IL-6 and COX-2 and, to a lesser extent, iNOS and TNF. These findings suggest that intestinal inflammation after HS/R is dependent on the presence of one or more microbial products that are produced by *E. coli* C25 (as well as the microbes found in the intestine of coventional mice) but are not produced by the microflora in gnotobiotic mice carrying the altered Schaedler flora. Our data are insufficient to determine whether enteral contamination of gnotobiotic mice with strains of bacteria other than *E. coli* C25 would have produced similar effects. Furthermore, it is noteworthy that the 70% increase in ileal mucosal permeability induced by HS/R in gnotobiotic mice contaminated with *E. coli* C25 was less than the 350% increase in permeability induced by HS/R in conventional mice. Thus, it is likely that acute enteral contamination with a single Gram-negative strain was not sufficient to completely normalize the ileal mucosal response to HS/R in gnotobiotic animals. Other bacterial strains or other unrelated factors may contribute to the failure of HS/R to cause ileal mucosal hyperpermeability in gnotobiotic mice.

Although it is well-established that ileal mucosal permeability increases in conventional mice subjected to hemorrhage (49) or HS/R (10;11;58;60;63;65), the fundamental mechanisms that are responsible for this phenomenon remain to be elucidated. Some data suggest that oxidant stress may be an important factor, since HS/R-induced structural or functional mucosal damage can be ameliorated in some models by timely administration of compounds capable of scavenging ROS (10;37;58;65). Other data suggest that complement activation, even without ROS-mediated injury, is a key event leading to gut mucosal injury due to HS/R (56). Secretion of the pro-inflammatory cytokine, IL-6, may be another important factor. Wang et al. reported that HS/R is associated with increased systemic arterial and portal venous plasma levels of IL-6 (60). Moreover, these authors showed that plasma IL-6 levels correlate with gut mucosal permeability in this model, suggesting that IL-6 may be an important factor contributing to intestinal epithelial dysfunction secondary to HS/R. This notion is supported by data obtained by Wang et al., showing that sepsis induces in gut mucosal hyperpermeability in wild-type but not IL-6 knock-out (KO) mice (59) and that induction of the heat shock response with arsenite. Furthermore, preliminary findings in our laboratory indicate that IL-6 KO mice are also protected from gut mucosal hyperpermeability induced by HS/R (unpublished observations). Prompted by these data, we hypothesize that a bacterial factor that is present in the lumen of conventional but not gnotobiotic mice leads to increased mucosal expression of IL-6 following HS/R. IL-6 release then promotes tight junction dysfunction and epithelial hyperpermeability via mechanisms that remain to be elucidated. Recently reported data suggest that activation of the heat shock response might be important in the pathogenesis of this phenomenon (43)

Our findings regarding the effects of the gnotobiotic state on intestinal mucosal permeability after HS/R are inconsistent with data previously reported by Deitch et al. (11). These authors used a combination of antibiotics to decrease intestinal populations of aerobic and facultative anaerobic bacteria in rats. Normal and antibiotic-decontaminated animals were subjected to HS/R and intestinal permeability to a macromolecular marker, horseradish peroxidase (HRP), was determined using electron microscopy 2 h later. In this study, HRP was detected in the channels between adjacent enterocytes in normal and antibiotic-decontaminated rats subjected to HS/R, but not in sham-hemorrhaged animals. These findings were interpreted as showing that gut-derived microflora are not a factor in the development of mucosal hyperpermeability after HS/R. A number of reasons might account for the variance in the results from this earlier study and the results reported by us here. First, in the earlier study, the period of shock was only 30 min as compared to the 2.0 h-long period used by us. Second, Deitch et al. studied rats, whereas we employed mice for our experiments. Third, Deitch et al used a 40 kDa marker (HRP) for their studies, whereas we used a 4 kDa marker (FD4) for ours. Fourth, the earlier study used a histological read-out to detect changes in permeability, whereas we used a functional assay. Finally, and perhaps most important, antibiotic decontamination achieved only about a 10,000-fold reduction in the total number of aerobic and facultative anaerobic bacteria in the cecal contents of rats. Thus, in the study by Deitch et al., the cecal contents of antibiotic-decontaminated rats still contained more than 1,000 cfu/g of normal intestinal micro-organisms. In other words, the strategy of using antibiotics to alter the intestinal flora may not have achieved a sufficient change to alter the intestinal inflammatory response to HS/R.

Ileal mucosal permeability tended to be higher in sham-treated gnotobiotic as compared to conventional mice. Although these differences were not quite consistent enough to achieve statistical significance, a strong trend was apparent. To our knowledge, this observation has not been reported before. We speculate that normal maturation of the tight junctions between adjacent intestinal epithelial cells may be dependent in some way on the interaction between the gut's endogenous microflora and the cells forming the mucosa. Alternatively, the stress associated with general anesthesia and surgical manipulation might alter gut mucosal permeability in the sham-hemorrhaged gnotobiotic animals. It was beyond the scope of the present study to pursue the mechanism(s) responsible for this effect. Circulating ALT levels also tended to be higher in gnotobiotic as compared to conventional mice subjected to the sham procedure.

Again this observation appears to be novel, but is also unexplained by the present data and might reflect the stress associated with the sham procedure.

In summary, we showed herein that HS/R was associated with the development of hepatocellular injury and the activation of several pro-inflammatory genes in the liver tissue of both conventional and gnotobiotic mice. In conventional mice, HS/R was also associated with the development of ileal mucosal hyperpermeability and the activation of pro-inflammatory genes in intestinal mucosa. These changes were not observed in gnotobiotic mice, but were observed in gnotobiotic mice contaminated with a Gram-negative organism, *E. coli* C25. These data support the view that a bacterial factor contributes the development of gut mucosal hyperpermeability and inflammation after HS/R.

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Figure legends

Figure 1. Effect of HS/R on ileal mucosal permeability assessed 4 h after the end of shock (or sham shock) in conventional and gnotobiotic mice. Conventional (n = 8) and gnotobiotic (n = 9) mice in the Sham groups were subjected to anesthesia and vascular cannulation but not subjected to hemorrhagic shock. Conventional (n = 12) and gnotobiotic (n = 9) mice in the HS/R groups were subjected to hemorrhagic shock for 2 h and then resuscitated with remaining shed blood plus Ringer's lactate solution (2× the shed blood volume). Results are means ± SE. * indicates $P < 0.05$ versus Sham group.

Figure 2. Effect of HS/R on circulating alanine aminotransferase (ALT) concentration assessed 4 h after the end of shock (or sham shock) in conventional and gnotobiotic mice. Groups and symbols are the same as in Figure 1.

Figure 3. Effects of HS/R on DNA binding of NF-κB in liver and ileal mucosal tissue samples obtained from conventional or gnotobiotic mice. EMSA was performed using nuclear extracts prepared from tissues obtained 4 h after starting resuscitation or 4 h after the end of the sham procedure. Shown as well are the results of supershift assays using antibodies against the p65 and p50 subunits of NF-κB and cold competition experiments using a 100-fold molar excess of either unlabeled (specific) NF-κB duplex oligonucleotide or unlabeled (nonspecific) hypoxia inducible factor (HIF)-1 duplex oligonucleotide. Results depicted are representative autoradiographs from representative EMSAs. Similar results were obtained at least three times using samples from replicate animals.

Figure 4. Hepatic expression of IL-6, iNOS, COX-2, and TNF mRNA in conventional or gnotobiotic mice subjected to HS/R or the sham procedure. Results were obtained using semi-quantitative RT-PCR as described in the Methods. Bands visualized after agarose gel electrophoresis of PCR reaction products were scanned using a NucleoVision imaging workstation and quantified using GelExpert™ release 3.5. Data in bar graphs are means ± SE (n = 3 or 4 per condition). Representative gels are depicted.

Figure 5. Ileal mucosal expression of IL-6, iNOS, COX-2, and TNF mRNA in conventional or gnotobiotic mice subjected to HS/R or the sham procedure. Results were obtained using semi-quantitative RT-PCR as described in the Methods. Data in bar graphs are means ± SE (n = 3 or 4 per condition). Representative gels are depicted.
Figure 6. Expression of iNOS in hepatic tissue samples from conventional or gnotobiotic mice subjected to HS/R or the sham procedure. Green areas indicate immunoreactive iNOS. Blue areas have been stained with DAPI, which binds to DNA and delineates nucelei. Original magnification was 20× for all the images.

Figure 7. Expression of iNOS in ileal mucosal tissue samples from conventional or gnotobiotic mice subjected to HS/R or the sham procedure. Green areas indicate immunoreactive iNOS. Blue areas have been stained with DAPI, which binds to DNA and delineates nucelei. Original magnification was 40× for all the images.

Figure 8. Effect of HS/R on ileal mucosal permeability assessed 4 h after the end of shock gnotobiotic mice (n = 9) and gnotobiotic mice contaminated 18 earlier with *E. coli* C25 (n = 9). * indicates $P<0.05$ for the between-group comparison.

Figure 9. Effect of HS/R on ileal mucosal expression of IL-6, iNOS, COX-2, and TNF mRNA in gnotobiotic mice mice and gnotobiotic mice contaminated 18 earlier with *E. coli* C25. Samples were obtained 4 h after the end of shock. Results were obtained using semi-quantitative RT-PCR as described in the Methods. Data in bar graphs are means ± SE (n = 3 or 4 per condition). Representative gels are depicted.

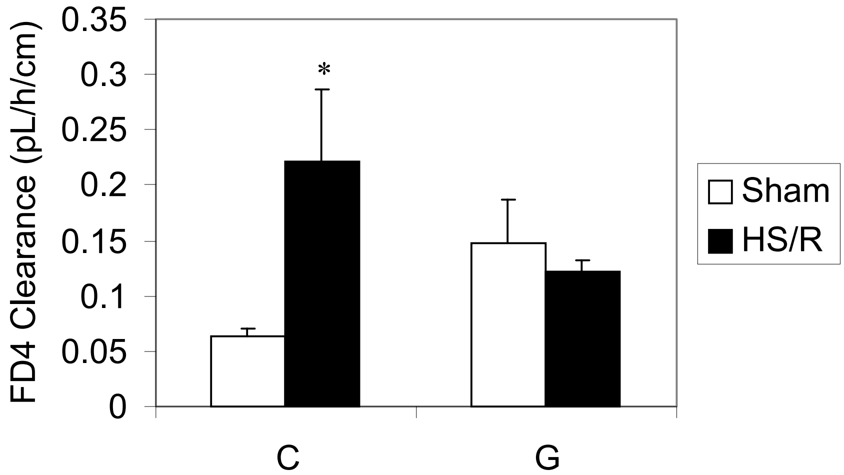


Figure 1

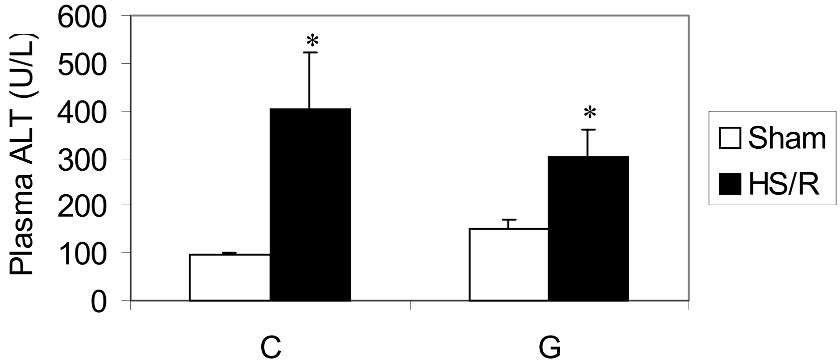


Figure 2

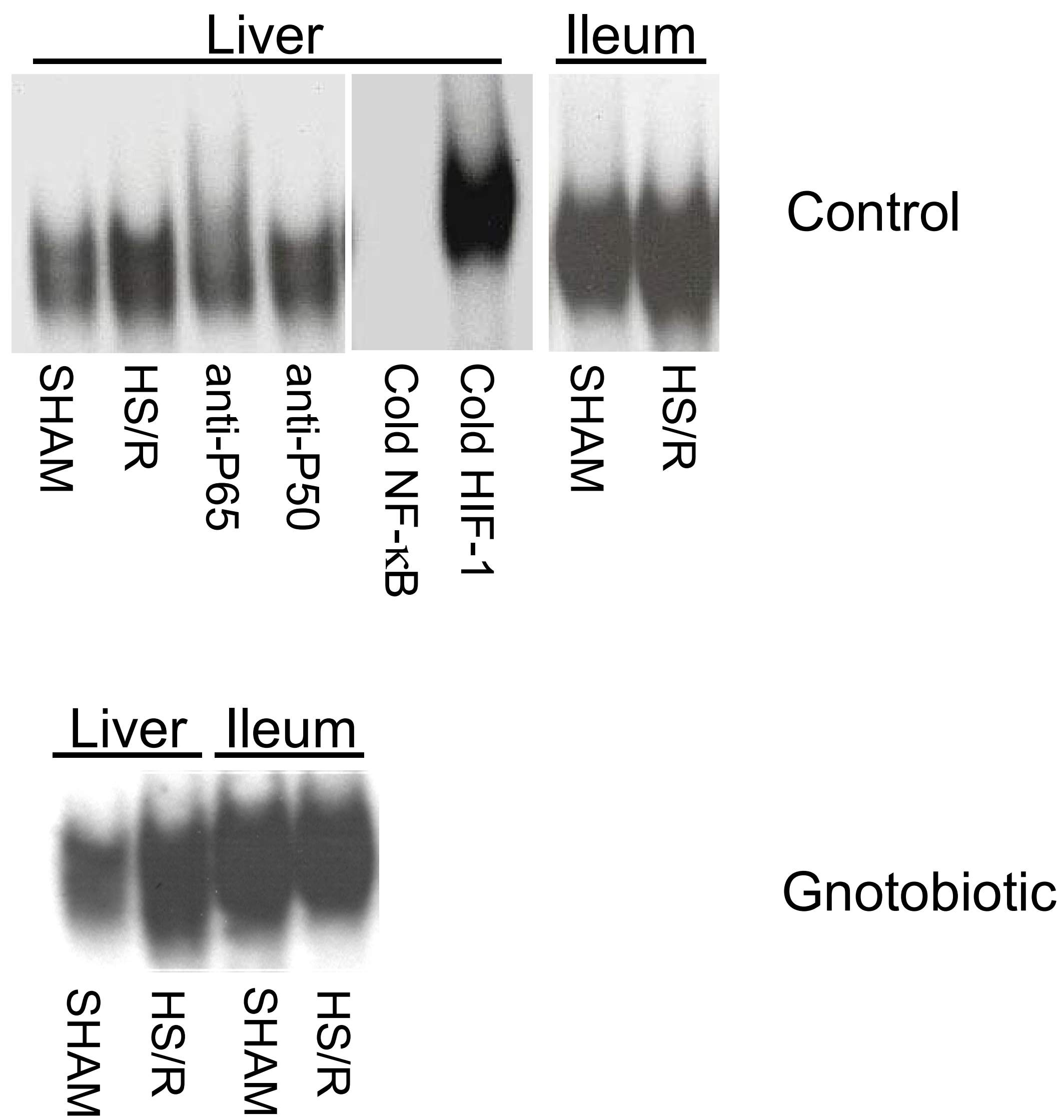
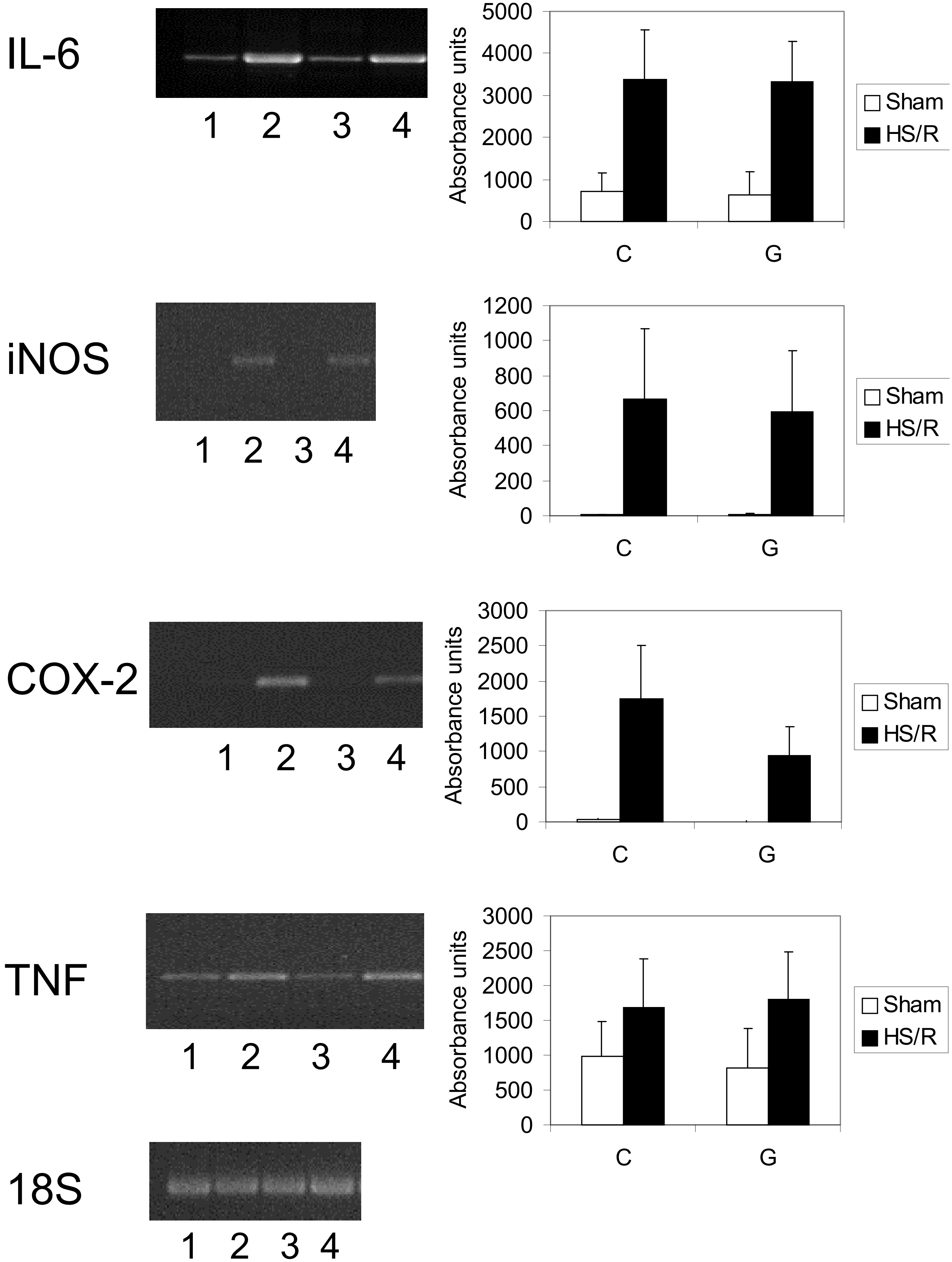
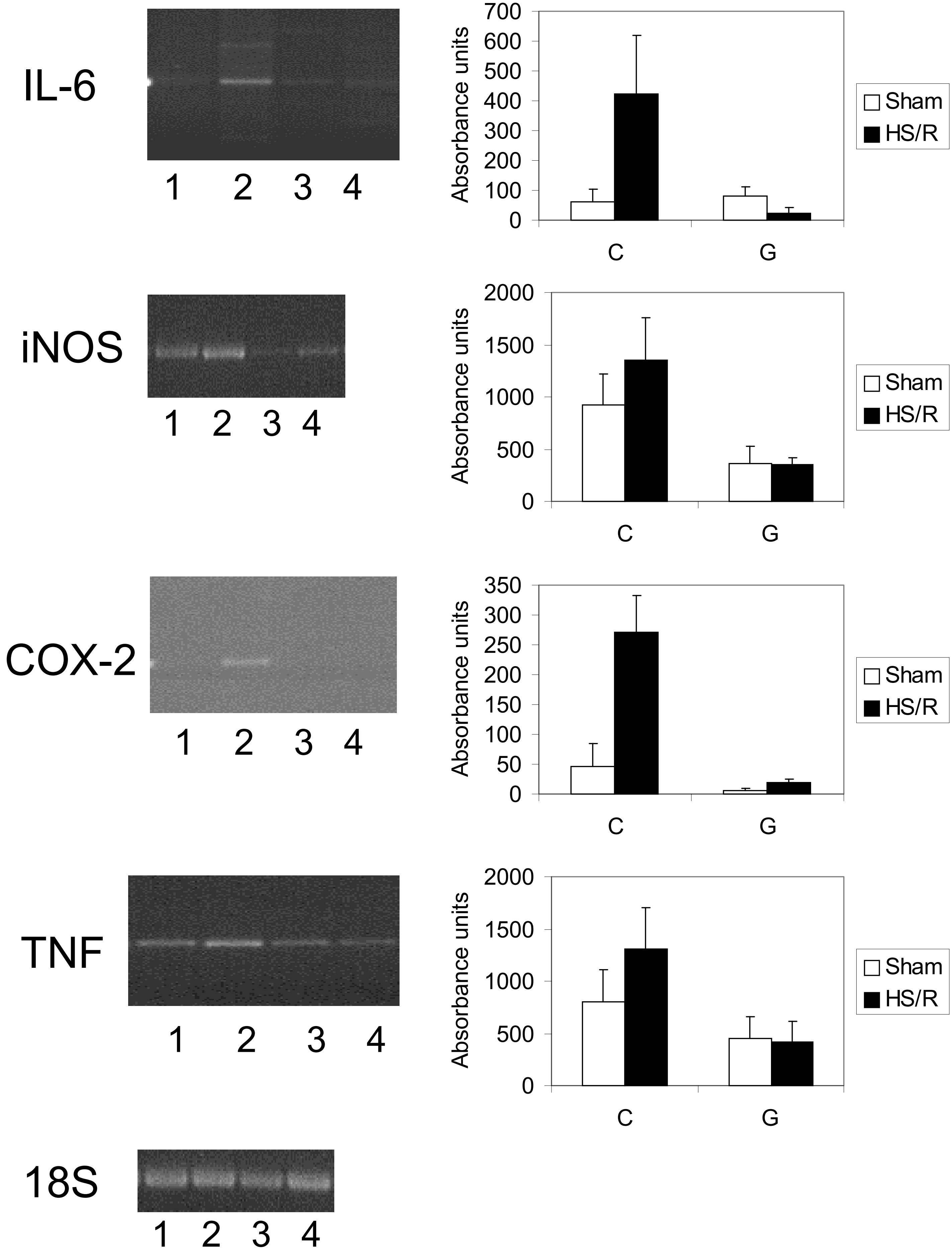

Liver
Ileum
Control
SHAM
HS/R
anti-P65
anti-P50
Cold NF-κB
Cold HIF-1
SHAM
HS/R
Liver
Ileum
Gnotobiotic
SHAM
HS/R
SHAM
HS/R

Figure 3


IL-6
1 2 3 4
Absorbance units
5000
4000
3000
2000
1000
0
C
G
Sham
HS/R
iNOS
1 2 3 4
Absorbance units
1200
1000
800
600
400
200
0
C
G
Sham
HS/R
COX-2
1 2 3 4
Absorbance units
3000
2500
2000
1500
1000
500
0
C
G
Sham
HS/R
TNF
1 2 3 4
Absorbance units
3000
2500
2000
1500
1000
500
0
C
G
Sham
HS/R
18S
1 2 3 4


IL-6
1 2 3 4
Absorbance units
700
600
500
400
300
200
100
0
C
G
Sham
HS/R
iNOS
1 2 3 4
Absorbance units
2000
1500
1000
500
0
C
G
Sham
HS/R
COX-2
1 2 3 4
Absorbance units
350
300
250
200
150
100
50
0
C
G
Sham
HS/R
TNF
1 2 3 4
Absorbance units
2000
1500
1000
500
0
C
G
Sham
HS/R
18S
1 2 3 4

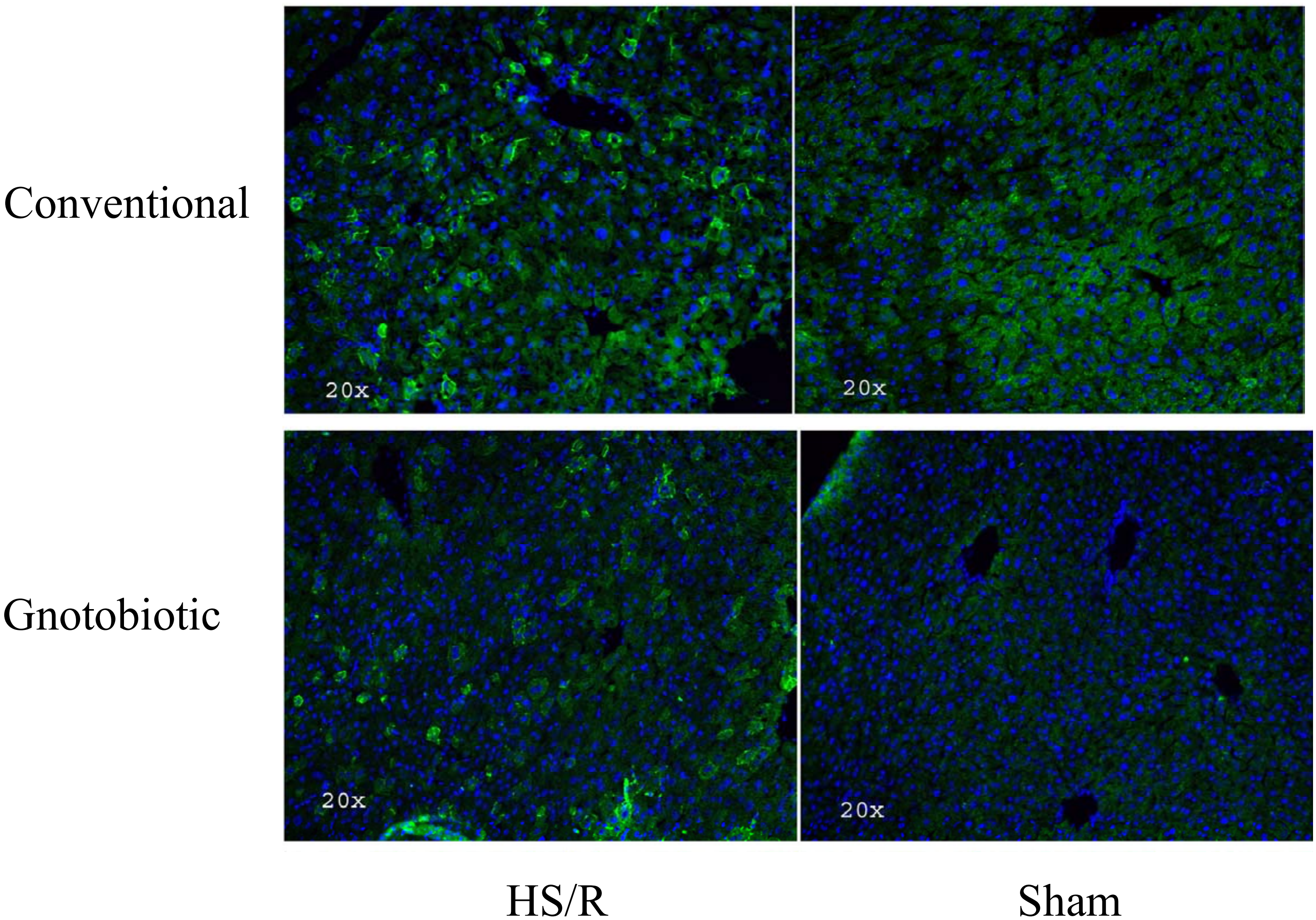


Figure 6

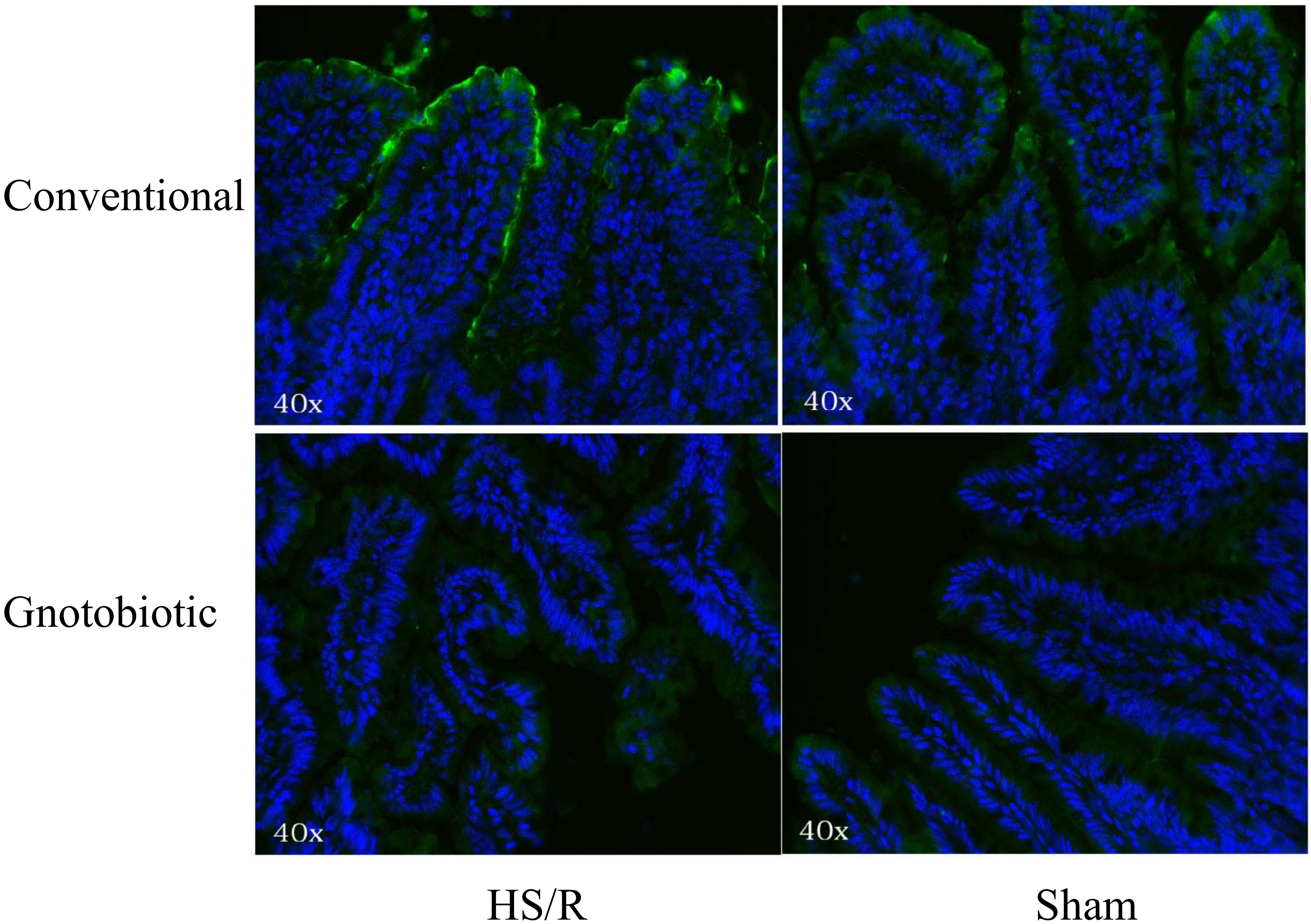


Figure 7

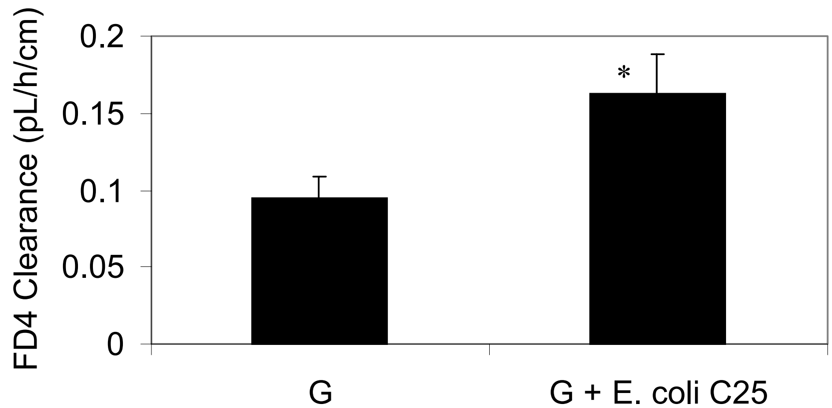
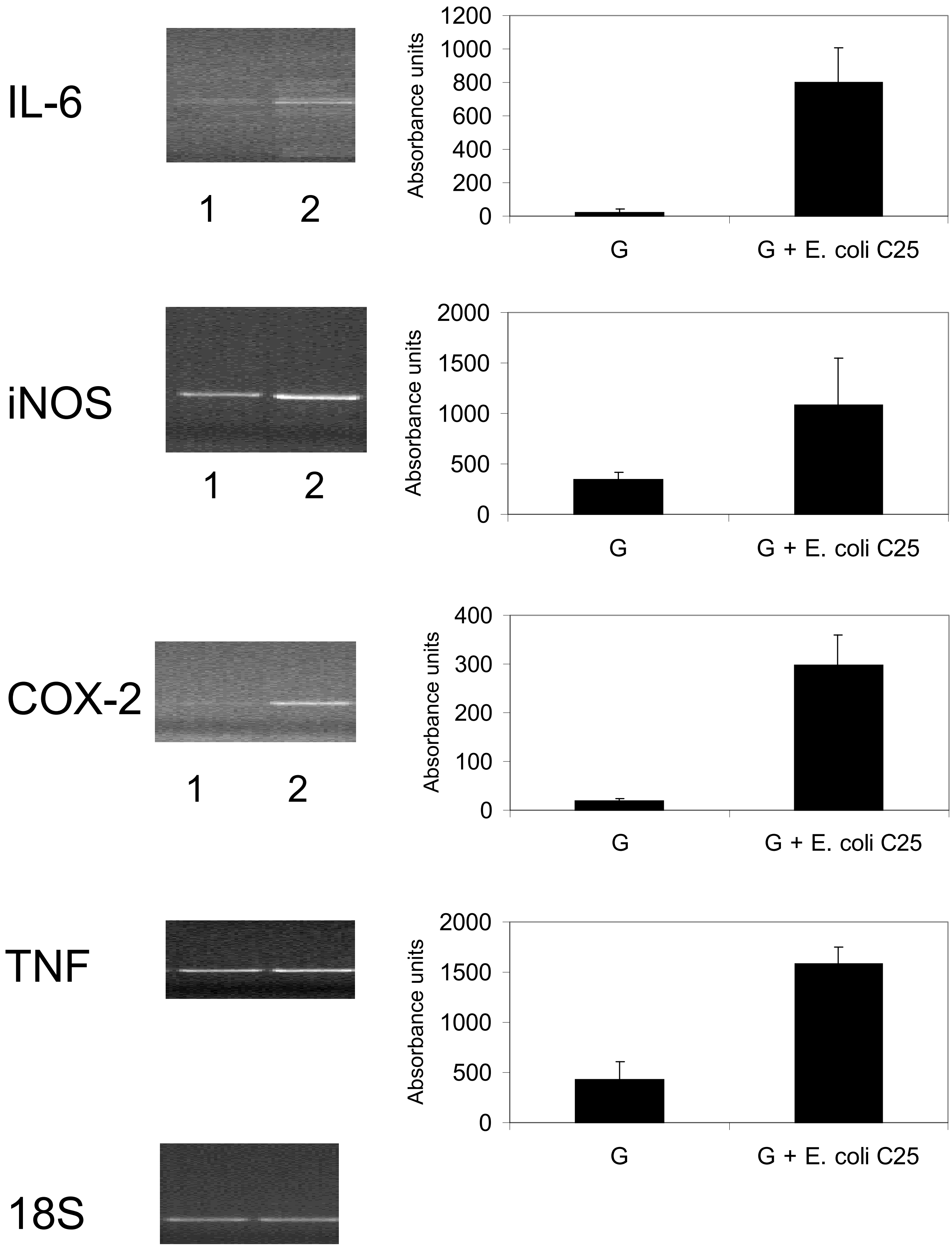


Figure 8


IL-6
1
2
Absorbance units
1200
1000
800
600
400
200
0
G
G + E. coli C25
iNOS
1
2
Absorbance units
2000
1500
1000
500
0
G
G + E. coli C25
COX-2
1
2
Absorbance units
400
300
200
100
0
G
G + E. coli C25
TNF
Absorbance units
2000
1500
1000
500
0
G
G + E. coli C25
18S