Am J Physiol Gastrointest Liver Physiol 299: G1187–G1197, 2010.
First published September 9, 2010; doi:10.1152/ajpgi.00452.2009.

Mechanical strain and TLR4 synergistically induce cell-specific inflammatory gene expression in intestinal smooth muscle cells and peritoneal macrophages

Sven Wehner,[1] Bettina M. Buchholz,[1,2] Silke Schuchtrup,[1] Anatol Rocke,[1] Nico Schaefer,[1] Mariola Lysson,[1] Andreas Hirner,[1] and Joerg C. Kalff[1]

[1]*Department of Surgery, Division of Transplantation and Hepatobiliary Surgery, University of Bonn, Bonn, Germany; and* [2]*Department of Medicine, Division of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh, Pittsburgh, Pennsylvania*

Submitted 3 November 2009; accepted in final form 3 September 2010

Wehner S, Buchholz BM, Schuchtrup S, Rocke A, Schaefer N, Lysson M, Hirner A, Kalff JC. Mechanical strain and TLR4 synergistically induce cell-specific inflammatory gene expression in intestinal smooth muscle cells and peritoneal macrophages. *Am J Physiol Gastrointest Liver Physiol* 299: G1187–G1197, 2010. First published September 9, 2010; doi:10.1152/ajpgi.00452.2009.—]?>Mechanical trauma of the gut is an unavoidable event in abdominal surgery. Former studies demonstrated that intestinal manipulation induces a strong inflammation within the tunica muscularis. We hypothesized that mechanical strain initiates or aggravates proinflammatory responses in intestinal smooth muscle cells (iSMC) or macrophages. First, an appropriate isolation and culture method for neonatal rat iSMC was established. Purified iSMC and primary peritoneal macrophages (pMacs) were subjected to static or cyclic strain, and gene expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), IL-6, and IL-1β was analyzed by quantitative PCR. Supernatants from stretched iSMC were transferred to untreated pMacs or contrariwise, and medium transfer-triggered inflammatory gene expression was measured in unstretched cells. Finally, we investigated the synergistic effect of static strain on LPS-induced proinflammatory gene expression. Although cyclic strain failed, static strain significantly induced iNOS, COX-2, and IL-1β mRNA in iSMC. pMacs showed an increase in all inflammatory genes investigated as well as macrophage inflammatory protein (MIP)-1α and MIP-2 mRNA after static strain. Both cell entities liberated unknown mediators in response to stretch that mutually stimulated iNOS gene expression. Finally, mechanostimulation amplified LPS-induced iNOS and IL-1β gene expression in iSMC as well as COX-2 and IL-6 mRNA in pMacs. In conclusion, static strain initiates proinflammatory gene expression in iSMC and pMacs and triggers a bidirectional paracrine communication between both cultured cell entities via the liberation of unknown mediators. Furthermore, static strain synergistically operates with Toll-like receptor 4 ligation in a cell-specific manner. Hence, this study demonstrates that mechanical strain functions as an immunomodulatory stimulus in abdominal cells.

postoperative inflammation; Toll-like receptor

INTESTINAL MECHANICAL TRAUMA is an inevitable component of abdominal surgery. In the past, our group has clearly demonstrated that the degree of intestinal trauma directly correlates to both the extent of impairment of muscarinic stimulated jejunal contractility and the magnitude of the inflammatory response as measured by cellular muscularis infiltration and cytokine production (14). With IL-6 and IL-1β being described as key cytokines, a plethora of other proinflammatory cytokines, chemokines, adhesion molecules, and enzymes [such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2)] contribute to gut muscularis inflammation, subsequently causing sustained intestinal dysmotility/postoperative ileus (POI) (3, 10, 16). Yet, despite extensive work within the last decade, the primary trigger of the inflammatory response remains to be identified.

Abnormal physical forces applied to intestinal cells during abdominal surgery and tonic stretch of the dilated gut wall during ileus might result in or prolong inflammation of the tunica muscularis, respectively. The concept of mechanical strain-induced expression of proinflammatory mediators has been identified in other organs like lung and bladder or the vascular system, specifically in tissue-specific parenchymal cells (3, 16), in smooth muscle cells (10, 24, 35), or in macrophages (9, 34). Within the multicellular syncytium of the tunica muscularis, two cell types are the most abundant: intestinal smooth muscle cells (iSMC) that function as the motor of gut motility, and muscularis macrophages, which reside as a dense network of highly immunocompetent cells within the muscularis (15). The latter are supposed to serve as a second line of defense against invading pathogens (1) but, importantly, are also primarily activated by mechanical intestinal manipulation (14). On the one hand, many studies identified this network of muscularis macrophages as a key player in the initial phase of POI; on the other hand, the effector cells of intestinal motility are iSMC and less is known about the immunological potential of smooth muscle cells (SMC) in general. However, investigations on whether iSMC or abdominal macrophages respond to mechanical strain in a proinflammatory manner are lacking.

Another consequence of the intestinal mechanical trauma and the subsequent inflammation is the transient loss of epithelial integrity (2, 5, 27) leading to translocation of luminal contents like microbes or bacterial parts into the tissue (22, 26, 29). It is well known that bacterial endotoxins, e.g., gram-negative bacteria-derived lipopolysaccharide (LPS), are potent inducers of severe inflammation and, furthermore, cause gastrointestinal dysmotility. Primary recognition of LPS is efficiently mediated by the innate immune receptor Toll-like receptor 4 (TLR4). During early endotoxemia, the nonhematopoietic cell system possesses the intrinsic machinery to primarily and independently initiate TLR4-triggered ileus but dual molecular activation of both the hematopoietic and nonhematopoietic lineage takes place (2). However, it is unknown whether the surgical trauma and the subsequent endotoxin challenge operate synergistically in inducing gut inflammation.

Undoubtedly, the in vitro reproduction of complex in vivo processes like POI is not possible. However, investigations on isolated abdominal cells might be very helpful in dissecting out

Address for reprint requests and other correspondence: J. Kalff, Dept. of Surgery, Division of Transplantation and Hepatobiliary Surgery, Univ. of Bonn, Sigmund-Freud-Str. 25, 53105 Bonn, Germany (e-mail: kalff@uni-bonn.de).

 0193-1857/10 Copyright © 2010 the American Physiological Society

possible in vivo mechanisms or individual pathways involved. Therefore, we thought to determine whether mechanical strain induces proinflammatory gene expression and mediator release in cell cultures of primary iSMC and peritoneal macrophages (pMacs). Moreover, we analyzed how mechanical strain alters LPS-induced gene transcription in both cell entities.

MATERIALS AND METHODS

Animals

Lewis rats and C57BL6/J mice were purchased from Harlan Winkelmann (Borchen, Germany) and were housed and bred in the specific pathogen-free environment of the animal facility of the University of Bonn. Animal housing and all experiments were performed in accordance with the national guidelines of laboratory animal care and the federal law regarding the protection of animals. The committee for animal experiments of the University of Bonn approved the protocol before the indicated procedures were performed.

Cell Cultures

Muscularis cell isolation. Muscularis cells were isolated from neonatal Lewis rats (day 6–10) by collagenase digestion. In brief, the mesentery was transected and the muscularis isolated by microdissection. Minced muscularis pieces were washed in Hanks buffered salt solution (HBSS) containing 1,000 U/ml penicillin G, 1,000 μg/ml streptomycin, and 50 mg/ml amphotericin B and further digested for 30 min under gentle shaking in HBSS containing 5 mg/ml collagenase type-II (Worthington, Lakewood, NJ), 1 mg/ml DNAse I, and 25 mg Dispase II (Roche, Heidelberg, Germany). Enzymatic digestion was repeated until the tissue was completely disaggregated. This cell suspension was filtered through a sterile 100 μM nylon gauze and depleted of $CD163^+$ macrophages by magnetic cell separation (MACS). Depletion was achieved by incubation of the cell suspension with anti-CD11b-biotinylated antibody (Serotec, Duesseldorf, Germany) and anti-biotin magnetic beads (Miltenyi Biotech, Moenchengladbach, Germany) according to the manufacturer's recommendations. MACS was performed by using the Possel-S program with an AutoMACS (Miltenyi Biotech). Negative and positive cell selection underwent fluorescence-activated cell sorting (FACS) against CD163 to validate the efficacy of tissue macrophage depletion. CD163 antibody was detected via biotinylated primary anti-CD163 antibody (Serotec, 1:100) and streptavidin-phycoerythrin (1:300 dilution, Immunotools, Friesoythe, Germany). FACS recording was performed with a Canto II (BD Biosciences, Heidelberg, Germany) and data were evaluated with the Flow Jo software (Tree Star, Ashland, OR).

$CD163^-$ muscularis cells were plated onto precoated 100-mm tissue culture dishes [collagen type IV (2.5 $\mu g/cm^2$), laminin V (10 $\mu g/cm^2$), poly-D-lysine (5 $\mu g/cm^2$), or Matrigel (6–10 $\mu g/cm^2$) or left untreated] with a density of 3,000 cells/cm^2. Cells were cultured in SmGM-II (Lonza, Verviers, Belgium), and morphology was analyzed microscopically 18 h after seeding. These cells are further referred to as iSMC, and the third cell passage was used for experiments.

pMac isolation. With optimal cell adherence on collagen type IV-coated dishes, a quarter of the primary cells obtained from the multicellular syncytium tunica muscularis expressed the CD163 antigen, indicating that these cells are resident muscularis macrophages. However, despite extensive studies we were not able to culture an adequate amount of adherent $CD163^+$ resident muscularis macrophages for more than 24 h. Complementing this, Ozaki et al. (29) showed similar quantitative results with a different isolation technique in mice, but they failed also to establish primary intestinal macrophage cultures with sufficient number of cells suitable for mass-cell in vitro experiments (H. Ozaki, personal communication). Therefore, we decided to utilize pMacs that are associated with the intestine and show similar properties (i.e., CD11b and F4/80 expression, phagocytosis, and endotoxin-induced cytokine production) as resident muscularis macrophages (results not shown). Four days after intraperitoneal injection of 8 ml of 3% sterile thioglycollate medium (Sigma, Taufkirchen, Germany), pMacs were isolated by peritoneal lavage with PBS from 6- to 8-wk-old Lewis rats. Contaminating erythrocytes, granulocytes, and dead cells were removed from the lavage medium by density gradient centrifugation (Accuspin histopaque 1077 medium, Sigma) for 20 min at 800 *g*. Purified pMacs were washed three times and cultured in Dulbecco's modified Eagle medium containing 10% FCS and 2 mM glutamine (DMEM-K).

Immunohistochemistry. Muscularis cells from adult mice were isolated by using the same protocol as described above for rats. Cells were seeded onto coverglasses and cultivated for up to 7 days until confluence. Cultures were examined by light and fluorescence microscopy for the presence of resident muscularis macrophages (F4/80 antibody, Invitrogen, Karslruhe, Germany). Furthermore, the cytoskeletal SMC markers α-actin (IgG2a antibody, Dianova, Hamburg, Germany) and calponin (IgG1 antibody, Sigma) were immunolabeled with appropriate secondary antibodies (Alexa-568 labeled anti-rat antibody, FITC-labeled anti-mouse IgG2a, and Alexa-568 labeled anti-mouse IgG2a; Invitrogen). In addition, mucosa-free whole mount muscularis specimens from rats were immunostained for resident tissue macrophages (CD163, 1:200, Serotec), glia [glial fibrillary acidic protein (GFAP), Z0334, 1:600, Dako, Hamburg, Germany] or neurons (PGP 9.5, ab10404, 1:300, Abcam, Cambridge, UK). Mice whole mounts were stained for F4/80 (MF48000, 1:200, Invitrogen). All specimens were mounted in ProLong antifade medium (Invitrogen) and observed under a Nikon TE-2000 microscope.

Immunoblotting. TLR4 detection in rat iSMC cultures and muscularis externa specimen was performed by immunoblotting of RIPA lysates with TLR4 antibody (Santa Cruz Biotechnology, Heidelberg, Germany) and secondary horseradish peroxidase-coupled antibody.

Mechanical strain. iSMC (2×10^5) or pMacs (5×10^5) were plated onto collagen type I-coated BioFlex wells (Flexcell International, Hillsborough, NC) and cultivated for 3 days in SMGM-II or DMEM-K, respectively. Both cell types were serum starved 1 h before the experiment. Cells were stretched for up to 6 h as indicated either with a static strain regimen (repetitive 30 min cycles with 20% elongation for 23 min followed by a 7-min break) or a cyclic strain regimen (20% elongation, 0.5 Hz, half sinus curve shape). Continued cell adherence was confirmed microscopically at the end of the experiment. Control cells were cultured in BioFlex wells but left unstretched. Total RNA was isolated from the cells, and cell culture supernatants from the 6-h stretched groups or unstretched cells were used subsequently for medium transfer experiments.

In another experiment, iSMC and pMacs were stretched or left unstretched in the presence or absence of 100 ng/ml LPS. Cells were harvested after a 6-h treatment period and analyzed for gene expression following RNA isolation.

Cytotoxicity assay. Cytotoxicity of mechanical strain was measured by lactate dehydrogenase (LDH) release into culture supernatants with the LDH PLUS Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Cytotoxicity was expressed in % and LDH concentration in supernatants of completely lysed cells was defined as 100%.

Medium transfer of stretch-conditioned media. iSMC were serum starved for 1 h and medium was replaced by conditioned culture supernatant from stretched or unstretched macrophages. In the same manner, pMacs were stimulated with culture supernatants from stretched or unstretched iSMC. After 6 h, cells were harvested and kept frozen until further mRNA analysis.

Quantitative PCR. RNA from cells was isolated by using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. cDNA was reverse transcribed with the High Fidelity cDNA kit and expression of mRNA was quantified in triplicate by a RT-PCR with gene expression assays for iNOS (assay ID no. Rn00561646), COX-2 (no.

Rn01483828), IL-6 (no. Rn00561420), IL-1β (no. Rn00580432), macrophage inflammatory protein (MIP)-1α (no. Rn00564660), MIP-2 (sense primer 5′-aacatccagagcttgacggtga-3′; reverse primer 5′-ggtagcctgccttttgttcagtatc-3′), and TLR-4 (sense primer 5′-acctggctggtttacacgtc-3′; reverse primer 5′- ctgccagagacattgcagaa-3′) and normalized to the endogenous control gene 18srRNA (no. 4319413E). The PCR reaction was performed in either Gene expression or Power SYBR green master mix by amplification of 10 ng cDNA for 40 cycles (95°C × 15 s, 60°C × 1 min) on an ABI Prism 7900HT. Data quantification was performed by the $\Delta\Delta C_T$ method and all results were normalized to unstretched cells. All PCR reagents and assays were obtained from Applied Biosystems (Darmstadt, Germany). Primers were synthesized by Metabion (Martinsried, Germany).

Drugs and Solutions

PBS, HBSS, and cell culture media were purchased from Lonza (Verviers, Belgium). All other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany) unless otherwise indicated.

Data Analysis

All investigations performed were repeated at least in three independent experiments. The individual group size for every experiment is noted in the figure legends. Statistical analysis was performed by one- or two-way ANOVA followed by a Bonferroni posttest where appropriate (Prism5, GraphPad, San Diego, CA). Significance levels were $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***). All values are presented as means ± SD.

RESULTS

Isolation, Purification, and Culture of iSMC and Resident Muscularis Macrophages

Within the tunica muscularis, iSMC and resident macrophages are the most abundant cell populations. For this study, the isolation and characterization of these cells was a basic requirement and the validation of cell purity was a prerequisite for the reliability of the in vitro model. Pilot studies demonstrated that both cell types could only be retrieved with an insufficient yield from adult rodent muscularis with poor viability of the adult cells. However, digestion of the tunica muscularis from neonatal rodents resulted in a very high yield (~9–10 × 10^6 cells/small bowel) of vital cells (vitality ~99–100%). Matrigel coating promoted the differentiation of functional neuronal and pacemaker cells (Fig. 1*E*) leading to the formation of several contracting multicellular aggregates (Supplemental Video S1; the online version of this article contains supplemental data). In contrast to Laminin 5 and poly-D-lysine coating (Fig. 1, *C* and *D*), collagen

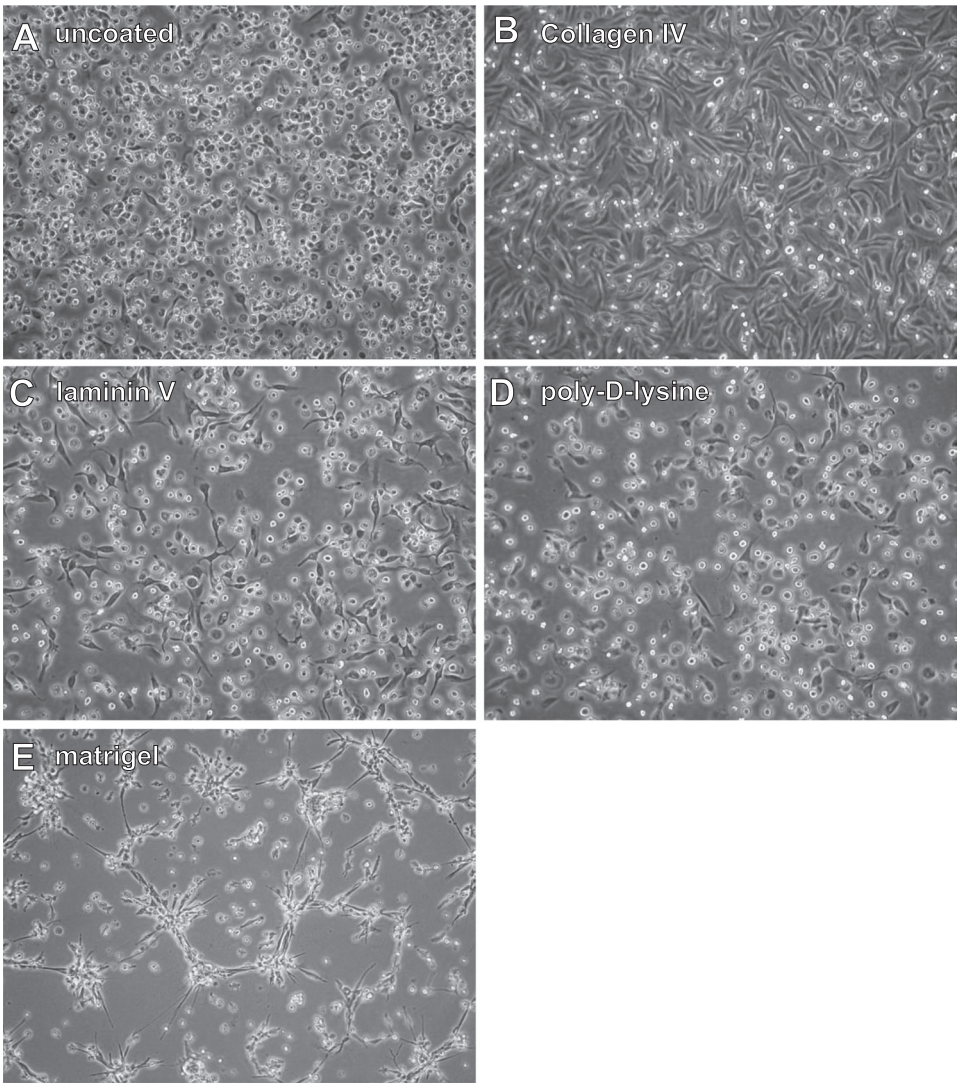


Fig. 1. Cultures of intestinal smooth muscle cells (iSMC) on different extracellular matrix proteins. Muscularis externa of 6- to 10-day-old Lewis rats was enzymatically digested and seeded on uncoated (*A*), collagen type IV (*B*), laminin V (*C*), poly-D-lysine (*D*), or Matrigel (*E*)-coated cell culture dishes. Representative phase contrast microscopy pictures were taken at ×100 magnification. Optimal adherence and cell differentiation was observed on the collagen IV matrix.

type IV coating of the tissue culture dishes proved to be optimal, allowing sufficient attachment and rapid spreading of the isolated smooth muscle cells within the first 18 h (Fig. 1*B*), and was therefore routinely used for cultivation of freshly isolated iSMC. However, subcultivation of iSMC after cell passaging did not require culture dish coating because all cells adhered within 2 h after seeding with immediate spreading.

Figure 2 demonstrates a dense network of $CD163^+$ (Fig. 2*A*) and $F4/80^+$ (Fig. 2*B*) resident muscularis macrophages in whole mount preparations of rat and murine gut muscularis, respectively. Complementing this, 27% of cells obtained from collagenase-digested rat muscularis specimen stained positive for CD163, subdivided in the subpopulations of $CD163^{high+}$ and $CD163^{low+}$ cells as detected by flow cytometry (Fig. 2*C*). After MACS depletion of $CD11b^+$ myeloid lineage cells, the muscularis cell suspension was near completely devoid of both $CD163^+$ cell populations (Fig. 2*D*).

Purification and phenotype of these iSMC cultures was positively confirmed by staining for the typical iSMC immunohistochemical markers α-actin and calponin (Supplemental Fig. S1, *A–C*). Contaminating neuronal/glial cells as detected by PGP 9.5/GFAP staining were nearly absent (results not shown). However, in muscularis cell cultures from mice that did not undergo $F4/80^+$ purification, macrophages with the typical dendriform-like cell shape could be rarely detected (Fig. 2, *E* and *F*). TLR4 expression on iSMC was not detectable by immunohistochemical techniques, but immunoblotting demonstrated TLR-4 expression in both muscularis specimen and cultured iSMC (Supplemental Fig. S1*D*), indicating that iSMC are able to directly respond to innate immune stimulation with TLR4 agonists like LPS.

Static Mechanical Strain Induces Proinflammatory Gene Expression in iSMC

We investigated whether mechanical stress alone could be a trigger of proinflammatory gene expression in iSMC and compared a static vs. a cyclic strain regimen. Expression of the

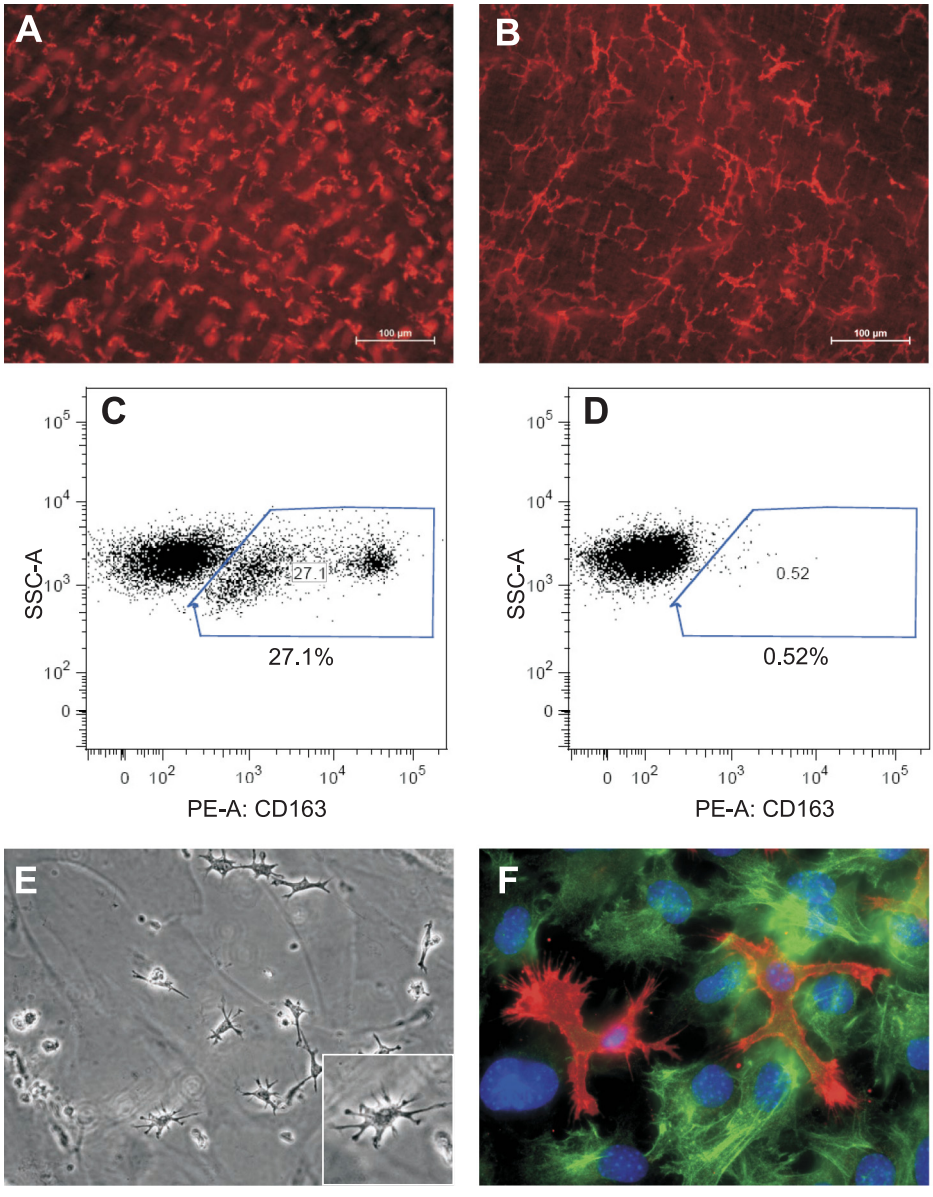


Fig. 2. Purification of iSMC from resident muscularis macrophages. Within the tunica muscularis, tissue macrophages form a dense network of resident cells. Resident muscularis macrophages of muscularis whole mount preparations stain positive for the cell surface markers CD163 in rats (*A*) and F4/80 in mice (*B*). *C*: scatterplot of fluorescence-activated cell sorting (FACS) detection of CD163-positive macrophages in an enzymatically digested muscularis cell suspension from rats. PE, phycoerythrin; SSC, sideward scatter. *D*: scatterplot showing the effective depletion of resident macrophages by $CD163^+$-directed magnetic cell separation (MACS). *E*: micrograph depicting an in vitro culture from murine muscularis cells with several dendriform macrophages on a feeder layer of iSMC at 200-fold magnification. *Inset*: single resident macrophage at high magnification. *F*: resident macrophages in murine muscularis cell cultures (630-fold magnification) express the cell surface marker F4/80 (red). iSMC stain positive for α-actin (green) and cell nuclei are marked by 4,6-diamidino-2-phenylindole (DAPI; blue).

analyzed genes in unstretched iSMC did not change over time (data not shown). After 2 h of static mechanical strain, iNOS, COX-2, IL-6, and IL-1β gene expression remained at baseline levels (Fig. 3, *A–D*). After 4 and 6 h, iNOS (15.1 ± 5.8 and 14.0 ± 3.7-fold), COX-2 (2.3 ± 0.5 and 2.5 ± 0.9-fold), and IL-1β (25.0 ± 4.5 and 2.9 ± 0.8-fold) mRNA levels significantly increased in iSMC subjected to the static strain regimen. IL-6 mRNA levels in stretched iSMC were unchanged over time. Measurement of LDH release into the supernatant of stretched iSMC demonstrated that static strain did not result in increased cytotoxicity (Fig. 3*E*). Interestingly, the cyclic strain regimen failed to induce any alterations in iSMC gene transcription for all genes of interest and at all time points investigated. Therefore, all further experiments were carried out by utilizing the static strain regimen.

Mechanical Strain Induces Proinflammatory Gene Expression in pMacs

Former studies by our group demonstrated that macrophages play a key role in the initial process of POI pathogenesis. Therefore, we further investigated whether mechanical stress is capable to induce a proinflammatory response also in macrophages. In pMacs, we observed a significant upregulation of iNOS at 6 h (4.6 ± 1.7-fold), COX-2 at 4 and 6 h (2.6 ± 0.1 and 2.3 ± 0.3-fold), IL-6 at 4 and 6 h (3.7 ± 0.9 and 4.5 ± 1.4-fold), IL-1β at 4 h (1.7 ± 0.1-fold), and the macrophage activations markers MIP-1α at 4 and 6 h (1.7 ± 0.2 and 1.9 ± 0.2-fold) and MIP-2 at 4 h (3.5 ± 0.5-fold) after mechanical static strain compared with unstretched cultured pMacs (Fig. 4, *A–F*). By analogy with our observations in iSMC cultures, static strain did not result in increased cytotoxicity in pMacs (Supplemental Fig. S2).

Mediator Release from iSMC and pMacs After Mechanical Strain

The above-demonstrated immunological potential of both abdominal cell entities led to the question whether a paracrine trauma-triggered cross talk of iSMC and pMacs could possibly occur. Hence, we transferred conditioned cell-free culture supernatants from stretched iSMC to pMacs cultures or vice

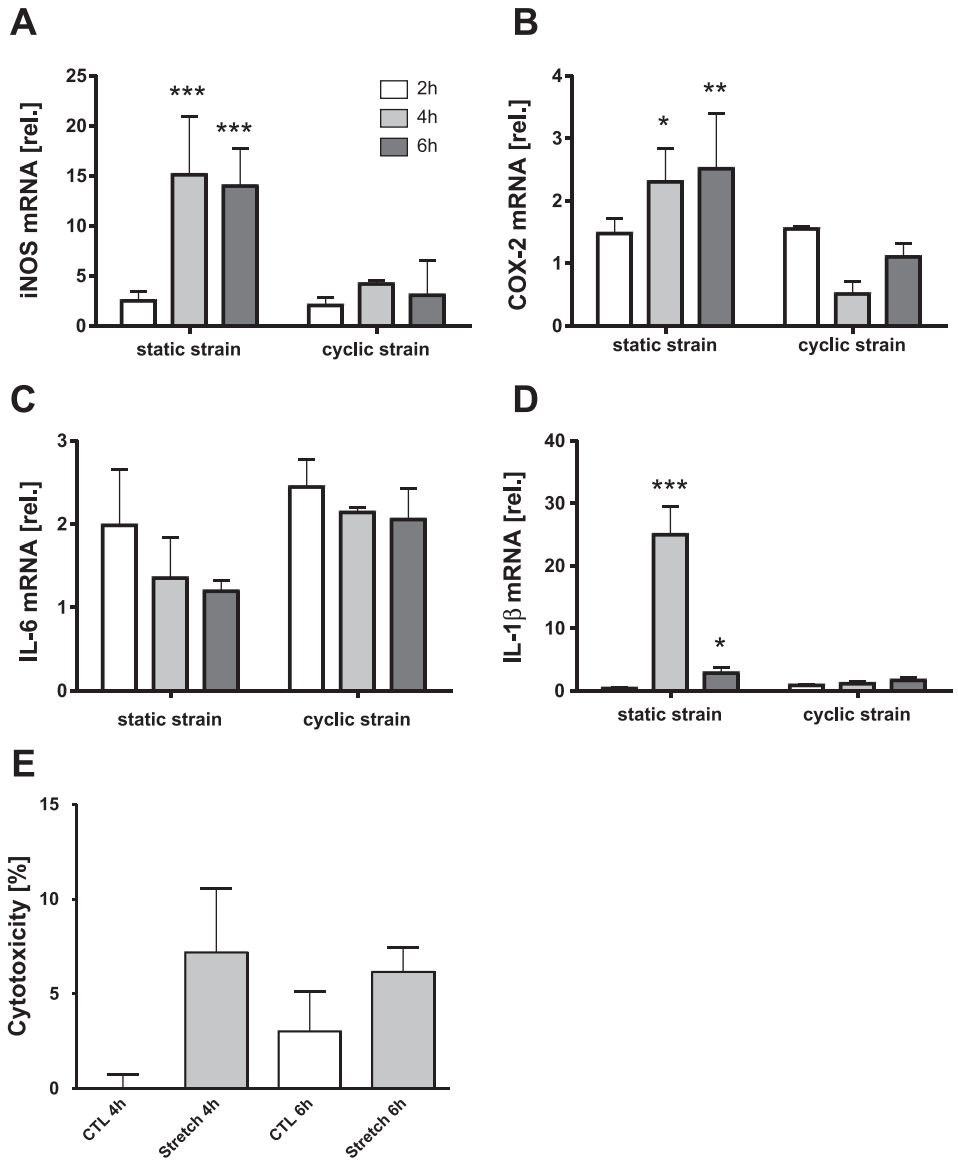


Fig. 3. *A–D*: static mechanical strain induced inducible nitric oxide synthase (iNOS; *A*), cyclooxygenase-2 (COX-2; *B*), IL-6 (*C*), and IL-1β (*D*) mRNA expression in iSMC. iSMC were cultured on collagen type I-coated BioFlex plates and mechanically stretched for 2, 4, and 6 h with 20% elongation with a static or cyclic strain regimen. Relative (rel.) gene expression levels of iNOS, COX-2, IL-1β, and IL-6 were determined by quantitative PCR (qPCR) and normalized to unstretched iSMC (not shown). *E*: cytotoxicity of mechanical strain was analyzed 4 and 6 h after mechanical strain by lactate dehydrogenase (LDH) release from cells into culture supernatant. LDH concentration in supernatants from totally lysed cells was set as 100% cytotoxicity. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. unstretched controls; 2-way ANOVA and Bonferroni posttest. Bars represent means ± SD; $n = 3$; CTL, control unstretched.

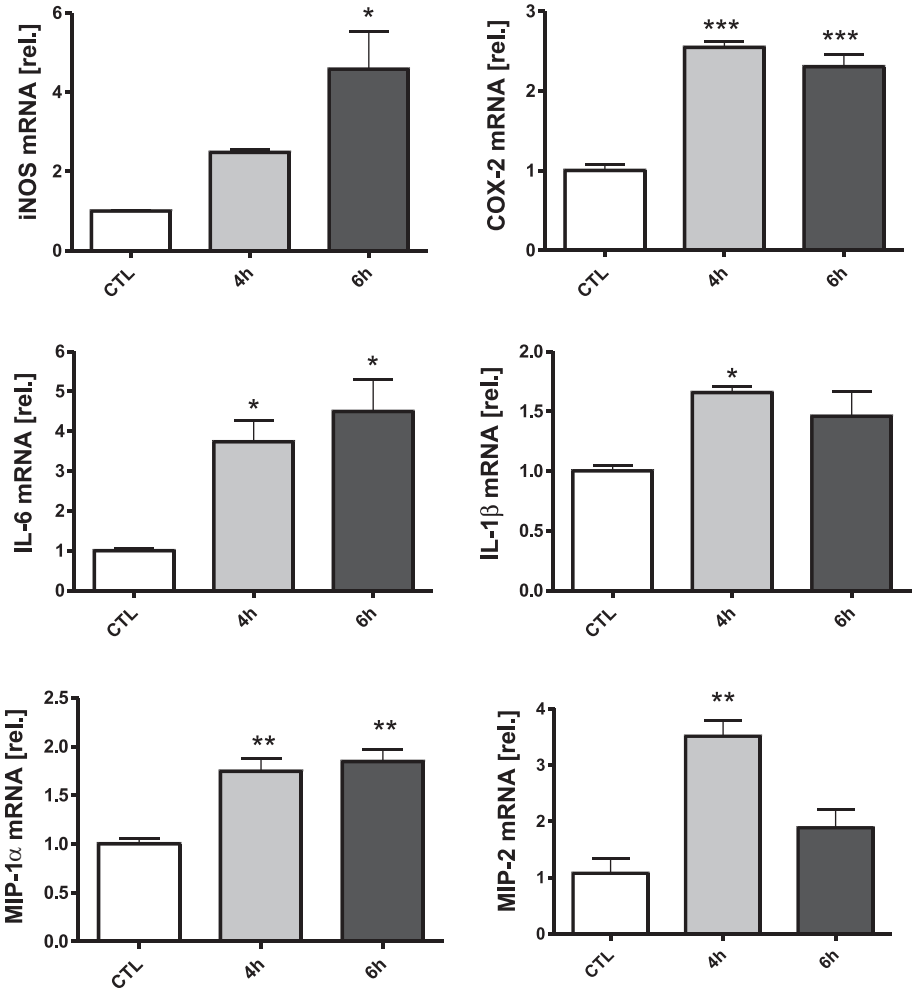


Fig. 4. Mechanical strain mediated proinflammatory gene expression in peritoneal macrophages (pMacs). pMacs were cultured on collagen type I-coated BioFlex plates and mechanically stretched for 4 and 6 h with 20% elongation or left unstretched (CTL). Gene expression levels were determined by qPCR. MIP, macrophage inflammatory protein. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. unstretched controls; 1-way ANOVA and Bonferroni posttest. Bars represent means ± SD; $n = 3$.

versa and analyzed the cells for the expression of proinflammatory genes. Culture supernatants from stretched pMacs induced a significant upregulation of iNOS gene expression (9.8 ± 3.1) in iSMC 3 h after medium transfer (Fig. 5 whereas COX-2 and IL-6 gene expression remained unaffected. In turn, when cell culture supernatant from stretched iSMC was transferred to pMacs (Fig. 6),) several proinflammatory genes were induced in unstretched pMacs compared with medium exposure from unstretched iSMC. At 2 and 4 h after medium transfer, iNOS expression was amplified 3.9 ± 1.6-fold and 11.8 ± 0.2-fold. COX-2 slightly increased 1.7 ± 0.2-fold and 2.2 ± 0.3-fold, respectively. Although IL-6 and IL-1β remained unchanged in pMacs, MIP-2 expression increased 2.2 ± 0.3-fold at 2 h and 2.2 ± 0.7-fold at 4 h after medium transfer. Interestingly, the macrophage activation marker MIP-1α, which was immediately upregulated in the muscularis externa after the surgical trauma in vivo, decreased significantly to 62% of control levels. Taken together, these results identify iSMC as nonclassical immunocytes with a potent immunomodulatory function.

Mechanical Strain Increases Endotoxin-Induced Proinflammatory Gene Expression

In vivo, bacterial translocation and transference of bacterial products like endotoxins are a transient consequence of intestinal manipulation contributing to muscularis inflammation. It has been demonstrated that this effect is in part mediated via activation of resident muscularis macrophages. However, it is unknown whether mechanical stress alters the well-known potent endotoxin-induced inflammatory response in macrophages.

In addition, recent immunohistochemical work discovered the expression of the pathogen recognition receptor TLR4, which detects, i.e., the important endotoxin LPS, on rodent and human intestinal smooth muscle cells, implicating a potential contribution of iSMC to the translocation-triggered inflammatory response. Although localization of cellular TLR4 expression in our iSMC cultures was not observed by immunohistochemistry, we successfully detected TLR4 mRNA in macrophage-depleted iSMC (results not shown) and TLR4 protein expression in macrophage-depleted iSMC cells and muscularis specimen (Supplemental Fig. S1*D*). Hence, we investigated the effect of the combined effect of in vitro LPS treatment and mechanical strain in both cell entities.

LPS potently induced iNOS (244 ± 111-fold) and IL-1β (88 ± 51-fold) gene expression in iSMC compared with unstimulated controls (Fig. 7). IL-6 mRNA was only slightly upregulated (3.0 ± 1.0) and COX-2 gene expression remained at base levels (Fig. 7). Simultaneous static strain of LPS-stimulated iSMC resulted in impressively and significantly amplified gene expression of iNOS (1,249 ± 435-fold), IL-1β (421 ± 153-

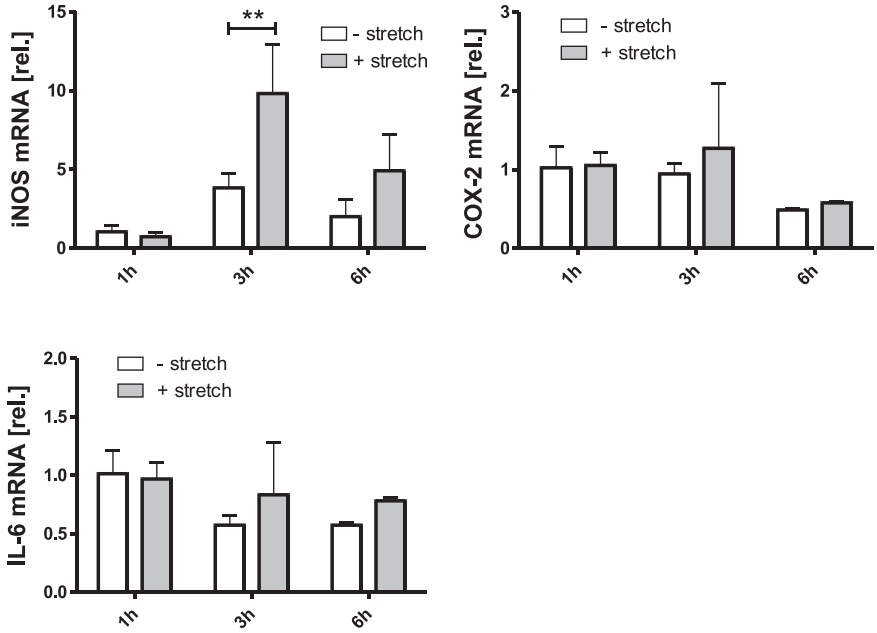


Fig. 5. Culture medium from stretched pMacs triggered iNOS expression in iSMC. pMacs were stretched (+stretch) or left untreated (−stretch) for 6 h, and cell-free culture supernatants were transferred to untreated iSMC cultures. After the indicated times, iNOS, COX-2, and IL-6 gene expression in iSMC was measured by qPCR. Stretch-conditioned medium from pMacs significantly increased iNOS expression in iSMC after 3 h. $^{**}P < 0.01$ vs. media from unstretched pMacs; 2-way ANOVA and Bonferroni posttest. Bars represent means ± SD; $n = 3$.

fold), IL-6 (5.1 ± 2.0-fold), and COX-2 (2.0 ± 0.7-fold) (Fig. 7). Interestingly, in pMacs, we observed a different expression pattern. Although LPS stimulation alone resulted in significantly higher expression of all genes in pMacs compared with iSMC, the LPS-induced gene expression of IL-1β (85 ± 16-fold vs. 68 ± 10-fold), iNOS (380 ± 46 vs. 306 ± 11) (Fig. 7), and the macrophage activation marker MIP-1a (32 ± 4.8 vs. 24) (data not shown) was not further increased by addi-

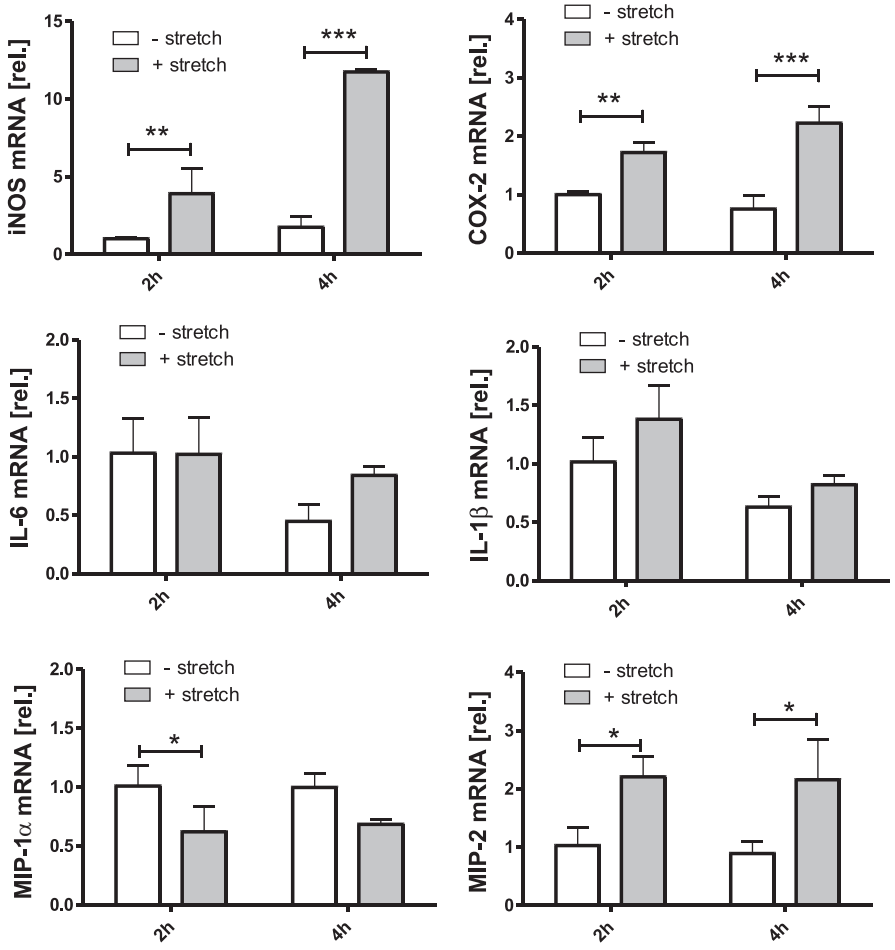


Fig. 6. Culture medium from stretched iSMC induced iNOS, COX-2, and MIP-2 expression in pMacs. iSMC were stretched or left untreated for 6 h and cell-free culture supernatants were transferred to untreated pMacs cultures. After the indicated times, gene expression was measured by qPCR. Stretch-conditioned medium from iSMC significantly induced iNOS, COX-2, and MIP-2 expression in pMacs after 2 and 4 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. unstretched controls; 2-way ANOVA and Bonferroni posttest. Bars represent means ± SD; $n = 3$.

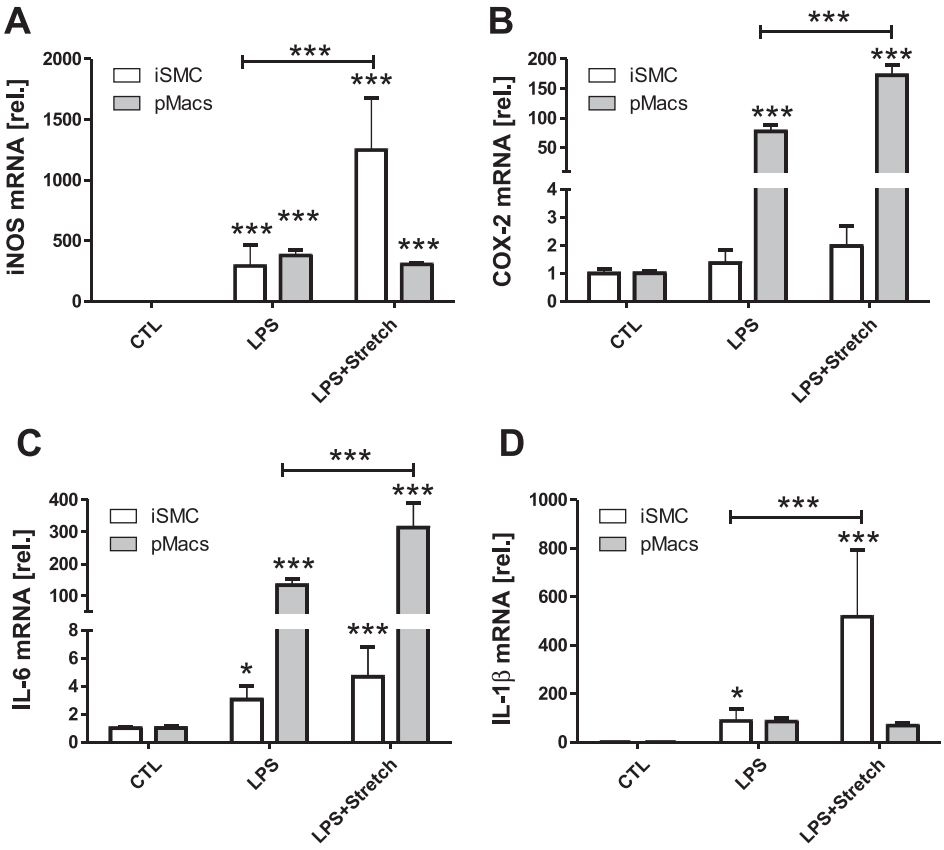


Fig. 7. Mechanical strain enhanced LPS-induced proinflammatory expression in iSMC and pMacs. iSMC and pMacs were either stimulated with 100 ng/ml LPS or stretched in the presence of LPS. Control cells (CTL) were left untreated. After 6 h, gene expression was measured by qPCR. LPS alone significantly induced iNOS, IL-6, and IL-1β gene expression in iSMC and transcription of all inflammatory genes in pMacs. Mechanical strain selectively potentiates the LPS-induced gene expression of iNOS and IL-1β in iSMC and of IL-6 and COX-2 in pMacs. $*P < 0.05$, $***P < 0.001$ vs. CTL or indicated probes; 1-way ANOVA and Bonferroni posttest. Bars represent means ± SD; $n = 3$ for pMacs and $n = 8$–10 for iSMC.

tional mechanical strain of pMacs. However, IL-6 (134 ± 20 vs. 313-fold), COX-2 (77 ± 10-fold vs. 172 ± 18-fold) (Fig. 7), and MIP-2 (83 ± 27 vs. 171 ± 24) (data not shown) significantly differ between the LPS and the dual LPS/stretch-stimulated pMacs, respectively. These results demonstrate that mechanical strain aggravates LPS induced proinflammatory gene expression in iSMC and macrophages. However, each cell entity exhibited a distinct expression pattern of proinflammatory genes.

DISCUSSION

In the present study, we demonstrated that in vitro static strain initiates proinflammatory gene expression in both isolated cultured iSMC and pMacs. Moreover, stretch-induced liberation of mediators contrariwise stimulated a paracrine proinflammatory reaction in both unstimulated cell entities. Furthermore, we have shown that static strain strongly increased LPS sensitivity of iSMC and macrophages. In conclusion, our results suggest that mechanical strain is an immunomodulatory trigger for abdominal cells.

Considerable evidence obtained from animal models of POI indicates that mechanical intestinal manipulation results in severe inflammation of the tunica muscularis (12, 14). Therefore, it is our hypothesis that mechanical strain *1*) might be a causative mechanism of POI by sparking off an inflammatory cascade and/or *2*) does occur during POI, acting as an inflammatory amplifier and subsequently contributing to the prolongation of postoperative gastrointestinal dysmotility.

Substantial progress in the understanding of POI was made with the observation that experimental inhibition of macrophage function forestalls intestinal trauma-triggered inflammation (32). Also, the readiness by which the dense resident network of muscularis macrophages is activated after intestinal manipulation further strengthens the central role of these immunoregulatory cells during POI, probably in response to mechanical strain. Mechanistically, macrophages were described to contain stretch-sensitive potassium channels (20). We therefore investigated stretch-induced activation of abdominal macrophages. As outlined in MATERIALS AND METHODS, cultivation of resident muscularis macrophages proved to be technically difficult, which necessitated the use of peritoneal macrophages as a substitute. The functions of pMacs have never been addressed in detail in in vivo models of intestinal trauma, but pMacs reside on the serosal surface of the intestine and it is therefore plausible that they also convert into a stretch-activated phenotype during intestinal manipulation or gut wall distention. Additionally, unpublished data from our group and electrophysiological single cell ion channel expression studies (23) showed that resident peritoneal macrophages operate in many aspects, e.g., phagocytosis and gene receptor expression, similarly to muscularis macrophages and other tissue macrophages. Herein, we demonstrated that static strain increased gene expression of the proinflammatory cytokines IL-6 and IL-1β, the macrophage activation markers MIP-1α and MIP-2, as well as the enzymes iNOS and COX-2 in adherent pMacs cultures. In parallel, Pugin et al. (25) have reported that pressure-cyclic mechanical strain activated alve-

olar macrophages are a major source of proinflammatory cytokines like IL-6, IL-8, and TNF-α. Conversely, other studies reported a lack of cyclic strain on alveolar macrophage IL-6 and TNF-α gene expression (18), which might be explained by the heterogeneity of the cell cultures or by the strain regimens used as further discussed below.

Nevertheless, iSMCs comprise another abundant cell entity of the multicellular muscularis syncytium, and mechanical strain was also described to induce proinflammatory mediator and enzyme release in several SMC subpopulations like pulmonary and vascular SMC (10, 16). A potential mechanism for the transduction of the inflammatory signal in iSMC in response to mechanical stress is the activation of mechanosensitive ion channels (8, 17, 19), which are believed to be important in the modulation of physiological gastrointestinal motility. Yet it is ill defined whether mechanosensation also triggers inflammatory responses in vivo, and more indicatively a paucity of data exists on the effect of static strain on iSMC.

First, we established a culture method for neonatal iSMC. The limiting step in culturing iSMC derived from adult rodents was the achievable number of viable cells during the isolation procedure. But, as indicated by the loss of the requirement of collagen IV culture dish coating for subcultivated iSMC as opposed to freshly isolated iSMC, cell differentiation occurred during cell passaging and, immunohistochemically, cultured iSMC expressed typical smooth muscle differentiation markers like α-actin and calponin.

Similar to stretched pMacs, static strain activated proinflammatory gene transcription of iNOS, COX-2 and IL-1β in iSMC. Consistently, these genes are strongly upregulated in vivo within the tunica muscularis after intestinal manipulation (11, 13, 30, 31), and leukocyte-derived iNOS has been shown to play a major role in mediating smooth muscle dysfunction in vivo (13, 31). However, the hypothetical contribution of nonhematopoietic derived surgery-triggered iNOS to the pathophysiology of POI has not been investigated yet, but iNOS message can be generated in vivo by cells of nonhematopoietic origin in response to TLR4 stimulation by LPS (2). Interestingly, the lack of IL-6 mRNA induction in the gene expression pattern of iSMC is pointing toward cell-specific stretch-triggered responses. In addition, the literature suggests a tissue-specific stretch susceptibility of SMC given that cyclic mechanical strain increased IL-6 expression in vascular SMC whereas in general our results indicate unresponsiveness of iSMC to cyclic strain (35). Indeed, it is important to state that intestinal or intestine-associated cells might be tolerant to cyclic strain since cyclic cell shape alterations happen naturally with peristalsis and the passage of food bolus. In contrast, tonic cell stretch occurring in dilated bowel loops is not physiological and might exceed a certain threshold of mechanosensitivity, resulting in stretch-activation of the gut wall.

It is believed until today that translocation of bacteria or bacterial products from the injured intestine invokes gut-associated immune and nonimmune intestinal cells to locally generate inflammatory factors (4, 5). Specifically, Schwarz et al. (29) have clearly shown that intestinal manipulation opens a transient pathway for the transference of orally fed luminal microspheres by increasing mucosal permeability. LPS-mediated intestinal paralysis has been studied in depth (6, 7) and iSMC express functional TLR4 receptors (2, 28). Herein we demonstrate that indeed our cultured iSMC transcribe TLR4 mRNA, and, although cellular TLR4 protein localization was not detectable by immunohistochemistry, we successfully demonstrated TLR4 protein expression in purified iSMC cultures by immunoblotting. Contaminating TLR-4-expressing neuronal cells of the myenteric plexus could only rarely be detected by PGP 9.5 and GFAP staining within the cell cultures (results not shown).

TLR4 engagement by LPS resulted in significant upregulation of iNOS and IL-1β but not IL-6 and COX-2 message in iSMC, and, most impressively, dual treatment with LPS and mechanical strain further amplified this specific inflammatory response. Interestingly, a LPS/stretch-mediated boosting-effect was also observed in macrophages. However, compared with iSMC, pMacs displayed a reversed inflammatory gene expression pattern with high mRNA induction of COX-2 and IL-6, which products are also known to participate in rodent POI (30, 33). A similar amplification of LPS-triggered inflammation by mechanical strain was described in human alveolar macrophages. Pugin et al. (25) demonstrated within a different model of mechanical trauma that LPS-induced IL-6 and TNF-α expression was augmented by mechanical strain. Furthermore, LPS-induced TNF-α expression was magnified in alveolar macrophages by large tidal ventilation stretch (21). Summarized, iSMC and pMacs respond to combined mechanical strain and TLR4 ligation in a cell-specific manner. To us, the cell-specific gene expression pattern demonstrates that both cell entities have a distinct and not interchangeable function in inflammation. Still, the underlying pathway by which mechanical strain enhances LPS-induced gene expression remains yet to be elucidated and no direct conclusions can be drawn by the individual gene expression profiles.

Admittedly, gene transcription does not necessarily equate functional protein expression. Translation of the molecular inflammatory response and active mediator secretion into the microenvironment or the systemic circulation is mandatory to orchestrate inflammation in multicellular organisms. In this study, static strain induced the liberation of currently unknown mediators from iSMC that subsequently initiated selective proinflammatory gene expression in resting macrophage cultures. In turn, stretched macrophages release unexplored molecules that triggered the transcription of selective inflammatory genes in untreated iSMC cultures. Hence, within the artificial environment of our cell culture, bidirectional cell-cell communication between iSMC and pMacs occurred in a paracrine fashion. Cell death and thereby the unspecific release of intracellular mediators could not be detected during strain application. In theory, both cell entities investigated might therefore become primarily activated during intestinal manipulation or gut wall distention and subsequently initiate a mutual paracrine cross talk within the abdominal cavity.

In conclusion, this study provides experimental evidence that mechanical strain is capable to induce *1*) selective cell-specific proinflammatory molecular fingerprints and *2*) liberation of unidentified, proinflammatory, paracrine-acting mediators in both iSMC and pMacs. Moreover, mechanical strain synergizes with TLR4-sensitive cell-specific immunoregulation. Thus further knowledge about the underlying pathways of mechanoimmunomodulation could open a new perspective in understanding the molecular triggers of intestinal diseases like POI.

Limitations of This Study

The initial idea of this study was to analyze the effect of surgical (mechanical) manipulation on cells of the intestinal muscularis during abdominal surgery. Therefore, we established the presented in vitro cell culture and stretch methodology. However, the following points have to be taken in consideration. *1*) The static stretch regimen is one kind of mechanical strain that can be applied to cells. Our results clearly demonstrate that static strain but not cyclic strain induces gene transcription in iSMC and pMacs. Nevertheless, it remains to be demonstrated whether this is indeed an adequate model to mimic the surgical trauma in humans. *2*) Although pMacs were isolated from adult rats, iSMC were isolated from ME preparation from neonatal rats. We were not able to isolate a sufficient amount of iSMC from adult animals because most of the cells died during the collagenase digestion procedure. Although the cells were passaged before the experiments and expressed typical smooth muscle cell markers, i.e., α-actin and calponin, one should be aware of possible differences between adult and neonatal cells. *3*) Although our results clearly demonstrate a significant stretch-induced gene expression, we were not able to detect changes in protein levels or nitric oxide (as a product of iNOS) in culture supernatants. One appropriate explanation is the low cell density within the BioFlex stretch wells. Probably, the cell number was too low to release detectable concentrations of IL-1β or nitric oxide into the cell culture medium. Another reason could be the absence of costimulatory factors as they exist in vivo. A good example is the activation of a complex and not fully understood enzyme cascade (inflammasome) for the posttranslational maturation and secretion of IL-1β. *4*) Additionally, the choice of pMacs instead of resident muscularis macrophages is also a limitation to this study. When isolated from neonatal rat ME, only a very small amount of viable macrophages (3×10^3 cells/intestine) can be purified. Although both macrophage types behave in the same manner in several functional (i.e., phagocytosis) and gene expression studies, our results also demonstrate that macrophages from different tissues show different responses to mechanical strain (i.e., compared with alveolar macrophages). On the other hand, the use of primary cells is also a great advantage compared with the use of cell lines. Further studies are needed to prove whether the findings of our in vitro work are retransferable into the in vivo situation.

GRANTS

This study was supported by grants from the Deutsche Forschungsgemeinschaft (Ka1270/3-1/2).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

1. **Bauer AJ, Boeckxstaens GE.** Mechanisms of postoperative ileus. *Neurogastroenterol Motil* 16, *Suppl* 2: 54–60, 2004.
2. **Buchholz BM, Chanthaphavong RS, Bauer AJ.** Nonhemopoietic cell TLR4 signaling is critical in causing early lipopolysaccharide-induced ileus. *J Immunol* 183: 6744–6753, 2009.
3. **Copland IB, Post M.** Stretch-activated signaling pathways responsible for early response gene expression in fetal lung epithelial cells. *J Cell Physiol* 210: 133–143, 2007.
4. **Deitch EA.** Bacterial translocation or lymphatic drainage of toxic products from the gut: what is important in human beings? *Surgery* 131: 241–244, 2002.
5. **Deitch EA, Bridges WM, Ma JW, Ma L, Berg RD, Specian RD.** Obstructed intestine as a reservoir for systemic infection. *Am J Surg* 159: 394–401, 1990.
6. **Eskandari MK, Kalff JC, Billiar TR, Lee KKW, Bauer AJ.** Intestinal macrophage inducible nitric oxide suppresses circular smooth muscle activity during endotoxemia. *Surgical Forum* 48: 80–82, 1997.
7. **Eskandari MK, Kalff JC, Billiar TR, Lee KKW, Bauer AJ.** Lipopolysaccharide activates the muscularis macrophage network and suppresses circular smooth muscle activity. *Am J Physiol Gastrointest Liver Physiol* 273: G727–G734, 1997.
8. **Farrugia G, Holm AN, Rich A, Sarr MG, Szurszewski JH, Rae JL.** A mechanosensitive calcium channel in human intestinal smooth muscle cells. *Gastroenterology* 117: 900–905, 1999.
9. **Frank JA, Wray CM, McAuley DF, Schwendener R, Matthay MA.** Alveolar macrophages contribute to alveolar barrier dysfunction in ventilator-induced lung injury. *Am J Physiol Lung Cell Mol Physiol* 291: L1191–L1198, 2006.
10. **Halka AT, Turner NJ, Carter A, Ghosh J, Murphy MO, Kirton JP, Kielty CM, Walker MG.** The effects of stretch on vascular smooth muscle cell phenotype in vitro. *Cardiovasc Pathol* 17: 98–102, 2008.
11. **Hori M, Kita M, Torihashi S, Miyamoto S, Won KJ, Sato K, Ozaki H, Karaki H.** Upregulation of iNOS by COX-2 in muscularis resident macrophage of rat intestine stimulated with LPS. *Am J Physiol Gastrointest Liver Physiol* 280: G930–G938, 2001.
12. **Kalff JC, Carlos TM, Schraut WH, Billiar TR, Bauer AJ.** Surgically induced leukocytic infiltrates within the rat intestinal muscularis mediate postoperative ileus. *Gastroenterology* 117: 378–387, 1999.
13. **Kalff JC, Schraut WH, Billiar TR, Simmons RL, Bauer AJ.** Role of inducible nitric oxide synthase in postoperative intestinal smooth muscle dysfunction in rodents. *Gastroenterology* 118: 316–327, 2000.
14. **Kalff JC, Schraut WH, Simmons RL, Bauer AJ.** Surgical manipulation of the gut elicits an intestinal muscularis inflammatory response resulting in postsurgical ileus. *Ann Surg* 228: 652–663, 1998.
15. **Kalff JC, Schwarz NT, Walgenbach KJ, Schraut WH, Bauer AJ.** Leukocytes of the intestinal muscularis externa: their phenotype and isolation. *J Leukoc Biol* 63: 683–691, 1998.
16. **Kanefsky J, Lenburg M, Hai CM.** Cholinergic receptor and cyclic stretch-mediated inflammatory gene expression in intact ASM. *Am J Respir Cell Mol Biol* 34: 417–425, 2006.
17. **Kraichely RE, Farrugia G.** Mechanosensitive ion channels in interstitial cells of Cajal and smooth muscle of the gastrointestinal tract. *Neurogastroenterol Motil* 19: 245–252, 2007.
18. **Lang CJ, Barnett EK, Doyle IR.** Stretch and CO_2 modulate the inflammatory response of alveolar macrophages through independent changes in metabolic activity. *Cytokine* 33: 346–351, 2006.
19. **Lyford GL, Farrugia G.** Ion channels in gastrointestinal smooth muscle and interstitial cells of Cajal. *Curr Opin Pharmacol* 3: 583–587, 2003.
20. **Martin DK, Bootcov MR, Campbell TJ, French PW, Breit SN.** Human macrophages contain a stretch-sensitive potassium channel that is activated by adherence and cytokines. *J Membr Biol* 147: 305–315, 1995.
21. **Moriyama K, Ishizaka A, Nakamura M, Kubo H, Kotani T, Yamamoto S, Ogawa EN, Kajikawa O, Frevert CW, Kotake Y, Morisaki H, Koh H, Tasaka S, Martin TR, Takeda J.** Enhancement of the endotoxin recognition pathway by ventilation with a large tidal volume in rabbits. *Am J Physiol Lung Cell Mol Physiol* 286: L1114–L1121, 2004.
22. **O'Boyle CJ, MacFie J, Mitchell CJ, Johnstone D, Sagar PM, Sedman PC.** Microbiology of bacterial translocation in humans. *Gut* 42: 29–35, 1998.
23. **Ozaki H, Kawai T, Shuttleworth CW, Won KJ, Suzuki T, Sato K, Horiguchi H, Hori M, Karaki H, Torihashi S, Ward SM, Sanders KM.** Isolation and characterization of resident macrophages from the smooth muscle layers of murine small intestine. *Neurogastroenterol Motil* 16: 39–51, 2004.
24. **Park JM, Yang T, Arend LJ, Schnermann JB, Peters CA, Freeman MR, Briggs JP.** Obstruction stimulates COX-2 expression in bladder smooth muscle cells via increased mechanical stretch. *Am J Physiol Renal Physiol* 276: F129–F136, 1999.
25. **Pugin J, Dunn I, Jolliet P, Tassaux D, Magnenat JL, Nicod LP, Chevrolet JC.** Activation of human macrophages by mechanical ventilation in vitro. *Am J Physiol Lung Cell Mol Physiol* 275: L1040–L1050, 1998.

26. **Reddy BS, Gatt M, Sowdi R, MacFie J.** Surgical manipulation of the large intestine increases bacterial translocation in patients undergoing elective colorectal surgery. *Colorectal Dis* 8: 596–600, 2006.
27. **Reddy BS, MacFie J, Gatt M, Macfarlane-Smith L, Bitzopoulou K, Snelling AM.** Commensal bacteria do translocate across the intestinal barrier in surgical patients. *Clin Nutr* 26: 208–215, 2007.
28. **Rumio C, Besusso D, Arnaboldi F, Palazzo M, Selleri S, Gariboldi S, Akira S, Uematsu S, Bignami P, Ceriani V, Menard S, Balsari A.** Activation of smooth muscle and myenteric plexus cells of jejunum via Toll-like receptor 4. *J Cell Physiol* 208: 47–54, 2006.
29. **Schwarz NT, Beer-Stolz D, Simmons RL, Bauer AJ.** Pathogenesis of paralytic ileus: intestinal manipulation opens a transient pathway between the intestinal lumen and the leukocytic infiltrate of the jejunal muscularis. *Ann Surg* 235: 31–40, 2002.
30. **Schwarz NT, Kalff JC, Turler A, Engel BM, Watkins SC, Billiar TR, Bauer AJ.** Prostanoid production via COX-2 as a causative mechanism of rodent postoperative ileus. *Gastroenterology* 121: 1354–1371, 2001.
31. **Turler A, Kalff JC, Moore BA, Hoffman RA, Billiar TR, Simmons RL, Bauer AJ.** Leukocyte-derived inducible nitric oxide synthase mediates murine postoperative ileus. *Ann Surg* 244: 220–229, 2006.
32. **Wehner S, Behrendt FF, Lyutenski BN, Lysson M, Bauer AJ, Hirner A, Kalff JC.** Inhibition of macrophage function prevents intestinal inflammation and postoperative ileus in rodents. *Gut* 56: 176–185, 2007.
33. **Wehner S, Schwarz NT, Hundsdoerfer R, Hierholzer C, Tweardy DJ, Billiar TR, Bauer AJ, Kalff JC.** Induction of IL-6 within the rodent intestinal muscularis after intestinal surgical stress. *Surgery* 137: 436–446, 2005.
34. **Yamamoto K, Ikeda U, Shimada K.** Role of mechanical stress in monocytes/macrophages: implications for atherosclerosis. *Curr Vasc Pharmacol* 1: 315–319, 2003.
35. **Zampetaki A, Zhang Z, Hu Y, Xu Q.** Biomechanical stress induces IL-6 expression in smooth muscle cells via Ras/Rac1-p38 MAPK-NF-κB signaling pathways. *Am J Physiol Heart Circ Physiol* 288: H2946–H2954, 2005.

