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RESEARCH PAPER

IL-1β reduces tonic contraction of mesenteric lymphatic muscle cells, with the involvement of cycloxygenase-2 and prostaglandin E_2

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BACKGROUND AND PURPOSE

The lymphatic system maintains tissue homeostasis by unidirectional lymph flow, maintained by tonic and phasic contractions within subunits, 'lymphangions'. Here we have studied the effects of the inflammatory cytokine IL-1β on tonic contraction of rat mesenteric lymphatic muscle cells (RMLMC).

EXPERIMENTAL APPROACH

We measured IL-1β in colon-conditioned media (CM) from acute (AC-CM, dextran sodium sulfate) and chronic (CC-CM, T-cell transfer) colitis-induced mice and corresponding controls (Con-AC/CC-CM). We examined tonic contractility of RMLMC in response to CM, the cytokines h-IL-1β or h-TNF-α (5, 10, 20 ng·mL^{-1}), with or without COX inhibitors [TFAP (10^{-5} M), diclofenac (0.2×10^{-5} M)], PGE_2 (10^{-5} M)], IL-1-receptor antagonist, Anakinra (5 μg·mL^{-1}), or a selective prostanoid EP_4 receptor antagonist, GW627368X (10^{-6} and 10^{-7} M).

KEY RESULTS

Tonic contractility of RMLMC was reduced by AC- and CC-CM compared with corresponding control culture media, Con-AC/CC-CM. IL-1β or TNF-α was not found in Con-AC/CC-CM, but detected in AC- and CC-CM. h-IL-1β concentration-dependently decreased RMLMC contractility, whereas h-TNF-α showed no effect. Anakinra blocked h-IL-1β-induced RMLMC relaxation, and with AC-CM, restored contractility to RMLMC. IL-1β increased COX-2 protein and PGE_2 production in RMLMC.. PGE_2 induced relaxations in RMLMC, comparable to h-IL-1β. Conversely, COX-2 and EP_4 receptor inhibition reversed relaxation induced by IL-1β.

CONCLUSIONS AND IMPLICATIONS

The IL-1β-induced decrease in RMLMC tonic contraction was COX-2 dependent, and mediated by PGE_2. In experimental colitis, IL-1β and tonic lymphatic contractility were causally related, as this cytokine was critical for the relaxation induced by AC-CM and pharmacological blockade of IL-1β restored tonic contraction.

Abbreviations

AA, arachidonic acid; AC-CM, acute colitis-conditioned media; CBA, cytometric beads array; CC-CM, chronic colitis-conditioned media; CD, Crohn's disease; CM, conditioned media; Con-AC-CM, control acute colitis-conditioned media; Con-CC-CM, control chronic colitis-conditioned media; DSS, dextran sodium sulfate; IBD, inflammatory bowel disease; LECs, lymphatic endothelial cells; RMLMC, rat mesenteric lymphatic muscle cells; TFAP, N-(5-amino-2-pyridinyl)-4-trifluoromethylbenzamide; UC, ulcerative colitis

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GPCRs[a]
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Catalytic receptors[b]
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Anakinra, IL-1 receptor antagonist
Diclofenac
GW627368X
IL-1β
IL-4
IL-13
PGE_2 (dinoprostone)
TNF-α

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

The common major characteristic and cardinal clinical symptom of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC) is a severe, and chronic, relapsing intestinal inflammation (Baumgart and Sandborn, 2012; Ordas *et al.*, 2012). Although the precise aetiology of IBD is still unknown, the intestinal microvasculature (arterioles, capillaries, venules) has been identified as a major contributor to the initiation and perpetuation of intestinal inflammatory processes by triggering leukocyte recruitment and extravasation (Chidlow *et al.*, 2007; Danese, 2011). However, the role of the lymphatic components of the vascular system in the pathophysiology of IBD still remains unclear (Van Kruiningen and Colombel, 2008).

The lymphatic system maintains tissue homeostasis by returning interstitial fluid, digested lipids and perivascularly infiltrated immune cells to the venous side of the bloodstream. Lymph uptake in the intestine begins within the villi, where initial lymphatics facilitate the passive transport of cells and fluids across a single layer of lymphatic endothelial cells (LECs), which are covered by a discontinuous basement membrane (Alitalo, 2011). Subsequently, lymph drains through precollector vessels into collecting lymphatic vessels, which are invested with a muscle cell layer, a continuous basement membrane and unidirectional valves, which prevent lymph backflow (Alitalo, 2011). In collecting lymphatics, propulsive lymph flow is generated and regulated by complex interactions of phasic, and sustained, tonic contractions of the lymphatic muscle within a complicated network of connected valve-containing contractile subunits, called 'lymphangions' (Smith, 1949; Mislin, 1961; Mislin and Rathenow, 1962; Zawieja *et al.*, 1993). Recent studies have shown that force and frequency of lymphatic contractions can be positively and negatively influenced by a variety of autocrine and paracrine signals, including nervous innervation, NO, prostanoids, chemokines and cytokines (Becker *et al.*, 2014). Among these, inflammatory cytokines have been strongly associated with IBD pathophysiology, as the persistent and uncontrolled imbalance between pro- and anti-inflammatory cytokines is a central event of the overwhelming immune response in CD and UC (Neurath, 2014). Thereby, inflammatory cytokines (including IL-1β and TNF-α) affect multiple functions of the vascular system by initiating diverse downstream inflammatory pathways (Hatoum *et al.*, 2003; Danese, 2011). One important inflammation-induced vascular effect mediated only by IL-1β is the up-regulation of the two major angio- and lymphangiogenic mediators, VEGF-C and -D, following NF-κB activation (Ristimaki *et al.*, 1998; Watari *et al.*, 2008). Both events have consistently been implicated in inflammation-induced angiogenesis and lymphangiogenesis (Huggenberger *et al.*, 2011; Achen and Stacker, 2012; Kim *et al.*, 2012a).

Although these alterations display structural reorganization of the lymphatic system in response to inflammation, important functional changes in lymph transport have also been recognized. Whereas many studies reveal an increase in lymph flow as an acute response to inflammation (which serves to balance an increased cell and fluid load), others have reported a reduction in lymph transport performance under chronic conditions (Huggenberger *et al.*, 2010; von der Weid and Muthuchamy, 2010; Lachance *et al.*, 2013). Some of our recent work has shown that, in a model of intestinal inflammation, lymph flow from the ileum fell significantly within 24 h of induction and remained depressed for days (Cromer *et al.*, 2015). Additionally, increasing evidence has demonstrated an important capacity of inflammatory cytokines to depress lymphatic pumping function, consistent with reports of reduced pumping in different states of inflammation (von der Weid and Muthuchamy, 2010; Aldrich and Sevick-Muraca, 2013). This effect has been shown in animal models of intestinal inflammation, which revealed impairment of the innate active contractile capacity within collecting mesenteric lymphatics (Wu *et al.*, 2006; Mathias and von der Weid, 2013). In these models, diminished phasic and tonic contractile responses in collecting lymphatic vessels were correlated with lymphatic dysfunction, lymphatic vessel dilation and mucosal inflammation (Von Der Weid and Rehal, 2010). Accordingly, cytokines, including ILs and TNF-α, have been proposed to mediate these effects, and IL-1β in particular has been the focus of several recent experimental studies. Using *in vitro* and *in vivo* models of lymphatic transport function, it has been shown that IL-1β potently inhibits lymphatic propulsion and thus lymph flow, through a failure of lymphatic contractility (Hanley *et al.*, 1989; Aldrich and Sevick-Muraca, 2013).

Although the earlier studies provide important insights into the roles of inflammatory cytokines on lymphatic transport function, they did not unequivocally differentiate between a direct influence of these mediators on lymphatic muscle cells, or indirect effects on lymphatic endothelial or resident and infiltrated inflammatory cells, on lymphatic pumping. Given the close association of immune/inflammatory cells with the mesenteric-collecting lymphatics, this differentiation is important (Bridenbaugh *et al.*, 2013; Chatterjee and Gashev, 2014). As an important link between vascular and immune responses to inflammation, the understanding of interstitial fluid and especially antigen and immune cell clearance, via lymph transport, underscores the importance to gain further mechanistic insights into these phenomena. Although currently available lymphatic muscle cell *in vitro* models do not reproduce all of the phasic contractile responses seen *in vivo*, our studies show that rat lymphatic muscle cells exhibit tonic contraction and show many similar contractile responses to those reported *in vivo*.

In this study, we have demonstrated that exposure to the inflammatory cytokine IL-1β, which is known to contribute to IBD aetiology, depressed tonic lymphatic muscle contraction in an *in vitro* model of lymphatic muscle function. We also demonstrated that this effect can be reproduced using conditioned media (CM) from acute and chronic murine models of experimental colitis, which is IL-1β dependent and associated with a COX-2 and PGE_2 pathway.

Methods

Ethical approval

All animal care and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of LSUHSC-S. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 16 animals were used in the experiments described here.

Mice

We used 6- to 8-week-old male C57BL/6J and recombinase activating gene-1-deficient (Rag-1 –/–, B6.129S7-Rag1) mice, which lack T cells and B cells. All mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and kept in a controlled environmental room at 25°C with a 12/12 h light/dark cycle at the animal care facility at LSUHSC-S, with free access to standard pellet diet and tap water before induction of colitis.

Induction of experimental colitis

We used two complementary models of experimental colitis: the acute model of erosive injury in the dextran sodium sulfate (DSS)-induced colitis and the T-cell-dependent model of chronic colitis following adoptive transfer of $CD4+CD45RB^{high}$ T-cell transfer. Acute colitis was induced in C57BL/6J mice by feeding 3% DSS (MW 36–50 kDa; ICN Biomedicals, Costa Mesa, CA, USA) in drinking water for 7 days. Chronic colitis was induced by reconstituting Rag-1 –/– mice with $CD4+CD45RB^{high}$ T cells, as previously described (Ostanin *et al.*, 2009).

Cells

Rat mesenteric lymphatic muscle cells (RMLMC) were obtained as previously described (Chakraborty *et al.*, 2011), and cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Atlanta Biologicals, Flowery Branch, GA, USA), 1% penicillin-streptomycin-amphotericin B (PSA, Coring Cellgro, Herndon, VA, USA) and 200 μM glutamine (HyClone Laboratories, Logan, UT, USA). These cells retain their contractile apparatus and regulation and were used from passage 12 to 28. Cells were cultured at 37°C in 7.5% CO_2. Cell culture medium was changed weekly and cells split every 14 days at a 1:5 ratio.

CM

CM were prepared using colon tissues from untreated, acute and chronic colitis-induced mice. Mice were anaesthetized by i.p. administration of ketamine (50 $mg \cdot mL^{-1}$) and xylazine (2.85 $mg \cdot mL^{-1}$), and killed by cardiac puncture, followed by removal of the entire colon. Colons were longitudinally opened, washed in ice-cold PBS, 1 cm^2 of each colon manually chopped and incubated in 10% FBS with 3% PSA supplemented DMEM for 24 h at 37°C. Subsequently, these media were collected, centrifuged at 485 x *g* for 15 min (min) at 4°C, filtered (0.22 μm, Puradisc 25 AS, Thermo/Fisher) and stored at –80°C.

Collagen gel contraction assay

Preparation of rat tail type 1 collagen. Rat tail type 1 collagen matrices were prepared by a modification of the protocol previously published by Benoit *et al*. (2008). Briefly, rat tail tendons were manually excised, washed with 100% isopropanol (Thermo/Fisher) and dissolved in sterile 4 mM acetic acid for 24 h at 4°C under constant agitation. Collagen solution was filtered through a 250 µm nylon filter (Spectrum Labs, Rancho Dominguez, CA, USA), centrifuged at 19 x *g* for 20 min at 4°C and snap frozen. Using a bench-top manifold freeze-dryer (Millrock Technology, Kingston, NY, USA), frozen aliquots were dried and stored at –20°C for future use.

Preparation of RMLMC/collagen gel. Twenty-four hours prior to experiments, freeze-dried collagen was resolubilized in cold 0.012 M hydrochloric acid (HCl) at 2.5 mg·mL^{-1} final collagen concentration and incubated overnight at 4°C with gentle agitation. On the day of the experiment, 0.8 mL of cold 5× PBS was added to 3.2 mL of dissolved collagen gel and the pH was titrated with 0.5 M sodium hydroxide (NaOH) to 7.4. Cultured RMLMC were washed twice with PBS and then harvested with trypsin-EDTA (Sigma-Aldrich). These cells were centrifuged at 485 x *g* for 5 min, resuspended in DMEM (supplemented with 10% FBS, PSA and glutamine) and counted. A total of 1.2×10^6 cells (50 000 cells per well) were resuspended in 8 mL of supplemented DMEM. The final RMLMC/collagen mixture (8 mL of cell suspension in 4 mL of collagen gel solution) was seeded in 500 µL aliquots into 24-well plates (Thermo/Fisher) and incubated at 37°C for 1 h, to polymerize. In our tonic contraction, assay gels maintained established levels of tonic contractility and did not exhibit relaxation or 'fatigue' once contracted.

Cytokines and CM treatment

RMLMC incorporated into collagen gels were stimulated with 1 mL DMEM (plus 10% FBS, PSA and glutamine) supplemented to a final concentration with cytokines: h-IL-1β (5, 10, 20 ng·mL^{-1}), h-TNF-α (5, 10, 20 ng·mL^{-1}), with or without COX inhibitors, TFAP [10× half maximal inhibitory concentration (IC_{50}) dose = 10^{-5} M], diclofenac (10× IC_{50} dose = 0.2×10^{-5} M), PGE_2 (10^{-5} M), IL-1 receptor antagonist, Anakinra (5 µg·mL^{-1}, optimized in a dose–response assay; see Supporting Information Fig. S2), EP_4 receptor antagonist [GW627368X (10^{-6} and 10^{-7} M)] or Con-AC, Con-CC-, AC-, CC-CM and 10-fold diluted CC-CM (1 mL·well^{-1}). Gels were then gently detached from the edges of the plates to allow unimpeded contraction, and incubated for 4 days at 37°C in 7.5% CO_2. In these experiments, RMLMC incorporated into gels attach to collagen fibres in three dimensions and promote progressive gel contraction as a function of time. To monitor time and treatment-dependent changes in contraction, digital photographs of gels were recorded daily over 4 days using a camera (Nikon D40, Tokyo, Japan). Experiments with CM were performed using three mice per group (Con-AC-, AC-, CC-, Con-CC-CM) in duplicate, and CM from each mouse was used in quadruplicate. Gel contractions in four replicate wells (quadruplicates) were averaged for each single *n* value, with $n = 3$ per group (i.e. 12 gels analysed for $n = 3$). Single stimulation/inhibition experiments were performed using five different RMLMC set-ups. Each culture of RMLMC was used to set up a minimum of four replicates per treatment concentration and the average of gel contraction in four wells averaged for each single *n* value for an $n = 5$ per treatment concentration (20 gels analysed for $n = 5$).

Gel contraction image analysis

Gel contraction was defined as the change in gel surface area on day 4 as a fraction of its area measured on day 0, and normalized to internal controls. All measurements were made using the NIH ImageJ analysis program (Schneider *et al*., 2012). At day 0, gel surface areas were initially equal to the well surface area as immediately after gel polymerization, contraction had not yet begun. Over 4 days, RMLMC contraction reduced the respective gel area, which was found to show the greatest differences at this time point. On day 4, gel surface areas were measured and area changes were determined as the gel area divided by the initial gel surface area on day 0. This value was subtracted from 1 to express it as a fractional change in area and then normalized to the contraction in control gels within each experiment, which was set as 100% contraction (see Supporting Information Fig. S4).

COX-2 and PGE_2 induction

RMLMC were split at a ratio of 1:3 into 6-well plates and incubated for 3 days until fully confluent. Media were then replaced with media supplemented with recombinant h-IL-1β (20 ng·mL^{-1}), recombinant h-TNF-α (20 ng·mL^{-1}), Con-, AC- or CC-CM, respectively, for 24 h. Afterwards, supernatant media were collected and stored at –80°C. RMLMC were placed on ice, washed with PBS and detached by scraping in 100 µL reducing sample buffer [45% Milli-Q water, 12% 0.5 M Tris-HCl, pH 6.8, 2% SDS, 15% glycerol, 2% phenol red (Sigma-Aldrich), 10% β-mercaptoethanol and protease inhibitor cocktail (Sigma-Aldrich)]. The cell lysate was homogenized, boiled for 1 min at 100°C and stored at –80°C.

Western blotting for COX-2

Total protein concentration was measured in all samples using a 660 nm protein assay (Thermo/Fisher). Cell lysate samples (40 µg per sample) were separated on 7.5% SDS polyacrylamide gels and transferred to nitrocellulose membranes using a transblot apparatus (Idea Scientific, Minneapolis, MN, USA). Protein transfer was verified with Ponceau-S (Sigma-Aldrich) staining. Membranes were blocked with 5% non-fat milk and were incubated with anti-COX-2 antibody (Cayman Chemical, 1:1000 in 0.1% milk powder) overnight at 4°C. An anti-actin N-terminal antibody (Sigma-Aldrich, 1:2000 in 0.1% milk powder) was used as loading control. Anti-rabbit IgG (whole molecule)-peroxidase antibody (Sigma-Aldrich, 1:1500 in 0.1% milk) was added as secondary antibody for 1 h at 25°C. Blots were visualized using enhanced chemiluminescence (Pierce ECL Western Blotting Substrate, Rockford, IL, USA). Densitometry of the resulting bands was performed using NIH ImageJ analysis program.

PGE_2 ELISA

PGE_2 concentrations in control and cytokine-treated RMLMC supernatants were determined using a commercially available PGE_2 ELISA kit (ENZO Life Science®, Farmingdale, NY, USA) performed according to the manufacturer's protocol.

BD™ cytometric bead array (CBA)

IL-1β and TNF-α concentrations in CM were measured using the CBA Flex Set System, Cell Signaling Master Buffer Kit, the CBA Mouse IL-1β and CBA TNF-α Flex Sets (all BD Bioscience, San Diego, CA, USA). The assay was performed according to the manufacturer's instructions, and all samples were run in duplicate.

Data analysis

Data are presented as mean ± SEM. When comparing three or more experimental groups, one-way ANOVA with Bonferroni's *post hoc* testing was used. Differences between two sets of data were determined using the unpaired two-tailed Student's *t*-test (GraphPad Instat 3 software, San Diego, CA, USA). Comparisons were considered statistically significant at $P < 0.05$.

Materials

Recombinant human (h) TNF-α and IL-1β were purchased from Thermo/Fisher (Waltham, MA, USA). PGE_2 was obtained from Cayman Chemical (Ann Arbor, MI, USA). Diclofenac (as the sodium salt) was purchased from Sigma-Aldrich and the selective COX-1 inhibitor IV [N-(5-amino-2-pyridinyl)-4-trifluoromethylbenzamide] TFAP was purchased from EMD Millipore (Billerica, MA). Anakinra (Kineret®; Sobi, Inc., Waltham, MA, USA), a recombinant IL-1 receptor antagonist (IL-1Ra), was obtained from Sobi, Inc. (Ardmore, PA, USA). The selective competitive antagonist of the prostanoid EP_4 receptor, GW627368X, was purchased from Cayman Chemical. All reagents were added to supplemented DMEM prior to the experiments.

Results

Tonic contraction of RMLMC was decreased following stimulation with CM from acute and chronic colitis-induced mice

First, we evaluated whether tissue-derived mediators, originating from acute and chronic colitis-induced mice, could influence the tonic contraction of RMLMC. RMLMC were stimulated with control-conditioned media from either control C57BL/6J (Con-AC-CM) or Rag-1 –/– (Con-CC-CM) mice, and with AC- and CC-CM from acute and chronic colitis-induced mice respectively. We found that after 4 days the tonic contractility (expressed in % of contraction vs. control) of RMLMC was significantly reduced by both AC-CM and CC-CM (Figure 1A and B).

IL-1β and TNF-α were increased in CM from murine models of acute and chronic colitis

Because IL-1β and TNF-α have been suggested to contribute to experimental and human colitis and IL-1β has also been proposed to depress lymphatic transport capacity, we tested whether IL-1β and TNF-α were present in CM from acute and chronic colitis-induced mice. Although IL-1β and TNF-α were not detectable in Con-AC/CC-CM, IL-1β and TNF-α were clearly present in AC-CM (Figure 2A and C) and in CC-CM (Figure 2B and D).

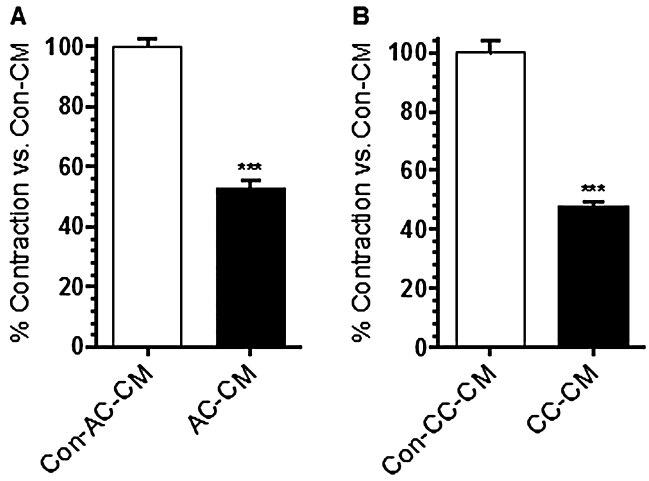

Figure 1

AC- and CC-CM decreased RMLMC contractility. (A) RMLMC treated with AC-CM for 4 days showed a significantly lower contractility (expressed in % of contraction vs. control) compared with control (Con-AC-CM, C57BL/6J mice) treated RMLMC. (B) CC-CM decreased RMLMC contractility at day 4 significantly compared with control (Con-CC-CM from Rag-1 –/– mice) treated RMLMC; $n = 3$, ***$P < 0.001$ versus control; Student's *t*-test; data are mean ± SEM.

IL-1β, but not TNF-α, decreased tonic RMLMC contractility

Although IL-1β and TNF-α have been associated with changes in the lymphatic vasculature under inflammatory conditions, the direct effect of these cytokines on tonic mesenteric lymphatic muscle cell contraction has not been previously evaluated. Therefore, we examined the effect of recombinant h-TNF-α (20 ng·mL^{-1}) and h-IL-1β (20 ng·mL^{-1}) on isolated RMLMC contractility, using the *in vitro* collagen gel contraction assay; Figure 3A shows a representative photograph of the collagen gel contraction assay, in which RMLMC were incubated with IL-1β (20 ng·mL^{-1}) or TNF-α (20 ng·mL^{-1}) over the period of 4 days. As shown in Figure 3B, h-TNF-α had no significant inhibitory effect, whereas h-IL-1β significantly impaired RMLMC contractility at day 4. Competitive inhibition of IL-1β from binding to IL-1 type 1 receptor (IL-1R1) using Anakinra (5 μg·mL^{-1}) maintained RMLMC contractility in gels co-treated with h-IL-1β (Figure 3B). To test whether the observed effects were dose dependent, we next examined different concentrations of h-IL-1β and found that 5 ng·mL^{-1}, 10 ng·mL^{-1} and 20 ng·mL^{-1}, all significantly impaired RMLMC/collagen gel contraction, showing a concentration-dependent effect of IL-1β on RMLMC contractility (Figure 3C). We then stimulated RMLMC with various doses of h-TNF-α (5, 10 and 20 ng·mL^{-1}), but none of these treatments had a significant influence on gel contractility (Figure 3D). We further tested if IL-1β + TNF-α could exhibit synergistic effects and found an enhanced relaxing effect, when cells we co-treated with IL-1β and TNF-α (see Supporting Information Fig. S3).

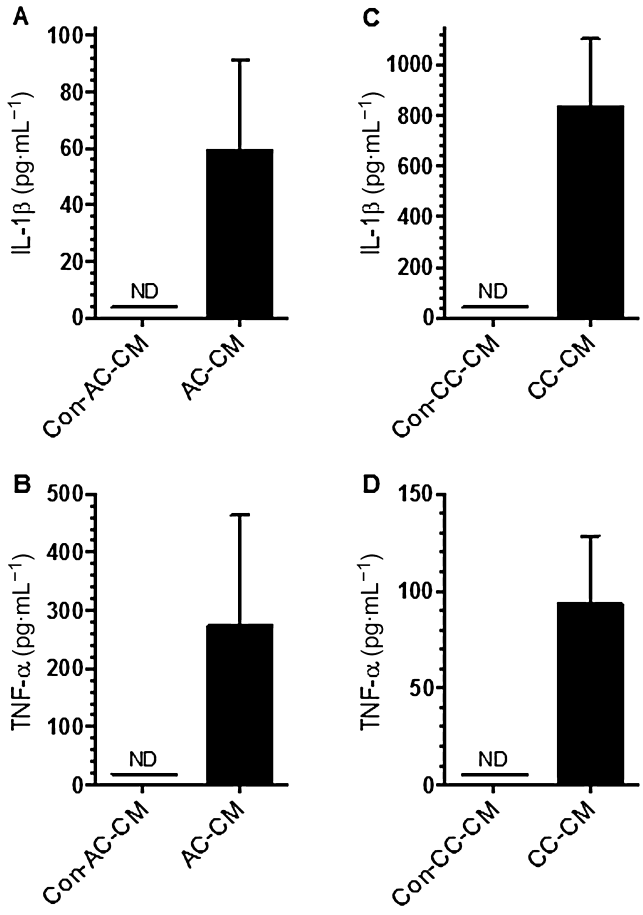

Figure 2

Concentration of IL-1β and TNF-α in CM measured by cytometric bead array. The concentration (pg·mL^{-1}) of IL-1β (A and C) and TNF-α (B and D) was measured in CM from AC-CM and CM from untreated controls (Con-AC-CM) as well as in CC-CM and respective untreated controls (Con-CC-CM) $n = 4$, ND, not detectable; data are mean ± SEM.

IL-1 receptor antagonist restored RMLMC contractility depressed by CM from acute colitis-induced mice

To further examine the influence of IL-1β on the relaxation effect seen in RMLMC after stimulation with AC- and CC-CM, we added Anakinra to test whether this treatment could restore lymphatic muscle contraction. We treated RMLMC with Con-AC-, AC-, Con-CC- and CC-CM supplemented with Anakinra at a dose of 5 μg·mL^{-1}. As shown in Figure 4, when Anakinra blocked IL-1 receptors, AC-CM-stimulated RMLMC showed a nearly complete restoration of normal contractility, compared with AC-CM alone (Figure 4A). However, blockade of the IL-1β receptor in RMLMC stimulated with CC-CM did not reverse the decrease in contractility produced by CC-CM (Figure 4B). This failure of Anakinra to restore contractility to CC-CM stimulated RMLMC could have been due to the greatly increased levels of IL-1β in CC-CM (14-fold levels in AC-CM). We therefore used the same concentration of Anakinra (5 μg·mL^{-1}), which reflects a frequently used, appropriate and therapeutic concentration, with 10-fold diluted CC-CM, where IL-1β levels were expected to be similar to levels found in AC-CM. Under these conditions, Anakinra (5 μg·mL^{-1}) was able to restore RMLMC contractility suppressed by the diluted CC-CM, compared with the corresponding control treatment (Figure 4C). To exclude potential relaxing effects caused by Anakinra itself, we also tested its effects on RMLMC contractility and found no significant differences between Anakinra and control treatments (Figures 4A and B).

IL-1β, but not TNF-α, increased COX-2 expression in RMLMC

IL-1β and TNF-α can each elicit inflammation-associated vasodilatation by activating endothelial COX-2 followed by the biosynthesis of vasodilator PGs from arachidonic acid (AA), resulting in smooth muscle relaxation. Additionally, IL-1β can produce similar effects on endothelium-denuded blood vessels, consistent with COX-2 production in smooth muscle cells (Beasley, 1999). However, these effects have so far only been addressed in blood vascular smooth muscle cells and not in lymphatic muscle cells. To test whether differences described here between the relaxing properties of IL-1β and TNF-α and between Con- and AC- or CC-CM may reflect differences in COX-2 protein levels in lymphatic cells, we evaluated the ability of these inflammatory cytokines, and CM, to induce COX-2 protein levels in RMLMC using Western blotting. Stimulation with h-TNF-α (20 ng·mL^{-1}) did not induce COX-2 protein levels in RMLMC (Figure 5A). However, h-IL-1β (20 ng·mL^{-1}) significantly increased COX-2 protein levels (measured as scan density values) in RMLMC, by approximately five-fold (Figure 5A). Also, AC- and CC-CM induced COX-2 protein levels in RMLMC after 24 h of treatment, compared with relevant Con-AC/CC-CM (Figure 5B and C).

Inhibition of COX-2, but not COX-1, restored RMLMCs contractility depressed by IL-1β

Because we found that IL-1β induced COX-2 protein levels in RMLMC, we investigated whether IL-1β-mediated relaxation was dependent on COX-2 activity. To differentiate between mediators derived from COX-1 and COX-2, we used the non-selective COX-1/-2 inhibitor diclofenac, and the selective COX-1 inhibitor TFAP, to attempt to restore RMLMC gel contractility decreased by IL-1β. RMLMC/collagen gels were treated with DMEM (plus 10% FBS, PSA and glutamine) as control or DMEM (plus with 10% FBS, PSA and glutamine) with h-IL-1β (20 ng·mL^{-1}), h-IL-1β (20 ng·mL^{-1}) + TFAP (10^{-5} M) or IL-1β (20 ng·mL^{-1}) + diclofenac (0.2×10^{-5} M). The non-selective COX-1/-2 inhibitor (diclofenac) blocked the relaxation effects of h-IL-1β, whereas TFAP (the selective COX-1 inhibitor) had no significant effect on IL-1β-stimulated RMLMC contractility (Figure 5D). To rule out possible relaxing effects caused by diclofenac or TFAP itself, we tested the influence of these inhibitors alone on RMLMC contractility. Figure 5D shows no significant differences between diclofenac, TFAP or control treatment.

PGE_2 was induced in RMLMC by IL-1β

AA metabolites are important regulators of lymphatic vessel contractility, and lymphatic vessels are capable of producing

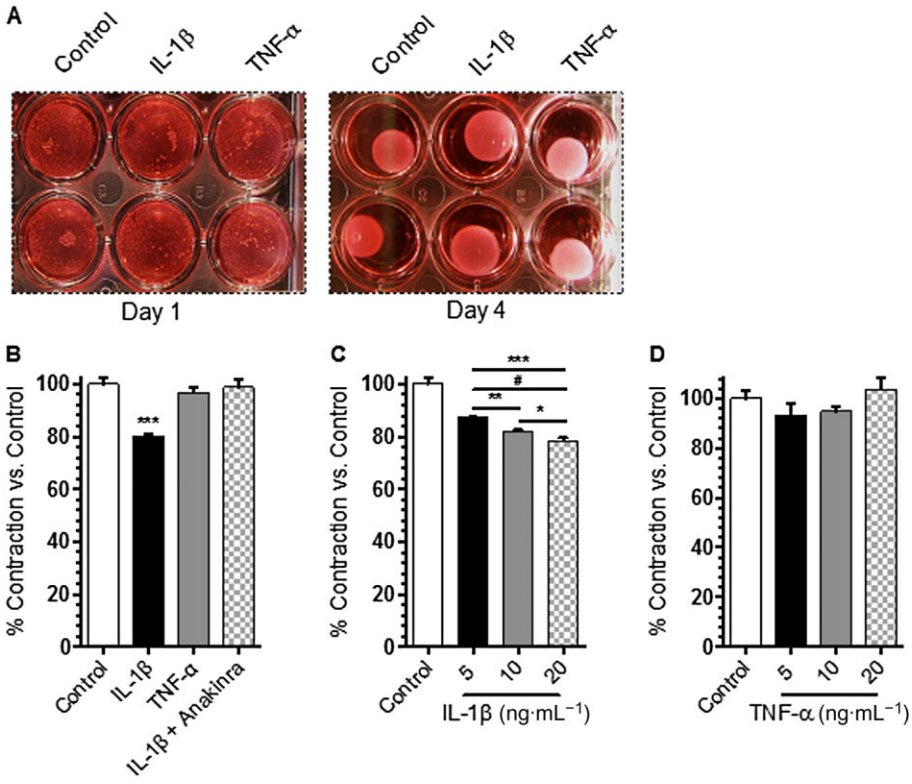

Figure 3

IL-1β decreased RMLMC contractility. (A) A representative photograph of RMLMC/collagen gels incubated with IL-1β (20 ng·mL^{-1}) and TNF-α (20 ng·mL^{-1}) over the period of 4 days. (B) IL-1β (20 ng·mL^{-1}), but not TNF-α (20 ng·mL^{-1}), decreased RMLMC contractility (expressed in % of contraction vs. control) while IL-1 receptor antagonist (Anakinra 5 μg·mL^{-1}) preserved RMLMC contractility in the presence of IL-1β. (C) IL-1β concentration-dependently decreased RMLMC contraction. (D) Different concentrations of TNF-α had no effect on RMLMC contraction. Contractility of RMLMC was measured after 4 days of incubation with cytokines; $n = 5$, ***$P < 0.001$ versus control, **$P < 0.01$ IL-1β 5 versus 10 ng·mL^{-1}, #$P < 0.001$ IL-1β 5 versus 20 ng·mL^{-1}, *$P < 0.05$ IL-1β 10 versus 20 ng·mL^{-1}; one-way ANOVA, with Bonferroni's *post hoc* test; data are mean ± SEM.

AA metabolites, which may influence their contractility in an autocrine fashion. COX-2 catalyses the conversion of AA to several PGs, of which PGE_2 is the most potent vasodilator. As we had found that COX-2 was highly expressed in RCLSMCs in response to IL-1β, which can increase PG levels. We measured PGE_2 in supernatants from RMLMC after stimulation with h-IL-1β (20 ng·mL^{-1}) and h-TNF-α (20 ng·mL^{-1}). Only IL-1β, not TNF-α, significantly increased PGE_2 levels (Figure 6A). Although we attempted to quantify PGE_2 in CM, we were not able to generate reliable data because of several technical difficulties (e.g. presence of mouse IgG in CM). Thus, we conducted additional experiments to test a possible relaxing effect of the pre-existing PGE_2 in CM (see Supporting Information Fig. S1). Our results suggest that the amount of PGE_2 in CM had negligible affects.

Exogenous PGE_2, dinoprostone, decreased RMLMC contractility

We tested if direct stimulation of RMLMC with exogenous PGE_2 could produce similar effects on tonic contractility as direct stimulation by IL-1β. Earlier studies on isolated lymphatic vessel contractile activity have shown that PGE_2-induced maximum inhibition around 30 μM, with a calculated EC_{50} of $10^{-5.9}$ M (Rehal *et al.*, 2009). Ren *et al.* (1995) reported that rat vascular smooth muscle cells were stimulated by PGE_2 with EC_{50} of 4.9×10^{-6} M. Hence, RMLMC collagen gels were treated with exogenous PGE_2 (dinoprostone; 10^{-5} M). As shown in Figure 6B, dinoprostone induced relaxation effects on RMLMC, similar to those of h-IL-1β.

EP4 receptor blockade restored RMLMC contractility depressed by IL-1β

We further determined the contribution of EP_4 receptors in mediating IL-1β-induced decreases in RMLMC contraction. RMLMC were treated with h-IL-1β (20 ng·mL^{-1}) alone or in co-treatment with the EP_4 receptor antagonist (GW627368X, 10^{-7} and 10^{-6} M, $IC_{50} = 10^{-6}$ M) (Wilson *et al.*, 2006; Rehal *et al.*, 2009). After 4 days, co-incubation with the EP_4 receptor antagonist blocked the relaxation effects of h-IL-1β stimulation and RMLMC contractility was restored to control values (Figure 6C).

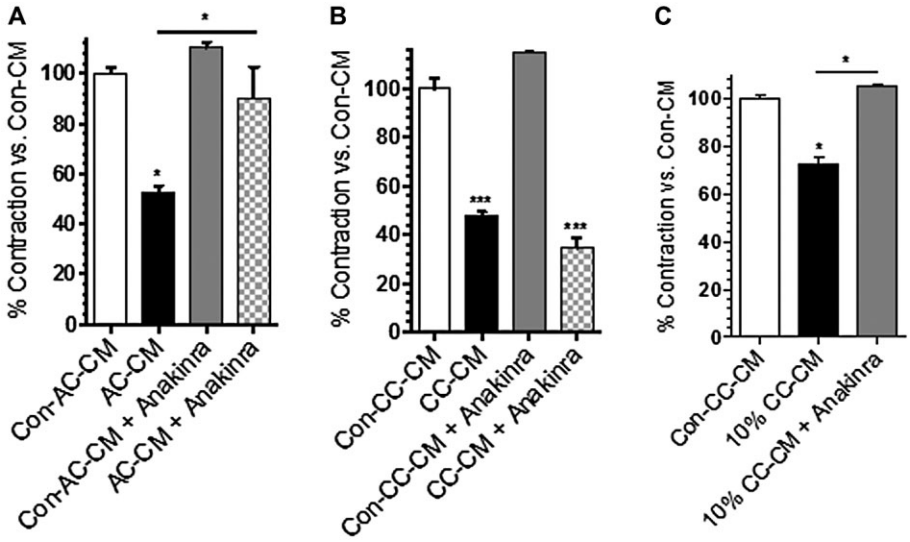

Figure 4

The IL-1 receptor antagonist Anakinra restored contractility of RMLMC treated with AC-CM. (A) Treatment with an IL-1 receptor antagonist inhibited the relaxation effect of AC-CM on RMLMC and restored their physiological contractility. When treated with AC-CM + IL-1 receptor antagonist RMLMC contractility showed no significant difference compared with Con-AC-CM (C57BL/6J mice). (B) Treatment with the IL-1 receptor antagonist did not restore RMLMC contractility suppressed by CC-CM. Contractility in RMLMC treated with CC-CM + IL-1 receptor antagonist showed a significant decrease compared with Con-CC-CM (Rag-1 −/− mice). (C) Anakinra inhibited the relaxation effect of 10-fold diluted (d) CC-CM on RMLMC and restored their physiological contractility. No significant difference in RMLMC contractility treated with 10% (d) CC-CM + IL-1 receptor antagonist; $n = 3$, $^{*}P < 0.05$, $^{***}P < 0.001$ versus control; one-way ANOVA, with Bonferroni's *post hoc* test; data are mean ± SEM.

Discussion

We used an *in vitro* collagen gel contraction assay, to demonstrate crucial pathways for the reduction in tonic RMLMC contraction, mediated by IL-1β. We demonstrated that this cytokine was essential for producing the relaxation effects caused by CM from acute experimental colitis, as IL-1β receptor blockade restored tonic contraction. Direct measurement of IL-1β, direct effects of recombinant IL-1β and the pharmacological blockade of IL-1β receptors with Anakinra further supported these findings. Despite potential influences of testing the effect of CM (mouse) and recombinant cytokines (human) on cells from a different species (rat), our results remain consistent with IL-1β as a potent contraction-limiting cytokine. The results presented here indicate that IL-1β stimulation up-regulated COX-2 expression in RMLMC, to increase local levels of the vasodilator PGE_2. We further showed that this effect is cytokine specific as TNF-α neither altered COX-2 expression nor had any effects on the tonic contraction of RMLMC.

Lymph transport *in vivo* is a complicated system involving lymphatic pumping, interstitial and venous pressures, lymph formation and lymphatic vascular resistance. So, the mechanisms that alter lymph flow during inflammation can alter any of these transport mechanisms and are variably dependent on the type of inflammation. Additionally because lymphatic muscle contains both smooth and striated muscle contractile and regulatory components, they exhibit both tonic and phasic contractile characteristics (Muthuchamy *et al.*, 2003). These different functional and molecular components work together and interact in a complex process that is not fully understood. Additionally many (although not all) inflammatory agents inhibit phasic contractile frequency and pumping, leaving lymph outflow resistance as one of the principal elements regulating lymph transport. This is one of the first descriptions of an *in vitro* assay of tonic lymphatic muscle contractile function. To date no one has developed a cultured lymphatic muscle cell model that reproduces phasic contractile function. This issue is also true for other striated muscle cell types. Thus, although these cultures of lymphatic muscle cells are not a model of phasic lymphatic contractility, they do represent a model of lymphatic muscle tonic activity. Our model is currently the only such lymphatic model available.

Inflammatory cytokines, including IL-1β and TNF-α, are expressed within the inflamed gut in animal models of colitis, which are characterized by a reduced lymphatic function (Egger *et al.*, 2000; Neurath, 2014). Accordingly, our data on AC- and CC-CM showed that IL-1β and TNF-α were generated in substantial levels. However, little is known about the direct mechanisms and effects of inflammatory cytokines on lymphatic muscle cell contractile function in collecting lymphatic vessels. Thus, to assess the role of these cytokines in regulating tonic lymphatic muscle cell contraction, we determined the effects of AC- and CC-CM on RMLMC contractility. Previously, Hanley *et al.* (1989) reported an inhibition of fluid pumping and tone in isolated mesenteric lymphatic vessels in response to IL-1β, an effect, which they attributed to the production of COX metabolites. Cytokine-induced dysregulation of lymphatic contractile function could in part account for the tonic and phasic contractile inhibition observed in mesenteric lymphatic vessels from an experimen-

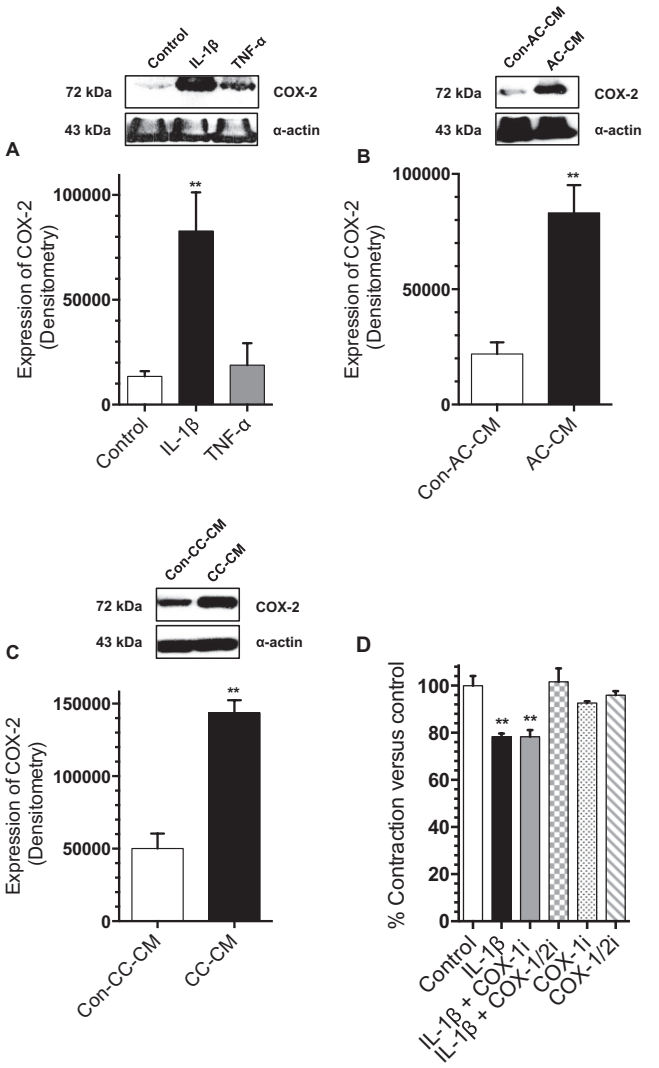

Figure 5

IL-1β increased COX-2 expression in RMLMC while COX-2 inhibition restored RMLMC contractility in the presence of IL-1β. (A) IL-1β (20 ng·mL^{-1}), but not TNF-α (20 ng·mL^{-1}), significantly induced COX-2 expression in RMLMC after 24 h of treatment. (B and C) AC-CM and CC-CM induced COX-2 expression in RMLMC after 24 h of treatment, compared with corresponding Con-AC/CC-CM. (D) COX-2 inhibition restored RMLMC contraction in combination with IL-1β as the COX-1/2 inhibitor (diclofenac), but not the COX-1 inhibitor (TFAP), restored the relaxation effect of IL-1β on RMLMC at day 4: $n = 3$ for Figure 5A–C, $n = 5$ for Figure 5D, $^{**}P < 0.01$ versus control; one-way ANOVA, Bonferroni's *post hoc* test was used for experiments, shown in Figure 5A and D. Student's *t*-test was used for experiments in Figure 5B and C; data are mean ± SEM.

tal model of ileitis. Wu *et al*. (2006) reported that the lymphatic vessel diastolic diameter increased with the severity of inflammation (reflecting a loss of tonic contraction), and that this effect could be seen *in vivo* and *in vitro*, thus indicating a substantial influence of intestinal inflammation on tonic contraction in mesenteric lymphatic vessels.

To further ascertain the effects seen in response to CM, we tested the direct effect of exogenous inflammatory cytokines on RMLMC tonic contractility. Because recombinant h-IL-1β and TNF-α have revealed sufficient cross-reactivity and can stimulate cell lines of rat origin, we used h-IL-1β and TNF-α in our model to further achieve a more clinically relevant and translational application (Kehrer *et al*., 1988). IL-1β, but not TNF-α, showed a dose-dependent inhibition of RMLMC contraction. Although IL-1β stimulation alone did not produce the same level of RMLMC contractility compared with that following AC- or CC-CM, we believe this is due to other mediators present in AC- and CC-CM, which contribute to RMLMC relaxation (Becker *et al*., 2014). However, our data clearly demonstrate that IL-1β is both necessary and sufficient to induce lymphatic muscle relaxation. Further studies are warranted to elucidate other mediators present in AC- and CC-CM that either contribute to lymphatic relaxation alone or enhance IL-1β-induced relaxation. Cytokines modulate vascular smooth muscle function through binding to specific receptors expressed on their surface (Sprague and Khalil, 2009). In our model, IL-1β receptor blockade with Anakinra restored RMLMC contractility suppressed by AC- and d-CC-CM. The lack of effect of Anakinra on undiluted CC-CM could be due to insufficient IL-1 receptor blockade by the used dose of 5 μg·mL^{-1} as CC-CM contained 14-fold more IL-1β than AC-CM. However, the same dose of Anakinra did restore RMLMC contractility suppressed by a 10-fold dilution of CC-CM, supporting the possibility that IL-1β is one of the critical lymphatic 'relaxant' cytokines released during experimental colitis. Treatment with an IL-1 receptor antagonist has been shown to reduce the severity of experimental immune complex colitis in rats (Cominelli *et al*., 1990). Our findings indicate that IL-1 receptor blockade may also partly reduce the inflammatory responses by restoring lymphatic transport capacity.

We demonstrated that IL-1β markedly induced COX-2 protein levels in RMLMC, as has been observed in several other cell types, including vascular and bronchial smooth muscle cells (Vigano *et al*., 1997; Beasley, 1999). Furthermore, IL-1β-induced inhibition of the tonic contractility in RMLMC was restored by the non-selective COX inhibitor diclofenac, whereas the selective COX-1 inhibitor TFAP alone had no effect on RMLMC contractility, reduced by IL-1β. This suggests that inhibition of COX-2-, but not of COX-1, was responsible for restoring tonic RMLMC contractility. Together, these results demonstrated that IL-1β inhibition of RMLMC contraction is COX-2 dependent. It is also likely that this effect was related to generation of COX-2 products, in particular, PGE_2. Vascular and bronchial smooth muscle cells produce PGE_2 in response to treatment with inflammatory cytokines (Feng *et al*., 1993; Belvisi *et al*., 1997) and lymphatic vessels themselves are also capable of producing PGs (Johnston and Gordon, 1981). Karnezis *et al*. (2012) have shown that LECs have the capacity to produce PGE_2, although it was unclear whether lymphatic muscle cells could be a possible source of PG production. In the present study, we detected high levels of PGE_2 in RMLMC culture media treated with IL-1β, showing that PGE_2 was produced directly by lymphatic muscle cells. Although these data do not rule out other PGs, and other PG sources, being involved in these responses, our results are the first demonstration of PGE_2 production by mesenteric lymphatic muscle cells in response to IL-1β, in the absence of endothelial cells.

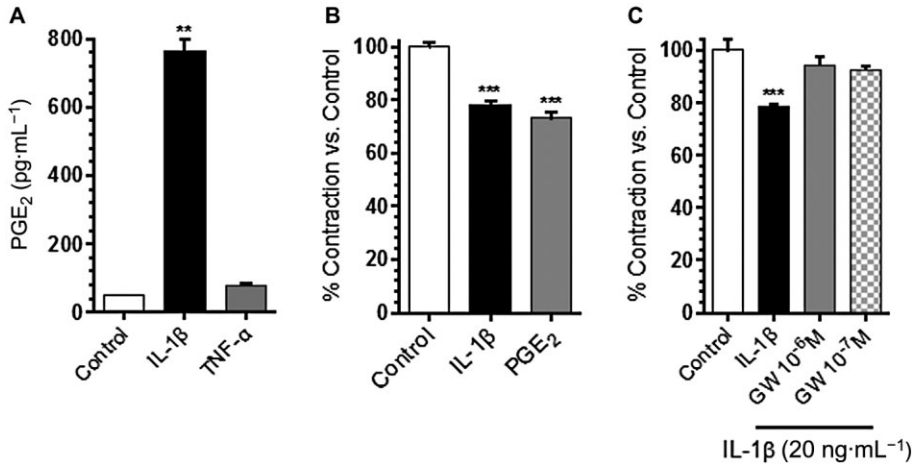

Figure 6

RMLMC produced PGE_2 in response to IL-1β stimulation, while treatment with an EP4 receptor antagonist maintained their physiological contractility. (A) PGE_2 levels in RMLMC supernatant were increased in response to IL-1β (20 ng·mL^{-1}), but not TNF-α (20 ng·mL^{-1}). (B) RMLMC contraction was significantly inhibited by PGE_2 (10^{-5} M) stimulation (similar to the relaxation effect to IL-1β), compared with control. (C) The EP4 receptor antagonist (GW627368X) restored RMLMC contraction that had been suppressed by IL-1β. GW627368X at 10^{-7} and 10^{-6} M reversed the relaxation effect of IL-1β on RMLMC at day 4; n = 3 for Figure 6A, n = 5 for Figure 6B and C, ***P < 0.001 versus control; one-way ANOVA, Bonferroni's *post hoc* test; data are mean ± SEM.

Studies on isolated mesenteric lymphatic vessels have shown that direct application of PGE_2 can induce lymphatic vessel relaxation, which could be mediated by the EP receptors, EP1-4 (Hata and Breyer, 2004; Rehal *et al.*, 2009). We found that EP_4 receptor mRNA was present in RMLMC (data not shown). The decrease in lymphatic muscle contraction could thus be produced by direct PGE_2 receptor binding in an autocrine fashion. Studies on isolated mesenteric lymphatic vessels from TNBS-treated animals also showed that the EP_4 receptor was the main receptor involved in the PGE_2-induced decrease in lymphatic pumping, and the downstream signalling involved the cAMP/PKA pathway (von der Weid and Muthuchamy, 2010). Our experiments showed that EP_4 receptors played a major role in IL-1β-induced decrease in RMLMC tonic contraction as a selective EP_4 receptor antagonist blocked the relaxation effect of Il-1β and restored RMLMC contractility. This suggests that PGE_2 mediates IL-1β-induced decrease in RMLMC contraction via binding to EP_4 receptors. Collectively, these data demonstrate that RMLMC are capable of generating and responding to PGE_2 in the absence of endothelial cells and confirmed PGE_2 as one of major regulators of lymphatic vessel contractility during inflammation.

Previously, we have demonstrated that inflammatory cytokines suppressed several LEC functions (Chaitanya *et al.*, 2010). In response to IL-1β and TNF-α, LECs showed alterations in barrier function, up-regulation of cell adhesion molecules and changes in proliferation rate, all of which are believed to play central roles in the interstitial fluid and immune cell uptake, within the initial lymphatics (Johnson *et al.*, 2006; Chaitanya *et al.*, 2010). The effects of inflammatory cytokines on both lymphatic endothelial and muscle cell types will also alter antigen and cytokine delivery to the node and thus inflammatory/immune reactions. Because IL-1β increased permeability and decreased lymph transport in mesenteric lymphatic vessels, one possible consequence could be the interstitial retention of fluids (oedema), lipids as well as immune cells and even the retrograde drainage from lymph from the vessel back into the interstitial space (Cromer *et al.*, 2014). This is compatible with one of our recent reports, in which we demonstrated that transgenic FoxC2 +/– mice, which are characterized by diminished intestinal lymph flow including valvular insufficiency, are highly susceptible to acute DSS-induced colitis and show signs of lymphatic transport failure as displayed by structural (dilated torturous lymphatic vessels) and functional (greater submucosal oedema, higher immune cell burden) changes in the intestinal lymphatic vasculature (Becker *et al.*, 2015). Additionally, Prox1+/– mice, which are characterized by lymphatic leakage, displayed an abnormal accumulation of lipids within the intestinal wall, partly reproducing the unique feature of fat wrapping in patients with CD (Harvey *et al.*, 2005; von der Weid *et al.*, 2011).

The shared molecular target of functional alterations in lymphatic muscle cells is their contractile apparatus, specifically their myosin light and heavy chains (MLC, MHC) and the corresponding phosphorylation status responsible for purposive muscle contraction. The influence of cytokines in general and specifically IL-1β on myosin light chain phosphorylation has previously been addressed. Although the critical influence of IL-1β on gastrointestinal motility disorders is long recognized, it has only recently been shown that IL-1β reduces the activity of important regulatory proteins such as CPI-17 (a phosphorylation-dependent inhibitor of myosin phosphatase), resulting in decreased phosphorylation of MLC (Cao *et al.*, 2005; Hu *et al.*, 2007; Ohama *et al.*,

2007). Although this concept has been successfully transferred to vascular smooth muscle cells, so far little is known about the molecular pathways, in which cytokines alter lymphatic muscle contractility at a molecular level (Kim *et al.*, 2012b). It is worth mentioning that even lymphatic muscle cells from different origins of the lymphatic vasculature (thoracic duct vs. mesentery) show significant differences in their contractile behaviour and MHC isoforms, which may further complicate the translation of results obtained from other than lymphatic muscle cells, to the lymphatic contractile apparatus and its molecular response to inflammatory stimuli (Muthuchamy *et al.*, 2003; Gashev *et al.*, 2004).

One possible limitation of our study is the focus on pro-inflammatory cytokines (IL-1β and TNF-α), which does not take the important role of Th2 and Th17 cytokines into account. Various studies have recently shown differential results, when reporting an increase in intestinal smooth muscle contractility, triggered by CD3+ T-cell-derived Th2 cytokines (IL-4, IL-13), a decrease in intestinal contraction induced by the Th17 cytokine IL-17 and a dual (activation vs. inhibition) dose-dependent modulation of airway smooth muscle cells by IL-4 (Akiho *et al.*, 2005b; Ohta *et al.*, 2008; Mori *et al.*, 2014). These findings are especially interesting as both hypo- as well as hyper-contractility of intestinal smooth muscle cells are displayed in clinical symptoms of IBD patients (e.g. strictures and constipation), while it is long recognized that human and experimental IBD are mainly accompanied by an impaired mesenteric lymphatic activity and diminished lymphatic transport function (Von Der Weid and Rehal, 2010). In line with the Il-1β / COX2 / PGE_2 pathway, described in this study, Akiho *et al.* (2005a) showed an increase in PGE_2 levels after exposure to the Th2 cytokines IL-4 and IL-13, which resulted in an enhanced intestinal muscle hyper-contractility. These results may indicate that the PGE_2-triggered increase in contractility can be induced by both Th1 and Th2 cytokines and that its relaxing effect is similar in different muscle cells. However, based on structural and functional differences, the results obtained from smooth muscle cells of origins other than the lymphatic vasculature have yet to be translated to the unique lymphatic muscle function and thus may lead towards further investigations in this field.

In summary, we have demonstrated a pathway in which IL-1β-induced PGE_2 production via up-regulation of COX-2 expression inhibits RMLMC tonic contraction. This study established one potential causal relationship between inflammatory cytokines and lymphatic muscle cells in the regulation of tonic lymphatic contractility in experimental colitis. Thus, IL-1β may play an important role in the pathophysiology of experimental colitis and human IBD, by depressing lymphatic transport capacity, leading to retention of inflammatory cells and mediators in the intestine, exacerbating the inflammatory state in a cascade reaction. Our findings indicate that lymphatic muscle contractility can be impaired independently of endothelial cells, suggesting that impaired lymphatic transport function represents an important component of the complex pathophysiology of experimental and human IBD. Overall, these data expand our understanding of the molecular mechanisms mediating lymphatic pump failure in experimental colitis and provide valuable mechanistic insights, which can exploit the lymphatic system as a target for the management of IBD.

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Author contributions

All experiments were undertaken at Louisiana State University Health Sciences Center – Shreveport, Louisiana. J. S. A. conceived and designed the research. M. A., F. B., M. W. and D. O. performed the experiments. M. A., M. W. and I. T. analysed the data. M. A., F. B., M. W., I. T., Y. W. and J. S. A. interpreted the results of the experiments. D. C. Z. and M. M. contributed essential reagents or tools. M. A., F. B., F. N. E. G., I. T., P. Y. W. and J. S. A. drafted the manuscript. All authors contributed to the critical review of drafts of the manuscript and gave final approval of the version to be published. M. A. and F. B. contributed equally to this work.

Conflict of interest

The authors declare no conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 PGE_2 decreased rat mesenteric lymphatic muscle cell (RMLMC) contractility resistant to Anakinra treatment. RMLMC treated with PGE_2 (10^{-5} M) for 4 days showed a significantly lower contractility (expressed in % of contraction vs. control) compared with control-treated RMLMC, while Anakinra (5 $\mu g \cdot mL^{-1}$) treatment showed no significant effect on the PGE_2-induced relaxation. $n = 4$, $^{**}P < 0.01$ versus control, one-way ANOVA, with Bonferroni's *post hoc* test; data are mean ± SEM.

Figure S2 Different doses of Anakinra restored IL-1β-induced decreased rat mesenteric lymphatic muscle cell (RMLMC) contractility. RMLMC treated with IL-1β (20 ng·mL^{-1}) for 4 days showed a significantly lower contractility (expressed in % of contraction vs. control) compared with control-treated RMLMC while co-treatment with Anakinra (0.5, 5, 50 μg·mL^{-1}) restored RMLMC relaxation. n = 5, ***P < 0.001 versus control, one-way ANOVA, with Bonferroni's *post hoc* test; data are mean ± SEM.

Figure S3 IL-1β + TNF-α exhibit synergistic effects in decreasing RMLMC contractility. IL-1β (20 ng·mL^{-1}), but not TNF-α (20 ng·mL^{-1}), decreased RMLMC contractility (expressed in % of contraction vs. control) while IL-1β (20 ng·mL^{-1}) + TNF-α (20 ng·mL^{-1}) decrease RMLMC contractility to a greater extent than IL-1β (20 ng·mL^{-1}) alone. IL-1 receptor antagonist (Anakinra 5 μg·mL^{-1}) maintained RMLMC contractility in the presence of IL-1β or IL-1β + TNF-α at day 4. n = 4, ***P < 0.001 versus control, ***P < 0.01 IL-1β (20 ng·mL^{-1}) versus IL-1β (20 ng·mL^{-1}) + TNF-α (20 ng·mL^{-1}). One-way ANOVA, with Bonferroni's *post hoc* test; data are mean ± SEM.

Figure S4 Equation for gel contraction. *exp*. represents the values of the treated gels, whereas *con*. represents the values for the untreated internal control gels. t0 = day 0, t4 = day 4. As we observed slightly different contractile response within our control groups, an own control for each single experimental treatment was set. These individual controls were set as 100% and the results of parallel conducted treatment studies were normalized to this internal control. When, for example, the control group produced a 70% contraction and a treatment a 35% contraction, then the data are presented as 100% contraction for control and 50% contraction for the treatment group.