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Novel Noncompetitive IL-1 Receptor–Biased Ligand Prevents Infection- and Inflammation-Induced Preterm Birth

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Preterm birth (PTB) is firmly linked to inflammation regardless of the presence of infection. Proinflammatory cytokines, including IL-1β, are produced in gestational tissues and can locally upregulate uterine activation proteins. Premature activation of the uterus by inflammation may lead to PTB, and IL-1 has been identified as a key inducer of this condition. However, all currently available IL-1 inhibitors are large molecules that exhibit competitive antagonism properties by inhibiting all IL-1R signaling, including transcription factor NF-κB, which conveys important physiological roles. We hereby demonstrate the efficacy of a small noncompetitive (all-d peptide) IL-1R–biased ligand, termed rytvela (labeled 101.10) in delaying IL-1β–, TLR2-, and TLR4-induced PTB in mice. The 101.10 acts without significant inhibition of NF-κB, and instead selectively inhibits IL-1R downstream stress-associated protein kinases/transcription factor c-jun and Rho GTPase/Rho-associated coiled-coil–containing protein kinase signaling pathways. The 101.10 is effective at decreasing proinflammatory and/or prolabor genes in myometrium tissue and circulating leukocytes in all PTB models independently of NF-κB, undermining NF-κB role in preterm labor. In this work, biased signaling modulation of IL-1R by 101.10 uncovers a novel strategy to prevent PTB without inhibiting NF-κB. ***The Journal of Immunology*****, 2015, 195: 3402–3415.**

Preterm birth (PTB; delivery before 37 wk of gestation, also referred to as prematurity) affects >1 of 10 infants worldwide, and is the leading cause of infant death in the United States and globally (1, 2). The onset of labor is a gradual process that begins several weeks before delivery and is characterized by changes in myometrium contractility and in cervical composition. Many causes have been suggested to explain preterm labor; in this context inflammation has been firmly linked to PTB (3–6).

Of various inflammatory cytokines implicated in PTB, IL-1 in particular has been identified as a key inducer of inflammation in PTB by binding to its ubiquitously expressed receptor IL-1RI, thus promoting activation and amplification of the inflammatory cascade. The major role of IL-1 in the onset of preterm labor is substantiated by the following evidence: 1) IL-1 alone is sufficient to induce labor in several animal models, and inhibition of its receptor prevents labor induction (7–9); 2) elevated IL-1β blood concentrations in humans is associated with PTB (10); 3) polymorphisms in human IL-1β gene (*IL1B*) and endogenous IL-1R antagonist gene (*IL1RN*) are associated with spontaneous preterm deliveries (11); and 4) IL-1β stimulates uterine activation protein (UAP) expression (12), and this effect is markedly amplified in the presence of $PGF_{2\alpha}$ in human myometrial cells (13).

Currently available tocolytics are at best only modestly effective compared with placebo; additionally, some of them present undesired maternal and/or fetal side effects (14, 15). Despite scientific evidence pointing to a major role for IL-1 in labor, preclinical studies using

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Abbreviations used in this article: G, gestational day; IKK, inhibitor of NF-κB kinase; LTA, lipoteichoic acid; PTB, preterm birth; ROCK, Rho GTPase/Rho-associated coiled-coil–containing protein kinase; SAPK, stress-associated protein kinase; SMC, smooth muscle cell; UAP, uterine activation protein.

IL-1–targeting agents reveal modest efficacy (16–18). At present there are three large molecule anti–IL-1 drugs approved for clinical use, as follows: the IL-1R antagonist Anakinra (Kineret), the soluble decoy receptor Rilonacept (Arcalyst), and the neutralizing anti–IL-1β mAb Canakinumab (Ilaris). As anticipated, these IL-1–targeting therapies inhibit all IL-1–signaling pathways, including NF-κB (19, 20). However, NF-κB, a major transcription factor for proinflammatory cytokines including IL-1, conveys important physiological roles such as cytoprotection and immune surveillance, particularly relevant in the vulnerable fetus. A recent study has reported deleterious (proapoptotic) effects of inhibiting NF-κB in pregnancy (21); accordingly, it has been suggested that complete blockade of NF-κB action would be undesirable (22).

Over the past few years, a new class of pharmacological agents termed allosteric modulators has been described. Allosteric compounds show functional selectivity by differently modulating signaling pathways induced by the binding of a natural ligand on a receptor, inhibiting some signals and/or preserving or enhancing others. Functional selectivity is a desirable approach in developing IL-1–targeting therapies in pregnancy because it does not inhibit all receptor-coupled response, contrary to that seen with orthosteric antagonists (23). Hence, functional selectivity could potentially minimize NF-κB inhibition and still inhibit other relevant IL-1 signaling. The host laboratory recently developed a small stable (all-d peptide) biased ligand modulator of IL-1R, specifically rytvela (labeled 101.10), which selectively binds to IL-1R and displays noncompetitive properties and functional selectivity toward specific pathways (24). The peptide rytvela has also been shown to be effective in numerous models of inflammation-linked diseases, including inflammatory bowel disease, contact dermatitis, hypoxic–ischemic newborn brain injuries, and ischemic retinopathies (24, 25). We hereby propose a hitherto unexplored strategy of delaying infection- and inflammation-induced PTB using 101.10, which selectively inhibits IL-1R downstream stress-associated protein kinase (SAPK)/c-jun and Rho/Rho GTPase/Rho-associated coiled-coil–containing protein kinase (ROCK) pathways without significantly affecting NF-κB activation.

Materials and Methods

Animals

Timed-pregnant CD-1 mice were obtained from Charles River at gestational day 12 and were allowed to acclimatize for 4 d prior to experiments. Animals were used according to a protocol of the Animal Care Committee of Hôpital Sainte-Justine along the principles of the *Guide for the Care and Use of Experimental Animals* of the Canadian Council on Animal Care. The animals were maintained on standard laboratory chow under a 12:12 light:dark cycle and allowed free access to chow and water.

Chemicals

Chemicals were purchased from the following manufacturers: human rIL-1β (200-01B; PeproTech), lipoteichoic acid (LTA; L3265; Sigma-Aldrich), LPS (L2630; Sigma-Aldrich), murine M-CSF (315-02; PeproTech), 101.10 (Elim Biopharmaceuticals, Hayward, CA), Kineret (Sobi, Biovitrum Stockholm, Sweden), SC-514 (10010267; Cayman Chemical), SR-11302 (2476; Tocris Bioscience), Y27632 (Y0503; Sigma-Aldrich), β-estradiol (2758; Sigma-Aldrich), and human rIL-1α (200-01A; PeproTech).

Cell culture

The myometrial smooth muscle cell (SMC) line (hTERT-C3) was provided by S. Laporte (University of McGill, Montréal, Canada). The RAW-Blue mouse macrophage reporter cell line and the HEK-blue IL-33/IL-1β cells were purchased from InvivoGen (San Diego, CA) and used at passages under 15. RAW-Blue mouse macrophages and HEK-BLUE cells were cultured in DMEM growth medium supplemented with 10% serum, 50 U/ml penicillin, 50 mg/ml streptomycin, and 200 μg/ml zeocin. Myometrial cells were cultured in DMEM/F12 growth medium supplemented with 10% FBS, 50 U/ml penicillin, 50 mg/ml streptomycin, and 0.1 mg/ml gentamicin. Cells were propagated in regular conditions (37˚C, 5% CO_2). For in vitro experiments, cells were serum starved overnight and treated with 1 μg/ml IL-1β, LPS, or LTA for 15 min. The 101.10, Kineret (1.5 mg/ml), SC-514 (10 μM), or Y27632 (1 μM) was allowed to reach equilibrium for 30 min prior to the experiments. Cell lysis was performed in ice-cold radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors. Samples were stored in Laemmli buffer at −20˚C or used fresh for Western blotting.

Intrauterine IL-1β–induced PTB model and i.p. LPS- and LTA-induced PTB models

Timed-pregnant CD-1 mice at 16.5 d of gestation were anesthetized with isoflurane and received an i.p. injection of either LTA (3 × 3-h interval injections of 12.5 mg/kg in 100 μL saline), LPS (a single dose of 0.5 μg in 100 μL saline), or a single intrauterine injection of IL-1β (1 μg). Doses of IL-1β, LPS, and LTA and frequencies of administration used were selected on the basis of reported documentation (8, 16, 26, 27) and on in vivo dose-response experiments we performed that would induce PTB in a reproducible manner. For the IL-1β–induced PTB model, animals were steadily anesthetized with an isoflurane mask. After body hair removal from the peritoneal area, a 1.5-cm–tall median incision was performed with chirurgical scissors in the lower abdominal wall. The lower segment of the right uterine horn was then exposed, and 1 μg IL-1β was injected between two fetal membranes with care of not entering the amniotic cavity. The abdominal muscle layer was sutured, and the skin was closed with clips. A total of 100 μL 101.10 (1 mg/Kg/12 h), Kineret (4 mg/Kg/12 h), SR-11302 (1 mg/Kg/12 h), Y27632 (0.5 mg/Kg/12 h), or vehicle was injected s.c. in the neck 30 min before stimulation with IL-1β, LPS, or LTA (to allow distribution of drugs to target tissues, in line with a first efficacy preclinical study); all doses used were based on reported efficacy (17, 24, 25, 28, 29). Mice delivery was assessed every hour until term (G19–G19.5). Immediately after delivery (<30 min postpartum), female adults were anesthetized and an intracardiac puncture was performed to collect systemic blood in heparin to prevent blood clotting. Blood plasma was isolated by centrifugation and immediately snap frozen in liquid nitrogen. The remaining blood cell pellet was treated with RBC lysis buffer (Norgen Biotek) and EDTA, according to the manufacturer protocol, and then centrifuged to isolate WBCs. Resulting WBC pellet in addition to myometrium fragments cut from the lower part of the right uterine horn was snap frozen in liquid nitrogen and kept at −80˚C for subsequent RNA purification or protein extraction.

RNA extraction and real-time quantitative PCR

Myometrium fragments were thawed and rapidly preserved in RIBOzol (AMRESCO), whereas cells from in vitro experiments were treated for 6 h with IL-1β with or without 101.10 or Kineret and collected directly into RIBOzol. RNA was extracted according to manufacturer's protocol, and RNA concentration and integrity were measured with a NanoDrop 1000 spectrophotometer. A total of 500 ng RNA was used to synthetize cDNA using iScript Reverse Transcription SuperMix (Bio-Rad, Hercules, CA). Primers were designed using National Center for Biotechnology Information Primer Blast (Table I). Quantitative gene expression analysis was performed on Stratagene MXPro3000 (Stratagene) with SYBR Green Master Mix (Bio-Rad). Gene expression levels were normalized to 18S universal primer (Ambion Life Technology, Burlington ON, Canada). Dissociation curves were also acquired to test primer specificity, and amplicon length was verified by electrophoresis of product on a 2% agarose gel (data not shown). Genes analyzed include the following: *IL1B*, *IL4*, *IL6*, *IL8*, *IL10*, *TNFA*, *CCL2* (chemokine ligand 2), *CRP* (C-reactive protein), *MMP1A*, *MMP3*, *MMP9*, *PTGHS2* (PG H synthetase 2 or cyclooxygenase-2), *PTGFR* (PG F receptor), *OXTR* (oxytocin receptor), *IL1R1* (IL-1R1), *GJA1* (connexin 43), *IL1RA* (IL-1R endogenous antagonist), and *IFNB1* (IFN β1). Detailed primer sequences are shown in Table I.

Semiquantitative PCR

Cells were pretreated with 10^{-6} M 101.10 or vehicle for 30 min and then stimulated with 50 ng/ml IL-1α for 24 h. Total RNA was isolated with RNeasy mini kit (Qiagen, Germantown MD). RT-PCR was performed, as described previously (30). QuantumRNA universal 18S standard primers (Ambion) were used as internal standard references.

Western blotting

Proteins from homogenized myometrium fragments and cell samples lysed in radioimmunoprecipitation assay buffer (containing protease and phosphatase inhibitors) were quantified using Bradford's method (Bio-Rad). A total of 50 μg protein sample was loaded onto SDS-PAGE gel and electrotransferred onto polyvinylidene difluoride membranes. After blocking, membranes were incubated with either an Ab against IL-1R1 (sc-689; Santa Cruz Biotechnology, Dallas, TX), OxtR (ab101617; Abcam, Toronto, ON, Canada), α-actin

Table I. Primers used for real-time quantitative PCR

Mouse Primers	
IL1B-F: 5′-AGATGAAGGGCTGCTTCCAAA-3′	IL1B-R: 5′-GGAAGGTCCACGGGAAAGAC-3′
IL4-F: 5′-AACGAAGAACACCACAGAGAG-3′	IL4-R: 5′-GTGATGTGGACTTGGACTCA-3′
IL6-F: 5′-CAACGATGATGCACTTGCAGA-3′	IL6-R: 5′-TCTCTCTGAAGGACTCTGGCT-3′
IL8-F: 5′-TGCTTTTGGCTTTGCGTTGA-3′	IL8-R: 5′-GTCAGAACGTGGCGGTATCT-3′
IL10-F: 5′-TAACTGCACCCACTTCCCAG-3′	IL10-R: 5′-AGGCTTGGCAACCCAAGTAA-3′
TNFA-F: 5′-GCCTCTTCTCATTCCTGCTTG-3′	TNFA-R: 5′-CTGATGAGAGGGAGGCCATT-3′
CRP-F: 5′-TCTGCACAAGGGCTACACTG-3′	CRP-R: 5′-ATCTCCGATGTCTCCCACCA-3′
IFNB1-F: 5′-AGCACTGGGTGGAATGAGAC-3′	IFNB1-R: 5′-GAGTCCGCCTCTGATGCTTA-3′
MMP1A-F: 5′-CAGGACTTATATGGACCTTCCC-3′	MMP1A-R: 5′-TAAATTGAGCTCAGGTTCTGGC-3′
MMP3-F: 5′-GTGACCCCACTCACTTTCTC-3′	MMP3-R: 5′-TTGGTACCAGTGACATCCTCT-3′
MMP9-F: 5′-TCAAGGACGGTTGGTACTGG-3′	MMP9-R: 5′-CTGACGTGGGTTACCTCTGG-3′
OXTR-F: 5′-TGTGTCTCCTTTTGGGACAA-3′	OXTR-R: 5′-GGCATTTCAGAATTGGCTGT-3′
PGHS2-F: 5′-ACCTCTCCACCAATGACCTGA-3′	PGHS2-R: 5′-CTGACCCCCAAGGCTCAAAT-3′
PTGFR-F: 5′-AGCTGGACTCATCGCAAACA-3′	PTGFR-R: 5′-GTGGGCACAAGCCAGAAAAG-3′
GJA1-F: 5′-GCACTTTTCTTTCATTGGGGG-3′	GJA1-R: 5′-GGGCACCTCTCTTTCACTTA-3′
IL1R1-F: 5′-CTTGAGGAGGCAGTTTTCGT-3′	IL1R1-R: 5′-ACATACGTCAATCTCCAGCG-3′
IL1RA-F: 5′-TGGGAAGGTCTGTGCCATA-3′	IL1RA-R: 5′-CCAGATTCTGAAGGCTTGCAT-3′
CCL2-F: 5′-GCTCAGCCAGATGCAGTTA-3′	CCL2-R: 5′-TGTCTGGACCCATTCCTTCT-3′
Human Primers	
IL1B-F: 5′-AGCTGGAGAGTGTAGATCCCAA-3′	IL1B-R: 5′-ACGGGCATGTTTTCTGCTTG-3′
IL6-F: 5′-TTCAATGAGGAGACTTGCCTGG-3′	IL6-R: 5′-CTGGCATTTGTGGTTGGGTC-3′
IL8-F: 5′-CTCTGTGTGAAGGTGCAGTTTT-3′	IL8-R: 5′-TGCACCCAGTTTTCCTTGGG-3′
MMP1-F: 5′-AGAATGATGGGAGGCAAGTTGA-3′	MMP1-R: 5′-TGGCGTGTAATTTTCAATCCTGT-3′
MMP3-F: 5′-TGCTGTTTTTGAAGAATTTGGGTT-3′	MMP3-R: 5′-AGTTCCCTTGAGTGTGACTCG-3′
CCL2-F: 5′-CAGCCAGATGCAATCAATGCC-3′	CCL2-R: 5′-TTTGCTTGTCCAGGTGGTCC-3′
PGHS2-F: 5′-ATATTGGTGACCCGTGGAGC-3′	PGHS2-R: 5′-GTTCTCCGTACCTTCACCCC-3′

(ab5694; Abcam), F4/80 (ab6640; Abcam), Lamin B1 (ab16048; Abcam), NF-κB p65 (sc-372; Santa Cruz Biotechnology), IL-1R accessory protein (ab8110; Abcam), or β-actin (sc-47778; Santa Cruz Biotechnology). After washing, membranes were incubated for 1 h with their respective secondary Abs conjugated to HRP (Sigma-Aldrich). For kinases, membranes were incubated with an Ab against either phospho-JNK (9251; Cell Signaling Technology, Whitby, ON, Canada), phospho–c-jun (9261; Cell Signaling Technology), phospho-p38 (4511; Cell Signaling Technology), phospho-ROCK2 (PA5-34895; Thermo Fisher Scientific), phospho-IκBα (2859; Cell Signaling Technology), JNK (9252; Cell Signaling Technology), c-jun (9165; Cell Signaling Technology), p38 (9212; Cell Signaling Technology), or ROCK2 (PA5-21131; Thermo Fisher Scientific, Waltham, MA). ECL (GE Healthcare, Little Chalfont, U.K.) was used for detection using the ImageQuant LAS-500 (GE Healthcare), and densitometric analysis was performed using ImageJ. Resulting values were normalized first with total proteins and then with the control sample.

Rhotekin-rho binding domain bead pull-down assay

Rho activation was assessed using a Rho Activation Assay Biochem Kit (Cytoskeleton). hTERT-C3 cells were plated in 150-mm petri dishes and serum starved at ∼50% confluence for 16 h prior to the experiment. The 101.10 or Kineret was administered 30 min before the IL-1β stimulation to allow the system to equilibrate. After 15 min of IL-1β stimulation, cells were rapidly lysed with ice-cold lysis buffer, and cell debris were removed by centrifugation at 4°C. A small amount of every sample was collected on ice for protein quantitation using Bradford's method, and the remaining cell lysate was snap frozen in liquid nitrogen and conserved at −80°C for ∼1 h during protein quantitation. After thawing, 800 μg of each sample was incubated on a rocking platform with 50 μg rhotekin-rho binding domain beads (high affinity for GTP-bound RhoA) for 1 h at 4°C. As a positive control, 800 μg cell lysate was incubated for 15 min with 200 μM GTPγS (a nonhydrolyzable GTP analog) prior to the bead pull down. After washing steps, samples were centrifuged and bead lysates were loaded on SDS-PAGE gel in 2× Laemmli buffer. Samples were electrotransferred on polyvinylidene difluoride membranes, blocked, and incubated with an anti-RhoA mAb (ARH03; Cytoskeleton) overnight at 4°C. The membrane was then incubated with a HRP-conjugated anti-mouse secondary Ab (Sigma-Aldrich) and revealed using an ECL solution (GE Healthcare). Total RhoA expression and β-actin were assessed using 50 μg of the samples that were set aside on ice before pull down. Densitometric analysis was performed using ImageJ.

Circulating leukocyte RNA purification

As described before, WBCs were isolated from systemic blood of female mice (<30 min postpartum) and total leukocyte RNA was extracted using a leukocyte RNA purification kit (Norgen Biotek, Thorold, ON, Canada). Briefly, the WBC pellet was lysed and passed through a RNA-binding column. After several washing procedures, the RNA was eluted from the column and equal amount of RNA was used to synthetize cDNA using iScript Reverse Transcription SuperMix (Bio-Rad). Quantitative RT-PCR was then performed on the samples, as previously described.

NF-κB QUANTI-Blue assay

Hek-Blue cells (InvivoGen) were pretreated with different concentrations of 101.10 (10^{-9}–10^{-5} M) and Kineret (1.5 mg/ml) for 30 min, followed by treatment with constant concentration of IL-1β (1 μg/ml), and then incubated at 37°C for 4 h. Levels of secreted alkaline phosphatase in cell culture supernatant were determined by the use of QUANTI-Blue, according to manufacturer instruction (InvivoGen). Alkaline phosphatase activity was assessed by reading the OD at 620–655 nm with a micro plate reader (EnVision Multilabel reader; PerkinElmer, Waltham, MA). Data are representative of five experiments (each with $n = 6$).

Ex vivo uterine contraction experiment

Timed-pregnant CD-1 mice at G18.5 were given a single dose of either saline or 101.10 (1 mg/kg in 100 μL saline). Within 30 min, mice were injected i.p. with IL-1β (1 μg/mouse). Seventeen hours after, uterine tissues were collected under anesthesia (2.5% isoflurane). Briefly, a midline abdominal incision was made, and the uterine horns were rapidly excised and carefully cleansed of surrounding connective tissues. Longitudinal myometrial strips (2–3 mm wide and 10 mm long) were dissected free from uterus and mounted isometrically in organ tissue baths, and initial tension was set at 2 g. The tissue baths contain 20 ml Krebs buffer of the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 0.9 $MgSO_4$, 1 KH_2PO_4, 11.1 glucose, and 23 $NaHCO_3$ (pH 7.4). The buffer was equilibrated with 95% oxygen/5% carbon dioxide at 37°C. Isometric tension was measured by a force transducer and recorded by BIOPAC data acquisition system (BIOPAC MP150). Experiments began after 1-h equilibration. Mean tension of spontaneous contractions was measured using a BIOPAC digital polygraph system (AcqKnowledge); the same parameters were also determined after addition of $PGF_{2\alpha}$. At the start of each experiments, mean tension of spontaneous myometrial contractions was considered as a reference response. Increase in mean tension (%) was expressed as percentages of (X/Y) − 100, where X is changes in mean tension (g) induced by $PGF_{2\alpha}$ and Y is the initial reference response (g).

Primary myometrial SMC isolation and culture

Primary myometrial SMC were isolated using modifications of a method previously described (31). Briefly, a single s.c. injection of 50 μg 17β-estradiol was administered to mice 24 h prior to the experiment. The day

after, mice were sacrificed by cervical dislocation and sprayed with 70% ethanol. The whole uterus was excised under sterile hood and placed in buffer A (HBSS [pH 7.4], 0.098 g/L magnesium sulfate, 0.185 g/L calcium chloride, 2.25 mmol/L I-HEPES, 100 U/ml penicillin-streptomycin [Life Technologies, Grand Island, NY], and 2.5 μg/ml amphotericin B [Sigma-Aldrich]). The uterine horns were cleansed of fat and vessels and then transferred into buffer B (buffer A without magnesium sulfate or calcium chloride) for several washes by gentle flushing. Afterward, the uterine horns were cut into 1-mm–wide fragments and transferred into a volume of 10 ml/g tissue of digestion buffer (1 mg/ml collagenase type II [Sigma-Aldrich], 0.15 mg/ml DNase I [Roche Diagnostics, Mannheim, Germany], 0.1 mg/ml soybean trypsin inhibitor [Sigma-Aldrich], 10% FBS, and 1 mg/ml BSA [Sigma-Aldrich] in buffer B). Enzymatic digestion was performed at 37˚C with agitation (100 rev/min) for 30 min. The homogenate (still containing undigested myometrium fragments) was then poured through a 100-μm cell strainer. The resulting filtered solution was centrifugated at 200 × *g* for 10 min, and the pellet was resuspended in complete DMEM and plated in a T-25 dish. The remaining myometrium fragments were reused in an enzymatic digestion, and the whole digestion–centrifugation process was repeated for a total of five times. First two digestion results were discarded because they contained mostly fibroblasts. The three other SMC-containing dishes were subjected to a differential adhesion technique to selectively enrich for uterine myocytes. Briefly, 30–45 min after the cells were first plated, the medium was removed and dispensed in another T-25 culture dish to separate quickly adhering fibroblast from slowly adhering myocytes. Cells were further analyzed in immunohistochemistry to assess culture purity with the SMC marker α-actin.

Primary bone marrow–derived macrophage isolation and culture

CD-1 mice were sacrificed with cervical dislocation and then sprayed with 70% ethanol. Both femurs and tibias were prelevated under sterile hood by gently removing the muscles and then cutting the epyphyses. Bone marrow was extruded by flushing it with a 25-gauge syringe containing sterile RPMI 1640 culture medium supplemented with 10% FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin. Resulting medium containing the bone marrow–derived progenitor cells was then homogenized, filtered through a 70-μm nylon web, and seeded in T-25 plates. A total of 20 ng/ml rM-CSF was added prior to incubation, and cells were allowed to differentiate for 6 d. Cells were further analyzed in immunohistochemistry to assess culture purity with the macrophage marker F4/80.

Murine IL-1β ELISA

The ELISA was performed using a mouse IL-1β Quantikine ELISA kit (R&D Systems), according to the manufacturer's protocol. Briefly, 50 μL either plasma samples, mouse rIL-1β positive control, or decreasing concentrations of a mouse rIL-1β standard were loaded into a 96-well plate precoated with an anti-mouse IL-1β mAb and incubated for 2 h at ambient temperature. Wells were washed five times and incubated with an enzyme-linked mouse polyclonal Ab specific to murine IL-1β for 2 h. After another washing step, a substrate solution was added. The enzymatic reaction was stopped after 30 min, and the plate was read at 450 nm, with wavelength correction set to 570 nm.

Immunohistochemistry

Cells were plated on coverslips precoated with poly-D-lysine and fixed in 4% paraformaldehyde. After blocking, cells were incubated overnight with 101.10-FITC or FITC alone (Sigma-Aldrich) and a primary Ab of rabbit anti–IL-1RI, rabbit anti–α-actin, or rat anti-F4/80, and then for 1 h at ambient temperature with a secondary Ab conjugated with Alexa Fluor 594 (red) or 647 (white) (Sigma-Aldrich). For tissue immunohistochemistry, mice were treated with a single s.c. 1 mg/Kg 101.10-FITC injection and animals were euthanized after 1 h of incubation. Uterine tissues were cleansed of fat and vessels. Myometrium fragments and placentas were fixed in 4% paraformaldehyde for 1 d and transferred in 30% sucrose for another day. Localization of 101.10 was determined on 14-μm uterine sagittal cryosections or longitudinal placenta cryosections. Sections blocked with 1% BSA, 1% goat serum, and 0.1% Triton X-100 (T-8787; Sigma-Aldrich) in PBS were subsequently incubated overnight with the primary Abs. Secondary Abs conjugated with Alexa Fluor (Molecular Probes) directed against rabbit or rat were incubated for 2 h at ambient temperature. Nuclei were stained with DAPI (Invitrogen; 1/5000). Images were captured using 10× (for myometrium tissues) or 30× (for cells and magnified placenta images) objective with Eclipse E800 (Nikon) fluorescence microscope. Whole placenta images were captured at 10× using a Zeiss AxioObserver.Z1 (Zeiss, San Diego, CA). Images were merged into a single file using the MosiaX option in the AxioVision software version 4.6.5 (Zeiss).

Statistical analysis

Groups were compared using one-way ANOVA. Dunnett's multiple comparison method was employed when treatments were compared with a single control. Tukey's multiple comparison test was used in Fig. 1G. A p value <0.05 was considered statistically significant. Data are presented as means ± SD.

Results

The 101.10 prevents IL-1β–induced preterm birth and associated inflammatory-triggered uterine activation

We first determined whether 101.10 was effective at delaying PTB induced specifically by intrauterine IL-1β. A total of 1 μg IL-1β was injected in the right uterine horn of pregnant mice at G16.5 to induce PTB; births between G16.5 and G18.5 were considered premature because normal term for CD-1 mice is G19.2 based on data of our group (32) (Fig. 1A). Twenty-four hours after the intrauterine injection, mice uterine horns were inspected to confirm the presence of macroscopic inflammation (edema, hemorrhage). Notably, IL-1β–treated mice exhibited frankly observable inflammation of uteri (Fig. 1B, *middle panel*) in comparison with sham animals (Fig. 1B, *left panel*); this inflammation was alleviated by 101.10 (1 mg/Kg/12-h s.c. injections; Fig. 1B, *right panel*). Accordingly, IL-1β–treated mice receiving vehicle ($n = 16$) rapidly went into premature labor, with 56% delivering within 24 h after IL-1β administration, whereas only 12% of IL-1β–treated mice receiving 101.10 (n = 17) delivered before G19 (Fig. 1C, *bottom panel*). In contrast, systemic (s.c.) administration of the competitive IL-1 inhibitor Kineret ($n = 11$) was ineffective at reducing prematurity (Fig. 1C, *top panel*) and increasing gestational duration (Supplemental Fig. 1A). A group simply treated with 101.10 (without IL-1) served for gross toxicity evaluation; there were no gross teratogenic changes detected in all major organs examined.

Analysis of myometrium samples collected within 30 min of pup delivery revealed that 101.10 diminished IL-1β–triggered induction of mRNA of numerous proinflammatory and/or prolabor genes (see Table I for primer sequences), including many UAP genes (such as *CCL2*, *OXTR*, *PTGFR*, *MMP9*, *GJA1*, and *PTGS2*; see Fig. 1D); 101.10 also decreased IL-1β–induced (protein) expression of IL-1R (Supplemental Fig. 2A) and oxytocin receptor (Supplemental Fig. 2B), but not of the IL-1R accessory protein (Supplemental Fig. 2C). Two genes of relevance to myometrial activation drew our attention, *OXTR* (Fig. 1F, *left panel*) and *PTGFR* (Fig. 1F, *right panel*), which respectively encode for oxytocin receptor and PG $F_{2\alpha}$ receptor; both were significantly suppressed by 101.10 in the myometrium of IL-1β–treated mice. Concordantly, 101.10 ($n = 4$) attenuated contractile tension in response to oxytocin (Fig. 1G, *left panel*) and $PGF_{2\alpha}$ (Fig. 1G, *right panel*) in myometrium of IL-1β–treated mice compared with controls ($n = 6$).

Consistent with its functional inefficacy, Kineret was ineffective in altering IL-1β–induced myometrial gene expression (Fig. 1D, 1F); similar results were observed on gene expression in placenta (Supplemental Fig. 3A). In contrast, gene expression profile of circulating leukocytes collected <30 min postpartum revealed comparable inhibition of intrauterine IL-1β–induced genes with 101.10 and Kineret (Fig. 1E). These observations support the concept that activated leukocytes responding to an inflammatory locus (uteroplacental unit in this case) are a significant source of IL-1, which in turn amplifies the inflammatory response (25, 33); accordingly, the systemically administered large molecule Kineret (17.5 kDa) is effective on blood leukocytes, but contrary to 101.10 seems to have limited access to intrauterine/placental IL-1R wherein inflammation is triggered (by IL-1), consistent with documentation on IL-1 (~17.5 kDa) (34, 35).

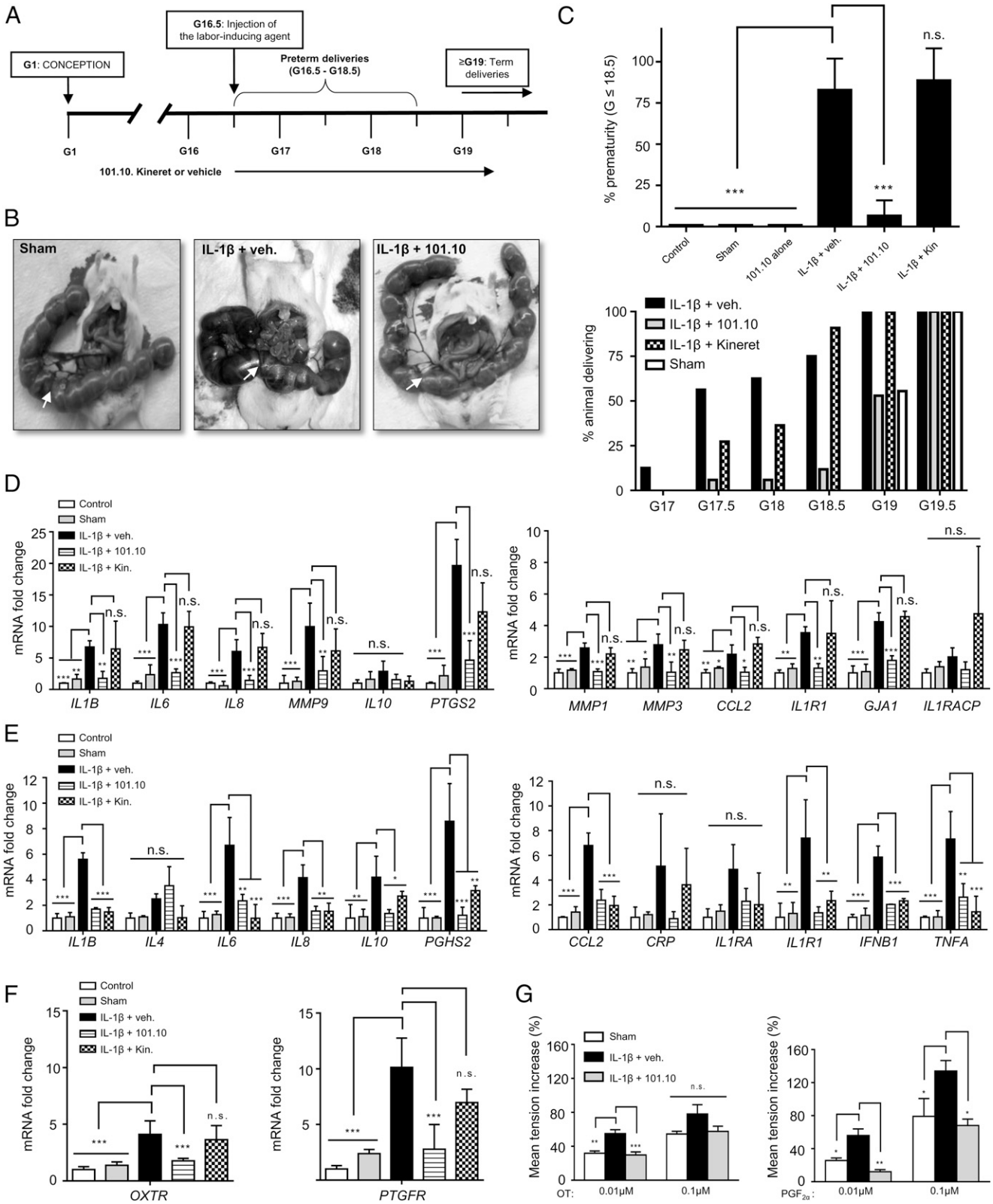

FIGURE 1. The 101.10 prevents IL-1β–induced preterm birth and curbs inflammation-induced uterine activation. (**A**) The labor-inducing agent is injected at G16.5, and spontaneous deliveries happening between G16.5 and G18.5 are considered as premature. Subcutaneous injections of 101.10 (1 mg/Kg/12 h), Kineret (4 mg/Kg/12 h), or vehicle are given twice per day until delivery. (**B**) Representative picture of uteri 24 h after the intrauterine IL-1β injection. *Left panel*, Sham; *middle panel*, IL-1β–induced uterine inflammation; *right panel*, 101.10 decreases clinical signs of IL-1β–induced uterine inflammation. (**C**) The 101.10 prevents IL-1β–induced preterm birth in mice. *Top panel*, Percentage of prematurity (≤G18.5) following 1 μg intrauterine IL-1β injection; *bottom panel*, percentage of animals having delivered plotted against gestational age. Control mice did not receive any treatment, whereas sham animals received an intrauterine dose of vehicle at G16.5. (**D**) Quantitative PCR from myometrium tissue of mice treated in (C), collected postpartum (<30 min following parturition). Results are normalized with 18S and are relative to control. (**E**) Quantitative PCR from leukocytes isolated from systemic blood of mice treated in (C) and collected postpartum (<30 min). Results are normalized with 18S and are relative to control. (**F**) The 101.10 decreases the (*Figure legend continues*)

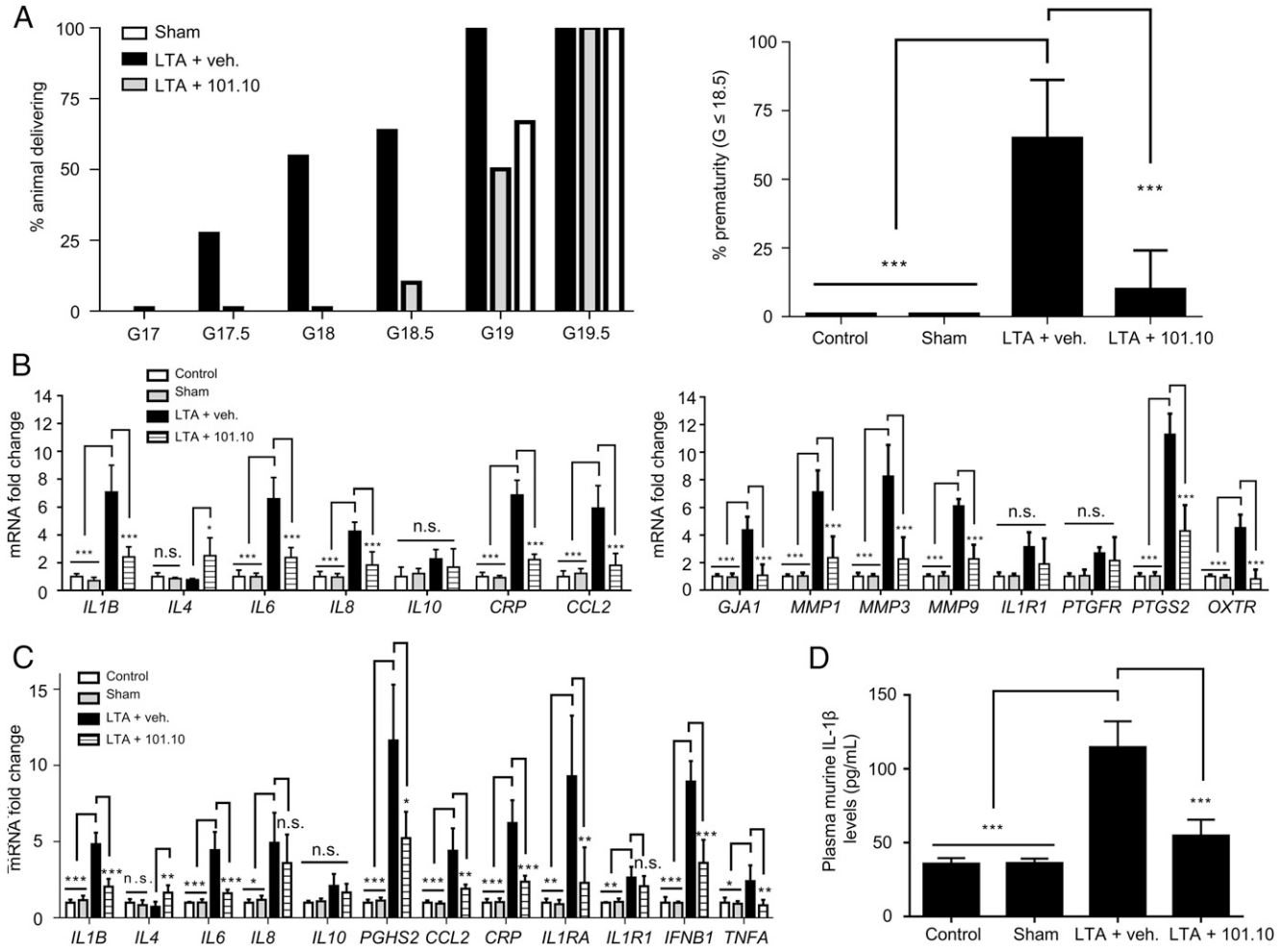

FIGURE 2. The 101.10 decreases TLR2-induced preterm birth. (**A**) The 101.10 decreases preterm birth induced by the TLR2 agonist LTA in mice. *Left panel*, Percentage of animals having delivered following three 3-h interval i.p. LTA injections (12.5 mg/Kg); *right panel*, percentage of premature deliveries. Control mice did not receive any treatment, whereas sham animals received three i.p. doses of vehicle over a period of 9 h at G16.5. The 101.10 (1 mg/Kg/12 h) or vehicle was injected s.c. twice per day until delivery. (**B**) Quantitative PCR from myometrium tissue of mice treated in (A) and collected postpartum (<30 min). Results are normalized with 18S and are relative to control. (**C**) Quantitative PCR from leukocytes isolated from systemic blood of mice treated in (A) and collected postpartum (<30 min). Results are normalized with 18S and are relative to control. (**D**) Murine IL-1β ELISA performed on plasma from mice treated in (A) and collected postpartum (<30 min). Values are presented as mean ± SD. Data are representative of 3–11 animals per group. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$ by one-way ANOVA with Tukey's multiple comparison test compared with LTA + vehicle group.

The 101.10 distributes to myometrial SMC, macrophages, and placenta

We next determined whether 101.10 localized in blood leukocytes, myometrium, and, more importantly, placenta. The 101.10 labeled with FITC was injected s.c.; no loss of function was ensued by the labeling, as 101.10-FITC was still efficient at delaying LTA-induced PTB (data not shown). The 101.10-FITC localized on SMC (colocalization with SMC marker α-actin) (Supplemental Fig. 4A), on macrophage (marker F4/80) (Supplemental Fig. 4C), as well as in placenta (Supplemental Fig. 3D); FITC-alone fluorescence was not detected on these cells and tissues, suggesting binding specificity (Supplemental Figs. 3C, 4B, 4D); of note, fluorescence in placentas from FITC-treated mice did not differ from the autofluorescence of unlabeled placentas (Supplemental Fig. 3B).

We previously showed that actions of 101.10 required presence of the ubiquitous IL-1RI (25). Accordingly, in this study again 101.10-FITC colocalized by immunohistochemistry with IL-1RI on the myometrial cell line hTERT-C3 and the macrophage cell line RAW-Blue mouse macrophages (Supplemental Fig. 4E–H); FITC alone did not colocalize with IL-1R1.

The 101.10 delays TLR2- and TLR4-induced preterm birth (by acting downstream of TLR signaling)

The efficacy of 101.10 was also tested in PTB models that mimic relevant $Gram^+$ and $Gram^-$ infections, by stimulating corresponding TLR2 and TLR4, respectively, with LTA and LPS. The 101.10 was particularly effective in (i.p.) LTA-induced PTB (Fig. 2A), as it prolonged gestation (Supplemental Fig. 1B). The 101.10 also nearly normalized LTA-induced expression of all genes screened

expression of oxytocin receptor (*left panel*) and FP receptor (*right panel*) in the myometrium of mice treated in (C). (**G**) Ex vivo myometrium contraction in pharmacological baths performed with uterine tissues from mice treated as indicated. Uterotonic agents oxytocin (*left panel*) and $PGF_{2\alpha}$ (*right panel*) were used to induce dose-dependent contractions of the myometrium. Values are presented as mean ± SD. Data are representative of 3–17 animals per group. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$ by one-way ANOVA with Tukey's multiple comparison test compared with IL-1β + vehicle group.

in myometrium (Fig. 2B) and blood leukocytes (Fig. 2C), with the exception of IL-1RI and the anti-inflammatory IL-4, which was increased; plasma levels of IL-1β were also decreased by 101.10 (Fig. 2D).

The 101.10 also reduced prematurity rate and prolonged gestation shortened by TLR4 stimulation with (i.p.) LPS (Fig. 3A, Supplemental Fig. 1C) and reduced LPS-induced gene induction on myometrium and blood leukocytes (Fig. 3B, 3C), as well as albeit modestly, plasma levels of IL-1β (Fig. 3D).

The 101.10 acts independently of IL-1β–induced NF-κB activation

To better understand how 101.10 regulates IL-1 activity, we determined the effects of 101.10 on IL-1R–coupled intracellular signaling in myometrial and macrophage cell lines hTERT-C3 and RAW-Blue macrophages. NF-κB is often been regarded as a key pathway for IL-1 signaling; the translocation of NF-κB to the nucleus is constitutively inhibited by IκB proteins in the cytosol, which when phosphorylated by inhibitor of NF-κB kinases (IKKs) results in its ubiquitination and subsequent degradation, hence promoting NF-κB activation (36). The 101.10 (dose dependently) did not affect IL-1β–induced IκBα phosphorylation in myometrial cells, whereas Kineret completely inhibited its activation (Fig. 4A, 4B). Likewise, in HEK-Blue cells engineered with a NF-κB–dependent promoter for secretory alkaline phosphatase, 101.10, contrary to Kineret, was again ineffective in altering IL-1β–induced secretion of alkaline phosphatase, and thus was NF-κB independent (Fig. 4C). Moreover, the critically important nuclear translocation of NF-κB upon IL-1 stimulation in myometrial cells was unaffected by 101.10, but was markedly inhibited by Kineret and the IKKβ inhibitor SC-514 (positive control) (Fig. 4D, 4E). Collectively, these data indicate that effects of 101.10 are independent of NF-κB.

The 101.10 inhibits SAPK p38 and JNK, transcription factor c-jun, and Rho/ROCK pathways in myometrial cells and in macrophages

The effect of 101.10 on other IL-1–triggered signaling pathways was investigated. Given their reported involvement in labor (37, 38), we

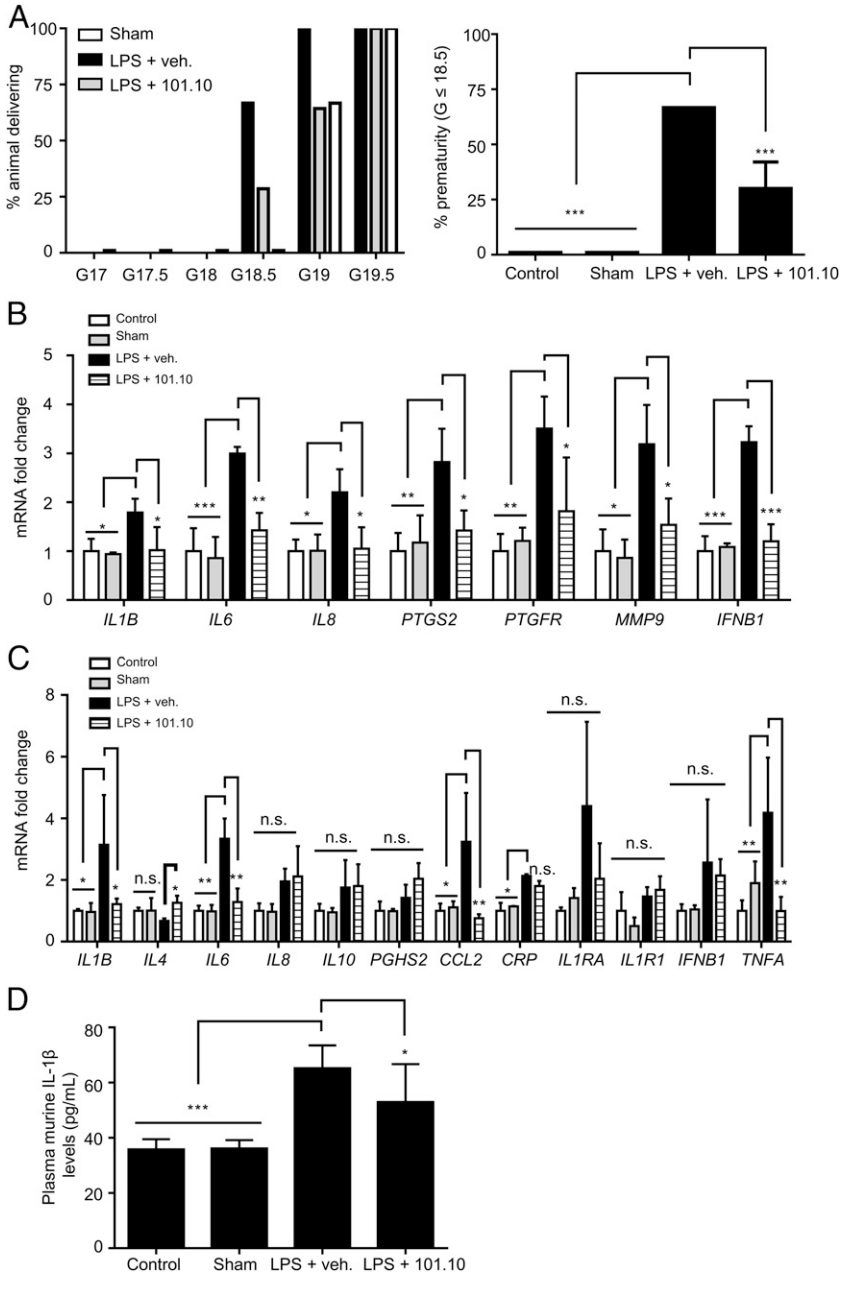

FIGURE 3. The 101.10 decreases TLR4-induced preterm birth. (**A**) The 101.10 decreases preterm birth induced by the TLR4 agonist LPS in mice. *Left panel*, Percentage of animals having delivered following a single i.p. LPS injection (0.5 μg per mice); *right panel*, percentage of premature deliveries. Control mice did not receive any treatment, whereas sham animals received a single i.p. dose of vehicle at G16.5. The 101.10 (1 mg/Kg/12 h) or vehicle was administered s.c. twice per day until delivery. (**B**) Quantitative PCR from myometrium tissue of mice treated in (A) and collected postpartum (<30 min). Results are normalized with 18S and are relative to control. (**C**) Quantitative PCR from circulating leukocytes isolated from systemic blood of mice treated in (A) and collected postpartum (<30 min). Results are normalized with 18S and are relative to control. (**D**) Murine IL-1β ELISA performed on plasma from mice treated in (A) and collected postpartum (<30 min). Values are presented as mean ± SD. Data are representative of 3–14 animals per group and of 4 in vitro experiments. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$ by one-way ANOVA with Tukey's multiple comparison test compared with LPS + vehicle group.

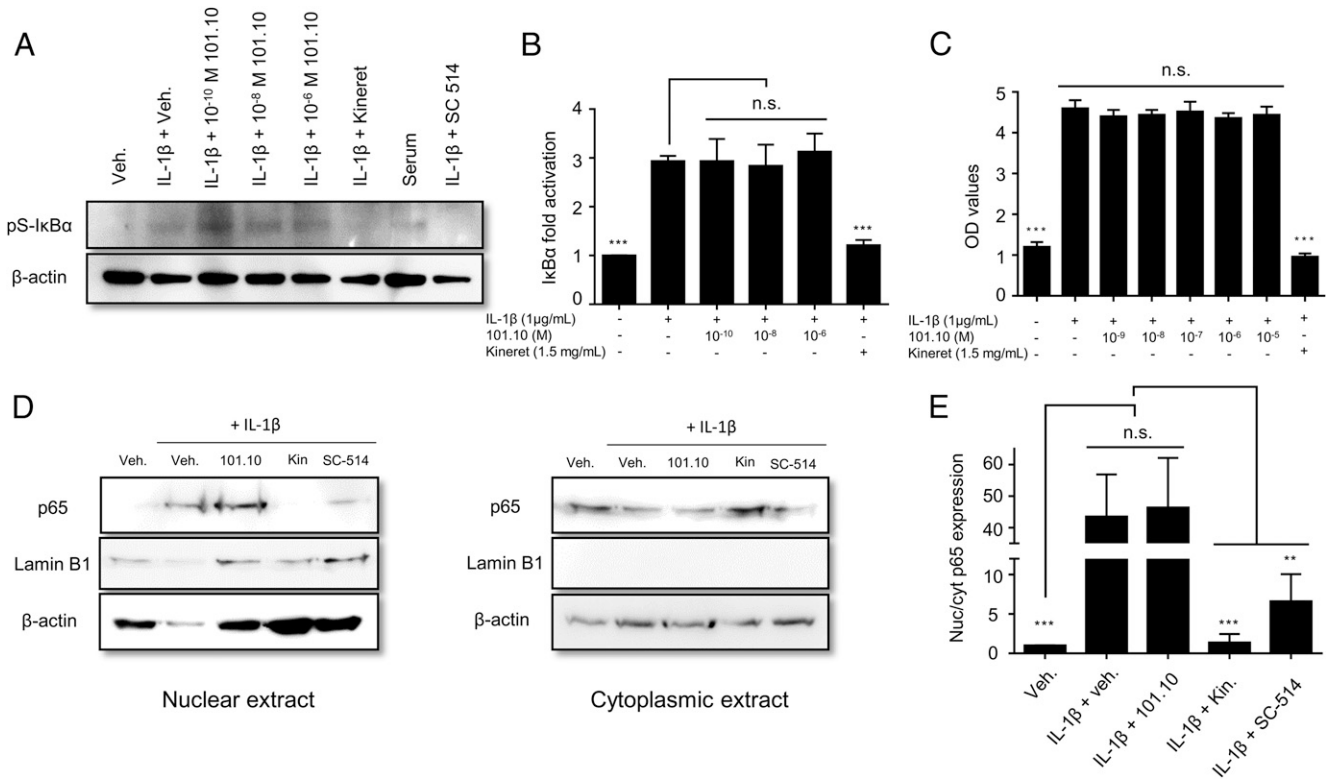

FIGURE 4. The 101.10 has no significant effect on IL-1β–induced NF-κB activation. (**A**) Myometrial SMC (hTERT-C3 cell line) were treated with IL-1β (1 μg/ml) in presence or absence of increasing doses of 101.10 for 1 h, and lysates were run on SDS-PAGE and blotted against pS-IκBα or β-actin. Kineret (1.5 mg/ml) and SC-514 (10 μM) were used as negative controls, and FBS (10%) was used as a metabolic positive control. (**B**) Densitometric analysis of protein bands showing no significant effect of 101.10 on IL-1β–induced NF-κB activity. (**C**) HEK-Blue cells were treated with IL-1β in presence or absence of increasing doses of 101.10 for 4 h, and levels of secreted alkaline phosphatase in cell culture supernatant were assessed by reading the absorbance (OD values) at 620–655 nm. Kineret was used as a negative control. (**D**) hTERT-C3 cells were treated with IL-1β (1 μg/ml) with or without 101.10 (10^{-6} M), and Western blot was performed on extracted nuclei or cytoplasmic lysate and blotted against NF-κB p65 or the nuclear maker lamin B1. Presence of NF-κB p65 in the nucleus was used as a measurement of NF-κB activation. (**E**) p65/β-actin quantification values from nuclear extracts were normalized with those for cytoplasmic extract. Kineret (1.5 mg/ml) and SC-514 (10 μM) were used as negative controls, and β-actin was used as a loading control. Values are presented as mean ± SD. Data are representative of 3–5 experiments. $^{**}p < 0.005$, $^{***}p < 0.001$ by one-way ANOVA with Tukey's multiple comparison test compared with IL-1β + vehicle group.

examined SAPK/c-jun and small GTPase Rho/ROCK pathways, which both lead to the activation of the transcriptional factor AP-1 (Fig. 5A), respectively, using myometrial and macrophage cell lines described above. The 101.10 dose dependently decreased IL-1β–induced phosphorylation of p38, JNK, and the transcription factor c-jun in both cell types (Fig. 5B–E); Kineret was also effective. The 101.10 (like Kineret) also decreased IL-1β–triggered induction of several proinflammatory and/or prolabor genes in vitro (Fig. 5F, 5G), as previously observed in vivo (Figs. 1–3); IL-1β–triggered induction of *PGHS2* was dose dependently inhibited by 101.10 (IC_{50} = 15.1 nM; see Fig. 5H). Additionally, in myometrial cells where RhoA is important in cell function, 101.10 inhibited RhoA activation and decreased downstream ROCK2 phosphorylation (Fig. 5I, 5J).

Based on data obtained in cell lines (Fig. 5), we proceeded to study the effects of 101.10 on IL-1 signaling in primary myometrial SMC. Primary myometrial SMC were obtained by digesting CD-1 mice uterine horns and cultured; immunohistochemical staining with α-actin assessed purity at >95% of cells (Supplemental Fig. 4I, 4J). The 101.10 dose dependently inhibited the activation of p38, JNK, and c-jun (Fig. 6A, 6B) and decreased the induction of several proinflammatory and/or prolabor genes in primary myometrial SMC (Fig. 6C); effects of Kineret were comparable. Moreover, 101.10 inhibited IL-1β–induced p38, JNK, and c-jun activation in myometrial tissue freshly isolated from pregnant mice (Fig. 6D, 6E).

We performed similar experiments on primary bone marrow–derived macrophages; >95% of the cells positively stained for the macrophage marker F4/80 (Supplemental Fig. 4L, 4M). Once again, 101.10 inhibited the activation of p38, JNK, and c-jun in primary bone marrow–derived macrophages (Fig. 6F, 6G) and decreased the induction of several proinflammatory genes triggered by IL-1β (Fig. 6H). Finally, 101.10 (and Kineret) was selective to these signaling pathways induced by IL-1, but not by LTA and LPS, whereupon 101.10 (and Kineret) was ineffective (Supplemental Fig. 1E, 1F).

Inhibiting AP-1 delays inflammation-induced preterm birth

Because our in vitro and ex vivo studies suggest that 101.10 acts by inhibiting IL-1R SAPK/c-jun and Rho/ROCK pathways leading to AP-1 assembly without modulating NF-κB activity, we wanted to validate this mechanism of action in vivo. Therefore, we subjected pregnant mice to intrauterine IL-1β–induced PTB model with a group of mice receiving a selective AP-1 inhibitor, SR-11302 (n = 9) and another group receiving SR-11302 in combination with the ROCK inhibitor Y27632 (n = 7), to mimic the proposed signaling mechanism of action of 101.10. SR-11302 alone or in combination with Y27632 was comparably effective to 101.10 in reducing preterm delivery (Fig. 7A, 7B) and increasing gestational length (Supplemental Fig. 1D).

Discussion

Inflammation plays a critical role in labor (39). Various major proinflammatory cytokines, including IL-1, upregulate UAP in gestational tissues and are associated with the onset of labor in animal models and in humans. However, available IL-1–targeting agents all cause a nonselective inhibition of the entire IL-1R–coupled

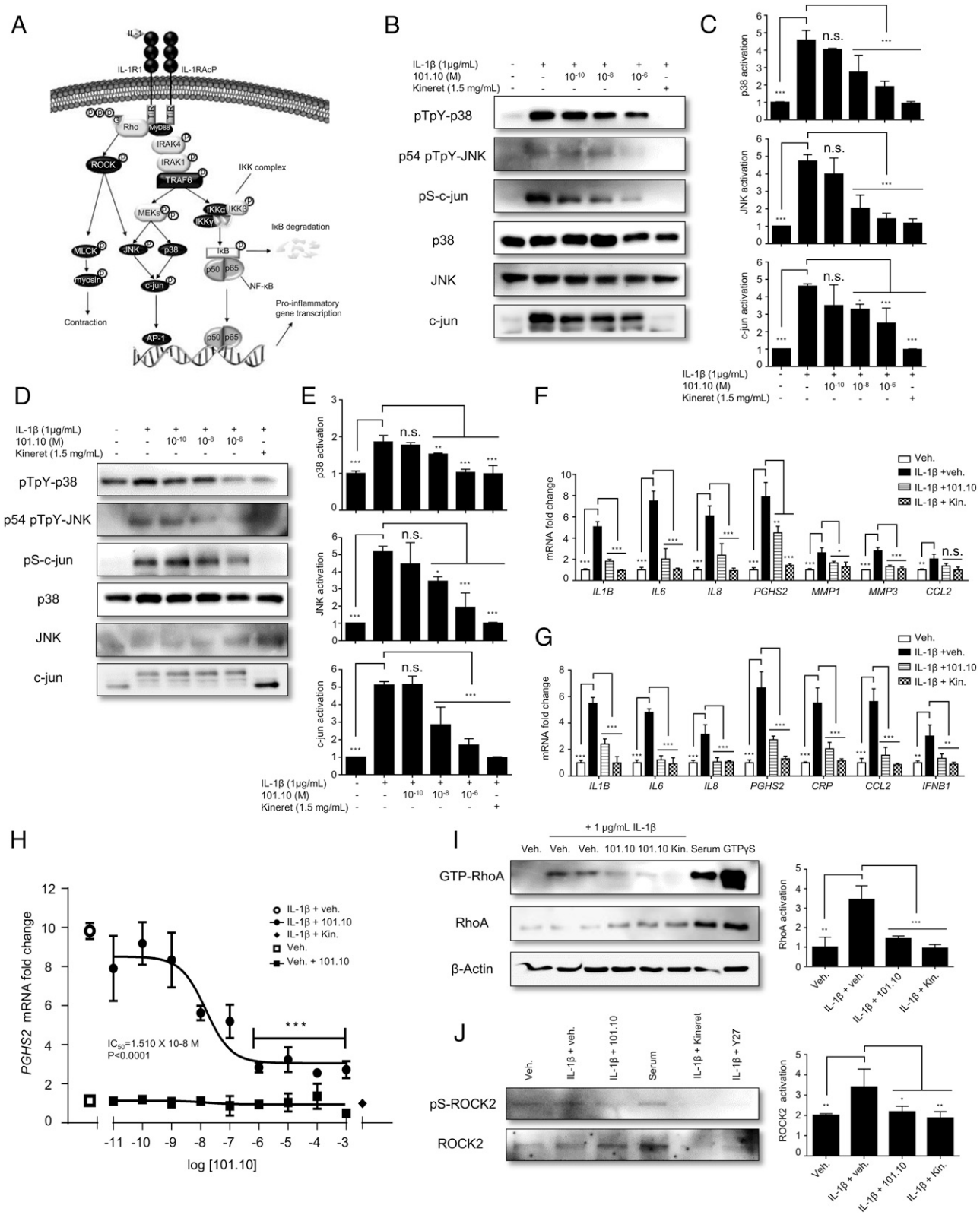

FIGURE 5. The 101.10 inhibits SAPK/c-jun and RhoA/ROCK signaling pathways in both myometrial and macrophage cell lines. (**A**) Simplified IL-1R intracellular signaling pathways. The activation of p38, JNK, or Rho/ROCK leads to the phosphorylation and translocation of the transcriptional factor c-jun to the nucleus and further assembling of the heterodimeric transcriptional factor AP-1. (**B** and **C**) Myometrial SMC (hTERT-C3 cell line) were treated with IL-1β or vehicle with or without increasing concentrations of 101.10, and Western blot was performed on lysates and blotted against indicated Abs (B). Densitometric analysis was used to quantify protein bands, and results were normalized with total proteins and plotted as fold over control (C). Kineret was used as a negative control. (**D** and **E**) RAW-Blue macrophages were treated with IL-1β (1 μg/ml) or vehicle with or without increasing concentrations of 101.10, and Western blot was performed on lysates and blotted against indicated Abs (D). Densitometric analysis was used to quantify protein bands, and results were normalized with total proteins and plotted as fold over control (E). Kineret (1.5 mg/ml) was used as a negative control. (**F**) Quantitative PCR of hTERT-C3 cells treated with IL-1β (1 μg/ml) or vehicle with or without 101.10 (10^{-6} M) or Kineret (1.5 mg/ml) for 6 h. Results are normalized with 18S and are relative (*Figure legend continues*)

signaling pathways, including NF-κB, which has an important role in cytoprotection and immune surveillance (40–42). In this work, we describe the efficacy of a noncompetitive stable (all-d peptide) modulator of IL-1R at delaying murine PTB models induced by IL-1β, LTA (TLR2 ligand), and LPS (TLR4 ligand). The 101.10 exhibited biased ligand properties by inhibiting IL-1–triggered SAPK/c-jun and Rho/ROCK pathways, without affecting NF-κB activity.

Inflammation is now considered a converging pathway toward labor (43, 44). It is believed that the initial inflammatory stimulus, such as pathogen-associated molecular patterns or danger-associated molecular patterns, activates innate immunity by binding on TLRs. This signal promotes cytokine production from cells of the innate immune response, which in turn activates adaptive immunity. Resulting inflammatory cascade leads to the induction of UAP and promotes the onset of labor. Accordingly, data from our laboratory and others show that acute inflammatory events increase UAP expression in the myometrium and other uterine tissue (12, 15, 45). This notion is supported by data obtained in the current study; IL-1β and TLR ligands induced various UAP, including *OXTR*, *PTGFR*, *PGHS2*, *CCL2*, and *GJA1* in myometrium. Products of these proinflammatory genes amplify the initial insult. Hence, targeting proinflammatory cytokines and their receptors accountable for expansion of the initial inflammatory trigger is a justifiable approach to prevent/arrest premature induction of UAP and ensued onset of PTB.

The present study focused on the role of IL-1β, a major mediator of inflammation, which can sustain the inflammatory cascade that results in preterm labor (46–49). Effects of IL-1β were antagonized by 101.10 (in a NF-κB–independent manner). Another IL-1R agonist ligand is IL-1α, which remains mostly intracellular and is released in the extracellular milieu upon cell lysis; IL-1α has been linked to sterile intra-amniotic inflammation (50). Of note, as observed for IL-1β, 101.10 is also capable of inhibiting actions of IL-1α (Supplemental Fig. 2E).

In this study, to our knowledge, we report for the first time the efficacy of a small noncompetitive inhibitor of IL-1R termed 101.10, in PTB. The peptide showed better efficacy than the competitive IL-1R antagonist Kineret in delaying IL-1β–induced PTB (Fig. 1C). Accordingly, intrauterine IL-1β triggered an inflammatory response locally, in the placenta/myometrial unit and systemically (increase in leukocyte cytokines), implicating mediators other than IL-1 partaking in amplified myometrial induction of various inflammatory factors; the dose of IL-1β used is consistent with that reported (8, 16), and, although higher than that used to stimulate human tissue, the exact concentration in human is not known, but most likely several fold higher in the immediate vicinity of cytokine-releasing cells. Contrary to 101.10, Kineret did not interfere with myometrial gene induction. Because Kineret did reduce blood leukocyte induction of inflammatory genes, the selective IL-1R antagonist Kineret is pharmacologically effective, but as a molecule as large as IL-1 per se (≈17.5 kDa), which does not cross the placental barrier (34, 35) (and with whom it competes for the ligand binding site on IL-1RI), Kineret has limited bioavailability to the placenta—the trigger locus of inflammation that in turn affects myometrium and systemic inflammation. In counterpart, the small molecule 101.10 (≈0.85 kDa) does distribute to placenta and myometrium, as seen with 101.10-FITC, and is able to diminish amplified inflammation in those tissues and in turn delay birth induced by IL-1β. The findings also infer that the local utero/placental inflammation surpasses in importance systemic inflammation in stimulating PTB.

NF-κB is a prominent downstream signal of inflammatory mediators. NF-κB has been implicated in the normal process of labor (22, 44), but its inhibition may be deleterious. Hence, reluctance to develop a NF-κB–targeted therapy to prevent PTB includes the following: 1) NF-κB plays an important role in cytoprotection, and its inhibition can increase rates of apoptosis (51); accordingly, the antibiotic sulfasalazine, which also inhibits NF-κB, has been associated with an increase in proapoptotic cells in human chorionic membranes (21) and an increased risk of adverse pregnancy outcomes (52). 2) Hypoxia-induced NF-κB activation might be implicated in preventing sequelae from myometrial contraction-induced ischemia (22). 3) NF-κB inhibition can hamper immune surveillance and potentially increase the risk of infection including during pregnancy (51, 53). In this regard, 101.10 offers a unique alternative to currently available IL-1 inhibitors by avoiding NF-κB inhibition while still interfering with other IL-1R–coupled pathways involved in the assembly of the transcription factor AP-1.

The notion that AP-1 partakes in labor is relatively new. Recent data demonstrate that labor is associated with changes in the AP-1 family members in the uterus and fetal membranes (54–56). Moreover, a causal role of JNK/AP-1 was recently described wherein AP-1 activation alone was sufficient to induce labor and inhibition of JNK was sufficient to delay LPS-induced PTB (37). Correspondingly, SAPK and their target c-jun/c-fos (AP-1) have been shown to be activated in human uterine cervix at term and after delivery, suggesting a concomitant function for AP-1 in cervical ripening (57). Our study markedly bolsters the evidence toward a crucial role of AP-1 in labor: we showed that 101.10 prevented PTB without significantly affecting NF-κB, but rather by inhibiting pathways upstream of AP-1, including c-jun. We further confirmed that inhibiting AP-1 alone was sufficient to delay IL-1β–induced PTB in mice. Notwithstanding that NF-κB controls expression of numerous genes implicated in inflammation, many proinflammatory and/or prolabor genes have both AP-1 and NF-κB binding sites, including *PGHS2* (58), *IL6* (58), *IL8* (59), and *CCL2* (60); in addition, the regulatory region of human *OXTR* displays binding sites for AP-1 (61), and AP-1 is a key regulator of MMP (62) and *CX43* (63–65). Hence, inhibition of either AP-1 or NF-κB appears to be sufficient to interfere with expression of these genes implicated in labor; this claim is supported by the comparable efficacy of 101.10 and AP-1 inhibitor SR-11302.

This study has some limitations, particularly as it relates to translation of all findings in rodents to humans. We focused on IL-1

to control. (**G**) Quantitative PCR of RAW-Blue macrophages treated with IL-1β (1 μg/ml) or vehicle with or without 101.10 (10^{-6} M) or Kineret (1.5 mg/ml) for 6 h. (**H**) Quantitative PCR of *PGHS2* induction in hTERT-C3 cells treated with IL-1β (●; 1 μg/ml) or vehicle (■) with increasing concentrations of 101.10 or with Kineret (▲; 1.5 mg/ml) for 2 h. Results are normalized with 18S and are relative to control. ***$p < 0.001$ relative to higher plateau. (**I**) hTERT-C3 cells were treated with IL-1β or vehicle with or without 101.10 (10^{-6} M), and lysates were incubated with affinity beads specific to GTP-bound RhoA. Beads and total proteins were then loaded on SDS-PAGE and blotted against RhoA or β-actin. Kineret (1.5 mg/ml) was used as a negative control, and FBS (10%) and GPTγS (200 μM) were used as positive controls. Quantification of protein bands was normalized with total RhoA and plotted as fold over control. (**J**) Western blot of hTERT-C3 cells treated with IL-1β (1 μg/ml) or vehicle with or without 101.10 (10^{-6} M) and blotted against pS-ROCK2 or ROCK2. Kineret (1.5 mg/ml) and Y27632 (10^{-6} M) were used as a negative control and FBS (10%) as a positive control. Quantification of protein bands was normalized with ROCK2 and plotted as fold over control. Values are presented as mean ± SD. Data are representative of three to four experiments. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ by one-way ANOVA with Tukey's multiple comparison test compared with IL-1β + vehicle group.

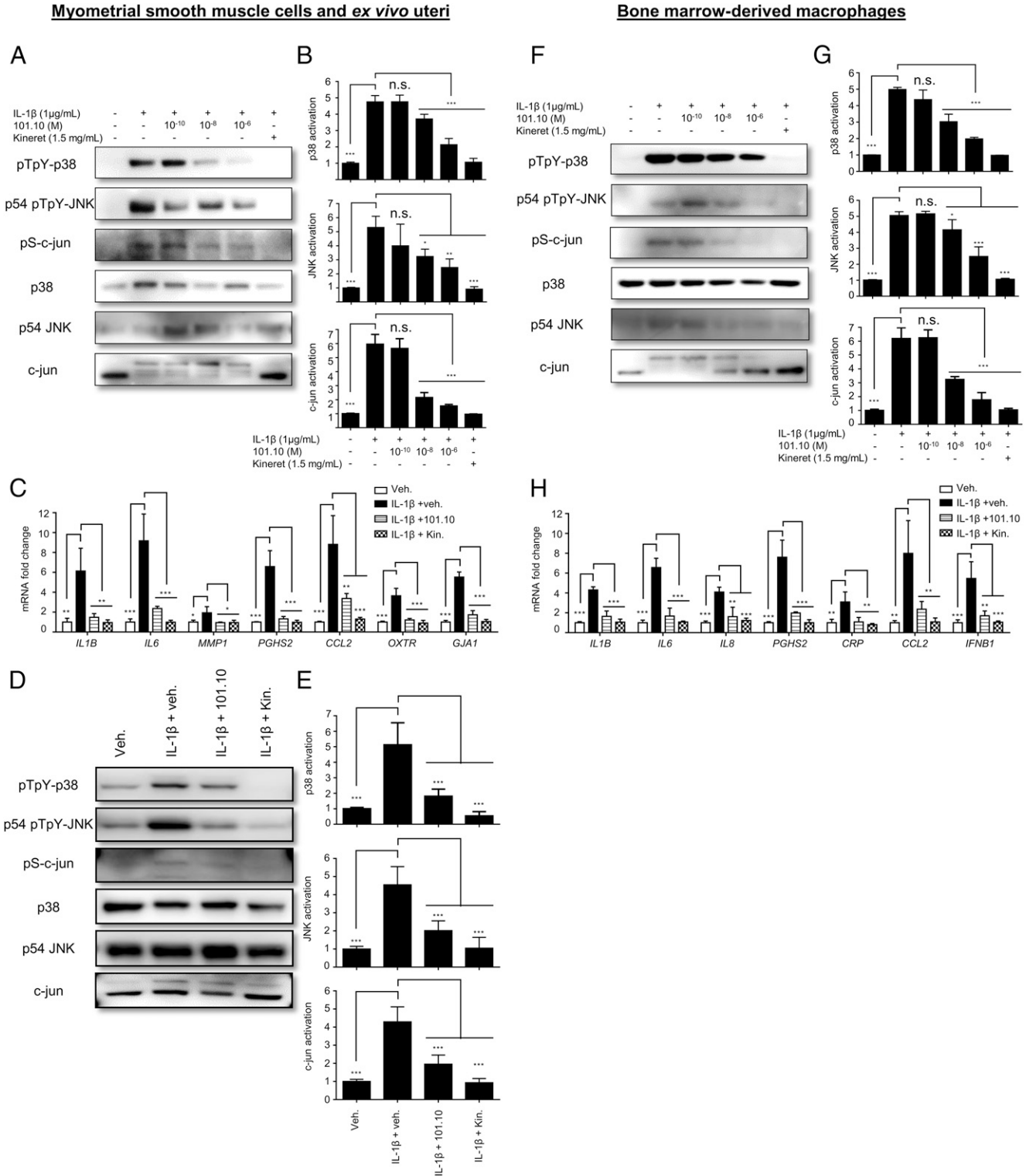

FIGURE 6. The 101.10 inhibits SAPK/c-jun signaling pathway in CD-1 mice primary myometrial SMC, in ex vivo myometrium fragments, and in bone marrow–derived macrophages. (**A** and **B**) Primary myometrial SMC were treated with IL-1β or vehicle with or without increasing concentrations of 101.10, and Western blot was performed on lysates and blotted against indicated Abs (A). Densitometric analysis was used to quantify protein bands, and results were normalized with total proteins and plotted as fold over control (B). Kineret was used as a negative control. (**C**) Quantitative PCR of primary myometrial SMC treated with IL-1β (1 μg/ml) or vehicle with or without 101.10 (10^{-6} M) or Kineret (1.5 mg/ml) for 6 h. Results are normalized with 18S and are relative to control. (**D** and **E**) Myometrium fragments were collected from CD-1 mice and incubated in serum-free medium for 1 h prior to stimulation with IL-1β (1 μg/ml) or vehicle with or without increasing concentrations of 101.10. Western blot was performed on lysates and blotted against indicated Abs (D). Densitometric analysis was used to quantify protein bands, and results were normalized with total proteins and plotted as fold over control (E). Kineret (1.5 mg/ml) was used as a negative control. (**F** and **G**) Primary bone marrow–derived macrophages were treated with IL-1β or vehicle with or without increasing concentrations of 101.10, and Western blot was performed on lysates and blotted against indicated Abs (F). Densitometric analysis was used to quantify protein bands, and results were normalized with total proteins and plotted as fold over control (G). Kineret was used as a negative control. (**H**) Quantitative PCR of primary bone marrow–derived macrophages treated with IL-1β (1 μg/ml) or vehicle with or without 101.10 (10^{-6} M) or Kineret (1.5 mg/ml) for 6 h. Results are (*Figure legend continues*)

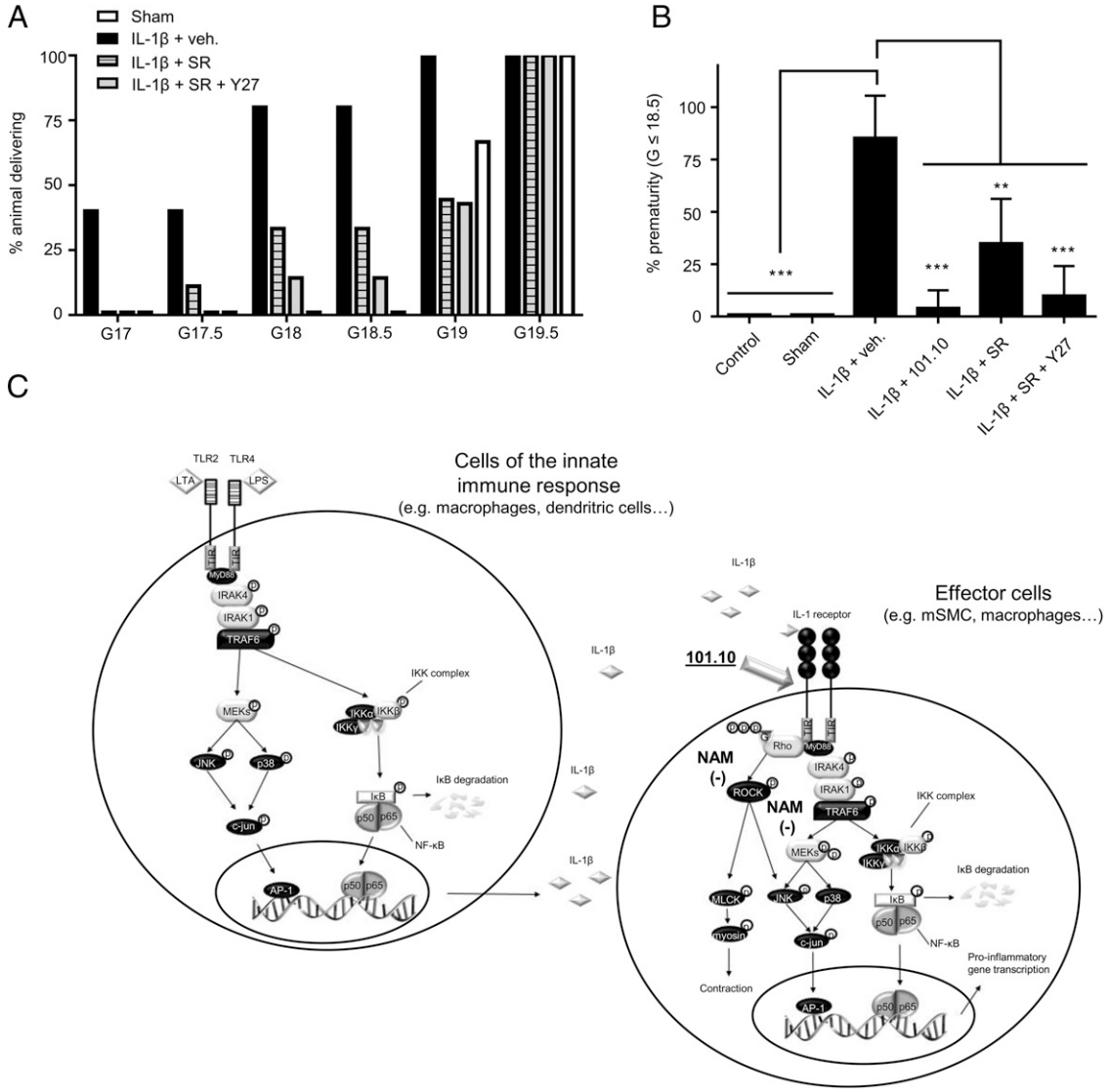

FIGURE 7. Inhibiting AP-1 protects against inflammation-induced preterm birth. (**A**) Percentage of animals having delivered following 1 μg intrauterine IL-1β injection and (**B**) percentage of prematurity. Control mice did not receive any treatment, whereas sham animals received an intrauterine dose of vehicle at G16.5. The 101.10 (1 mg/Kg/12 h), SR11302 (1 mg/Kg/12 h), Y27632 (1 mg/Kg/12 h), or vehicle was administered s.c. twice per day until delivery. (**C**) Proposed mechanism of action of 101.10. Effector cells comprise a wide range of possible cells, although the focus has been made on myometrial SMC and macrophages in this study. Values are presented as mean ± SD. Data are representative of 3–10 animals per group. $^{**}p < 0.005$, $^{***}p < 0.001$. NAM, negative allosteric modulator by one-way ANOVA with Tukey's multiple comparison test compared with IL-1β + vehicle group.

and upstream TLR4 and TLR2 (66) pathways proposed by an abundance of literature to be implicated in triggering human PTB (67–70); also, efficacy of 101.10 was shown in a relevant human cell line. Concordantly, in the current study, several mediators of inflammation were induced in our rodent models (e.g., IL-6, IL-8, and cyclooxygenase-2); yet, specific inhibition of IL-1R by 101.10 reduced PTB induced by every stimulus (IL-1, LPS, LTA) tested, highlighting its critical role. Extrapolation of these findings to humans does not exclude a role for other pathways. This inference has been proposed for IL-6 (71), IL-8 (72), FOX01 (73), and other mediators implicated in labor of humans. Although biologic effects of (heat-inactivated) Gram$^+$ bacteria are not fully reproduced by LTA (74), the latter do elicit many features of the bacteria (75); a similar argument can be made for (heat-inactivated) Gram$^-$ bacteria and LPS (76), including as it applies to placental/fetal biology (66). Ideally, anti-inflammatory drugs should be administered at an earlier time point than currently applied; accordingly, appropriate diagnostic markers are also needed for effective prevention of PTB in humans. Overall, our findings on the role of IL-1 concur with those previously reported by authors of this paper (17, 77).

Small biased ligands offer therapeutic advantages, which cannot be mimicked by currently available orthosteric inhibitors. Small peptide or peptidomimetics are likely to exhibit better bioavailability and a therapeutic index due to selective and partial modulation of specific (and not all) receptor-coupled signaling pathways. Advantages of 101.10 over available IL-1–targeting therapies in PTB comprise the following: 1) 101.10 avoids the inhibition of IL-1–induced NF-κB activation and therefore offers a novel way to prevent premature uterine activation, by acting as a negative allosteric biased ligand, in line with its reported actions on other cells (24);

normalized with 18S and are relative to control. Values are presented as mean ± SD. Data are representative of three to four experiments. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$ by one-way ANOVA with Tukey's multiple comparison test compared with IL-1β + vehicle group.

2) due to enhanced pharmacological selectivity, 101.10 could be deprived of major adverse effects; 3) 101.10 is more likely to have increased bioavailability and less invasive route of administration [101.10 has been reported to exhibit enteral bioavailability (24)]; and 4) cost of goods for 101.10 is most likely less compared with recombinant proteins and Abs; the latter provides a more suitable therapeutic option for developing countries, where prematurity is a main cause of mortality (78).

In summary, to our knowledge, we hereby describe the first noncompetitive biased modulator of a cytokine receptor showing efficacy in delaying the onset of preterm birth. The 101.10 acts desirably without inhibiting IL-1β–induced NF-κB activation, albeit by dose dependently inhibiting relevant IL-1β–induced phosphorylation of SAPK p38 and JNK, transcription factor c-jun, as well as Rho/ROCK pathway. Hence, 101.10 acts independently of NF-κB in delaying IL-1β–, TLR2- and TLR4-induced PTB in mice, thus undermining the role of NF-κB activation in labor.

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Disclosures

S.C., C.Q., and W.L. hold a patent on composition of matter for the use of 101.10 (Interleukin-1 receptor antagonists, compositions, and methods of treatment, United States patent no. USPTO8618054, 2005, May 05). The other authors have no financial conflicts of interest.

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