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Abstract

Chronic inflammation and persistent oxidative stress contribute to the development and progression of vascular proliferative diseases. We hypothesized that the proinflammatory cytokine interleukin (IL)-17A induces oxidative stress and amplifies inflammatory signaling in human aortic smooth muscle cells (SMC) via TRAF3IP2-mediated NLRP3/caspase-1-dependent mitogenic and migratory proinflammatory cytokines IL-1β and IL-18. Further, we hypothesized that these maladaptive changes are prevented by empagliflozin (EMPA), an SGLT2 (Sodium/

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Glucose Cotransporter 2) inhibitor. Supporting our hypotheses, exposure of cultured SMC to IL-17A promoted proliferation and migration via TRAF3IP2, TRAF3IP2-dependent superoxide and hydrogen peroxide production, NLRP3 expression, caspase-1 activation, and IL-1β and IL-18 secretion. Furthermore, NLRP3 knockdown, caspase-1 inhibition, and pretreatment with IL-1β and IL-18 neutralizing antibodies and IL-18BP, each attenuated IL-17A-induced SMC migration and proliferation. Importantly, SMC express SGLT2, and pre-treatment with EMPA attenuated IL-17A/TRAF3IP2-dependent oxidative stress, NLRP3 expression, caspase-1 activation, IL-1β and IL-18 secretion, and SMC proliferation and migration. Importantly, silencing SGLT2 attenuated EMPA-mediated inhibition of IL-17A-induced cytokine secretion and SMC proliferation and migration. EMPA exerted these beneficial antioxidant, anti-inflammatory, anti-mitogenic and anti-migratory effects under normal glucose conditions and without inducing cell death. These results suggest the therapeutic potential of EMPA in vascular proliferative diseases.

Keywords

Hyperplasia; Inflammasome; Interleukin Converting Enzyme; Migration; Mitogenesis

1. Introduction

Vascular proliferative diseases, including restenosis following angioplasty, intermittent hypoxia, and atherosclerosis, are characterized by smooth muscle cell (SMC) migration and proliferation, and are exacerbated in diabetic subjects. Persistent inflammation and oxidative stress have been shown to play causative roles in their pathology in both diabetic and non-diabetic individuals.

Interleukin (IL)-17A is proinflammatory cytokine that belongs to a unique IL-17 family of cytokines. The IL-17 family consists of 6 ligands (A-F) and 5 receptors (IL-17RA-E). Originally, T helper 17 (Th17) cells are the only cell type to express IL-17A. However, it is now known that multiple immune and non-immune cells, including cells of cardiac origin, express IL-17A (e.g., [1]). IL-17A plays a pathological role in various inflammatory and autoimmune diseases, including atherosclerosis and restenosis. However, published reports have indicated either a causal role or a protective effect for this unique cytokine in the pathophysiology of atherosclerosis. For example, it's increased expression has been shown to promote a more fibrous and stable plaque in a preclinical mouse model [2], demonstrating that IL-17 is atheroprotective. In contrast, a positive correlation was identified between its enhanced expression and inflammation and plaque vulnerability in humans [3], suggesting that its expression is proatherogenic. Recently, it has also been shown that targeting the IL-17RA pathway normalizes arterial inflammation even in advanced atherosclerosis in a preclinical model [4], suggesting again a proatherogenic role for IL-17A.

IL-17 signals predominantly via TRAF3IP2 [(TRAF3 Interacting protein 2, also known as CIKS (Connection to IKK and SAPK/JNK) or Act1 (Nuclear Factor NF-Kappa-B Activator 1)], a cytoplasmic adapter molecule. We have previously reported that IL-17A promotes migration and proliferation of human primary aortic SMC in a TRAF3IP2-dependent manner [5]. We have also reported that oxidative stress plays a role in TRAF3IP2 induction

[6–8]. Interestingly, TRAF3IP2 also increases oxidative stress, as evidenced by the increased generation of superoxide and hydrogen peroxide in cells of cardiac and renal origin in response to various stressors, including high glucose, cytokines, angiotensin II and aldosterone [8–11]. More importantly, deletion of *TRAF3IP2* in *ApoE* knockout mice blunts the development of atherosclerosis [12], a known contributor to myocardial injury, inflammation, and heart failure. In fact, *TRAF3IP2* deletion blunts ischemia/reperfusion-induced oxidative stress, inflammation, myocardial injury, and heart failure development [13]. These reports thus indicate a causal role for TRAF3IP2 in atherosclerotic and other cardiovascular diseases.

Recently, several clinical trials have reported the cardiovascular protective effects of the SGLT2 (Sodium/Glucose Cotransporter 2; also known as SLC5A2 for Solute Carrier Family 5 Member 2) inhibitors empagliflozin (EMPA), canagliflozin, and dapagliflozin [14] [15] [16]. A majority of their protective effects have been ascribed to their blood sugar lowering properties independent of insulin [17]. In fact, SGLT2 transcripts are expressed mainly in proximal tubular epithelial cells in the S1 segment of the kidney ([18]), where SGLT2 plays a major role in glucose reabsorption, and thus in glucose homeostasis. However, the expression of SGLT2 has also been reported in other tissues and cell types. For example, epicardial adipose tissue (EAT) from subjects with cardiovascular diseases expresses SGLT2 and responds to dapagliflozin treatment *ex vivo* as measured by reduced glucose uptake and inflammatory cytokine expression [19], suggesting a functional role for SGLT2 in EAT. In the same study [19], it has also been reported that SGLT2 inhibitor treatment of differentiated stromal vascular cells from fat pads enhances glucose uptake, indicating SGLT2 expression in stromal vascular cells. SGLT2 expression has also been reported in isolated cultured rat mesangial cells [20]. Aortic SMC also express SGLT2, albeit at a lower level than in kidney [21]. These reports suggest that cell types other than proximal tubular epithelial cells, such as aortic SMC, are direct targets of SGLT2 inhibitors and contribute to some of their non-glycemic vascular protective effects.

Since administration of EMPA exerts pleiotropic antioxidant and anti-inflammatory effects that are independent of its anti-hyperglycemic effects, and since IL-17A is a potent pro-oxidant and proinflammatory cytokine, we investigated whether EMPA blunts IL-17A-induced TRAF3IP2/oxidative stress-dependent proliferation and migration of cultured primary human aortic SMC under normal glucose conditions. Further, since IL-1β and IL-18 exert mitogenic and migratory effects [22] [23] [24]) we investigated their role in IL-17-induced SMC proliferation and migration, and inhibition by EMPA. Since both IL-1β and IL-18 are synthesized as inactive precursors and cleaved by caspase-1 into biologically active forms [25], we also determined whether EMPA negatively affects caspase-1 expression and activation. Previously, EMPA was shown to inhibit NLRP3 (**N**ucleotide-binding domain and **L**eucine-**r**ich repeat containing Family **P**yrin Domain Containing **3**) expression in liver and kidney, but not heart, in a murine model of diet-induced obesity and insulin resistance ([26]). Since the NLRP3 inflammasome forms a molecular platform for recruitment and activation of pro-caspase-1 to caspase-1 [27], we also determined whether EMPA inhibits NLRP3 expression.

Indeed, our results demonstrate that SMC express SGLT2, but at a lower level compared to human kidney and human proximal tubule epithelial cell line, and its expression is induced by IL-17A in a time-dependent manner. Moreover, pretreatment with the SGLT2 inhibitor EMPA inhibits SMC proliferation and migration by targeting IL-17A/TRAF3IP2-dependent superoxide and hydrogen peroxide generation, NLRP3 expression, caspase-1 activation, and the release of mitogenic and migratory IL-1β and IL-18, all in the presence of normal glucose and without inducing cell death. Importantly, silencing EMPA attenuates, but not abrogates, IL-17A-induced cytokine expression and SMC proliferation and migration. Together, these results further support the growing evidence of pleiotropic protective effects of EMPA and suggest its therapeutic potential in vascular proliferative diseases.

2.   Materials and methods

2.1.   Reagents

Empagliflozin (EMPA; 1-chloro-4-(β-D-glucopyranos-1-yl)-2-[4-((S)-tetrahydrofuran-3-yl-oxy)-benzyl]-benzene; #S8022) was purchased from Selleckchem (Houston, TX). MCC950 (#S8930; 10μM in ethanol for 1 h), a selective NLRP3 inhibitor that does not inhibit NLRC4, AIM2, TLR2 signaling, or priming of NLRP3 was also purchased from Selleckchem. In initial experiments, SMC were treated with EMPA at concentrations ranging between 0.1 to 5 μM for 15 min prior to IL-17A addition. Since significant inhibitory effects were observed at 1μM, this concentration was used in all subsequent experiments. In fact, pharmacokinetics revealed its Cmax to be between 0.5 and 1.11 μM at 25 and 50 mg dose, respectively ([28]). Recombinant human (rh) IL-17A protein, carrier-free (#317-ILB), human/primate IL-17/IL-17A neutralizing antibodies (Monoclonal Mouse IgG$_{2B}$; #MAB317–100), mouse IgG$_{2B}$ isotype control (#MAB004), monoclonal mouse anti-human IL-1beta/IL-1F2 neutralizing antibodies (MAB601), monoclonal mouse anti-human IL-18/IL-1F4 neutralizing antibodies (D044–3), polyclonal goat anti-human IL-18/IL-1F4 propeptide antibody (#AF646; 0.5μg/ml), anti-human IL-18 antibody (#D043–3; 0.5μg/ml) that specifically detects mature IL-18, goat polyclonal IL-17RA neutralizing IgG antibodies (#AF177), normal goat IgG isotype control (#AB-108-C), protein A or G purified normal goat IgG isotype control (#AB-108-C), recombinant IL-18BPa:Fc (#119-BP), the Fc portion of the immunoglobulin, recombinant human PDGF-BB protein, carrier-free (#220-BB), cell permeable fluoromethyl ketone (FMK)-derivatized non-toxic Caspase-1/Interleukin Converting Enzyme inhibitor Z-WEHD-FMK (#FMK002; 50 μM in DMSO for 30 min), the pan-caspase inhibitor Z-VAD-FMK (#FMK001; 50 μM in DMSO for 30 min), caspase inhibitor control Z-FA-FMK (#FMKC01; 50 μM in DMSO for 30 min), the NLRP3 inflammasome disruptor Dapansutrile (#6902; 3-(methylsulfonyl)propanenitrile; also known as OLT1177®; 1μM in ethanol along with ATP (5mM, Millipore-Sigma) following 30 min of IL-17A addition; [29]), Caspase-1/ICE Colorimetric Assay Kit (#K111–100), human IL-1 beta/IL-1F2 Quantikine ELISA Kit (#DLB50, sensitivity: 1 pg/ml) and human IL-18/IL-1F4 ELISA Kit (#7620, sensitivity: 12.5 pg/ml) were all purchased from R&D Systems (Minneapolis, MN). We have previously demonstrated the functionality of these neutralizing antibodies and IL-18BP in an *in vitro* cell system ([30]). Anti-IL-1β antibody (#ab9722, 0.2 μg/ml) that recognizes both proform and mature forms in Western blotting and human MIP2 ELISA Kit (CXCL2; ab184862) were bought from abcam (Cambridge, MA). Since

rhIL-17A contained <0.10 EU/1μg of protein by the Limulus Amebocyte Lysate (LAL) method (manufacturer's technical data sheet), in a subset of experiments the recombinant protein (200 ng/ml) was incubated with the endotoxin inhibitor Polymyxin B sulfate (10μg/ml for 1 h), and SMC proliferation and migration were used as readouts. Polymyxin B sulfate ((#BP1028), N-acetyl-L-cysteine (NAC, 5mM in water for 30 min; #A7250), Amicon Ultra-2 Centrifugal Filter Units ($UFC200324) and other biochemicals were purchased from Millipore-Sigma (St. Louis, MO, USA). gp91 ds-tat, a peptide inhibitor of NOX2 assembly (YGRKKRRQRRRCSTRIRRQL- NH_2; #AS-63818) and its scrambled peptide (RKKRRQRRRCLRITRQSR-NH_2; #AS-63855) were purchased from AnaSpec (Fremont, CA), and used at a concentration of 1μM for 1 h prior to IL-17A addition as has been previously described ([9]). The NOX1/4 inhibitor GKT137831 (2–2(chlorophenyl)-4-[3-(dimethylamino)phenyl]-5-methyl–1 H-pyrazolo[4,3-c]pyridine-3,6(2H,5H)-dione; #17164; 5μM in DMSO 15 min prior to IL-17A addition) was purchased from Cayman Chemical (Ann Arbor, MI) and used as before ([9],[31]). DMSO was purchased from EMD Biosciences (San Diego, CA). BioCoat™ Matrigel™ invasion chambers (# 354481) were from BD/Discovery Labware (Bedford, MA). Pierce™ BCA Protein Assay Kit (#23227), prestained protein molecular weight markers (#26612 and 26616) and SuperSignal® West Femto Maximum Sensitivity Substrate (#34096) were purchased from Thermo Fisher Scientific (Waltham, MA). Human kidney whole tissue lysate (adult whole normal; #NB820–59231) was purchased from polyclonal rabbit anti-human MIP2/CXCL2 antibodies (NBP3–03233; 1:2500) Novus Biologicals (Centennial, CO). LDH (Lactate Dehydrogenase) Cytotoxicity Assay Kit (#601170) was purchased from Cayman Chemical, Ann Arbor, MI).

2.2. Cell culture

Human aortic SMC, purchased from LONZA *CC-2571), were grown in SmGM-2 basal medium with SmGM™–2 SingleQuots™ supplements (LONZA, CC-4149) as previously described ([5]). The cells were positive for αSMA (α smooth muscle actin) and SM-MHC (smooth muscle myosin heavy chain), but not for VWF (Von Willebrand Factor) by RT-qPCR using validated TaqMan® probes (data not shown). At 70–80% confluency, the culture medium was replaced with basal medium containing 0.5% bovine serum albumin (conditioning medium). After 48 h incubation, recombinant human IL-17A was added and SMC were cultured for the indicated time periods. In our previous report, we tested IL-17A at various concentrations, ranging from 1 to 50 ng/ml, and found that IL-17A at 25 ng/ml induced maximal SMC proliferation ([5]). In studies examining the effect of IL-17A on IL-1β and IL-18 mRNA expression, SMC were treated with rhIL-17A at 25 ng/ml for 2 h. In studies examining the effect of IL-17A on IL-1β and IL-18 secretion, SMC were treated with rhIL-17A at 25 ng/ml for 24 h. At the end of the experimental period, culture supernatants were collected into slick tubes, snapfrozen, and stored at –80°C for subsequent analysis by ELISA.

HK-2 (the human kidney-2) cells were purchased from ATCC® (CRL-2190 Manassas, VA). HK-2 is an immortalized human proximal tubule cell line derived from a single cell isolated from an adult male normal kidney. The cells were authenticated by ATCC®. The cells were cultured in DMEM/F12 medium as previously described by us ([32]).

2.3. Adeno and lentiviral infection

Adenoviral vector expressing short hairpin RNA (shRNA) targeting human TRAF3IP2 (ad.TRAF3IP2 shRNA) was custom generated at Vector Biolabs (Malvern, PA). Ad.GFP (#1060, Vector Biolabs) served as a non-targeting control. For adenoviral transduction, cells were traduced with adenoviral vectors at moi10 for 1 h in basal medium and then switched to complete medium for 24 h. Lentiviral vectors expressing shRNA targeting human NLRP3 (Product type: SHCLNV-NM_004895, TRC# TRCN0000432208; Target sequence: CCGGGTGGATCTAGCCACGCTAATGCTCGAGCA TTAGCGTGGCTAGATCCACTTTTTTG) and SGLT2 (SLC5A2; Product type: SHCLNV-NM_003041, TRC# TRCN0000043603, Target sequence: CGGGCATATTTCCT GCTGGTCATTCTCGAGAATGACCAGCAGGAAATATGCTTTTTG) were purchased from Millipore-Sigma. Lentiviral shRNA targeting p65 subunit of NF-κB (#sc-29410-V) and c-Jun subunit of AP-1 (#sc-29223-V) were purchased from Santa Cruz Biotechnology, Inc., and have all been previously described ([11]). For lentiviral infection, SMC at 50–60% confluency were infected with the indicated shRNA at a multiplicity of infection (moi) of 0.5 for 48 h in complete media. To increase transfection efficiency, cells were cotreated with Polybrene® (5 μg/ml in water), a cationic polymer. Neither adenoviral or lentiviral shRNA nor Polybrene® modulated SMC adherence, shape, or viability (trypan blue-dye exclusion; data not shown).

2.4. Superoxide and hydrogen peroxide production

The generation of superoxide ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) was quantified as previously described ([33]). In brief, $O_2^{-\bullet}$ generation was quantified using the lucigenin-enhanced chemiluminescence assay as previously described ([6]). After subtracting background luminescence, results are expressed as pmol superoxide/min/mg protein. Studies were also performed after treating cells with the NOX2 inhibitor gp91 ds-tat (1 μM for 1 h). A corresponding scrambled peptide (sgp91 ds-tat) served as a control. H_2O_2 production was measured according to the manufacturer's instructions using a commercially available kit in the presence of horseradish peroxidase (0.1 unit/ml, Amplex Red: and 50 μM) according to the manufacturer's instruction and described in our previous report ([10]). Fluorescence was recorded at 530 nm excitation and 590 nm emission wavelengths (CytoFluor II; Applied Biosystems, Foster City, CA). Standard curves were generated using known concentrations of H_2O_2. Studies were also performed after treatment with the Nox1/4 dual inhibitor GKT137831. The results are expressed as μM H_2O_2 produced/10^6 cells.

2.5. mRNA expression

DNA-free total RNA was prepared using the RNeasy Plus Micro Kit (#74034; Qiagen, Germantown, MD). RNA quality was assessed by capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). All RNA samples had RNA integrity greater than 9.0 (scale = 1–10) as assigned by default parameters of the Expert 2100 Bioanalyzer software package (v2.02). mRNA expression was analyzed by RT-qPCR using the following Applied Biosystems™ TaqMan™ probes: NLRP3 (Assay ID: Hs00918082_m1), caspase-1 (Assay ID: Hs00354836_m1), IL1B (Assay ID: Hs01555410_m1), IL18 (Assay ID: Hs01038788_m1), SGLT2 (Hs00894642_m1 [34] and

Hs00894634_g1), MIP2 (CXCL2; Hs00601975_m1), and 18S (Hs99999901_s1). No template controls were used for each assay, and samples processed without the reverse transcriptase step served as a negative control. Each cDNA sample was run in triplicate. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method. All data were normalized to corresponding 18S rRNA expression and presented as fold change from untreated control.

2.6. Protein expression, secretion, and activity

Preparation of whole cell homogenates, Western blotting, detection of the immunoreactive bands by enhanced chemiluminescence (ECL Plus; GE Healthcare), and their quantification by densitometry were all previously described ([5, 13, 32, 33]. Briefly, Cell lysates obtained with Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 3 mM p-amidinophenylmethanesulfonyl fluoride, 5 mg/ml aprotinin, 2 mM sodium orthovanadate, 5 mM EDTA). Samples were separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with primary antibodies diluted in 2% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TTBS) overnight at 4 °C. The blots were rinsed in TTBS for 30 min and then incubated in species-specific HRP-conjugated secondary antibodies in 5% milk in TTBS for 1 h and developed using the SuperSignal® West Femto Maximum Sensitivity chemiluminescent substrate. The following primary antibodies at indicated dilutions/concentrations were used: TRAF3IP2 (1:400; NB100–56740, Novus Biologicals, Centennial, CO), ASK1 (1:1000; #3762, Cell Signaling Technology/CST, Danvers, MA), Tubulin (1:1000; #2144, CST), p-p65 (1:1000; #3031, CST), p65 (1:1000; #3033, CST), cleaved Caspase-1 (1:1000; #4199, CST), NLRP3 (2.5 µg/ml; #NBP2–12446, NOVUS Biologicals), and SGLT2 (1:100; #sc-393350; Santa Cruz Biotechnology, Inc., Dallas, TX). Secreted IL-1β IL-18 levels in equal amounts of culture supernatants were quantified by ELISA. Secreted cytokine levels were also analyzed by Western blotting using equal amounts of culture supernatants after concentrating with Amicon Ultra-2 Centrifugal Filter Units. Caspase-1 activity was analyzed according to the manufacturer's instructions using the Caspase-1/ICE Colorimetric Assay Kit.

2.7. Cell proliferation

SMC proliferation was analyzed as described previously ([5, 9, 33]. In brief, SMC were seeded at a concentration of 1×10^3 cells/well in 200 µl of complete medium in 96-well clear bottom, black-sided flat-bottom plates (VWR Scientific Products, West Chester, PA). After 24 h incubation, the complete medium was replaced with medium containing 0.5% BSA and no fetal bovine serum (conditioning medium) and incubated for an additional 48 h (quiescence). The quiescent cells were then incubated with IL-17A for 48 h. Afterward the medium was removed and plates were frozen at −80°C for 2 h before assay. Plates were then thawed, stained with CyQUANT GR dye according to manufacturer's protocol (Molecular Probes, Eugene, OR), and read on a FLX800 microplate fluorescence reader (Bio- Tek Instruments, Winooski, VT) using 485/20 excitation and 528/20 emission filters, and analyzed using KC^4 software (Bio- Tek Instruments).

2.8. Cell migration

SMC migration was quantified as described previously using BioCoat™ Matrigel™ invasion chambers and 8.0-µm pore polyethylene terephthalate membranes with a thin layer of

Matrigel™ basement membrane matrix ([5, 8, 31, 33]. Cultured SMC were trypsinized and suspended in medium containing 0.5% bovine serum albumin, and 1 ml containing 2.0×10^5 cells/ml was layered on the coated insert filters. Cells were stimulated with IL-17A (25 ng/ml). The lower chamber contained IL-17A at the same concentration. Plates were incubated at 37 °C for 18 h. Membranes were washed with phosphate-buffered saline, and non-invading cells on the upper surface were removed using cotton swabs. Cells migrating to the lower surface of the membrane were determined at $A_{540 \text{ nm}}$ using MTT assay.

2.9. Cell vitality

To determine the potential effects of IL-17A, phramacologiocal inhibitors, neutralizing antibodies and shRNA on cell damage and death, we analyzed LDH (lactate dehydrogenase) activity at 8 h in culture supernatants according to the manufacturer's instructions using the LDH Cytotoxicity Assay Kit. In brief, the assay analyzes the integrity of cell membrane as damage to the cell membrane permits the leakage of LDH into culture supernatants. This is a two-step assay; in the first step, LDH catalyzes the reduction of NAD^+ to NADH and H^+ by oxidation of lactate to pyruvate, and in the second step, diaphorase uses the newly formed NADH and H^+ to catalyze the reduction of a tetrazolium salt (INT) to highly colored formazan which absorbs strongly at 490–520 nm. Hydrogen peroxide (100 μM for 24 h) served as a positive control. Each LDH activity value was compared with that of LDH released into culture supernatant after incubation with 0.1% Triton X-100, a nonionic surfactant that permeabilizes cell membranes (total LDH release) and expressed as a percentage of total release.

2.10. Statistical analysis

All data are expressed as mean ± SE. Statistical significance was determined by one-way analysis of variance followed by Tukey's post hoc test (GraphPad Prism software, San Diego, CA). Differences are considered significant if the P value is <0.05. Further, though a representative Western blot is shown in the main figures, changes in target protein expression from three to four independent experiments were semi-quantified by densitometry, and presented at the bottom or side of respective panels as fold changes over control, which was set at a value of 1. The numbers at the bottom of each panel in figures denote lane numbers.

3. Results

3.1. IL-17A stimulates human aortic smooth muscle cell (SMC) migration and proliferation via TRAF3IP2-dependent ROS generation

Exposure to IL-17A, as detailed in Fig. 1A, at 25 ng/ml, stimulated proliferation of SMC (Fig. 1B) as reported by us previously ([5]). PDGF-BB (10 ng/ml) served as a positive control, and significantly increased SMC proliferation (Fig. 1B). TRAF3IP2, a critical downstream signaling intermediate of IL-17A, and its knockdown by an adenoviral vector encoding a short hairpin RNA directed against TRAF3IP2 (Ad.TRAF3IP2 shRNA) led to a marked inhibition in IL-17A-mediated SMC proliferation (Fig. 1C) and migration (Fig. 1D). Importantly, these anti-mitogenic and anti-migratory effects of silencing TRAF3IP2 in IL-17A treated cells, did not result from reduced cell viability, as evidenced by the low

levels of LDH activity in equal amounts of culture supernatants (Fig. 1E). Additionally, adenoviral transduction of GFP shRNA modulated neither proliferation (Fig. 1C), migration (Fig. 1D) nor cell viability (Fig. 1E). However, hydrogen peroxide (100μM), used as a positive control, induced cell death as evidenced by a marked increase in LDH activity in culture supernatants (Fig. 1E).

Since TRAF3IP2 is an oxidative stress-responsive cytoplasmic adapter molecule ([32], [8], [7], we next investigated whether IL-17A induces oxidative stress. We quantified superoxide and hydrogen peroxide as markers of oxidative stress. Indeed, IL-17A induced $O_2^{-\bullet}$ generation (Fig. 1F), an effect markedly inhibited by the broad-spectrum antioxidant NAC and the NOX2 inhibitor gp91 ds-tat, but not by the scramble peptide, sgp91 ds-tat. Exposure to IL-17A also stimulated hydrogen peroxide production (Fig. 1G), an effect inhibited by NAC and the NOX1/4 dual inhibitor GKT137831. Importantly, NAC, gp91 ds-tat and GKT137831 each attenuated IL-17-induced upregulation in TRAF3IP2 expression (Fig. 1H). Recombinant IL-17A contained trace amounts of endotoxin. Therefore, to exclude the possibility that the mitogenic and migratory effects of IL-17A, and TRAF3IP2 induction, are mediated by these trace amounts of endotoxin (<0.10 EU/1μg of recombinant protein), we next exposed SMC to recombinant human IL-17A preincubated with the endotoxin inhibitor polymyxin B. These experiments revealed that exposure to IL-17A pretreated with polymyxin B failed to affect SMC proliferation and migration (Fig. 1I, 1J), suggesting that the low levels of contaminating endotoxin in the recombinant protein samples had no significant effect on IL-17A's mitogenic and migratory effects. Taken together, these results indicate that the proinflammatory cytokine IL-17A exerts mitogenic and migratory effects in SMC via oxidative stress-responsive TRAF3IP2 expression (Fig. 1).

3.2. IL-17A induces SMC proliferation and migration via IL-1β and IL-18

Both IL-1β and IL-18 are transcriptionally regulated proinflammatory cytokines and their increased expression plays a pathological role in vascular proliferative diseases, including atherosclerosis ([22] [23],[24]). Results show that SMC exposure to IL-17A, as detailed in Fig. 2A, upregulated IL-1β and IL-18 mRNA expression by 6- to 8-fold (Fig. 2B, 2C). Consistent with increased mRNA expression, IL-17A also enhanced their protein expression, as shown in insets in Fig. 2B and 2C. Since both cytokines are secreted proteins, we next measured their secreted levels in equal amounts of culture supernatants by ELISA. The data show that IL-17A significantly enhanced their secretion (Fig. 2D and 2E). In addition to ELISA, we also measured secreted cytokine levels in concentrated culture supernatants by Western blotting. The results show that the majority of cytokines detected in culture supernatants is the mature form, with trace to undetectable amounts of respective proforms (Fig. 2D and 2E). Importantly, silencing TRAF3IP2 inhibited their mRNA and protein expression, and secretion (Fig. 2B–2E). Furthermore, pre-incubation with IL-1β neutralizing antibodies (experimental design in Fig. 2F) attenuated IL-17A-induced SMC proliferation (Fig. 2G) and migration (Fig. 2H). Similarly, pretreatment with IL-18 neutralizing antibodies or its naturally occurring endogenous inhibitor, IL-18BP, as shown in Fig. 2F, attenuated IL-17A's mitogenic and migratory effects (Fig. 2G and 2H). However, the inhibitory effects of these neutralizing antibodies and IL-18-BP are not as a consequence of reduced cell viability, as evidenced by the low levels of LDH activity in equal amounts of

culture supernatants. Together, these results indicate that IL-17A-induced SMC proliferation and migration are mediated, in part, via TRAF3IP2-dependent IL-1β and IL-18 (Fig. 2).

3.3. IL-17A induces NLRP3 expression via NF-κB, AP-1 and p38MAPK

We have demonstrated that IL-17A induces SMC proliferation and migration, in part, via IL-1β and IL-18 (Fig. 2). Both IL-1β and IL-18 are synthesized as precursors and released as biologically active cytokines by the cytosolic NLRP3 inflammasome ([27, 35, 36]). Therefore, we next investigated whether IL-17A induces NLRP3 expression as well as the signaling intermediates that play a role in its induction. The data show that, exposure to IL-17A, as shown in Fig. 3A, increased NLRP3 expression (Fig. 3B), an effect significantly inhibited by TRAF3IP2 knockdown. We have previously demonstrated that NF-κB, AP-1 and p38MAPK are downstream targets of TRAF3IP2 ([11], [9]). Therefore, we next investigated whether IL-17A-mediated NLRP3 expression is dependent on these signaling intermediates. We targeted the p65 subunit of NF-κB and the c-jun subunit of AP-1 by lentiviral transduction of validated shRNA, and p38MAPK by SB239063 or SB202190, two different, but highly specific, pharmacological inhibitors. Silencing p65 and c-Jun markedly attenuated IL-17A-induced NLRP3 expression (Fig. 3C). Further, pharmacological inhibition of p38 MAPK significantly inhibited IL-17A-induced NLRP3 expression (Fig. 3D). However, knockdown of p65 or c-Jun, or pharmacological inhibition of p38 MAPK did not increase LDH activity in culture supernatants (Fig. 3E). Together, these results indicate that IL-17A induces NLRP3 expression in SMC via NF-κB, AP-1 and p38 MAPK (Fig. 3).

3.4. IL-17A induces caspase-1 expression and activation via NLRP3

Both IL-1β and IL-18 are synthesized as inactive precursors and cleaved by caspase-1 to become biologically active ([25, 36]). In general, caspase-1, also known as IL-1β converting enzyme (ICE), is activated via an autocatalytic pathway due to its proximity and recruitment to the inflammasome ([35]). Since IL-17A upregulated NLRP3 expression and stimulated the expression both IL-1β and IL-18 (Fig. 2 and 3), we next investigated whether IL-17A induces caspase-1 activation via NLRP3. NLRP3 expression was targeted by MCC950 and dapansutrile. Our results demonstrate that pretreatment with the NLRP3 inhibitors MCC950 and dapansutrile prior to IL-17A addition (experimental design is shown in Fig. 4A) markedly inhibited IL-17A-induced caspace-1 activation, as evidenced by a significant decrease in cleaved caspase-1 levels (Fig. 4B). Using a colorimetric assay, we further confirmed inhibition of caspase-1 activity by the NLRP3 inhibitors (Fig. 4C). However, neither MCC950 nor dapansutrile modulated MIP2 mRNA and protein levels (Fig. 4D) or LDH activity in culture supernatants (Fig. 4E), suggesting that IL-17A-induced IL-1β and IL-18, but not MIP2 expression, is NLRP3-dependent. Together, these results indicate that IL-17A induces caspase-1 activation via NLRP3 (Fig. 4).

3.5. IL-17A induces SMC proliferation and migration via NLRP3 and caspase-1

Since NLRP3 inflammasome serves as a platform for caspase-1 recruitment and activation that cleaves pro-IL-1β and pro-IL-18 to their respective mature and functionally active forms ([36], [37]), we next investigated whether IL-17A induces cytokine expression, and SMC proliferation and migration via NLRP3 and caspase-1. Caspase-1 was targeted by a cell permeable irreversible pan-caspase inhibitor Z-WEHD-FMK and a caspase-1-specific

inhibitor Z-VAD-FMK ([38]). Consistent with the results shown in Fig. 2, exposure to IL-17A, as described in Fig. 5A, significantly increased IL-1β (Fig. 5B) and IL-18 (Fig. 5C) secretion quantified by ELISA and Western blotting, an effect markedly inhibited by pharmacological inhibition of NLRP3, as well as NLRP3 knockdown. Further, pre-treatment with Z-WEHD-FMK and Z-VAD-FMK, at indicated concentrations, each inhibited IL-17A-induced IL-1β (Fig. 5B) and IL-18 (Fig. 5C) secretion. Importantly, targeting NLRP3 and caspase-1 each attenuated IL-17A-induced SMC proliferation (Fig. 5D) and migration (Fig. 5E), without increasing LDH activity (Fig. 5F). These results indicate that IL-17A induces proinflammatory IL-1β and IL-18 expression, and SMC proliferation and migration, in part, via NLRP3-dependent caspase-1 activation (Fig. 5).

3.6. EMPA inhibits IL-17A-induced oxidative stress and TRAF3IP2 expression without inducing cell death

Because SGLT2 inhibitors, including EMPA, exert pleiotropic antioxidant and anti-inflammatory effects independent of their blood sugar lowering properties, we next investigated whether EMPA modulates IL-17A-induced oxidative stress and TRAF3IP2 expression. At first, we investigated whether SMC express SGLT2 and whether IL-17A regulates its expression. Confirming an earlier report ([21]), results show that SMC (experimental design in Fig. 6A) express SGLT2 mRNA and protein at basal conditions (Fig. 6B and 6C), albeit at a lower level than in whole human kidney homogenates and HK-2 cell lysates (~1/10th of that seen in whole kidney and ~1/4th seen in HK-2 cell homogenates) (Fig. 6B). Moreover, its expression was not modulated when culture media was switched from complete medium to conditioning medium. However, when exposed to IL-17A, SGLT2 expression increased both at mRNA and protein levels (Fig. 6C and 6D) in a time dependent manner, with significantly increased protein levels detected after 30 min (Fig. 6D). Its levels remained high even at 24h compared to its basal expression. Further, pre-treatment with EMPA, as described in Fig. 6E, inhibited IL-17A-induced superoxide generation in a dose-dependent manner, with a marked, but similar levels of inhibition seen at both 1 and 5μM concentrations (Fig. 6F). Therefore, in all subsequent experiments, EMPA was used at a clinically relevant 1 μM concentration. At this concentration, EMPA also inhibited IL-17A-induced hydrogen peroxide production (Fig. 6G) and TRAF3IP2 induction (Fig. 6H), without increasing LDH activity in culture supernatants (Fig. 6I), indicating that the observed inhibitory effects of EMPA on oxidative stress and TRAF3IP2 expression are not due to reduced cell viability. Together, these results indicate that SMC express SGLT2 at a low basal level, and increased following exposure to IL-17A. Moreover, EMPA inhibits IL-17A-induced oxidative stress and TRAF3IP2 expression without modulating cell viability (Fig. 6).

3.7. EMPA inhibits IL-17A-induced SMC proliferation and migration

Since IL-17A induced SMC proliferation and migration in part via NLRP3/caspase-1-dependent IL-1β and IL-18 expression (Fig. 2 and Fig. 4), we next investigated whether EMPA exerts anti-mitogenic and anti-migratory effects by targeting NLRP3, caspase-1, and cytokine expression. Consistent with results in Fig. 3B, exposure to IL-17A, as described in Fig. 7A, upregulated NLRP3 expression (Fig. 7B), and pretreatment with EMPA suppressed this effect in a dose-dependent manner (Fig. 7B), with maximal inhibition seen at 1 and 5μM

concentrations, and without inducing cell death at either concentration as evidence by no significant change in LDH activity (Fig. 7C). At 1μM concentration, EMPA also suppressed caspase-1 activation, as evidenced by a marked reduction in cleaved caspase-1 levels (Fig. 7D) and activity (Fig. 7E). EMPA also inhibited IL-1β (Fig. 7F) and IL-18 (Fig. 7G) protein secretion. Importantly, EMPA inhibited IL-17A-induced SMC proliferation (Fig. 7H) and migration (Fig. 7I). Together, these results indicate that EMPA suppresses IL-7A-induced SMC proliferation and migration by targeting NLRP3/caspase-1/IL-1β/IL-18 axis (Fig. 7).

3.8. Silencing SGLT2 attenuates the inhibitory effects of EMPA on IL-17A-induced cytokine secretion and SMC proliferation and migration.

Since EMPA exerted anti-inflammatory, antimotogenic and anti-migratory effects in SMC, and as EMPA signals mainly via SGLT2, we next investigated whether silencing SGLT2 reverses the protective effects of EMPA on IL-17A-induced cytokine secretion and SMC proliferation and migration. Therefore, we silenced SGLT2 using lentiviral transduction of SGLT2-specific shRNA as described in Fig. 8A. Knockdown of SGLT2 was confirmed by Western blotting (Fig. 8B), with MyD988 serving as an off-target. The SGLT2-silenced SMC were then exposed to IL-17A. The secreted IL-1β and IL-18 levels were quantified by ELISA. We also estimated the secreted cytokine levels by Western blotting after concentrating the culture supernatants. The results in Fig. 8C and 8D show that silencing SGLT2 partially blunted the inhibitory effects of EMPA on secreted cytokine levels. Silencing SGLT2 also partially reversed the inhibitory effects of EMPA on IL-17A-induced SMC proliferation (Fig. 8E) and migration (Fig. 8F). Together, these results indicate that EMPA exerts its inhibitory effects on IL-17A-induced cytokine secretion, and SMC proliferation and migration in part via SGLT2 (Fig. 8).

4. Discussion

Persistent inflammation and oxidative stress contribute to the development and progression of vascular proliferative diseases, with SMC proliferation and migration playing a critical role in their pathogenesis. In this mechanistic *in vitro* study, we show that human aortic smooth muscle cells (SMC) express SGLT2 mRNA and protein, and treatment with EMPA, a potent and selective SGLT2 inhibitor used to treat hyperglycemia in diabetic patients ([39–42], attenuates IL-17A/TRAF3IP2-mediated oxidative stress, NLRP3 expression, caspase-1 activation, IL-1β and IL-18 secretion, and SMC migration and proliferation. Interestingly, EMPA exerted these antioxidant, anti-inflammatory, anti-migratory and anti-mitogenic effects under normal glucose conditions and without inducing cell death (Fig. 9). These results support the therapeutic potential of EMPA in the treatment of vascular proliferative diseases.

Chronic oxidative stress contributes to SMC proliferation and migration [43–45]. Here we show that, EMPA inhibits IL-17A-mediated oxidative stress at a clinically relevant dose, as indicated by a significant reduction in superoxide and hydrogen peroxide production, and TRAF3IP2 induction. These results support earlier reports that demonstrated that the SGLT2 inhibitors inhibit oxidative stress both *in vitro* and *in vivo* ([46–49], via inhibition of pro-oxidant enzyme systems or potentiation of antioxidant defenses. NADPH oxidases (NOX)

play a major role in ROS generation and oxidative stress ([50, 51]), and the initial generation of ROS by NOXes amplifies oxidative stress by further enhancing ROS generation by other pro-oxidant systems [52]. Here we show that EMPA inhibits IL-17A-induced NOX2-dependent superoxide generation in SMC. Similar results were reported previously, where EMPA improved chronic hyperglycemia-induced vascular dysfunction by targeting NOX2 activity ([53]), further confirming the antioxidant effects of EMPA. Here we report that, in addition to inhibition of NOX2, EMPA also inhibits IL-17A-mediated NOX4-dependent hydrogen peroxide production in SMC. This confirms our earlier results, where EMPA inhibited hyperglycemia-induced NOX4-mediated hydrogen peroxide production in a human renal proximal tubular epithelial cell line ([32]). Though we focused on NOX2- and NOX4-mediated oxidative stress in the current investigation, SGLT2 inhibitors have also been shown to inhibit ROS generation and oxidative stress by targeting the polyol pathway, mitochondrial dysfunction, iNOS induction, NO generation, and peroxynitrite formation in diverse cell types (reviewed in [54]).

In fact, the outcomes of the EMPA-REG (Empagliflozin Multicenter, International, Randomized, Parallel Group, Double Blind Cardiovascular Safety Study) and CANVAS trials have suggested that suppression of oxidative stress by SGLT2 inhibitors is one of the underlying mechanisms that contributed to reduced cardiovascular events, emphasizing the pleiotropic antioxidant potential of EMPA [55, 56]. In addition to inhibition of oxidative stress, we also report for the first time that EMPA inhibits TRAF3IP2 expression in SMC. These results are consistent with our previous report demonstrating inhibition of TRAF3IP2 in high glucose-treated human proximal tubule epithelial cells ([32]). TRAF3IP2 is a signaling intermediate critical in IL-17A signaling. TRAF3IP2 physically associates with IL-17 receptors through SEFIR (**S**imilar **E**xpression to **F**ibroblast growth factor genes and **I**nterleukin-17 **R**eceptor)-SEFIR interaction and activates multiple downstream oxidative stress-responsive proinflammatory pathways [57]. We and others have demonstrated TRAF3IP2-dependent activation of proinflammatory transcription factors and stress-activated kinases, including NF-κB, AP-1, and p38 MAPK, in cells of vascular and non-vascular origin. Of note, NF-κB, AP-1, and p38 MAPK have all been shown to regulate NOX2 and NOX4 expression and oxidant stress in various cell types. Moreover, SMC-specific inhibition of NF-κB by overexpressing a truncated version of IκB attenuated phenotypic switching of SMC *in vitro* and neointima formation *in vivo* following injury ([58]). Interestingly, TRAF3IP2 is an oxidative stress-responsive adapter molecule, indicating that it acts both upstream and downstream of oxidative stress. In fact, the promoter region of TRAF3IP2 contains binding sites for several oxidative stress-responsive cis-regulatory elements, including AP-1, IRF-1, and c/EBP. High glucose-induced endothelial dysfunction is mediated via AP-1-, IRF-1-, c/EBP-1-dependent TRAF3IP2 expression as we reported previously [11]. The results from this and other studies indicate that EMPA exerts pleiotropic antioxidant and anti-inflammatory effects in vascular and other cell types under both high and normal glucose conditions.

In general, oxidative stress and inflammation co-exist and their persistent induction contributes to the pathogenesis of various chronic inflammatory diseases, including vascular proliferative diseases. Of note, inflammatory mediators also contribute to oxidative stress;

they increase free radical generation, including NOX2- and NOX4-dependent superoxide and hydrogen peroxide production, respectively [59, 60]. Here we show that EMPA inhibits the proinflammatory and pro-atherogenic cytokines IL-1β and IL-18 in SMC by inhibiting NLRP3 expression. These results support a prior report demonstrating EMPA-mediated inhibition of NLRP3 inflammasome activation in liver and kidney, but not heart, in a murine model of diet (high fat/high sugar)-induced obesity and insulin resistance [26]. Herein, we have not specifically investigated the molecular mechanisms underlying EMPA-mediated downregulation of NLRP3 expression and activation. As such, we hypothesize that reduced oxidative stress and inhibition of NF-κB and AP-1 activation contributed to EMPA-mediated NLRP3 downregulation, given that the *NLRP3* promoter region contains multiple NF-κB-binding sites [61] and putative binding sites for AP-1, Sp1, cMyb, and c-ETS [62].

Consequent to inhibition of NLRP3 expression, EMPA attenuated IL-17A-mediated IL-1β and IL-18 expression. Both cytokines are induced following vascular injury and play a role in intimal hyperplasia, either alone or in combination with growth factors [63, 64]. We previously reported that IL-18 enhances IL-18R/NOX1 physical association, TRAF3IP2 induction, and SMC migration [65], indicating that oxidative stress and TRAF3IP2 each contribute to the promigratory effects of IL-18. We further demonstrated that targeting NF-κB and AP-1 inhibit IL-18-induced MMP-9 expression and SMC migration [66]. Of note, *Il18* is also an NF-κB and AP-1-responsive gene, indicating that induction of TRAF3IP2, activation of NF-κB and AP-1, and upregulation of inflammatory cytokines collectively perpetuate inflammation and oxidative stress in vasculature, ultimately resulting in vascular proliferative diseases, characterized by SMC proliferation and migration.

IL-17A stimulated IL-1β secretion in an NLRP3-dependent manner, and like IL-18, *Il1b* is also an AP-1 and NF-κB responsive gene. IL-1β has been previously shown to play a role in porcine artery response to injury [67] and carotid artery ligation-induced neointima formation [68]. Moreover, high levels of IL-1β were detected in human atherosclerotic coronary arteries [69]. IL-1β is also shown to promote SMC migration [70], and promote SMC proliferation by enhancing oxidative stress and the induction of multiple inflammatory mediators in SMC [71]. Consistent with published reports, our data show that IL-17A induced SMC migration and proliferation, in part, via IL-1β. Importantly, we show that EMPA inhibits inducible IL-1β expression and secretion. Since EMPA also inhibits the expression of multiple inflammatory mediators that are known to contribute to vascular proliferative diseases, such as TNF-α, IL-6, and MCP-1, one can argue that EMPA has the potential to inhibit the progression of vascular proliferative diseases by targeting oxidative stress-responsive pro-inflammatory mediators, including IL-18 and IL-1β in SMC.

In the current in vitro investigation, we have demonstrated that IL-17A-induced SMC proliferation and migration are mediated in part via NLRP3/Caspase-1-dependent IL-1β and IL-18 expression. However, in vivo, monocyte/macrophage activation is evident in vascular proliferative diseases, and these leukocytic cell types are generally regarded as more efficient sources of mature IL-1β and IL-18 as compared to SMC. For example, in human atherectomy samples, IL-18 expression was predominantly seen in macrophages and correlated with plaque instability [72]. In another study, macrophages in atheroma were shown to express the mature form of IL-18, while SMC in the lesions expressed its receptors

[73], indicative of paracrine effects of macrophage-secreted mature IL-18 on IL-18R expressing SMC. Similarly, macrophages are shown to be the predominant source of IL-1β in vascular proliferative diseases. For example, macrophages isolated from subjects with restenosis following angioplasty expressed high levels of IL-1β [74]. Increased IL-1β has also been demonstrated in human atherosclerotic lesions [69], localized predominantly to macrophages and endothelial cells. Its increased circulating levels also serve as a risk factor for coronary artery disease in hypertensive subjects [75]. Together, data from the current investigation and published reports indicate that IL-1β and IL-18 expressed and secreted by multiple cell types contribute to vascular proliferative diseases.

Here we have demonstrated that EMPA exerts its beneficial effects in SMC in part via SGLT2, as silencing SGLT2 did not abrogate its protective effects, indicating that some of its protective effects are mediated in SGLT2-independent manner. In fact, in many studies where SGLT2 inhibitors have shown protective effects, whether their beneficial effects are mediated via SGLT2 are not investigated. For example, the SGLT2 inhibitors EMPA and dapagliflozin are shown to inhibit stearic acid-induced oxidative stress and inflammatory cytokine expression in myeloid angiogenic cells and platelets [76]. Interestingly, neither cell type expressed SGLT2 mRNA or protein. However, both cell types expressed Sodium/ Hydrogen Exchanger 1 (NHE-1), a member of the solute carrier family 9, that plays a role in pH homeostasis and cell migration [77]. Amiloride, a broad spectrum NHE inhibitor and Cariporide, an NHE-1-specific inhibitor, each suppressed stearic acid-induced inflammatory markers [76], indicating that EMPA and dapagliflozin rather functioned through inhibition of NHE, but not SGLT2, in those cell types. Further, using *in silico* analysis of SGLT2 inhibitor binding to a homology model of NHE, those authors also reported that EMPA and other SGLT2 inhibitors could efficiently bind the Na^+-binding pocket of NHE. Of note, vascular SMC express NHE-1, but not NHE-2, NHE-3 or NHE-4 [78], and inhibition of NHE-1 by amiloride or dimethyl amiloride blunts lysophosphatidic acid-induced vascular SMC proliferation [79]. Therefore, it appears that EMPA and other SGLT2 inhibitors act via SGLT2 and/or NHE in cell types other than renal proximal tubule epithelial cells. Interestingly, however, dapagliflozin has been shown to inhibit NHE-1 expression in LPS-treated cultured cardiac fibroblasts [80], suggesting cell type-specific effects of SGLT2 inhibitors on NHE-1 expression and/or activation.

Our study has some limitations: (i) This is an *in vitro* mechanistic study using primary human aortic SMC and this model may not recapitulate all the complex pathophysiological process regulated in a diseased vessel. However, it can also be considered a strength, as we have investigated the effects of IL-17A and EMPA in a pure population of SMC, ruling out the potential paracrine effects and cell-cell interactions with other vascular cells on SMC proliferation and migration. (ii) Confirming an earlier report [21], we have demonstrated the expression of SGLT2 by RT-qPCR and Western blotting in SMC, but at a level lower than in whole kidney and a proximal tubule epithelial cell line. Importantly, we have also demonstrated that IL-17A induces its expression. Interestingly, the inhibitory effects of EMPA were partially attenuated when SGLT2 was silenced. Since SMC also express NHE-1 [78], an off-target of SGLT2 inhibitors, it is plausible that EMPA might function via both SGLT2 and NHE-1 in SMC (Fig. 9). Our future studies will determine whether amiloride potentiates the inhibitory effects of EMPA on IL-17A-induced NLRP3 inflammasome

activation, inflammatory cytokine expression, and SMC proliferation and migration. Another limitation of the study is that (iii) though we have demonstrated for the first time that IL-17A induces SGLT2 expression at both mRNA and protein levels, we have not investigated whether this increased expression is due to enhanced transcription or mRNA stability or both. Of note, administration of LPS, a known inducer of pro-inflammatory mediators, has been shown to suppress SGLT2 expression in mouse kidneys [81]. In that study, injection of cytokines, such as IL-1, IL-6, or TNF-α, also suppressed SGLT2 expression in kidneys [81]. In contrast, IL-6 and TNF-α were shown to induce SGLT2 expression in cultured porcine kidney epithelial cells under normal glucose conditions, but in a delayed manner [82]. Moreover, insulin has been shown to induce SGLT2 expression via oxidative stress in proximal tubule epithelial cells [82]. These conflicting results suggest that the regulation of SGLT2 is complex, and the signaling pathways underlying its expression and regulation need a thorough investigation. While we focused mainly on EMPA-mediated inhibition of IL-17A-induced oxidative stress, reduced oxidative stress also results from enhanced antioxidant defenses. Therefore, in future studies, (iv) we will investigate whether EMPA, which inhibited NOX2- and NOX4-mediated superoxide and hydrogen peroxide production, also blunts IL-17A-induced oxidative stress by enhancing or restoring the expression and activity of antioxidant enzymes, such as catalase, glutathione peroxide, Mn-SOD, and Cu/ZN SOD, that are shown to be enhanced in diabetic mice treated with a SGLT2 inhibitor [47].

In summary, our results demonstrate that EMPA exerts pleiotropic effects, and inhibits SMC migration and proliferation by targeting IL-17A-mediated oxidative stress, NLRP3 expression, and inflammation under normal glucose conditions and without inducing cell death. These results suggest the therapeutic potential of EMPA in vascular proliferative diseases.

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Abbreviations

Act1	Nuclear Factor NF-Kappa-B Activator 1
ApoE	Apolipoprotein E
ASK1	Apoptosis Signal Regulated Kinase 1
CIKS	Connection to IKK and SAPK/JNK
CXCL2	C-X-C Motif Chemokine Ligand 2
DMSO	Dimethyl Sulfoxide
EMPA	Empagliflozin

IL, IKK	IκB Kinase; Interleukin
LDH	Lactate Dehydrogenase
MIP	Macrophage Inflammatory Protein
MMP	matrix metallopeptidase
moi	multiplicity of infection
NHE	Na^+/H^+ Exchanger
NLRP3	Nucleotide-binding domain and Leucine-rich repeat containing Family Pyrin Domain Containing 3
PDGF	Platelet Derived Growth Factor
ROS	Reactive oxygen species
SAPK	Stress-Activated Protein Kinase
SEFIR	similar expression to fibroblast growth factor genes) and IL-17R
SGLT2	Sodium/Glucose Cotransporter 2
SLC5A2	Solute Carrier Family 5 Member 2
αSMA	alpha smooth muscle actin
SMC	Human aortic smooth muscle cells
SM-MHC	Smooth Muscle Myosin Heavy Chain
TRAF	Tumor Necrosis Factor Receptor Associated Factor
TRAF3IP2	TRAF3 Interacting Protein 2
VWF	Von Willebrand Factor

References

[1]. Venkatachalam K, Mummidi S, Cortez DM, Prabhu SD, Valente AJ, Chandrasekar B, Resveratrol inhibits high glucose-induced PI3K/Akt/ERK-dependent interleukin-17 expression in primary mouse cardiac fibroblasts, American journal of physiology. Heart and circulatory physiology 294(5) (2008) H2078–87. [PubMed: 18310510]

[2]. Brauner S, Jiang X, Thorlacius GE, Lundberg AM, Östberg T, Yan ZQ, Kuchroo VK, Hansson GK, Wahren-Herlenius M, Augmented Th17 differentiation in Trim21 deficiency promotes a stable phenotype of atherosclerotic plaques with high collagen content, Cardiovascular research 114(1) (2018) 158–167. [PubMed: 29016728]

[3]. Erbel C, Dengler TJ, Wangler S, Lasitschka F, Bea F, Wambsganss N, Hakimi M, Böckler D, Katus HA, Gleissner CA, Expression of IL-17A in human atherosclerotic lesions is associated with increased inflammation and plaque vulnerability, Basic research in cardiology 106(1) (2011) 125–34. [PubMed: 21116822]

[4]. Nordlohne J, Helmke A, Ge S, Rong S, Chen R, Waisman A, Haller H, von Vietinghoff S, Aggravated Atherosclerosis and Vascular Inflammation With Reduced Kidney Function Depend

on Interleukin-17 Receptor A and Are Normalized by Inhibition of Interleukin-17A, JACC. Basic to translational science 3(1) (2018) 54–66. [PubMed: 30062194]

[5]. Mummidi S, Das NA, Carpenter AJ, Yoshida T, Yariswamy M, Mostany R, Izadpanah R, Higashi Y, Sukhanov S, Noda M, Siebenlist U, Rector RS, Chandrasekar B, RECK suppresses interleukin-17/TRAF3IP2-mediated MMP-13 activation and human aortic smooth muscle cell migration and proliferation, Journal of cellular physiology 234(12) (2019) 22242–22259. [PubMed: 31074012]

[6]. Valente AJ, Irimpen AM, Siebenlist U, Chandrasekar B, OxLDL induces endothelial dysfunction and death via TRAF3IP2: inhibition by HDL3 and AMPK activators, Free radical biology & medicine 70 (2014) 117–28. [PubMed: 24561578]

[7]. Valente AJ, Yoshida T, Clark RA, Delafontaine P, Siebenlist U, Chandrasekar B, Advanced oxidation protein products induce cardiomyocyte death via Nox2/Rac1/superoxide-dependent TRAF3IP2/JNK signaling, Free radical biology & medicine 60 (2013) 125–35. [PubMed: 23453926]

[8]. Somanna NK, Yariswamy M, Garagliano JM, Siebenlist U, Mummidi S, Valente AJ, Chandrasekar B, Aldosterone-induced cardiomyocyte growth, and fibroblast migration and proliferation are mediated by TRAF3IP2, Cellular signalling 27(10) (2015) 1928–38. [PubMed: 26148936]

[9]. Padilla J, Carpenter AJ, Das NA, Kandikattu HK, López-Ongil S, Martinez-Lemus LA, Siebenlist U, DeMarco VG, Chandrasekar B, TRAF3IP2 mediates high glucose-induced endothelin-1 production as well as endothelin-1-induced inflammation in endothelial cells, American journal of physiology. Heart and circulatory physiology 314(1) (2018) H52–h64. [PubMed: 28971844]

[10]. Valente AJ, Sakamuri SS, Siddesha JM, Yoshida T, Gardner JD, Prabhu R, Siebenlist U, Chandrasekar B, TRAF3IP2 mediates interleukin-18-induced cardiac fibroblast migration and differentiation, Cellular signalling 25(11) (2013) 2176–84. [PubMed: 23872479]

[11]. Venkatesan B, Valente AJ, Das NA, Carpenter AJ, Yoshida T, Delafontaine JL, Siebenlist U, Chandrasekar B, CIKS (Act1 or TRAF3IP2) mediates high glucose-induced endothelial dysfunction, Cellular signalling 25(1) (2013) 359–71. [PubMed: 23085260]

[12]. Sakamuri S, Higashi Y, Sukhanov S, Siddesha JM, Delafontaine P, Siebenlist U, Chandrasekar B, TRAF3IP2 mediates atherosclerotic plaque development and vulnerability in ApoE(−/−) mice, Atherosclerosis 252 (2016) 153–160. [PubMed: 27237075]

[13]. Erikson JM, Valente AJ, Mummidi S, Kandikattu HK, DeMarco VG, Bender SB, Fay WP, Siebenlist U, Chandrasekar B, Targeting TRAF3IP2 by Genetic and Interventional Approaches Inhibits Ischemia/Reperfusion-induced Myocardial Injury and Adverse Remodeling, The Journal of biological chemistry 292(6) (2017) 2345–2358. [PubMed: 28053087]

[14]. Packer M, Anker SD, Butler J, Filippatos G, Zannad F, Effects of Sodium-Glucose Cotransporter 2 Inhibitors for the Treatment of Patients With Heart Failure: Proposal of a Novel Mechanism of Action, JAMA cardiology 2(9) (2017) 1025–1029. [PubMed: 28768320]

[15]. Täger T, Atar D, Agewall S, Katus HA, Grundtvig M, Cleland JGF, Clark AL, Fröhlich H, Frankenstein L, Comparative efficacy of sodium-glucose cotransporter-2 inhibitors (SGLT2i) for cardiovascular outcomes in type 2 diabetes: a systematic review and network meta-analysis of randomised controlled trials, Heart failure reviews (2020).

[16]. Verma S, Sharma A, Zinman B, Ofstad AP, Fitchett D, Brueckmann M, Wanner C, Zwiener I, George JT, Inzucchi SE, Butler J, Mazer CD, Empagliflozin reduces the risk of mortality and hospitalization for heart failure across Thrombolysis In Myocardial Infarction Risk Score for Heart Failure in Diabetes categories: Post hoc analysis of the EMPA-REG OUTCOME trial, Diabetes, obesity & metabolism 22(7) (2020) 1141–1150.

[17]. Steven S, Frenis K, Oelze M, Kalinovic S, Kuntic M, Bayo Jimenez MT, Vujacic-Mirski K, Helmstädter J, Kröller-Schön S, Münzel T, Daiber A, Vascular Inflammation and Oxidative Stress: Major Triggers for Cardiovascular Disease, Oxidative medicine and cellular longevity 2019 (2019) 7092151. [PubMed: 31341533]

[18]. Chen J, Williams S, Ho S, Loraine H, Hagan D, Whaley JM, Feder JN, Quantitative PCR tissue expression profiling of the human SGLT2 gene and related family members, Diabetes therapy : research, treatment and education of diabetes and related disorders 1(2) (2010) 57–92.

[19]. Díaz-Rodríguez E, Agra RM, Fernández Á L, Adrio B, García-Caballero T, González-Juanatey JR, Eiras S, Effects of dapagliflozin on human epicardial adipose tissue: modulation of insulin

resistance, inflammatory chemokine production, and differentiation ability, Cardiovascular research 114(2) (2018) 336–346. [PubMed: 29016744]

[20]. Wakisaka M, Nagao T, Yoshinari M, Sodium Glucose Cotransporter 2 (SGLT2) Plays as a Physiological Glucose Sensor and Regulates Cellular Contractility in Rat Mesangial Cells, PloS one 11(3) (2016) e0151585. [PubMed: 26999015]

[21]. Takahashi H, Nomiyama T, Terawaki Y, Horikawa T, Kawanami T, Hamaguchi Y, Tanaka T, Motonaga R, Fukuda T, Tanabe M, Yanase T, Combined treatment with DPP-4 inhibitor linagliptin and SGLT2 inhibitor empagliflozin attenuates neointima formation after vascular injury in diabetic mice, Biochemistry and biophysics reports 18 (2019) 100640. [PubMed: 31032431]

[22]. Libby P, Interleukin-1 Beta as a Target for Atherosclerosis Therapy: Biological Basis of CANTOS and Beyond, Journal of the American College of Cardiology 70(18) (2017) 2278–2289. [PubMed: 29073957]

[23]. Merhi-Soussi F, Kwak BR, Magne D, Chadjichristos C, Berti M, Pelli G, James RW, Mach F, Gabay C, Interleukin-1 plays a major role in vascular inflammation and atherosclerosis in male apolipoprotein E-knockout mice, Cardiovascular research 66(3) (2005) 583–93. [PubMed: 15914123]

[24]. Whitman SC, Ravisankar P, Daugherty A, Interleukin-18 enhances atherosclerosis in apolipoprotein E(−/−) mice through release of interferon-gamma, Circulation research 90(2) (2002) E34–8. [PubMed: 11834721]

[25]. Keller M, Rüegg A, Werner S, Beer HD, Active caspase-1 is a regulator of unconventional protein secretion, Cell 132(5) (2008) 818–31. [PubMed: 18329368]

[26]. Benetti E, Mastrocola R, Vitarelli G, Cutrin JC, Nigro D, Chiazza F, Mayoux E, Collino M, Fantozzi R, Empagliflozin Protects against Diet-Induced NLRP-3 Inflammasome Activation and Lipid Accumulation, The Journal of pharmacology and experimental therapeutics 359(1) (2016) 45–53. [PubMed: 27440421]

[27]. Franchi L, Eigenbrod T, Muñoz-Planillo R, Nuñez G, The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis, Nature immunology 10(3) (2009) 241–7. [PubMed: 19221555]

[28]. Scheen AJ, Pharmacokinetic and pharmacodynamic profile of empagliflozin, a sodium glucose co-transporter 2 inhibitor, Clinical pharmacokinetics 53(3) (2014) 213–225. [PubMed: 24430725]

[29]. Marchetti C, Swartzwelter B, Gamboni F, Neff CP, Richter K, Azam T, Carta S, Tengesdal I, Nemkov T, D'Alessandro A, Henry C, Jones GS, Goodrich SA, St Laurent JP, Jones TM, Scribner CL, Barrow RB, Altman RD, Skouras DB, Gattorno M, Grau V, Janciauskiene S, Rubartelli A, Joosten LAB, Dinarello CA, OLT1177, a β-sulfonyl nitrile compound, safe in humans, inhibits the NLRP3 inflammasome and reverses the metabolic cost of inflammation, Proceedings of the National Academy of Sciences of the United States of America 115(7) (2018) E1530–e1539. [PubMed: 29378952]

[30]. Reddy VS, Harskamp RE, van Ginkel MW, Calhoon J, Baisden CE, Kim IS, Valente AJ, Chandrasekar B, Interleukin-18 stimulates fibronectin expression in primary human cardiac fibroblasts via PI3K-Akt-dependent NF-kappaB activation, Journal of cellular physiology 215(3) (2008) 697–707. [PubMed: 18064631]

[31]. Somanna NK, Valente AJ, Krenz M, Fay WP, Delafontaine P, Chandrasekar B, The Nox1/4 Dual Inhibitor GKT137831 or Nox4 Knockdown Inhibits Angiotensin-II-Induced Adult Mouse Cardiac Fibroblast Proliferation and Migration. AT1 Physically Associates With Nox4, Journal of cellular physiology 231(5) (2016) 1130–41. [PubMed: 26445208]

[32]. Das NA, Carpenter AJ, Belenchia A, Aroor AR, Noda M, Siebenlist U, Chandrasekar B, DeMarco VG, Empagliflozin reduces high glucose-induced oxidative stress and miR-21-dependent TRAF3IP2 induction and RECK suppression, and inhibits human renal proximal tubular epithelial cell migration and epithelial-to-mesenchymal transition, Cellular signalling 68 (2020) 109506. [PubMed: 31862399]

[33]. Higashi Y, Mummidi S, Sukhanov S, Yoshida T, Noda M, Delafontaine P, Chandrasekar B, Minocycline inhibits PDGF-BB-induced human aortic smooth muscle cell proliferation and

migration by reversing miR-221- and −222-mediated RECK suppression, Cellular signalling 57 (2019) 10–20. [PubMed: 30716386]

[34]. Uthman L, Homayr A, Juni RP, Spin EL, Kerindongo R, Boomsma M, Hollmann MW, Preckel B, Koolwijk P, van Hinsbergh VWM, Zuurbier CJ, Albrecht M, Weber NC, Empagliflozin and Dapagliflozin Reduce ROS Generation and Restore NO Bioavailability in Tumor Necrosis Factor α-Stimulated Human Coronary Arterial Endothelial Cells, Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology 53(5) (2019) 865–886.

[35]. Schroder K, Tschopp J, The inflammasomes, Cell 140(6) (2010) 821–32. [PubMed: 20303873]

[36]. Martinon F, Burns K, Tschopp J, The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta, Molecular cell 10(2) (2002) 417–26. [PubMed: 12191486]

[37]. Wannamaker W, Davies R, Namchuk M, Pollard J, Ford P, Ku G, Decker C, Charifson P, Weber P, Germann UA, Kuida K, Randle JC, (S)-1-((S)-2-{[1-(4-amino-3-chloro-phenyl)-methanoyl]-amino}-3,3-dimethyl-butanoyl)-pyrrolidine-2-carboxylic acid ((2R,3S)-2-ethoxy-5-oxo-tetrahydro-furan-3-yl)-amide (VX-765), an orally available selective interleukin (IL)-converting enzyme/caspase-1 inhibitor, exhibits potent anti-inflammatory activities by inhibiting the release of IL-1beta and IL-18, The Journal of pharmacology and experimental therapeutics 321(2) (2007) 509–16. [PubMed: 17289835]

[38]. Chandrasekar B, Vemula K, Surabhi RM, Li-Weber M, Owen-Schaub LB, Jensen LE, Mummidi S, Activation of intrinsic and extrinsic proapoptotic signaling pathways in interleukin-18-mediated human cardiac endothelial cell death, The Journal of biological chemistry 279(19) (2004) 20221–33. [PubMed: 14960579]

[39]. Kluger AY, Tecson KM, Lee AY, Lerma EV, Rangaswami J, Lepor NE, Cobble ME, McCullough PA, Class effects of SGLT2 inhibitors on cardiorenal outcomes, Cardiovascular diabetology 18(1) (2019) 99. [PubMed: 31382965]

[40]. Zinman B, Wanner C, Lachin JM, Fitchett D, Bluhmki E, Hantel S, Mattheus M, Devins T, Johansen OE, Woerle HJ, Broedl UC, Inzucchi SE, Empagliflozin, Cardiovascular Outcomes, and Mortality in Type 2 Diabetes, The New England journal of medicine 373(22) (2015) 2117–28. [PubMed: 26378978]

[41]. Cherney DZI, Zinman B, Inzucchi SE, Koitka-Weber A, Mattheus M, von Eynatten M, Wanner C, Effects of empagliflozin on the urinary albumin-to-creatinine ratio in patients with type 2 diabetes and established cardiovascular disease: an exploratory analysis from the EMPA-REG OUTCOME randomised, placebo-controlled trial, The lancet. Diabetes & endocrinology 5(8) (2017) 610–621. [PubMed: 28666775]

[42]. Neal B, Perkovic V, Mahaffey KW, de Zeeuw D, Fulcher G, Erondu N, Shaw W, Law G, Desai M, Matthews DR, Canagliflozin and Cardiovascular and Renal Events in Type 2 Diabetes, The New England journal of medicine 377(7) (2017) 644–657. [PubMed: 28605608]

[43]. San Martín A, Griendling KK, Redox control of vascular smooth muscle migration, Antioxidants & redox signaling 12(5) (2010) 625–40. [PubMed: 19737088]

[44]. Laurindo FR, da Luz PL, Uint L, Rocha TF, Jaeger RG, Lopes EA, Evidence for superoxide radical-dependent coronary vasospasm after angioplasty in intact dogs, Circulation 83(5) (1991) 1705–15. [PubMed: 1850666]

[45]. Souza HP, Souza LC, Anastacio VM, Pereira AC, Junqueira ML, Krieger JE, da Luz PL, Augusto O, Laurindo FR, Vascular oxidant stress early after balloon injury: evidence for increased NAD(P)H oxidoreductase activity, Free radical biology & medicine 28(8) (2000) 1232–42. [PubMed: 10889453]

[46]. Ishibashi Y, Matsui T, Yamagishi S, Tofogliflozin A Highly Selective Inhibitor of SGLT2 Blocks Proinflammatory and Proapoptotic Effects of Glucose Overload on Proximal Tubular Cells Partly by Suppressing Oxidative Stress Generation, Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme 48(3) (2016) 191–5. [PubMed: 26158396]

[47]. Sugizaki T, Zhu S, Guo G, Matsumoto A, Zhao J, Endo M, Horiguchi H, Morinaga J, Tian Z, Kadomatsu T, Miyata K, Itoh H, Oike Y, Treatment of diabetic mice with the SGLT2 inhibitor TA-1887 antagonizes diabetic cachexia and decreases mortality, NPJ aging and mechanisms of disease 3 (2017) 12. [PubMed: 28900540]

[48]. Tahara A, Kurosaki E, Yokono M, Yamajuku D, Kihara R, Hayashizaki Y, Takasu T, Imamura M, Li Q, Tomiyama H, Kobayashi Y, Noda A, Sasamata M, Shibasaki M, Effects of SGLT2 selective inhibitor ipragliflozin on hyperglycemia, hyperlipidemia, hepatic steatosis, oxidative stress, inflammation, and obesity in type 2 diabetic mice, European journal of pharmacology 715(1–3) (2013) 246–55. [PubMed: 23707905]

[49]. Terami N, Ogawa D, Tachibana H, Hatanaka T, Wada J, Nakatsuka A, Eguchi J, Horiguchi CS, Nishii N, Yamada H, Takei K, Makino H, Long-term treatment with the sodium glucose cotransporter 2 inhibitor, dapagliflozin, ameliorates glucose homeostasis and diabetic nephropathy in db/db mice, PloS one 9(6) (2014) e100777. [PubMed: 24960177]

[50]. Konior A, Schramm A, Czesnikiewicz-Guzik M, Guzik TJ, NADPH oxidases in vascular pathology, Antioxidants & redox signaling 20(17) (2014) 2794–814. [PubMed: 24180474]

[51]. Szöcs K, Lassègue B, Sorescu D, Hilenski LL, Valppu L, Couse TL, Wilcox JN, Quinn MT, Lambeth JD, Griendling KK, Upregulation of Nox-based NAD(P)H oxidases in restenosis after carotid injury, Arteriosclerosis, thrombosis, and vascular biology 22(1) (2002) 21–7.

[52]. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, Harrison DG, Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension, The Journal of clinical investigation 111(8) (2003) 1201–9. [PubMed: 12697739]

[53]. Oelze M, Kröller-Schön S, Welschof P, Jansen T, Hausding M, Mikhed Y, Stamm P, Mader M, Zinßius E, Agdauletova S, Gottschlich A, Steven S, Schulz E, Bottari SP, Mayoux E, Münzel T, Daiber A, The sodium-glucose co-transporter 2 inhibitor empagliflozin improves diabetes-induced vascular dysfunction in the streptozotocin diabetes rat model by interfering with oxidative stress and glucotoxicity, PloS one 9(11) (2014) e112394. [PubMed: 25402275]

[54]. Yaribeygi H, Atkin SL, Butler AE, Sahebkar A, Sodium-glucose cotransporter inhibitors and oxidative stress: An update, Journal of cellular physiology 234(4) (2019) 3231–3237. [PubMed: 30443936]

[55]. Edwards JL, Empagliflozin, Cardiovascular Outcomes, and Mortality in Type 2 Diabetes, The New England journal of medicine 374(11) (2016) 1093.

[56]. Rajagopalan S, Brook R, Canagliflozin and Cardiovascular and Renal Events in Type 2 Diabetes, The New England journal of medicine 377(21) (2017) 2098–9.

[57]. Liu C, Swaidani S, Qian W, Kang Z, Sun P, Han Y, Wang C, Gulen MF, Yin W, Zhang C, Fox PL, Aronica M, Hamilton TA, Misra S, Deng J, Li X, A CC' loop decoy peptide blocks the interaction between Act1 and IL-17RA to attenuate IL-17- and IL-25-induced inflammation, Science signaling 4(197) (2011) ra72. [PubMed: 22045852]

[58]. Yoshida T, Yamashita M, Horimai C, Hayashi M, Smooth muscle-selective inhibition of nuclear factor-κB attenuates smooth muscle phenotypic switching and neointima formation following vascular injury, Journal of the American Heart Association 2(3) (2013) e000230. [PubMed: 23702880]

[59]. Sies H, Jones DP, Reactive oxygen species (ROS) as pleiotropic physiological signalling agents, Nature reviews. Molecular cell biology 21(7) (2020) 363–383. [PubMed: 32231263]

[60]. Meier B, Radeke HH, Selle S, Younes M, Sies H, Resch K, Habermehl GG, Human fibroblasts release reactive oxygen species in response to interleukin-1 or tumour necrosis factor-alpha, The Biochemical journal 263(2) (1989) 539–45. [PubMed: 2556998]

[61]. Wang S, Xie X, Lei T, Zhang K, Lai B, Zhang Z, Guan Y, Mao G, Xiao L, Wang N, Statins Attenuate Activation of the NLRP3 Inflammasome by Oxidized LDL or TNFα in Vascular Endothelial Cells through a PXR-Dependent Mechanism, Molecular pharmacology 92(3) (2017) 256–264. [PubMed: 28546421]

[62]. Anderson JP, Mueller JL, Misaghi A, Anderson S, Sivagnanam M, Kolodner RD, Hoffman HM, Initial description of the human NLRP3 promoter, Genes and immunity 9(8) (2008) 721–6. [PubMed: 18719602]

[63]. Nathe TJ, Deou J, Walsh B, Bourns B, Clowes AW, Daum G, Interleukin-1beta inhibits expression of p21(WAF1/CIP1) and p27(KIP1) and enhances proliferation in response to platelet-derived growth factor-BB in smooth muscle cells, Arteriosclerosis, thrombosis, and vascular biology 22(8) (2002) 1293–8.

[64]. Liu W, Liu Y, Jiang H, Ding X, Zhu R, Li B, Zhao Y, Plasma levels of interleukin 18, interleukin 10, and matrix metalloproteinase-9 and −137G/C polymorphism of interleukin 18 are associated with incidence of in-stent restenosis after percutaneous coronary intervention, Inflammation 36(5) (2013) 1129–35. [PubMed: 23636637]

[65]. Valente AJ, Yoshida T, Izadpanah R, Delafontaine P, Siebenlist U, Chandrasekar B, Interleukin-18 enhances IL-18R/Nox1 binding, and mediates TRAF3IP2-dependent smooth muscle cell migration. Inhibition by simvastatin, Cellular signalling 25(6) (2013) 1447–56. [PubMed: 23541442]

[66]. Chandrasekar B, Mummidi S, Mahimainathan L, Patel DN, Bailey SR, Imam SZ, Greene WC, Valente AJ, Interleukin-18-induced human coronary artery smooth muscle cell migration is dependent on NF-kappaB- and AP-1-mediated matrix metalloproteinase-9 expression and is inhibited by atorvastatin, The Journal of biological chemistry 281(22) (2006) 15099–109. [PubMed: 16554298]

[67]. Morton AC, Arnold ND, Gunn J, Varcoe R, Francis SE, Dower SK, Crossman DC, Interleukin-1 receptor antagonist alters the response to vessel wall injury in a porcine coronary artery model, Cardiovascular research 68(3) (2005) 493–501. [PubMed: 16099441]

[68]. Chamberlain J, Evans D, King A, Dewberry R, Dower S, Crossman D, Francis S, Interleukin-1beta and signaling of interleukin-1 in vascular wall and circulating cells modulates the extent of neointima formation in mice, The American journal of pathology 168(4) (2006) 1396–403. [PubMed: 16565512]

[69]. Galea J, Armstrong J, Gadsdon P, Holden H, Francis SE, Holt CM, Interleukin-1 beta in coronary arteries of patients with ischemic heart disease, Arteriosclerosis, thrombosis, and vascular biology 16(8) (1996) 1000–6.

[70]. Nomoto A, Mutoh S, Hagihara H, Yamaguchi I, Smooth muscle cell migration induced by inflammatory cell products and its inhibition by a potent calcium antagonist, nilvadipine, Atherosclerosis 72(2–3) (1988) 213–9. [PubMed: 2850808]

[71]. Lin SJ, Yen HT, Chen YH, Ku HH, Lin FY, Chen YL, Expression of interleukin-1 beta and interleukin-1 receptor antagonist in oxLDL-treated human aortic smooth muscle cells and in the neointima of cholesterol-fed endothelia-denuded rabbits, Journal of cellular biochemistry 88(4) (2003) 836–47. [PubMed: 12577317]

[72]. Mallat Z, Corbaz A, Scoazec A, Besnard S, Lesèche G, Chvatchko Y, Tedgui A, Expression of interleukin-18 in human atherosclerotic plaques and relation to plaque instability, Circulation 104(14) (2001) 1598–603. [PubMed: 11581135]

[73]. Gerdes N, Sukhova GK, Libby P, Reynolds RS, Young JL, Schönbeck U, Expression of interleukin (IL)-18 and functional IL-18 receptor on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for atherogenesis, The Journal of experimental medicine 195(2) (2002) 245–57. [PubMed: 11805151]

[74]. Tashiro H, Shimokawa H, Sadamatsu K, Aoki T, Yamamoto K, Role of cytokines in the pathogenesis of restenosis after percutaneous transluminal coronary angioplasty, Coronary artery disease 12(2) (2001) 107–13. [PubMed: 11281299]

[75]. Dalekos GN, Elisaf M, Bairaktari E, Tsolas O, Siamopoulos KC, Increased serum levels of interleukin-1beta in the systemic circulation of patients with essential hypertension: additional risk factor for atherogenesis in hypertensive patients?, The Journal of laboratory and clinical medicine 129(3) (1997) 300–8. [PubMed: 9042815]

[76]. Spigoni V, Fantuzzi F, Carubbi C, Pozzi G, Masselli E, Gobbi G, Solini A, Bonadonna RC, Dei Cas A, Sodium-glucose cotransporter 2 inhibitors antagonize lipotoxicity in human myeloid angiogenic cells and ADP-dependent activation in human platelets: potential relevance to prevention of cardiovascular events, Cardiovascular diabetology 19(1) (2020) 46. [PubMed: 32264868]

[77]. Stock C, Schwab A, Role of the Na/H exchanger NHE1 in cell migration, Acta physiologica (Oxford, England) 187(1–2) (2006) 149–57.

[78]. Lucchesi PA, DeRoux N, Berk BC, Na(+)-H+ exchanger expression in vascular smooth muscle of spontaneously hypertensive and Wistar-Kyoto rats, Hypertension (Dallas, Tex. : 1979) 24(6) (1994) 734–8.

[79]. Liu Y, Chen F, Ji L, Zhang L, Xu YJ, Dhalla NS, Role of lysophosphatidic acid in vascular smooth muscle cell proliferation, Canadian journal of physiology and pharmacology 98(2) (2020) 103–110. [PubMed: 31369714]

[80]. Ye Y, Jia X, Bajaj M, Birnbaum Y, Dapagliflozin Attenuates Na(+)/H(+) Exchanger-1 in Cardiofibroblasts via AMPK Activation, Cardiovascular drugs and therapy 32(6) (2018) 553–558. [PubMed: 30367338]

[81]. Schmidt C, Höcherl K, Bucher M, Regulation of renal glucose transporters during severe inflammation, American journal of physiology. Renal physiology 292(2) (2007) F804–11. [PubMed: 17032938]

[82]. Maldonado-Cervantes MI, Galicia OG, Moreno-Jaime B, Zapata-Morales JR, Montoya-Contreras A, Bautista-Perez R, Martinez-Morales F, Autocrine modulation of glucose transporter SGLT2 by IL-6 and TNF-α in LLC-PK(1) cells, Journal of physiology and biochemistry 68(3) (2012) 411–20. [PubMed: 22351116]

Highlights

1. Human aortic smooth muscle cells (SMC) express SGLT2

2. The SGLT2 inhibitor empagliflozin inhibits IL-17-induced oxidative stress in SMC

3. Empagliflozin inhibits NLRP3 expression, caspase-1 activation, and inflammatory cytokine expression

4. Empagliflozin inhibits IL-17A-induced SMC proliferation and migration.

5. Empagliflozin has therapeutic potential in vascular proliferative diseases.
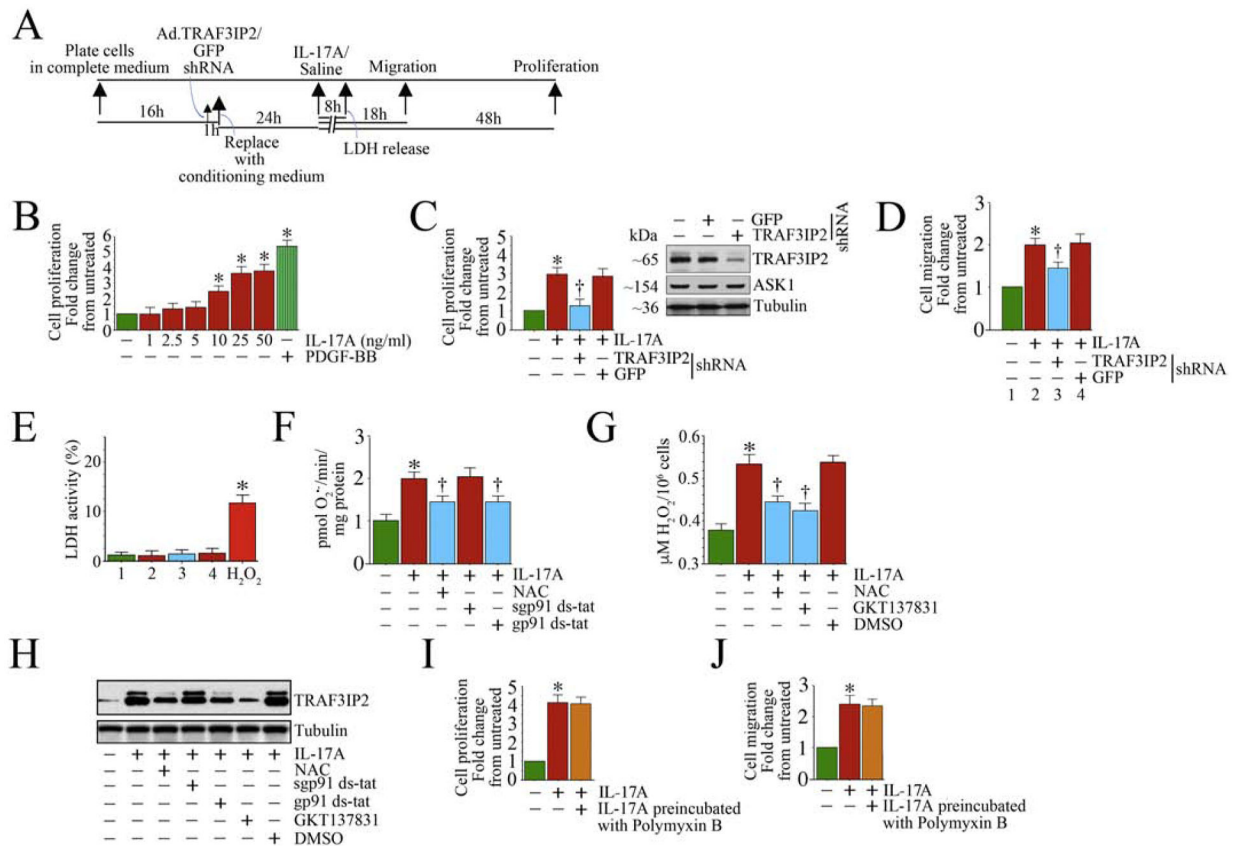
Figure 1. IL-17A induces human aortic smooth muscle cell (SMC) proliferation and migration.
(a) Experimental design. (b) IL-17A at 25 ng/ml stimulated SMC proliferation, while recombinant human PDGF-BB served as a positive control. (c) Adenoviral shRNA-mediated TRAF3IP2 knockdown markedly attenuates IL-17A-induces SMC proliferation. Adenoviral transduction of GFP shRNA failed to modulate IL-17A's pro-mitogenic effects. The inset on the left confirms downregulation in TRAF3IP2 expression by TRAF3IP2-specific shRNA, but not by the non-specific GFP shRNA at a similar multiplicity of infection. (d) TRAF3IP2 silencing leads to inhibition of IL-17A-mediated SMC migration. Boyden chamber assay demonstrating inhibition of IL-17A-mediated SMC migration by adenoviral-mediated TRAF3IP2 silencing. (e) A quantitative colorimetric assay to determine LDH activity in equal amounts of culture supernatants to determine cell death. Hydrogen peroxide (100 μM) served as a positive control. (f,g) IL-17A treatment leads to increased superoxide ($O_2^{-•}$; f) and hydrogen peroxide (H_2O_2; g) production in SMC via NOX2 and NOX4. NAC (5mM in water for 30 min) served as a broad-spectrum antioxidant. NOX2 was targeted by gp91 ds-tat (1μM for 1 h). NOX4 was targeted by GKT137831 (5μM in DMSO 15 min). (h) Importantly, targeting NOX2 and NOX4 attenuated IL-17A-induced TRAF3IP2 expression. (i,j) Polymyxin B pretreatment of IL-17A had no modulatory effect on IL-17A-induced proliferation (i) or migration (j). Quiescent SMC were exposed to IL-17A that has been preincubated with polymyxin B for 1h. SMC proliferation and migration were analyzed as in b and d. Data represent mean ±SE of 8 (proliferation) or 6 (migration) independent experiments. The data presented in (e) is a representative of three independent experiments. *P<at least 0.01 versus untreated; †P<0.05 versus IL-17A.
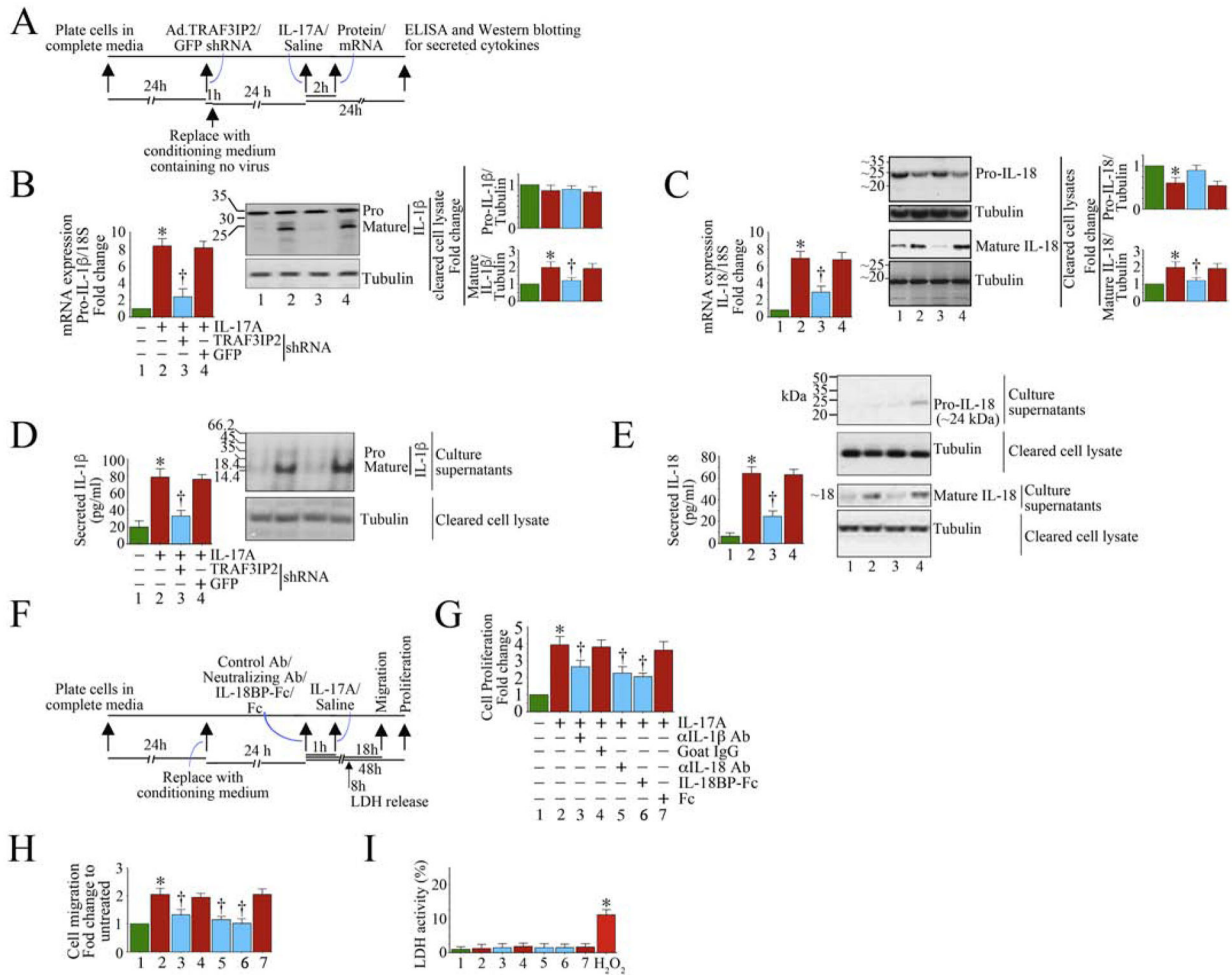
Figure 2. IL-17A induces aortic SMC proliferation and migration via IL-1β and IL-18.
(a) Experimental design for IL-17A-induced cytokine expression and secretion. (b-e, IL-17A induces IL-1β and IL-18 expression and secretion by SMC. Quiescent SMC treated with IL-17A were analyzed for IL-1β and IL-18 mRNA expression by RT-qPCR and protein levels (b,c) by Western blotting at 2 h, and secreted levels by ELISA (d,e) at 24h. In addition, IL-1b and IL-18 levels were analyzed by Western blotting after concentrating culture supernatants (d,e, insets). In a subset of experiments, TRAF3IP2 was silenced by adenoviral transduction of a validated shRNA as in Fig. 1B, prior to IL-17A treatment. In the knockdown studies, GFP served as a non-targeting control. (f) Experimental design for IL-1β and IL-18 neutralization. (g,h) Anti-IL-1β and anti-IL-18 neutralizing antibodies blunt IL-17A-induced SMC proliferation (g) and migration (h). In addition to neutralizing antibodies, IL-18 expression was targeted by IL-18BP. In these studies, species-specific normal IgG and Fc served as respective controls. (i) LDH activity in equal amounts of culture supernatants in the presence of neutralizing antibodies or IL-18BP-Fc to determine cell death. Treatment with hydrogen peroxide (100 μM) served as a positive control. Data represent mean ±SE of 6 (mRNA expression, cytokine secretion, proliferation and migration) independent experiments. Western blotting in b,c,i, and j were a representative of three independent experiments. *P<at least 0.01 versus untreated; †P<0.05 versus IL-17A.
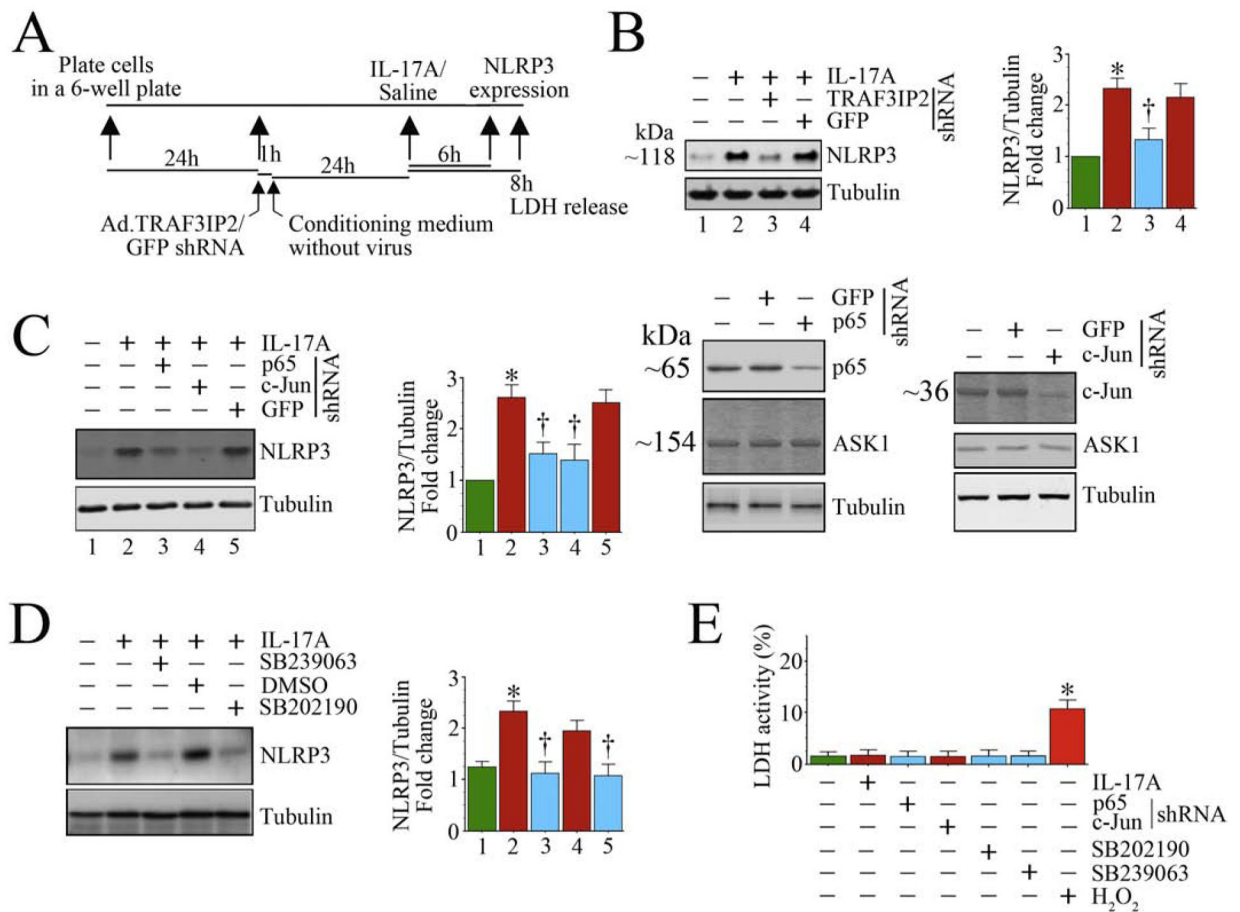
Figure 3. IL-17A induces NLRP3 expression via TRAF3IP2, NF-κB, AP-1 and p38 MAPK.
(a) Experimental design to analyze IL-17A-mediated NLRP3 expression. (b) IL-17A induces NLRP3 expression in part via TRAF3IP2. Quiescent SMC were treated with IL-17A for 6 h and analyzed for NLRP3 expression by Western blotting. In a subset of experiments, TRAF3IP2 was silenced as in Fig. 1A. In the knockdown experiments, GFP served as a non-targeting control. Data from three independent experiments is summarized on the right. (c) NF-κB p65 and AP-1 c-Jun are involved in IL-17A-induced NLRP3 expression. Both p65 and c-Jun were silenced by lentiviral transduction of validated shRNA in SMC. GFP served as a non-targeting control. Data from three independent experiments is summarized on the right. Knockdown of p65 and c-Jun was confirmed by Western blotting as shown in the right most panels. In those knockdown studies, ASK1 served as a non-targeting control. (d) Pharmacological inhibition of p38 MAPK inhibits IL-17A-induced NLRP3 expression. p38 MAPK was targeted by SB239063 or SB202190. DMSO served as a solvent control. Data from three independent experiments is summarized on the right. (e) Silencing p65 or c-Jun or pharmacological inhibition of p38 MAPK did not affect cell viability. Cell death was analyzed by quantifying LDH activity in culture supernatants at 8 h by a quantitative colorimetric assay. Data represent mean ±SE of 3 independent experiments. *P<at least 0.01 versus untreated; †P<0.05 versus IL-17A.
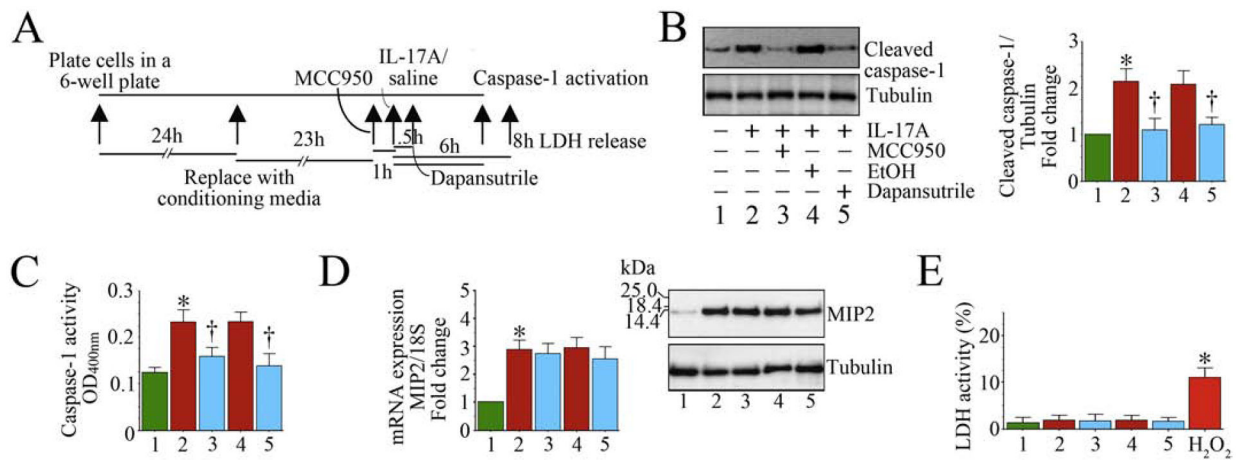
Figure 4. IL-17A enhances the expression and activity of caspase-1.
(a) Experimental design. (b) MCC950 and dapansutrile inhibit IL-17A-induced NLRP3 expression. Quiescent SMC were treated with MCC950 or dapansutrile prior to IL-17A addition. Ethyl alcohol (EtOH) served as a solvent control. NLRP3 expression at 6 h was analyzed by Western blotting. Data from three independent experiments is summarized on the right. (c,d,e) Targeting NLRP3 inflammasome by MCC950 or dapansutrile inhibit caspase-1 cleavage (c) or activity (d) without compromising cell survival (e). The experiments in c-e were performed three times, and a representative Western blot is shown. However, data from three independent experiments is summarized in the respective right hand panel. *P<at least 0.05 versus untreated; †P<at least 0.05 versus IL-17A.
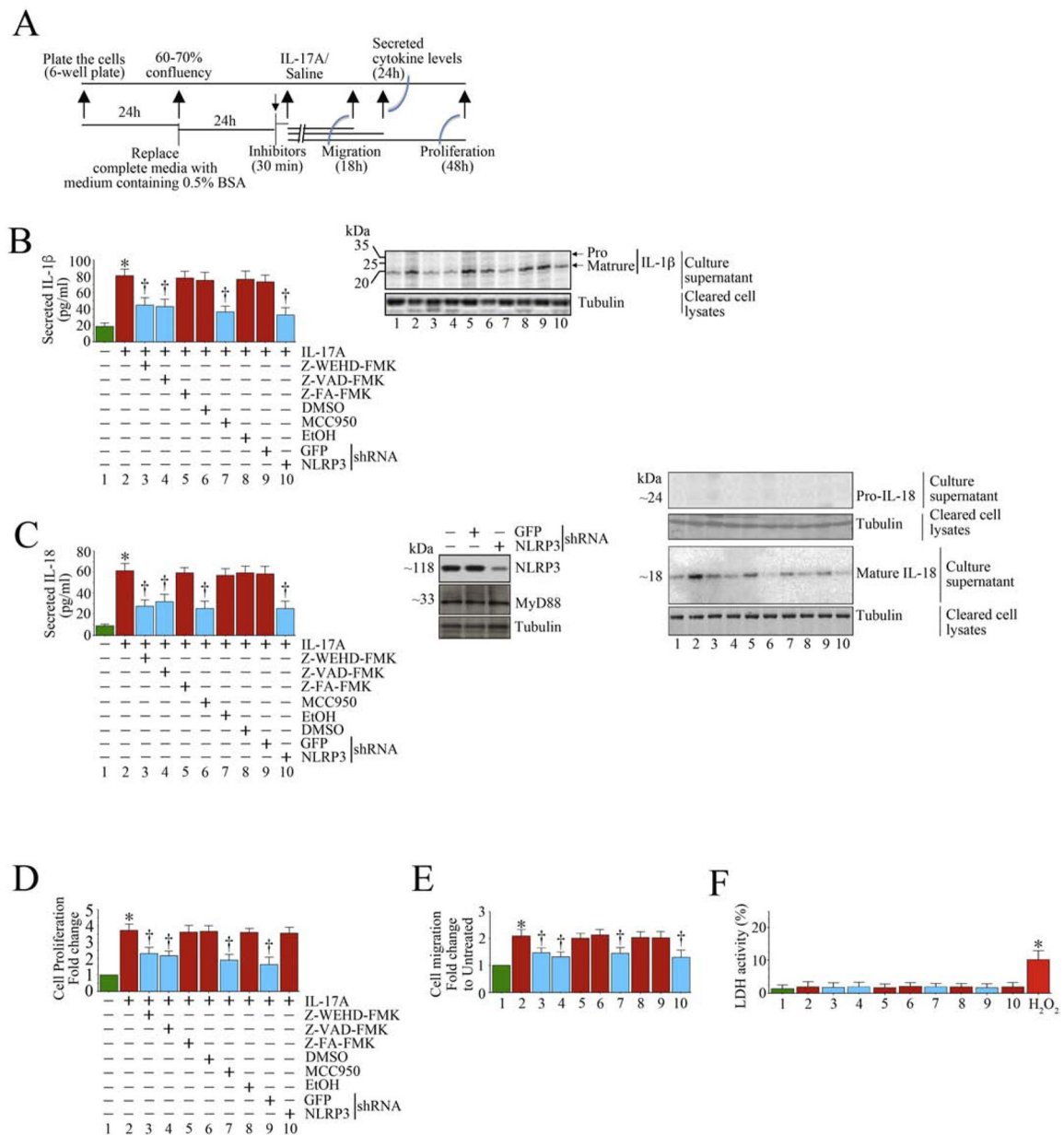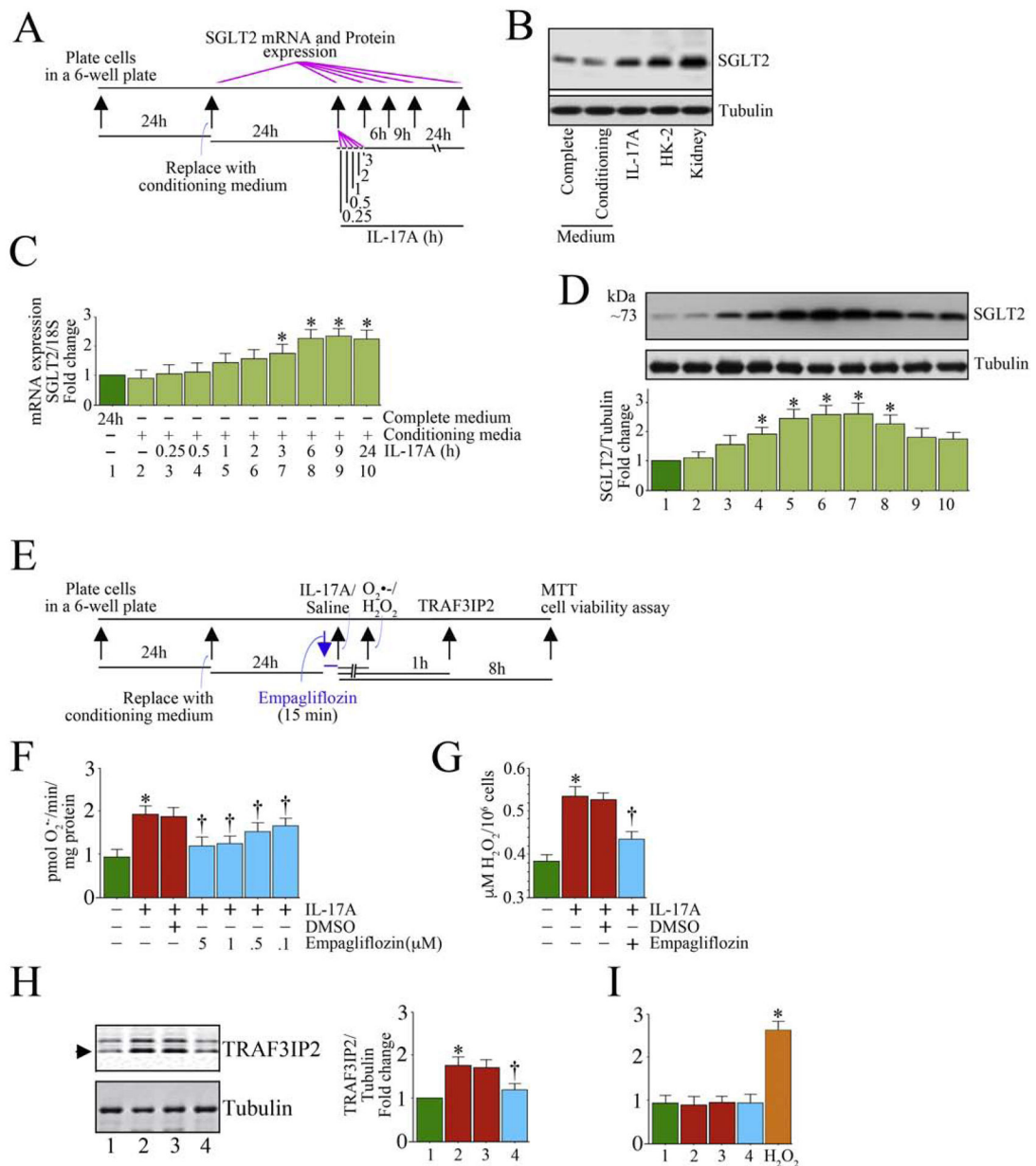
Figure 5. Inhibition of NLRP3 and caspase-1 attenuate IL-17A-induced IL-1β and IL-18 secretion, and SMC migration and proliferation.

(a) Experimental design. (b) Targeting caspase-1 by a specific inhibitor or pan-caspase inhibitor or pharmacological inhibition of NLRP3 inflammasome or its knockdown attenuate IL-17A-induced IL-1β (b) or IL-18 (c) secretion. Cytokine levels in culture supernatants at 24 h were quantified by ELISA. Knockdown of NLRP3 was confirmed by Western blotting (Fig. 5C, right hand panel). In the knockdown studies, MyD88 served as a non-targeting control. (d,e,f) Targeting caspase-1 or NLRP3 attenuates IL-17A-induced SMC proliferation and migration, without inducing cell death. Cell death was analyzed by quantifying LDH activity at 8 h in equal amounts of culture supernatants by a quantitative colorimetric assay. H_2O_2 served as a positive control. Data represent mean ±SE of 6 (b,c,d,e) or 3 (f) independent experiments. $P<0.01$ versus untreated; †$P<0.05$ versus IL-17A.

Figure 6. Empagliflozin inhibits IL-17A-induced oxidative stress and TRAF3IP2 expression without affecting cell viability.

(a) Experimental design. (b) SMC express SGLT2 transcript. Quiescent SMC were analyzed for SGLT2 mRNA expression by RT-qPCR, and normalized to 18S expression. SGLT2 protein levels were analyzed by Western blotting. Human kidney homozenates and HL-2 cleared cell lysates served as positive controls. (c, d) IL-17A induces SGLT2 mRNA (c) and protein (d) expression in a time-dependent manner. Quiescent SMC were incubated with IL-17A for the indicated periods. SGLT2 mRNA expression was analyzed by RT-qPCT and protein levels by Western blotting. (e) Experimental design to investigate empagliflozin's effects. (f, g) Empagliflozin inhibits IL-17A-induced superoxide (f) and hydrogen peroxide (g) production. Quiescent SMC were treated with empagliflozin ranging from 0.1 to 5 μM for 15 min prior to IL-17A addition. Superoxide production was analyzed at 15 min by

lucigenin-enhanced chemiluminescence assay (f). In experiments analyzing hydrogen peroxide production (g), empagliflozin was used at a concentration of 1μM for15 min prior to IL-17A addition for 30 min. Hydrogen peroxide production was analyzed by Amplex Red assay. (h) Empagliflozin inhibits IL-17A-induced TRAF3IP2 expression. Quiescent SMC treated as in (g) were analyzed for TRAF3IP2 expression at 1 h by Western blotting. (i) Empagliflozin did not affect cell survival. Cell death was analyzed by quantifying LDH activity in equal amounts of culture supernatants using a quantitative colorimetric assay. Data represent mean ±SE of 6 (c,f,g,i) or 3 (b,d,h) independent experiments. P<0.01 versus untreated; †P<at least 0.05 versus IL-17A.
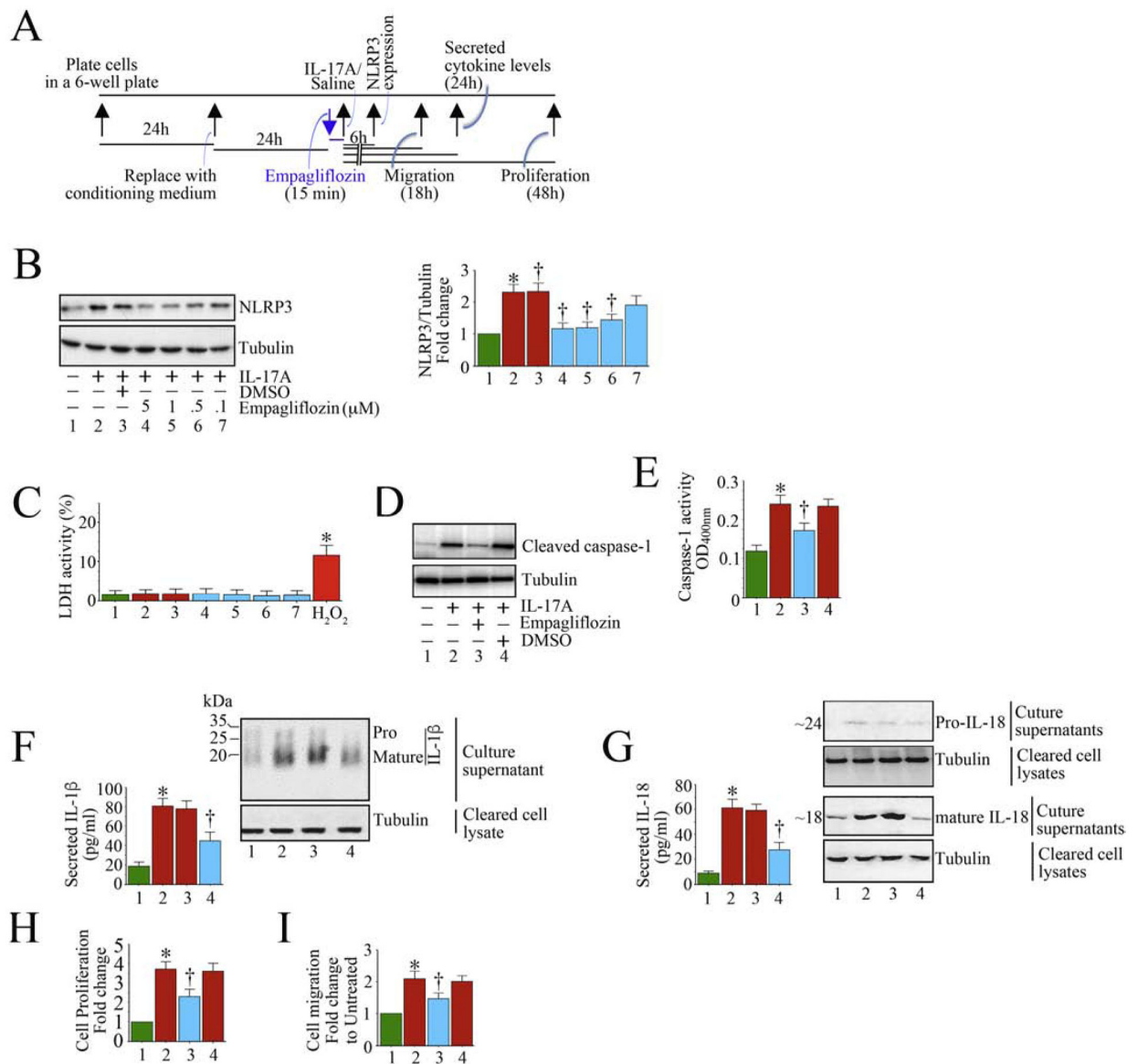
Figure 7. Empagliflozin inhibits IL-17A-induced NLRP3 inflammasome expression, caspase-1 activation, cytokine secretion, and SMC proliferation and migration.
(a) Experimental design. (b) Empagliflozin dose-dependently inhibits IL-17A-induced NLRP3 expression. Quiescent SMC were incubated with empagliflozin at the indicated concentrations for 15 min prior to IL-17A addition. DMSO served as a solvent control. NLRP3 expression was analyzed at 6h by Western blotting. (c) Empaglioflozinn does not affect cell viability, as evidenced by no significant changes in LDH activity at 8 h in equal amounts of culture supernatants. (d,e) Empagliflozin inhibits caspase-1 activation. SMC treated as in (a) but with 1μM empagliflozin and analyzed for cleaved caspase-1 levels at 6h by Western blotting and its activity by a colorimetric assay. (f,g) Empagliflozin inhibits IL-17A-induced cytokine secretion. Quiescent SMC treated with empaglifloziingn (1 μM for 15 min) prior to IL-17A addition were analyzed for secreted cytokine levels at 24h by ELISA. Secreted cytokine levels were also analyzed by Western blotting using equal

amounts of concentrated culture supernatants. Tubulin in whole cell lysates served as a loading control. (h,i) Empagliflozin inhibits IL-17A-induced SMC proliferation and migration. Quiescent SMC were incubated with 1 μM empagliflozin for 15 min prior to IL-17A addition. Cell proliferation was quantified at 48h using the CyQUANT assay. SMC migration was analyzed after 18h using the Boyden chamber assay. Data represent mean ±SE of 3 (b,c,d, and insets in f, g) or 6 (f-i) independent experiments. *P<at least 0.01 versus untreated; †P<0.05 versus IL-17A.
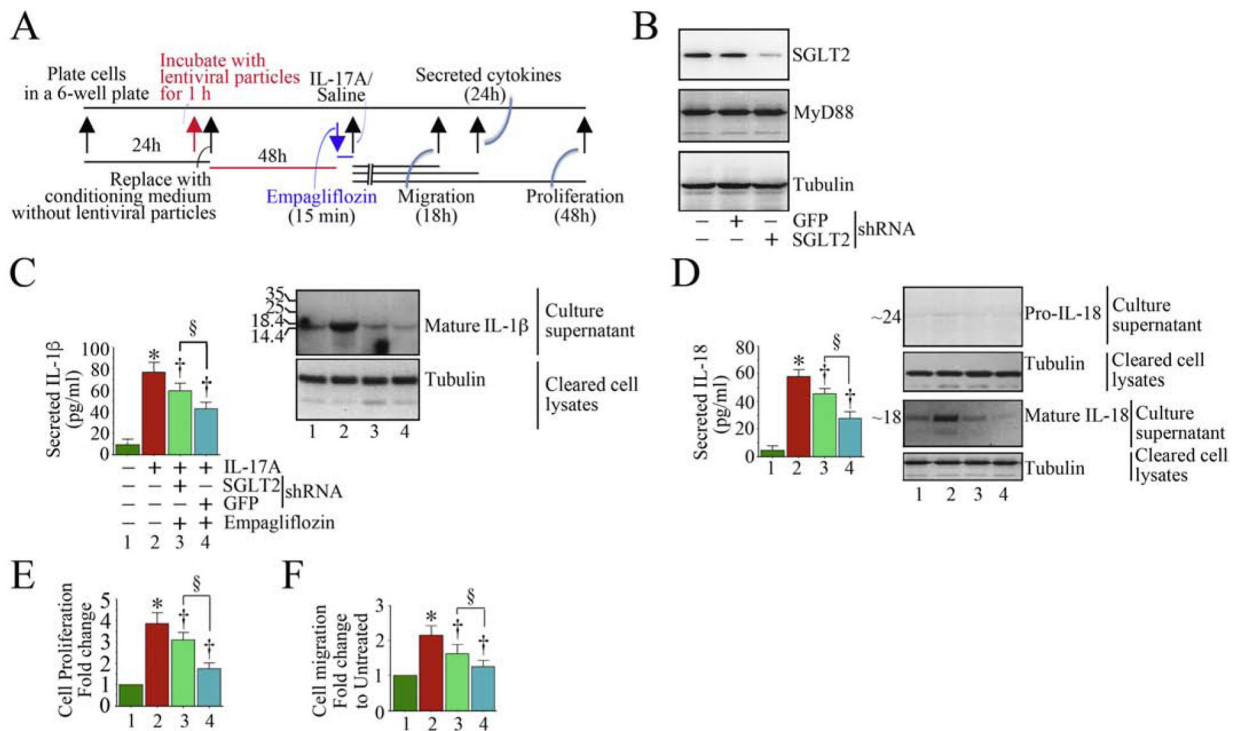
Figure 8. Silencing SGLT2 attenuates IL-17A-induces cytokine secretion, and SMC proliferation and migration.

(a) Experimental design. (b) SGLT2 knockdown. SMC transduced with lentiviral SGLT2 shRNA for 48 h were analyzed for SGLT2 expression by Western blotting. MyD88 served as an off target. (c,d) SGLT2 knockdown attenuates empagliflozin-mediated inhibition of IL-17-induced IL-1b (c) and IL-18 (d) secretion. SMC silenced for SGLT2 were made quiescent, treated with expagliflozin (1mM for 15 min) and then exposed to IL-17A for 24 h. Secreted cytokine levels were analyzed by ELISA using equal amounts of culture supernatants. Secreted cytokine levels were also analyzed by Western blotting using equal amounts of concentrated culture supernatants (insets). (e,f) SGLT2 knockdown attenuates empagliflozin-mediated inhibition of SMC proliferation (e) and migration (f). SMC silenced for SGLT2 treated as in c and d, but for 48 h (e) and 18 h (f) were analyzed for proliferation (e) and migration (f). Proliferation was analyzed by the CyQUANT assay and migration by the Boyden chamber assay. Data represent mean ±SE of 3 (b,insets in c and d) or 6 (c,d,e,f) independent experiments. *P<at least 0.05 versus untreated; †P<0.05 versus IL-17A; §P<0.05 versus SGLT2 knockdown.
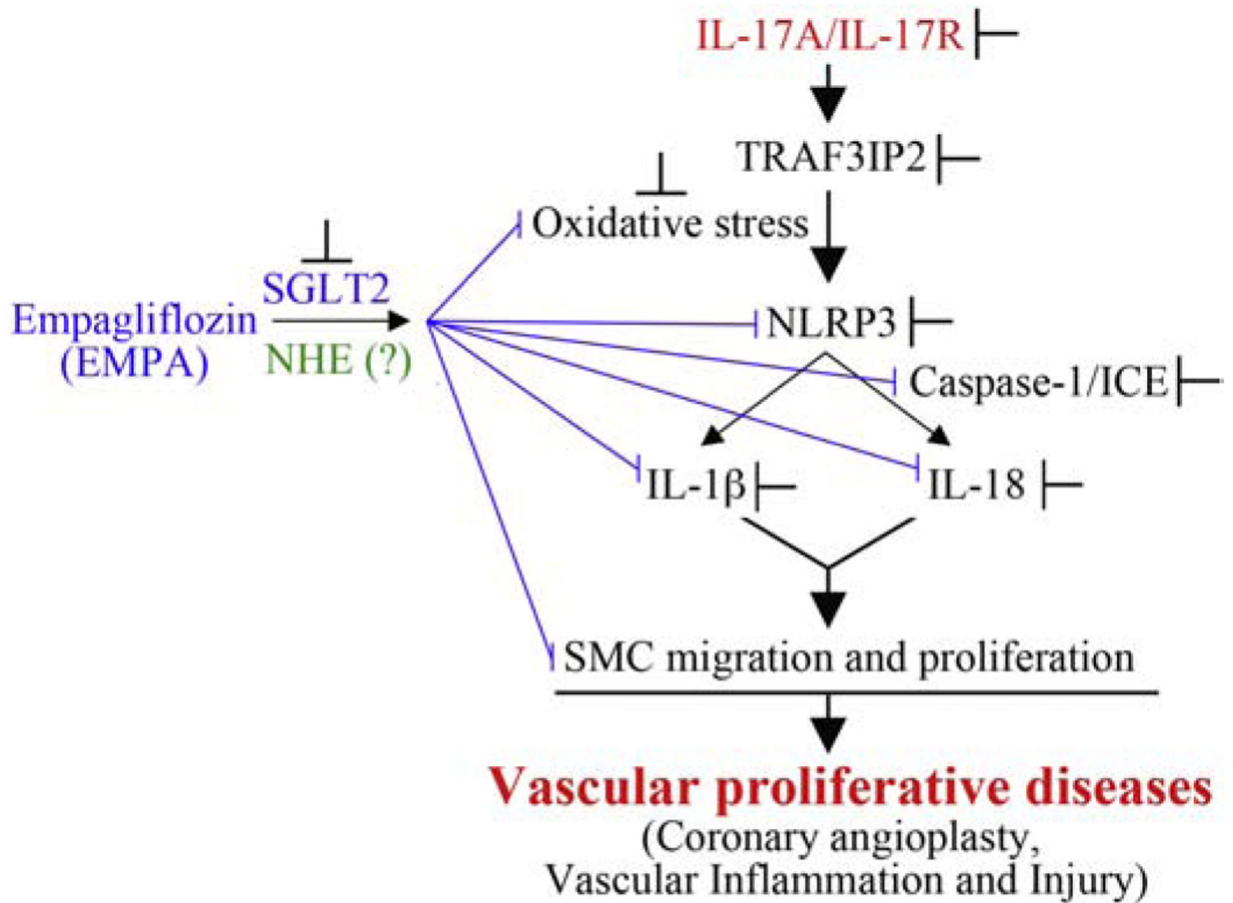
Figure 9. A working model describing the molecular mechanisms underlying IL-17A-induced SMC migration and proliferation, and those targeted by empagliflozin.
IL-17A induces primary human aortic smooth muscle cell migration and proliferation via TRAF3IP2-dependent ROS generation, NLRP3 expression, caspase-1 activation, and proinflammatory and pro-mitogenic IL-1β and IL-18 expression. Transduction of short hairpin RNA (shRNA) by adeno- or lentivirus, pharmacological or peptide inhibitors and neutralizing antibodies targeted TRAF3IP2, NF-κB, AP-1, NOX2, NOX4, NLRP3, caspase-1, IL-1β and IL-18. Importantly, SMC express SGLT2, and the SGLT2 inhibitor empagliflozin (EMPA) attenuated IL-17A-induced maladaptive changes by exerting anti-oxidant, anti-inflammatory, anti-migratory and anti-mitogenic effects without inducing cell death. It is also plausible that empagliflozin may exert its beneficial inhibitory effects in part via NHE (in green). Together, these mechanistic *in vitro* results suggest the therapeutic potential of EMPA in vascular proliferative diseases.