

## ORIGINAL RESEARCH

## BK Channels Regulate LPS-induced CCL-2 Release from Human Pulmonary Endothelial Cells

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## Abstract

We recently established a role for the stretch-activated two-pore-domain K<sup>+</sup> (K2P) channel TREK-1 (K2P2.1) in inflammatory cytokine secretion using models of hyperoxia-, mechanical stretch-, and TNF- $\alpha$ -induced acute lung injury. We have now discovered the expression of large conductance, Ca<sup>2+</sup>-activated K<sup>+</sup> (BK) channels in human pulmonary microvascular endothelial cells and primary human alveolar epithelial cells using semiquantitative real-time PCR, IP and Western blot, and investigated their role in inflammatory cytokine secretion using an LPS-induced acute lung injury model. As expected, LPS induced IL-6 and CCL-2 secretion from pulmonary endothelial and epithelial cells. BK activation with NS1619 decreased LPS-induced CCL-2 but not IL-6 secretion from endothelial cells and had no effect on epithelial cells, although fluorometric assays revealed that BK activation hyperpolarized the plasma membrane potential (Em) of both cell types. Interestingly, BK inhibition (Paxilline) did not alter cytokine secretion or the Em in either cell type. Furthermore, LPS treatment by itself did not affect the Em or intracellular Ca<sup>2+</sup> concentrations. Therefore, we propose BK channel activation as a novel targeted

approach to counteract LPS-induced CCL-2 secretion from endothelial cells. This protective effect appears to occur via Em hyperpolarization but independent of intracellular Ca<sup>2+</sup> concentrations.

**Keywords:** acute lung injury; large conductance potassium channels; LPS; cytokines; inflammation

## Clinical Relevance

Our data show for the first time the functional expression of large conductance K<sup>+</sup> (BK) channels in human pulmonary microvascular endothelial cells and primary human alveolar epithelial cells. Furthermore, we propose a role for the BK channel activator NS1619 in inflammatory cytokine secretion in an LPS-induced lung injury model. These results will contribute to the development of new therapeutic approaches against LPS-induced lung injury by highlighting BK channels as potentially new targets.

Infectious stimuli are the most common triggers for lung injury in humans, which in its most severe form may progress to acute respiratory distress syndrome (ARDS). Decades of intense research have focused on creating experimental acute lung injury (ALI) models to dissect the underlying mechanisms and identify potential

therapeutic targets. LPS-induced ALI models are routinely used to experimentally mimic gram-negative infections, and the LPS-mediated proinflammatory effects on cytokine secretion from immune and inflammatory cells are well documented (1). However, less is known about the mechanism of LPS-induced injury in lung

resident cells, including alveolar epithelial and endothelial cells. Nevertheless, upregulation of inflammatory mediators such as CCL-2 and IL-6 is consistently observed in the serum and BAL fluid from lung injury patients, and in multiple experimental ALI models (2). Besides antibacterial therapies to treat underlying

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infections, currently no targeted interventions exist against ALI/ARDS that translate into improved patient outcomes.

Using different *in vivo* and *in vitro* ALI models, we have previously shown that regulation of inflammatory cytokine secretion by  $K^+$  channels plays an important role in the development of alveolar inflammation (3–6). In general, one of the main functions of  $K^+$  channels is the regulation and stabilization of the plasma membrane potential (Em), and our lab is particularly interested in exploring mechanisms that regulate the Em as a tool to counteract alveolar inflammation and injury. We previously found that TREK-1  $K^+$  channels, which are characterized by a single channel conductance in the range of 40–150 pS (7), participate in the regulation of inflammatory cytokine secretion from alveolar epithelial cells (3, 4, 8). Based on these findings, it is intriguing to speculate that manipulation of channels with a larger unitary conductance, such as the large conductance,  $Ca^{2+}$ - and voltage-activated  $K^+$  channels (BK, KCa1.1, MaxiK), may constitute an even more powerful tool to regulate alveolar epithelial and endothelial inflammation.

The functional BK channel is a homotetramer of four pore-forming  $\alpha$ -subunits (BK- $\alpha$ ) associated with modulatory  $\beta$ -subunits (BK- $\beta$ ), which can modify the  $Ca^{2+}$ - and voltage dependence of the channels and its gating properties (9). Four types of BK- $\beta$  auxiliary subunits have been identified thus far in mammals, of which BK- $\beta$ 2 and - $\beta$ 3 are expressed in lung tissue (10). Interestingly, the vascular BK- $\beta$ 1-subunit has been implicated in LPS-induced hypotension, organ damage, and mortality (11). Overall, despite differences in its subunit composition, BK channels are ubiquitously expressed in the body and represent a unique class of  $K^+$  channels, not only because of their large single channel conductance ( $\sim$ 250 pS in symmetrical  $K^+$  solutions) but also given their individual and synergistic sensitivity to intracellular  $Ca^{2+}$  ( $iCa^{2+}$ ) levels and membrane depolarization (12). Consequently, BK channels are involved in key physiological roles, including regulation of neurotransmitter release, neuronal and cardiac excitability, smooth muscle tone, endocrine secretion, cell proliferation, and renal electrolyte regulation, and dysregulation of BK channels has been linked to various disease processes such as

schizophrenia, cardiac arrhythmias, and diabetes mellitus (13).

It is unknown whether human pulmonary microvascular endothelial cells (HULEC5a) and human primary alveolar epithelial cells (HPAEPiC) express BK channels, and thus, their potential role in inflammatory cytokine secretion from these cells remains unclear. This study was designed to determine the expression and contribution of BK channels to LPS-induced cytokine secretion from alveolar epithelial and microvascular endothelial cells, and to establish the underlying mechanisms as potential targets for future therapeutic interventions. We hypothesize that pharmacological BK activation counteracts LPS-induced IL-6 and CCL-2 secretion through Em- and  $iCa^{2+}$ -dependent mechanisms. Of note, some of the results of this study have been previously reported in the form of an abstract (14).

## Methods

### Cells

HULEC5a (ATCC-CRL-3244), human alveolar epithelial A549 (ATCC-CRM-CCL-185), and NCI-H441 (ATCC-HTB-174) cells were purchased from American Type Culture Collection. HPAEPiC were purchased from ScienCell. HEK293 cells were obtained from Dr. Olcese (University of California Los Angeles). All cells were used at passages 6–15.

### Gene Expression

Total RNA was isolated using a Qiagen RNeasy Mini Kit; 1–8  $\mu$ g RNA were reverse transcribed with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and amplified by semiquantitative real-time PCR (TaqMan) with primers for *KCNMA1* (BK- $\alpha$ 1), *KCNMB1-4* (BK- $\beta$ 1–4), or *GAPDH* (Applied Biosystems).

### Protein Expression

BK- $\alpha$ 1 protein was immunoprecipitated in cell lysates with 10  $\mu$ g mouse monoclonal anti-BK- $\alpha$  antibody (UC Davis/NeuroMab) or nonspecific rabbit polyclonal IgG isotype (negative control; Cell Signaling). Immunocomplexes were captured with 100  $\mu$ l A/G PLUS agarose beads (Santa Cruz Biotechnology). Dissociated samples were separated on a NuPAGE Bis-

Tris 10% gel, transferred onto 0.45- $\mu$ m Immuno-Blot PVDF membranes (BioRad), and probed with a primary mouse monoclonal anti-BK- $\alpha$  antibody (1:200; UC Davis/NeuroMab) and a goat anti-mouse-Cy3 secondary antibody (1:1000; Millipore).

As a positive control for Western blot, the BK- $\alpha$ 1-subunit (*KCNMA1*) was overexpressed in HEK293 cells using the human plasmid BK- $\alpha$ 1-YFP-pcDNA3.1.

### Em Measurements

Em changes were analyzed by fluorescence-based plate reader (FLIPR) Membrane Potential Assay (Molecular Devices) (15–17). Relative fluorescence of Em red dye-loaded cells was recorded every 7 seconds using a Synergy-2 Multi-Mode Microplate Reader (Biotek; excitation 525 nm, emission 590 nm, at 37°C) after adding LPS (2  $\mu$ g/ml; Cell Signaling; *E. coli* serotype O111:B4), NS1619 (30  $\mu$ M; Millipore), NS19504 (30  $\mu$ M; Alomone Labs), Paxilline (10  $\mu$ M; Alomone Labs), or equimolar vehicle controls.

### Cytokine Measurements

CCL-2 and IL-6 concentrations in 5% FBS-containing supernatants were quantified using ELISA assays (BD Biosciences) after treatment of cells with 2  $\mu$ g/ml LPS, NS1619 (30  $\mu$ M), Paxilline (10  $\mu$ M), or equimolar vehicle controls.

### $Ca^{2+}$ Measurements

$iCa^{2+}$  concentrations were quantified by Fluo-4 NW Calcium Assay (Fisher Scientific; excitation 488 nm, emission 516 nm). Baseline fluorescence was recorded for 1 minute before adding LPS (2  $\mu$ g/ml), NS1619 (30  $\mu$ M), Paxilline (10  $\mu$ M), Ionomycin (5  $\mu$ M; Adipogen), or equimolar vehicle controls. Relative fluorescence was recorded every 11 seconds. All buffers contained 1.8 mM  $CaCl_2$ .

### Gene Microarray Experiments

NS1619-mediated alterations in LPS signaling were analyzed using a TaqMan 94-gene Array Human Inflammation kit (#4414074; Fisher Scientific). RNA isolation and DNA synthesis were performed as above. Target gene amplification was performed using a QuantStudio-5 system (Fisher Scientific) and normalized to *GAPDH*. Canonical pathway analysis was performed by Ingenuity Pathway Analysis (Qiagen) (18) with a threshold

for significance set at  $\geq 1.2$ -fold change (FC).

### Statistics

Data are expressed as mean  $\pm$  SEM, or median with minimum and maximum values. Comparisons were performed using Mann-Whitney *U* or Student's *t* tests (GraphPad 8.3), with  $P \leq 0.05$  considered significant.

## Results

### BK Channels Are Expressed in Human Pulmonary Microvascular Endothelial and Primary Human Alveolar Epithelial Cells

Using semiquantitative real-time PCR, we measured mRNA expression of the pore-forming  $\alpha 1$ -subunit *KCNMA1* and the auxiliary  $\beta$ -subunits *KCNMB1*, *KCNMB2*, *KCNMB3*, and *KCNMB4* in cultured HULEC5a and human alveolar type-2 (A549 and H441), and in HPAEpiC cells (Figures 1A and E1A). Interestingly, *KCNMA1*, *KCNMA3*, and *KCNMA4* subunit expression was detected in all cell types tested, whereas the *KCNMB2* was only detected in HPAEpiC and H441 but not HULEC5a and A549 cells. Because BK channel mRNA is expressed in both transformed (A549, H441) and primary HPAEpiC cells, for the rest of the study, we focused on HPAEpiC cells.

Similar to gene expression, we also detected protein expression of the pore-forming *KCNMA1* subunit by IP and Western blot in endothelial HULEC5a and epithelial HPAEpiC cells (Figure 1B). To control for nonspecific antibody binding, we replaced the primary antibody with a rabbit polyclonal IgG isotope as a negative control. As a positive control for Western blot analysis, we used HEK293 cells overexpressing the BK- $\alpha 1$ -subunit (*KCNMA1*). Treatment of endothelial HULEC5a and epithelial HPAEpiC cells with LPS (2  $\mu\text{g}/\text{ml}$ ) did not affect *KCNMA1* expression levels (Figure 1C). Taken together, these findings demonstrate that human microvascular endothelial cells and alveolar epithelial cells express BK channels, and that BK expression in these cells is not altered by LPS exposure.

### BK Channel Activity Regulates the Em of Human Pulmonary Microvascular Endothelial Cells during LPS-induced Inflammation

To determine the functional role of BK channels in regulating the Em in human endothelial and epithelial cells, we activated or inhibited BK channels with NS1619 (19) and NS19504 (20), or Paxilline (21), respectively, and measured Em changes using fluorometric FLIPR assays. We also performed an internal validation of the assay for both endothelial and epithelial cells by incrementally increasing extracellular  $\text{K}^+$  concentrations using 2- to 45-mM  $\text{K}_2\text{SO}_4$  to gradually depolarize the Em (Figures E2A–E2D).

Interestingly, LPS treatment by itself did not alter the Em in either endothelial or epithelial cells (Figures 2A–2E). In endothelial cells, activation of BK channels with both BK agonists, NS1619 (30  $\mu\text{M}$ ) and NS19504 (30  $\mu\text{M}$ ), resulted in Em hyperpolarization (reflected by a decrease in relative fluorescence) in both untreated and LPS-treated (2  $\mu\text{g}/\text{ml}$ ) cells (Figures 2A and 2B), suggesting that both drugs target BK channels and indicating the functional presence of BK channels in these cells. Despite the difference between the two drugs not reaching statistical significance, the hyperpolarizing effect of NS1619 appeared stronger than with NS19504, and for this reason NS1619 was used for the rest of this study. Furthermore, inhibition of BK channels with Paxilline (10  $\mu\text{M}$ ) did not alter the Em in endothelial cells, suggesting a low level of BK activity in untreated cells (Figure 2C).

Similarly to endothelial cells, activation of BK channels with NS1619 also hyperpolarized the Em of HPAEpiC, whereas BK channel inhibition with Paxilline had no effect on the Em (Figures 2D and 2E), suggesting a similarly low level of BK activity at baseline as seen in endothelial cells.

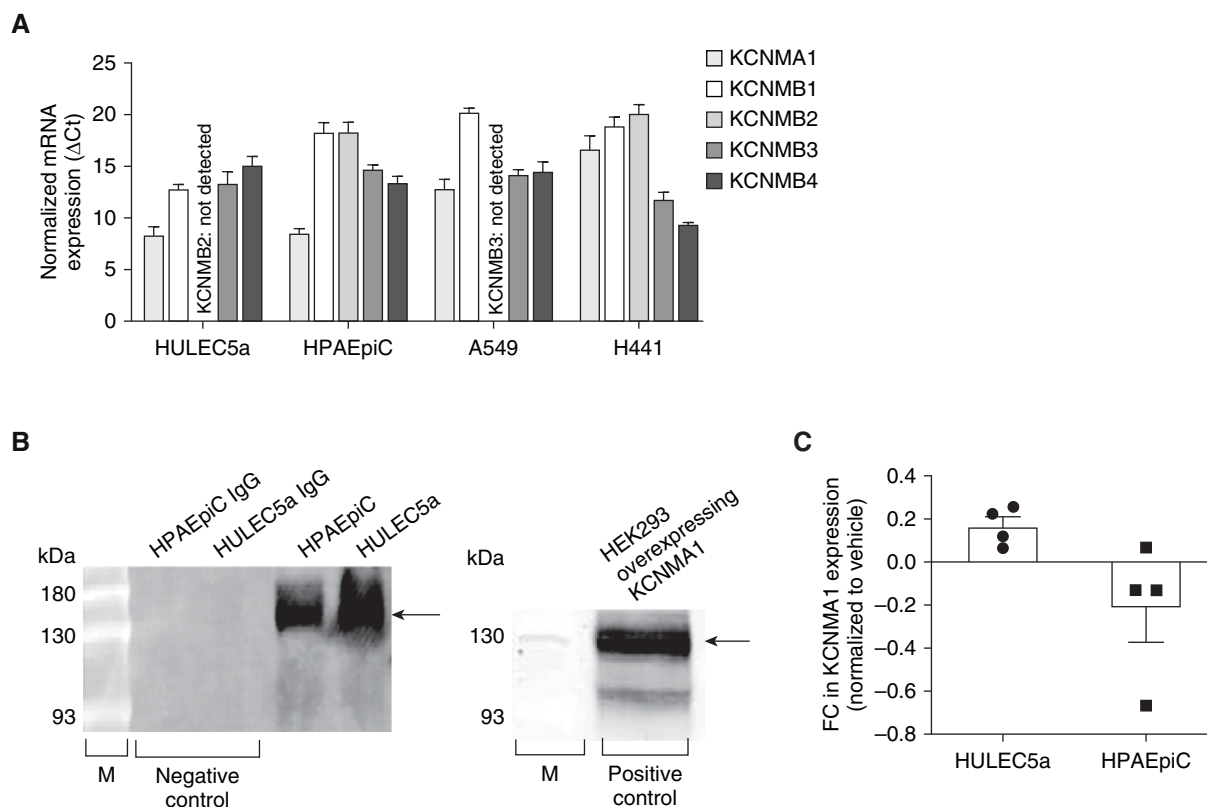
Taken together, these findings demonstrate 1) the functional presence of BK channels on endothelial and epithelial cells, 2) the potential for pharmacological hyperpolarization of both cell types, and 3) a lack of direct LPS effects on the Em of endothelial and epithelial cells.

### BK Channel Activation with NS1619 Decreases LPS-induced CCL-2 but Not IL-6 Secretion from HULEC5a Cells

To determine whether BK channel activation regulates inflammatory cytokine

secretion from HULEC5a and HPAEpiC cells, we quantified LPS-induced CCL-2 and IL-6 secretion using ELISA assays (Figure 3). Neither BK channel activation with NS1619 (30  $\mu\text{M}$ ) nor inhibition with Paxilline (10  $\mu\text{M}$ ) affected baseline CCL-2 or IL-6 secretion from these two cell types (Figures 3A–3D). Treatment of endothelial cells with LPS (2  $\mu\text{g}/\text{ml}$ ) increased both CCL-2 and IL-6 secretion compared with untreated controls (Figures 3A and 3B). Pilot experiments revealed that 2  $\mu\text{g}/\text{ml}$  and 10  $\mu\text{g}/\text{ml}$  of LPS evoke a similar increase in CCL-2 and IL-6 levels (data not shown), and therefore, we used the lower 2  $\mu\text{g}/\text{ml}$  LPS dose for the rest of this study. Specifically, LPS treatment increased mean endothelial CCL-2 concentrations from  $166 \pm 13$  to  $855 \pm 196$  pg/ml ( $P \leq 0.001$ ), and mean IL-6 concentrations from  $170 \pm 24$  to  $2,753 \pm 694$  pg/ml ( $P \leq 0.001$ ), representing an 81% and 94% increase over baseline, respectively (Figures 3A and 3B; red horizontal bars). Importantly, BK channel activation with NS1619 inhibited LPS-induced CCL-2 secretion, as indicated by a decrease in mean LPS-induced CCL-2 levels decreased from  $855 \pm 196$  to  $322 \pm 59$  pg/ml after BK activation with NS1619 ( $P \leq 0.05$ ), representing a 62% decrease (Figure 3A, red horizontal bars). Interestingly, LPS-induced IL-6 secretion was not affected by BK channel activation with NS1619 (Figure 3B). In contrast to BK channel activation, BK channel inhibition with Paxilline did not affect LPS-induced CCL-2 or IL-6 secretion from endothelial cells (Figures 3A and 3B), supporting the idea that most BK channels are closed at baseline.

Similar to endothelial cells, LPS treatment also increased IL-6 secretion from HPAEpiC cells from  $144 \pm 14$  pg/ml to  $277 \pm 57$  pg/ml, representing a 48% increase compared with untreated cells ( $P \leq 0.05$ ), but had no effect on CCL-2 secretion from epithelial cells (Figures 3C and 3D, red horizontal bars). In contrast to endothelial cells, neither BK channel activation (NS1619) nor inhibition (Paxilline) affected LPS-induced CCL-2 or IL-6 secretion (Figures 3C and 3D). Taken together, these data suggest a novel link between BK-mediated regulation of the endothelial Em and downstream LPS-induced CCL-2 but not IL-6 secretion, which is not observed in alveolar epithelial cells.



**Figure 1.** Large conductance potassium (BK) channels are expressed in human pulmonary microvascular endothelial cells (HULEC5a) and human primary alveolar epithelial cells (HPAEpiC). (A) Relative mRNA expression ( $\Delta C_t$ ) of the pore-forming  $\alpha$ -1 subunit *KCNMA1* and the auxiliary *KCNMB1*, -2, -3, -4  $\beta$ -subunits in HULEC5a ( $n=6$ ) and HPAEpiC (A549 and H441;  $n=4-7$ ). (B) On the left: a representative IP and Western blot experiment: pore-forming *KCNMA1* protein expression in HULEC5a and HPAEpiC cells ( $n=3$ ). On the right: a representative Western blot experiment showing that the anti-*KCNMA1* BK- $\alpha$ 1 antibody detected the protein at the expected size (100–130 kD; right lane) in HEK293 cells overexpressing the *KCNMA1* BK- $\alpha$ 1-subunit. Arrows depict expected band size. (C) Fold change in *KCNMA1* gene expression in LPS-treated (6 h, 2  $\mu$ g/ml) HULEC5a and HPAEpiC cells ( $n=4$ ). FC=fold change; M=molecular weight marker.

### LPS-induced CCL-2 and IL-6 Secretion from HULEC5a Cells Occurs Independently of $iCa^{2+}$ Concentrations

To determine whether BK channel-mediated Em alterations (Figure 2) and downstream inflammatory cytokine secretion (Figure 3) were dependent on  $Ca^{2+}$  influx via voltage-gated  $Ca^{2+}$  channels ( $Ca_V$ ), we quantified LPS-induced CCL-2 and IL-6 secretion from HULEC5a cells after blocking L-type  $Ca_V$  channels with Nifedipine (10  $\mu$ M; Figure 4). Interestingly, in unstimulated endothelial cells, Nifedipine decreased baseline secretion of CCL-2 but not IL-6 (Figures 4A and 4B), but following LPS stimulation,  $Ca_V$  inhibition with Nifedipine did not affect CCL-2 or IL-6 secretion.

Because LPS-induced CCL-2 and IL-6 secretion appears to occur independently of extracellular  $Ca^{2+}$  influx via  $Ca_V$  channels,

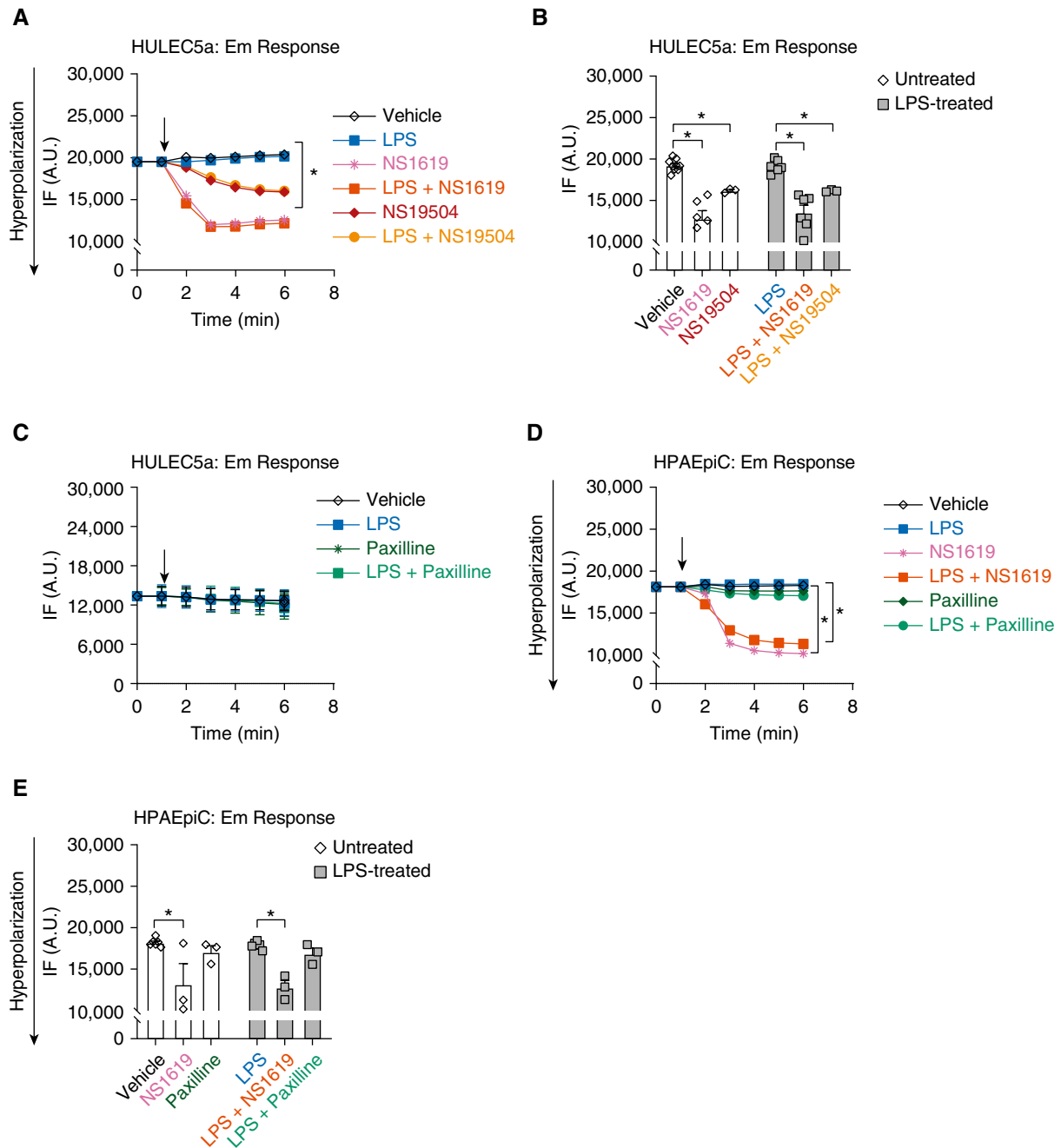
we determined whether LPS stimulation itself affects  $iCa^{2+}$  concentrations in HULEC5a cells (Figures 4C and 4D). Using fluorometric Fluo-4 assays, we measured the immediate effect of LPS treatment on endothelial  $iCa^{2+}$  concentrations, as well as  $iCa^{2+}$  concentrations after 24 hours of LPS exposure (Figure 4D). These studies showed that LPS treatment had neither short- nor long-term effects on endothelial  $iCa^{2+}$  concentrations. Furthermore, BK inhibition with Paxilline did not increase  $iCa^{2+}$  concentrations, supporting the idea that in endothelial cells at baseline most BK channels are closed, and do not mediate any changes in Em or  $iCa^{2+}$  homeostasis. The  $Ca^{2+}$  ionophore Ionomycin was used as positive control to validate the responsiveness of cells and the sensitivity of the Fluo-4 assay (Figures 4C–4F). Taken together, these findings suggest that baseline CCL-2 secretion from pulmonary endothelial cells is dependent on  $Ca_V$

activity, but LPS-induced CCL-2 and IL-6 secretion occurs independently of alterations in  $iCa^{2+}$  concentrations.

### BK-mediated Changes in LPS-induced Cytokine Secretion Alter Specific Downstream Signaling Pathways in HULEC5a Cells

To further explore the electrochemical coupling processes and signaling mechanisms linking BK-mediated Em alterations to downstream LPS-induced cytokine secretion, we used a human inflammatory 94-gene microarray and confirmatory real-time PCR (Tables 1 and E1). For this purpose, we compared gene expression patterns between endothelial cells stimulated with LPS alone (2  $\mu$ g/ml) for 8 hours and cells treated with LPS + NS1619 (BK activator). We calculated fold changes in relative gene expression (FC; a quantitative parameter for scored genes, indicative of the ratio between the



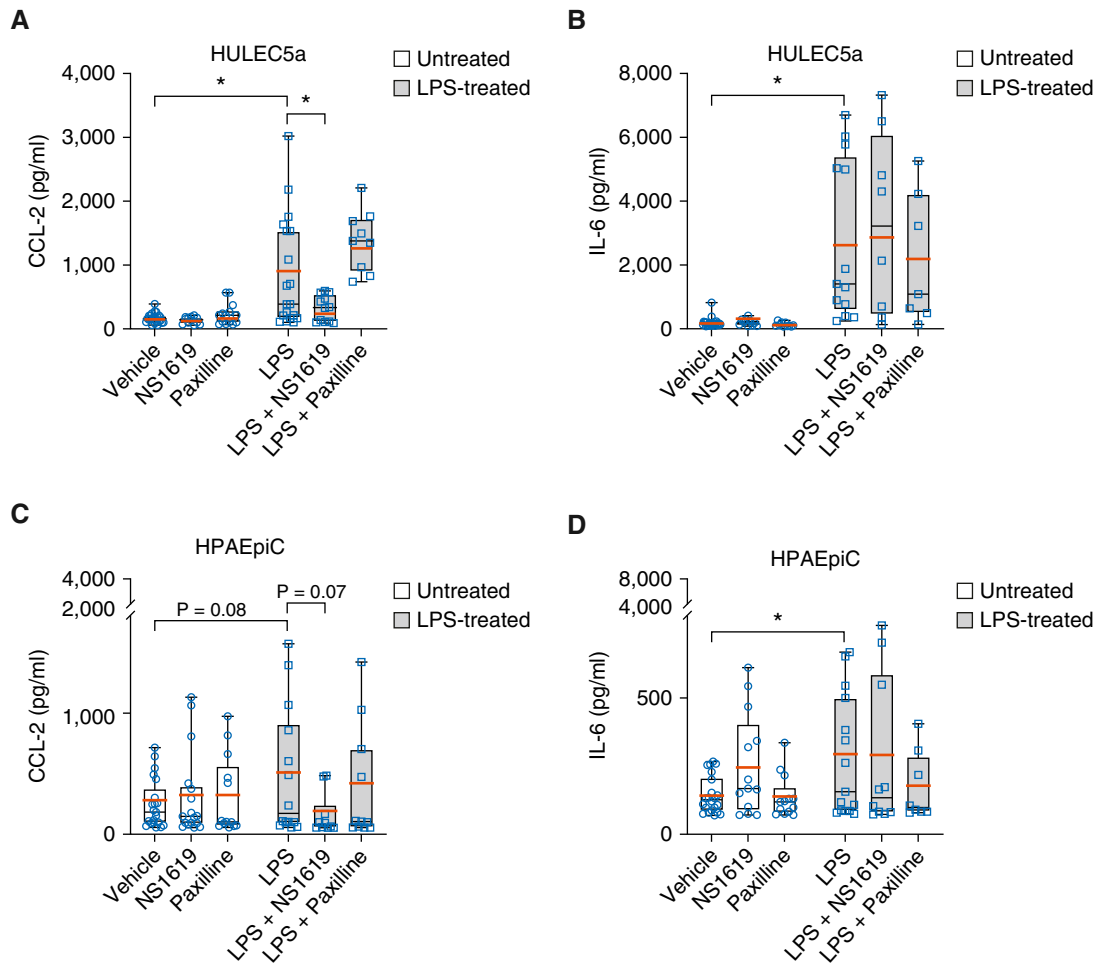


**Figure 2.** BK channels regulate the plasma membrane potential ( $E_m$ ) in HULEC5a during LPS-induced inflammation. Fluorescence intensity (IF) measured by fluorescence-based plate reader (FLIPR) assay depicts  $E_m$  changes in endothelial (HULEC5a) and epithelial (HPAEpiC) cells. Cells were treated with LPS ( $2 \mu\text{g}/\text{ml}$ ), the BK activators NS1619 ( $30 \mu\text{M}$ ) and NS19504 ( $30 \mu\text{M}$ ), the BK inhibitor Paxilline ( $10 \mu\text{M}$ ), or a combination of LPS with these agents. (A) A representative recording showing NS1619- and NS19504-induced  $E_m$  hyperpolarization. (B) NS1619 and NS19504 cause a decrease in IF in HULEC5a cells representing  $E_m$  hyperpolarization, both at baseline and after LPS treatment ( $2 \mu\text{g}/\text{m}$ ,  $n = 6$ ,  $*P \leq 0.05$ ). (C) Treatment of endothelial cells with LPS, Paxilline, or LPS+Paxilline did not change the  $E_m$  ( $n = 6$ ). (D and E) Treatment of epithelial cells with LPS, Paxilline, or LPS+Paxilline had no effect on the  $E_m$ , whereas NS1619 alone and in combination with LPS caused  $E_m$  hyperpolarization ( $n = 3-6$ ,  $*P \leq 0.05$ ). Black arrows indicate the timing of drug delivery. A.U. = arbitrary units.

experimental and control values) to quantify the effects of BK activation with NS1619 on specific signaling pathways in LPS-treated cells. As shown in Table 1, using an FC  $\geq 1.2$  as the

cutoff criterion, our PCR results identified top gene candidates based on the highest FC values: NS1619 treatment downregulated two proinflammatory genes (*TNFSF13B*, FC  $-3.3$ ; *CASP1*,

FC  $-2.0$ ) and upregulated one proinflammatory gene (*PTGS2*, FC 2.2) and one antiinflammatory gene (*IL1RI*, FC 1.2). Importantly, *JNK-1* (FC 1.4), a kinase that has both pro- and



**Figure 3.** BK activation with NS1619 decreased CCL-2 but not IL-6 secretion from HULEC5a cells during LPS-induced inflammation. (A and B) Treatment of HULEC5a cells with LPS (2  $\mu$ g/ml; 24 h) increased CCL-2 and IL-6 secretion. BK activation with NS1619 decreased LPS-induced CCL-2 but not IL-6 secretion, whereas BK inhibition with Paxilline had no effect on LPS-induced CCL-2 or IL-6 secretion ( $n = 7-26$ ,  $*P \leq 0.05$ ) from HULEC5a cells. (C and D) LPS treatment (2  $\mu$ g/ml; 24 h) also increased CCL-2 and IL-6 secretion from HPAEpiC cells, but neither BK activation with NS1619 nor BK inhibition with Paxilline affected CCL-2 or IL-6 secretion from these cells ( $n = 8-22$ ,  $*P \leq 0.05$ ). Mean values are represented in red horizontal bars; black bars indicate medians.

antiinflammatory properties, was also upregulated by NS1619. Importantly, real-time PCR also revealed that *TLR-4* expression (not included in the microarray panel) was downregulated by NS1619 (FC  $-1.2$ ). In total, in the microarray assay, 8 genes were downregulated and 21 upregulated (FC  $\geq 1.2$  or  $\leq -1.2$ ; Table E1). Canonical pathway analysis revealed that the Eicosanoid signaling pathway was particularly affected by BK activation.

Collectively, these data identify signaling pathways with a high likelihood of involvement in BK-mediated regulation of LPS-induced cytokine secretion by reducing proinflammatory and promoting antiinflammatory signals.

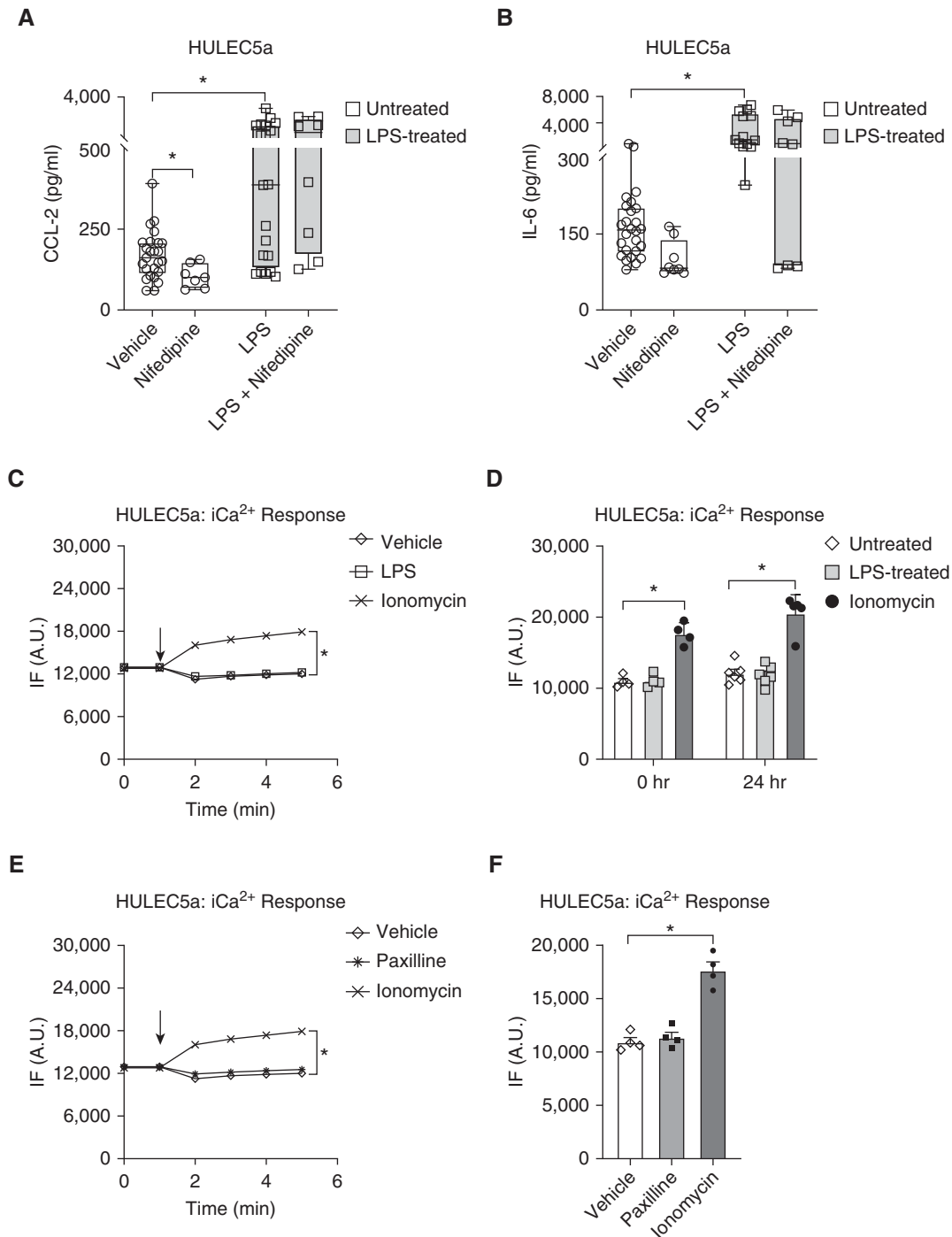
## Discussion

In this study, we provide the first evidence for the expression of functional BK channels (pore-forming  $\alpha 1$  subunit *KCNMA1* and the auxiliary *KCNMB1*, -3, -4  $\beta$ -subunits) in human microvascular endothelial cells and in primary human alveolar epithelial cells by demonstrating pharmacological hyperpolarization with two BK activators (NS1619 and NS19504), and propose a potential role for BK channels in LPS-induced cytokine secretion.

In tissues other than the lung, a role for BK channels in LPS-induced inflammation has previously been implicated by a few other groups. In fact, in LPS-infected mouse urinary bladders, inhibition of BK with

Iberitoxin prevents inflammatory cytokines secretion, including IL-6 (22), and in rat hippocampal neurons, BK activation promotes IL-1- $\beta$  release (23). BK-mediated regulation of cytokine secretion from macrophages appears dependent of the cellular phenotype. In RAW264.7 murine macrophages, genetic (siRNA) or pharmacological (Iberitoxin) inhibition of BK channels increases TNF- $\alpha$  release (24), whereas in bone marrow-derived macrophages, BK inhibition suppresses TNF- $\alpha$  and IL-6 release (25, 26).

Our findings expand our knowledge on the role of BK channels in regulation of inflammatory processes by demonstrating an important role for BK activity in cytokine secretion from human lung resident cells, such as primary alveolar epithelial cells and



**Figure 4.** LPS-induced CCL-2 and IL-6 secretion from HULEC5a cells does not require intracellular  $\text{Ca}^{2+}$  ( $\text{iCa}^{2+}$ ). (A and B) Inhibition of L-type  $\text{Ca}_v$  channels with Nifedipine ( $10 \mu\text{M}$ ; 24 h) decreased baseline CCL-2 but not IL-6 levels. Although LPS ( $2 \mu\text{g}/\text{ml}$ ; 24 h) induced both CCL-2 and IL-6 secretion, these effects were not inhibited by Nifedipine ( $n = 7-26$ ,  $*P \leq 0.05$ ). (C) Representative recording of  $\text{iCa}^{2+}$  kinetics represented by changes in relative IF using a Fluo-4 assay. Treatment of endothelial cells with LPS ( $2 \mu\text{g}/\text{ml}$ ) did not affect  $\text{iCa}^{2+}$  concentrations. (D)  $\text{iCa}^{2+}$  levels were recorded either immediately after  $2 \mu\text{g}/\text{ml}$  LPS treatment (time 0 h) or after 24 hours of LPS treatment ( $n = 4-6$ ). BK inhibition with Paxilline ( $10 \mu\text{M}$ ) did not affect  $\text{iCa}^{2+}$  levels as shown in a representative recording in E and as a summary of  $n = 4$  in F. Ionomycin served as a positive control for the  $\text{iCa}^{2+}$  signal ( $n = 4-6$ ,  $P \leq 0.05$ ). Black arrows indicate the timing of drug delivery.

**Table 1.** Summary of Top Gene Candidates Affected by NS1619 in LPS-treated Human Pulmonary Microvascular Endothelial Cells

Gene Symbol	Gene Description	FC by Microarray	FC by Confirmatory RT-PCR
<i>TNFSF-13B</i>	Tumor necrosis factor ligand superfamily member 13B/B-cell activating factor ( <i>BAFF</i> )	-3.1	-3.3
<i>CASP-1</i>	Caspase-1	-2.3	-2.0
<i>NF-κB-1</i>	Nuclear factor kappa B subunit-1	2.0	1.0
<i>ALOX-5</i>	Arachidonate 5-lipoxygenase	-1.5	0.1
<i>TNF</i>	Tumor necrosis factor	1.3	-0.8
<i>PTGS-2</i>	Prostaglandin-endoperoxide synthase-2, cyclooxygenase-2 or <i>COX-2</i>	4.3	2.2
<i>IL1R-1</i>	Interleukin 1 receptor, type I/Cluster of differentiation 121a ( <i>CD121a</i> )	2.4	1.2
<i>TLR-4</i>	Toll-like receptor-4	Not tested	-1.2
<i>MAPK-8</i>	Mitogen-activated protein kinase-8 ( <i>JNK-1</i> )	1.2	1.4
<i>MAPK-14</i>	Mitogen-activated protein kinase-14 ( <i>p38</i> )	-0.1	0.0
<i>MAPK-3</i>	Mitogen-activated protein kinase-3 ( <i>ERK-1</i> )	0.1	-0.2
<i>MAPK-1</i>	Mitogen-activated protein kinase-1 ( <i>ERK-2</i> )	0.2	0.4

Definition of abbreviation: FC = fold change in gene expression.

pulmonary microvascular endothelial cells. Remarkably, BK channel activation with NS1619, the most commonly used BK agonist, inhibited CCL-2 but not IL-6 secretion from endothelial but not epithelial cells. Conversely, BK channel inhibition with a classic BK blocker, Paxilline, had no effect on LPS-induced cytokine secretion from either cell type (27), suggesting that most BK channels are likely closed under those conditions. It was also interesting to observe a larger variability in baseline CCL-2 and IL-6 secretion from epithelial cells than endothelial cells, which could be explained by the fact that the HULEC5a cells consist of a fairly homogenous cell population, whereas the primary alveolar epithelial cells represent a mixture of type-1 and type-2 cells and may derive from more than one donor.

Based on current knowledge, we initially anticipated that LPS stimulation would increase  $iCa^{2+}$  levels, resulting in an increase in inflammatory cytokine secretion, and that we could counteract this process by hyperpolarizing the Em with a BK activator (NS1619 and NS19504). Of note, NS1619, a benzimidazolone, was introduced as a synthetic BK opener in 1994 and is the most commonly used pharmacological tool to activate functional BK channels (19). To our surprise, we found no effect of LPS exposure on the Em in either endothelial or epithelial cells. Previous literature reports suggest that the effects of LPS on the Em may vary among different cell types. In fact, LPS activates BK channels in murine vascular smooth muscle

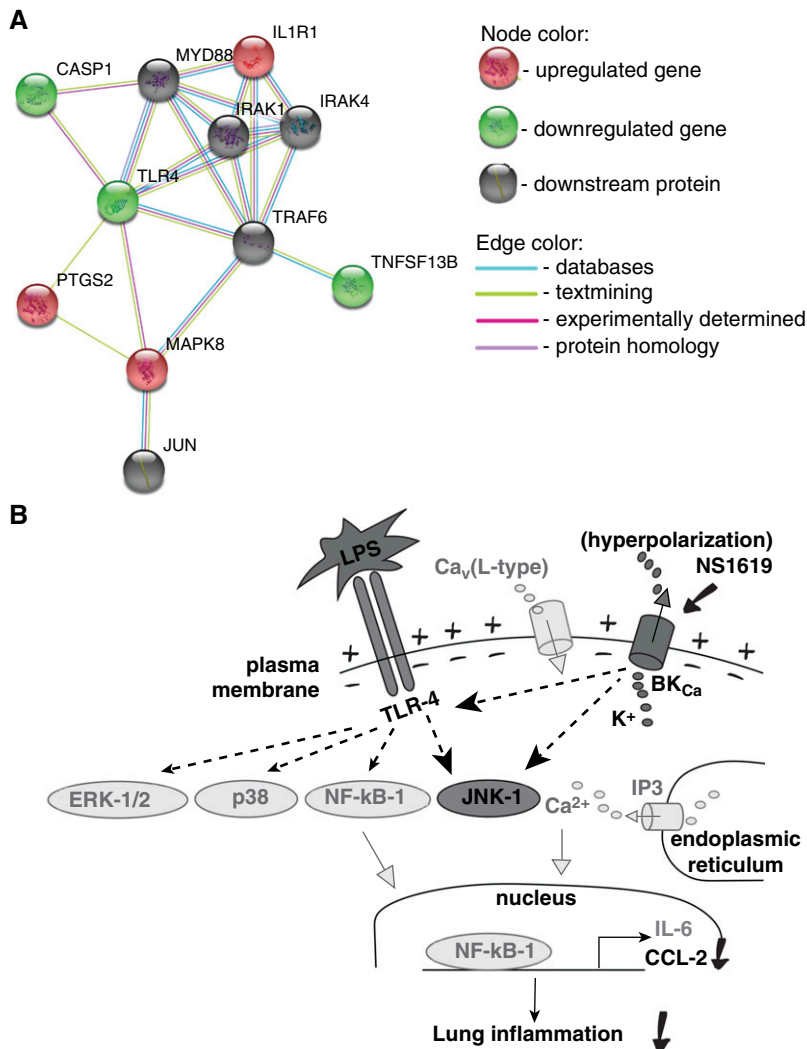
cells (28) but causes a delay in rectifier potassium currents in rabbit left atrial myocytes (29). Importantly, both cell types were susceptible to pharmacological Em hyperpolarization with two BK activators, NS1619 and NS19504, suggesting the presence of functional BK channels in both cell types and supporting the specificity of both drugs toward BK channels. Previous multiple parameter optimization analysis, which predicts drug-like properties of small molecules (30), revealed that NS19504 stands out from earlier reported compounds because of its low molecular weight and absence of an acidic function and, therefore, represents an interesting lead in the search for new BK channel modulators. Importantly, NS19504 (5-[(4-bromophenyl) methyl]-1,3-thiazol-2-amine), introduced in 2014 by Nausch and colleagues in HEK293 cells, has very little effect on IK, SK3, and  $Na_v1.2$  channels, and virtually no effect on high-threshold  $Ca_v$  channels (20). Furthermore, NS19504 also lacks any effects on 65 other channels and receptors, including L- and N-type  $Ca^{2+}$ -channels;  $Na_v$ ,  $K_{ATP}$ , and hERG channels; muscarinic M1, M2, and M3 receptors; neuropeptide Y1 and Y2 receptors; NK1 receptor; and purinergic P2X and P2Y receptors (20). Together with our NS1619 data, these observations provide strong experimental support for the selectivity profile of NS19504 for BK channels and limit concerns for off-target effects. Nevertheless, as with all ion channel modulators, it is always important to remain vigilant for potential off-target

effects. In fact, inhibitory effects of NS1619 have been reported on L-type  $Ca^{2+}$  channels in rat ventricular myocytes (31) and human neuroblastoma cells (SH-SY5Y) (32), but the lack of effect of the specific L-type  $Ca^{2+}$  channel inhibitor Nifedipine on cytokine secretion in our study argues against a contribution of such an off-target effect to our results. Another potential off-target effect of NS1619 observed in cardiac and neuronal cells is inhibition of the mitochondrial electron transport chain and ROS production, resulting in protection from ischemia and reperfusion injury (33). In an initial set of experiments, we found no effect of NS1619 or LPS on endothelial ROS production, and therefore, we chose to rather explore in more detail the effects of NS1619 on CCL-2 and IL-6 signaling cascades (Tables 1 and E1).

One of our most interesting findings is that BK activation (NS1619) decreases LPS-induced CCL-2 secretion (Figure 3A). CCL-2 is a proinflammatory mediator found in high concentrations in the BAL fluid and plasma of patients with ARDS and LPS-induced ALI mouse models (34). Secretion of CCL-2 by LPS-activated endothelial and epithelial cells results in the selective recruitment of leukocytes to inflammatory foci, thus promoting inflammation (35), but can also accelerate bacterial clearance during sepsis (36).

Although multiple studies have linked ion channel activity to changes in cytokine secretion (37), the molecular mechanisms by which  $K^+$  channels regulate secretory events in alveolar epithelial and microvascular





**Figure 5.** (A) Functional protein association network (STRING) model based on our microarray and confirmatory real-time PCR data on HULEC5a cells treated with LPS and NS1619. (B) Putative model of NS1619-mediated LPS signaling downstream pathways in HULEC5a cells. More-likely pathways are labeled in black, while less-likely pathways are labeled in gray.

endothelial cells are still unclear. Our data highlight important differences in the Em dependency of CCL-2 and IL-6 secretion between epithelial and endothelial cells, by showing that once LPS-stimulated endothelial cells are exposed to a hyperpolarized environment (BK channel activation), CCL-2 secretion is impaired, whereas IL-6 secretion appears to occur unharmed. In contrast, in epithelial cells, neither CCL-2 nor IL-6 secretion are altered by Em hyperpolarization with NS1619. Therefore, in the case of endothelial CCL-2 secretion, it is reasonable to assume that at least one voltage-dependent step exists in the LPS signaling cascade. Because classical LPS signaling occurs via the Toll-like receptor-4

(TLR-4), NF- $\kappa$ B and MAPKs pathways (38), it is intriguing to speculate that activation of one of these molecules is voltage-dependent and plays a key role in the electrochemical signal conversion process. Such a finding would be a very important, novel, and potentially paradigm-shifting concept and needs to be further explored. Notably, TLR-4 stimulation can also activate BK channels directly by elevating intracellular  $iCa^{2+}$  concentrations because BK is highly  $Ca^{2+}$  sensitive (39). However, such an  $iCa^{2+}$ -dependent, LPS-TLR-4-mediated effect on BK channels is unlikely in endothelial cells because we saw no changes in  $iCa^{2+}$  levels with LPS treatment. Furthermore, although LPS is known to increase  $iCa^{2+}$

concentrations in several cell types (22, 40, 41), the lack of such an effect in HULEC5a cells helps explain why inhibition of voltage-gated  $Ca_v$  channels with Nifedipine had no effect on CCL-2 or IL-6 secretion (Figure 4). Notably, expression of Nifedipine-sensitive, L-type ( $Ca_v$  1.2) channels in pulmonary endothelial cells has previously been reported (42). We also found that the BK inhibitor Paxilline had no effect on the Em (Figure 2C) or  $iCa^{2+}$  levels (Figures 4E and 4F), suggesting that in both endothelial and epithelial cells, most BK channels are constitutively closed.

Interestingly, and in contrast to our findings, in certain cell types, BK channel expression and activity appear to be upregulated by LPS. In RAW264.7 murine macrophages, LPS upregulates BK channel expression (24), and in mouse bladder epithelium (umbrella cells), BK channel activity is increased after LPS exposure (43). In murine microglia, plasma membrane BK currents can be activated by LPS at a very early stage through TLR-4, leading to nuclear translocation of NF- $\kappa$ B-1 and production of proinflammatory cytokines such as TNF- $\alpha$  and IL-6 (44). Similarly, studies in murine monocytes/macrophages found that BK channels are opened by LPS, leading to inhibition of NF- $\kappa$ B-1 translocation to the nucleus, and a decrease in TNF- $\alpha$  and IL-8 secretion (45).

Our microarray and real-time PCR results in HULEC5a cells highlight several pro- and antiinflammatory genes involved in LPS-induced signaling pathways that are regulated by the BK activator NS1619 and could be responsible for the observed decrease in LPS-induced CCL-2 secretion (Table 1). Interestingly, although our top candidates (*TNFSF-13B*, *CASP-1*, *PTGS-2*, *IL1R-1*) confirm NF- $\kappa$ B-1-dependent signaling cascades, NF- $\kappa$ B-1 expression itself was not changed by NS1619. This is a particularly interesting finding because NF- $\kappa$ B is known for both its pro- and antiinflammatory properties (46). Of note, TNFSF13B (BAFF), a member of the TNF ligand superfamily, acts downstream of the IL1R1 and TLR4-pathway through an NF- $\kappa$ B pathway (47), whereas CASP-1 is recruited by the NLRP3-inflammasome and promotes secretion of proinflammatory cytokines ("proinflammatory" caspase) (48), and IL1R-1 facilitates TLR-driven antiinflammatory cytokine production (49, 50). The effects of BK activation

(NS1619) on members of the eicosanoid signaling pathway are also interesting (upregulation of PTGS-2) and need further investigation. Furthermore, we also found that NS1619 downregulates TLR-4 expression, the main LPS-binding receptor, which might contribute to the observed decrease in endothelial CCL-2 secretion. Similarly, it appears that among the numerous MAP kinases, the *JNK-1* pathway is particularly involved in the regulation of CCL-2 secretion, whereas other main pathway candidates (p38 and ERK-1/2) are less likely contributors.

Taken together, our results demonstrate that BK channel activation reduces LPS-induced CCL-2 release from human pulmonary microvascular endothelial cells but not alveolar epithelial cells, and that these events occur independently of  $\text{Ca}^{2+}$  influx. Based on our microarray and real-time PCR data, we propose a model where NS1619 downregulates TLR-4 expression and subsequently (or simultaneously) activates JNK-1-mediated signaling cascades that ultimately result in a decrease in LPS-induced CCL-2 secretion (Figure 5). For future studies, it is

particularly important to focus on Em-dependent versus Em-independent mechanisms of inflammatory cytokine secretion. These processes remain poorly understood but may constitute novel targets for rational antiinflammatory drug design. ■

**Author disclosures** are available with the text of this article at [www.atsjournals.org](http://www.atsjournals.org).

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