Inhibition of endotoxin-induced airway epithelial cell injury by a novel family of pyrrol derivates

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Inflammation and apoptosis are crucial mechanisms for the development of the acute respiratory distress syndrome (ARDS). Currently, there is no specific pharmacological therapy for ARDS. We have evaluated the ability of a new family of 1,2,3,5-tetrasubstituted pyrrol compounds for attenuating lipopolysaccharide (LPS)-induced inflammation and apoptosis in an *in vitro* LPS-induced airway epithelial cell injury model based on the first steps of the development of sepsis-induced ARDS. Human alveolar A549 and human bronchial BEAS-2B cells were exposed to LPS, either alone or in combination with the pyrrol derivatives. Rhein and emodin, two representative compounds with proven activity against the effects of LPS, were used as reference compounds. The pyrrol compound that was termed DTA0118 had the strongest inhibitory activity and was selected as the lead compound to further explore its properties. Exposure to LPS caused an intense inflammatory response and apoptosis in both A549 and BEAS-2B cells. DTA0118 treatment downregulated Toll-like receptor-4 expression and upregulated nuclear factor-κB inhibitor-α expression in cells exposed to LPS. These anti-inflammatory effects were accompanied by a significantly lower secretion of interleukin-6 (IL-6), IL-8, and IL-1β. The observed antiapoptotic effect of DTA0118 was associated with the upregulation of antiapoptotic Bcl-2 and downregulation of proapoptotic Bax and active caspase-3 protein levels. Our findings demonstrate the potent anti-inflammatory and antiapoptotic properties of the pyrrol DTA0118 compound and suggest that it could be considered as a potential drug therapy for the acute phase of sepsis and septic ARDS. Further investigations are needed to examine and validate these mechanisms and effects in a clinically relevant animal model of sepsis and sepsis-induced ARDS.
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The acute respiratory distress syndrome (ARDS) develops as a result of an acute and intense inflammation of the lung.[1,2] Sepsis is the most common predisposing factor and sepsis-associated ARDS has a higher disease severity, poorer recovery from acute lung injury, and higher mortality compared with non-sepsis-related ARDS.[3] Bacterial lipopolysaccharide (LPS), also termed endotoxin, is the major component of the outer membrane of Gram-negative bacteria and one of the major pathogen-associated pattern recognition molecules that activate the innate immunity. LPS has a crucial role in the inflammatory response triggered by Gram-negative bacteria in epithelial and other cells of the lung. Toll-like receptor-4 (TLR4) recognizes LPS and initiates a signaling cascade by several downstream adapter proteins that leads to the activation of nuclear factor-κB (NF-κB),[4] involving phosphorylation and

degradation of the inhibitor of NF-κB (IκBα), and the translocation of NF-κB heterodimers into the nucleus of the cells. Eventually, the NF-κB system exerts transcriptional regulation on the expression of genes related to innate immunity and inflammation, and other gene products.[5]

LPS can induce apoptosis in lung endothelial and epithelial cells[6,7] and contributes to the pathogenesis of ARDS. Recent studies strongly suggested a role for epithelial apoptosis as a key profibrotic event in lung fibrogenesis.[8] Thus, there is a rationale to manipulate apoptosis therapeutically. Currently, there is no specific pharmacological treatment of proven benefit in ARDS. Based on a well-established protocol of the National Cancer Institute of the United States,[9] we used a validated screening strategy to identify potential therapeutic molecules by examining the effects of a novel family of

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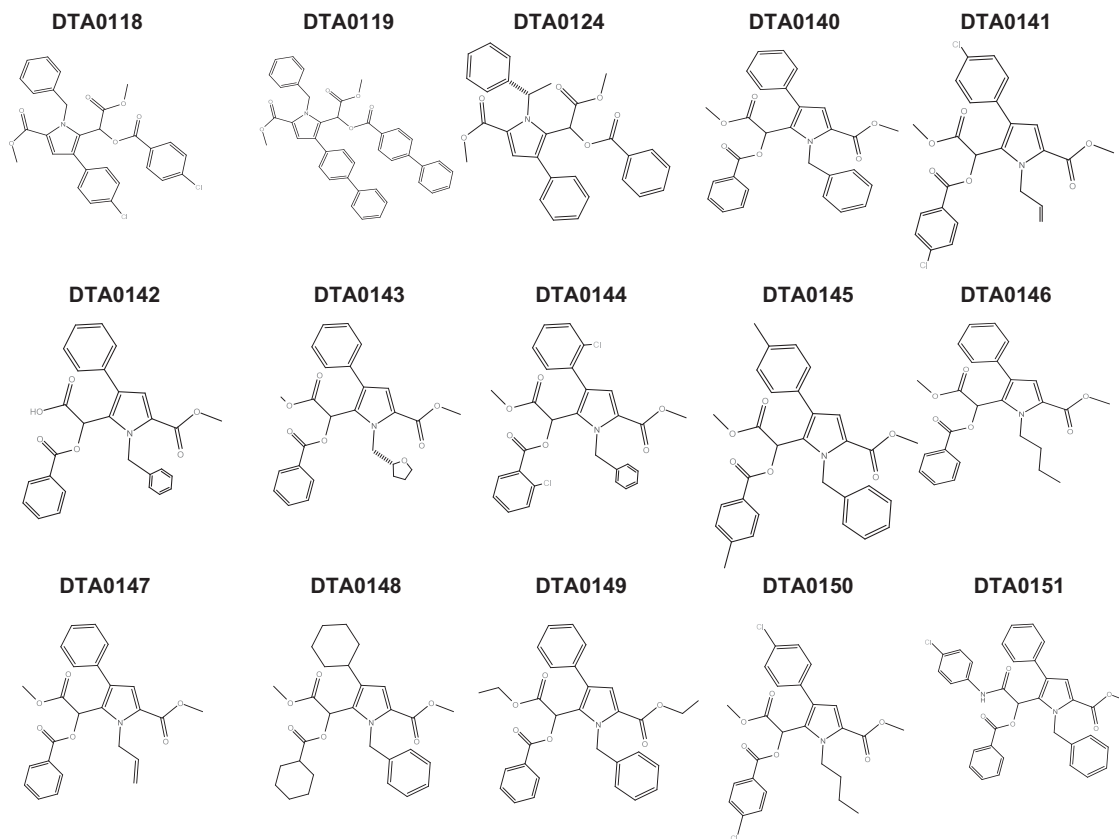E-mail: jesus.villar54@gmail.com

Figure 1 Chemical structures of 1,2,3,5-tetrasubstituted pyrrols derivatives.

tri- and tetrasubstituted pyrrols, which were previously studied by our group.[10] In the present study, we report the marked anti-inflammatory and antiapoptotic properties of a compound termed by us as DTA0118 in a well-established *in vitro* LPS-induced airway epithelial cell injury model,[11–16] based on the initial steps of the development of sepsis and sepsis-induced ARDS, using human alveolar A549 and human bronchial BEAS-2B cells. We used rhein and emodin, two anthracene-derived anthraquinones with proven activity against the effects of LPS,[17] as reference compounds. Our results demonstrate that the compound DTA0118 prevents LPS-induced cell death and inhibits TLR4 upregulation and IκBα downregulation.

MATERIALS AND METHODS
Cell Lines, Cell Cultures, and Reagents
We used human lung alveolar type-II A549 cell line and human bronchial epithelial BEAS-2B cells (American Type Culture Collection, Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) inactivated fetal bovine serum (FBS) and in antibiotics (0.1 mg/ml streptomycin, 100 U/ml penicillin) in a humidified incubator at 37 °C with 5% CO_2 and 95% humidified air (see Electronic Supplementary Material (ESM) for details).

Survival Assays and Cell Growth
A total of 3×10^4 A549 and BEAS-2B cells per well were seeded in a 96-well plate in a volume of 100 μl of DMEM supplemented with 2% (v/v) inactivated FBS and in antibiotics. After 24 h, cells were exposed to 100 ng/ml LPS either alone or in combination with 10 μM rhein, emodin, or 1,2,3,5-tetrasubstituted pyrrol compounds (Figure 1). After 18 h, cell survival was analyzed by precipitation with 25 μl ice-cold TCA 50% (w/v) and fixed for 60 min at 4 °C. Then, we performed the sulforhodamine B (SRB) assay.[18] We then studied the anti-inflammatory and antiapoptotic properties of the most active compound (termed by us as DTA0018). The SRB assay was used to test the effects of various concentrations of DTA0118 (0.1, 1, 10, 100, and 1000 μM) in the presence of A549 and BEAS-2B cells (see ESM for details).

Western Blot Analysis
After 18 h of compound incubation (control-vehicle: 100 ng/ml *Escherichia coli* LPS alone or in combination with 10 μM rhein, emodin, or DTA0118), A549 and BEAS-2B cells from each experimental group were harvested and centrifuged at 1200 r.p. m. for 7 min using a protocol described previously by our group.[5] Protein concentrations in extracts were determined
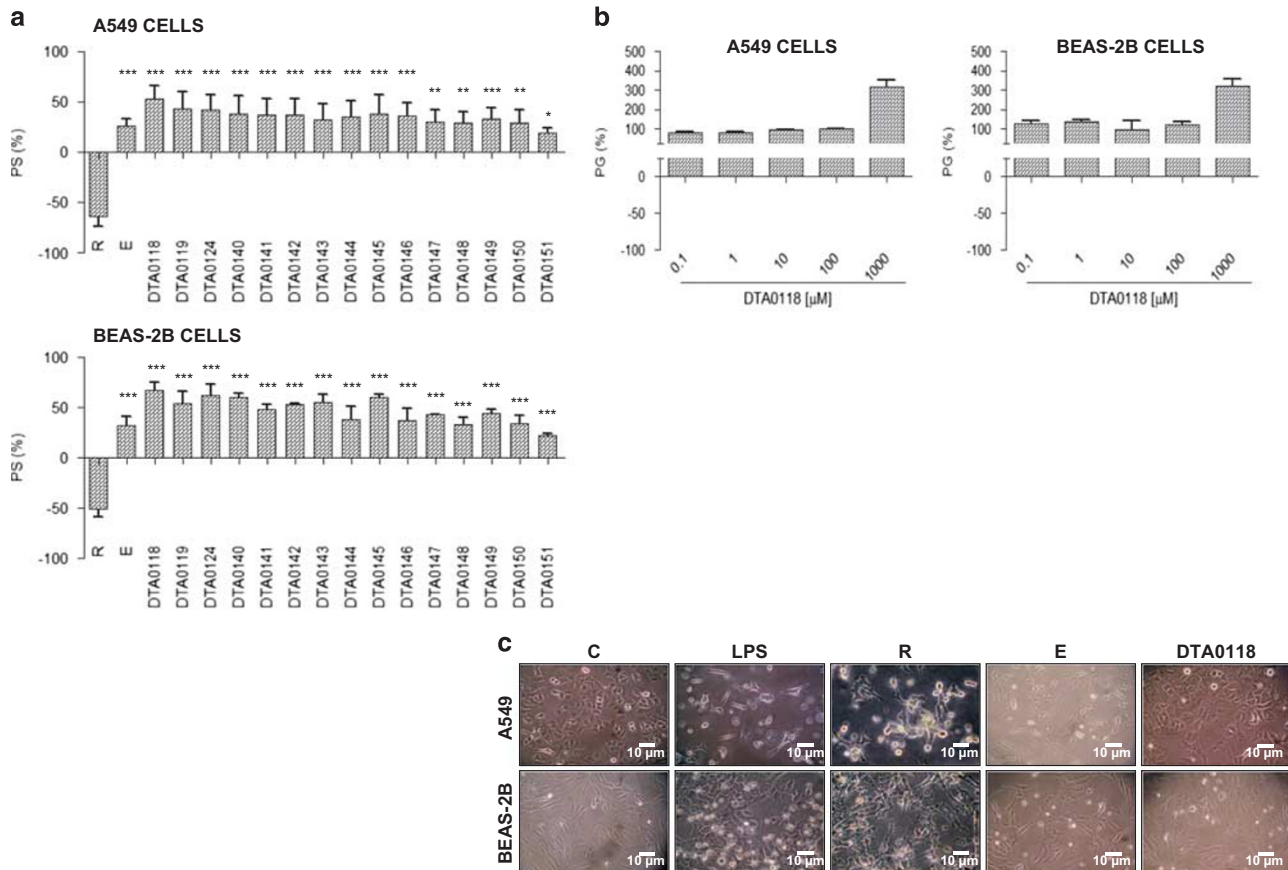
Figure 2 Effects of pyrrol compounds on A549 and BEAS-2B cells survival. (**a**) Percentage of survival of 100 ng/ml lipopolysaccharide (LPS)-stimulated A549 and BEAS-2B cells in combination with 10 μM rhein (R), emodin (E) and 15 1,2,3,5-tetrasubstituted pyrrol synthetic compounds. (**b**) Percentage of A549 and BEAS-2B cell growth under the effects of 0.1, 1, 10, 100, and 1000 μM of compound DTA0118. In (**a** and **b**), A549 and BEAS-2B cells were stimulated with 100 ng/ml LPS alone or in combination with 10 μM rhein (R), emodin (E) and 15 1,1,3,5-tetrasubstituted pyrrol synthetic compounds (A) or with 0.1, 1, 10, 100, and 1000 μM of lead compound DTA0118 for 18 h (B). (**c**) Morphological features of human A549 and BEAS-2B cells following the stimulation with 100 ng/ml of LPS (LPS) plus R (LPS+rhein), E (LPS+emodin), and DTA0118(LPS+DTA0118) for 18 h. Panels correspond to x200 objective. C = untreated control-vehicle cells. *$P < 0.05$ *vs* R; **$P < 0.01$ *vs* R; ***$P < 0.001$ *vs* R. Note the marked growth of A549 and BEAS-2B cells treated with 1000 μM of lead compound DTA0118.

with the Bio-Rad DC protein assay (Bio-Rad DC, Hercules, CA, USA). For immunoblotting, 30 μg of samples were electrophoresed and transferred to polyvinylidene difluoride (PVDF) membranes, blocked for 1 h at room temperature, and probed with primary antibodies to TLR4, IκBα, Bax, Bcl-2, caspase-3, and β-actin in all experimental groups (see ESM for details). Chemiluminiscence detection was performed with the ECL Kit (Thermo Fisher Scientific, Waltham, MA, USA). Densitometry in each band was performed and measured using the Scion Image software package. Western blots were repeated three times in each experimental condition.

Detection of Apoptosis

We used an Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) for measuring early apoptosis and cell death according to the manufacturer's instructions. A549 and BEAS-2B cells were seeded at 2×10^6 in DMEM supplemented with 2% inactivated FBS and 1% antibiotics for 24 h. Then,

cells were treated during 18 h with or without 100 ng/ml *E. coli* LPS alone or in combination with 10 μM rhein, emodin, or DTA0118. After the treatment, A549 and BEAS-2B cells were analyzed by means of flow cytometry according to 100 000 cells (events). Results were analyzed using the WinMDI 2.8 software (Scripps Institute, La Jolla, CA, USA) (see ESM for details).

Measurement of Cytokines

A total of 3×10^5 A549 and BEAS-2B cells were cultured in 96-well plates. After 24 h, cells were treated with 100 ng/ml LPS alone or in combination with 10 μM rhein, emodin, or DTA0118 for 18 h. Afterwards, the medium of each well was collected and stored at − 80 ℃. Then, we measured the levels of interleukin-6 (IL-6), IL-8, and IL-1β in A549 and BEAS-2B cell culture supernatants using Cytometric Bead Array Human Inflammatory Cytokines Kit (BD Biosciences) in a FACSCanto II Flow Cytometer (see ESM for details).

Statistical Analysis

Data are expressed as mean ± s.d. and analyzed for significant differences among groups using one-way analysis of variance followed by *post hoc* testing using the Bonferroni corrections for multiple comparisons. Densitometric data were normalized to β-actin as loading controls. Data are from three independent experiments. A two-sided *P*-value < 0.05 was considered statistically significant.

RESULTS

Effects of Pyrrol Compounds on Cell Survival and Growth

Mean percentage of cell survival (PS) with rhein was $-64.2 \pm 20.1\%$ in A549 cells and $-51.0 \pm 15.2\%$ in BEAS-2B cells, and with emodin it was $26.5 \pm 12.6\%$ in A549 cells and $32.2 \pm 18.9\%$ in BEAS-2B cells. Cells treated with pyrrol compounds showed higher PS values. The best results were obtained for the derivative DTA0118 with a PS of $53.3 \pm 25.8\%$ in A549 cells and $67.1 \pm 15.4\%$ in BEAS-2B cells (Figure 2a and Table 1). No A549 and BEAS-2B cell death occurred at any concentration of DTA0118 (Figure 2b).

DTA0118 Prevented LPS-Induced Cellular Morphological Changes

Control-vehicle cells showed an intact appearance, whereas the LPS-stimulated cells displayed morphological changes, such as stretching. Additionally, LPS- plus rhein- and emodin-treated cells displayed a round morphology as well as floating round shapes. Of note, LPS-induced morphological changes were prevented after treatment with DTA0118 (Figure 2).

DTA0118 Decreased Cytokine Levels

IL-6, IL-8, and IL-1β levels increased in LPS-treated cells compared with untreated control-vehicle cells ($P < 0.001$ in all cases; $P < 0.01$ for IL-8 in A549 cells). This effect was reduced after cotreatment with 10 μM rhein or emodin ($P < 0.001$ for all cases; $P < 0.01$ for IL-8) in cotreatment with 10 μM rhein and A549 cells ($P < 0.05$ for IL-1β in cotreatment with 10 μM rhein and A549 cells). Inhibition of LPS-induced effects was marked after cotreatment with 10 μM DTA0118 ($P < 0.01$ for IL-6, $P < 0.05$ for IL-8, and $P < 0.001$ for IL-1β in A549 cells; $P < 0.001$ for all cytokines in BEAS-2B cells) (Figure 3).

Effects of DTA0118 on TLR4 and IκBα Protein Levels

The expression of TLR4 increased in A549 and BEAS-2B cells after LPS stimulation ($P < 0.001$) (Figure 4) (see Supplementary Table E3 in ESM for mean ± s.d. values of densitometric analysis in each experimental group). Down-regulation of TLR4 protein levels was observed when both cell types were cotreated with LPS plus 10 μM emodin or rhein, but these effects were maximum with cotreatment with 10 μM DTA0118 ($P < 0.001$). IκBα protein levels decreased after LPS stimulation compared with control-vehicle cells ($P < 0.001$) and increased after cotreatment with LPS plus 10 μM rhein or

emodin ($P < 0.01$ for rhein in A549 cells; $P < 0.001$ for the rest of experimental conditions). These changes were inhibited after treatment with DTA0118 ($P < 0.001$) (Figure 4) (see Table in the ESM for mean ± s.d. values of densitometric analysis in each experimental group). These results were replicated with TLR4 and IκBα immunocytochemical staining (see ESM for further details). We found a high intensity of IκBα staining in the nuclei and low-intensity staining in the cytoplasm of A549 and BEAS-2B cells stimulated with LPS, consistent with the low transcript level of NF-κB (see ESM for details).

LPS-Mediated Apoptosis was Prevented by DTA0118

After LPS incubation, the percentage of apoptosis and cell death increased when compared with untreated cells ($P < 0.001$ in both cell types) (Figure 5). This effect was prevented after treatment with DTA0118 ($P < 0.001$) (Figures 5a and b).

DTA0118 Inhibited LPS-Induced Changes of Bax, Bcl-2, and Caspase-3 Proteins

We performed additional western blot assays (Figure 6) for evaluating the mechanisms induced by DTA0118. Stimulation
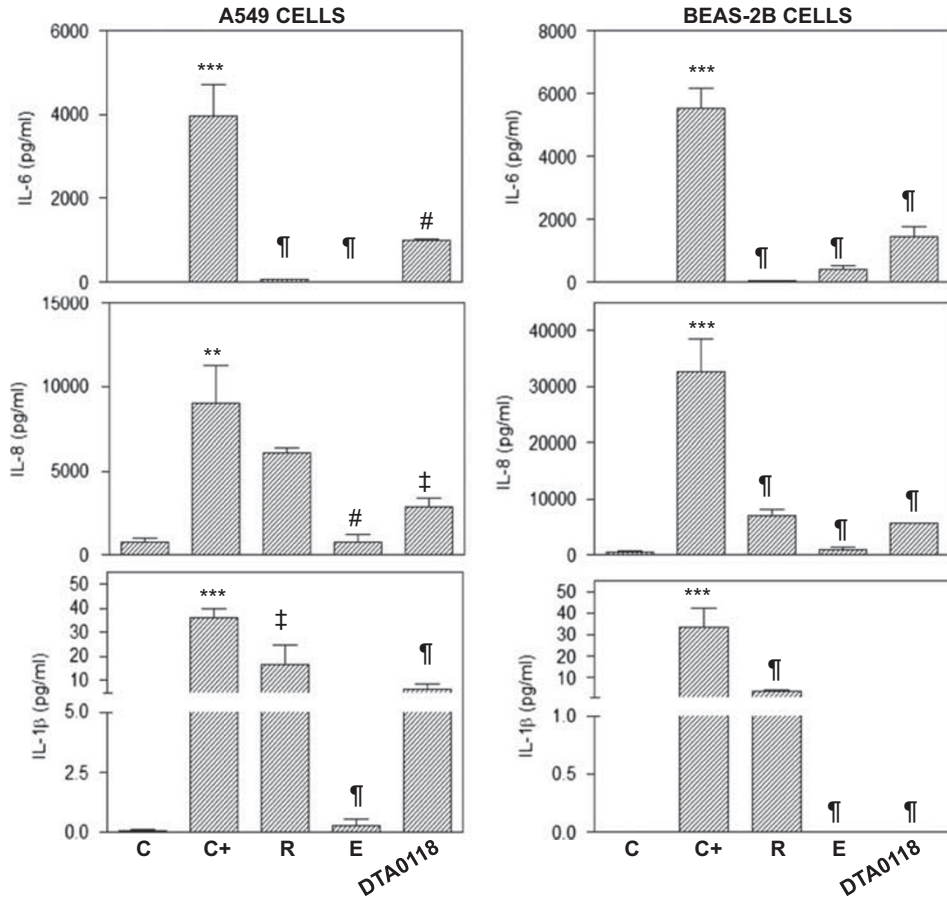
Table 1 Percentage of survival of human A549 and BEAS-2B cells[a]

Compound (10 μM)	A549 cells	BEAS-2B cells
	PS (± s.d.)	PS (± s.d.)
Rhein	−64.25 ± 20.07	− 51 ± 15.18
Emodin	26.50 ± 12.61	32.20 ± 18.9
DTA0118	53.33 ± 25.8	67.11 ± 15.39
DTA0119	42.96 ± 35.10	54.21 ± 24.49
DTA0124	42.59 ± 28.77	62.44 ± 22.32
DTA0140	37.91 ± 31.80	60.16 ± 6.97
DTA0141	37.17 ± 26.92	48.40 ± 7.95
DTA0142	36.90 ± 27.38	52.73 ± 3.21
DTA0143	32.53 ± 26.78	55.30 ± 12.89
DTA0144	34.80 ± 28.86	38.30 ± 22.81
DTA0145	38.55 ± 31.66	60.22 ± 5.92
DTA0146	35.79 ± 22.36	37.46 ± 19.47
DTA0147	29.96 ± 21.88	42.72 ± 0.76
DTA0148	29.07 ± 19.26	32.94 ± 12.64
DTA0149	33.33 ± 19.29	43.83 ± 6.84
DTA0150	29.27 ± 21.87	34.60 ± 12.68
DTA0151	19.45 ± 8.18	22.47 ± 3.09

LPS, lipopolysaccharide; PS, percentage of cell survival.
[a]Stimulated with 100 ng/ml LPS in combination with 1,2,3,5-tetrasubstituted pyrrol compounds.

Figure 3 Cytometric bead array analyses to determine interleukin-6 (IL-6), IL-8 and IL-1β. C: untreated control-vehicle cells, C+: cells treated with lipopolysaccharide (LPS), R: LPS+rhein, E: LPS+emodin, DTA0118: LPS+DTA0118 for 18 h. **$P < 0.01$ *vs* C; ***$P < 0.001$ *vs* C; ‡$P < 0.05$ *vs* LPS; #$P < 0.01$ *vs* LPS; ¶$P < 0.001$ *vs* LPS.

with LPS induced upregulation of proapoptotic Bax protein when compared with control-vehicle cells ($P < 0.001$). This effect was prevented in cells cotreated with emodin and DTA0118 compared with LPS-stimulated cells ($P < 0.001$). Furthermore, a significant downregulation of the antiapoptotic Bcl-2 protein was observed in LPS-stimulated cells ($P < 0.001$). Emodin and DT0118 prevented the LPS-induced downregulation of Bcl-2 protein ($P < 0.001$). Stimulation with LPS induced an upregulation of procaspase-3 and downregulation of caspase-3 when compared with control-vehicle cells ($P < 0.001$ for A549 cells and $P < 0.01$ for BEAS-2B cells). This effect was maximum with DTA0118 ($P < 0.001$ for both cell types) (Figure 6). See Supplementary Table E3 in the ESM for mean ± s.d. values of densitometric analysis in each experimental group.

DISCUSSION

This is the first report showing that 1,2,3,5-tetrasubstituted pyrrol compounds are able to prevent lung epithelial cell injury induced by bacterial LPS. The main findings of our study are: (i) our screening method identified a pyrrol compound that inhibited LPS-induced cell death in two well-validated *in vitro* models of endotoxin-induced airway epithelial cell injury; (ii) DTA0118, the candidate drug, inhibited the secretion of proinflammatory cytokines; (iii) this inhibition was associated with downregulation of TLR4 and upregulation of IκBα; (iv) DTA0118 prevented apoptosis by modulating Bax, Bcl-2, and caspase-3 protein levels; and (v) inhibition of LPS effects was seen while human cell integrity and viability was preserved during DTA0118 treatment.

LPS initiates an inflammatory and immune defense response in the lung through recognition by TLR4.[4] Our results demonstrate that pyrrol compound DTA0118 inhibited changes in TLR4 and IκBα expression levels in human A549 and BEAS-2B cells stimulated by LPS, and this inhibition was much more effective than the effects produced by the reference compounds. Furthermore, DTA0118 significantly inhibited the synthesis of cytokines. IL-1β was generated presumably by activation of the inflammasome,[19] which has been linked to the pathogenesis of ARDS.[20] This inhibition of proinflammatory cytokines has also been
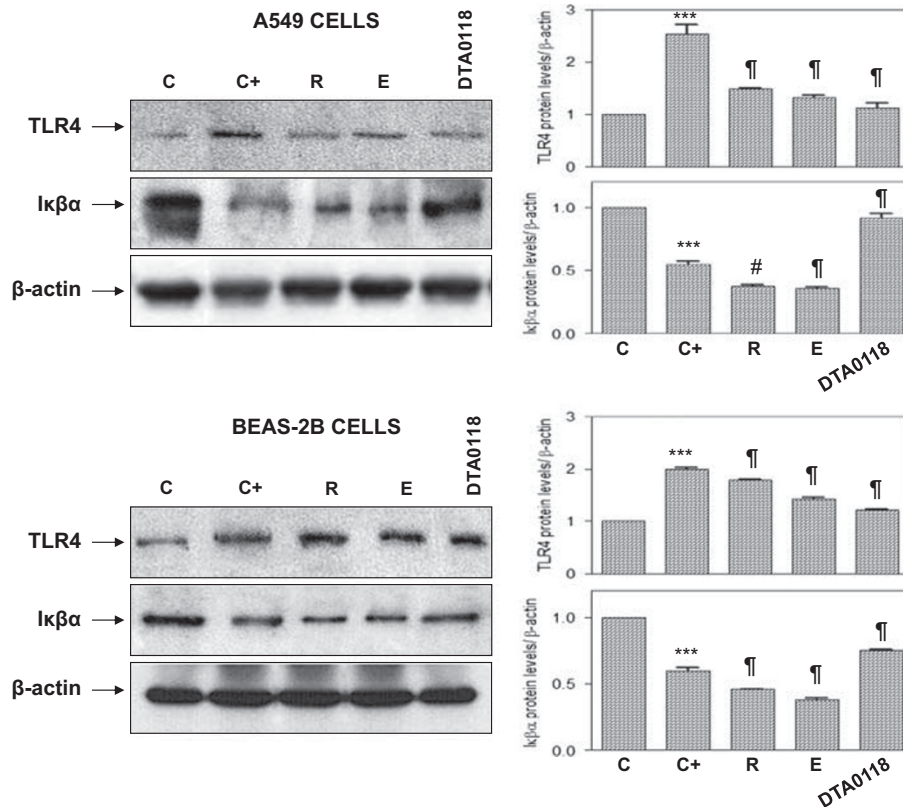
Figure 4 Effects of DTA0118 on lipopolysaccharide (LPS)-induced changes of Toll-like receptor-4 (TLR4) and IκBα. Representative blots from each experimental condition and histograms exhibiting the changes in TLR4 and IκBα protein levels. C: untreated control-vehicle cells; C+: cells treated with LPS; R: LPS+rhein; E: LPS+emodin; DTA0118: LPS+DTA0118 for 18 h. ***$P < 0.001$ *vs* C; #$P < 0.01$ *vs* LPS; ¶$P < 0.001$ *vs* LPS.

reported by other compounds, such as inosine,[14] bupleurum polysaccharides,[21] proxylin A,[22] and quercetin,[23] and by other pyrrol compounds, such as T-686, an inhibitor of plasminogen activator inhibitor-1 that was associated with a mortality reduction in mice exposed to LPS.[24] However, none of those compounds had described any antiapoptotic activity in sepsis-induced lung injury.

We chose A549 and BEAS-2B cell lines as representative human alveolar and bronchial epithelial cells because they have been implicated in the pathogenesis of sepsis-induced ARDS.[11–16] One of the most important determinants of lung severity is the magnitude of injury to the epithelial barrier.[2] Epithelial cells generate cytokines, chemokines, and anti-microbial peptides in response to inflammatory stimuli,[25,26] and airway epithelium controls lung inflammation and injury through NF-κB.[27] Numerous experimental studies have used human A549 alveolar and BEAS-2B bronchial epithelial cell lines to examine the acute lung inflammatory response induced by LPS, as an acceptable, validated, and suitable *in vitro* airway epithelial injury model based on the initial steps of the development of sepsis and ARDS[11–16,25,28–30] and continue being extensively used.[31–35] We selected *E. coli* LPS because it has been used in most endotoxin-induced lung injury models[36,37] and LPS is a key pathogen recognition

molecule for sepsis[27] that induces apoptosis in lung cells.[38] Previous reports examining the efficacy of molecules on the LPS-induced activation of inflammatory markers in the lung, or before evaluating the potential *in vivo* anti-inflammatory properties, have used a similar *in vitro* epithelial cell injury model to the one we used.[14,39]

The activation of apoptosis following LPS-induced lung inflammation have a key role in the development of sepsis-induced ARDS.[38] The induction of apoptosis-mediated DNA damage is an important characteristic in the development of acute lung injury during sepsis and ARDS.[40,41] Apoptosis occurred in airway epithelial cells after LPS administration in an *in vivo* rat model.[42] While proliferation of type-II alveolar cells occurs during the early phase of ARDS as a reparative phenomenon, extensive apoptosis is responsible for the loss of these cells in the resolution phase. Two main pathways are involved in the induction of apoptosis:[42] (i) the mitochondrial pathway, which involves cytochrome *c* release from the mitochondria and the activation of caspase-9, which then cleaves and activates caspase-3, caspase-6, and caspase-7; and (ii) the death domain receptor pathway that involves activation of Fas or tumor necrosis factor receptors followed by the activation of caspase-8 and subsequent activation of downstream caspases. Cytochrome *c*
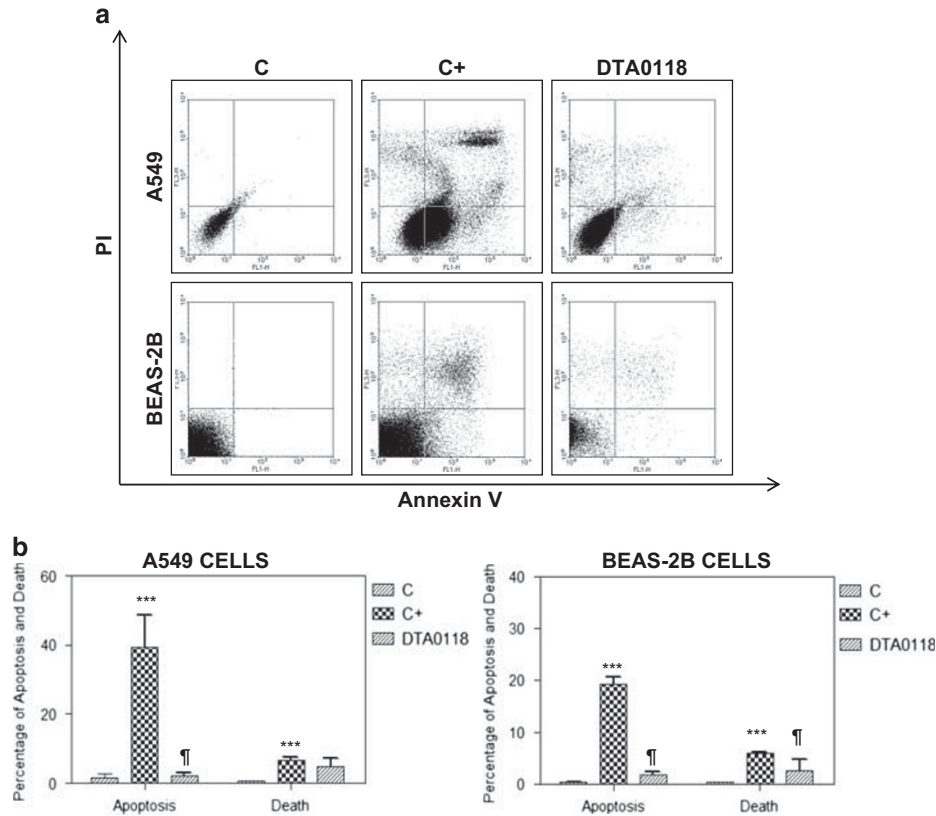
Figure 5 Lipopolysaccharide (LPS)-induced apoptosis is prevented by DTA0118. (**a**) Representative dot-plot diagrams demonstrating typical apoptotic and death in A549 and BEAS-2B cells populations detected by Annnexin V-FITC and propidium iodide (PI) staining. (**b**) Percentages of early apoptosis and death of A549 and BEAS-2B under all experimental conditions. C: untreated control-vehicle cells; C+: cells treated with LPS; DTA0118: LPS+DTA0118 for 18 h. ***$P < 0.001$ *vs* C; ¶$P < 0.001$ *vs* LPS.

release is determined by the ratio of antiapoptotic (Bcl-2 and Bcl-xL) and proapoptotic (Bax, Bad, Bik, and Bid) members of the Bcl-2 family proteins.[43] In our study, LPS stimulation induced apoptosis that was associated with changes in protein levels of antiapoptotic Bcl-2 and Bax and caspase-3. Previous studies have tested the hypothesis that an increased duration of sepsis stimulates apoptosis in lung tissue,[44,45] whereas others have suggested a biphasic response where changes in Bax/Bcl-2 protein levels were found after sepsis onset.[40] Although NF-κB could have a role in apoptosis by regulating the expression of genes involved in cell death, a specific role of NF-κB on the apoptotic process cannot be ascertained from our data. As reported by several investigators, apoptosis is a major pathway responsible for the resolution of alveolar cells in ARDS.[6] In our study, LPS induced a significant apoptotic effect in both lung epithelial cell lines and this effect was prevented by the cotreatment with 10 μM of the pyrrol compound DTA0118. Further investigations are needed to determine NF-κB-dependent and/or -independent apoptosis mechanisms.

Although our data may imply a role for pyrrol compounds in the therapy of the acute phase of sepsis and septic ARDS, we acknowledge some limitations to this study. First, we describe *in vitro* studies using micromolar concentrations; further studies are necessary to prove the relevance of these findings using nanomolar concentrations and *in vivo* models. Second, we did not use primary alveolar epithelial cells; however, there is a plethora of published studies validating the use of human BEAS-2B and A549 cells as an acceptable model of epithelial cell injury and for studying the LPS-induced effects in the airway epithelium.[11–16,25,28–35] Third, further studies are needed to elucidate whether there are other mechanisms by which the pyrrol compound DTA0118 elicits its anti-inflammatory and antiapoptotic activities. Fourth, TLR4, Ik$\beta\alpha$, and β-actin were not adjusted for the cell number. Furthermore, although we did not test whether these compounds modulate pulmonary vascular function by impacting endothelial cell function, Wang *et al*[32] have recently reported that the presence of A549 cells or primary human alveolar epithelial cells in a coculture model with endothelial cells attenuated septic pulmonary endothelial cell permeability by 40–100% using an *in vitro* model of LPS-induced ARDS. Based on these results, it is plausible that our pyrrol compound would have an additive effect by modulating vascular permeability during sepsis and sepsis (LPS)-induced ARDS. More studies are needed to explore the specific role of
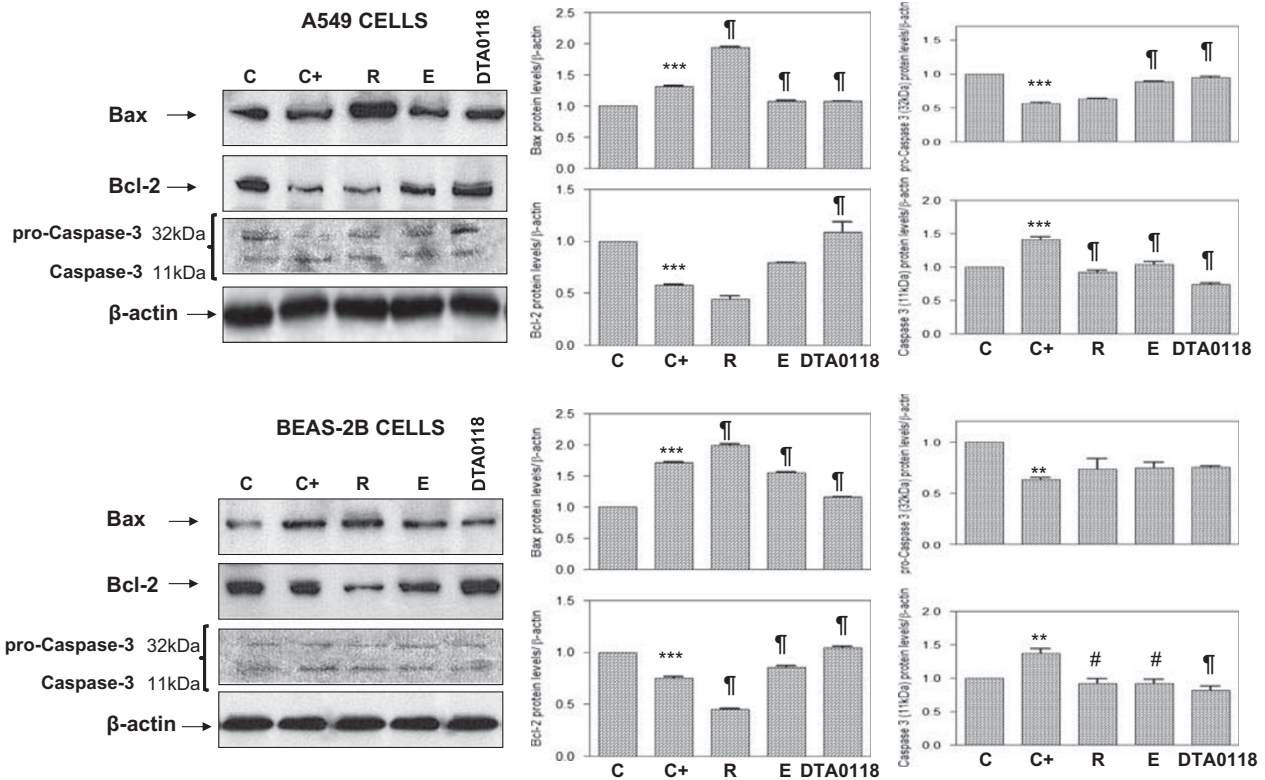
Figure 6 Immunobloting analyses for Bax, Bcl-2, and procaspase-3/caspase-3 in A549 and BEAS-2B cells. Immunobloting and β-actin-normalized densitometry of Bax, Bcl-2, procaspase-3 (32 kDa)/caspase-3 (11 kDa) western blots from protein extracts of A549 and BEAS-2B cells treated or not for 18 h with 100 ng/ml lipopolysaccharide (LPS) (C+) in combination or not with rhein (R), emodin (E), or DTA0118. C: untreated control-vehicle cells. **$P<0.01$ *vs* C; ***$P<0.001$ *vs* C; #$P<0.01$ *vs* LPS; ¶$P<0.001$ *vs* LPS.

pyrrol compound DTA0118 in the context of sepsis and septic ARDS.

In conclusion, this study showed *E. coli* LPS stimulated the production of proinflammatory mediators in an *in vitro* human airway epithelial cell injury model, and provided evidence that DTA0118, a novel pyrrol compound, inhibited the cytokine overproduction through the modulation of TLR4/NF-κB pathway. LPS induced an apoptotic response that was prevented by DTA0118 through the modulation of antiapoptotic proteins. Our study suggests that DTA0118 can be considered as a potential therapy in the acute phase of sepsis and septic ARDS. Further investigations are needed to examine and validate these new effects and mechanisms in a clinically, relevant animal model of sepsis and sepsis-induced ARDS.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

AUTHOR CONTRIBUTIONS
All authors participated in the design, interpretation of the studies and analysis of data, and review of the manuscript. JV and JMP obtained funding for the study. NECB, ARN, EPR, IS, and CRG performed the experiments and the statistical analysis. JV, NECB, EPR, and ASS conceived the study and drafted the first manuscript. JV coordinated the study. All authors participated in the writing process of the manuscript and read and approved the final manuscript.

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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