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Novel Role for the Liver X Nuclear Receptor in the Suppression of Lung Inflammatory Responses*

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The liver X receptors (LXRα/β) are part of the nuclear receptor family and are believed to regulate cholesterol and lipid homeostasis. It has also been suggested that LXR agonists possess anti-inflammatory properties. The aim of this work was to determine the effect of LXR agonists on the innate immune response in human primary lung macrophages and a pre-clinical rodent model of lung inflammation. Before profiling the impact of the agonist, we established that both the human macrophages and the rodent lungs expressed LXRα/β. We then used two structurally distinct LXR agonists to demonstrate that activation of this transcription factor reduces cytokine production in THP-1 cells and lung macrophages. Then, using the expression profile of ATP binding cassettes A1 (ABCA-1; a gene directly linked to LXR activation) as a biomarker for lung exposure of the compound, we demonstrated an LXR-dependent reduction in lung neutrophilia rodents *in vivo*. This inhibition was not associated with a suppression of c-Fos/c-Jun mRNA expression or NF-κB/AP-1 DNA binding, suggesting that any anti-inflammatory activity of LXR agonists is not via inhibition of NF-κB/AP-1 transcriptional activity. These data do not completely rule out an impact of these agonists on these two prominent transcription factors. In summary, this study is the first to demonstrate anti-inflammatory actions of LXRs in the lung. Chronic innate inflammatory responses observed in some airway diseases is thought to be central to disease pathogenesis. Therefore, data suggest that LXR ligands have utility in the treatment of lung diseases that involves chronic inflammation mediated by macrophages and neutrophils.

The liver X receptors (LXR)[2] are part of the nuclear receptor super family and exist in two forms known as LXRα and LXRβ. These receptors are coded by two separate genes, Nr1h3 and Nr1h2, respectively (1). LXRs form obligate heterodimers with another orphan nuclear receptor, RXR, and regulate cholesterol and lipid homeostasis. Originally it was believed that LXRs were orphan receptors until the discovery of endogenous ligands; that is, the oxysterols (1–4). LXR directly controls the transcription of several genes involved in the cholesterol efflux pathway including ATP binding cassettes A1 (ABCA-1) and G1 (5–8). The nuclear receptors LXRα and LXRβ have been implicated in the control of cholesterol and fatty acid metabolism, and in the intestine ligand activation of LXR/RXR heterodimers dramatically reduces dietary cholesterol absorption, an effect postulated to be mediated by ABCA1 (6). A dual LXRα/β agonist has been shown to reduce the development of atherosclerosis (9), and in a similar murine model the same authors found that two LXRα/β agonists reduced inflammatory mRNA expression. This data suggest that LXR agonists may reduce atherosclerosis not only by promoting cholesterol efflux but also by limiting the production of inflammatory mediators in the artery wall (10). This anti-inflammatory profile has also been shown in two murine models of contact dermatitis (11).

In cultured mouse macrophages, human blood-derived macrophages, and a macrophage cell line, LXRα/β agonists have been shown to reduce inflammatory mediator production (*e.g.* nitric oxide (10, 12), inflammatory cytokines (10, 13–16), and matrix metalloproteinase 9 (17). Therefore, there is a clear rationale for evaluating the ability of LXR ligands to evoke anti-inflammatory activity in the lung (18–20).

The aim of this study was to determine the effect of two structurally distinct LXR agonists, GW3965 and T1317, on the response to LPS in the airways. The innate inflammatory response to endotoxin is central to host defense processes, but in disease conditions, this chronic inflammatory response can become detrimental to the airways. As yet there are no published studies on the impact of LXR agonists in the airways; therefore, the first challenge was to determine whether LXR receptors are expressed in the *in vitro* and *in vivo* systems we were to use. Having demonstrated the presence of the receptors in both cell types, we profiled the impact of the two LXR agonists in the LPS-driven cytokine release in the human cell-based assays. In parallel, having shown that the airways of the rat strain used in the pre-clinical model expressed LXR, we used the expression of ABCA-1 as a guide for determining the dose of compound to be used to profile their effect *in vivo*.

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[2] The abbreviations used are: LXR, liver X receptor; ABC, ATP binding cassette; LPS, lipopolysaccharide; HLTM, human lung tissue macrophage; TNF, tumor necrosis factor; IL, interleukin; EMSA, electrophoretic mobility shift assay; AP-1, apoprotein 1; iNOS, inducible nitric-oxide synthase; GRO, growth-related oncogene; MIP, macrophage inflammatory protein; CINC, cytokine-induced neutrophil chemoattractant.

EXPERIMENTAL PROCEDURES

Collection of Human Lung Tissue Macrophages (HLTMs)—HLTMs were obtained from recipient tissue from lung transplant surgery. Ethical approval for the study was obtained along with relevant consent from the patient, or in the case of tissues obtained from brain-dead donors, consent from the relatives. The details of the collection technique used are published in Birrell *et al.* (21). Once collected and purified, the cell suspension was diluted in RPMI 1640 with glutamax I and supplemented with 10% fetal calf serum and 1% antibiotic and antimycotic solution (penicillin/streptomycin/amphotericin B; Sigma-Aldrich), and 400,000 cells per well were added to 24-well plates. These plates were then incubated for 60 min at 37 °C in a humidified atmosphere (95% air, 5% (v/v) CO_2). After 60 min, the non-adherent cells were removed by washing, and fresh medium was added. The adherent, purified macrophages (consistently > 99%) were incubated overnight for use/treatment the following day.

Culture of THP-1 Monocytic Cells—The human monocytic cell line THP-1 was purchased from the European Collection of Cell Cultures (ECACC, Health Protection Agency, Salisbury, Wiltshire, UK). Cells were cultured in accordance with the methods detailed in Birrell *et al.* (21). On experiment days the cells (10×10^6 cells/ml) were centrifuged at $800 \times g$ for 5 min at room temperature (Mistral 3000i, MSE). The supernatant was discarded, and the pellet of cells was washed in 10 ml of RPMI 1640 with glutamax I (Invitrogen) supplemented with 1% antibiotic and antimycotic solution, with no fetal calf serum. The cells were then centrifuged under the same conditions, then the supernatant was discarded, and the cells were resuspended in RPMI 1640 with glutamax I, supplemented with 3% fetal calf serum and 1% antibiotic and antimycotic solution.

LXRα and -β mRNA Expression in THP-1 Cells and HLTMs—Supernatants were removed from the cultured THP-1 cells and HLTMs, and the cells were then collected and retained at −80 °C.

Total cellular RNA was isolated from the cells using Tri Reagent (Sigma) and RNA reverse-transcribed to cDNA as detailed in Birrell *et al.* (21). Amplification of the cDNA and detection of target PCR product was carried out by real-time PCR, which involves a 5′-nuclease assay using fluorescent-labeled TaqMan probes (Applied Biosystems, Warrington, UK). Pre-developed assays (Assay on Demand) were purchased from Applied Biosystems for the investigation of mRNA expression of LXRα and -β. Reactions were internally controlled with the 18 S rRNA internal control (Applied Biosystems). PCR reactions were performed in a 25-μl reaction volume containing 3 μl of sample cDNA (2.5 ng/μl), with the Assay on Demand of the target gene, TaqMan universal master mix, and 18 S rRNA internal control. Amplification and detection of specific products were carried out in an ABI PRISM 7000 sequence detection system (Applied Biosystems) using an amplification protocol consisting of 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Results were analyzed using the Sequence Detection Software(Applied Biosystems), and the relative amount of target gene transcript was normalized to the amount of 18 S rRNA internal control transcript in the same cDNA sample. The data were then converted from the exponential form into linear data by using the calculation $2^{-(\text{target ct} - 18\text{ S ct}}$, ct is threshold cycles) and then arbitrarily multiplied by 10^6 to change the values into whole numbers. Before performing the analysis, each assay was validated according to instructions from Applied Biosystems. Assay validations were performed to ensure that the threshold cycles of both the target and internal control, determined in the linear exponential phase of the amplifications, had equal efficiencies.

Compound Treatment of THP-1 Cells and HLTMs—Cells were treated with vehicle (Me_2SO, final concentration 0.1% v/v) or compound (GW3965 or T1317, 10^{-11}–10^{-6} M) synthesized at GlaxoSmithKline by previously described methods (6, 14) 1 h before stimulation with a submaximal concentration of LPS (0.1 μg/ml). The potency (EC_{50}) of GW3965 and T1317 in a LXR cell-based reporter gene assay has been published at 190 ± 30 and 85 ± 10 nM, respectively (22). Dexamethasone (10^{-6} M) was included as an intra-assay positive control. After 24 h of culturing the supernatants were collected and stored for subsequent assessment of cytokine levels. Protein levels of TNFα, IL-8, IL-6, and MIP-1α (plus IL-1β and GROα for experiments with THP-1 cells) were determined by enzyme-linked immunosorbent assay using human DuoSets® according to manufacturer's instructions (R&D Systems Europe, Oxfordshire, UK).

LXRα and -β mRNA Expression in Human and Rat Tissues—Human RNA extracted from a range of human tissues was bought from Clontech Laboratories (*n* numbers of these samples were varied for each tissue type, and where there was more than one source, the RNA was pooled at the place of manufacture). A similar range of tissues were collected from 3 male naïve Wistar rats (180–200 g of the same strain used in the LPS-challenged model below), and RNA was extracted as outlined above and in Birrell *et al.* (22). LXRα and -β mRNA expression was established as outlined above in the human cells.

Establishing an Appropriate Dose of LXR Ligand to Use in the Rodent Model of Endotoxin-driven Airway Inflammation—To establish an appropriate dose of LXR agonist to use in the preclinical model of endotoxin-driven inflammation, it was decided to measure the mRNA expression of ABCA-1, a gene shown to be regulated by LXR agonists (10) in rats treated with the agonist. Vehicle or T1317 (1, 3, 10, and 30 mg/kg, twice a day for 4 days and 7 and 4 h before sampling) was orally dosed to Wistar rats, and 4 h after the last dose the animals were culled, and the lung was collected and stored at −80 °C. ABCA-1 mRNA expression was assessed using real-time TaqMan reverse transcription-PCR as outlined above and in Birrell *et al.* (22). The data were expressed as -fold difference from vehicle-treated samples.

Determination of the Effect of GW3965 and T1317 on LPS-induced Airway Inflammation in the Rat—Male Wistar rats (180–200 g) were purchased from Harlan-Olac (Bicester, UK) and housed for at least 5 days before use. Food and water were supplied *ad libitum*. UK Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act 1986 were strictly observed.

Rats were challenged with an aerosol of endotoxin-free saline (for 30 min) or LPS (0.3 mg/ml) as previously described (22). Vehicle (0.5% methylcellulose and 0.2% Tween 80 in water, 2 ml/kg), GW3965 (30 mg/kg), or T1317 (30 mg/kg) were administered orally 1 h before and 2 h post-challenge. This dose was chosen because it was shown to increase the expression of ABCA-1, a gene linked to activation of LXR (10). The positive

standard, dexamethasone (1 mg/kg), was included and administered using the same treatment regimen. Six hours after challenge cellular burden in the airway tissue was determined using methodology detailed in Birrell *et al.* (22). Briefly, animals were euthanized with sodium pentobarbitone (200 mg/kg, intraperitoneal), the left lobe was removed, and the vasculature was perfused with RPMI to remove any blood and then finely chopped. A 300-mg aliquot of the lung tissue went through an enzymatic digest to extract the white cells. The remaining lung tissue was kept for ABCA-1 mRNA expression analysis. Total white blood cell counts in the lung samples were determined using an automated cell counter (Sysmex F-820, Sysmex UK Ltd., Linford Wood, Buckinghamshire, UK). Cytospins of these samples were prepared by centrifugation of 100-μl aliquots in a cytospin centrifuge (Shandon, Runcorn, UK) at 700 rpm for 5 min, low acceleration at room temperature. Slides were fixed and stained on a Hema-tek 2000 (Ames Co., Elkhart, IN) with modified Wrights-Giemsa stain. Three part differential counts on 200 cells per slide were performed following standard morphological criteria, and the numbers of neutrophils were established from the total white cell counts.

To determine whether the anti-inflammatory action of the LXR ligands was mediated by inhibition of the NF-κB/AP-1 pathway as previously reported in alternative assay systems (10, 14, 16, 17, 23), we first analyzed previous time course samples (vehicle or LPS challenged rat lung samples collected 1, 2, 4, 6, and 8 h post-challenge) for c-Jun and c-Fos mRNA expression (used as a marker of AP-1 pathway activation). Then, having chosen an appropriate time point, we repeated the *in vivo* phase described above and collected lung tissue for assessment of NF-κB/AP-1 pathway activation. In addition to c-Jun/c-Fos mRNA expression, we also performed EMSA analysis on lung tissue nuclear extracts using the appropriate NF-κB and AP-1 DNA sequence-labeled probes as detailed in Birrell *et al.* (24).

To explore other possible anti-inflammatory mechanisms of the LXR agonists, we measured a range of inflammatory biomarkers thought to be involved in the development of the LPS-induced airway neutrophilia. Lung tissue taken from the 6-h LPS study was analyzed for levels of inducible nitric-oxide synthase (iNOS) mRNA expression (by real-time TaqMan reverse transcription-PCR as described above) and cytokines (IL-1β and rat equivalent of IL-8/GROα-CINC-1 and CINC-3 by rats specific enzyme-linked immunosorbent assay).

Data Analysis—All the values in the figures and text are expressed as the mean ± S.E. mean of n observations. The effect of LPS exposure compared with saline control was analyzed using a Mann-Whitney U post-test for unpaired data. Statistical analysis of multiple comparisons was made using one-way analysis of variance with an appropriate post-test. All treatments were compared with relevant vehicle control groups; differences were deemed significant when $p < 0.05$.

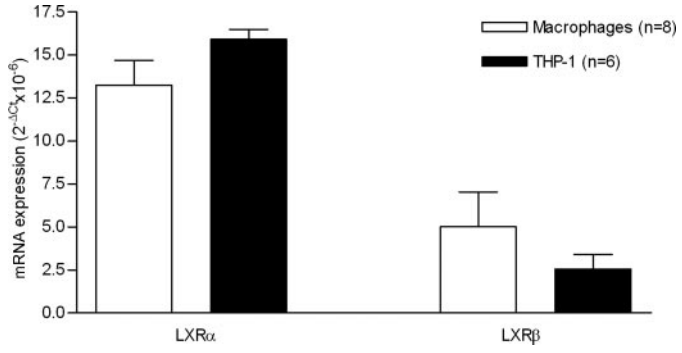

FIGURE 1. **LXRα/β mRNA expression in THP-1 cells and primary human lung tissue macrophages.** Levels of LXRα/β mRNA expression as determined by real-time TaqMan PCR in THP-1 cells (n = 6) and primary human lung macrophages (n = 8).

RESULTS

LXRα and -β mRNA Expression in THP-1 Cells and HLTMs—The level of LXR mRNA expression appears to be comparable in THP-1 cells and HLTMs (Fig. 1). The semiquantitative expression levels may suggest that there is more LXRα compared with LXRβ at the gene level at least. Direct comparison, however, between genes is not recommended, as actual levels of genes will depend on many factors including efficiency of the assay and design of the primers and probes.

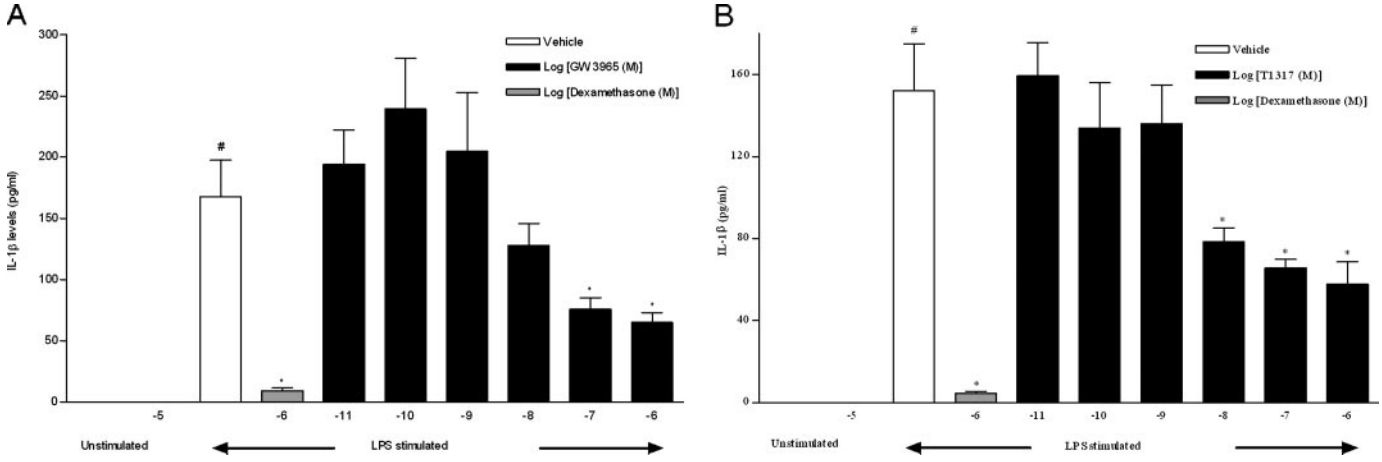

FIGURE 2. **Effect of LXRα/β agonists on LPS-nduced cytokine release in cultured THP-1 cells.** Cultured THP-1 cells were treated with vehicle (Me_2SO, 0.1% v/v), compound, or intra-assay positive control dexamethasone before stimulation with LPS. Cytokine levels were determined by enzyme-linked immunosorbent assay (*A*, GW3965, IL-1β; *B*, T1317, IL-1β). Data represent n = 6, with the # symbol indicating significant ($p < 0.05$) differences between vehicle/non-stimulated values and vehicle/LPS-stimulated values, and the *asterisk* indicating significant differences between vehicle/LPS-stimulated values and compound-treated/LPS-stimulated values.

TABLE 1
Effect of GW3965 on LPS-induced cytokine release from THP-1 cells

Cytokine	Non-stimulated vehicle	LPS-stimulated		
		Vehicle	GW3965 (1 μM)	Dexamethasone (1 μM)
	μg/ml	*μg/ml*	*% Inhibition*	*% Inhibition*
GROα	0.08 ± 0.01	1.2 ± 0.3	70 ± 2[a]	98 ± 2[a]
IL-6	0	0.8 ± 0.07	52 ± 4[a]	100 ± 0[a]
IL-8	0	3.0 ± 0.9	60 ± 9[a]	94 ± 2[a]
MIP-1α	0	34.9 ± 6.5	66 ± 4[a]	96 ± 0[a]
TNFα	0.03 ± 0.01	1.9 ± 0.4	14 ± 3	45 ± 8[a]

[a] Significant difference from the vehicle/LPS-treated group.

TABLE 2
Effect of T1317 on LPS-induced cytokine release from THP-1 cells

Cytokine	Non-stimulated vehicle	LPS-stimulated		
		Vehicle	T1317 (1 μM)	Dexamethasone (1 μM)
	μg/ml	*μg/ml*	*% Inhibition*	*% Inhibition*
GROα	0.04 ± 0.007	1.1 ± 0.2	68 ± 7*	99 ± 1[a]
IL-6	0	0.9 ± 0.1	56 ± 2*	100 ± 0[a]
IL-8	0	1.9 ± 0.3	52 ± 10*	94 ± 1[a]
MIP-1α	0	53.0 ± 8.9	64 ± 4*	96 ± 0[a]
TNFα	0.05 ± 0.02	2.0 ± 0.3	19 ± 5	47 ± 11[a]

[a] Significant difference from the vehicle/LPS-treated group.

Compound Treatment of THP-1 Cells and HLTMs—LPS exposure to THP-1 cells caused a significant increase in IL-1β, GROα, IL-6, IL-8 MIP-1α, and TNFα (Fig. 2 and Tables 1 and 2). Both LXR agonists caused a significant, concentration-related inhibition of cytokine production, with a maximal inhibition of around 50 to 70% (Fig. 2 and Tables 1 and 2). Neither compound appeared to have any toxic effect on the cells as shown by the similar cell viability readouts compared with the relevant vehicle group (data not shown). Although one caveat to this is that because the agonists are known to induce multidrug transports (ABC family), trypan blue exclusion may not accurately reflect true viability. The intra-assay positive control, dexamethasone, caused a significant inhibition of all cytokines measured (Fig. 2 and Tables 1 and 2).

In the cultured HLTMs, exposure to endotoxin caused an increase in IL-6, TNFα, IL-8, and MIP-1α (Fig. 3 and Tables 3 and 4; note that we did not obtain a suitable LPS-induced signal for IL-1β and GROα with cells from these patients). Both LXR agonists caused a concentration-related inhibition of cytokine production, with a maximal inhibition of around 40–65% (Fig. 3 and Tables 3 and 4). The intra-assay-positive control, dexamethasone, inhibited all of the cytokines measured (Fig. 3 and Tables 3 and 4).

LXRα and -β mRNA Expression in Human and Rat Tissues—Fig. 4 depicts the levels of LXR mRNA expression in a range of human and rat tissues. It would appear that there are some similarities between human and rat tissue *i.e.* low levels of LXRα in the brain and skeletal muscle and low levels of LXRβ in the liver (Fig. 4). It is clear from these data that there is measurable expression of both receptors in human and rat lung, which is the tissue of interest in this manuscript (Fig. 4, *highlighted* in *gray*).

Establishing an Appropriate Dose of LXR Ligand to Use in the Rodent Model of Endotoxin-driven Airway Inflammation—To aid in determining an appropriate dose of LXR ligand to profile

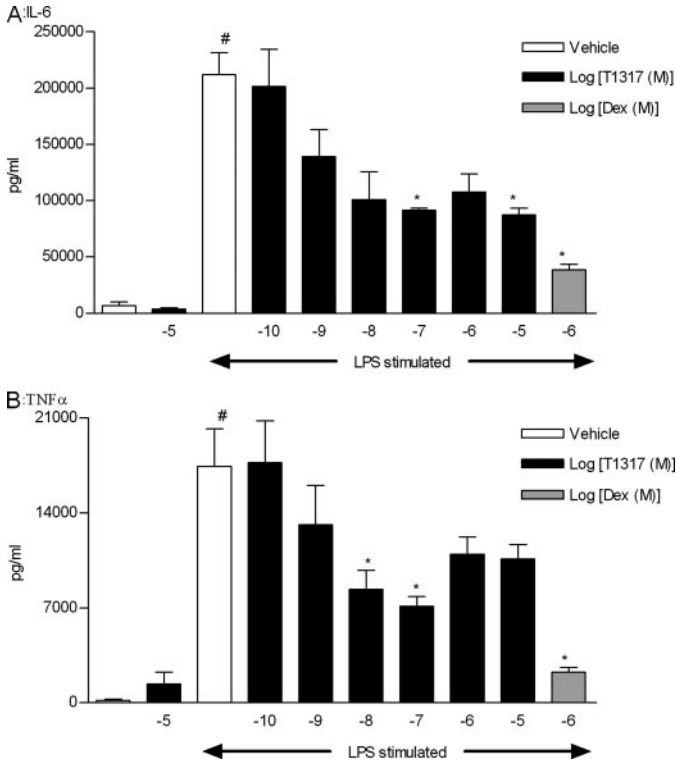

FIGURE 3. **Effect of LXRα/β agonist on LPS induced cytokine release in cultured primary human lung tissue macrophages.** Isolated macrophages were treated with vehicle (Me_2SO, 0.1% v/v), compound, or intra-assay positive control dexamethasone (*Dex*) before stimulation with LPS. Cytokine levels were determined by enzyme-linked immunosorbent assay. *A*, IL-6. *B*, TNFα. Data represent $n = 6$, with the # symbol indicating significant ($p < 0.05$) differences between vehicle/non-stimulated values and vehicle/LPS-stimulated values, and the *asterisk* indicating significant differences between vehicle/LPS-stimulated values and compound-treated/LPS-stimulated values.

TABLE 3
Effect of GW3965 on LPS-induced cytokine release from human lung tissue macrophages

Cytokine	Non-stimulated vehicle	LPS-stimulated		
		Vehicle	GW3965 (100 nM)	Dexamethasone (1 μM)
	μg/ml	*μg/ml*	*% Inhibition*	*% Inhibition*
IL-6	2.2 ± 0.9	214.8 ± 14.4	58	83
IL-8	37.2 ± 9.4	222.2 ± 11.8	40	87
MIP-1α	16.8 ± 10.5	280.9 ± 39.5	35	76
TNFα	0.4 ± 0.3	13.6 ± 1.8	43	84

TABLE 4
Effect of T1317 on LPS-induced cytokine release from human lung tissue macrophages

Cytokine	Non-stimulated vehicle	LPS-stimulated		
		Vehicle	T1317 (100 nM)	Dexamethasone (1 μM)
	μg/ml	*μg/ml*	*% Inhibition*	*% Inhibition*
IL-6	6.8 ± 3.2	211.9 ± 19.8	59	85
IL-8	35.3 ± 14.4	199.2 ± 24.4	42	97
MIP-1α	29.5 ± 18.1	306.5 ± 60.1	47	86
TNFα	0.2 ± 0.1	17.4 ± 2.8	60	88

in the preclinical rodent model of endotoxin-induced neutrophilia, a satellite study was performed in which levels of ABCA-1 mRNA expression were measured after ligand administration. An increase in ABCA-1 mRNA expression in the lung tissue would imply activation of the LXR receptor by the deliv-

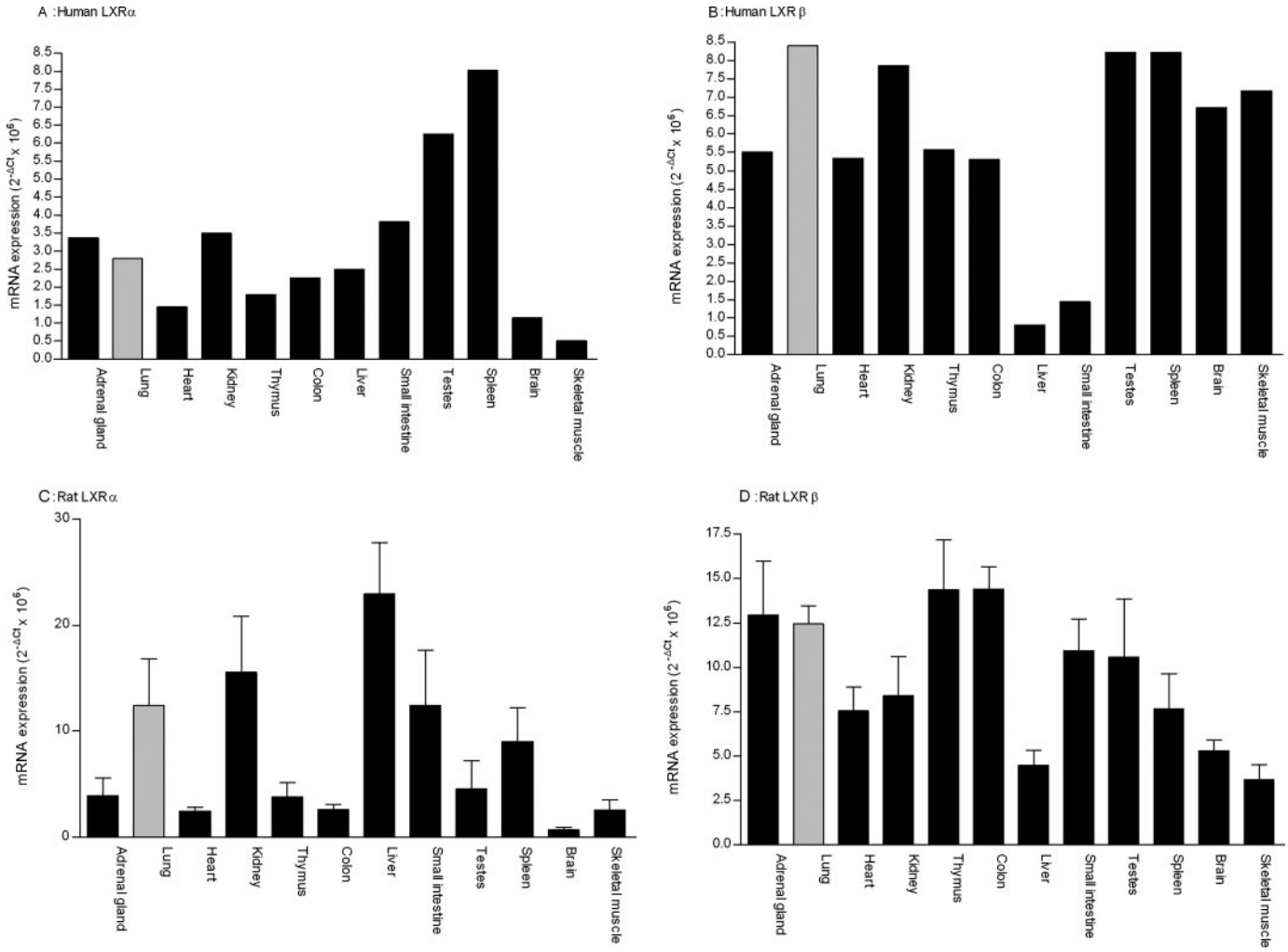

FIGURE 4. **LXRα/β mRNA expression in human and rat (male Wistar, $n = 3$) tissues.** Levels of LXRα/β mRNA expression were determined by real-time TaqMan PCR in various tissues; levels in the lung are highlighted. *A*, human LXRα; *B*, human LXRβ; *C*, rat LXRα; *D*, rat LXRβ.

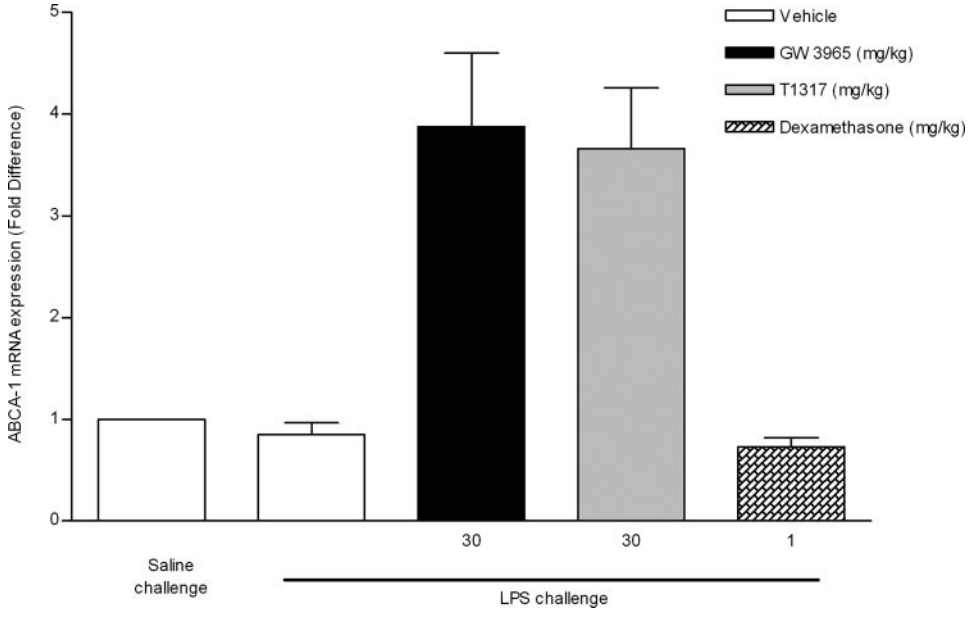

FIGURE 5. **ABCA-1 mRNA expression in rat lung tissue after treatment with LXRα/β agonist.** Male Wistar rats were orally dosed with vehicle, GW3965 (30 mg/kg), T1317 (30 mg/kg), or intra-assay positive control, dexamethasone (1 mg/kg) 1 h before and 2 h after saline, or LPS challenge (0.3 mg/ml, aerosolized for 30 min). ABCA-1 gene expression was determined by real-time TaqMan PCR in the lung tissue ($n = 8$) taken 4 h after the last dose of compound.

ered ligand. It can be clearly seen from the values that 30 mg/kg of the LXR agonist increases the levels of ABCA-1 mRNA (0.73 ± 0.08-, 0.96 ± 0.14-, 1.26 ± 0.22-, and 3.01 ± 0.39-fold difference from vehicle group, for 1, 3, 10, and 30 mg/kg, respectively), and therefore, this dose was chosen to be used in the main profiling study.

Determination of the Effect of GW3965 and T1317 on the LPS-induced Airway Inflammation in the Rat—Fig. 5 shows levels of ABCA-1 mRNA expression in the main profiling study, clearly indicating that both agonists were delivered appropriately to activate LXR. This activation of LXR by both compounds was associated with a reduction in the endotoxin-induced increase in neutrophil numbers in the lung tissue, although the inhibition by

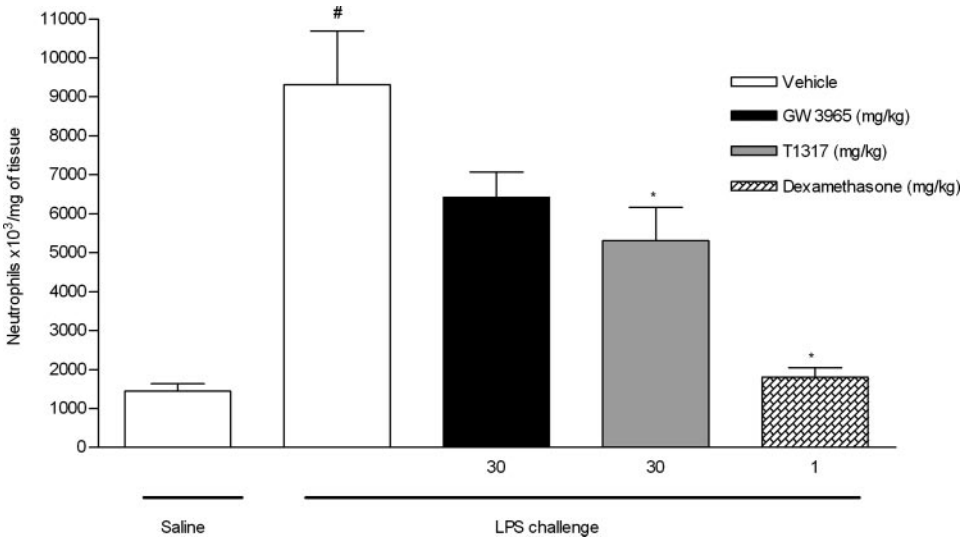

FIGURE 6. **Effect of LXRα/β agonists on LPS induced airway neutrophilia in rat airways.** Male Wistar rats were orally dosed with vehicle, GW3965 (30 mg/kg), T1317 (30 mg/kg), or intra-assay positive control, dexamethasone (1 mg/kg) 1 h before and 2 h after saline, or LPS challenge (0.3 mg/ml, aerosolized for 30 min). Six hours after challenge the lungs were removed, and the numbers of neutrophils were determined. Data represent $n = 8$, with the # symbol indicating significant ($p < 0.05$) differences between vehicle/non-stimulated values and vehicle/LPS-stimulated values, and the *asterisk* indicating significant differences between vehicle/LPS-stimulated values and compound-treated/LPS-stimulated values.

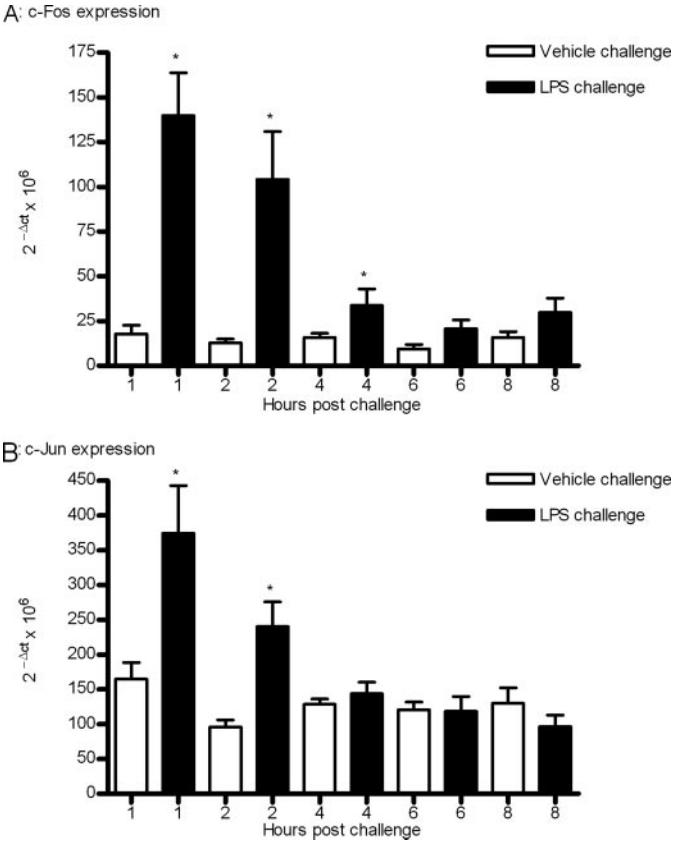

FIGURE 7. **c-Jun and c-Fos mRNA expression after LPS stimulation in the rat lung.** Male Wistar rats were saline or LPS challenge (0.3 mg/ml, aerosolized for 30 min). At different times after challenge (1, 2, 4, 6, and 8) the lung tissue was collected and assessed for mRNA expression determined by real-time TaqMan PCR. *A* shows c-Fos mRNA levels, and *B* the c-Jun expression. The *asterisk* indicates significant ($p < 0.05$) differences between time-matched vehicle controls and LPS-stimulated values.

GW3965 failed to reach statistical significance (Fig. 6). The positive control dexamethasone also caused a significant inhibition of airway neutrophilia (Fig. 6). To explore if the LXR agonists have their anti-inflammatory action through inhibition of the NF-κB/AP-1 pathway, we assessed samples from a previously performed time course for c-Fos and c-Jun mRNA expression (marker of AP-1 pathway activation) and showed an increase in mRNA levels 2 h post-LPS challenge (Fig. 7). The *in vivo* phase was repeated with lung tissue samples collected and assessed for c-Fos/c-Jun mRNA expression (Fig. 8). In parallel, specific EMSAs were performed to assess NF-κB and AP-1 DNA binding (Fig. 9). These data suggest that the LXR agonists do not have their anti-inflammatory activity via impacting on the transcriptional activity of NF-κB or AP-1 in this system.

To explore other possible anti-inflammatory mechanisms of the LXR agonists, we measured a range of inflammatory biomarkers. Fig. 10 shows an increase in expression of iNOS mRNA and IL-1β, CINC-1, and CINC-3 protein in the lung tissue after exposure to LPS. Although treatment with the LXR ligands failed to impact on IL-1β or CINC-1/3, there did appear to be a reduction in the level of iNOS expression (Fig. 10).

DISCUSSION

LXR agonists have been shown to possess anti-inflammatory properties in cell-based assays and in a rodent model. As yet, however, the impact of LXR agonists has not been studied in the lung. The aim of this study was to examine the effect of two structurally distinct LXR agonists in endotoxin-driven inflammation in human cell-based assays and a pre-clinical rat model. Before profiling the impact of the LXR ligands in the primary lung macrophages, it was decided to determine whether the gene for the receptors was expressed in these primary cells, as they may be different from other non-lung-derived sources of macrophages. The results showed that these macrophages express the gene for both receptor subtypes. Furthermore, the level of expression appeared to be similar to that of the THP-1 cell, a cell type previously shown to express the receptors (25). Treatment of LPS-stimulated THP-1 cells and primary human macrophages with either LXR ligand caused a concentration-related reduction in cytokine production. Although the maximal effect on cytokine release was less than a glucocorticoid receptor agonist, dexamethasone, it would appear that the results obtained here parallel previously published *in vitro* data. For example, in mouse macrophages, LXR ligands have been shown to reduce LPS-, IL-1β-, or TNFα-induced metalloproteinase-9, but not metalloproteinase 12 (MMP-12) and MMP-13, expression at the gene level (17), LPS or bacterial infection

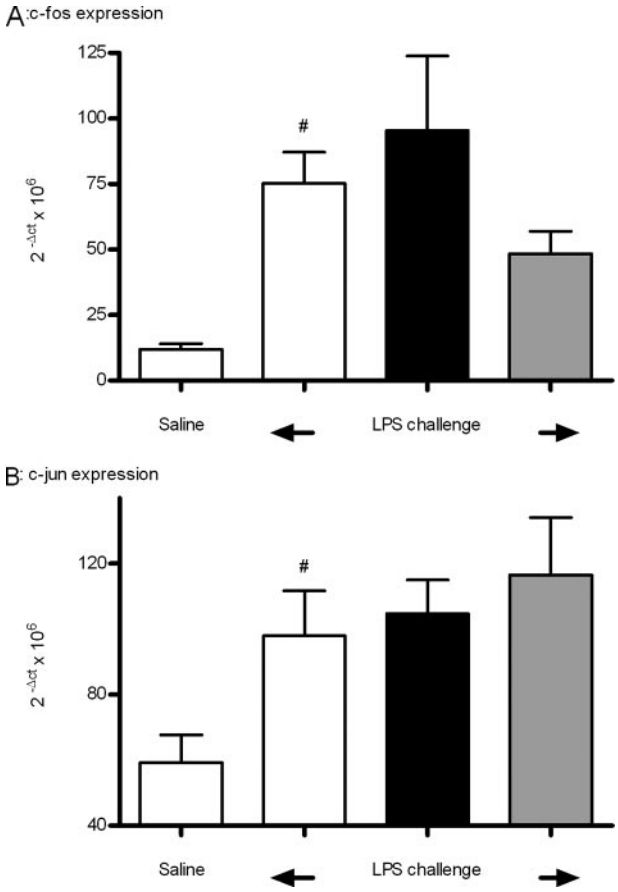

FIGURE 8. **Effect of LXRα/β agonists on LPS-induced AP-1 pathway activation in rat airways.** Male Wistar rats were orally dosed with vehicle, GW3965 (30 mg/kg), or T1317 (30 mg/kg) 1 h before and 2 h after saline or LPS challenge (0.3 mg/ml, aerosolized for 30 min). Two hours after challenge the lungs were removed, and mRNA expression was determined by real-time TaqMan PCR. *A* shows c-Fos mRNA levels, and *B* shows the c-Jun expression. Data represent $n = 8$, with the symbol # indicating significant ($p < 0.05$) differences between vehicle/non-stimulated values and vehicle/LPS-stimulated values, and the *asterisk* indicating significant differences between vehicle/LPS-stimulated values and compound-treated/LPS-stimulated values.

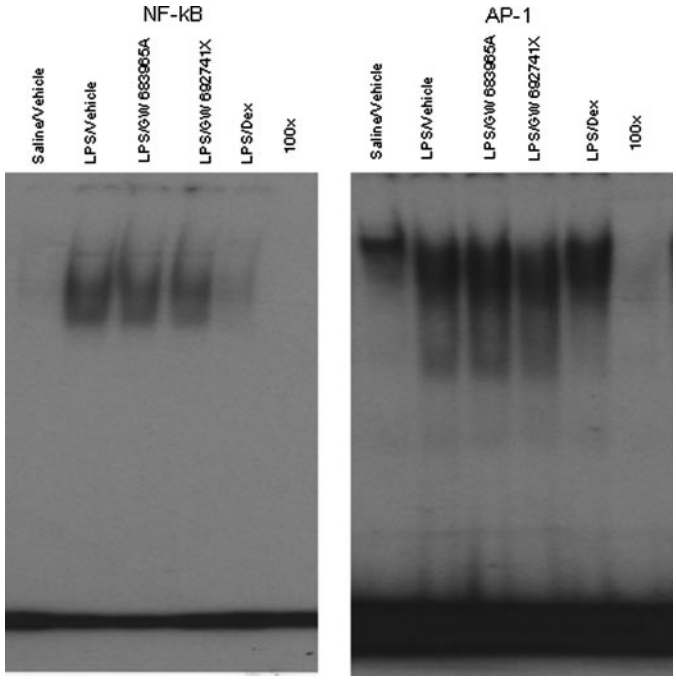

FIGURE 9. **Effect of LXRα/β agonists on LPS-induced NF-κB/AP-1 pathway activation in rat airways.** Male Wistar rats were orally dosed with vehicle, GW3965 (30 mg/kg), T1317 (30 mg/kg), or intra-assay positive control, dexamethasone (*Dex*, 1 mg/kg), 1 h before and 2 h after saline, or LPS challenge (0.3 mg/ml, aerosolized for 30 min). Two hours after challenge the lungs were removed, and EMSAs were performed on the nuclear extract. The *left panel* is a representative autoradiograph obtained with EMSA analysis of NF-κB DNA binding, and the *right panel* AP-1 DNA binding.

induced gene transcription such as iNOS, cyclooxygenase-2, and IL-6 (10), LPS induced NO production (12), and LPS and polyriboinocinic:polyribocytidylic acid induced inflammatory cytokines (13). Similarly, in human blood monocyte-derived macrophages LXR agonists have been shown to inhibit TNFα and IL-1β expression at the mRNA and protein level (14, 16).

To determine whether the anti-inflammatory actions of the LXR agonists observed in the human cell-based assays were also apparent in a more biologically complex system, the aim was to profile the ligands in a preclinical rat model of endotoxin-induced lung inflammation. However, as with the *in vitro* assay, there is as yet no published data on LXR ligands in the rat lung. Therefore, we determined the mRNA expression profile of LXRα/β in human tissues and compared them to the same tissue taken from the strain of rats used in the preclinical *in vivo* model. It was shown that the expression profile was comparable between human and male Wistar rat tissues, and importantly, there were both receptor types in the lung tissue. To establish an appropriate dose level of the LXR agonist, we measured the expression of ABCA-1, a gene directly linked to the activation of the transcription factor (5–8), in the lung tissue after compound delivery. The dose needed to increase the expression of ABCA-1 was then used in the LPS-induced model of lung inflammation. In the main study we confirmed activation of LXR with both ligands, *i.e.* increase in ABCA-1 mRNA expression, and showed that this was associated with a reduction in neutrophil burden in the lung.

It is not known how LXR agonists are anti-inflammatory, but published work has suggested that activation of the receptor may impact on other inflammatory transcription factors. Indeed, reciprocal regulation of ABCA-1 and inflammatory mRNA expression has been shown suggesting a link (10). Groups have suggested that LXR agonists impact on NF-κB transcription factor signaling (10, 13, 14, 16, 17), whereas others have suggested that LXR agonists have anti-inflammatory actions through inhibition of AP-1 transcription (16, 23). To investigate this possibility in our preclinical rodent model we measured the impact of the LXR agonists on the expression of c-Jun/c-Fos (markers of AP-1 transcriptional activity) and on NF-κB/AP-1-DNA binding using specific EMSAs. The data suggests that the LXR agonists do not block LPS-induced NF-κB/AP-1 nuclear translocation or its ability to bind to DNA (under the conditions used for the EMSA technique). These data do not completely rule out an impact of these agonists on these two prominent transcription factors. Previous *in vitro* studies have demonstrated that LXR agonists can inhibit NF-κB-dependent transcription from a reporter gene assay (9). Therefore, it seems most likely that LXR agonists inhibit NF-κB activity through protein/protein interactions at the promoter of

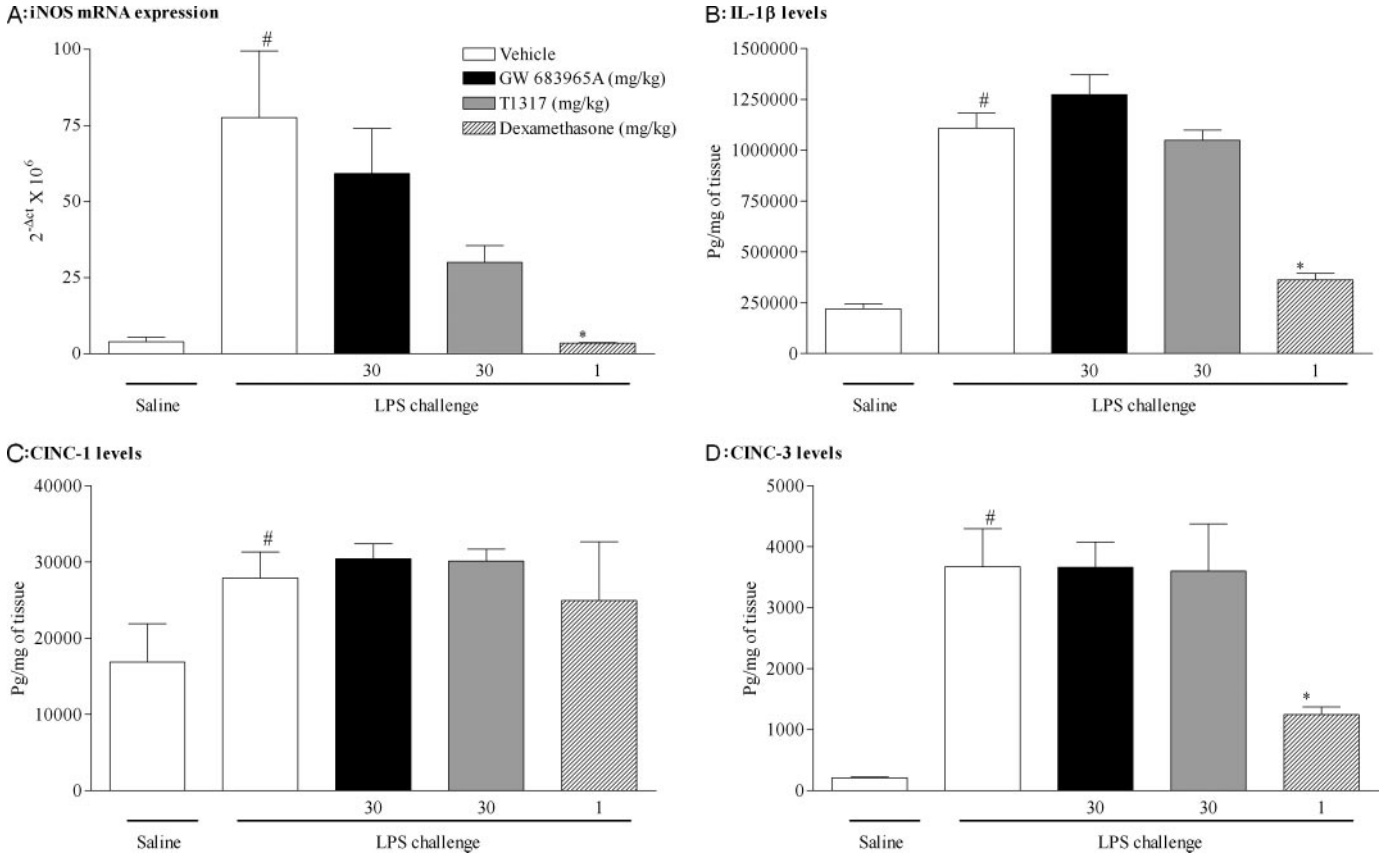

FIGURE 10. **Effect of LXRα/β agonists on LPS-induced airway inflammatory biomarkers in rat airways.** Male Wistar rats were orally dosed with vehicle, GW3965 (30 mg/kg), T1317 (30 mg/kg), or the intra-assay positive control, dexamethasone (*Dex*; 1 mg/kg) 1 h before and 2 h after saline, or LPS challenge (0.3 mg/ml, aerosolized for 30 min). Six hours after challenge the lungs were removed, and the levels of iNOS mRNA expression (*A*) and cytokines were measured (*B*, IL-1β; *C*, CINC-1; *D*, CINC-3). Data represent $n = 8$, with the symbol indicating significant ($p < 0.05$) differences between vehicle/non-stimulated values and vehicle/LPS-stimulated values, and the *asterisk* indicating significant differences between vehicle/LPS-stimulated values and compound-treated/LPS-stimulated values.

target genes in a similar manner to the glucocorticoid receptor. Alternatively, LXR agonists may recruit repressive cofactors to induce anti-inflammatory effects or act by impacting on other inflammatory signal pathways/transcription factors or by inducing the production of anti-inflammatory mediators. During further investigation into the possible mechanism of action of these LXR ligands we showed that whereas there was no impact on inflammatory cytokines such as IL-1β or the rat equivalent of IL-8/GROα, there was a decrease in iNOS mRNA expression. Although we cannot say conclusively that the reduction in airway neutrophilia is linked to the apparent reduction in iNOS and presumably subsequent reduction in NO, it is interesting to note that previous work from other laboratories has shown inhibition of this mediator with LXR agonists (9), and we have shown that NO plays a key role in the neutrophilia observed in this preclinical model (26).

In summary, we have for the first time shown the mRNA expression of LXRα/β in primary human lung tissue macrophages, a range of different human tissues, and equivalent rat tissues. Using two structurally distinct LXR agonists, we showed a reduction in endotoxin-induced inflammatory cytokine production in a human primary lung cell-based assay and lung neutrophilia in a more complex biological rodent model using the expression of ABCA-1 as a guide to an appropriate dose. The anti-inflammatory actions of the LXR agonists appear to be not associated with a reduction in NF-κB/AP-1 transcriptional activation in this assay system.

The innate inflammatory response to endotoxin is central to the defense of invading pathogens in the airways; however, in disease this chronic inflammatory response can become detrimental to the airways. These data would suggest that LXR ligands may be of use in the treatment of lung diseases which involve innate inflammation and feature macrophage and neutrophil infiltration.

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