ESTABLISHMENT OF AN IN VITRO INTESTINAL INFLAMMATORY MODEL USING HUMAN INTESTINAL EPITHELIAL AND IMMUNE CELLS VIA THE TLR4/NF-κB SIGNALING PATHWAY

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ABSTRACT

Inflammatory bowel disease (IBD) is a chronic condition marked by persistent inflammation of the digestive tract, in which the interaction between intestinal epithelial and immune cells plays a pivotal role through key signaling pathways. To investigate the molecular mechanisms underlying intestinal inflammation, we examined the effects of lipopolysaccharide (LPS) on human intestinal epithelial (HIEC-6) and pro-monocytic (U937) cells to establish an *in vitro* model resembling IBD. Cells were treated with varying concentrations of LPS for different incubation periods, and molecular changes were assessed by RT-PCR and western blotting. In HIEC-6 cells, LPS markedly enhanced IL-6 and COX-2 gene expression and increased iNOS and TLR4 protein levels. In U937 cells, LPS strongly induced IL-6, IL-1β, TNF-α, and COX-2 transcripts and elevated the protein expression of TLR4, TNF-α, and IL-6. Furthermore, nuclear translocation of NF-κB was prominently detected in both cell types, confirming the activation of the TLR4/NF-κB signaling pathway. These findings demonstrate that LPS stimulation effectively reproduces hallmark features of intestinal inflammation, pro-inflammatory cytokine induction, and NF-κB activation. The established *in vitro* system provides a robust and reproducible platform for elucidating the molecular mechanisms of intestinal inflammation and for evaluating therapeutic interventions targeting TLR4/NF-κB signaling in IBD and related disorders.

Keywords:

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INTRODUCTION

Inflammatory bowel disease (IBD), which encompasses conditions such as Crohn's disease and ulcerative colitis, presents a significant challenge in gastroenterology because of its chronic and relapsing nature, which is driven by dysregulated immune responses and environmental factors. Research has shown that gut microbiota dysbiosis is closely associated with the pathogenesis of IBD (Alsulaiman et al., 2023). A balanced microbiota is crucial for maintaining human health, with a stable microbial ecosystem characterized by high taxonomic diversity, microbial gene richness, and a resilient core microbiota (Fan & Pedersen, 2021). However, alterations in the composition of gut microbiota have been linked to IBD, primarily through their impact on inflammation. One of the key microbial changes observed in IBD is a reduction in beneficial bacteria, such as *Bacteroides*, *Firmicutes*, and *Lactobacillus* (Khan et al., 2019), alongside an increased prevalence of gram-negative bacteria, including *Escherichia coli*, and members of the *Proteobacteria phylum* (Petersen, 2022). These gram-negative bacteria produce lipopolysaccharides (LPS), which are potent endotoxins that trigger pro-inflammatory immune responses by activating Toll-like receptors (TLRs), leading to sustained inflammation, endothelial dysfunction, and epithelial barrier impairment. Additionally, adherent-invasive *E. coli* (AIEC), which is particularly enriched in Crohn's disease, invades intestinal epithelial cells and further exacerbates inflammation. Despite extensive research, the complex interactions among the intestinal epithelium, immune cells, and inflammatory mediators remain incompletely understood. The development of robust and replicable models to study these interactions is essential for advancing therapeutic strategies. In recent years, the Toll-like receptor 4 (TLR4)/nuclear factor-κB (NF-κB) signaling pathway has emerged as a critical axis in the regulation of intestinal inflammation. TLR4, a key pattern recognition receptor, responds to microbial-associated molecular patterns (MAMPs), such as lipopolysaccharides, by activating downstream NF-κB signaling. This activation triggers the production of pro-inflammatory cytokines, chemokines, and other mediators, amplifying the inflammatory response and contributing to the pathogenesis of IBD.

In vitro cell models of IBD serve as valuable tools for studying the complex interactions between intestinal epithelial cells, immune cells, and inflammatory mediators under controlled laboratory conditions (Mobbs et al., 2024). These models help researchers investigate the molecular mechanisms underlying IBD pathogenesis, including epithelial barrier dysfunction, immune activation, and microbial interactions. Commonly used *in vitro* models include monolayer cultures of human intestinal epithelial cells, such as Caco-2 and HT-29 cells, which mimic the intestinal barrier. More advanced models, such as co-culture systems with immune cells or the gut microbiota, allow for a better understanding of inflammatory responses.

In this study, we describe the establishment of an *in vitro* cell model to simulate intestinal inflammation by leveraging human intestinal epithelial cells (HIEC-6) and immune cells (U937), which are the cells found in the microenvironment. This culture system is designed to mimic the cellular and molecular interactions observed in the inflamed gut by stimulating the system with LPS, a potent inflammatory agent which increased by dysbiosis of gram-negative bacteria in the gut. The inflammatory-responsive mRNA and protein expression levels were examined by RT–PCR and western blotting, respectively. These results clarify the potential of LPS as inducers of inflammation in an intestinal inflammatory model. This research aimed to investigate the inflammatory effects of LPS on human intestinal epithelial and monocytic cells via activation of the TLR4/NF-κB pathway, thereby replicating the oxidative and inflammatory milieu characteristic of IBD.

LITERATURE REVIEWS

Pathophysiology of IBD and the Role of Gut Microbiota

Inflammatory bowel disease (IBD), encompassing Crohn's disease (CD) and ulcerative colitis (UC), is a chronic relapsing-remitting inflammatory disorder of the gastrointestinal tract. Its etiology is multifactorial, involving genetic susceptibility, dysregulated immune responses, environmental triggers, and microbial imbalance (Alatab et al., 2020). A hallmark feature of IBD is gut microbiota dysbiosis, where commensal microbial diversity is diminished, and pathogenic bacteria are enriched. Several large-scale metagenomic analyses reveal significant depletion of beneficial species such as *Faecalibacterium prausnitzii* and *Roseburia hominis*, alongside enrichment of *Escherichia coli* pathobionts, which promote pro-inflammatory signaling (Wiredu Ocansey et al., 2023). Beyond compositional changes, functional alterations in the microbiome contribute to IBD pathogenesis. For instance, reduced production of short-chain fatty acids (SCFAs) such as butyrate compromises epithelial integrity and impairs mucosal immune regulation (Parada Venegas et al., 2019). These findings highlight dysbiosis not merely as an epiphenomenon but as a driving force in the disruption of epithelial barrier function and the amplification of mucosal immune activation.

Lipopolysaccharide (LPS) as a Key Pro-Inflammatory Trigger

Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, is a crucial microbial product associated with intestinal inflammation. Elevated systemic and mucosal LPS levels have been reported in IBD patients, correlating with disease activity and severity (Candelli et al., 2021). LPS disrupts epithelial tight junction proteins, thereby impairing intestinal permeability and facilitating microbial translocation. This phenotype is a central event in IBD, allowing microbial components to perpetuate inflammatory cascades. Mechanistically, LPS binds Toll-like receptor 4 (TLR4) on intestinal epithelial and immune cells, activating downstream signaling cascades leading to phosphorylation and nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB is a transcriptional master regulator of inflammation, driving the expression of cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), as well as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Excessive and sustained NF-κB activity has been consistently documented in inflamed intestinal tissues from IBD patients, linking this pathway directly to disease persistence and relapse (Neurath, 2019). Importantly, therapies that suppress NF-κB activation, such as corticosteroids or biologicals targeting TNF-α, have proven effective in IBD management, thereby validating NF-κB as a therapeutic target (Fakhoury et al., 2014). Thus, dissecting the TLR4/NF-κB axis in controlled experimental systems remains central to developing novel therapeutics.

In Vitro Models for Studying Intestinal Inflammation

Animal models have historically provided insights into IBD, but species-specific differences in immune and microbial interactions limit their translational relevance (Wen et al., 2024). *In vitro* models, therefore, represent essential tools for dissecting specific mechanisms under controlled conditions. Traditional models utilize intestinal epithelial cancer-derived lines such as Caco-2 or HT29, which form tight junctions and can be used to assess barrier permeability and cytokine responses (Donetti et al., 2025). However, their tumorigenic origin and lack of immune cell components reduce physiological accuracy. To address this limitation, advanced co-culture systems incorporating epithelial and immune cells have been developed. For example, co-cultures of Caco-2 with THP-1-derived macrophages or peripheral blood mononuclear cells (PBMCs) allow simultaneous evaluation of epithelial barrier integrity and immune responses (Paul et al., 2023). Despite these advances, a significant gap remains in modeling TLR4/NF-κB-driven epithelial-immune interactions *in vitro*. Here, the use of HIEC-6 (non-tumorigenic human intestinal epithelial cells) and U937 monocytic cells offers a more

physiologically relevant system. Such models enable precise investigation of LPS-induced oxidative stress, protein expression, and NF-κB activation in two cell models, providing valuable insights for translational IBD research.

RESEARCH METHODOLOGY

Chemicals and reagents

OptiMEM l, Roswell Park Memorial Institute (RPMI)-1640, GlutaMax, penicillin–streptomycin, and trypsin 0.25% (1X) solutions were purchased from Hyclone (Thermo Fisher Scientific, MA, USA). Fetal bovine serum was purchased from Hi-Media (Maharashtra, India). Lipopolysaccharide and anti-β-actin were purchased from Sigma Chemical Co. (Massachusetts, USA). Goat anti-rabbit IgG secondary antibodies, HRP conjugates, rabbit anti-mouse IgG secondary antibodies, HRP conjugates, and anti-TRL4, anti-TNF-α, and anti-NF-κB antibodies were obtained from Invitrogen, Thermo Fisher Scientific (Massachusetts, USA).

Cell culture

Human intestinal epithelial cells (HIEC-6) and human monocytic cells (U937) were purchased from the American Type Culture Collection (ATCC, Virginia, USA). HIEC-6 cells were cultured in OptiMEM medium supplemented with 4% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mM GlutaMAX, and 10 ng/mL epidermal growth factor. In parallel, U937 were maintained in RPMI-1640 with 10% FBS and antibiotics. Both cell types were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% relative humidity.

Assessment of protein expression

Total protein was extracted using NP-40 buffer. Protein concentrations were measured by the BCA assay. Equal amounts of protein were mixed with SDS sample buffer and denatured by boiling at 95°C for 5 min. Proteins were then separated by SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The membranes were blocked with skim milk and subsequently incubated with the following primary antibodies, followed by HRP-conjugated secondary antibodies. Detection was performed by enhanced ECL, and protein bands were imaged using an ImageQuant™. All experiments were performed in triplicate.

Assessment of mRNA expression

Total RNA was extracted using the RNeasy Mini Kit, and RNA concentration and purity were assessed by NanoDrop spectrophotometer and TapeStation analysis. cDNA was synthesized from RNA using the SuperScript™ VILO™ Kit, and RT-qPCR was performed on a QuantStudio™ system with Power SYBR Green Master Mix and gene-specific primers (Table 1). The cycling protocol was 95 °C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s, with melting curve analysis. Relative expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method, normalized to GAPDH. All assays were performed in triplicate.

Table 1 Sequences of the RT–PCR oligonucleotide primers.

Primer	Direction	Sequence	Length (bp)	PCR Product (bp)
IL-6	forward	5’-TGACAAACAAATTCGGTACATCCT-3’	24	144
	reverse	5’-CATCCATCTTTTTCAGCCATCTTT-3’	24	
IL-1β	forward	5’-CCTATCTTCTTCGACACATGGGATA-3’	25	141
	reverse	5’-CTGGAGGTGGAGACTTTCAGTT-3’	22	
TNF-α	forward	5’-GGCTCCAGGCGGTGCTTGTTC-3’	21	409
	reverse	5’-AGACGGCGATGCGGCTGATG-3’	20	
COX-2	forward	5’-TCACGCATCAGTTTTTCAAGA-3’	21	94
	reverse	5’-TCACCGTAAATATGATTTAAGTCCAC-3’	26	
GAPDH	forward	5’-TCAAGGCTGAGAACGGGAAGG-3’	21	87
	reverse	5’-CGCCCCACTTGATTTTGGAG-3’	20	

Data analysis

The data are presented as the mean ± standard deviation (S.D.). Statistical analyses were performed via GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA), and p-values ≤ 0.05 were considered statistically significant.

RESEARCH RESULTS

Effects of LPS on the protein expression of intestinal epithelial cells (HIEC-6)

We investigated LPS-induced NF-κB signaling in HIEC-6 cells by monitoring NF-κB nuclear translocation over stimulation periods ranging from 0.5 to 24 h. Treatment with 1 μg/mL LPS, previously identified as an optimal dose for NF-κB activation, led to a rapid redistribution of NF-κB from the cytoplasm to the nucleus. Cytoplasmic NF-κB expression decreased within the first hour, while nuclear accumulation became evident as early as 30 min and progressively increased with prolonged stimulation (Fig. 1). These findings are consistent with the activation of toll-like receptor (TLR) signaling, since TLR4 serves as the major receptor for LPS recognition in epithelial cells. Indeed, LPS treatment markedly elevated TLR4 expression, with a significant increase detected at 6 h. In parallel, we assessed the expression of iNOS, a well-known downstream target of NF-κB. Western blotting confirmed strong iNOS induction in LPS-stimulated cultures, appearing as a distinct ~130 kDa band, whereas control cells showed minimal expression. Together, these results demonstrate that LPS activates TLR4 in HIEC-6 cells, driving NF-κB nuclear translocation and the subsequent upregulation of proinflammatory mediators such as iNOS.

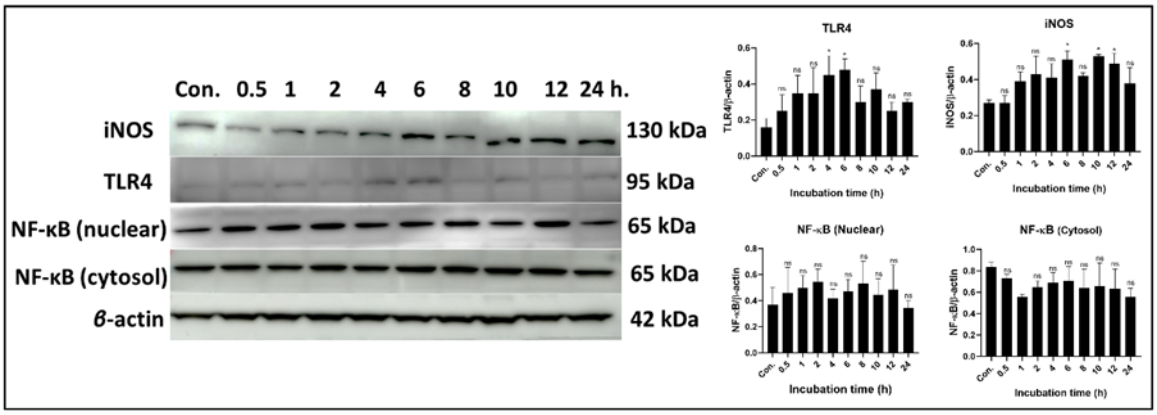

Figure 1 Western blot analysis of the effects of LPS stimulation in HIEC-6 cells. The data are representative of three independent experiments (means ± S.D. of triplicate samples). *, $p < 0.05$ vs control. The error bars represent the S.D.

Effects of LPS on protein expression in human pro-monocytic cells (U937)

Western blot analysis of U937 cells treated with 1 or 10 μg/mL LPS revealed a dose-dependent activation of inflammatory signaling pathways (Fig. 2). Treatment with 10 μg/mL LPS significantly upregulated NF-κB and IL-6 expression compared with untreated controls, whereas 1 μg/mL LPS produced only a modest, non-significant increase. Nuclear fraction analysis further confirmed NF-κB activation, showing a marked accumulation of NF-κB in the nucleus following LPS treatment, particularly at 10 μg/mL, with a corresponding decrease in cytosolic NF-κB. This nuclear translocation represents a critical step in NF-κB activation and was consistent with the enhanced IL-6 expression observed under the same conditions. Collectively, these results demonstrate that LPS induces inflammatory signaling in a concentration-dependent manner, with robust pathway activation achieved only at higher doses. The findings highlight the importance of NF-κB nuclear translocation in initiating downstream cytokine expression and underscore its central role in regulating LPS-mediated

inflammatory responses (Fig. 2A). Western blot analysis was conducted to examine the temporal expression of TLR4, NF-κB, and IL-6 in pro-monocytic cells treated with 10 μg/mL LPS over 3, 6, 12, 24, 48, and 72 h. The results showed a time-dependent increase in all three markers from 3 to 24 h, with peak expression observed at 24 h, followed by a gradual decline at 48 and 72 h (Fig. 2B). The early upregulation of TLR4 suggests enhanced cellular recognition of LPS, which likely triggers downstream signaling pathways, including NF-κB activation. Correspondingly, NF-κB levels progressively increased up to 24 h, consistent with its role as a central transcription factor in initiating inflammatory responses. IL-6, a downstream effector of NF-κB, showed a similar temporal pattern, indicating coordinated regulation of the inflammatory cascade. The peak expression of TLR4, NF-κB, and IL-6 at 24 h likely represents the maximal cellular inflammatory response, while the subsequent decline after 48 h may reflect activation of negative feedback mechanisms or cellular adaptation to prolonged LPS exposure. Overall, these findings highlight the dynamic, time-dependent modulation of inflammatory signaling in pro-monocytic cells and emphasize the pivotal roles of TLR4 and NF-κB in orchestrating early responses to LPS. This temporal profile provides insight into the kinetics of inflammatory activation and may inform the optimal timing of therapeutic strategies targeting TLR4 or NF-κB in inflammatory and infectious diseases.

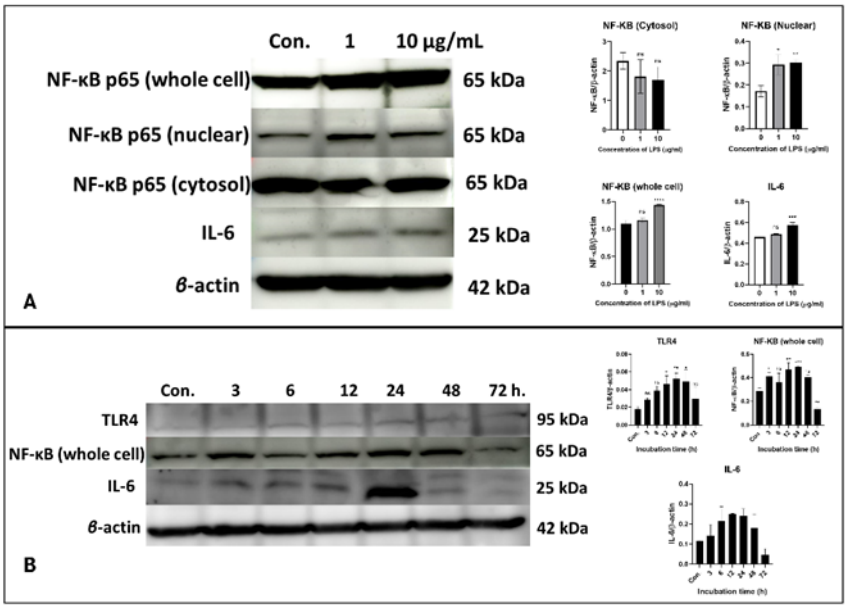

Figure 2 (A) Western blot analysis of the effects of 0, 1, and 10 μg/mL LPS in U937. (B) Western blot analysis of the effects of LPS stimulation in U937 at various incubation times. The data are representative of three independent experiments (means ± S.D. of triplicate samples). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs control. The error bars represent the S.D.

Effects of LPS on protein expression in human macrophage-like cells (U937)

U937 macrophage-like cells were treated with 100 ng/mL LPS to examine the dynamics of inflammatory signaling at the protein level (Fig. 3). TLR4 expression increased steadily over time, indicating continuous activation of LPS recognition pathways throughout the treatment period. Total cellular TNF-α levels did not show significant changes; however, analysis of specific isoforms revealed distinct temporal patterns. The transmembrane form of TNF-α (tmTNF-α) was significantly elevated at 3 h, followed by a gradual decline from 6 to 72 h, suggesting an early role in surface-mediated signaling. In contrast, the soluble monomeric form of TNF-α increased progressively, with significant accumulation between 48 and 72 h, reflecting delayed secretion and maturation processes. IL-6 protein levels also increased in a

time-dependent manner, consistent with its function in sustained inflammatory signaling. NF-κB expression in whole-cell extracts rose from 3 to 24 h, with statistically significant increases observed at 48 and 72 h, indicating prolonged activation. Subcellular fractionation further revealed that NF-κB nuclear translocation was significantly elevated at 3 and 6 h, while cytosolic levels remained largely unchanged, highlighting early activation and rapid nuclear signaling. Together, these findings indicate that LPS induces a coordinated and temporally distinct inflammatory response in macrophage-like cells, with early events mediated by tmTNF-α and NF-κB translocation, followed by later increases in soluble TNF-α, IL-6, and TLR4 expression. This dynamic regulation underscores the complexity of macrophage activation and the interplay of multiple signaling pathways in orchestrating inflammatory responses.

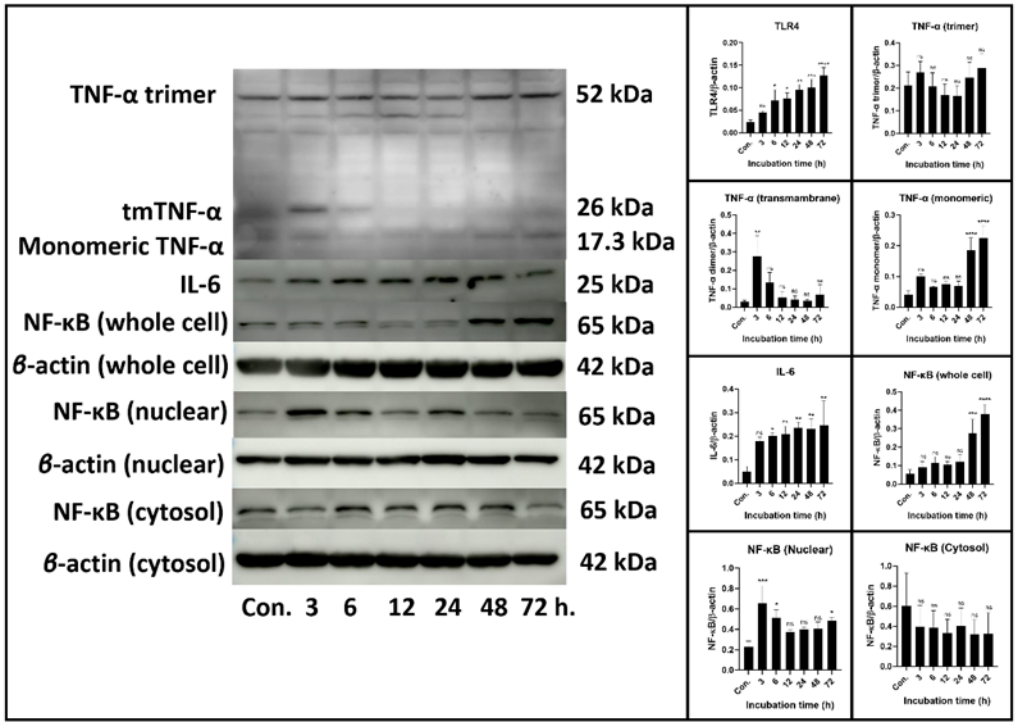

Figure 3 Western blot analysis of the effects of LPS stimulation in U937 (macrophage-like) cells at various incubation times (3, 6, 12, 24, 48, and 72 h). The data are representative of three independent experiments (means ± S.D. of triplicate samples). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs control. The error bars represent the S.D.

LPS-stimulated mRNA expression

To investigate LPS-induced inflammatory responses, HIEC-6 and U937 cells were treated with LPS for 3 and 6 h, and mRNA expression of key cytokines was analyzed by RT–qPCR (Fig. 4). In HIEC-6 intestinal epithelial cells, IL-6 and COX-2 mRNA were significantly upregulated, peaking at 3 h before declining at 6 h (Fig. 4A and 4J), whereas IL-1β and TNF-α showed no significant changes (Fig. 4D and 4G). This indicates selective activation of IL-6 and COX-2 pathways in epithelial cells, while other cytokines may require different conditions or longer stimulation. In contrast, U937 pro-monocytic cells showed a broader response, with all markers (IL-6, IL-1β, TNF-α, and COX-2) significantly upregulated, and with expression levels generally higher at 3 h than 6 h, consistent with a transient activation profile (Fig. 4B, 4E, 4H, and 4K). To further assess dose effects, U937 macrophage-like cells were treated with 100 ng/mL LPS for 3 and 6 h (Fig. 4C, 4F, 4I, and 4L). IL-6, IL-1β, and COX-2 expression increased in a time-dependent manner, confirming that even lower LPS concentrations robustly

induce inflammatory genes in these cells. Interestingly, TNF-α mRNA rose at 3 h but declined by 6 h, suggesting feedback regulation or rapid turnover at the lower dose. Overall, these results reveal distinct patterns of LPS responsiveness between epithelial and immune cell types. HIEC-6 cells preferentially activated IL-6 and COX-2, while U937 cells mounted a more comprehensive inflammatory response. The higher expression levels observed at 3 h in both cell types highlight the importance of temporal dynamics in regulating inflammation. Together, these findings underscore cell-type-specific differences in LPS signaling and provide insight into the differential regulation of epithelial versus monocytic inflammatory responses.

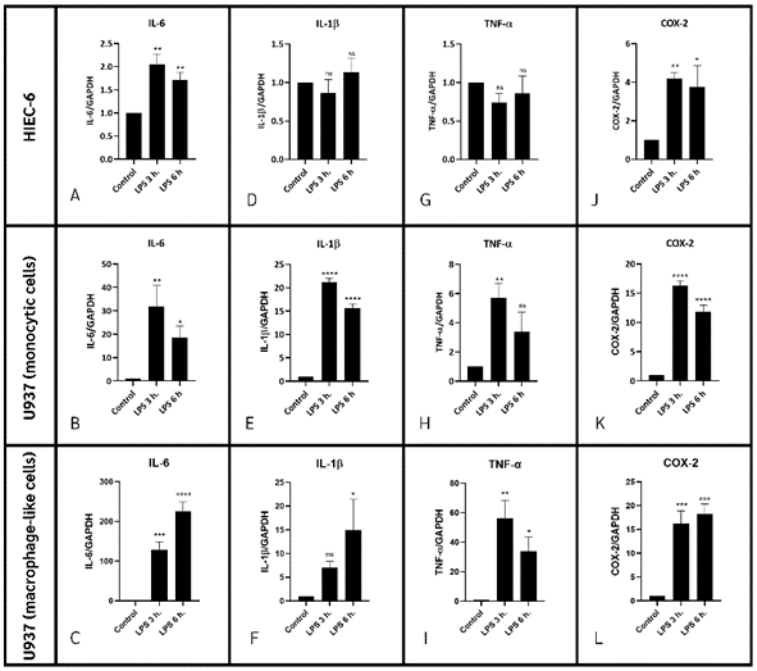

Figure 4 (A, D, G, J) LPS-induced mRNA expression in HIEC-6 cells. (B, E, H, K) LPS-induced mRNA expression in U937 (pro-monocytic) cells. (C, F, I, L) LPS-induced mRNA expression in U937 (macrophage-like) cells. The data are representative of three independent experiments (means ± S.D. of triplicate samples). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs control. The error bars represent the S.D.

DISCUSSION & CONCLUSION

Human intestinal epithelial cells and human monocytic leukemia cells were selected to study the interaction of cellular functions. Although other cell lines, such as Caco-2, are popular models for studying IBD-related experiments (Schnur et al., 2022), HIEC-6 is also an option. Caco-2 cells are derived from human colorectal adenocarcinoma and exhibit many characteristics of small intestinal epithelial cells. While Caco-2 cells appear to have a cancerous phenotype, HIEC-6 cells are more similar to normal intestinal cells. Induction with substances such as LPS drives the inflammatory process (Lopez-Escalera & Wellejus, 2022). Therefore, we established an LPS-induced HIEC-6 model and examined several inflammatory parameters as a baseline. Another cell type that plays a role in the inflammatory microenvironment is immune cells. U937 pro-monocytic cells were also used to investigate the inflammatory response. Hence, our research focused on understanding the effects of LPS on HIEC-6 and U937 cells to establish an intestinal inflammatory model.

At the receptor level, Toll-like receptor 4 (TLR4) serves as the primary pattern recognition receptor for LPS. Our results showed that LPS stimulation increased TLR4 expression, consistent with its role in pathogen sensing and initiation of inflammatory signaling. Engagement of TLR4 triggered downstream cascades leading to NF-κB activation. Normally

retained in the cytoplasm in an inactive form, NF-κB was observed to translocate into the nucleus following LPS treatment, highlighting its role as a transcriptional regulator of immune and inflammatory genes (Albensi, 2019). Our experimental results revealed that, in the cytoplasmic fraction of cell lysates, the mean expression of NF-κB decreased sharply after LPS treatment. In contrast, the levels of NF-κB in the nuclear lysate fractions were increased after LPS treatment. Inducible nitric oxide synthase (iNOS) was also significantly upregulated by LPS treatment. As a key enzyme responsible for nitric oxide production, iNOS contributes to antimicrobial activity but is also strongly implicated in tissue damage when excessively expressed (Zamora et al., 2000). This finding indicates that our model not only activates cytokine responses but also pathways contributing to oxidative stress, which is relevant to chronic inflammatory conditions. Two pro-inflammatory cytokines, TNF-α and IL-6, displayed distinct temporal and functional patterns. TNF-α was rapidly induced in U937 cells at early time points, before declining with prolonged LPS exposure. This profile reflects its well-established role as an early inflammatory trigger, initiating leukocyte recruitment, endothelial activation, and amplification of downstream cytokine networks (Parameswaran & Patial, 2010). The subsequent decline may represent negative feedback mechanisms or receptor desensitization to prevent uncontrolled inflammation. In contrast, IL-6 expression rose progressively and remained elevated, consistent with its function as a mediator of sustained inflammatory activity. IL-6 not only drives acute-phase protein synthesis in hepatocytes but also supports B-cell differentiation and T-cell survival, linking innate and adaptive immune responses (Tanaka et al., 2014). In our model, the sequential activation of TNF-α followed by IL-6 suggests a division of labor: TNF-α initiates the response, while IL-6 prolongs and stabilizes it. Taken together, the interplay among TLR4, NF-κB, TNF-α, IL-6, and iNOS highlights the complexity of LPS-induced signaling. Upon LPS recognition, TLR4 activates NF-κB, which in turn induces transcription of TNF-α, IL-6, and iNOS. TNF-α amplifies NF-κB activity through positive feedback, IL-6 provides sustained pro-inflammatory signaling, and iNOS contributes nitric oxide as an effector molecule. This interconnected network ensures a strong antimicrobial defense but, when dysregulated, may result in chronic inflammation and contribute to IBD pathogenesis. This study establishes an LPS-induced intestinal inflammatory model using both epithelial and monocytic cells. While this system is based on single-factor induction, it provides important insights into cell-specific inflammatory pathways and their temporal regulation. Future refinements, such as co-cultures with gut microbiota, better mimic the complex microenvironment of the intestine.

In summary, our findings demonstrate that LPS stimulation activates a coordinated inflammatory network involving TLR4, NF-κB, TNF-α, IL-6, and iNOS, with TNF-α acting as a rapid initiator, IL-6 sustaining the response, and iNOS mediating downstream antimicrobial effects. This *in vitro* model captures the essential features of intestinal inflammation, providing a valuable platform for drug screening and mechanistic studies aimed at targeting cytokine networks and signaling pathways in inflammatory diseases.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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