The FASEB Journal

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RESEARCH ARTICLE OPEN ACCESS

SARS-CoV-2 Nucleocapsid Protein Does Not Induce Inflammation in Endothelial Cells or Monocytes

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Received: 2 June 2025 | **Revised:** 26 September 2025 | **Accepted:** 6 October 2025

Funding: This work was supported by the Canadian Government, CIHR, Institute of Infection and Immunity (III) (PJT-178398).

Keywords: COVID-19 | endothelial cells | endotoxin contamination | inflammation | monocytes | nucleocapsid protein

ABSTRACT

The nucleocapsid (N) protein of SARS-CoV-2 is critical for viral replication and genome packaging. However, whether it induces inflammation through endothelial cell and monocyte activation remains controversial. One aspect that has been overlooked is the consideration of residual endotoxins in recombinant proteins when conducting immune response studies. We aimed to assess whether N-protein induces an inflammatory response in mouse and human microvascular endothelial cells (MEC and HMEC) and THP-1 cells, independent of endotoxin contamination. MEC, HMEC, and THP-1 cells were treated with vehicle, lipopolysaccharide (LPS), or recombinant N-protein in the presence or absence of the LPS-neutralizing agent polymyxin B. The inflammatory response was assessed through quantification of mRNA and protein levels of inflammatory markers and monocyte adhesion to endothelial cells. In MEC, treatment with N-protein resulted in at least a 15-fold increase in inflammatory marker levels. Similarly, in THP-1 cells, N-protein caused a significant increase in mRNA inflammatory marker levels, which were brought back to control levels with polymyxin B. LPS or N-protein alone resulted in a tripling of monocyte adhesion to MEC, and this was reduced to control levels with polymyxin B. In addition, in HMEC, endotoxin-depleted N-protein did not cause a significant increase in inflammatory marker levels. In conclusion, our data show that N-protein does not induce inflammation in endothelial cells or monocytes. Some of its observed inflammatory effects may be due to endotoxin contamination, suggesting that it does not directly induce inflammation.

1 | Introduction

Coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) and has led to over 7 million deaths worldwide. Acute respiratory distress syndrome (ARDS) and sepsis due to systemic inflammation are the leading causes of death in COVID-19 [1, 2]. Sepsis results in the upregulation of proinflammatory factors like tumor necrosis factor alpha (TNFα), interleukins (ILs) such as IL-6, IL-1, IL-12, as well as interferons (IFNs) [3]. Patients with COVID-19 have significantly higher levels of these cytokines as compared to healthy controls [4]. ARDS is a severe lung injury that occurs during an uncontrolled inflammatory process, leading to diffuse alveolar damage, capillary membrane leakage, and progressive respiratory

Abbreviations: AAV, adeno-associated virus; ARDS, acute respiratory distress syndrome; COVID-19, coronavirus disease 2019; HMEC, human microvascular endothelial cells; ICAM-1, Intracellular adhesion molecule 1; IFN, interferon; IL, interleukin; IL-1Ra, interleukin-1 receptor antagonist; LPS, lipopolysaccharide; LVV, lenti-viral vector; MCP-1, monocyte chemoattractant protein-1; MEC, mouse microvascular endothelial cells; MERS-CoV, middle east respiratory syndrome coronavirus; NP, nucleocapsid protein; NPd, nucleocapsid protein, endotoxin-depleted; NPnon, nucleocapsid protein, endotoxin nondepleted; PMB, polymyxin B; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TLR, toll-like receptor; TNFα, tumor necrosis factor-alpha.

failure [5]. The most common etiology of ARDS is severe sepsis [6]. The release of proinflammatory cytokines leads to recruitment of neutrophils in the lungs, where they can release toxic mediators and damage the capillary endothelium and alveolar epithelium, resulting in pulmonary edema and inflammation [5, 7].

The nucleocapsid (N) protein of SARS-CoV-2 is a multifunctional RNA-binding protein essential for the viral life cycle. It plays a central role in packaging the viral genome into a helical ribonucleoprotein complex, thereby maintaining genome stability and facilitating efficient replication and transcription [8, 9]. Beyond its structural functions, N-protein may also influence host immune responses, though this remains a subject of ongoing investigation. A particularly controversial aspect is whether the N-protein directly induces inflammation in the host. For example, adeno-associated viral expression of N-protein resulted in lung injury in mice by promoting activation of NLRP3 inflammasomes and causing hyperinflammation, as seen by increased mRNA levels of various cytokines and chemokines [10]. Recombinant N-protein produced from *E. coli* also induced the expression and secretion of IL-6 in human monocytes and macrophages [11]. Preliminary evidence from Urbán and colleagues showed that recombinant N-protein produced from *E. coli* did not induce a primary proinflammatory response in human monocytes. However, N-protein pre-exposure primes monocytes to produce elevated levels of the anti-inflammatory cytokine IL-1Ra upon a subsequent LPS challenge [12]. Additionally, SARS-CoV-2 N-protein may play a dual role in immune response regulation. In HEK293T and HepG2 cells, transfection of N-protein expression plasmids at a relatively low dose (0.25 μg) inhibited type 1 interferon (IFN-1) signaling and repressed production of inflammatory cytokines. However, it facilitated IFN-1 signaling and inflammatory cytokine expression when the dose was increased to 1 μg [13]. Similarly, Qian and colleagues found that the recombinant N-protein produced from HEK293 cells activated human endothelial cells and led to increased expression of proinflammatory cytokines [14]. On the contrary, other studies have suggested that the N-protein may not contribute to the inflammatory state observed in COVID-19. Singh and colleagues found that recombinant N-protein and Spike-protein failed to activate human monocyte-derived dendritic cells [15]. Similarly, Khan et al. showed that N-protein does not stimulate macrophages, monocytes, or lung epithelial cells [16]. Notably, both studies used recombinant proteins produced in mammalian HEK293T cells, rather than *E. coli* [15, 16].

Commercially available recombinant proteins are often produced in *E. coli* [17]. This process leaves proteins highly susceptible to contamination from LPS, a molecule found in the outer bacterial membrane [18]. LPS initiates an immune response through recognition by TLR4 receptors and can lead to pro-inflammatory cytokine production, complement activation, activation of the coagulation cascade, and ultimately septic shock and organ dysfunction [19]. Polymyxin B (PMB) is an antibiotic that neutralizes the effects of LPS through binding to lipid A and has been used to assess the effects of recombinant proteins independent of endotoxin contamination [20].

We aimed to determine whether the SARS-CoV-2 N-protein alone, independent of the potential effects of endotoxin contamination, induces inflammation in mouse and human vascular endothelial cells and human monocytes.

2 | Materials and Methods

2.1 | Animals and Ethics

All mice were handled according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (8th edition, 2011). All procedures were performed in accordance with the Canadian Council on Animal Care guidelines and approved by the Animal Care Committee at Western University. C57BL/6 wild-type mice were purchased from Charles River Laboratories (Laval, Quebec, Canada). Animals were housed in a 12-h light/dark cycle and given *ad libitum* access to normal chow and water.

2.2 | Cells

Primary microvascular endothelial cells (MEC) were isolated from the skeletal muscle of male C57BL/6 mice aged approximately 2–3 weeks [21]. Mice were euthanized via intraperitoneal injection of ketamine (264 μg/g body mass of ketamine, 13.2 μg/g body mass acepromazine, 13.2 μg/g body mass xylazine, 660 ng/g body mass atropine) and subsequent cervical dislocation. Skeletal muscle tissue was collected from the hind limbs, minced, and washed with 1X phosphate-buffered saline (PBS). The tissue was then digested in Dulbecco's Modified Eagle Medium (DMEM) containing 0.8 mg/mL collagenase II, 1.6 mg/mL BSA, and 0.13 mg/mL dispase II for 45–60 min at 37°C. Between digestions, tissues were dissociated by aspiration with a pipette. The supernatant from the solution was collected and strained using a 200 μm filter, followed by a 70 μm filter, and was subsequently centrifuged at 400 × g for 5 min to obtain an endothelial cell pellet. The pellet was washed with 1X PBS and then resuspended in endothelial cell media containing 20% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine, 5 U/mL heparin, and endothelial cell growth supplements (Lonza).

Human pulmonary artery microvascular endothelial cells (HMEC, American Type Culture Collection (ATCC)) were cultured in endothelial cell media as described above, in an incubator at 37°C with 5% CO_2. The cells were grown to confluence, subcultured, and used in experiments.

THP-1 cells (human monocytes, ATCC) were cultured in RPMI-1640 medium (Wisent) containing 10% FBS, 1% P/S, and 1% β-mercaptoethanol (5 mM) at a density between 1×10^5 and 1×10^6 cells/ml. To pass the cells, they were collected in a 50 mL tube and centrifuged at 200 × *g* for 10 min. The old medium was removed, and fresh medium was added, after which the cells were plated at 6×10^5 cells/ml. For experiments, cells were replated in 24-well plates at a density of 1×10^6 cells/ml per well.

2.3 | Recombinant N-Proteins

Recombinant SARS-CoV-2 N-proteins with a polyhistidine tag were either purchased from ThermoFisher Scientific (RP-87707, Mississauga, ON) or expressed and purified using an *E. coli* cell line as follows.

The pET-28a (+) vector containing the SARS-related Coronavirus 2, Wuhan-Hu-1 nucleocapsid gene, NR-53507 (BEI Resources,

NIAID, NIH) was transformed into *E. coli* BL21 (DE3) competent cells. A total of 0.5 μg plasmid DNA was added to 100 μL of cells, heat shocked at 42°C for 45 s, and incubated on ice for 2 min. After adding 900 μL of Luria-Bertani (LB) medium, cells were incubated at 37°C for 1 h with shaking and plated on LB agar containing 60 μg/mL kanamycin. Plates were incubated overnight at 37°C. Single colonies were cultured overnight in 20 mL LB-kanamycin (60 μg/mL) at 37°C. The next day, 10 mL of culture was transferred into 1 L of LB-kanamycin and grown at 37°C until OD_{600} reached 0.6–0.8. Protein expression was induced with 400 μM IPTG, and cultures were incubated overnight at 20°C. Cells were harvested by centrifugation at 8500×*g* for 30 min at 8°C. Pellets were re-suspended in lysis buffer (50 mM Tris–HCl pH 7.5, 500 mM NaCl, 5% glycerol, 5 mM β-mercaptoethanol, 1 mM PMSF) and lysed by sonication. Lysates were incubated at room temperature for 30 min, then centrifuged at 12000×*g* for 30 min at 4°C. The supernatant was incubated with HIS-Select Nickel affinity gel (Sigma-Aldrich) for 90 min on a rotator, then loaded onto a gravity column pre-equilibrated with water and lysis buffer. After washing with 10–15 column volumes of wash buffer (same composition without PMSF), proteins were eluted with 300 mM imidazole in elution buffer and dialyzed overnight in 1X PBS using 6–8 kDa MWCO tubing (Spectrum Laboratories). Endotoxins were removed from lab-purified recombinant N-protein using the Genscript endotoxin eraser kit. The protein was passed through the endotoxin removal column a total of four times. N-protein concentrations were determined before and after endotoxin removal using a Nanodrop Lite spectrophotometer (ThermoFisher Scientific, Mississauga, ON). Endotoxin levels in N-proteins were assessed using a ToxinSensor chromogenic LAL endotoxin assay kit (L00350C, GenScript, New Jersey, USA).

2.4 | In Vitro N-Protein Treatments

HMEC, MEC, and THP-1 monocytes were treated in 24 or 96-well plates at varying times and doses and assessed for cytokine mRNA levels, monocyte adhesion to the endothelial monolayer, and cytokine protein levels. Doses of LPS, N-protein, and polymyxin B (PMB) were titrated and optimized based on the assay and the cell type being used.

2.5 | RNA Isolation and qPCR

After the designated treatment time, the medium was removed from the endothelial cells, and they were rinsed with 1X PBS three times. TRIzol reagent (500 μL, Invitrogen) was added to each well, and a pipette tip was used to scrape cells from the wells. For THP-1 cells, the cell suspension was collected and centrifuged at 5000×g for 10 min to obtain a cell pellet. The pellet was then suspended in 500 μL of TRIzol reagent.

For RNA isolation, all samples were thawed on ice and vortexed for 10 s before adding 100 μL of chloroform. After mixing for 15 s, samples were incubated on ice for 10 min and centrifuged at 14000×*g* for 15 min at 4°C. The aqueous phase was transferred to clean tubes, mixed with 250 μL of 100% isopropanol, and incubated on ice for 15 min. Samples were centrifuged again under the same conditions. The supernatant was discarded, and RNA pellets were washed with 500 μL of 70% ethanol in 0.1% DEPC-treated water, followed by centrifugation at 12000×*g* for 10 min at 4°C. Pellets were air-dried in a fume hood for 15–30 min, then resuspended in 30–50 μL of 0.1% DEPC water and heated at 60°C for 5 min. RNA concentrations were measured using a Nanodrop Lite spectrophotometer (Thermo Fisher Scientific).

To each RNA sample, 1 μL of random primer (20 μM) was added, followed by heating at 65°C for 5 min. Samples were then briefly incubated on ice before adding 7.5 μL reverse transcription master mix (4 μL of 5X First Strand buffer, 2 μL of 0.1 M DTT, 1 μL of 10 mM dNTPs, and 0.5 μL of MMLV reverse transcriptase (200 U/μL)). Reverse transcription was carried out at 37°C for 4 h, followed by inactivation at 95°C for 5 min. The resulting cDNA was diluted in 40–60 μL of nuclease-free water and stored at −20°C.

For qPCR, 2 μL of cDNA was mixed with 8 μL of master mix (5 μL of 2X EvaGreen qPCR buffer, 2.5 μL of ddH_2O, and 0.5 μL of gene-specific primer mix containing both forward and reverse primers at 20 μM each) in a 96-well PCR plate. Amplification was performed using an Eppendorf Realplex Mastercycler for 35 cycles, with annealing temperatures of 60°C–61°C. Ct values were analyzed using the standard curve method to calculate expression ratios and fold changes. Housekeeping genes, 28S or GAPDH, were used for normalization.

Mouse Gene Primers (Sigma)	
TNFα:	*Forward = 5′CGGCATCCATCTCAAAGACA3′* *Reverse = 5′CTTGACGGCAGAGAGGAGGT3′*
IL-6:	*Forward = 5′CAAAGCCAGAGTCCTTCAGAG3′* *Reverse = 5′ATGGTCTTGGTCCTTAGCCAC3′*
ICAM-1:	*Forward = 5′GTGATGCTCAGGTATCCATCCA3′* *Reverse = 5′CACAGTTCTCAAAGCACAGCG3′*
E-Selectin:	*Forward = 5′ATGCCTCGCGCTTTCTCTC3′* *Reverse = 5′GTAGTCCCGCTGACAGTATGC3′*
Human Gene Primers (Invitrogen)	
TNFα:	*Forward = 5′ AACCTCCTCTCTGCCATCAA3′* *Reverse = 5′ CCAAAGTAGACCTGCCCAGA3′*
IL-6:	*Forward = 5′ AGGAGACTTGCCTGGTGAAA3′* *Reverse = 5′ GCATTTGTGGTTGGGTCAG3′*
ICAM-1:	*Forward = 5′ CGACTGGACGAGAGGGATT3′* *Reverse = 5′ GAGAGCACATTCACGGTCAC3′*
E-Selectin:	*Forward = 5′ CTTTCCAGTGCACAGCCTTG3′* *Reverse = 5′ GCATCGCATCTCACAGCTTC3′*

2.6 | Monocyte Isolation and Adhesion Assay

C57BL/6 mice were euthanized through intraperitoneal injection of ketamine, followed by a cervical dislocation. The femurs were collected and cut from both ends. Bone marrow was obtained from the femurs through flushing with 1X PBS, using a 1 mL syringe and 26-gauge needle. After thoroughly flushing the femurs, the marrow-derived suspension was filtered and centrifuged at 250×g for 10 min. The resulting pellet was resuspended in a red blood cell lysis buffer for 10 min to remove the red blood cells. The cell suspension was centrifuged at 250×g

for 10 min, and a monocyte cell pellet was obtained. The monocytes were resuspended in 1X PBS and counted using a hemocytometer, with a yield of 20–25 million monocytes on average.

MEC that were grown to 100% confluence were treated with recombinant N-protein with or without endotoxin neutralizing reagent PMB for 3 h, after which isolated monocytes were added to each well. After 1 h, the medium was removed, and the cells were washed with PBS to remove any unadhered monocytes. The cells were visualized using a light microscope, and random field images were obtained from multiple parts of the well. Adhered monocytes were quantified from a 100×100 μm area, and values were expressed as adhered monocytes per 10000 μm^2 cultured MEC area [21].

2.7 | Cytokine Analysis in THP-1 Culture Supernatant

THP-1 monocytes were treated for 24 h, after which the cell suspension was collected in 1.5 mL Eppendorf tubes and centrifuged at 5000×g for 10 min. The supernatant was collected and stored at −80°C until ready for shipping to Eve Technologies (Calgary, Alberta) for analysis through the "Human Cytokine Proinflammatory Focused 15-Plex Discovery Assay Array". This assay used Luminex xMAP technology for multiplexed quantification of 15 human cytokines, chemokines, and growth factors. The analysis was performed using the Luminex 200 system (Luminex, Austin, USA). The markers included GM-CSF, IFNγ, IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, MCP-1, and TNF-α.

2.8 | Statistical Analysis

Data were presented as mean ± SEM. mRNA, cytokine, and monocyte adhesion data were analyzed using a one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. All data were analyzed and graphed using GraphPad Prism version 9.4.1 for Windows (GraphPad Software, La Jolla, California, USA). A p value of less than 0.05 was considered statistically significant.

3 | Results

3.1 | N-Protein (Endotoxin Nondepleted) Does Not Increase mRNA Levels of Inflammatory Cytokines and Adhesion Molecules in Human Microvascular Endothelial Cells

Recombinant proteins produced in *E. coli* were contaminated with endotoxins [17, 22], which were quantified using a chromogenic LAL assay. Endotoxin levels in recombinant N-proteins (10 μg/mL) were 6.9 EU/ml for nondepleted samples and decreased to 4.0 and 0.6 EU/ml after two and four rounds of endotoxin depletion, respectively, confirming effective endotoxin removal. As a reference, endotoxin levels detected in the LPS used in this study at 100 pg/mL and 1 ng/mL were 0.3 and 4.6 EU/ml, respectively.

Based on pilot experiments assessing dose–response and time-course effects, N-protein at 10 μg/mL for 4 h served as the treatment conditions for our study (see Figures S1–S3). Human pulmonary artery microvascular endothelial cells were treated with LPS (1 μg/mL) or N-protein, endotoxin nondepleted (NPnon, 10 μg/mL) for 4 h. RT-qPCR analysis revealed that NPnon did not cause a significant increase in mRNA levels of TNFα, IL-6, E-selectin, and ICAM-1. However, LPS stimulation led to a significant increase in inflammatory marker gene expression (Figure 1).

3.2 | Polymyxin B Inhibits N-Protein (Endotoxin Nondepleted, NPnon) Induced mRNA Levels of Inflammatory Cytokines and Adhesion Molecules in Mouse Microvascular Endothelial Cells

Mouse microvascular endothelial cells were treated with LPS (1 μg/mL), NPnon (10 μg/mL), and combination treatments with an endotoxin neutralizing reagent polymyxin B (PMB, 250 μg/mL) for 4 h. RT-qPCR analysis showed that both LPS and NPnon caused significant increases in mRNA levels of TNFα, IL-6, E-selectin, and ICAM-1 in mouse microvascular endothelial cells. However, in the presence of PMB, the inflammatory marker levels induced by LPS or NPnon were reduced to control levels (Figure 2).

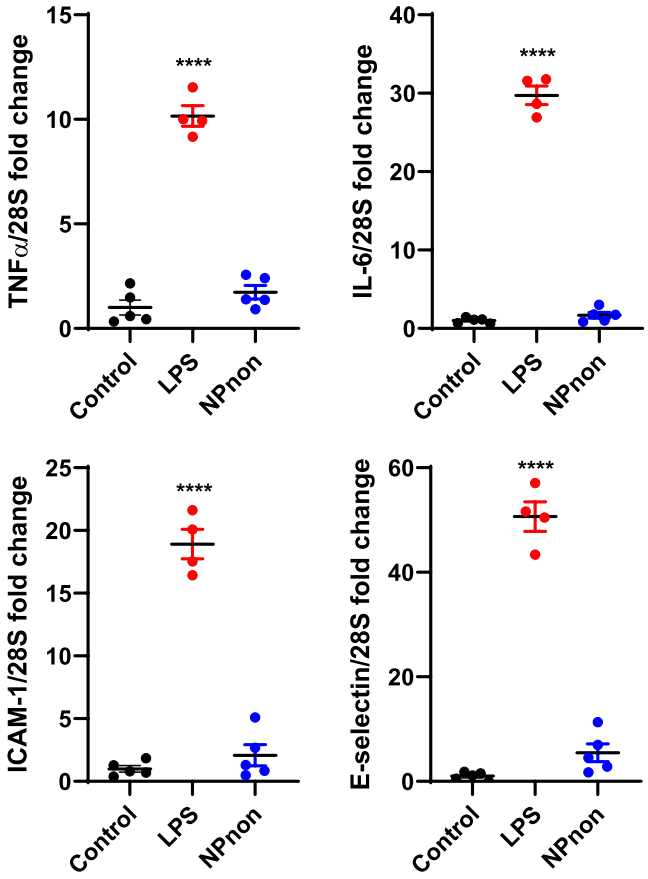

FIGURE 1 | Effects of recombinant N-protein (endotoxin nondepleted, NPnon) on inflammatory marker mRNA levels in human microvascular endothelial cells. Cells were treated with vehicle, LPS (1 μg/mL), or NPnon (10 μg/mL, ThermoFisher) for 4 h. The total RNA was isolated, and qPCR analysis was performed to measure the mRNA levels of TNFα, IL-6, E-selectin, and ICAM-1. Data are presented as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's multiple comparisons test. $n = 4–5$ independent cell cultures per group. ****$p < 0.0001$ vs. control.

3.3 | Polymyxin B Inhibits N-Protein (Endotoxin Nondepleted, NPnon) Induced Monocyte Adhesion to Mouse Microvascular Endothelial Cells

During endotoxemia, monocyte adhesion to endothelial cells is a key process promoting inflammation and disease progression [23, 24]. In this experiment, mouse microvascular endothelial cells were treated with LPS (1 μg/mL), NPnon (10 μg/mL), and combination treatments with an endotoxin-neutralizing reagent PMB (250 μg/mL) for 3 h. Mouse monocytes were added to the cultured endothelial cells for 1 h, and monocyte adhesion to endothelial cells was assessed through phase contrast light microscopy. Representative images are shown in Figure 3. Quantitative analysis showed that LPS or NPnon caused a significant increase in monocyte adhesion to mouse endothelial cells, which was abolished by PMB treatment (Figure 4).

3.4 | Endotoxin Depleted N-Protein (NPd) Does Not Increase mRNA Levels of Inflammatory Cytokines or Adhesion Molecules in Mouse Microvascular Endothelial Cells

To assess endotoxin-independent effects, recombinant N-proteins were depleted from endotoxin using a Genscript endotoxin eraser kit, repeated a total of four times. Additionally, PMB was used to neutralize the effect of endotoxin. Mouse microvascular endothelial cells were treated with vehicle, LPS (1 μg/mL), or endotoxin-depleted N-protein (NPd, 50 μg/mL) either with or without PMB (250 μg/mL) for 4 h. RT-qPCR analysis showed that LPS stimulation led to a significant increase in mRNA levels of TNFα, IL-6, E-selectin, and ICAM-1. However, NPd did not cause a significant increase in any of the inflammatory markers, either with or without PMB (Figure 5). These data suggest that endotoxin-depleted N-proteins do not induce an inflammatory response in mouse microvascular endothelial cells.

3.5 | Endotoxins Drive the Inflammatory Response of Endotoxin-Depleted N-Protein (NPd) in Human THP-1 Monocytes

Human THP-1 monocytes were treated with LPS (100 pg/mL), endotoxin-depleted and laboratory-purified N-protein (NPd, 1 μg/mL), and combination treatments with an endotoxin neutralizing reagent PMB (1 μg/mL) for 4 h. LPS stimulation led to a significant increase in inflammatory marker levels. Notably, NPd caused a significant increase in mRNA levels of TNFα and IL-6 (Figure 6). This increase could be attributed to the higher sensitivity of THP-1 cells to LPS. Nonetheless, when combined with PMB treatment, NPd did not cause a significant increase in inflammatory marker levels.

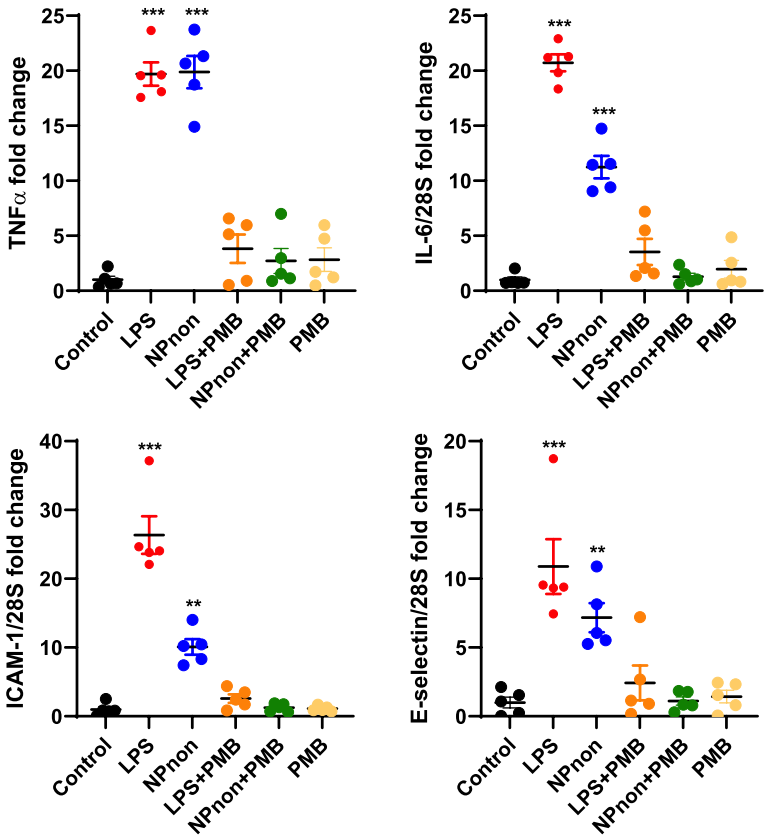

FIGURE 2 | Effects of recombinant N-protein (endotoxin nondepleted, NPnon) on inflammatory marker mRNA levels in mouse microvascular endothelial cells. Cells were treated with vehicle, LPS (1 μg/mL), NPnon (10 μg/mL, ThermoFisher), LPS + polymyxin B (PMB) (1.25 mg/mL), NPnon + PMB (250 μg/mL), or PMB (250 μg/mL) for 4 h. The total RNA was isolated and qPCR analysis was performed to measure the mRNA levels of TNFα, IL-6, E-selectin, and ICAM-1. Data are presented as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's multiple comparisons test. $n = 5$ independent cell cultures per group. ***$p < 0.001$, **$p < 0.01$ vs. control.

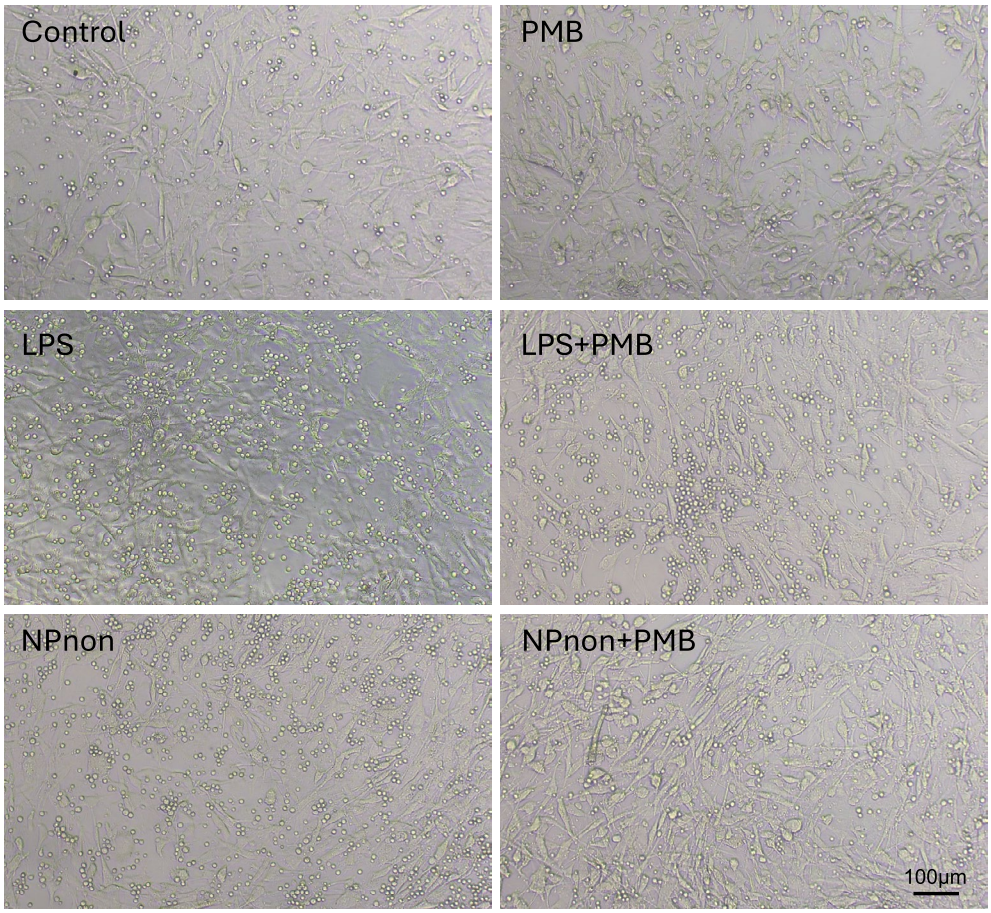

FIGURE 3 | Representative images of monocyte adhesion to mouse endothelial cells. Cells were treated with vehicle, LPS (1 μg/mL), N-protein, endotoxin nondepleted (NPnon) (10 μg/mL, ThermoFisher), LPS + polymyxin B (PMB) (1.25 mg/mL), NPnon + PMB (250 μg/mL), or PMB (250 μg/mL) for 3 h. Mouse monocytes were added for 1 h, and adhesion was visualized with a light microscope.

3.6 | Endotoxin-Depleted N-Proteins (NPd) do Not Increase Inflammatory Cytokine or Chemokine Levels in THP-1 Monocyte Cell Culture Supernatants

THP-1 monocytes were treated with LPS (1 ng/mL), endotoxin-depleted N-protein (NPd, 1 μg/mL), and combination treatments with an endotoxin neutralizing reagent PMB (1 μg/mL), for 24 h. The cell culture supernatant was analyzed for protein levels of inflammatory cytokines and chemokines through Luminex multiplex analysis. LPS stimulation resulted in significant increases in all measured inflammatory markers, while TNFα and IL-8 levels also trended upward but did not reach statistical significance. However, endotoxin-depleted N-proteins did not significantly increase protein levels of TNFα, IL-8, IL-10, IL-6, IL-1β, IL-1Ra, or monocyte chemoattractant protein-1 (MCP-1), either with or without PMB treatment (Figure 7).

4 | Discussion

Although much progress has been made in elucidating the pathogenesis of COVID-19, the underlying molecular mechanisms that mediate the immune response to SARS-CoV-2 infection are still not fully understood. Initially, SARS-CoV-2 infection leads to the activation of cells such as macrophages, monocytes, and dendritic cells, all of which can contribute to a cytokine storm [16]. Endothelial cells have also been shown to be dysregulated, which can further exacerbate this response [25, 26]. The N-proteins of earlier coronaviruses, such as SARS-CoV and MERS-CoV, have been known to play a role in host immune response regulation; therefore, studies have aimed to explore the role of N-protein in SARS-CoV-2 infection [27, 28].

Previous reports have described both extracellular and intracellular actions of N protein, with outcomes varying by cell type and delivery method: endothelial activation via cell surface TLR2 receptors [14], lack of dendritic cell activation attributed to contaminants [15], and hyperinflammation in respiratory epithelial cells following endocytosis [29]. Studies using transfection or viral expression suggest intracellular mechanisms [10, 30], though findings remain debated [31]. Notably, endothelial cells appear less permissive to N protein entry, as evidenced by the absence of N protein in these cells during infection despite its presence in neighboring epithelial or pneumocyte populations [32]. Thus, although intracellular effects cannot be ruled out, in the absence of transfection, recombinant N protein appears to act primarily through extracellular interactions in endothelial cells.

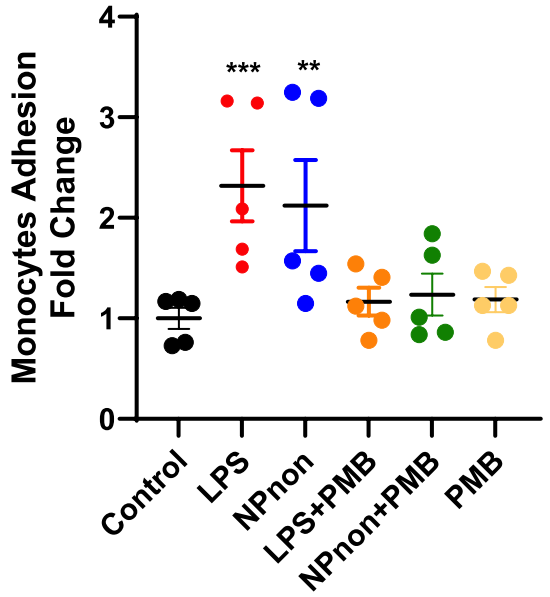

FIGURE 4 | Monocyte adhesion to mouse endothelial cells following recombinant N-protein (nondepleted, NPnon) treatment. Cells were treated with vehicle, LPS (1 μg/mL), NPnon (10 μg/mL, ThermoFisher), LPS + Polymyxin B (PMB) (1.25 mg/mL), NPnon + PMB (250 μg/mL), or PMB (250 μg/mL) for 3 h. Monocytes were added to each treatment for 1 h, and adhesion was visualized with a light microscope. Quantification was done using a 100 × 100 μm area, and fold change relative to the control was calculated. Data are presented as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's multiple comparisons test. $n = 5$ independent cell cultures per group. ***$p < 0.001$, **$p < 0.01$ vs. control.

Our work shows that the N-protein of SARS-CoV-2 does not itself elicit an increase in inflammatory marker levels in mouse or human microvascular endothelial cells or human monocytes. Furthermore, we found that N-protein on its own did not induce monocyte adhesion to mouse microvascular endothelial cells. In fact, any proinflammatory response observed with N-protein treatment was reduced to control levels when combined with the LPS-binding and neutralizing reagent PMB, suggesting that residual LPS from the bacteria used to generate the recombinant protein is responsible for the proinflammatory effect. This is also supported by our observation that endotoxin-depleted N-proteins did not elicit any measurable response, with or without PMB. These observations are in contrast to some studies, which suggested that N-protein can activate human endothelial cells and monocytes [11, 14], but support other studies that N-protein may not contribute to inflammation in dendritic cells, monocytes, or lung epithelial cells [15, 16].

The differing responses to N protein (nondepleted) between mouse and human endothelial cells likely arise from a combination of species-specific factors and endothelial heterogeneity. Endothelial cells are not uniform; their phenotype and immune responsiveness vary depending on the tissue of origin [33]. Previous studies have reported variable endothelial responses to SARS-CoV-2 proteins and attributed this to differences in the source of primary endothelial cells (e.g., lung vs. brain) [32, 34]. In our study, the human endothelial cells were isolated from pulmonary arteries, whereas the mouse endothelial cells were derived from skeletal muscle, which could partly explain the divergent outcomes.

Our study vigorously examined the impact of N-protein on endothelial cells and monocytes with more stringent controls for LPS contamination, with the use of an LPS-neutralizing agent. Our results show that endotoxin-depleted N protein did not induce inflammation in endothelial cells but did increase cytokine expression in human monocytes. This discrepancy likely reflects the higher sensitivity of monocytes, which can respond to extremely low levels of LPS [35]. Although N proteins were subjected to endotoxin depletion, trace amounts persist, in agreement with previous studies [22]. Importantly, when THP-1 cells were treated with N protein in the presence of PMB, cytokine induction was abolished, supporting the interpretation that the observed effects were due to residual endotoxin rather than N protein itself.

Xia and colleagues also used PMB when investigating the effects of N-protein in bone-marrow-derived macrophages [36]. They pretreated N-protein with PMB and still observed proinflammatory effects, as indicated by increased IL-1β, IL-6, and TNFα mRNA levels. Our work, however, did not show similar patterns in mouse or human endothelial cells or human monocytes. This disparity may relate to varying sensitivity to residual LPS that escapes neutralization with PMB, depending on cell type and species. On the other hand, two studies observed activation of human endothelial cells and monocytes with N-protein treatment; however, this was seen without the use of PMB in their recombinant proteins [11, 14]. Furthermore, the findings of Karwaciak et al. were in opposition to those of Khan et al., who did not observe any measurable N-protein effects on inflammatory markers in human monocytes [11, 16]. Other studies examined the inflammatory effects of N-protein with the use of vectors such as plasmids, adeno-associated virus (AAV), and lentiviral vectors (LVV) for expression. For instance, one study reported that N-protein promoted NLPR3 inflammasome activity in both mice and cultured cells, such as HEK293T, A549, and THP-1-derived macrophages [10]. These researchers used viral vectors (AAV and LVV) to induce N-protein expression, presumably eliminating the risk of LPS' confounding effects that are associated with recombinant proteins. However, AAV and LVV vectors are well known to activate innate immune responses [37].

Our work highlights the significant impact LPS contamination can have as a confounding variable when investigating immune responses. Since immune dysregulation and inflammation are prominent in COVID-19, it is important that any study investigating this response take into account the effects of endotoxin contamination. We attempted to do so using PMB and endotoxin depletion in all recombinant proteins. The use of PMB may have some limitations, including potential toxicity to cells and activation of proinflammatory cytokines with higher doses in certain cells [38]. To circumvent these limitations, we optimized PMB doses to avoid cell death or proinflammatory activation.

Further work assessing the inflammatory effects of recombinant proteins could be conducted with the use of other vectors

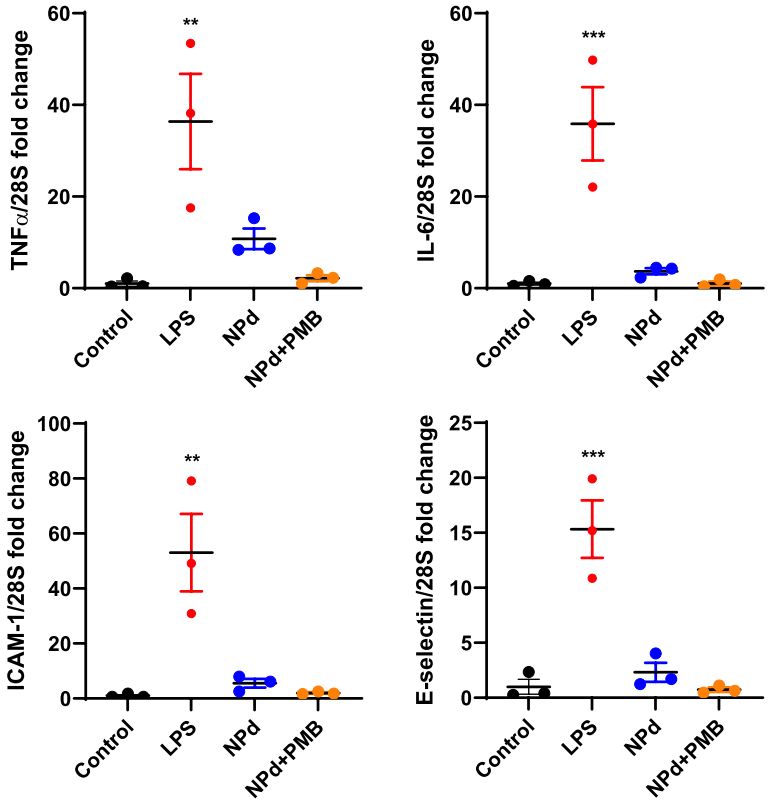

FIGURE 5 | Effects of endotoxin-depleted N-protein (NPd) on inflammatory marker mRNA levels in mouse microvascular endothelial cells. Cells were treated with vehicle, LPS (1 μg/mL), NPd (50 μg/mL), NPd + PMB (250 μg/mL), or PMB (250 μg/mL) alone (control) for 4 h. Total RNA was isolated and RT-qPCR analysis was performed to measure mRNA levels of TNFα and E-selectin. Data are presented as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's multiple comparisons test. $n = 3$ independent cell cultures per group. ***$p < 0.001$, **$p < 0.01$ vs. control.

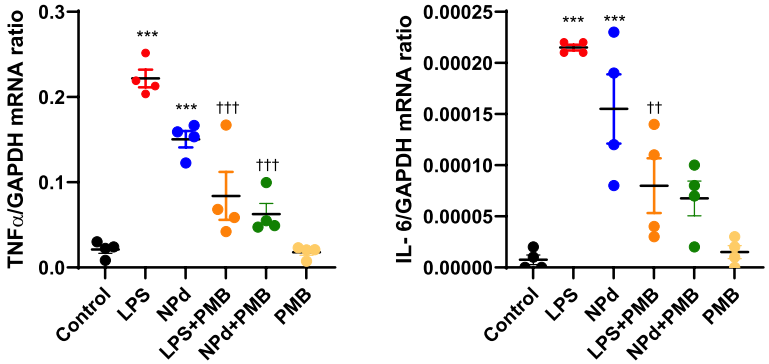

FIGURE 6 | Effects of endotoxin-depleted N-protein (NPd) on inflammatory marker mRNA levels in THP-1 cells. Cells were treated with vehicle, LPS (100 pg/mL), endotoxin-depleted N-protein (NPd, 1 μg/mL), LPS + PMB (1 μg/mL), NPd + PMB (1 μg/mL), or PMB (1 μg/mL) for 4 h. The total RNA was isolated, and qPCR analysis was performed to measure mRNA levels of TNFα and IL-6. Data are presented as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's multiple comparisons test. $N = 4$ independent cell cultures per group. ***$p < 0.001$ vs. control, †††$p < 0.001$, ††$p < 0.01$ vs. respective LPS or NPd alone.

or proteins that have substantially lower LPS contamination. However, few, if any, currently available LPS removal methods are 100% efficient, owing to the stability of LPS and its ability to form aggregates of various sizes. Furthermore, interactions between LPS and recombinant proteins can be strong, resulting in some residual LPS presence even after several rounds of LPS removal [22].

A possible extension of our work could explore potential synergistic effects between N-protein and LPS, similar to what Petruk and colleagues observed with Spike-protein. When Spike-protein was added to THP-1 cells in addition to LPS, it led to a boost in proinflammatory activity, as compared to LPS treatment alone [39]. However, proper validation of this claim with N-protein would require minimal LPS contamination, comparable to the level used with Spike-protein by Petruk and colleagues (30 fg/μg of residual LPS) [39]. One way to achieve this could be to use endotoxin-free strains of bacteria, such as ClearColi BL21, to express N-protein, as Singh and colleagues did in their work [15].

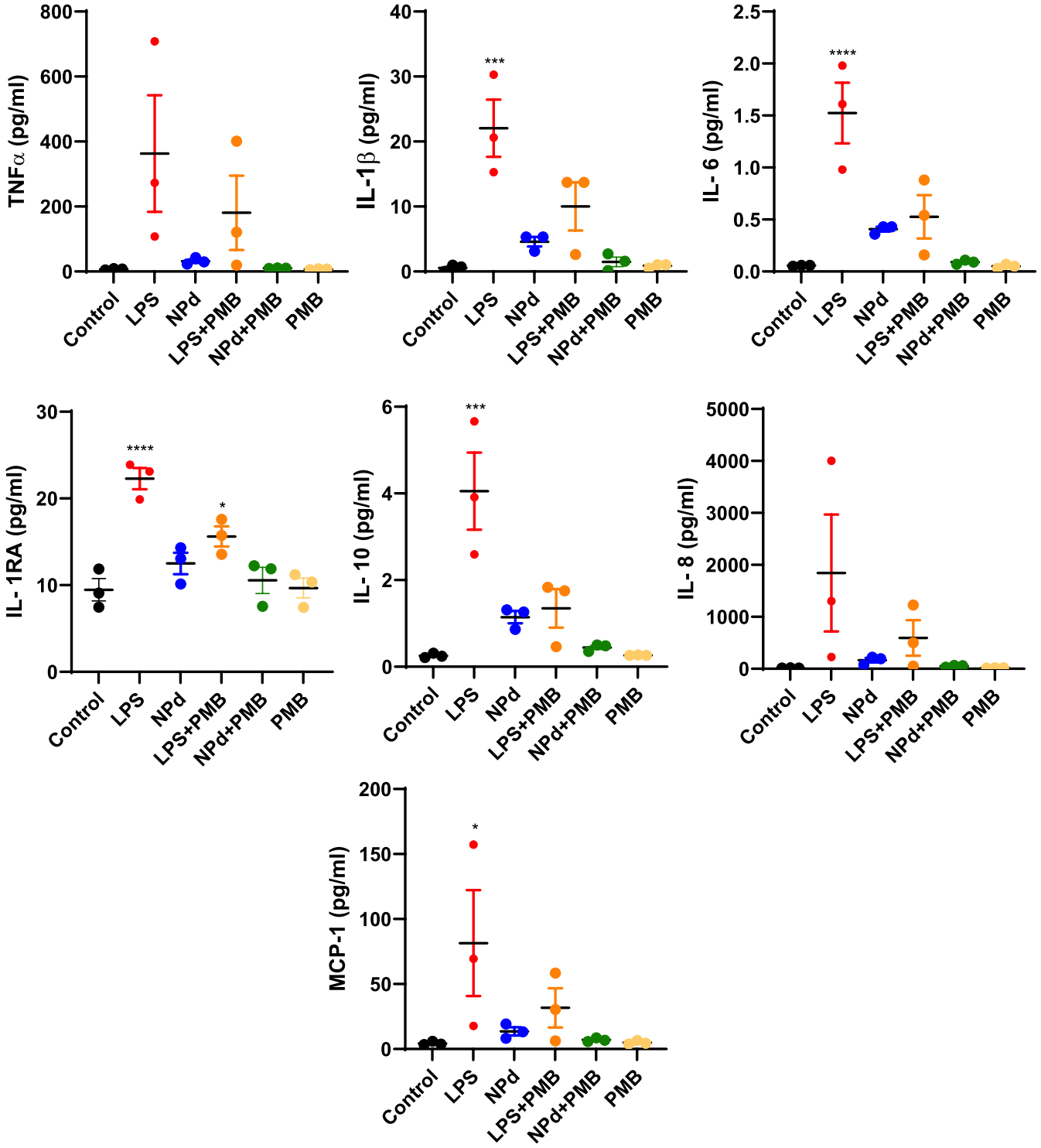

FIGURE 7 | Effects of endotoxin-depleted N-protein (NPd) on inflammatory marker protein levels in THP-1 cells. Cells were treated with vehicle, LPS (1 ng/mL), endotoxin-depleted N-protein (NPd, 1 μg/mL), LPS + PMB (1 ng/mL + 5 μg/mL), NPd + PMB (1 μg/mL for both), and PMB (1 μg/mL) for 24 h. The total cell culture supernatant was collected and analyzed by Luminex multiplex assay for cytokines and chemokines. Data are presented as mean ± SEM and analyzed using one-way ANOVA, followed by Bonferroni's multiple comparisons test. $N = 3$ independent cell cultures per group. ****$p < 0.0001$, ***$p < 0.001$, *$p < 0.05$ vs. control. Undetectable cytokines and chemokines are not shown here.

To conclude, our findings show that the N-protein of SARS-CoV-2 does not contribute to the inflammatory response in mouse and human microvascular endothelial cells or human monocytes. Furthermore, this work confirms that endotoxin contamination is a confounding variable that can contribute to the proinflammatory effects seen in immune response studies. Overall, our work helps reconcile the conflicting data that exist on the role of the N-protein in COVID-19 inflammation.

Author Contributions

M.T. contributed to study design and performed experiments and data analysis, and participated in manuscript preparation and revision. Q.F. and X.L. contributed to study design and conceptualization. Q.F. also contributed to manuscript writing and revision. X.L. contributed to experiments and data analysis. N.B. and S.B. provided essential reagents for experiments, commented on, and edited the manuscript. All authors reviewed the manuscript.

Acknowledgments

We thank Dr. Rithwik Ramachandran and Dr. Peter Chidiac for their constructive comments and edits to this manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Included in article and its supporting information.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figures S1–S3.** fsb271142-sup-0001-Figures.pdf. **Dataset S1.** fsb271142-sup-0002-DatasetS1.xlsx