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Neutralizing *Staphylococcus aureus* PAMPs that Trigger Cytokine Release from THP-1 Monocytes

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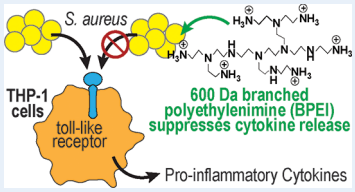
ABSTRACT: Innate immunity has considerable specificity and can discriminate between individual species of microbes. In this regard, pathogens are "seen" as dangerous to the host and elicit an inflammatory response capable of destroying the microbes. This immune discrimination is achieved by toll-like receptors on host cells recognizing pathogens, such as *Staphylococcus aureus*, and microbe-specific pathogen-associated molecular pattern (PAMP) molecules, such as lipoteichoic acid (LTA). PAMPs impede wound healing by lengthening the inflammatory phase of healing and contributing to the development of chronic wounds. Preventing PAMPs from triggering the release of inflammatory cytokines will counteract the dysregulation of inflammation. Here, we use ELISA to evaluate the use of cationic molecules branched polyethylenimine (BPEI), PEGylated BPEI (PEG-BPEI), and polymyxin-B to neutralize anionic LTA and lower levels of TNF-α cytokine release from human THP-1 monocytes in a concentration-dependent manner. Additional data collected with qPCR shows that BPEI and PEG-BPEI reduce the expression profile of the TNF-α gene. Similar effects are observed for the neutralization of whole-cell *S. aureus* bacteria. In vitro cytotoxicity data demonstrate that PEGylation lowers the toxicity of PEG-BPEI (IC_{50} = 2661 μm) compared to BPEI (IC_{50} = 853 μM) and that both compounds are orders of magnitude less toxic than the cationic antibiotic polymyxin-B (IC_{50} = 79 μM). Additionally, the LTA neutralization ability of polymyxin-B is less effective than BPEI or PEG-BPEI. These properties of BPEI and PEG-BPEI expand their utility beyond disabling antibiotic resistance mechanisms and disrupting *S. aureus* biofilms, providing additional justification for developing these agents as wound healing therapeutics. The multiple mechanisms of action for BPEI and PEG-BPEI are superior to current wound treatment strategies that have a single modality.



INTRODUCTION

Each year, millions of pressure ulcers and diabetic chronic wounds occur. Chronic wounds with high morbidity and mortality rates affect millions of people around the world, emerging as a threat to healthcare systems. Approximately, 6.5 million have chronic skin ulcers in the United States and there is an extensive economic burden on the healthcare setting and society due to skin repair failing and nonhealing ulcers.[1,2] In diabetic patients, these ulcers lead to lower limb amputation and inflict significant pain, which results in reducing the quality of life for patients.

The slow healing of diabetic wounds and pressure ulcers is exacerbated by excessive inflammation when tissue cells detect danger signal molecules and, in response, cause the activation and accumulation of immune cells.[3−5] Inflammation is the first response to infection and injury caused by harmful stimuli, such as infected pathogens, damaged cells, or irritants.[6,7] It is an essential protective immune response involving immune cells, blood vessels, and molecular mediators to eliminate infectious pathogens, remove necrotic cells, and clear damaged tissues.[7] However, dysregulated immune activation results in hyper-production of cytokines and systemic inflammation culminating in recurrent infection and tissue necrosis, septic shock, multiorgan failure, and death.[8−10] *Staphylococcus aureus* (*S. aureus*) is a Gram-positive bacterium that can cause serious infections such as skin and soft tissue infections, bloodstream infections, and life-threatening septicemia. It is also an aggravator of the inflammatory skin disease atopic dermatitis (AD).[11−13] *S. aureus* infections cause approximately 20,000 deaths a year in the United States.[12] When the skin barrier is breached and exposed to *S. aureus* pathogens, an efficient immune response is initiated. This response involves both innate and recruited immune cells and eventual clearance of infection.[11,12]

The immunostimulant agents or pathogen-associated molecular pattern molecules (PAMPs) of *S. aureus* bacteria are cell wall components lipoteichoic acid (LTA) and peptidoglycan (PGN).[14] LTA is the major PAMP of *S. aureus* bacteria that is released from bacterial cells following

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bacteriolysis induced by immune cells lysozymes, cationic bactericidal peptides, phospholipase A2, elastase, and cathepsins, or beta lactam antibiotics.[10,15] LTA is an amphiphilic polymer composed of a hydrophilic chain of poly glycerol-phosphate units, substituted with D-alanine and/or sugars that are covalently attached to the cytoplasmic membrane of *S. aureus* via a hydrophobic glycolipid anchor.[14−17] The backbone chain of LTA extends into the peptidoglycan to the outer surface of the bacterial cell.[18] As an adhesion molecule, LTA facilitates the binding of bacteria to eukaryotic cells, their colonization, and tissue invasion.[10]

During infection, LTA binds to immune cells either nonspecifically via establishing phosphodiester units with membrane phospholipids or specifically via LPS binding protein (LBP), glycosylphosphatidylinositol-anchored membrane protein CD14, nuclear protein high-mobility group box 1 (HMGB1), and TLR2 interaction.[19,20] By binding to the target cells, LTA activates the innate immune system by inducing the production of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α).[15] After interaction with TLR2, LTA activates mitogen-activated protein kinase (MAPK), which induces associations with myeloid differentiation primary response 88 (MYD88), interleukin-1 receptor (IL-1R)-associated kinases (IRAKs), and TNF-receptor-associated factor 6 (TRAF6). LTA activates target genes using the extracellular signal-regulated kinase (ERK) pathway by the phosphorylation of ERK-1, MEK1/2, and c-Raf.[19] LTA can trigger the production of proinflammatory cytokines, nitric oxide (NO), the activation of nuclear transcription factor NF-kB, and other proinflammatory mediators.[17,21,22] Through these mechanisms, LTA is involved in the pathophysiology of inflammation and postinfectious outcome, thus suggesting that LTA is one of the major virulence factors of *S. aureus*.[15]

There is an unmet need for developing technology that alleviates wound infection and improves healing. Ideal wound healing drugs should be effective against factors that impair the healing process such as reducing the chances of a prolonged inflammation at the wound site, restoring the optimal inflammatory response, and minimizing the development of antibiotic resistance and biofilm formation. However, successful compounds are elusive. Recent studies in our lab show that 600 Da branched polyethylenimine (BPEI) and its less cytotoxic derivative PEG-BPEI can kill bacterial pathogens, facilitate the uptake of drugs by lowering drug influx barrier, and eradicate biofilms of methicillin-resistant *S. aureus* (MRSA), in addition to methicillin-resistant *S. epidermidis*, *P. aeruginosa*, *E. coli*, and carbapenem-resistant *Enterobacteriaceae* bacteria.[23−33]

We have previously identified low molecular weight 600 Da branched polyethylenimine (600 Da BPEI) (Figure 1 top) as a broad-spectrum antibiotic potentiator that can overcome antibiotic resistance and eradicate biofilms of multidrug-resistant strains of Gram-positive and Gram-negative bacteria including methicillin-resistant *Staphylococcus aureus*, methicillin-resistant *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Escherichia coli*, and carbapenem-resistant *Enterobacteriaceae*.[23−25,27−30,33−35] However, the toxicity concerns arising from the presence of primary amines 600 Da BPEI are significant and need to be addressed. The in vivo toxicity concerns are resolved by covalently conjugating a low-molecular-weight polyethylene glycol (350 MW PEG) moiety to 600 Da BPEI, resulting in PEG-BPEI (Figure 1 bottom).[26,30−33] Additionally, we have shown that the PEGylated derivative possesses the potentiator characteristics of BPEI against the MDR strains and their biofilms.[27,30−33] Regardless of the BPEI ability to facilitate the uptake of drugs and lower drug influx barriers as an antibiotic potentiator against bacterial biofilms and planktonic cells, therapeutic benefits of 600 Da BPEI and PEG-BPEI can be expanded. In this work, we continue to investigate 600 Da BPEI and its PEGylated derivative as anti-PAMP molecules to help mitigate the overproduction of inflammatory response. Both BPEI and PEG-BPEI function as anti-PAMP molecules to reduce the production of TNF-α cytokine by human THP-1 monocyte cells exposed to *S. aureus* LTA and intact *S. aureus* bacterial cells. Overall, we envision BPEI and PEG-BPEI as topical agents for acute and chronic wounds because they are multifunctional agents that can address bacterial infection, disperse biofilms, and reduce inflammation.

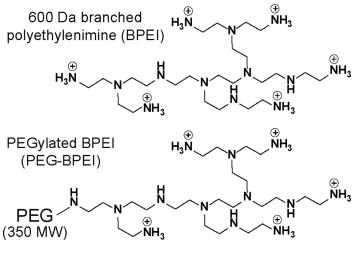

Figure 1. Structure of 600 Da BPEI (top) and PEG-BPEI (bottom).

METHODS

Cell Culture. The THP-1 human monocyte-like cell line was purchased from Sigma (St. Louis, MO, ECACC, 88081201) and maintained in RPMI 1640 medium containing L-glutamine and sodium bicarbonate (Sigma, St. Louis, MO, R8758), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT, SH30066.03), and 1% pen strep (PS) (10,000 U/mL penicillin and 10,000 μg/mL streptomycin, Gibco 15140122), at 37 °C and 5% CO_2 (v/v) in a humidified incubator. The cells were suspended and grown in T-75 cm^2 culture flasks (Corning, 431464) and subcultured every 5−6 days by three to five times dilution. The cells were adherent and grown in tissue culture dishes (Fisherbrand, FB012924) and subcultured every 2−3 days.

PAMP Neutralizing Agents. Branched polyethylenimine, 600 Da, (BPEI) was purchased from Polysciences, Inc. The synthesis and characterization of PEG350-BPEI has been described elsewhere.[28] Briefly, 600 Da BPEI was dried overnight with lyophilization, and the final mass was used to determine the amount of monofunctionalized PEG-epoxide (Nanocs, Inc., mPEG-epoxide, 350 molecular weight) required to react with 600 Da BPEI in a 1-to-1 stoichiometric ratio. Both 600 Da BPEI and mPEG-epoxide were dispersed in anhydrous ethanol at 60 °C for 24 h. The success of the epoxide ring-opening reactions was determined through ^{1}H NMR spectroscopy. Chemicals were purchased from Sigma-Aldrich, and polymyxin B was purchased from Gold Biotechnology.

LTA, PGN, and HKSA Exposure to THP-1 Cells. The THP-1 cells were seeded onto 96-well plates (Greiner Bio-one, Stuttgart, Germany) at 2 × 10^6 cells/mL in RPMI 1640 complete medium (1% PS and 10% FBS) and incubated

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overnight at 37 °C and 5% CO_2. The day after, the THP-1 cells were stimulated with 25 μg/mL of LTA (purified lipoteichoic acid from *S. aureus*; TLR2 ligand, InvivoGen, tlrl-pslta) for 4 h or 25 μg/mL of PGN (peptidoglycan from *S. aureus*; TLR2 ligand, InvivoGen, tlrl-pgns2) for 6 h. Whole-cell heat-killed *S. aureus* bacteria (HKSA, heat-killed *S. aureus*; TLR2 ligand, InvivoGen, tlrl-hksa) were also used for stimulation at 10^8 cells/mL final concentration for 6 h. Concentrations of treatments were selected based on the optimum production of TNF-α cytokine. LTA, PGN, and HKSA were reconstituted in LAL-grade water (InvivoGen). After stimulation, supernatants were collected at certain time points by centrifugation. To protect TNF-α cytokine protein from degradation by endogenous proteases released during protein extraction, a halt protease inhibitor cocktail (Thermo Scientific, 87786) was used. Immediately after supernatant collection, 10 μL of protease inhibitor was added per 1 mL of supernatant to produce a 1× final concentration. Cell medium was frozen at −80 °C until analysis. Untreated cells were used as controls.

Time Point Assays of LTA, PGN, and HKSA. To determine the time at which the *S. aureus* PAMPs have the most inflammatory effect on TNF-α cytokine secretion, a time point assay was performed. THP-1 cells were plated in 96-well plates at 2×10^6 cells/mL in RPMI complete medium. After 24 h incubation, the THP-1 cells were treated with 25 μg/mL of LTA, 25 μg/mL of PGN, or 10^8 cells/mL of HKSA cells. All the solutions were prepared in endotoxin-free water. The plates were then incubated for 2, 4, 6, 8, and 24 h, and supernatants were collected at the end of each time point. To protect TNF-α cytokine protein from degradation by endogenous proteases released during protein extraction, a halt protease inhibitor cocktail was used at the final 1× concentration. Time durations of treatments were selected based on the optimum production of TNF-α cytokine. Cell medium was frozen at −80 °C until analysis. Untreated cells were used as controls.

Neutralizing Immune Response Induced by *S. aureus* PAMPs by BPEI, PEG-BPEI, and Polymyxin B. The effect of BPEI and its derivative PEG-BPEI against PAMP-induced TNF-α cytokine production was determined. Briefly, THP-1 cells were plated in 96-well plates at 2×10^6 cells/mL in RPMI complete medium and incubated overnight. Then, different concentrations of BPEI were prepared in endotoxin-free water. For the LTA neutralizing experiment, the THP-1 cells were treated with either LTA alone, combinations of an equivalent amount (25 μg/mL) of LTA with each of the BPEI concentrations, and BPEI concentrations alone. The latter was used as negative control, and the cells treated with LTA alone represented the positive control. The combinations were incubated for 30 min before being added to THP-1 cells. Untreated cells were also prepared as control. Then, supernatants were collected after 4 h of incubation. A similar methodology was used for experiments with PGN or HKSA except that the supernatants were collected at a 6 h time point. In the HKSA neutralizing experiment, combinations were added to the cells immediately without prior incubation. We performed the same experiments to investigate the mitigating effects of PEG-BPEI and polymyxin B. Three independent experiments (n = 3) were performed for analysis.

Enzyme-Linked Immunosorbent Assay Measurements. Concentrations of TNF-α cytokine were determined using DuoSet ELISA kits (R&D Systems, DY210) in THP-1 cell supernatants treated by each of the *S. aureus* PAMPs along with either BPEI, PEG-BPEI, or polymyxin B. A 96-well plate was coated with 100 μL per well of TNF-α specific capture antibody diluted in endotoxin-free PBS (Endotoxin-free Dulbecco's PBS 1×, Millipore Sigma, TMS-012-A) and incubated overnight at room temperature. The day after, each well was washed 3 times with 300 μL of washing buffer. Next, the plate was blocked with 300 μL of reagent diluent (R&D Systems, DY995) and incubated at room temperature for a minimum of 1 h and then washed three times with washing buffer. Subsequently, 100 μL of standards or collected supernatants was added to the plate and incubated at room temperature for 2 h, followed by washing. Then, 100 μL of TNF-α specific detection antibody was added and the plate was incubated for 2 h at room temperature and then washed. After that, 100 μL of streptavidin-HRP was added, and the plate was incubated for a minimum of 30 min at room temperature in dark, followed by washing. Then, 100 μL of substrate solution (equal volume of hydrogen peroxide and tetramethylbenzidine (R&D Systems, DY999) was added, and the plate was once again incubated in dark at room temperature until the color developed. The reaction was stopped by adding 50 μL of 2 N sulfuric acid as a stop solution. The absorbance of each well was immediately determined using a microplate reader set at 450 nm. For wavelength correction, the reading was set to 540 nm as well. Optical imperfections in the plate were corrected by subtracting readings at 540 nm from readings at 450 nm.

Cell Stimulation, RNA Extraction, and cDNA Synthesis. For total RNA isolation, THP-1 cells were seeded at 2×10^6 cells/mL in RPMI 1640 complete medium and incubated overnight at 37 °C and 5% CO_2. After 17 h of incubation, the cells were stimulated by LTA alone, LTA combined with either BPEI or PEG-BPEI, BPEI alone, or PEG-BPEI alone. The solutions were prepared in endotoxin-free water. The combo conditions were incubated for 30 min before being added to THP-1 cells. Untreated cells were used as control. Each sample consisted of 12 wells. After stimulation, total cellular RNA was extracted from THP-1 cells using the Quick RNA MiniPerp Kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's protocol. DNase I treatment (Zymo Research, Irvine, CA, USA) was executed to remove any genomic DNA. The concentration of the RNA samples was determined spectrophotometrically using Nanodrop (Thermo Fisher Scientific Inc., Waltham, MA, USA), and then the RNAs were reverse-transcribed to cDNA using a Verso cDNA synthesis kit (ThermoFisher, USA) based on the manufacturer's protocol. Two independent experiments (n = 2) were performed for all analyses.

qPCR. Quantitative real-time PCR analysis was performed using the Roche 480 Lightcycler detection system, and the qPCR products were detected with the PowerTrack SYBR Green master mix (appliedbiosystems). Human GAPDH primers (housekeeping gene) (Hs02786624_g1) and human TNF-a primers (Hs00174128_m1) were purchased from ThermoFisher. Three independent experiments (n = 2) were performed for all analyses. Fold-change values were calculated according to the delta delta Ct method ($\Delta\Delta$Ct) to compare gene expression levels between GAPDH and TNF-α genes.

Cytotoxicity Assay. THP-1 monocyte cells were seeded onto 96-well plates (5000−10,000 cells/cm^2) and allowed to incubate overnight. The cells were then treated with different concentrations of BPEI (7576, 3030, 1515, 151.5, and 15.15 μM), PEG-BPEI (4785, 1914, 957, 95.7, and 9.57 μM), and

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polymyxin B (1511, 1134, 756, 75.6, and 7.56 μM) for 48 h. All the tested compounds were prepared in a 10 mM Tris−HCl buffer. Polymyxin B was used as a positive control, and cells that were only treated with Tris−HCl were considered as negative control. At the end of the incubation period, 10 μL of 0.15 mg/mL of Resazurin dye was added to the wells as a redox indicator. The plate was then incubated for 3 h, and fluorescence signals were detected at 570 nm (excitation) and 600 nm (emission). Six replicate measurements were performed for each compound concentration condition, and the average was reported.

Statistical Analysis. All experiments were performed in triplicate, and the presented data are representative results of the means ± standard error of the mean (SEM). Differences in cytokine production were analyzed using one-way ANOVA with Tukey's post-test. A 95% confidence value with a p-value consisting of $p < 0.05$ was considered statistically significant. Data were analyzed using GraphPad Prism 6.01 software (GraphPad Software Inc., USA) and Adobe Illustrator.

RESULTS

Optimization of TNF-α Protein Production Induced by LTA, PGN, and HKSA. Lipoteichoic acid (LTA) and peptidoglycan (PGN) are *S. aureus* PAMPs that are known to be TLR2 receptor agonists. To mimic inflammation propagated by the PAMPs, we treated THP-1 cells with various concentrations of LTA or PGN (100 ng/mL, 1, 10, and 25 μg/mL) for 24 h. Then, levels of TNF-α cytokine were measured in cell supernatants. Treatment of THP-1 cells with either 25 μg/mL of LTA or PGN provoked strong TNF-α secretion. However, when cells were treated with lower concentrations of the PAMPs, the release of TNF-α was similar to nontreated cells (data is not shown). Therefore, the concentration of 25 μg/mL of LTA was selected to induce the highest secretion of TNF-α cytokine. Likewise, heat-killed *S. aureus* (HKSA) was used to stimulate TNF-α production. HKSA bacteria retain the structural components of the cell wall, including LTA and PGN. This structural integrity can activate different components of the immune system, including toll-like receptors (TLRs) and pattern recognition receptors (PRRs) that provide a more comprehensive and complex immune stimulus compared to isolated components such as LTA. This can lead to a broader and more representative immune response.

Different concentrations of HKSA were used to stimulate THP-1 cells, and we found the concentration of 10^8 cells/mL of HKSA to produce the highest TNF-α protein level. Next, we wanted to explore if the production of TNF-α cytokine was time-dependent. We treated THP-1 cells with 25 μg/mL of LTA for 4, 8, and 24 h. For cells stimulated with either 25 μg/mL of PGN or 10^8 cells/mL of HKSA, we investigated 2, 4, 6, 8, and 24 h time points. Supernatants were collected at the end of each time point and the level of TNF-α was measured using ELISA. Untreated cells were incubated for each of the time points as control. As shown in Figure 2, LTA, PGN, and HKSA showed a time-dependent manner for inducing TNF-α cytokine secretion. For LTA, cytokine levels were highest at 4 h incubation. Therefore, 4 h of incubation for THP-1 cells treated with 25 μg/mL of LTA was used for subsequent studies. For PGN, the incubation time was 4 h and for HKSA, the 6 h time point was used to evaluate TNF-α secretion. In all the time point assays, levels of TNF-α protein were the lowest at 24 h, which is due to the TNF-α protein turnover to maintain the balance of the protein within cells and ensure its proper function and cellular homeostasis.

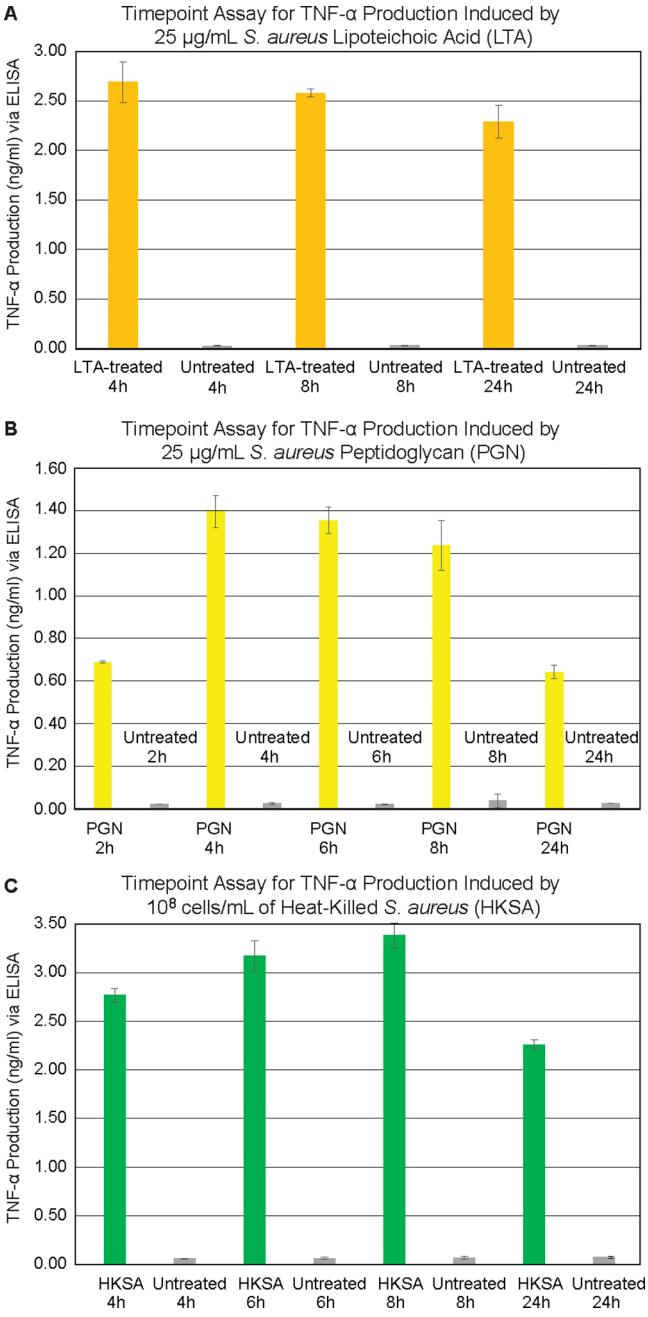

Figure 2. Results for time point assay of TNF-α production induced by *S. aureus* PAMPs. THP-1 cells were treated with (A) 25 μg/mL of LTA, (B) 25 μg/mL of PGN, and (C) 10^8 cells/mL of HKSA. The monocytes were then incubated for 2, 4, 6, 8, and 24 h, and supernatants were collected at the end of each time point. Levels of TNF-α cytokines were quantified using ELISA. Untreated cells were used as controls. Time points that resulted in the highest secretion of TNF-α protein were selected as the optimum time points for stimulating THP-1 cells with the respective PAMPs.

Given the reported synergistic effect of lipoteichoic acid and peptidoglycan in inducing inflammation,[10,18,36,37] we anticipated a stronger and more pronounced inflammatory response for TNF-α production induced by heat-killed *S. aureus* bacteria (HKSA) compared to their individual effects. The findings presented in Figure 2C suggest that the coexistence of LTA

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and peptidoglycan in HKSA significantly amplifies inflammatory signaling pathways and subsequently enhances the immune response in THP-1 cells. None of the untreated cells in each time point showed any signal for TNF-α production in ELISA.

Modulation of Inflammatory Effects of *S. aureus* PAMPs with Different Cationic Compounds. To examine whether BPEI and PEG-BPEI have an antagonistic effect on the production of the immune response, THP-1 cells were treated with combinations of various *S. aureus* PAMPs including LTA, PGN, or HKSA, and either BPEI or PEG-BPEI. First, human monocyte cells were stimulated using LTA alone, as well as combinations of LTA with varying concentrations of BPEI. Each concentration of BPEI was applied to the cells independently to see if BPEI itself can induce any TNF-α cytokine production. Results in Figure 3A demonstrate that treatment of THP-1 cells with LTA provoked strong TNF-α secretion. However, the interaction between LTA and all concentrations of BPEI was antagonistic since preneutralization significantly lowered TNF-α secretion compared to LTA alone. The mixture of 25 μg/mL of LTA and 25 μg/mL of BPEI resulted in an 86% reduction in TNF-α release. Notably, the treatment of cells with different concentrations of BPEI itself yielded TNF-α release levels comparable to those of nontreated cells (Figure 3A).

Next, we investigated if LTA can be neutralized with the PEGylated derivative of BPEI as well. We observed lower cytokine secretion levels, and the effects were greater with higher amounts of PEG-BPEI (Figure 3B). Although PEGylation of the amines of 600 Da BPEI reduced the neutralization ability of 600 BPEI against LTA, PEG-BPEI shows a significant reduction in TNF-α secretion from 25 to up to 60% at its highest concentration level (100 μg/mL) and PEG-BPEI itself did not stimulate cytokine release.

Figure 3C shows ELISA data for cytokine expression and LTA neutralizing ability of polymyxin B, an FDA-approved cationic antibiotic. While polymyxin B is primarily an antibiotic rather than an anti-inflammatory drug, it does possess endotoxin-binding effects. Therefore, polymyxin B can disrupt TLR activation and can suppress TNF-α activity.[38] We investigated polymyxin B and LTA interactions on TNF-α secretion (Figure 3C). Combinations of polymyxin B and LTA showed a significant reducing effect in cytokine production. Despite polymyxin B nearly completely blocking the immune response at the highest concentrations, its neutralizing effect at 25 μg/mL was only 40%, which is less than half of the effect exhibited by BPEI at the same concentration.

Subsequently, we evaluated the impact of BPEI on attenuating the TNF-α production induced by peptidoglycan (PGN), another cell wall component of *S. aureus*. As illustrated in Figure 2B, monocyte cells treated solely with PGN displayed notable activation of TLR after 6 h of incubation. Although BPEI concentrations partially neutralized PGN and mitigated the secretion of TNF-α, the overall reduction in cytokine levels did not significantly differ from the immune response elicited by PGN (Figure 4A). Treatment of THP-1 cells with different concentrations of PEG-BPEI and an equivalent amount of PGN resulted in a greater TNF-α reduction than its precursor molecule, 600 Da BPEI (Figure 4B). To our surprise, the outcomes of neutralizing PGN with polymyxin B were unfavorable (Figure 4C). We hypothesize that all the cationic compounds examined in this study, including BPEI, PEG-BPEI, and polymyxin B, employ electrostatic interactions to

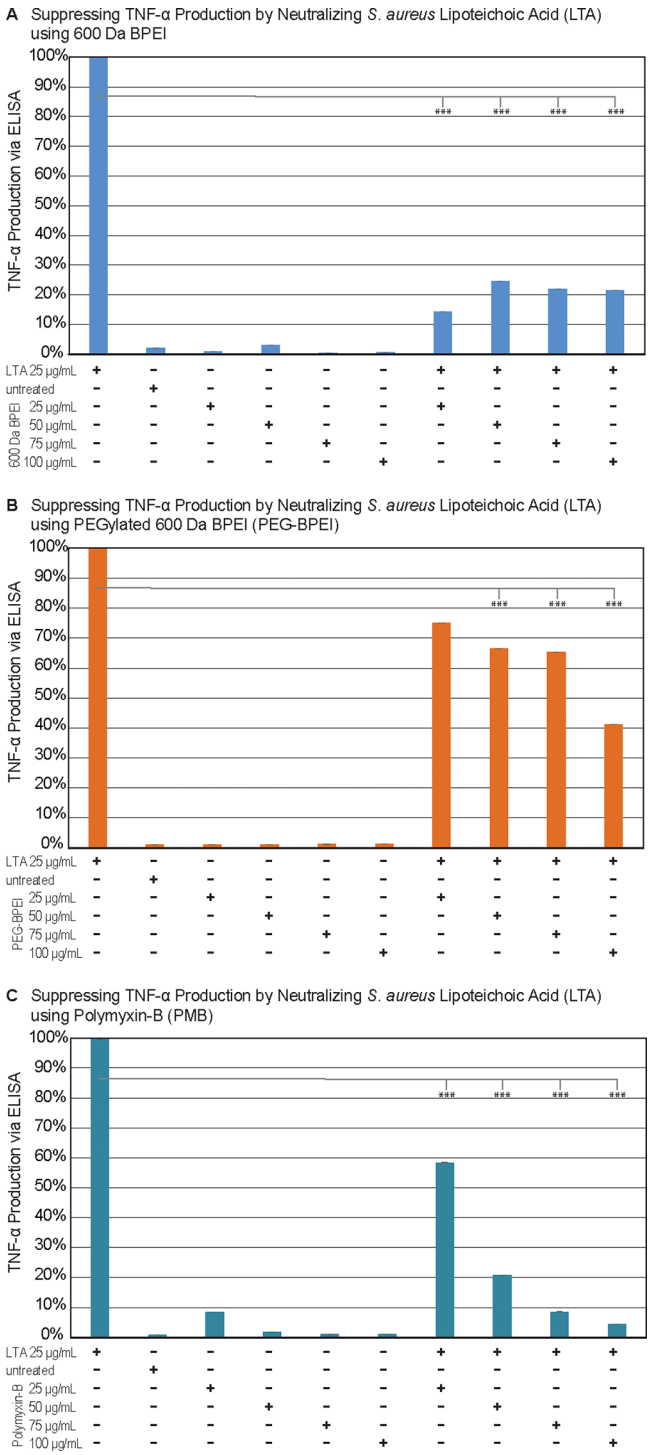

Figure 3. Results for neutralizing immune response induced by *S. aureus* LTA. THP-1 cells were treated with an equivalent amount of LTA and concentrations of (A) BPEI, (B) PEG-BPEI, and (C) polymyxin B. Levels of TNF-α cytokines were quantified using ELISA. Untreated cells were used as controls. All experiments were performed in triplicate, and the presented data are representative results of the means ± standard error of the mean (SEM). A 95% confidence value with a p-value consisting of $p < 0.05$ was considered statistically significant.

bind with LTA and PGN and modify the characteristics of the Gram-positive cell wall components that activate TLRs and induce cytokine production. However, as evidenced by the results presented in Figure 4, it appears that BPEI, PEG-BPEI,

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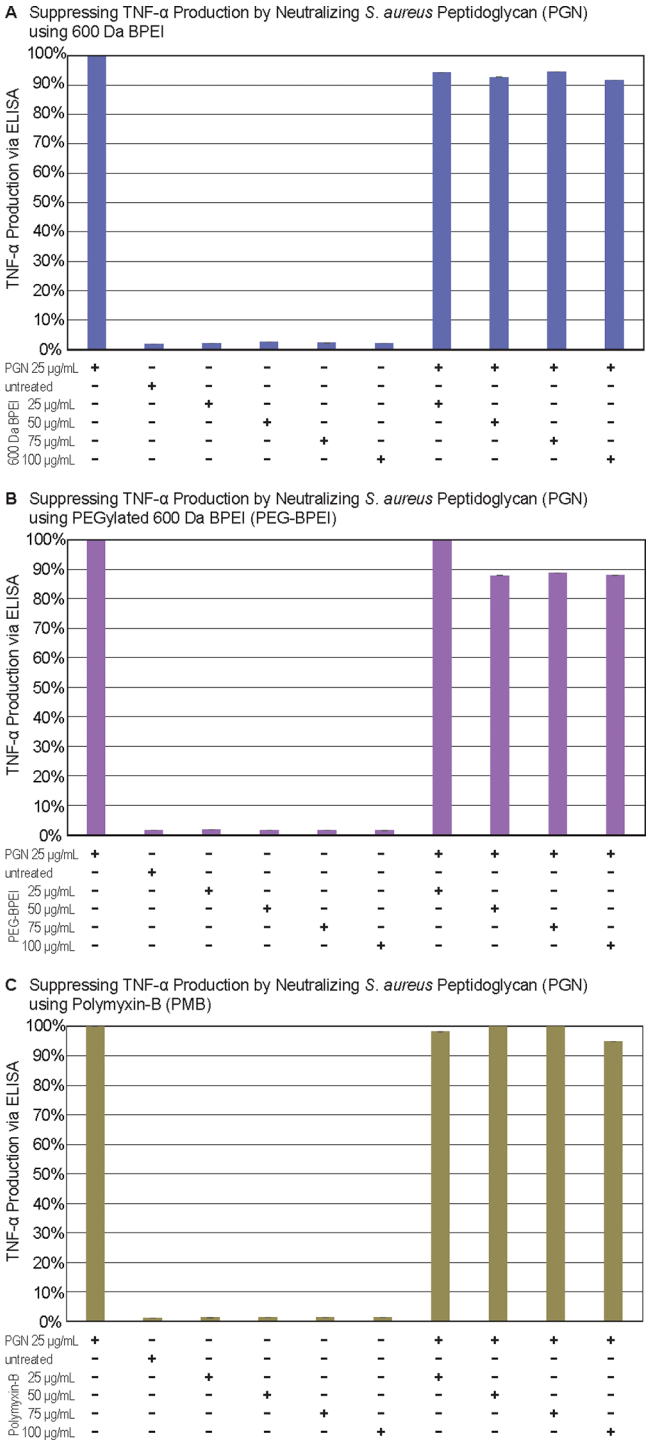

Figure 4. Results for neutralizing immune response induced by *S. aureus* peptidoglycan (PGN). THP-1 cells were treated with an equivalent amount of PGN and concentrations of (A) BPEI, (B) PEG-BPEI, and (C) polymyxin B. Levels of TNF-α cytokines were quantified using ELISA. Untreated cells were used as controls. All experiments were performed in triplicate, and the presented data are representative results of the means ± standard error of the mean (SEM). A 95% confidence value with a *p*-value consisting of $p < 0.05$ was considered statistically significant.

and polymyxin B may not form robust interactions with PGN, hindering their ability to effectively neutralize PGN.

We also evaluated the ability of BPEI and PEG-BPEI to neutralize the induction of TNF-α production by heat-killed *S. aureus* bacteria (HKSA). We applied concentrations of 0.1−15 μg/mL of BPEI and PEG-BPEI to neutralize HKSA-triggered TNF-α production in THP-1 cells. Considering that lipoteichoic acid and peptidoglycan together may have a greater effect in inducing inflammatory responses (Figure 2), the data from Figures 3 and 4 suggest that the neutralizing ability of BPEI and PEG-BPEI on TNF-α secretion induced by heat-killed *S. aureus* bacteria would be diminished in comparison to the individual PAMPs. Unexpectedly, a concentration of 15 μg/mL of BPEI resulted in a 40% decrease in TNF-α production, as depicted in Figure 5A. Moreover, BPEI exhibited a 10% reduction in the inflammatory response at concentrations of 0.1 and 10 μg/mL, also shown in Figure 5A. On the other hand, PEG-BPEI demonstrated a more robust mitigating effect in HKSA-induced TNF-α production compared to BPEI, resulting in reductions of 60% and approximately 20% at concentrations of 15 and 10 μg/mL, respectively (Figure 5B). We did not pursue further investigations involving higher concentrations of BPEI and PEG-BPEI to neutralize the immune response stimulated by HKSA. While it was not feasible to quantify the concentrations of LTA and PGN present in 10^8 cells/mL of HKSA bacteria for direct comparison with 25 μg/mL of each of LTA and PGN used in this study (Figures 3 and 4), we consider the observed neutralizing effects of BPEI and PEG-BPEI on HKSA-induced inflammation response to be of value. This is because heat-killed whole *S. aureus* bacteria contain a wide range of antigens and molecular patterns capable of activating various components of the immune system, including toll-like receptors (TLRs) and pattern recognition receptors (PRRs). This can lead to a wider range of immune responses that may occur in situ, unlike the utilization of isolated components such as LTA and PGN. Additionally, the structural components and antigens present in heat-killed *S. aureus* bacteria remain intact, reducing the risk of utilizing chemically isolated LTA and PGN that may be degraded during isolation. Finally, we utilized polymyxin B as a reference cationic antibiotic to mitigate the inflammation response triggered by HKSA, allowing us to compare the results with those obtained using BPEI and PEG-BPEI. In contrast to what we observed with LTA alone (Figure 3), polymyxin B exhibited a slight reduction of only 12% in the HKSA-induced TNF-α production (Figure 5C).

BPEI and PEG-BPEI Mitigation of LTA-Induced Inflammation Response by RT-PCR and qPCR Assays. The ability of BPEI and PEG-BPEI to mitigate the release of TNF-α cytokines was confirmed by measuring the expression of mRNA responsible for the TNF- α gene. Initially, THP-1 cells were exposed to 25 μg/mL of LTA for varying durations (1, 2, 6, and 8 h) to determine the time point at which LTA could induce the highest levels of TNF-α gene expression. Figure 6A illustrates the initial expression of TNF-α mRNA within 1 h of THP-1 cell incubation. This early gene expression notably declined at the 6-h time point and was completely absent after 8 h of incubation. Hence, we focused on a 1 h incubation period to reach the highest levels of TNF-α mRNA levels induced by LTA. Subsequently, THP-1 cells were exposed to various treatments, including LTA alone and combinations of LTA with different concentrations of BPEI. Cells treated with BPEI concentrations and untreated cells were used as negative controls. Following a 1-h stimulation, total cellular RNA was extracted, reverse-transcribed into cDNA, and subjected to quantitative real-time PCR analysis. As depicted in Figure 6B, BPEI treatment exhibited a

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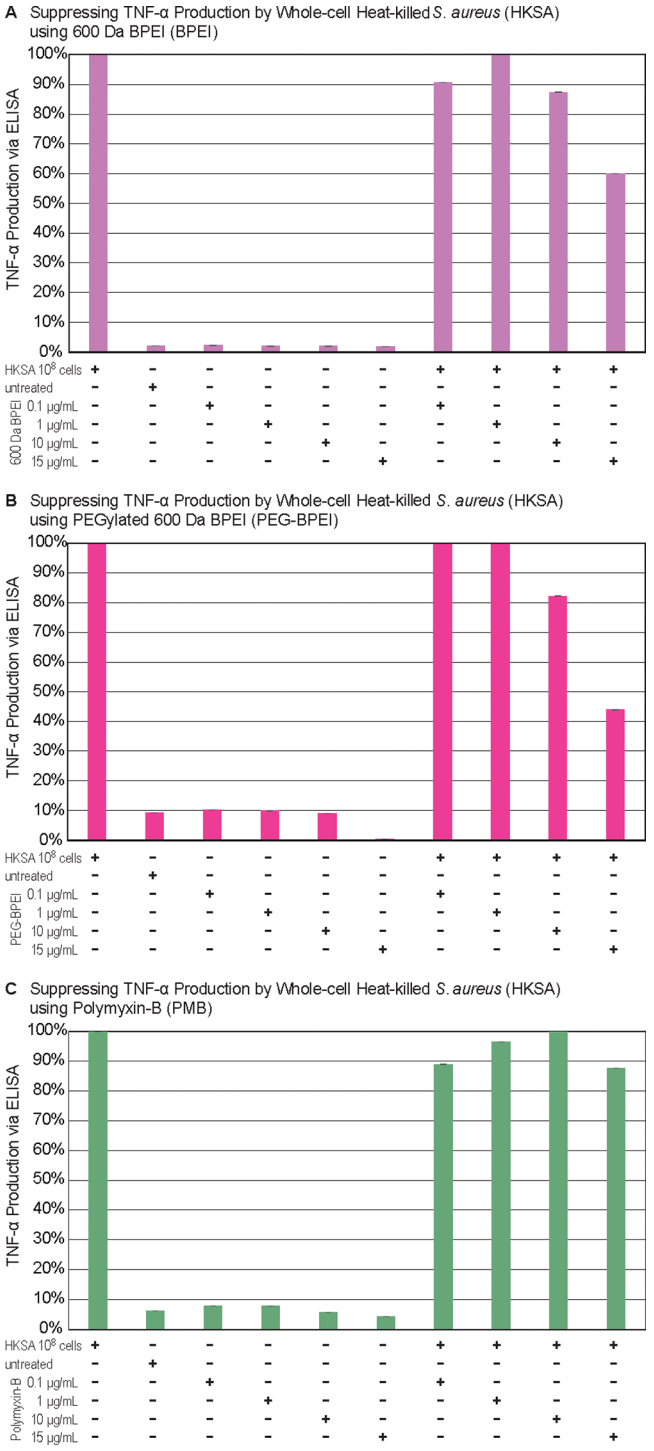

Figure 5. Results for neutralizing immune response induced by heat-killed *S. aureus* bacteria (HKSA). THP-1 cells were treated with an equivalent amount of HKSA and concentrations of (A) BPEI, (B) PEG-BPEI, and (C) polymyxin B. Levels of TNF-α cytokines were quantified using ELISA. Untreated cells were used as controls. All experiments were performed in triplicate, and the presented data are representative results of the means ± standard error of the mean (SEM). A 95% confidence value with a *p*-value consisting of $p < 0.05$ was considered statistically significant.

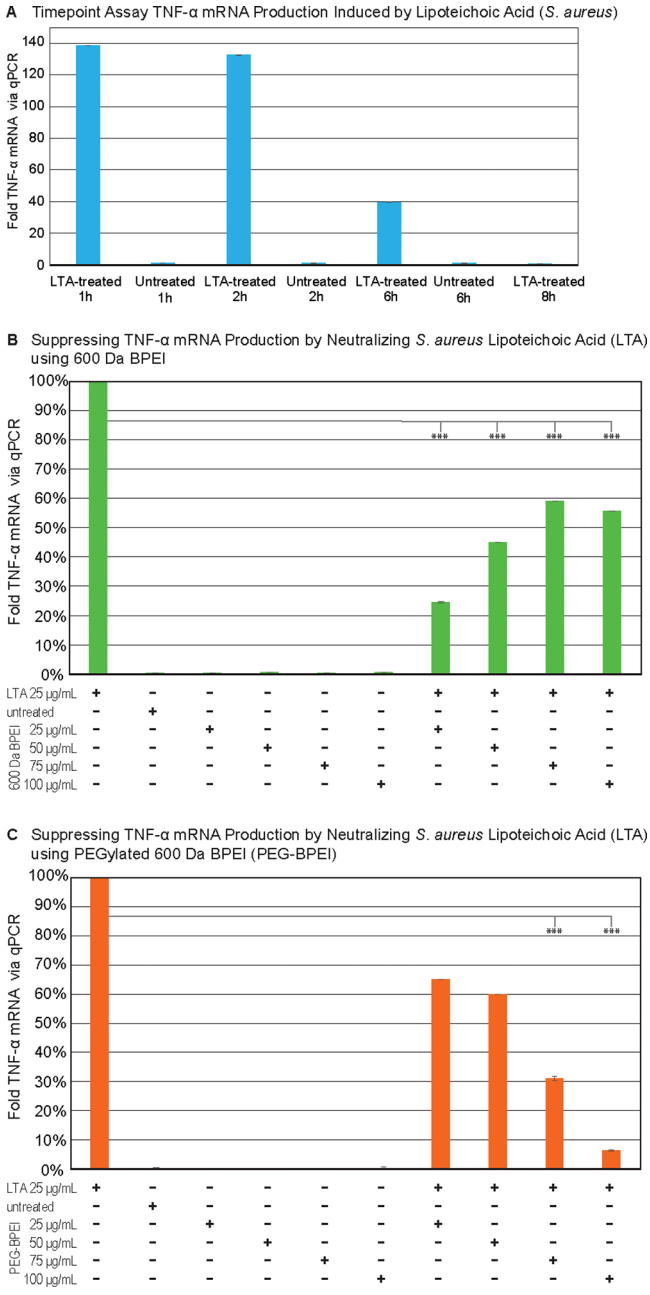

Figure 6. qPCR results for neutralizing immune response induced by *S. aureus* LTA. (A) THP-1 cells were treated with 25 μg/mL of LTA and incubated for 1, 2, 6, and 8 h. Total cellular RNA was extracted and reverse-transcribed to cDNA, and then quantitative real-time PCR analysis was performed to detect the levels of TNF-α mRNA. (B) THP-1 cells were treated with an equivalent amount of LTA and concentrations of BPEI. Then, TNFα gene expression was quantified using qPCR. (C) THP-1 cells were treated with an equivalent amount of LTA and concentrations of PEG-BPEI. Then, TNF-α gene expression was quantified using qPCR. Untreated cells were used as controls. All experiments were performed in duplicate, and the presented data are representative results of the means ± standard error of the mean (SEM). A 95% confidence value with a *p*-value consisting of $p < 0.05$ was considered statistically significant. To normalize the fold-changes between different experiments, their values were expressed as percentages.

significant inhibitory effect on the expression profile of the TNF-α gene. These qPCR findings were consistent with the results obtained from the ELISA experiments (Figure 3A). According to the data in Figure 6B, BPEI at a concentration of 25 μg/mL suppressed TNF-α mRNA production by 75%. Although higher concentrations of BPEI resulted in a lesser reduction in TNF-α gene expression, they still caused a

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considerable decrease of 44−55% in the expression of the gene (Figure 6B). The RT-PCR experiments were also conducted for PEG-BPEI. The results, as depicted in Figure 6C, demonstrated that PEGylated BPEI effectively inhibited the expression profile of the TNF-α gene in a dose-dependent manner, in accordance with the findings obtained from the ELISA assays (Figure 3B). At the lowest PEG-BPEI concentrations, there was a noticeable decrease of up to 40% in the mRNA levels of the TNF-α cytokine gene. Intriguingly, at a concentration of 75 μg/mL of PEG-BPEI, the down-regulation reached 70% of mRNA production, and at 100 μg/mL, only a minimal 6% expression of the TNF-α gene was observed (Figure 6C). Taken together, these results verified the results of ELISA analysis and suggested that BPEI and PEG-BPEI can mitigate the production of inflammation response via the downregulation of signal transduction associated with the TNF-α cytokine production.

BPEI and PEG-BPEI Cytotoxicity in Human Monocytes. To test the cytotoxicity of 600 Da BPEI, PEG 350-BPEI, and polymyxin B used in the previous experiments, THP-1 human monocyte cells were incubated for 2 days in the presence of 0.01−5 mg/mL of BPEI, PEG-BPEI, and polymyxin B. The latter compound was used as a positive control due to its known cytotoxicity effects on eukaryotic cells. The concentration at which each compound reduced cell viability by 50%, IC_{50}, was calculated to determine the dose−response relationship (Figure 7). As anticipated from previous

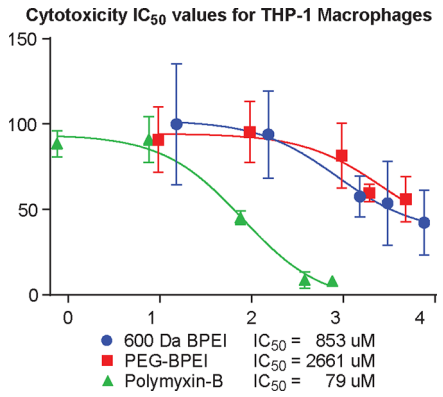

Figure 7. Cytotoxic effects of BPEI, PEG-BPEI, and polymyxin B on THP-1 human monocytes as determined by a cell viability inhibition assay.

work with HeLa cells, kidney cells, and colon cells,[23,26,29,34] the findings from the resazurin assay indicated that BPEI had minimal impact on cell viability, and likewise, the treatment with PEG-BPEI also exhibited negligible cytotoxicity effects (Figure 7). The IC_{50} was determined for BPEI and PEG-BPEI, resulting in values of 853.3 μM (340 μg/mL) and 2661 μM (833 μg/mL), respectively. These results show that the BPEI and PEG-BPEI do not cause any appreciable cytotoxicity against eukaryotic cells until they reach a threshold of 340 μg/mL for BPEI and 833 μg/mL for PEG-BPEI, which are much higher than the concentrations that used in TNF-α neutralization experiments in this study (Figures 3−5).

DISCUSSION

From a clinical perspective, chronic wounds are characterized by a disruption in the typical sequence of healing phases.[39] Instead, they become trapped in a state of pathological inflammation, persistent infections, and necrosis and exhibit resistance to the normal repair process. Because of the ongoing inflammation in chronic wounds, the clinical management for these wounds continues to present a challenge[40] and current technologies do not have a specific focus on the treatment of chronic nonhealing wounds.[41] The FDA acknowledges that there is a lack of available innovative products for treating nonhealing chronic wounds and emphasizes that it is crucial to overcome barriers to product development in this area.[41] To date, the FDA has only approved one product called becaplermin gel, along with two moderately effective cell-based therapies, Dermagraft and Apligraf (developed by Organogenesis, Canton, MA), for the treatment of chronic nonhealing wounds. Furthermore, there is currently no small-molecule drug approved by the FDA specifically for treating nonhealing chronic wounds.[42−45]

Considering the substantial impact of the inflammatory phase on the progression of chronic wound healing,[46−48] the extracellular and intracellular components of the inflammatory response are attractive therapeutic targets for novel immunomodulation approaches.[49] These approaches hold the potential for counteracting dysregulation of the immune response that is often characterized by abnormal tissue remodeling, such as chronic inflammatory disorders, healing disorders, and even cancer.[50] *S. aureus* is a Gram-positive bacterium that causes serious infections in skin and soft tissues.[11−13] It can also initiate a large variety of proinflammatory cytokines in wounds by its immunostimulatory pathogen-associated molecular pattern components, LTA and PGN.[14] The PAMP molecules bind to TLR2 in monocyte cells to induce activation of nuclear factor kappa B, a transcription factor involved in TNF-α, IL-1, IL-6, IL-8, and IL-10 cytokine generation.[10,51] Therefore, as expected, the treatment of THP-1 cells with LTA or PGN resulted in the induction of TNF-α cytokine production, with LTA showing a more pronounced effect (Figures 3 and 4).

A recent study demonstrated that heat-killed whole-cell preparations derived from *S. aureus* can activate the transcription factor NF-kB in a TLR2 and CD14-dependent manner.[36] In Figure 2C, we confirmed this finding by demonstrating that whole *S. aureus* bacteria induce the production of TNF-α cytokines in THP-1 cells expressing TLR2. These results align with previous reports highlighting the synergistic effects of LTA and PGN in enhancing the generation of proinflammatory agonists.[10] The inhibition of TLR signaling pathways has been identified as a promising therapeutic approach to attenuate undesirable chronic inflammatory loops associated with various diseases such as nonhealing wounds.[39,52] The ongoing clinical development of TLR blockade strategies encompasses several approaches[39,53] such as comprehensive inhibition of TLR activity through the utilization of neutralizing antibodies, soluble TLR extracellular domains (ECDs), natural antagonists, and small molecule inhibitors;[49] targeting downstream signaling pathways that are activated as a result of TLR stimulation by using small molecules and microRNA inhibitors that hinder MyD88/TRAF/IRAK complex formation, MAPK, or IKK activity; and employing PAMP antagonists, including LTA inhibitors such as antibodies, phospholipids, cationic immunoglobulins, and cationic peptides.[10,39,54] Some of these compounds have reached phase II clinical trials, and the results are currently awaited, while others, particularly those targeting common signaling pathways such as MAPK, have proved to be of limited efficacy.[53,55] Regarding the LTA inhibitors, there are

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currently no established clinical guidelines for employing proven strategies to neutralize LTA activity in vivo. It is advised to avoid the exclusive use of antibiotics, such as beta-lactams, that trigger bacteriolysis and induce the release of LTA.[10,56,57]

The currently available therapeutic adjuvants for the treatment of inflammatory disorders include nonsteroidal anti-inflammatory drugs and anti-TNF-α and anti-IL-1β antibodies such as infliximab, alemtuzumab, adalimumab, itanercept, anakinra, etc. However, these are narrow-spectrum macromolecules, which have severe interactions and side effects such as sepsis, increase in upper respiratory disorders, reactivation of latent tuberculosis, and lupus-like syndrome.[7,58] On the other hand, an effective treatment for chronic and nonhealing wounds should encompass not only the eradication of bacterial infections and existing biofilms but also the establishment of a favorable wound microenvironment for wound healing by mitigating inflammation and oxidative stress. This involves a universal therapeutic that can address factors that impede wound healing.[40,59,60] Previous studies in our lab have utilized laser scanning confocal microscopy images to illustrate the attachment of BPEI to teichoic acids within the cell wall of methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria.[34] According to the microscopy data, BPEI was located within the cell wall region of the bacteria, indicating a potential interaction between BPEI and the major components of the Gram-positive cell wall, teichoic acids. The interaction was similarly detected through nuclear magnetic resonance (NMR) investigations involving combined solutions of BPEI and wall teichoic acids (WTA) extracted from Gram-positive *Bacillus subtilis* bacteria, as compared to NMR spectra of teichoic acid alone. The NMR data indicated a phosphate:-amine binding resulting from the WTA:BPEI interactions, likely facilitated by the electrostatic attraction between the abundant cationic primary amines of BPEI and the anionic phosphate groups of WTA.[34]

In general, teichoic acids encompass two types: lipoteichoic acids (LTAs), which are secured in the bacterial membrane through a glycolipid anchor, and WTAs, which are covalently linked to peptidoglycan. These integral cell wall constituents consist of an anionic chain comprising repeating units of polyglycerolphosphate or polyribitolphosphate. As a result, the Gram-positive envelope acquires a significantly negative surface net charge.[61] Taking this into account, this research focused on harnessing the polycationic characteristics of BPEI and PEG-BPEI, aiming to establish robust electrostatic interactions with the polyanionic phosphate groups found in lipoteichoic acid molecules (LTA). We suggest the same mechanism of interaction involving the primary amines of BPEI with the phosphate groups of LTA, which aligns with the findings of the earlier study.[34] As shown in Figure 3, both BPEI and its PEGylated derivative demonstrated the ability to neutralize LTA molecules and alleviate the production of TNF-α cytokines. Our rationale is that the cationic polymers can modify the characteristics of Gram-positive cell wall components by modifying the molecular structure and/or introducing steric bulk through the branched polymer. Such interference can impede the attachment of LTA to TLR receptors, block the signal transduction through TLR, and consequently prevent the initiation of TNF-α cytokine production (Figure 8). Additionally, we examined the impact of BPEI and PEG-BPEI binding to another constituent of the Gram-positive cell wall, peptidoglycan, on the generation of an

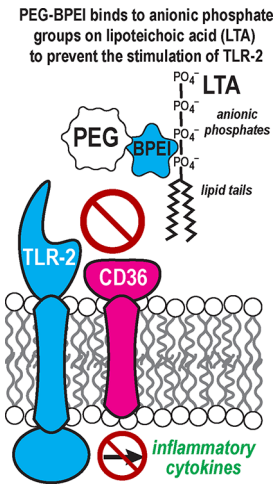

Figure 8. Proposed mechanism of action of LTA and PEG-BPEI interactions. LTA molecules activate TLR2 receptor and induce the downstream signaling cascade that produces inflammatory cytokines. Cationic PEG-BPEI binds with LTA through electrostatic interactions and prevents it from activating TLR2 receptor. This eventually results in reduced levels of cytokine production.

inflammatory response. Nevertheless, as depicted in Figure 4, peptidoglycan activation of TLR signaling was only partially neutralized by the polyethylenimines. This partial neutralization can be attributed to the structural composition of peptidoglycan, which contains fewer ionic groups in comparison to teichoic acids. Consequently, there was an insufficient formation of electrostatic interactions between the cationic components of BPEI and PEG-BPEI polymers and the anionic acidic groups of peptidoglycan, hindering the effective neutralization of the molecule.[62]

Moreover, our findings demonstrate that heat-killed whole *S. aureus* bacteria (HKSA) elicit a more pronounced inflammatory response in THP-1 cells compared to LTA and PGN individually, as shown in Figure 5. This can be attributed to the presence of both LTA and PGN in the cell wall of *S. aureus* bacteria, which could have a greater effect in inducing TNF-α production. It is intriguing to note that combining HKSA with either BPEI or PEG-BPEI resulted in a substantial reduction of the immune response in THP-1 cells (Figure 5A,B). The neutralization of intact bacterial cells in wound environments holds great significance, as it may facilitate the creation of a conducive healing environment. This neutralization process may help to minimize inflammation, foster tissue regeneration, inhibit biofilm formation, and reduce the potential for secondary infections. Typically, wounds are exposed to a mixture of pathogenic bacteria and PAMPs. Hence, the development of a therapeutic agent capable of targeting all invading components that can cause wound infection and trigger immune responses becomes imperative. Likewise, intact bacterial cells inhibit tissue regeneration as they release toxins within wounds that can damage tissue cells and hinder their proliferation and migration capacities.[63,64] Therefore, killing these cells is also important. An added benefit to killing bacteria is reducing biofilm formation, which impedes the healing process and enhances bacterial resistance against antibiotics and immune defenses.[33]

Lastly, we assessed the impact of polymyxin B, an FDA-approved cationic antibiotic known to possess certain anti-inflammatory properties. Polymyxin B, a cationic polypeptide,

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contains five primary amine groups that, at physiological pH, are protonated and bear a positive charge. This charge enables the antibiotic to bind effectively to the negatively charged endotoxin components of the Gram-negative bacterial cell wall. The inhibitory effects of polymyxin B on the release of inflammatory mediators, such as TNF-α and interleukin-6, have been observed in previous studies.[38,65] In this study, while polymyxin B demonstrated a notable decrease in TNF-α cytokine release induced by LTA (Figure 3C), its impact on TLR activation triggered by PGN and HKSA was not substantial (Figures 4C and 5C). Studies to evaluate the anti-PAMP properties of polymyxin B against Gram-negative bacterial PAMPs are underway. The cytotoxic effects of polymyxin B are significant, and it is categorized as a drug of last resort.[66−69] Our study further confirmed this fact by demonstrating the pronounced cytotoxic effect of polymyxin B on human monocyte cells, while simultaneously observing negligible cytotoxicity in the cells treated with low-molecular-weight BPEI and PEG-BPEI, as illustrated in Figure 7.

BPEI, along with the safer derivative PEG-BPEI, exhibit several beneficial properties[23−35] These compounds have shown the potential to enhance the uptake of drugs, reduce barriers to drug influx, and counteract antibiotic resistance mechanisms. Moreover, BPEI and PEG-BPEI potentiators possess an additional advantage in their ability to disrupt biofilms formed by *Staphylococci*, *P. aeruginosa*, and carbapenem-resistant *Enterobacteriaceae* bacteria expressing KPC and NDM-1.[23−35]

CONCLUSIONS

In this study, we present compelling evidence demonstrating the capacity of 600 Da BPEI and PEG-BPEI to effectively mitigate the production of inflammatory responses in human THP-1 monocyte cells, while exhibiting minimal cytotoxicity. Our findings indicate that both BPEI and PEG-BPEI possess anti-inflammatory properties against the Gram-positive cell wall components and intact *S. aureus* bacteria. While PBEI can reduce cytokine response at all concentrations studied, it appears to be less effective at higher concentrations for reasons that are not fully understood. The polycationic nature of BPEI and the polyanionic nature of LTA may result in oligomeric complexes composed of multiple BPEI and LTA molecules. In this scenario, the alkyl tails could have greater exposure to the MD2 and LPB factors, leading to increased signaling. This scenario would be harder to create with PEG-BPEI because the PEG groups would provide steric barriers to the formation of oligomeric complexes. Thus, PEG-BPEI is likely superior to BPEI. Nevertheless, based on these outcomes, we envision BPEI and PEG-BPEI as versatile topical agents for application in acute and chronic wounds. Not only do these agents actively prevent the release of cytokines, but they also disrupt antibiotic resistance mechanisms and interfere with the biofilm matrix. This multifaceted approach holds the potential to speed up the wound healing process. We envision BPEI and PEG-BPEI as broad-spectrum multipurpose agents against inflammatory PAMPs, biofilms, pathogenic bacteria, and antibiotic resistance mechanisms. The modalities are likely to find the most benefit toward novel strategies to treat nonhealing chronic wounds and pressure ulcers.

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Notes

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