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ORIGINAL RESEARCH

IRF3 Promotes Production of IL-6 and Nitric Oxide but Represses CCL22 in RAW264.7 Macrophage Cells Exposed to Lipopolysaccharides in Culture

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Introduction: Macrophage responses to lipopolysaccharides (LPS) drive inflammatory diseases, such as periodontitis, with production of IL-6 and Nitric Oxide (NO). However, anti-inflammatory macrophages counter inflammation with the production of CCL22. Interferon regulatory factor 3 (IRF3) plays a significant role in expression of both IL-6 and NO during macrophage responses through Interferon-stimulated Response Elements (ISREs) of promoters.

Methods: To determine the role of IRF3 in LPS-induced pro- and anti-inflammatory macrophage responses, we used the macrophage cell line RAW264.7 modified with an ISRE promoter driving secreted luciferase (RAW264.7-Lucia) to assess IRF3 activity in response to *Escherichia coli* and *Porphyromonas gingivalis* LPS. For comparison, responses to poly I:C and IFN-gamma and responses from RAW264.7 cells deficient in IRF3 were also assessed.

Results: Herein, LPS of *P. gingivalis*, significantly enhanced production of IL-6 and NO that was induced by *E. coli* LPS but significantly decreased poly I:C-induced ISRE promoter activity. Moreover, IRF3 deficiency depressed the LPS-induced ISRE promoter activity and NO production but increased IL-6 and CCL22 in response to LPS. Restoration of IRF3 expression in IRF3KO RAW cells increased IL-6, restored NO, and decreased CCL22 production in response to LPS of *E. coli*.

Discussion: Therefore, IRF3 is critical to the expression of pro- and anti-inflammatory factors produced by macrophages responding to LPS and could be a target during periodontitis treatment.

Keywords: IRF3, lipopolysaccharide, macrophages, RAW264.7 cells, cytokines

Introduction

Periodontitis is a chronic inflammation at the gingiva that often leads to alveolar bone resorption and tooth loss. Epidemiological estimates indicate that a majority of the US population will develop some form of periodontitis within their lifetime.[1] This is an important health issue because chronic periodontitis is also associated with certain systemic diseases such as cardiovascular disease,[2] rheumatoid arthritis,[3] and even early Alzheimer's disease.[4] Thus, understanding the immunological components of periodontitis is vital to prevent tooth loss and several systemic diseases.

Numerous reports have indicated that inflammatory macrophages infiltrate the gingiva and drive chronic periodontitis.[5,6] Intriguingly, macrophages are also components of each systemic disease linked to periodontitis.[7] Therefore, evaluation of macrophage responses to periodontal pathogens could suggest treatments for chronic periodontitis, as well as related systemic diseases.

The lipopolysaccharide of *P. gingivalis* (Pg-LPS) is a virulence factor that contributes significantly to periodontitis because it stimulates production of inflammatory cytokines from macrophages.[8] This is not surprising since LPS of gram-negative bacteria, such as *E. coli* (Ec-LPS), are prototypical agonists of macrophage Toll-like receptor 4 (TLR4) Pattern Recognition Receptors.[9] However, compared with prototypical Ec-LPS, which contains bis-phosphorylated Lipid A, Pg-LPS is atypical with four different patterns of Lipid A phosphorylation.[8] In contrast, other Gram negative periopathogens, such as

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Fusobacterium nucleatum, produce bis-phosphorylated Lipid A similar to Ec-LPS.[10] In addition, Pg-LPS prepared in the conventional manner, as opposed to ultra-purified Pg-LPS, was shown to also stimulate macrophage through TLR2 pathways.[11] This atypical response to Pg-LPS is likely due to contamination with other outer membrane components of *P. gingivalis*.[12] Therefore, evaluating both conventional and ultra-purified Pg-LPS during stimulation of macrophages should be provide a more accurate assessment of its role in inflammation.

While it is clear that macrophage responses to periopathogens contribute to periodontitis, macrophages themselves exhibit plasticity in their phenotypes.[13] The basic mature macrophage (M0) differentiates in response to LPS plus IFN-γ towards a pro-inflammatory M1 phenotype, producing higher levels of IL-6 and reactive oxygen species, including NO.[14] In contrast, M0 macrophages differentiate in response to IL-4 (plus IL-13 in humans) towards an M2 anti-inflammatory phenotype for tissue repair with significantly less anti-microbial capability.[15] Moreover, M2 macrophages produce chemokines such as CCL22[16] that stimulate CD4 Treg cell entry into inflamed tissue.[17] There is evidence that gingival tissue from periodontitis patients is enriched in pro-inflammatory M1 macrophages.[18] Moreover, resolution of periodontal inflammation is associated with increased M2 markers, including CCL22.[19] Using the RAW264.7 macrophage cell line, Lam et al[20] indicates that whole-cell *P. gingivalis* stimulates macrophages differentiation towards the M1 more than the M2 phenotype. Therefore, understanding the cell-signaling components of macrophage responses to *P. gingivalis* LPS is critical to developing effective treatments for periodontitis.

One of the outcomes of the MyD88-independent TLR4 signaling pathway is activation of the transcription factor IRF3,[21] which ultimately controls many downstream genes during macrophage responses. Interestingly, several reports identify IRF3 as a significant factor in inflammatory cytokine production[22] and several inflammatory diseases,[23] including periodontitis.[24] Therefore, pharmaceutical targeting of IRF3 activation may provide an approach to control chronic inflammatory diseases, such as periodontitis. The RAW264.7 macrophage cell line has been used in periodontitis research to understand macrophage responses to periopathogens[25] and has been used by our group to understand the activation of IRF3 during the response of macrophages to viruses and TLR agonists, such as poly I:C.[26] Therefore, the response of RAW264.7 cells provides a rational approach to assess IRF3 activity during periodontitis. The aim here was to use the same approach to assess the role for IRF3 during the response of macrophages to Pg-LPS and Ec-LPS to determine if pharmaceutical control of macrophage IRF3 activity could treat periodontitis driven by LPS stimulation of macrophage pro-inflammatory responses. Inability to modulate inflammatory cytokines in the presence or absence of IRF3 would suggest that pharmaceuticals that target IRF3 would not be effective in treating periodontitis.

Materials and Methods

Cell Lines and Reagents

RAW264.7-Lucia and RAW264.7-IRF3KO-Lucia cell lines (Invivogen, San Diego, CA) were incubated at 37 °C in 5% CO_2 in DMEM cell culture media with 10% FBS and 50 µg/mL gentamycin, as previously reported.[27] IRF3 deficiency in the RAW264.7-IRF3KO-Lucia cell line is confirmed by Invivogen with an IRF3 Western blot. Ultrapure LPS from *E. coli* O55:B5, standard LPS from *P. gingivalis*, and ultrapure LPS from *P gingivalis* were obtained from Invivogen. Poly I:C was obtained from Invivogen. We obtained an IRF3 expression vector from Genecopia (Ex-Mm7218-M56).

ISRE Promoter Activity

To evaluate ISRE-IRF3 promoter reporter activity in RAW-Lucia and IRF3KO RAW-Lucia, cells were seeded into 24-well cell culture plates at 2×10^5/well in 0.5 mL of complete DMEM and incubated overnight at 5% CO_2 and 37 °C. Cells were then stimulated with Ec-LPS (100 ng/mL or 500 ng/mL), Pg-LPS (100 ng/mL or 500 ng/mL), ultrapure Pg-LPS (10 µg/mL), poly I:C (10 µg/mL), IFN- γ (50 ng/mL), or poly I:C plus IFN-γ. Preliminary experiments with several concentrations of LPS showed that 500 ng/mL of Ec-LPS and Pg-LPS was the optimum concentration to stimulate cytokine production from RAW-Lucia cells within 24h. After 24 h, 10 ul of supernatant from stimulated cells was mixed with 50 µL of QuantiLuc (Invivogen), and luminescence was measured with a Turner Biosystems Luminometer TD20/20. Supernatants were also used for Nitric Oxide assays and cytokine ELISAs.

Nitric Oxide Assay

For NO measurements, the Griess Assay kit of Invitrogen was used. Briefly, 150 µL of 24 h supernatant was mixed with 20 µL of Griess reagent plus 130 µL of deionized water. Using an ELISA spectrophotometric plate reader, absorbencies at 548 nm were used to determine the concentration of NO using a standard curve generated with several concentrations of nitrite.

Cytokine ELISAs

IL-6 concentrations in supernatants were determined using the mouse IL-6 ELISA kit obtained from ThermoFisher and CCL22 concentrations in supernatants using the mouse CCL22 ELISA kit of Peprotech (Cranbury, NJ). Briefly, anti-mouse IL-6 or anti-mouse CCL22 capture antibody in coating buffer was applied to 96-well ELISA plates, sealed, and incubated at 4 °C overnight. After removing capture-antibody and washing with PBS/0.05% Tween 20, 10% FBS blocking agent was applied for 1 h at RT. After washing with PBS/Tween, supernatants from stimulated cells or dilutions of cytokine standards were applied and incubated for 2 h at RT. After 3 washes, biotinylated anti-IL-6 or anti-CCL22 was applied for 1 h at RT. Following 3 washes, avidin peroxidase was added for 30 min. Plates were then washed again, TMB substrate was then added, followed by the addition of acidified stopping reagent. Measurement of ODs at 450 nm with OD 570 nm reference was made with an ELISA spectrophotometric plate reader, after which concentrations of cytokine per mL of individual supernatants were determined using the standard curve as reference.

Transfections

To restore WT-IRF3 in IRF3KO RAW Lucia cells, 2×10^5 cells in complete DMEM were incubated overnight in 24-well plates. Cells were then transfected with 0.5 µg pEGFP (empty vector) or 0.5 µg WT pIRF3 (Genecopia) using Lipofectamine 3000 (ThermoFisher). After 24 h, transfected cells were stimulated with the LPS preparations, rIFN- γ, or poly I:C as described above. Supernatants were assessed for ISRE-promoter activity (secreted Luciferase), NO, IL-6, and CCL22 as described above.

Statistical Analysis

All experiments in 24-well plates were done once and then repeated at least once to achieve sufficient statistical power (n=8). Cytokine and NO production from at least two experiments (n=8) were analyzed using Student's *t*-test to determine the significance of differences between means; p-values of ≤0.05 were considered significant.

Results

IRF3 Promotes Nitric Oxide Production and Represses CCL22 Production During Macrophage Responses to Pg or Ec LPS

The results indicate that like the response to poly I:C, the ISRE/ISG54 promoter response to Ec-LPS is highly dependent on IRF3 (Figure 1A). The ISRE/ISG54 promoter response to cPg-LPS, which was near the promoter activity in unstimulated RAW264.7-Lucia cells, was significantly lower than that for Ec-LPS. Moreover, the minimal response to cPg-LPS did not appear to be dependent on IRF3. Induction of IL-6 was much higher with Ec-LPS (Figure 1C) compared with cPg-LPS (Figure 1B). As previously reported,[26] production of IL-6 in response to poly I:C is also dependent on IRF3 (Figure 1C). However, production of IL-6 in response to both Ec and cPg LPS was significantly elevated in IRF3KO RAW Lucia cells, suggesting that IRF3 is involved in regulating IL-6 expression in response to LPS. Moreover, like our previous report,[28] NO production in response to poly I:C is also driven by IRF3 activity (Figure 1D). Production of NO, which was much higher in response to Ec-LPS than cPg-LPS, was also highly dependent on IRF3 activity. Thus, cPg-LPS and Ec-LPS have different levels of activity in stimulating inflammatory factors associated with periodontitis, some of which are dependent on IRF3 activity. Moreover, it appears that IRF3 activation may dampen inflammatory responses with cytokine production under certain conditions, such as LPS stimulation.

Herein, production of CCL22 in response to Ec-LPS (Figure 1E) was substantially greater than that to Pg-LPS (Figure 1F), while poly I:C failed to induce production of CCL22 (Figure 1E). However, deficiency of IRF3 resulted in a significant increase in CCL22 during the response to either Ec-LPS or Pg-LPS. Overall, these data suggest that IRF3 is a definitive transcription factor stimulating expression of certain M1 factors and repressing expression of certain M2 macrophage factors during the response of macrophages to LPS.

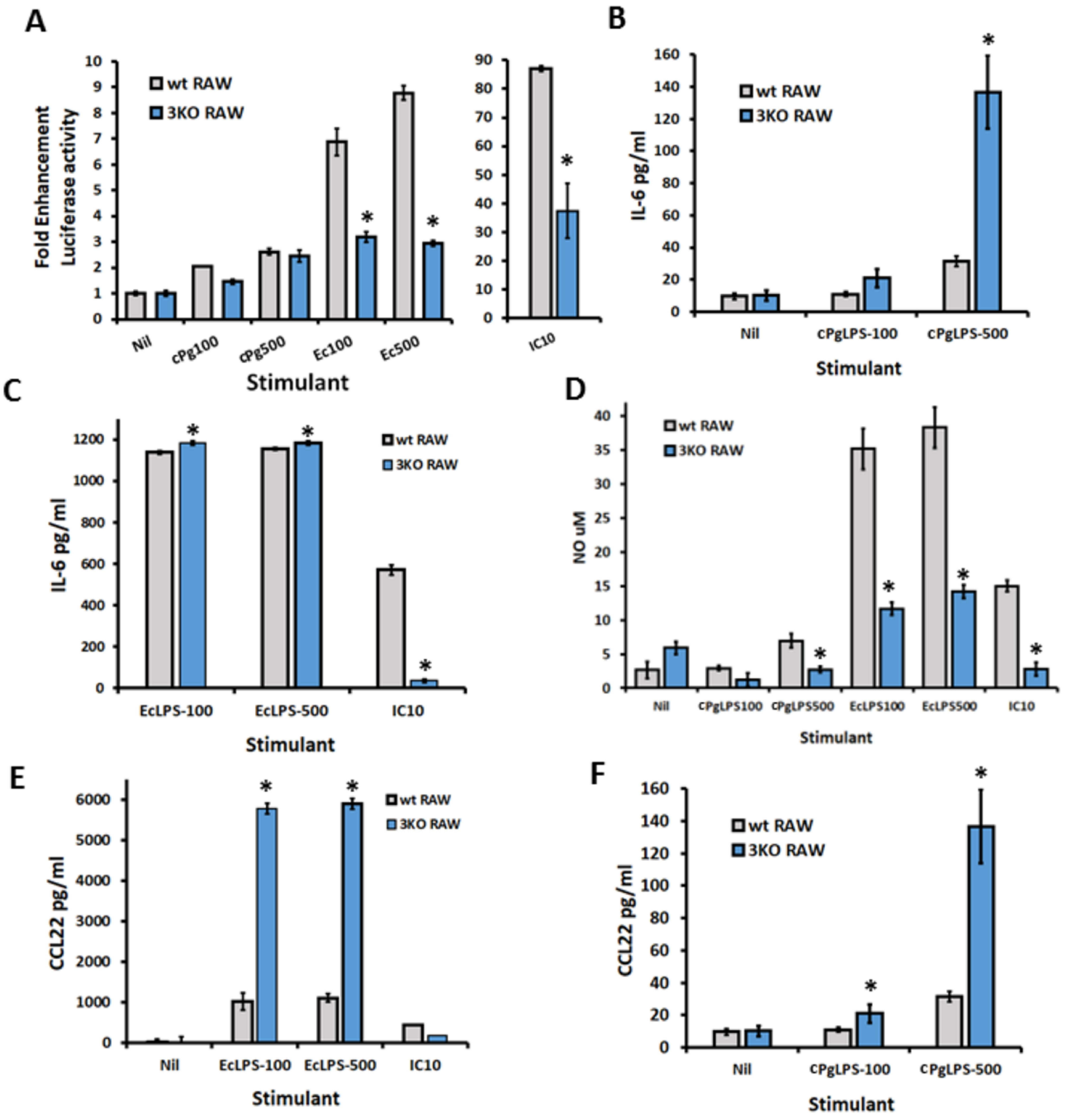

Figure 1 IRF3 deficiency in RAW264.7 cells enhances IL-6 and CCL22 production but decreases ISRE promoter activity and Nitric Oxide (NO) production in response to LPS. RAW-Lucia (wt RAW) or IRF3KO-RAW-Lucia cells (3KO RAW) at 5×10^5 per well incubated overnight were stimulated with 100 ng or 500 ng *E. coli* LPS (Ec-LPS) or *P. gingivalis* LPS (Pg-LPS) or stimulated with 10 μg/mL of poly (I)C (IC10) for 24 h at 37 °C after which secreted luciferase activity (**A**), IL-6 (**B** and **C**), Nitric Oxide (NO) (**D**) and CCL22 (**E** and **F**) production were evaluated. Bars represent mean ± standard error of mean (SEM) of quadruplicate samples from a representative experiment. * indicates significantly different from RAW Lucia response, $p< 0.01$, as determined by Student's *t*-test.

Conventional Pg LPS Preparations Significantly Enhance the Macrophage Nitric Oxide and IL-6 Responses to Ultrapure Pg LPS, Ec LPS and IFN- γ

Addition of cPg-LPS to Ec-LPS or uPg-LPS did not increase the response of the ISRE/ISG54 promoter (Figure 2A) but it significantly diminished poly I:C-induced ISRE/ISG54 promoter activity. In contrast, cPg-LPS significantly increased IL-6 and NO from RAW Lucia cells responding to Ec- or uPg-LPS (Figure 2B and C). Likewise, addition of cPg-LPS

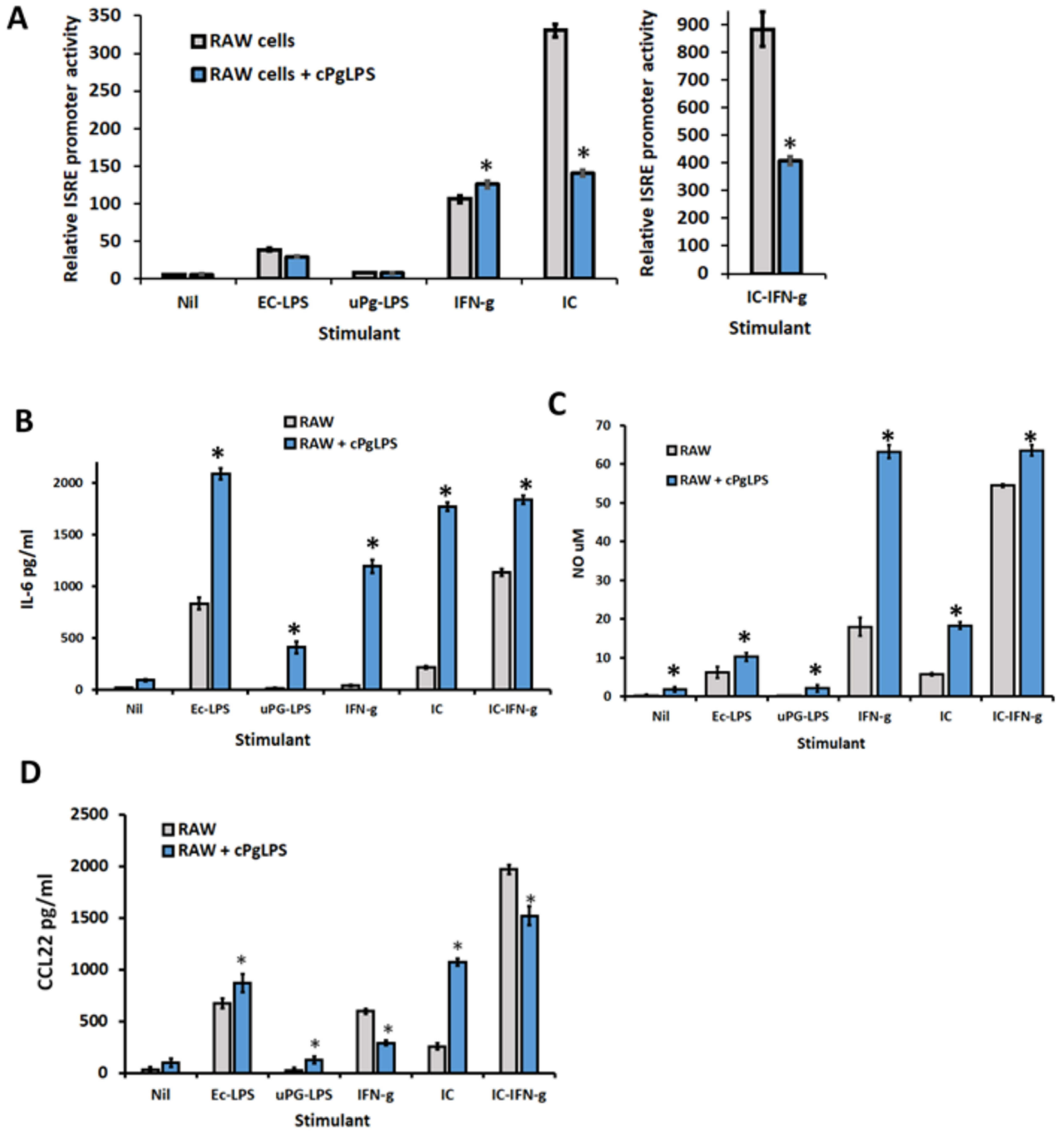

Figure 2 *P. gingivalis* LPS significantly augments inflammatory cytokines from macrophages. RAW-Lucia cells (RAW cells) at 2×10^5 per well were incubated overnight and then stimulated with 500 ng *E. coli* LPS (Ec-LPS), 10 μg/mL ultrapure *P. gingivalis* LPS (uPg-LPS), 10 μg/mL of poly (I)C (IC), 50 ng/mL IFN-γ (IFN-g), or poly (I)C/IFN-γ (IC-IFN-g) in the presence or absence of 5 μg/mL conventional Pg-LPS (cPg-LPS) for 24 h at 37 °C. Then secreted luciferase activity (**A**), IL-6 (**B**), Nitric Oxide (NO) (**C**) and CCL22 (**D**) were evaluated. Bars represent mean ± standard error of mean (SEM) of quadruplicate samples from a representative experiment. * indicates significantly different from RAW Lucia response without cPg-LPS, $p< 0.01$, as determined by Student's *t*-test.

slightly but significantly increased production of CCL22 in response to Ec- or uPg-LPS (Figure 2D). However, cPg-LPS significantly increased poly I:C-induced CCL22 (Figure 2D). Therefore, the lipoprotein components of conventionally prepared Pg-LPS (cPg-LPS) can contribute significantly to inflammatory factors associated with periodontitis.

Addition of cPg-LPS to IFN-γ slightly but significantly increased ISRE/ISG54 promoter activity compared with IFN-γ alone. However, cPg-LPS significantly diminished poly I:C/IFN-γ-induced promoter activity (Figure 2A). In contrast,

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cPg-LPS significantly enhanced IFN-γ-, poly I:C- and poly I:C/IFNγ-induced IL-6 (Figure 2B) and NO (Figure 2C) production. However, cPg-LPS significantly decreased CCL22 production (Figure 2D) from RAW264.7 Lucia cells stimulated with IFN-γ or poly I:C/IFN-γ. Therefore, cPg-LPS influences macrophage production of M1 factors made in response to IFN- γ in an opposite manner compared with M2 factors (CCL22) made in response to IFN-γ.

Because the response of macrophages to LPS through TLR4 is dependent upon activation of IRF3, we evaluated responses to Ec-LPS and cPg-LPS in the absence of IRF3 by using IRF3KO RAW Lucia cells. The data here confirm that the response of the ISRE/ISG54 promoter to various LPSs or poly I:C is totally dependent on IRF3 (Figure 3A). However, IFN-γ significantly stimulated ISRE/ISG54 promoter activity in the absence of IRF3, demonstrating that the ISRE-response to IFN-γ is not dependent on IRF3. Interestingly in the absence of IRF3, cPg-LPS significantly decreased the response of this promoter to IFN-γ. In contrast, cPg-LPS significantly increased IL-6 production in response to Ec-LPS or uPg-LPS, IFN-γ and poly I:C from IRF3KO RAW Lucia cells (Figure 3B). Similarly, cPg-LPS increased IFN-induced production of NO from IRF3KO RAW Lucia cells (Figure 3C). In the absence of IRF3, production of CCL22 in response to Ec-LPS, uPg-LPS, or poly I:C with cPg-LPS was increased (Figure 3D), but cPg-LPS with IFN-γ diminished CCL22 production. Therefore, cPg-LPS significantly upregulates the M1 factors, IL-6 and NO, during the responses to Ec-LPS, poly I:C, and/or IFN-γ.

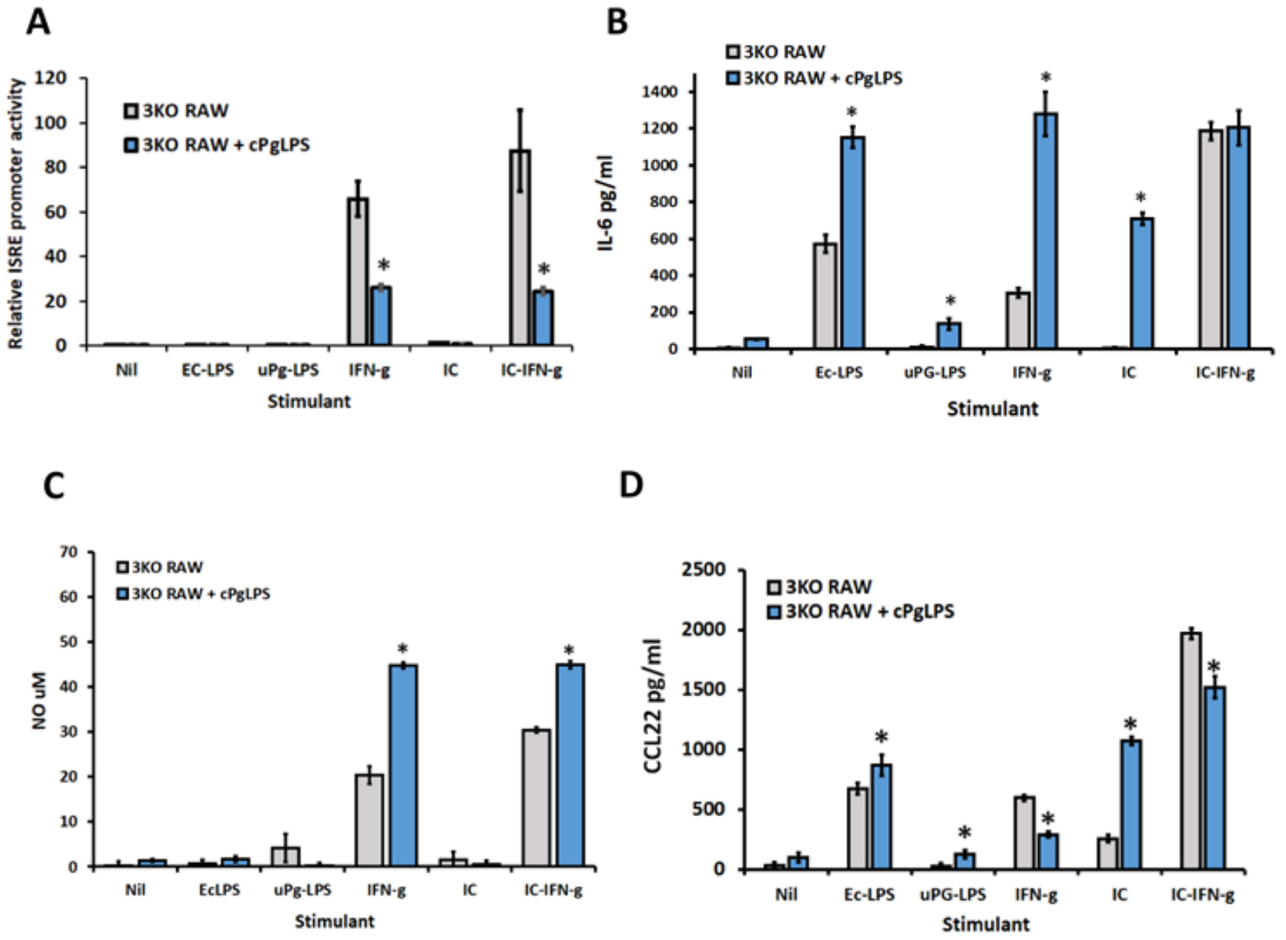

Figure 3 *P. gingivalis* LPS significantly augments inflammatory cytokine responses from macrophages with IRF3 deficiency. IRF3KO-RAW-Lucia cells (3KO RAW) at 2×10^5 per well were incubated overnight and then stimulated with 500 ng *E. coli* LPS (Ec-LPS), 10 μg/mL ultrapure *P. gingivalis* LPS (uPg-LPS), 10 μg/mL of poly (I)C (IC), 50 ng/mL IFN-γ (IFN-g), or poly (I)C/IFN-γ (IC- IFN-g) in the presence or absence of 5 μg/mL conventional Pg-LPS (cPg-LPS) for 24 h at 37 °C. Then Relative ISRE promoter activity (secreted luciferase activity) (**A**), IL-6 (**B**), Nitric Oxide (NO) (**C**) and CCL22 (**D**) were evaluated. Bars represent mean ± standard error of mean (SEM) of quadruplicate samples from a representative experiment. * indicates significantly different from IRF3KO-RAW Lucia response without cPg-LPS, $p< 0.01$, as determined by Student's *t*-test.

Restoring IRF3 Expression in IRF3KO RAW Lucia Cells, Augments IL-6 and NO Production and Decreases CCL22 Production in Response to LPS

To confirm the role of IRF3 in M1 vs M2 macrophage phenotypes, we transfected an IRF3 expressing plasmid (pIRF3) or an empty pEGFP vector into IRF3KO RAW-Lucia cells prior to stimulation with cPg-LPS, Ec-LPS, polyI:C, IFN-γ, or IFN-γ/poly I:C. As expected, ISRE-ISG54 promoter activity in IRF3KO RAW cells in response to all stimulants was significantly enhanced by transfection of pIRF3 (Figure 4A). Unexpectedly, transfection of pIRF3 significantly enhanced IL-6 production from IRF3KO RAW cells in response to all stimulants (Figure 4B). In contrast, production of NO from IRF3KO RAW cells increased significantly with pIRF3 during the response to Ec-LPS but none of the other stimulants. Interestingly, restoring IRF3 significantly diminished the production of CCL22 in response to Ec-LPS, IFN-γ, and IFN-γ/poly I:C (Figure 4C and D), thereby confirming the role of IRF3 in regulating CCL22 expression seen in Figure 1. These data confirm that IRF3 activity has a duplicitous role in M1 cytokine, IL-6, but clearly regulates a critical M2 factor, CCL22, during the response of macrophages to Ec-LPS or IFN-γ.

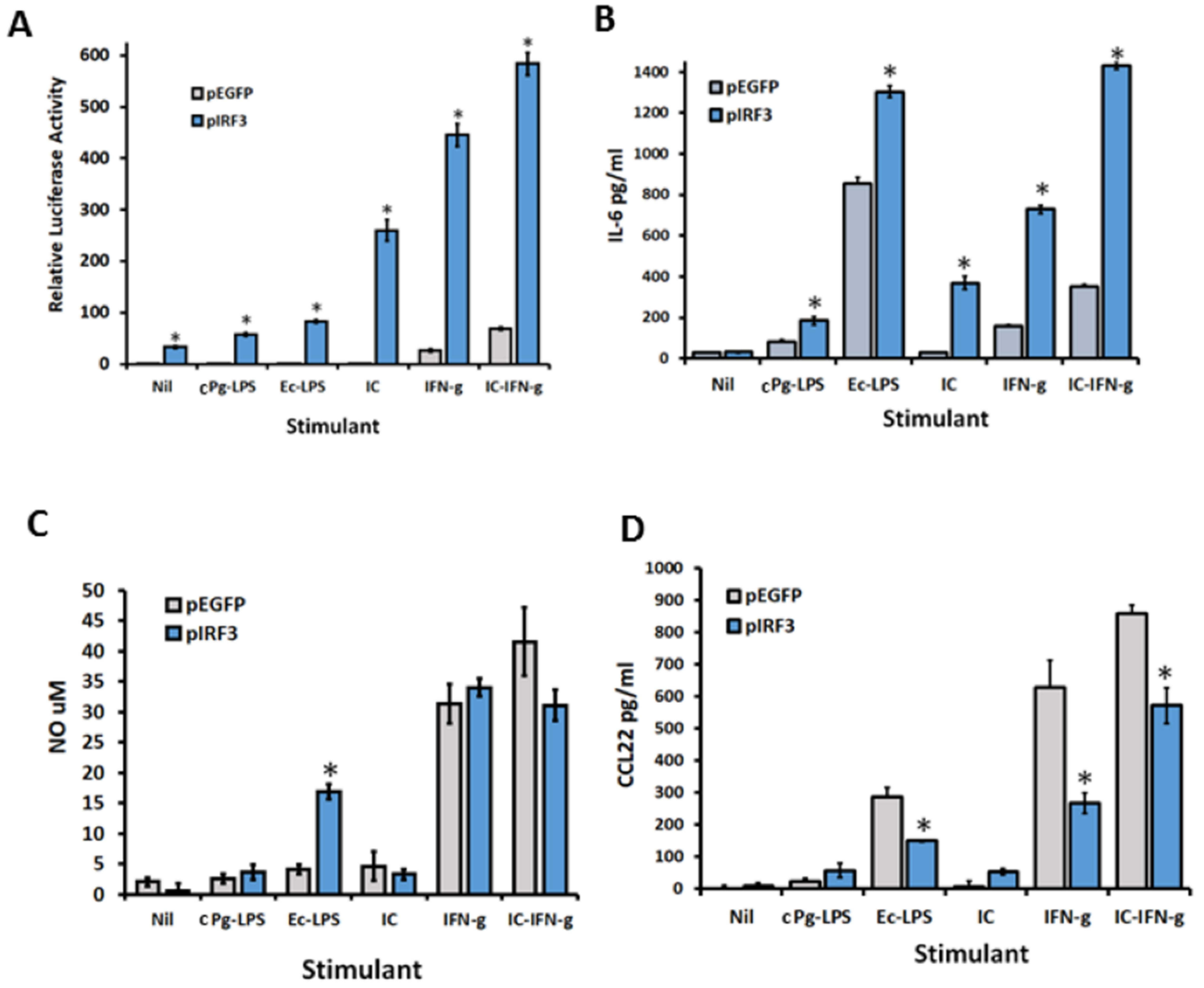

Figure 4 IRF3 augments inflammatory and diminishes anti-inflammatory responses from macrophages. IRF3KO-RAW-Lucia cells at 1×10^5 per well were incubated overnight and then transfected with an empty plasmid (pEGFP) or a mouse IRF3 expressing plasmid (pIRF3). After 24 h, transfected cells were stimulated with 500 ng *E. coli* LPS (Ec-LPS), 5 μg/mL *P. gingivalis* LPS (uPg-LPS), 10 μg/mL of poly (I)C (IC), 50 ng/mL IFN-γ (IFN-g), or poly (I)C/IFN-γ (IC- IFN-g) in the presence or absence of 5 μg/mL conventional Pg-LPS (cPg-LPS) for 24 h at 37 °C. Then Relative ISRE promoter activity (secreted luciferase activity) (**A**), IL-6 (**B**), Nitric Oxide (NO) (**C**) and CCL22 (**D**) were evaluated. Bars represent mean ± standard error of mean (SEM) of quadruplicate samples from a representative experiment. * indicates significantly different from IRF3KO-RAW Lucia response without cPg-LPS, $p< 0.01$, as determined by Student's *t*-test.

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Discussion

The RAW264.7 macrophage cell line is used extensively to examine the response of macrophages to LPS.[29] During these responses, RAW264.7 cells will activate or produce several factors that are involved in periodontitis, such as IRF3,[24] IL-6, NO, and IFIT2 (aka ISG54).[26,30] RAW264.7 Lucia macrophages, derived from the RAW264.7 cell line, were modified to express secreted luciferase under the direction of an ISRE/ISG54 promoter. Moreover, the promoter response of these cells to PRR ligands is a measure of IRF3 activity. A second RAW264.7 Lucia cell line with the IRF3 gene deleted (IRF3KO RAW Lucia) serves as a control. Since *P. gingivalis* and many other periodontal pathogens produce LPS for their outer membrane, these two RAW264.7 Lucia cell lines are ideal to determine the role of IRF3 in the response of macrophages to LPS. The research described here using the RAW264.7 Lucia cell line shows that IRF3 is a pivotal transcription factor promoting production of IL-6 and NO and restraining the production of CCL22 from macrophages in response to LPS from periopathogenic *P. gingivalis*. It has long been suspected that LPS from *P. gingivalis* and other Gram negative periopathogens contribute to chronic inflammation of periodontitis.[8] This suspicion originated from reports showing that injection of LPS into the gingiva of experimental animals stimulates expression of inflammatory cytokines and results in experimental periodontitis.[31] In addition, experimental animal models include one in which multiple injections of *P. gingivalis* LPS results in experimental periodontitis.[32] However, LPS also stimulates other factors such as ISG54[30] and IFN-β[24] from gingival macrophages during periodontitis. Expression of this second set of LPS-induced factors is known to be highly dependent on the LPS activation of IRF3. More recently, evidence points to a particular macrophage differentiated subset termed M1 as the responding subset producing inflammatory cytokines, such as IL-6 and inflammatory factors, such as NO.[13,18] Experimental development of the M1 macrophage subset is usually accomplished by culturing mature macrophages with LPS and IFN-γ, while the alternate macrophage M2 subset develops in response to IL-4 with or without IL-13.[33] However, in vivo development of M1 or M2 can take place with other PAMPs and cytokines.[33] The M2 subset produces much lower levels of inflammatory factors in response to LPS but does produce a chemokine, CCL22, that stimulates migration of anti-inflammatory CD4 Tregs into inflamed tissue[34] that would help to control experimental periodontitis.[35] The conclusion then is that M2 macrophages are involved in dampening inflammation and promoting healing. The results herein suggest that decreased activation of IRF3 results in reduced M1 factors, such as NO, and increased M2 factors, such as CCL22, in response to LPS, thereby suggesting a therapeutic approach to periodontitis treatment.

Many microbial components trigger activation of IRF3 in macrophages leading to inflammatory cytokines and interferons.[36] Microbial Pathogen Associated Molecular Patterns (PAMPs) bound to by cell membrane Pattern Recognition Receptors (PRRs) drive macrophage inflammatory responses. Among the PRRs, TLR4 recognizes bacterial LPS, such as that from *Porphyromonas gingivalis*, while TLR2 recognizes bacterial lipoproteins, also found with *P. gingivalis*.[37] In contrast to other LPS preparations that stimulate macrophage responses through TLR4, conventional preparations of Pg LPS (cPg LPS) were found to stimulate macrophages through both the TLR2 and TLR4 pathways due to outer membrane lipoproteins.[11,37] More recently, ultra-purified (u) Pg LPS (uPg-LPS), devoid of membrane lipoproteins, stimulates only through TLR4. Nevertheless, cPg LPS preparations with lipoproteins are relevant to the response of macrophages. Therefore, the strength of our study is that we mixed cPg-LPS with Ec-LPS or uPg-LPS during stimulations of RAW Lucia cells to gain a more complete understanding of IRF3 in the inflammatory cytokine response of macrophages relevant to periodontitis.

In addition to bacterial periopathogens, several human viruses have been linked to the development of periodontitis, including Hepatitis C Virus (HCV),[38] which is an enveloped RNA virus and thus stimulates macrophages through TLR3[39] recognition of dsRNA. In addition, periodontitis is also associated with IFN-γ at inflamed gingiva.[40] To evaluate these potential interactions, Pg-LPS, Ec-LPS, or poly I:C were mixed with IFN-γ. Our data confirm that IFN-γ synergizes with Pg-LPS, Ec-LPS, or poly I:C during the response of macrophages to produce inflammatory cytokines very likely through pathways that activate IRF3.

TLR3 and certain TLR4 signaling components are part of MyD88-independent, TRIF-dependent signaling pathways that activate downstream TBK1/IKKε for IRF3 hyper-phosphorylation[41] at its Signal Response Domain.[42] Hyper-phosphorylated IRF3 then homo-dimerizes enters the nucleus, associates with CBP/p300, and participates in transcriptional activity for cytokines, type I interferons, and ISGs. We have shown that IRF3 activity is downregulated by phosphorylation at IRF3 S123 that is catalyzed by ERK MAP kinases.[26] In addition, Pin1 isomerase downregulates IRF3

activity by isomerizing proline-333 of murine IRF3 when serine-332 is phosphorylated,[43] which is likely due to ERK MAP kinase activation. Ultimately, Pin1-isomerized IRF3 has decreased activity, thereby preventing interferonopathies or chronic inflammation. The research here suggests that down-regulating IRF3 activity may decrease expression of certain pro-inflammatory and increase expression of anti-inflammatory factors of macrophages. Up to now, there have only been several attempts to control periodontitis pharmacologically with JAK/STAT inhibitors.[44] We postulate, based on the data here and our previous report,[26] that decreased IRF3 activity possibly through phosphorylation of IRF3 S332 and Pin1 isomerization could be an approach to control LPS-induced inflammatory macrophage cytokines. Recently, a very specific Pin1 inhibitor was discovered, Sulfopin, that blocks Pin1 isomerase activity[45] and could be used to verify its role in regulating IRF3 activity. In contrast, IL-33 preserves Pin1 activity.[46] This leads to the possibility that clinical local modulation of IRF3 activity through Pin1 with IL-33 could alter macrophage responses to LPS, thereby dampening IRF3-dependent inflammatory cytokine production during chronic inflammations such as periodontitis.

In addition to macrophage inflammatory responses that destroy pathogens, macrophages also participate in controlling inflammation and promoting repair of tissue following infection.[47] LPS, which is a stimulant for the M1 macrophage phenotype associated with inflammation, is not a stimulant of the M2 macrophage phenotype associated with anti-inflammatory tissue repair responses. Several reports indicate that the gingival tissue of periodontitis patients is enriched in M1 macrophages, which produce IL-6 and NO.[18] In contrast, CCL22 is a chemokine that is a product of M2 macrophages that are involved in recruiting Tregs to infected tissue for immunoregulation.[16,48] Moreover, CCL22 deficiency promotes periodontitis and subsequent alveolar bone loss.[34,35] The results here show that while activation of IRF3 involves some critical pro-inflammatory factors from macrophages it is also involved in down-regulating CCL22 expression. This points to additional outcomes that are likely associated with pharmaceutical dampening of IRF3 activity that diminishes M1 pro-inflammatory macrophage phenotypes but increases certain M2 anti-inflammatory macrophage factors, such as CCL22. Therefore, the null hypothesis that decreasing IRF3 activity during macrophage responses to LPS or poly I:C with or without IFN-γ will have no effect on periodontitis is incorrect. However, one of the limitations of the present study is the use of macrophage cell line, RAW264.7. It is possible that primary macrophages from humans respond differently than RAW264.7 cells to decreased IRF3 activity during the responses to LPS or poly I:C with or without IFN-γ. On the other hand, many investigations have used RAW264.7 cells to model macrophage inflammatory responses. Moreover, because it may be crucial to manipulate IRF3 activity in human macrophages clinically, testing pharmaceuticals to diminish IRF3 activity is expected to be challenging but essential. Therefore, RAW264.7 cells deficient in IRF3 allows for a more consistent first approach to gain information on ways to control IRF3 activity. In conclusion, the key findings of the present investigation indicate that LPS-stimulated activation of IRF3 promotes certain M1 macrophage factors, such as NO, suppresses M2 factors, such as CCL22, but has an ambiguous role in expression of the M1 factor IL-6. These effects due to IRF3 activation with LPS are different than during poly I:C stimulation.

Abbreviations

CCL22, CC motif chemokine ligand 22; IRF3, Interferon Regulatory Factor 3; IFN, Interferon; ISGs, Interferon Stimulated Genes; ISRE, Interferon Stimulated Response Element; IL-6, Interleukin-6; LPS, Lipopolysaccharide; NO, Nitric Oxide.

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Disclosure

The authors declare that they have no known competing interests for the work reported in this paper.

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