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Mol Oral Microbiol. Author manuscript; available in PMC 2023 June 01.

Published in final edited form as:
Mol Oral Microbiol. 2022 June ; 37(3): 109–121. doi:10.1111/omi.12366.

Interleukin-34 permits Porphyromonas gingivalis survival and NF-κB p65 inhibition in macrophages

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Abstract

Interleukin-34 (IL-34) is a cytokine that supports the viability and differentiation of macrophages. An important cytokine for the development of epidermal immunity, IL-34, is present and plays a role in the immunity of the oral environment. IL-34 has been linked to inflammatory periodontal diseases, which involve innate phagocytes, including macrophages. Whether IL-34 can alter the ability of macrophages to effectively interact with oral microbes is currently unclear. Using macrophages derived from human blood monocytes with either the canonical cytokine colony-stimulating factor (CSF)1 or IL-34, we compared the ability of the macrophages to phagocytose, kill, and respond through the production of cytokines to the periodontal keystone pathogen *Porphyromonas gingivalis*. While macrophages derived from both cytokines were able to engulf the bacterium equally, IL-34-derived macrophages were much less capable of killing internalized *P. gingivalis*. Of the macrophage cell surface receptors known to interact with *P. gingivalis*, dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin was found to have the largest variation between IL-34- and CSF1-derived macrophages. We also found that upon interaction with *P. gingivalis*, IL-34-derived macrophages produced significantly less of the neutrophil chemotactic factor IL-8 than macrophages derived in the presence of CSF1. Mechanistically, we identified that the levels of IL-8 corresponded with *P. gingivalis* survival and dephosphorylation of the major transcription factor NF-κB p65. Overall, we found that macrophages differentiated in the presence of IL-34, a dominant cytokine in the oral gingiva, have

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

SUPPORTING INFORMATION
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a reduced ability to kill the keystone pathogen *P. gingivalis* and may be susceptible to specific bacteria-mediated cytokine modification.

Keywords

DC-SIGN; interleukin-34; interleukin-8; periodontal disease; phagocytosis

1 | INTRODUCTION

Tissue-resident macrophages have multiple roles, including non-inflammatory uptake and removal of apoptotic cells during development, remodeling of the cellular matrix during tissue repair, and inflammatory engulfment, digestion and presentation of antigens to adaptive immune cells of foreign substances, cancerous cells, and microbes (Wynn et al., 2013). Although tissue macrophages have many features in common, including extensive lysosomes, stellate morphology, and location relative to epithelia, they are nevertheless extremely heterogeneous in terms of function and surface marker expression. This heterogeneity is due to the diversity of the local cytokines and chemokines within the surrounding microenvironments of various tissues.

During homeostasis as well as inflammation, recruitment of circulating monocytes into tissues can occur where, following acclimatization by local growth factors, cytokines, and microbial products, the monocytes differentiate into macrophages to supplement the local population (Geissmann et al., 2010; Gentek et al., 2014; Wynn et al., 2013). Generally, the survival and differentiation of monocytes into tissue macrophages depends upon signaling through the colony stimulating factor-1 receptor (CSF1R; Geissmann et al., 2010). The canonical cytokine for this receptor is macrophage CSF1, which plays an essential role in the proliferation, differentiation, and survival of monocytes, macrophages, and bone marrow progenitor cells. However, a novel 39 kDa homodimer cytokine designated interleukin-34 (IL-34) has been identified as an additional ligand for CSF1R (Lin et al., 2008). IL-34 is capable of supporting the viability and differentiation of macrophages from monocytes in the absence of CSF1 but appears to elicit some different signaling downstream from CSF1R than CSF1 (Barve et al., 2013; Chihara et al., 2010; Grayfer & Robert, 2015; Liu et al., 2012; Wei et al., 2010). Interestingly, IL-34 is tissue-restricted and is principally required over CSF1 for the development of the brain- and skin-resident microglia and Langerhans cells, respectively (Greter et al., 2012; Y. Wang et al., 2012).

As an important cytokine for the development of epidermal immunity, IL-34 likely also plays a role in the immunity of the oral environment. Indeed, IL-34 is constitutively expressed by gingival fibroblasts and is increased earlier than CSF1 in oral tissue in response to inflammatory conditions (Bostrom & Lundberg, 2013; Ciccia et al., 2013), with results from studies examining IL-34 levels in advanced periodontal disease having mixed levels of IL-34 (Guruprasad & Pradeep, 2018a, 2018b; Martinez et al., 2017). Thus, while cytokines are present in the oral environment, the exact role IL-34 may play in monocyte and macrophage function in gingival tissue under homeostatic conditions or during the initiation of oral inflammatory diseases is unclear. Given recent studies suggesting that IL-34 plays

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a role in other chronic inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease and kidney disease (Baek et al., 2015; Franze et al., 2015; Masteller & Wong, 2014; Zwicker et al., 2015), an understanding of how IL-34 alters the ability of macrophages to effectively interact with oral pathogens will provide insight into the role this cytokine has in defining the ability of the immune system to respond to oral inflammatory conditions.

The oral pathogen we chose to examine was *Porphyromonas gingivalis*, a keystone pathogen that can have a disproportionate effect on a commensal microbial community and promote microbial community dysbiosis (Darveau et al., 2012; Hajishengallis & Lamont, 2014). *Porphyromonas gingivalis* also simultaneously interferes with host immune surveillance while promoting a selective increase in inflammatory responses (Lamont & Hajishengallis, 2015), fostering inflammatory periodontal disease. *Porphyromonas gingivalis* has the capability to survive within a number of cell types, including dendritic cells (DCs) and macrophages (El-Awady et al., 2015; M. Wang et al., 2007) and appears capable of traveling to extraoral sites where it may adversely affect a number of diseases, including atherosclerosis (Beck & Offenbacher, 2005; Beck et al., 1996; Carrion et al., 2012; Hayashi et al., 2011; Rivera et al., 2013; Zeituni et al., 2010).

We found that IL-34 gives rise to macrophages that, while capable of equivalent phagocytic activity toward *P. gingivalis*, had a significantly lower ability to kill the bacteria once they were engulfed. We also found that IL-34-derived macrophages have significantly more DC-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN) expression, a receptor important for *P. gingivalis* survival within DCs (El-Awady et al., 2015). IL-34-differentiated macrophages also released significantly lower levels of the chemokine IL-8 upon interaction with *P. gingivalis* than CSF1-differentiated macrophages, and we found that this lower production of IL-8 in response to *P. gingivalis* correlated with dephosphorylation of NF-κB (Nuclear Factor kappa-light-chain-enhancer of activated B cells) subunit p65, similar to what has been reported in epithelial cells (Takeuchi et al., 2013). Concurrently, we found IL-8 production by IL-34-differentiated macrophages to be more sensitive to inhibition of the NF-κB p65 subunit than CSF1-derived macrophages. Overall, our results indicate that *P. gingivalis* may be primed to take advantage of subtle differences in IL-34-differentiated macrophages present in the gingival environment to enhance the bacterium's ability to subvert the oral immune response.

2 | METHODS

2.1 | Ethics statement

Human peripheral blood was obtained from healthy donors of the University at Buffalo (UB) general population using an Institutional Review Board (IRB)-approved protocol and written consent form in accordance with UB IRB-approved protocols (Protocol 6267142).

2.2 | Generation of peripheral blood monocyte-derived macrophages

Macrophages were generated from monocytes as previously described (Croft et al., 2018). Briefly, peripheral blood was obtained from healthy donors, which was then layered on top of an equal volume of a 1-step polymorphic dextran gradient (Accurate Chemical). The

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samples were spun at 730 RCF for 35 min at room temperature, and the mononuclear cells (monocytes) were isolated from the uppermost band. Macrophages were generated from blood monocytes in six-well culture plates in culture medium (Roswell Park Memorial Institute (RPMI)) 1640 medium [Corning] containing 10% Fetal Bovine Serum (FBS) (Caisson Labs), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin) for 6 days supplemented with 50 ng/ml CSF1 or 50 ng/ml IL-34 (GenScript; Foucher et al., 2013). In all experiments, the two types of macrophages (IL-34- or CSF1-derived) were generated in parallel from monocytes of the same donor, although different donors were used for different replicates.

2.3 | *Porphyromonas gingivalis* internalization assay

Human monocyte-derived macrophages on coverslips in multiwell plates were incubated at 37°C with biotin-labeled *P. gingivalis* (biotin-hydrazide [Thermo Fisher Scientific] as previously described; Croft et al., 2018) at a multiplicity of infection (MOI) of 10:1 for 30 min to allow phagocytosis to occur. The cells were then washed with cold phosphate-buffered saline (PBS) and incubated with Alexa 594-conjugated streptavidin at 4°C for 5 min to label external *P. gingivalis*. The cells were washed again, fixed with 4% paraformaldehyde, permeabilized, and stained with 4'-6-diamidino-2-phenylindole (DAPI). The coverslips were then mounted onto slides and analyzed by fluorescence microscopy (Figure S1). The average number of internalized bacteria (DAPI positive but Alexa 594 negative) per macrophage was recorded by manual counting and reported as the phagocytic index, with a minimum of 100 macrophages examined per condition per experiment.

2.4 | Bacterial growth and macrophage killing assays

Porphyromonas gingivalis (ATCC 33277) was grown anaerobically at 37°C in tryptic soy broth supplemented with menadione (1 μg/ml) and hemin (10 μg/ml). The intracellular *P. gingivalis* clearance capability of macrophages was determined by a modified antibiotic protection-based survival assay as previously reported (Lamont et al., 1995). Briefly, following incubation of *P. gingivalis* with macrophages (at an MOI of 10:1) for 30 min in RPMI without antibiotics, extracellular nonadherent bacteria were removed by extensive washing with PBS. The macrophages were then returned to RPMI with the antibiotic gentamicin (300 μg/ml) to kill external bacteria for 30 min, followed by antibiotic-free replacement media (Croft et al., 2018). After 1 and 2 h, internalized bacteria were released by lysis of macrophages in sterile distilled water. Serial dilutions of the lysates were plated onto blood agar dishes, and colony-forming units of live *P. gingivalis* released from the macrophages were determined after growth for 1 week in anaerobic conditions, giving the number of "surviving bacteria." The phagocytic index (described above) was used to determine the amount of engulfed bacteria by multiplying the number of macrophages per condition by the phagocytic index. The percent survival of *P. gingivalis* was calculated by dividing the number of "surviving bacteria" after 2 h post phagocytosis by the calculated "engulfed bacteria" (i.e., "surviving bacteria"/"engulfed bacteria" × 100). In this way, the percent survival accounts for only survival differences of the bacteria actually internalized by the macrophages.

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2.5 | Flow cytometry

Macrophages were blocked with human Fc Block (BD Biosciences) for 10 min at 4°C in fluorescence-activated cell sorting (FACS) buffer (PBS with 2% bovine serum albumin (BSA)). Cells were subsequently stained at 4°C, washed three times with FACS buffer, fixed with 2% PFA in PBS (containing 2% BSA) for 30 min at 4°C and washed three times. Flow cytometry was performed using a BD Fortessa flow cytometer, and all data were analyzed using FlowJo, version 9.9 or higher. The antibodies used for this study were CD14 (clone M5E2, BD Bioscience), CD282 (Toll-like receptor 2 [TLR2], clone 11G7), CD284 (TLR4, clone HTA125), CD88 (clone D53-1473), and CD209 (DC-SIGN, clone DCN46) along with the appropriate isotype controls. Mean fluorescence intensities were calculated by subtracting the mean fluorescence signal of the isotype control signal from the signal of the antibody label.

2.6 | Cytokine release assay

Human monocyte-derived macrophages (IL-34 or CSF1-differentiated) were incubated with or without *P. gingivalis* (MOI: 10:1) for 24 h, and media samples were taken at 2, 6, and 24 h. Broad-spectrum protease inhibitor cocktail (Pierce) was added to the media supernatant, and enzyme-linked immunosorbent assays (ELISAs) were performed using the commercial ELISA kit Bio-Plex Pro human cytokine 17-plex (Bio-Rad) according to the manufacturer's instructions. The cytokines included in the kit were CSF3 (G-CSF), CSF2 (GM-CSF), interferon (IFN)-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17A, MCP-1, macrophage inflammatory protein (MIP)-1β and tumor necrosis factor alpha (TNFα). The cytokines that were released in significant amounts above the control (medium alone) were calculated and presented.

2.7 | NF-kB p65 functional blocking assay

Human monocyte-derived macrophages (IL-34 or CSF1-differentiated) were preincubated with 100 μM NFkB p65 inhibitor (NBP2-29321, Novus Biologicals) or control peptide (NBP2-29334) overnight. Cells were then stimulated with lipopolysaccharide (LPS) (0.1 μg/ml) (from *Salmonella enterica* serotype Minnesota strain Re595) for 16 h. Cell supernatants were collected, broad-spectrum protease inhibitor cocktail (Pierce) was added to the media supernatant, and ELISA was performed by using a human IL-8/CXCL8 DuoSet ELISA kit (R&D Systems) according to the manufacturer's instructions. The levels of IL8 above the control were calculated and presented.

2.8 | Western blot analysis

Western blot analysis for detecting NF-κB (p65) activation was performed as described (H. -T. Cheng et al., 2009) using total p65 and p65 phospho-Ser536 specific antibodies along with appropriate secondary antibodies (Cell Signaling Technology). Anti-mouse actin was used as an internal control. Briefly, CSF1- and IL-34-differentiated macrophages were stimulated with *P. gingivalis* at an MOI of 100 for 2 h, with LPS used as a positive control. Cells were lysed, clarified by centrifugation, separated by SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with TBST (1X TBS (Tris-buffered saline) and 0.1% Tween 20) containing

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2% skim milk for 1 h at room temperature and were then incubated overnight at 4°C with primary antibodies diluted in TBST. The membranes were washed three times with TBST, incubated for 1 h at room temperature with a 1:2000 dilution of horseradish peroxidase (HRP)-conjugated secondary antibodies in TBST, and washed three times with TBS. Immunoreactive bands were detected using Pierce ECL Western Blotting Substrate (Thermo Scientific). Images were acquired with a Chemi Doc XRS Plus (Bio-Rad) and analyzed with FIJI (Schindelin et al., 2012).

2.9 | Luminol reactive oxygen species (ROS) detection assay

Human monocytes were isolated and differentiated as described above in 24-well tissue culture plates. For luminol ROS detection experiments, cells were preincubated with 250 μg/ml HRP for 15 min to allow for fluid-phase uptake into the endosomal system. The cells were then placed in Hanks balanced salt solution with 250 μg/ml HRP and 50 μM luminol. Cells were stimulated with phorbol myristate acetate (PMA; 100 nm), and luminescence was read on a Synergy temperature-controlled plate reader (Bio-Tek). Readings were taken once per minute, with a 1000 ms integration time, for 90 min at 37°C.

2.10 | Statistical analysis

Data are shown as the mean ± standard error (SEM) and were tested by analysis of variance (ANOVA), paired or unpaired *t*-test, as appropriate (and indicated in the figure legends) using GraphPad Prism. All statistical tests used an $\alpha = 0.05$ to determine significance.

3 | RESULTS

3.1 | IL-34-derived macrophages have reduced *P. gingivalis* killing ability

The differentiation of monocytes to macrophages in the presence of CSF1 results in the maintenance of CD14 expression and an increase in CD163 as does differentiation in the presence of IL-34 (Chihara et al., 2010; Foucher et al., 2013). We initially confirmed that IL-34 was capable of producing macrophages from peripheral blood monocytes as efficiently as CSF1 in our hands by measuring the surface expression of CD14 and CD163 by flow cytometry (Figure 1) and confirmed that the cells maintained CD14 expression and acquired CD163 similarly upon differentiation with both CSF1 and IL-34.

Macrophages are important immune cells during the development of periodontal disease (Ebersole et al., 2017; Lam et al., 2014), and *P. gingivalis* is capable of surviving within macrophages (Hajishengallis et al., 2017; M. Wang et al., 2007); we next examined the ability of *P. gingivalis* to be taken up by and survive within IL-34- and CSF1-differentiated macrophages. Previous reports have indicated that gingipains from *P. gingivalis* can remove CD14 from mouse macrophages and reduce the phagocyte's ability to engulf the bacterium (Wilensky et al., 2015); however, we observed no differences in CD14 in these human macrophages as a result of *P. gingivalis* incubation (Figure S2). Indeed, upon measuring the average number of bacteria phagocytosed, we found that both types of macrophages had a similar ability to internalize *P. gingivalis* (Figure 2a). However, CSF1-differentiated macrophages were significantly better at killing *P. gingivalis* than IL-34-differentiated macrophages after both 60 and 120 min (Figure 2b).

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3.2 | IL-34-differentiated macrophages have reduced ROS production

The active production of ROS within phagosomes is an important mechanism for killing engulfed bacteria (Flannagan et al., 2009), including *P. gingivalis* (Choi et al., 2013). To compare the ROS production capabilities of IL-34- and CSF1-matured macrophages, we performed a luminol ROS detection assay in the presence of the broad activator PMA. We found that IL-34-differentiated macrophages consistently resulted in a lower production of ROS than CSF1-derived macrophages, both during the time course of interaction (Figure 3a), as well as calculated peak ROS production over multiple experiments (Figure 3b). This reduced ROS production aligned with the higher survival of *P. gingivalis* in IL-34-differentiated macrophages (Figure 2).

3.3 | IL-34-derived macrophages express increased DC-SIGN

Porphyromonas gingivalis is capable of interacting with a number of pattern recognition receptors (PRRs) on professional phagocytes to promote its own survival. Specific receptors that have been implicated include CD11b, CD14, C5a receptor (CD88), DC-SIGN (CD209), TLR2 (CD282), and TLR4 (CD284; El-Awady et al., 2015; Hajishengallis et al., 2017; Hajishengallis, Tapping, et al., 2006; Holden et al., 2014; Maekawa et al., 2014; Papadopoulos et al., 2013; M. Wang et al., 2007; Wilensky et al., 2015). While IL-34 and CSF1 maturation of monocytes leads to macrophages with broadly similar phenotypes, there have been some significant differences reported (Barve et al., 2013; Chihara et al., 2010; Foucher et al., 2013), including in interactions with pathogens (Boulakirba et al., 2018; S. T. Cheng et al., 2017; Xu et al., 2015). Therefore, based on the results of the internalization and killing assays (Figure 2), we hypothesized that there may be differences between IL-34- and CSF1-matured macrophages in terms of relevant receptor expression. We then analyzed the cell surface levels of receptors expressed by macrophages that have been directly linked to interactions with *P. gingivalis* by flow cytometry (Figure 4). While we found no significant differences in the expression of the majority of the PRRs tested, two receptors did show differences: TLR4 trended lower while DC-SIGN was significantly increased on IL-34 differentiated macrophages compared to macrophages matured in the presence of CSF1.

3.4 | Production of inflammatory cytokines

Porphyromonas gingivalis induces the production of proinflammatory cytokines by macrophages, including IL-6, TNFα, IL-8, IL-1β, and G-CSF (Bodet et al., 2005; Huang et al., 2016; Papadopoulos et al., 2013; Shapira et al., 1998; Varanat et al., 2017), and while the expression of the anti-inflammatory cytokine IL-10 can be induced, it is at much lower levels (Fleetwood et al., 2017; Foey et al., 2017). As the survival of the bacterium was significantly different between the two macrophage types (Figure 2), we also wanted to determine whether there were cytokine production differences between the macrophages. We incubated *P. gingivalis* with IL-34 or CSF1 macrophages for 24 h at an MOI of 10:1, with samples taken at 2, 6, and 24 h and the levels of cytokines released from the macrophages determined by multiplex ELISA. The majority of the cytokines produced by IL-34 and CSF1-differentiated macrophages were not released at significantly different rates upon incubation with *P. gingivalis*, with the exception of IL-8 (CXCL8; Figure 5). IL-8 was

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released at significantly lower levels after both 6 and 24 h by IL-34-matured macrophages than by CSF1-differentiated macrophages.

3.5 | Dephosphorylation of NF-κB p65

Previous studies have found that the *P. gingivalis* serine phosphatase SerB can inhibit the production of IL-8 by gingival epithelial cells via interference with the phosphorylation of NF-κB p65 (Sasaki et al., 2005; Takeuchi et al., 2013). We hypothesized that p65 might more readily be a target for dephosphorylation by *P. gingivalis* in IL-34 differentiated macrophages as we observed increased bacterial survival in these cells (Figure 2). We, therefore, tested cell lysates of IL-34- and CSF1-derived macrophages for phosphorylated NF-κB p65 by western blotting. Immunoblots (Figure 6) showed that after 2 h of *P. gingivalis* stimulation, the levels of p65 phosphorylation in IL-34-differentiated macrophages were significantly lower than those in CSF1-differentiated macrophages.

Given the increased survivability of *P. gingivalis* we observed in IL-34 macrophages (Figure 2) correlated with the reduced NF-κB p65 phosphorylation and IL-8 production (Figures 6 and 5, respectively), we next wanted to also confirm that IL-8 production requires NF-κB p65 activation equally in both types of macrophages. To this end, we treated IL-34- and CSF1-differentiated macrophages with a p65-specific peptide inhibitor (NBP2-29321). After incubation of the macrophages with the general inflammatory stimulator LPS, we found IL-8 production to be significantly reduced in IL-34-, but not CSF1-, differentiated macrophages in the presence of the p65 inhibitor (Figure 7).

4 | DISCUSSION

Periodontal disease is a common, progressive disease that can disturb the attachment apparatus of the teeth and cause bone loss in the jaw, often resulting in tooth loss (Armitage, 2004; Hajishengallis & Korostoff, 2017). *Porphyromonas gingivalis* is present at greater frequency and at higher levels at sites of inflammation (Bostanci & Belibasakis, 2012; Yang et al., 2004), and while recent evidence indicates that *P. gingivalis* acts by enhancing the dysbiosis of commensal microbes to perpetuate disease (Hajishengallis et al., 2011; Irie et al., 2014), interactions with and inhibition of the immune system are also an important virulence strategy of this keystone pathogen (Hajishengallis et al., 2017; Kramer & Genco, 2017; Maekawa et al., 2014). Macrophages have an important role in periodontal disease, not only for controlling periodontal pathogens such as *P. gingivalis* but also in the pathology of the disease, including the bone loss seen during severe periodontal disease (Lam et al., 2014). The receptor CSF1R is also known to be important in driving gingival inflammation (Clark et al., 2021).

Previous reports have shown that the cytokine IL-34 is capable of differentiating monocytes into macrophages, that IL-34 is expressed by gingival fibroblasts, and that this expression is enhanced by the proinflammatory cytokines TNFα and IL-1β (Bostrom & Lundberg, 2013). Furthermore, IL-34 is the dominant CSF1R ligand during homeostasis in the oral cavity, and while both IL-34 and CSF1 are increased in inflammatory oral tissues, IL-34 is initially dominant (Bostrom & Lundberg, 2013). As all previous studies investigating *P. gingivalis* and macrophage interactions have used macrophages differentiated with CSF1,

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the purpose of this study was to determine whether there are differences in *P. gingivalis* and macrophage interactions that may be important for the development or progression of periodontal disease.

Our initial differentiation results were consistent with earlier publications (Foucher et al., 2013; Wei et al., 2010), and our results of the phagocytic uptake of *P. gingivalis* by both macrophage types are similar to those reported using *Escherichia coli* (Boulakirba et al., 2018), with both macrophages capable of engulfing similar numbers of bacteria.

Surprisingly, however, when we examined the ability of the macrophages to kill the phagocytosed oral bacterium, *P. gingivalis* was capable of surviving significantly better within macrophages derived from monocytes in the presence of IL-34. *Porphyromonas gingivalis* has some defenses against ROS production (Hajishengallis & Lamont, 2014), but it remains a potent inhibitor of the bacterium. Indeed, traditionally activated (M1) macrophages that have increased phagosomal ROS production, compared to resting or naïve macrophages (Canton et al., 2014), have an increased ability to kill internalized *P. gingivalis* (Lam et al., 2016). Our findings add to the knowledge of differential macrophage ROS production and suggest that when interacting with *P. gingivalis*, IL-34-differentiated macrophages are less well equipped than CSF1-derived macrophages to kill *P. gingivalis* via this mechanism.

While the exact mechanisms behind the observed *P. gingivalis* survival within and differential ROS production by IL-34- and CSF1-differentiated macrophages have not been fully elucidated, there are a number of PRRs that activate or inhibit intracellular inflammatory signaling and are known to play a role in allowing for increased *P. gingivalis* survival within professional phagocytes, including CD14, CD11b, TLR2 (CD282), TLR4 (CD284), DC-SIGN (CD209), and C5aR (CD88; El-Awady et al., 2015; Hajishengallis et al., 2017; Hajishengallis et al., 2007; Hajishengallis, Wang et al., 2006; Hou et al., 2017; Jotwani et al., 2010; M. Wang et al., 2010; M. Wang et al., 2007; Zeituni et al., 2009). We observed no differences in the levels of C5aR, CD14, TLR2, TLR4, or CD11b between IL-34- and CSF1-matured macrophages, which is consistent with other reports where the majority of genes and surface marker levels are similar between these two macrophage types (Barve et al., 2013; Bostrom & Lundberg, 2013; Foucher et al., 2013). However, we did see differences in the expression of DC-SIGN (CD209), a C-type lectin receptor that recognizes and binds to pattern-associated molecular patterns commonly found on viruses, bacteria, and fungi (van Kooyk & Geijtenbeek, 2003). The minor fimbriae of *P. gingivalis* have been shown to bind DC-SIGN and result in phagosomes containing *P. gingivalis* obtaining autophagocytic properties, allowing for extended survival of the bacterium within DCs (El-Awady et al., 2015; Zeituni et al., 2009; Zeituni et al., 2010). Increased DC-SIGN on the surface of IL-34-matured macrophages was the only PPR known to interact with *P. gingivalis* we found to change between IL-34 and CSF1 macrophages, and this is consistent with gene expression (Barve et al., 2013). As this increase correlates with greater *P. gingivalis* survival, it suggests that in addition to taking advantage of DC-SIGN within DCs, *P. gingivalis* is also able to do so in macrophages when they express DC-SIGN as a result of IL-34 maturation, such as in the oral environment (Bostrom & Lundberg, 2013; Ciccia et al., 2013).

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In general, we also observed that IL-34- and CSF1-matured macrophages had very similar cytokine and chemokine responses to incubation with *P. gingivalis*, with the significant exception of IL-8 production. The chemokine IL-8, while produced by macrophages in response to stimulation, is also produced by oral epithelial cells and gingival fibroblasts (Odake et al., 1993; Takeuchi et al., 2013). Reports measuring IL-8 levels in gingival crevicular fluid of patients with periodontal disease have had mixed results, with some patients having higher levels than controls (Ertugrul et al., 2013; Tsai et al., 1995), while other studies found lower concentrations in patients with the disease (Chung et al., 1997; Mathur et al., 1996). However, the microbiome makeup of the patients in these studies was not examined and may have involved different pathogenic organisms. This is an important caveat because while a number of pathogens, including *P. gingivalis*, secrete proteases capable of degrading cytokines (Bodet et al., 2005; Bradshaw et al., 2018; Klapproth et al., 1995), *P. gingivalis* has an additional ability to cause "localized cytokine paralysis" by selectively reducing the ability of epithelial cells to produce IL-8 (Darveau et al., 1998), which may account for some of the reported IL-8 discrepancies. Indeed, our results suggest that the ability of *P. gingivalis* to cause cytokine inhibition via dephosphorylation of NF-κB p65 is not limited to epithelial cells but extends to macrophages as well if the bacterium is able to survive within the macrophage.

Macrophages are the primary producers of IL-6 and TNFα (Gordon & Martinez-Pomares, 2017), and these cytokines are consistently elevated in periodontal tissue and gingival crevicular fluid in patients with chronic periodontitis (Ertugrul et al., 2013; Okada & Murakami, 1998). We observed no significant changes in IL-6 or TNFα release from IL-34 macrophages despite the reduction in both IL-8 production and NF-κB p65 phosphorylation during *P. gingivalis* incubations. IL-6 and TNFα production are generally dependent on NK-κB signaling via p50 subunit-containing dimers (Chen & Greene, 2004), which are not dephosphorylated by *P. gingivalis* (Takeuchi et al., 2013). Indeed, other cytokines that are not differentially released between IL-34- and CSF1-matured macrophages incubated with *P. gingivalis* (Figure 4) also tend to depend on p50-containing NF-κB components (Chen & Greene, 2004). While IL-8 production is generally thought to be dependent on p65 homodimers (Chen & Greene, 2004; Kunsch & Rosen, 1993; Takeuchi et al., 2013), we found that inhibition of p65 in CSF1-differentiated macrophages had no significant effect on IL-8 release, although it did inhibit the production of IL-8 in IL-34 differentiated macrophages (Figure 7). It is possible that c-Rel signaling is more important than p65 in CSF1-matured macrophages (Chen & Greene, 2004) and that in addition to *P. gingivalis* surviving at lower rates within CSF1-derived macrophages, the cells are less sensitive to inhibition of p65 by bacterial SerB, although this signaling is still to be confirmed. However, it is clear that reduced IL-8 cytokine production by IL-34-matured macrophages in the presence of *P. gingivalis* is another potential point of disruption of the immune response initiated by the bacterium leading to periodontitis (Lamont et al., 2018).

In summary, IL-34 is becoming recognized as an important cytokine in the oral environment (Bostrom & Lundberg, 2013; Martinez et al., 2017), and we have begun to examine the functional consequences of IL-34 on macrophage interactions with oral bacteria. We found that IL-34-derived macrophages (i) express significantly more DC-SIGN than CSF1-derived macrophages, (ii) are less capable of killing the oral pathogen *P. gingivalis*,

possibly although reduced ROS production, and (iii) simultaneously release less IL-8 when interacting with *P. gingivalis* than CSF1-matured macrophages in a likely NF-κB p65-dependent manner. When taken together with previous findings that the healthy oral environment has significant IL-34 (Martinez et al., 2017), which is increased during the early response to inflammatory conditions (Bostrom & Lundberg, 2013; Ciccia et al., 2013), there is potential for the keystone pathogen *P. gingivalis* to take advantage of the increased DC-SIGN expression on IL-34 macrophages during initiation of disease to survive within (El-Awady et al., 2015) and cause selective inhibition of IL-8 production (Takeuchi et al., 2013), adding one more point of perturbation in the balance of protective and destructive immunity within the periodontium (Lamont et al., 2018). As macrophages contribute to the control of inflammation, metabolism, and tissue homeostasis (Gordon et al., 2014), identifying the role IL-34 plays in altered interactions of macrophages with *P. gingivalis*, as well as other oral microbiome members, will lead to a better understanding of the role this cytokine plays in the pathogenesis of periodontitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by grants R03DE025062 (JGK), R01DE028307 (JGK), F31DE029400 (SM) and T32DE02526 from the National Institute for Dental and Craniofacial Research. Flow cytometry and microscopy were performed in the Optical Imaging and Analysis Facility in the School of Dental Medicine at the University at Buffalo. Graphical abstract was made with Biorender.com.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abbreviations:

CSF1R	colony stimulating factor-1 receptor
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
HRP	horseradish peroxidase
IFN-γ	interferon gamma
IL-34	interleukin-34
IRB	Institutional Review Board

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LPS	lipopolysaccharide
MFIs	mean fluorescence intensities
MOI	multiplicity of infection
NF-*κ*B	Nuclear Factor kappa-light-chain-enhancer of activated B cells
PBS	phosphate-buffered saline
PMA	phorbol myristate acetate
PRRs	pattern recognition receptors
ROS	reactive oxygen species
RLU	relative luminescence units
TLR	Toll-like receptor
TNF*α*	tumor necrosis factor alpha
UB	University at Buffalo

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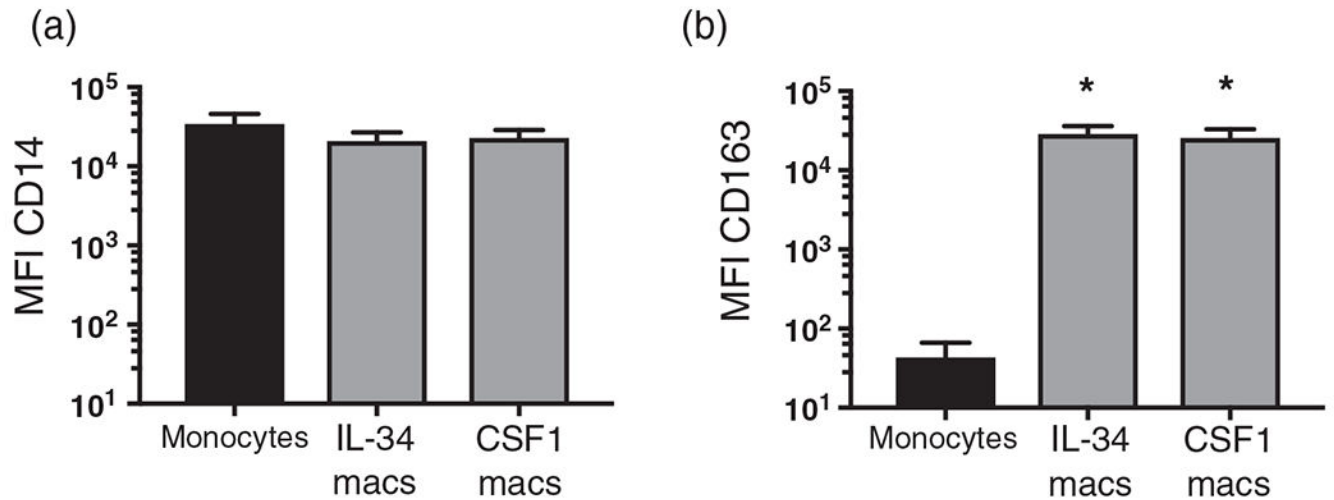

FIGURE 1.

Interleukin-34 (IL-34) and colony-stimulating factor 1 (CSF1) equally differentiate blood monocytes into macrophages. Flow cytometry analysis of (a) CD14 and (b) CD163 on undifferentiated monocytes as well as CSF1 and IL-34 differentiated macrophages. The mean fluorescence intensity (MFI) of CD14 is not significantly different between all three cell types, while CD163 expression is increased significantly upon differentiation of macrophages in the presence of both IL-34 and CSF1. In all cases, 95% of differentiated macrophages were positive for CD14 and CD163. Shown is the average ± standard error of 4–9 independent experiments, * indicates $p < 0.05$ by one-way ANOVA with Dunnett's multiple comparison

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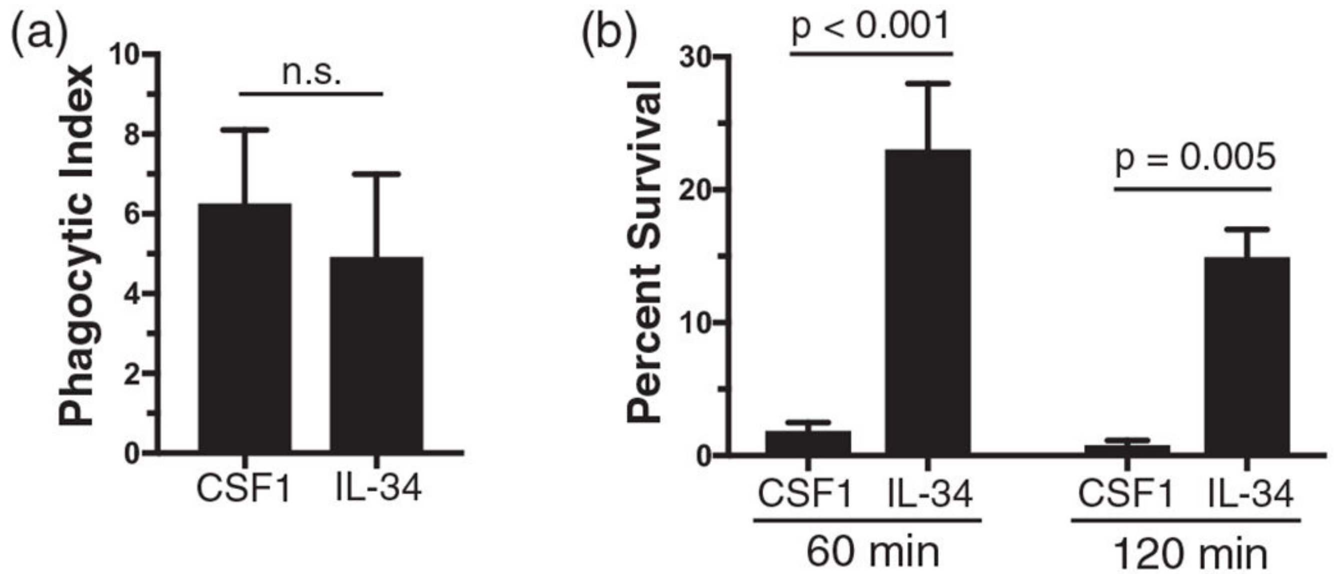

FIGURE 2.

IL-34 macrophages are less efficient at killing internalized *Porphyromonas gingivalis.* (a) CSF1 and IL-34 macrophages are equally efficient at internalizing *P. gingivalis.* Shown are the average (± standard error) internalized bacteria per macrophage after 30 min co-incubation (MOI 10:1) of four independent experiments with bacteria quantified within a minimum of 100 macrophages per experiment. (b) Percent survival of internalized *P. gingivalis* within macrophages for 60 or 120 min post-uptake using an antibiotic protection assay. Shown is the mean (± standard error) of 3–4 independent experiments, p-value calculated with (a) an unpaired t-test and (b) a two-way ANOVA followed by Šídák’s multiple comparison test of survival of *P. gingivalis* within IL-34 and CSF1-differentiated macrophages at each timepoint

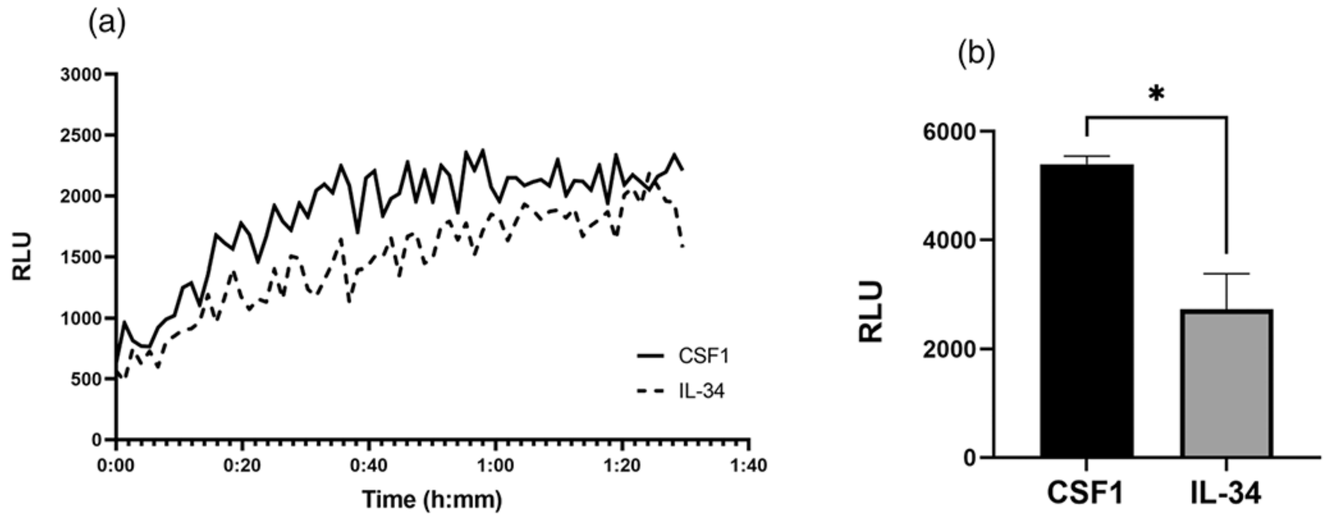

FIGURE 3.

IL-34 downregulates intracellular reactive oxygen species (ROS) production from macrophages. Luminol-based ROS detection was used to quantify the ROS response by CSF1- and IL-34-differentiated macrophages during the activation with phorbol myristate acetate (100 nm). (a) Representative panel for the time course of luminol luminescence detection, shown in relative luminescence units (RLU). (b) Mean peak RLU (with standard error) was detected over three independent experiments. * $p < 0.01$ (paired t-test)

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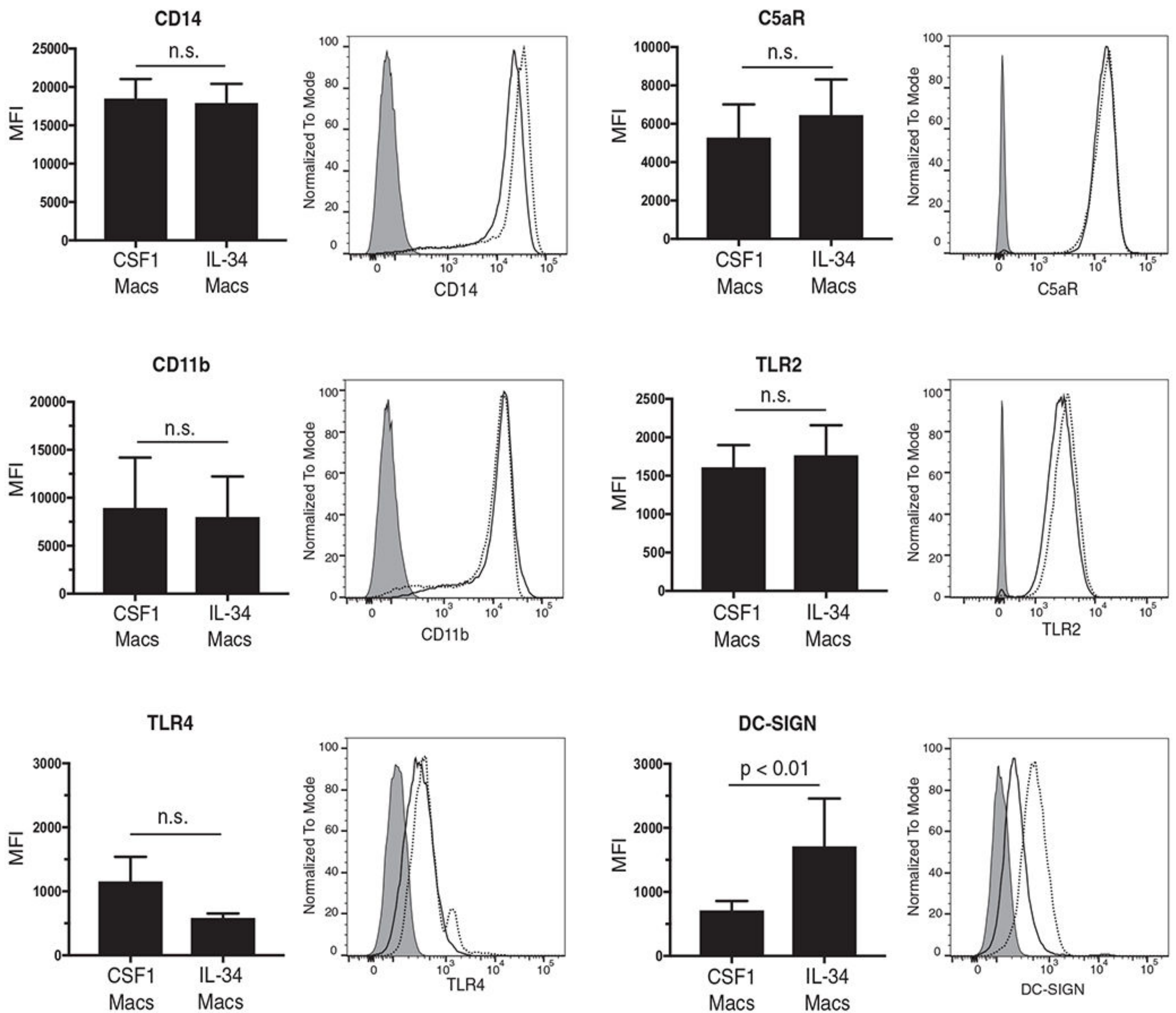

FIGURE 4.

Comparison of the surface expression of markers with known *P. gingivalis* interactions on IL-34 and CSF1 macrophages. The majority of markers with known interactions with *P. gingivalis* are not significantly different between CSF1- and IL-34-differentiated macrophages, with the exception of dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN). Macrophages matured in the presence of either CSF1 or IL-34 were labeled with CD14 and one or more of C5aR, CD11b, TLR 2, TLR 4, or DC-SIGN and analyzed by flow cytometry. The cells were gated for positive CD14 (90% of differentiated cells), and the MFI of the additional markers was determined. Representative histograms are shown, along with the mean (± standard error) of at least four independent experiments per marker tested. *p*-values were determined by paired (IL-34 vs. CSF1) *t*-tests.

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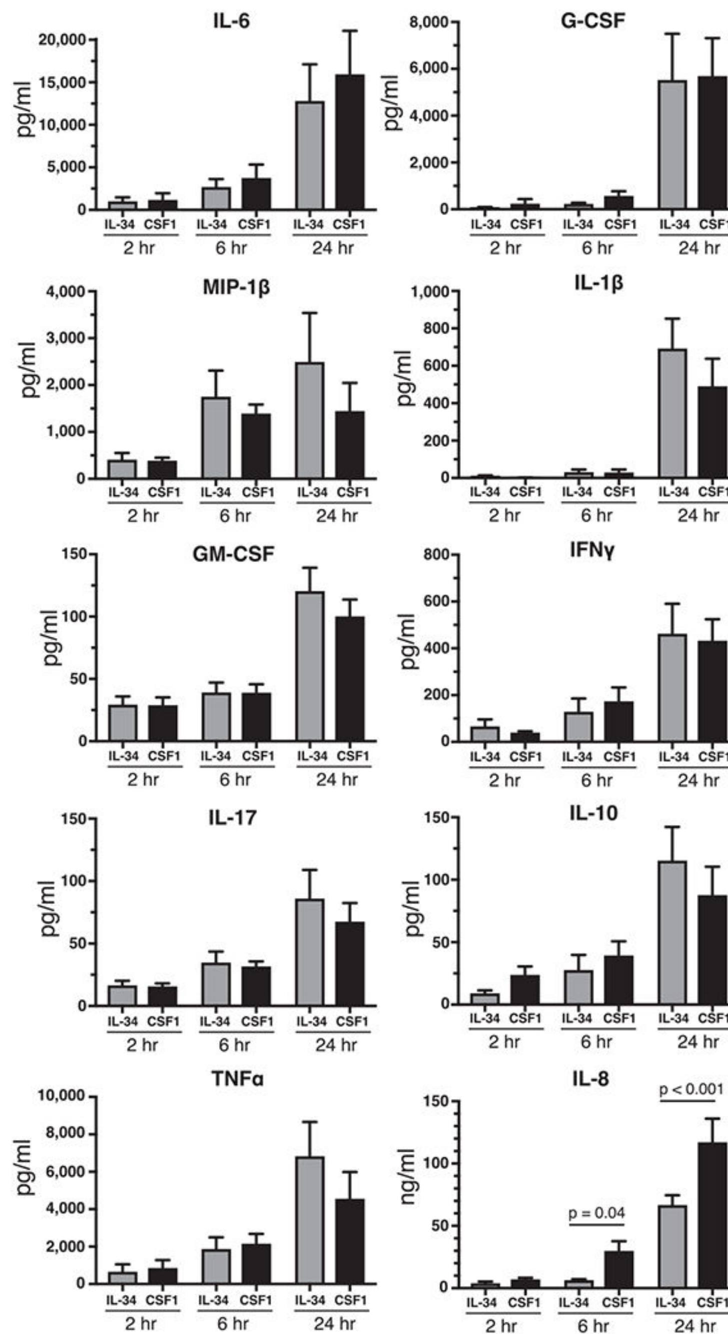

FIGURE 5.
IL-34-matured macrophages produce less IL-8 than CSF1-matured macrophages. IL-34- or CSF1-differentiated macrophages were co-incubated with *P. gingivalis* for 24 h, with media samples removed at 2, 6, and 24 h and subsequently analyzed by multiplex ELISA. The analytes that had significant release above control macrophages are shown. Four independent experiments were performed, mean ± standard error is shown. *p*-values were determined by two-way ANOVA followed by Šídák's multiple comparison test of IL-34 versus CSF1-differentiated macrophages at each timepoint: those without *p*-value indicated

have a $p > 0.05$. Significantly less IL-8 was produced by IL-34-differentiated macrophages than by CSF1-differentiated macrophages

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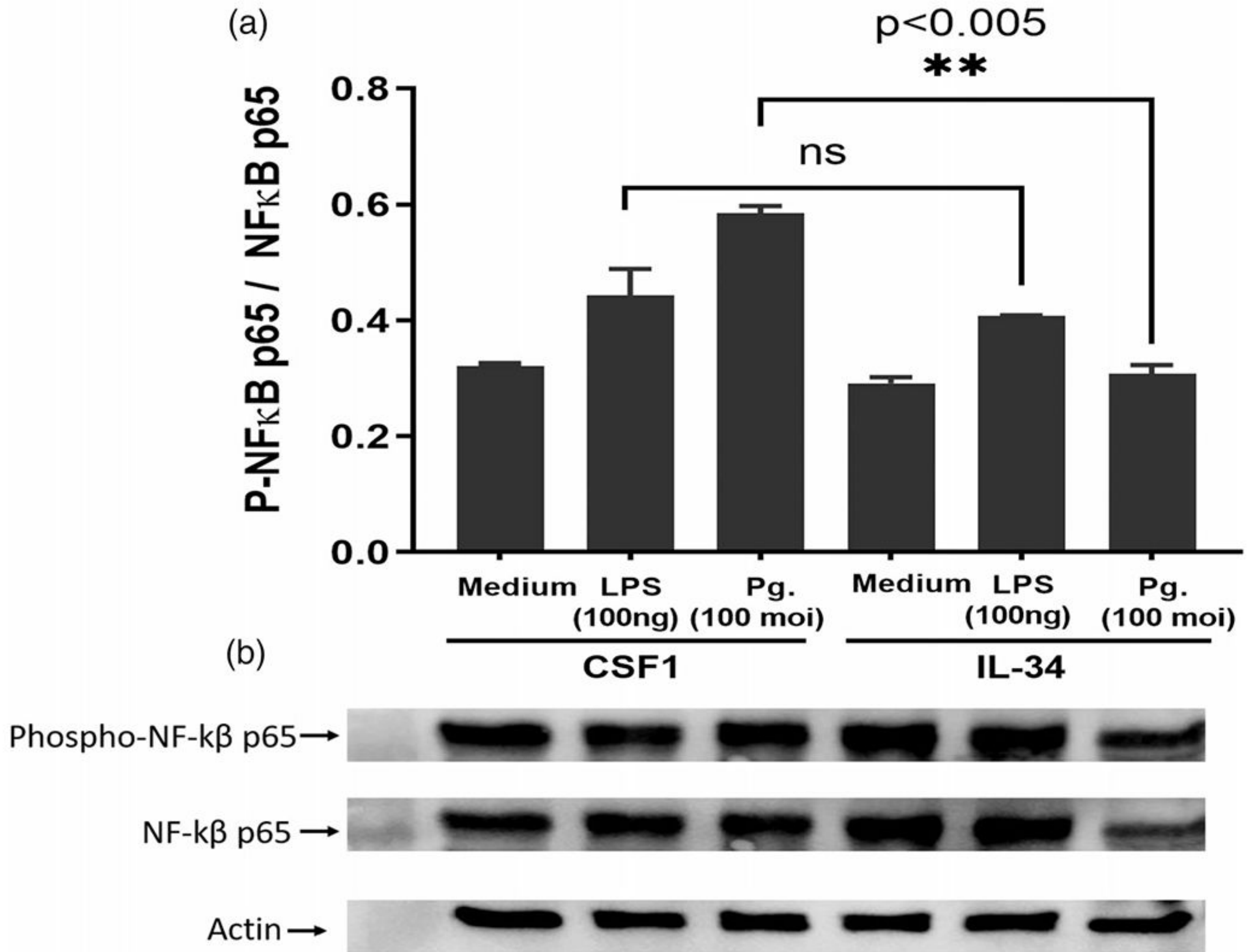

FIGURE 6.

Dephosphorylation of NF-*κ*B p65 is associated with lower levels of IL-8 in IL-34-differentiated macrophages. IL-34- or CSF1-differentiated macrophages were co-incubated with *P. gingivalis* for 2 h Total cell lysates were assessed by western blot using phospho-NF-*κ*B p65 (Ser-536) or total NF-*κ*B p65 antibodies. Anti-mouse actin was used as an internal control. Data shown in Panel B are representative of three independent experiments with similar results; Panel A shows the resulting densitometry analysis of the ratio between phosphorylated and total NF-*κ*B. Statistical analysis (one-way ANOVA with pairwise comparison) indicates a significant difference in the phosphorylated NF-*κ*B between IL-34- and CSF1-differentiated macrophages incubated with *P. gingivalis* but not control or LPS-incubated macrophages.

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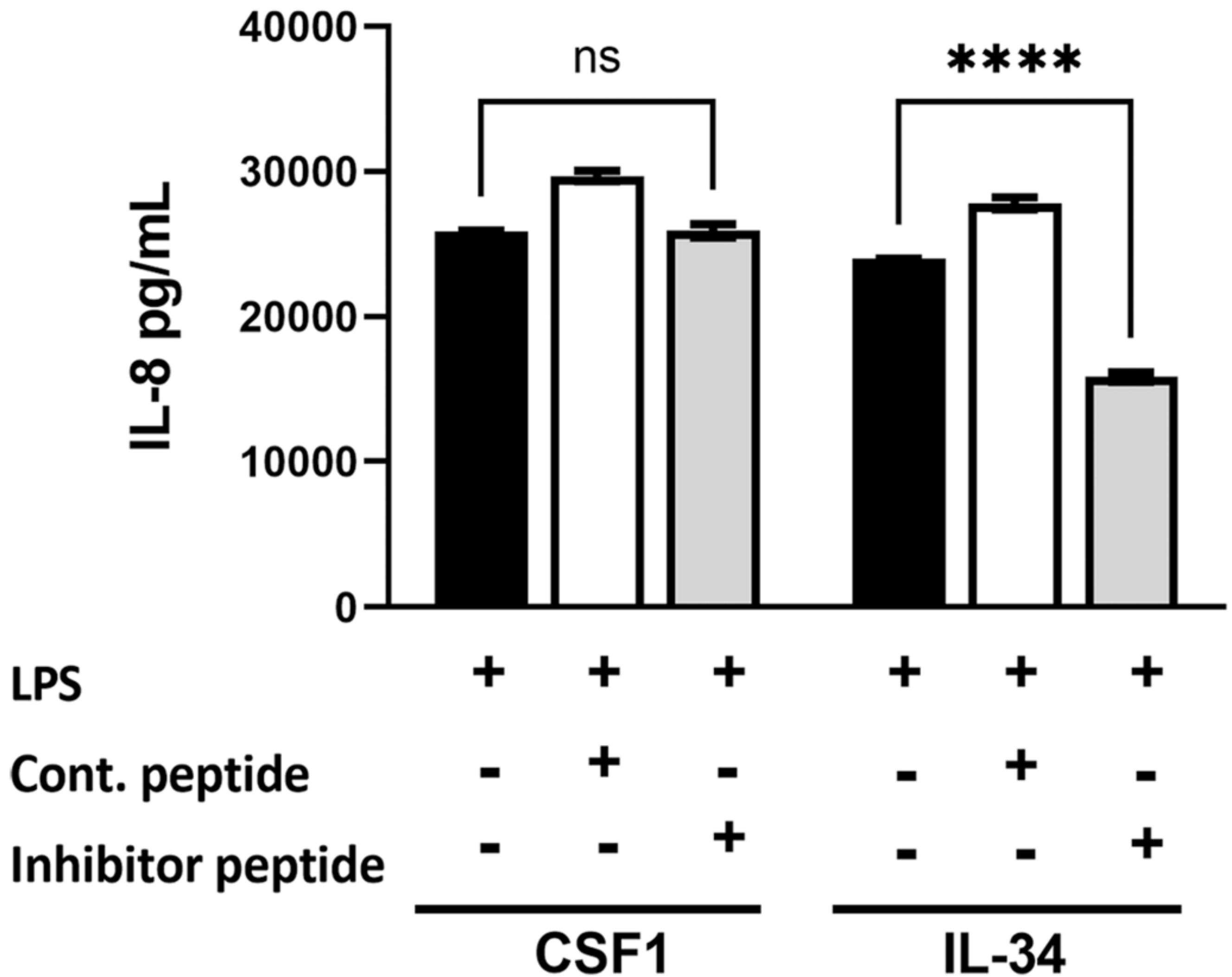

FIGURE 7.

IL-34-matured macrophages produce less IL-8 upon NF-kB p65-specific inhibition. Human peripheral blood monocytes were differentiated with CSF1 (50 ng/ml) and IL-34 (50 ng/ ml)for 6 days in 24-well tissue culture plates (2×10^5 cells/well). The differentiated macrophages were pre-incubated with NFkB p65 inhibitor (NBP2-29321, 100, μM) and control peptide (NBP2-29334, 100 μM) overnight. Cells were then stimulated with LPS (0.1 μg/ml) for 16 h. The cell supernatants were analyzed for the production of IL-8 by ELISA. Shown is the average ± standard error, **** indicates $p < 0.0001$ by one-way ANOVA with Dunnett’s multiple comparison test