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Minocycline hydrochloride as a PAD4 inhibitor suppresses NETs formation: attenuated inflammation with enhanced neutrophil antibacterial capacity in vitro

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Abstract

Background Periodontitis and peri-implantitis are driven by dysregulated host immune responses to periodontal pathogens. Minocycline hydrochloride (MH), widely used in periodontal therapy, has been reported to reversibly inhibit peptidylarginine deiminase 4 (PAD4), a key enzyme in neutrophil extracellular trap (NET) formation. Whether MH suppresses NETs in a cellular context and its downstream biological consequences remain unknown. This study investigated whether MH reduces NETs formation via PAD4 inhibition in vitro and evaluated its anti-inflammatory and antibacterial effects.

Methods HL-60 cells were differentiated into neutrophil-like cells (dHL-60) using all-trans retinoic acid or N,N-dimethylformamide, confirmed by flow cytometry. NETs formation was induced by phorbol 12-myristate 13-acetate and verified by immunofluorescence co-localization of neutrophil elastase and DNA. MH cytotoxicity was assessed by CCK-8. PAD4 inhibition was quantified by measuring citrullinated histone H3 via ELISA, with calcium ionophore as a PAD4 agonist control; NETs suppression was validated by MPO-DNA complex quantification. Anti-inflammatory effects were assessed by measuring IL-1 β , IL-6, IL-17, and TNF- α . Antibacterial capacity was evaluated by co-culture with *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Prevotella intermedia*, followed by colony-forming unit counting and two-way ANOVA.

Results Over 90% of HL-60 cells were successfully differentiated. MH at 600 µg/mL significantly reduced citrullinated histone H3 and MPO-DNA complex levels (both $p < 0.05$); these effects were partially or fully reversed by calcium ionophore, confirming PAD4-dependent NETs suppression. Immunofluorescence demonstrated markedly reduced extracellular NETs at this concentration. MH significantly decreased IL-1 β , IL-6, IL-17, and TNF- α secretion (all $p < 0.0005$); reductions in IL-6 and TNF- α were partially NETs-dependent, while IL-1 β and IL-17 reductions appeared NETs-independent. MH also significantly enhanced neutrophil-like cell antibacterial efficacy against all three periodontopathogens, with a significant interaction between MH and neutrophil-like cells confirmed by two-way ANOVA (all $p < 0.0001$).

Conclusions This study provides the first cellular evidence that MH suppresses NETs formation through PAD4 inhibition, attenuates pro-inflammatory cytokine secretion, and enhances antibacterial capacity against key periodontopathogens. These findings suggest MH may function as a dual-action immunomodulatory antibiotic with optimized therapeutic potential for periodontitis and peri-implantitis.

KEY WORDS neutrophil extracellular traps, minocycline hydrochloride, PAD4 inhibitor, anti-inflammatory, antibacterial

Background

Periodontitis is one of the most prevalent oral diseases worldwide[1]. Peri-implantitis, a similar inflammatory condition affecting dental implants, has become an increasingly common complication as implant-supported restorations grow in popularity[2, 3]. Both conditions arise from the interplay between microbial challenge by dental plaque and the susceptibility of the host immune response[4]. An ideal therapeutic agent for these conditions should combine direct antibacterial activity to eliminate the primary etiological factors with anti-inflammatory properties to limit collateral tissue damage caused by excessive host immune responses.

Minocycline hydrochloride (MH), a second-generation tetracycline antibiotic, is widely used in the clinical management of periodontitis and peri-implantitis. It demonstrates broad-spectrum inhibitory activity against major periodontal pathogens, including *Porphyromonas gingivalis* (Pg), *Fusobacterium nucleatum* (Fn), *Aggregatibacter actinomycetemcomitans* (Aa), and spirochetes[5-8]. Its high lipophilicity[9] and carrier-mediated transmembrane transport[10] facilitate intracellular accumulation, where it exerts direct antibacterial effects by binding to the bacterial 30S ribosomal subunit and inhibiting protein synthesis[11]. MH enters neutrophils via a sodium-dependent mechanism and accumulates intracellularly at concentrations up to 64-fold higher than extracellular levels at low doses[10]. Its local sustained-release formulation for subgingival delivery has been clinically validated to improve periodontal treatment outcomes[12-15].

Beyond direct antibacterial activity, certain antibiotics can modulate immune cell function to exert indirect antibacterial effects. For example, the macrolide azithromycin accumulates within immune cells and enhances neutrophil antibacterial capacity[16-18]; the fluoroquinolones pefloxacin, norfloxacin, and enoxacin augment neutrophil phagocytosis[19]; the β -lactam cefodizime enhances phagocytosis by neutrophils and monocytes[20]; rifampicin, despite inhibiting neutrophil chemotaxis, increases intracellular killing of *Staphylococcus aureus*[20]; and the glycopeptide dalbavancin

has been reported to impair neutrophil bactericidal activity[21]. To date, no such immunomodulatory antibacterial mechanism has been reported for MH.

MH also possesses intrinsic anti-inflammatory properties, though these have received comparatively less attention than its antibacterial effects. MH has been shown to suppress the expression of extracellular matrix metalloproteinase inducer (EMMPRIN) and matrix metalloproteinase-9 (MMP-9)[22, 23], inhibit collagenase activity[24], reduce IL-1 β production at the transcriptional level[22, 25], and downregulate inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), thereby decreasing nitric oxide and prostaglandin E₂ production[25, 26]. Liu et al. demonstrated that MH loaded into nanoliposomes suppressed TNF- α production in lipopolysaccharide-stimulated macrophages in vitro[27]. Notably, Knuckley et al. identified MH as a potent reversible mixed-type inhibitor of peptidylarginine deiminase 4 (PAD4)[28], opening a new avenue for investigating its anti-inflammatory mechanisms.

PAD4 is a calcium-dependent post-translational modification enzyme located in the nucleus of human cells[29] and plays a pivotal role in the formation of neutrophil extracellular traps (NETs)[30]. Upon inflammatory stimulation, activated PAD4 citrullinates arginine residues in histones H3, H2A, and H4, triggering chromatin decondensation and extracellular release of a web-like structure composed of a chromatin scaffold decorated with antimicrobial components including citrullinated histone H3 (cit H3), myeloperoxidase (MPO), neutrophil elastase (NE), and cathepsin G — collectively termed NETs[31, 32]. Key NETs components, including cit H3[33], MPO[34-36], and cathepsin G[37, 38], have been associated with periodontitis and peri-implantitis[39]; NE in particular has been shown to exacerbate periodontal inflammation[40, 41]. NETs release is also accompanied by the extracellular secretion of multiple pro-inflammatory cytokines, including IL-1 α/β , IL-4, IL-5, and IL-17[42, 43]. NETs are therefore often described as a "double-edged sword": while capable of trapping and neutralizing pathogens[44, 45], they simultaneously amplify downstream

immune responses and promote disease progression[43, 46]. As an ancient component of innate immunity, the bactericidal efficacy of NETs is relatively limited — many periodontal bacteria have evolved countermeasures[47-49] — and NETs in most inflammatory conditions are primarily pro-inflammatory rather than antimicrobial[50-56].

PAD4 inhibitors generally attenuate inflammation by suppressing NETs formation[57, 58], but may concurrently impair certain neutrophil antibacterial functions. MH, which combines potent PAD4 inhibitory activity[28] with intrinsic antibacterial properties, therefore represents a particularly promising candidate for infectious inflammatory conditions such as periodontitis and peri-implantitis. However, no study has yet demonstrated that MH can suppress NETs formation in a cellular model or characterized the downstream biological consequences of such suppression. This study therefore aimed to investigate whether MH, acting as a PAD4 inhibitor, can reduce NETs production in neutrophil-like cells, attenuate NETs-associated inflammatory cytokine secretion, and preserve or enhance neutrophil antibacterial capacity against key periodontopathogens.

Materials and methods

1. HL-60 cell culture, differentiation, and NETs induction

HL-60 cells (Procell, CL-0110) were maintained at 37°C in 5% CO₂ in Iscove's Modified Dulbecco's Medium (IMDM; Procell, GPM150510) supplemented with 20% fetal bovine serum (FBS; Servicebio, G8003) and 1% penicillin-streptomycin (P-S; NCM Biotech, C100C5). Differentiation into neutrophil-like cells (dHL-60) was induced by culturing HL-60 cells in complete medium containing either 1 µmol/L all-trans retinoic acid (ATRA; Beyotime, ST1627) or 70 mmol/L N,N-dimethylformamide (DMF; Abmole, M31181) for 5 days with medium renewal every other day.

Differentiation efficiency was assessed by flow cytometry measuring surface expression of CD11b and CD14. A CD11b positivity rate exceeding 80% was

considered indicative of successful differentiation. Two groups were defined: Blank (undifferentiated HL-60), dHL-60(ATRA/DMF induced HL-60).

For NETs induction, dHL-60 cells were adjusted to a density of 10^6 – 10^7 cells/mL and stimulated with 250 ng/mL phorbol 12-myristate 13-acetate (PMA; MCE, HY-18739) for 4 hours.

2. CCK-8 cytotoxicity assay

Complete medium containing MH at concentrations of 100, 200, 400, 600, 800, and 1000 μ g/mL was prepared. dHL-60 cells were resuspended in medium at each corresponding concentration and incubated for 3 hours, with MH-containing medium without cells serving as blank controls. CCK-8 reagent (Procell, P-CA-001) was then added and cells were incubated at 37°C for 1 hour, followed by absorbance measurement at 450 nm (OD_{450}). Groups were named by MH concentration (e.g., 0, 100, 200).

3. ELISA quantification of cit H3 to determine effective PAD4-inhibitory concentration

dHL-60 cells were resuspended in medium containing MH at each concentration described above and stimulated with 250 ng/mL PMA for 4 hours. Cells were lysed by sonication and centrifuged at $1500 \times g$ for 10 minutes; supernatants were collected and diluted 2-fold prior to quantification of citrullinated histone H3 (cit H3) using a commercially available ELISA kit (Enzyme-linked Biotechnology, ml105339) to establish the effective PAD4-inhibitory concentration of MH.

Groups were defined as follows: the 0 group consisted of dHL-60 cells treated with MH-free medium and stimulated with PMA; drug groups consisted of dHL-60 cells treated with MH-containing medium and stimulated with PMA, named by MH concentration.

To further validate the effective concentration, calcium ionophore A-23187 (CI; MCE, HY-N6687) at 4 μ mol/L was added to the effective concentration group as a

PAD4 agonist control. The 0 group, effective concentration group, and CI group were all stimulated for 4 hours, and cit H3 was quantified as described above.

4. ELISA quantification of MPO-DNA complexes to validate NETs suppression

dHL-60 cells were resuspended in MH-containing medium at each concentration and stimulated with 250 ng/mL PMA for 4 hours. Cell supernatants were collected by centrifugation at $1000 \times g$ for 20 minutes, diluted 1000-fold, and quantified for MPO-DNA complexes — a validated NETs marker — using an ELISA kit (JONLBIO, JL47452). Grouping and subsequent procedures were identical to those described for the cit H3 assay.

5. Immunofluorescence visualization of NETs

HL-60 cell suspensions or dHL-60 cell suspensions were divided into equal aliquots and resuspended in either MH-free or MH-containing (effective concentration) complete medium at approximately 5×10^5 cells/mL. Cells were seeded onto 14 mm poly-D-lysine-coated coverslips (LABSELECT, 18122) and stimulated with 250 ng/mL PMA for 4 hours.

Following fixation and blocking, cells were incubated overnight at 4°C in the dark with a 1:150 dilution of recombinant CoraLite® Plus 488-conjugated rabbit anti-human NE antibody (Proteintech, CL488-27642). Coverslips were mounted using an anti-fade mounting medium containing DAPI (Beyotime, P0131). Cells were gently washed three times with PBS at 4°C between each step. Fluorescence was observed using a 10× eyepiece and 40× objective under light-protected conditions, with excitation wavelengths selected according to manufacturer specifications.

Three groups were defined: Blank (HL-60 + PMA), NETs group (dHL-60 + PMA), and Mino group (dHL-60 + PMA + MH at effective concentration).

6. ELISA quantification of cytokine secretion

dHL-60 cells were resuspended in medium containing MH at the effective

concentration and stimulated with 250 ng/mL PMA, with or without 4 μ mol/L CI, for 4 hours. Cell supernatants were collected by centrifugation at $1500 \times g$ at 4°C. Extracellular concentrations of IL-1 β , IL-6, TNF- α (Arigo, ARG82862), and IL-17 (Multi Sciences, EK1177) were quantified by ELISA.

Groups were defined as: NETs group (dHL-60 + MH-free medium + PMA), Mino group (dHL-60 + MH at effective concentration + PMA), and CI group (dHL-60 + MH at effective concentration + PMA + CI).

7. Bacterial culture and CFU-OD600 standard curve generation

Porphyromonas gingivalis (Pg), *Fusobacterium nucleatum* (Fn), and *Prevotella intermedia* (Pi) (kindly provided by the laboratory of Prof. Dongqing Wang) were maintained on tryptic soy blood agar (TSBA) and subcultured into brain heart infusion (BHI) broth two days prior to each experiment.

Bacterial suspensions were collected, washed with phosphate-buffered saline (PBS), and resuspended in PBS. The OD₆₀₀ of the original suspension was measured using a spectrophotometer (ALLSHENG, Nano-300), and the suspension was diluted with PBS to OD₆₀₀ $\approx$ 0.8, followed by serial dilutions to obtain four additional OD₆₀₀ values. Each of the five suspensions was then serially diluted, and 10 μ L aliquots were spotted in triplicate onto agar plates. Plates were left open until droplets had dried, then incubated under fully anaerobic conditions at 37°C for 18 hours. Colonies numbering between 3 and 30 CFU per spot, without spreading growth, were counted. CFU/mL for each suspension was calculated by multiplying the mean colony count by the corresponding dilution factor $\times 100$. A standard curve was generated by linear regression of OD₆₀₀ against CFU/mL values for each bacterial species.

8. Colony-forming unit assay for antibacterial capacity

Co-culture medium was prepared using IMDM supplemented with 20% FBS, with or without 600 μ g/mL MH. dHL-60 cells were collected by centrifugation, washed with PBS, resuspended in co-culture medium, and counted using an automatic cell counter

(BodBoge, JSY-SC-031N).

Bacterial suspensions were collected, resuspended in PBS, and diluted to $OD_{600} < 0.8$. CFU/mL was calculated from the OD_{600} value using the respective standard curve. Four equal aliquots of the bacterial suspension were divided and centrifuged: two were resuspended in standard co-culture medium and two in MH-containing co-culture medium. Each suspension was further diluted to a density 100 times greater than the cell density ($MOI = 1:100$). Equal volumes of cell and bacterial suspensions were mixed and incubated at $37^{\circ}C$ on an orbital shaker for 4 hours. Colony counting was performed as described above.

Four groups were defined: Blank ($-/0$; bacteria + co-culture medium), dHL-60 ($+/0$; bacteria + dHL-60 + co-culture medium), Mino ($-/600$; bacteria + MH-containing co-culture medium), and dHL-60 + Mino ($+/600$; bacteria + dHL-60 + MH-containing co-culture medium).

9. Statistical analysis

CCK-8 and ELISA data were first assessed for normality (Shapiro-Wilk test) and homogeneity of variance (Brown-Forsythe test). Normally distributed data with equal variances were analyzed by one-way ANOVA; normally distributed data with unequal variances were analyzed by Welch's one-way ANOVA; non-normally distributed data were analyzed by non-parametric tests. All CCK-8 and ELISA data in this study satisfied the assumptions of normality and homogeneity of variance and were therefore analyzed by one-way ANOVA.

Fluorescence images were processed and analyzed using ImageJ (NIH, Bethesda, MD, USA).

Standard curves for *Pg*, *Pi*, and *Fn* were generated by simple linear regression, with R^2 values reported.

For the co-culture antibacterial assay, CFU data were $\log_{10}$ -transformed prior to normality testing (Shapiro-Wilk test) and residual plot analysis for variance homogeneity. Data satisfying both assumptions were analyzed by two-way ANOVA

with Šídák correction for multiple comparisons; non-normally distributed data were analyzed by non-parametric tests. All co-culture data in this study met the assumptions for two-way ANOVA.

Results

1. Successful differentiation of HL-60 cells into dHL-60

Following 5 days of induction, the proportion of CD11b-positive cells in dHL-60 group was markedly higher than in the Blank group, reaching 95.4%, exceeding the 80% threshold for successful differentiation. CD14 surface expression was negligible in both groups (Fig. 1).

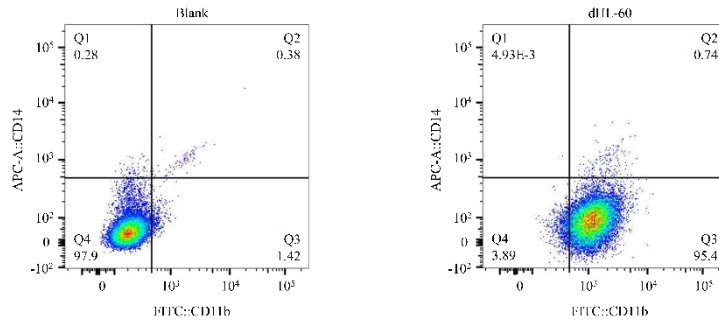


Fig. 1 Flow cytometry analysis of CD11b and CD14 surface expression in Blank (undifferentiated HL-60), and dHL-60 groups following 5-day differentiation induction. CD11b positivity rate was 1.42% (Blank) and 95.4% (dHL-60).

2. Cytotoxicity of MH toward dHL-60: toxic concentration at 1000 µg/mL

After 4 hours of incubation, cell viability in the 100-600 µg/mL groups showed a slight, non-significant decline compared to the 0 group, but remained above 80% in all cases. Cell viability in the 800 µg/mL group decreased further to just above 80% and, although not statistically different from the 0 group, approached significance ($p = 0.0514$). The 1000 µg/mL group demonstrated a marked reduction in cell viability to approximately 50%, which was significantly lower than both the 80% threshold and the 0 group ($***p < 0.0001$) (Fig. 2).

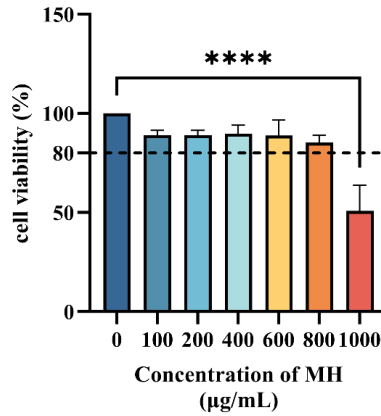


Fig. 2 Cell viability of dHL-60 following 4-hour incubation with MH at concentrations of 0-1000 µg/mL, assessed by CCK-8 assay. Cell viability is expressed as a percentage relative to the 0 µg/mL group. Data are presented as mean $\pm$ SD ($n > 3$). Statistical comparisons were performed by one-way ANOVA. **** $p < 0.0001$.

3. MH at 600 µg/mL inhibits PAD4 activity in dHL-60

Following 4 hours of PMA stimulation, cit H3 production was detectable across all groups. From the 100 µg/mL group onward, cit H3 levels declined progressively with increasing MH concentration. No significant difference was observed between the 100-400 µg/mL groups and the 0 group. A further reduction was observed in the 600-1000 µg/mL groups, which were significantly lower than the 0 group ($*** p < 0.005$).

Upon addition of CI, cit H3 production in the CI group was significantly elevated compared to the 600 µg/mL group ($** p = 0.007$), yet remained significantly lower than the 0 group ($* p = 0.0107$) (Fig. 3).

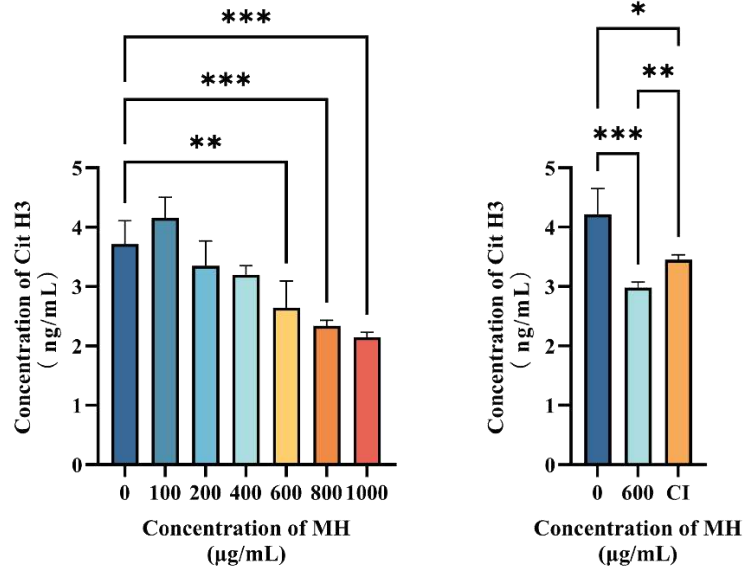


Fig. 3 Cit H3 production by dHL-60 following 4-hour PMA stimulation in medium containing MH at 0–1000 $\mu\text{g/mL}$ (left panel), and in the 0, 600 $\mu\text{g/mL}$, and calcium ionophore (CI; 4 $\mu\text{mol/L}$) groups (right panel). Data are presented as mean $\pm$ SD ($n > 3$). One-way ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4. MH at 600 $\mu\text{g/mL}$ suppresses NETs formation in dHL-60

Following PMA stimulation, NETs production was detected across all groups. NETs levels in the 100 and 200 $\mu\text{g/mL}$ groups were comparable to the 0 group, and those in the 400 $\mu\text{g/mL}$ group showed a downward trend without reaching statistical significance. NETs production in the 600–800 $\mu\text{g/mL}$ groups was significantly reduced compared to the 0 group (* $p < 0.05$).

Addition of CI significantly increased NETs production relative to the 600 $\mu\text{g/mL}$ group (* $p = 0.0242$), restoring levels to a point no longer significantly different from the 0 group ($p = 0.1945$) (Fig. 4).

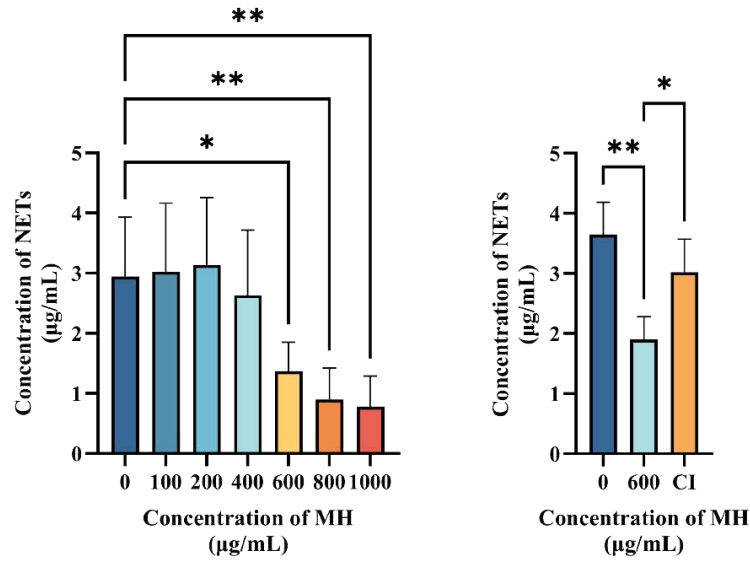


Fig. 4 NETs production, quantified by MPO-DNA complex ELISA, in dHL-60 following 4-hour PMA stimulation in medium containing MH at 0-1000 µg/mL (left panel), and in the 0, 600 µg/mL, and calcium ionophore (CI; 4 µmol/L) groups (right panel). Data are presented as mean ± SD (n > 3). One-way ANOVA. * p < 0.05; ** p < 0.01.

5. Immunofluorescence confirms reduction in NETs at 600 µg/mL MH

Following 4 hours of PMA stimulation, no extracellular NE or DNA was detected in the Blank group (Fig. 5a, d). In the NETs group, cells displayed irregular morphology with visible extracellular NE and web-like DNA structures showing substantial co-localization (Fig. 5b, e). Green fluorescence intensity was visibly higher in the NETs group than in the Blank group. Notably, the NETs group exhibited cellular aggregation surrounding the extracellular NE and DNA structures (Fig. 5e).

In the Mino group, extracellular DNA (blue fluorescence) and NE (green fluorescence) were largely absent. Green fluorescence intensity was visibly reduced compared to the NETs group, and cellular aggregation was no longer observed (Fig. 5c, f).

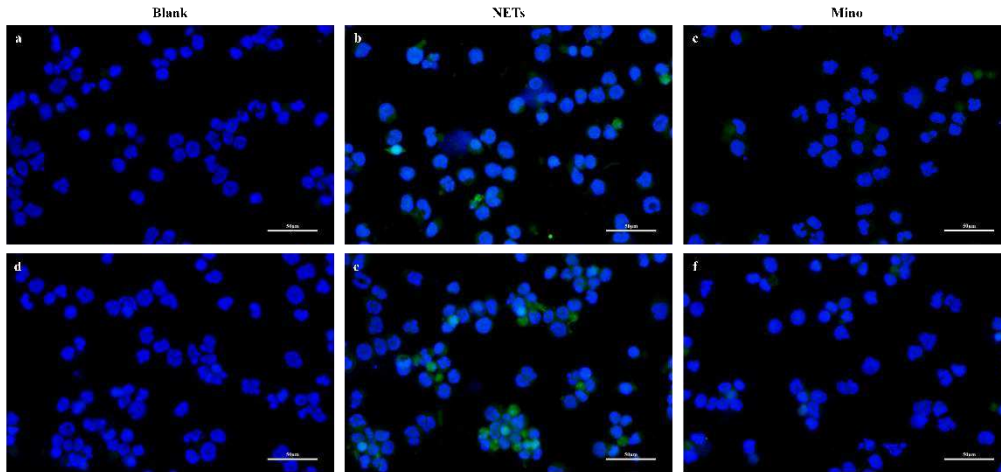


Fig. 5 Immunofluorescence staining following 4-hour PMA stimulation (two representative fields shown per group). Green: anti-neutrophil elastase (NE) antibody (CoraLite® Plus 488); blue: DAPI (DNA). (a, d) Blank group (undifferentiated HL-60 + PMA): only intracellular blue fluorescence detected, with no extracellular NE or DNA signal. (b, e) NETs group (dHL-60 + PMA): extracellular green (NE) and blue (DNA) signals with substantial co-localization, indicating NETs formation; cellular aggregation is observed in panel (e). (c, f) Mino group (dHL-60 + PMA + 600 µg/mL MH): extracellular NE and DNA signals largely absent; green fluorescence markedly reduced compared to the NETs group; cellular aggregation no longer observed. Scale bar: 50 µm.

6. MH suppresses IL-1 β , IL-6, IL-17, and TNF- α secretion

Addition of 600 µg/mL MH to the NETs group resulted in significant reductions in dHL-60 secretion of IL-1 β , IL-6, IL-17, and TNF- α (all $^{***}p < 0.0005$).

Upon addition of CI, IL-6 and TNF- α levels were significantly elevated compared to the Mino group. IL-6 showed a significant increase ($^{**}p = 0.0012$) but remained significantly lower than the NETs group ($^{****}p < 0.0001$). TNF- α increased markedly ($^{****}p < 0.0001$) and, upon CI addition, was even significantly higher than in the NETs group ($^{*}p = 0.0326$). In contrast, IL-1 β and IL-17 levels showed no significant change following CI addition compared to the Mino group, and remained significantly lower than the NETs group (both $^{**}p < 0.005$) (Fig. 6).

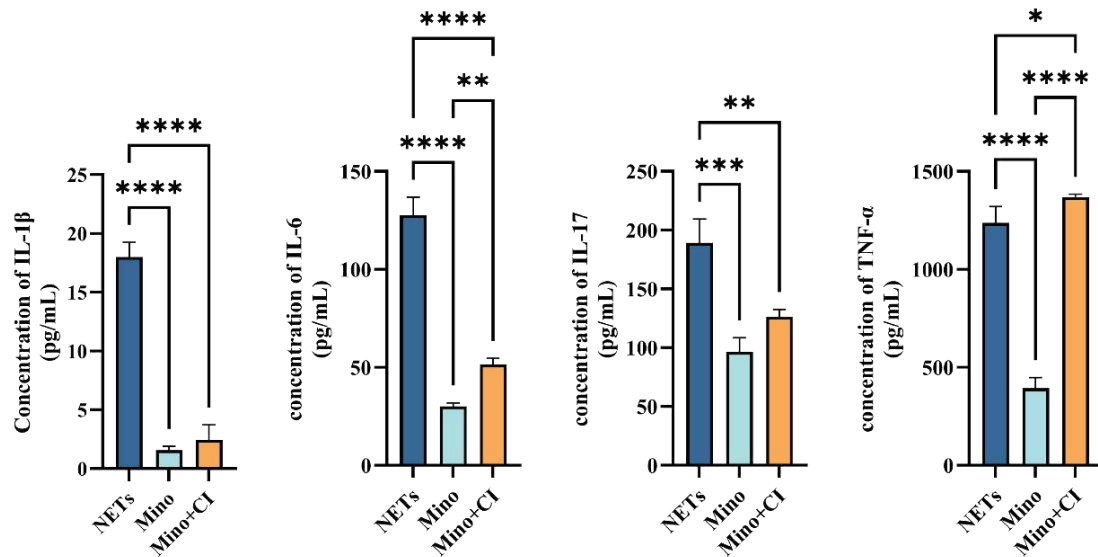


Fig. 6 Secretion of IL-1 β , IL-6, IL-17, and TNF- α (all in pg/mL) by dHL-60 in the NETs group (dHL-60 + PMA), Mino group (dHL-60 + PMA + 600 μ g/mL MH), and Mino+CI group (dHL-60 + PMA + 600 μ g/mL MH + 4 μ mol/L CI). Data are presented as mean $\pm$ SD (n > 3). One-way ANOVA. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

7. CFU-OD600 standard curves for *Pg*, *Pi*, and *Fn*

Standard curves were successfully generated for *Pg*, *Pi*, and *Fn* by simple linear regression, with R^2 values of 0.9952, 0.9982, and 1.000, respectively, indicating excellent linearity across the tested range (Fig. 7).

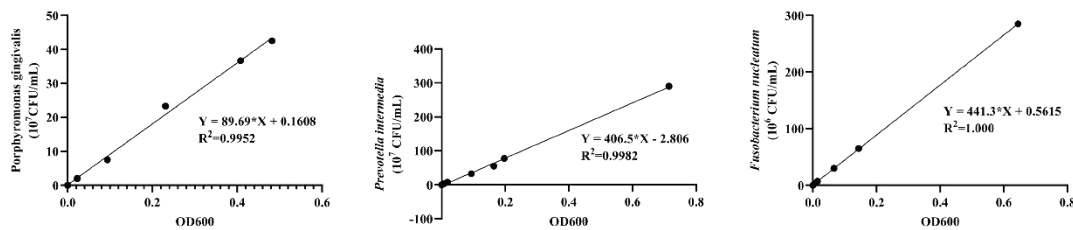


Fig. 7 CFU-OD600 standard curves for *Pg*, *Pi*, and *Fn* generated by simple linear regression. *Pg*: $Y = 89.69X + 0.1608$, $R^2 = 0.9952$; *Pi*: $Y = 406.5X - 2.806$, $R^2 = 0.9982$; *Fn*: $Y = 441.3X + 0.5615$, $R^2 = 1.000$. X-axis: OD₆₀₀; Y-axis: CFU/mL ($\times 10^7$ for *Pg* and *Pi*; $\times 10^6$ for *Fn*).

8. MH enhances the antibacterial capacity of dHL-60

Two-way ANOVA demonstrated that both MH and dHL-60 independently exerted highly significant effects on bacterial CFU counts (all **** p < 0.0001 across all three species). Critically, a significant interaction between MH and dHL-60 was detected for

all three periodontopathogens (all **** $p < 0.0001$). The proportion of total variance attributable to each factor is summarized in Table 1.

In terms of variance contribution, MH was the dominant factor driving changes in bacterial CFU counts, followed by dHL-60, with the MH–dHL-60 interaction accounting for a smaller but statistically significant proportion of total variance.

Table 1 Two-way ANOVA results for *Pg*, *Pi*, and *Fn*

		Sum of				% of	
		squares	df	MS	F (DFn, DFd)	total	<i>p</i> value
						variance	
<i>Pg</i>	Interaction	1.174	1	1.174	F (1, 12) = 70.68	6.677%	**** $p < 0.0001$
	dHL-60	5.706	1	5.706	F (1, 12) = 343.6	32.45%	**** $p < 0.0001$
	MH	10.5	1	10.5	F (1, 12) = 632.4	59.74%	**** $p < 0.0001$
	Residual	0.1993	12	0.01661			
<i>Pi</i>	Interaction	1.068	1	1.068	F (1, 12) = 60.37	5.374%	**** $p < 0.0001$
	dHL-60	6.22	1	6.22	F (1, 12) = 351.4	31.29%	**** $p < 0.0001$
	MH	12.38	1	12.38	F (1, 12) = 699.5	62.27%	**** $p < 0.0001$
	Residual	0.2124	12	0.0177			
<i>Fn</i>	Interaction	2.903	1	2.903	F (1, 12) = 150.7	7.55%	**** $p < 0.0001$
	dHL-60	3.814	1	3.814	F (1, 12) = 198.0	9.92%	**** $p < 0.0001$
	MH	31.5	1	31.5	F (1, 12) = 1635	81.93%	**** $p < 0.0001$
	Residual	0.2312	12	0.01926			

Post-hoc multiple comparisons with Šídák correction revealed that MH significantly potentiated the antibacterial effect of dHL-60. After 4 hours of co-culture, CFU counts in both the dHL-60 and Mino groups were significantly lower than in the Blank group for all three bacterial species (all **** $p < 0.0001$), and the Mino group showed significantly lower CFU counts than the dHL-60 group. The dHL-60 + Mino group achieved the greatest bacterial reduction, with CFU counts significantly lower than all other groups for *Pg* and *Pi*, and significantly lower than the Blank and dHL-60 groups for *Fn* (Fig. 8).

Representative plate images of colony counts for each group are shown in Fig. 9.

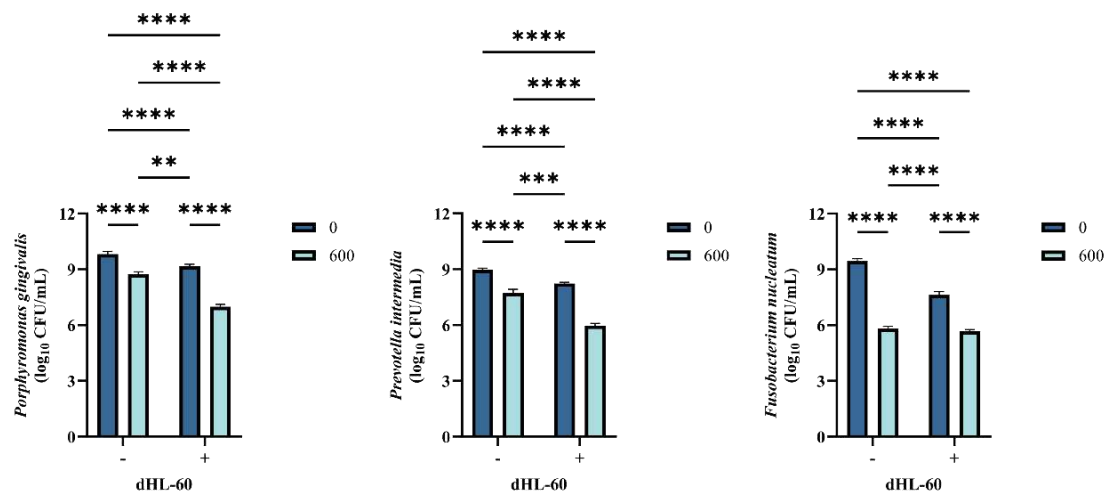


Fig. 8 Two-way ANOVA multiple comparison results for *Pg*, *Pi*, and *Fn*. X-axis: absence (–) or presence (+) of dHL-60; bar color: MH concentration (0 or 600 µg/mL). Y-axis: log₁₀-transformed CFU/mL. Data are presented as mean ± SD (n > 3). Two-way ANOVA with Šidák correction. ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.

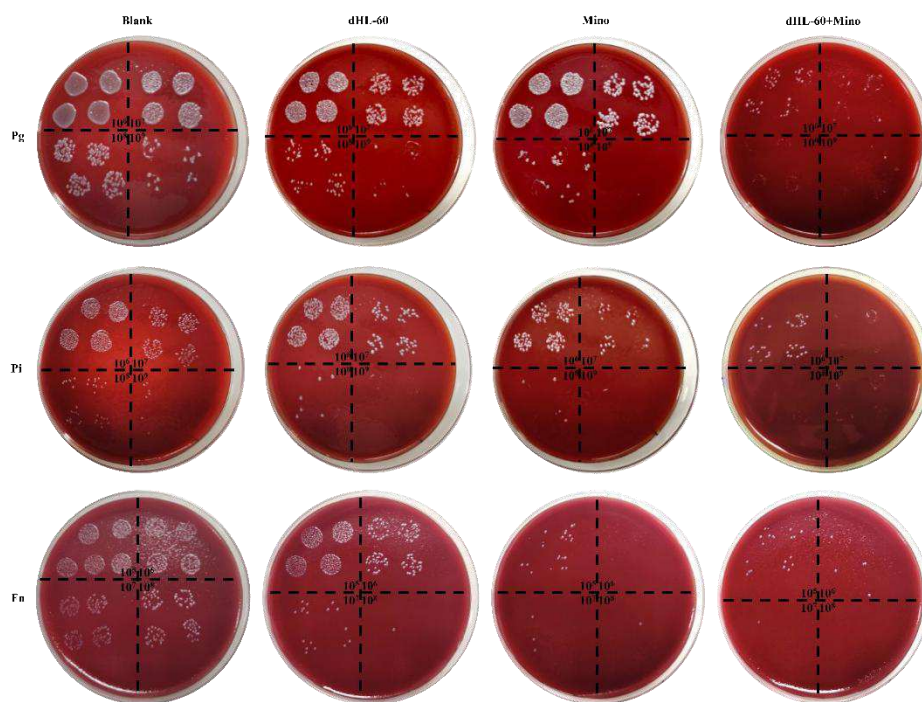


Fig. 9 Representative TSBA plate images of colony counts following 18-hour anaerobic culture at 37°C. Rows represent bacterial species: *Pg*, top row; *Pi*, middle row; and *Fn*, bottom row.

Columns represent treatment groups from left to right: Blank (bacteria + medium), dHL-60 (bacteria + dHL-60 + medium), Mino (bacteria + 600 µg/mL MH + medium), and dHL-60 + Mino (bacteria + dHL-60 + 600 µg/mL MH + medium). Each plate is divided into quadrants

corresponding to serial dilutions (10^{-4} - 10^{-7} for *Pg* and *Pi*, 10^{-3} - 10^{-6} for *Fn*). Progressive reduction in colony density from Blank to dHL-60 + Mino groups is consistent with quantitative CFU data shown in Fig. 8.

Discussion

1. The effective NETs-inhibitory concentration of MH falls within a physiologically plausible range

Knuckley et al. were the first to identify MH as a mixed-type inhibitor of PAD4 using a novel activity-based protein profiling (ABPP) approach, reporting an IC_{50} of 0.62 mM (approximately 306 μ g/mL) against PAD4 with benzyl-L-arginine ethyl ester (BAEE) as substrate[28]. However, no subsequent study has examined whether MH can inhibit PAD4 activity or suppress NETs formation in a cellular or in vivo model.

The present study established an in vitro NETs model and first determined the upper safe concentration of MH during the NETs induction period using CCK-8, thereby excluding confounding effects of differential cell viability on downstream assays. Within this concentration range, PAD4 activity was assessed by quantifying its enzymatic product cit H3, and NETs formation was validated by quantifying MPO-DNA complexes, with CI serving as a PAD4 agonist control to confirm the effective inhibitory concentration. The results demonstrated that MH at 600-800 μ g/mL significantly suppressed both cit H3 and MPO-DNA complex production, and these effects were partially or fully reversed by CI. Combined with immunofluorescence visualization of NE, which provided qualitative confirmation of NETs suppression, the present study employed multiple established NETs markers to characterize NETs formation both qualitatively and quantitatively, demonstrating for the first time that MH can inhibit PAD4 activity and consequently suppress NETs formation in a cellular model, with a minimum effective concentration of 600 μ g/mL.

This effective concentration is approximately twice the IC_{50} reported by Knuckley et al., a discrepancy that is attributable to differences in both the experimental readout

and the model system. First, Knuckley et al. measured IC_{50} against PAD4 enzymatic activity directly, whereas the present study focused on the minimum concentration required to achieve statistically significant suppression of NETs formation as a downstream biological outcome. Second, Knuckley et al. conducted a biochemical assay in the absence of cellular barriers or intracellular metabolism, allowing direct and efficient drug–target contact. In contrast, the present cellular model requires MH to first traverse the plasma membrane via transmembrane transport. Although MH is known to accumulate intracellularly in neutrophils at low concentrations, the intracellular-to-extracellular concentration ratio decreases progressively as extracellular concentrations rise[10], meaning that elevating extracellular MH yields diminishing returns in intracellular drug accumulation. MH must then reach PAD4 within the nucleus while contending with non-specific protein binding and intracellular metabolism that further reduce the effective intracellular concentration. The effective cellular concentration of approximately twice the biochemical IC_{50} is therefore within a physiologically plausible range, and the two findings are mutually corroborating: MH can inhibit PAD4 not only in a biochemical protein profiling assay, but also in a cellular model through suppression of the downstream biological process of NETs formation.

2. MH attenuates inflammation and may reduce bone resorption through NETs suppression

The present study examined changes in the secretion of four cytokines — IL-1 β , IL-6, IL-17, and TNF- α — following MH-mediated NETs suppression. These cytokines were selected because of their well-established roles in periodontitis and peri-implantitis[59-61]: their concentrations are markedly elevated in the gingival crevicular fluid and saliva of affected patients[62, 63], correlate with disease severity, and their blockade with antagonists or receptor inhibitors has been shown to significantly reduce lesion size[64-66]. They are accordingly regarded as established biomarkers of periodontitis and peri-implantitis[67-71]. Notably, IL-1 β , IL-6, and TNF- α can themselves stimulate neutrophils to produce NETs[56], establishing a self-amplifying

inflammatory loop that MH may interrupt at multiple points.

Upon inflammatory stimulation, neutrophils undergo cell lysis to release NETs, accompanied by the concurrent release of various cytokines. Several studies have reported associations between cytokine secretion and NETs production: Zhang et al. found that protein levels of IL-1 β , IL-36 α , IL-36 γ , caspase-1, and HMGB1 largely paralleled NETs production in an ex vivo skin explant model[72]. Curren et al. demonstrated that HpARI, an immunomodulatory protein secreted by intestinal parasites, reduced rhinovirus-induced NETs in the lungs of chronic asthmatic mice while simultaneously decreasing airway levels of IL-1 α , IL-1 β , IL-4, and IL-5[42]. However, although these studies attempted to investigate the mechanisms of cytokine release following NETs depletion using PAD4 inhibitors or DNase, they were conducted primarily in vivo, making it difficult to distinguish whether the observed reduction in cytokine release resulted directly from decreased neutrophil secretion or from attenuated downstream responses in other cell types secondary to reduced NETs. Consequently, few studies have specifically examined the relationship between these neutrophil-derived pro-inflammatory cytokines and NETs formation — that is, whether their secretion constitutes an intrinsic part of the NETs formation process itself, or merely co-occurs with NETs release following neutrophil activation by inflammatory stimuli.

The present study addressed this question by quantifying cytokine levels following MH-mediated NETs suppression, and by using CI to restore PAD4 activity and thereby distinguish NETs-dependent from NETs-independent cytokine secretion. The results showed that 600 μ g/mL MH significantly reduced IL-1 β , IL-6, IL-17, and TNF- α secretion. Notably, TNF- α secretion tracked closely with NETs production and was restored beyond NETs-group levels upon CI addition, while IL-6 suppression was only partially reversed by CI. In contrast, IL-1 β and IL-17 secretion was unaffected by CI addition, remaining significantly lower than the NETs group. These findings indicate that MH suppresses IL-6 and TNF- α secretion at least in part through NETs-dependent

pathways, whereas the reduction in IL-1 β and IL-17 secretion is mediated through NETs-independent mechanisms. In other words, IL-6 and TNF- α secretion is coupled to the NETs formation pathway, while IL-1 β and IL-17 secretion in this model is not.

Beyond pro-inflammatory cytokine regulation, NETs have been shown to promote M1 macrophage polarization[73-77], accelerate osteoclast differentiation[78, 79], and enhance osteoclast-mediated bone resorption[80]. Suppression of NETs formation — whether by PAD4 inhibitors, Padi4-deficient mouse models, or DNase treatment — has been shown to significantly reduce bone resorption[80, 81] and accelerate fracture healing[82]. Taken together, these findings suggest that MH may confer additional benefits in periodontitis and peri-implantitis through direct attenuation of NETs-driven tissue and bone destruction, beyond its suppressive effects on pro-inflammatory cytokine secretion.

3. MH enhances neutrophil antibacterial capacity: evidence of immunomodulatory activity

The present study selected three bacterial species with well-established roles in the pathogenesis of periodontitis[83] and peri-implantitis[84-86]: *Pg*, a member of the red complex, and *Pi* and *Fn*, members of the orange complex. Standardized CFU-OD₆₀₀ standard curves were generated for each species to ensure a consistent MOI across experiments. Two-way ANOVA was then applied to dissect the independent and interactive effects of dHL-60 and MH on bacterial killing.

The results demonstrated that, despite suppressing NETs formation, MH did not impair dHL-60 antibacterial capacity; rather, MH and dHL-60 together exhibited superior antibacterial efficacy compared to either agent alone. Critically, two-way ANOVA revealed a significant interaction between MH and dHL-60 across all three bacterial species, and post-hoc multiple comparisons confirmed that MH significantly enhanced dHL-60 antibacterial efficacy — an effect that cannot be explained by the additive contributions of MH and dHL-60 alone.

MH is a widely used tetracycline-class antibiotic characterized by high

lipophilicity[9], enabling it to diffuse directly across the bacterial cell membrane and bind to the bacterial 30S ribosomal subunit, thereby inhibiting protein synthesis and exerting its antibacterial effect[11]. In terms of pharmacodynamic/pharmacokinetic (PD/PK) properties, MH is classified as an AUC (area under the curve)/MIC (minimum inhibitory concentration)-dependent antibiotic, or alternatively as a time-dependent antibiotic with a prolonged post-antibiotic effect: its antibacterial activity does not strictly correlate with the duration for which plasma concentration exceeds the MIC, but rather depends on the total drug exposure to the bacteria. Among PD/PK indices, the AUC/MIC ratio best predicts its clinical antibacterial efficacy[87]. Consequently, although MH already exhibits excellent antibacterial activity against periodontal pathogens, with an $MIC_{90} \leq 2 \mu\text{g/mL}$ [6, 7], substantially increasing its concentration — even up to $500 \mu\text{g/mL}$ — can further enhance its antibacterial potency, significantly reducing the viability of preformed Pg biofilms[5].

Building on these findings, the present study demonstrates that MH exerts antibacterial effects not only through its direct antimicrobial action, but also indirectly through modulation of immune cell function. Previous studies have reported that other classes of antibiotics can influence neutrophil antibacterial function through diverse mechanisms: azithromycin accumulates within neutrophil lysosomes and cytoplasmic granules and fuses with bacteria-containing phagosomes, thereby enhancing intracellular bactericidal capacity[16-18]; cefodizime may enhance neutrophil phagocytosis via the LPS–TLR4–NF- κ B pathway[20]; and fluoroquinolones have also been shown to potentiate antibacterial activity by stimulating neutrophil phagocytosis[19]. No such immunomodulatory antibacterial mechanism has previously been reported for MH, and the present study is the first to identify this phenomenon. Given that MH similarly accumulates within neutrophils, it may act through a mechanism analogous to that of azithromycin — enhancing intracellular bactericidal capacity and thereby acting synergistically to achieve superior antibacterial efficacy. However, the precise underlying mechanism and signaling pathway remain to be

confirmed through further investigation.

Taken together, the present study successfully established a stable and reproducible in vitro NETs model. Using this model, qualitative and quantitative analysis of PAD4 products and multiple established NETs markers demonstrated for the first time that MH suppresses NETs formation through PAD4 inhibition, while simultaneously enhancing — rather than impairing — neutrophil antibacterial capacity against key periodontopathogens, and exerting additional anti-inflammatory effects through both NETs-dependent and NETs-independent pathways.

4. Significance and Limitations

1. Significance

This study provides the first demonstration that MH at 600-800 µg/mL can suppress NETs formation through inhibition of intracellular PAD4 activity in neutrophils, thereby exerting anti-inflammatory and immunomodulatory antibacterial effects.

As noted above, the clinical application of MH in periodontitis and peri-implantitis has historically focused on its antibacterial properties, with dosing guided by MIC₉₀ values that exploit its potency at relatively low concentrations. The present findings suggest that elevating the effective extracellular concentration to 600 µg/mL unlocks additional therapeutic benefits beyond direct antibacterial activity.

With regard to anti-inflammatory effects, MH at this concentration not only exerts the intrinsic anti-inflammatory actions previously described — including suppression of IL-1β, nitric oxide, and prostaglandin E₂ production[25, 26] — but additionally inhibits PAD4 activity, thereby reducing NETs formation and attenuating downstream inflammatory progression.

With regard to antibacterial effects, increasing the MH concentration to 600 µg/mL amplifies its direct antibacterial potency: while retaining inhibitory activity against planktonic bacteria, MH at this concentration has also been shown to effectively reduce

the viability of bacterium in biofilms[5]. Beyond these direct effects, MH at 600 $\mu\text{g/mL}$ further enhances antibacterial capacity through modulation of neutrophil function.

When the effective extracellular concentration reaches 600 $\mu\text{g/mL}$, MH therefore exhibits a broader and more potent therapeutic profile that more closely aligns with the complex pathophysiological demands of periodontitis and peri-implantitis. These findings provide a novel rationale and consideration for future optimization of MH dosing strategies in clinical practice.

2. Limitations

According to the present findings, the effective concentration of MH solution (600–800 $\mu\text{g/mL}$) is relatively close to its cytotoxic threshold for neutrophils (1000 $\mu\text{g/mL}$), raising concerns regarding the likelihood of adverse effects and the feasibility of direct clinical application at this concentration. As this study was conducted in vitro, MH in solution form was distributed directly in the extracellular environment, lacking cellular targeting and exhibiting low efficiency of transmembrane delivery. Moreover, the simplified in vitro conditions differ substantially from the complexity of the in vivo environment, and these findings therefore remain at some distance from direct clinical translation.

In practical applications, drug delivery could be improved through the development of transmembrane carrier systems that selectively target neutrophils — or even intracellular PAD4 within neutrophils — to enable selective delivery of MH into the cell, thereby enhancing delivery efficiency and reducing the minimum effective concentration required. Alternatively, appropriate administration routes and formulations, such as local delivery via microneedle injection, could improve drug utilization efficiency and reduce the required dosage, thereby improving the safety profile of MH.

Furthermore, given that MH acts as a mixed-type PAD4 inhibitor binding to an allosteric site distinct from the enzyme's active center, it may be co-administered with other PAD4 inhibitors that act through mechanisms different from that of MH,

potentially achieving comparable or superior anti-inflammatory and antibacterial effects at lower, safer drug doses. Should additional antibiotics beyond the tetracycline class be identified with PAD4-inhibitory activity, their combined use with MH could further enhance antibacterial efficacy while reducing the risk of resistance development.

The mechanism underlying the immunomodulatory effect of MH on neutrophils during its antibacterial action warrants further investigation, and whether MH exerts similar regulatory effects on other immune cells within the immune system merits future exploration.

Conclusions

This study provides the first cellular evidence that MH suppresses NETs formation in an in vitro model through inhibition of PAD4 activity. MH significantly reduced secretion of the pro-inflammatory cytokines IL-1 β , IL-6, IL-17, and TNF- α ; the suppression of IL-6 and TNF- α was at least partially mediated through NETs-dependent pathways, while that of IL-1 β and IL-17 operated through NETs-independent mechanisms. Furthermore, MH was found to interact synergistically with neutrophil-like cells to enhance antibacterial efficacy against key periodontopathogens, demonstrating an immunomodulatory antibacterial function not previously described for MH.

List of abbreviations

Abbreviations	Definition
ABPP	Activity-Based Protein Profiling
ANOVA	Analysis of Variance
ATRA	All-trans-retinoic Acid
AUC	Area under the Curve
BAEE	N-benzoyl-L-arginine-ethylester
BHI	Brain-Heart Infusion

CCK8	Cell Counting Kit-8
CFU	Colony-Forming Unit
CI	Calcium Ionophore
Cit H3	Citrullinated Histone H3
COX-2	Cyclooxygenase-2
dHL-60	Differentiated HL-60
DMF	N,N-Dimethylformamide
ELISA	Enzyme Linked Immunosorbent Assay
EMMPRIN	Extracellular Matrix Metalloproteinase Inducer
FBS	Fetal Bovine Serum
<i>Fn</i>	<i>Fusobacterium nucleatum</i>
HMGB1	High Mobility Group Box 1
HpARI	Heligmosomoides polygyrus Alarmin
	Release Inhibitor
IL-1 α/β	Interleukin-1 α/β
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-17	Interleukin-17
IMDM	Iscove's Modified Dulbecco's Medium
iNOS	Inducible Nitric Oxide Synthase
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
MMP-9	Matrix Metalloproteinase 9
MOI	Multiplicity of Infection
MPO	Myeloperoxidase
NE	Neutrophil Elastase
NETs	Neutrophil Extracellular Traps

OD	Optical Density
PAD4	Peptidyl Arginine Deiminase 4
PBS	Phosphate Buffered Saline
PD	Pharmacodynamic
<i>Pg</i>	<i>Porphyromonas gingivalis</i>
<i>Pi</i>	<i>Prevotella intermedia</i>
PK	Pharmacokinetic
PMA	Phorbol 12-myristate 13-acetate
P-S	Penicillin-Streptomycin
TNF- α	Tumor Necrosis Factor- α
TSBA	Tryptic Soybean Blood Agar

Declarations

1. Ethics approval and consent to participate

This study did not involve human participants, human data, or human tissue; commercially available cell lines (HL-60, Procell) and laboratory bacterial strains were used. As no human or animal subjects were directly involved, consent to participate was not applicable.

2. Consent for publication

Not applicable.

3. Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

4. Competing interests

The authors declare that they have no competing interests.

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6. Authors' contributions

W.Y. and S.C. contributed to the conceptualization and design of the study. All experimental work was carried out by W.Y. under the supervision of S.C. N.W., Y.Y., J.Z. and Z.G. assisted with data acquisition of bacterial and cellular experiments respectively. W.L., T.L. and X.C. assisted with data analysis. H.G. and Z.W. were responsible for project administration. W.Y. drafted the manuscript. S.C. reviewed and revised the manuscript. All authors read and approved the final manuscript.

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References

1. Sanz M, Herrera D, Kebschull M, Chapple I, Jepsen S, Berglundh T, Sculean A, Tonetti MS. Treatment of Stage I-III Periodontitis-the EFP S3 Level Clinical Practice Guideline. J CLIN PERIODONTOL. 2020; 47 Suppl 22:4-60. doi:10.1111/jcpe.13290.
2. Herrera D, Berglundh T, Schwarz F, et al. Prevention and Treatment of Peri-Implant Diseases-the EFP S3 Level Clinical Practice Guideline. J CLIN PERIODONTOL. 2023; 50 Suppl 26:4-76. doi:10.1111/jcpe.13823.
3. Reis INRD, Huamán-Mendoza AA, Ramadan D, Honório HM, Naenni N, Romito GA, Holzhausen M, Pannuti CM. The Prevalence of Peri-Implant Mucositis and Peri-Implantitis Based On the World Workshop Criteria: A Systematic Review and Meta-Analysis. J DENT. 2025; 160:105914. doi:10.1016/j.jdent.2025.105914.
4. Herrera D, Sanz M, Kebschull M, Jepsen S, Sculean A, Berglundh T, Papapanou PN, Chapple I,

- Tonetti MS. Treatment of Stage IV Periodontitis: The EFP S3 Level Clinical Practice Guideline. J CLIN PERIODONTOL. 2022; 49 Suppl 24:4-71. doi:10.1111/jcpe.13639.
5. Noiri Y, Okami Y, Narimatsu M, Takahashi Y, Kawahara T, Ebisu S. Effects of Chlorhexidine, Minocycline, and Metronidazole On Porphyromonas Gingivalis Strain 381 in Biofilms. J PERIODONTOL. 2003; 74:1647-1651. doi:10.1902/jop.2003.74.11.1647.
 6. Walker CB, Pappas JD, Tyler KZ, Cohen S, Gordon JM. Antibiotic Susceptibilities of Periodontal Bacteria. In Vitro Susceptibilities to Eight Antimicrobial Agents. J PERIODONTOL. 1985; 251:190. doi:10.1016/s0174-3031(81)80057-1.
 7. Hagiwara S, Takamatsu N, Tominaga Y, Umeda M. Subgingival Distribution of Periodontopathic Bacteria in Adult Periodontitis and their Susceptibility to Minocycline-HCl. J PERIODONTOL. 1998; 69:92-99. doi:10.1902/jop.1998.69.1.92.
 8. Walker CB, Gordon JM, McQuilkin SJ, Niebloom TA, Socransky SS. Tetracycline: Levels of Achievable in Gingival Crevice Fluid and in Vitro Effect On Subgingival Organisms. Part II. Susceptibilities of Periodontal Bacteria. J PERIODONTOL. 1981; 52:613-616. doi:10.1902/jop.1981.52.10.613.
 9. Bastos LFS, de Oliveira ACP, Watkins LR, Moraes MFD, Coelho MM. Tetracyclines and Pain. N-S ARCH PHARMACOL. 2012; 385:225-241. doi:10.1007/s00210-012-0727-1.
 10. Walters JD. Characterization of Minocycline Transport by Human Neutrophils. J PERIODONTOL. 2006; 77:1964-1968. doi:10.1902/jop.2006.060096.
 11. Asadi A, Abdi M, Kouhsari E, et al. Minocycline, Focus On Mechanisms of Resistance, Antibacterial Activity, and Clinical Effectiveness: Back to the Future. J GLOB ANTIMICROB RE. 2020; 22:161-174. doi:10.1016/j.jgar.2020.01.022.
 12. Jones AA, Kornman KS, Newbold DA, Manwell MA. Clinical and Microbiological Effects of Controlled-Release Locally Delivered Minocycline in Periodontitis. J PERIODONTOL. 1994; 65:1058-1066. doi:10.1902/jop.1994.65.11.1058.
 13. Arnett MC, Chanthavisouk P, Costalonga M, Blue CM, Evans MD, Paulson DR. Effect of Scaling and Root Planing with and without Minocycline HCl Microspheres On Periodontal Pathogens and Clinical Outcomes: A Randomized Clinical Trial. J PERIODONTOL. 2023; 94:1133-1145. doi:10.1002/JPER.23-0002.
 14. Laza GMRA, Sufaru IG, Martu MRAA, Martu C, Diaconu-Popa DA, Jelihovschi I, Martu S. Effects of Locally Delivered Minocycline Microspheres in Postmenopausal Female Patients with Periodontitis: A Clinical and Microbiological Study. DIAGNOSTICS. 2022; 12:1310. doi:10.3390/diagnostics12061310.
 15. Williams RC, Paquette DW, Offenbacher S, et al. Treatment of Periodontitis by Local Administration of Minocycline Microspheres: A Controlled Trial. J PERIODONTOL. 2001; 72:1535-1544. doi:10.1902/jop.2001.72.11.1535.
 16. Lai PC, Schibler MR, Walters JD. Azithromycin Enhances Phagocytic Killing of Aggregatibacter Actinomycetemcomitans Y4 by Human Neutrophils. J PERIODONTOL. 2015; 86:155-161. doi:10.1902/jop.2014.140183.
 17. Silvestri M, Oddera S, Eftimiadi C, Rossi GA. Azithromycin Induces in Vitro a Time-Dependent Increase in the Intracellular Killing of Staphylococcus Aureus by Human Polymorphonuclear Leucocytes without Damaging Phagocytes. J ANTIMICROB CHEMOTH. 1995; 36:941-950.

doi:10.1093/jac/36.6.941.

18. Wildfeuer A, Laufen H, Zimmermann T. Uptake of Azithromycin by Various Cells and its Intracellular Activity Under in Vivo Conditions. *ANTIMICROB AGENTS CH.* 1996; 40:75-79. doi:10.1128/aac.40.1.75.
19. Desnottes JF. Quinolones and Phagocytosis. *Pathol Biol (Paris).* 1987; 35:1426-1430.
20. Grassi GG, Fietta A. Antibiotics and their Interaction with the Host Defense System in Vivo. *J CHEMOTHERAPY.* 1991; 3 Suppl 1:112-115.
21. Ettel P, Sehgal ANA, Harrison N, Pickl WF, Grabmeier-Pfistershammer K. Glycopeptide Antibiotics Impair Neutrophil Effector Functions. *INT ARCH ALLERGY IMM.* 2023; 184:932-948. doi:10.1159/000530865.
22. Homsí S, Federico F, Croci N, Palmier B, Plotkine M, Marchand-Leroux C, Jafarian-Tehrani M. Minocycline Effects On Cerebral Edema: Relations with Inflammatory and Oxidative Stress Markers Following Traumatic Brain Injury in Mice. *BRAIN RES.* 2009; 1291:122-132. doi:10.1016/j.brainres.2009.07.031.
23. Liu Y, Li Z, Khan S, Zhang R, Wei R, Zhang Y, Xue M, Yong VW. Neuroprotection of Minocycline by Inhibition of Extracellular Matrix Metalloproteinase Inducer Expression Following Intracerebral Hemorrhage in Mice. *NEUROSCI LETT.* 2021; 764:136297. doi:10.1016/j.neulet.2021.136297.
24. Rifkin BR, Vernillo AT, Golub LM. Blocking Periodontal Disease Progression by Inhibiting Tissue-Destructive Enzymes: A Potential Therapeutic Role for Tetracyclines and their Chemically-Modified Analogs. *J PERIODONTOL.* 1993; 64:819-827. doi:10.1902/jop.1993.64.8s.819.
25. Yrjänheikki J, Keinanen R, Pellikka M, Hokfelt T, Koistinaho J. Tetracyclines Inhibit Microglial Activation and are Neuroprotective in Global Brain Ischemia. *P NATL ACAD SCI USA.* 1998; 95:15769-15774. doi:10.1073/pnas.95.26.15769.
26. Yrjänheikki J, Tikka T, Keinänen R, Goldsteins G, Chan PH, Koistinaho J. A Tetracycline Derivative, Minocycline, Reduces Inflammation and Protects Against Focal Cerebral Ischemia with a Wide Therapeutic Window. *P NATL ACAD SCI USA.* 1999; 96:13496-13500. doi:10.1073/pnas.96.23.13496.
27. Liu D, Yang PS. Minocycline Hydrochloride Nanoliposomes Inhibit the Production of TNF- α in LPS-Stimulated Macrophages. *INT J NANOMED.* 2012; 7:4769-4775. doi:10.2147/IJN.S34036.
28. Knuckley B, Luo Y, Thompson PR. Profiling Protein Arginine Deiminase 4 (PAD4): A Novel Screen to Identify PAD4 Inhibitors. *BIOORGAN MED CHEM.* 2008; 16:739-745. doi:10.1016/j.bmc.2007.10.021.
29. Jones JE, Causey CP, Knuckley B, Slack-Noyes JL, Thompson PR. Protein Arginine Deiminase 4 (PAD4): Current Understanding and Future Therapeutic Potential. *Current opinion in drug discovery & development.* 2009; 12:616.
30. Lewis HD, Liddle J, Coote JE, et al. Inhibition of PAD4 Activity is Sufficient to Disrupt Mouse and Human NET Formation. *NAT CHEM BIOL.* 2015; 11:189-191. doi:10.1038/nchembio.1735.
31. Mondal S, Thompson PR. Protein Arginine Deiminases (PADs): Biochemistry and Chemical Biology of Protein Citrullination. *ACCOUNTS CHEM RES.* 2019; 52:818-832. doi:10.1021/acs.accounts.9b00024.
32. Tan C, Aziz M, Wang P. The Vitals of NETs. *J LEUKOCYTE BIOL.* 2021; 110:797-808.

doi:10.1002/JLB.3RU0620-375R.

33. Magán Fernández A, O'Valle F, Abadía Molina F, Muñoz R, Puga Guil P, Mesa F. Characterization and Comparison of Neutrophil Extracellular Traps in Gingival Samples of Periodontitis and Gingivitis: A Pilot Study. *J PERIODONTAL RES.* 2019; 54:218-224. doi:10.1111/jre.12621.
34. Dionigi C, Larsson L, Carcuac O, Berglundh T. Cellular Expression of DNA Damage/Repair and Reactive Oxygen/Nitrogen Species in Human Periodontitis and Peri-Implantitis Lesions. *J CLIN PERIODONTOL.* 2020; 47:1466-1475. doi:10.1111/jcpe.13370.
35. Carcuac O, Berglundh T. Composition of Human Peri-Implantitis and Periodontitis Lesions. *J DENT RES.* 2014; 93:1083-1088. doi:10.1177/0022034514551754.
36. Nizam N, Gümüş PN, Pitkänen J, Tervahartiala T, Sorsa T, Buduneli N. Serum and Salivary Matrix Metalloproteinases, Neutrophil Elastase, Myeloperoxidase in Patients with Chronic Or Aggressive Periodontitis. *INFLAMMATION.* 2014; 37:1771-1778. doi:10.1007/s10753-014-9907-0.
37. Tervahartiala T, Konttinen YT, Ingman T, Häyrynen-Immonen R, Ding Y, Sorsa T. Cathepsin G in Gingival Tissue and Crevicular Fluid in Adult Periodontitis. *J CLIN PERIODONTOL.* 1996; 23:68-75. doi:10.1111/j.1600-051X.1996.tb00537.x.
38. Kunimatsu K, Ozaki Y, Hara Y, Aoki Y, Yamamoto K, Kato I. Immunohistochemical Study of Cathepsin G and Medullasin in Inflamed Gingival Tissues From Periodontal Patients. *J PERIODONTAL RES.* 1997; 32:264-270. doi:10.1111/j.1600-0765.1997.tb00533.x.
39. Altman LC, Baker C, Fleckman P, Luchtel D, Oda D. Neutrophil-Mediated Damage to Human Gingival Epithelial Cells. *J PERIODONTAL RES.* 1992; 27:70-79. doi:10.1111/j.1600-0765.1992.tb02088.x.
40. Hiyoshi T, Domon H, Maekawa T, et al. Neutrophil Elastase Aggravates Periodontitis by Disrupting Gingival Epithelial Barrier Via Cleaving Cell Adhesion Molecules. *SCI REP-UK.* 2022; 12:8159. doi:10.1038/s41598-022-12358-3.
41. Tseng HY, Chen YW, Lee BS, Chang PC, Wang YP, Lin CP, Cheng SJ, Kuo MYP, Hou HH. The Neutrophil Elastase-Upregulated Placenta Growth Factor Promotes the Pathogenesis and Progression of Periodontal Disease. *J PERIODONTOL.* 2022; 93:1401-1410. doi:10.1002/JPER.21-0587.
42. Curren B, Ahmed T, Howard DR, et al. IL-33-Induced Neutrophilic Inflammation and NETosis Underlie Rhinovirus-Triggered Exacerbations of Asthma. *MUCOSAL IMMUNOL.* 2023; 16:671-684. doi:10.1016/j.mucimm.2023.07.002.
43. Warnatsch A, Ioannou M, Wang Q, Papayannopoulos V. Neutrophil Extracellular Traps License Macrophages for Cytokine Production in Atherosclerosis. *SCIENCE.* 2015; 349:312-316. doi:10.1126/science.1261040.
44. Guimarães-Costa AB, Nascimento MTC, Froment GS, Soares RPP, Morgado FN, Conceição-Silva F, Saraiva EM. *Leishmania Amazonensis* Promastigotes Induce and are Killed by Neutrophil Extracellular Traps. *Proceedings of the National Academy of Sciences - PNAS.* 2009; 106:6748-6753. doi:10.1073/pnas.0900226106.
45. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. Neutrophil Extracellular Traps Kill Bacteria. *Science (American Association for the Advancement of Science).* 2004; 303:1532-1535. doi:10.1126/science.1092385.
46. Mikhalechik E, Basyreva LY, Gusev SA, et al. Activation of Neutrophils by Mucin-Vaterite

- Microparticles. *INT J MOL SCI*. 2022; 23:10579. doi:10.3390/ijms231810579.
47. Beiter K, Wartha F, Albiger B, Normark S, Zychlinsky A, Henriques-Normark B. An Endonuclease Allows *Streptococcus Pneumoniae* to Escape From Neutrophil Extracellular Traps. *CURR BIOL*. 2006; 16:401-407. doi:10.1016/j.cub.2006.01.056.
 48. Palmer LJ, Chapple ILC, Wright HJ, Roberts A, Cooper PR. Extracellular Deoxyribonuclease Production by Periodontal Bacteria. *J PERIODONTAL RES*. 2012; 47:439-445. doi:10.1111/j.1600-0765.2011.01451.x.
 49. Berends ETM, Horswill AR, Haste NM, Monestier M, Nizet V, von Köckritz-Blickwede M. Nuclease Expression by *Staphylococcus Aureus* Facilitates Escape From Neutrophil Extracellular Traps. *J INNATE IMMUN*. 2010; 2:576-586. doi:10.1159/000319909.
 50. Mutua V, Gershwin LJ. A Review of Neutrophil Extracellular Traps (NETs) in Disease: Potential Anti-NETs Therapeutics. *CLIN REV ALLERG IMMUN*. 2020; 61:194-211. doi:10.1007/s12016-020-08804-7.
 51. Boeltz S, Amini P, Anders HJ, et al. To NET Or Not to NET:Current Opinions and State of the Science Regarding the Formation of Neutrophil Extracellular Traps. *CELL DEATH DIFFER*. 2019; 26:395-408. doi:10.1038/s41418-018-0261-x.
 52. Kapellos TS, Baßler K, Fujii W, et al. Systemic Alterations in Neutrophils and their Precursors in Early-Stage Chronic Obstructive Pulmonary Disease. *CELL REP*. 2023; 42:112525. doi:10.1016/j.celrep.2023.112525.
 53. Liu X, Arfman T, Wichapong K, Reutelingsperger CPM, Voorberg J, Nicolaes GAF. PAD4 Takes Charge During Neutrophil Activation: Impact of PAD4 Mediated NET Formation On Immune-Mediated Disease. *J THROMB HAEMOST*. 2021; 19:1607-1617. doi:10.1111/jth.15313.
 54. Coffelt SB, Wellenstein MD, de Visser KE. Neutrophils in Cancer: Neutral No More. *NAT REV CANCER*. 2016; 16:431-446. doi:10.1038/nrc.2016.52.
 55. Luo J, Thomassen JQ, Nordestgaard BG, Tybjaerg-Hansen A, Frikke-Schmidt R. Neutrophil Counts and Cardiovascular Disease. *EUR HEART J*. 2023; 44:4953-4964. doi:10.1093/eurheartj/ehad649.
 56. Huang J, Hong W, Wan M, Zheng L. Molecular Mechanisms and Therapeutic Target of NETosis in Diseases. *MEDCOMM*. 2022; 3:e162. doi:10.1002/mco2.162.
 57. Willis VC, Gizinski AM, Banda NK, et al. N-Alpha-Benzoyl-N5-(2-Chloro-1-Iminoethyl)-L-Ornithine Amide, a Protein Arginine Deiminase Inhibitor, Reduces the Severity of Murine Collagen-Induced Arthritis. *J IMMUNOL*. 2011; 186:4396-4404. doi:10.4049/jimmunol.1001620.
 58. Knight JS, Luo W, O Dell AA, et al. Peptidylarginine Deiminase Inhibition Reduces Vascular Damage and Modulates Innate Immune Responses in Murine Models of Atherosclerosis. *CIRC RES*. 2014; 114:947-956. doi:10.1161/CIRCRESAHA.114.303312.
 59. Papatheanasiou E, Conti P, Carinci F, Lauritano D, Theoharides TC. IL-1 Superfamily Members and Periodontal Diseases. *J DENT RES*. 2020; 99:1425-1434. doi:10.1177/0022034520945209.
 60. Mazurek-Mochol M, Bonsmann T, Mochol M, Poniewierska-Baran A, Pawlik A. The Role of Interleukin 6 in Periodontitis and its Complications. *International Journal of Molecular Sciences*. 2024; 25:2146. doi:10.3390/ijms25042146.
 61. Bonsmann T, Mochol M, Bonsmann E, Jablonowski L, Pawlik A, Raśławska-Socha J, Lipski M, Mazurek-Mochol M. The Role of IL-17 in Periodontitis and its Systemic Connections. *International*

Journal of Molecular Sciences. 2025; 26:10902. doi:10.3390/ijms262210902.

62. Oliveira JA, de Oliveira Alves R, Nascimento IM, Hidalgo MAR, Scarel-Caminaga RM, Pigossi SC. Pro- And Anti-Inflammatory Cytokines and Osteoclastogenesis-Related Factors in Peri-Implant Diseases: Systematic Review and Meta-Analysis. BMC ORAL HEALTH. 2023; 23:420. doi:10.1186/s12903-023-03072-1.
63. Malmqvist S, Clark R, Johannsen G, Johannsen A, Boström EA, Lira-Junior R. Immune Cell Composition and Inflammatory Profile of Human Peri-Implantitis and Periodontitis Lesions. CLIN EXP IMMUNOL. 2024; 217:173-182. doi:10.1093/cei/uxae033.
64. Zhang J, Wang AX, Wu Y, Zhang S. IL-1 Receptor Antagonist (IL-1RA) Suppresses a Hyper-IL-17 Response-Mediated Bone Loss in a Murine Experimental Periodontitis. ARCH ORAL BIOL. 2022; 144:105555. doi:10.1016/j.archoralbio.2022.105555.
65. Oates TW, Graves DT, Cochran DL. Clinical, Radiographic and Biochemical Assessment of IL-1/TNF- α Antagonist Inhibition of Bone Loss in Experimental Periodontitis. J CLIN PERIODONTOL. 2002; 29:137-143. doi:10.1034/j.1600-051x.2002.290208.x.
66. Vieira GHA, Rivas ACA, Costa KF, Oliveira LFF, Suzuki KT, Messoria MR, Ricoldi MS, de Almeida ALG, Taba M. Specific Inhibition of IL-6 Receptor Attenuates Inflammatory Bone Loss in Experimental Periodontitis. J PERIODONTOL. 2021; 92:1460-1469. doi:10.1002/jper.20-0455.
67. Wang HL, Pazmino CG, Collins A, Ong HS, Chudri R, Giannobile WV. Protein Biomarkers and Microbial Profiles in Peri-Implantitis. CLIN ORAL IMPLAN RES. 2015; 27:1129-1136. doi:10.1111/clr.12708.
68. Ghassib I, Chen Z, Zhu J, Wang HL. Use of IL-1 B, IL-6, TNF- α , and MMP-8 Biomarkers to Distinguish Peri-Implant Diseases: A Systematic Review and Meta-Analysis. CLIN IMPLANT DENT R. 2019; 21:190-207. doi:10.1111/cid.12694.
69. Relvas M, Mendes-Frias A, Gonçalves M, Salazar F, López-Jarana P, Silvestre R, Viana Da Costa A. Salivary IL-1B, IL-6, and IL-10 are Key Biomarkers of Periodontitis Severity. INT J MOL SCI. 2024; 25:8401. doi:10.3390/ijms25158401.
70. Teles F, Martin L, Patel M, et al. Gingival Crevicular Fluid Biomarkers During Periodontitis Progression and After Periodontal Treatment. J CLIN PERIODONTOL. 2025; 52:40-55. doi:10.1111/jcpe.14061.
71. Teles F, Chandrasekaran G, Martin L, et al. Salivary and Serum Inflammatory Biomarkers During Periodontitis Progression and After Treatment. J CLIN PERIODONTOL. 2024; 51:1619-1631. doi:10.1111/jcpe.14048.
72. Zhang ZH, Zhan ZY, Jiang M, Wang XY, Quan SL, Wu YL, Nan JX, Lian LH. Casting NETs On Psoriasis: The Modulation of Inflammatory Feedback Targeting IL-36/IL-36R Axis. INT IMMUNOPHARMACOL. 2024; 142:113190. doi:10.1016/j.intimp.2024.113190.
73. Cui X, Zhao L, Su Q, Feng Y, Guo J, Zhu J, Yang J, Zhang S. Neutrophil Extracellular Traps Mediate Macrophage Polarization and Pyroptosis through the TLR9-NF- κ B/AIM2 Pathway Exacerbating Myositis-Associated Interstitial Lung Disease. INT IMMUNOPHARMACOL. 2025; 165:115431. doi:10.1016/j.intimp.2025.115431.
74. Tan H, Zhang S, Zhang Z, Zhang J, Wang Z, Liao J, Qiu X, Jia E. Neutrophil Extracellular Traps Promote M1 Macrophage Polarization in Gouty Inflammation Via Targeting Hexokinase-2. FREE RADICAL BIO MED. 2024; 224:540-553. doi:10.1016/j.freeradbiomed.2024.09.009.

75. Liu B, Deng Y, Duan Z, Chu C, Wang X, Yang C, Li J, Ding W. Neutrophil Extracellular Traps Promote Intestinal Barrier Dysfunction by Regulating Macrophage Polarization During Trauma/Hemorrhagic Shock Via the TGF- β Signaling Pathway. *CELL SIGNAL*. 2024; 113:110941. doi:10.1016/j.cellsig.2023.110941.
76. Song C, Li H, Li Y, et al. NETs Promote ALI/ARDS Inflammation by Regulating Alveolar Macrophage Polarization. *EXP CELL RES*. 2019; 382:111486. doi:10.1016/j.yexcr.2019.06.031.
77. Liu L, Lan Z, Liu X, Chen Y, Chen Z, Cheng L, Hu T. NETs Accelerate Aortic Valve Calcification by Promoting M1 Macrophage Polarization through the TLR9 Signaling Pathway. *MOL CELL BIOCHEM*. 2025; 480:6225-6238. doi:10.1007/s11010-025-05375-z.
78. Guo Y, Zhou H, Wang Y, Gu Y. Activated NETosis of Bone Marrow Neutrophils Up-Regulates Macrophage Osteoclastogenesis Via CGAS-STING/AKT2 Pathway to Promote Osteoporosis. *EXP CELL RES*. 2025; 446:114477. doi:10.1016/j.yexcr.2025.114477.
79. Carmona-Rivera C, Kaplan MJ, O Neil LJ. Neutrophils in Inflammatory Bone Diseases. *CURR OSTEOPOROS REP*. 2024; 22:280-289. doi:10.1007/s11914-024-00865-3.
80. Schneider AH, Taira TM, Públio GA, et al. Neutrophil Extracellular Traps Mediate Bone Erosion in Rheumatoid Arthritis by Enhancing RANKL-Induced Osteoclastogenesis. *BRIT J PHARMACOL*. 2024; 181:429-446. doi:10.1111/bph.16227.
81. Kim TS, Silva LM, Theofilou VI, et al. Neutrophil Extracellular Traps and Extracellular Histones Potentiate IL-17 Inflammation in Periodontitis. *J EXP MED*. 2023; 220:e20221751. doi:10.1084/jem.20221751.
82. Zhao X, Wang Q, Wang W, Lu S. Increased Neutrophil Extracellular Traps Caused by Diet-Induced Obesity Delay Fracture Healing. *FASEB J*. 2024; 38:e70126. doi:10.1096/fj.202401523R.
83. Jun HK, Jung YJ, Choi BK. *Treponema Denticola*, *Porphyromonas Gingivalis*, and *Tannerella Forsythia* Induce Cell Death and Release of Endogenous Danger Signals. *ARCH ORAL BIOL*. 2017; 73:72-78. doi:10.1016/j.archoralbio.2016.09.010.
84. Alves CH, Russi KL, Rocha NCO, Bastos F, Darrieux M, Parisotto TM, Girardello R. Host-Microbiome Interactions Regarding Peri-Implantitis and Dental Implant Loss. *J TRANSL MED*. 2022; 20:425. doi:10.1186/s12967-022-03636-9.
85. Belibasakis GN, Manoil D. Microbial Community-Driven Etiopathogenesis of Peri-Implantitis. *J DENT RES*. 2021; 100:21-28. doi:10.1177/0022034520949851.
86. Carvalho ÉBS, Romandini M, Sadilina S, Sant'Ana ACP, Sanz M. Microbiota Associated with Peri-Implantitis-a Systematic Review with Meta-Analyses. *CLIN ORAL IMPLAN RES*. 2023; 34:1176-1187. doi:10.1111/clr.14153.
87. Craig WA. Pharmacokinetic/Pharmacodynamic Parameters: Rationale for Antibacterial Dosing of Mice and Men. *CLIN INFECT DIS*. 1998; 26:1-10. doi:10.1086/516284.