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Original Article

Modulation of Pro-Inflammatory Cytokine Release by Anti-Rheumatic Drugs in Oral Cells During Microbial Interactions

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ABSTRACT

Individuals with rheumatoid arthritis (RA) who also have periodontitis often show reduced inflammation in periodontal tissues, likely as a consequence of anti-rheumatic therapy. This in vitro investigation explored whether common RA medications could affect the secretion of IL-8 and IL-1β by both professional and non-professional immune cells when exposed to microbial stimuli. Periodontal ligament (PDL) fibroblasts, MONO-MAC-6 monocytes, and gingival keratinocytes were treated with ibuprofen, prednisolone, or methotrexate, either alone or in combination with lysates of Fusobacterium nucleatum or Candida albicans. Cytokine levels in the supernatants were then assessed, with IL-1β measured exclusively in MONO-MAC-6 cells. Stimulation with F. nucleatum lysate triggered the most pronounced pro-inflammatory cytokine release in PDL fibroblasts and MONO-MAC-6 cells, whereas the impact of the anti-rheumatic drugs on cytokine production was generally limited. Interestingly, prednisolone enhanced IL-8 release from MONO-MAC-6 cells exposed to F. nucleatum, whereas methotrexate reduced it. Furthermore, anti-inflammatory drugs promoted the adherence of C. albicans to epithelial cells. These results highlight that, in RA patients, controlling subgingival microbial load remains essential, but anti-rheumatic therapies may influence the immune response to oral microorganisms.

Keywords: Oral cells, Rheumatoid arthritis, Periodontitis, Proinflammatory cytokines, Anti-inflammatory drugs

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Introduction

Periodontitis ranks among the most prevalent chronic diseases globally, with severe cases affecting roughly 11% of the population [1]. The disease arises from a persistent inflammatory response to an imbalanced subgingival microbiota, ultimately leading to the breakdown of the tissues supporting teeth [2]. Fusobacterium nucleatum, an opportunistic bacterium, often acts as a bridge between commensal and pathogenic species, playing a central role in microbial pathogenicity [3]. Inflammasome activation is a key factor in periodontal tissue destruction, driving the maturation of pro-inflammatory cytokines such as interleukin (IL)-1β and IL-18 [4]. IL-8 (CXCL9), a chemokine of the CXC family, recruits neutrophils and triggers degranulation, with its expression levels correlating with disease severity [5, 6].

Periodontitis is not confined to local tissue damage but is associated with systemic conditions that can influence disease progression and treatment outcomes. Diabetes mellitus was among the first systemic disorders clearly linked to periodontal attachment loss [7]. More recently, rheumatoid arthritis (RA) has received significant attention due to its association with periodontitis. Analysis of health insurance data from over 500,000 individuals in South Korea revealed a higher prevalence of periodontitis among RA patients (19.6%) compared to non-RA individuals (16.6%), as well as a higher prevalence of RA in patients with

periodontitis (6.2%) compared to those without (5.2%) [8].

RA is a chronic systemic inflammatory disease, characterized by joint stiffness, swelling, and pain, often accompanied by autoantibodies against rheumatoid factor and citrullinated proteins [9]. In affected joints, inflammatory cell infiltration is accompanied by elevated concentrations of IL-1, IL-6, IL-8, IL-10, and MCP-1, potentially linked to neutrophil autophagy, which may contribute to disease pathogenesis [10, 11]. Historically, IL-1 and tumor necrosis factor (TNF) were identified as key mediators of bone and tissue destruction in RA, with IL-1 acting locally and TNF exerting systemic effects. Therapeutic strategies aim to control inflammation and reduce disease activity by limiting IL-1 and TNF activity [12]. Common treatments include disease-modifying anti-rheumatic drugs (DMARDs) and nonsteroidal anti-inflammatory drugs (NSAIDs) [9]. This study specifically examines methotrexate, a DMARD that reduces inflammation and enhances TNF inhibitor efficacy [12, 13]; ibuprofen, an NSAID that limits local inflammation by reversibly inhibiting COX-1 and COX-2 enzymes [13]; and prednisolone, a glucocorticoid that suppresses neutrophil migration and normalizes increased capillary permeability [14].

Porphyromonas gingivalis, a well-known periodontal pathogen, has been suggested as a potential link between periodontitis and RA, as its peptidyl arginine deiminase can citrullinate peptides, and RA patients show increased antibodies against this bacterium [15, 16]. However, recent animal studies indicate that other bacteria may also play important roles. Microbiome analyses have shown an enrichment of F. nucleatum in periodontitis compared to healthy periodontal tissues [17]. In murine models, exposure to F. nucleatum or Aggregatibacter actinomycetemcomitans accelerated arthritis onset and progression [18], and DNA from F. nucleatum has been detected in the synovial fluid of arthritis patients [19].

Previous investigations by our group revealed that tooth loss in RA patients did not always correlate with classical signs of periodontitis, such as deep probing depths or bleeding on probing [16, 20, 21]. This observation suggests that long-term use of anti-rheumatic drugs might suppress typical periodontal inflammation, prompting the question of whether these medications interfere with local immune responses. Additionally, Candida albicans was frequently detected in subgingival biofilm samples from RA patients, unlike in periodontally healthy individuals or periodontitis patients without systemic disease, justifying its inclusion in this study.

The current in vitro study aimed to address two primary questions: (i) whether anti-inflammatory drugs commonly used in RA therapy can modulate IL-8 and IL-1β release from professional and non-professional oral immune cells, and (ii) how these drugs influence the interaction between these cells and microorganisms.

Materials and Methods

Cells

For the experiments, three cell types were utilized: periodontal ligament (PDL) fibroblasts, the human monocytic cell line MONO-MAC-6 (DSMZ no. ACC 124), and telomerase-immortalized gingival keratinocytes (TIGK; ATCC-CRL-3397). PDL fibroblasts were obtained from extracted teeth of three individual donors. All donors provided written consent for research use of their tissues, in line with the Cantonal Ethics Committee (KEK) guidelines. As the collected samples were fully anonymized and could not be traced back to the donors, formal ethical approval was not required. The isolation and culture of these cells followed established protocols [22].

PDL fibroblasts were maintained in DMEM, whereas MONO-MAC-6 cells were cultured in RPMI 1640, with both media supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). TIGK cells were grown in specialized Keratinocyte Growth Medium (KGM-Gold; Lonza, Basel, Switzerland).

Anti-rheumatic drugs

The following drugs were applied at the specified final concentrations in the experiments.

- ibuprofen (Brufen 600 mg, BGP Products GmbH, Baar, CH): 2, 10, and 50 µg/ml
- prednisolone (Spricort 20, Spirig Health Care AG, Egerkingen, CH): 20, 100, and 500 ng/ml
- Methotrexate (Methotrexat Farmos 10 mg, Orion Pharma AG, Zug, CH): 1, 5, and 25 µg/ml.

The selected drug concentrations were based on previously reported levels in plasma or tissues [23-25]. Cytotoxic effects at these concentrations were assessed using trypan blue exclusion assays. For the experiments, stock solutions of each drug were prepared in phosphate-buffered saline (PBS) and serially diluted at a 1:4 ratio. The initial dilution contained ten times the highest target concentration. PBS alone served as the negative control.

Microorganisms

For the study, lysates of Fusobacterium nucleatum (ATCC 25586) and Candida albicans (ATCC 76615)

were generated. Bacterial or fungal suspensions in PBS were adjusted to an OD600 of 1 and subjected to 20 minutes of ultrasonication at 280 W. The homogenized mixture was subsequently centrifuged at 10,000 × g for 10 minutes at 20°C, and the clarified supernatant was passed through a 0.4 µm filter. This treatment produced a preparation corresponding to an approximate MOI of 20:1 (bacteria:cells), allowing cell stimulation with microbial components rather than whole organisms.
In the case of TIGK cells, C. albicans was prepared at an OD600 of 0.2 in PBS, resulting in a MOI of roughly 5:1 (fungi:cells) for experimental stimulation.

Methods MONO-MAC-6-cells
MONO-MAC-6 cells were first centrifuged at 250 × g for 5 minutes, after which the supernatant was discarded. The cell pellet was washed twice with PBS and resuspended in RPMI 1640 medium containing 0.5% FBS to a final density of 1×10^6 cells/ml. For the assay, the cell suspension was combined with microbial lysate and the respective drug solution in an 8:1:1 ratio, and 1 ml of this mixture was added to each well of a 24-well plate. After plating, the cells were incubated for 18 hours. Following incubation, the contents of each well were transferred to 1.5 ml tubes and centrifuged at 10,000 × g for 5 minutes at 20°C. The resulting supernatants were collected and stored at −80°C until subsequent analysis by ELISA.

Methods PDL fibroblasts
Prior to the assays, PDL fibroblasts were seeded into 24-well plates and cultured until they reached confluence. The culture medium was then removed, and the cells were washed twice with PBS. Subsequently, 1 ml of the drug–microbe–cell mixture, prepared at a 1:1:8 ratio, was added to each well. For these experiments, the FBS content in the medium was reduced to 0.5%, and cells were seeded at a density of 3×10^5 cells/cm². Only fibroblasts within five passages were used. The remaining steps followed the same protocol as described for MONO-MAC-6 cells. All experiments were performed in triplicate and repeated independently three times.

Methods TIGK cells
TIGK cells were prepared at a concentration of 1×10^5 cells/ml in culture medium, and 1 ml of this suspension was plated into each well of a 24-well plate 24 hours prior to the start of the experiments. On the day of the assay, the cells were examined to ensure appropriate confluency and then washed twice with PBS. Subsequently, 1 ml of the drug–microbe–cell mixture, prepared at a 1:1:8 ratio, was added to each well containing TIGK cells. After 6 hours of incubation, the culture medium was collected, processed as previously described (centrifugation and storage of supernatants at −80°C).
Following this, the cells were washed twice with PBS to remove non-adherent C. albicans. Next, 1 ml of ice-cold distilled water was added to each well, and the plates were left for 15 minutes with thorough mixing to release adherent (including invasive) fungal cells. The resulting suspensions were then plated onto agar to quantify colony-forming units of C. albicans.

ELISA
Preliminary experiments were conducted to determine which biomarkers were most suitable for analysis. IL-8 was consistently released by all cell types, whereas IL-1β was specifically measured in supernatants from MONO-MAC-6 cells following microbial exposure. Cytokine levels were quantified using ELISA kits (R&D Systems, Minnesota, MN, USA) in accordance with the manufacturer's guidelines. The assays had a minimum detection threshold of 1 pg/ml for both IL-8 and IL-1β.

Statistical analysis
Statistical analyses were performed using SPSS version 24.0 (IBM, Armonk, NY, USA). One-way ANOVA followed by Bonferroni post-hoc tests was applied to assess differences between groups. Prior to analysis, the normality of the data was confirmed through evaluation of skewness and kurtosis. Post-hoc comparisons were limited to the effects of each drug versus the corresponding microbial control or, at specified drug concentrations, versus cells cultured without microbes. A p-value of less than 0.05 was considered statistically significant.

Results and Discussion

MONO-MAC-6 cells
In the absence of any stimulation, the cells released an average of 42.47 ± 24.29 pg/ml of IL-8 after 18 hours of incubation. Treatment with prednisolone or ibuprofen at the tested concentrations did not significantly alter IL-8 secretion. In contrast, exposure to methotrexate at 1, 5, and 25 µg/ml resulted in significantly elevated IL-8 levels compared to the control ($p = 0.001$, $p = 0.020$, and $p < 0.001$, respectively; **Figure 1a**).

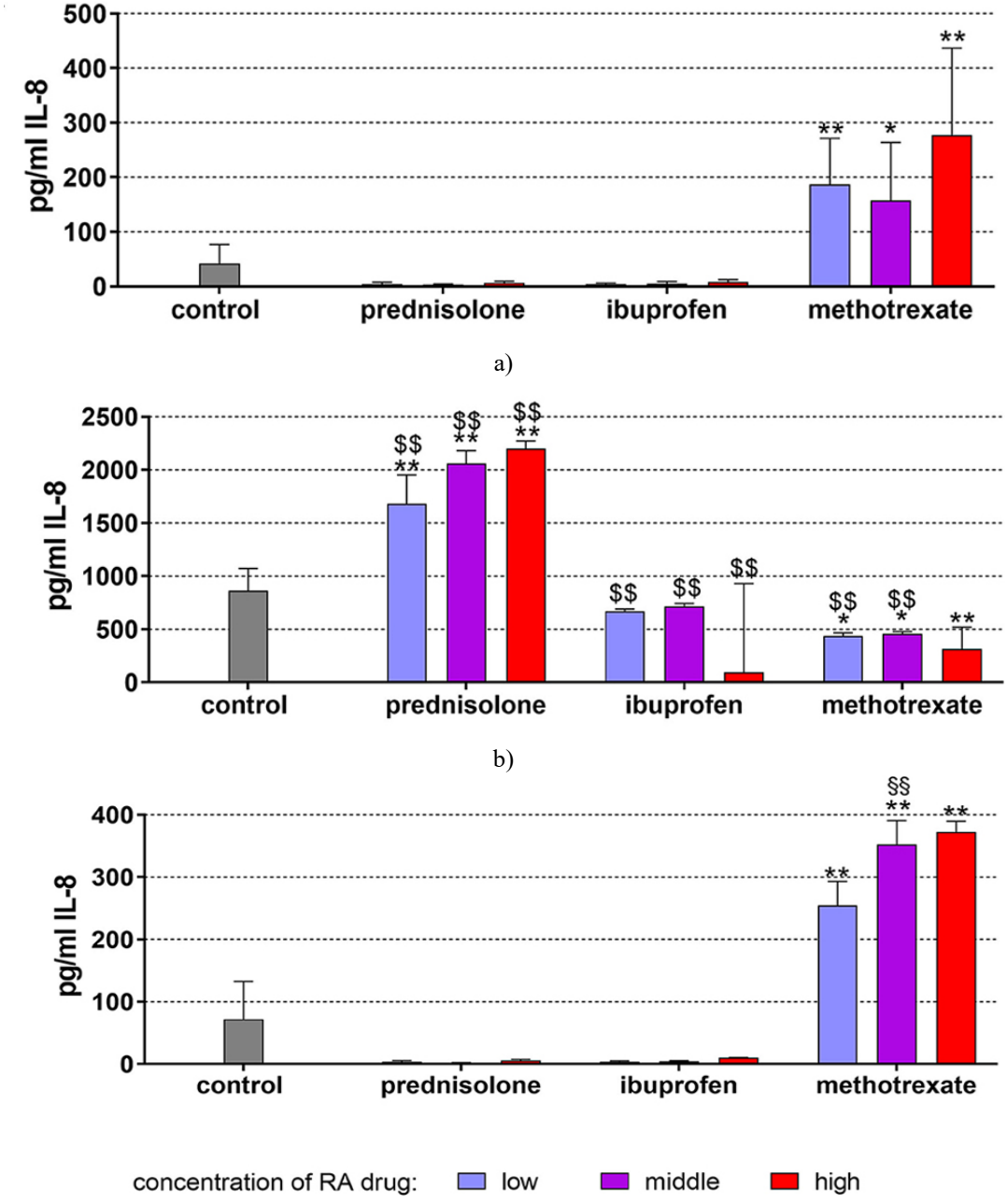

Figure 1. IL-8 production by MONO-MAC-6 cells after 18 hours of treatment with prednisolone (low: 20 ng/ml, medium: 100 ng/ml, high: 500 ng/ml), ibuprofen (low: 2 µg/ml, medium: 10 µg/ml, high: 50 µg/ml), and methotrexate (low: 1 µg/ml, medium: 5 µg/ml, high: 25 µg/ml) under three experimental conditions: (a) without microbial stimuli, (b) following stimulation with Fusobacterium nucleatum lysate, and (c) after exposure to Candida albicans lysate. Bars indicate standard deviations, and columns represent mean values. Statistical significance: *p < 0.05 vs. control, **p < 0.01 vs. control, $^{\$\$}$p < 0.01 vs. the respective group without microbial stimulation.

Stimulation with F. nucleatum lysate induced a strong increase in IL-8 secretion, reaching 862.73 ± 209.00 pg/ml ($p < 0.001$). When anti-rheumatic drugs were added, IL-8 levels remained higher than in non-stimulated controls. In comparison to cells exposed to F. nucleatum alone, ibuprofen did not significantly alter cytokine release. Prednisolone further amplified IL-8 production, with the highest concentration (500 ng/ml) yielding 2,201.46 ± 70.60 pg/ml ($p < 0.001$). Methotrexate, on the other hand, attenuated IL-8 secretion relative to the bacterial lysate alone, but levels still exceeded those in cells not exposed to F. nucleatum **(Figure 1b)**.

In contrast, stimulation with C. albicans lysate did not significantly affect IL-8 release (73.23 ± 60.53 pg/ml). Methotrexate significantly increased IL-8 secretion across all tested doses ($p < 0.001$), and at 5 µg/ml, IL-8 levels were higher in the presence of C. albicans than in cells cultured without microbial stimulation ($p = 0.006$; **Figure 1c**).

For IL-1β, unstimulated MONO-MAC-6 cells released low amounts, averaging 2.70 ± 2.65 pg/ml after 18 hours. None of the drug treatments significantly altered these basal levels **(Figure 2a)**.

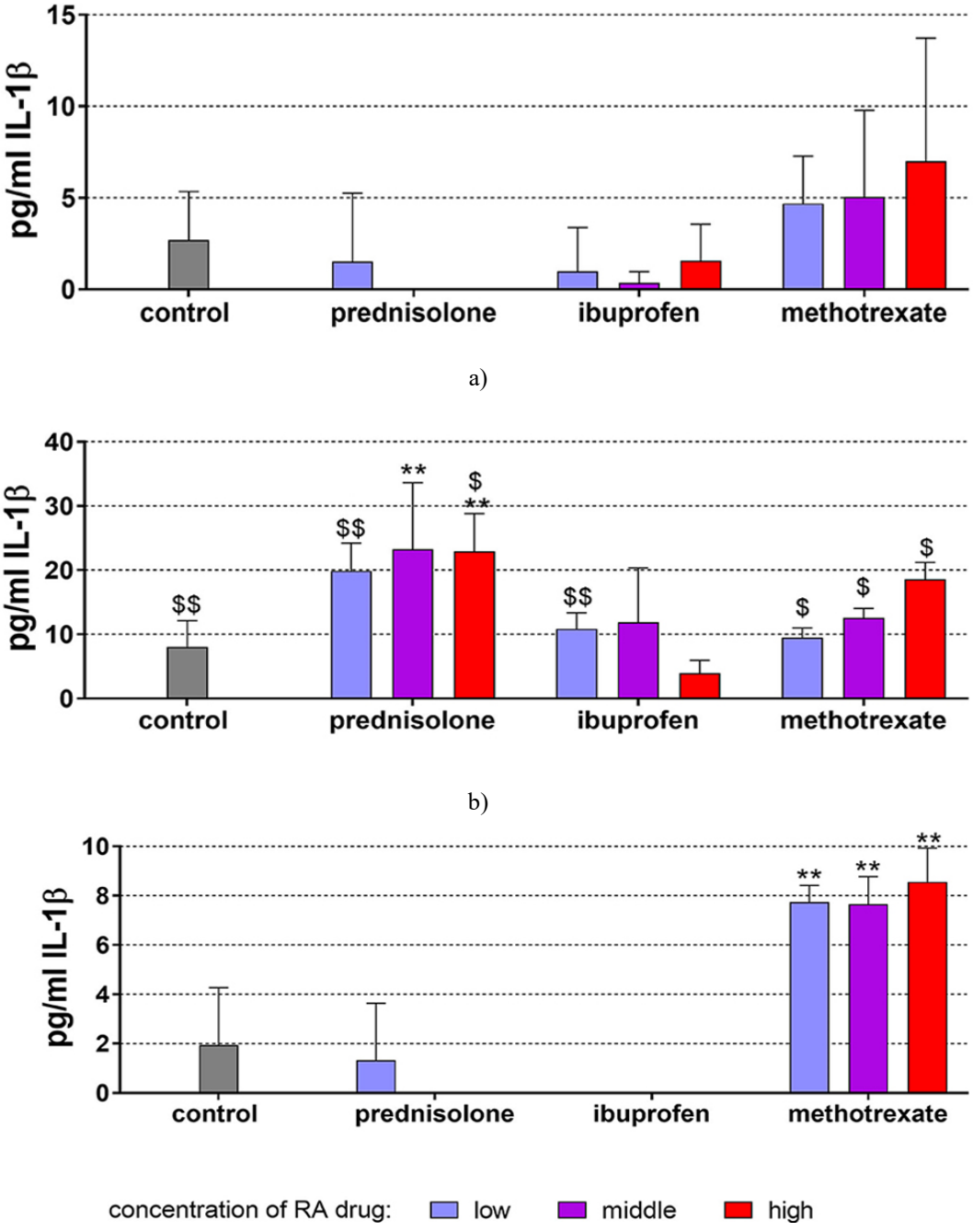

Figure 2. IL-1β secretion by MONO-MAC-6 cells following 18 hours of treatment with prednisolone (low: 20 ng/ml, medium: 100 ng/ml, high: 500 ng/ml), ibuprofen (low: 2 µg/ml, medium: 10 µg/ml, high: 50 µg/ml), and methotrexate (low: 1 µg/ml, medium: 5 µg/ml, high: 25 µg/ml) under three conditions: (a) without microbial stimuli, (b) with Fusobacterium nucleatum lysate, and (c) with Candida albicans lysate. Mean values are represented by columns and standard deviations by error bars. Statistical significance: *$p <$ 0.05 vs. control, **$p < 0.01$ vs. control, $^{\$/\$\$}p < 0.05/p < 0.01$ vs. the corresponding group without microbial components.

Stimulation with F. nucleatum lysate significantly elevated IL-1β secretion to 8.06 ± 4.10 pg/ml compared with unstimulated cells ($p < 0.001$; **Figure 2b**). When anti-rheumatic drugs were present, IL-1β levels remained higher than in cells without microbial exposure. Specifically, prednisolone at 100 and 500 ng/ml further increased IL-1β release ($p = 0.004$ and $p = 0.006$, respectively).

In contrast, C. albicans lysate did not significantly affect IL-1β secretion. However, methotrexate significantly enhanced IL-1β release at all tested concentrations ($p < 0.001$ for each dose; **Figure 2c**).

PDL fibroblasts

In the absence of any stimulation, the cells produced an average of 3.17 ± 2.66 pg/ml of IL-8 after 18 hours of incubation. Treatment with the tested drugs did not result in a statistically significant change in IL-8 secretion **(Figure 3a)**.

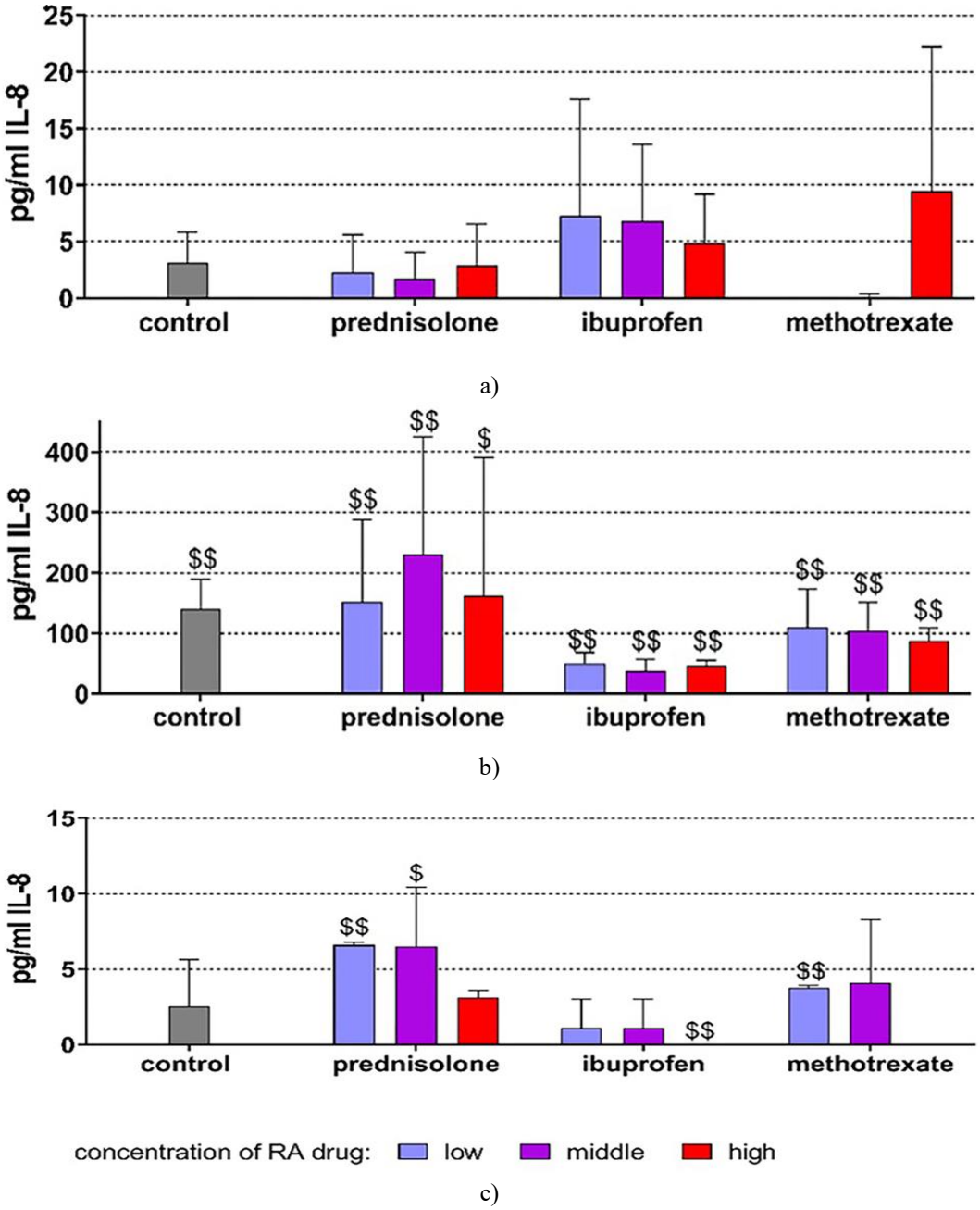

Figure 3. IL-8 secretion by PDL fibroblasts following 18 hours of treatment with prednisolone (low: 20 ng/ml, medium: 100 ng/ml, high: 500 ng/ml), ibuprofen (low: 2 µg/ml, medium: 10 µg/ml, high: 50 µg/ml), and methotrexate (low: 1 µg/ml, medium: 5 µg/ml, high: 25 µg/ml) under three conditions: (a) without microbial components, (b) after stimulation with *Fusobacterium nucleatum* lysate, and (c) after exposure to

Candida albicans lysate. Columns indicate mean values, and error bars represent standard deviations. Statistical significance: $^{\$/\$\$}p < 0.05/p < 0.01$ vs. the respective group without microbial stimulation.

Stimulation with *F. nucleatum* lysate significantly increased IL-8 release to 140.33 ± 49.18 pg/ml ($p < 0.001$). Elevated IL-8 levels compared with non-stimulated controls were also observed in the presence of the tested drugs **(Figure 3b)**. In contrast, exposure to *C. albicans* lysate did not significantly affect IL-8 secretion (2.52 ± 3.14 pg/ml). Nevertheless, low doses of prednisolone ($p = 0.003$) and methotrexate ($p = 0.001$) caused a significant increase in IL-8, while high-dose ibuprofen (50 µg/ml) led to a reduction compared with cells without microbial stimulation ($p = 0.002$; **Figure 3c**).

TIGK cells

In this set of experiments, whole C. albicans cells rather than lysates were applied to TIGK cells. On average, 4.42 ± 0.67 $\log_{10}$ fungal cells adhered per well. The presence of anti-rheumatic drugs consistently increased fungal adherence, ranging from 0.31 $\log_{10}$ (100 ng/ml prednisolone) to 1.01 $\log_{10}$ (50 µg/ml ibuprofen). These increases reached statistical significance for ibuprofen at 2 µg/ml and 50 µg/ml ($p = 0.013$ and $p < 0.001$, respectively) and for methotrexate at 25 µg/ml ($p = 0.048$; **Figure 4a**).

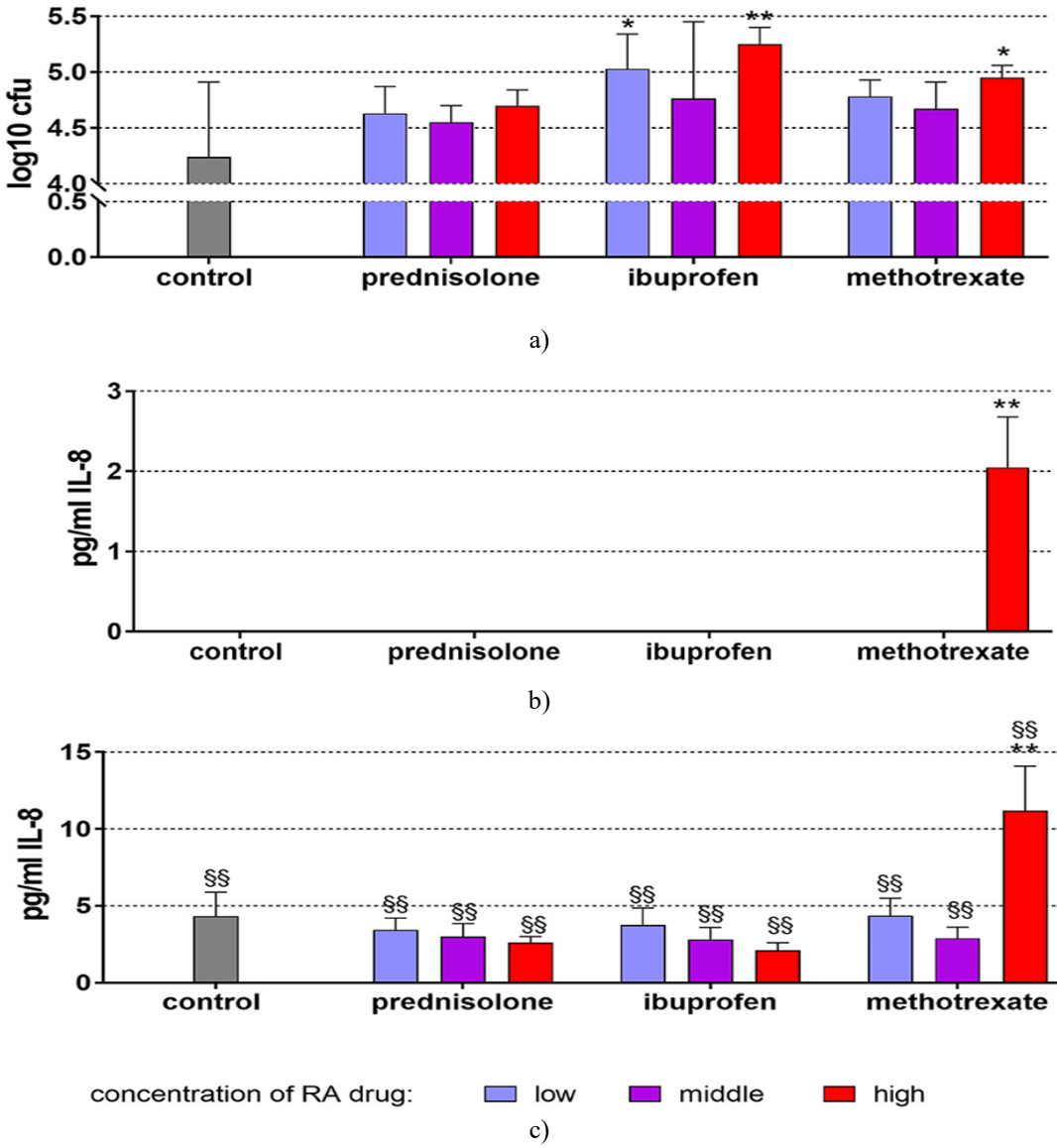

Figure 4. (a) Adherence (including invasion) of Candida albicans to TIGK cells and (b, c) IL-8 release by TIGK cells following 6 hours of treatment with prednisolone (20, 100, 500 ng/ml), ibuprofen (2, 10, 50 µg/ml), and methotrexate (1, 5, 25 µg/ml) under different conditions: (B) without microbial stimulus and (C) in the presence of C. albicans. Mean values are shown as columns, with standard deviations indicated by

error bars. Statistical significance: */**$p < 0.05/p < 0.01$ vs. control, $^{\$/\$\$}p < 0.05/p < 0.01$ vs. the corresponding group without microbial stimulation.

In the absence of microbial cells, IL-8 secretion was minimal and only detectable following treatment with 25 µg/ml methotrexate after 6 hours of incubation **(Figure 4b)**. Exposure to C. albicans led to increased IL-8 production regardless of the presence of anti-rheumatic drugs **(Figure 4c)**. Notably, IL-8 levels were further elevated when 25 µg/ml methotrexate was added compared with C. albicans alone ($p < 0.001$).

In this study, we investigated how anti-rheumatic drugs, with or without microbial stimulation, affect oral cells. Among the microbial stimuli, Fusobacterium nucleatum lysate elicited the strongest pro-inflammatory cytokine release from PDL fibroblasts and monocytic cells, whereas the effects of the tested anti-rheumatic drugs were generally minor.

Porphyromonas gingivalis has been suggested as a key link between periodontitis and rheumatoid arthritis (RA) [26]. This pathogen expresses peptidyl-arginine deiminase, which can citrullinate both host and bacterial proteins [27]. Outer membrane vesicles from P. gingivalis may contain up to 50 citrullinated proteins [28]. In preliminary experiments using P. gingivalis ATCC 33277 lysate, IL-8 was undetectable in PDL fibroblast supernatants, both with and without anti-rheumatic drugs, likely due to active gingipains that degrade IL-8 [29]. Consequently, subsequent experiments employed F. nucleatum, which is enriched in gingivitis and periodontitis compared with periodontal health [30, 31] and is a potent inducer of cytokine production in gingival fibroblasts [32]. This study confirmed these findings, particularly for PDL fibroblasts and MONO-MAC-6 monocytic cells.

In PDL fibroblasts, a non-professional immune cell type, exposure to F. nucleatum in the absence of drugs increased IL-8 release, though to a lesser extent than in MONO-MAC-6 cells. Candida albicans did not significantly alter IL-8 secretion, which may be attributable to the use of blastospores rather than germinated forms, as previous studies showed that germinated C. albicans rather than blastospores stimulate IL-8 in gingival fibroblasts [33]. MONO-MAC-6 cells, representing professional immune cells, produced high levels of IL-8 and IL-1β, particularly following F. nucleatum stimulation, but remained unresponsive to C. albicans. The method used for C. albicans preparation likely contained cell wall components such as β-glucans, which, together with LPS, are known to induce IL-10 without affecting pro-inflammatory cytokines in monocytes [34]. This may explain the limited influence of C. albicans on pro-inflammatory cytokine release. Similarly, Candida biofilm supernatants do not alter IL-8 mRNA or protein in blood cells, unless combined with mixed bacterial species, which can dramatically enhance IL-8 expression and secretion [35]. A limitation of the current study is that stimulation with multi-species lysates was not performed.

MONO-MAC-6 cells produced high IL-1β levels in response to F. nucleatum lysate, whereas C. albicans had no effect. Glucocorticoids, commonly used in RA therapy, reduce inflammatory cell infiltration and lower levels of TNFα, IL-1β, IL-6, and IL-17 [36]. Clinically, prednisolone decreases IL-8 expression in RA synovium but does not alter IL-1β levels [37, 38]. Consistently, in this in vitro study, prednisolone alone did not significantly affect cytokine release, though it enhanced IL-8 and modestly IL-1β release when combined with F. nucleatum in MONO-MAC-6 cells.

Ibuprofen had no detectable effect on IL-8 or IL-1β release, which contrasts with studies in bovine fibroblast-like synoviocytes showing decreased IL-1β following LPS stimulation [39]. Clinically, ibuprofen did not affect IL-1β levels in gingival crevicular fluid during orthodontic treatment [40], and short-term adjunctive use in periodontitis modestly reduced clinical inflammation [41].

Methotrexate, which acts on folate pathways, adenosine, leukotrienes, and cytokines, is used not only in RA but also in chronic sarcoidosis [42]. In RA, it reduces neutrophil counts [43]. Methotrexate has been reported to increase IL-1 expression and secretion in U937 monocytic cells via JUN and FOS activation [44]. In our study, methotrexate increased cytokine release in the absence of microbial stimulation, but levels were reduced when cells were exposed to F. nucleatum, suggesting that bacterial-induced inflammation may be dampened. Clinically, methotrexate alone or with anti-TNF therapy lowers systemic inflammation in RA but does not significantly impact periodontal inflammation as measured by PISA [45]. The severity of periodontitis in RA patients appears to be influenced by the type of RA medication: patients treated with methotrexate combined with rituximab or leflunomide had fewer bleeding sites than those receiving methotrexate plus TNFα inhibitors [46]. Among DMARDs, only methotrexate demonstrated antimicrobial activity against oral pathogens, including F. nucleatum and Viridans streptococci [47].

Studies report conflicting findings regarding IL-1β levels in gingival crevicular fluid (GCF) of RA patients with periodontitis, with some showing higher levels [16, 48], others lower [49], and some no difference [50]. Anti-TNF therapy decreased IL-1β and IL-8 levels in GCF [51], and salivary IL-1β levels were lower in RA patients receiving anti-TNF therapy compared with other DMARDs [52].

In our prior microbiota analyses, yeasts were frequently detected in RA patients' subgingival biofilm but not in periodontitis patients without RA [20]. Accordingly, C. albicans was included in this study. Methotrexate and ibuprofen increased its adhesion to gingival epithelial cells, though the underlying mechanism remains unclear. In C. albicans, Als3 mediates adhesion to E-cadherin and host cell endocytosis [53].

This study has several limitations. Only isolated cell types were examined, whereas in vivo immune responses involve complex interactions. The model used F. nucleatum lysate rather than a multi-species biofilm, and cytokine measurements were performed at a single time point. Finally, lysates were used instead of live bacteria.

Conclusion

In summary, bacterial stimuli were the primary drivers of pro-inflammatory cytokine release from professional and non-professional immune cells. Among the anti-inflammatory drugs tested, prednisolone and methotrexate modulated the interaction between microorganisms and monocytic cells: prednisolone enhanced IL-8 release following F. nucleatum stimulation, whereas methotrexate reduced it. Both methotrexate and ibuprofen increased C. albicans adhesion to epithelial cells. Clinically, in RA patients, reducing subgingival microbial load is crucial to control inflammation, although anti-rheumatic drugs may alter typical periodontal signs.

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Conflict of Interest: None

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Ethics Statement: Written informed consent was obtained from all participants for their participation in this study.

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