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## Polybacterial challenge effects on cytokine/chemokine production by macrophages and dendritic cells

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### Abstract

**Objective**—To investigate the polymicrobial infection of periodontal disease, which elicits inflammatory mediators/cytokines/chemokines in the local gingival tissues, and a polybacterial challenge of antigen-presenting cells, e.g. macrophages and dendritic cells (DCs), at the mucosal surface.

**Materials and methods**—The cytokine/chemokine profiles of human macrophages and DCs in response to polybacterial challenges were investigated.

**Results**—Oral Gram-negative bacteria elicited significantly greater IL-8 levels from macrophages, compared to Gram-positive bacteria. Gram-positive bacteria did not show synergism in inducing this chemokine from macrophages. In contrast, pairs of oral Gram-negative bacteria elicited synergistic production of IL-8 by macrophages. Similar results were not observed with TNF $\alpha$ , which only appeared additive with the polybacterial challenge. Selected Gram-negative bacterial pairs synergized in IL-6 production by immature DCs. In mature DCs (mDCs), a *Porphyromonas gingivalis/Fusobacterium nucleatum* and *Porphyromonas intermedia/F. nucleatum* polybacterial challenge resulted in significant synergism for IL-6 and TNF $\alpha$  levels. However, only the *Pi/Fn* combination synergized for IL-12 production and there appeared to be no polybacterial effect on IL-10 production by the mDCs.

**Conclusions**—These results indicate that a polybacterial challenge of cells linking innate and adaptive immune responses results in varied response profiles that are dependent upon the characteristics of the microorganisms that are components of the polybacterial complex.

### Keywords

Polymicrobial; Cytokine; Macrophage; Dendritic cells; Oral bacteria

## Introduction

The lesion of the polymicrobial disease, periodontitis, is a result of a complex host response to a polybacterial challenge derived from subgingival biofilms [1, 2]. It remains unclear how the resident cells, e.g. epithelial, fibroblast, and immune cells that emigrate into the infected tissues interface with the complex oral microbiome to discriminate symbiotic commensal bacteria from bacterial species consistent with pathogenic biofilms [3-7]. Specific bacteria and bacterial consortia have been implicated in the oral disease biofilms [8-10]; however, it remains to be determined how this range of phyla, genera, and species presents as a polybacterial challenge to host innate and adaptive immune cells in the periodontal tissues.

It would be predicted that a polybacterial infection would engage a range of cellular receptors, including pathogen-associated molecular pattern (PAMP) receptors. These ligand-receptor interactions could transduce a range of complementary and competing intracellular signals that would directly impact the profile of individual host cell responses to this complex challenge. Clear evidence demonstrates that the polymicrobial infection of periodontitis induces inflammatory and innate immune responses in the gingival tissues and link to the adaptive immune response, represented by the production of elevated local and systemic antibody responses to members of the oral microbiota [11-17]. However, the detailed characteristics of the local and systemic antibody responses (e.g. level, avidity, isotype, subclass) vary substantially to individual oral bacteria, and are not necessarily commensurate with the estimated oral burden of the individual species [1, 18-24]. Consequently, it could be interpreted that those local cellular mechanisms responsible for bacterial uptake, processing, and presentation of antigens to the adaptive immune system must interact with the individual microbes in the context of a polybacterial challenge, and may be differentially affected in their engagement of individual species by the complexity of the challenge. However, even recent reports of APC cellular interactions, particularly dendritic cells (DCs), and intracellular molecular recognition and signaling mechanisms continue to emphasize studies limited to a single microorganism or isolated microbial components [25-29].

Due to the chronic microbial colonization of the mucosal surfaces of the oral cavity, the contiguous host tissues are infiltrated with both acute and chronic inflammatory cells, including macrophages and DCs, to control the microbial challenge and present antigen to the adaptive immune system [30-34]. The presence of both macrophages and DCs has been documented for gingival tissues, with the quantity and phenotypic changes occurring during progressing disease [30, 35, 36]. This study evaluated the characteristics of cytokines produced by macrophages and DCs in response to a polybacterial challenge with oral microorganisms.

## Materials and methods

### Cell lines and culture

The BF24 is a monocyte/macrophage cell line that is a subclone of the monocytic leukemia cell line THP-1, which was obtained through the NIH AIDS Research and Reference Program, Division AIDS, NIAID, NIH reagents program (cat# 1296) (<http://www.aidsreagent.org>). These cells were cultured in 175 cm<sup>2</sup> flasks in RPMI 1640 with L-glutamine and 10% fetal bovine serum in a 5% CO<sub>2</sub> atmosphere at 37°C.

The THP-1 cell line (THP-1) was used to generate DCs, a subclone of the monocytic leukemia cell line. Specifically, monocyte THP-1 cell, a gift from Dr. Seymour Klebanoff, University of Washington, was cultivated in RPMI 1640 (Gibco) supplemented with 10%

FBS. THP-1 was cultured for 6 days at  $10^5$  cells/ml in medium supplemented with 50 ng of recombinant GM-CSF (rGM-CSF, R&D Systems, Minneapolis, MN, USA) per ml and 1,000 U of recombinant IL-4 (rIL-4; Promega, WI, USA) per ml. After 6 days in culture, immature DCs (iDCs) were obtained. At day six, 50 ng of TNF $\alpha$  was added to the iDCs for an overnight incubation and mature DCs (mDCs) were obtained.

### Microorganisms

The bacterial strains used in this study were *Porphyromonas gingivalis* ATCC 33277, *Aggregatibacter actinomycetemcomitans* JP2, *Fusobacterium nucleatum* ATCC 25586, *Streptococcus mutans* ATCC 33535, *S. gordonii* ATCC 10558, and *S. sanguis* ATCC 10556. All bacteria were grown as we have described previously [37]. The bacterial suspensions were washed three times with sterile PBS after centrifugation at 10,000g for 20 min at 4°C. The pellet was finally resuspended in 15 ml PBS with complete EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany), and bacteria were sonicated using an ultrasonic disrupter (Branson Sonifier model 450-Branson). The crude extract after sonication was centrifuged at 13,000g for 10 min at 4°C and protein concentration of supernatants was determined by BCA assay (Pierce, Rockford, IL, USA).

### In vitro models

BF24 or THP-1 cells were placed into 48-well plates at a cell density of  $2.5 \times 10^5$  cells/well in 900  $\mu$ l RPMI 1640 media supplemented with 2% FBS. The BF24 cells were treated with 100  $\mu$ l of various amounts of the bacterial sonicates in duplicate for all of the comparisons. THP-1 cells were differentiated into DCs first as described previously [38, 39] and followed by treatment of various bacterial sonicates. The cells and bacteria ( $1 \times 10^9$ /ml) were incubated overnight (16 h), supernatants harvested and debris removed by centrifugation (13,000g for 5 min). The supernatants were then aliquoted and stored frozen at  $-80^\circ\text{C}$  until analyzed.

### Cytokine assays

Culture supernatants were evaluated for IL-8, TNF $\alpha$ , IL-6, IL-12 heterodimer (p70) or IL-10 by standard sandwich ELISA (eBioscience) according to the manufacturer's instructions. All samples were tested in triplicate.

### Statistical analyses

Statistical analyses were performed using a Mann–Whitney *U* or Kruskal–Wallis analysis of variance on ranks with a post hoc Dunn's test for multiple testing (SigmaStat 3.5, Point Richmond, CA, USA). An alpha value of  $p < 0.05$  was accepted as statistically significant when comparing the mediator levels under test conditions to media derived from untreated cells.

## Results

### Polybacterial effects on cytokine production by macrophages

Macrophages are crucial cells for interacting with various microbes in tissues and contribute to innate immunity and wound healing processes in these tissues, generally employing various cytokines and chemokines as cell communication, maturation, and functional expression factors. Figure 1a demonstrates significant increases in IL-8 production with increasing doses of each of the three Gram-negative bacteria (i.e. *Aa*, *Pg*, *Pf*). While the Gram-positive bacteria also increased IL-8 production by the macrophages, there was a minimal increase of levels with increasing challenge by these bacterial sonicates (Fig. 1c). Minimal levels of TNF $\alpha$  were elicited from the cell line by these Gram-positive

microorganisms (data not shown). As was noted with IL-8 for the Gram-negative bacteria, these species stimulated significant increases in TNF production by the macrophages (Fig. 1b). Interestingly, *P. gingivalis*, a species most frequently associated with the pathogenic biofilms in periodontitis, was the least effective of this group in stimulating the pro-inflammatory factors and, at the highest dose of this sonicate, appeared to minimize the levels of IL-8 and TNF in the cell supernatants.

Figure 2 shows no synergism in IL-8 production by a polybacterial challenge by the Gram-positive streptococcal species. Additionally, these species demonstrated a minimal additive effect when used to challenge the macrophages. Significant effects on cytokine/chemokine profiles were observed in challenging the macrophages with Gram-negative polybacterial complexes (Fig. 3). Stimulation of the macrophages with varying amounts of *Aa* sonicate combined with *Pg* sonicates demonstrated a significant synergism in production of IL-8 and TNF in selected dosage combinations. Interestingly, higher doses of *Pg* appeared to inhibit the supernatant levels of IL-8 and TNF that would have been expected to be elicited by the *Aa* challenge. Similarly, an even greater significant synergism in both IL-8 and TNF were detected when *Aa* was paired with *Pi* for the polybacterial challenge.

### Polybacterial effects on cytokine production by DCs

DCs are critical for engaging microbes to enable antigen processing and presentation. These biologic processes generally are dependent upon a range of cytokines and chemokines produced by these cells, once activated. We examined the variation in ability of individual oral bacteria, or pairs of bacteria, to stimulate cytokine responses by the DCs. The responses of iDCs to the polymicrobial challenges are shown in Fig. 4. With multiple combinations of Gram-negative bacteria, we observed synergistic production of IL-6, as a marker of the iDC responses to a polymicrobial challenge. A combination of *Pg* and *Fn* resulted in a significant synergism in production of IL-6, as did the *Pi* and *Fn* combination. In contrast, the combination of *Pg* and *Pi* did not show any synergism for IL-6 production by the iDCs.

Figure 5a and b depict the results of studies examining synergism of the polybacterial challenge with mDCs. A synergism of the polymicrobial challenge was observed for induction of both TNF and IL-6 from the mDCs, using *Pg* and *Fn*, and *Pi* and *Fn* as the experimental stimuli (Fig. 5a). In contrast, no synergistic stimulation was noted for IL-10, nor with the *Pg* and *Fn* combination for IL-12. A synergistic response was observed for IL-12 induction using a polybacterial pair of *Pi* and *Fn* (Fig. 5b).

## Discussion

This investigation hypothesized that cytokine and chemokine responses of macrophages and DCs to a polybacterial challenge would be different than the responses to the individual microbial components of the mixture. We emphasized selected pro-inflammatory mediators that were representative of standard responses of each of these cell types related to their role in innate and adaptive immunity; however, these findings do not cover the broad array of response capacity for these cells when challenged with microbial stimuli. This concept is even more important since much of the literature is limited to examination of host-bacterial interactions with a single microbial species, or in many cases defined ligand/agonists for microbial associated molecular pattern (MAMP) receptors [40-43]. In our studies, selected polybacterial pairs demonstrated a synergism in stimulation of cytokines/chemokines. However, there were clear differences in the capacity of Gram-positive and Gram-negative bacteria in this synergistic response. The responses of the macrophages and DCs in the production of cytokines and chemokines were generally similar for the various microbial challenges. This suggests that it is likely that similar receptors were engaged by the individual bacteria, and polybacterial pairs on each of the host cell types, leading to these

response profiles. Nevertheless, there were significantly different levels of the various pro-inflammatory mediators that were related to the specific bacteria. Selected observations showed that high levels of *P. gingivalis* extracts related to lower levels of cytokines produced by these cells, and at least with macrophages, these higher *Pg* amounts appeared to adversely affect the levels of cytokines produced by the cells in response to other more potent stimuli. This is not totally unexpected based upon the literature suggesting that *P. gingivalis* components have somewhat unique characteristics in how they bind to and stimulate various host cells [44-49], as well as inhibiting host cell responses to other stimuli [50-53]. These findings could support the idea that an important strategy used by this opportunistic pathogen was to “bind and hide” from aggressive host inflammatory and innate immune responses crucial for managing the colonization and emergence of this pathogen at disease sites.

We observed with the macrophages that synergism was detected with both IL-8 and TNF levels with *Pg* and *Aa* combinations, while no synergism was detected for TNF production. In contrast, combinations of *Pi* and *Aa* synergized in inducing both elevated IL-8 and TNF. Interestingly, high levels of *Pg* sonicates significantly inhibited the ability of *Aa* sonicates to induce by IL-8 and TNF by macrophages. Summarizing these findings emphasizes that a polybacterial challenge of macrophages results in differences in cytokine/chemokine response profiles, and that these differences are regulated by the character and concentration of the polybacterial mixture.

As we have noted previously, differences in the magnitude of responses to oral bacteria by iDCs suggested different receptors and/or intracellular circuits are engaged for activation [54]. We have also observed that generally the Gram-negative bacteria pairs synergized for HIV promoter activation, which was not observed with Gram-positive microorganisms or combinations of a Gram-negative and Gram-positive microorganism [55]. As was noted with the macrophage responses, selected pairs of the Gram-negative bacteria synergized significantly for eliciting IL-6 production by iDCs. While both *Pg* and *Pi* worked in this fashion with *F. nucleatum*, the combination of *Pg* with *Pi* did not synergize. A potential molecular mechanism for this would be that similar receptors are engaged by *Pg* and *Pi* to trigger these cells, thus, they act as competing ligands. In contrast, *Fn* components are primarily ligands for other receptors distinct from these species and, as such, these combinations more robustly elicited intracellular signaling pathways resulting in IL-6 transcription and translation.

We have shown, previously, different responses of iDCs and mDCs to individual oral bacteria [54]. Since iDCs and mDCs have distinctive cell surface receptor patterns, and specific roles for these receptors in engaging antigens (i.e. iDC) and presenting antigens (i.e. mDC), as well as a portfolio of cytokines/chemokines related to their primary functions in innate and adaptive immunity, it would be expected that they might respond differently to a polybacterial challenge [32, 56-58]. Interestingly, we could not demonstrate any bacterial synergism in mDCs with respect to HIV reactivation in a model of latent infection [55]. However, the current study demonstrated under similar circumstances that a Gram-negative polybacterial challenge will synergize in inducing some cytokine responses from mDCs, including IL-6, TNF, and IL-12. However, this synergism did not appear to be simply a general upregulation of the mediators, since this was not observed for all bacterial combinations with IL-12 production and no synergism was observed for IL-10 production by the mDCs. Consequently, the significant decrease in TLRs during maturation from iDC to mDC [59] suggests that the polybacterial triggering of cytokines, such as TNF and IL-6, may also be via non-TLR engagement and thus more targeted in the cell response profiles [32, 33, 60].

The results of this study are some of the first to demonstrate polybacterial alterations in the profile of cytokine/chemokine responses of critical host cells that interface with both the innate and adaptive immune system. As we attempt to elucidate the characteristics of host–bacterial interactions in chronic diseases and diseases with a polymicrobial etiology, it will be vital to move forward from simply reductionist approaches to define what types of responses may be expected, to systems that incorporate more components of these interactions to best assess the net result of challenge to the host.

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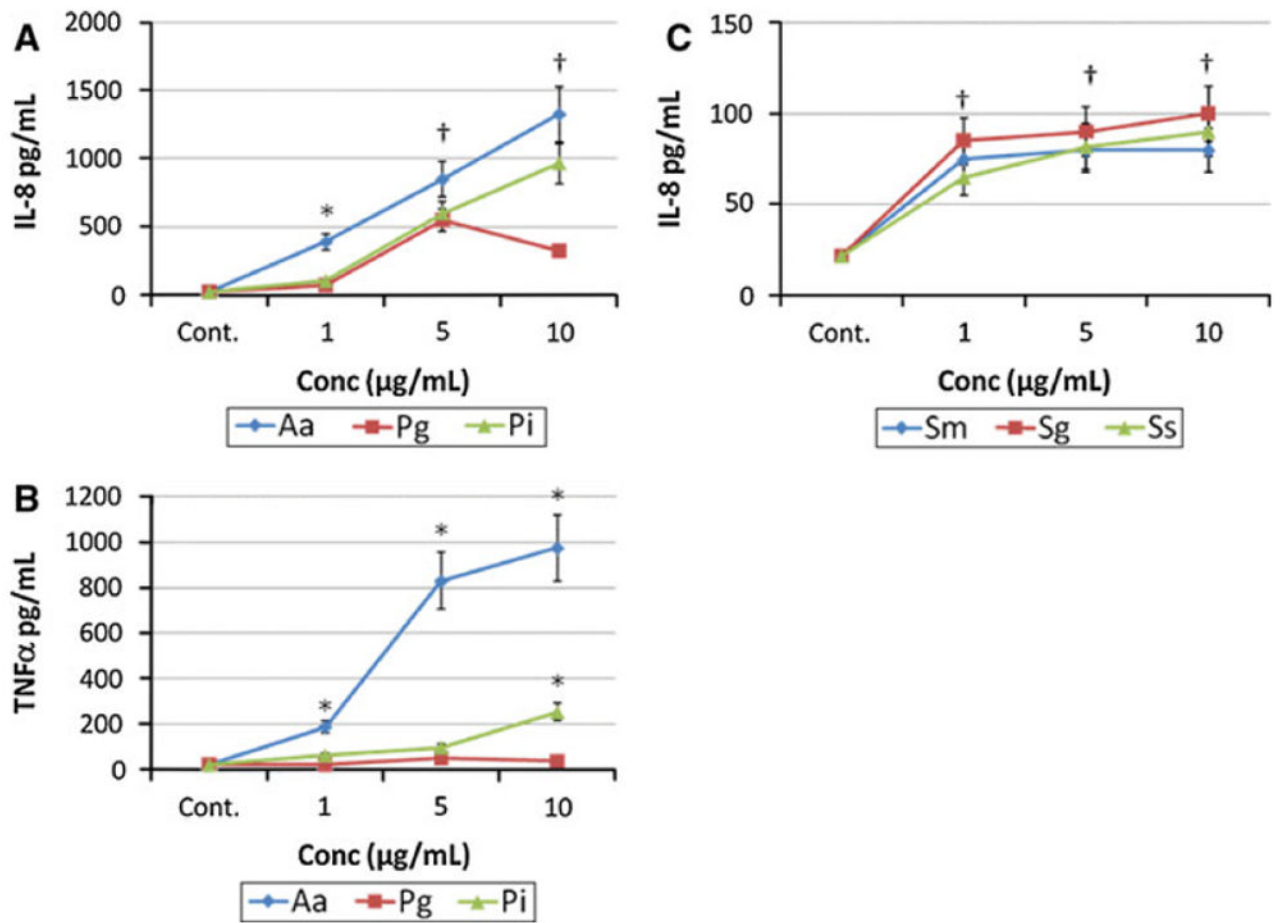


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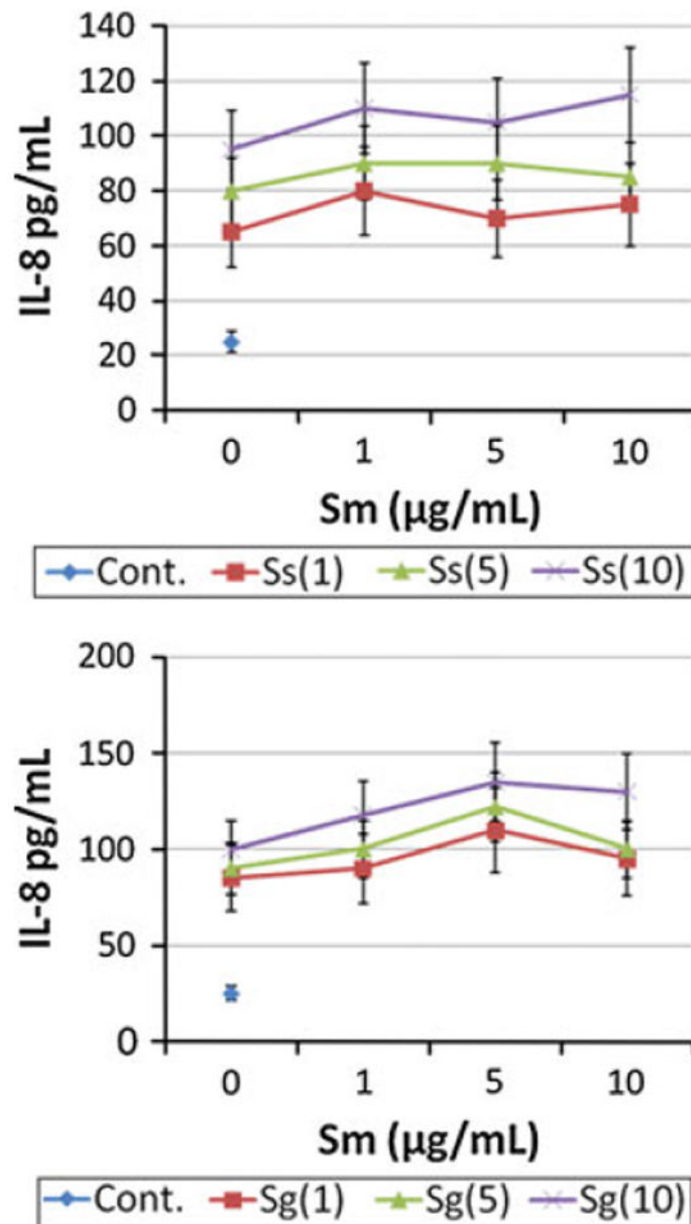
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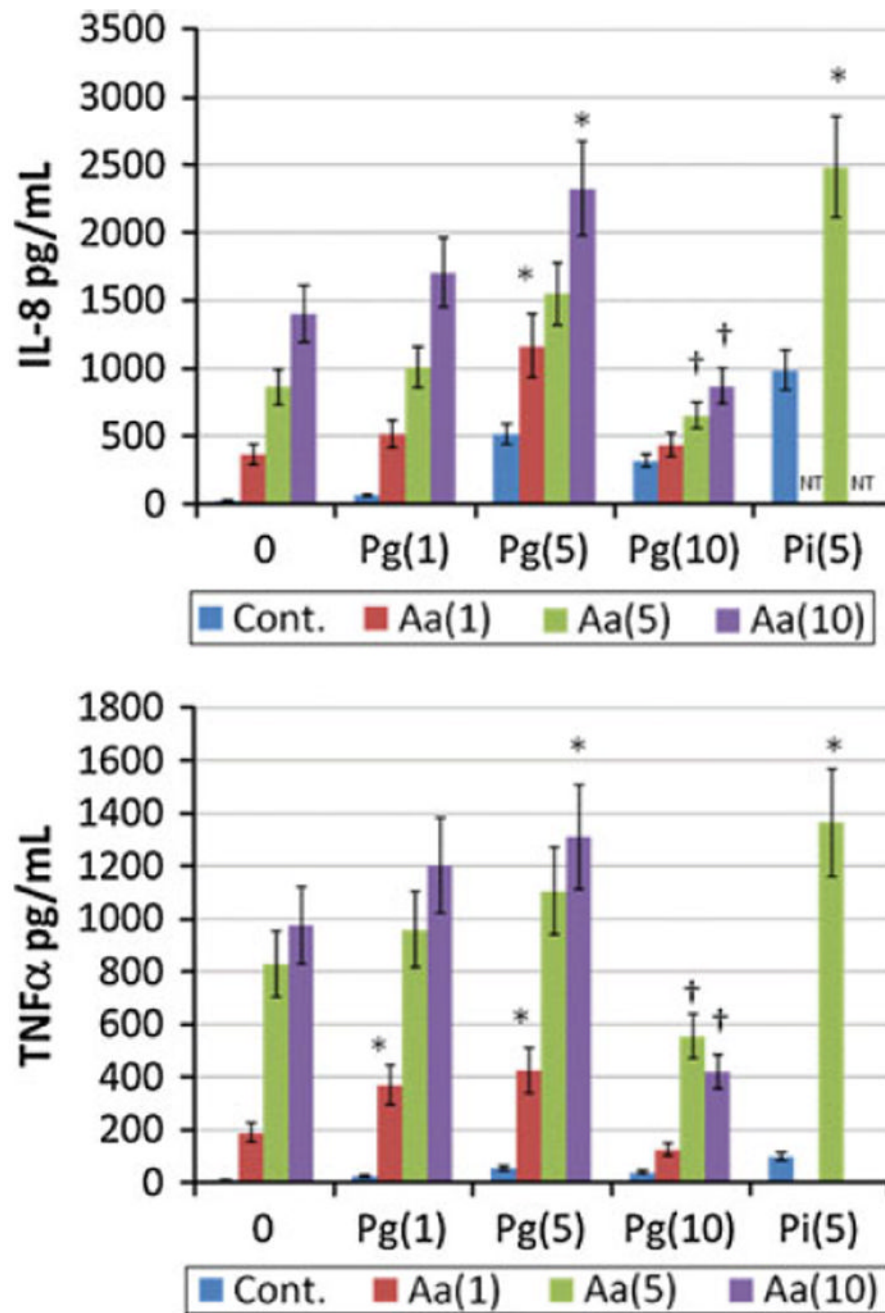
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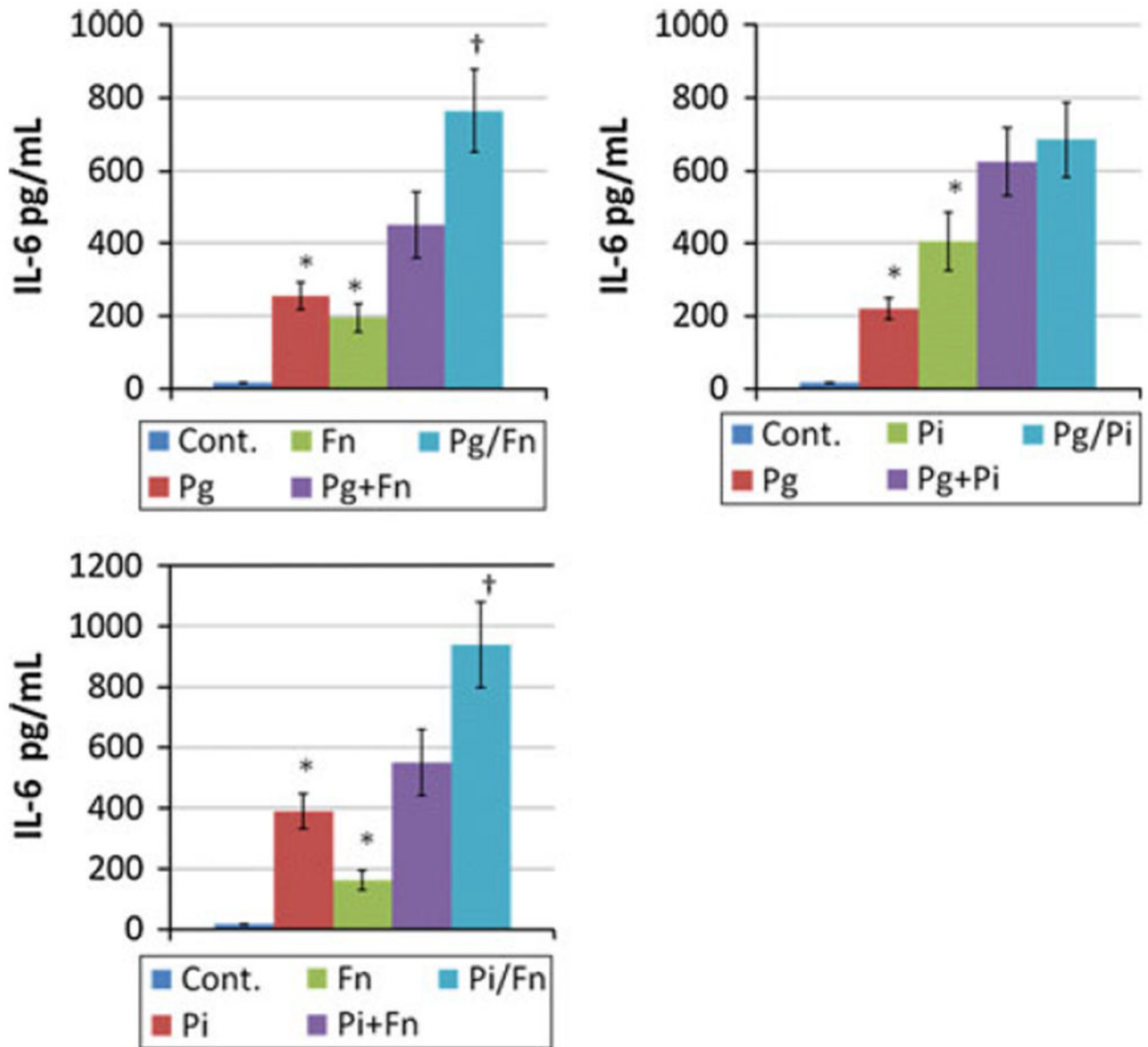
**Fig. 1.** Macrophages (BF24) were stimulated in triplicate with various concentrations of sonicates from Gram-negative (**a, b**) and Gram-positive (**c**) bacteria. Supernatant levels of IL-8 (**a, c**) or TNF (**b**) were determined at 24 h. The *points* denote the mean of at least triplicate determinations and the *brackets* enclose 1 SD. The *asterisk* denotes statistically different from the control levels at least at  $p < 0.05$ . The *dagger* denotes that response levels with all bacterial stimuli were significantly different than control levels at least at  $p < 0.05$



**Fig. 2.** Macrophages (BF24) were stimulated in triplicate with various combinations of oral Gram-positive bacterial sonicates. Supernatant levels of IL-8 were determined at 24 h. The *points* denote the mean of at least triplicate determinations for each stimulant combination. The designations in the graphs identify the microorganism and concentration used [i.e. Ss(1) or Sg(5)]. All stimuli elicited levels of IL-8 significantly greater than the media control at least at  $p < 0.05$ . No significant differences were noted with any of the combinations

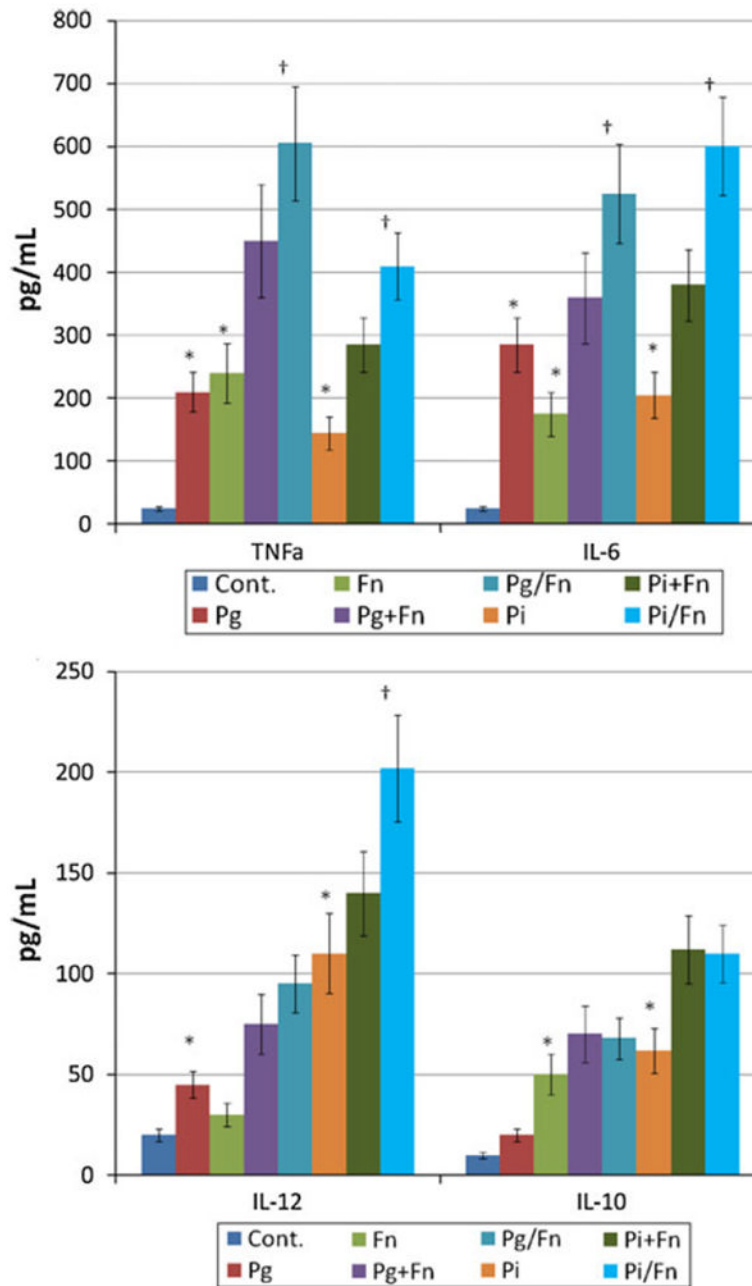


**Fig. 3.** Cytokine levels produced by macrophages (BF24) when challenged with various combinations of oral bacteria. The *bars* denote the mean of at least triplicate determinations and the *brackets* enclose 1 SD. The designations in the graphs identify the microorganism and concentration used [i.e. Aa(1) or Pg(5)]. The *asterisk* denotes statistically greater than additive levels with the bacterial combination and concentration at least at  $p < 0.05$ . The *dagger* denotes that response levels were significantly lower than additive levels with the bacterial combination and concentration at least at  $p < 0.05$



**Fig. 4.** Cytokine levels produced by iDCs when challenged with various combinations of oral bacteria. The *bars* denote the mean of at least triplicate determinations and the *brackets* enclose 1 SD. *Pg + Fn* denotes addition of the values from the two individual challenge conditions and *Pg/Fn* denotes an experimental challenge with the combination of the bacteria. The *asterisk* denotes statistically greater than control levels at least at  $p < 0.05$ . The *dagger* denotes statistical difference from the additive levels of the two bacteria, at least at  $p < 0.05$





**Fig. 5.** Mature DCs were challenged with 5  $\mu$ l ( $1 \times 10^9$ /ml) combinations of *P. gingivalis* and *F. nucleatum* or *P. intermedia* and *F. nucleatum*. The mDCs were defined by flow cytometry phenotypically as CD80<sup>hi</sup>, CD83<sup>hi</sup>, CD86<sup>hi</sup>, and HLA-DR<sup>hi</sup> compared to the original THP-1 cells [54]. The *bars* denote the mean of at least triplicate determinations and the *brackets* enclose 1 SD. *Pg + Fn* or *Pi + Fn* denotes addition of the values from the two individual challenge conditions and *Pg/Fn* or *Pi/Fn* denotes an experimental challenge with the combination of the bacteria. The *asterisk* denotes statistically greater than control levels at least at  $p < 0.05$ . The *dagger* denotes statistical difference of the bacterial combination when compared to the additive levels of the two bacteria, at least at  $p < 0.05$