ARTICLE IN PRESS

Cytokine xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Converging findings from linkage between periodontal pathogen with atopic and allergic immune response

Sindy Cornelia Nelwan[a,*], Ricardo Adrian Nugraha[b], Anang Endaryanto[c], Asti Meizarini[d], Udijanto Tedjosasongko[a], Seno Pradopo[a], Haryono Utomo[e]

[a] Department of Pediatric Dentistry, Faculty of Dentistry Universitas Airlangga, Surabaya, Indonesia
[b] Department of Medicine, Faculty of Medicine Universitas Airlangga, Surabaya, Indonesia
[c] Department of Child Health, Faculty of Medicine Universitas Airlangga, Surabaya, Indonesia
[d] Department of Dental Materials Science and Technology, Faculty of Dentistry Universitas Airlangga, Surabaya, Indonesia
[e] Department of Forensic Dentistry, Faculty of Dentistry Universitas Airlangga, Surabaya, Indonesia

ARTICLE INFO

Keywords:
Atopy
Allergy
Oral pathogen
Periodontitis
Porphyromonas gingivalis

ABSTRACT

This study aims to explore a relationship between exposures of whole-cell *Porphyromonas gingivalis* in various doses with atopic inflammatory responses at experimental mice. A pretest-posttest controlled group design, with 16 Wistar rats (*Rattus novergicus*) randomized into four groups. Group 1 was the control group. Group 2 was given low-dose (9×10^7 colony-forming unit) of *P. gingivalis*. Group 3 was given medium-dose (9×10^9 colony-forming unit) of *P. gingivalis*. Group 4 was given high-dose (9×10^{11} colony-forming unit) of *P. gingivalis*. Interleukin-4, Interleukin-5, Interleukin-17F, Interleukin-21, Immunoglobulin-E, Immunoglobulin-G_4, and γ-Interferon were measured by direct-sandwich ELISA just before the treatments began, day-4, and day-11 after treatments. There is a sudden increase of Interleukin-4 in the group 4 (23.79 ± 0.91 pg/ml to 54.17 ± 0.79 pg/ml; $p = 0.01$) and slight increase of Interleukin-5 in the group 4 (207.60 ± 11.15 pg/ml to 243.40 ± 9.33 pg/ml; $p = 0.03$). No change was observed for Interleukin-17F in all groups. Serum concentration of Immunoglobulin-E was decreased in group 2 (−10.44 ± 8.13 pg/ml), but increased in group 4 (+1.03 ± 4.57 pg/ml). Taken together, some cytokines are up-regulated and others are down-regulated after exposure to whole-cell *P. gingivalis*. Moreover, study of host responses during periodontal infection may offer critical key insight that contribute to the development of atopy.

Clinical implications: We introduced and explained the potential role of periodontal pathogen *Porphyromonas gingivalis* in systemic immune responses, along with its virulence factor inside the oral cavity. Our results consider several changes and differences of cytokines and immunoglobulins following whole-cell *Porphyromonas gingivalis* exposure. However, results of the study need to be interpreted with caution due to its limitations.

Capsule summary: Interleukin (IL)-4 and IL-5 had been found increase after exposure to the periodontal pathogens *Porphyromonas gingivalis*, whereas no or minimal change had been found in the level of IL-17F, Ig-G_4, and IFN-γ. The various cytokines and immunoglobulins shown in this study do not prove a causal relationship, and the precise role of *Porphyromonas gingivalis* in the regulation of atopic immune response warrants further investigation. Nevertheless, these findings may provide some critical key insight into the host responses following *Porphyromonas gingivalis* infection.

1. Introduction

Atopic and allergic diseases including bronchial asthma, hay fever, eczema, and food allergies have suddenly increased over the decade in Indonesia, initially in urban communities but now elsewhere [1]. The concept of hygiene hypothesis – a more infection in early childhood protects against later allergies – is believed by most people, although reasons behind those immunological properties are speculative [2]. In Indonesia, allergies are commonly found in the slum and poverty area where children are often exposed to the infections [3]. In spite of the fact that allergy is not perceived in slum area in Indonesia as serious health problem, previous study estimated a relatively high prevalence

Abbreviations: ANOVA, analysis of variant; CD-48, cluster differentiation-48; CFU, colony-forming unit; IFN-γ, gamma-interferon; Ig-E, Immunoglobulin-E; Ig-G, Immunoglobulin-G; IL, interleukin; LPS, lipopolysaccharide; NK, Natural Killer cells; PAMP, pathogen-associated molecular patterns; Pg, *Porphyromonas gingivalis*; Th, T-helper cell; TNF, tumor necrosis factor

* Corresponding author at: Department of Pediatric Dentistry, Universitas Airlangga, Jalan Mayjend Prof. Dr. Moestopo 47, Surabaya, Indonesia.
E-mail address: sindy-c-n@fkg.unair.ac.id (S.C. Nelwan).

https://doi.org/10.1016/j.cyto.2018.06.015
Received 5 March 2018; Received in revised form 30 May 2018; Accepted 11 June 2018
1043-4666/ © 2018 Elsevier Ltd. All rights reserved.

of atopic and allergic diseases among them. Hygiene hypothesis-related phenomena, seemingly conflicting in Indonesia. Even though, some convincing epidemiological data are supporting hygiene hypothesis, it often fails to explain why the incidence of atopic and allergic diseases are doubling in the slum and poverty area in Indonesia, where they got exposure to various kinds of microbes [4].

Majority of children living in the slum and poverty area in Indonesia didn't have any access to clean water, less educated about personal hygiene including oral hygiene. Therefore, they were much exposed to dirt and microbes, compare to the others who live in the clean environment. A previously discussed hypothesis suggests that environmental exposures during the first years of life play an important role in immune homeostasis and in determining allergic risk throughout childhood [5]. As a contrary to that hypothesis, child with poor oral hygiene results in a skewed development of the T-helper 2 (Th-2) immune system with specific cytokine profiles resulting an abnormal immune responses to various environmental allergens which are otherwise innocuous [6]. Even though the reasons for these trends remain unclear, however allergic disease prevalence rates have increased among children with poor oral hygiene, strongly suggests that oral cavity with their microbes' diversity may play a role [7].

Oral cavities are the home of many unique microbiomes whose interactions with host immune responses are less frequently studied compared to the intestinal microbiomes. In children with poor oral hygiene, periodontitis – an oral infection process resulting from an interaction between *Porphyromonas gingivalis* bacterial attack with host local inflammatory response – will cause inflammation of the supporting tissues of the teeth leading to stimulate pathogen-associated molecular patterns (PAMPs) and recognize this atopic inflammatory pathway. Upon activation of PAMPs, interleukin-4 (IL-4) and interleukin-5 (IL-5) - cytokines that induce differentiation of naïve helper T cells (Th-0 cells) to Th-2 cells - become maladaptive to atopic inflammatory pathway [8].

A relationship between periodontal pathogens and various atopic-allergic diseases has been widely reported. Our preliminary data suggest that *Porphyromonas gingivalis* – pathogens which commonly found in the oral cavity – influences the development of atopy in children with poor oral hygiene [9]. *Porphyromonas gingivalis* is a gram-negative, anaerobic bacterium considered as an important pathogen of periodontal disease that is also implicated in initiation and progression of allergies in the early life period [9]. By modulating immune system and changing their epitopes, researchers believe that these kind of bacteria are responsible to the development of Immunoglobulin-E (Ig-E) mediated inflammatory responses [10]. The propensity to produce Ig-E antibodies in response to lipopolysaccharides – a core oligosaccharides and a lipid component of bacteria wall cell – is considered a significant risk factor for childhood allergies development [11].

For more than two decades, *Porphyromonas gingivalis* lipopolysaccharide has been proposed to explain the phenomenon of periodontal disease-induced allergy [12], and our current understanding of the link between oral hygiene and Ig-E mediated inflammatory responses suggests that any associated risk of allergies in the early childhood is likely precipitated by early or preëxisting oral disease. Nevertheless, *Porphyromonas gingivalis* as a direct causative agent of atopic immune response is sometimes viewed with skepticism due to the several confounding factors [13]. For instance, periodontal disease in of itself is a poor predictor of dysbiosis in microbiomes. Dysbiosis in the oral microbiome (For instance: caries, periodontitis, and chronic rhinosinusitis) is associated with changes in the cutaneous microbiome and gut microbiome. This hypothesis suggests that the association between periodontal pathogens with altered immune system can only be happened indirectly [14].

On the other hand, some researchers strongly support the concept of hygiene hypothesis, believed that *Porphyromonas gingivalis* might have a protective role to prevent allergic diseases [15]. Friedrich reported a significant inverse correlation between periodontal diseases and inflammatory airway diseases in responses to house dust mites, with a borderline significant inverse association between periodontitis and asthma also observed [16]. Very few studies attempted to explore the concept of association between periodontitis, bronchial asthma, allergic rhinitis, atopic dermatitis, and food allergy which could neither support such a protective factor nor a risk factor for that, nor reject such an association [17]. This fact suggests a necessity in starting an experiment to understanding the association between oral hygiene and allergies, in term of direct association between exposures of periodontal pathogens with potential inflammatory markers for atopy.

Since IL-4 and IL-5 contribute to induce differentiation of naïve helper T-cells (Th-0) to become Th-2 cells, thus their roles in atopy remain inevitable. Recent study found that IL-17 – cytokine family induced by microbial lipopolysaccharides – propagating a positive feedback loop between innate and adaptive immunity in mediating pro-inflammatory response to aerosol allergen during asthma and airway inflammation [18]. Among six members in the IL-17 cytokine family, the presence of IL-17F plays the most vital role in to the amplification of allergic inflammation [18]. Dysregulated IL-17F but not IL-17 can result in excessive pro-inflammatory cytokine expression, which lead to tissue damage and systemic inflammation [19]. On the contrary to the others cytokines explained above, IL-21 – a newly emerging member of the type I cytokine family – is found to be involved in plasma cell differentiation from both naive and memory B cells [20]. In general, IL-21 induces survival, proliferation, isotype switching, and differentiation to Ig-secreting plasma cells [20], but might help to diminish B cells that are activated through an antigenic non-specific fashion during acute infection without the cognate antigen-specific or co-stimulatory signals [21]. Therefore, it is important to understand the characteristics and consequences of IL-4, IL-5, IL-17F, and IL-21 to regulate immunoglobulin-E (Ig-E), immunoglobulin G_4 (Ig-G_4), interferon gamma (IFN-γ), and in particular to several features of this atopic pattern of immunological reactivity, depending on the immune setting and environmental system after exposure of periodontal pathogens [22].

There are several limited hypothesis about the relationship between periodontitis, periodontal pathogen, and the developing of allergy in the early childhood. *Porphyromonas gingivalis*, is a well-known periodontopathic pathogen, which may provoke allergies. The aim of this study was to explore the relationship between administrations of whole-cell *Porphyromonas gingivalis* in various doses with atopic and ectopic inflammatory conditions at experimental mice. We did measure level of interleukin-4 (IL-4), IL-5, IL-17F, IL-21, immunoglobulin-E (Ig-E), immunoglobulin G_4 (Ig-G_4), and gamma-interferon (IFN-γ) in responses to the exposure of whole-cell *Porphyromonas gingivalis* at various doses in the intra-sulcular coronal surface of experimental mice.

2. Methods

2.1. Materials

2.1.1 Whole cell of *Porphyromonas gingivalis* (Astarte Biologics, WA, USA, in three different dosages: low-dose 9×10^7 colony-forming unit (CFU), medium-dose 9×10^9 CFU, and high-dose 9×10^{11} CFU). These strains were cultured on blood agar plates supplemented with 5 μg of hemin and 1 μg vitamin K1 mL^{-1}. Bacterial cultures were maintained in a Coy anaerobic chamber (Ann Arbor, MI) at an atmosphere of 10% H_2, 5% CO_2, and 85% N_2.

2.1.2 Level of IL-4, IL-5, IL-17F, and IL-21 were determined with a Bio-Plex mouse cytokine kit (Bio-Rad, Hercules, CA), using fluorescently labeled microsphere beads and a Bio-Plex suspension array system (Bio-Rad) according to the manufacturer's instructions.

2.1.3 Total serum of Ig-E, Ig-G_4, and IFN-γ were measured in blood serum by direct sandwich ELISA (R&D System Europe Ltd., Abingdon, UK) according to manufacturer's instructions. Ig-E, Ig-G_4, and IFN-γ concentration was quantified by staining with an AP-conjugated anti-IgE polyclonal Ab and SIGMAFAST™ p-

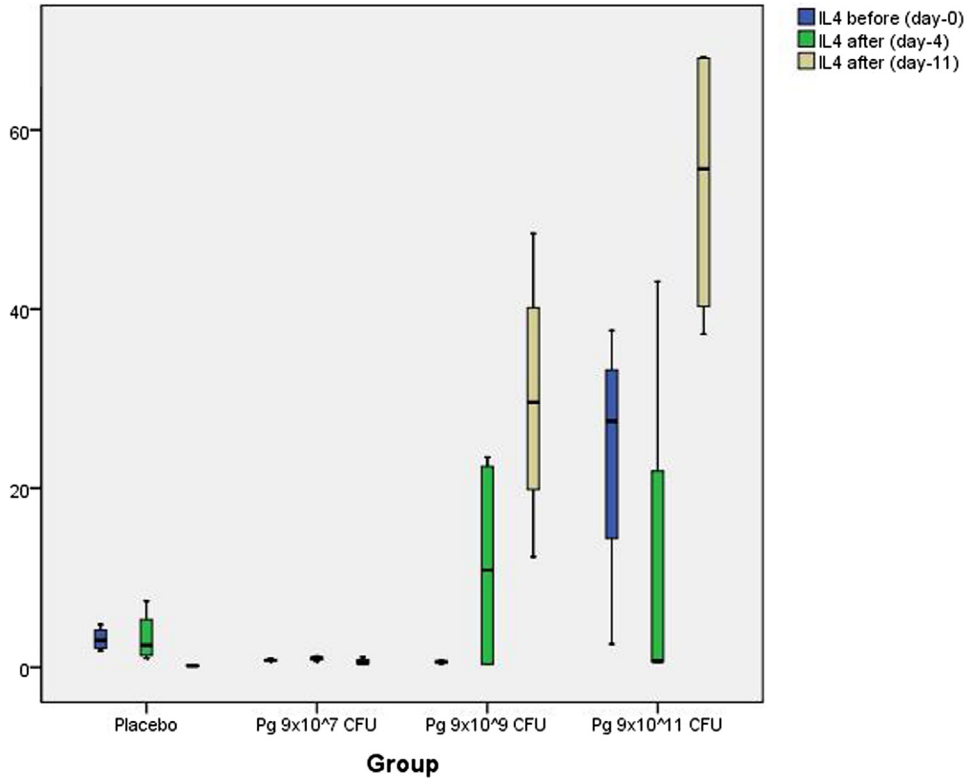

Fig. 1. Analysis of IL-4 as a pro-inflammatory cytokines before (day 0) and after (day 4 & day 11) treatment. Fold increase of IL-4 in the group 4 [high dose of whole cell *Porphyromonas gingivalis* administration (9×10^{11} CFU)] was compared to decreased of IL-4 in the placebo group.

Nitrophenyl phosphate Tablets (Sigma-Aldrich) and by measuring the absorbance at 405 nm.

2.2. Ethics statement

Universitas Airlangga conferred ethical clearance for this study. Compliance with ethical standards, this research proposal was approved by the ethical committees of Airlangga Oral and Dental Hospital in collaboration with College of Dentistry Research Ethics Committee and College of Medicine Research Council (Ref: 50 /KKEPK.FKG/IV/2015) under the name of Nelwan SC as the Principal Investigator.

2.3. Animal studies

All studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. 16 healthy male Wistar rats (*Rattus norvegicus*; age 8–10 weeks, weight 120–150 g) were randomized and divided into four groups of intervention:

- Group 1: Placebo intra-sulcular injected
- Group 2: 9×10^7 CFU of *P. gingivalis* intra-sulcular injected
- Group 3: 9×10^9 CFU of *P. gingivalis* intra-sulcular injected
- Group 4: 9×10^{11} CFU of *P. gingivalis* intra-sulcular injected.

Administration of these strains had been done by intra-sulcular/subgingival injection at the coronal intra-sulcular surface of primary molar teeth.

2.4. Experimental procedures

The entire sample was taken blood serum at day 0 (before intra-sulcular injection), day 4 (after intra-sulcular injection), and day 11 (after intra-sulcular injection). We measured the level of IL-4, IL-5, IL-17F, IL-21, Ig-E, Ig-G_4, and IFN-γ with fluorescently labeled microsphere beads and direct sandwich enzyme-linked immunosorbent assay (ELISA). Samples were conducted with euthanasia protocols after research ended.

2.5. Study design

Pretest-posttest controlled group design was done in this study. We extracted 16 Wistar rats (*Rattus norvegicus*) and randomized them into 4 groups. There were no significant age and body weight differences. Within each group, we measured serum sample of IL-4, IL-5, IL-17F, IL-21, Ig-E, Ig-G_4, and IFN-γ before experiments began (day 0). After 4 days and 11 days, serum samples were also taken in both groups. The level of IL-4, IL-5, IL-17F, IL-21, Ig-E, Ig-G_4, and IFN-γ were measured and compared between groups and between periods.

2.6. Statistical analysis

Data analysis has been performed using SPSS version 17.0 (IBM Corp., Chicago, IL, USA). The assumption of normality distribution data has been tested by Shapiro–Wilk (SW) normality test. SW test showed W = 0.892516, V = 3.416357, P = 0.055, means that the data are normally distributed. Statistical analyses were done using analysis of variant (ANOVA). Results were presented as means ± standard deviation. The repeated measure ANOVA were used to test differences between level of IL-4, IL-5, IL-17F, IL-21, Ig-E, Ig-G4, and IFN-γ in each period among single groups of Wistar rats. The level of statistical significance was denoted at $p < 0.05$.

3. Results

We investigated average serum level of IL-4, IL-5, IL-17F, IL-21, Ig-E, Ig-G_4 and IFN-γ in the four groups, before and after 11 days administrations of whole-cell *Porphyromonas gingivalis*. Data are the

Table 1
Comparisons of mean IL-4 (ng/ml) before and after treatment (mean ± SD).

Time-point	n	Control	Pg 9×10^7 CFU	Pg 9×10^9 CFU	Pg 9×10^{11} CFU
Day-0 Before treatment	4	3.15 ± 1.29	0.79 ± 0.15	0.59 ± 0.15	23.79 ± 14.96
Day-4 After treatment	4	3.33 ± 2.88	0.98 ± 0.24	11.38 ± 12.77	11.27 ± 21.22
Day-11 After treatment	4	0.18 ± 0.07	0.62 ± 0.35	30.00 ± 14.86	54.17 ± 16.20
p value*	/	0.012	0.223	0.004	0.002

Note: df hypothesis = 2, df error = 11, F table 15.711; significant at $p < 0.05$.
* Measured by repeated measure ANOVA.

representative of four independent samples between each groups. Results of the study include comparison between periods and groups. Data expressed as mean ± SD of experiments made in quadruplicate.

We first investigated the effect of whole-cell *Porphyromonas gingivalis* administration at various doses of a colony forming unit (CFU). As has been shown in the figure below, group with high dose of whole-cell *Porphyromonas gingivalis* administration (9×10^{11} CFU) correlates with the increase of IL-4 (23.79 ± 14.96 ng/ml to 54.17 ± 16.20 ng/ml; $p = 0.02$). Nevertheless, serum IL-4 didn't change significantly with the administration of low-dose Pg (0.79 ± 0.15 ng/ml to 0.62 ± 0.35 ng/ml; $p = 0.223$) and were found decrease in placebo group (3.15 ± 1.29 ng/ml to 0.18 ± 0.07 ng/ml; $p = 0.012$) (see Fig. 1) (see Table 1).

As reported in the past, there are slight increases of serum IL-5 in groups with exposure of *Porphyromonas gingivalis* during 11 days of observation. In the group 4 [High dose of Pg (9×10^{11} CFU)], serum IL-5 had been observed increase slightly (207.60 ± 78.56 pg/ml to 243.40 ± 124.37 pg/ml; $p = 0.039$). Similar increase had been observed in the group 3 [medium dose of Pg (9×10^9 CFU)] and group 2 [low dose of Pg (9×10^7 CFU)], but no significant increase had been reported in control group/placebo (37.65 ± 3.37 pg/ml to 59.86 ± 5.83 pg/ml; $p = 0.058$) (see Fig. 2) (see Table 2).

Our results next confirmed that serum IL-17F weren't affected by treatment of the whole-cell Pg. Compared to each periods, no significant change had been observed in all groups. Compared to the other groups, the various doses of the whole-cell Pg treatment group demonstrated no significant differences between group 4 and the control group at day-11 ($p = 0.31$) (see Fig. 3) (see Table 3).

We next examined whether IL-21 is affected by administration of whole-cell Pg at various doses. Fig. 4 helped us to show whether the decrease of serum IL-21 occurred in all groups, in which the greatest decrease had been found in the group 4 [High dose of Pg (9×10^{11} CFU)]. Significant differences were found among the proportions of IL-21 decrements between group 4 and other group at day-11 ($p = 0.03$). Group 2 showed less decrements among other groups (see Fig. 4) (see Table 4).

Next, we compared serum Ig-E levels at the baseline with those at day-11 in subjects assigned to whole-cell Pg treatment. The results are summarized in Fig. 5 and show significant differences between each group for serum Ig-E level at the baseline (F statistic 14.988 > F table 3.49; $p = < 0.001$). After 4 days of experiments, there is a significant difference in net change between levels of serum Ig-E at the different dose of whole-cell Pg exposure (F statistic 3.623 > F table 3.49; $p = 0.045$). Nevertheless, a non-significant net Ig-E changes were shown by each group at day-11 (see Fig. 5) (see Table 5).

Before the experiments and until 4 days after the experiments, no

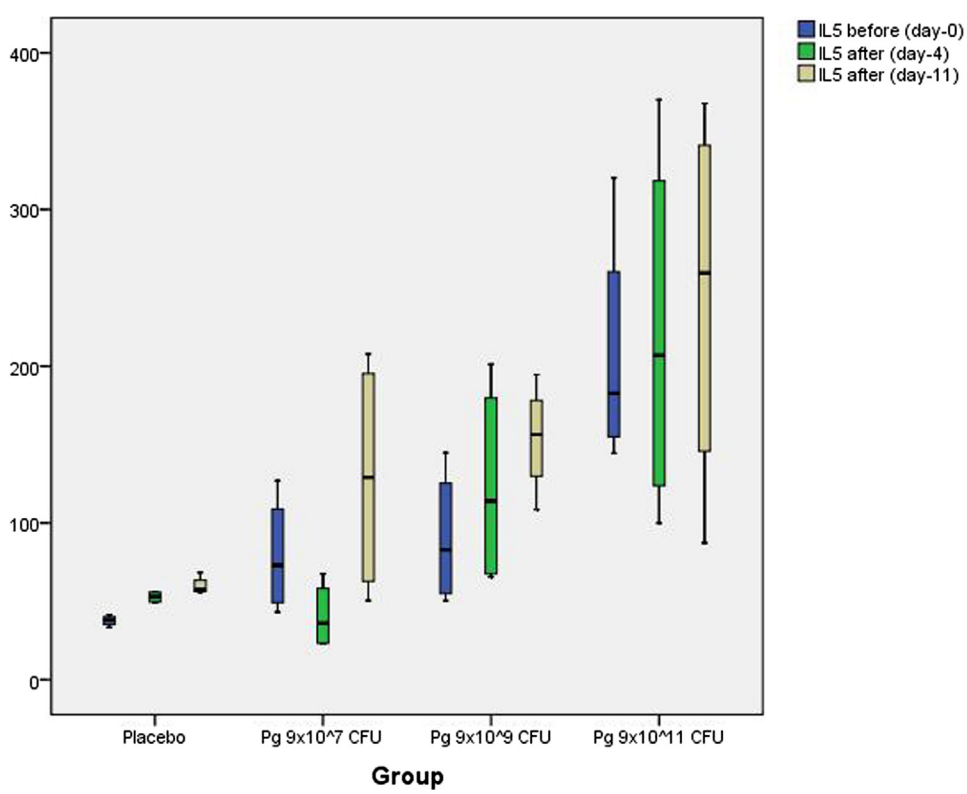

Fig. 2. Analysis of IL-5 as a pro-inflammatory cytokines before (day 0) and after (day 4 & day 11) treatment. Slight increase of IL-5 had been observed in all group of treatments. The proportions of these increment in all groups showed no statistically different.

Table 2
Comparisons of mean IL-5 (pg/ml) before and after treatment (mean ± SD).

Time-point	n	Control	Pg 9 × 10^7 CFU	Pg 9 × 10^9 CFU	Pg 9 × 10^{11} CFU
Day-0 Before treatment	4	37.65 ± 3.37	79.03 ± 37.78	90.16 ± 43.76	207.60 ± 78.56
Day-4 After treatment	4	52.74 ± 3.69	40.68 ± 21.63	123.66 ± 67.17	221.00 ± 121.57
Day-11 After treatment	4	59.86 ± 5.83	129.06 ± 77.89	153.99 ± 35.56	243.40 ± 124.37
p value*	/	0.058	0.062	0.003	0.039

Note: df hypothesis = 2, df error = 11, F table 15.711; significant at $p < 0.05$.
* Measured by repeated measure ANOVA.

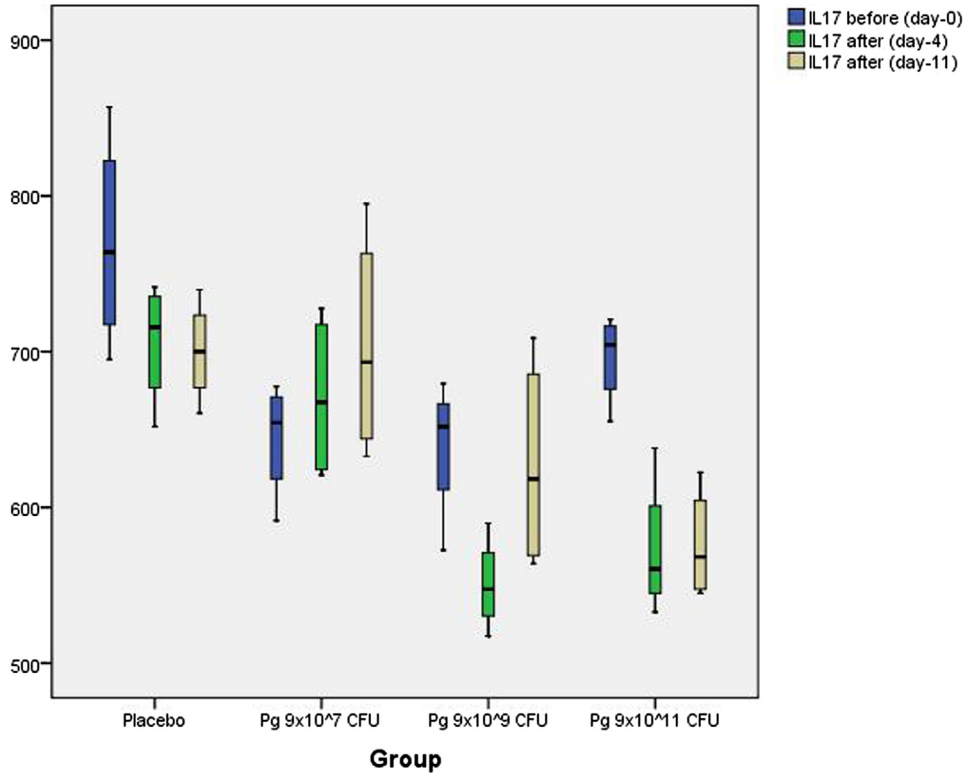

Fig. 3. Analysis of IL-17F as a pro-inflammatory cytokines before (day 0) and after (day 4 & day 11) treatment. Little decrease had been observed in all group of treatments. No statistically differences among IL-17F levels between all groups at day-0, day-4, and day-11.

Table 3
Comparisons of mean IL-17F (pg/ml) before and after treatment (mean ± SD).

Time-point	n	Control	Pg 9 × 10^7 CFU	Pg 9 × 10^9 CFU	Pg 9 × 10^{11} CFU
Day-0 Before treatment	4	769.9 ± 69.4	644.5 ± 37.8	638.9 ± 46.2	696.2 ± 29.1
Day-4 After treatment	4	706.2 ± 39.8	670.8 ± 54.5	550.5 ± 29.9	572.9 ± 45.4
Day-11 After treatment	4	700.1 ± 32.9	703.6 ± 74.0	627.2 ± 69.9	575.9 ± 36.0
p value*	/	0.072	0.059	0.225	0.317

Note: df hypothesis = 2, df error = 11, F table 15.711; significant at $p < 0.05$.
* Measured by repeated measure ANOVA.

significant difference was observed in the average serum of Ig-G_4. After 11 days of experiments, there was a reduction tendency in the group 2 (23.97 ± 3.77 ng/ml to 18.08 ± 3.01 ng/ml; $p = 0.01$), but no change in the group 3 (18.78 ± 2.95 ng/ml to 17.61 ± 2.79 ng/ml; $p = 0.17$) and group 4 (21.71 ± 3.53 ng/ml to 21.17 ± 3.39 ng/ml; $p = 0.43$). An increasing tendency was found in control group (8.34 ± 2.05 ng/ml to 13.91 ± 2.79 ng/ml; $p = 0.02$). (see Fig. 6)

As seen in Fig. 7, analysis of within-subject effects showed a slight but not significant increase in serum concentration of IFN-γ in the high-dose exposure of the whole-cell Pg across the days (140.40 ± 21.79 pg/ml at day-0 to 171.21 ± 23.07 pg/ml at day-11; $p = 0.08$). There is a pattern of decreases across the days between the control groups (181.44 ± 25.39 pg/ml at day-0 to 167.69 ± 22.81 pg/ml at day-11; $p = 0.34$). Test of between-subject effects showed no significant difference at day-4 and day-11. ($p = 0.23$ & $p = 0.47$, respectively). With respect to the group 4 and the control group, serum concentration of IFN-γ didn't change significantly in the group 2 and group 3. (see Fig. 7)

4. Discussion

In all experimental research, international guidelines highlight the necessity for standardized and validated measurements to quantify laboratory parameters for atopy and allergy. European Academy of

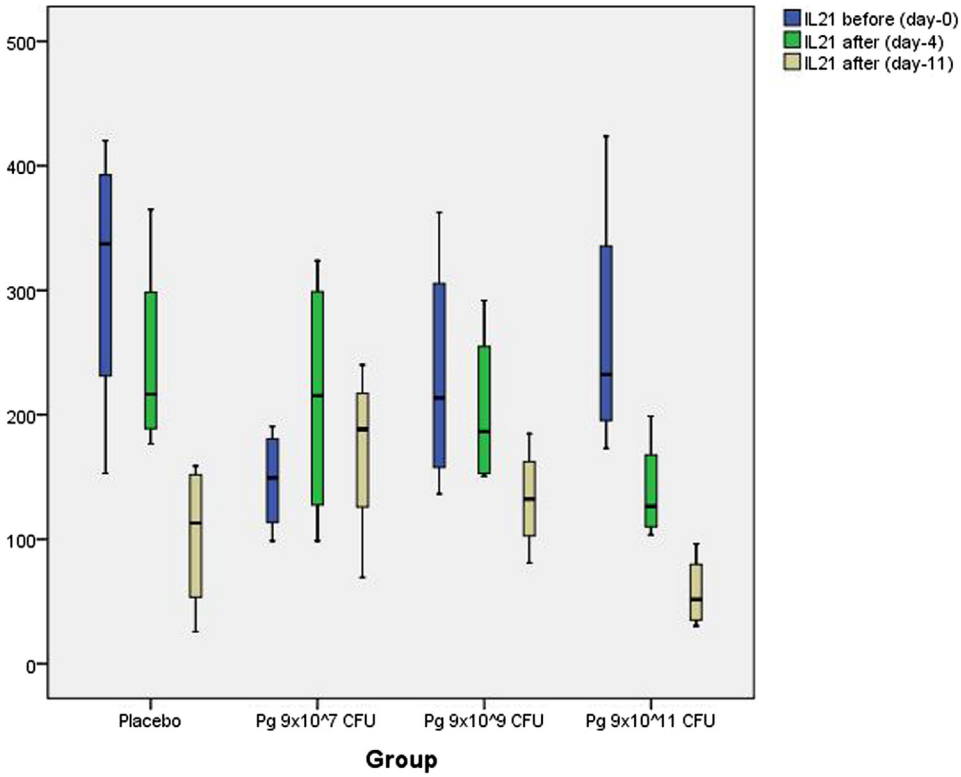

Fig. 4. Analysis of IL-21 as an anti-inflammatory cytokines before (day 0) and after (day 4 & day 11) treatment. Significant decrease had been observed in all group, however greatest decrease had been observed in the group 4. Statistically differences were found among the proportions of IL-21 decrements between group 4 and control group at day-11.

Allergy and Clinical Immunology Task Force reported a consensus statement on potential laboratory parameters for atopy. These were classified into 7 parameters: (1) Ig-E (both total and allergen-specific Ig-E); (2) Ig-G subclasses (both allergen-specific IgG [Ig-G_1] and blocking antibody [Ig-G_4]); (3) Interleukin-4 (IL-4), (4) IL-5, (5) IL-17F, (6) IL-21; and (7) in vivo biomarkers, which include provocation tests [23].

Porphyromonas gingivalis induces periodontitis through the disruption of the host adaptive immune response. Without alarming the innate immune system, *Porphyromonas gingivalis* is allowed to get an uncontrolled growth in oral cavity [24]. Several studies showed that high doses of *Porphyromonas gingivalis* does stimulate dendritic cells, but seems to prime T cells for subsequent production of IL-5 rather than IFN-γ [25]. Upon *Porphyromonas gingivalis* infection, periodontal tissues of infected mice readily expressed IL-17F within 12 h. IL-17F was found elevated in the sputum and bronchoalveolar lavage of infected mice, whereas the level of IL-17F in the blood remains similar [26]. Several studies demonstrated an increase of IL-21 would stimulate CD4 + T cells to produce IL-17F also [27,28]. This fact may explain why level of IL-17F in the blood wasn't significantly changed after exposure of various doses of *Porphyromonas gingivalis.*

As opposed to Ig-E, Ig-G_4 antibodies are recognized as a blocking antibody, which is capable to regulate the maladaptive immune response and atopic parameters towards allergy. Our study doesn't address a direct association between serum of Ig-G_4 levels and with serum Ig-E levels. Our results are in accordance with the literature whether increases of pro-inflammatory cytokines, such as IL-4 and IL-5. Even though our results are not followed by significant change of Ig-E and Ig-G_4 level respectively. This fact can be explained by common regulations of Ig-E and Ig-G_4 that is dependent to Th-2 cells [29]. Recent studies have clearly defined the differential roles of Ig-E and Ig-G_4 in atopic and allergic diseases; Ig-E stimulates mast cell degranulation resulting an occurrence of allergy, whereas Ig-G_4 mediates the pathophysiological processes that control them [30]. Thus, the Ig-E/Ig-G_4 balance could hypothetically have some value as a predictor or allergy severity or disease evolution. However, the Ig-G_4 binding-profile to allergen extracts can differ substantially from the Ig-E binding pattern, and these differences can be associated to clinical symptoms [31].

This research highlighted gram-negative anaerobic periodontal pathogen, *Porphyromonas gingivalis* as a specific contributor to atopic and allergic diseases like hay fever and bronchial asthma [32]. As a paradox to hygiene hypothesis, *Porphyromonas gingivalis* reveals low inflammatory potency during early childhood periods. Each cytokine produced in oral cavity exerted synergistic effects with those infection of *Porphyromonas gingivalis* to enhance ectopic and atopic immune responses [33–35]. Spontaneous allergy with co-morbid *Porphyromonas gingivalis* bacteremia has been reported in children with low-grade periodontal disease, and the frequency of detection of these pathogens in the oral cavity increases in children with allergies [36]. Following exposure of whole-cell *Porphyromonas gingivalis*, atopic immune response is initiated at an extralymphoidal site, typically in the airways'

Table 4
Comparisons of mean IL-21 (pg/ml) before and after treatment (mean ± SD).

Time-point	n	Control	Pg 9 × 10^7 CFU	Pg 9 × 10^9 CFU	Pg 9 × 10^{11} CFU
Day-0 Before treatment	4	311.9 ± 115.2	147.0 ± 41.4	231.5 ± 98.8	265.4 ± 109.9
Day-4 After treatment	4	243.6 ± 83.9	213.2 ± 103.7	203.8 ± 66.2	138.8 ± 42.3
Day-11 After treatment	4	102.5 ± 61.4	171.4 ± 72.5	132.6 ± 42.8	57.5 ± 29.4
p value*	/	0.002	0.039	0.017	< 0.001

Note: df hypothesis = 2, df error = 11, F table 15.711; significant at $p < 0.05$.
* Measured by repeated measure ANOVA.

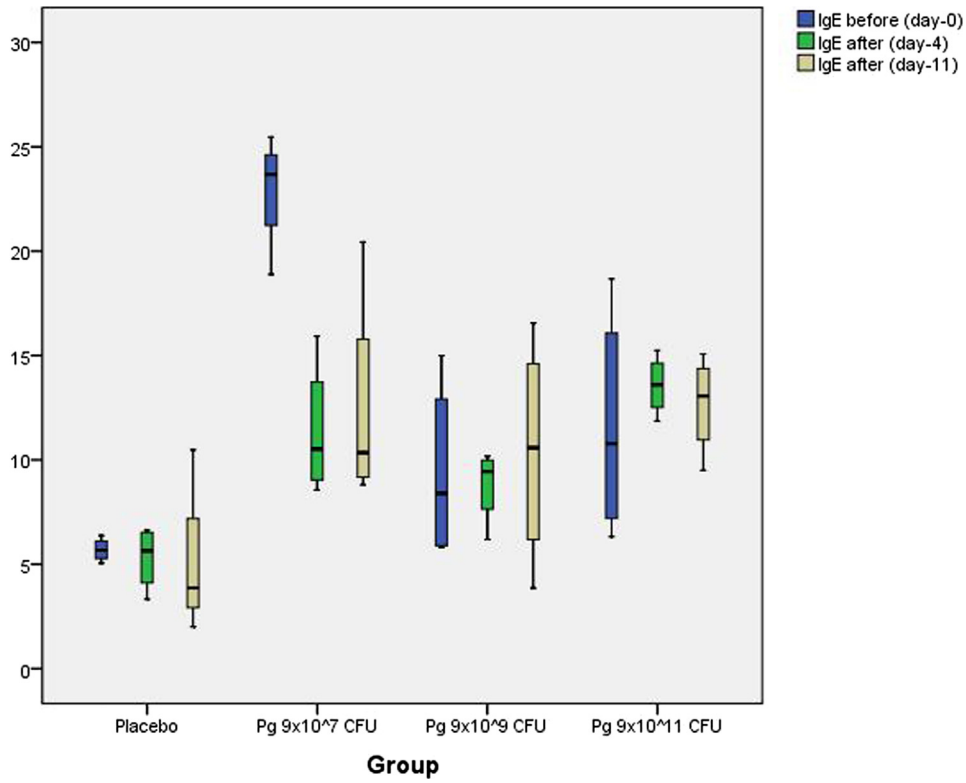

Fig. 5. Comparison of serum Ig-E level during 11 days of experiments. At day-4 and day-11 after treatments, we found statistically differences between group 4 and control group.

mucosa [37]. Hence, we could recognize why bronchial asthma is common among children with inadequate oral hygiene.

Our study had several limitations. Firstly, this study was conducted in Wistar rats, and the evolution of the laboratory parameters for atopy was not quite similar to human. Classic serum and blood biomarkers have not consistently correlated with the occurrence of allergies. Results from various experimental models can be difficult to interpret; for example, the protocols used for the investigation of the IgG_4 response in Wistar rats produce results that differ in several ways from human immune response. The close to 100% response rate in the mouse models contrasts with < 25% response rate in humans [38]. IgG_4 antibody responses in the rats are typically transient, whereas the atopic IgG_4 response in a human persists for many years, although its function for blocking antibody is practically similar [39]. Secondly, our subjects were unsupported with very similar baseline characteristic. As a consequence, we did subtract baseline data and require an adjustment to make clearer findings. Thirdly, our observations need being interpreted with caution because of the risk of the inflation of false positive tests related to multiple comparisons. However, our data were accurate and reproducible, because all parameters were assessed using the same equipment.

5. Conclusions

The presence finding suggests no significant inter-group differences in the levels of serum Ig-E, Ig-G_4, and IFN-γ; but a significant inter-group difference in the level of IL-4. In conclusion, results have demonstrated that some cytokines are up-regulated and others are down-regulated following exposure of various doses of whole-cell *Porphyromonas gingivalis*. Due to its limitations, this study needs to be interpreted with caution. However, this study may offer critical key insights that contribute to the rationale for clinical evaluation of *Porphyromonas gingivalis* in the mucosal and systemic immune responses.

6. Declaration of all sources of funding

All authors have nothing to declare regarding this work.

Table 5
Comparisons of mean Ig-E level (pg/ml) before and after treatment (mean ± SD).

Time-point	n	Control	Pg 9 × 10^7 CFU	Pg 9 × 10^9 CFU	Pg 9 × 10^{11} CFU	*F stat* *	*p* value*
Day-0 Before treatment	4	5.69 ± 0.56	22.92 ± 2.82	9.40 ± 4.39	11.64 ± 5.59	14.988	< 0.001
Day-4 After treatment	4	5.31 ± 1.53	11.38 ± 3.27	8.81 ± 1.80	13.57 ± 1.42	10.993	0.001
Day-11 After treatment	4	5.06 ± 3.72	12.48 ± 5.39	10.39 ± 5.46	12.67 ± 2.37	2.573	0.103
Δ net Ig-E changes day-4 from day-0	4	−0.38 ± 1.98	−11.54 ± 2.74	−0.59 ± 3.62	+1.94 ± 4.23	3.623	0.045
Δ net Ig-E changes day-11 from day-0	4	−0.64 ± 3.77	−10.44 ± 8.13	+0.99 ± 3.92	+1.03 ± 4.57	1.329	0.311
p value***	/	0.046	0.009	0.392	0.046	/	/

Note:
* Measured by one-way ANOVA (df1-3, df2 = 12, F table = 3.49; significant at p < 0.05).
*** Measured by repeated measure ANOVA (df hypothesis = 2, df error = 11, F table = 15.711; significant at p < 0.05; adjustment for multiple comparisons: Bonferroni).

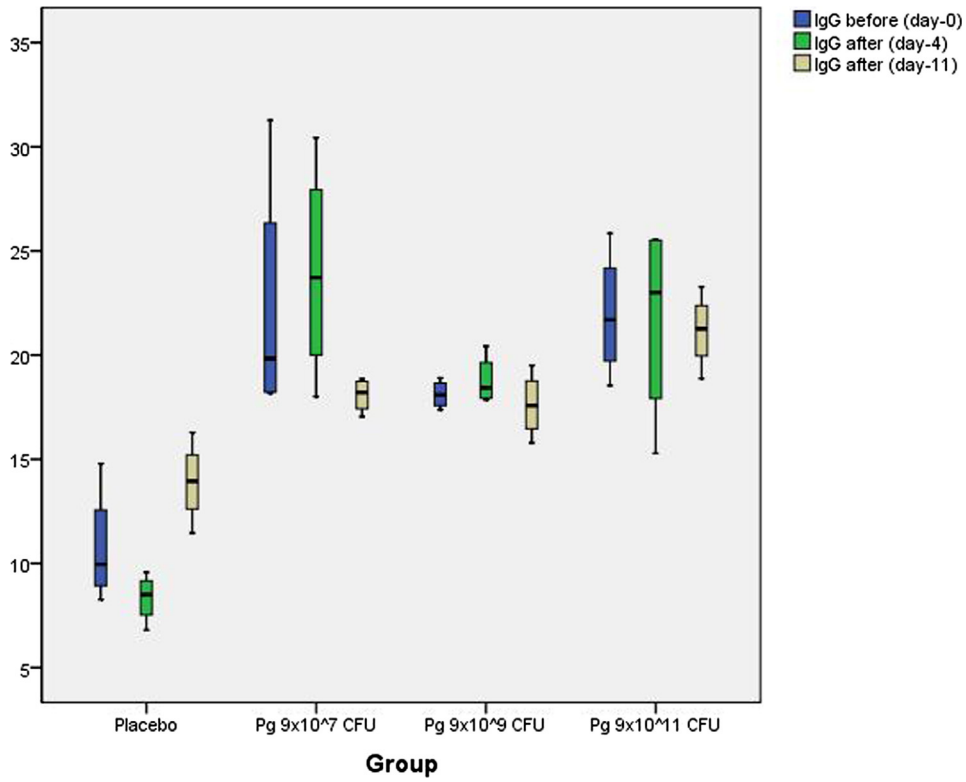

Fig. 6. Comparison of serum Ig-G_4 during 11 days of experiments. Group 2 shows slight decrease of serum Ig-G_4, whereas no significant change in the group 3 and group 4. Slight increase found in the control group. No significant inter-group differences had been found in day-11.

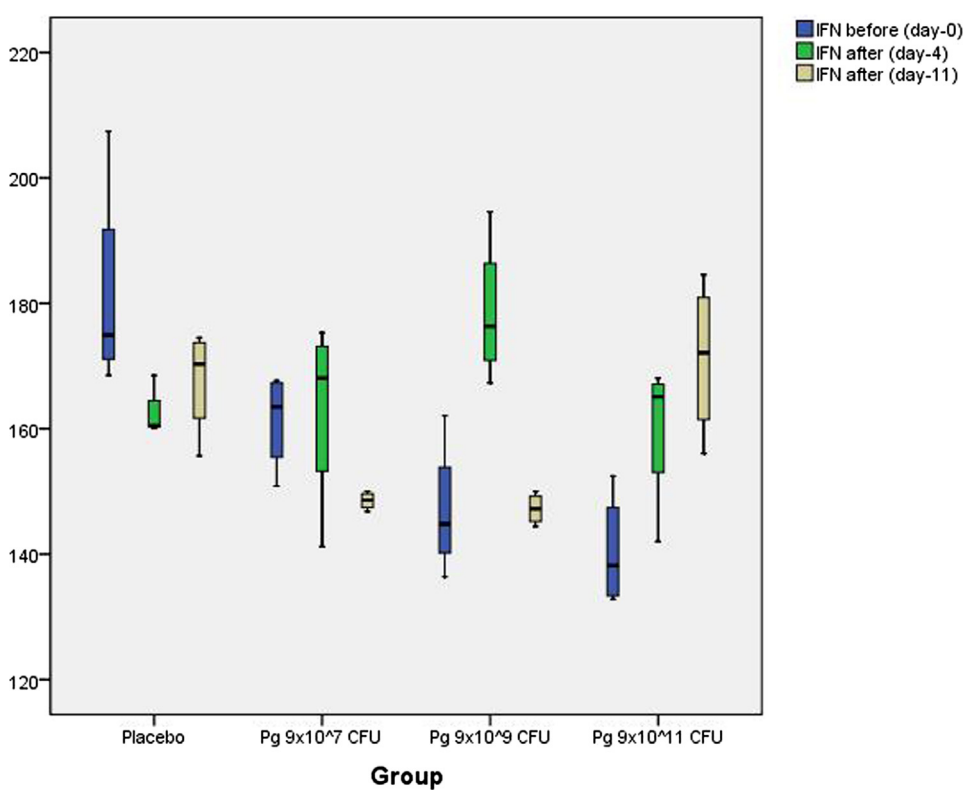

Fig. 7. Comparison of serum IFN-γ during 11 days of experiments. Slight decrease for control group and slight increase for group 4 in each period, but no statistical significance were measured. Inter-group analysis shows no significant differences at day-0, day-4, and day-11.

7. Availability of data and materials

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

8. Consent for publications

Not applicable.

9. Financial support and sponsorship

Nil.

Acknowledgment

We wish to thank Prof. Dr. Soetojo as Dean of Faculty of Medicine and Dr. Darmawan Setijanto as Dean of Faculty of Dentistry Universitas Airlangga for their support during the initiation of this study. The authors also gratefully thank all staffs and students from Universitas Airlangga, for their assistance and technical contribution.

Author contributions

S.C.N. (Pediatric Dentist/ Principal Investigator) conceived the presented ideas and carried out the experiment, R.A.N (Medical Doctor/ Research Scientist) analyzed the data and led the writing manuscript, A.E. (Allergist/ Clinical Assistant Professor) developed the theory, H.U. (Forensic Odontologist) performed the computation and gave critical revision of the manuscript, A.M. (Dental Biomaterials Scientist) contributed to the materials and sample preparation, U.T. and S.P. (Instructor/ Pediatric Dentists Consultants) helped supervise the project. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2018.06.015.

References

[1] M.I. Asher, S. Montefort, B. Bjorksten, C.K. Lai, D.P. Strachan, WS Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood, Int. Study Asthma Allerg. Child. 368 (2006) 733–743.
[2] A.B. Fishbein, R.L. Fuleihan, The hygiene hypothesis revisited: does exposure to infectious agents protect us from allergy? Curr. Opin. Pediatr. 24 (1) (2012 Feb) 98–102.
[3] Sally F. Bloomfield, Graham A.W. Rook, Elizabeth A. Scott, Fergus Shanahan, Rosalind Stanwell-Smith, Paul Turner, Time to abandon the hygiene hypothesis: new perspectives on allergic disease, the human microbiome, infectious disease prevention and the role of targeted hygiene, Perspect. Public Health 136 (4) (2016) 213–224.
[4] Bernardes E van Tilburg, M.C. Arrieta, Hygiene hypothesis in asthma development: is hygiene to blame? Arch. Med. Res. (2017).
[5] K. Tse, A.A. Horner, Allergen tolerance versus the allergic march: the hygiene hypothesis revisited, Curr. Allergy Asthma Rep. 8 (6) (2008) 475–483 Review.
[6] A. Patki, Eat dirt and avoid atopy: the hygiene hypothesis revisited, Indian J. Dermatol. Venereol. Leprol. 73 (1) (2007) 2–4.
[7] T.H. Lin, H.H. Su, H.Y. Kang, T.H. Chang, The interactive roles of lipopolysaccharides and dsRNA/viruses on respiratory epithelial cells and dendritic cells in allergic respiratory disorders: the hygiene hypothesis, Int. J. Mol. Sci. 18 (10) (2017).
[8] S.T. Sale, H. Parvez, R.K.R. Yeltiwar, G. Vivekanandan, A.J. Pundir, P. Jain, A comparative evaluation of topical and intrasulcular application of coenzyme Q10 (Perio Q™) gel in chronic periodontitis patients: a clinical study, J. Indian Soc. Periodontol. 18 (4) (2014) 461–465.
[9] S.C. Nelwan, A. Endaryanto, J.M. Harjanto, S. Pradopo, N. Prawati, Shifting immune response and cytokine profiles after porphyromonas gingivalis lipopolysacaride exposure, J. Int. Dent. & Med. Res. 10 (1) (2017) 95–99.
[10] S.C. Nelwan, R.A. Nugraha, A. Endaryanto, I. Retno, Modulating toll-like receptor-mediated inflammatory responses following exposure of whole cell and lipopolysaccharide component from *Porphyromonas gingivalis* in wistar rat models, Eur. J. Dent. 11 (4) (2017) 422–426.
[11] B. Lapin, J. Piorkowski, D. Ownby, S. Freels, N. Chavez, E. Hernandez, et al., Relationship between prenatal antibiotic use and asthma in at-risk children, Ann. Allergy Asthma Immunol. 114 (2015) 203–207.
[12] B.V. Reddy, V.K. Chava, S. Nagarakanti, S. Gunupati, P. Samudrala, Hygiene hypothesis and periodontitis–a possible association, Med. Hypotheses 82 (1) (2014) 60–63.
[13] S.K. Arora, P. Dewan, P. Gupta, Microbiome: Paediatricians' perspective, Indian J. Med. Res. 142 (5) (2015) 515–524.
[14] D.M. Saulnier, K. Riehle, T.A. Mistretta, M.A. Diaz, D. Mandal, S. Raza, et al., Gastrointestinal microbiome signatures of pediatric patients with irritable bowel syndrome, Gastroenterology 141 (2011) 1782–1791.
[15] C. Villeneuve, H.H. Kou, H. Eckermann, A. Palkar, L.G. Anderson, E.A. McKenney, R.R. Bollinger, W. Parker, Evolution of the hygiene hypothesis into biota alteration theory: what are the paradigms and where are the clinical applications? Microbes Infect. S1286–4579 (17) (2017) 30185–30195.
[16] N. Friedrich, H. Volzke, C. Schwahn, A. Kramer, M. Junger, T. Schafer, U. John, T. Kocher, Inverse association between periodontitis and respiratory allergies, Clin. Exp. Allergy. 36 (2006) 495–502.
[17] T.A. Platts-Mills, The allergy epidemics: 1870–2010, J. Allergy Clin. Immunol. 136 (1) (2015) 3–13.
[18] S.H. Chang, C. Dong, IL-17F: regulation, signaling and function in inflammation, Cytokine 46 (1) (2009) 7–11.
[19] W. Jin, C. Dong, IL-17 cytokines in immunity and inflammation, Emerg. Microbes Infect. 2 (2013) e60.
[20] S. Kuchen, R. Robbins, G.P. Sims, C. Sheng, T.M. Phillips, P.E. Lipsky, R. Ettinger, Essential role of IL-21 in B cell activation, expansion, and plasma cell generation during CD4+ T cell-B cell collaboration, J. Immunol. 179 (9) (2007) 5886–5896.
[21] Y. Wu, N.M. van Besouw, Y. Shi, M.J. Hoogduijn, L. Wang, C.C. Baan, The biological effects of IL-21 signaling on B-cell-mediated responses in organ transplantation, Front. Immunol. 7 (2016) 319.
[22] G.L. Piacentini, S. Guerresi, A. Kantar, L. Lubrano, F. Olivieri, A.L. Boner, D.G. Peroni, A comparison between IgE and IgG4 as markers of allergy in children: an experimental trial in a model of natural antigen avoidance, Int. J. Immunopathol. Pharmacol. 24 (4) (2011) 1049–1056.
[23] M.T. Ventura, M. Casciaro, S. Gangemi, R. Buquicchio, Immunosenescence in aging: between immune cells depletion and cytokines up-regulation, Clin. Mol. Allergy 15 (2017) 21.
[24] L. Gölz, S. Memmert, B. Rath-Deschner, A. Jäger, T. Appel, G. Baumgarten, et al., LPS from *P. gingivalis* and hypoxia increases oxidative stress in periodontal ligament fibroblasts and contributes to periodontitis, Mediators Inflamm. 2014 (2014) 986264.
[25] K. Abdi, T. Chen, B.A. Klein, A.K. Tai, J. Coursen, X. Liu, J. Skinner, S. Periasamy, Y. Choi, B.M. Kessler, R.J. Palmer, Mechanisms by which *Porphyromonas gingivalis* evades innate immunity, PLoS One 12 (8) (2017) e0182164.
[26] K.I. Happel, P.J. Dubin, M. Zheng, N. Ghilardi, C. Lockhart, L.J. Quinton, A.R. Odden, J.E. Shellito, G.J. Bagby, S. Nelson, J.K. Kolls, Divergent roles of IL-23 and IL-12 in host defense against *Klebsiella pneumoniae*, J. Exp. Med. 202 (2005) 761–769.
[27] H. Piyadasa, A. Altieri, S. Basu, J. Schwartz, A.J. Halayko, N. Mookherjee, Biosignature for airway inflammation in a house dust mite-challenged murine model of allergic asthma, Biol Open. 5 (2) (2016) 112–121.
[28] C. Jandl, S.M. Liu, P.F. Cañete, J. Warren, W.E. Hughes, A. Vogelzang, K. Webster, M.E. Craig, G. Uzel, A. Dent, P. Stepensky, B. Keller, K. Warnatz, J. Sprent, C. King, IL-21 restricts T follicular regulatory T cell proliferation through Bcl-6 mediated inhibition of responsiveness to IL-2, Nat. Commun. 8 (2017) 14647.
[29] K.D. Stone, C. Prussin, D.D. Metcalfe, IgE, mast cells, basophils, and eosinophils, J. Allergy Clin. Immunol. 125 (2 Suppl 2) (2010) S73–S80.
[30] S.G. Gao, J.Q. Yang, Z.K. Ma, X. Yuan, C. Zhao, G.C. Wang, H. Wei, X.S. Feng, Y.J. Qi, Preoperative serum immunoglobulin G and A antibodies to *Porphyromonas gingivalis* are potential serum biomarkers for the diagnosis and prognosis of esophageal squamous cell carcinoma, BMC Cancer. 18 (1) (2018) 17.
[31] N.M. O'Brien-Simpson, J.A. Holden, J.C. Lenzo, Y. Tan, G.C. Brammar, K.A. Walsh, W. Singleton, R.K.H. Orth, N. Slakeski, K.J. Cross, I.B. Darby, D. Becher, T. Rowe, A.B. Morelli, A. Hammet, A. Nash, A. Brown, B. Ma, D. Vingadassalom, J. McCluskey, H. Kleanthous, E.C. Reynolds, A therapeutic *Porphyromonas gingivalis* gingipain vaccine induces neutralising IgG1 antibodies that protect against experimental periodontitis, NPJ Vaccines. 1 (2016) 16022.
[32] J. Carrion, E. Scisci, B. Miles, G.J. Sabino, A.E. Zeituni, Microbial carriage state of peripheral blood dendritic cells (DCs) in chronic periodontitis influences DC differentiation, atherogenic potential, J. Immunol. 189 (2012) 3178–3187.
[33] R. Cheng, T. Hu, N.A. Bhowmick, Be resistant to apoptosis: a host factor from gingival fibroblasts, Cell Death Dis. 6 (2015) e2009.
[34] R.P. Darveau, G. Hajishengallis, M.A. Curtis, *Porphyromonas gingivalis* as a potential community activist for disease, J. Dent. Res. 91 (2012) 816–820.
[35] R. Cheng, W. Liu, R. Zhang, Y. Feng, N.A. Bhowmick, T. Yu, *Porphyromonas gingivalis*-derived lipopolysaccharide combines hypoxia to induce caspase-1 activation in periodontitis, Front. Cell. Infect. Microbiol. 7 (2017) 474.
[36] C. Hayashi, C.V. Gudino, F.C. Gibson III, C.A. Genco, Review: Pathogen-induced inflammation at sites distant from oral infection: bacterial persistence and induction of cell-specific innate immune inflammatory pathways, Mol. Oral. Microbiol. 25 (2010) 305–316.

[37] J.W. Card, M.A. Carey, J.W. Voltz, J.A. Bradbury, C.D. Ferguson, E.A. Cohen, S. Schwartz, G.P. Flake, D.L. Morgan, S.J. Arbes Jr, D.A. Barrow, S.P. Barros, S. Offenbacher, D.C. Zeldin, Modulation of allergic airway inflammation by the oral pathogen *Porphyromonas gingivalis*, Infect. Immun. 78 (6) (2010) 2488–2496.

[38] T.A. Platts-Mills, J.W. Vaughan, K. Blumenthal, S. Pollart Squillace, R.B. Sporik, Serum IgG and IgG4 antibodies to Fel d 1 among children exposed to 20 microg Fel d 1 at home: relevance of a nonallergic modified Th2 response, Int. Arch Allergy Immunol. 124 (2001) 126–129.

[39] F. Ramadani, H. Bowen, N. Upton, P.S. Hobson, Y.C. Chan, J.B. Chen, T.W. Chang, J.M. McDonnell, B.J. Sutton, D.J. Fear, H.J. Gould, Ontogeny of human IgE-expressing B cells and plasma cells, Allergy 72 (1) (2017) 66–76.

Data Profile - Data Sets

Author: sindy-c-n@fkg.unair.ac.id

Name	Caption	Description	File type	File Size	Source	Access Type
Porphyromonas gingivalis and Atopy	Converging Findings from Linkage between Periodontal Pathogen with Atopic and Allergic Immune Response	Interleukin (IL)-4 and IL-5 had been found increase after exposure to the periodontal pathogens Porphyromonas gingivalis, whereas no or minimal change had been found in the level of IL-17F, Ig-G4, and IFN-γ. The various cytokines and immunoglobulins shown in this study do not prove a causal relationship, and the precise role of Porphyromonas gingivalis in the regulation of atopic immune response warrants further investigation.	XLS	22 KB	Original data	Data available on request Email address of the person that can be contacted with requests for data: sindy-c-n@fkg.unair.ac.id