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Differential human Th22-lymphocyte response triggered by *Aggregatibacter actinomycetemcomitans* serotypes

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ABSTRACT

Objective: In *Aggregatibacter actinomycetemcomitans*, different serotypes have been described based on lipopolysaccharide (LPS) antigenicity. When T lymphocytes were stimulated with these serotypes, different patterns of T-helper (Th)1 and Th17-type of immune responses were reported. Recently, two new Th phenotypes have been described and named Th9 and Th22 lymphocytes; however, their role in the pathogenesis of periodontitis remains unclear. This study aimed to investigate the potential Th9 and/or Th22 lymphocyte responses when stimulated with autologous dendritic cells infected with different *A. actinomycetemcomitans* serotypes.
Methods: Monocyte-derived dendritic cells and *naïve* $CD4^+$ T lymphocytes were obtained from healthy donors and stimulated with different serotypes of *A. actinomycetemcomitans* at a multiplicity of infection MOI = 10^2 or their purified LPS (10–50 ng/ml). The levels for the Th9 and Th22-associated cytokines, as well as the transcription factor master-switch genes implied in their differentiation Spi-B and AhR, were quantified by qPCR and ELISA.
Results: When stimulated with the serotype *b* of *A. actinomycetemcomitans*, higher levels of interleukin (IL)-6 and tumor necrosis factor (TNF)-α were detected in dendritic cells, as well as higher levels of IL-22 and AhR were detected in T lymphocytes, when compared with stimulation with the other serotypes.
Conclusions: The serotype *b* of *A. actinomycetemcomitans* has a higher capacity of trigger Th22-type of immune response in both dendritic cells and T lymphocytes. These data allow us to suggest that, when the serotype *b* of *A. actinomycetemcomitans* is a significant part of the subgingival biofilm, the Th22 polarization might be triggered within the periodontal lesion.

1. Introduction

Aggregatibacter actinomycetemcomitans is a highly pathogenic bacteria strongly associated with periodontitis (Fine et al., 2007; Herbert et al., 2016; Könönen & Müller, 2014; Schacher et al., 2007; Slots & Ting, 1999; Socransky & Haffajee, 2005). Although it has been associated mostly with the aggressive forms of the disease, it can also be found in patients with chronic periodontitis as well as in healthy individuals, what suggests that different clones of the same species may have different degrees of virulence (Schacher et al., 2007; van der Reijden et al., 2008). In fact, different serotypes of *A. actinomycetemcomitans* have shown distinct immunogenicity, and our research group has reported that the serotype *b* of *A. actinomycetemcomitans*, when exposed to human dendritic cells and T lymphocytes, induced significantly higher levels of T-helper (Th)1 and Th17-associated cytokines, chemokines, and transcription factors, when compared with the other *A. actinomycetemcomitans* serotypes, thus inducing a pattern of immune response associated with the pro-inflammatory and destructive events characteristic of periodontitis (Alvarez et al., 2015; Díaz-Zúñiga, Melgar-Rodríguez et al., 2015; Díaz-Zúñiga, Monasterio et al., 2015; Díaz-Zúñiga et al., 2014; Melgar-Rodríguez et al., 2015).

Recently, two new lineages of Th lymphocytes have been described and named Th9 and Th22 lymphocytes. The Th9 subset, which differentiates in presence of interleukin (IL)-4 and transforming growth factor (TGF)-β1 during antigen presentation,

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expresses the transcription factor master-switch PU.1-related that binds to PU-box (Spi-B), and displays immuno-suppressor functions by producing IL-9 (Akdis, Palomares, van de Veen, van Splunter, & Akdis, 2012; Trifari, Kaplan, Tran, Crellin, & Spits, 2009). Otherwise, the Th22 subset, which differentiates in presence of IL-6 and tumor necrosis factor (TNF)-α during the antigen presentation, expresses the transcription factor master-switch aryl hydrocarbon receptor (AhR), and displays pro-inflammatory functions by producing IL-22 (Jabeen & Kaplan, 2012; Kaplan, 2013). The heterogeneity in the immuno-stimulatory potential attributed to the different serotypes of *A. actinomycetemcomitans*, however, has not yet been tested with these newly described Th lymphocyte subpopulations.

The aim of this investigation was, therefore, to evaluate whether different serotypes of *A. actinomycetemcomitans* when exposed to human dendritic cells and T lymphocytes trigger a differential Th9 and Th22 responses. We hypothesized that the serotype *b* of *A. actinomycetemcomitans* induces higher levels of the Th22-associated cytokines in human dendritic cells and higher levels of IL-22 and AhR in human T lymphocytes, compared with the other serotypes.

2. Materials and methods

2.1. Study population

Blood cells were obtained during platelet-apheresis processes from healthy donors consecutively enrolled at the Blood Bank of the Hospital Del Salvador in the Eastern Metropolitan Health Service, Santiago, Chile. The study group consisted of 10 adults (five males and five females, aged 30 to 36; mean age 32.4 ± 2.07 years) who did not have periodontal disease as determined by absence of gingival inflammation, no clinical attachment level (CAL) loss, and probing depth (PD) <4 mm. Further exclusion criteria were being positive for human immunodeficiency virus and hepatitis B or C virus, history of manifest infections during the last month, fever, symptomatic allergies, abnormal blood cell counts, increased liver enzymes, or intake of any kind of medication, except vitamins and oral contraceptives. The study design (#2010/14) was approved by the Ethics Committee of Faculty of Dentistry, Universidad de Chile, and conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000. The protocol of the study was clearly explained to all the individuals, who agreed to participate in it by signing an IRB-approved informed consent.

2.2. A. actinomycetemcomitans *strains*

The *A. actinomycetemcomitans* strains ATCC® 43717™ (serotype *a*), ATCC® 43718™ (serotype *b*), and ATCC® 43719™ (serotype *c*) were cultured on agar brain-heart infusion medium (Oxoid Ltd, Hampshire, England) at 37 °C under capnophilic conditions (8% O_2 and 12% CO_2) using an appropriate micro-aerobic condition generator (CampyGen™; Oxoid Ltd, Hampshire, England). Growth curves were made in liquid brain-heart infusion medium (Oxoid Ltd, Hampshire, England) until a reliable number of colony-forming units for the *in vitro* stimulation of dendritic cells was obtained (Vernal et al., 2008). Viable bacteria were taken during the exponential growth phase of the bacterial culture and used for dendritic cell stimulation. Lipopolysaccharide (LPS) was purified from the different *A. actinomycetemcomitans* strains as described previously (Díaz-Zúñiga et al., 2014).

2.3. Dendritic cell differentiation and stimulation

Immature monocyte-derived dendritic cells were obtained and stimulated as described previously (Vernal et al., 2008). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from platelet-apheresis filters following standard procedures (Ficoll-Paque Plus®; GE Healthcare, Uppsala, Sweden). Monocytes ($CD14^+$ cells) were purified from PBMCs by magnetic-cell-sorting using an anti-CD14 monoclonal antibody conjugated to magnetic beads (MACS®; Miltenyi Biotec, Bergisch Gladbach, Germany) and then differentiated to dendritic cells by culture at 1×10^6 cells/ml in RPMI-1640 containing 10% fetal calf serum (Gibco Invitrogen Corp., Grand Island, NY, USA) and 20 ng/ml of recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) and recombinant human interleukin-4 (rhIL-4) (R&D Systems Inc., Minneapolis, MN, USA) for 6 d at 37 °C. Differentiated dendritic cells were stimulated at a multiplicity of infection MOI = 10^2 (bacteria/dendritic cells ratio) with different *A. actinomycetemcomitans* strains or 10, 20, or 50 ng/ml of their purified LPS for 2 d. For each individual, the experiment was performed separately. Dendritic cells stimulated with 10 ng/ml *Escherichia coli* strain 0111:B4 LPS (Fluka, Sigma-Aldrich Chemie, Buchs, Switzerland) and non-induced dendritic cells were used as controls.

2.4. T-lymphocyte stimulation

A purified population of *naïve* $CD4^+$ T lymphocytes was obtained by magnetic-cell-sorting from the $CD14^-$ cell fraction of the PBMCs as described previously (Díaz-Zúñiga, Melgar-Rodríguez et al., 2015). Briefly, both non-T lymphocytes and memory Th lymphocytes were depleted using a cocktail of biotin-conjugated monoclonal antibodies and anti-biotin monoclonal antibody conjugated to magnetic beads (MACS®; Miltenyi Biotec, Bergisch Gladbach, Germany). For T-lymphocyte stimulation, 1×10^6 cells/ml were cultured with primed autologous dendritic cells (50:1) in RPMI-1640 containing 10% fetal calf serum (Gibco Invitrogen Corp., Grand Island, NY, USA) for 5 d at 37 °C. Previous to each co-culture, dendritic cells were washed twice in RPMI-1640 supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MI, USA). For each individual, the experiment was performed separately. T-lymphocyte cultures devoid of dendritic cells or exposed to non-induced autologous dendritic cells were used for comparison. In each experimental step, dendritic cell and T-lymphocyte counting was performed with a hemocytometer and using a phase contrast microscopy (Axiovert 100®; Zeiss Co., Göttingen, Germany) and cell viability equal to or greater than 95% was calculated by Trypan blue dye exclusion.

2.5. Expression of cytokines and transcription factors

Total cytoplasmic RNA was isolated from dendritic cells and T lymphocytes as described previously (Vernal et al., 2008). Synthesis of first-strand cDNA was performed using a reverse transcription kit following the manufacturer's recommendations (SuperScrip™ III; Invitrogen, Grand Island, NY, USA). The mRNA expression levels for the cytokines IL-4, IL-6, IL-9, IL-22, TNF-α, and TGF-β1, and the transcription factor master-switch Spi-B and AhR were quantified by qPCR using the appropriate forward and reverse primers (Table 1). In a qPCR equipment (StepOnePlus®; Applied Biosystems, Singapore), 50 ng of cDNA were amplified using a qPCR reagent (KAPA™ SYBR® Fast qPCR; KAPA Biosystems, Woburn, MA, USA) as follows: a first step of 95 °C for 3 min, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. For detection of non-specific product formation and false-positive amplification, a final melt curve of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s was performed. As an endogenous control, 18S rRNA expression levels were determined.

Table 1
Forward and reverse primers used for cytokine and transcription factor mRNA and 18S rRNA amplifications by qPCR.

Target	Forward Primer	Reverse Primer
IL-4	caccgagttgaccgtaacag	gccctgcagaaggtttcc
IL-6	gcccagctatgaactccttct	gaaggcagcaggcaacac
IL-9	catccttgcctctgttttgct	gggagagacacaagcagctgg
IL-22	acctttccttgaccaaactca	agcttcttctcgctcagacg
TNF-α	cagcctcttctccttcctgat	gccagagggctgattagaga
TGF-β1	cacgtggagctgtaccagaa	cagccggttgctgaggta
Spi-B	caacatcacctacgccagtc	ctatgccgcttggaaggat
AhR	gcataccccacggagaact	cggtaagtcttcctcctctgatag
18S rRNA	ctcaacacgggaaacctcac	cgctccaccaactaagaacg

2.6. Secretion of cytokines

After bacterial or LPS stimulation for 5 d, T-lymphocyte culture supernatants were collected and the secretion of IL-9 and IL-22 was quantified by ELISA according to manufacturer's protocols (Quantikine®; R&D Systems Inc., Minneapolis, MN, USA) and using an automatic microplate spectrophotometer (Synergy™ HT; Bio-Tek Instrument Inc., Winooski, VT, USA).

2.7. Statistical analysis

The qPCR data were presented as relative fold-change quantities by normalizing the cytokine or transcription factor mRNA expression to 18S rRNA expression using the $2^{-\Delta\Delta Ct}$ method (StepOne version 2.2.2; Applied Biosystems, Singapore). The ELISA data were presented as pg/ml and calculated with a four-parameter logistic equation. Using a statistical software (SPSS version 15.0; Lead Technologies Inc., Charlote, NC, USA), the normality of data distribution was determined using the Kolmogorov-Smirnov test. Differences among groups and within each group regarding cytokine and transcription factor mRNAs expression as well as cytokine secretion were analyzed using the ANOVA and Tukey post-hoc tests. Correlation coefficients were obtained using the Pearson test. Statistical significance was assumed when p-value <0.05.

3. Results

3.1. Expression of IL-4, IL-6, TNF-α, and TGF-β1 in bacteria-stimulated dendritic cells

Due to IL-4 and TGF-β1 determine the Th9 polarization, and IL-6 and TNF-α determine the Th22 polarization when dendritic cells prime *naïve* $CD4^+$ T lymphocytes during the antigen presentation, the mRNA expression for these cytokines was quantified in dendritic cells stimulated at a MOI = 10^2 with different serotypes of *A. actinomycetemcomitans* (Fig. 1A). An increment in the expression levels for IL-4, IL-6, TNF-α, and TGF-β1 mRNAs was detected when dendritic cells were stimulated with the different serotypes of *A. actinomycetemcomitans* compared with non-induced cells. In particular, when serotype *b* (strain ATCC® 43718™) of *A. actinomycetemcomitans* was used for dendritic cell stimulation, higher expressed levels of IL-6 ($p = 0.01$ and $p = 0.043$) and TNF-α ($p = 0.006$ and $p = 0.007$) were detected compared with using either serotype *a* (strain ATCC® 43717™) or serotype *c* (strain ATCC® 43719™), respectively. No differences were detected in the mRNA expression for IL-6 and TNF-α in dendritic cells after challenge with *A. actinomycetemcomitans* serotypes *a* or *c*. The levels of IL-4 and TGF-β1 mRNAs were no different when the same dendritic cells were stimulated with *A. actinomycetemcomitans* serotypes *a*, *b*, or *c*.

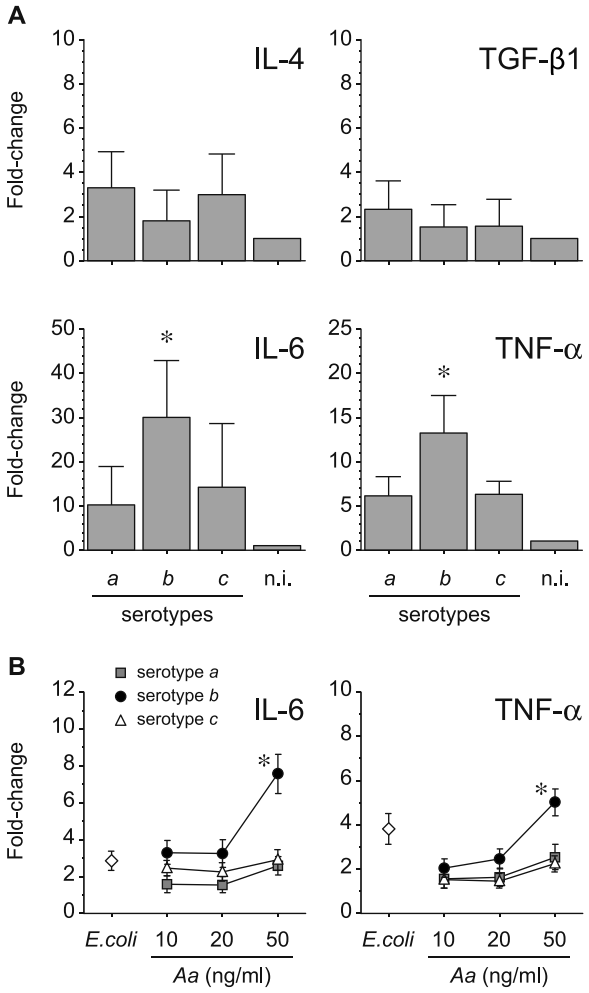

Fig. 1. Production of IL-4, IL-6, TNF-α, and TGF-β1 in dendritic cells. (A) Expression levels for the cytokines IL-4, IL-6, TNF-α, and TGF-β1 in dendritic cells stimulated at a MOI = 10^2 with the serotypes *a*, *b*, or *c* of *A. actinomycetemcomitans*. For relative expression, the cytokine expression in non-induced (n.i.) dendritic cells was considered as 1, as a reference for fold-change in expression. (B) Expression levels for the cytokines IL-6 and TNF-α in dendritic cells stimulated with 10, 20, or 50 ng/ml of LPS purified from the serotypes *a*, *b*, or *c* of *A. actinomycetemcomitans*. Dendritic cells stimulated with 10 ng/ml of the *Escherichia coli* LPS (*E. coli*) were used as positive control. Comparisons were done between the different *A. actinomycetemcomitans* serotypes (*$p < 0.05$).

3.2. Expression of IL-4, IL-6, TNF-α, and TGF-β1 in LPS-stimulated dendritic cells

The increased expression levels of Th22-associated cytokines in dendritic cells upon stimulation with serotype *b* of *A. actinomycetemcomitans* was confirmed when the mRNA expression levels of the analyzed cytokines were quantified in the same cells stimulated with LPS purified from the different serotypes of *A. actinomycetemcomitans* (Fig. 1B). A dose-dependent increase in the expression levels for IL-6 and TNF-α mRNAs was elicited in dendritic cells stimulated with 10, 20, or 50 ng/ml of LPS purified from each of the serotypes of *A. actinomycetemcomitans*. The dendritic cells stimulated with 50 ng/ml of LPS purified from the serotype *b* of *A. actinomycetemcomitans* had a higher expression of IL-6 ($p = 0.003$ and $p = 0.004$) and TNF-α ($p = 0.021$ and $p = 0.012$) than cells stimulated with 50 ng/ml of LPS purified from bacterial strains belonging to either the serotype *a* or *c*, respectively. No differences were detected in the mRNA expression for IL-6 and TNF-α in dendritic cells after challenge with 50 ng/ml of LPS purified from *A. actinomycetemcomitans* serotypes *a* or *c*. The levels

of IL-4 and TGF-β1 mRNAs were no different when dendritic cells were stimulated with any concentration of LPS purified from *A. actinomycetemcomitans* serotypes *a*, *b*, or *c* (data not shown). Overall, an increased response biased towards a Th22-pattern of cytokine expression was detected in *A. actinomycetemcomitans* serotype *b*-stimulated dendritic cells compared with the same cells stimulated with the other serotypes.

3.3. Expression and secretion of IL-9 and IL-22 by bacteria-stimulated T lymphocytes

The levels of IL-9 and IL-22 quantified at mRNA and protein levels in $CD4^+$ T lymphocytes stimulated with autologous dendritic cells primed at a MOI = 10^2 with different serotypes of *A. actinomycetemcomitans* were expressed as fold-change (Fig. 2A) and pg/ml (Fig. 2B) for each condition. The T lymphocytes stimulated with serotype *b*-primed dendritic cells showed higher expression levels of IL-22 mRNA than cells stimulated with the other *A. actinomycetemcomitans* serotypes ($p < 0.001$). Similarly, when stimulated with serotype *b*, T lymphocytes secreted higher levels of IL-22 than the same cells stimulated with the *A. actinomycetemcomitans* serotypes *a* or *c* ($p < 0.001$). When IL-9 was analyzed at protein level, higher secretion was detected in T lymphocytes stimulated with serotype *b*-primed dendritic cells than cells stimulated with the other *A. actinomycetemcomitans* serotypes ($p < 0.001$); however, no differences were detected at mRNA level. No differences were detected for IL-9 and IL-22 mRNA expression and secretion in T lymphocytes after challenge with dendritic cells stimulated with *A. actinomycetemcomitans* serotypes *a* or *c*.

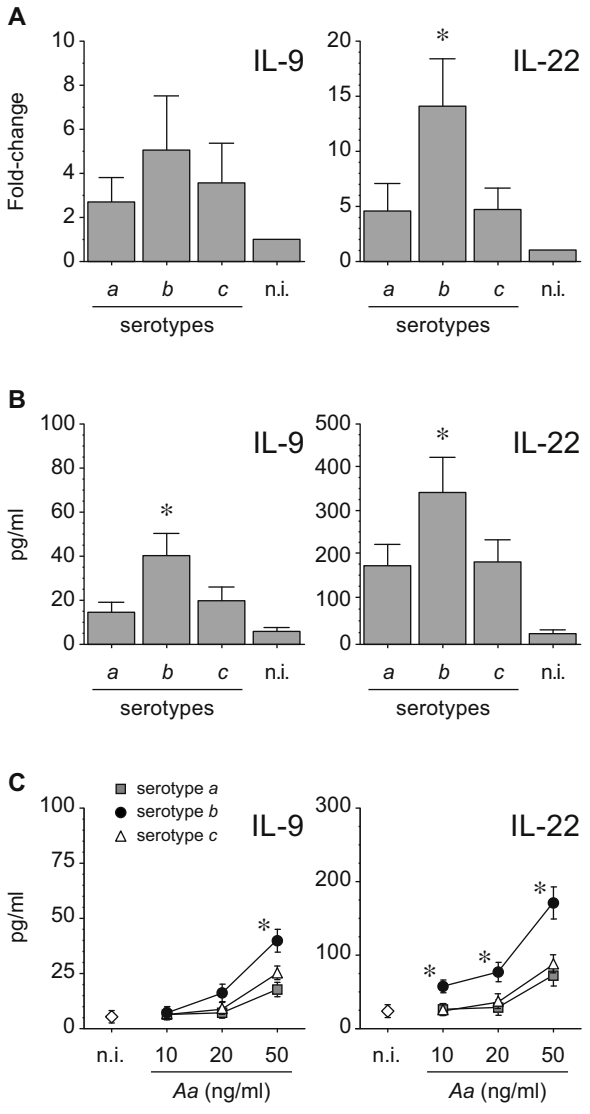

Fig. 2. Production of IL-9 and IL-22 in T lymphocytes. (A) Expression levels for the cytokines IL-9 and IL-22 in *naïve* $CD4^+$ T lymphocytes activated with dendritic cells primed at a MOI = 10^2 with the serotypes *a*, *b*, or *c* of *A. actinomycetemcomitans*. For relative expression, the cytokine expression in T lymphocytes exposed to non-induced (n.i.) dendritic cells was considered as 1, as a reference for fold-change in expression. (B) Secretion levels for the cytokines IL-9 and IL-22 in *naïve* $CD4^+$ T lymphocytes activated in the same conditions as described in Fig. 2A. T-lymphocytes exposed to non-induced (n.i.) dendritic cells were used as control. (C) Secretion levels for the cytokines IL-9 and IL-22 in *naïve* $CD4^+$ T lymphocytes activated with dendritic cells primed with 10, 20, or 50 ng/ml of LPS purified from the serotypes *a*, *b*, or *c* of *A. actinomycetemcomitans*. T-lymphocytes exposed to non-induced (n.i.) dendritic cells were used as control. Comparisons were done between the different *A. actinomycetemcomitans* serotypes (*$p < 0.05$).

3.4. Secretion of IL-9 and IL-22 by LPS-stimulated T lymphocytes

To ascertain whether the higher capacity to trigger Th22-type cytokine production of the serotype *b* of *A. actinomycetemcomitans* may be explained by a higher immunogenicity of its LPS, the cytokine production was analyzed in T lymphocytes activated by dendritic cells primed with LPS purified from the different serotypes of *A. actinomycetemcomitans* (Fig. 2C). A dose-dependent increase in the secretion levels of IL-9 and IL-22 was elicited in T lymphocytes following the activation with autologous dendritic cells stimulated with 10, 20, or 50 ng/ml of LPS purified from each of the serotypes of *A. actinomycetemcomitans*. The T lymphocytes induced by dendritic cells stimulated with 10, 20, or 50 ng/ml of LPS purified from the serotype *b* of *A. actinomycetemcomitans* produce higher levels of IL-22 ($p < 0.001$) than cells stimulated with 10, 20, or 50 ng/ml of LPS purified from bacterial strains belonging to either the *A. actinomycetemcomitans* serotypes *a* or *c*. Similarly, higher secreted levels of IL-9 were detected in T lymphocytes exposed to dendritic cells stimulated with 50 ng/ml of LPS purified from serotype *b* of *A. actinomycetemcomitans* compared with cells stimulated with 50 ng/ml of LPS purified from serotype *a* or *c* ($p < 0.001$). At lower LPS concentration, no differences were detected in the secreted levels of IL-9 among the different serotypes of *A. actinomycetemcomitans*. No differences were detected in the secreted levels of IL-9 and IL-22 in T lymphocytes challenged with any concentration of LPS purified from *A. actinomycetemcomitans* serotypes *a* or *c*. Overall, an increment in a T-lymphocyte response biased towards a Th22-pattern of cytokine production was detected under stimulation with serotype *b* of *A. actinomycetemcomitans* compared with the other serotypes.

3.5. Expression of Spi-B and AhR in bacteria-stimulated T lymphocytes

To confirm whether there was an association between the Th22 phenotype and specific function after being stimulated with serotype *b* of *A. actinomycetemcomitans*, the expression of mRNA for Spi-B and AhR was quantified in $CD4^+$ T lymphocytes stimulated with autologous dendritic cells primed at a MOI = 10^2 with different serotypes of *A. actinomycetemcomitans* (Fig. 3A). T lymphocytes stimulated with serotype *b*-primed dendritic cells showed higher expression levels of AhR ($p = 0.013$ and $p = 0.015$) than cells stimulated with the *A. actinomycetemcomitans* serotypes *a* or *c*, respectively. No differences were detected in the mRNA expression for Spi-B in T lymphocytes after challenge with *A. actinomycetemcomitans* serotypes *a*, *b*, or *c*. Similarly, no differences were detected in the mRNA expression for the analyzed transcription factors in T lymphocytes after challenge with

autologous dendritic cells stimulated with *A. actinomycetemcomitans* serotypes *a* or *c*. The analysis of correlation between IL-9 and Spi-B and between IL-22 and AhR yielded significant positive correlations when T lymphocytes were activated by dendritic cells primed with serotype *a* ($p = 0.001$ and $p = 0.023$), serotype *b* ($p = 0.004$ and $p = 0.001$), or serotype *c* ($p = 0.001$ and $p = 0.017$) of *A. actinomycetemcomitans* (Fig. 3B).

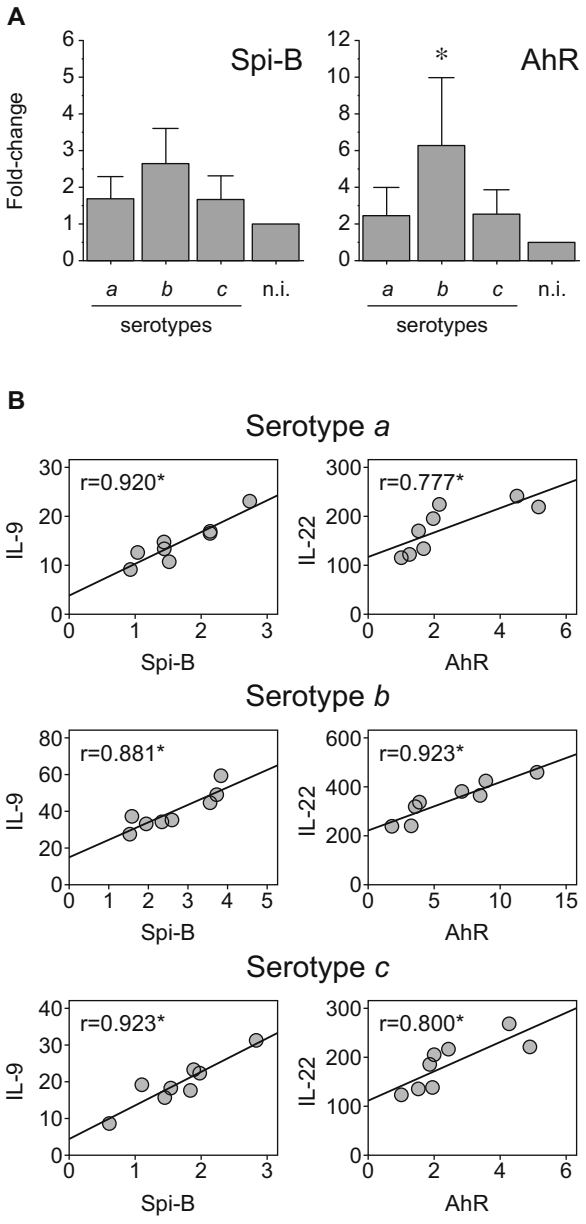

Fig. 3. Expression of Spi-B and AhR in T lymphocytes. (A) Expression levels for the transcription factors Spi-B and AhR in *naïve* $CD4^+$ T lymphocytes activated with dendritic cells primed at a MOI = 10^2 with the serotypes *a*, *b*, or *c* of *A. actinomycetemcomitans*. For relative expression, the transcription factor expression in T lymphocytes exposed to non-induced (n.i.) dendritic cells was considered as 1, as a reference for fold-change in expression. (B) Correlation among the expressed levels for the transcription factors Spi-B and AhR and the secreted levels for the cytokines IL-9 and IL-22 in *naïve* $CD4^+$ T lymphocytes activated with dendritic cells primed at a MOI = 10^2 with the serotypes *a*, *b*, or *c* of *A. actinomycetemcomitans*. Comparisons were done between the different *A. actinomycetemcomitans* serotypes ($^*p < 0.05$).

4. Discussion

There is clear evidence that several pathogens resident in the subgingival biofilm, such as *A. actinomycetemcomitans*, possess a series of virulence factors that facilitate the bacterial breakthrough of natural tissue barriers or their evasion of the host immuno-inflammatory defence mechanisms, as well as the direct stimulation of metabolic cascades leading to destruction of periodontal connective tissues and alveolar bone (Cochran, 2008; Socransky & Haffajee, 2005). These metabolic cascades are mediated by the different T lymphocyte subpopulations through the Th1, Th2, Th17, and T regulatory (Treg) paradigm of the adaptive immune response (Díaz-Zúñiga, Melgar-Rodríguez et al., 2015; Dutzan, Gamonal, Silva, Sanz, & Vernal, 2009; Gaffen & Hajishengallis, 2008; Garlet, 2010; Gemmell et al., 2007; Graves, Oates, & Garlet, 2011; Hernandez et al., 2011; Hofbauer & Heufelder, 2001; Houri-Haddad et al., 2007; Taubman & Kawai, 2001; Vernal & Garcia-Sanz, 2008).

In this investigation, we analyzed the dendritic cell and T lymphocyte responses when stimulated with the different serotypes of *A. actinomycetemcomitans*. Dendritic cells, the most potent antigen presenting cells, are responsible of activating *naïve* $CD4^+$ T lymphocytes and regulating the development of the distinct T-cell lineages, thus influencing the direction of the immune response against infections (Banchereau et al., 2000; Iwasaki & Medzhitov, 2004). We demonstrated significant quantitative variations in the IL-6 and TNF-α production when dendritic cells were stimulated with different bacterial strains belonging to the serotypes *a*, *b*, or *c* of *A. actinomycetemcomitans*. In particular, upon stimulation with the serotype *b* of *A. actinomycetemcomitans* significantly higher amounts of IL-6 and TNF-α were detected, thus demonstrating a role in the Th22 polarization. Furthermore, when T lymphocytes were activated by these serotype *b*-primed dendritic cells, higher expression levels for the transcription factor AhR, which is the master-switch gene implied in Th22 differentiation, and the cytokine IL-22, which is specifically produced by Th22 lymphocytes, were detected. Conversely, the expression levels of IL-4, TGF-β1, and IL-9 were no different when dendritic cells or T-lymphocytes, respectively, were stimulated with *A. actinomycetemcomitans* serotypes *a*, *b*, or *c*. In addition, no differences were detected in the expression levels of Spi-B in T lymphocytes after challenge with the different serotypes of *A. actinomycetemcomitans*. Thus, none of the *A. actinomycetemcomitans* serotypes was associated with a Th9-pattern of immune response. Taken together, these data indicate that the most immunogenic serotype of *A. actinomycetemcomitans*, the serotype *b*, induces not only the activation of the Th1 and Th17-pattern on immune responses as previously described, but also a Th22-pattern in human immune cells (Alvarez et al., 2015; Chen et al., 2010; Díaz-Zúñiga, Melgar-Rodríguez et al., 2015; Díaz-Zúñiga, Monasterio et al., 2015; Díaz-Zúñiga et al., 2014; Melgar-Rodríguez et al., 2015; Shimada et al., 2008; Takahashi et al., 1991; Yamaguchi, Kawasaki, Yamashita, Nakashima, & Koga, 1995; Yamaguchi, Kawasaki, Yamashita, Nakashima, & Koga, 1995).

Differences in the sugar composition and structure of the O-polysaccharide component of the LPS between the distinct *A. actinomycetemcomitans* serotypes may help to explain the detected differential immuno-stimulatory potential in dendritic cells and T lymphocytes (Perry, MacLean, Brisson, & Wilson, 1996; Perry, MacLean, Gmur, & Wilson, 1996). However, together with LPS, other virulence factors may play a crucial role in the increased pathogenicity attributed to the serotype *b* of *A. actinomycetemcomitans*, such as cytolethal distending toxin (Cdt), leukotoxin (Ltx), fimbriae, outer membrane protein-34 (Omp34), and extracellular matrix protein adhesin A (EmaA), which may induce modulation of inflammation, destruction of soft tissues, and resorption of

alveolar bone (Haubek & Johansson, 2014; Herbert et al., 2016; Li et al., 2010; Raja, Ummer, & Dhivakar, 2014). Consequently, the presence of the serotype *b* of *A. actinomycetemcomitans* in the periodonto-pathogenic biofilm could be associated with a cooperative Th1, Th17, and Th22 differentiation and response on periodontal tissues and, thus, could play a role in the initiation, progression and/or severity of periodontitis.

Increased expression of IL-22 has been reported in periapical granulomas as compared with healthy samples; however, these augmented IL-22 levels were not associated with the production of receptor activator of nuclear factor-κB ligand (RANKL), a potent stimulator of bone resorption (Aranha et al., 2013; Araujo-Pires et al., 2014). In rheumatoid arthritis, however, the increased IL-22 levels detected in affected articular tissues induced directly the production of RANKL in human synovial fibroblasts, leading to osteoclastogenesis and bone resorption (Kim et al., 2012; Zhao et al., 2013). In this sense, IL-22 has been also associated with the amount and extent of periodontal breakdown during periodontitis, in particular, the salivary levels of IFN-γ and IFN-γ/IL-22 ratio significantly correlated with the CAL loss, being proposed as indicators of periodontal destruction (Isaza-Guzman et al., 2015).

When the IL-22 receptor has been experimentally blocked, Th9 lymphocytes were over-expressed and Th17 lymphocytes were down-expressed (Elyaman et al., 2009; Marijnissen et al., 2011). It is remarkable that Th9 lymphocytes have a common differentiation pathway with Th17 and Treg lymphocytes, what has led to speculate that Th9 cells could represent an intermediate phenotype in the differentiation towards Th17 or Treg lymphocytes. Thus, depending on the concentration levels of IL-4, IL-6, TNF-α, or TGF-β1, Th9 lymphocytes could remain in this cell phenotype or, in turn, differentiate to either Th17 or Treg lymphocytes (Elyaman et al., 2009; Jager et al., 2009; Nowak et al., 2009; Veldhoen et al., 2008). In this context, Th17 and Th22 lymphocytes differentiate in the presence of IL-6 and low levels of TGF-β1, and Th9 and Treg lymphocyte polarization require TGF-β1 and low levels of IL-6 (Dardalhon et al., 2008; Singh et al., 2013; Vernal & Garcia-Sanz, 2008). In this investigation, increased levels of IL-6 and low levels of TGF-β1 were detected in dendritic cells after *A. actinomycetemcomitans* stimulation, explaining at least in part the higher expression (3-times) and secretion (8.5-times) of IL-22 than IL-9 in serotype *b*-induced T lymphocytes.

Serotype *b* of *A. actinomycetemcomitans* has been found more frequently associated with patients with chronic and aggressive periodontitis when compared with healthy individuals and has demonstrated a higher immuno-stimulatory potential in different immune and non-immune cells when compared with the other serotypes (Alvarez et al., 2015; Bandhaya et al., 2012; Chen et al., 2010; Díaz-Zúñiga, Melgar-Rodríguez et al., 2015; Díaz-Zúñiga, Monasterio et al., 2015; Díaz-Zúñiga et al., 2014; Jentsch et al., 2012; Melgar-Rodríguez et al., 2015; Roman-Torres et al., 2010; Sakellari et al., 2011; Shimada et al., 2008; Takahashi et al., 1991; Yamaguchi et al., 1995; van der Reijden et al., 2008). Taken together all these information, allow us to suggest that the increased periodonto-pathogenic potential of the serotype b of *A. actinomycetemcomitans* could be explained by the activation of the Th1/Th17/Th22 axis of the adaptive immune response.

In periodontitis, Th1 and Th17 lymphocytes have been associated with the onset and progression of the disease, while Th2 and Treg lymphocytes have been associated with suppression of the Th1 and Th17 responses and periodontal health (Garlet et al., 2010; Garlet, 2010; Gemmell et al., 2007; Graves et al., 2011; Hernandez et al., 2011; Houri-Haddad et al., 2007; Vernal & Garcia-Sanz, 2008). In this context, Th9 lymphocytes could have immune-modulatory activities by inhibiting the Th1 and/or Th17 differentiation and function, and Th22 lymphocytes could have a similar pro-inflammatory and periodontal destructive role that as Th1 and Th17 lymphocytes (Ikeuchi et al., 2005; Khan et al., 2009; Kim et al., 2012; Marijnissen et al., 2011). Consequently, when the serotype *b* of *A. actinomycetemcomitans* plays a significant role in the pathogenic subgingival biofilm, the Th22 polarization might be triggered within the periodontal lesion, promoting inflammation and tooth-supporting alveolar bone resorption.

5. Conclusion

To our knowledge, this is the first report identifying quantitative variations in the Th9 and Th22-associated cytokine production in dendritic cells and T lymphocytes upon stimulation with *A. actinomycetemcomitans*. These results let us to propose that Th22 lymphocytes could play a role in the pathogenesis of periodontitis.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

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Ethical approval

The study design (Protocol #2010/14) was approved by the Ethics Committee of Faculty of Dentistry, Universidad de Chile, Santiago, Chile.

Author's contributions

Jaime Díaz-Zúñiga and Samanta Melgar-Rodríguez organized the study, processed the blood samples, performed the cell cultures, carried out the qPCR experiments, and critically evaluated and supplemented the manuscript. Gustavo Monasterio, Myriam Pujol, Leticia Rojas, and Carla Alvarez carried out the qPCR and ELISA experiments, performed the data analysis, and were involved in drafting the manuscript. Paola Carvajal examined the study individuals. Rolando Vernal designed and implemented the research protocol, performed the data analysis, and prepared the figures and the manuscript for submission. All the authors read and approved the final manuscript.

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