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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.