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ORIGINAL ARTICLE

Expression and secretion levels of Th1 and Th2 cytokines in patients with aggressive periodontitis

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Abstract The role of Th1 and Th2 cytokines in the pathogenesis of aggressive periodontitis has not been previously examined. The aim of this study was to analyse the expression and production of IL-2, IFN-γ, IL-4 and IL-13 in CD4+ cells from the peripheral blood of patients with aggressive periodontitis (AgP) and periodontally healthy controls. Gene expression was analysed in inactivated and activated CD4+ cells by real-time PCR. Cells were activated for 4, 8 and 24 h with anti-CD3/CD28 antibody, phytohemagglutinin (PHA), and *Porphyromonas gingivalis* (*P.g.*) outer membrane protein (OMP). Protein levels were measured in supernatants of activated CD4+ cells by bead-based immunoassay (CBA). Statistics were performed using *U* test ($p<0.05$). In controls, IL-4 expression was increased in inactivated CD4+ cells ($p=0.05$), and IFN-γ and IL-2 expressions were increased in activated CD4+ cells: IFN-γ with anti-CD3/anti-CD28, *P.g.* OMP and PHA ($p<0.05$); IL-2 with *P.g.* OMP and PHA ($p<0.05$). In patients, although IL-4 and IL-13 expressions were higher in activated CD4+ cells, there were no differences compared to controls. The production of IL-4 and IL-2 was higher in the patients' CD4+ cells activated with PHA ($p<0.05$). Although the results showed a predominantly Th1 mRNA profile in activated CD4+ cells of controls, protein concentrations showed no clear Th1 or Th2 profiles. The functional pathways of the Th cell immune response in aggressive periodontitis are still not well understood in order to develop individualised diagnostic and treatment plans.

Keywords Th cells · Cytokines · Aggressive periodontitis · Expression · Protein

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Introduction

The adaptive response is under the control of T cells. They are necessary for specific antibody production and polyclonal B cell activation. Protection against extracellular infectious agents is mainly provided by the soluble antibodies which are produced by B cells via the collaboration with activated CD4+ Th cells and can neutralise the infectious agents in every part of the body (humoral immunity) [1]. However, the form of the specific immune response mediated by Th cells is diversified. The original classification of Th1 and Th2 pathways has recently been expanded to include additional effector Th cell subsets, i.e. Th17, Th9 and T-regulatory (Treg) cells. Th1 cells produce IFN-γ, IL-2 and TNF-α and promote the production of IgG2a opsonising and complement-fixing antibodies, activation of macrophages, antibody-dependent cell cytotoxicity and delayed type hypersensitivity (DTH) [2, 3]. Th2 cells produce IL-4, IL-5, IL-9, IL-10 and IL-13 and provide optimal help for humoral immune responses, including IgE and IgG1 isotype switching, and mucosal immunity, through the production of mast cell and eosinophil growth and differentiation and facilitation to IgA synthesis [4]. Some Th2-derived cytokines, such as IL-4, IL-10 and IL-13, inhibit several macrophage functions [5, 6]. Other cytokines, such as IL-3, granulocyte/macrophage colony-stimulating factor (GM-CSF) and TNF-α, are produced by

both Th1 and Th2 cells. T cells expressing cytokines of both patterns have been designated Th0 and usually mediate intermediate effects depending upon the ratio of lymphokines produced and the nature of the responding cells. T-regulatory cell subset produces a different pattern of cytokines than Th1 or Th2 as they produce IL-10 and TGF-β but low IFN-y and no IL-4 [7, 8]. These cells are thought to downregulate the antigen-presenting cell, possibly via TGF-β [9]. Finally, IL-17-secreting CD4+ T cells arise in the absence of Th1- and Th2-induced transcription factors and cytokines [10, 11]. It is now known that IL-23 is required for Th17 cell expansion, survival and pathogenicity and that the key differentiative cytokines for Th17 are TGFβ, IL-6, IL-1 and IL-21 [12].

With regard to periodontitis, it is now clear that the factors of the innate immune response are involved in determining susceptibility to periodontitis. As a consequence, the balance between Th cytokines has led to the formulation of several hypotheses as to which T-cell subsets are associated with the disease [13–15]. However, due to the complexity of the mechanisms involved, no conclusive results have been reported so far. One group of investigators have repeatedly proposed that non-susceptibility to periodontitis involves IL-2 and IFN-γ-producing Th1 cells that induce a delayed-type hypersensitivity response, synonymous with the putative T-cell stable lesion. In contrast, susceptibility to periodontitis is promoted by IL-4-producing Th2 cells, providing B-cell activation and expansion. Thus, failure of the innate immune response to clear the infection leads to the development of a B cell/plasma cell lesion. After polyclonal B cell activation, an expansion of the B cells will take place, with the production of non-specific and/or low-avidity specific antibodies, and large amounts of IL-1, mediating tissue destruction. The production of non-protective antibodies will result in continuous connective tissue breakdown [13, 16].

However, only two studies have so far analysed the mRNA Th1 and Th2 cytokine expression profiles in cells isolated from patients with AgP [17, 18]. Whereas Suarez et al. [17] did not find a definitive pattern of Th1 or Th2 response in unstimulated CD4+ and CD8+ cells, Sigusch et al. [18] reported a higher expression of Th2 cytokines (IL-5 and GM-CSF) compared with the expression of Th1 cytokines (IL-2 and IFN-γ) in stimulated peripheral blood mononuclear cells (PBMC) of patients with early-onset periodontitis (EOP).

In view of this evidence, and considering the lack of data that exist on Th1/Th2 cytokine responses in AgP, we analysed the expression of Th1 cytokines (IL-2 and IFN-γ) and Th2 cytokines (IL-4 and IL-13) in inactivated and activated CD4+ cells isolated from patients with aggressive periodontitis and healthy controls. In addition, protein levels were analysed in the supernatants of the activated CD4+ cells.

Methods

The protocol of the study was written in conformity with the ethical guidelines of the Helsinki Declaration and according to the validated Good Clinical Practice guidelines [19]. The protocol of the study was approved by the Ethic Committee of the University of Giessen (No. 113/05). Before inclusion in the study, all subjects gave their freely written, informed consent.

Subjects

The patients included in the present work were all patients presenting at the Department of Periodontology, Dental School of the Justus-Liebig University of Giessen, Germany. In order to be considered for inclusion in the study, the patients had to be of North European heritage presenting with a diagnosis of generalised aggressive periodontitis. This diagnosis was made based on the clinical and radiographic criteria that were proposed at the 1999 International Workshop for a Classification in Periodontal Diseases and Conditions [20, 21] and the 5th European Workshop in Periodontology where definitions of a periodontitis case and disease progression for use in risk factor research were proposed [22].

Prior to inclusion, the following standard clinical parameters were registered in patients and controls at six sites/tooth using a periodontal probe (PCP-UNC15): probing pocket depth (PPD) as the distance from the gingival margin to the base of the periodontal pocket and clinical attachment level (CAL) as the distance from the cement–enamel junction to the base of the periodontal pocket [23]. Bleeding on probing (BOP) was registered as present or absent in percent of the total of sites (6*x*/tooth) [24]. Additionally, modified plaque and papillary bleeding indices, registered as present or absent in percent of the total of sites (4*x*/tooth) were assessed [25, 26].

The patients had to be clinically healthy except for the presence of generalised aggressive periodontitis and be younger than 35 years. However, older patients could be included if it was clear from the historical data or radiographies that the onset of the disease was prior to this age. The periodontal status of the patients had to be characterised by a severe loss of attachment in relation to their age. This condition was assured in that patients had a total of at least 20 teeth, with PPD and CAL of ≥5 mm present on at least one interproximal site affecting at least three teeth other than the first molars and incisors [27].

The radiographic status of the patients had to be characterised by severe bone destruction in relation to their age. Panoramic radiographs of diagnostic quality were taken and evaluated for interproximal bone loss measurements from the cement–enamel junction of the tooth to the bone crest, expressed as % of the total root length. All patients had at

least three permanent teeth other than the first molars and incisors with interproximal sites with 30% bone loss [27, 28].

Exclusion criteria of patients were systemic diseases and syndromes, medication at the time of inclusion and/or 6 months prior to the inclusion in the study, heavy smokers (>10 cigarettes per day), pregnancy and patients unwilling to participate.

The control subjects included in the present work were all volunteer students and staff of the Dental School of the Justus-Liebig University of Giessen, Germany. In order to be considered for inclusion in the study, the control subjects had to be healthy, North European individuals without signs of periodontitis and be non-smokers or light smokers (<10 cigarettes/day). As far as possible, their age and gender were matched with that of the patients. The clinical parameters previously described were also assessed in this group. To ensure that controls had no signs of periodontitis, these subjects had to have presented with a minimum of 26 teeth, ≥95% of the tooth sites with ≤4 mm PPD without BOP, no PPD ≥5 mm and no loss of CAL [29].

Blood sampling and magnetic cell separation

Peripheral blood was obtained using vacutainer tubes (Sarstedt, Nümbrecht, Germany): 3.5 ml citrate blood for the erythrocyte sedimentation rate (ESR), 22.5 ml lithium heparin for the gene expression assays with unstimulated cells and 60 ml lithium heparin for the gene expression assays with stimulated cells. ESR was determined in our laboratory using the method described by Westergren, with slight modifications [30].

PBMC were isolated using standard Ficoll-Hypaque density gradient centrifugation (Ficoll-Paque Plus, Amersham Biosciences). Cells were counted to 10^7 cells on a semi-automated microcell counter (F-800, Sysmex Deutschland GmbH, Norderstedt, Germany) using the instructions of the manufacturer.

CD4+ lymphocytes were separated using the MiniMACS magnetic cell sorting technique, employing the autoMACS® separator for positive selection of labelled cells (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cells were labelled with CD4 microbeads conjugated to monoclonal anti-human CD4 (isotype: mouse IgG1; clone: M-T466) according to the manufacturers (CD4 MicroBeads human, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Briefly, 20 μl of MicroBeads per 10^7 total cells was added, mixed well and incubated for 15 min at 4°C. Thereafter, cells were washed by adding 1–2 ml of buffer (PBS ph 7.2 supplemented with 0.5% BSA and 2 mM EDTA) and centrifuged for 10 min at 1,500 rpm with brake. The supernatant was completely removed and the cells resuspended in 500 μl of PBS/EDTA/BSA buffer. After magnetic separation, the cells were counted as previously described.

Immunofluorescent staining

The purity of the positively selected CD4+ T cells was evaluated by immunostaining followed by FACScan analysis by flow cytometry (Becton Dickinson Biosciences, Heidelberg, Germany) using the manufacturers' protocols (CD4-FITC human, CD8-FITC, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Briefly, 10 μl monoclonal anti-human CD4 (isotype: mouse IgG1; clone: M-T321) antibody conjugated to fluorescein isothiocyanate was added to 107 cells resuspended in 50 μl of buffer (PBS/EDTA/BSA). Cells were mixed well and incubated for 15 min in the dark at room temperature. Thereafter, the cells were washed by adding 2 ml PBS per 10^7 cells and centrifuged at 1,500 rpm for 5 min. The supernatant was removed and a post-labelling fixation with 500 μl CellFIX (BD) was performed. Immediately afterwards, the samples were analysed using a BD FACScan flow cytometer (BD Germany, Heidelberg) and the CellQuest software programme for acquisition and analysis. CD4-positive events were identified and gated.

Real-time polymerase chain reaction for gene expression assays

Total RNA was isolated from CD4+ T lymphocytes using the RNeasy procedure (RNeasy Mini Kit, Qiagen, Hilden, Germany). All centrifugation steps were performed at 20–25°C in a standard microcentrifuge. Biological samples were first lysed by adding a highly denaturing guanidine–thiocyanate-containing buffer and β-mercaptoethanol (β-ME) (10 μl β-ME per 1 ml RLT buffer) to the total cells. Immediately afterwards, samples were homogenised using QIAshredder homogenisers. The lysate (350–600 μl) was loaded onto a QIAshredder spin column placed in a 2-ml collection tube and spun for 2 min at maximum speed (13,000 rpm) in a microcentrifuge. Homogenised cell lysates were either stored at −70°C for later use or RNA was immediately isolated with the RNeasy spin column technique. Samples not processed immediately were stored at −70°C.

cDNA was synthesised from 1.5 to 2 μg of total RNA using the Ready-To-Go You-Prime First-Strand Beads kit (Amersham Biosciences Europe GmbH, Freiburg, Germany). The reaction beads utilise Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase to generate first-strand cDNA. Briefly, first, the RNA samples were heated at 65°C for 10 min and chilled on ice for 2 min. Then, 30 μl of total RNA were added to the tube containing the reaction beads. The buffer and dNTP conditions in the completed first-strand cDNA reaction were: 50 mM Tris (pH 8.3), 75 mM KCL, 7.5 mM DTT, 10 mM MgCl2, 0.08 mg/ml BSA and 2.4 mM each of dNTP and M-MuLV. Thereafter, 2 μl containing 0.2 μg of the random primer pd(N)6 was added to the mixture

and allowed to sit at room temperature for approximately 1 min. The contents of the tube were mixed by gently vortexing and were briefly centrifuged. An incubation at 37°C for 60 min followed. Samples not processed immediately were stored at −70°C.

Prior to real-time PCR, the cDNA samples were diluted at a concentration of 1:3 with RNase-free water. The real-time PCR was performed using the TaqMan® gene expression assays (Applied Biosystems, Darmstadt, Germany). Standards (RNase-free water), controls and target samples were pipetted in triplicate in a 96-well real-time PCR plate. Each reaction had a final volume of 20 μl, containing 9 μl of cDNA, 10 μl of TaqMan® universal PCR master mix (with AmpErase® UNG), 18 μM of each primer and 5 μM of each TaqMan® probe. The human PPIA (peptidylprolyl isomerase A or cyclophilin A) was used as housekeeping gene. Polymerase chain reaction amplification and detection was performed using an ABI 7000 Sequence Detection System (Applied BioSystems) with initial denaturation for 5 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For relative quantification of mRNA, the comparative C_t method was applied (User Bulletin #2: ABI Prism 7700 Sequence Detection System). Automatic analysis settings were selected to determine the C_t values and baseline settings.

Cell culture and cell stimulation

Preliminary assays were needed in order to optimise the conditions for cell number, antigen concentration and kinetics. CD4+ T cells were stimulated for 24 h with one of three different agents in separated experiments. Firstly, cells were cultured in the presence of anti-CD3 antibody (purified mouse IgG2a, clone: HIT3a, Becton Dickinson GmbH, Heidelberg, Germany) at a concentration of 1.0 μg/ml. Costimulation involved the addition of soluble anti-CD28 antibody (purified mouse IgG, clone: CD28.2, Becton Dickinson GmbH, Heidelberg, Germany) at the same concentration. Secondly, CD4+ T cells were cultured in the presence of PHA-L (Sigma-Aldrich Chemie GmbH, Germany) at a concentration of 1.0 μg/well (200 μl). Finally, CD4+ T cells were cultured in the presence of *Porphyromonas gingivalis* ATCC 33277 OMP (courtesy of Prof. Yamazaki, Niigata, Japan) at a concentration of 1.0 μg/well (200 μl). A negative (media alone) control was performed with each culture. After incubation with the different stimuli, the cells were pelleted, washed and subjected to RNA isolation as previously described. The culture medium was collected after 24 h and assessed for cytokine protein secretions.

Analysis of soluble cytokines

For the analysis of the proteins in the supernatants of the activated cells, double cultures in separate wells were performed in each experiment. A bead-based immunoassay (Cytometric Bead Array, BD™ CBA, Heidelberg, Germany) was used. This system uses the sensitivity of amplified fluorescence detection by flow cytometry to measure soluble analytes in a particle-based multiplex immunoassay, using beads that have various sizes or that are labelled with varying levels of fluorochrome. For the present experiments, bead populations with distinct fluorescence intensities were coated with capture antibodies specific for IL-4, IL-13, IL-2 and IFN-γ proteins. The experiments were performed using the Human Soluble Protein Master Buffer Kit for each protein tested, following the instructions of the manufacturer (BD Biosciences, Heidelberg). The bead populations were then mixed together to form the CBA, which was resolved in the FL3 channel of the Cyan® flow cytometer (DakoCytomation, Hamburg). Quantitative analysis was used to determine the analyte concentration in a sample file based on the known concentration values in the set of standards.

Statistical analysis

Data were entered into a computer database. Data and test were computed using a commercial statistics programme (SPSS, version 10.0 for Windows 98). The form of the statistical analyses of the data was performed according to the explorative nature of the investigations.

Descriptive statistics were used for the demographic data, age, number of teeth and five clinical parameters (PPD, CAL, BOP, PLI, PBI) of patients and controls. Since these parameters contributed to the inclusion criteria, no statistical analysis of differences between the groups was performed. The distribution of the data is described by the median and the inter-quartile range. A non-parametric test (Mann–Whitney U test) was used nonetheless to provide better information on the clinical data of the groups.

In order to analyse the differences between the controls and the patients with regard to gene expression and protein production, the Mann–Whitney U test was used. These statistical procedures were performed at a level of significance of $\alpha<0.05$. In the case of a confirmatory analysis, the Mann–Whitney U test would have a significance level of $\alpha=0.016$.

Results

Subject demographics

Data of demographical and clinical parameters are demonstrated in Table 1. Number of subjects, number of teeth per subject, age and sex showed a similar distribution. As expected, all clinical parameters showed higher levels in the group of patients in comparison with the group of controls (Mann–Whitney test, $p<0.01$).

Table 1 Subject, sample demographics and clinical parameters in the study population

	Aggressive periodontitis	Non-periodontitis
Number of subjects	20	20
Age (years)[a]	30 [27–32]	28 [25–30]
Number of teeth (per subject)[b]	28 [26–29]	28
Female	11	12
Male	9	8
PPD (mm)[b]	3.94 [3.3–4.4][c]	1.76 [1.6–1.9]
CAL (mm)[b]	4.52 [3.4–5.3][d]	1.76 [1.6–1.9]
BOP+ (%)[b]	53 [28–72][d]	4.2 [2–6]
PLI (%)[b]	47 [26–66][d]	27 [24–30]
PBI (%)[b]	19 [4–42][d]	3.5 [0–5]

PPD pocket probing depth, *CAL* clinical attachment level, *BOP+* positive sites for bleeding upon probing, *PLI* plaque index, *PBI* papillary bleeding index

[a] Numbers represent median with interquartiles

[b] Numbers represent the median, with the interquartiles in square brackets

[c] Compared to controls, $p<0.001$, Mann–Whitney test

[d] Compared with healthy subjects, $p<0.01$, Mann–Whitney U test

Cell counting, magnetic cell separation and immunofluorescence

The semi-automated cell count of the freshly isolated white blood cells and the magnetically separated CD4+ cells demonstrated similar results for controls and patients. The mean proportion of the freshly separated CD4+ cells for the controls was 95.3%, with a SD of 2.3%. In the patients, the mean proportion of the CD4+ cells after separation was 96.4%, with a SD of 1.6%. This result demonstrates similarly high proportions of CD4+ cells in controls and patients (>95%).

Expression of target genes in inactivated CD4+ cells

The mean RNA concentration of the CD4+ cells of the controls ($n=20$) was 62.2 ng/50 µl, with a SD of 27.5 ng/50 µl. In the patients ($n=20$), the mean RNA concentration of the CD4+ cells was 67.6 ng/50 µl, with a SD of 34.2 ng/50 µl.

Mean ΔC_t values of unstimulated CD4+ cells differed between patients and controls for all the genes (data not shown).

Figure 1 demonstrates the expression of target genes in the inactivated CD4+ cells. In the group of patients, the expression of IL-4 (17.8±3.6), IL-13 (19.1±2.8) and IL-2 (18.6±2.8) was higher than the expression of IFN-γ (4.9±0.2). Expression of IL-4 was increased in the group of controls (41.5±2.1). There was a difference in IL-4 expression between the controls and the patients (Mann–Whitney U test, $p=0.05$). Expression of IL-2 (10.5±2.6) and IFN-γ (5.7±1.8) was lower than expression of IL-4 in the cells of the controls. Expression of IL-13 could not be calculated.

Expression of target genes in activated CD4+ cells

The mean RNA concentration of the CD4+ cells stimulated with anti-CD3+ anti-CD28 of the controls ($n=12$) was 58.4 ng/50 µl, with a SD of 22.3 ng/50 µl. In the group of patients ($n=12$), the mean RNA concentration was 49.6 ng/50 µl, with a SD of 16.7 ng/50 µl. Mean ΔC_t values of stimulated CD4+ cells differed between controls and patients for all the genes (data not shown).

Figures 2, 3, 4 and 5 show the relative expression of the target genes. In the group of patients, gene expression assays showed a higher expression of IL-4 in comparison with IL-2 (Figs. 2 and 4) and of IL-13 in comparison with IFN-γ (Figs. 3 and 5). Only the expression of IFN-γ was higher than the expression of IL-4 in the cells of the patients. However, no differences in IL-4 and IL-13 gene expression between patients and controls were detected (Figs. 2 and 3). The highest IL-4 expression was detected in

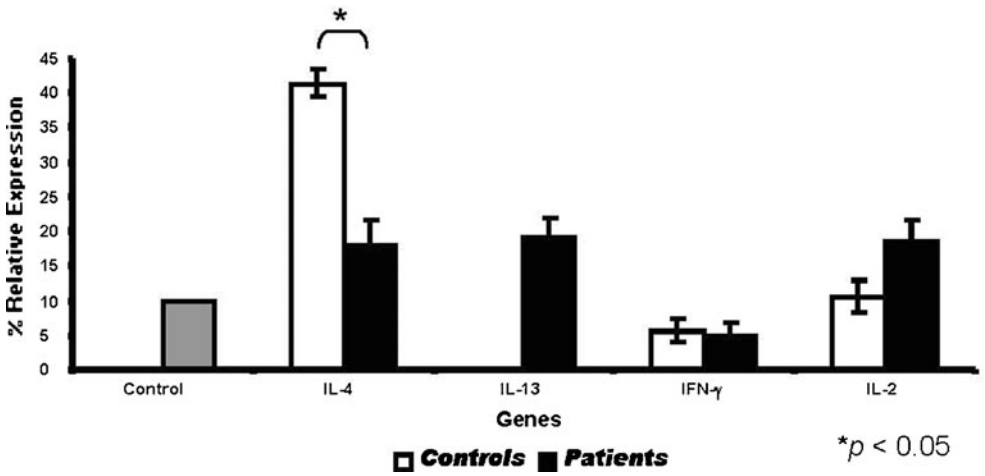

Fig. 1 Relative mRNA quantification for IL-4, IL-13, IFN-γ and IL-2 after normalisation to the endogenous control PPIA in unstimulated CD4+ cells isolated from controls ($n=20$) and patients ($n=20$). The data are expressed as mean (%)±SD in relation to the control (100%): gene expression in CD8+ cells

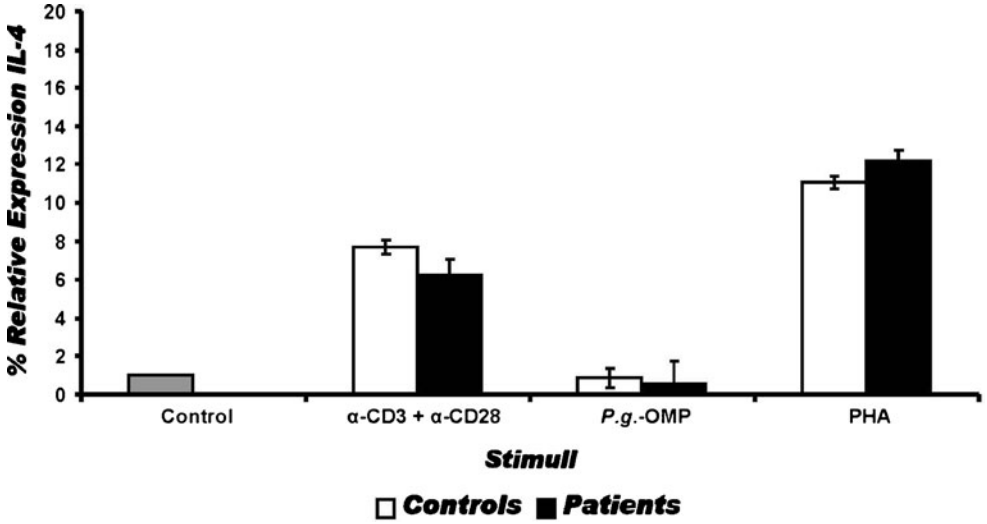

Fig. 2 Relative mRNA quantification for IL-4 after normalisation to the endogenous control PPIA. CD4+ cells were isolated from controls (n=12) and patients (n=12) and stimulated with anti-CD3+ anti-CD28, *P.g.* OMP and PHA for 24 h. Data are expressed as mean ± SD. The control (100%) shows IL-4 expression by unstimulated CD4+ cells. In controls, IL-4 expression (%) was 7.72±0.4 in cells stimulated with anti-CD3+ anti-CD28, 0.84±0.5 in cells stimulated with OMP and 11.06±0.3 in cells stimulated with PHA. In patients, IL-4 expression was 6.25±0.8 in cells stimulated with anti-CD3+ anti-CD28, 0.55±1.16 in cells stimulated with OMP and 12.18±0.6 in cells stimulated with PHA

cells stimulated with PHA; the lowest IL-4 expression in cells stimulated with *P.g.* OMP (Fig. 2). Similar results were observed for the expression of IL-13 (Fig. 3). Interestingly, increased IFN-γ expression was detected in the cells of the healthy controls stimulated with anti-CD3+ anti-CD28, *P.g.* OMP and PHA in comparison with the IFN-γ expression in the cells of the patients (p<0.05) (Fig. 4). The higher IFN-γ gene expression was detected in cells stimulated with anti-CD3+ anti-CD28 and the lowest in cells stimulated with *P.g.* OMP. Similar to IFN-γ expression, high IL-2 expression was detected in the cells of the controls stimulated with *P.g.* OMP and PHA in comparison with the IL-2 expression in the cells of the patients (p<0.05) (Fig. 5). The highest IL-2 expression was detected in cells stimulated with PHA and the lowest in cells stimulated with *P.g.* OMP.

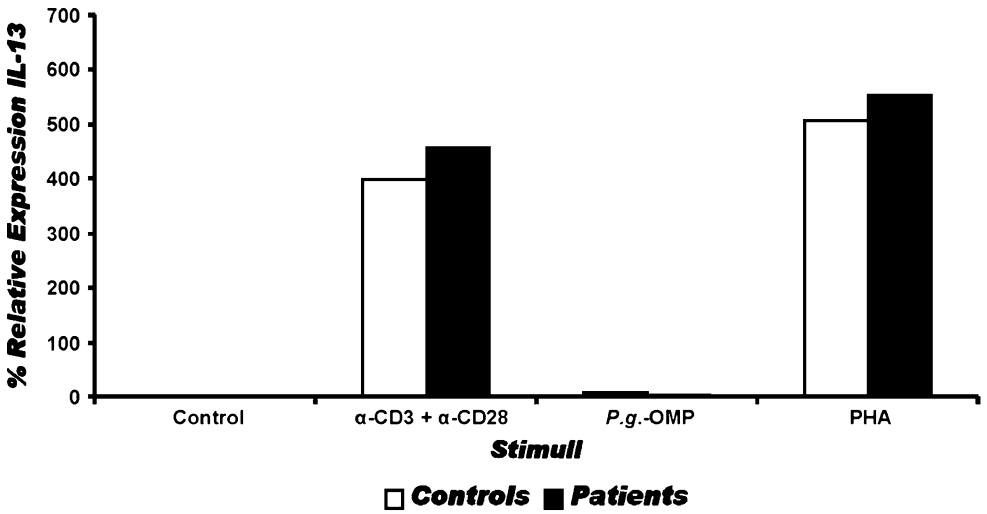

Fig. 3 Relative mRNA quantification for IL-13 after normalisation to the endogenous control PPIA. CD4+ cells were isolated from controls (n=12) and patients (n=12) and stimulated with anti-CD3+ anti-CD28, *P.g.* OMP and PHA for 24 h. Data are expressed as mean ± SD. The control (100%) shows the IL-13 expression by unstimulated CD4+ cells. In controls, the relative expression (%) of IL-13 was 398.1±2.3 in cells stimulated with anti-CD3+ anti-CD28, 7.8±0.9 in cells stimulated with OMP and 508.3±2.3 in cells stimulated with PHA. In patients, the IL-13 expression was 456.4±2.2 in cells stimulated with anti-CD3+ anti-CD28, 2.1±0.7 in cells stimulated with OMP and 552.7±1.8 in cells stimulated with PHA

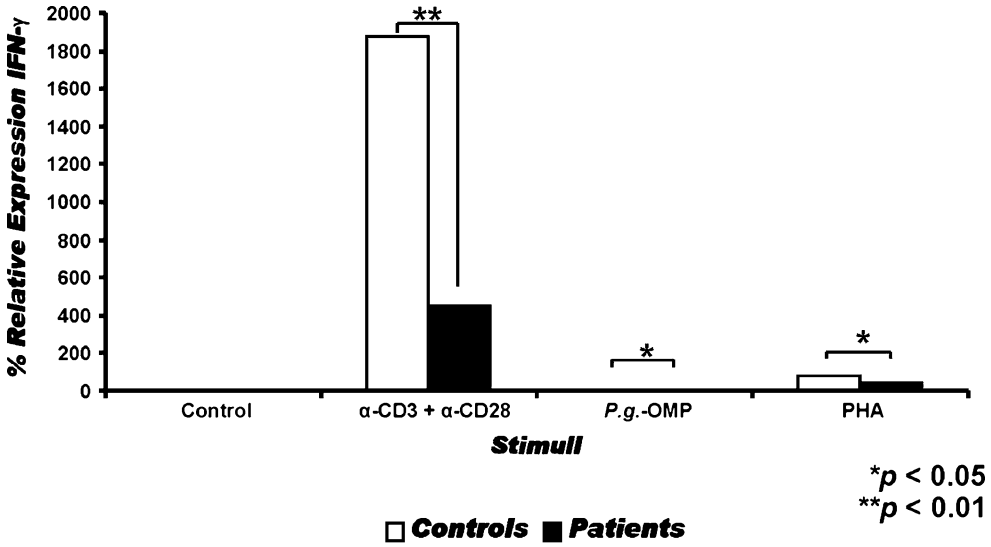

Fig. 4 Relative mRNA quantification for IFN-γ after normalisation to the endogenous control PPIA. CD4+ cells were isolated from controls (n=12) and patients (n=12) and stimulated with anti-CD3+ anti-CD28, *P.g.* OMP and PHA for 24 h. Data are expressed as mean ± SD. The control (100%) shows the IFN-γ expression by unstimulated CD4+ cells. In controls, the relative expression (%) of IFN-γ was 1875.3±2.4 in cells stimulated with anti-CD3+ anti-CD28, 3.0±0.3 in cells stimulated with OMP and 79.6±0.3 in cells stimulated with PHA. In patients, the IFN-γ expression was 451.3±0.3 in cells stimulated with anti-CD3+ anti-CD28, 1.4±0.6 in cells stimulated with OMP and 41.7±0.2 in cells stimulated with PHA

Cytokine production by activated CD4+ cells

Table 2 shows the production of the cytokines in the supernatants by the stimulated CD4+ cells of controls and patients. The median protein levels and their interquartile ranges are demonstrated (pg/ml). All cytokines showed higher levels in the group of patients in comparison with the group of controls. These levels were significant for the production of IL-4 and IL-2, after cells were stimulated with anti-CD3+ anti-CD28 (IL-4) and with anti-CD3+ anti-CD28 and PHA (IL-2) (Mann–Whitney test, p<0.05). Th1 cytokine levels were higher than Th2 cytokine levels in both groups.

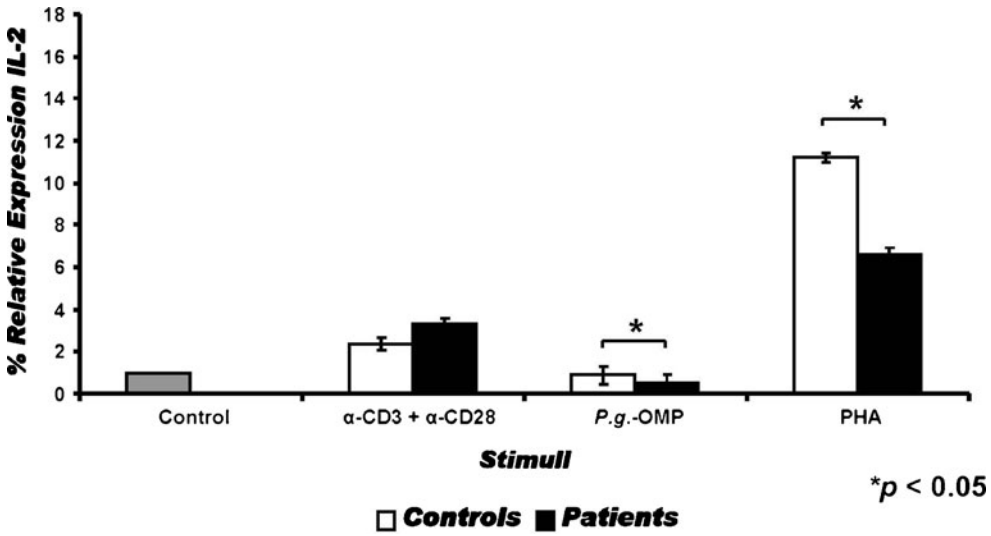

Fig. 5 Relative mRNA quantification for IL-2 after normalisation to the endogenous control PPIA. CD4+ cells were isolated from controls (n=12) and patients (n=12) and stimulated with anti-CD3+ anti-CD28, *P.g.* OMP and PHA for 24 h. Data are expressed as mean ± SD. The control (100%) shows the IL-2 expression by unstimulated CD4+ cells. In controls, the relative expression of IL2 (%) was 2.4±0.3 in cells stimulated with anti-CD3+ anti-CD28, 0.9±0.4 in cells stimulated with OMP and 11.2±0.2 in cells stimulated with PHA. In patients, the IL-2 expression was 3.3±0.3 in cells stimulated with anti-CD3+ anti-CD28, 0.5±0.3 in cells stimulated with OMP and 6.6±0.4 in cells stimulated with PHA

Table 2 Cytokine production by stimulated CD4+ cells in association with the different stimuli

Cytokine	Stimulus	Aggressive periodontitis (pg/ml)	Non-periodontitis (pg/ml)
IL-4	a-CD3 + a-CD28[a]	14.55 [4.42–27.85]	7.83 [3.1–10.25]
	PHA[a]	63.45 [43.41–99.84][b]	40.65 [8.32–47.20]
	P.g. OMP[a]	n.d.	n.d.
IL-13	a-CD3 + a-CD28[a]	294.04 [88.27–352.18]	160.6 [36.97–292.91]
	PHA[a]	1016.29 [697.4–1188.32]	855.3 [446.28–1014.14]
	P.g. OMP[a]	n.d.	n.d.
IFN-γ	a-CD3 + a-CD28[a]	1146.54 [423.59–2189.9]	927.15 [450.1–1284.03]
	PHA[a]	426.9 [207.53–1027.66]	456.58 [179.57–596.27]
	P.g. OMP[a]	n.d.	n.d.
IL-2	a-CD3 + a-CD28[a]	853.72 [172.98–1601.26][b]	278.48 [73.36–532.95]
	PHA[a]	680.51 [330.8–1551.98][b]	61.01 [15.23–348.02]
	P.g. OMP[a]	16.13 [3.11–32.81]	n.d.

a-CD3 anti-CD3, *a-CD28* anti-CD28, *PHA* phytohemagglutinin, *P.g. Porphyromonas gingivalis*, *OMP* outer membrane protein, *IL* interleukin, *IFN* interferon, *n.d.* not detected

[a]Numbers represent the median (pg/ml), with the interquartiles (pg/ml) in square brackets

[b]Compared with non-periodontitis subjects, $p<0.05$, Mann–Whitney *U* test

Discussion

Several investigators have analysed the Th1/Th2 profile in periodontal disease [13, 31, 32]. These studies have analysed the cytokine responses using different approaches, like cells in situ, cells extracted from gingival tissues, peripheral blood mononuclear cells, T-cell lines, purified T-cell populations and clones. The methodologies used were also different, including flow cytometry, enzyme-linked immunosorbent assay, in situ hybridization and reverse transcriptase/polymerase chain reaction. The results have been inconsistent. Whereas several studies have shown an increased Th1 response in periodontitis, increased Th2 responses have also been reported.

Despite the recent finding that Th17 cells are further involved in the pathogenesis of aggressive periodontitis [33], to our knowledge, only two studies have so far analysed the mRNA Th1 and Th2 cytokine expression profiles in T cells isolated from the peripheral blood of patients with aggressive periodontitis [17, 18]. Whereas Suarez et al. [17] did not find a definitive pattern of Th1 or Th2 response in unstimulated CD4+ and CD8+ cells, Sigusch et al. [18] reported a higher expression of Th2 cytokines (IL-5 and GM-CSF) compared with the expression of Th1 cytokines (IL-2 and IFN-γ) in stimulated peripheral blood mononuclear cells of patients with EOP. Our results are similar to those of Sigusch et al. [18]; however, they did not analyse IL-4 and IL-13 in their samples, and we did not analyse IL-5 and GM-CSF in our samples.

The majority of the studies analysing Th1/Th2 mRNA profiles were instead performed either using inflamed gingival tissues or polymorphonuclear blood cells of patients with chronic periodontitis. Mostly, the results did not show a definitive pattern of Th1 or Th2 response, or they suggested a bias towards a Th2-type cytokine response [13, 34–37]. In contrary, Bartova et al. showed that peripheral blood mononuclear cells of patients with EOP stimulated with *Escherichia coli* produced significantly higher levels of IL-4 and IgM and significantly lower levels of IFN-γ compared with their healthy siblings [38]. In view of these results, it was recently suggested that the dominance of B cells and plasma cells in periodontitis lesions cannot entirely be explained by enhanced Th2 functions but most probably by an imbalance in this dynamic [39]. This situation, however, might apply to the adaptive host response in chronic periodontitis as the majority of the studies indicated. In contrast, in aggressive periodontitis, the few studies conducted demonstrated a shift towards the Th2 phenotype. We are aware that the results of the mRNA analyses of the present study are not conclusive enough to support this observation. Nevertheless, the expression of IFN-γ and IL-2 was elevated in the cells of the group of controls, compared with the group of patients: IFN-γ in the cells stimulated with anti-CD3/anti-CD28 ($p<0.01$), *P.g.* OMP and PHA ($p=0.05$) and IL-2 in the cells stimulated with PHA and *P.g.* OMP ($p<0.05$). Conversely, the expression of the Th2 cytokines in the group of controls was not increased.

In this study, CD4+ T cells were activated through different pathways, including the TCR–CD3 and CD28 receptor complex, phytohemagglutinin (PHA) and *P. gingivalis* OMP. Engagement of the CD3–T cell receptor complex initiates a complex signalling cascade involving coordinated regulation and recruitment of tyrosine and lipid kinases to specific regions or microdomains in the plasma membrane [40]. However, the activation of T lymphocytes is thought to require at least two signals, one delivered by the TCR/CD3 complex after antigen recognition and one provided on engagement of costimulatory receptors [41]. Signals initiated by simultaneous binding of soluble mAb to CD3 and CD28 molecules induce functional IL-4R on

the surface of human T cells [42]. CD28, constitutively expressed on the surface of T cells, is the ligand for CD80 and plays a very important role in T cell–B cell interaction [43]. PHA is used for the stimulation of cell proliferation in lymphocyte cultures [44]. PHA-L consists only of L-type subunits (isolectin L4, "leukoagglutinin") and is especially suitable for high-efficiency induction of T-lymphocytes. Outer membrane protein isolated from *P. gingivalis* evokes strong humoral immune responses in many periodontitis patients [45]. The OMPs of *P.gingivalis* have been isolated from different strains (e.g. ATCC 33277, 381, ESO 75, ESO 101, HW24D-1, OMZ 409, ATCC 53977, HG 184, 6/26, FAY19M-1, ATCC 49417, W50, W83 and HNA-99). Recently, several major outer membrane proteins have been identified from the ATCC 33277 strain. These consist of RagA (Pgm1), RagB (Pgm4), a 75-kDa major protein (Pgm2), gingipains (Lys-gingipain, Pgm3; Arg-gingipain, Pgm5) and a mixture of immunoreactive 42- and 43-kDa antigens, named Pgm6/7 OmpA-like proteins because they share homology with *E. coli* OmpA protein at the C-terminal region [46, 47].

The results of cytokine production in the supernatants were different between the patients and the healthy subjects, showing higher levels in the group of patients. In the supernatants of the CD4+ cells of the patients that were stimulated with PHA, the IL-13 production was higher than the production of IFN-γ. Conversely, the production of IFN-γ was higher than IL-13 production in the supernatants of all cells that were stimulated with anti-CD3 and anti-CD28 and higher than the IL-4 production in the supernatants of the cells stimulated with anti-CD3/anti-CD28 and PHA. Also, the production of IL-2 was higher than that of IL-4 in the supernatants of all cells, irrespective of the stimuli.

In addition to the expression analyses in stimulated CD4+ cells, mRNA expression of IL-4, IL-13, IL-2 and IFN-γ was analysed in unstimulated CD4+ cells. The expression of the unstimulated and stimulated CD4+ cells showed different patterns, which is consistent with previously reported data [48–51]. Expression levels are lower in unstimulated T cells compared with stimulated T cells [52, 53]. The IL-4 expression was higher in the CD4+ T cells of the controls than in the cells of the patients. Conversely, IL-13 expression was not detected in the unstimulated CD4+ cells of the controls. This indicates that the expression of IL-13 is only strong if regulated by signalling mechanisms after activation [54]. Therefore, one plausible explanation for these results might be the lower mRNA expression of IL-4 and IL-13 that naïve T cells produce in comparison to Th2 cells [55, 56]. Although naïve T cells themselves are the cellular source of Th2-initiating IL-4, they must interact with a costimulatory APC, rather than a resting B cell, for initiation of the primary response [57]. Thereafter, they are able to produce enough IL-4 to initiate their own Th2 differentiation, especially under conditions of IFN-γ neutralisation [52, 57]. This situation correlates with the lower expression of IFN-γ that was detected in the unstimulated CD4+ cells of patients and controls in comparison to the stimulated cells. Since IL-4 also blocks the specific IL-2-induced proliferation of naïve CD4+ cells, this might explain the lower expression of IL-2 compared with IL-4 in the unstimulated cells of the controls [58].

In summary, this is the first report analysing the gene expression profiles of Th1/Th2 cytokines in inactivated CD4+ and activated CD4+ T lymphocytes of patients with aggressive periodontitis. The results showed clear differences in expression patterns between unstimulated and stimulated cells. A predominantly Th1 mRNA profile in activated CD4+ cells of controls was detected. Additionally, the increased IFN-γ expression in the cells of the healthy controls is an interesting finding which is consistent with the early immunoregulatory role of this cytokine, necessary for the immediate response with high IFN-γ secretion to immature antigen-presenting cells. Although the activated CD4+ cells of AgP patients expressed and produced higher levels of Th2 cytokines, results from direct comparisons between Th1 versus Th2 profiles did not show domination of either Th1- or Th2-related cytokines.

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Conflicts of interest The authors declare that there are no conflicts of interest in this study.

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