Reactivity of neutrophils, monocytes and platelets in periodontitis

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Chapter 3

Expression of FcγRs and mCD14 on polymorphonuclear neutrophils and monocytes may determine periodontal infection

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Expression of FcγRs and mCD14 on polymorphonuclear neutrophils and monocytes may determine periodontal infection

Abstract

Variance in expression of receptors for IgG (FcγRs), complement (CR3) and LPS (mCD14) on polymorphonuclear neutrophils (PMNs) and monocytes might affect susceptibility for infection with certain pathogens in periodontitis, a chronic infectious disease of tooth-supportive tissues. Levels of FcγRI, IIa, III, CR3, and mCD14 on PMNs and monocytes were measured in 19 periodontitis patients and 18 healthy controls. Subgingival infection with A. actinomycetemcomitans (Aa) and P. gingivalis (Pg) was determined. Activation of PMNs and monocytes in response to stimulation with A. actinomycetemcomitans and P. gingivalis was assessed by means of change in mCD14 expression. Periodontitis is associated with an enrichment of the FcγRIII+ monocytes (P=0.015) with concomitant low mCD14 (P=0.001). Unadjusted data showed that the subjects culture-positive for A. actinomycetemcomitans (Aa+) had significantly lower expression of monocytic FcγRI (P=0.005) and FcγRIIa (P=0.015) than Pg+ subjects. The FcγRI was still lower on monocytes from Aa+ subjects after adjusting for the background factors (P=0.037). PMNs from Aa+ subjects responded in a hyper-reactive manner, in particular when stimulated with A. actinomycetemcomitans (P=0.011). Lower FcγRs expression by monocytes is related to a higher susceptibility of a subject to become infected with A. actinomycetemcomitans. The higher proportion of FcγRIII+ monocytes may be involved in the chronicity of this condition. Hyper-reactive PMNs in Aa+ subjects may contribute to accelerated breakdown of tooth-supportive tissues.
Chapter 3

Introduction

Periodontitis is a chronic infectious disease of the supportive tissues of the teeth characterized by gradual loss of periodontal attachment and alveolar bone. Approximately 10% of the population suffers from the severe form and, if untreated, it may result in tooth loss. The major pathogens associated with periodontitis are *Aggregatibacter actinomycetemcomitans* (Aa) and *Porphyromonas gingivalis* (Pg) (1,2). In conjunction with the bacterial challenge, an important role in the onset and progression of periodontitis is played by the host immune response (3). A phagocytic response represents the first barrier to the penetration of bacteria into periodontal tissue (4). However, in addition to their defensive role, the phagocytes (polymorphonuclear neutrophils [PMNs] and monocytes) may also be responsible for collateral damage to the periodontal tissues, thus the host defense may be at the cost of periodontal attachment and alveolar bone (5,6).

One aspect of the host-derived breakdown of periodontal tissues is related to a hyper-reactive trait of PMNs in response to immunoglobulin G (IgG)-opsonized antigens (7,8,9). The nature of this hyper-reactivity might be related either to the genetically-determined increased binding capacity of the receptors for IgG (FcγR) on PMNs or to the increased expression levels of Fcγ-receptors.

There are several types of FcγRs identified on phagocytes: FcγRI (CD64), FcγRIIa (CD32) and FcγRIII (CD16) (10). Previous studies have shown that single-nucleotide polymorphisms, affecting the IgG-binding domain of the genes of FcγRIIa and IIIb, have functional consequences. PMNs from periodontitis patients bearing the more reactive genotype (i.e. FcγRIIa-131H/H and FcγRIIIb-NA1/NA1) show hyper-reactivity in response to stimulation with *A. actinomycetemcomitans* or *P. gingivalis* (11,9). Furthermore, periodontitis patients with FcγRIIa-131H/H genotype have more severe periodontal breakdown than the patients with FcγRIIa-131H/R or 131R/R genotype (9,12,13,14). There are several studies that reported the expression of FcγRs on peripheral PMNs in periodontitis (15,16), but a difference of expression levels between patients and controls could not be demonstrated.

In contrast to the PMN, little data exist on the expression of FcγR by monocytes in periodontitis. Nagasawa et al. (17) described an increased percentage of FcγRII⁺ monocytes in chronic periodontitis, but did not analyze expression of other FcγRs on monocytes. Percentages of FcγRII⁺ monocytes are also increased in other chronic inflammatory conditions, such as rheumatoid arthritis (18). Moreover, the monocytes and
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Macrophages of rheumatoid arthritis patients express increased levels of FcγRI and FcγRIIa. It is conceivable that similar changes in the FcγR expression would occur in periodontitis, as periodontitis and rheumatoid arthritis appear to share many pathologic features (19).

In addition to the FcγRs, there are other classes of immune receptors important for the antibacterial functions of PMNs and monocytes. CD14 is a lipopolysaccharide (LPS) co-receptor and involved in the recognition of gram-negative bacteria and clearance of circulating LPS. Membrane-bound CD14 (mCD14) is mainly expressed on mature monocytes, macrophages and activated neutrophils (20). The β2-integrins are heterodimeric receptors consisting of a common β-subunit (CD18) associated with a unique α-subunit (CD11a, b, c, or d). These receptors are involved in infection and inflammation by mediating cell-cell, cell-extracellular matrix and cell-pathogen interactions (21).

The first aim of the present study was to assess the expression of different FcγRs by PMNs and monocytes from untreated periodontitis patients and healthy controls. In addition we analyzed the expression levels of two other molecules important for phagocyte-bacterial interactions: the complement receptor CR3 (CD11b/CD18) and membrane-bound CD14. The second aim of the study was to explore the activation of PMNs and monocytes in response to stimulation with two important periodontal pathogens, A. actinomycetemcomitans and P. gingivalis.

Materials and methods
Study population
We recruited 19 periodontitis patients who were referred to the Department of Periodontology of the Academic Center for Dentistry Amsterdam (ACTA) for diagnosis and treatment of periodontitis. All patients had ≥8 teeth with radiographic bone loss beyond 30% of the root length. Age-, gender-, race- and smoking status-matched controls were selected among subjects registered for restorative dental procedures or who visited the dental school for regular dental check-ups. Control subjects were selected if they were not missing more than one tooth per quadrant (3rd molar excluded) and if they showed on dental bitewing radiographs ≤1 year old a distance between the cemento-enamel junction and the alveolar bone crest of ≤3 mm. Besides their periodontal condition, all participants
were otherwise healthy and had not taken any antibiotics during the last 6 months and no medication that could influence the immune response during the last 2 weeks. All patients were initially screened in the departmental clinic and had agreed to accept the proposed treatment plan. All samples were taken before the start of the periodontal therapy. All subjects were informed verbally and written about the purposes of the study and had signed an informed consent. The Medical Ethical Committee of the Academic Medical Center of the University of Amsterdam approved the study.

**Blood sampling**
All participants were asked to fast for at least 12 hours before the clinical visit. Fasting blood samples were obtained between 8.30 and 10.30 AM by venipuncture in the antecubital fossa. The venous blood was collected into evacuated tubes (BD Vacutainer System, Plymouth, UK) with anti-coagulant (one 5 mL - EDTA tube for automated leukocyte counting and leukocyte differential count and one 5 mL Na-citrate tube for flow cytometric analysis).

**Bacterial sampling**
Subgingival microbiological samples were taken from all subjects to determine the presence of the periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis*. Samples were taken after venous blood collection to avoid possible phagocyte activation. Sampling, laboratory procedures, and identification of *A. actinomycetemcomitans* and *P. gingivalis* were performed as described before (2).

**Flow cytometric analysis**
For cell surface staining, citrated whole blood (40 µl) was incubated for 30 min on ice with saturated concentrations (1-10 µg/mL) of the fluorochrome-conjugated antibodies. CD14-PE (8G3), CD16-FITC (5D2), and CD11b-FITC (B2) were purchased from Sanquin (Amsterdam, The Netherlands). CD32-FITC (AT10) and CD64-FITC (10.1) were obtained from Serotec (Marseille, France). Appropriate isotype controls were used: PE-IgG2a, FITC-IgG1, FITC-IgG2a from Sanquin and FITC-IgM from Beckman-Coulter (Fullerton, CA, USA). After incubation, 3 mL of ice-cold NH₄Cl buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA at pH 7.4) was added to lyse the erythrocytes, samples placed on ice for 15 min and then centrifuged at 500g, 4°C. The cell pellet was washed
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two times (500g, 4°C) in HEPES-buffer (137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl₂, 5.6 mM glucose, 20 mM HEPES, 1 mg/mL bovine serum albumin, 3.3 mM NaH₂PO₄, pH 7.4) and resuspended in HEPES-buffer containing 0.3% formaldehyde (final concentration). Flow cytometric analysis was performed within 2h of sample preparation. Expression of CD11b, CD14, CD16, CD32 and CD64 is indicated as the geometric mean fluorescence intensity (MFI).

Flow cytometric analysis was performed in a FACScan flow cytometer with CellQuest software (Becton Dickinson, San Jose, CA, USA). The PMNs and the monocytes were identified based on their forward and sideward scatter light patterns (Fig. 3.1A). Based on the binding of the corresponding isotype control antibody, a fluorescence threshold was set (≤1% of cells being positive for the isotype control antibody)(22); monocytes were considered to be FcγRIII⁺ when fluorescence was above this threshold (Fig. 3.1B, C). The fraction (%) of FcγRIII⁺ was calculated from the total monocyte population.

**Bacterial stimulation assays**

Brain-heart infusion broth enriched with hemin (5 mg/L) and menadione (1 mg/L) was used for the bacterial culturing. *A. actinomycetemcomitans* Y4 was grown aerobically for 18h at 37°C in humidified 5% CO₂, whereas *P. gingivalis* W83 was grown anaerobically (80% N₂, 10% H₂, 10% CO₂) for 48h at 37°C. The bacterial suspensions were washed,
reduced to an optical density of 1 at 600 nm in HEPES buffer, and stored in aliquots at -20°C.

Fresh citrated blood (25 µl) was added to a mix containing 55 µl of A. actinomycetemcomitans- or P. gingivalis- suspension, 4 µg/mL CD14-PE. The mix was incubated for 60 min at room temperature. In the control tubes, whole blood was incubated with HEPES containing no bacteria. After the incubation period, 1 mL of lysis solution was added to the samples, thoroughly mixed and placed on ice for 15 min. The cells were mildly fixed by addition of 1 mL of HEPES containing 0.3% PFA.

The same FACS-settings were used to determine the expression of mCD14 on PMNs and monocytes (See above: Flow cytometric analysis). As the mCD14 baseline values were different among subjects within our study group, the effect of stimulation was expressed as the percent change of mCD14-MFI: [the % change in MFI = (the MFI of stimulated cells – MFI of untreated cells)/MFI of untreated cells]*100]; the value of untreated cells was taken as 0% change (23).

Statistical analysis
Data analyses were performed with the SPSS package, version 14.0 (SPSS Inc., Chicago, IL, USA). Means ± standard deviations (SD), and frequency distributions were calculated. Prior to the analyses normal distributions of data were confirmed by Kolmogorov-Smirnov goodness-of-fit test. Differences in background characteristics between patients and controls were statistically analyzed with the Student’s t-test or the χ²-test (or Fisher exact test), where appropriate. Differences in the receptor expression levels between patients and controls were compared using t-tests. Furthermore, differences in the analyzed parameters were explored in a general linear model using periodontal condition and colonization with A. actinomycetemcomitans or P. gingivalis as fixed factors, and age, gender, race, and smoking status as co-variates. Colonization with A. actinomycetemcomitans and P. gingivalis appeared to significantly influence the expression of some receptors. Therefore the study population was grouped according to the subgingival presence of A. actinomycetemcomitans or P. gingivalis (i.e. Pg Aa⁻ [n=18], Aa⁺ [n=9], Pg⁺ [n=7] and Pg⁺Aa⁺ [n=3] donors) and receptor expression patterns and cell activation after bacterial stimulation were compared in a one-way ANOVA with the Tukey-Kramer post-test for uneven groups (after confirming equal variances between subgroups using the Levene’s test). A Pearson’s correlation coefficient and a partial correlation coefficient correcting for
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Periodontal condition, colonization with *A. actinomycetemcomitans* or *P. gingivalis*, age, gender, race, smoking status, between mCD14 on monocytes and % FcγRIII+ monocytes were calculated. The effects of *A. actinomycetemcomitans-* vs. *P. gingivalis-* stimulation were compared with paired t-tests; *P*-values <0.05 were considered statistically significant.

**Results**

**Characteristics of the study population.** On the basis of the radiographic bone loss criterion (less than 3 mm between the alveolar bone crest and the cemento-enamel junction on all teeth) one control subject was excluded from the analysis. The culturing results revealed that 12 subjects were *Aa*+, of which 9 periodontitis patients and 3 controls. Nine patients were *Pg*+, whereas only 1 control tested positive for *P. gingivalis* (*P*=0.004, Table 3.1). The *Aa*+ subjects were younger (mean age 34.8 ± 11.2) than the *Pg*+ individuals (mean 48.3 ± 6.4, *P*=0.033).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Periodontitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>40.8 ± 10.1</td>
<td>42.0 ± 9.9</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6 (33 %)</td>
<td>7 (37 %)</td>
</tr>
<tr>
<td>Female</td>
<td>12 (67 %)</td>
<td>12 (63 %)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Caucasian</td>
<td>4 (22 %)</td>
<td>4 (21 %)</td>
</tr>
<tr>
<td>Caucasian</td>
<td>14 (78 %)</td>
<td>15 (79 %)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smoker</td>
<td>13 (72 %)</td>
<td>13 (68 %)</td>
</tr>
<tr>
<td>Smoker</td>
<td>5 (28 %)</td>
<td>6 (32 %)</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>2.2 ± 3.9</td>
<td>3.6 ± 5.1</td>
</tr>
<tr>
<td>Leukocytes (x 10^9/L)</td>
<td>5.8 ± 1.6</td>
<td>6.5 ± 2.1</td>
</tr>
<tr>
<td>Neutrophils (x 10^9/L)</td>
<td>3.3 ± 1.1</td>
<td>3.8 ± 1.5</td>
</tr>
<tr>
<td>Monocytes (x 10^9/L)</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Lymphocytes (x 10^9/L)</td>
<td>2.0 ± 0.6</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>Subgingival colonization</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aa Pg</em></td>
<td>14 (78 %)</td>
<td>4 (21 %)</td>
</tr>
<tr>
<td><em>Aa</em>+<em>Pg</em></td>
<td>3 (17 %)</td>
<td>6 (32 %)</td>
</tr>
<tr>
<td><em>Aa</em>+<em>Pg</em>+</td>
<td>1 (5 %)</td>
<td>6 (32 %)</td>
</tr>
<tr>
<td><em>Aa</em>+<em>Pg</em>+</td>
<td>0</td>
<td>3 (15 %)</td>
</tr>
<tr>
<td>Number of teeth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>28.7 ± 1.6</td>
<td>27.2 ± 2.5</td>
</tr>
<tr>
<td>With bone loss</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 30 %</td>
<td>0</td>
<td>17.1 ± 5.1</td>
</tr>
<tr>
<td>≥ 50 %</td>
<td>0</td>
<td>6.7 ± 4.2</td>
</tr>
</tbody>
</table>

*Table 3.1* Characteristics of the study population. *Aa, A. actinomycetemcomitans; Pg, P. gingivalis*

Values are means ± standard deviations or numbers (%) of subjects; *P*=0.004 (χ²-test).
Receptors expression by PMNs and monocytes. The expression of all tested receptors by PMNs was comparable in patients and controls (Fig. 3.2A-E). Periodontitis patients had a higher percentage of FcγRIII⁺ monocytes and lower monocytic mCD14 expression than controls (Fig. 3.2E, F). Overall, the level of mCD14 was inversely correlated with the percentage of FcγRIII⁺ monocytes (r=−0.473, P=0.003). This correlation was not found in
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control subjects (r=-0.124, P=0.624; Fig. 3.3A), but was strong within periodontitis patients (r=-0.618, P=0.005; Fig. 3.3B).

In Tables 3.2 and 3.3 we present adjusted data from a general linear model (GLM), for which periodontal condition and colonization with A. actinomycetemcomitans/P. gingivalis were entered as fixed factors and age, gender, race and smoking status as covariates. Neither colonization with A. actinomycetemcomitans/P. gingivalis nor other potential confounding factors were associated with expression levels by the PMNs of any of the receptors tested (Table 3.2). Further, in the GLM we observed that a lower expression of FcγRI by monocytes was present in the Aa+ subjects (Table 3.3). After correcting for periodontal condition, colonization with A. actinomycetemcomitans/P. gingivalis, age, gender, race, smoking status, the correlation between mCD14 and % FcγRII+ monocytes was still apparent (r_adj=-0.382, P_adj=0.034).
Table 3.2. Receptor expression by PMNs. Aa = A. actinomycetemcomitans, Pg = P. gingivalis

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Control n=18</th>
<th>Periodontitis n=19</th>
<th>Aa Pg₀ n=18</th>
<th>Aa⁺ n=9</th>
<th>Pg⁺ n=7</th>
<th>Aa⁺ Pg⁺ n=3</th>
<th>P_adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcγRI</td>
<td>2.4 (0.3-4.4)</td>
<td>2.5 (1.1-3.8)</td>
<td>0.960</td>
<td>0.427</td>
<td>4.9 (2.4-7.3)</td>
<td>0.7 (0-4.4)</td>
<td>0.157</td>
</tr>
<tr>
<td>FcγRIIa</td>
<td>116.9 (103.1-130.7)</td>
<td>105.4 (96.2-114.6)</td>
<td>0.159</td>
<td>0.382</td>
<td>16.3 (9.4-22.9)</td>
<td>0.44</td>
<td>0.049</td>
</tr>
<tr>
<td>FcγRIII</td>
<td>508.9 (398.2-619.6)</td>
<td>522.9 (449.1-596.8)</td>
<td>0.828</td>
<td>0.177</td>
<td>41.7 (27.6-60.7)</td>
<td>0.668</td>
<td></td>
</tr>
<tr>
<td>CR3</td>
<td>96.9 (2.9-191.0)</td>
<td>153.1 (90.4-215.8)</td>
<td>0.308</td>
<td>0.134</td>
<td>88.8 (0-186.3)</td>
<td>0.352</td>
<td></td>
</tr>
<tr>
<td>mCD14</td>
<td>3.1 (1.8-4.4)</td>
<td>3.9 (3.0-4.8)</td>
<td>0.301</td>
<td>0.177</td>
<td>4.1 (2.7-5.5)</td>
<td>0.204</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3. Receptor expression by monocytes. Aa = A. actinomycetemcomitans, Pg = P. gingivalis.

* Values are adjusted means (confidence intervals) for MFI and corresponding adjusted P-values obtained from a general linear model using periodontal condition and colonization with A. actinomycetemcomitans or P. gingivalis as fixed factors and age, gender, race, smoking status as covariates.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Control n=18</th>
<th>Periodontitis n=19</th>
<th>Aa Pg₀ n=18</th>
<th>Aa⁺ n=9</th>
<th>Pg⁺ n=7</th>
<th>Aa⁺ Pg⁺ n=3</th>
<th>P_adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcγRI</td>
<td>42.5 (30.6-54.3)</td>
<td>47.8 (39.9-55.7)</td>
<td>0.441</td>
<td>0.177</td>
<td>59.6 (45.5-73.7)</td>
<td>0.105</td>
<td></td>
</tr>
<tr>
<td>FcγRIIa</td>
<td>113.7 (96.0-131.5)</td>
<td>107.0 (95.2-118.8)</td>
<td>0.515</td>
<td>0.134</td>
<td>126.8 (105.7-147.8)</td>
<td>0.177</td>
<td></td>
</tr>
<tr>
<td>FcγRIII</td>
<td>2.4 (0-7.4)</td>
<td>5.6 (2.2-8.9)</td>
<td>0.285</td>
<td>0.177</td>
<td>6.4 (0.4-12.3)</td>
<td>0.269</td>
<td></td>
</tr>
<tr>
<td>CR3</td>
<td>136.3 (69.8-202.7)</td>
<td>175.9 (131.6-220.2)</td>
<td>0.309</td>
<td>0.134</td>
<td>166.9 (88.1-245.8)</td>
<td>0.707</td>
<td></td>
</tr>
<tr>
<td>mCD14</td>
<td>83.9 (62.6-105.2)</td>
<td>52.1 (37.9-66.4)</td>
<td>0.015</td>
<td>0.134</td>
<td>62.9 (37.6-88.2)</td>
<td>0.470</td>
<td></td>
</tr>
</tbody>
</table>

% FcγRII⁺ c

| % FcγRII⁺ | 16.3 (11.4-21.2) | 21.7 (18.4-25.0) | 0.068       | 0.134   | 21.8 (15.9-27.6) | 0.470 |

Table 3.3. Receptor expression by monocytes. Aa = A. actinomycetemcomitans, Pg = P. gingivalis.

* Values are adjusted means (confidence intervals) for MFI and proportion of FcγRII⁺ cells and corresponding P_adj-values obtained from a general linear model using periodontal condition and colonization with A. actinomycetemcomitans or P. gingivalis as fixed factors and age, gender, race, smoking status as covariates.

* Aa⁺ subjects had lower levels of monocytic FcγRI than Aa Pg⁺ (P_adj=0.026) and Pg⁺ subjects (P_adj=0.007) in post-hoc testing.

* % FcγRII⁺ is inversely correlated with mCD14 on monocytes; partial correlation coefficient (corrected for periodontal condition, colonization with Aa/Pg, age, gender, race, smoking status) r = -0.382, P=0.034
Expression of FcγRs and mCD14 on polymorphonuclear neutrophils and monocytes may determine the periodontal infection.

**Receptor expression on PMNs and monocytes in Aa−Pg−, Aa+, Pg+ and Aa+Pg+ subjects.** The significance of colonization with *A. actinomycetemcomitans* or *P. gingivalis* for some of the receptor expression levels prompted us to further explore expression of receptors in subgroups of subjects (patients and controls) based on their colonization with *A. actinomycetemcomitans* or *P. gingivalis*.

Receptor expression levels on PMNs did not significantly differ between Aa−Pg−, Aa+, Pg+ and Aa+Pg+ subjects (Fig. 3.4A-E). On monocytes we observed that FcγRI and

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**Fig. 3.4** PMN and monocyte expression of (A) FcγRI, (B) FcγRIIa, (C) FcγRIII, (D) CR3 and (E) mCD14 for Aa−Pg− subjects (open bars, n=18), Aa+ subjects (bright grey bars, n=9), Pg+ subjects (dark grey bars, n=7), and Aa+Pg+ subjects (closed bars, n=3). Values are means ± standard deviation; Aa (A. actinomycetemcomitans), Pg (P. gingivalis).

*a* On panel A – Monocytes: *P*=0.005 for the overall ANOVA; post-hoc *P*=0.004.

*b* On panel B – Monocytes: *P*=0.015 for the overall ANOVA; post-hoc *P*=0.009.
FcγRIIa were present at lower levels in Aa+ subjects than in the other three subgroups (overall ANOVA $P=0.005$ and $P=0.015$, respectively). In particular, the FcγRI and FcγRIIa expression levels on monocytes from Aa+ subjects were lower than on monocytes from Pg+ subjects ($P=0.004$ and $P=0.009$, respectively, Fig. 3.4A, B).

Expression levels of FcγRIII, CR3, and mCD14 were not significantly different between the monocytes from Aa−Pg−, Aa+, Pg+ and Aa+Pg+ donors (Fig. 3.4C, D, E), even though the same trend of lower monocytic expression in Aa+ subjects was visible for FcγRIII and CR3 (overall ANOVA $P=0.168$ and $P=0.149$, respectively). The expression profile in the subjects colonized with both A. actinomycetemcomitans and P. gingivalis (Aa+Pg+ subjects) seemed to be the result of a combined effect of the two bacterial species; the levels of FcγRI, FcγRIIa, FcγRIII and CR3 resembled the levels of Aa+ subjects, whereas the mCD14 in Aa+Pg+ subjects seemed more similar to the Pg+ subjects.

**PMN and monocyte activation in response to A. actinomycetemcomitans or P. gingivalis.** The above results indicated that the subjects having different periodontal infection patterns show variability in the numbers of phagocytic receptors. The question arose whether A. actinomycetemcomitans and P. gingivalis can activate PMNs and monocytes differentially. Thus, we tested the reactivity of PMNs and monocytes from all donors to these periodontal pathogens. The change in mCD14 expression was used as a measure of cell activation. Both stimulation with A. actinomycetemcomitans and P. gingivalis resulted in activation of PMNs and monocytes. Important to note was that the % change in mCD14-MFI on PMNs and monocytes after P. gingivalis or A. actinomycetemcomitans stimulation did not differ significantly between patients and controls (data not shown).
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The % change in mCD14 expression by PMNs induced by *A. actinomycetemcomitans* was of a higher magnitude than after *P. gingivalis* stimulation (*P*<0.0001). PMNs from *A. actinomycetemcomitans*-culture positive subjects, showed an enhancement of mCD14 after *A. actinomycetemcomitans* or *P. gingivalis* stimulation in contrast to PMNs from *Pg*−*Aa*−, *Pg*+ and *Aa*+*Pg*+ subjects who showed downregulation or less upregulation of mCD14 (*P*=0.011 and *P*=0.053, respectively; Fig. 3.5A, B). In particular, *A. actinomycetemcomitans* stimulation resulted in increased mCD14 on PMNs from *Aa*+ subjects, thus higher than on PMNs from *Pg*+ and *Pg*′*Aa*− subjects (*P*=0.022 and *P*=0.021, respectively).

On monocytes, *A. actinomycetemcomitans* induced also a higher % change in mCD14 than *P. gingivalis* (*P*<0.0001). The activation of monocytes by *A. actinomycetemcomitans* was strong, as evidenced by the up-regulation of mCD14 up to 200%, whereas *P. gingivalis* induced hardly any increase in mCD14 on monocytes (<25%). Monocytes from *Pg*−*Aa*−, *Aa*+, *Pg*+ or *Aa*+*Pg*+ subjects had comparable response to *A. actinomycetemcomitans* and *P. gingivalis* (Fig. 3.5A, B).

**Discussion**

This study was undertaken to investigate expression patterns of some major receptors by phagocytic cells in periodontitis and health to further elucidate susceptibility, infection patterns and biological pathways in this inflammatory condition. On PMNs we found no
differences between patients and controls in the expression patterns of the tested receptors. On monocytes we found also a comparable expression of FcγRI, II, and CR3 in patients and controls. However, an intriguing finding of this study is the heterogeneity of receptor expression levels between individuals. In the general linear models, bacterial infection patterns appeared as a major determinant for expression levels by monocytes. When we subdivided the study population into \( Pg^-Aa^-, Aa^+, Pg^+ \) and \( Aa^+Pg^+ \) subjects, the expression of FcγRI and IIa by monocytes was significantly lower in \( Aa^+ \) subjects than in \( Pg^+ \) subjects (Fig. 3.4); after correcting for periodontal condition and potential confounding factors, the expression of FcγRI monocytes was still decreased in \( Aa^+ \) subjects (Table 3.3).

One possible explanation for the lower levels of FcγRs on monocytes from \( Aa^+ \) subjects might be due to genotypic differences, possibly making these individuals more susceptible to become infected with \textit{A. actinomycetemcomitans}. Similarly, genetically-defined deficiencies in various components of the innate immune system (mannose-binding lectin, vitamin D receptor, mannose-associated serine-protease-2, Toll-like receptors, etc.) have been associated with a greater risk of infection with \textit{Mycobacterium tuberculosis}, meningococci or gram-negative bacteria (24,25,26,27,28). In periodontitis it has been shown that polymorphisms in the FcγRs and IL-6 genes are associated with increased odds of detecting \textit{A. actinomycetemcomitans}, \textit{P. gingivalis} and \textit{Tannerella forsythia} after adjustment for age, ethnicity, smoking and periodontitis extent (29). However, in our study neither the FcγRIIa 131H\(^+\) (131 H/R or H/H) nor the CD14 -260T\(^+\) (-260 C/T or T/T) genotypes showed increased odds of detecting \textit{A. actinomycetemcomitans} or \textit{P. gingivalis} in subgingival plaque samples (data not shown).

Alternatively, the lower expression of FcγRs on monocytes in \( Aa^+ \) subjects might be a consequence of the infection with \textit{A. actinomycetemcomitans}. In order to elucidate this, future studies are needed evaluating the effects of therapeutic elimination of \textit{A. actinomycetemcomitans} from the subgingival flora on the expression of FcγRs. In line with this possibility there are studies demonstrating the effects of medication on FcγRs expression levels in rheumatoid arthritis. Expression levels of FcγRs with cell activation potential on peripheral monocytes in patients suffering from rheumatoid arthritis were \textit{increased} before therapy in comparison to healthy subjects (30,31,32,33). The levels of FcγRs are changed to “healthy” levels due to treatment (33,34,35). This change in expression levels of FcγRs correlates with clinical improvement, thus restoring expression
levels of FcγRs can have beneficial effects. Similarly, it is conceivable that periodontal therapy may have an effect on expression levels of FcγRs.

Normally, the majority of monocytes (90%) do not express FcγRIII and show high levels of mCD14 (36). However, we found an increased % of FcγRIII+ monocytes and a lower level of mCD14 on monocytes from periodontitis patients compared to healthy controls (Fig. 3.2). These results are in line with previous studies showing an increased % of FcγRIII+ monocytes in chronic periodontitis and lower mCD14 on peripheral monocytes from early-onset periodontitis patients (37,17). Furthermore, we demonstrated a correlation between the mCD14 on monocytes and the % of FcγRIII+ monocytes, suggesting that periodontal infection affects the monocyte phenotype leading to differentiation of the CD14low-FcγRIII+ cells (36). The CD14low-FcγRIII+ monocytes account for about 10% of monocytes in healthy adults and are expanding up to 40% in inflammatory conditions such as rheumatoid arthritis (18,31), sepsis (38) or Kawasaki disease (39). In our study mCD14 was lowest on monocytes from Pg+ subjects (51.8 ± 13.8 MFI, Fig. 3.4E; adjusted mean 62.9 MFI, Table 3.3) and not significantly different than the overall value noted for the whole periodontitis patient group (53.1 ± 20.9 MFI, Fig. 3.2E; adjusted mean 52.1 MFI, Table 3.3). This suggests that individuals infected with P. gingivalis show the most marked changes in the monocyte phenotype and they account for the difference in mCD14 expression noted between patients and controls.

The association between P. gingivalis and a lower mCD14 expression on monocytes is supported by in vitro findings where P. gingivalis LPS was able to induce maturation of CD14low-FcγRIII+ dendritic cells (40). Compared to the CD14bright-monocytes, the CD14low-FcγRIII+ monocyte subset can differentiate into dendritic cells that produce lower amounts of IL-1β, IL-6, IL-12, TNF-α and IL-8, but have higher phagocytic and oxidative activity (41). These dendritic cells are capable of antigen presentation, but fail to efficiently stimulate T-cells, possibly due to lack of co-stimulatory molecules (40). These characteristics of the dendritic cells originating from the CD14low-FcγRIII+ monocytes are possibly contributing to the perpetuation of chronic inflammation in periodontitis, as they might be unable to efficiently coordinate the antibacterial defense in the inflamed periodontium. We hypothesize that during periodontal infection frequent bacteremic episodes, especially of P. gingivalis origin, lead to the early selection of the CD14low-FcγRIII+ precursors of dendritic cells, which are ultimately unable to clear the periodontal infection.
PMN receptor expression was essentially not different between patients and controls and a possible relation to the infection pattern was not found. Thus, in contrast to monocytes for which we suggest a role in the susceptibility to certain infection patterns, the PMNs may be solely involved in bacterial clearance. It is commonly accepted that during their antibacterial functions PMNs induce collateral damage in inflamed periodontal tissues, which is largely attributable to the production of proteolytic enzymes (i.e. matrix-metalloproteinases, elastase) and reactive oxygen species (42). In particular this process is relevant, since hyper-reactive PMNs in periodontitis have been demonstrated by several research groups and several mechanisms have been proposed (7,43,8,9). The PMNs from Aa+ subjects showed a stronger response to both P. gingivalis and A. actinomycetemcomitans (measured by the change in mCD14), further providing evidence for a hyper-reactive trait of the PMNs from A. actinomycetemcomitans-infected subjects, possibly aggravating the periodontal inflammatory reactions in this individuals (7,8,9). This hypothesis may be strengthened by the observations that A. actinomycetemcomitans induced stronger PMN activation than P. gingivalis (Fig. 3.5A, B). A. actinomycetemcomitans might be in general a stronger stimulator of phagocytes than P. gingivalis (44,45,46,47). A. actinomycetemcomitans LPS is inducing higher amounts of IL-1β, TNF-α and IL-8 in PMNs than P. gingivalis LPS (48), evoking a more acute, E.coli - like inflammatory immune response. This might help explaining the severe and relative early onset of periodontal breakdown associated with A. actinomycetemcomitans infection, especially in patients with aggressive periodontitis (49).

An interesting aspect of the periodontal pathogen P. gingivalis is its capability to produce bacterial cysteine proteinases (gingipains) which are able to proteolyse human monocyte mCD14 (50,51). However, protease inhibitors present in serum inhibit the gingipain activity (51), since in our bacterial stimulation assays A. actinomycetemcomitans and P. gingivalis were similarly able to induce mCD14 upregulation on monocytes, although of different amplitude.

In conclusion, we suggest that receptor expression patterns by monocytes may be related to the susceptibility of a subject to become infected with certain periodontal pathogens. The enrichment of the FcγRIII+ monocytes in periodontitis, in particular in patients that are culture-positive for P. gingivalis, may result in formation of a dendritic cell type that can stimulate T cells less efficiently. As monocytes and their progeny, the dendritic cells, are expected to orchestrate the immune response in periodontitis, the
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FcγRIII* monocytes may be involved in the chronicity of the infection. PMNs from A. actinomycetemcomitans-culture positive subjects respond in a hyper-reactive fashion to the infection, and in particular seem to get strongly activated when stimulated with A. actinomycetemcomitans. In this way PMNs may contribute to advanced breakdown of tooth-supportive tissues through an enhanced release of a variety of proteolytic enzymes and reactive oxygen species.

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