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Mature Dendritic Cells Infiltrate the T Cell-Rich Region of Oral Mucosa in Chronic Periodontitis: In Situ, In Vivo, and In Vitro Studies

Ravi Jotwani,* Anna Karolina Palucka,† Montasr Al-Quotub,‡ Mahyar Nouri-Shirazi,† Jay Kim,‡ Diana Bell,† Jacques Banchereau,† and Christopher W. Cutler²*

Previous studies have analyzed the lymphoid and myeloid foci within the gingival mucosa in health and chronic periodontitis (CP); however, the principal APCs responsible for the formation and organizational structure of these foci in CP have not been defined. We show that in human CP tissues, CD1a⁺ immature Langerhans cells predominantly infiltrate the gingival epithelium, whereas CD83⁺ mature dendritic cells (DCs) specifically infiltrate the CD4⁺ lymphoid-rich lamina propria. In vivo evidence shows that exacerbation of CP results in increased levels of proinflammatory cytokines that mediate DC activation/maturation, but also of counterregulatory cytokines that may prevent a Th-polarized response. Consistently, in vitro-generated monocyte-derived DCs pulsed with Porphyromonas gingivalis strain 381 or its LPS undergo maturation, up-regulate accessory molecules, and release proinflammatory (IL-1β, PGE₂) and Th (IL-10, IL-12) cytokines. Interestingly, the IL-10:IL-12 ratio elicited from P. gingivalis-pulsed DCs was 3-fold higher than that from Escherichia coli-pulsed DCs. This may account for the significantly (p < 0.05) lower proliferation of autologous CD4⁺ T cells and reduced release of IFN-γ elicited by P. gingivalis-pulsed DCs. Taken together, these findings suggest a previously unreported mechanism for the pathophysiology of CP, involving the activation and in situ maturation of DCs by the oral pathogen P. gingivalis, leading to release of counterregulatory cytokines and the formation of T cell-DC foci. The Journal of Immunology, 2001, 167: 4693–4700.

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Materials and Methods

Human subject use

The Institutional Review Board approved all protocols involving human subjects. Informed consent was obtained from all subjects before commencement of the study. All subjects were in good general health and were not on antibiotic, anti-inflammatory, or anticoagulant therapy during the
month preceding the baseline exams. Also excluded from the study were subjects with a history of drug allergies, rheumatic fever or other conditions requiring prophylactic antibiotic treatment, gross caries or other dental pathology, fewer than 20 teeth, and pregnant or nursing females.

Collection and handling of clinical specimens, disease categories, clinical protocols

Gingival tissue specimens from 29 adult subjects, including 8 periodontally healthy, 7 with gingivitis, and 14 with CP, were excised under nerve block anesthesia by sharp dissection. The clinical criteria for these disease categories have been described previously (5, 31, 32). Gingival tissue was properly oriented in OCT medium by inserting a tooth landmark (3-mm strip of filter paper) alongside the tissue specimen, was flash frozen, and then was stored at −80 °C. Cryostat sections measuring 7 μm thick were cut and fixed in cold acetone for 10 min. Sections were stained with HE to confirm the clinical diagnosis by histological means (not shown). The experimental design for the induction of active (n = 12) or “quiescent” (n = 20) gingival inflammation (Table I) in subjects with preexisting mild CP (minimum of three probing pocket depths of ≥ 5 mm, clinical attachment levels of ≥ 5 mm, bleeding on probing, and horizontal bone loss at the same sites), as well as the technique for sampling gingival crevicular fluid (GCF), have been described previously (32). All four periopaper strips from each tooth were placed in one glass screw-capped vial and stored at −80 °C.

Analysis of GCF cytokines by ELISA

Sterile saline (400 μl) was added to vials containing strips, and proteins were eluted by placing the vials on a rotator (Roto-Mix; Barnstead/Thermolyne, Dubuque, IA) at 250 ± 10 rpm for 1 h in a 4 °C cold room. Initial studies (not shown) established the dilution of test samples that yielded sufficient volume and sensitivity for detection of the four cytokines within the linear range of their respective standard curves (32). Dilutions ranged from 1/2,5 to 1/5 v/v. The diluted samples then were aliquoted into new vials and frozen at −80 °C for subsequent analysis. Commercial ELISA kits (R&D Systems, Minneapolis, MN.) were used to analyze the levels of IL-1β, IFN-γ, IL-10, and PGE2 within the GCF samples. All reagents were brought to room temperature (30–37 °C) and incubated (30 min) with H&E buffer. The standard or unknown sample then was added to the wells (200 μl volume), and analysis was performed as described by manufacturer. Color development and intensity of the color were measured with an ELISA plate reader (Molecular Devices, Sunnyvale, CA.). A standard curve was prepared, plotting the optical density vs the concentration of the cytokine expressed as pg/30 s.

Single and double immunohistochemistry (enzyme-linked)

Single immunohistochemistry was performed on prefixed frozen sections by indirect method, as described previously (33). Sections were stained with the biotin-streptavidin-peroxidase method (Vectastain ABC Elite kit, Burlingame, CA). The primary Abs used are listed (Table II). The specificity was confirmed by substituting the respective isotype controls for the primary Abs. The sections were counterstained with hematoxylin. For double staining, after the first staining, sections were washed and labeled by the biotin-streptavidin-glucose oxidase method (Vectastain ABC GO kit). The sections were counterstained with Vector MethylGreen (Vector Laboratories). The specificity of the secondary Ab was confirmed by substituting the respective isotype control for the secondary Abs. A blinded examiner using light microscopy enumerated the immunoreactive cells. The area of the grid was calculated to obtain the number of cells per square millimeter. Two different areas were randomly selected by a blinded examiner in the epithelium and two in the LP to determine the number of cells per square millimeter. The number of positively stained cells in the epithelium and LP were also compared with each other. Statistical analysis is described below.

Purification of autologous CD4+ cells

CD4+ cells were purified from PBMC of the same donor that were used for DC generation (autologous), as described previously (34). Cells bearing CD4 Ag were isolated from mononuclear fraction through positive selection with anti-CD4 mAb and goat anti-mouse IgG-coated microbeads (Miltenyi Biotec, Gladbach, Germany). Isolation of CD4+ cells was achieved with Minimacs separation columns (Miltenyi Biotec) as described by the manufacturer. In all of the experiments, the isolated cells were 80–90% CD4+ as determined by staining with FITC-conjugated anti-CD4 mAb followed by flow cytometry analysis (not shown).

LPS purification

The methodology for isolation and purification of LPS from P. gingivalis 381 and E. coli American Type Culture Collection (ATCC, Manassas, VA) type strain 25922 was as described previously in our laboratory (35). Briefly, whole cell pellets were subjected to hot-phenol water extraction; the aqueous phase was subjected to deionization by distillation water, followed by lyophilization and then isopycnic density gradient centrifugation. The LPS-containing fractions were dialyzed extensively against distilled water, lyophilized, and subjected to biochemical analysis for purity (34).

Cytokines from DCs and T cells

To study cytokines produced by MDDCs, culture supernatants were collected at 24 h from DC pulsed with P. gingivalis 381, E. coli ATCC type strain 25922 (25:1 bacteria-to-DC ratio), or 100 ng/ml of P. gingivalis or E. coli LPS. For T cell cytokines (IFN-γ), culture supernatants were collected after DCs were cocultured with autologous CD4+ T cells for 5 days. The percentage of positive cells in the relevant population defined by forward scatter and side scatter characteristics. Expression levels were evaluated by assessing mean fluorescence intensity indices calculated by relating mean fluorescence intensity noted with the relevant mAb to that with the isotype control mAb for samples labeled in parallel and acquired by using the same setting.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Active (n = 12)</th>
<th>Quiescent (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>224 ± 26 b</td>
<td>80 ± 27</td>
</tr>
<tr>
<td>PGE2</td>
<td>396 ± 44 b</td>
<td>228 ± 44</td>
</tr>
<tr>
<td>IL-10</td>
<td>21.34 ± 6 b</td>
<td>8.13 ± 6</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>46 ± 12</td>
<td>31 ± 6</td>
</tr>
</tbody>
</table>

* The experimental protocol is described in Materials and Methods.

b Significant increase, as determined by Proc Mixed covariate analysis (p < 0.05).

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**T cell proliferation assay**

The ability of bacteria-pulsed DCs to stimulate autologous T cell proliferation was performed as described previously (36). Day 6 MDDCs were pulsed with either *P. gingivalis* (25:1 multiplicity of infection (MOI)); *E. coli* (25:1 MOI); 100 ng/ml *P. gingivalis* LPS; or 100 ng/ml *E. coli* LPS, for 24 h at 37°C. DCs in complete RPMI with no bacteria or LPS were used as controls. DCs were washed extensively and cultured at graded doses (5000 DC/200 μl, 1000 DC/200 μl, and 300 DC/200 μl) in complete RPMI 1640 medium with 10% human AB serum with autologous CD4+ T cells (50,000 cells/200 μl). Peak proliferative response was achieved with 1000 DC, so this number was used for subsequent assays. Proliferation was determined after 5 days by uptake of tritiated thymidine (1 μCi/ml) for the last 16 h. Assay was repeated 10 times on separate days, and the mean results are shown in Table III.

**Statistical analyses**

Descriptive statistics, means, and SE for the numbers of immunoreactive cells with each cell surface marker in healthy, gingivitis, and CP tissues (see Fig. 2) were calculated and analyzed for statistical significance by Tukey’s multiple comparisons test (p < 0.05; Minibit, State College, PA). Differences within epithelium and LP for each individual cell marker were analyzed by Student’s t test (p < 0.05). Descriptive statistics, means, and SE of the clinical indices (not shown) and GCF cytokine levels (Table I) were calculated by using SAS 6.12 (Cary, NC) and Proc Mixed with experimental group and genotype as the factors and the baseline levels as the covariate, as described previously (31). Proc Mixed covariate analysis was used to determine which means were statistically significant, with the output being least squares means and least significant difference. Differences in IL-10, IL-1β, PGE2, and IFN-γ between-group means were declared only if the p value for the F statistic in the analysis of covariance was <0.05, and the least significant difference also was significant at p <0.05. Results of in vitro cytokine levels and T cell proliferation (Table III) were analyzed by Kruskall-Wallis test (p < 0.05; Minibit).

**Results**

**Mature DCs specifically infiltrate the T cell-rich LP in CP**

We have enumerated immature CD1a+ Langerhan cells (LCs) and mature CD83+ DCs by using immunohistochemistry (Fig. 1) within epithelium and LP of the gingiva in health, gingivitis, and CP (Fig. 2A). Macrophage/myeloid cells (CD14+) and lymphocyte subsets also were enumerated within these same tissue compartments for comparison (Fig. 2B).

Microscopic analysis of healthy epithelium revealed the presence of large numbers of CD1a+ LCs, but only a few CD83+ mature DCs or CD14+ cells (Fig. 2A). In gingivitis, there was a slight increase in the numbers of CD1a+ -labeled cells in the epithelium. However, the transition from health to CP was associated with a significant increase (over 3-fold increase, from 90 cells/mm² to 312 cells/mm²; p < 0.05) in numbers of LCs in the epithelium. Moreover, the transition from health to CP was associated with a significant increase (6-fold increase, from 8 cells/mm² to 48 cells/mm²; p < 0.05) in numbers of mature DCs in the LP.
CD86, CD40, and HLA-Dr also were up-regulated. The morphologic changes as immature DCs become matured by LPS also are shown (Fig. 3, B and C).

P. gingivalis-matured DCs release relevant cytokines and stimulate a limited T cell response

Based on in situ, in vivo, and in vitro correlates detailed above, we had reason to expect that DCs matured by the oral pathogen P. gingivalis would be potent at stimulating T cell responses, as determined by IFN-γ/H9253 production and proliferation. However, we had reservations in this regard because of the GCF evidence that IFN-γ/H9253 did not increase significantly in active CP. This was possibly attributable to the presence of counterregulatory Th2-biasing cytokines, such as IL-10 (27) and PGE2 (38). Therefore, we analyzed the supernatants from the DCs pulsed as above with P. gingivalis, E. coli and their LPS moieties for proinflammatory cytokines (IL-1β/H9262, PGE2), the Th2-biasing cytokine IL-10, and the Th1-biasing cytokine IL-12. DCs pulsed with either P. gingivalis or E. coli release IL-1β, PGE2, IL-10, and IL-12, although P. gingivalis-pulsed DCs released significantly less (p < 0.05) of all four cytokines (Table III). The ratio of IL-10:IL-12 elicited by P. gingivalis was 3-fold higher than that by E. coli (7:1 vs 2:1, respectively). This elevation in IL-10:IL-12 ratio induced by P. gingivalis may be reflected in the significantly (p < 0.05) lower IFN-γ levels from CD4+ T cells cocultured with DC-P. gingivalis, relative to DC-E. coli (35 vs 1031 pg/ml, respectively) and in the limited proliferation of autologous T cells elicited by P. gingivalis-pulsed DCs as compared with E. coli-pulsed DCs (Table III).

Discussion

Our results show a marked change in the tissue localization of DCs from health to CP. Indeed, immature CD1a+ DCs (by definition, Ag capture cells) increased significantly in numbers in the diseased epithelium, whereas CD83+ mature DCs (by definition, APCs) increased in numbers within the lymphoid-rich diseased LP. Interestingly, when the data for all the markers was expressed as a percentage of change from health to CP (not shown), the following cells changed by >100%: mature DCs in LP ( ↑ 164%); immature DCs in LP ( ↓ 198%); and CD8+ cells in epithelium ( ↑ 169%).
Although these percentages were not tested statistically, we have interpreted the increase in mature DCs and decrease in immature DCs in the same tissues as evidence for local redistribution of LCs to LP, and subsequent maturation in situ (Fig. 4); however, the actual source of mature DCs is unknown presently. Our results further show that although certain cytokines released in this microenvironment (i.e., IL-1β and PGE₂) could promote in situ DCs activation/maturation and T cell expansion, the counterregulatory cytokine IL-10 also is released. Finally, the oral pathogen P. gingivalis is able to induce cultured MDDCs to undergo maturation and to release relevant proinflammatory (i.e., IL-1β and PGE₂) and Th cytokines (IL-10 and IL-12), but elicits a limited T cell response compared with E. coli-pulsed DCs.

Based on these results, we have developed a novel working model for the pathophysiology of CP involving the formation of what we have termed “oral lymphoid follicles” or OLF (Fig. 4) within the LP around the dentition. We acknowledge that the term “follicle” might imply a proliferating B cell lymphoid organ and hence may be misleading, but nonetheless propose this terminology. We propose that the development of OLF is initiated in gingivitis by LCs homing to the gingival epithelium in response to bacteria/Ags in the oral biofilm (39), as well as a source of the earliest signals for lymphoid/myeloid trafficking, i.e., epithelium/keratinocytes (40). That the CD1a⁺ cells enumerated in this study were in fact LCs was confirmed by scanning laser confocal microscopy and double immunofluorescence with CD1a and the LC-specific marker of Birbeck granules, LAG (41), as well as single immunoenzyme staining with langerin (Ref. 43; data not shown). Earlier studies have shown the dynamics of LCs trafficking in the respiratory mucosa in response to bacterial Ags (15), as well as LCs in oral mucosa/gingiva in response to plaque/allergen accumulation (10, 39, 42, 44, 45). One study identified increased numbers of LCs in the gingival epithelium of CP patients and also identified P. gingivalis (i.e., Bacteroides gingivalis) and Actinobacillus actinomycetemcomitans within the same tissues by immunohistochemistry (47). Our previous studies have shown by scanning laser confocal microscopy that LCs are infected with P. gingivalis in situ (6). In this same study, the pathophysiology of chronic gingivitis/mild CP was equated with a bacterially induced contact hypersensitivity response (46), wherein Ag (i.e., P. gingivalis)-sensitized gingival epithelial LCs engage CD8⁺ T cells homing to the gingival epithelium (6). This supposition is consistent with the cellular infiltrates within the epithelium but is too simplistic to account for all of our findings, particularly those observed within the LP. The foci of myeloid and lymphoid cells within the LP of the diseased periodontium have been characterized by other investigators (8–14). Some have interpreted the predominance of CD4⁺ memory T cells and B cells within the LP as evidence that the CP “lesion” is in fact a tertiary lymphoid organ (48). It is necessary to differentiate, within OLF, expression of primary immune response markers from those consistent with recall responses expected within a tertiary lymphoid organ.
FIGURE 3. The oral mucosal pathogen *P. gingivalis* or its LPS activate DCs to undergo maturation and up-regulate costimulatory molecule expression in vitro. Day 6 immature MDDCs were generated as described in Materials and Methods. **A**, Immature MDDC phenotype (CD1a−, CD83−), as confirmed by multiparameter flow cytometry analysis. CD14 expression (not shown) was typically 80–95% negative. **B**, Immature MDDCs stained by H&E. **C**, LPS-matured MDDCs stained by H&E. **D** and **E**, FACS histograms showing up-regulation (red histogram) of, from left to right, CD40, CD80, CD83, CD86, and HLA-Dr on MDDCs after coculture with *P. gingivalis* whole cells for 4 and 14 h, respectively. **F**, MDDCs cocultured with *P. gingivalis* LPS (100 ng/ml) for 14 h. **G**, MDDCs cocultured with *E. coli* LPS (100 ng/ml) for 14 h. Green histograms are DC controls and black histograms are isotype controls. The primary mAbs used are given in Table II and in Materials and Methods.
We propose that OLF are not fully developed unless and until sufficient antigen challenge is provided by specific pathogens/commensals in the subgingival flora such as *P. gingivalis* and *Fusobacterium nucleatum* (1, 2, 40). In like manner, lymphoid follicles in Peyer’s patches do not develop normally in germ-free animals, but are restored when animals are mono-infected (49, 50). Our in vitro results indicate that DCs matured by *P. gingivalis* or its LPS have been shown to stimulate human gingival epithelial cells in vitro to produce β-defensins, proinflammatory cytokines, and chemokines in vitro that are required for leukocyte recruitment (40). We further propose that although T cell expansion and IFN-γ production (Table I) occurs in OLF, the development of a protective cell-mediated response is limited by the presence of elevated IL-10 and PGE2 levels in vivo (Table III).

In our vitro results indicate that DCs matured by *P. gingivalis* are able to stimulate a very limited autologous CD4+ T cell response, as compared with DCs matured by \textit{E. coli}. Our efforts are now being directed to understanding the mechanism(s) of this disparity. One possibility being investigated in the human and murine (51) systems is that *P. gingivalis* or its LPS induce a Th2-biased response in DCs. This is supported by the data in Table III showing that DCs matured by limited by the presence of elevated IL-10 and PGE2 levels in vivo that are re-proinflammatory in animals, but are restored when animals are monoinfected (49, 50).

In conclusion, our results support the role of DCs in the pathogenesis of periodontal disease. DCs are implicated in the initial recognition of periodontopathogens and in the promotion of immune responses in the subgingival area. DCs matured by *P. gingivalis* or other periodontal pathogens stimulate a Th2-biased response in DCs, which may be counterregulatory to a protective T cell-mediated response.

**References**


**FIGURE 4.** Working model of DC/lymphoid foci and local milieu in CP.


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