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IL-23-producing CD68$^+$ macrophage-like cells predominate within an IL-17-polarized infiltrate in chronic periodontitis lesions

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Abstract

Aim: To analyse antigen-presenting cells (APCs), such as dendritic cells (DCs), macrophages (Mo) or B cells depending on the regional site of chronic periodontitis (CP), and to investigate their relation to Th17 cells.

Material and Methods: Biopsies from oral mucosa as well as the coronal and bottom regions of CP were analysed by immunhistochemistry, immunofluorescence, flow cytometry and real-time PCR.

Results: A predominance of CD68$^+$ Mo-like cells and CD20$^+$ B cells and strong Th17 infiltration was observed in the bottom region of CP lesions, while CD1a$^+$ DCs were only detected in the coronal regions, where Th17 infiltration was low. Furthermore, CD68$^+$ Mo-like cells displayed CD163 expression as a typical Mo-marker, but expressed in parallel typical DCs markers, such as CD11c or CD209 and TLR4. Interestingly, Th17-inducing cytokine IL-23p19 was produced by CD68$^+$ Mo-like cells, but not CD20$^+$ B cells. Moreover, the stimulation of in vitro generated CD68$^+$ Mo-like cells by *Porphyromonas gingivalis*-derived (*Pg*) lipopolysaccharide resulted in the upregulation of their IL-23p19 mRNA expression, which was inhibited by the blockage of TLR4.

Conclusions: In view of these data, a picture emerges that IL-17-producing cells in CP could be in part directed by CD68$^+$ Mo-like cells, which produce IL-23p19 upon TLR4 activation by *Pg*.

Key words: *Chronic periodontitis*; *IL-23, IL-17*; *Langerhans cells*; *macrophages*; *oral mucosa*

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Chronic periodontitis (CP) is a long-lasting inflammatory condition of the periodontium. CP has a high prevalence and represents the major cause for tooth loss in adults (Papapanou, 1996, Irfan et al. 2001, Pihlstrom et al. 2005). Bacterial species such as *Porphyromonas gingivalis* (*Pg*) and their products are thought to play a central role in the pathogenesis of CP (Rylev & Kilian 2008). Furthermore, several published studies linked CP to autoimmunity and it has been postulated that interleukin (IL)-17-producing T cells – so-called Th17 cells – are critically involved in mediating autoimmune reactions (Gemmell et al. 2007, Gutcher & Becher 2007). Indeed, recent publications have demonstrated an upregulation of IL-17 in CP lesions as well as in the gingival cervicular fluid (Vernal et al. 2005, Cardoso et al. 2009, Dutzan et al. 2009, Ohyama et al. 2009). In this context, it is most likely that antigen-presenting cells (APCs) orchestrate Th17 immune responses in CP, as APCs are not only capable of capturing and processing antigens but also of

Conflict of interest and source of funding statement

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inducing antigen-specific immune responses (Novak et al. 2004, Steinman, 2007). To date, several different APC populations have been described, including dendritic cells (DCs) and macrophages (Mo), which both represent heterogeneous populations. In this regard, DCs are further subdivided into plasmacytoid DCs (pDCs) and myeloid DCs (mDCs), while Mo are classified into different subtypes as well (Novak et al. 2004, Benoit et al. 2008). These APCs differ in surface marker expression and carry out distinct tasks within the innate and adaptive arm of the immune system (Novak et al. 2010, Hoebe et al. 2004). However, recent publications suggest that especially mDCs and Mo share some common features (Ferenbach & Hughes 2008, Kwan et al. 2008). With regard to Th17 cells, it has been shown that APCs are critically involved in the production of IL-23 – a heterodimeric cytokine composed of a specific p19 subunit and the p40 chain, which is also a component of IL-12 (Oppmann et al. 2000, Gutcher & Becher 2007). Until now, data from several studies suggest an involvement of mDCs and Mo in addition to B cells, which may also display APC properties in CP (Donati et al. 2009, Ford et al. 2010). Nevertheless, only little is known about the impact of APC subtypes in Th17 cell recruitment and polarization in CP. Therefore, the aim of this study was to investigate the presence of different APC subtypes, such as DCs, Mo and B cells, as well as Th17 cells in different regions of CP lesions and oral mucosal tissue from patients with CP. Furthermore, we sought to investigate the potential of *Pg*-LPS to induce IL-23p19 production in APCs.

Material and Methods

Patients and periodontitis samples

Patients ($n = 16$; age (mean ± SD) 56.5 ± 10.6 years; female: $n = 9$; age 57.1 ± 11.9 years; male: $n = 7$; age 55.7 ± 9.6 years) suffering from CP (pocket depth > 6 mm) were included in the study after informed written consent. In each patient, gingival biopsies were taken from oral mucosa sites as well as the coronal and bottom regions of CP lesions (Fig. 1). The study was conducted after approval from the local ethics committee.

Immunhistochemistry (IHC)

IHC was performed on 4 μm sections of the archival oral tissue biopsies, which had been fixed in 4% formalin fixative and embedded in paraffin. The sections were deparaffinized, hydrated and washed in phosphate-buffered saline (PBS, pH 7.2). Antigen retrieval was carried out with the Dako Target Retrieval Solution (pH 6.0, Dako, Denmark), and then the sections were covered with a 3% H_2O_2 solution to block endogenous peroxides at room temperature (RT) for 10 min. treated with 10% normal goat serum containing 5% bovine serum albumin (BSA) at RT for 30 min. (Jackson Immunoresearch, West Grove, PA, USA) and incubated with the primary antibodies at the appropriate optimal dilutions overnight at 4°C in a humidified chamber (Table 1). The Dako REAL™ Detection system (Alkaline Phosphatase/RED; Dako, Denmark, Code K5005) was used for the detection of the primary antibody binding. Chromogen (RED) was carried out with Substrate-Batch and the sections were counterstained with Mayer's haematoxylin. In addition, sections of psoriatic skin served as a positive control, while the negative controls were obtained by substitution of the primary antibodies with isotype control IgG_1 or IgG_{2a}, respectively, in the staining procedure. The immunohistochemical image data were analysed and quantified using stereological image processing software Image-Pro Plus (Version 6.0; Media Cybernetics Inc., Bethesda, MD, USA).

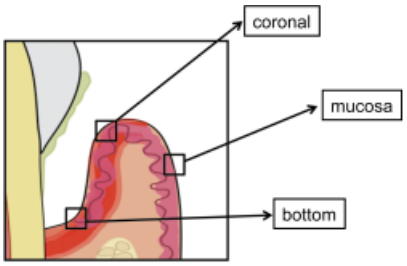

Fig. 1. Schematic drawing of a chronic periodontitis lesion, i.e. periodontal pocket, and the regions, from which biopsies were taken. Three biopsies were taken from oral mucosa as well as the coronal and the bottom regions of CP lesions from each donor.

Immunofluorescence (double staining)

Paraffin-embedded slides were deparaffinized by immersion in xylene, followed by dehydration in ethanol. Antigen retrieval was performed using Dako Target Retrieval Solution (pH 6.0, Dako, Hamburg, Germany). Following a 5-min. wash with Tris-buffered saline-Tween 20 (TBS-T), sections were incubated for 30 min. at RT with 10% normal goat serum. Tissue sections were incubated overnight at 4°C with the mixture of two primary antibodies (anti-CD68 or anti-CD20 and anti-IL-23p19). Following a 5-min. wash with TBS-T, slides were incubated for 30 min. at RT with the mixture of two secondary antibodies cyanine (Cy)2-conjugated affinipure goat anti-mouse IgG and Cy3-conjugated affinipure goat anti-rabbit IgG; Jackson Immunoresearch), and counterstaining was performed with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Merck, Darmstadt, Germany). Again, sections of psoriatic skin served as a positive control, while negative controls were obtained by substitution of the primary antibodies with mouse IgG_1 or rabbit serum in the staining procedure. All slides were mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA) to preserve fluorescence. Images were acquired using a Leica DMLB fluorescence microscope with a JVC digital device camera and collected using DISKUS software (Version 4.60, Hilgers, Germany).

Flow cytometry

Biopsies were trypsinized overnight at 4°C and further processed for a single-cell suspension as described in detail

Table 1. Antibodies used for immunohistochemistry

Antibody against	Dilution	Clone	Source
CD1a	1:200	010	Dako, mouse anti-human mAb
CD3	1:200	PS1	Novocastra, mouse anti-human mAb
CD20	1:100	L26	Dako, mouse anti-human mAb
CD68	1:200	KP1	Dako, mouse anti-human mAb
IL-17	1:100	Polyclonal	Santa Cruz, rabbit anti-human pAb
IL-23	1:1000	Polyclonal	Lifespan, rabbit anti-human pAb
IFN-γ	1:1000	Polyclonal	Abcam, rabbit anti-human pAb
IL-4	1:100	NYPhIL4	Santa Cruz, mouse anti-human mAb

elsewhere (Allam et al. 2011). Cells were then processed for extracellular and intracellular staining as described before (Kraft et al. 1998). Mouse monoclonal antibodies labelled to either peridinin chlorophyll protein (PerCP), phycoerythrin (PE), allophycocyanin (APC), APC-Cy7 or fluoresceinisothiocyanat (FITC) were used following the manufacturer's manual as depicted in Table 2. Respective isotype control FITC-, PE- and APC-labelled 27-35 (IgG2b), PE-Cy7-labelled MOPC-21 (IgG1), PE-labelled MOPC-21 (IgG1) and PerCP-labelled X39 (IgG2a) were from BD Biosciences (Heidelberg, Germany) and PE-labelled IgG2a was from eBiosciences (San Diego, CA, USA).

In vitro generation of Mo-like cells

$CD68^+$ Mo-like cells were generated in vitro from peripheral blood monocytes, which were isolated from healthy volunteers after informed written consent ($n = 6$) as described in detail elsewhere (Jockers & Novak 2006). Isolated monocytes were cultured in the presence of 500 U/ml granulocyte-Mo-colony stimulating factor (GM-CSF) in RPMI medium supplemented with FCS, antibiotics and antimycotics. After 3 days, cells were harvested and analysed for CD68, CD163 and TLR4 expression.

Stimulation of Mo-like cells and ELISA

10^6 in vitro generated Mo-like cells were placed in 1 ml RPMI culture medium. Cells were left untreated or stimulated with 1 μg/ml *Pg*-LPS, which was purified from Pg as described in detail elsewhere (Cryz et al. 1984). Moreover, cells were incubated with blocking anti-TLR4 (clone HTA125, mIgG2a, eBioscience) as well as the IgG2a isotype control (eBioscience) before stimulation with *Pg*-LPS. After 16 h of culture, cells were harvested and further processed for real-time PCR analysis. In a further set of experiments, Mo-like cells were left untreated or stimulated with 1 μg/ml *Pg*-LPS for 18 h. Then supernatants were collected and processed for IL-23p19 ELISA (eBioscience) following the manufacturer's manual.

RNA isolation and quantitative real-time PCR analysis

For mRNA analysis, lysis of in vitro generated Mo-like cells and RNA clean-up was performed using the Nucleospin RNA-II Kit (Macherey-Nagel, Dueren, Germany). Following phase separation by the addition of chloroform and centrifugation, the aqueous phase containing RNA was selected and washed in 70% ethanol. Then mRNA was subjected to cDNA synthesis with TaqMan reverse transcription reagents with random hexamers according to the manufacturer's instructions (Applied Biosystems, Darmstadt, Germany). The prepared cDNA was amplified using the TaqMan assay Master Mix (Applied Biosystems, Foster City, CA, USA) according to the recommendations of the manufacturer in an ABI Prism 7300 Sequence Detection System (Applied Biosystems). Primers for IL23p19 (Hs00372324_m1) as well as endogenous controls (18s rRNA) were obtained from Applied Biosystems. All assays were performed according to the manufacturer's instructions. Relative quantification and calculation of the range of confidence was performed using the comparative $\Delta\Delta C_T$ methods. All analyses were conducted in duplicate.

Cell analysis and statistics

The immunohistochemical image data were analysed and quantified using stereological image processing software Image-Pro Plus (Version 6.0; Media

Table 2. Antibodies used for flow cytometry

Antibody against	Conjugation	Clone	Source
CD1a	PE	HI149	BD Biosciences
HLA-DR	PerCP	L243	BD Biosciences
CD83	APC	HB15e	BD Biosciences
CD3	APC-Cy7	SK7	BD Biosciences
CD68	FITC	Y1/82A	eBiosciences
CD11c	PE-Cy7	B-Ly6	BD Biosciences
CD209	PE	DCN46	BD Biosciences
CD163	PE	eBioGHI/61	eBiosciences
TLR2	PE	TL2.1	eBiosciences
TLR4	PE	76B357.1	Imgenex
IL-17	PE	SCPL1362	BD Biosciences

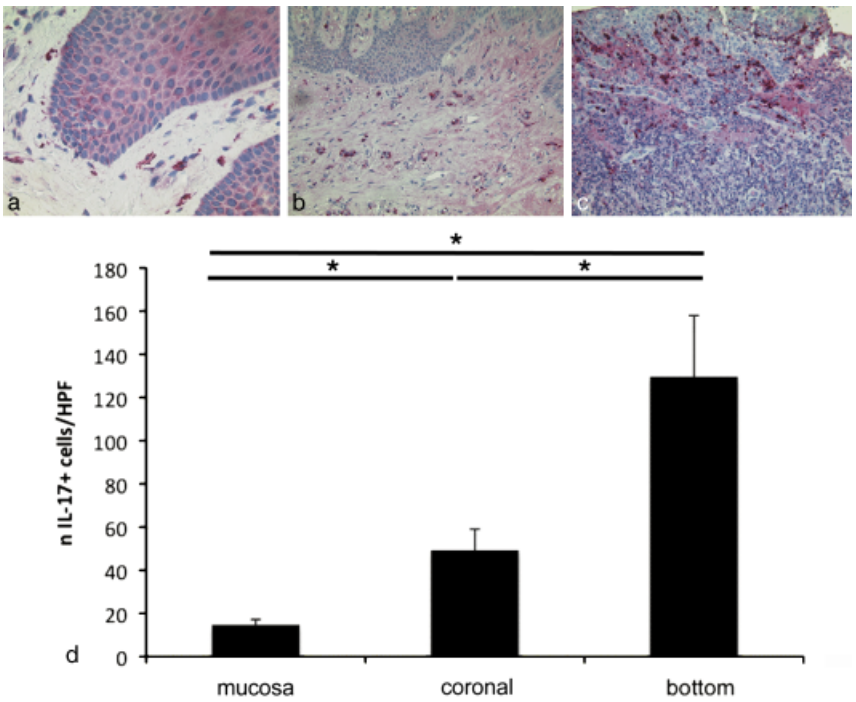

Fig. 2 Distribution of $IL\text{-}17^+$ cells in different regions of chronic periodontitis (CP) and oral mucosa. $IL\text{-}17^+$ cells were observed in (a) oral mucosa as well as in (b) the coronal and (c) the bottom regions of CP (representative immunohistochemical staining of $n = 5$). (d) Compared with the oral mucosa, the number of $IL\text{-}17^+$ cells was significantly higher in all regions of CP, with the highest number in the bottom region ($n = 5$).

Cybernetics Inc.) following the instruction manual. Briefly, five random microscope fields were selected from each slide (× 200). The number of immunopositive cell/field was counted by means of the five fields. Data were expressed as mean ± SEM. For the statistical evaluation of significances, the Wilcoxon (signed-rank) test or Pearson test was performed using SPSS 17.0 software (SPSS GmbH Software, an IBM Company, Munich, Germany), $^{**}p<0.01$; $^{*}p<0.05$; no indication = not significant unless otherwise indicated.

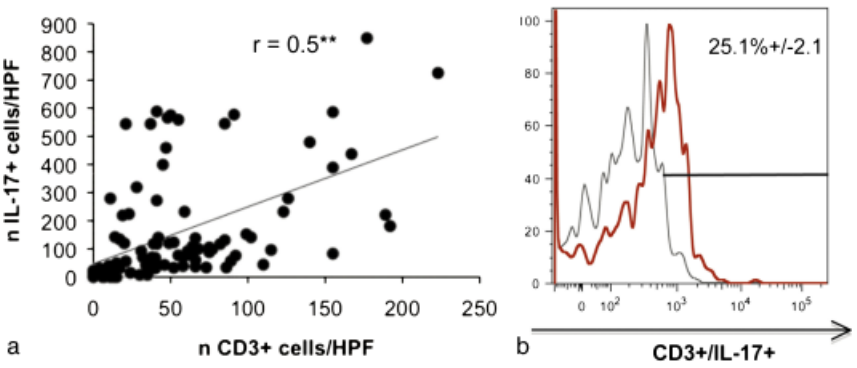

Fig. 3. $CD3^+$ T cells in chronic periodontitis (CP) produce IL-17. (a) A significant correlation could be detected between $CD3^+$ T cells and IL-17 ($n = 100$). (b) Moreover, IL-17 could be detected in $CD3^+$ T cells isolated from CP tissue ($n = 3$).

Results

Number of IL-17^+ cells correlates with the severity of inflammation

To further characterize the presence of IL-17^+ cells in CP, biopsies were taken from the oral mucosa as control tissue and different regions of CP lesions, i.e. periodontal pockets, to gain a picture of the inflammatory status within different sites. Thus, biopsies from the oral mucosa, coronal and bottom regions of CP lesions were taken (Fig. 1). The highest number of IL-17^+ cells was detected within the bottom region, followed by the coronal region, and the lowest number within the oral mucosa (Fig. 2). Moreover, the number of IL-17^+ cells significantly correlated with the number of $CD3^+$ T cells in the infiltrate and IL-17 production could be demonstrated in $CD3^+$ cells by flow cytometry (Fig. 3). On the other hand, the Th1 cytokine interferon (IFN)-γ was only detected in a few cells, while expression of the Th2

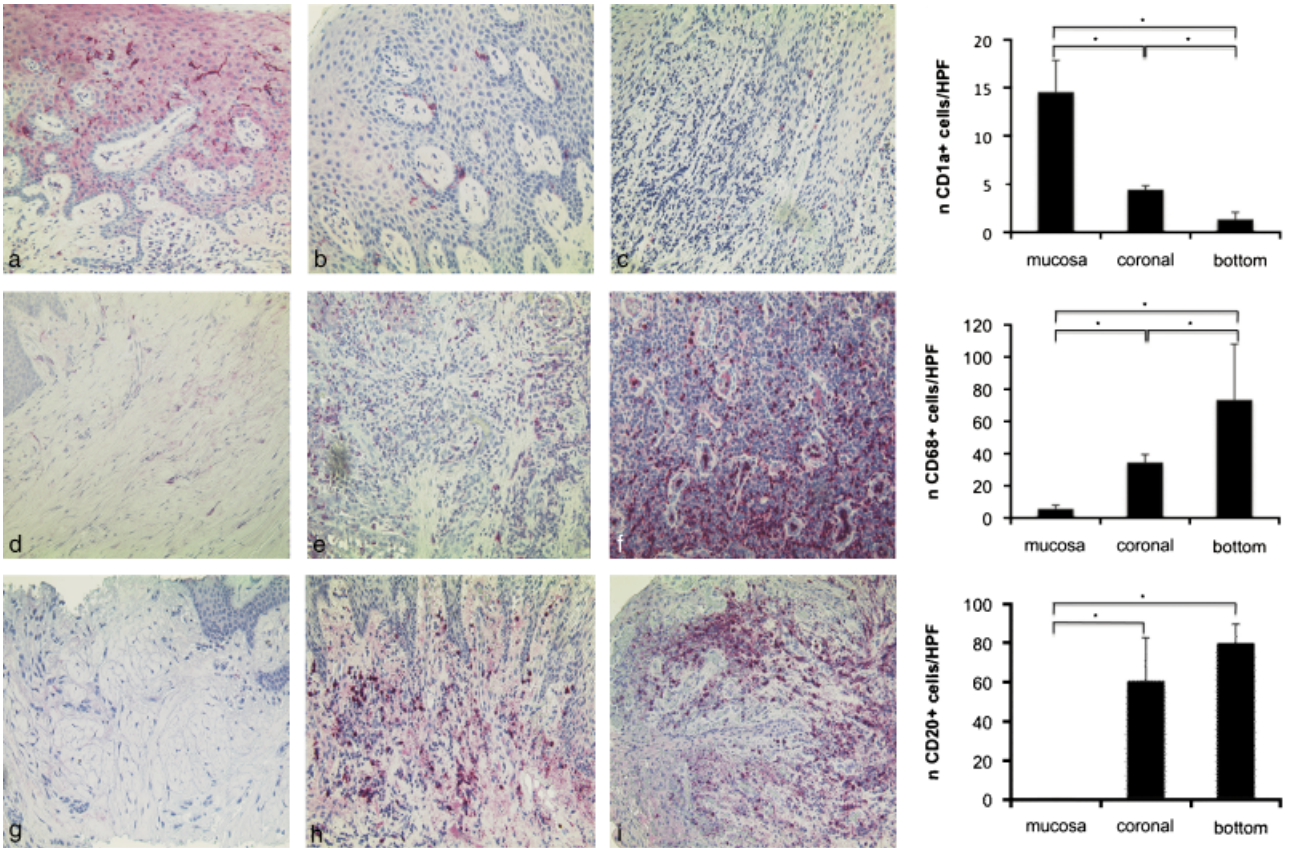

Fig. 4. Distribution of $CD1a^+$ dendritic cells (DCs), $CD68^+$ macrophage-like cells and $CD20^+$ B cells in chronic periodontitis (CP) and oral mucosa. Detection of (a–c) $CD1a^+$ DCs, (d–f) $CD68^+$ macrophage-like cells and (h–j) $CD20^+$ B cells in (a, d, h) oral mucosa ($n = 5$), (b, e, i) coronal ($n = 5$) as well as (c, f, j) bottom CP region ($n = 5$). Upper row, compared with (a) the oral mucosa, the number of $CD1a^+$ DCs was reduced in the (b) coronal region of CP, while in (c) the bottom region of CP, only a few $CD1a^+$ DCs could be detected ($n = 5$); middle row, in contrast, a significant increase of $CD68^+$ macrophages (Mo)-like cells was observed in CP tissue, with (f) the highest numbers in the bottom region of CP ($n = 5$); lower row, the same distribution pattern could be detected in view of $CD20^+$ B cells, with increased numbers in (i) the coronal and (j) bottom region of CP, while (h) only a few $CD20^+$ B cells were detected in the oral mucosa.

cytokine IL-4 was not observed at all (data not shown).

From this set of experiments, we conclude that severity of inflammation is the strongest in the bottom region of CP lesions, where infiltration of Th17 cells predominates.

Mo-like cells and B cells are the main APCs in CP lesions, while DCs are virtually absent

It is well known that T cell responses are induced and mediated by APCs (Banchereau & Steinman 1998). Moreover, $CD1a^+$ DCs have been suspected to play a central role in controlling CP (Cutler et al. 2001, Jotwani et al. 2001). For this reason, the presence of $CD1a^+$ DCs was investigated in different regions of CP lesions and compared with oral mucosa. Thereby, a decreased number of $CD1a^+$ DCs was detected in the coronal region, while in the bottom region of CP lesions, $CD1a^+$ DCs were virtually absent (Fig. 4a–c). Therefore, the presence of other APCs, such as $CD68^+$ Mo-like cells (Fig. 4d–f) and $CD20^+$ B cells (Fig. 4h–j), was investigated. A significant increase of $CD68^+$ Mo-like cells and $CD20^+$ B cells could be demonstrated in the coronal as well as in the bottom region of CP lesions (Fig. 4d–j).

In view of these data, we conclude that $CD68^+$ Mo-like cells and $CD20^+$ B cells, but not $CD1a^+$ DCs, predominate CP lesions.

The number of $CD68^+$ Mo-like cells and $CD20^+$ B cells correlates with IL-23 and IL-17 expression in the tissue

Furthermore, a significant correlation could be demonstrated between the presence of $CD68^+$ Mo-like cells as well as $CD20^+$ B cells and IL-17^+ cells, while a reciprocal correlation was detected between the number of IL-17^+ cells and $CD1a^+$ DCs (Fig. 5). It has been shown that IL-23 production by APCs might skew naïve as well as memory T cells towards Th17 (Gutcher & Becher 2007). Thus, correlation analysis of IL-23p19 expression with the number of $CD68^+$ Mo-like cells and $CD20^+$ B cells in different regions of CP lesions was performed. In this regard, a significant correlation between the number of $CD68^+$ Mo-like cells and $CD20^+$ B cells to IL-23p19 expression and a reciprocal correlation between the numbers of $CD1a^+$ DC to IL-23p19 expression could be detected (Fig. 5). In the next set of experiments, the production of IL-23p19 by $CD68^+$ Mo-like cells and $CD20^+$ B cells was investigated. In this regard, immunofluorescence staining could demonstrate that $CD68^+$ Mo-like cells but not $CD20^+$ cells

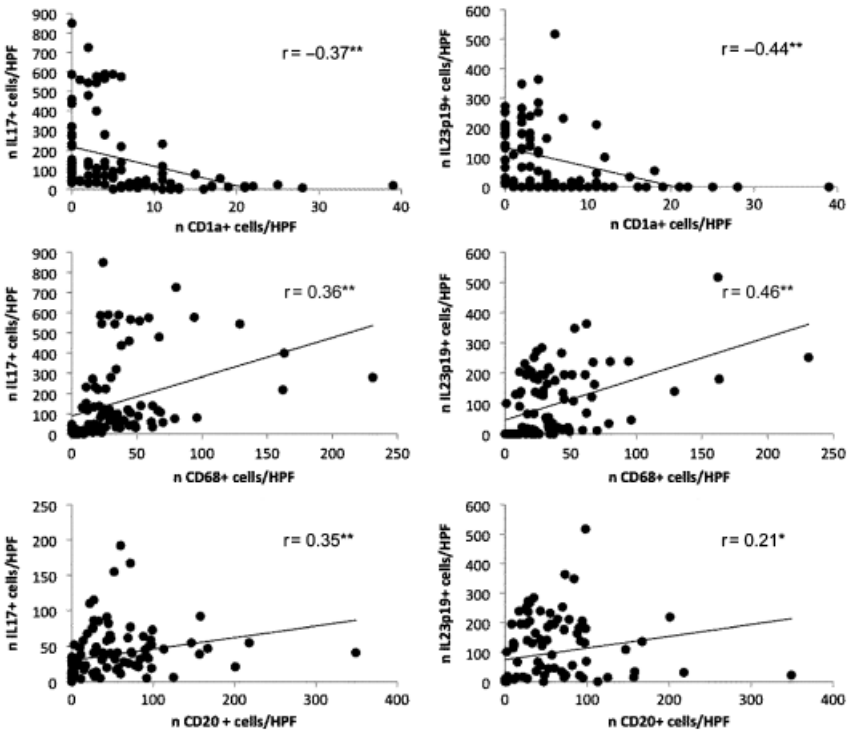

Fig. 5. Correlation between the number and type for antigen-presenting cells (APCs) and IL-17 as well as IL-23 expression in the tissue. Upper row, reciprocal correlation was detected between $CD1a^+$ dendritic cells (DCs) and IL-17 (first column) and IL-23p19 expression (second column) ($n = 100$). Middle row, in contrast, a significant correlation between $CD68^+$ macrophages (Mo)-like cells and IL-17 (first column) and IL-23p19 expression (second column) could be demonstrated in chronic periodontitis (CP) tissue ($n = 100$); lower row, the same correlation could be detected in view of $CD20^+$ B cells and IL-17 (first column) and IL-23p19 expression (second column) ($n = 100$).

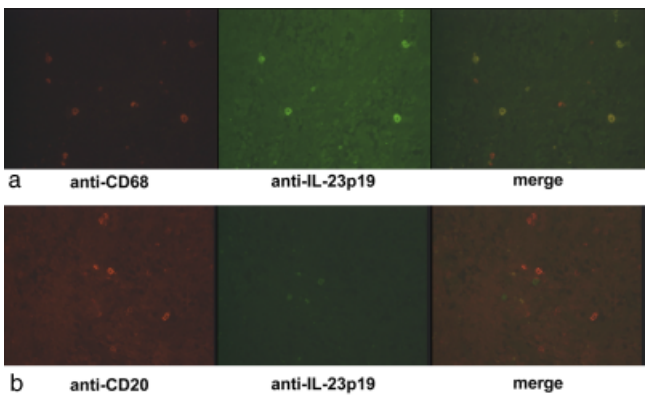

Fig. 6. Colocalization of different antigen-presenting cells (APCs) with IL-17 as well as IL-23 expressing $CD68^+$ macrophage (Mo)-like cells and $CD20^+$ B cells were stained with Cy2-labelled antibody (first column), while IL-23p19 was detected by Cy3-labelled antibody (second column). Pictures were merged in the third column. (a) $CD68^+$ expression of Mo-like cells colocalizes with IL-23p19-expressing cells (representative pictures from $n = 5$). (b) $CD20^+$ expression of B cells did not colocalize with IL-23p19-expressing cells (representative pictures from $n = 5$).

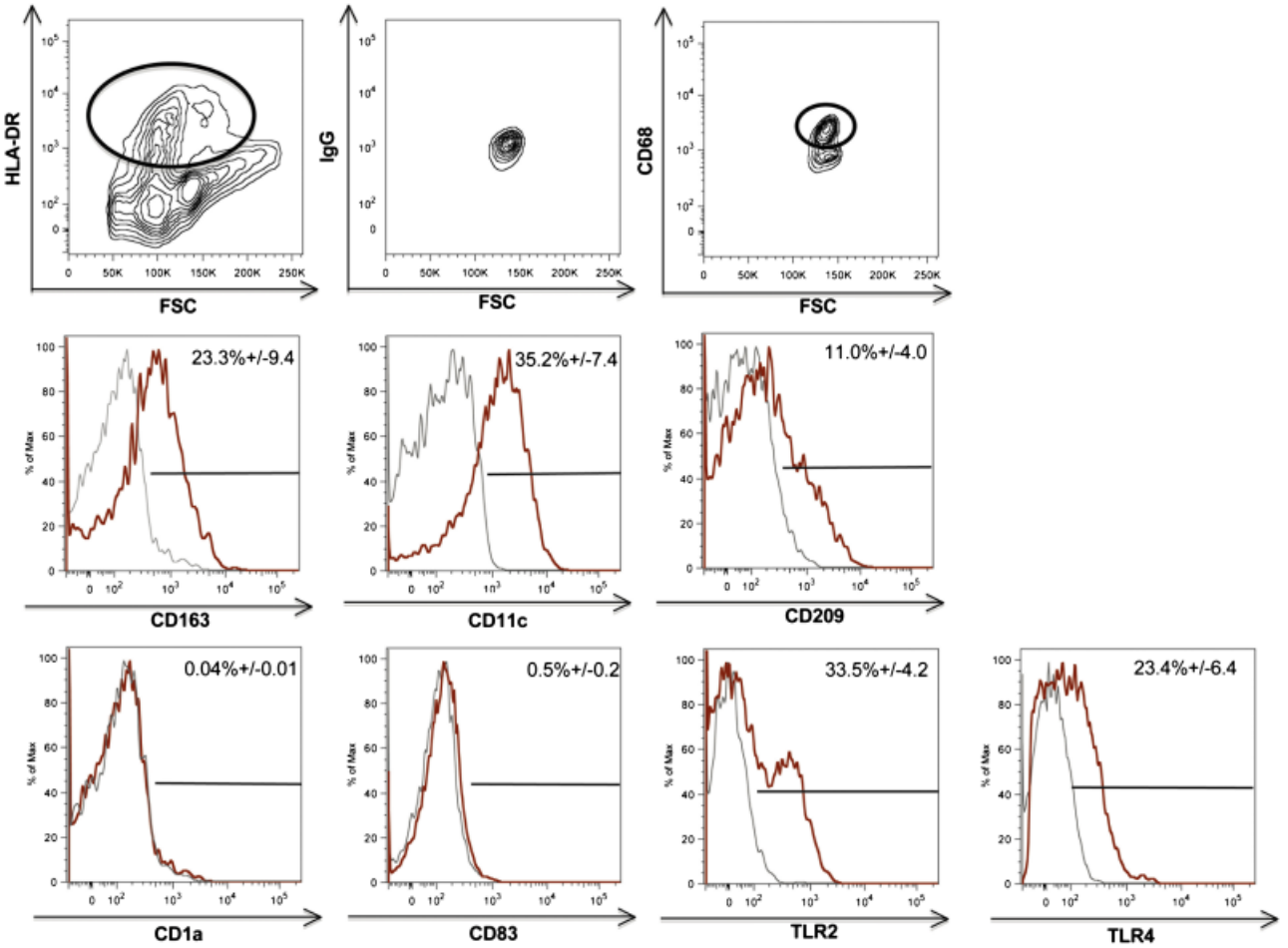

Fig. 7. CD68$^+$ cells express macrophage (Mo)- and dendritic cell (DC)-marker. CD68$^+$ Mo-like cells were gated by HLA-DR and CD68 expression (dot plots; upper row) and further investigated for Mo- and DC-markers (representative histograms; percentage of positive gated cells ± standard SEM depicted in the upper corner). CD68$^+$ Mo-like cells expressed the Mo-marker CD163, but also the DC-marker CD11c and CD209, while CD1a and CD83 could not be detected. Further on CD68$^+$ Mo-like cells expressed TLR2 and TLR4 (grey lines = isotype control; red lines = investigated marker; $n = 5$).

expressed IL-23p19, as demonstrated by immunofluorescence staining (Fig. 6a and b).

From these data, a picture emerged that CD68$^+$ Mo-like cells may orchestrate the Th17 infiltrate in CP.

CD68$^+$ cells display typical Mo- but also DC-markers

It has been suggested that CD68 expression is not only restricted to Mo but is also expressed in some DC subsets (Nakamura et al. 1998, Strobl et al. 1998). Thus, ex vivo isolated CD68$^+$ cells from the bottom region of CP lesions were investigated for Mo-markers such, as CD163, as well as DC markers, namely CD1a, CD83, CD11c and CD209. In this context, a significant expression of the Mo-marker CD163 and a concomitant expression of CD11c and CD209 was detected on CD68$^+$ cells. However, CD68$^+$ cells did not express CD1a, the typical mDC marker, and CD83, the maturation marker of DCs, while these cells expressed TLR2 and TLR4 (Fig. 7). CD123$^+$ pDCs were not detected at any of the investigated sites (data not shown).

From this set of experiments, we conclude that CD68$^+$ cells represent a heterogeneous population of Mo-like DCs.

Pg-LPS induces IL-23 production in in vitro generated Mo-like cells

Pg has been considered as a major pathogen involved in CP (Ford et al. 2010). In this regard, it has been shown that *Pg*-LPS is able to signal through TLR2 and TLR4, which is also expressed by CD68$^+$ cells in CP (Darveau et al. 2004, Ogawa et al. 2007). Thus, we used an in vitro model to generate CD68$^+$ cells (95.57% ± SEM 0.73; $n = 3$) coexpressing CD163 (54.33% ± SEM 3.58; $n = 3$), which expressed TLR2 (64.00% ± SEM 4.53; $n = 3$) and TLR4 (42.50% ± SEM 8.35; $n = 3$). Stimulation of in vitro generated Mo-like cells with *Pg*-LPS resulted in a significant upregulation of IL-23p19 mRNA transcripts, compared with unstimulated mock-control cells. Blocking of TLR2, TLR4 or TLR2 and TLR4 abrogated *Pg*-LPS induced upregulation of IL-23p19 in Mo-like cells (Fig. 8a). Moreover, Pg-LPS stimulation of Mo-like cells also upregulated IL-23 production on the protein level compared with unstimulated control cells as detected by ELISA (Fig. 8b).

In conclusion, *Pg*-LPS is able to induce IL-23p19 production in Mo-like cells in response to TLR2 and TLR4 activation and might thereby propagate the Th17-predominated infiltrate in CP.

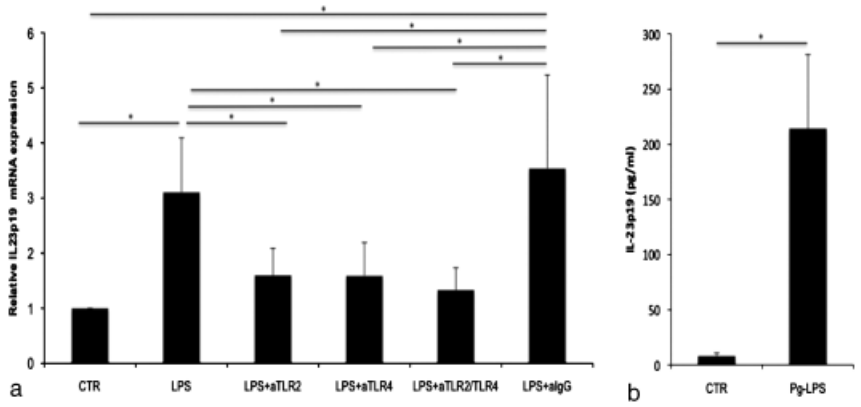

Fig. 8. CD68$^+$ macrophages (Mo)-like cells produce IL-23p19 upon TLR2 and TLR4 activation by Pg-LPS. CD68$^+$ Mo-like cells were blocked with ant-TLR2 (aTLR2), anti-TLR4 (aTLR4), IgG2a as well as anti-TLR2 and anti-TLR4 (aTLR2/aTLR4) before Pg-LPS stimulation or were left unstimulated. (A) Pg-LPS simulation upregulated IL-23p19 mRNA expression in CD68$^+$ Mo-like cells. Blocking of TLR2 or TLR4 as well as TLR2 and TLR4 in combination inhibited IL-23p19 mRNA expression of Mo-like cells, while stimulation with the isotype control antibody did not influence their IL-23p19 mRNA expression ($n = 5$). (b) CD68$^+$ Mo-like cells significantly upregulated IL-23p19 protein expression upon stimulation by Pg-LPS ($n = 5$).

Discussion

CP represents a long-lasting inflammatory condition of periodontal tissue, in which microbes, such as *Pg*, are considered a major aetiological factor for periodontitis (Ford et al. 2010). Next to mechanisms of the innate arm of the immune system, it is most likely that the adaptive immune responses critically drive the course of CP. In this context, several studies have investigated the role of APCs, such as DCs, Mo and B cells, as well as T cell subsets including Th1, Th2 and Th17 in CP (Ford et al. 2010). However, data on the role of adaptive immune responses in CP and in particular on the role of DCs are conflicting so far. This study shows for the first time that (I) Th17 cells represent the predominant T cell subset in severe inflammatory CP lesions and their number correlates with the state of inflammation, (II) the number of TLR2- and TLR4-expressing CD68$^+$ Mo-like cells and CD20$^+$ B cells correlates with the number of IL-17$^+$ cells, while classical CD1a$^+$ DCs are virtually absent, (III) Mo-like cells, but not B cells produce Th17 propagating IL-23 in vivo and (IV) in vitro generated Mo-like cells produce IL-23 in response to TLR2 and TLR4 activation by *Pg*-LPS.

Previous studies have demonstrated the upregulation of IL-17 mRNA expression and proteins in gingival crevicular fluid (GCF), periapical lesions and active CP lesions (Vernal et al. 2005, Xiong et al. 2009, Cardoso et al. 2009, Ford et al. 2010). Nevertheless, in view of the data presented here, it appears that IL-17 plays a major role especially at severe inflammatory sites of CP, such as the bottom region of the lesions, while other sites of the same CP tissue, such as the coronal region, are characterized by both less Th17 infiltration and less inflammation. Thus, it is tempting to speculate that IL-17 plays mainly a role in the destruction of periodontal tissue, which characterizes severe CP. This hypothesis is further supported by the detection of IL-23p19-producing Mo-like cells within sites of severe inflammation only, which might amplify Th17 immune response in CP, and by the absence of classical CD1a$^+$/CD207$^+$ DCs, so-called Langerhans cells (LCs) from these sites. The absence of oral LCs (oLCs) is of critical immunological importance, as it has been shown that LCs by default rather act as pro-tolerogenic than pro-inflammatory cells (Novak et al. 2008). Moreover, it is believed that oLCs are capable of maintaining to some degree the immunohomeostasis by actively counteracting inflammatory immune mechanisms (Romani et al. 2010). In the context of oLCs, a recent publication has demonstrated that the constitutive pro-tolerogenic properties of oLCs are further emphasized by TLR4 stimulation. TLR4-stimulated oLCs produce high amounts of pro-tolerogenic IL-10 and transforming-growth factor-β1 and are capable of inducing regulatory T cells in addition to Th1 cells (Allam et al. 2008). In this regard, it is tempting to speculate that the specific pattern of cells infiltrating CP lesions as well as the nature of the microenvironement of CP together with the absence of oLCs and TLR4-mediated anti-inflammatory mechanisms might promote excessive inflammation in CP. However, recent publications have also demonstrated the presence of oLCs as well as CD83$^+$ DCs in CP lesions (Jotwani et al. 2001, Cutler & Jotwani 2006). As we observed in the present study that the distribution of oLCs and other APC subsets is critically dependent on both the lesional site investigated as well as the state of inflammation, heterogenous results might be based most likely on different lesional CP sites analysed in the studies. Mo-like cells as well as B cells predominate at severe inflammatory lesions at the bottom of the CP lesions. In contrast to the pro-tolerogenic immune responses induced by TLR4 activation on oLCs isolated from oral mucosal tissue, we demonstrated that the stimulation of TLR4 on oral mucosal Mo-like cells by *Pg*-LPS resulted in the upregulation of their Th17 propagating IL-23p19 production. In view of CP, the presence of IL-23p19-producing Mo-like cells upon *Pg*-LPS stimulation implies that these APCs might enhance pro-inflammatory cytokine production of epithelial cells by increasing the number of IL-17-producing T cells in the gingival tissue. The absence of regulatory APCs subtypes such as oLCs might further promote the manifestation of severe chronic inflammatory reactions in CP. Furthermore, it has been postulated that Th1 cells producing IFN-γ play a major role during the initial steps of CP, while Th2 cells expressing IL-4 are considered as critical cells in the progressive course of CP (Ford et al. 2010). Nevertheless, in our study, we could only detect a few IFN-γ producing cells. This in line with a previous study that could reveal similar amounts of IFN-γ in CP lesions and control tissues (Ohyama et al. 2009).

Taken together, the data presented in this study suggest that IL-17-producing T cells predominate at severe inflammatory sites of CP and that the amount of Th17 cells in the CP tissue is directly related to the number of IL-23p19-producing Mo-like cells. Further studies are needed to focus on the potential clinical

benefit of IL-23p19 and IL-17 blockage as a potential novel therapeutic approach to counteract pro-inflammatory mechanisms in CP.

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Clinical Relevance

Scientific rationale for the study: As stated at the recent EuropeanWorkshop (2010), there is a lack of data from human studies on the role of cytokines in immune regulation and response to microbial challenge in periodontal pathogenesis.

Principal findings: IL-17-producing T cells predominated at severe inflammatory sites of CP. The amount of Th17 cells in the CP lesion was directly related to the number of IL-23p19-producing Mo-like cells. *Pg*-LPS was able to induce IL-23p19 production in Mo-like cells in response to TLR4 activation and might thereby propagate the Th17-predominated infiltrate in CP.

Practical implications: A potential benefit of IL-23p19 and IL-17 blockage as a novel therapeutic approach to counteract pro-inflammatory mechanisms in CP warrants further study.