Immature, but not Mature, Dendritic Cells are More Often Present in Aggressive Than Chronic Periodontitis: An Immunohistochemical Study

Raphael J. G. da Motta†‡, Camila Tirapelli*‡, Roberto Juns da Silva†‡, Kelly R. V. Villafuerte†§, Luciana Y. Almeida*||||, Alfredo Ribeiro-Silva*¶, Jorge E. León*#

*PhD.

†PhD Students.

‡Integrated Dental Clinic, Department of Dental Materials and Prosthodontics, University of São Paulo, School of Dentistry of Ribeirão Preto (FORP/USP), Ribeirão Preto, Brazil.

§Department of Oral &Maxillofacial Surgery and Periodontology, University of São Paulo, School of Dentistry of Ribeirão Preto (FORP/USP), Ribeirão Preto, Brazil.

||||Oral Medicine, Department of Diagnosis and Surgery, Araraquara Dental School, Universidade Estadual Paulista (UNESP), Araraquara, Sao Paulo, Brazil.

¶Department of Pathology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto – Brazil.

#Oral Pathology, Department of Stomatology, Public Oral Health and Forensic Dentistry, University of São Paulo, School of Dentistry of Ribeirão Preto (FORP/USP), Ribeirão Preto, Brazil.

Background: Dendritic cells (DCs) form a key link between innate and adaptive immune responses. The aim of this study was to analyze the presence and distribution of immature (imDCs) and mature DCs (mDCs) in gingival tissue samples obtained from patients diagnosed with aggressive periodontitis (AgP), chronic periodontitis (ChP), and clinically healthy periodontium (Control group).

Methods: Gingival tissue samples obtained from patients with AgP (aged <35), ChP (aged >35) and Control group (aged >18) (n=10 per group) were collected. We used Two-way analyses of variance and posterior Fisher´s LSD Test to observe differences between the means of cells positively marked for imDC (S100, CD1a and CD207) and mDC (CD208) immunomarkers.

Results: imDCs were more numerous in AgP than ChP and Control groups, being statistically significant only the S100+ cells. Conversely, mDCs were visualized in higher number in ChP than AgP and Control groups (both p<0.05). Considering the frequency of immunostained cells, the number of S100+ cells was greater than CD207+ and CD1a+ cells, followed by a lesser number of CD208+ cells, in all groups.

Conclusions: Considering that the ability of DCs to regulate immunity is dependent on DC maturation, our results suggest that predominance of imDCs appear to be involved in AgP pathogenesis, probably due to lack of ability to induce immune cell activation. Further studies are necessary to elucidate the role of DC maturation in regulating immune responses in periodontal disease.

KEY WORDS:

Aggressive periodontitis, Immunology, Oral Pathology.

The two principal clinical phenotypes of periodontal disease are chronic periodontitis (ChP) and aggressive periodontitis (AgP), which present distinctive clinicopathological features, such as: i) age of onset, ii) progression rate, iii) destruction pattern, iv) clinical

signs of inflammation, and v) presence and formation of dental biofilm and calculus.[1, 2] Different from ChP, early onset and rapid course of periodontal tissue destruction, usually attributed to an altered immune response against periodontal pathogens,[3] are characteristics of AgP.[1, 3-9] In this context, the host immune response has a central role in the pathogenesis of periodontitis.[6, 7, 10-12]

The innate immune system includes neutrophils, macrophages and dendritic cells (DCs), while the adaptive immune system is represented by lymphocyte subsets, which infiltrate the infected periodontal tissue and efficiently mediate pathogen clearance, promoting immunological memory.[13,14] It is known that DCs are the most potent antigen-presenting cells (APCs), activating naive T-cells, thus showing their critical function in induction of adaptive immunity.[3] The periodontal tissue contains three types of immature DCs (imDCs): Langerhans cells (LCs), submucosal DCs (subDCs) and plasmacytoid DCs (pDCs).[3, 13] LCs constitute a distinct population, which following antigen capture, differentiate into mature DCs (mDCs).[14, 15] Depending on the activation and maturation state, DCs play an important role in the initiation and amplification of adaptive immune responses, modulating the activation and differentiation of both B-cells and T-cells, as well as regulating critical interactions with macrophage populations.[3, 4] Therefore, the knowledge of the activation and maturation states of immune cells present in periodontal disease is fundamental in order to better understand of their immunological mechanisms.

By considering periodontal disease, several studies have assessed the infiltration of lymphocyte subsets,[7, 8, 16-21] as well as macrophages[8, 10, 19-22] in ChP and/or AgP; whereas there are no studies observing imDC and mDC subsets in AgP. In fact, in ChP tissues, DCs were analyzed through S100,[10, 12] CD1a,[10, 16, 22-31] CD83[19, 23-25, 27, 31] and CD208[21, 25] immunomarkers. In AgP tissues, DCs were evaluated only through CD1a[9] and CD83[19] immunomarkers. Remarkably, to date, no study has comparatively evaluated maturation and/or activation states of DC subsets between ChP and AgP cases.

Thus, regarding the central role of immune system in the pathogenesis of periodontitis, it remains to be determined the balance between imDC and mDC subsets in ChP and AgP. In the current study, we have comparatively analyzed the presence and distribution of imDCs (S100+/CD1a+/CD207+) and mDCs (CD208+) in gingival tissue samples obtained from patients diagnosed with AgP, ChP, and clinically healthy periodontium. To the best of our knowledge, immunoexpression pattern of CD207 in ChP and both CD207 and CD208 in AgP, is unknown to date.

MATERIALS AND METHODS

Participant's Selection and Samples Collection

This clinical study received the approval of the Ethics Committee of the School of Dentistry of Ribeirão Preto (CAAE: 12945713.1.0000.5419) on March 28th, 2013; and it was conducted in accordance with the Declaration of Helsinki. All participants signed a consent statement prior the beginning of the study.

We selected 30 patients (n=10 per group): Control (clinically health gingiva), AgP and ChP groups, in accordance with Armitage.[1] The inclusion criteria were:

   - AgP group: 1) healthy patients with bleeding on probing (BOP), 2) bone loss, 3) probing depth (PD) >5.0mm, 4) clinical attachment loss (CAL) >5.0mm, 5) age under 35, 6) microbial deposits inconsistent with the severity of periodontal breakdown, 7)

familial aggregation (that is, at least one other family member presenting or with a history of periodontitis).[1] Generalized AgP (GAgP) was defined as generalized interproximal attachment loss affecting at least three permanent teeth other than the first molars and incisors, whereas localized AgP (LAgP) as interproximal attachment loss affecting no more than two teeth other than the first molars and incisors.[1]

- ChP group: 1) healthy patients with BOP, 2) bone loss, 3) PD >5.0mm, 4) CAL >5.0mm, 5) age over 35, 6) presence of dental biofilm, supragingival and subgingival calculus consistent with the severity of periodontal breakdown.[1] Generalized (GChP) and localized ChP (LChP) were classified depending on whether >30% or <30% of sites, respectively, are involved.[1]

- Control group: healthy patients without bone loss visible on radiographs, with PD ≤3.0mm, CAL <1.0mm, <10% of sites exhibiting BOP, no extensive caries or restorations and at least 28 permanent teeth.

- Additionally, all patients showed absence of any local or systemic oral pathology.

The exclusion criteria were:

Patients with less than 24 teeth, orthodontic or prosthetic appliances, pregnancy, chronic diseases (such as *autoimmune diseases*, diabetes and other endocrine disorders), (former) smokers, drug-induced gingival hyperplasia and use of antibiotics, corticosteroids or non-steroidal anti-inflammatory drugs in the preceding 6-month.

Clinical parameters were recorded at baseline by one trained periodontist (KRVV). Plaque index (PI) was used to assess the oral hygiene status of the patients, determined by the presence or absence of plaque at the gingival margin of the four faces of the tooth and it was expressed as a percentage of biofilm on the total of tooth surfaces. BOP was recorded based on the presence or absence of bleeding up to 30 seconds after probing on four sides of each tooth, and it was expressed as a percentage of bleeding sides on the total of sides examined. PD was measured from the free gingival margin to the bottom of the periodontal pocket and CAL was measured from the cementum-enamel junction (CEJ) to the bottom of periodontal pocket. PD and CAL were measured at six sites per tooth (mesio-buccal, buccal, disto-buccal, disto-lingual, lingual, and mesio-lingual). Bone loss was assessed radiographically.

All probing measurements were performed using a manual probe[#].

We collected gingival tissue samples (epithelium and connective tissue) from 30 patients (n=10 per group). Gingival biopsies were obtained around the teeth from either mesial or distal aspect of the periodontal lesion (AgP and ChP) including the lateral wall of the periodontal pockets (mucoperiosteal flaps were raised in order to obtain gingival tissue). All these procedures were performed under local anaesthesia; therefore, the patient does not feel any discomfort related with pain. Samples were collected with adequate depth including epithelium and connective tissue, being fundamental the understanding of the anatomy of the blood supply of the gingival tissue.

In the Control group, the gingival tissue samples were obtained during surgical procedures involving exodontias due to orthodontic reasons or esthetic gingivectomy. In the ChP and AgP groups, gingival specimens (one biopsy specimen per patient) were obtained from sites with deep periodontal pocket >5.0mm. The gingival specimens were collected in the first consultation after the diagnosis and prior to the beginning of the periodontal treatment plan proposed (periodontal therapy). No patient had indication to

receive medication (antibiotics and/or anti-inflammatory drugs), prior to the procedures involved in the gingival tissue sample collection.

The degree of inflammation in the gingival tissue was defined according Rivera et al.[32] study: no inflammation (score 0), mild inflammation (score 1), 2-4 inflammatory cells/x400 microscopic field (high-power field, HPF); moderate inflammation (score 2), 5-10 inflammatory cells/HPF); severe inflammation (score 3), >10 inflammatory cells/HPF. A final score in each case was obtained after analysis of whole-specimen.

Immunohistochemistry

All tissue specimens were fixed in 10% neutral-buffered formalin for 24 h at room temperature, embedded in paraffin at 55ºC, and cut into parallel consecutive 3-μm thick sections. For the immunohistochemical (IHC) reactions, sections were attached over organosilane revested slides[**]. The slides were hydrated and treated with hydrogen peroxide (3%). For the antigen retrieval of the S100, CD1a, and CD208 immunomarkers, the tissue sections received a pretreatment on pressure cooker containing 10mM of sodium citrate buffer (pH 6.0), and for CD207 immunomarker, sections were pretreated on eletric pressure cooker containing buffer solution of 10mM Tris/1mM EDTA (pH 9.0).

Sections were successively incubated with the primary antibodies against S100[††], CD1a[‡‡], CD207[§§], and CD208[||||||]. [1]

After incubation with the primary antibody, secondary antibodies conjugated with streptavidin-biotin-peroxidase[¶¶], developed with diaminobenzidine (DAB) and counterstained with Carazzi's hematoxylin, were used.

Appropriated sections of human tonsil were used to provide a positive control for all primary antibodies. Negative controls were obtained using phosphate-buffered saline instead of primary antibody, which did not reveal non-specific background signal.

Immunohistochemistry Staining Analyses

The immunoexpression of each marker was evaluated utilizing image representation by computerized system, consisting of a light microscope[##], adapted to a high resolution camera[***] and a colored video display. Images were obtained using an image manager program[†††] and the processing was done by a specific program[‡‡‡]. [2]

It was considered immunopositive staining one that showed brownish coloration, assessed by DAB cromogen. After evaluation of the slides at x100 microscopic field, areas with higher density of immunostaining were selected and the density of positive cells were registered at HPF ($0.785mm^2$) (positive cells/mm^2). To evaluate the distribution pattern of the immunostained cells, the epithelium and lamina propria of each gingival sample was registered. The mean ± standard deviation of ten HPF counts of both epithelium and lamina propria, for each immunomarker, was obtained. Additionally, the shape of the immunostained cells was also evaluated.

Statistical Analyses

We analyzed the data with statistical software[§§§]. Shapiro-Wilk test showed the normal distribution of the data and Bartlett test the homogeneity of variances. We used Two-way analyses of variance and posterior Fisher´s LSD Test to observe differences between the means and unpaired Student t test to observe differences between

epithelium and lamina propria. To observe association between demographic characteristics, microscopical features and periodontal diagnoses we used Spearman correlation test. Confidence level adopted was 95% (p<0.05).

RESULTS

The epidemiologic and clinical data are shown in the Table 1. Inflammatory cells were observed more often in both AgP (score 3, n=4; score 2, n=4; score 1, n=2) and ChP (score 3, n=6; score 1, n=4) than Control (score 2, n=3; score 1, n=3, score 0, n=4) groups (both p<0.05), usually constituted by chronic lymphoplasmacytic infiltrate. Moreover, a significant positive correlation was observed between the intensity of the inflammatory infiltrate and imDC immunomarkers.

The immunostained cells in the epithelium showed preferentially a dendritic morphology, whereas in the lamina propria a round to oval shaped was frequently observed (Figure 1). Interestingly, these latter cells displayed focally a perivascular distribution pattern. All cases were positive for S100, CD1a and CD207; whereas 20% of both ChP and AgP and 40% of Control group were negative for CD208.

All imDC immunomarkers were more numerous in AgP than ChP and Control groups, being statistically significant only the S100+ cells (both p<0.001). Conversely, mDCs were visualized in higher number in ChP than AgP and Control groups (both p<0.05) (Table 2 and Figure 2).

Considering the frequency of imDC immunomarkers, the number of S100+ cells was significantly greater than CD207+ cells, followed by CD1a+ cells, in each group. Moreover, it was observed a lower number of CD208+ mDCs than imDCs, in all groups (Table 2 and Figure 2).

Taking into account the tissular distribution, different from S100+ cells, CD207+ and CD1a+ cells showed preferential intraepithelial location, while that CD208+ cells exhibited preferential connective tissue location, sometimes in perivascular pattern.

DISCUSSION

Several researches have been developed to assess the presence and distribution of immune cells in periodontal disease, in attempt to elucidate their immunologic mechanisms that could eventually be used in treatment planning, as well as identify potential therapeutic targets.[6, 7, 10, 12] Overall, these studies have shown variable results regarding number and density of DC subsets in different stages of periodontal disease. However, the immunomarkers used did not provide information about the DC maturation and/or activation states in these periodontal lesions, and even less considering AgP group.[4, 9] In the current study, to the best of our knowledge, we show for the first time that imDCs are more numerous in AgP than ChP and Control groups, and that mDCs can be visualized in higher number in ChP than AgP and Control groups. These findings are relevant since it is known that the ability of DCs to regulate immunity is dependent on DC maturation. Thus, our results suggest that predominance of imDCs and scarce amount of mDCs in AgP than ChP appear to be involved in their etiopathogenetic mechanisms, probably indicating lack or impairment of ability to induce lymphocyte and/or macrophage activation.[14]

It is known that T-cells represent the predominant type of inflammatory cells present in early stages of periodontal disease, while B-cells and even macrophage populations predominate in more advanced lesions, where periodontal tissue destruction

is evident.[8, 20, 22, 33, 34] Moreover, it has been showed that higher levels of interleukin (IL)-2, IL-10, IL-17, tumor necrosis factor (TNF)-α and interferon (IFN)-γ can be detected in ChP. [23] On the above, it is evident that maturation and/or activation states of DCs are critical in regulating the presence and distribution of inflammatory cells as well as the production of specific cytokines. In fact, several studies have suggested that DC activation occurs after coming into contact with bacterial lipopolysaccharide or immune complexes.[27, 35-37] As a result, mDCs become apparent which express co-stimulatory molecules and distinct cytokine patterns, determining selective migration of T-cell subsets in ChP.[23, 27] Thus, at least in part, our results suggest defective or impaired immunological mechanisms in AgP group.

In the last years, a fascinating field named osteoimmunology has received particular interest, especially because it provides a molecular framework between immune cells and periodontal disease pathogenesis.[38,39] In the concept of osteoimmunology, DCs participate within an alternate pathway associated with enhancing osteoclastogenesis of the periodontal tissue.[5] Thus, it has been suggested that imDCs serve as an ancillary reservoir that can convert or differentiate into mature osteoclasts participating in bone destruction during periodontal inflammation process.[5, 38-40] It is probable that the higher amount of imDCs detected in our gingival samples of AgP group may explain the high rates of bone destruction observed in these patients.

As previously commented, LCs are a distinct subtype of imDCs (S100+/CD1a+/CD207+), which after antigen capture and subsequent displacement to the regional lymph nodes, differentiate into mDCs, these latter characterized by gain expression of CD83 and CD208.[15,21,27,40,45,46] In the current study, we have also observed CD208 expression, preferentially on gingival lamina propria, indicating that DC maturation in situ occurs, corroborating the Jotwani et al.[27] study. Moreover, interestingly, it was visualized focal intraepithelial location of CD208+ cells, probably on LCs (Fig. 2). Curiously, some studies have shown that the number of LCs in the gingival epithelium may to be increased [30] or decreased [29], or to show no quantitative change [31] during inflammation in periodontal disease. In the current study, we have observed a gradual increase of LC immunomarkers, having the AgP group the greatest number of immunostained cells. Moreover, our results have showed that CD207+ than CD1a+ cells were more frequently visualized, on preferential intraepithelial location, in almost all cases. It is noteworthy that the current study seems to be the first to assess CD207 expression in ChP and AgP, and according to Seguier et al.[41] study, we suggest heterogeneity of CD1a+ or CD207+ cells in gingival location, being that CD207 seems the most specific LC marker. In fact, it can be likely that LCs (CD1a+/CD207+), indeterminate DCs (CD1a+/CD207-) and subDCs (CD1a-/CD207+) have been randomly detected in our gingival samples. It is for this reason that, and unlike most studies,[16,25,26,29,30,41] we have preferred to identify these DCs as CD1a+ or CD207+ cells instead of LCs. A detail analysis in human gingival tissues obtained from patients with ChP and AgP and involving accurate identification of LCs (e.g., flow cytometry, confocal laser scanning microscopy or high-content screening), as well as to define their functional aspects, are necessary in order to understand the true participation of LCs in the pathogenesis of the human periodontal disease. It would help understand the recent findings using a mice model which show a protective immunoregulatory role for LCs in inflammation-induced alveolar bone resorption.[42]

Thus, when assessing LCs in oral mucosal samples it is necessary to known that both CD1a and CD207 immunomarkers may identify distinctive DC subpopulations influenced by functional stages and presence of specific cytokines in the local milieu.[15,

[41] On the other hand, the S100 marker, when assessed individually, is highly unspecific for imDCs, because other cells, such as melanocytes and histiocytes, can also be S100 positive. Therefore, S100 expression should be interpreted with caution and in conjunction with both CD1a and CD207 markers, in accordance with the current study.

CD83 is a member of the immunoglobulin superfamily, which is upregulated during the maturation of DCs.[15, 43] Moreover, it is known that CD83 may be expressed by monocytes/macrophages, weakly by germinal center lymphocytes and at low levels by B-cell and T-cell populations.[44] Remarkably, Gemmell et al.[31] analyzing the presence and distribution of APCs in periodontal disease tissues, reported positivity for CD83 in APCs and endothelial cells. Thus, it becomes evident that CD83 is a marker with a relative degree of specificity when assessing maturation state of DCs. Interestingly, several studies have been developed to detect mDCs through CD83 [19, 23-25, 27, 31] and CD208 [21, 25] markers in ChP; whereas only a single study has assessed CD83 expression in AgP.[19] It is noteworthy that, to date, there is no study analyzing CD208 expression in AgP group. CD208 is a member of the lysosome-associated membrane protein (LAMP) family, an integral membrane protein, highly glycosylated and located in the lumen of lysosomes. CD208 is expressed on all mDCs but not on imDCs or other immune cells.[45, 46] For this reason, in the current study, we have preferred using CD208 instead CD83, to define mDC populations. Moreover, an evident cytoplasmic granular labeling pattern in association with adequate cellular morphology was considered to establish CD208 positivity. We found CD208+ cells in 80% of both ChP and AgP, as well as in 60% of Control group, which showed a perivascular distribution pattern. In relation to age-related changes in DC subpopulations, and regarding that decline in immune responses is associated with aging (this is, immunosenescence),[47] we expected to find that AgP (mean age, 32 years), followed by ChP (mean age, 51 years) group, has a great number of mDCs. Interestingly, and such as evidenced in the current study, this did not happen. In fact, we observe that ChP than AgP group exhibited a higher number of CD208+ mDCs, and it is probable that these findings are involved in their pathogenesis. In fact, knowing that the interaction of DCs with pathogenic bacteria may lead to DC maturation well as influence the DC production of immunomodulating cytokines, thereby contributing to the definition of the T-cell responses,[48] our results suggest defective and/or impairment maturation mechanism on DC populations in AgP group. It is supported by findings from Bodineau et al.[21,25] studies, which show that elderly patients with ChP present an increased number of gingival CD208+ mDCs.

As previously commented, the predominance of imDCs appears to be involved in AgP pathogenesis. Interestingly, there are evidences suggesting imDC transdifferentiation into osteoclasts,[40] as well as presence of DC-derived osteoclasts during immune interactions with CD4+ T-cells and microbial products under inflammatory conditions,[38,39,49] which could indicate DC involvement in inflammation-induced osteoclastogenesis and bone loss in periodontal disease. In this context, functional studies are required to determine the significance of DC subsets in periodontal disease.

CONCLUSIONS

Our results suggest that predominance of imDCs appear to be involved in AgP pathogenesis, probably due to lack of ability to induce immune cell activation. This is supported by the fact that DCs as professional APCs, and during maturation and activation processes after bacterial interactions, can activate and drive the host immune reaction.[14]

ACKNOWLEDGMENTS

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Corresponding author: Prof. Dr. Camila Tirapelli, Faculdade de Odontologia de Ribeirão Preto, USP. Avenida do Café, S/N, 14040-904 Ribeirão Preto, São Paulo, Brasil. E-mail: catirapelli@forp.usp.br; Tel: 0055-16-33150479; Fax: 0055-16-33150479.

Figure 1.

Immunohistochemical findings of gingival samples regarding the distribution of immature (imDCs) and mature dendritic cells (mDCs) in the epithelium (Ep) and lamina propria (LP). It can be observed a higher number of S100+, CD1a+, and CD207+ cells in aggressive periodontitis; and, inversely, a higher number of CD208+ cells in chronic periodontitis. The most DCs are highlighted by black arrows (original magnification, x400; scale bar, 100 µm).

Figure 2.

Graphic illustration: on the y axis, the numbers indicate the mean of total (Ep + LP) immunostained cells per field at x400· magnification; on the x axis, periodontal diagnoses grouped by immunomarkers. Errors bars show standard deviation; the four point stars indicate statistically significant differences between periodontal diagnoses for the same immunomarker.

Table 1.

Clinical and demographic characteristics of the periodontal disease groups.

Patient group	L	G	n	Gender (%)	Age (yr): Range / Mean	Teeth per patient *	Affected teeth per patient**	PD	CAL
Aggressive periodontitis	6	4	10	9(90%) female 1 (10%) male	15 to 34 / 32	26	5	5.5	7.5
Chronic periodontitis	2	8	10	6(60%) female 4(40%) male	35 to 65 / 51	21	9	5	6
Control group	-------		10	7 (70%) female 3 (30%) male	18 to 38 / 25	28	0	1.8	1.8

L=localized, G=generalized, n=number, PD= Probing depth (mean/mm); CAL= clinical attachment loss (mean/mm).

*Mean within the group obtained by determining the mean of the number of teeth per patient.

**Mean within the group obtained by determining the mean of the number of affected teeth per patient.

PD and CAL means were obtained by taking the sum of all PD and CAL values of affected teeth divided by the number of affected teeth per patient. The obtained values for each patient were then used to determine the mean within the groups.

Spearman's rank correlation coefficient showed an association between periodontal diagnoses and age (p<0.05) but not with gender (p>0.05).

Table 2.

Distribution (mean and standard deviation) of imDC and mDC subsets regarding S100, CD1a, CD207 and CD208 immunomarkers in Ep and LP of gingival samples obtained from patients with AgP, ChP and Control (clinically healthy gingiva) groups.

DC subtype	Immuno-markers	Periodontal diagnoses								
		Aggressive Periodontitis			Chronic Periodontitis			Control group		
		Ep	LP	Total	Ep	LP	Total	Ep	LP	Total
imDC	S100	12.4(±0.8)a	11.4(±1.1)a	23.5(±1.8)a	7.1 (±2.3)a	6.8(±1.7)a	13.9(±5.6)a	9.0(±2.2)a	5.5(±2.4)a	14.5(±4.2)a
	CD1a	7.8(±10)b	1.4(±1.2)b	9.2(±2.0)b	5.9(±1.4)a	1.4(±1.0)b	7.3(±2.9)b	4.1(±1.0)b	1.8(±1.9)b	5.9(±1.7)b
	CD207	9.8(±1.6)b	2.9(±0.8)b	12.7(±1.1)b	8.2(±0.7)a	2.0(±1.9)b	10.2(±4.2)b	4.8(±0.8)b	2.0(±1.5)b	6.8(±2.2)b
mDC	CD208	0.4(±0.4)c	0.5(±0.6)c	0.9(±0.7)c	0.7(±0.5)b	1.1(±1.2)b	1.8(±1.2)c	0.3(±0.4)c	0.4(±0.7)c	0.7(±0.5)c

Legends: DC, dendritic cell; imDC, immature DC; mDC, mature DC; AgP, aggressive periodontitis; ChP, chronic periodontitis, Ep, epithelium; LP, lamina propria.

In accordance with 2-Way analyses of Variance and Fischer LSD Test (p<0.05):

In columns, different letters indicate statistically different means (p<0.05) considering the immunomarkers S100, CD1a, CD207 and CD208 in Ep, LP or Total for each periodontal diagnosis.

** Sigma-Aldrich, St Louis, MO.

†† Polyclonal, 1:10000 dilution; DakoCytomation, Glostrup, Denmark.

‡ ‡ Clone 010, 1:400 dilution; DakoCytomation, Glostrup, Denmark.

§§ Clone 12D6, 1:200 dilution; Monosan, Uden, The Netherlands.

|||||||| Clone 104G4, 1:500 dilution; Dendritics, Lyon, France.

¶ ¶ K0690; Universal Dako LSAB® + Kit, Peroxidase, Carpinteria, CA.
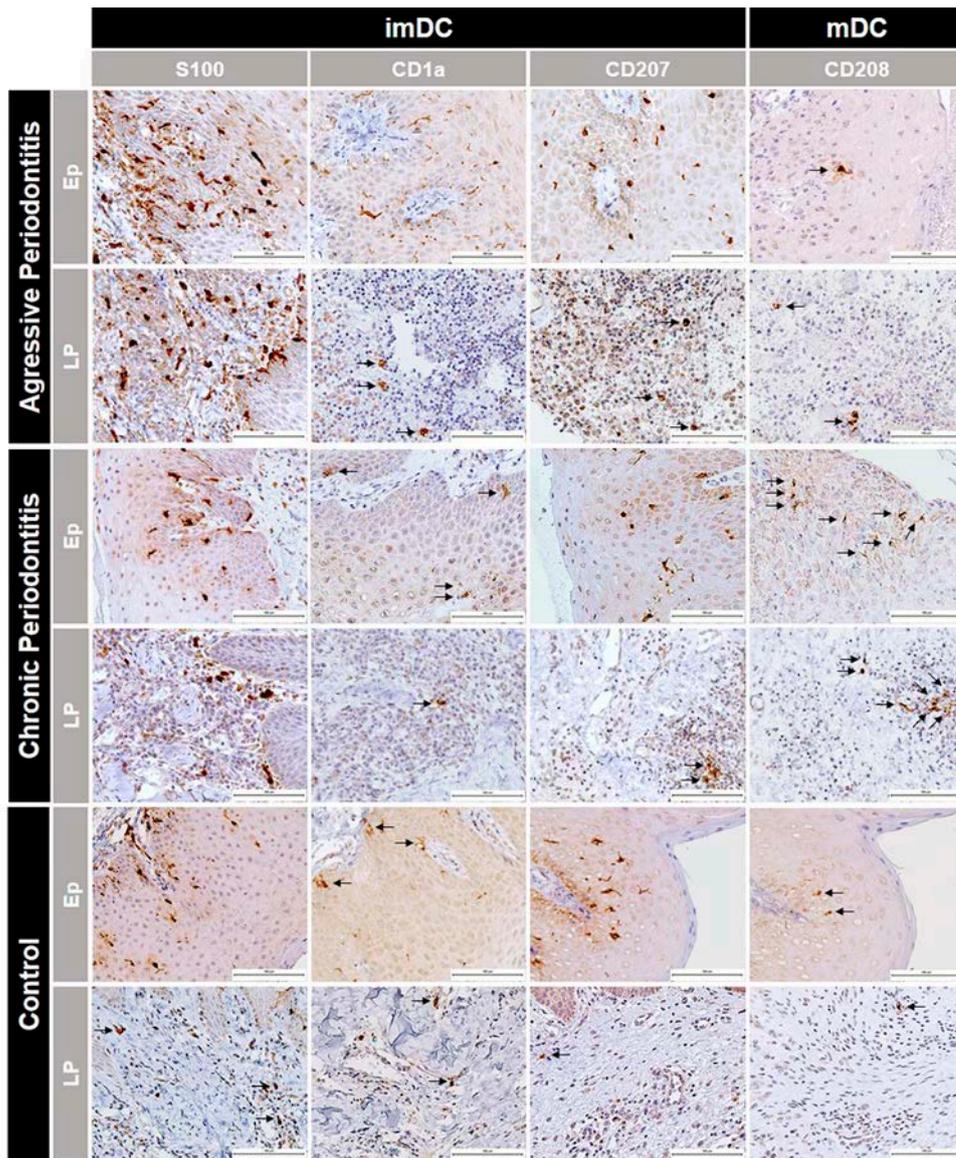
Leica DM500, Wetzlar, Germany.

*** Leica ICC50, Wetzlar, Germany.

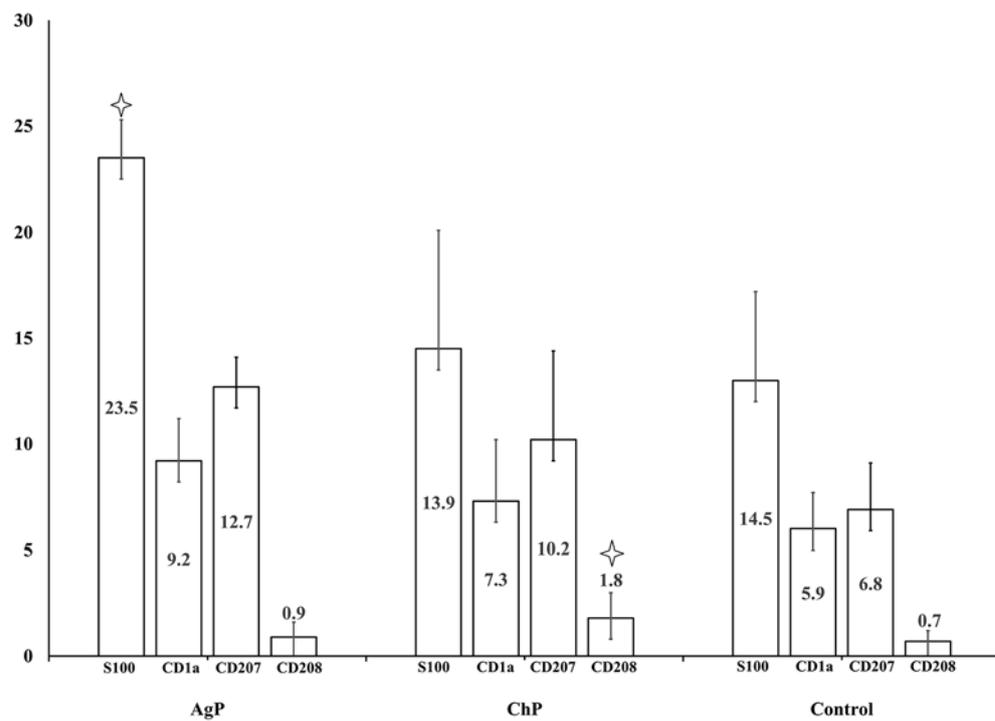††† Leica IM50 Image Manager, Wetzlar, Germany.

‡‡‡ Leica QWin Image Processing and Analysis System, Wetzlar, Germany.

§§§ Prism, version 6.0, Graphpad, CA.