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Characterization of Macrophage Polarization in Periodontal Disease

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DISCLOSURE

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ABSTRACT

AIM: To explore the M1/M2 status of macrophage polarization from healthy, gingivitis and periodontitis patient samples.

MATERIALS AND METHODS: Gingival biopsies were collected from 42 individuals (14 gingivitis, 18 periodontitis, and 10 healthy samples) receiving periodontal therapy. Histomorphology analysis was performed with hematoxylin & eosin staining. Immunofluorescence was performed using a combination of CD68 (macrophages), iNOS (M1), and CD206 (M2) in order to acquire changes in macrophage polarization at a single cell resolution. Macrophages were quantified under microscopy using narrow wavelength filters to detect Alexa 488, Alexa 568, Alexa 633 fluorophores and Hoechst 33342 to identify cellular DNA content.

RESULTS: Gingivitis and periodontitis samples showed higher levels of macrophages compared with healthy samples. Unexpectedly, periodontitis samples displayed lower levels of macrophages dispersed in the stromal tissues compared to gingivitis samples; however, it remained higher than healthy tissues. The polarization of macrophages appears to be reduced in periodontitis and showed similar levels to those observed in healthy tissues.

CONCLUSIONS: Our study found that gingivitis and periodontitis differ one from each other by the levels of macrophage infiltrate, but not by changes in macrophage polarization.

Clinical Relevance (100 words)

Scientific rationale for the study: Oral dysbiosis may lead to significant changes in the host immune response. The balance of M1/M2 signaling could reflect a response to periodontal therapy and host susceptibility for inflammatory and immunosuppression events.

Principal findings: Healthy tissues, gingivitis, and periodontitis samples were characterized by a mixed population of CD68 positive macrophages presenting M1, M2, and the combination/transition of M1 and M2 polarization.

Practical implications: Future development of locally-delivered host-modulation drugs could target macrophages to reverse or enhance immunosuppressive events for treatment and prevention of periodontal diseases.

INTRODUCTION

Periodontal diseases are infectious and inflammatory conditions that disrupt the periodontium homeostasis, collectively affecting the gingiva, alveolar bone, periodontal ligament, and cementum. Periodontitis is characterized by a spread of inflammatory infiltrate progressively into the periodontal tissues, resulting in loss of attachment and alveolar bone together with the apical migration of the junctional epithelium (Kinane et al. 2008; Page and Schroeder 1976). A high prevalence of periodontitis was confirmed from a recent National Health and Nutrition Examination Survey (NHANES) study reporting that nearly 50% of the United States population is affected (Eke et al. 2015). The more severe form of periodontitis affects about 10% of all patients (Kassebaum et al. 2014) suggesting the existence of contributing factors such as gene polymorphisms (Divaris et al. 2013) and increased host susceptibility for disease progression (Eke et al. 2015; Eke et al. 2016; Loe et al. 1986).

Over the past decades, different disease progression models for periodontitis have been described addressing bacterial biofilm as the primary etiology (Jeffcoat and Reddy 1991; Socransky et al. 1984; Teles et al. 2016). However, limited evidence on the transition from established-to-advanced stages of periodontitis is available. Experimental human models have provided valuable information on the key role of biofilm and

patterns of adaptive-innate immune responses (Loe et al. 1965; Seymour et al. 1983). Conversely, a particular group of individuals does not display signs of progressive attachment and/or bone loss despite the presence of biofilm and gingival inflammation (Hugoson et al. 2008). In other instances, long-standing gingivitis lesions exhibit different cellular composition to periodontitis lesions (Thorbert-Mros et al. 2015). The hypothesis of immunological mechanisms down-regulating the destructive nature could explain why some established lesions might not progress into advanced forms.

Several mechanisms may influence disease progression of periodontitis like epigenetic deregulation of the periodontium homeostasis resulting in dysfunctional host response to local microbiota, and the development of an endotoxin tolerance phenotype elicited by chronic exposure of periodontal tissues to bacterial endotoxins (Martins et al. 2016; Seeley and Ghosh 2017; Zhang et al. 2013). In fact, activation of the endotoxin tolerance mechanism constitutes an emerging area of interest in chronic inflammatory diseases (CID) and may hold the promise to better understand disease progression of patients at high risk for the development of periodontitis.

The proportional distribution of inflammatory cells in periodontitis lesion has been described in reviews on studies reporting on histological evaluations of human samples (Berglundh and Donati 2005; Berglundh et al. 2011). Thus, B cells and plasma cells together occupy about 60%, T helper and T-cytotoxic cells 17%, while macrophages and neutrophils represent 5-7% of the inflammatory cell population. Similar results were reported in recent assessments of human periodontitis lesions (Carcuac and Berglundh 2014; Thorbert-Mros et al. 2015). Although occurring in relatively small proportions; macrophages exhibit essential defense and regulatory functions. The phagocytic abilities of macrophages as resident cells, or as monocyte-derived cells recruited upon inflammation are key players in the development of acquired immunity (Martinez and Gordon 2014). Macrophages are endowed with high cellular plasticity capable of responding to distinct environmental signals. Upon activation, macrophages can differentiate into M1 (classical) or M2 (alternative) phenotypes, with M1 being pro-inflammatory and involved in bacterial killing and promoting inflammation by an increase in production of interleukin-6 (IL-6), tumor necrosis factor–alpha (TNF-α) and inducible nitric oxide synthase (iNOS).

In contrast, the M2 phenotype plays a role in the resolution of inflammation and tissue repair being characterized by the production of IL-10 and a decreased expression of in IL-6 (Das et al. 2015; Garlet and Giannobile 2018; Yu et al. 2016). Furthermore, macrophage polarization to an M2 phenotype has been associated with the presence of chronic infections (Mills and Ley 2014). Animal models for experimental periodontitis have shown high levels of TGF-β, CD80, and TNF-α mRNA expression during early inflammatory process of ligature-induced periodontitis (M1), while high CD206 expression level was found during tissue healing (M2) (Viniegra et al. 2018). Despite the importance of M1/M2 polarization in inflammatory diseases, little is known about the macrophage polarization status of periodontitis and gingivitis in humans. In this investigation, we explored the macrophage content and polarization of periodontitis compared to gingivitis and healthy gingiva tissues to better understand macrophage polarization in vivo.

MATERIALS AND METHODS

Subject selection

Forty-two patients (38.08% females and 61.89% males) provided a total of 10 healthy, 14 gingivitis and 18 periodontitis biopsies. Overall, the patients mean age was 56.75 years old (Range: 19-75 years old). Six patients (14.28%) reported a history of smoking (> 1 year). Analysis of the effects of smoking, age or gender were not accessed in this study. Based on the periodontal clinical and radiographic parameters, demographic data of all included subjects were subdivided into 3 groups according to the previously described disease categories as depicted in Table 1.

Research subjects were recruited from patients seeking dental treatment or receiving active/supportive therapy at the Graduate Periodontics Clinic from the University of Michigan School of Dentistry (n=32, 10 healthy, 10 gingivitis, and 12 periodontitis) and the Clinic of Periodontics, Public Dental Service and Clinic for undergraduate training, Institute of Odontology, Sahlgrenska Academy, University of Gothenburg (n=10, 4 gingivitis and 6 periodontitis). The study protocols were reviewed and approved by the University of Michigan Health Science Institutional Review Board (HUM00097548) and the local Human Review Board at University of Gothenburg (Dnr

677-05) to ensure participants' rights were protected. This study was performed in accordance with the Helsinki Declaration of 1964, as revised in 2013.

To be eligible for this study, patients possessing 20 or more teeth received a complete oral examination. The inclusion criteria combined periodontal probing depths (PD) (< 4mm for gingivitis and ≥ 4mm for periodontitis), clinical attachment level (CAL) (< 3mm for gingivitis and ≥ 3mm for periodontitis), radiographic marginal bone loss (MBL) (≥ 50% for periodontitis) and bleeding on probing (BOP) assessed at six sites per tooth. Gingivitis samples were used here as controls for Periodontitis, therefore we paid special attention to the selection of the biopsy site. Tissue harvesting was exclusively removed from areas presenting no clinical history of periodontitis, along radiograph data with lack of bone loss. All inclusion criteria were accessed at six sites per tooth for gingivitis patients and 4 sites per tooth for periodontitis patients. Other measurements included furcation involvement (FI), gingival bleeding index (GBI), bleeding with exudate (BE), mobility, fremitus, and zone of keratinized gingiva.

Patients were excluded if they possessed any of the following conditions: 1) uncontrolled systemic disease or condition is known to alter bone metabolism (e.g., osteoporosis, osteopenia, hyperparathyroidism, Paget's disease); 2) pregnancy; 3) history of oral cancer, sepsis or adverse outcomes to oral procedures; 4) long-term use of antibiotics (>2 weeks in the past two months); 5) patients taking medications known to modify bone metabolism (e.g., bisphosphonates, corticosteroids, hormone replacement therapy), past (<1 year) and current smokers. Before enrollment, all subjects received information about the study and signed informed consent. Patients requiring at least one surgical procedure within the periodontium whereby a gingival biopsy could be harvested were identified.

Sample collection and processing

Samples were obtained from a single-site around common dental and periodontal procedures displaying the most evident clinical signs of chronic inflammation (deepest PD site, edema, red/purple-colored gingiva, profuse bleeding). Gingivectomies after orthodontic treatment and esthetic crown lengthening procedures were employed for control subjects for esthetic purposes. Patients affected with gingivitis; clinical crown

lengthening was used to reflect chronic gingival inflammation for restorative and biologic purposes. Ultimately, open flap debridement and/or resective approaches were selected for periodontitis-affected patients. Removal of these tissue biopsies did not interfere with regular (internal beveled) incisions or procedures as presented in the initial treatment plan or influence upon the expected clinical outcomes. After collection, biopsies were fixed in a 4% formalin solution for 24h of fixation, dehydrated and embedded in paraffin. The samples were stored as coded specimens not to reveal patient-related information.

Identification of single-cell macrophage polarization using immunofluorescence

Sections of approximately 4 μm in thickness were dewaxed, hydrated and incubated in antigen retrieval solution of 10mM/L sodium citrate buffer (pH 6). The sections were incubated with 3% bovine serum albumin (BSA) in PBS (phosphate buffered saline) for blocking of unspecific binding followed by incubation overnight at 4°C with the following primary antibodies (Dilution 1:50): anti-CD68 (rabbit monoclonal antibody, Thermo Scientific, Waltham, MA, USA), anti-iNOS (MAB9502, R&D system, USA), anti-CD206 (goat AF2534, R&D system, USA). The sections were then incubated with Alexa-conjugated secondary antibodies: Alexa-488 anti-goat (dilution 1:200), Alexa-568 anti-rabbit (dilution 1:200) and Alexa-633 anti-mouse (dilution 1:50) for 1 hour at room temperature. The sections were counterstained with Hoechst 33342 (Sigma-Aldrich Corp., St. Louis, MO, USA) to visualize DNA content. The omission of the primary antibody was used as negative controls. Images were taken using a QImaging® EXi Aqua™ monochrome digital camera attached to a Nikon Eclipse 80i Microscope (Nikon, Melville, NY) and visualized with QCapturePro software.

Quantification of M1 (CD68/iNOS) and M2 (CD68/CD206) Macrophage Polarization

Representative areas of each coded sample were photographed (10 independent fields per sample) including the area of the sub sulcular/junctional epithelium and at the inflamed/infiltrated connective tissue (ICT)(40X objective). Images from each sample were extracted using fluorophore channels correspondent to CD68 (total macrophages), CD68/iNOS (M1 polarization) or CD68/CD206 (M2 polarization). Only cells presenting double staining for CD68 and iNOS or CD68 and CD206 were counted to avoid the

quantification of non-macrophages cells expressing iNOS or CD206. All pictures were further analyzed as monochromatic images using the NIH Image J software (https://imagej.nih.gov/ij/) split-channel mode and further quantified using the cell counting plugin app. The results were expressed as a percentage of positively double stained cells among the total number of CD68 positive cells. The samples were evaluated by two independent trained and calibrated examiners (LL and CGP).

Statistical Analysis

Statistical analyses were performed by Student *t*-test using GraphPad Prism (GraphPad Software, San Diego, CA). Spearman rank correlation test was used to measure the strength of the correlation between variables (CD68/iNOS and CD68/CD206). *P* value ≤0.05 was considered statistically significant and differences were noted by asterisks (*) or *P* values (* P ≤0.05, ** P ≤0.01, *** P ≤0.001, **** P≤0.0001, and NS P >0.05, not significant). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests was also used for each group (healthy, periodontitis and gingivitis).

RESULTS

Single Cell Resolution of Macrophage Polarization in Healthy Tissues

Here, we explored the macrophage content of the gingival connective tissue localized juxta-epithelia from healthy samples as a baseline for the analysis of tissues from gingivitis or periodontitis patients. In order to establish a normal baseline of macrophage content and subsequently polarization, we evaluated healthy gingival tissues for CD68, iNOS (M1) and CD206 (M2) expression levels. We found that normal tissues negative for BOP exhibit a density of CD68 positive macrophages at a mean concentration of 5.38 cells per microscopic field at a magnification of 200X (Fig. 1A). During the polarization analysis, macrophages demonstrated co-expression of iNOS signifying M1 polarization, with a mean concentration of 0.93 cells per field. Healthy tissues also presented macrophages undergoing M2 polarization (CD68/CD206 positive staining) at significantly higher levels (mean of 3.4 cells/field) when compared with macrophages under M1 polarization (Fig. 1A, P≤0.0001). Such differences became more

evident when gingivitis and periodontitis samples were baseline corrected versus healthy tissues. Under these circumstances, macrophages undergoing M1 and M2 polarization represented a mean of 22.14% and 72.24% of the total number of tissue macrophages respectively (Fig. 1B).

Gingivitis contain a higher number of macrophages when compared with periodontitis tissues

Similar to normal healthy tissues, gingivitis and periodontitis tissues are characterized by the presence of CD68 positive macrophages expressing iNOS or CD206 (Fig. 2A to 2D), and in some instances, the co-expression of all 3 markers was observed (Fig. 2B, box# 4). Such diversity of the expression pattern of macrophages is well observed in Figures 2B and 2D that illustrates macrophages exclusively expressing the surface marker CD68 and thereby demonstrating M0 polarization (Fig. 2B and 2D, box #1), the presence of M1 polarization (Fig. 2B and 2D, box #2), the presence of M2 polarization (Fig. 2B and 2D, box #3), and the presence of macrophages co-expressing markers for M1 and M2 polarization shown here exclusively in the gingivitis samples (Fig. 2B box #4). Overall, tissue samples from gingivitis and periodontitis showed high levels of M2 polarization as represented by the heat map distribution containing all quantified histological fields per condition (Fig. 2E and 2F).

Interestingly, however, gingivitis tissues differ from periodontitis concerning the total number of macrophages distributed within the connective tissues. Gingivitis tissues showed nearly doubled number of macrophages presented per field over periodontitis samples (Fig. 3A and 3B, mean of 15.12 cells and 8.04 cells respectively-red circles). Furthermore, gingivitis tissues showed significantly higher number of M2 polarization compared with periodontitis (mean of 7.6 cells/field for gingivitis vs. 3.8 cells/field for periodontitis-green squares), while little difference was observed in M1 polarized cells (mean of 2.64 cells/field for gingivitis vs 1.52 cells/field for periodontitis-blue triangles)(Fig. 3A and 3B). Most interestingly is that upon baseline correction for a total number of macrophages, the ratio of M1 and M2 polarization among gingivitis and periodontitis samples is maintained. In this way, M2 polarization observed in gingivitis samples represents 45% of all detected CD68 positive cells, while M1 polarization

corresponds to 18% of all macrophages (Fig. 3C). Similarly, periodontitis macrophages present 48% of macrophages undergoing M2 polarization and 20% of all macrophages undergoing M1 polarization (Fig. 3D). Like M1 or M2 polarization, we also found macrophages presenting a transition between M1 and M2 polarization identified by the co-labeling of CD68, iNOS, and CD206. These macrophages are relatively common within soft tissues of gingivitis and periodontitis representing 16% of all stained macrophages in gingivitis and 14.8% in periodontitis samples (Fig. 3E).

The polarization of macrophages in periodontitis share similarities with healthy tissues

The screening of all histological fields from health, gingivitis, and periodontitis patients revealed a common trend on M2 macrophage polarization (Fig. 4A and 4B). Immunofluorescence analysis also revealed that gingivitis tissues presented the highest total numbers of macrophages as well as highest M1 and M2 polarization (Fig. 4B). In fact, it became very evident that gingivitis samples showed higher loading levels of macrophages compared with healthy or periodontitis tissues (Fig. 4C, $P<0.0001$). Although displaying lower numbers of macrophages, periodontitis lesions present overall higher counts of macrophages compared to healthy tissues (Fig. 4C, $P<0.01$). From an M1 polarization perspective, gingivitis tissues demonstrated significantly greater polarized macrophages than periodontitis ($P<0.001$) or healthy ($P<0.0001$) samples (Fig. 4D). Interestingly, there were no statistical differences between healthy tissues and periodontitis tissues related to M1 polarization ($P>0.05$). M2 polarization followed a similar trend in which gingivitis samples were once again highly identified in the soft tissues compared with healthy or periodontitis samples (Fig. 4E, $P<0.0001$). Nonetheless, there were no statistical differences between the M2 polarization of periodontitis or healthy tissues ($P>0.05$).

All in all, our study found that gingivitis and periodontitis differ one from each other by the levels of macrophage infiltrate, but not by changes in macrophage polarization.

DISCUSSION

Based on the concept of host-modulation, macrophages play important roles as mediators and effector in the immune response mediated by Th1 and Th2 cells (Mills et al. 2000). The role of M1/M2 macrophage polarization in the pathogenesis of periodontal disease has proven difficult to define. Much of the complications of the field rely on the individual analysis of M1 or M2 polarization of macrophages using immunohistochemistry techniques, while only a few studies explore the presence of both phenotypes, especially at a single cell resolution. For example, M1 focused studies have noted an M1-polarization phenotype in periodontitis (Gheren et al. 2008; Gorska et al. 2003; Gullu et al. 2005; Holden et al. 2014; Hussain et al. 2016; Lam et al. 2014; Lappin et al. 2000; Navarrete et al. 2014; Ozmeric et al. 2000), while other studies have shown enhanced accumulation of an M2 phenotype (Gheren et al. 2008; Navarrete et al. 2014). Adding to the M1/M2 paradigm complexity, a reduction of M2 polarization found in periodontitis lesions has also been demonstrated (Gullu et al. 2005; Lam et al. 2014).

To address this shortcoming in the literature, we designed a well-controlled experiment using a representative cohort of patients ranging from 19 to 75 years old presenting no clinical history of uncontrolled systemic disease or use of drugs that could have an impact over the bone metabolism. We also decided to carefully exclude pregnant as well as patients with a history of oral cancer, sepsis, use of antibiotics, and smokers. As our study design aims to explore a more accurate picture from a pathogenesis standpoint and minimize potential environmental factors (e.g., smoking), we focused on a cohort of patients presenting stable periodontal disease progression.

Some other methodological improvements were included in this study aiming at reducing the bias of using a single antibody per histological sections. By implementing four different fluorescent channels, we gain single-cell resolution of macrophages undergoing polarization, along with reduced bias in quantifying non-macrophage cells expressing M1 and M2 polarization markers and gaining in the ability to identify macrophages undergoing an M1-M2 transition. At last, we decided to evaluate the global macrophage polarization throughout the gingival tissue samples by accounting for the total number of macrophages located at the ICT and distributed at the subjacent mucosa.

Our strategy demonstrated that macrophages are more frequently found in gingivitis samples compared to periodontitis. Furthermore, the ratio of macrophages

under M1 or M2 cellular polarization followed similar patterns of displaying larger numbers in gingivitis compared to periodontitis. From a polarization perspective, macrophages were often observed in M2 polarization for both conditions, gingivitis, and periodontitis. Much of these findings suggest an overall presence of a chronic phase of inflammation in both inflammatory conditions compared to an acute process represented by an M1 polarization. This observation may elute to the observed overall reduction in the total number of macrophages observed in periodontitis samples and suggests a reset of macrophages influx to levels observed in normal tissues. Down-regulation of macrophage influx may, to some degree, be a weak level of local immunosuppression at the chronic inflammatory anatomical site in an attempt to mitigate tissue destruction. Although unexpected, our findings align with the observations from Chapple and colleagues that observed an apparent failure of recruitment and activation of macrophages in destructive periodontitis (Chapple et al. 1998). Another study from Lins and collaborators also demonstrated the presence of a higher density of macrophages in gingivitis samples compared to chronic periodontitis (Lins et al. 2008). Conversely, Thorbert-Mros et al. showed evidence on the accumulation of macrophages within the composition of the inflammatory infiltrate from severe generalized periodontitis compared to long-standing gingivitis (Thorbert-Mros et al. 2015). All studies present robust methodologies but differ on the approach and patient cohort composition.

In the context of cellular phenotype, the polarization of macrophages might be part of both inductive and resolving mechanisms of tissue inflammation (Das et al. 2015; Viniegra et al. 2018). Yu and coworkers described a phenotypic switch from M2 to M1 from bone marrow-derived macrophages after *P. gingivalis* lipopolysaccharide (LPS) stimulation in a ligature-induced periodontitis murine model (Yu et al. 2016). We also observed the presence of a cohort of macrophages presenting a combined polarization phenotype simultaneously expressing M1 and M2 markers. Such finding was consistently observed in gingivitis (16.01%) and periodontitis (14.87%) samples suggesting greater plasticity of macrophages capable of transition between M1 and M2 polarization.

Macrophage polarization-modulating agents might be considered in the future as immune regulation drugs for the prevention, treatment, and reduction of patient susceptibility for periodontal diseases (Di Paola et al. 2006; Hassumi et al. 2009; Sima

and Glogauer 2013). Recently, rosiglitazone-induction of pro-resolving macrophages have shown to reduce bone resorption and increase bone formation during healing in experimental ligature-induced periodontitis (Viniegra et al. 2018). Advancements in immunology and a complete understanding of the role of macrophages in the transition of established to advanced stages of periodontal disease could open new fields for the development of diagnostics and therapeutic tools in periodontics.

In summary, our ability to identify the polarization of macrophages at a single cell resolution provides a unique landscape of healthy tissues, gingivitis, and periodontitis. Our study found that gingivitis and periodontitis differ one from each other by the levels of macrophage infiltrate, but not by changes in macrophage polarization.

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Table 1. Demographic Characteristics of Study Participants

Characteristic (total)	Healthy	Gingivitis	Periodontitis
Inclusion criteria	No CAL PD ≤ 4mm No BOP (GBI ≤ 1) No MBL	CAL <3mm PD ≤ 4mm BOP (GBI ≥2) No MBL	CAL ≥ 3mm PD ≥ 4mm BOP (GBI ≥2) MBL (≥ 50%)
Subjects (n=42)	10	14	18
Males (61.89%)	14.28%	21.42%	26.19%
Females (38.08%)	9.52%	11.90%	16.66%
Past Smokers (n=6)	4.76%	0%	9.52%
Mean age (yrs) (56.75 yrs old [19-75])	49.10 (19-72)	61.50 (39-64)	59.16 (30-75)

CAL: Clinical attachment level; PD: Probing depths; BOP: Bleeding on probing; GBI: Gingival bleeding index; MBL: Marginal bone loss

Figure Legends:

Figure 1. Macrophage polarization profile of healthy gingival tissues. ***A.*** Graphic display total number of macrophages positive for CD68, CD206, and iNOS observed per histological field. Note statistically relevant accumulation of macrophages undergoing M2 compared with M1 polarization (**** P<0.001, SE of difference 0.3375). ***B***. Graphical representation of M1 and M2 polarization adjusted for the total number of CD68 positive cells (macrophages) displayed as a percentage of total cells. Note higher levels of M2 polarization in healthy tissues compared to M1 polarization.

Figure 2. M1 and M2 macrophage polarization in gingivitis and periodontitis lesions. ***A to D.*** Representative tissue samples from gingivitis and periodontitis presenting single-color staining for CD68 (red), iNOS (cyan), CD206 (green) and Hoechst (blue), and merge channels. Dashed line delineates epithelial from stromal tissues. Boxed numbers of merged staining depict all observed composition of markers including the presence of exclusive staining for CD68 positive cells without polarization (1), the presence of M1 polarization (CD68/iNOS) (2), the presence of M2 polarization (CD68/CD206) (3), and macrophages presenting a combination of all markers (CD68/iNOS/CD206) (4). ***E and F.*** Heatmap representing all observed histological fields from gingivitis (n=132) and periodontitis (n=168) fields depicting the observed histological polarization pattern. Note prevalence of macrophages undergoing M2 polarization. Colorimetric scale bar from 0 to 40 cells per field.

Figure 3. Overall polarization of macrophages from gingivitis and periodontitis samples. ***A and B*****.** Quantification of total number of macrophages from gingivitis and periodontitis samples presenting exclusive staining for CD68 (red), or double staining for CD68 and iNOS (blue), or CD68 and CD206 (green) per field. Note reduced levels of M1 and M2 polarization along with low levels of total macrophages in periodontitis disease tissues compared with gingivitis. ***C and D.*** Expression of the baseline corrected percentage of total number of macrophages presenting M1 and M2 polarization from gingivitis and periodontitis samples. Macrophages found in gingivitis (mean of 45.84%, SEM 2.19) and periodontitis (mean of 48.34%, SEM 1.68) tissues show higher numbers of M2 polarization. ***E.*** Representation of $CD68^{+}$ macrophage distribution presenting M1 polarization (iNOS) (18.24% for gingivitis and 20.02% for periodontitis), and M2 polarization (CD206) (45.84% for gingivitis and 48% for periodontitis), along with macrophage cells expressing concomitant M1 and M2 polarization (16.01% for gingivitis and 14.87% for periodontitis).

Figure 4. Comparative expression of M1 and M2 polarization among healthy and gingivitis and periodontitis tissues. ***A.*** Heatmap representation of all observed histological fields from healthy (n=128), gingivitis (n=132) and periodontitis (n=168) samples depicting the observed histological polarization pattern. Note prevalence of macrophages undergoing M2 polarization for all groups. Colorimetric scale bar from 0 to 40 cells per field. ***B.*** Dispersion graphic of macrophage polarization from healthy, gingivitis and periodontitis samples. ***C.*** Macrophage density comparison observed in healthy, gingivitis, and periodontitis groups demonstrating high levels of macrophage accumulation in gingivitis samples compared with healthy and periodontitis (**** $P<0.0001$). Note also higher levels of macrophages in periodontitis samples when compared with healthy subjects (** $P<0.01$). ***D.*** Dispersion graphic depicting enhanced M1 polarization in gingivitis samples compared with healthy and periodontitis tissues (**** $P<0.0001$), while periodontitis samples do not present statistical differences with healthy group (NS $P>0.05$). ***E.*** M2 polarization of macrophages is observed in gingivitis samples compared to healthy and periodontitis samples (**** $P<0.0001$). Note that macrophage levels in periodontitis samples do not present statistical differences when compared to healthy tissues (NS $P>0.05$).

Figure 1

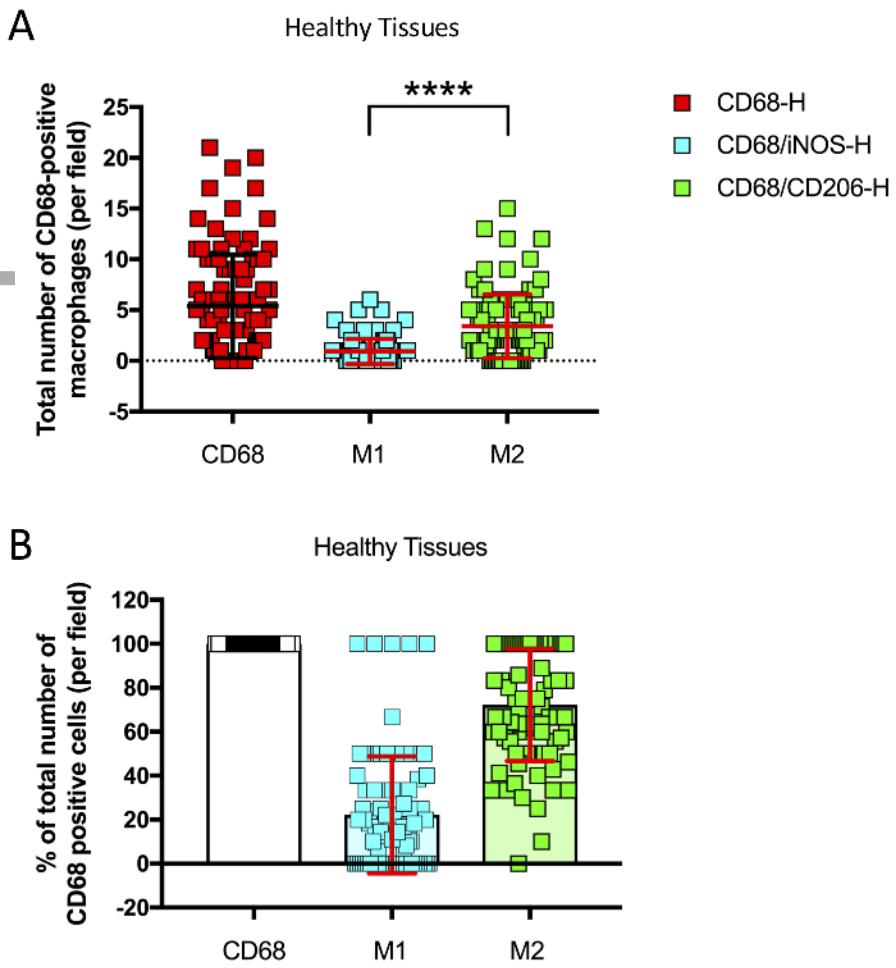

Figure 2

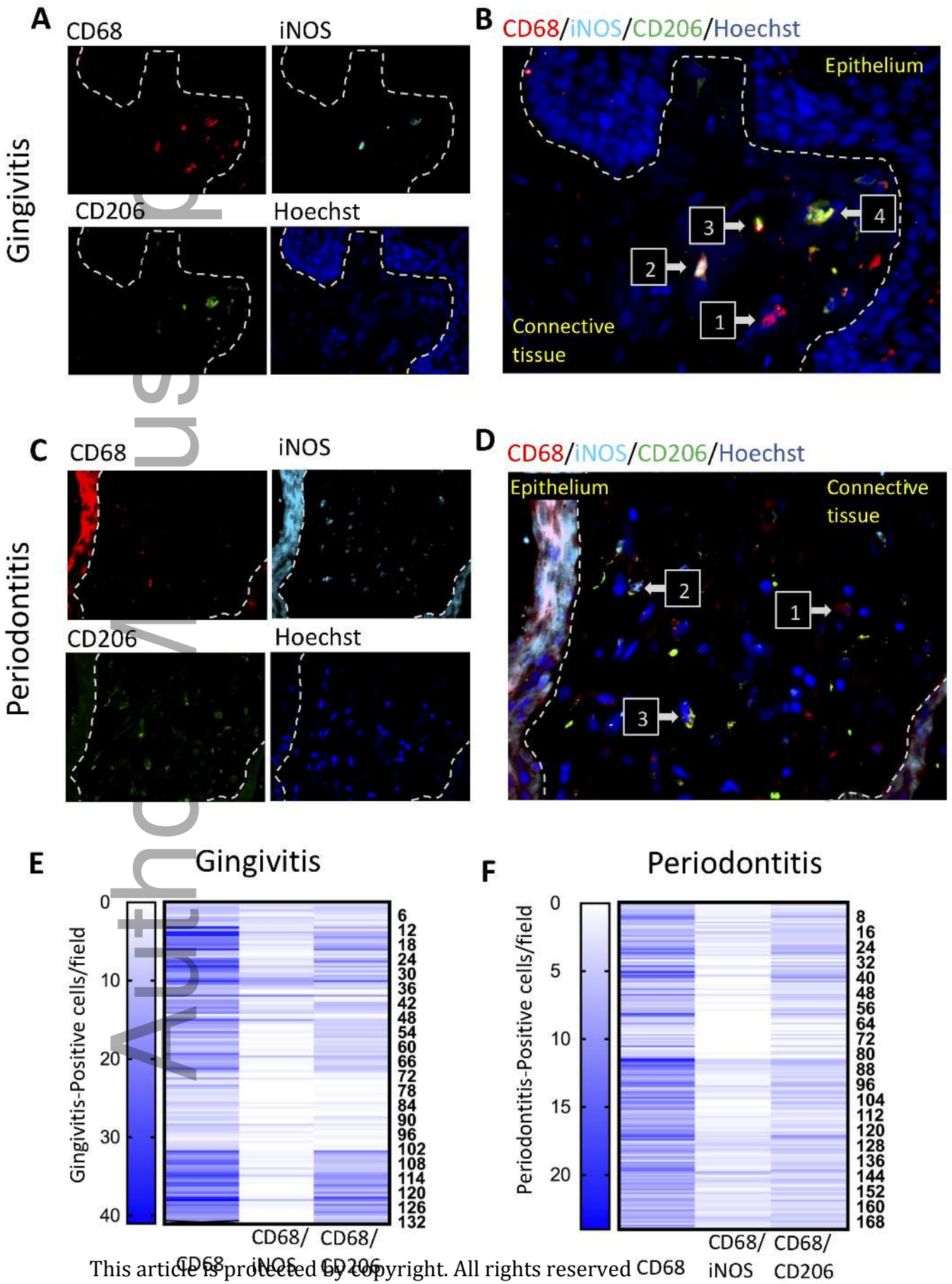

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Figure 3

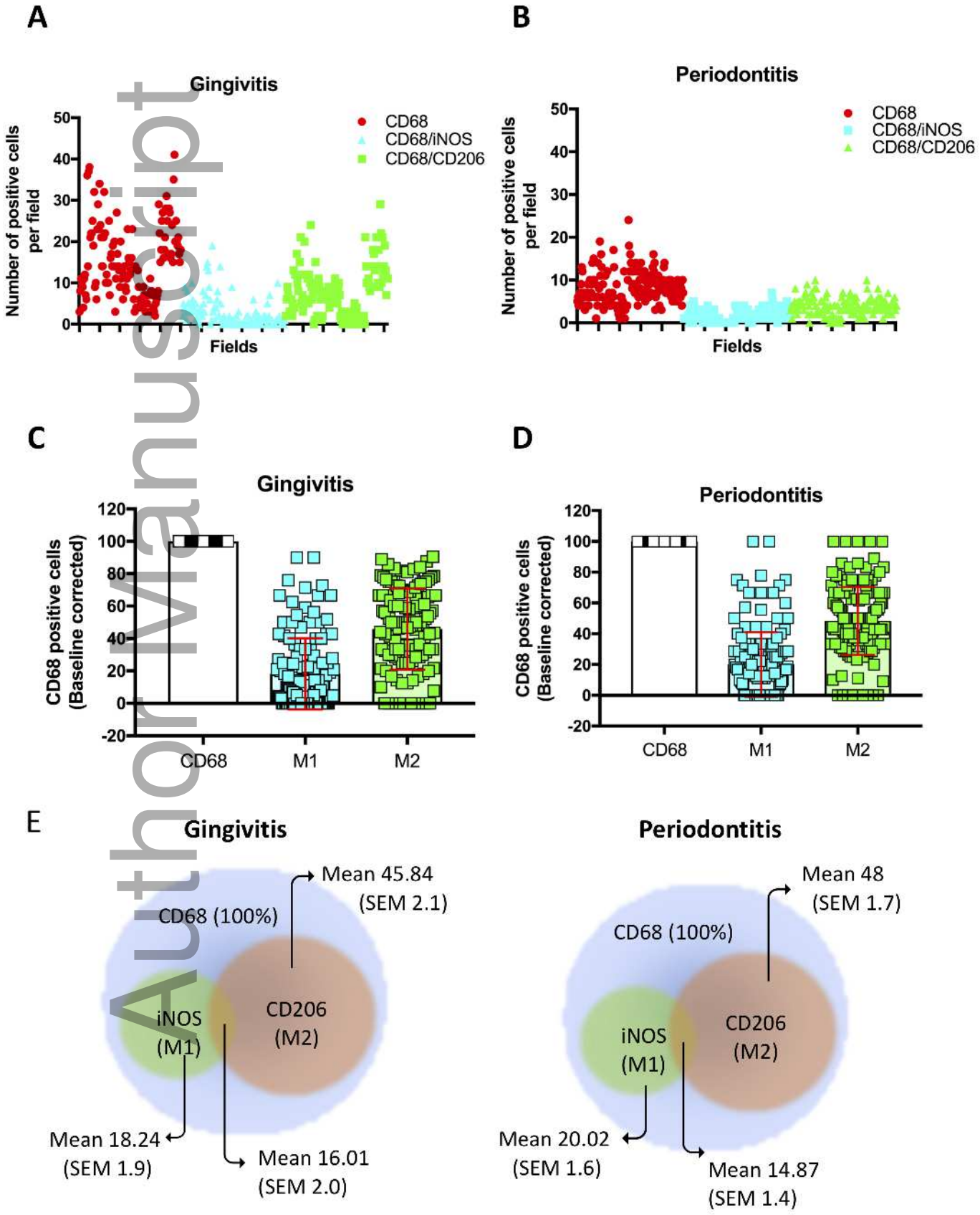

Figure 4