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Original article

Gingival crevicular fluid infiltrating CD14+ monocytes promote inflammation in periodontitis

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ABSTRACT

Periodontitis is a condition that occurs because of inflammation-mediated tissue degeneration. Many studies have been conducted to identify inflammatory molecules in periodontitis, but the well-defined role of cells from the immune system in the progression of periodontitis as well as in gingival tissue degeneration has not been appropriately established. The objective of the present study was to characterize the monocytes isolated from the gingival crevicular fluid (GCF) in patients with periodontitis. GCF was obtained from periodontitis patients and healthy controls. Cytokine levels of CCL2 were evaluated by ELISA in GCF samples. CD14+ monocytes were separated using magnetic sorting from GCF. RT-qPCR was performed to assess the gene expression. Cytometric bead array analysis was performed to analyze the levels of cytokines and chemokines in the secretome of cells. CD14+ monocytes from GCF secreted higher levels of CCL2 and showed elevated expression of genes responsible for monocyte migration. Additionally, upon lipopolysaccharide stimulation, these monocytes secreted higher levels of inflammatory cytokines and chemokines. This investigation aids in understanding the inflammatory microenvironment of periodontitis by characterizing GCF in terms of infiltrated CD14+ monocytes, cytokines, and molecules secreted by these monocytes, which are specific for cellular differentiation.

1. Introduction

Periodontitis is a periodontal disease with chronic inflammatory pathological conditions in tissues that support the dental complex, including soft and hard periodontal tissues (Kumar and Prakash, 2012; Cheng et al., 2016). It causes irreversible damage to the supporting bones of the teeth, which ultimately results in tooth loss (Kumar and Prakash, 2012; Gürkan et al., 2016; Zein Elabdeen et al., 2017). The bacterial colonies in the teeth trigger host immune responses in the surrounding periodontal tissues (Anil et al., 2013; Gunpinar et al., 2017). This further leads to the recruitment of cells producing inflammatory responses, and these cells secrete inflammatory molecules such as IL-8, TNF-α, CCL2, IL-6, and IL-1β (Cheng et al., 2016; Anil et al., 2013; Weber et al., 2000; Cros et al., 2010; Cardoso et al., 2018; Kapellos et al., 2019). This signaling cascade leads to the secretion of multiple chemokines and chemoattractants to recruit other cells of the immune system, resulting in the deterioration of the tissues of

Abbreviations: CCL2, C-C motif chemokine ligand 2; STAT2, Signal transducer and activator of transcription 2; CCR2, C-C chemokine receptor type 2; CD11b (ITGAM), Integrin alpha M; CCR1, C-C chemokine receptor type 1; CCR5, C-C chemokine receptor type 5; CXCR5/BLR1, C-X-C chemokine receptor type 5; IL-8, Interleukin 8; IL-6, Interleukin 6; CCL3, C-C motif chemokine ligand 3; CCL5, C-C motif chemokine ligand 5; IL-1β, Interleukin 1 beta; STAT6, Signal transducer and activator of transcription 6; TNF-α, Tumor necrosis factor-alpha; STAT1, Signal transducer and activator of transcription 1.

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the dental complex (Cheng et al., 2016; Zein Elabdeen et al., 2017; Ancuta et al., 2003). The gingival crevicular fluid (GCF) is a fluid system that is in constant contact with the periodontal tissue and the abrogation of its constituents, in terms of cells and secretory signaling, can result in local inflammation (Miyajima et al., 2014; Nagasawa et al., 2004; Pradeep et al., 2009; Wong et al., 2011). High levels of chemokines specific for monocytes have been reported in the GCF of periodontitis patients (Miyajima et al., 2014; Nagasawa et al., 2004). These chemokines regulate the secretion of pro-inflammatory cytokines (Pradeep et al., 2009; Wong et al., 2011; Kapellos et al., 2019; Sander et al., 2017; Carneiro et al., 2012). Moreover, these chemokines potentially activate basophils, which further induce degranulation of the tissue and result in the release of histamines (Zein Elabdeen et al., 2017; Meyle et al., 2017). This contributes to the progressive inflammatory responses experienced by patients with periodontitis.

The migration of CD14+ monocytes towards inflamed or injured tissues is known to create a long sustainable inflammatory microenvironment in many chronic inflammatory diseases, as the injured tissue has chemokine receptors, including CCR2, CXCR5, CCR5, and CCR1, which migrate along with CCL2 and CCL3 gradients, as well as secrete many inflammatory cytokines and chemokines such as CCL2, IL-8, IL-6, IL-1β, and TNF-α (Kornman et al., 2017; Gunpinar et al., 2017; Garlet, 2010). To understand the characteristics and inflammatory potential of GCF infiltrating monocytes of periodontitis origin in this study, we characterized the CD14+ monocytes that create an inflammatory microenvironment in GCF from periodontitis patients.

2. Materials and methods

2.1. Sample collection and ethical permissions

GCF was collected from periodontitis patients (n = 5) (Male: 2, Female: 3) (Age: 38–52 years) and healthy individuals (n = 5) (Male: 4, Female: 1) (Age: 36–45 years). Informed consent was obtained according to institutional ethical considerations. The samples were kept in a sterile container and transported to the laboratory for further experimentation.

2.2. ELISA for analysis of protein levels of CCL2 in GCF

CCL2 protein levels were analyzed using the KRIBIOLISA human ELISA kit (Krishgen Biosystems, Los Angeles, CA, USA). All the GCF samples were diluted 10 times with sterile phosphate buffered saline. The protocol set by the manufacturer were strictly adhered. The absorbance was read at 450 nm using a spectrophotometer (Multiskan FC, Thermo Scientific, San Jose, CA, USA).

2.3. Magnetic sorting and flow cytometry analysis of CD14+ cells

Adhereing to the manufacturers instructions, GCF-derived monocytes were subjected to magnetic sorting using CD14 (anti-human) MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) by positive selection method. The sorted CD14+ cells were labeled with monoclonal Anti-CD14-APC antibody (Miltenyi Biotec, Auburn, CA, USA), acquired on a flow cytometer to check the purity, and then seeded with complete media with DMEM (Invitrogen, Carlsbad, CA, USA + 10% FBS (Gibco, Rockville, MD, USA) for further experimentation.

2.4. Analysis of the gene expression with Real-time qPCR

Total RNA was extracted using GeneJet purification columns (Invitrogen, Thermo Scientific, Lithuania). cDNA synthesis (High Capacity, Applied Biosystems, Carlsbad, CA, USA) was used to reverse-transcribe 1 μg of the total RNA. Quantitative analyses of gene expression were performed using the SYBR Green PCR master mix (Applied Biosystems, Austin, TX, USA) on a QuantStudio 5 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Expression of the target genes (CCR2, CD11b, CCR1, CCR5, CXCR5, STAT1, STAT2, and STAT6) was normalized to β-actin using the ΔΔCt technique. The quantification of the data obtained by RT-PCR was performed by calculating $2^{-\Delta\Delta Ct}$ values. The list of primers is given in Table 1.

2.5. Cytometric bead array for the detection of cytokines and chemokines

Cytometric bead arrays were used to determine the levels of the selected cytokines and chemokines (IL-8, IL-6, CCL2, CCL3, CCL5, IL-1β, and TNF-α) in conditioned media. LEGENDplex Human Essential Immune Response Panel and Human Proinflammatory Chemokine Panel 1 (BioLegend, San Diego, CA, USA) were used for the detection of the cytokines and chemokines. Adhering to the protocol set by the manufacturer further experiments were performed. CD14+ cells were treated with 15 ng/mL lipopolysaccharide (LPS) (Sigma-Aldrich, Taufkirchen, Germany), and after incubation for 24 h, the cell culture supernatant was analyzed for selected cytokine and chemokine levels. Briefly, 25 μL of the cell culture supernatant was incubated with microbeads for 2 h. After the incubation, the detection antibodies were introduced subsequently to the tests and incubated for 30 min. A wash buffer was used to wash the samples, which was followed by a 5 min centrifugation at 2000 rpm. Following removal of the supernatant, resuspension of the rellet was carried out in 200 μL of sheath fluid. The samples were then acquired on a flow cytometer (Attune NxT, Thermo Fisher Science, Waltham, MA, USA), and analysis was performed using the LEGENDplex Data Analysis Software (BioLegend, San Diego, CA, USA).

2.6. Statistical analysis

The data generated from the three independent experimental values are depicted as mean ± standard deviation. GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA) was used to perform the unpaired *t*-test (two-tailed) analysis of the data for each cytokine. Data was designated to be significant for $^{*}p < 0.05$, and $^{**}p < 0.01$.

Table 1
List of primers.

Gene	Forward primer	Reverse primer
CCR2	5′-CAG GTG ACA GAG ACT CTT GGG A-3′	5′-GGC AAT CCT ACA GCC AAG AGC T-3′
CD11b (ITGAM)	5′-GGA ACG CCA TTG TCT GCT TTC G-3′	5′-ATG CTG AGG TCA TCC TGG CAG A-3′
CCR1	5′-CAA CTC CGT GCC AGA AGG TGA A-3′	5′-GTT CAG GAG GTA GAT GCT GGT C-3′
CCR5	5′-TCT CTT CTG GGC TCC CTA CAA C-3′	5′-CCA AGA GTC TCT GTC ACC TGC A-3′
CXCR5/ BLR1	5′-TGA AGT TCC GCA GTG ACC TGT C-3′	5′-GAG GTG GCA TTC TCT GAC TCA G-3′
STAT1	5′-CTA GTG GAG TGG AAG CGG AG-3′	5′-CAC CAC AAA CGA GCT CTG AA-3′
STAT2	5′-TTT TGG GTG GTC GAA CG-3′	5′-AAA AAC AAC GCC AAA CCG-3′
STAT6	5′-AGT CAC TAT AAG CCC GAA CAG-3′	5′-GCC ATT CCA AGA TCA TAA GGT-3′
GAPDH	5′-GTC TCC TCT GAC TTC AAC AGC G-3′	5′-ACC ACC CTG TTG CTG TAG CCA A-3′

3. Results

3.1. Higher CCL2 concentration and CD14+ cells percentage in GCF from periodontitis patients

ELISA and flow cytometry results showed that GCF samples from periodontitis patients had a higher presence of CCL2, a chemoattractant chemokine for migrating monocytes (Fig. 1A). The percentage of CD14+ was also high in GCF samples originating from periodontitis, ranging from 6 to 25% (Fig. 1B–H).

3.2. CD14+ sorted cells from GCF of periodontitis express genes responsible for characteristic migratory markers of monocytes

GCFs were pooled from five samples of periodontitis patients and normal donors, and CD14+ cells were sorted using magnetic cell sorting, and the results showed that the cells were enriched to nearly 80% (Fig. 2A and B). Real-time PCR results showed that sorted CD14+ cells expressed mRNA of CCR2, CD11b, CCR1, CCR5, and CXCR5, which are characteristic markers of migrating monocytes (Fig. 2C–G).

3.3. CD14+ sorted cells from the GCF of periodontitis patients secrete inflammatory cytokines and chemokines when challenged with LPS

Sorted CD14+ cells were incubated for 24 h with the treatment of 15 ng/mL of LPS. The culture supernatant was analyzed for IL-8, IL-6, CCL2, CCL3, CCL5, IL-1β, and TNF-α using a flow cytometry-based bead array system. The results showed that sorted CD14+ monocytes secreted a significantly higher amount of all inflammatory molecules compared to CD14− cells (Fig. 3A–G).

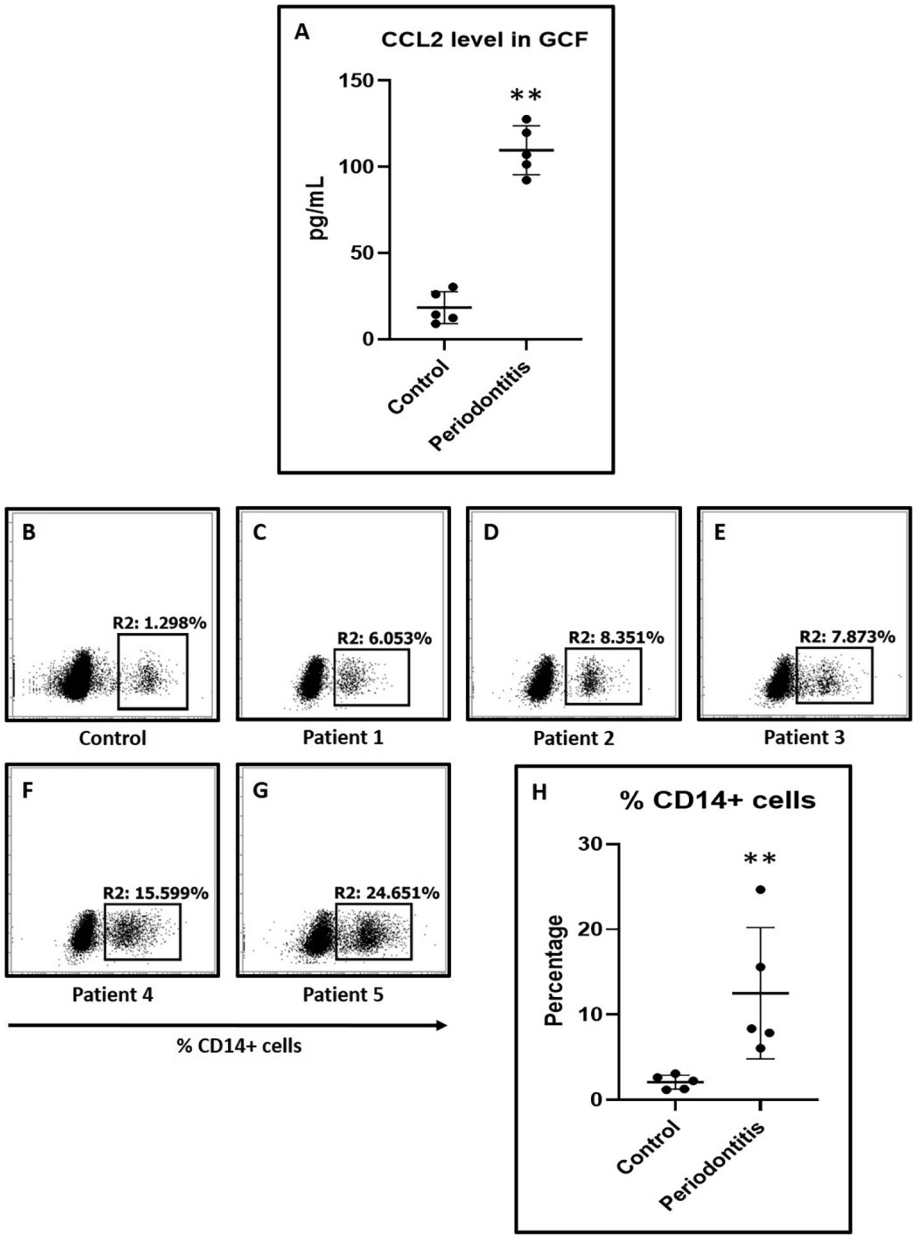

Fig. 1. CCL2 levels in GCF and Isolation of CD14+ monocytes. (A) ELISA assay was performed to assess the protein levels of CCL2 in GCF from normal subjects and periodontitis patients. (B–H) Flow Cytometry based characterization of CD14+ cells from GCF and comparative analysis of CD14+ cells in GCF from normal subjects and periodontitis patients. ns not significant, *p < 0.05, **p < 0.001. CCL2: C–C motif chemokine ligand 2.

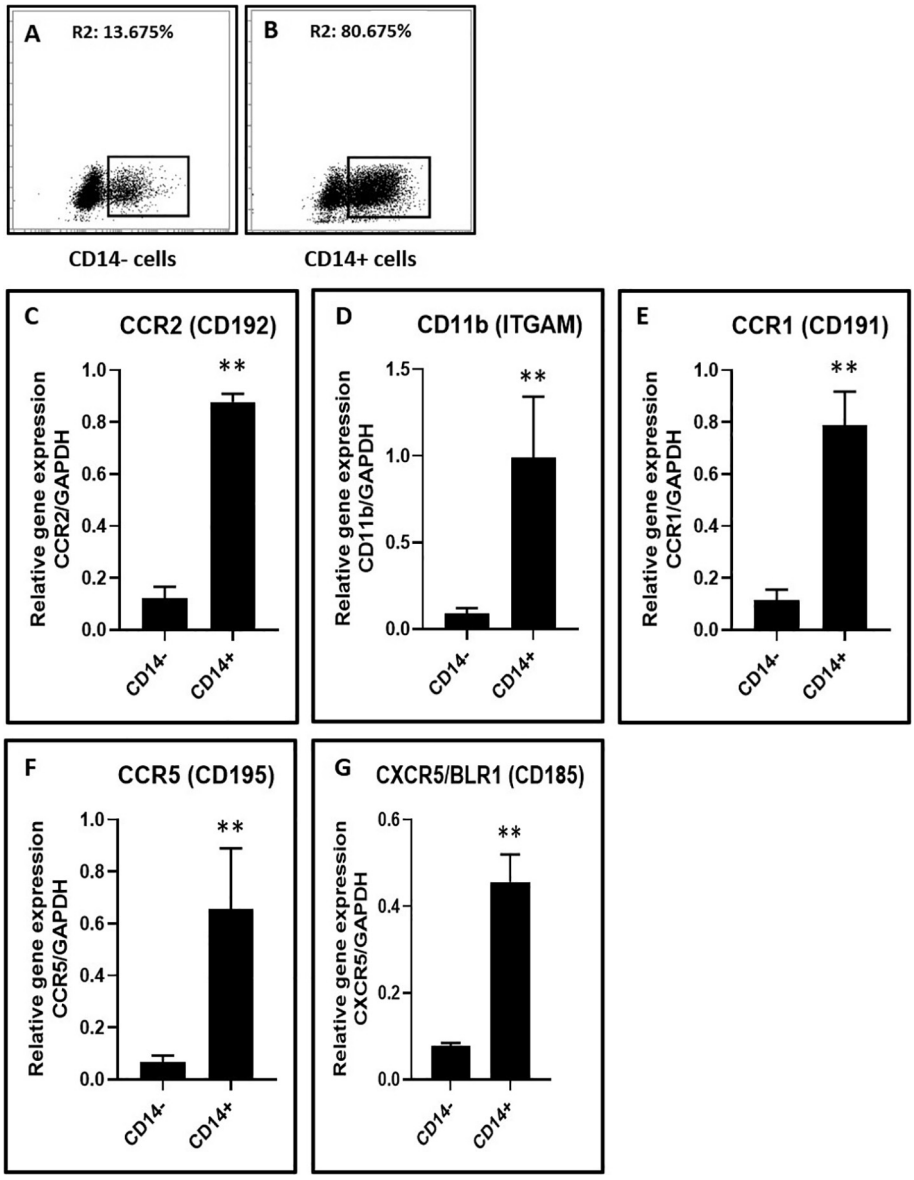

Fig. 2. Flow Cytometry analysis of sorted CD14+ monocytes and relative gene expression of chemokine receptors in the sorted CD14+ cells. (A and B) Percentage of CD14+ cells in the sorted and unsorted cells. (C-G) Relative gene expression analysis for chemokine receptors in the CD14− and CD14+ cells. ns not significant, $^{*}p < 0.05$, $^{**}p < 0.001$. CCR2: C-C chemokine receptor type 2, CD11b (ITGAM): Integrin alpha M, CCR1: C-C chemokine receptor type 1, CCR5: C-C chemokine receptor type 5, CXCR5: C-X-C chemokine receptor type 5.

3.4. CD14+ sorted cells from GCF of periodontitis patients express slightly higher levels of M1 type of macrophage-specific transcription factor genes

The sorted CD14+ cells from pooled GCF showed slightly higher expressions of STAT1 and STAT2 transcription factor genes (Fig. 4A and B) which are M1 macrophage-specific in comparison to the STAT6 gene which is M2 specific transcription factor (Fig. 4C). This difference in gene expression indicates that monocyte infiltrating the GCF in periodontitis is possible predetermined to differentiate in an M1 macrophage biased way.

4. Discussion

Periodontitis is a significant pathological condition in the class of chronic diseases worldwide (Kumar and Prakash, 2012; Kapellos et al., 2019). It is an inflammatory condition that arises from microbial dysbiosis and a broad spectrum of cells and signaling molecules from the innate and adaptive immune responses contribute to its occurrence (Gürkan et al., 2016; Kramer and Genco, 2017). This inflammatory microenvironment is similar to that of other chronic inflammatory diseases and ultimately causes erosion and loss of soft periodontal tissue and, in worse cases, periodontal bone tissue (Sander et al., 2017; Carneiro et al., 2012). The periodontal inflammatory microenvironment mainly consists of pro-inflammatory cytokines, including IL-1β, TNF-α, IL-6, IL-17, and IL-8, which are secreted by cells such as monocytes, macrophages, and other cells of the adaptive and innate immune system (Carneiro et al., 2012). Many targeted approaches are being tested by various research groups to use the information of the inflammatory microenvironment to resolve the pathology of periodontitis (Ancuta et al., 2003; Meyle et al., 2017).

The migration of classical CD14+ monocytes in injured or inflamed tissues is a significant aspect of the creation of an

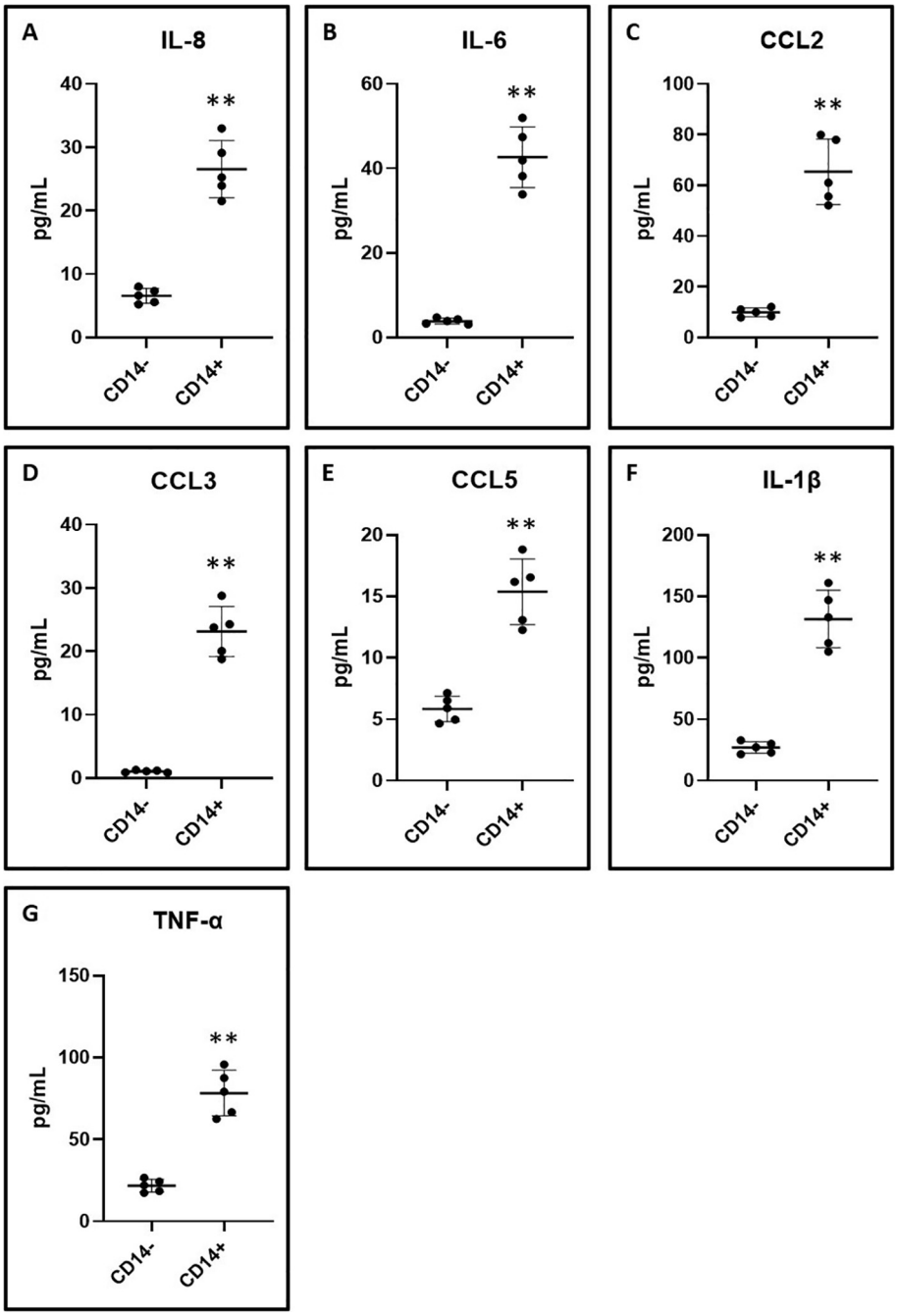

Fig. 3. Cytometric bead array for the quantification of cytokines secreted by CD14− and CD14+ cells cultured *in vitro*. (A-G) Comparative analysis of cytokines in conditioned media from CD14− cells and CD14+ cells. ns not significant, *$p < 0.05$, **$p < 0.001$. IL-8: Interleukin 8, IL-6: Interleukin 6, CCL2: C–C motif chemokine ligand 2, CCL3: C–C motif chemokine ligand 3, CCL5: C–C motif chemokine ligand 5, IL-1β: Interleukin 1 beta, TNF-α: Tumor necrosis factor alpha.

inflammatory microenvironment (Gunpinar et al., 2017). Classical CD14+ monocytes show chemotactic movement along the gradient of chemokine CCL2 (Garlet, 2010; Kramer and Genco, 2017; Liu et al., 2016) and express chemokine receptors, such as CCR2, CCR1, CCR5, CXCR1, and CXCR2, which are responsible for monocyte migration (Garlet, 2010; Liu et al., 2016). These monocytes also have the potential to differentiate into macrophages in the inflamed tissue (Serbina et al., 2009). Periodontitis is also a pathological condition with a highly pro-inflammatory microenvironment. To understand the role of monocytes in this particular pathology, in this study, we characterized the monocytes in GCF from periodontitis patients and their potential to participate in inflammation.

As a first step, when we compared the levels of CCL2 ligand, which is a chemoattractant to CD14+ CCR2+ monocytes, it was observed that the levels of CCL2 were significantly higher in the GCF from periodontitis patients than in normal GCF, which is a clear indicator that GCF from periodontitis has a prerequisite signaling environment for the migration of monocytes (Fig. 1A). Furthermore, the healthy and periodontitis GCF were compared in terms of the percentage of CD14+ cells, and it was found that GCFs from periodontitis origin had significantly higher percentages of CD14+ monocytes ranging from 6 to 25% of the total single-cell suspension in GCF (Fig. 1B–H). Both findings regarding high levels of CCL2 and a high percentage of CD14+ cells indicate that GCF contains a perfect milieu for

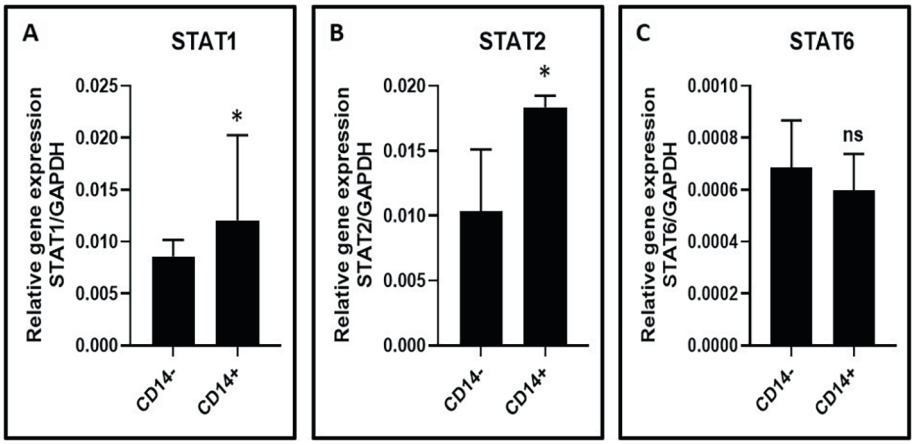

Fig. 4. Relative gene expression of M1 and M2 macrophage differentiation-related transcription factors in GCF-infiltrated monocytes. (A and B) Relative gene expression analysis for STAT1 and STAT2 which are highly expressed during M1 macrophage differentiation than M2 macrophage differentiation. (C) Relative gene expression analysis for STAT6 which is highly expressed during M2 macrophage differentiation than M1 macrophage differentiation. ns not significant, $^{*}p < 0.05$, $^{**}p < 0.001$. STAT1: Signal transducer and activator of transcription 1, STAT2: Signal transducer and activator of transcription 2, STAT6: Signal transducer and activator of transcription 6.

migration and a comfortable stay of infiltrating monocytes in GCF.

GCFs from five periodontitis patients were pooled and CD14+ cells were sorted using the magnetic cell sorting system (MACS). When compared for gene expression, sorted cells showed significantly higher mRNA expression of CCR2 (Fig. 2C), which is the receptor of CCL2, clearly indicating that cells use the common but classical signaling of the CCL2-CCR2 axis for migration in GCF. Sorted cells were also found to have a significant expression of CD11b, CCR1, CCR5, and CXCR5 (Fig. 2D–G), which are the characteristic markers of migrating monocytes towards inflamed or injured tissues, as mentioned above. Sorted cells were stimulated with LPS, and the next day, a flow cytometry-based multiplex bead array based quantification of inflammatory molecules (IL-8, IL-6, CCL2, CCL3, CCL5, IL-1β, and TNF-α) was carried out in the supernatant. We observed that sorted cells had a significantly higher release of inflammatory molecules in comparison to the CD14− set of cells (Fig. 3A–G). Findings regarding mRNA expression analysis and inflammatory molecule release strongly suggest that GCF-migrating CD14+ monocytes have great potential to create a strong and sustainable inflammatory microenvironment in periodontitis patient GCF. Finally, we have observed that the monocyte infiltrating the GCF of periodontitis has a characteristic slight difference in M1 macrophage-specific transcription factors STAT1 and STAT2. This expression towards M1 transcription factors is the indicator of the fact that even before the differentiation CD14+ cells are possibly biased to go towards the inflammatory M1 macrophage system which should be investigated further.

5. Conclusion

From the current study, we can conclude that GCF from periodontitis patients creates a conducive milieu for the attraction and migration of CD14+ monocytes, which express the characteristic markers of the classical category of monocytes capable of secreting inflammatory molecules. These monocytes should be further investigated for their potential to differentiate into macrophages in the presence of GCF and whether they show M1/M2 macrophage bias.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None.

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