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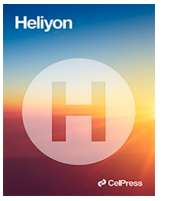

Immunomodulatory differences between mesenchymal stem cells from different oral tissues

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ABSTRACT

Mesenchymal stem cells (MSCs) have recently been identified as having potentially therapeutic immunomodulatory properties. MSCs isolated from different oral tissues have similar morphology and immunophenotypes, however, direct comparisons of their gene expression and immunomodulatory properties have not been conducted. We isolated alveolar bone-derived MSCs (aBMSCs), dental pulp stem cells (DPSCs) and gingiva-derived MSCs (GMSCs) from the same patients and compared their immunophenotypes and transcriptomes. Additionally, we compared their production of soluble immunomodulatory cytokines as well as their immunoregulatory properties in coculture with THP-1 human monocytic cells. RNA sequencing revealed distinct gene expression in DPSCs while aBMSCs and GMSCs had less differentially expressed genes. DPSCs also had significantly less secretion of osteopontin compared to aBMSCs and GMSCs. Finally, DPSCs did not exhibit an immunosuppresive effect on THP-1 cells to the same degree as aBMSCs and GMSCs. These findings demonstrate that MSCs from different oral tissues have distinct transcriptomes and immunoregulatory properties.

1. Introduction

Mesenchymal stem cells (MSCs), first characterized from bone marrow of the iliac crest (bone marrow stromal/stem cells, or BMSCs) are self-renewing, multipotent cells which have been well-studied for their tissue healing, reparative, and regenerative properties [1–6]. A number of other tissues have been identified as robust sources of MSCs, including adipose tissue (ASCs) [7], umbilical cord [8,9], and placenta [10], but even more recently, MSCs have been isolated from oral/dental tissues including dental pulp (dental pulp stem cells, DPSCs), gingiva (gingival MSCs, GMSCs), periodontal ligaments [11], and alveolar bone (aBMSCs) [12–14].

MSCs isolated from these various tissue sources have similarities in morphology and immunophenotype and are capable of differentiation into osteoblasts, adipocytes and chondroblasts *in vitro* [15]. Unfortunately, these criteria alone do not capture the diversity of MSCs from different tissues, particularly as it relates to their more recently identified immunomodulatory properties. BMSC and ASC immunomodulatory properties have been directly compared and have shown that allogeneic ASCs reduce immunoglobin production in peripheral blood mononuclear cells (PBMCs) to a greater extent than BMSCs [16]. Additionally, comparisons of BMSCs and ASCs have shown that relative to BMSCs, ASCs exhibit greater inhibition of the differentiation and maturation of human monocytes into dendritic

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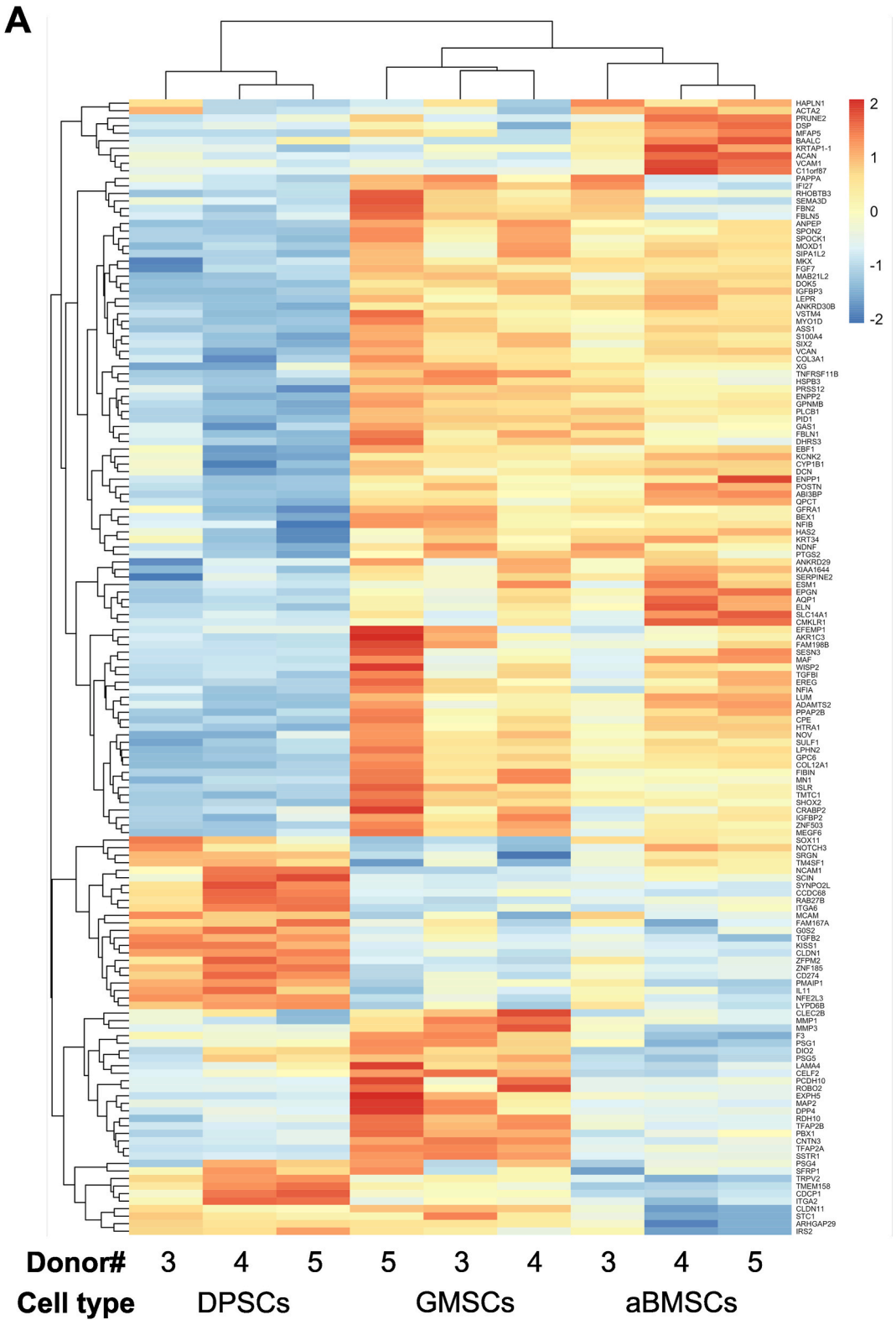

Fig. 1. DE genes in aBMSCs, DPSCs, and GMSCs. RNA was extracted from aBMSCs, DPSCs, and GMSCs at 70 − 80 % confluency, and sequenced for comparison of gene expression. (A) A heat map of the 151 genes that were DE in at least one comparison between aBMSCs, DPSCs, and GMSCs and with SD > 1. (B–G) Analyses by iPathwayGuide. (B) Venn diagram of the DE genes in the comparisons DPSCs vs. aBMSCs (in pink), GMSCs vs. aBMSCs (in yellow), and GMSCs vs. DPSCs (in blue). (C–E) Volcano diagrams of the DE genes in the comparisons DPSCs vs. aBMSCs (C), GMSCs vs.

aBMSCs (D), and GMSCs vs. DPSCs (E). (F) Venn diagram of the DE genes in the comparisons GMSCs vs. others (in pink), DPSCs vs. others (in yellow), and aBMSCs vs. others (in blue). (G) Expression levels of DE gene in the comparisons DPSCs vs. aBMSCs (in pink), GMSCs vs. aBMSCs (in yellow), and GMSCs vs. DPSCs (in blue) that are involved in the pathway "cytokine-cytokine receptor interactions" shown in log2 (fold change) (logFC). For additional information on RNA sequencing, see Data S1–S3.

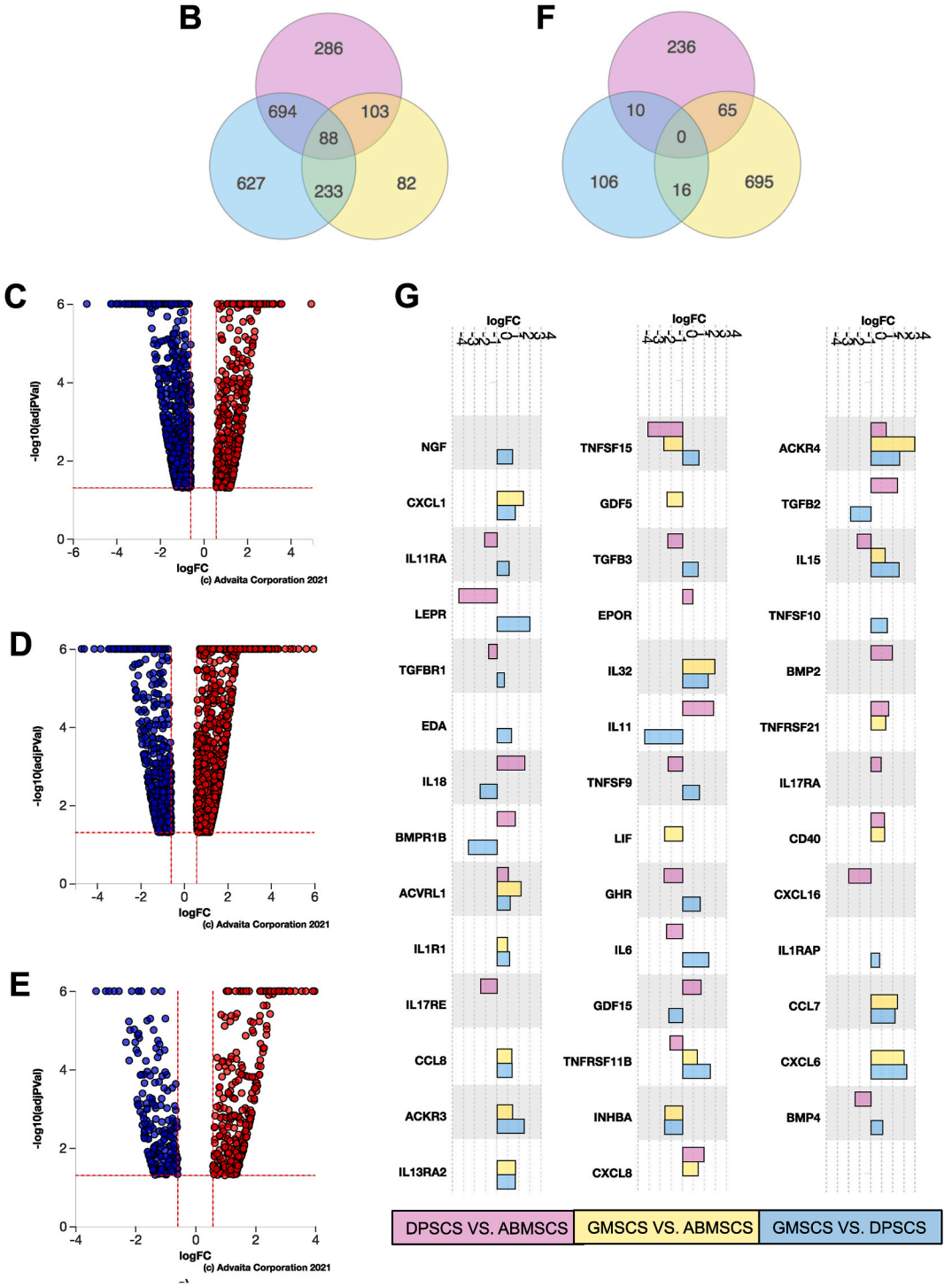

Fig. 1. (*continued*).

cells (DCs) and they produce more immunomodulatory cytokines such as interleukin-6 (IL-6) and transforming growth factor-β1 (TGF-β1) [17]. Conversely, BMSCs have been shown to inhibit allogeneic T cell proliferation to a greater extent than ASCs and express higher levels of interleukin-10 (IL-10) [17].

There are currently no studies comparing the immunomodulatory properties of MSCs derived from different oral-derived tissues, namely dental pulp (DPSCs), gingiva (GMSCs), and alveolar bone (aBMSCs). Considering that oral tissues are highly accessible and can provide abundant sources of MSCs, studies are needed to better understand the molecular properties that underlie differences in their immunological and immunomodulatory properties. In this study, we investigated these differences in human MSCs (from the same patients) derived from different oral tissues and the findings show that despite equivalence in their immunophenotypes, MSCs from these different oral tissues exhibit differences in their transcriptomes and immunomodulatory properties.

2. Results

2.1. aBMSCs, DPSCs, and GMSCs differentially expressed genes

aBMSCs, DPSCs, and GMSCs that we isolated exhibited the same immunophenotype as described by Dominici et al. [15], *i.e.*, stained positive for Von Kossa, Alcian Blue, and Oil Red O as proof of osteogenic, chondrogenic and adipogenic differentiation, respectively (Supplementary Fig. S1), and all achieved >93 % triple positive for CD 73, CD 90, and CD105 by flow cytometry (Supplementary Table S1 and Supplementary Fig. S2). However, expression of 17,085 genes was compared between them and showed there were 1176 differentially expressed (DE) genes between aBMSCs and DPSCs (Data S1), 512 DE genes between aBMSCs and GMSCs (Data S2), and 1659 DE genes between DPSCs and GMSCs (Data S3). Fig. 1a shows the top DE genes, *i.e.*, 151 genes DE in at least one comparison and with standard deviation (SD) greater than 1. iPathwayGuide [18] was then used in one to one comparisons and showed the following: a total of 1171 genes were DE in DPSCs vs. aBMSCs (Fig. 1B and C) and 1642 DE genes in GMSCs vs. DPSCs (Fig. 1B and D), whereas only 506 genes were DE in GMSCs vs. aBMSCs (Fig. 1B and E). Among the three types of MSCs, DPSCs are the most distinctive because 695 genes were recognized as DE only in DPSCs vs. the other two cell types, whereas the DE genes in aBMSCs only and GMSCs only were 106 and 236, respectively (Fig. 1F). Furthermore, 16 genes were DE in both aBMSCs and DPSCs (Fig. 1F), yet the expression levels were opposite, *i.e.*, genes expressed at significantly higher levels in aBMSCs were expressed at significantly lower levels in DPSCs, and vice versa. This reciprocal phenomenon was also seen for 65 DE genes among GMSCs and DPSCs. Interestingly, the 10 DE genes in both aBMSCs and GMSCs were more highly expressed in GMSCs than in aBMSCs.

iPathwayGuide computes *p*-values for KEGG pathways using the Impact Analysis method [18]. *p*-values for signaling pathways are calculated by combining the over-representation *p*-value and a *p*-value for the overall perturbation of a given pathway due to the connections of the DE genes in the pathway graph. Certain pathways (*e.g.*, metabolic pathways) are not amenable to the perturbation calculation, so those *p*-values were determined using only over-representation analysis. In the DPSCs-vs.-aBMSCs comparison, the top 5 impacted pathways are "AGE (advanced glycation end product)-RAGE (receptor for AGE) signaling pathway in diabetic complications", "ECM (extracellular matrix)-receptor interaction", "cell adhesion molecules", "protein digestion and absorption", and "cytokine-cytokine receptor interaction" (Table 1). The latter four pathways are also among the top 5 impacted pathways in the GMSCs-vs.-DPSCs comparison with the addition of "protein digestion and absorption" (Table 1). In the GMSCs-vs.-aBMSCs comparison, only "cytokine-cytokine receptor interaction" remains in the list of top 5 of most impacted pathways, whereas the other 4 are "complement and coagulation cascades", "PI3K (phosphoinositide 3-kinase)-Akt (also known as protein kinase B) signaling pathway", "viral protein interaction with cytokine and cytokine receptor", and "chemokine signaling pathway" (Table 1). Hence,

Table 1
Top 5 most impacted pathways between aBMSCs, DPSCs, and GMSCs.

Comparison	Most Impacted Pathways	*p*-value (FDR)	Count (DE/All)
DPSCs vs. aBMSCs	ECM-receptor interaction	1.424×10^{-4}	19/53
	AGE-RAGE signaling pathway in diabetic complications	4.184×10^{-4}	25/88
	Cell adhesion molecules[a]	5.971×10^{-4}	25/79
	Protein digestion and absorption	5.971×10^{-4}	17/47
	Cytokine-cytokine receptor interaction	0.001	26/117
GMSCs vs. aBMSCs	Cytokine-cytokine receptor interaction	7.384×10^{-5}	19/125
	Complement and coagulation cascades	7.236×10^{-4}	10/39
	PI3K-Akt signaling pathway	0.001	25/251
	Viral protein interaction with cytokine and cytokine receptor	0.001	7/38
	Chemokine signaling pathway	0.001	13/121
GMSCs vs. DPSCs	ECM-receptor interaction	4.839×10^{-4}	22/55
	Cytokine-cytokine receptor interaction	4.839×10^{-4}	31/121
	Complement and coagulation cascades	4.839×10^{-4}	16/37
	Cell adhesion molecules[a]	4.839×10^{-4}	27/80
	Protein digestion and absorption[a]	4.839×10^{-4}	20/49

The *p*-value corresponding to the pathway was computed using only over-representation analysis. aBMSCs, alveolar bone marrow-derived mesenchymal stem cells. AGE, advanced glycation end product. DE, differentially expressed. DPSCs, dental pulp stem cells. ECM, extracellular matrix. FDR, false discovery rate. GMSCs, gingival mesenchymal stem cells. PI3K, phosphoinositide 3-kinase. RAGE, receptor for advanced glycation end product.

"cytokine-cytokine receptor interaction" is a major pathway where aBMSCs, DPSCs, and GMSCs differ, and Fig. 1G shows these DE genes.

2.2. aBMSCs, DPSCs, and GMSCs release of inflammatory and immunomodulatory cytokines

Being that cytokine-cytokine receptor interaction is one of most impacted pathways in all comparisons between aBMSCs, DPSCs, and GMSCs, soluble production of 80 inflammatory and immunomodulatory growth factors and chemokines was measured. The secretome analysis determined that though most of the targeted cytokines were not highly produced by aBMSCs, DPSCs, or GMSCs, appreciable levels of osteoprotegerin (OPG; also designated as osteoclastogenesis inhibitory factor or tumor necrosis factor receptor superfamily member 11B/TNFRSF11B), tissue inhibitors of metalloproteinase (TIMP)-1 and 2, and insulin-like growth factor binding protein (IGFBP)-4 (Fig. 2a) were found. Quantification of these cytokines by ELISA showed that there was no statistically significant difference in the secretion of TIMP-1, and IGFBP-4 between aBMSCs, DPSCs, and GMSCs (Fig. 2B–D). However, aBMSCs released

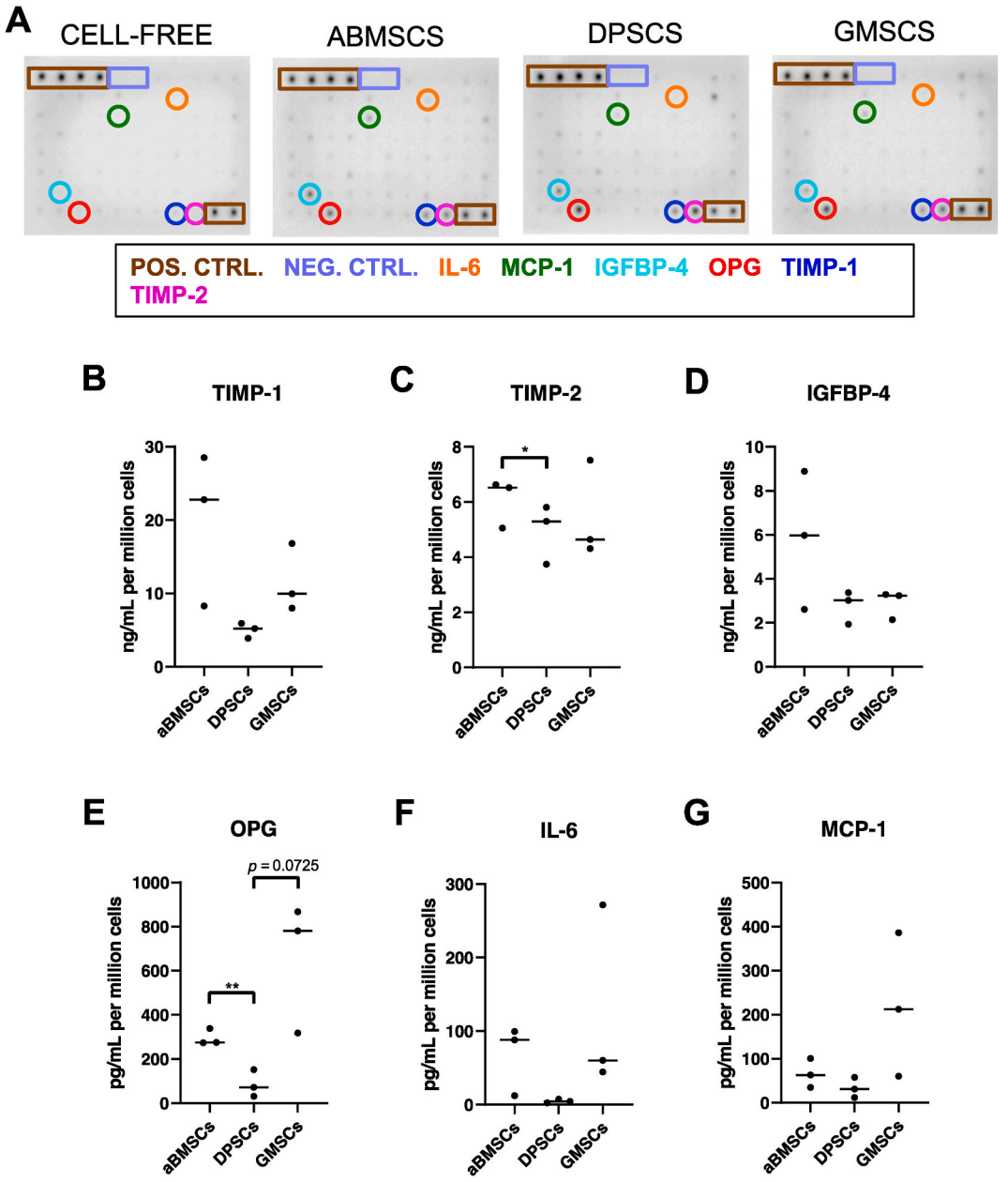

Fig. 2. Cytokines released from aBMSCs, DPSCs and GMSCs. aBMSCs, DPSCs and GMSCs were cultured in basal medium for 24 h, and the CM were analyzed by a cytokine array (A) as well as ELISA of indicated targets (B–G). *, $p < 0.05$; **, $p < 0.01$; $n = 3$ in each group.

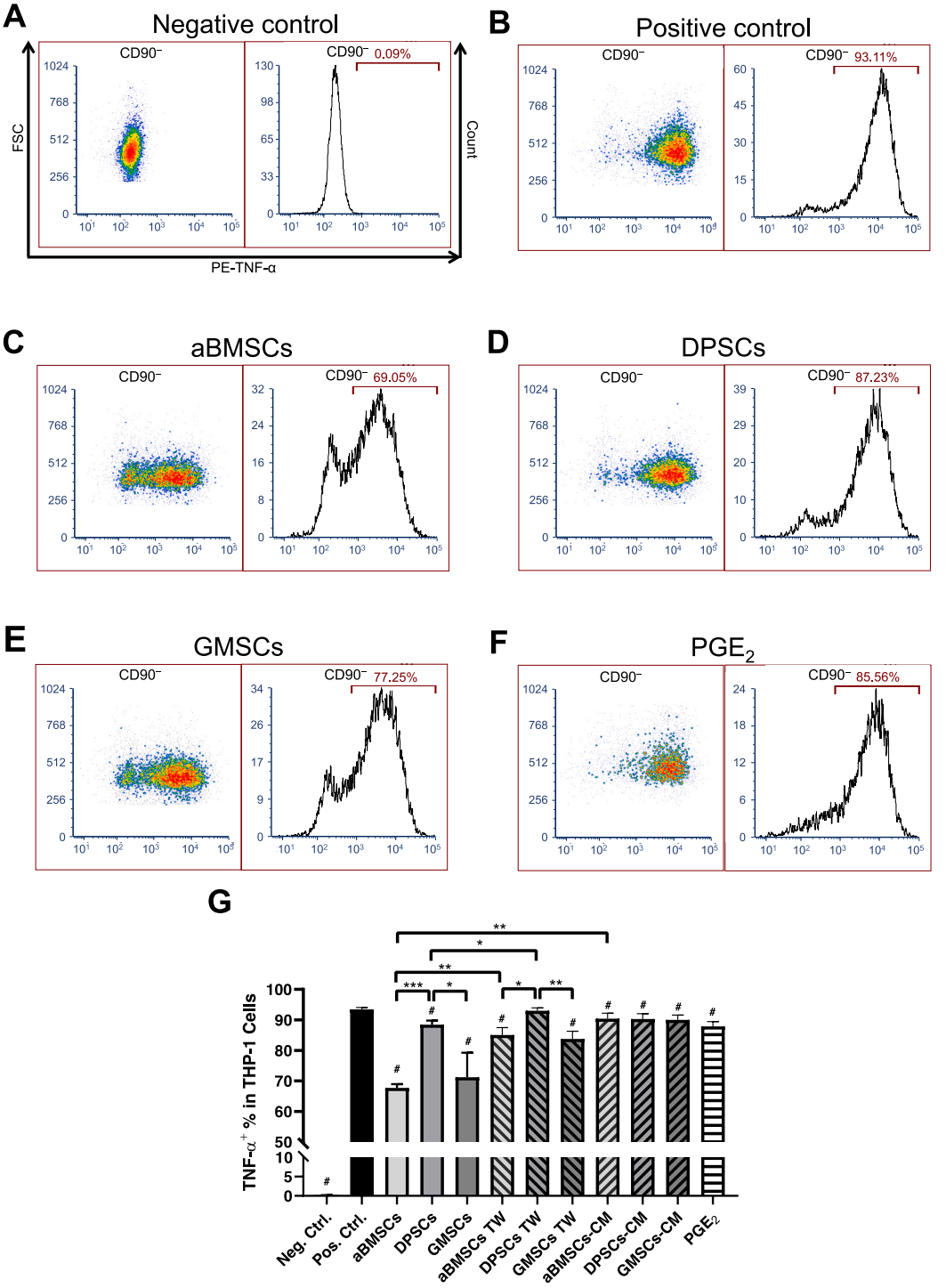

Fig. 3. aBMSCs, DPSCs, and GMSCs induced inhibition on TNF-α expression in **THP-1 cells in response to LPS stimulation.** THP-1 cells were cultured in the presence or absence of aBMSCs, DPSCs, or GMSCs in direct contact or in Transwell inserts for 72h, followed by 4-h treatment of 1 μg/mL LPS and 5 μg/mL brefeldin A. The non-adherent cells were collected and analyzed by flow cytometry using PerCP/Cy5.5-CD90 and PETNF-α antibodies. (A–F) Representative flow cytometric density plots and histograms of CD90− cells (THP-1 cells). (A) Naive THP-1 cells without LPS stimulation (negative control; $n = 12$). (B) THP-1 cells cultured alone and treated with LPS (positive control; $n = 13$). (C–D) THP-1 cells cocultured with aBMSCs (C), or DPSCs (D) or GMSCs (E) and treated with LPS ($n = 4$). (F) THP-1 cells cultured alone in the present of 100 nM PGE2 and treated with LPS ($n = 4$). (G) Quantification of TNF-α+ THP-1 cells in CD90−population of indicated treatment groups. #, $p < 0.0001$ comparing to positive control (Pos. Ctrl.) using unpaired *t*-test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; $n = 3$ in the groups not mentioned above.

significantly more TIMP-2 and OPG than DPSCs ($p = 0.0175$ and $p = 0.00065$, respectively; Fig. 2C–E) and GMSCs produced higher levels of OPG compared to DPSCs ($p = 0.0725$; Fig. 2E). These results are in alignment with the RNA sequencing data, which showed that aBMSCs and GMSCs had significantly higher expression of OPG relative to DPSCs (Data S1 and S2), while OPG mRNA levels were significantly higher in GMSCs than in aBMSCs (Data S3).

We previously reported that aBMSCs secreted low levels of IL-6 and MCP-1 under such basal conditions [19], and RNA sequencing results suggested that aBMSCs and GMSCs have significantly higher expression of *IL6* relative to DPSCs (Data S1 and S2); as such, their levels in the CM of DPSCs and GMSCs was also evaluated quantitatively using ELISA. The results showed that while IL-6 and MCP-1 were not highly produced by any of these cell populations, DPSCs had the lowest level of production (Fig. 2F–G).

2.3. aBMSCs, DPSCs and GMSCs immunomodulation of THP-1 monocytic cells

The immunosuppressive properties of aBMSCs, DPSCs, and GMSCs were evaluated in co-cultures with THP-1 monocytic cells, as we have previously described [19]. Naive THP-1 cells did not express the canonical pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) (0.185 ± 0.159 %; Fig. 3A–G). After stimulation with 1 μg/mL lipopolysaccharide (LPS) from *Escherichia coli* (*E. coli*) for 4 h, 93.455 ± 0.612 % THP-1 cells expressed TNF-α (Fig. 3B–G). aBMSCs, DPSCs, and GMSCs all produced an immunomodulatory effect on THP-1 cells in reducing the percentages of TNF-α-expressing THP-1 cells to 67.700 ± 1.253 % ($p < 0.0001$; Fig. 3C–G), 88.415 % ± 1.378 % ($p < 0.0001$; Fig. 3D–G), and 71.198 ± 8.046 % ($p < 0.0001$; Fig. 3E–G), respectively. However, DPSCs were significantly less potent in inhibiting expression of TNF-α in THP-1 cells relative to aBMSCs and GMSCs ($p < 0.0001$ and $p < 0.01$, respectively; Fig. 3G). Comparable inhibitory effects were observed when aBMSCs and GMSCs were co-cultured with THP-1 cells without cell-cell contact, although to a lesser degree (Fig. 3G). These data suggest that soluble factors are at least partially responsible for the immunosuppressive properties of aBMSCs and GMSCs. By contrast, DPSCs co-cultured with THP-1 cells without cell contact did not yield an immunomodulatory effect on THP-1 cells as measured by any reduction in the TNF-α-expressing THP-1 population (Fig. 3G).

2.4. Production of PGE_2 from aBMSCs, DPSCs, and GMSCs in coculture with THP-1 cells after LPS stimulation

PGE_2 is as an important immunomodulatory factor and addition of exogenous PGE_2 to THP-1 cultures significantly reduces the percentage of TNF-α expressing THP-1 cells to 87.888 ± 1.555 % ($p < 0.0001$; Fig. 3F–G). As such, PGE_2 levels from MSCs were measured in co-cultures of THP-1 cells with the three different MSC populations. In aBMSC and THP-1 co-cultures, it was determined that the PGE_2 levels were 605.4 ± 452.6 pg/mL (Fig. 4). By contrast, in DPSC co-culture with THP-1 cells, none of the DPSC populations produced PGE_2 at a detectable level (Fig. 4). There was high variability between patients in GMSC production of PGE_2 in THP-1 cocultures, with the highest level being 4645 pg/mL, lowest level being undetectable, and the mean production being 1523.1 pg/mL

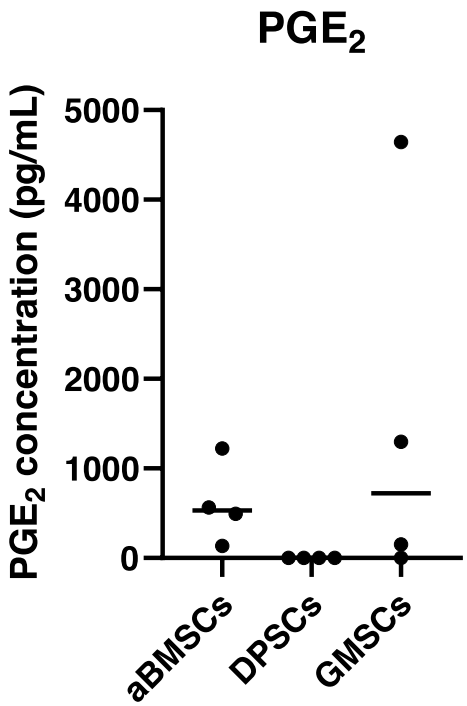

Fig. 4. PGE2 levels in coculture of aBMSCs, DPSCs, or GMSCs and THP-1 cells. THP-1 cells were cultured in the presence or absence of aBMSCs, DPSCs, or GMSCs for 72 h followed by 4-h treatment of 1 μg/mL LPS. The levels of PGE2 in the supernatants were quantified by PGE2 Parameter Assay. Samples of undetectable levels were plotted as 0. $n = 3$ in each group.

(Fig. 4).

2.5. Expression of PTGS1, PTGS2, IL6 and CCL2/MCP-1 in aBMSCs, DPSCs, and GMSCs when cocultured with THP-1 cells and/or stimulated with LPS

The RNA sequencing data revealed that aBMSCs, DPSCs, and GMSCs have differential expression of prostaglandin G/H synthase 1 and 2 (*PTGS1* and *PTGS2*), the upstream synthases for PGE_2 (Fig. 1A and Data S1–S3). This differential expression could ultimately translate to differences in PGE_2 production by aBMSCs, DPSCs, and GMSCs. To determine this, RT-PCR was used to determine gene expression of *PTGS1* and *PTGS2* from aBMSCs, DPSCs, and GMSCs co-cultured with THP-1 cells. These data showed that aBMSCs had significantly lower expression levels of *PTGS1* compared to DPSCs and GMSCs in both conditions, *i.e.*, higher ΔCt values relative to 18S rRNA (Fig. 5A). In contrast, there was no difference in *PTGS2* expression in aBMSCs and DPSCs co-cultured with THP-1 cells (Fig. 5B). aBMSCs also had the highest level of *PTGS2* among MSCs cultured alone ($p < 0.05$ compared to DPSCs, and $p < 0.01$ compared to GMSCs; Fig. 5B). Moreover, interaction with THP-1 cells without cell-cell contact resulted in increased expression of *PTGS2* expression in aBMSCs (Fig. 5B). By contrast, *PTGS1* levels remained the same with or without the presence of THP-1 cells (Fig. 5A). These data, together with the secreted levels of PGE_2 from aBMSCs in aBMSCs/THP-1 cocultures (Fig. 4) suggest that PTGS2 induction and resultant elevation of PGE_2 contribute to aBMSC-induced immunomodulatory effects on THP-1 monocytes.

Because IL-6 and MCP-1 are prominent regulators of monocytes, we also evaluated their expression levels in aBMSCs, DPSCs, and GMSCs co-cultured with or without THP-1 cells without cell contact. Soluble factors from THP-1 cells enhanced *IL6* expression in all three types of MSCs with DPSCs' levels being the lowest (Fig. 5C). This is in line with the differential expression of *IL6* in DPSCs vs.

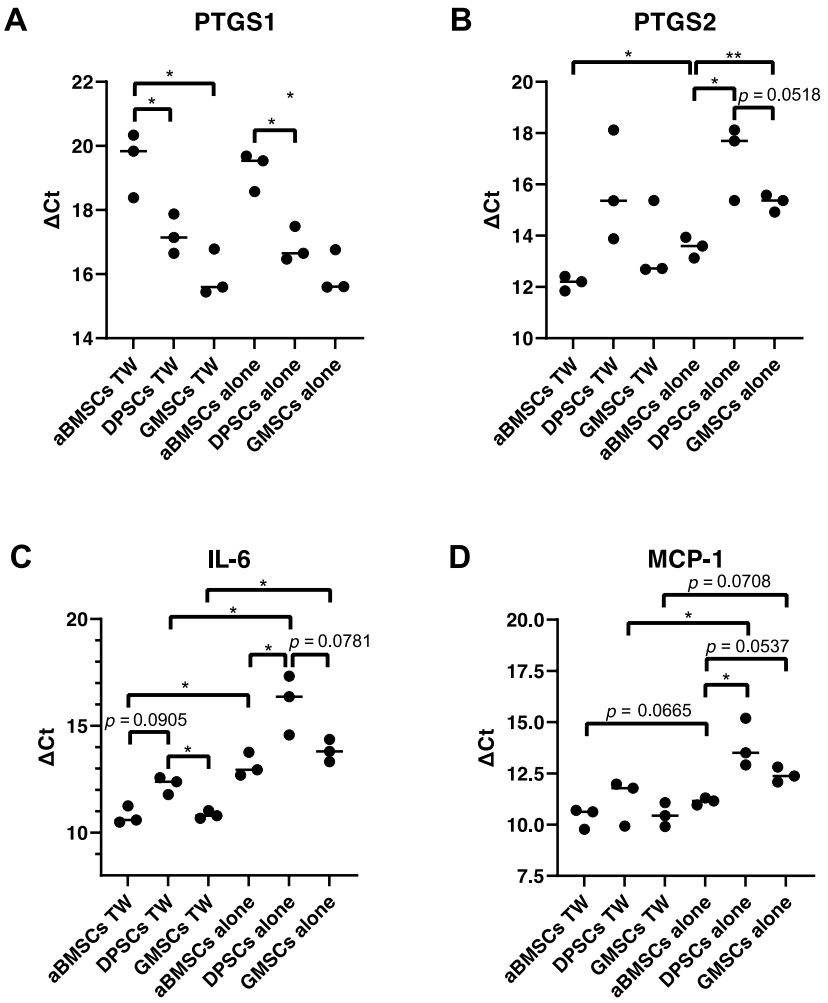

Fig. 5. Expression of PTGS1, PTGS2, IL-6, and MCP-1 in aBMSCs, DPSCs, and **GMSCs with or without coculture with THP-1 cells.** aBMSCs, DPSCs, or GMSCs were cultured in Transwell inserts with or without THP-1 cells cocultured in the bottom of wells for 72 h followed by 4-h treatment of 1 μg/mL LPS. Then RNA was extracted from the aBMSCs, DPSCs, or GMSCs on Transwell inserts, and the expression levels of indicated genes were quantified by RT-PCR and presented as ΔCt relative to 18S rRNA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; $n = 3$ in each group.

aBMSCs (Data S1) and GMSCs vs DPSCs (Data S3). In contrast, *MCP*-1 mRNA levels were not DE under basal conditions without stimulation (RNA sequencing data not shown), whereas aBMSCs were found to have higher levels of *CCL2/MCP-1* expression after LPS stimulation compared to DPSCs and GMSCs (Fig. 5D). Interestingly, the interaction with THP-1 cells induced expression of *CCL2/MCP-1* in DPSCs and GMSCs to levels seen in aBMSCs (Fig. 5D).

3. Discussion

There have been limited studies comparing human MSCs from different tissue sources and to our knowledge, none comparing immunomodulatory properties of MSCs from different oral tissues of the same patients, namely, aBMSCs, DPSCs, and DPSCs. In the present study, we harvested three types of tissues, namely alveolar bone, dental pulp, and gingiva, from the same patients to eliminate the influences of donors' biological differences on the results. Whole transcriptome analyses revealed many DE genes in aBMSCs, DPSCs, and GMSCs with DPSC gene expression being the most distinct. Additionally, in comparing aBMSCs, DPSCs and GMSCs, the DPSCs demonstrated the least potent immunomodulatory properties with respect to their production of soluble immunomodulatory cytokines and in their immunomodulation of monocytic cells.

There are a number of comparative studies showing that MSCs derived from various oral and dental tissues have tissue regenerative properties comparable to MSCs from bone marrow and adipose tissue [20–22]. In contrast, there have been limited comparative studies evaluating the immunomodulatory properties of oral derived MSCs relative to MSCs of other tissues [23–25]. Comparisons of BMSC and ASC differentiation and immunophenotype have been the most common analyses yet differences in these parameters between these two sources of MSCs has been attributed to variables such as donor age, sex, and culture medium conditions [16]. In studies designed to minimize biological background variables of the donor, where ASCs and BMSCs from the same donor were compared, it was determined that ASC were significantly more proliferative and adipogenic, whereas BMSCs exhibited significantly higher osteogenic and chondrogenic capacity [26].

Though our first comparative analysis in this study evaluated DE genes between the transcriptomes of aBMSCs, GMSCs, and DPSCs, a limitation with this analysis is that DE genes do not necessarily translate to differences in the protein levels or secretion levels. For example, *TIMP1* and *IL6* were expressed at a higher mRNA level in aBMSCs and GMSCs compared to DPSCs (Data S1 and S2); however, the differences in normalized concentrations of TIMP-1 or IL-6 in the CM did not reach statistical significance (Fig. 2). In fact, only four cytokines were expressed at appreciable levels among the 80 common cytokines in our cytokine array (Fig. 2A), and TIMP-2 and OPG (TNFRSF11B) were the only two whose concentrations in the CM differed significantly between cell types (Fig. 2C–E). As a member of the tumor necrosis factor receptor superfamily (TNFRSF), OPG is most highly recognized for its inhibition on osteoclastogenesis through disruption of the binding of receptor activator of nuclear factor-κB (RANK, or TNFRSF11A) and its ligand (RANKL, or TNFSF11), *i.e.*, OPG is a decoy receptor for RANKL [27,28]. Though RANKL-RANK-OPG signaling is well-established to be highly critical in bone homeostasis, this pathway also regulates immunity through modulation of T cell selection, differentiation, and their cell-cell interactions with antigen-presenting DCs [29,30]. In the present study, the basal level of OPG secretion as well as mRNA levels was found to be significantly higher in cultures of aBMSCs and GMSCs compared to DPSCs (Fig. 2E, Data S1 and S2). However, OPG expression can be regulated by a variety of cytokines including TNF (upregulation) and PGE_2 (downregulation) [30]. *In vivo* studies would be of interest to determine whether aBMSCs and GMSCs release greater levels of OPG in stem cell therapeutic contexts aimed to regenerate bone or modulate inflammation.

As inhibitors of matrix metalloproteinases (MMPs), TIMP-1 and TIMP-2 play key roles in maintaining the balance of extracellular matrix proteleolysis involved in a variety of physiological and pathological processes [31]. In addition, TIMP-1 interacts with β_1 integrin and CD63 to mediate angiogenesis [31]. Similarly, TIMP-2 has angio-inhibitory and anti-tumorigenic properties via binding to integrin $\alpha_3\beta_1$ [32]. Lozito et al. showed that BMSC secretion of TIMP-1 and TIMP-2 inhibits high levels of MMP-2 and MMP-9 in the perivascular niche. This secretion contributes to BMSC protection of vascular matrix molecules and endothelial cell structures from MMP-induced disruption in the perivascular niche [33]. Lozito et al. also investigated ASCs and MSCs isolated from traumatized muscle (TMD-MSCs): and found that MMP-2 inhibition by BMSCs and ASCs was predominantly mediated by TIMP-2 and that MMP-2 inhibition by TMD-MSCs was TIMP-1 dependent [34]. In the present study, we detected high levels of TIMP-1 and TIMP-2 secretion by all three types of oral tissue-derived MSCs, and TIMP-2 levels in aBMSCs were significantly higher than in DPSCs (Fig. 2C). Functional assays need to be performed to determine whether there are functional differences as a result of differential expression of TIMP-2.

The immunomodulatory functions of aBMSCs, DPSCs and GMSCs was evaluated in co-cultures with THP-1 monocytic cells and relative to aBMSCs and GMSCs, DPSCs did not reduce TNF-α-expression to the same extent in THP-1 cells (in response to LPS stimulation) (Fig. 3G). As such, these data suggest that aBMSCs and GMSCs would inhibit monocyte inflammatory responses to a greater degree than DPSCs in an immunomodulatory context. Interestingly, when conditioned medium from aBMSCs, DPSCs, and GMSCs was applied to THP-1 cells, there was a small but statistically significant reduction in the TNF-α-positive THP-1 cells in all CM groups (Fig. 3G). Therefore, cell-cell contact between DPSCs and THP-1 cells is required for DPSC-induced immunosuppression on THP-1 cells; alternatively, soluble factors from THP-1 cells may change the secretome of DPSCs and abolish their dependent inhibitory effects.

To determine if the observed immunosuppression was mediated through PGE_2 secretion, PGE_2 levels in the co-culture media were determined and showed that in the presence of LPS, PGE_2 was released from: aBMSCs from all 4 donors, GMSCs from 3 of 4 donors, and none of the DPSCs (Fig. 4). These findings could be attributed to the differential expression of PTGS1 and PTGS2 (Fig. 5A–B). PGE_2 is an important soluble factor that regulates multiple types of immune cells [35]. In particular, PGE_2 in THP-1 monocytic cells reduces TNF-α production and upregulates the release of two soluble TNF receptors (BP-55, BP-75), which may in turn bind to and inhibit the cytolytic activity of TNF-α [36]. Exogenous PGE_2 has been demonstrated to cause a dose-dependent reduction in LPS-induced TNF-α production in macrophages [37]. The lack of PGE_2 in DPSCs/THP-1 co-culture may partially explain why DPSCs had a lower

immunosuppressive effect on THP-1 cells (Fig. 3). Additionally, PGE_2 release may enable aBMSCs and GMSCs to regulate other immune responses as observed in MSCs derived from other tissues such as, ASCs and BMSCs [38–41]. Of note, *PTGS2* is one of the top DE genes between aBMSCs, DPSCs, and GMSCs, being high in both aBMSCs and GMSCs but low in DPSCs, whereas *PTGS1* was also DE in aBMSCs, DPSCs, and GMSCs with levels from low to high in this order (Data S1–3). Potential differences of aBMSCs, DPSCs, and GMSCs in modulation of other immune cells may provide insights for selecting specific MSC populations for therapeutic applications in various inflammatory conditions.

In addition to PTGS1 and PTGS2, expression of IL-6 and MCP-1 was also detected in aBMSCs, DPSCs, and GMSCs in response to THP-1 cells and LPS stimulation (Fig. 5C–D). IL-6 is a pleiotropic cytokine that acts on a variety of immune and non-immune cells, and it exhibits both pro- and anti-inflammatory properties depending on the context [42]. In its regulatory functions on human monocytes, IL-6 is found to favor anti-inflammatory polarization into M2 macrophages [42,43]. Additionally, it is well-established that MCP-1 recruits monocytes and is chemoattractive for T cells, B cells, natural killer cells, basophils, macrophages, DCs, myeloid-derived suppressor cells, and neutrophils under certain conditions [44]. Our results showed that *IL6* expression and its protein secretion in DPSCs is lower than those in aBMSCs and GMSCs under basal or stimulated conditions (Figs. 1, 2F and 5C). By contrast, *CCL2/MCP-1* was not DE under basal conditions (data not shown) or in THP-1 coculture with LPS treatment (Fig. 5D), although LPS treatment alone resulted in significantly higher mRNA levels of *CCL2/MCP-1* in aBMSCs compared to DPSCs and GMSCs (Fig. 5D).

While we conducted a comprehensive transcriptome comparison between aBMSCs, DPSCs, and GMSCs, a limitation in the present study is that we only evaluated the immunoregulatory effects by these oral-derived MSCs in an *in vitro* model of MSC/THP-1 monocytic cell coculture. More studies are required to determine whether and how different types of MSCs regulate the function of other immune cells such as differentiated macrophages and T lymphocytes.

4. Conclusion

In summary, aBMSCs, DPSCs, and GMSCs isolated from oral tissues of the same patients have many DE genes and differ in their immunoregulatory properties, with GMSCs and aBMSCs exhibiting the most potent immunomodulatory properties. These findings serve as important foundational studies upon which to develop preclinical studies to determine how these different MSCs can be used in a targeted manner in different immunoregulatory and inflammatory contexts.

5. Materials and methods

5.1. MSC sourcing and characterization

Following University of Michigan Institutional Review Board approval (IRB #HUM00142680), specimens of alveolar bone, gingiva and teeth were all obtained from the same patients (n = 4; male and female ages 18–25) undergoing routine oral surgical procedures requiring tooth removal. The tissues harvested were not inflamed nor were they malignant. DPSCs were isolated from dental pulp as described previously [45]. The preparation of aBMSCs from small quantities (<0.5 cc) of alveolar bone particulates was performed as we have previously described [13]. The isolation protocol of GMSCs from gingival tissue was adapted from that described by Zhang et al. [25]. In brief, gingival tissues were digested in 2 mg/mL dispase (MilliporeSigma) in DPBS at 4 °C overnight, and then minced into 1–3 mm fragments and further digested in collagenase II (Worthington Biochemical, Lakewood, NJ) in PBS at 37 °C for 2 h with agitation every 30 min, followed by two times of washing with DPBS. The tissue/cell suspension from dental pulp, alveolar bone marrow, or gingiva was placed to a T-25 flask with respective growth medium (see below) plus 1 × Antibiotic-Antimyotic (Thermo Fisher Scientific, Waltham, MA). After colonies of adherent cells formed in about a week with medium replaced every 2–3 days, the adherent cells were passed and cultured in their respective growth medium: aBMSCs and DPSCs were cultured in nucleosides-containing Minimum Essential Media α (MEM α; Thermo Fisher Scientific) supplemented with 15 % fetal bovine serum (FBS; MilliporeSigma, Burlington, MA), 0.1 mM L-ascorbic acid-2-phosphate (AA2P; MilliporeSigma), and 25 μg/mL gentamicin (Thermo Fisher Scientific); GMSCs growth medium was MEM α supplemented with 10 % FBS, 0.1 mM AA2P, non-essential amino acids (Thermo Fisher Scientific), GlutaMAX™ (Thermo Fisher Scientific), and 25 μg/mL gentamicin. The isolated cells were confirmed to be MSCs by performing mesodermal differentiation assays and immunophenotype characterization (as described by Dominici et al. [15]). In brief, aBMSCs, DPSCs and GMSCs cultured in various cell type specific differentiation media were stained positive for Von Kossa, Alcian Blue, and Oil Red O as proof of osteogenic, chondrogenic and adipogenic differentiation, respectively (Supplementary Fig. S1). The following were differentiation medium which were used: Osteogenic medium, MEM α supplemented with 15 % FBS, 50 μM AA2P, 10.224 mM β-Glycerol phosphate disodium (MilliporeSigma), 0.1 μM dexamethasome (MilliporeSigma), and 25 μg/mL gentamicin; Chondrogenic medium, MEM α supplemented with 15 % FBS, 50 μM AA2P, 0.1 μM dexamethasome, ~5.75 μg/mL insulin (MilliporeSigma), 10 ng/mL TGF-β1 (R&D Systems, Systems, Minneapolis, MN), 0.4 mM proline (MilliporeSigma), 1 × Non-Essential Amino Acid (Thermo Fisher Scientific), and 25 μg/mL gentamicin; Adipogenic mediuam, MEM α supplemented with 15 % FBS, 0.5 mM 3-Isobutyl-1-methylxanthine (MilliporeSigma), 1 μM dexamethasome, ~10.5 μg/mL insulin, 0.2 mM indomethacin (MilliporeSigma), and 25 μg/mL gentamicin. All MSCs isolated were stained with Brilliant Violet 421™ (BV421) conjugated anti-human CD73, fluorescein isothiocyanate (FITC)-conjugated anti-human CD90, and phycoerythrin (PE)-conjugated anti-human CD105 antibodies and achieved >96 % triple positive for CD 73, CD 90, and CD105 by flow cytometry (Supplementary Table S1 and Supplementary Fig. S2). aBMSCs, DPSCs, and GMSCs at Passage 3–5 were used in this study.

THP-1 monocytic cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA), and maintained between 2 and 8 × 10^5/mL in the RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10 % FBS, 50 U/mL penicillin and 50 μg/mL

streptomycin (Thermo Fisher Scientific), and 0.05 mM 2-mercaptoethanol (ATCC).

5.2. RNA extraction

Cultured cells were briefly rinsed with DPBS, and then lyzed with RLT Lysis Buffer from the RNeasy Mini Plus Kit or RNeasy Micro Plus Kit (Qiagen, Hilden, Germany) with addition of 1 % 2-mercaptoethanol. The lysates were kept at −80 °C until processed to extract RNA with the rest of the reagents from the kits according to the manufacturer's instructions. The RNA concentrations were determined by Qubit Assay using the Qubit RNA HS Assay Kit and the Qubit fluorometer (Thermo Fisher Scientific) according to the manufacturer's instructions.

5.3. RNA sequencing and analyses

aBMSCs, DPSCs, and GMSCs were cultured to averagely 70–80 % confluent in T flasks. After a brief rinse with DPBS, the cells were treated with TRI Reagent (MilliporeSigma), and the lysates were kept frozen at 80 °C until ready for RNA extraction using a RNeasy Mini Plus Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA sequencing was performed by the University of Michigan DNA Sequencing Core using the Lexogen QuantSeq library preparation. Data were pre-filtered to remove genes with 0 counts in all samples. Differential gene expression analysis was performed with DESeq2 [46], using a negative binomial generalized linear model and accounting for natural variation of patients by using a pair-sample regression design.

5.4. Collection of conditioned media (CM)

For analysis of released soluble factors, aBMSCs, DPSCs, and GMSCs were cultured to averagely 70–80 % confluent. After rinsing with DPBS for 3 times, the cells were incubated with serum-free basal medium (MEM α supplemented with 0.1 mM AA2P and 25 μg/mL gentamicin) for 24 h. Then the media were collected, centrifuged to remove any detached cells, frozen and kept at −80 °C. The cells were trypsinized and counted for normalization. To collect CM for use in THP-1 cultures, aBMSCs, DPSCs and GMSCs were plated at 1×10^4 per cm^2 growth area and incubated for 18 h overnight for the cells to adhere. Then the cells were rinsed with DPBS for 3 times followed by 24-h incubation in 1 mL THP-1 growth medium per cm^2 growth area. The CM were collected in the same way.

5.5. Analysis of soluble factors

To analyze the soluble factors secreted by aBMSCs, DPSCs, and GMSCs, 24-h MEM α conditioned medium (CM) were subjected to a human cytokine array with 80 targets (RayBiotech #AAH-CYT-5-4, Peachtree Corners, GA), including: angiogenin, BDNF, CCL1, CCL2 (MCP-1), CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL17, CCL18, CCL20, CCL22, CCL23, CCL24, CCL26, CSF1 (M-CSF), CSF2 (GM-CSF), CSF3 (G-CSF), CX3CL1, CXCL1, CXCL1/CXCL2/CXCL3, CXCL5, CXCL6, CXCL7 (PPBP), CXCL8 (IL-8), CXCL9, CXCL10, CXCL12α (SDF-1α), CXCL13, EGF, FGF-4, FGF-6, FGF-7, FGF-9, Flt3 ligand, GDNF, HGF, IFN-γ, IGF-1, IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12A, IL-13, IL-15, IL-16, leptin, LIF, MIF, NT-3, NT-4, OSM, PDGF-BB, PGF, OPN (SPP1), SCF (KIT ligand), TGF-β1, TGF-β2, TGF-β3, TIMP-1, TIMP-2, TNF-α, TNF-β (TNFSF1B), TNFSF14 (LIGHT), TNFRSF11B (OPG), TPO, VEGF-A. ELISA kits detecting MCP-1 (CCL2), IL-6 (Biolegend), TIMP- 1 and 2 (R&D), and OPG (TNFRSF11B) (MilliporeSigma), and the prostaglandin E2 (PGE_2) parameter assay (R&D Systems) were used to quantify the corresponding soluble factors in the CM following the manufacturers' instructions.

5.6. Coculture of MSCs and THP-1 cells

aBMSCs, DPSCs, or GMSCs (1×10^5) were cocultured with THP-1 cells (2×10^5) in 3 mL THP-1 growth medium in 6-well plates for 72 h. In some cocultures, aBMSCs, DPSCs, or GMSCs were cultured in 0.4 μm Transwell (TW) inserts (Corning) without cell-cell contact. In the CM group, THP-1 cells were cultured alone in 2 mL fresh THP-1 growth medium plus 1 mL MSC-conditioned THP-1 growth medium for 72 h. In the PGE_2 treatment group, THP-1 cells were cultured alone in the presence of 100 nM exogenous PGE_2 (MilliporeSigma). aBMSCs, DPSCs and GMSCs were also cultured alone in THP-1 growth medium as controls. After 72 h of culture, 1 μg/mL lipopolysaccharide (LPS) from *Escherichia coli* (*E. coli*; MilliporeSigma) was added to the cell cultures for additional 4 h of incubation. To block secretion of cytokines for detection of intracellular tumor necrosis factor α (TNF-α), 5 μg/mL brefeldin A (Biolegend, San Diego, CA) was added to the cell cultures 4 h before the end of incubation (at the same time when LPS was added). The non-adherent cells were harvested and subjected to flow cytometry detecting as previously described [47]. In brief, collected cells were first incubated with peridinin-chlorophyll-protein/cyanine5.5 (PerCP/Cy5.5)-conjugated anti-human CD90 antibody (Thermo Fisher Scientific) to label MSCs, if any, to be excluded from counting THP-1 cells, and then fixed and permeabilized using the Cytofix/Cytoper Fixation/Permeabilization kit from BD Biosciences (Hercules, CA), followed by incubation with phycoerythrin (PE)-conjugated anti-human TNF-α antibody. Cells were twice rinsed with Cell Staining Buffer (Biolegend), and analyzed by Bio-Rad ZE5 Cell Analyzer (San Jose, CA). The media in cell cultures without brefeldin A were collected and centrifuged, and the supernatant was saved and kept at −80 °C until being analyzed.

5.7. RT-PCR

cDNA was made from isolated RNA with High-Capacity cDNA. Reverse Transcription Kit (Applied Biosystems, Foster City, CA), and RT-PCR was performed on 7500 Real-Time PCR System (Applied Biosystems) using TaqMan™ Fast Advanced Master Mix and TaqMan™ primers for human *CCL2/MCP-1* (Hs00234140_m1), *IL6* (Hs00174131_m1), *CXCL8/IL8* (Hs00174103_m1), *IL10* (Hs00961622_m1), *PTGS1* (Hs00377726_m1), *PTGS2* (Hs00153133_m1), and 18S rRNA (Hs99999901_s1; Applied Biosystems) according to the manufacturer's instructions.

5.8. Data analysis

The flow cytometry data were analyzed with FCS Express 6 and 7 (De Novo Software, Pasadena, CA). With the exception of RNA sequencing results, all data are presented as mean ± standard deviation (SD), and statistical analyses were performed in Prism 8 and 9 (GraphPad Software, San Diego CA) using paired two-tailed *t*-test unless specified. Differences with a *p*-value less than 0.05 was considered statistically significant. For analysis of differential gene expression, *p*-values were adjusted for multiple comparisons using Benjamini-Hochberg false discovery rate (FDR) [48]. Genes expressed in different MSC types with a FDR of $\leq$0.05 and absolute fold-change $\geq$1.5 were reported as significantly differentially expressed (DE).

Ethics declarations

This study was reviewed and approved by University of Michigan Institutional Review Board with the approval number IRB #HUM00142680.

Data availability statement

Data associated with this study has not been deposited into a publicly available repository, however, all relevant and pertinent data to support this study is included in the article and supplemental materials.

CRediT authorship contribution statement

Chen Cao: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bartosz Maska:** Investigation, Conceptualization. **Malika A. Malik:** Investigation. **Rebecca Tagett:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis. **Darnell Kaigler:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

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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Appendix A. Supplementary data

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