Research Article

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Regenerative Medicine

Human mesenchymal stem cells alter the gene profile of monocytes from patients with Type 2 diabetes and end-stage renal disease

Aim: Macrophage infiltration contributes to the pathogenesis of Type 2 diabetes. Mesenchymal stem cells (MSCs) possess immunomodulatory properties, making them an ideal candidate for therapeutic intervention. This study investigated whether MSCs can modulate the phenotype of monocytes isolated from Type 2 diabetic patients with end-stage renal disease. **Materials & methods:** Monocytes from control (n = 4) and Type 2 diabetic patients with end-stage renal disease (n = 5) were assessed using flow cytometry and microarray profiling, following 48 h of co-culture with MSCs. **Results:** Control subjects had a greater proportion of $CD14^{++}CD16^{-}$ monocytes while diabetic patients had a higher proportion of $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ monocytes. MSCs promoted the proliferation of monocytes isolated from diabetic patients, reduced HLA-DR expression in both groups and promoted the expression of anti-inflammatory genes. **Conclusion:** MSC-derived factors alter the polarization of monocytes isolated from healthy and diabetic subjects toward an M2 phenotype.

Keywords: end-stage renal disease • macrophages • mesenchymal stem cells • monocytes • Type 2 diabetes

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Diabetic nephropathy (DN), a progressive kidney disease that results from enduring diabetes, is the leading cause of end-stage renal disease (ESRD) worldwide [1]. It is characterized by the accumulation of extracellular matrix and eventual glomerular and interstitial fibrosis, leading to declining renal function [2]. Chronic inflammation is a key factor promoting the development and progression of DN. The infiltration of monocytes and monocyte-derived macrophages is a hallmark of diabetic kidney disease, and the number of macrophages correlates with declining renal function in both humans and mice [3,4].

Monocytes show considerable heterogeneity in both phenotype and function [5]. The classification of human peripheral blood monocytes is centered on the expression of the LPS co-receptor, CD14 and the FcγReceptor III, CD16 [6]. Three major subsets exist: $CD14^{hi/++}CD16^{-}$ (classical), $CD14^{hi/++}CD16^{+}$ (intermediate) and $CD14^{dim/+}CD16^{hi/++}$ (nonclassical) [6]. Classical $CD14^{++}CD16^{-}$ monocytes traffic to the site of inflammation via a CCR2-dependent mechanism during the early stages of inflammation where they exhibit functions related to a typical inflammatory response, such as phagocytosis and reactive oxygen species (ROS) production [5,7]. By contrast, nonclassical $CD14^{+}CD16^{++}$ monocytes predominantly patrol blood vessel walls and are believed to contribute to resident macrophage populations [8]. These cells also accumulate in a variety of chronic inflammatory diseases where they are recruited at a later stage of the inflammatory response via a CX3CR1-dependent pathway [5,9]. The third subset, $CD14^{++}CD16^{+}$, is believed to be an intermediate phenotype between the classical and nonclassical subsets [6]. Importantly, although functionally and phenotypically distinct monocyte subsets exist, these subsets may denote the same cell at different stages of maturation. Supporting evidence confirms that classical $CD14^{++}CD16^{-}$ monocytes

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are the most immature phenotype but are capable of developing into the intermediate $CD14^{++}CD16^{+}$ subset, which in turn mature into the $CD14^{+}CD16^{++}$ non-classical subset, characterized by the downregulation of CD14 and CCR2 expression and the upregulation of CD16 and CX3CR1 [10].

Current therapeutic approaches have been able to reduce proteinuria in patients with DN, but have shown limited success in attenuating disease progression. Mesenchymal stem (stromal) cells (MSCs) have been used in several clinical trials including kidney transplantation, renal allograft rejection and Type 2 diabetes (T2D), demonstrating promising results with no adverse side effects [11–13]. Preclinical studies show that administered MSCs home to sites of injury where they ameliorate renal injury and accelerate repair through paracrine and/or endocrine mechanisms. It has been well documented that MSCs have remarkable immunomodulatory abilities, capable of altering dendritic cell phenotype and function, and macrophage phenotype and function [14,15].

Despite the interest in MSC-based approaches for the treatment of human kidney injury, there is limited information on the effects that MSCs have on circulating human monocytes, the progenitors of dendritic cells and macrophages, particularly under chronic inflammatory conditions. This study investigated the effects of human bone marrow-derived MSCs on human monocytes isolated from healthy (control) subjects and Type 2 diabetic patients with ESRD.

Materials & methods

Study population

Blood was obtained from five Type 2 diabetic patients with ESRD due to their diabetes. All patients received regular, three-times per week, hemodialysis at Monash Medical Centre using polysulphone high-flux dialyzers. All anticoagulation for dialysis was with low-molecular-weight heparin (enoxaparin). Control blood was obtained from four donors from the Australian Red Cross Blood Service. Donor details are summarized in Table 1. Informed consent was obtained from all participants. The research was carried out in accordance with the Declaration of Helsinki (2008) of the World Medical Association. Ethical approval was obtained from the Monash Health Human Research Ethics Committee (Monash 10179B) and Monash University Human Research Ethics Committee (CF07/3495 – 2007001798).

Monocyte purification

Peripheral blood mononuclear cells (PBMCs) were harvested from whole blood using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Uppsala, Sweden) according to the manufacturer's guidelines. $CD14^{+}$ monocytes were then sorted from the PBMCs using human anti-CD14 conjugated microbeads (Miltenyi Biotec, CA, USA), on a QuadroMACS (Miltenyi Biotec) separator. The purity of the $CD14^{+}$ sorted cells was greater than 90% when confirmed by flow cytometry.

Flow cytometry

Immunophenotypic analysis of monocytes on day 0 and 2 by flow cytometry was performed using the following fluorochrome-conjugated anti-human antibodies: CD45-FITC, CD14-APC, CD16-eFluor® 450 and HLA-DR-PE. All antibodies were purchased from eBioscience, Inc. (CA, USA). Cell population data were acquired using an FACSCanto II flow cytometer (BD Biosciences, CA, USA) with the FACS Diva acquisition software (BD Biosciences) and analyzed using FlowLogic FCS analysis software (Inivai Technologies, Melbourne, Australia).

Monocyte & MSC co-culture

Human bone marrow-derived MSCs purchased from the Tulane Centre for Stem Cell Research and Regenerative Medicine (Tulane University, LA, USA) were cultured and characterized as previously described [15]. For co-culture experiments, 3×10^5 $CD14^{+}$ monocytes were plated in six-well plates and 3×10^5 MSCs were plated indirectly on a 0.4 µm pore size Transwell (Millipore Corporation, MA, USA; Figure 1). Cells were co-cultured for 48 h in RPMI 1640 medium supplemented with 10% fetal bovine serum (Sigma-Aldrich, MO, USA), 1% L-glutamine and 1% penicillin-streptomycin (Gibco®, NY, USA). Monocytes were visualized on an Olympus IX81 microscope and images captured with an Olympus IX2-UCB camera.

RNA isolation & microarray analysis

RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. Any contaminating DNA was removed by using 50U DNase I (Qiagen). RNA quantity was measured using a Nanodrop ND 1000 spectrophotometer (Rockland, DE, USA), quality was determined with an Agilent 2100 electrophoresis bioanalyzer (Agilent, CA, USA) and an RNA integrity number threshold of ≥0.8 was used for all samples. Samples (5 ng RNA per sample) were prepared using an Ovation Pico WTA system V2 (NuGEN Technologies, Inc., Bemmel, The Netherlands) and Encore Biotin Module V2 (NuGEN Technologies, Inc.). 5µg of biotin-labeled fragmented cDNA was hybridized to an Affymetrix GeneChip® Human Gene 2.0 ST array (Affymetrix, CA, USA). Data

Table 1. Baseline characteristics of control and Type 2 diabetic subjects.

Patient group	Subject #	Gender	Age	Renal function	WC (×10⁹/l)	Monocyte (×10⁹/l)	CRP	Duration (years)	Disease
Control	1	Female	25	–	–	–	–	–	–
	2	Male	29	–	–	–	–	–	–
	3	Female	25	–	–	–	–	–	–
	4	Male	29	–	–	–	–	–	–
T2D	1	Male	63	Dialysis	7.3	0.63	25	30	Diabetic nephropathy
	2	Female	74	Dialysis	5.6	0.71	12	Unknown	Diabetic nephropathy
	3	Male	65	Dialysis	6.4	0.49	17	20	Diabetic nephropathy
	4	Male	64	Dialysis	7.1	0.6	23	21	Diabetic nephropathy
	5	Male	85	Dialysis	11.3	0.7	14	6	Diabetic nephropathy

CRP: C-reactive protein; T2D: Type 2 diabetes; WC: White cell.

were background corrected, $\log_2$ transformed and quantile normalized using the bioconductor packages for gene-expression profiling in R. A linear model was then fitted to the normalized data and differential gene expression was when samples had a $\log_2$-fold change of <-0.75 and >0.75. False discovery rate (FDR) correction was performed on the p-values according to methods described by Benjamini and Hochberg using the limma bioconductor package. An FDR p-value of ≤0.05 was considered statistically significant. The list of differentially expressed genes was then subjected to pathway analysis using Ingenuity Pathway Analysis software (Ingenuity Systems, CA, USA). Microarray data can be visualized at the stemformatic website [16].

Statistical analysis

All statistical analyses, except for the microarray analysis, were performed using GraphPad Prism software version 5.0 (GraphPad Software, Inc., CA, USA). Comparisons between two groups were made with a Student's *t*-test (unpaired, two-tailed). All data are expressed as means ± standard deviation (SD). A p-value of <0.05 was considered statically significant.

Results

The proportions of blood monocyte subsets are altered in Type 2 diabetic patients with ESRD

To understand the impact of T2D on blood monocytes, flow cytometry was used to assess and compare the phenotypes and relative proportions of freshly purified monocytes from control and diabetic subjects based on the expression of the monocyte markers CD14 and CD16. The baseline clinical parameters of the study participants are shown in Table 1. All diabetic subjects had T2D with ESRD and were receiving hemodialysis at the time of blood collection. Representative dot plots

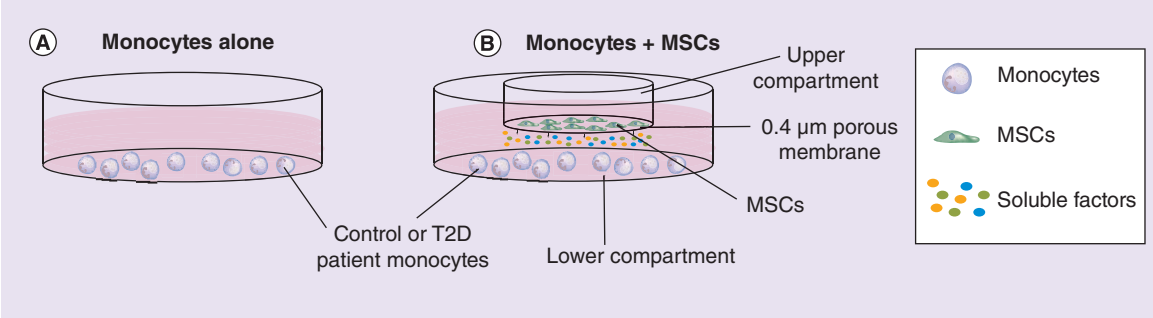

Figure 1. A diagram of the co-culture system used to culture human monocytes and mesenchymal stem cells.
$CD14^+$ control and Type 2 diabetic patient monocytes were cultured alone **(A)** and with MSCs **(B)** for 48 h.
MSC: Mesenchymal stem cell; T2D: Type 2 diabetes.

show $CD45^{+}CD14^{+}CD16^{+/-}$ monocytes from control and Type 2 diabetic subjects on the day of blood collection (Figure 2A). Three monocyte populations were identified in both patient groups. The $CD14^{++}CD16^{-}$ classical monocytes formed the predominant subset, while the transitioning intermediate and more mature nonclassical subsets constituted smaller proportions of the monocyte pool. Significant differences in the proportions of each of these subsets were observed when compared between the two subject groups (Figure 2B–D). Within the monocyte pool, there were a significantly greater proportion of classical monocytes from control subjects, compared with those from Type 2 diabetic patients (Figure 2B; T2D: 56.3 ± 11.0% vs control: 73.9 ± 7.2%; $p < 0.05$). By contrast, the Type 2 diabetic patients had a higher proportion of intermediate (Figure 2C; T2D: 32.2 ± 8.0% vs control: 19.2 ± 6.7%; $p < 0.05$) and nonclassical (Figure 2D; T2D: 7.5 ± 2.5 vs control: 2.8 ± 1.1%; $p < 0.05$) monocytes, compared with the control subjects. Purified monocytes were also cytospun and subjected to Giemsa staining. Representative bright field images of monocytes from both subject groups (included in the dot plots in Figure 2A) confirmed normal monocyte morphology. These data identified a shift in the proportions of the different monocyte subsets in patients with T2D-mediated ESRD.

MSCs alter the maturation state of monocytes

MSCs can exert their immunomodulatory and regenerative effects through the release of soluble factors. In order to test this, monocytes from healthy control subjects and Type 2 diabetic patients were co-cultured with MSCs for 48 h using a Transwell system. MSCs promoted the expansion of the T2D monocytes (T2D monocytes alone: $6.0 \times 10^4 \pm 2.2 \times 10^4$ vs T2D monocytes + MSCs: $19.4 \times 10^4 \pm 8.0^4$; $p < 0.01$), but this effect was not seen in the control monocytes co-cultured under the same conditions (Figure 3A & B). Both the control and T2D monocytes displayed a more uniform

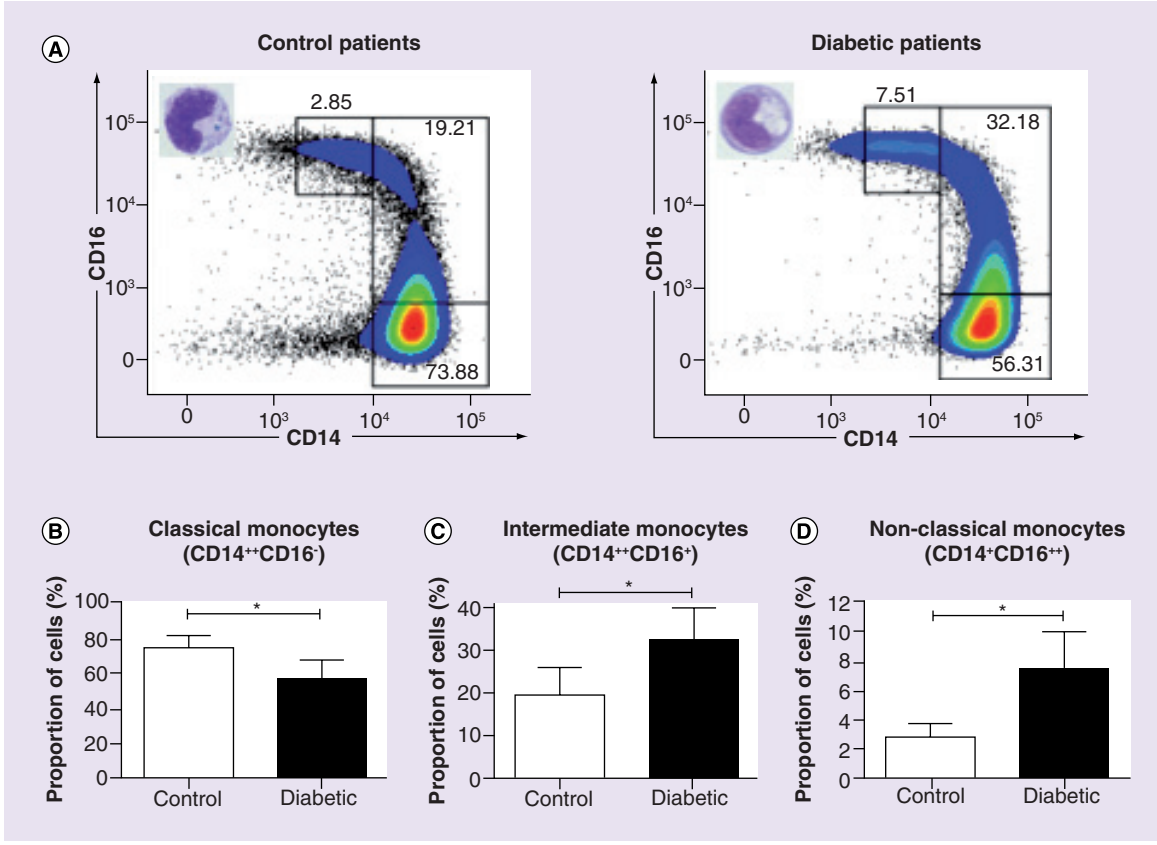

Figure 2. Comparison of CD14/CD16 monocyte populations isolated from control and Type 2 diabetic subjects.
Freshly isolated $CD14^{+}$ cells from control and Type 2 diabetic subjects were subjected to Giemsa staining to observe morphology (magnification ×1000) and analyzed by flow cytometry for their expression of CD14 and CD16 on day 0. Representative dot plots are shown. Numbers on dot plots represent the proportion of viable, sorted $CD14^{+}$ cells **(A)**. Proportion of $CD14^{++}CD16^{-}$ classical **(B)**, $CD14^{++}CD16^{+}$ intermediate **(C)** and $CD14^{+}CD16^{++}$ nonclassical **(D)** monocytes from control and Type 2 diabetic subjects. Data were analyzed with a student's *t*-test (unpaired, two-tailed). n = 4–5 per group.
Data are means ± standard deviation; *$p < 0.05$.

and mature $CD14^+CD16^+$ phenotype following MSC co-culture (Figure 3C). Co-culture of monocytes with MSCs caused a significant upregulation of CD14 and CD16 expression on the Type 2 diabetic patient monocytes, as depicted by an increased mean fluorescence intensity (MFI) of the CD14 (Figure 4D; T2D monocytes alone: $2.3 \times 10^4 \pm 1.5 \times 10^3$ vs T2D monocytes + MSCs: $3.3 \times 10^4 \pm 3.3 \times 10^3$; $p < 0.001$) and CD16 (Figure 4E; T2D monocytes alone: $2.5 \times 10^3 \pm 59.1$ vs T2D monocytes + MSCs: $3.0 \times 10^3 \pm 521.5$; $p < 0.05$) parameters. This trend was also observed in the control group (Figure 4A & B). By contrast, HLA-DR expression on co-cultured monocytes was significantly lower in both control (Figure 4C; $1.2 \times 10^4 \pm 4.8 \times 10^3$ vs $4.9 \times 10^3 \pm 1.1 \times 10^3$; $p < 0.05$) and T2D (Figure 4F; $1.4 \times 10^4 \pm 7.0 \times 10^3$ vs $5.6 \times 10^3 \pm 2.6 \times 10^3$; $p < 0.05$) monocytes. These results indicate that the MSCs promoted the maturation of monocytes without concurrent expression of class II antigen presentation complexes. We predicted the resulting macrophages would have a less inflammatory phenotype, so microarray expression profiling was undertaken to assess other changes in inflammatory signaling pathways resulting from MSC co-culture.

Monocytes co-cultured with MSCs have distinct gene-expression profiles

Monocytes from control subjects and patients with T2D and ESRD were profiled before and after MSC co-culture. Principal component analysis (PCA) revealed that the MSC treatment was the biggest driver of expression variation between the samples (Figure 5A). Following co-culture with MSCs, a total of 324 genes were differentially expressed (pairwise analysis, threshold of >twofold and adjusted p-value < 0.05), with 212 genes significantly upregulated (red) and 112 genes significantly downregulated (green) in both MSC-treated monocyte groups, compared with the monocyte alone groups (Figure 5B & Supplementary Tables 1 & 2).

Hierarchical clustering and heatmap visualization of the differentially expressed genes further demonstrated altered maturation profiles of the MSC co-cultured monocytes, with downregulation (blue) of the nonclassical monocyte markers *ITGAL* and *SPN* and the upregulation (red) of the classical monocyte markers *FCGR2A* and *FCGR2B* (both associated with CD64), *CD163*, *CD93*, *CD38*, *CLEC4D*, *HIF1A* and *FPR1* (Figure 5C). MSC treatment also significantly upregulated factors associated with a reparative macrophage phenotype, including the cytokines *IL-10*, *IGF1*, *CCL2* and *VEGF-A*; membrane receptors *MRC1*, *CD163*, *CD163L1*, *CD226*, *CD93*, *LILRB1* and *PTGER2* (also known as *EP2*); and enzymes *MMP9*, *MMP19*, *F13A1*, *SERPINB2* and *PTGS2* (also known as *COX2*; Figure 5D). These data were consistent with the changed patterns of CD14, CD16 and MHC class II protein expression detected previously (Figures 2–4), demonstrating that the alterations in monocyte phenotype are the result of an altered transcriptional program.

The top ten canonical signaling pathways significantly over-represented in the MSC-treated monocytes (Figure 6 & Table 2) shared several common genes indicative of cell maturation including FcϒR receptors (*FCGR1B*, *FCGR2A*, *FCGR2B* and *FCGR3A*), MHC class II molecules (*HLA-DMB* and *HLA-DQB1*) and adhesion molecules (*ITGAL*, *ITGAX*, *ITGA9* and *ICAM1*). Interestingly, the MHC class II molecules and many of the adhesion molecules were in fact downregulated by the MSC treatment (Supplementary Table 3). Genes commonly associated with an inflammatory macrophage phenotype were also downregulated, including *APOE*, *APOC1*, *LPL* and *PPARG* (Supplementary Table 3). IPA software was used to generate network hubs showing the common differentially expressed genes involved within the top ten signaling pathways (see Supplementary Figures 1 & 2), and these networks were dominated by the reparative factors *IL-10*, *IGF1*, *MMP9* and *VEGF* (Supplementary Figure 1) along with the classical monocyte and M2 macrophage associated phagocytic receptors *FCGR1B*, *FCGR2A*, *FCGR2B* and *CD163* (Supplementary Figure 2). Altogether, these results demonstrate that MSCs alter the maturation of monocytes toward a reparative macrophage phenotype.

Discussion

The current study explored the ability of MSCs to alter the phenotype of monocytes isolated from control and Type 2 diabetic subjects. We demonstrate that Type 2 diabetic patients displayed significantly higher proportions of the intermediate and nonclassical monocyte subsets contrasted to a higher proportion of circulating classical monocytes observed in control subjects. Although the nonclassical monocytes only constitute a minor subset, which is responsible for vessel patrolling during homeostasis, in a chronic inflammatory environment the $CD14^+CD16^{++}$ subset accumulate and can contribute to the pathogenesis of disease [9]. Our findings correlate with other studies reporting increased numbers of circulating monocytes in T2D which display a pro-inflammatory profile and secrete the pro-inflammatory cytokines IL-6, IL-8, TNF-α and IL-1β [17–19]. In rodents, it has been demonstrated that these pro-inflammatory cytokines induce insulin resistance and that blocking these cytokines reverses this phenomenon [20–22]. Furthermore, monocytes from diabetic patients have demon-

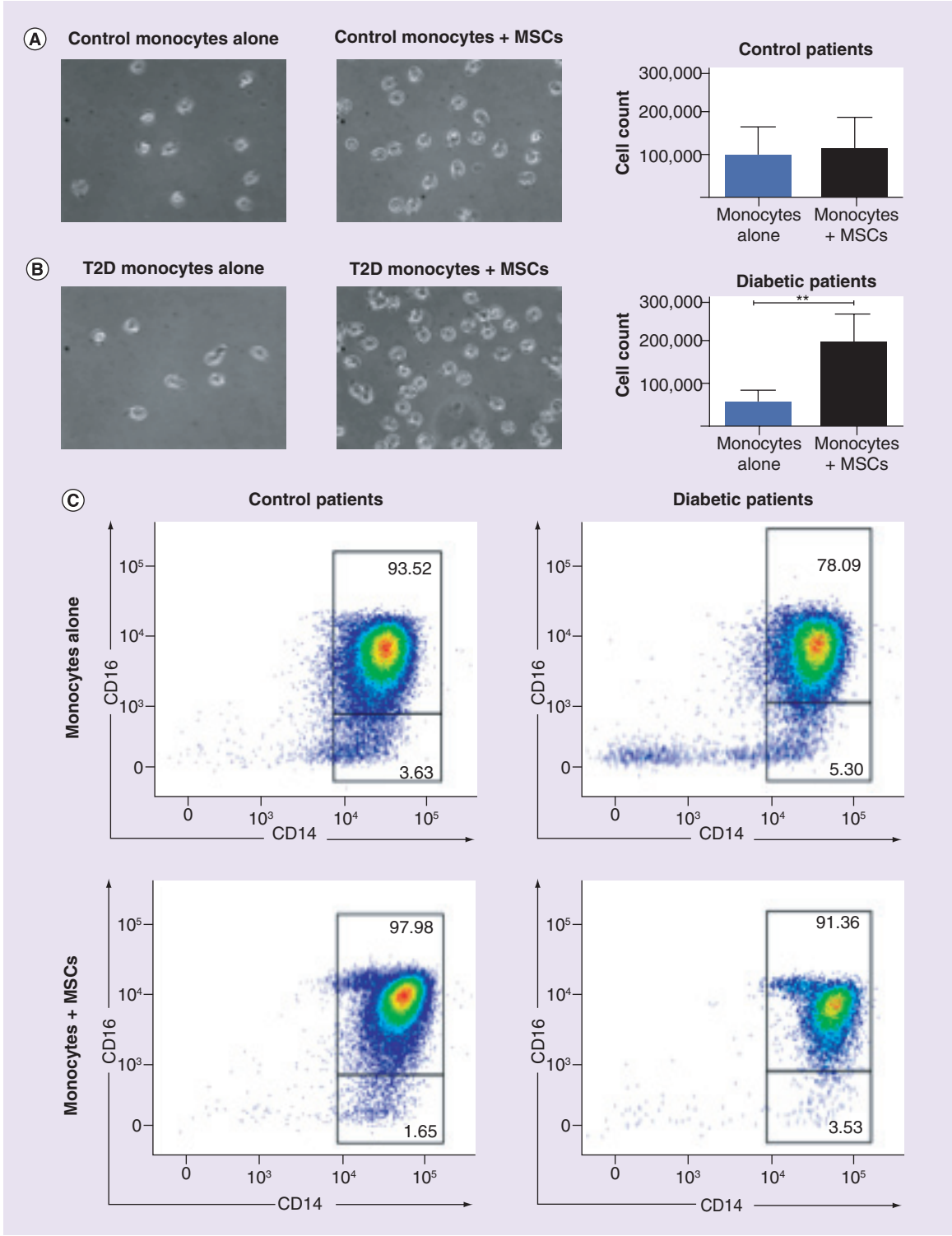

Figure 3. Control and Type 2 diabetic derived monocytes following 48 h of co-culture with mesenchymal stem cells. Representative photomicrographs (magnification ×400) and total cell counts of monocytes isolated from control **(A)** and Type 2 diabetic **(B)** subjects following 48 h of culture with and without MSCs. Representative flow cytometry dot plots showing the expression of CD14 and CD16 in monocytes following 48 h of co-culture with and without MSCs. Numbers on dot plots represent the proportion of viable, sorted $CD14^+$ cells **(C)**. Data were analyzed with a student's *t*-test (unpaired, two-tailed). n = 4–5 per group. Data are means ± standard deviation; $^{**}p < 0.01$.
MSC: Mesenchymal stem cell; T2D: Type 2 diabetes.

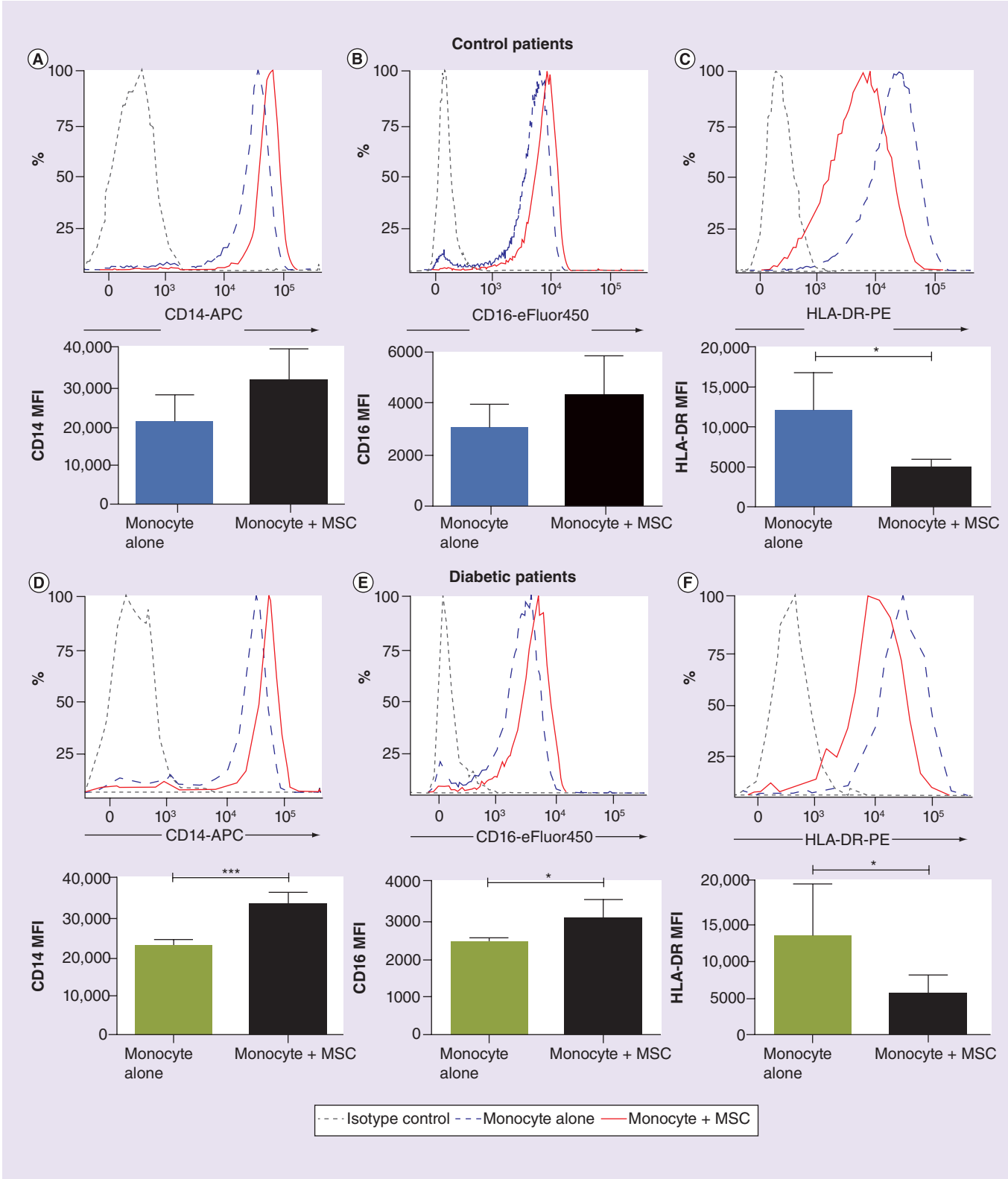

Figure 4. Mesenchymal stem cells alter the phenotype of human monocytes. The **(A)** CD14-APC, **(B)** CD16-eFluor450 and **(C)** HLA-DR-PE MFIs, representing surface marker expression level, of monocytes isolated from **(A–C)** control and **(D–F)** Type 2 diabetic subjects following 48 h of co-culture with and without MSCs. Data were analyzed with a student's *t*-test (unpaired, two-tailed). n = 4–5 per group. Data are means ± standard deviation; *p < 0.05; ***p < 0.001.
MFI: Mean fluorescence intensity; MSC: Mesenchymal stem cell.

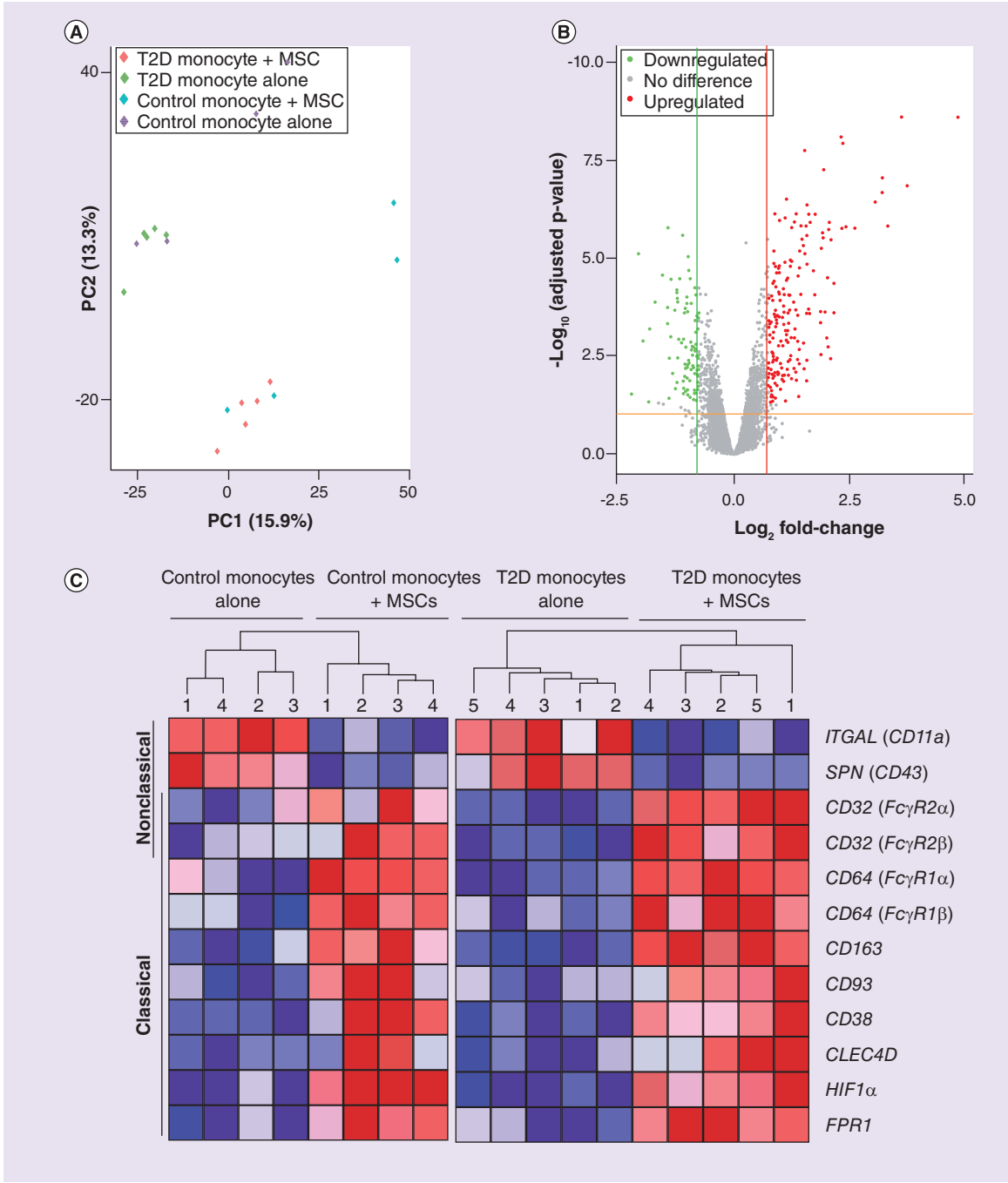

Figure 5. Mesenchymal stem cells alter the gene-expression profile of monocytes. Principal component anaylsis of monocytes isolated from control and Type 2 diabetic subjects, following 48 h of co-culture with and without MSCs, demonstrating the relationship between samples for the first two principal components. The variance explained by each principal component is shown within the brackets of the axis labels (pairwise analysis, threshold of >twofold and adjusted p-value < 0.05; **[A]**). Volcano plot showing the $\log_2$ fold-change and adjusted p-value of differentially expressed genes for the comparison of MSC-treated monocytes versus untreated monocytes (log-fold-change of <-0.75 or >0.75; **[B]**). Hierarchical clustering and heatmap visualization of known classical and nonclassical monocyte **(C)** and M2 macrophage **(D)** markers differentially expressed on monocytes co-cultured with and without MSCs for 48 h. Numbers at the top of the heatmap correspond to subject number (Defined in Table 1). Each gene is colored according to their expression; red indicates upregulated, blue indicates downregulated. The intensity of the color indicates the level of gene expression.
MSC: Mesenchymal stem cell; T2D: Type 2 diabetes.

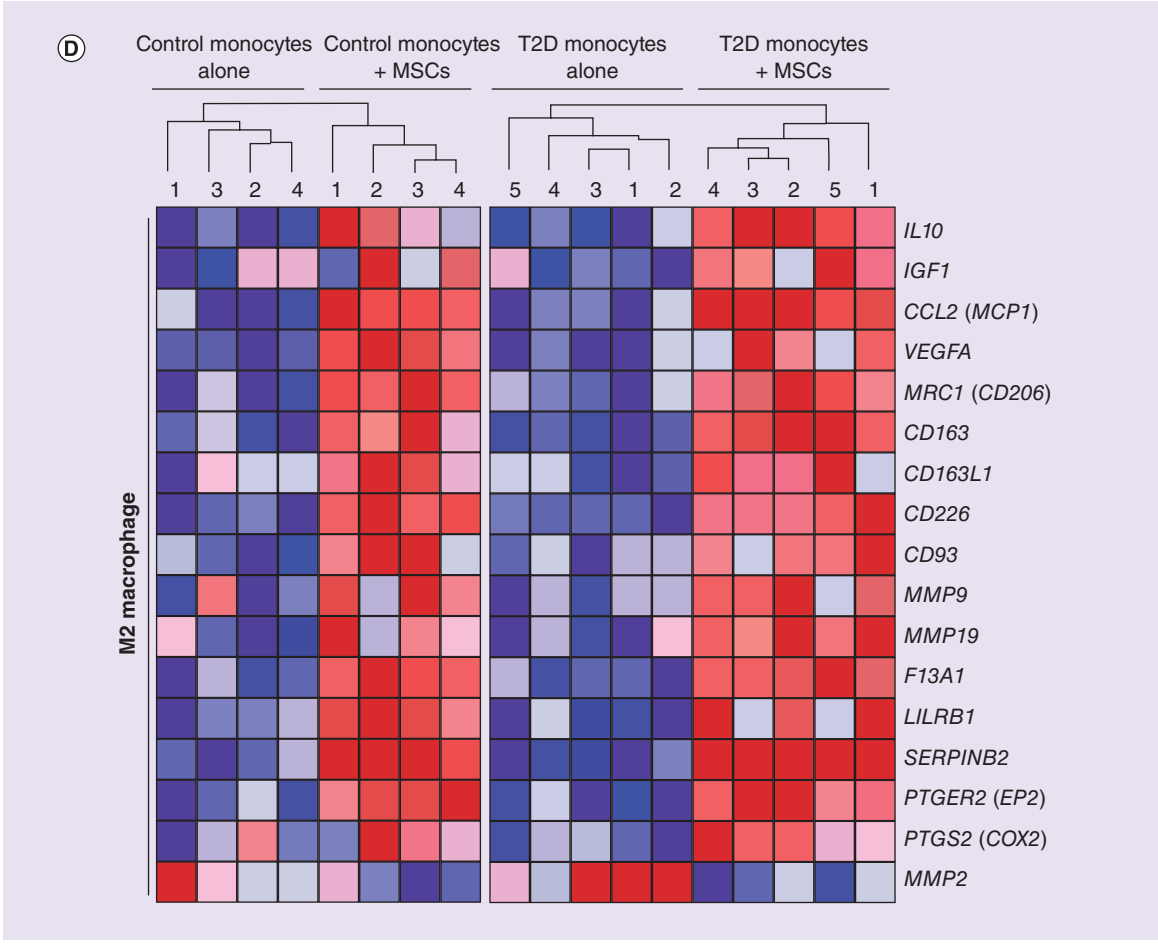

Figure 5. Mesenchymal stem cells alter the gene-expression profile of monocytes (cont.).

strated impaired functionality, involving chemotaxis and phagocytosis [23,24].

The present study provides evidence that soluble factors produced by MSCs promoted the growth of monocytes *in vitro* in the disease group, and upregulated CD14 and CD16 expression, with a concomitant reduction in HLA-DR expression on both T2D and control monocytes. The immunomodulatory properties of MSCs have been reported to be more profound in an inflammatory environment compared with steady-state conditions, whereby the MSCs' immunosuppressive properties are primed by inflammatory stimuli [25,26]. This was consistent with our own observations of more pronounced immunomodulatory effects of MSCs following co-culture with T2D monocytes. Our finding that MSCs have the capacity to modulate monocyte phenotype, is in accordance with other studies that have demonstrated MSC immunomodulatory abilities [27,28]. Rocher *et al.* [27] recently reported that MSCs impair the differentiation of $CD14^{++}CD16^{-}CD64^{+}$ classical monocytes, from mixed leukocyte reactions, into $CD14^{++}CD16^{+}CD64^{++}$ intermediate monocytes with decreased levels of MHC class II expression. In addition, Cutler *et al.* [28] revealed that MSCs decreased the expression of HLA-DR and increased CD14 expression on monocytes in nonactivated and alloantigen-activated PBMC cultures. These cells also had increased CD206 expression, a marker associated with an anti-inflammatory M2 macrophage phenotype. Furthermore, these findings could be replicated using a Transwell co-culture system or the MSC-conditioned medium alone, indicating the effects were mediated by the MSC-derived soluble factors [28]. However, the precise mechanisms in which these MSC-derived soluble factors modulate monocyte phenotype remain to be fully elucidated.

Manipulating monocyte phenotype in order to reduce inflammation and subsequently insulin resistance could be a promising therapeutic option for Type 2 diabetic patients. Macrophage-derived IL-10 can inhibit the pathological effects of TNF-α-induced insulin resistance in adipocytes [29]. In an experimental model of Type 2 diabetes, the reduction of pro-inflammatory cytokines along with the enhancement of a reparative macrophage phenotype resulted in improved insulin signaling and glycemic control [30]. Fadini *et al.* [31]

did not find significant differences in the proportion of monocyte subsets from control and diabetic patients. However, they reported that Type 2 diabetic patients have an imbalanced M1 ($CD68^+CCR2^+$)/M2 ($CX3CR1^+CD206^+CD163^+$) ratio, which was attributable to a reduction in M2 macrophages. This imbalance directly correlated to waist circumference, HbA1c and DN.

The present study demonstrated that MSCs upregulate several genes associated with an M2 macrophage phenotype, including the cytokines IL-10, IGF-1 and VEGF, known for their potent anti-inflammatory and regenerative abilities, and have the capacity to protect against diabetes in both mice and humans [32–34]. Others have shown that IGF-1 administration can improve glycemic control in Type 2 diabetic patients by enhancing insulin sensitivity and reducing hyperglycemia and HbA1c [35–37]. Furthermore, macrophages expressing IGF-1 and IL-10 have both been shown to dampen inflammation and promote extracellular matrix remodeling and repair of damaged kidneys [38,39]. During the early stages of diabetic nephropathy, elevated expression of VEGF is present within the kidney. However, as the disease advances, VEGF expression and activity declines [40,41]. In humans, Tumlin *et al.* [42] demonstrated that in patients with advanced DN, a reduction in proteinuria and stabilization of renal function correlated with increased levels of urinary VEGF.

The present study demonstrated that MSCs upregulated several scavenger receptor genes, which are key players mediating the remodeling functions of macrophages following tissue damage. An increase in MMP-9 and MMP-19 was also observed, which in the kidney also contributes to extracellular matrix degradation and the attenuation of fibrosis [15,43]. In addition, we observed an upregulation of the PTGER2–IL-10 signaling pathway in the MSC-treated monocytes regardless of whether the monocytes were from diabetic patients or healthy controls. MSCs enhanced *PTGER2* gene expression in the monocytes. It has been demonstrated that MSCs secrete PGE_2, which acts via the EP_2 receptor on monocytes, encoded by *PTGER2*. This secreted prostaglandin induces IL-10 production, resulting in immunosuppression [28,44]. Additionally, PTGS2, which also induces the production of PGE_2, was upregulated in the MSC-treated monocytes. Recent studies have shown that it is the MSC-derived PGE_2 that induces macrophage polarization toward a reparative phenotype [45–47]. However, this effect is lost as a result of PGE_2 ablation or blocking of the PGE_2 receptors, EP2 or EP4 [45,47]. Therefore our results suggest that a major factor that promotes the MSC modulation of monocyte phenotype is IL-10.

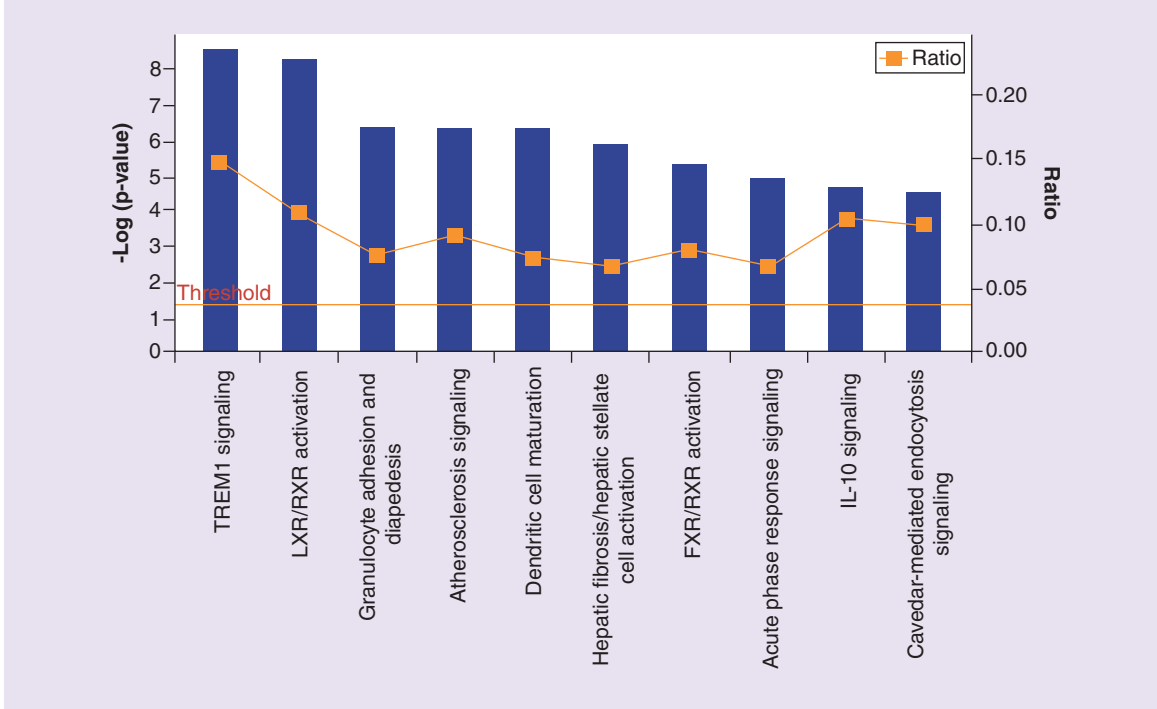

Figure 6. Signaling pathways upregulated in monocytes co-cultured with mesenchymal stem cells. The top ten canonical signaling pathways significantly over-represented in control and Type 2 diabetes monocytes co-cultured with mesenchymal stem cells compared with the monocyte alone groups. The threshold line refers to the cut-off probability ($p < 0.05$) and the ratio line indicates the number of genes from the gene list that pass the cut-off criteria in the pathway divided by the total number of genes in the pathway.

Table 2. Top ten canonical signaling pathways significantly over-represented in mesenchymal stem cell-treated monocytes.

Canonical signalling pathway	p-value	Differentially expressed genes
TREM1 signaling	2.83×10^{-9}	*CASP5*, *CCL2*, *CD83*, *CIITA*, *CXCL8*, *FCGR2B*, *ICAM1*, *IL-10*, *IL-1B*, *ITGAX*, *TREM1*
Liver X receptor/retinoid X receptor activation	1.55×10^{-9}	*APOC1*, *APOE*, *C3*, *CCL2*, *IL-1A*, *IL-1B*, *IL1RAP*, *LPL*, *MMP9*, *MSR1*, *PTGS2*, *SERPINA1*, *SERPINF1*
Granulocyte adhesion and diapedesis	4.74×10^{-7}	*CCL2*, *CXCL5*, *CXCL8*, *FPR1*, *FPR2*, *ICAM1*, *IL-1A*, *IL-1B*, *IL1RAP*, *ITGAL*, *MMP2*, *MMP9*, *MMP19*
Atherosclerosis signaling	5.20×10^{-7}	*APOC1*, *APOE*, *CCL2*, *CXCL8*, *ICAM1*, *IL-1A*, *IL-1B*, *LPL*, *MMP9*, *MSR1*, *SERPINA1*
Dendritic cell maturation	5.93×10^{-7}	*CD83*, *FCGR1B*, *FCGR2A*, *FCGR2B*, *FCGR3A/FCGR3B*, *HLA-DMB*, *HLA-DQB1*, *ICAM1*, *IL-10*, *IL-1A*, *IL-1B*, *STAT4*, *TREM2*
Hepatic fibrosis/hepatic stellate cell activation	1.59×10^{-6}	*A2M*, *CCL2*, *CXCL8*, *EDNRB*, *ICAM1*, *IGF1*, *IL-10*, *IL-1A*, *IL-1B*, *IL1RAP*, *MMP2*, *MMP9*, *VEGFA*
Farnesoid X receptor/retinoid X receptor activation	5.48×10^{-6}	*APOC1*, *APOE*, *C3*, *IL-1A*, *IL-1B*, *LPL*, *PPARG*, *SERPINA1*, *SERPINF1*, *VLDLR*
Acute phase response signaling	1.16×10^{-5}	*A2M*, *C2*, *C3*, *IL-1A*, *IL-1B*, *IL1RAP*, *RBP1*, *SERPINA1*, *SERPINF1*, *SOCS3*, *SOD2*
IL-10 signaling	2.55×10^{-5}	*FCGR2A*, *FCGR2B*, *IL-10*, *IL-1A*, *IL-1B*, *IL1RAP*, *SOCS3*
Caveolar-mediated endocytosis signaling	3.72×10^{-5}	*FLNA*, *FLNB*, *FLOT1*, *ITGA9*, *ITGAL*, *ITGAX*, *PRKCA*

MSCs may be capable of improving β-cell function and consequently insulin sensitivity, ameliorating hyperglycemia and glomerulosclerosis leading to significantly improved renal function in Type 1 and 2 diabetic rodents [48–51]. In a Phase I clinical trial, three intravenous infusions of MSCs administered 1 month apart, to patients with T2D diabetes, reduced blood glucose levels and Hb1Ac, increased C-peptide and insulin secretion, indicative of an improvement in β-cell function and improved renal function [12]. Together, these studies highlight the ability of MSCs to target the many facets of this multifactorial disease. Their capability to reduce fibrosis, remodel/repair the damaged kidney and improve renal function, make MSCs a prime candidate to treat many forms of kidney damage. The additional immunosuppressive properties, which can dampen the inflammatory environment resulting in a downstream improvement in glycemic control, make them an ideal therapy for Type 2 diabetic patients.

Conclusion

In summary, we report that monocytes from Type 2 diabetic patients show a different phenotype compared with control cells, based on CD14 and CD16 expression. The inflammatory monocyte populations from Type 2 diabetic patients could be either perpetuating the underlying inflammation, or may provide an indicator of disease state. Nonetheless, these data provide a useful benchmark for which to assess potential treatments for T2D and DN. In this regard, manipulating monocyte phenotype and the pro-inflammatory state of T2D patients with the use of MSCs could serve as a useful tool for the development of a novel therapeutic option for patients with T2D.

Future perspective

These findings provide novel insight into the feasibility of administering MSCs to alter the profile of inflammatory monocytes and macrophages in patients with diabetes. MSCs may therefore have the potential to be harnessed for cell-based therapies to reduce the progression of DN in a setting of ESRD. The recruitment of inflammatory monocytes plays an important role in disease progression. Modulation of these cells, with the use of MSCs, is a potential therapeutic approach.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/rme.15.74

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Monocyte subsets

- Control subjects had a significantly greater proportion of $CD14^{++}CD16^{-}$ classical monocytes compared with diabetic patients. By contrast, the diabetic patients had a higher proportion of $CD14^{++}CD16^{+}$ intermediate and $CD14^{+}CD16^{++}$ nonclassical monocytes.

Mesenchymal stem cells alter the maturation state of monocytes

- Mesenchymal stem cells (MSCs) treatment impaired the differentiation of monocytes toward a pro-inflammatory nonclassical phenotype.

MSCs alter the maturation state of monocytes

- Principal component analysis revealed that MSC-treated monocytes clustered separately from the untreated monocytes.
- MSC co-culture promoted expression of anti-inflammatory genes consistent with an M2-like macrophage phenotype.

Limitations of the study

- Control patient samples would be optimally age-matched to Type 2 diabetic subjects.
- Protein validation of the key genes involved would provide valuable insight into the mechanism driving MSC modulation of monocyte phenotype.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1 Atkins RC, Zimmet P. Diabetic kidney disease: act now or pay later. *Nat. Rev. Nephrol.* 6(3), 134–136 (2010).

2 Lane PH, Steffes MW, Fioretto P, Mauer SM. Renal interstitial expansion in insulin-dependent diabetes mellitus. *Kidney Int.* 43(3), 661–667 (1993).

3 Nguyen D, Ping F, Mu W, Hill P, Atkins RC, Chadban SJ. Macrophage accumulation in human progressive diabetic nephropathy. *Nephrology* 11(3), 226–231 (2006).

4 Chow F, Ozols E, Nikolic-Paterson DJ, Atkins RC, Tesch GH. Macrophages in mouse Type 2 diabetic nephropathy: correlation with diabetic state and progressive renal injury. *Kidney Int.* 65(1), 116–128 (2004).

5 Ingersoll MA, Platt AM, Potteaux S, Randolph GJ. Monocyte trafficking in acute and chronic inflammation. *Trends Immunol.* 32(10), 470–477 (2011).

6 Ziegler-Heitbrock L, Ancuta P, Crowe S *et al.* Nomenclature of monocytes and dendritic cells in blood. *Blood* 116(16), e74–e80 (2010).

• **This paper proposes a nomenclature for both human and mouse monocyte subsets that should be used in the field.**

7 Martinez FO, Gordon S, Locati M, Mantovani A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J. Immunol.* 177(10), 7303–7311 (2006).

8 Auffray C, Fogg D, Garfa M *et al.* Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science* 317(5838), 666–670 (2007).

9 Ziegler-Heitbrock L. The $CD14^{+}$ $CD16^{+}$ blood monocytes: their role in infection and inflammation. *J. Leukoc. Biol.* 81(3), 584–592 (2007).

• **Useful review that discusses the role of monocyte subsets in different disease settings.**

10 Ancuta P, Liu KY, Misra V, Wacleche VS. Transcriptional profiling reveals developmental relationship and distinct biological functions of $CD16^{+}$ and $CD16^{-}$ monocyte subsets. *BMC Genomics* doi:10.1186/1471-2164-10-403 (2009) Epub ahead of print).

11 Perico N, Casiraghi F, Introna M *et al.* Autologous mesenchymal stromal cells and kidney transplantation: a pilot study of safety and clinical feasibility. *Clin. J. Am. Soc. Nephrol.* 6(2), 412–422 (2011).

12 Jiang R, Han Z, Zhuo G *et al.* Transplantation of placenta-derived mesenchymal stem cells in Type 2 diabetes: a pilot study. *Front. Med.* 5(1), 94–100 (2011).

13 Reinders MEJ, de Fijter JW, Roelofs H *et al.* Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a Phase I study. *Stem Cells Transl. Med.* 2(2), 107–111 (2013).

14 Jiang X-X, Zhang Y, Liu B *et al.* Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood* 105(10), 4120–4126 (2005).

15 Wise AF, Williams TM, Kiewiet MBG *et al.* Human mesenchymal stem cells alter macrophage phenotype and promote regeneration via homing to the kidney following ischemia-reperfusion injury. *Am. J. Physiol. Renal Physiol.* 306(10), F1222–F1235 (2014).

16 Wells CA, Mosbergen R, Korn O *et al.* Stemformatics: visualisation and sharing of stem cell gene expression. *Stem Cell Res.* 10(3), 387–395 (2013).

17 Giulietti A, van Etten E, Overbergh L, Stoffels K, Bouillon R, Mathieu C. Monocytes from Type 2 diabetic patients have a pro-inflammatory profile. 1,25-dihydroxyvitamin D(3) works as anti-inflammatory. *Diabetes Res. Clin. Pract.* 77(1), 47–57 (2007).

18 Min D, Brooks B, Wong J *et al.* Alterations in monocyte CD16 in association with diabetes complications. *Mediators Inflamm.* doi:10.1155/2012/649083 (2012) (Epub ahead of print).

19 Yang M, Gan H, Shen Q, Tang W, Du X, Chen D. Proinflammatory $CD14^{+}CD16^{+}$ monocytes are associated with microinflammation in patients with Type 2 diabetes mellitus and diabetic nephropathy uremia. *Inflammation* 35(1), 388–396 (2012).

20 Ruan H, Lodish HF. Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-alpha. *Cytokine Growth Factor Rev.* 14(5), 447–455 (2003).

21 Cai D, Yuan M, Frantz DF *et al.* Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat. Med.* 11(2), 183–190 (2005).

22 Ehses JA, Lacraz G, Giroix M-H *et al.* IL-1 antagonism reduces hyperglycemia and tissue inflammation in the Type 2 diabetic GK rat. *Proc. Natl Acad. Sci. USA* 106(33), 13998–14003 (2009).

23 Katz S, Klein B, Elian I, Fishman P, Djaldetti M. Phagocytotic activity of monocytes from diabetic patients. *Diabetes Care* 6(5), 479–482 (1983).

24 Waltenberger J, Lange J, Kranz A. Vascular endothelial growth factor-A-induced chemotaxis of monocytes is attenuated in patients with diabetes mellitus: a potential predictor for the individual capacity to develop collaterals. *Circulation* 102(2), 185–190 (2000).

25 Ryan JM, Barry F, Murphy JM, Mahon BP. Interferon-γ does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. *Clin. Exp. Immunol.* 149(2), 353–363 (2007).

26 Sheng H, Wang Y, Jin Y *et al.* A critical role of IFN gamma in priming MSC-mediated suppression of T cell proliferation through up-regulation of B7-H1. *Cell Res.* 18(8), 846–857 (2008).

27 Rocher BD, Mencalha AL, Gomes BE, Abdelhay E. Mesenchymal stromal cells impair the differentiation of $CD14^{++}$ $CD16^{-}$ $CD64^{+}$ classical monocytes into $CD14^{++}$ $CD16^{+}$ $CD64^{++}$ activate monocytes. *Cytotherapy* 14(1), 12–25 (2012).

28 Cutler AJ, Limbani V, Girdlestone J, Navarrete CV. Umbilical cord-derived mesenchymal stromal cells modulate monocyte function to suppress T cell proliferation. *J. Immunol.* 185(11), 6617–6623 (2010).

29 Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J. Clin. Invest.* 117(1), 175–184 (2007).

30 Jadhav A, Tiwari S, Lee P, Ndisang JF. The heme oxygenase system selectively enhances the anti-inflammatory macrophage-M2 phenotype, reduces pericardial adiposity, and ameliorated cardiac injury in diabetic cardiomyopathy in Zucker diabetic fatty rats. *J. Pharmacol. Exp. Ther.* 345(2), 239–249 (2013).

31 Fadini GP, de Kreutzenberg SV, Boscaro E *et al.* An unbalanced monocyte polarisation in peripheral blood and bone marrow of patients with Type 2 diabetes has an impact on microangiopathy. *Diabetologia* 56(8), 1856–1866 (2013).

32 Hong E-G, Ko HJ, Cho Y-R *et al.* Interleukin-10 prevents diet-induced insulin resistance by attenuating macrophage and cytokine response in skeletal muscle. *Diabetes* 58(11), 2525–2535 (2009).

33 van Exel E, Gussekloo J, de Craen AJM *et al.* Low production capacity of interleukin-10 associates with the metabolic syndrome and Type 2 diabetes: the Leiden 85-Plus Study. *Diabetes* 51(4), 1088–1092 (2002).

34 Blüher M, Fasshauer M, Tönjes A, Kratzsch J, Schön MR, Paschke R. Association of interleukin-6, C-reactive protein, interleukin-10 and adiponectin plasma concentrations with measures of obesity, insulin sensitivity and glucose metabolism. *Exp. Clin. Endocrinol. Diabetes* 113(9), 534–537 (2005).

35 Moses AC, Young SC, Morrow LA, O'Brien M, Clemmons DR. Recombinant human insulin-like growth factor I increases insulin sensitivity and improves glycemic control in Type II diabetes. *Diabetes* 45(1), 91–100 (1995).

36 Cusi K, DeFronzo R. Recombinant human insulin-like growth factor I treatment for 1 week improves metabolic control in Type 2 diabetes by ameliorating hepatic and muscle insulin resistance. *J. Clin. Endocrinol. Metab.* 85(9), 3077–3084 (2000).

37 Clemmons DR, Moses AC, Sommer A *et al.* Rh/IGF-I/rhIGFBP-3 administration to patients with Type 2 diabetes mellitus reduces insulin requirements while also lowering fasting glucose. *Growth Hormone IGF Res.* 15(4), 265–274 (2005).

38 Alikhan MA, Jones CV, Williams TM *et al.* Colony-stimulating factor-1 promotes kidney growth and repair via alteration of macrophage responses. *Am. J. Pathol.* 179(3), 1243–1256 (2011).

39 Li W, Zhang Q, Wang M *et al.* Macrophages are involved in the protective role of human umbilical cord-derived stromal cells in renal ischemia-reperfusion injury. *Stem Cell Res.* 10(3), 405–416 (2013).

40 Cha DR, Kim NH, Yoon JW *et al.* Role of vascular endothelial growth factor in diabetic nephropathy. *Kidney Int. Suppl.* 77(Suppl. 77), S104–S112 (2000).

41 Hohenstein B, Hausknecht B, Boehmer K, Riess R, Brekken RA, Hugo CPM. Local VEGF activity but not VEGF expression is tightly regulated during diabetic nephropathy in man. *Kidney Int.* 69(9), 1654–1661 (2006).

42 Tumlin JA, Galphin CM, Rovin BH. Advanced diabetic nephropathy with nephrotic range proteinuria: a pilot

study of the long-term efficacy of subcutaneous ACTH gel on proteinuria, progression of CKD, and urinary levels of VEGF and MCP-1. *J. Diabetes Res.* doi:10.1155/2013/489869 (2013) (Epub ahead of print).

43 Mankhey RW, Wells CC, Bhatti F, Maric C. 17beta-estradiol supplementation reduces tubulointerstitial fibrosis by increasing MMP activity in the diabetic kidney. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 292(2), R769–R770 (2007).

44 Wang D, Chen K, Du WT *et al.* $CD14^+$ monocytes promote the immunosuppressive effect of human umbilical cord matrix stem cells. *Exp. Cell Res.* 316(15), 2414–2423 (2010).

45 Németh K, Leelahavanichkul A, Yuen PST *et al.* Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat. Med.* 15(1), 42–49 (2009).

46 Maggini J, Mirkin G, Bognanni I *et al.* Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. *PLoS ONE* 5(2), e9252 (2010).

•• **Breakthrough paper describing the mechanism in which mesenchymal stem cells reprogram macrophages toward an alternative phenotype.**

47 Furuhashi K, Tsuboi N, Shimizu A *et al.* Serum-starved adipose-derived stromal cells ameliorate crescentic GN by promoting immunoregulatory macrophages. *J. Am. Soc. Nephrol.* 24(4), 587–603 (2013).

48 Lee RH, Seo MJ, Reger RL *et al.* Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. *Proc. Natl Acad. Sci. USA* 103(46), 17438–17443 (2006).

49 Park JH, Hwang I, Hwang SH, Han H, Ha H. Human umbilical cord blood-derived mesenchymal stem cells prevent diabetic renal injury through paracrine action. *Diabetes Res. Clin. Pract.* 98(3), 465–473 (2012).

50 Si Y, Zhao Y, Hao H *et al.* Infusion of mesenchymal stem cells ameliorates hyperglycemia in Type 2 diabetic rats. *Diabetes* 61(6), 1616–1625 (2012).

51 Lv S-S, Liu G, Wang J-P *et al.* Mesenchymal stem cells transplantation ameliorates glomerular injury in streptozotocin-induced diabetic nephropathy in rats via inhibiting macrophage infiltration. *Int. Immunopharmacol.* 17(2), 275–282 (2013).