Exosomes derived from monocytes and from endothelial cells mediate monocyte and endothelial cell activation under high D-glucose conditions

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ABSTRACT

Diabetes mellitus type 2 (DMT2) is characterized by hyperglycemia and associated with low grade inflammation affecting both endothelial cells and monocytes. Exosomes are nanovesicles, allow communication between endothelial cells and monocytes and have been associated with diabetic complications. In this study we evaluated whether high glucose can activate monocytes and endothelial cells and whether exosomes play a role in this activation. Moreover, we studied whether endothelial cells and monocytes communicate with each other via exosomes under high and basal glncubation. In the first experiment, monomac 6 cells (MM6) were exposed to high glucose (HG; 25 mmol/L) or to exosomes from MM6 exposed to HG (exoMM6-HG) or basal glucose (5.5 mmol/L) (exoMM6-BG). In the second experiment, MM6 were exposed to exosomes from human umbilical vein endothelial cells (HUVECs) and HUVECs to exosomes from MM6. In the third experiment, MM6 and HUVECs were exposed to a mixture of exosomes from MM6 and HUVECs (exoMix). Cell activation was evaluated by measuring the protein surface expression of intracellular adhesion molecule-1 (ICAM-1) by flow cytometry. HG increased ICAM-1 expression in MM6 and monocytic exosomes from HG or BG shown similar effect in HG and BG MM6 cells. Exosomes from HUVECs increased ICAM-1 expression in MM6 cells, incubated under HG or BG, while also exosomes from MM6 increased ICAM-1 expression in HUVECs incubated under HG or BG. The combination of exosomes from both cell types (exoMixHG or exoMixBG) also increased ICAM-1 expression in both type cells in most conditions. However, the exoMixBG reversed the effect of HG in both MM6 and HUVECs. Our results show that HG activated monocytes and endothelial cells and that exosomes play a role in this HG-induced cell ICAM-1 expression. We hypothesize that during DMT2, exosomes may act as a communication mechanism between monocytes and endothelial cells, inducing and maintaining activating of both cell types in the presence of high glucose.

1. Introduction

Diabetes is one of the major health burdens in the world. It is estimated that 415 millions of adults suffer from this disease in 2015 (Ogurtsova et al., 2017). Moreover, the worldwide prevalence is increasing (Riddle et al., 2018). Diabetes mellitus Type 2 (DMT2) is the most common type of diabetes, since around 90% of all diabetes patients have type 2 diabetes (Ji et al., 2017). DMT2 is a complex disease, which is characterized by insulin resistance, beta-cell deficiency, resulting in decreased insulin secretion and hyperglycemia (Chénard et al., 2017). A combination of various factors, such as genetic, lifestyle (such as diet), environmental factors, is responsible for the development of the disease.

DMT2 is viewed as a chronic low-grade inflammatory disease, in which both immune cells and endothelial cells are involved (Kimball et al., 2017; Raygan et al., 2016). Hallmarks of the low-grade inflammatory process are endothelial and monocyte cell activation (Davison et al., 2017; Halvorsen et al., 2016). This cell activation leads to the endothelial and monocytes cells to secrete cytokines and express adhesion molecules such as intracellular adhesion molecule type I (ICAM-1) (Altannavch et al., 2004; Faas et al., 2010; Sáez et al., 2018; Štulc et al., 2014), which mediates the adhesion and transmigration of immune cells into vascular wall. Additionally, patients with DMT2 have increased circulating levels of ICAM-1 (Karimi et al., 2018), which is a marker of endothelial cell activation (Schnoor, 2015). Exosomes are extracellular nanovesicles released by various cell types including endothelial cells and monocytes (de Jong et al., 2012; Tang et al., 2016). Exosomal cargo includes proteins, mRNAs and microRNAs (Kowal et al., 2014), which are able to modulate endothelial cell and monocyte function (Rice et al., 2015). Exosomes have been recognized as communication mechanism(Simons and Raposo, 2009), leading to interactions between different cell types, such as endothelial cells and immune cells (as monocytes) (Müller, 2012) and modulate their cellular function.

In the present study, we hypothesize that monocytes and endothelial cells produce exosomes in the presence of high glucose and that the glucose-modulated exosomes activated monocytes and endothelial cells. We used ICAM-1 as a measure of activation of monocytes and endothelial cells. We also studied whether monocyte and HUVECs can communicate and interact with each other via exosomes under basal or high glucose conditions.

2. Material and methods

2.1. Cell culture and treatment

The monocyte cell line (monomac-6 (MM6)) was used for the experiments. MM6 cells show phenotypical and functional features of mature monocytes (Ziegler-Heitbroc et al., 1988). MM6 were cultured (250.000 cells/mL) in monocyte culture medium (RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 2 mmol/L L-Glutamine, 100 IE/mL Penicillin, 100 μg/mL Streptomycin, 10% heat-inactivated fetal calf serum, at 37 °C and 5% CO_2 (Corning® Costar®; Sigma-Aldrich, Zwijndrecht, The Netherlands). The MM6 cell line was cultured as described before (Faas et al., 2010). Cells were used for experiments under basal (5.5 mmol/L) and high (25 mmol/L) levels of glucose.

Human umbilical vein endothelial cells (HUVECs) were provided by the Endothelial Cell facility of the UMCG (Groningen, Netherlands). HUVECs were isolated by collagenase digestion (0.25 mg/mL Collagenase Type II from Clostridium histolyticum; Boehringer, Mannheim, Germany), as previously described (Faas et al., 2010). HUVECs were cultured in HUVECs culture medium (RPMI (Lonza, Basel, Switzerland) supplemented with 2 mmol/L L-Glutamine, 5 U/mL Heparin, 100 IE/mL Penicillin, 100 μg/mL Streptomycin, 50 μg/mL Crude ECGF solution and 20% FCS) at 37 °C, 5% CO_2 on gelatin-precoated tissue culture flasks (Corning® Costar®; Sigma-Aldrich,

Zwijndrecht, The Netherlands). Cells were used for experiments under basal (5.5 mmol/L) and high (25 mmol/L) levels of glucose.

Co-culture of HUVECs and MM6: confluent HUVECs in passage 2 in 12 wells-plate were co-cultured with MM6 cells (125.000 cells/mL). 3 days before the start of the co-culture, MM6 cells were transferred to endothelial cell medium. Cells in co-culture were exposed to high glucose (25 mmol/L) or basal glucose (5.5 mmol/L) in exosome-free medium, during 24 h. After this, the supernatant was collected and centrifuged at 1200 rpm for 10 min at 4 °C, to collect the MM6 cells. MM6 cells were not attached to endothelial cells. HUVECs were washed with PBS (3×) and trypsin was used to isolate HUVECs as described above. HUVECs and MM6 were prepared for flow cytometry. Dead cells were excluded of the analysis by centrifugation.

2.2. Exosome isolation and purification

Before isolation of exosomes, HUVECs and MM6 cells were exposed to high glucose (HG, 25 mmol/L) (HUVEC-HG and MM6-HG) or to basal glucose (5.5 mmol/L; HUVEC-BG and MM6-BG) during 24 h. Exosomes were isolated as we previously described (Sáez et al., 2018). Supernatants (40 mL) from MM6 and HUVECs (HG and BG conditions) were collected after 24 h incubation. Exosomes were isolated and purified by differential ultracentrifugation. In brief, supernatant was filtered through a 0.22 μm filter and then, centrifugation was initially performed at 2000 g at 4 °C for 30 min, followed by 12,000 g at 4 °C for 45 min. Then the supernatant was centrifuged at 110,000 g at 4 °C for 70 min (Centrikon T-1080 ultracentrifuge, Kontron Instruments). The pellet was resuspended in PBS (pH 7.4) and again centrifuged at 110,000 g at 4 °C for 70 min. The exosome pellet was resuspended in sucrose/HEPES 2 mmol/L and subsequently purified using a discontinuous sucrose gradient (following the protocol described by Théry et al (Théry et al., 2006); from 2 mmol/L to 0.25 mmol/L). Thus, exosomes were mixed with 2 mL of 2 M sucrose in HEPES and placed on the bottom of a SW41 centrifuge tube, overlaid with 1.5 mL 1.35 mmol/L sucrose, 0.5 mL 1.28 mmol/L sucrose, 1 mL 1.15 mmol/L sucrose and 5 mL 0.25 mmol/L sucrose, and ultracentrifuged for 16 h at 210,000 g at 4 °C. Thus, based on our previous study (Sáez et al., 2018), we collected the first 11 fractions, since exosomes were only present in fraction 11. Fractions were collected with 1 mL sucrose and refractive index was measured. After this, all fractions were washed and ultracentrifuged again with PBS at 110,000 g at 4 °C for 70 min. Fraction 11 showed floating density for exosomes (1.16 g/mL) and exosomal markers by western blotting (see results) and was used for the experiments described below. The final pellet was re-suspended in 300 μL of PBS and stored at −80 °C for later incubation experiments with HUVECs. The exosomal protein concentration was determined using the BSA™ Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Preparation of exosomes for TEM

10 μL of exosome suspension was allowed to adhere on freshly prepared Formvar coated 200 mesh EM grids for 2 min. After draining the liquid with a filter paper, samples were stained for 1 min on a drop of 2% ammonium molybdate in water. After draining the liquid again with filter paper and air-drying for 5 min, samples were examined in a FEI Cm100 transmission electron microscope operated at 80 KV. Pictures were taken with a Morada camera using Olympus-sis software.

2.4. Nanoparticle tracking analysis

Quantification and size distribution of exosomes were determined using the Nanoparticle Tracking Analysis (NTA) equipped with a 405 nm laser with LM14 module (NanoSight NTA 3.0 Nanoparticle Tracking, Version 3.0). Samples were diluted with PBS (1/10) prior to analysis in order to obtain particle distribution of particles per image (50 particles per image). Samples were measured using capture 60 and

repeat 3. Settings were kept constant in all samples. Each video was analyzed to get the mean particle size and the concentration of particles.

2.5. Western blotting of exosomes

Exosomal pellets isolated from cells exposed to exosomes were lysed with RIPA buffer (1X) (Radio-Immunoprecipitation Assay buffer, 25 mmol/L Tris·HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) (Pierce® RIPA Buffer, Thermo Fisher Scientific Inc, Landsmeer, Netherlands) at room temperature for 5 min and then loading buffer (5×) was added to the samples and incubated at 95 °C for 5 min. Twenty μg of exosomal protein per well was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon®-FL, Millipore, Amsterdam-Zuidoost, Netherlands). Membranes were probed with primary antibody, polyclonal rabbit anti-CD63 (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) After incubation at 4 °C overnight, membranes were washed in Tris buffered saline (pH 7.4) and incubated for 1 h at room temperature with secondary antibody IRDye® 800CW or 680CW Secondary Antibodies (LI-COR, Lincoln, Nebraska USA). Membranes were analyzed using LI-COR Odissey Scanner (LI-COR, Lincoln, Nebraska USA).

2.6. Treatment of MM6 and HUVECs with high and basal glucose

MM6 and HUVECs were exposed to high glucose (HG, 25 mmol/L; MM6-HG or HUVEC-HG) or to basal glucose (5.5 mmol/L; MM6-BG or HUVEC-BG) for 24 h. Thereafter, cells were exposed to exosomes and prepared for flow cytometry.

2.7. Experiment 1: treatment of MM6 with exosomes from MM6 treated with high and basal glucose

To study the role of exosomes in HG-induced MM6 cell activation, exosomes from MM6-HG (exoMM6-HG) and MM6-BG (exoMM6-BG) were isolated from the supernatant as described above. MM6-HG and MM6-BG cells (250.000 cells/mL) were washed and exposed to exoMM6-BG or exoMM6-HG (concentration of exosomes: 0.5 μg protein/cm^2 (equivalent to ~2.7 × 10^7 particles per cm^2) in exosome-free medium for another 12 h. Then MM6 cells were washed and prepared for flow cytometry (Fig. 1A).

2.8. Experiment 2: treatment of MM6 with exosomes from HUVECs and treatment of HUVECs with exosomes from MM6

2.8.1. Experiment 2a: Exposure of HUVECs to exoMM6

To study whether exosomes from MM6 can influence HUVECs activation, exosomes from MM6 incubated with HG (exoMM6-HG) or BG (exoMM6-BG) were isolated as described before. Confluent HUVECs in passage 2 were preincubated in high (25 mmol/L) (HUVEC-HG) of basal (5.5 mmol/L) (HUVEC-BG) glucose medium for 24 h. Then HUVECs were washed and exposed to exoMM6-BG or exoMM6-HG in exosome-free medium for another 12 h (exosome concentration: 0.5 μg protein/cm^2, equivalent to ~2.7 × 10^7 particles per cm^2). Then cells were washed with PBS and prepared for flow cytometry (Fig. 1B).

2.8.2. Experiment 2b: Exposure of MM6 to exoHUVEC

To evaluate the effect of exosomes from HUVECs on MM6 activation, exosomes from HUVEC-HG (exoHUVEC-HG) and exosomes from HUVEC-BG (exoHUVEC-BG) were isolated as described before. MM6 cells (250.000 cells/mL) were preincubated with high (25 mmol/L) or basal (5.5 mmol/L) glucose for 24 h, washed and exposed to exoHUVEC-BG or exoHUVEC-HG for another 12 h in exosome-free medium (exosome concentration: 0.5 μg protein/cm^2, equivalent to ~2 × 10^5 particles per cm^2). Finally, cells were washed with PBS and

prepared for flow cytometric analysis (Fig. 1C).

2.9. Experiment 3: Exposure of HUVECs and MM6 to exosomal mix from MM6 and HUVECs

Confluent HUVECs in passage 2 or MM6 cells (250.000 cells/mL) were preincubated with high (25 mmol/L) or basal (5.5 mmol/L) glucose during 24 h. After this, cells were exposed to a mixture of exoMM6-BG and exoHUVEC-BG (exoMix-BG) or exoMM6-HG and exoHUVEC-HG (exoMix-HG) for 12 h, in a 1:1 ratio in the same concentration as described above. After incubation, the cells were washed with PBS and prepared for flow cytometry (Fig. 1D).

For control, confluent HUVECs in passage 2 in 12 wells-plates were co-cultured with MM6 cells (125.000 cells/mL) as described above and were exposed to high (25 mmol/L) or basal glucose (5.5 mmol/L) in exosome-free medium, during 24 h as described above. HUVECs and MM6 were prepared for flow cytometry as described above. These cells were used as controls for incubation of MM6 or HUVECs with exoMix.

2.10. Flow cytometry analysis

To measure monocyte or HUVECs activation, ICAM-1 expression on these cells was measured using flow cytometry. Therefore, after different exposures (D-glucose and exosomes), MM6 cells were washed with PBS, while HUVECs were washed with PBS and trypsinized, in order to continue with flow cytometer protocol. Then both cell types were incubated with a solution of PBS-FCS 5% (PBS and Fetal calf serum 5%) and mouse fluorescein isothiocyanate (FITC) conjugated anti-ICAM-1 (BioLegend Inc. Uithoorn, The Netherlands). Mouse IgG1 κ (BioLegend Inc. Uithoorn, The Netherlands) was used as isotype control to test for non-specific antibody binding. HUVECs and MM6 cells exposed to LPS (lipopolysaccharide, 2 μg, for 4 h at 37 °C) served as positive control and on these cells increased ICAM-1 expression was always induced (data not shown). To measure dead cells (apoptotic cells) propidium iodide staining (PI) was performed. Flow cytometric analysis was performed using a BD FACSCalibur with two lasers (488 and 635 nm) (BD Biosciences, Breda, The Netherlands). ICAM-1 expression in cells was analyzed using Kaluza Flow Cytometry Analysis Software. HUVECs and MM6 cells were gated based on size and scatter in the forward-side scatter plot. In a forward scatter-PI plot, live cells were selected as cells negative for PI. These live cells were copied to a forwardscatter-FITC plot. The isotype of each sample was used to set a gate excluding all positive cells. This gate was copied to the sample stained for ICAM-1 and percentage positive cells were determined.

2.11. Statistical analysis

All data are expressed as median ± interquartile range. The data displayed a non-parametric distribution as tested by the Shapiro-Wilk normality test. Comparisons between groups were performed using Kruskal Wallis test, followed by Wilcoxon's signed rank test or Man Whitney *U* test where appropriate. P < 0.05 was considered to be significant.

3. Results

3.1. High glucose affects exosomes derived from MM6

Exosomes from MM6 and HUVECs were isolated after exposure to BG or HG in exosome-free culture medium for 24 h. From each supernatant, 12 fractions were collected after sucrose gradient isolation, however, only fractions from 4 to 11 were used to characterize exosomes by western blotting using anti-CD63 (following the protocol used in our previous study Sáez et al., 2018). Exosomes, characterized by expression of CD63, were only found in fraction 11 of both cell types (exosomes derived from HUVECs are shown in Sáez et al. (Sáez et al.,
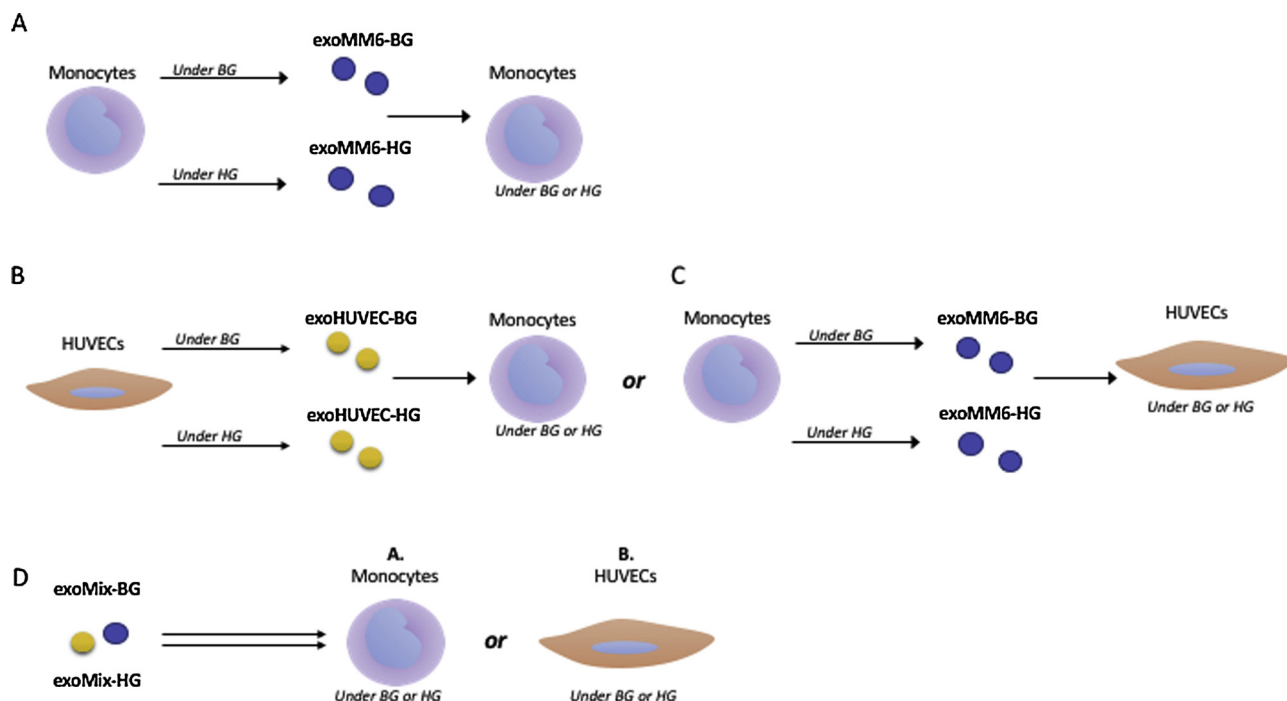
**Fig. 1.** Schematic representation of exosome isolation and exposure to MM6 and HUVECs. Exosomes were isolated from the supernatant of MM6 and HUVECs exposed to basal glucose (BG) and high glucose (HG), after 24 h incubation with exosome-free culture medium. Exosomes from all conditions were purified and characterized as described in Fig. 2. A) Experiment 1: MM6-BG and HG were exposed during 12 h with exoMM6-BG or exoMM6-HG. B) Experiment 2a: MM6-BG and HG were exposed during 12 h with exoHUVEC-BG or exoHUVEC-HG. C) Experiment 2b: HUVEC-BG and HUVEC-HG were exposed during 12 h with exoMM6-BG or exoMM6-HG. D) Experiment 3: HUVECs or MM6 (BG or HG) were exposed to exoMixBG or exoMixHG (exosomes from monocytes and HUVECs under BG or HG) during 12 h.
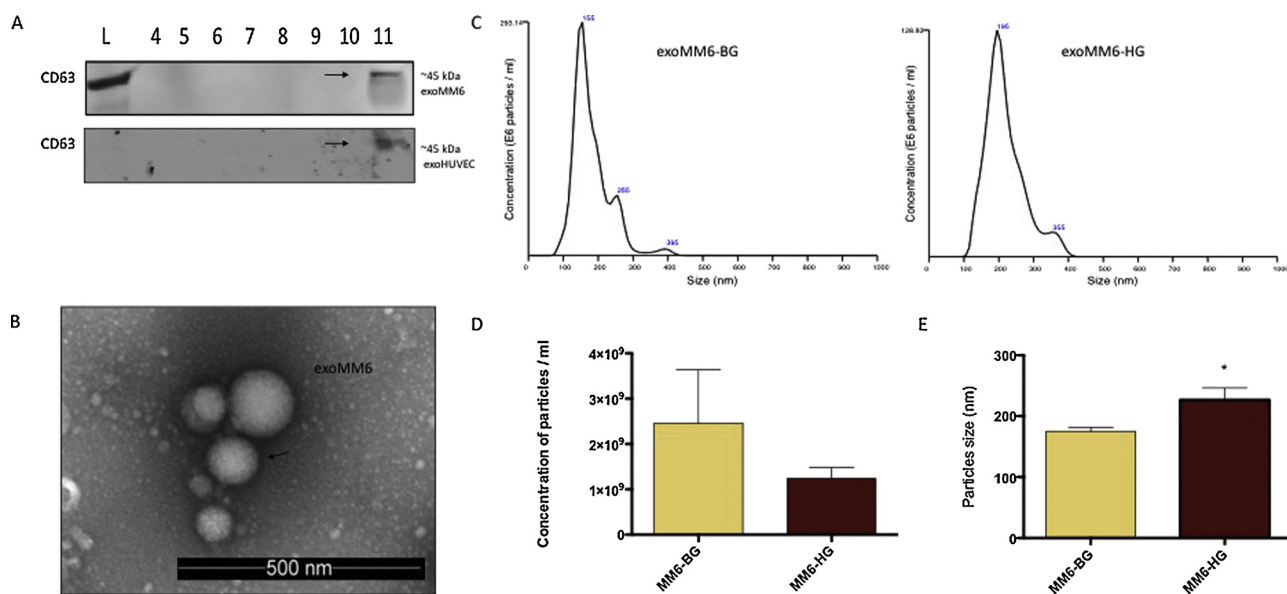
Fig. 2. Exosomes derived from MM6 cells under BG and HG. Exosomes were isolated by the ultracentrifugation protocol. A) After the sucrose gradient isolation, western blotting for each fraction (fractions 4–11 are shown) collected was performed. Only fraction 11 of MM6 showed expression of the exosomal marker CD63. Characterization for exosomes derived from HUVECs are shown in previous studies (Sáez et al., 2018). B) Fractions 11 were analyzed by electron microscopy and all fractions showed exosomes. C) NTA analysis was performed to determine the exosomal concentration and particle size derived from MM6 basal glucose (BG, 5.5 mmol/L, left panel) and high D-glucose (HG, 25 mmol/L, right panel). D) and E) Median (with interquartile range) exosomal concentration (D) and exosomal size (E) for exosomes from MM6 (n = 5), * p < 0.05, Wilcoxon signed rank test.

2018)) (Fig. 2A), so this fraction was used for all subsequent experiments. Fractions 11 were also characterized by electron microscopy and exosomes were found in all fractions 11 (Fig. 2B). Exosomes from fractions 11 were analyzed by NTA (Fig. 2C). Although the concentration of exosomes from MM6-HG appeared to be decreased as compared

with MM6-BG, this was not significant (Fig. 2D). The analysis of exosomal size showed that HG increased the size of exosomes as compared to BG in MM6 (205 nm vs 180 nm, respectively) (Fig. 2E).
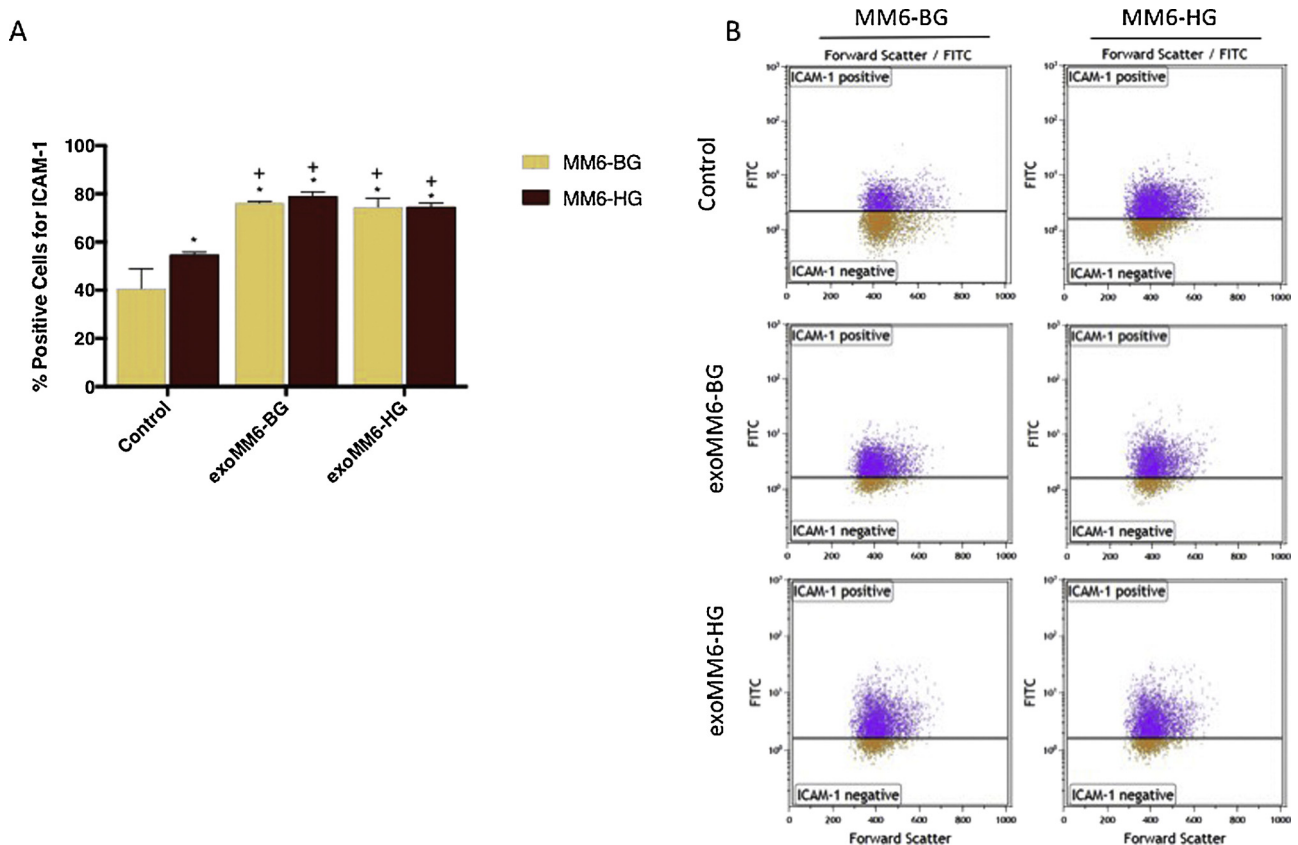
Fig. 3. Effect of high glucose and exosomes on ICAM-1 expression by MM6 exosomes. Percentage of positive cells for ICAM-1 was evaluated in A) MM6-BG and HG incubated with exosomes from HG (exoMM6-HG) or MM6-BG (exoMM6-BG) (n = 5) B) Representative flow cytometric dot plots from each condition. p < 0.05, Wilcoxon Signed rank test (* vs MM6-BG, + vs MM6-HG).

3.2. Experiment 1

3.2.1. High glucose and exosomes derived from MM6-BG and MM6-HG increased the protein surface expression of ICAM-1 in MM6

HG increased the expression of ICAM-1 in MM6 cells as compared to MM6-BG (Fig. 3A). To evaluate whether exosomes play a role in this HG-induced monocyte activation, ICAM-1 was analyzed in MM6-BG or MM6-HG exposed to exoMM6-BG or exoMM6-HG. exoMM6-HG increased ICAM-1 expression in MM6-BG as compared to MM6-BG and HG (Fig. 3A). In MM6-HG the exposure to exoMM6-BG also increased the expression of ICAM-1 as compared to MM6-HG and MM6-BG (Fig. 3A). In MM6-BG exposed to exoMM6-BG and in MM6-HG exposed to exoMM6-HG, exosomes also increased the expression of ICAM-1 (Fig. 3B).

3.3. Experiment 2a

3.3.1. Exosomes derived from MM6 modulate ICAM-1 protein surface expression in HUVECs

To evaluate whether monocytes can communicate with HUVECs via exosomes, we studied the expression of ICAM-1 in HUVECs exposed to exosomes from MM6. ExoMM6-HG increased the expression of ICAM-1 in HUVEC-BG as compared with HUVEC-BG and HUVEC-HG (Fig. 4A). ExoMM6-BG also increased the expression of ICAM-1 in HUVEC-BG as compared with HUVEC-BG (Fig. 4A). The exposure of HUVEC-HG to exoMM6-BG decreased the expression of ICAM-1 as compared with HUVEC-HG (Fig. 4A) to the level of ICAM-1 expression in HUVEC-BG. Exposure of HUVEC-HG to exoMM6-HG highly increased the expression of ICAM-1 as compared with HUVEC-HG and HUVEC-BG (Fig. 4A).

3.4. Experiment 2b

3.4.1. Exosomes derived from HUVECs modulate ICAM-1 protein surface expression in MM6

To study whether HUVECs communicate with monocytes by way of exosomes, MM6-BG and MM6-HG were exposed during 12 h to exoHUVEC-BG or exoHUVEC-HG. The exposure of MM6-BG cells to exoHUVEC-HG increased the expression of ICAM-1 as compared with MM6-BG to similar levels as seen in MM6-HG. Similarly, in MM6-BG exposed to exoHUVEC-BG ICAM-1 expression was increased as compared with MM6-BG to the level of ICAM-1 expression in MM6-HG (Fig. 4B). The expression of ICAM-1 was higher after the incubation of MM6-HG with exoHUVEC-BG as compared with MM6-HG (Fig. 4B). No differences were found after the incubation of MM6-HG incubated with exoHUVEC-HG as compared with MM6-HG (Fig. 4B).

3.5. Experiment 3

3.5.1. A mix of exosomes from monocytes and endothelial cells modulate the protein surface expression of ICAM-1 in monocytes and in endothelial cells

To study whether exosomes derived from monocytes and endothelial cells can interact with each other and modulate ICAM-1 expression in both cell types, we first evaluated ICAM-1 expression on MM6 and HUVECs after co-culture of the cells for 24 h. Thus, MM6 and HUVECs were co-cultured in presence of BG and HG and ICAM-1 expression in co-cultured cells was compared to expression of ICAM-1 in monocultures of MM6 and HUVECs (Fig. 5A and B). HG increased the expression of ICAM-1 in co-cultured MM6 and HUVECs (Fig. 5A). Also, in monocultures of MM6 and HUVEC HG increased ICAM-1 expression as compared to BG. The effect of HG seemed to be stronger in co-
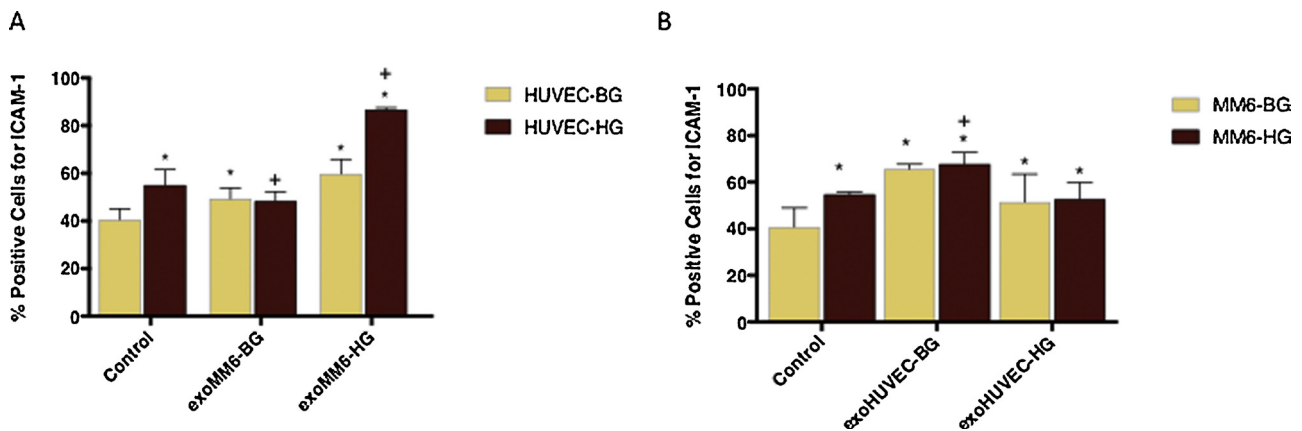
Fig. 4. Effect of exosomes derived from MM6 on HUVECs ICAM-1 expression. A: Percentage of positive cells for ICAM-1 was evaluated in HUVECs exposed to BG and to HG (HUVEC-BG and HUVEC-HG, respectively) and compared with percentage of positive cells for ICAM-1 in HUVEC-BG and HG cells exposed exosomes from MM6-HG (exoMM6-HG) and/or MM6-BG (exoMM6-BG). B: The Percentage of positive cells for ICAM-1 was evaluated in MM6 cells exposed to BG and HG and compared to percentage of positive cells for ICAM-1 in MM6-BG and HG cells exposed exosomes from HUVEC-HG (exoHUVEC-HG) and from HUVEC-BG (exoHUVEC-BG) (n = 5 in all conditions). $p < 0.05$, Wilcoxon signed rank test (* vs MM6-BG, + vs MM6-HG).

cultured cells as compared with monocultures (Fig. 5B), this was significant in MM6-HG but not in HUVEC-HG.

The question arose whether this increased ICAM-1 expression in the co-cultures could be due to a collaboration of exosomes released from both cell types. To test this, monocultures of HUVECs or MM6 cells were exposed to a mix of exoMM6-BG and exoHUVEC-BG or exoMM6-HG and exoHUVEC-HG (exoMix-BG or exoMix-HG, respectively) (Fig. 5C–F). Expression of ICAM-1 was compared to expression of this molecule in co-cultured cells. ExoMix-HG increased the expression of ICAM-1 in MM6-BG (Fig. 5C) and also in HUVEC-BG (Fig. 5E) as compared with the respective controls BG controls. exoMix-BG decreased the expression of ICAM-1 in MM6-HG (Fig. 5C) and in HUVEC-HG (Fig. 5E) as compared with their respective HG controls. The expression of ICAM-1 in MM6-BG and HUVEC-BG exposed to exoMix BG was increased as compared with the respective BG controls (Fig. 5D and F). The expression of ICAM-1 in MM6-HG and HUVEC-HG exposed with exoMix-HG was not different as compared with ICAM-1 expression in the respective HG controls (Fig. 5D and F).

4. Discussion

DMT2 is associated with monocyte and endothelial cell activation. One of the factors responsible for endothelial cell and monocyte activation is hyperglycemia (Davison et al., 2017; Manduteanu et al., 1999). In the present study, we evaluated whether monocytes and endothelial cells were activated by high glucose. This was measured by measuring the expression of ICAM-1, which is a well-known activation marker for both cell types. We also studied whether this effect was mediated by exosomes. Secondly, we evaluated whether endothelial cells and monocytes communicate with each other via exosomes under basal and high glucose conditions.

In the present study, we confirm production of exosomes induced by HG in endothelial cells, but to the best of our knowledge, this is the first study showing changes in exosomes produced by monocytes under the influence of HG. Rather than isolating monocytes from blood, which isolation procedure activates monocytes (Faas et al., 2010), we used the MM6 cell line as a model for monocytes because of its low basal activation. This cell line shows many characteristics of mature monocytes (Ziegler-Heitbroc et al., 1988). We showed that the exposure to HG modified the size of exosomes from MM6 but did not significantly alter the concentration of exosomes. This was different from the effect of HG on HUVEC that we described previously (Sáez et al., 2018), in which we observed that HG increased the concentration of exosomes but not the size. Moreover, our results suggest that HG can probably also alter the

content of the exosomes derived from both monocytes, since exosomes isolated from monocytes incubated in HG have different effects on monocyte ICAM-1 surface expression as compared with exosomes isolated from monocytes incubated in BG.

HG increased the expression of ICAM-1 in MM6 cells. Our results are in line with a study by Shanmugan et al. (Shanmugam et al., 2003), who showed that incubation of monocytes with high glucose activated monocytes. In this study monocyte activation was characterized by increased production of inflammatory cytokines such as TNF-α and MCP-1 (Shanmugam et al., 2003). In line with our previous study (Sáez et al., 2018), HG also increased endothelial cell activation, which is also in line with other studies (Takami et al., 1998). The cellular mechanisms by which HG induced monocyte and endothelial cell activation cannot be deduced from our study. However, previous studies have shown that HG induces the production of oxygen free radicals (Dasu and Jialal, 2011; Li et al., 2016). It seems likely that such oxygen free radicals induce monocyte and endothelial cell activation (Chiu et al., 1997; Kim et al., 2011).

We speculated that the effect of HG on monocytes (and endothelial cells as previously described) was induced by exosomes. Therefore MM6-BG cells were exposed to exoMM6-HG. This indeed caused increased ICAM-1 cell surface protein expression, suggesting that the effect of HG can indeed be induced by exosomes derived from cells incubated under HG. The exposure of MM6-HG to exoMM6-BG or exoMM6-HG, as well as the exposure of MM6-BG to exoMM6-BG, also increased the expression of ICAM-1. This may suggest that the effect of exosomes on ICAM-1 expression is an exosomal effect and not specific for exosomes derived from monocytes under HG condition.

In vivo, monocytes and endothelial cells are in close contact. Thus, we evaluated whether monocytes and endothelial cells are able to communicate with each other under basal and high glucose conditions via exosomes. The exposure of MM6-BG to exoHUVEC-HG or exoHUVEC-BG significantly increased the expression of ICAM-1 in these cells. Again, this may suggest that exosomes per se activate monocytes, i.e. increased their ICAM-1 expression, independent of the glucose concentration. Further studies are needed to evaluate whether (and how) exosomes from HUVECs may also influence MM6 in other ways, f.i by inducing cytokine expression. Interestingly, the exposure of HUVEC-BG to exoMM6-HG increased the expression of ICAM-1 as compared with HUVEC-BG and HUVEC-HG. Remarkably, exoMM6-BG incubated with HUVEC-HG reversed the effect of HG to almost normal values.

Our data of monocyte exosomes affecting endothelial cells are in line with the study of Tang et al. (Tang et al., 2016), who observed that
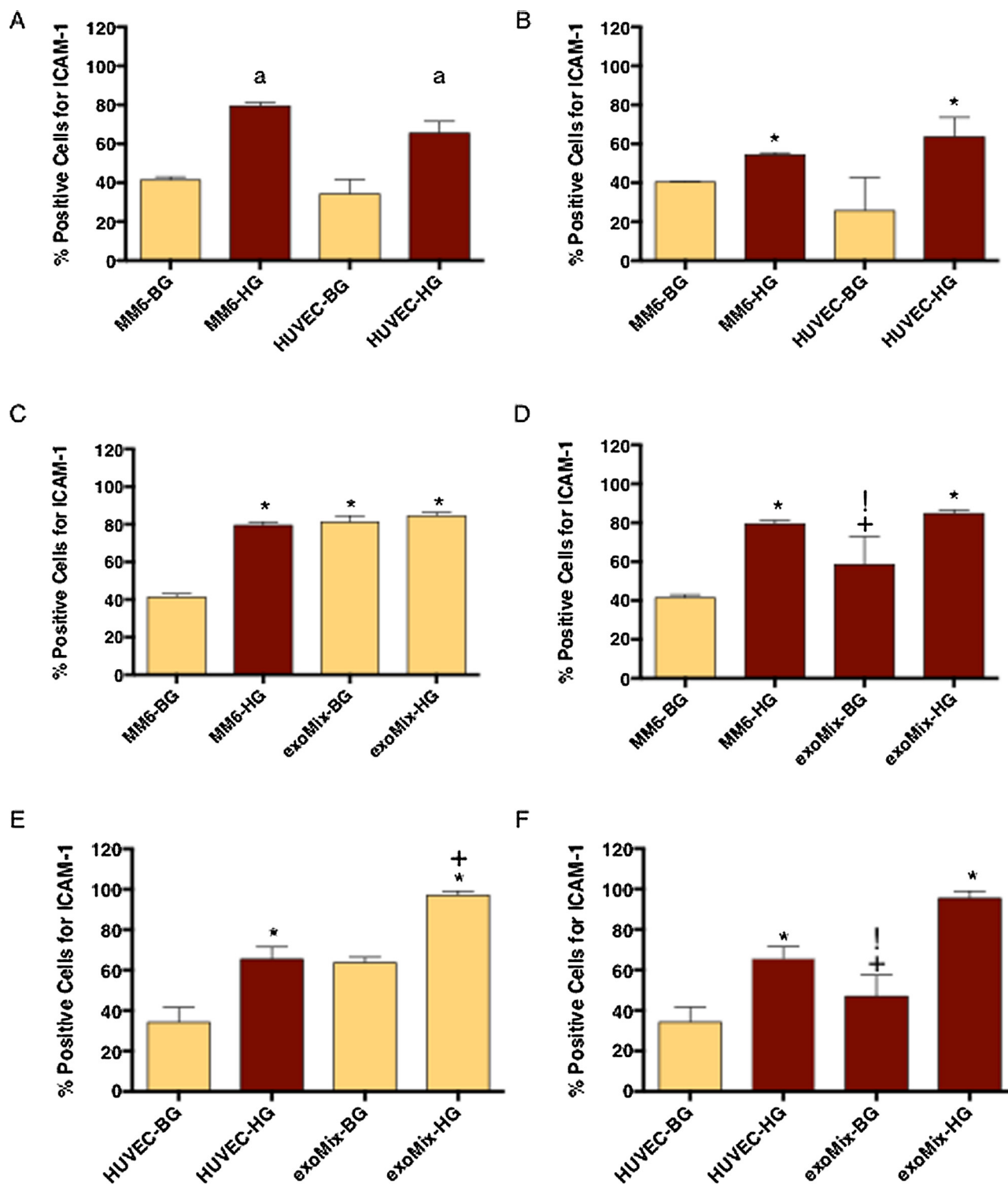
Fig. 5. Effect of a mix of exosomes derived from monocytes and endothelial cells on ICAM-1 expression in MM6 cells and HUVECs, under BG and HG condition. A) Percentage of positive cells for ICAM-1 was evaluated in MM6 cells and in HUVECs in co-cultures under BG or HG conditions. B) Percentage of positive cells for ICAM-1 was evaluated in MM6 cells and in HUVECs in monocultures under BG or HG conditions. C–D) Percentage of positive cells for ICAM-1 was evaluated in MM6-BG and in MM6-HG exposed to exosomes from MM6-HUVEC-BG or MM6-HUVEC-HG (exoMix-BG or exoMix-HG), respectively. E–F) Percentage of positive cells for ICAM-1 was evaluated in HUVEC-BG and in HUVEC-HG exposed to exosomes from MM6-HUVEC-BG or MM6-HUVEC-HG (exoMix-BG or exoMix-HG), respectively. (n = 5 in all conditions). p < 0.05, Wilcoxon Signed rank test (a vs MM6-BG or HUVEC-BG, * vs MM6-BG or HUVEC-BG, + vs MM6-HG or HUVEC-HG, ! vs exoMix-HG).

exosomes derived from LPS-activated monocytes induced the expression ICAM-1 and pro-inflammatory cytokines in HUVECs. Not only exosomes, but also microparticles derived from leukocytes stimulate the expression of ICAM-1 on endothelial cells (Mesri and Altieri, 1998).

Although, we did not investigate the mechanisms by which monocyte exosomes affect endothelial cells, other studies have shown that monocyte exosomes affected endothelial cells by activating NFκB and Toll like receptor signaling pathways (Bretz et al., 2013; Tang et al.,

Hyperglycemia
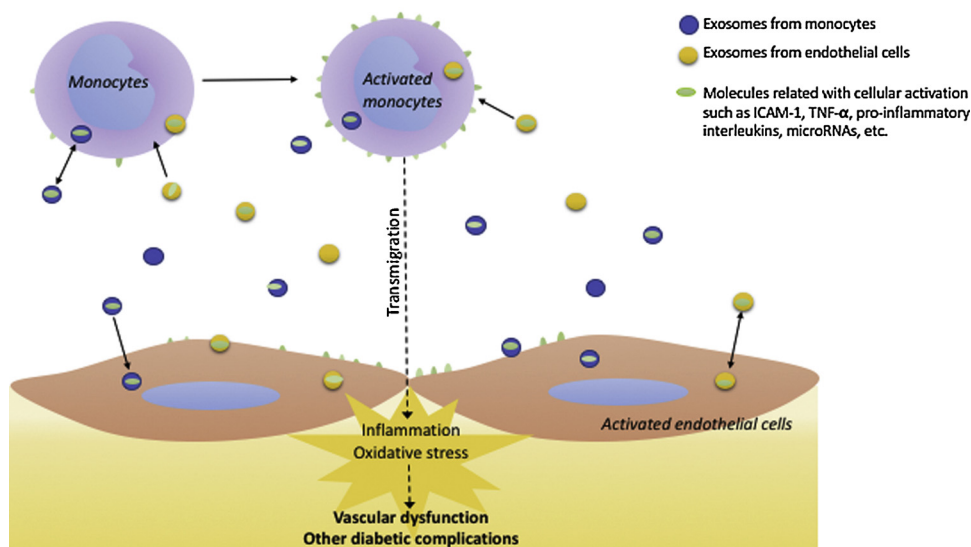
Fig. 6. Model of cell activation mediated by exosomes in monocytes and endothelial cells. Exosomes derived from monocytes and endothelial cells may carry molecules associated to cell activation such as intracellular adhesion molecule type 1 (ICAM-1). Due to exosomes can deliver exosome cargoes into cells, exosomes may increase the expression of these molecules, acting in an autocrine or paracrine manner. The increasing expression of molecules related to cell activation may facilitate the potential transmigration of monocytes and probably mediating pro-inflammatory process or atherosclerotic mechanism in diabetes.

2016). However, whether the increased ICAM-1 expression mediated by exosomes may affect the interaction between endothelial cells and monocytes, i.e. monocytes transmigration, was not evaluated in this study. Together, these data suggest that in vivo, exosomes derived from both cells types may be one of the mechanisms inducing endothelial cell and monocyte activation under hyperglycemic conditions.

In view of the effects of exosomes from MM6 and HUVECs on each other, we hypothesized that in a co-culture of MM6 and HUVECs, under HG or BG conditions, ICAM-1 expression would be increased as compared with monocultures of either HUVECs of MM6. This hypothesis appeared to be true: co-cultured HUVECs and MM6 showed higher ICAM-1 expression as compared with monocultures, however, only under high glucose conditions. We also observed that, similar to monocultures, both HUVECs and MM6 cells exposed to HG in co-culture increased the expression of ICAM-1 as compared to co-cultured cells exposed to BG. In line with the present data, we have previously shown communication between monocytes and endothelial cells (Faas et al., 2010). We hypothesized that in co-culture MM6 and HUVECs communicate by exosome production. Alternatively, but not mutually exclusive, HG may also cause increased release of pro-inflammatory cytokines by monocytes (Li-bo et al., 2011) or endothelial cells (Liu et al., 2012), by which these cells may also activate each other (Altannavch et al., 2004; Manna and Jain, 2014).

In accordance with our hypothesis, the exosomal mixture of HUVECs and MM6 exosomes from cells incubated under high glucose increased the expression of ICAM-1 in both HUVECs and MM6 cultured under basal glucose. Interestingly, ICAM-1 levels were increased to the same level as ICAM-1 in co-cultured MM6 cells, i.e. higher than in monocultures. This may suggest that the exosomes from HUVECs and MM6 under high glucose collaborate in inducing ICAM-1 protein surface expression in both HUVECs and MM6. These data thus confirm our hypothesis that in co-cultured HUVECs and MM6 cells exosomes from both cell types collaborate and that exosomes are, at least partly, responsible for increasing ICAM-1 expression in both cell types. Moreover, we observed that the exosomal mix from HUVECs and MM6 under BG decreased the expression of ICAM-1 in both HUVECs and MM6 incubated with HG, i.e. they reverted the effect of high glucose on both HUVECs and MM6. Further studies are needed to investigate the exact mechanisms of how the exosomes influence ICAM-1 expression and how MM6 and HUVECs exosomes collaborate.

In summary, our results indicate that high levels of glucose may activate monocytes. The present data also show that exosomes derived from both monocytes and endothelial cells can modulate the protein surface expression of ICAM-1 in endothelial cells and in monocytes. Exosomes can thus act as communication mechanism between monocytes and endothelial cells, both under BG and HG conditions. Despite, this study did not show whether exosomes derived from monocytes and endothelial cells may affect exosome cargo, this effect of HG could be in line with data showing that circulating microparticles from DMT2 patients are enriched with proteins involved in cell activation (Xu et al., 2016). Also exosomes derived from myocytes from patients with DMT2 contain higher levels of microRNA-1 and microRNA-133a, as compared with exosomes from healthy individuals (de Gonzalo-Calvo et al., 2017). Studies into the cargo of HG and BG exosomes are in progress. We propose that exosomes from both endothelial cells and monocytes have an important role endothelial and monocyte activation induced by high levels of glucose and may play a role in inflammatory cell activation in DMT2 (Fig. 6) or cardiovascular complications associated with diabetes.

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Declaration of interest

The authors declare no competing interests.

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