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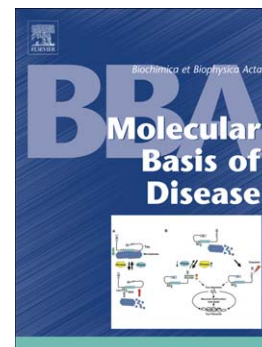
High glucose and hyperglycemic sera from type 2 diabetic patients impair DC differentiation by inducing ROS and activating Wnt/ $\beta$ -catenin and p38 MAPK

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High glucose and hyperglycemic sera from type 2 diabetic patients impair DC differentiation by inducing ROS and activating Wnt/ $\beta$ -catenin and p38 MAPK.

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**Keywords:** DC, hyperglycemia, ROS, Wnt/ $\beta$ -catenin, p38MAPK, Quercetin.

**Abbreviations:** Dendritic cells (DCs), type 2 diabetes (DM2), Reactive Oxygen Species (ROS), Mitogen-Activated Protein Kinases (MAPKs), Pathogen Associated Molecular Patterns (PAMPs), Damage Associated Molecular Patterns (DAMPs), T-Cell Factor/Lymphoid Enhancer Factor (TCF/LEF), Extracellular Regulated Kinase (ERKs), c-Jun N-terminal kinases (JNKs), High Glucose (HG), Hyperglycemic Sera (HYG), Normoglycemic Sera (NG), Primary Effusion Lymphoma (PEL), Quercetin (Q), 2', 7'-dichlorofluorescein diacetate (DCFDA), Interleukin 4 (IL-4), Granulocyte-Monocyte Colony Stimulator Factor (GMCSF), Cluster Differentiation (CD), Heat Shock Proteins (HSPs). FACS (Fluorescence-activated cell sorting), Phosphate-

Buffered Saline (PBS), Bovine Serum Albumin (BSA), FITC (Fluorescein Isothiocyanate), Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), Lipopolysaccharide (LPS), Mixed Lymphocyte Reaction (MLR), Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE)

## Abstract

Type 2 is the type of diabetes with higher prevalence in contemporary time, representing about 90% of the global cases of diabetes. In the course of diabetes, several complications can occur, mostly due to hyperglycemia and increased reactive oxygen species (ROS) production. One of them is represented by an increased susceptibility to microbial infections and by a reduced capacity to clear them. Therefore, knowing the impact of hyperglycemia on immune system functionality is of utmost importance for the management of the disease. In this study, we show that medium containing high glucose reduced the *in-vitro* differentiation of monocytes into functional DCs and their activation mediated by PAMPs or DAMPs. Most importantly, the same effects were mediated by the hyperglycemic sera derived by type 2 diabetic patients, mimicking a more physiologic condition. DC dysfunction caused by hyperglycemia may be involved in the inefficient control of infections observed in diabetic patients, given the pivotal role of these cells in both the innate and adaptive immune response. Searching for the molecular mechanisms underlying DC dysfunction, we found that canonical Wnt/ $\beta$ -catenin and p38 MAPK pathways were activated in the DCs differentiated either in the presence of high glucose or of

hyper-glycemic sera. Interestingly, the activation of these pathways and the DC immune dysfunction were partially counteracted by the anti-oxidant quercetin, a flavonoid already known to exert several beneficial effects in diabetes.

## 1. Introduction

Type 2 diabetes (DM2), the most common form of diabetes, is a metabolic disorder whose most typical feature is represented by hyperglycemia. It is a chronic inflammatory disease characterized by the occurrence of infectious diseases that are more frequent and more long lasting, suggesting a reduced efficiency of the immune system. Hyperglycemia might directly contribute to the immune dysfunction observed in diabetic patients [1, 2]. Indeed, it has been previously reported that the induction of hyperglycemia by intravenous administration of alloxan or by high fat diet in mice models impairs the neutrophil phagocytic activity, reducing their ability to clear bacterial infections [3]. Impaired phagocytosis has also been observed in leukocytes derived from diabetic patients [4]. Besides defects of the innate immune cells, also a delayed priming and impaired adaptive immunity has been described in mouse model of diabetes [5]. Macrophages and dendritic cells (DCs) play key roles in sensing infection and in orchestrating adaptive immune response, therefore a perturbation of monocyte/macrophage or DC functions could be responsible for the increased susceptibility to infections. Moreover, hyperglycemia and oxidative stress play a role in the insulin resistance, characteristic of DM2 and in most of the late

complications occurring in the course of this disease. Increased reactive oxygen species (ROS) production has been observed in the immune cells such as neutrophils derived from DM2 patients [6] and high levels of reactive oxygen intermediates have been detected also in intra-peritoneal macrophages derived from diabetic mice [7]. There are many sources of ROS production in diabetes, either of mitochondrial and non-mitochondrial origins. A relationship between ROS production and the activation of signaling pathways such as Wnt/ $\beta$ -catenin and p38 Mitogen-Activated Protein Kinases (MAPKs) have been described in previous studies [8-11]. Wnt/ $\beta$ -catenin is the “canonical” Wnt pathway whose activation leads to the accumulation of  $\beta$ -catenin that, once translocated into the nucleus, acts as co-activator of transcription factors belonging to the T-Cell Factor/Lymphoid Enhancer Factor (TCF/LEF) family. On the other hand, p38, belongs to the MAPK family that includes Extracellular Regulated Kinase (ERKs) and c-Jun N-terminal kinases (JNKs). These kinases transduce signals from the cell membrane to the nucleus in response to a variety of different stimuli and participate in various intracellular signaling pathways [12]. Wnt/ $\beta$ -catenin and p38 pathways are hyper-activated in different pathological conditions and are also tightly associated with diabetes [13]. They are up-regulated in several cell types including DCs [13, 14] and their activation in DCs has been linked to a tolerogenic phenotype [15-20].

In this study, we found that high glucose-containing medium or hyperglycemic sera, derived from type 2 diabetic patients, increased ROS production, activated Wnt/ $\beta$ -catenin and p38 MAPK pathways impairing DC differentiation and maturation.

Interestingly, quercetin, a flavonoid with antioxidant and anti-inflammatory properties, previously reported to inhibit multiple pathways [21] including p38 MAPK [22] and Wnt/ $\beta$ -catenin [23] efficiently counteracted the immune suppressive effect mediated by high glucose or hyperglycemic sera in differentiating monocytes.

## 2. Materials and Methods

### 2.1 Cells and human sera

BCBL1 (kindly provided by Prof. P. Monini), a human B-cell line derived from PEL carrying latent KSHV, was cultured in RPMI 1640 (Sigma, R0883), 10% Fetal Calf Serum (FBS) (Euroclone, ECLS0180L), L-glutamine and streptomycin (100 $\mu$ g/ml) and penicillin (100U/ml) (Gibco, 10378-016) in 5% CO<sub>2</sub> at 37°C. PEL cells were diluted at 2x10<sup>5</sup>/ml and kept in culture for one night before using for the experiments. Cells were maintained in culture for one month.

Human sera from type 2 diabetes patients (glycemia  $\geq$  300 mg/dl) or healthy donors (glycemia  $\leq$  90 mg/dl) were obtained from San Filippo Neri Hospital, previous informed consent of all participants. The ethical committee of Policlinico Umberto I has approved the experiments. 10 different hyperglycemic sera and 10 normoglycemic sera were used throughout the study. About 3 ml of serum was taken from each patient.

## 2.2 Immature DC generation

Human peripheral blood mononuclear cells (PBMCs) from buffy coats of healthy donors were isolated by Lympholyte cell separation medium (Cedarlane, CL5020). Briefly, 30 ml of diluted buffy coat was layered over 15 ml of Lympholyte cell separation medium and gradients were centrifuged at  $800 \times g$  for 30 min at room temperature in a swinging-bucket rotor without the brake applied. The PBMC interface was carefully removed by pipetting and washed 3 times with PBS-EDTA by centrifugation at  $600 \times g$  for 10 min.

Monocytes were isolated from PBMCs by immunomagnetic cell separation using anti-CD14-conjugated microbeads according to the manufacturer's instructions (Miltenyi Biotec, 1300-50-201). Purity of population was checked by FACS analysis (Fluorescence-activated cell sorting). To induce the differentiation of immature DCs (iDCs), purified monocytes (approximately 95% CD14<sup>+</sup>) were cultured at a density of  $2 \times 10^6$  cells/ml in 12-well plates for 6 days in RPMI 1640 (Euroclone) containing 10% FCS, 2mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (complete medium) with the addition of recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF) (50 ng/mL) (Miltenyi Biotec, 130-093-865) and interleukin-4 (IL-4) (20 ng/mL) (Miltenyi Biotec, 130-095-373) every two days. After the first 18 hrs 20% of culture volume was replaced by: the same complete medium as above reported (control medium), a complete medium with 25mM D-glucose (high glucose medium, HG medium), normoglycemic serum from healthy

donors (glycemic values between 80 and 100) (NG serum), hyperglycemic serum from hyperglycemic donors (glycemic values between 300 and 500) (HG serum). In some experiments, 10  $\mu$ M Quercetin (Q) (Q4951, Sigma-Aldrich, St. Louis, USA) or 20  $\mu$ M SB203580 (SB) (sc-3533, Santa Cruz) was added 30 min before treatments with high glucose medium and hyperglycemic serum. Cells were fed every two days with GM-CSF (50 ng/mL) and IL-4 (20 ng/mL) and, in some experiments with quercetin or SB too, for the remaining 5 days.

### *2.3 Immunofluorescence and FACS analysis*

For immunofluorescence, cells were washed with Phosphate-buffered saline (PBS) with 0.5 % bovine serum albumin (BSA) and incubated for 30 min at 4 °C with appropriate isotype control antibodies (Milteny Biotec, 130-092-213 and 130-092-212) or antibodies against CD1a (BD Pharmingen, 555807) and CD14 (Milteny Biotec, 130-080-701) on iDCs, and CD86 (Milteny Biotec, 130-094-878), CD83 (Milteny Biotec, 130-094-181) and CD80 (Milteny Biotec, 130-097-202) on DCs. Cells were analyzed by EPICS XL Coulter (Beckman Coulter, Hialeah, FL, USA) or by FACSCalibur, using CELLQuest software (BD Biosciences, San Jose, USA). DCs were gated according to their FSC and SSC properties. At least  $10 \times 10^3$  events were acquired for each sample.

### *2.4 FITC- dextran uptake*



After 6 days of culture iDCs, generated in the presence or in the absence of high glucose medium, were washed and incubated for 60 min with 1 mg/ml FITC-dextran (molecular weight 40 000; Sigma, St Louis, MO). Half of the cells was incubated at 37° C and the other half was incubated at 4° C to assess the background uptake. The cells were then washed and analyzed by FACScalibur flow cytometer (BD, USA). For each analysis 10,000 events were recorded.

### *2.5 Measurement of intracellular reactive oxygen species production*

To measure reactive oxygen species (ROS) production, we used two different oxidation-sensitive dyes: the 2',7'-dichlorofluorescein diacetate (DCFDA) (Molecular Probes, CA) and 5-amino-2,3-dihydro-1,4-phthalazinedione (Luminol) (Sigma-Aldrich St. Louis, USA). The DCFDA is a fluorogenic dye that, after diffusion in to the cell, is oxidized by ROS into 2',7'-dichlorofluorescein (DCF), a highly fluorescent compound which can be detected by fluorescence spectroscopy. The cell-permeable dye Luminol (250 mM), when excited by ROS, releases energy in the form of light (chemiluminescence), which can be measured by luminometry. To measure ROS production by DCFDA,  $5 \times 10^5$  CD14<sup>+</sup> cells treated with control and high-glucose media or normoglycemic and hyperglycemic sera for 2 hours were washed with pre-warmed PBS and then were incubated at 37°C with 10 µM DCFDA for 15 min in PBS. Then, the cells were washed and analyzed by FACScalibur flow

cytometer (BD, USA). For each analysis 10,000 events were recorded.

To measure ROS production by Luminol,  $15 \times 10^4$  CD14<sup>+</sup> cells were incubated in 0.15 ml of Hank's Balanced Salt Solution (HBSS) without phenol red in a 96 wells plate. Following the addition of glucose (25mM) chemiluminescence was measured after 1 hour using the automatic luminescence analyzer VICTOR X2 Multilabel Plate Reader (Wallac).

### *2.6 Maturation of DC*

To induce DC maturation, iDCs were incubated for 24hrs with Lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, USA, 100 ng/ml) or cocultured with Bortezomib (Sigma-Aldrich, St. Louis, USA, 25 nM) -treated BCBL1 tumor cells as previously described [24], at a 1:1 iDC/tumor cell ratio for 24 hrs.

### *2.7 Mixed Lymphocyte Reaction (MLR)*

MLR was performed by adding different number of irradiated (3000 rads) LPS activated DCs to allogeneic PBLs. PBL cells, obtained from buffy coat of healthy donors, were labeled with 5  $\mu$ M Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE, Molecular Probes, Leiden, The Netherlands) for 10 min at 37°C, then washed twice and seeded at  $4 \times 10^5$  in 48-well flat-bottom microtiter plates. Irradiated LPS activated DCs were used as stimulator cells in a ratio 1:10 and 1:100. At day 5 of

culture at 37°C, cell proliferation was assessed by FACScalibur flow cytometer. The mean of proliferating cells and the error bars, representing the standard deviation (SD) were calculated.

### *2.8 Antibodies for Western blotting*

In western blotting analysis, we used the following primary antibodies: rabbit polyclonal anti- $\beta$ -catenin (1:200; Santa Cruz Biotechnology, sc-7199), rabbit polyclonal anti-Phospho-p38 MAPK (Thr180/Tyr182) (pp38) (1:500; Cell Signaling Technology, 4631), rabbit polyclonal anti-p38 MAPK (p38) (1:1000; Cell Signaling Technology, 9212).

Monoclonal mouse anti- $\alpha$ -tubulin (1:1000; Sigma Aldrich, T6199), anti- $\beta$ -actin (1:10000; Sigma Aldrich, A5441) and anti-GAPDH (Santa Cruz Biotechnology, sc-13717) were used as loading controls. The goat polyclonal anti-mouse IgG-HRP (Santa Cruz Biotechnology, sc-2005) and anti-rabbit IgG-HRP (Santa Cruz Biotechnology, sc-2004) were used as secondary antibodies. All the primary and secondary antibodies were diluted in phosphate-buffered saline (PBS; Sigma Aldrich, D8537)-0.1% Tween 20 (Sigma Aldrich, P1379) solution containing 3% BSA (Serva, 11943.03)

### *2.9 Western blot analysis*

$1 \times 10^6$  cells were washed twice with 1X PBS-0.1% Tween 20 solution and centrifuged at 212 (g) for 5 min. The pellet fraction was lysed in a RIPA buffer containing 150 mM NaCl, 1% NP-40 (Sigma Aldrich, NP40S), 50 mM Tris-HCl, pH 8, 0.5% deoxycholic acid (Sigma Aldrich, D6750), 0.1% SDS (Sigma Aldrich, 71736), protease (Sigma Aldrich, S8830) and phosphatase inhibitors (Sodium Orthovanadate; Sigma Aldrich, S6508) (Sodium Fluoride; Sigma Aldrich, S7920). Then, 30  $\mu$ g of protein lysates were subjected to electrophoresis on 4-12% NuPage Bis-Tris gels (Life Technologies, N00322BOX) according to the manufacturer's instruction. The gels were transferred to nitrocellulose membranes (Bio-Rad, 162-0115) for 2 h in Tris-glycine buffer. The membranes were blocked in PBS-0.1% Tween 20 solution containing 3% BSA, probed with specific antibodies and developed using ECL Blotting Substrate (Advansta, K-12045-D20).

### *2.10 Densitometric analysis*

The quantification of proteins bands was performed by densitometric analysis using the Image J software, which was downloaded from NIH web site (<http://imagej.nih.gov>).

### *2.11 Statistical analysis*

Data are represented by the mean  $\pm$  standard deviation (SD) of at least three

independent experiments. Statistical significance was calculated with the Student *t* test.  $P \leq 0.05$  was considered a statistically significant difference.

### 3. Results

#### *3.1 Monocyte-differentiation and phagocytic ability are impaired in the presence of high glucose.*

Monocytes were isolated from healthy donors and cultured in the IL-4/GM-CSF differentiating cocktail in complete medium containing glucose at 11 mM (control medium) or in complete medium containing a higher amount of glucose (25 mM) (HG medium). After 6 days, *in-vitro* differentiation of monocytes, based on CD1a and CD14 expression, was evaluated by FACS analysis. As shown in Fig.1A, cells differentiated in the presence of HG medium showed reduced CD1a expression (in terms of percentage of positive cells and the mean of fluorescence intensity) that was accompanied by higher expression of CD14, in comparison to iDC obtained in the control medium. These results indicate an impairment of monocyte differentiation into DCs in the presence of HG. To evaluate whether this phenotypic impairment was

also accompanied by a reduced function, these cells were incubated in the presence of FITC-dextran, to assess their phagocytic ability. We found that also the FITC-dextran capture was reduced in DCs differentiated in the presence of HG (Fig.1B), suggesting their functional impairment. Altogether, these results indicate that monocytes differentiation under HG condition results in reduced differentiation into DCs with subsequent reduced phagocytic ability.

### *3.2 DCs differentiated in HG condition show reduced maturation in response to LPS.*

We then investigated the activation of iDCs differentiated in the presence of HG in response to LPS, a pathogen associated molecular pattern (PAMP) classically used to induce DC maturation. As shown in Fig.2A, DCs differentiated in the presence of HG displayed reduced expression of CD86 and CD83 (respectively activation and maturation markers) in comparison to the DCs differentiated in control medium. Accordingly, impairment of their ability to stimulate T cell proliferation in a Mixed Lymphocyte Reaction (MLR) assay was observed (Fig.2B). All together these results indicate that DC maturation was phenotypically and functionally impaired in the presence of HG.

### *3.3 Monocyte differentiation into DCs is inhibited by hyperglycemic sera derived from type 2 diabetic patients.*

Next, we investigated the DC differentiation in a more physiological system of HG condition. To this aim, monocytes, isolated from healthy donors, were cultured in GM-CSF/IL-4 differentiation cocktail the presence of 20% hyperglycemic sera (glycemia  $\geq 300$ ) from DM2 patients, or normo-glycemic sera (glycemia  $\leq 90$ ). We found that hyperglycemic sera impaired monocytes differentiation, based on their expression of CD1a and CD14 (Fig.3A). We next investigated whether DC maturation would be impaired also by hyperglycemic sera and found that DCs cultured in this condition displayed a reduced maturation after treatment with LPS (Fig. 3B and 3C). Accordingly, DCs matured in the presence of hyper-glycemic sera showed an altered MLR capacity in comparison with DC grown in the presence of normo-glycemic sera (Fig.3D).

### *3.4 DCs differentiated in HG condition show reduced maturation in response to bortezomib-induced immunogenic cell death.*

Chemotherapy-induced cancer cell death can be classified in immunogenic or not immunogenic, based on its ability to activate DCs [25, 26]. We have previously shown that bortezomib induced an immunogenic cell death in primary effusion lymphoma (PEL) cells that was characterized by surface exposure of calreticulin,

HSP70 and HSP90, proteins belonging to the damage associated molecular patterns (DAMPs). Due to DAMP exposure, apoptotic PEL cells promoted DC maturation, as they induced high expression of CD86 and CD83 in DCs, after co-culture [24, 27]. Therefore, iDCs differentiated in HG or control medium were challenged in co-culture assay with bortezomib-treated PEL cells, to investigate whether HG would reduce DC maturation induced by DAMPs. Similarly to what observed in response to LPS, a reduced expression of CD86 and CD83 was observed in DC differentiated in the presence of HG and co-cultured with apoptotic PEL cells (Fig.4A). Finally, the same experiments were performed using normo and hyperglycemic sera. We found that also hyperglycemic sera inhibited DC maturation induced by DAMPs, in comparison to normo-glycemic sera (Fig.4B).

### *3.5 High glucose or hyperglycemic sera increase ROS production and activate p38 MAPK and Wnt/ $\beta$ -catenin pathways.*

We next explored the production of ROS of iDCs differentiated in the presence of HG or in control medium, since it has been reported that hyperglycemia correlates with an increase of ROS production in phagocytic cells either in diabetic mouse models or in patients suffering this disease [6, 7]. We found that ROS were produced in higher amount in iDCs differentiated in the presence of the HG (Fig.5A and 5B). Searching for molecular pathways underlying DC dysfunction, we found that p38 MAPK and Wnt/ $\beta$ -catenin pathways were hyper-activated either in DCs differentiated in the



presence of HG and of hyperglycemic sera (Fig.5C and 5D). The activation of Wnt/ $\beta$ -catenin pathway has been reported to correlate with a tolerogenic phenotype in DCs, characterized by the release of immunosuppressive cytokines, while the activation of p38 MAPK correlated with an impairment of monocyte differentiation into DCs [16, 17]. Based on these previous studies, we hypothesize that their activation could be involved in DC dysfunction induced by HG.

*3.6 Quercetin counteracts the immune suppressive effects mediated by hyperglycemia, by inhibiting ROS production and reducing Wnt/ $\beta$ -catenin and p38MAPK activation.*

We then investigated whether quercetin, a flavonoid known to affect several pathways, including Wnt/ $\beta$ -catenin and p38MAPK, could revert their activation mediated by hyperglycemic sera in DCs. The results, shown in Fig.6A, indicate that quercetin reduced the activation of Wnt/ $\beta$ -catenin and p38MAPK and was also able to decrease ROS production induced by hyperglycemic sera (Fig.6B). Next, we asked whether quercetin could also counteract the immune suppressive effects mediated by HG. The results shown in Fig.6C and 6D indicate that this flavonoid, together with the reduction of ROS and Wnt/ $\beta$ -catenin and p38MAPK activation, induced a partial recovery of DC differentiation and maturation.

*3.7 SB203580 partially reverts the impairment of DC differentiation and maturation induced by HG.*

It has been reported that p38 MAPK activation plays critical role during monocytes differentiation into DC [28]. Moreover, p38 MAPK activation has been shown to lead to DC functional impairment [29, 30] that was restored by p38 MAPK inhibition [31]. Thus, the direct role of p38 MAPK activation in iDCs cultured with hyperglycemic serum was investigated using SB204580 p38MAPK specific inhibitor. Accordingly to the above mentioned studies, we found that SB partially rescued DC differentiation and maturation (Fig. 7A and 7B) indicating that p38 MAPK activation was involved in the immune-suppressive effects exerted by HG.

#### 4. Discussion

In this study we found that high glucose-containing medium or hyperglycemic sera affect the *in vitro* differentiation and maturation of DCs and this effect could be involved in the dysfunction of the immune system observed in diabetic patients [32-34]. We focused on DCs since they play a central role in the activation of an efficient immune response. Previous studies have reported that both myeloid and plasmacytoid

DCs are diminished in diabetic patients with poor metabolic control [35] and that the mature DCs are reduced in these patients [36]. In agreement, an impairment of the adaptive immune response has been observed in diabetes mouse model [5], and naïve T cells, originating from these mice, are activated like antigen-experienced T cells without expressing CD44 [37], a molecule regulating survival and memory development of Th1 cells [38]. The direct effect of high glucose on monocyte differentiation into functional DCs has not been investigated yet. The results obtained in this study suggest that high glucose-containing medium or the more physiological system represented by hyperglycemic sera derived from DM2 patients impaired monocyte differentiation into DCs, as assessed by reduction of the percentage of CD1a positive cells. The DCs cultured in HG condition also displayed reduced expression of activation and maturation markers upon exposure to LPS or after co-culture with PEL cells undergoing an immunogenic cell death upon bortezomib treatment and exposing DAMPs on their cell surface [24]. These results strongly indicate a defect in DC maturation in response to the classical activating stimuli represented by PAMPs or DAMPs. It has been reported that ROS production is increased in the diabetic patients, mainly due to the hyperglycemic status [39]. ROS are known to activate, in turn, a number of stress sensitive pathways including the nuclear factor- $\kappa$ B (NF- $\kappa$ B) and MAPKs such as ERK1/2, p38 MAPK and c-Jun NH2-terminal kinase (JNKs) [40, 41]. The activation of these pathways is linked not only to the development of the late complications of DM2, but also to the insulin resistance and  $\beta$ -cell dysfunction [42]. p38 MAPK, for example, activates serine

kinases that promote the degradation of insulin receptor substrate (IRS) reducing the insulin signaling [43]. Another pathway activated by ROS [44] and amplified by hyperglycemia is Wnt/ $\beta$ -catenin. Its activation, together with the chronic inflammation, may also constitute a possible link between diabetes and the higher risk of cancer in these patients [45]. Besides that, hyperglycemia has been shown to predispose to cancer development and progression [46] and to affect several molecules including tumor suppressor p53 [47]. In this study, we found that differentiating monocytes cultured in the presence of high glucose or hyperglycemic sera produced an increased amount of ROS and displayed a hyper-activation of Wnt/ $\beta$ -catenin and p38MAPK. Since these pathways have been previously associated to dysfunctional DCs [16, 19, 28-31], we next correlated their activation to DC function. At this purpose, we used quercetin, a flavonoid able to affect multiple pathways, including Wnt/ $\beta$ -catenin and p38MAPK, and found that it inhibited their activation and partially counteracted the DC functional impairment induced by HG. Quercetin has been previously shown to exert many beneficial effects in the diabetes, such as helping to obtain a better glycemic control and to reduce diabetic complications, mostly related to the oxidative stress, improving diabetic status in animal models [48]. There are several sources of increased ROS production in diabetes including NADPH oxidases, mitochondrial electron transport enzymes, xanthine oxidase, cyclooxygenase, lipoxygenase, and uncoupled nitric oxide synthase [49]. Quercetin, as other flavonoids, has been reported to protect pancreatic  $\beta$  cell from oxidative damage caused by ROS production [43], to decrease NF- $\kappa$ B activation

and iNOS overexpression in liver of rats in which diabetes was induced by streptozotocin [50] and to reduce ROS-induced p38 MAPK activation, protecting against diabetes-induced exaggerated vasoconstriction [51, 52]. p38 MAPK activation induced by phorbol ester and calcium ionophore A23187 in mast cells can also be attenuated by quercetin [53] and in this study, we found that p38 MAPK inhibitor SB203580 was able to partially counteract the HG-mediated effects in DC. Another signaling pathway reported to be inhibited by quercetin is Wnt/ $\beta$ -catenin pathway, either in B-1 lymphocytes [23] or in cancer cells [54, 55]. In this study, we found that the use of quercetin reduced ROS production, attenuated the activation of both Wnt and p38 MAPK pathways and partially counteracted the impairment of DC differentiation/maturation induced by high glucose and by the hyperglycemic sera. Similarly to quercetin, we recently showed that capsaicin, the principal component of hot peppers, restored DCs dysfunction induced by tumor released factors [56].

In conclusion, this study suggests that quercetin, besides the other beneficial effects previously described in DM2 patients, could be used to restore DC dysfunction induced by high glucose. These findings further encourage its use in the treatment of diabetes, especially in the prevention of the infective complications observed in these patients.

**Disclosure of Potential Conflicts of Interest**

We have no financial or commercial conflicts of interest to declare.

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## REFERENCES

- [1] L. J. Wheat, Infection and diabetes mellitus, *Diabetes care* 3 (1980) 187-197.
- [2] E. J. Rayfield, M. J. Ault, G. T. Keusch, M. J. Brothers, C. Nechemias H. Smith, Infection and diabetes: the case for glucose control, *The American journal of medicine* 72 (1982) 439-450.
- [3] U. S. Pettersson, G. Christoffersson, S. Massena, D. Ahl, L. Jansson, J. Henriksnas M. Phillipson, Increased recruitment but impaired function of leukocytes during inflammation in mouse models of type 1 and type 2 diabetes, *PloS one* 6 (2011) e22480.
- [4] M. Delamaire, D. Maugendre, M. Moreno, M. C. Le Goff, H. Allannic B. Genetet, Impaired leucocyte functions in diabetic patients, *Diabetic medicine : a journal of the British Diabetic Association* 14 (1997) 29-34.
- [5] T. Vallerskog, G. W. Martens H. Kornfeld, Diabetic mice display a delayed adaptive immune response to *Mycobacterium tuberculosis*, *Journal of immunology* 184 (2010) 6275-6282.
- [6] A. Gupta, A. K. Tripathi, R. L. Tripathi, S. V. Madhu B. D. Banerjee, Advanced glycosylated end products-mediated activation of polymorphonuclear neutrophils in diabetes mellitus and associated oxidative stress, *Indian journal of biochemistry & biophysics* 44 (2007) 373-378.
- [7] W. Ptak, M. Klimek, K. Bryniarski, M. Ptak P. Majcher, Macrophage function in alloxan diabetic mice: expression of adhesion molecules, generation of monokines and oxygen and NO radicals, *Clinical and experimental immunology* 114 (1998) 13-18.
- [8] J. W. Wen, J. T. Hwang G. M. Kelly, Reactive oxygen species and Wnt signalling crosstalk patterns mouse extraembryonic endoderm, *Cellular signalling* 24 (2012) 2337-2348.
- [9] S. Kajla, A. S. Mondol, A. Nagasawa, Y. Zhang, M. Kato, K. Matsuno, C. Yabe-Nishimura T. Kamata, A crucial role for Nox 1 in redox-dependent regulation of Wnt-beta-catenin signaling, *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 26 (2012) 2049-2059.
- [10] Y. Funato, T. Michiue, M. Asashima H. Miki, The thioredoxin-related redox-regulating protein nucleoredoxin inhibits Wnt-beta-catenin signalling through dishevelled, *Nature cell biology* 8 (2006) 501-508.
- [11] V. Sidarala, R. Veluthakal, K. Syeda, C. Vlaar, P. Newsholme A. Kowluru, Phagocyte-like NADPH oxidase (Nox2) promotes activation of p38MAPK in pancreatic beta-cells under glucotoxic conditions: Evidence for a requisite role of Ras-related C3 botulinum toxin substrate 1 (Rac1), *Biochemical pharmacology* 95 (2015) 301-310.
- [12] Z. Yao, K. Diener, X. S. Wang, M. Zukowski, G. Matsumoto, G. Zhou, R. Mo, T. Sasaki, H. Nishina, C. C. Hui, T. H. Tan, J. P. Woodgett J. M. Penninger, Activation of stress-activated protein kinases/c-Jun N-terminal protein kinases (SAPKs/JNKs) by a novel mitogen-activated protein kinase kinase, *The Journal of biological chemistry* 272 (1997) 32378-32383.
- [13] K. Rashid P. C. Sil, Curcumin ameliorates testicular damage in diabetic rats by suppressing cellular stress-mediated mitochondria and endoplasmic reticulum-dependent apoptotic death, *Biochimica et biophysica acta* 1852 (2015) 70-82.
- [14] H. Lu, K. Yao, D. Huang, A. Sun, Y. Zou, J. Qian J. Ge, High glucose induces upregulation of scavenger receptors and promotes maturation of dendritic cells, *Cardiovascular diabetology* 12 (2013) 80.
- [15] A. Jiang, O. Bloom, S. Ono, W. Cui, J. Unternaehrer, S. Jiang, J. A. Whitney, J. Connolly, J. Banchereau I. Mellman, Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation, *Immunity* 27 (2007) 610-624.
- [16] M. Cirone, L. Di Renzo, P. Trivedi, G. Lucania, G. Borgia, L. Frati A. Faggioni, Dendritic cell differentiation blocked by primary effusion lymphoma-released factors is partially restored by inhibition of P38 MAPK, *International journal of immunopathology and pharmacology* 23 (2010) 1079-1086.

- [17] C. Oderup, M. LaJevic E. C. Butcher, Canonical and noncanonical Wnt proteins program dendritic cell responses for tolerance, *Journal of immunology* 190 (2013) 6126-6134.
- [18] R. van de Ven, J. J. Lindenberg, D. Oosterhoff T. D. de Gruijl, Dendritic Cell Plasticity in Tumor-Conditioned Skin: CD14(+) Cells at the Cross-Roads of Immune Activation and Suppression, *Frontiers in immunology* 4 (2013) 403.
- [19] D. Swafford S. Manicassamy, Wnt signaling in dendritic cells: its role in regulation of immunity and tolerance, *Discovery medicine* 19 (2015) 303-310.
- [20] M. Cirone, G. Lucania, S. Aleandri, G. Borgia, P. Trivedi, L. Cuomo, L. Frati A. Faggioni, Suppression of dendritic cell differentiation through cytokines released by Primary Effusion Lymphoma cells, *Immunology letters* 120 (2008) 37-41.
- [21] F. H. Sarkar, Y. Li, Z. Wang D. Kong, Cellular signaling perturbation by natural products, *Cellular signalling* 21 (2009) 1541-1547.
- [22] Y. J. Choi, Y. J. Jeong, Y. J. Lee, H. M. Kwon Y. H. Kang, (-)Epigallocatechin gallate and quercetin enhance survival signaling in response to oxidant-induced human endothelial apoptosis, *The Journal of nutrition* 135 (2005) 707-713.
- [23] M. C. Novo, L. Osgui, V. O. dos Reis, I. M. Longo-Maugeri, M. Mariano A. F. Popi, Blockage of Wnt/beta-catenin signaling by quercetin reduces survival and proliferation of B-1 cells in vitro, *Immunobiology* 220 (2015) 60-67.
- [24] M. Cirone, L. Di Renzo, L. V. Lotti, V. Conte, P. Trivedi, R. Santarelli, R. Gonnella, L. Frati A. Faggioni, Activation of dendritic cells by tumor cell death, *Oncoimmunology* 1 (2012) 1218-1219.
- [25] O. Kepp, L. Senovilla, I. Vitale, E. Vacchelli, S. Adjemian, P. Agostinis, L. Apetoh, F. Aranda, V. Barnaba, N. Bloy, L. Bracci, K. Breckpot, D. Brough, A. Buque, M. G. Castro, M. Cirone, M. I. Colombo, I. Cremer, S. Demaria, L. Dini, A. G. Eliopoulos, A. Faggioni, S. C. Formenti, J. Fucikova, L. Gabriele, U. S. Gaip, J. Galon, A. Garg, F. Ghiringhelli, N. A. Giese, Z. S. Guo, A. Hemminki, M. Herrmann, J. W. Hodge, S. Holdenrieder, J. Honeychurch, H. M. Hu, X. Huang, T. M. Illidge, K. Kono, M. Korbelik, D. V. Krysko, S. Loi, P. R. Lowenstein, E. Lugli, Y. Ma, F. Madeo, A. A. Manfredi, I. Martins, D. Mavilio, L. Menger, N. Merendino, M. Michaud, G. Mignot, K. L. Mossman, G. Multhoff, R. Oehler, F. Palombo, T. Panaretakis, J. Pol, E. Proietti, J. E. Ricci, C. Riganti, P. Rovere-Querini, A. Rubartelli, A. Sistigu, M. J. Smyth, J. Sonnemann, R. Spisek, J. Stagg, A. Q. Sukkurwala, E. Tartour, A. Thorburn, S. H. Thorne, P. Vandenabeele, F. Velotti, S. T. Workenhe, H. Yang, W. X. Zong, L. Zitvogel, G. Kroemer L. Galluzzi, Consensus guidelines for the detection of immunogenic cell death, *Oncoimmunology* 3 (2014) e955691.
- [26] M. Cirone, A. Garufi, L. Di Renzo, M. Granato, A. Faggioni G. D'Orazi, Zinc supplementation is required for the cytotoxic and immunogenic effects of chemotherapy in chemoresistant p53-functionally deficient cells, *Oncoimmunology* 2 (2013) e26198.
- [27] M. Cirone, L. Di Renzo, L. V. Lotti, V. Conte, P. Trivedi, R. Santarelli, R. Gonnella, L. Frati A. Faggioni, Primary effusion lymphoma cell death induced by bortezomib and AG 490 activates dendritic cells through CD91, *PloS one* 7 (2012) e31732.
- [28] J. Xie, J. Qian, J. Yang, S. Wang, M. E. Freeman, 3rd Q. Yi, Critical roles of Raf/MEK/ERK and PI3K/AKT signaling and inactivation of p38 MAP kinase in the differentiation and survival of monocyte-derived immature dendritic cells, *Experimental hematology* 33 (2005) 564-572.
- [29] S. Wang, J. Yang, J. Qian, M. Wezeman, L. W. Kwak Q. Yi, Tumor evasion of the immune system: inhibiting p38 MAPK signaling restores the function of dendritic cells in multiple myeloma, *Blood* 107 (2006) 2432-2439.
- [30] J. F. Arrighi, M. Rebsamen, F. Rousset, V. Kindler C. Hauser, A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-alpha, and contact sensitizers, *Journal of immunology* 166 (2001) 3837-3845.



- [31] S. Wang, S. Hong, J. Yang, J. Qian, X. Zhang, E. Shpall, L. W. Kwak Q. Yi, Optimizing immunotherapy in multiple myeloma: Restoring the function of patients' monocyte-derived dendritic cells by inhibiting p38 or activating MEK/ERK MAPK and neutralizing interleukin-6 in progenitor cells, *Blood* 108 (2006) 4071-4077.
- [32] S. E. Geerlings A. I. Hoepelman, Immune dysfunction in patients with diabetes mellitus (DM), *FEMS immunology and medical microbiology* 26 (1999) 259-265.
- [33] J. Casqueiro, J. Casqueiro C. Alves, Infections in patients with diabetes mellitus: A review of pathogenesis, *Indian journal of endocrinology and metabolism* 16 Suppl 1 (2012) S27-36.
- [34] D. Pedicino, G. Liuzzo, F. Trotta, A. F. Giglio, S. Giubilato, F. Martini, F. Zaccardi, G. Scavone, M. Previtero, G. Massaro, P. Cialdella, M. T. Cardillo, D. Pitocco, G. Ghirlanda F. Crea, Adaptive immunity, inflammation, and cardiovascular complications in type 1 and type 2 diabetes mellitus, *Journal of diabetes research* 2013 (2013) 184258.
- [35] C. C. Seifarth, C. Hinkmann, E. G. Hahn, T. Lohmann I. A. Harsch, Reduced frequency of peripheral dendritic cells in type 2 diabetes, *Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association* 116 (2008) 162-166.
- [36] C. Musilli, S. Paccosi, L. Pala, G. Gerlini, F. Ledda, A. Mugelli, C. M. Rotella A. Parenti, Characterization of circulating and monocyte-derived dendritic cells in obese and diabetic patients, *Molecular immunology* 49 (2011) 234-238.
- [37] N. Martinez, T. Vallerskog, K. West, C. Nunes-Alves, J. Lee, G. W. Martens, S. M. Behar H. Kornfeld, Chromatin decondensation and T cell hyperresponsiveness in diabetes-associated hyperglycemia, *Journal of immunology* 193 (2014) 4457-4468.
- [38] B. J. Baaten, C. R. Li, M. F. Deiro, M. M. Lin, P. J. Linton L. M. Bradley, CD44 regulates survival and memory development in Th1 cells, *Immunity* 32 (2010) 104-115.
- [39] H. Kaneto, N. Katakami, M. Matsuhisa T. A. Matsuoka, Role of reactive oxygen species in the progression of type 2 diabetes and atherosclerosis, *Mediators of inflammation* 2010 (2010) 453892.
- [40] X. Ma, C. Dang, H. Kang, Z. Dai, S. Lin, H. Guan, X. Liu, X. Wang W. Hui, Saikosaponin-D reduces cisplatin-induced nephrotoxicity by repressing ROS-mediated activation of MAPK and NF-kappaB signalling pathways, *International immunopharmacology* 28 (2015) 399-408.
- [41] J. Xiao, J. Deng, L. Lv, Q. Kang, P. Ma, F. Yan, X. Song, B. Gao, Y. Zhang J. Xu, Hydrogen Peroxide Induce Human Cytomegalovirus Replication through the Activation of p38-MAPK Signaling Pathway, *Viruses* 7 (2015) 2816-2833.
- [42] P. J. Barnes M. Karin, Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases, *The New England journal of medicine* 336 (1997) 1066-1071.
- [43] K. Batumalaie, S. Zaman Safi, K. Mohd Yusof, I. Shah Ismail, S. Devi Sekaran R. Qvist, Effect of gelam honey on the oxidative stress-induced signaling pathways in pancreatic hamster cells, *International journal of endocrinology* 2013 (2013) 367312.
- [44] H. C. Korswagen, Regulation of the Wnt/beta-catenin pathway by redox signaling, *Developmental cell* 10 (2006) 687-688.
- [45] C. Garcia-Jimenez, J. M. Garcia-Martinez, A. Chocarro-Calvo A. De la Vieja, A new link between diabetes and cancer: enhanced WNT/beta-catenin signaling by high glucose, *Journal of molecular endocrinology* 52 (2014) R51-66.
- [46] W. Duan, X. Shen, J. Lei, Q. Xu, Y. Yu, R. Li, E. Wu Q. Ma, Hyperglycemia, a neglected factor during cancer progression, *BioMed research international* 2014 (2014) 461917.
- [47] A. Garufi G. D'Orazi, High glucose dephosphorylates serine 46 and inhibits p53 apoptotic activity, *Journal of experimental & clinical cancer research : CR* 33 (2014) 79.
- [48] N. Arias, M. T. Macarulla, L. Aguirre, M. G. Martinez-Castano M. P. Portillo, Quercetin can reduce insulin resistance without decreasing adipose tissue and skeletal muscle fat accumulation, *Genes & nutrition* 9 (2014) 361.

- [49] T. M. Paravicini R. M. Touyz, NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities, *Diabetes care* 31 Suppl 2 (2008) S170-180.
- [50] A. S. Dias, M. Porawski, M. Alonso, N. Marroni, P. S. Collado J. Gonzalez-Gallego, Quercetin decreases oxidative stress, NF-kappaB activation, and iNOS overexpression in liver of streptozotocin-induced diabetic rats, *The Journal of nutrition* 135 (2005) 2299-2304.
- [51] M. F. Mahmoud, N. A. Hassan, H. M. El Bassossy A. Fahmy, Quercetin protects against diabetes-induced exaggerated vasoconstriction in rats: effect on low grade inflammation, *PloS one* 8 (2013) e63784.
- [52] J. Liu, J. Li, W. J. Li C. M. Wang, The role of uncoupling proteins in diabetes mellitus, *Journal of diabetes research* 2013 (2013) 585897.
- [53] Y. D. Min, C. H. Choi, H. Bark, H. Y. Son, H. H. Park, S. Lee, J. W. Park, E. K. Park, H. I. Shin S. H. Kim, Quercetin inhibits expression of inflammatory cytokines through attenuation of NF-kappaB and p38 MAPK in HMC-1 human mast cell line, *Inflammation research : official journal of the European Histamine Research Society ... [et al.]* 56 (2007) 210-215.
- [54] H. Kim, J. Y. Moon, K. S. Ahn S. K. Cho, Quercetin induces mitochondrial mediated apoptosis and protective autophagy in human glioblastoma U373MG cells, *Oxidative medicine and cellular longevity* 2013 (2013) 596496.
- [55] B. E. Shan, M. X. Wang R. Q. Li, Quercetin inhibit human SW480 colon cancer growth in association with inhibition of cyclin D1 and survivin expression through Wnt/beta-catenin signaling pathway, *Cancer investigation* 27 (2009) 604-612.
- [56] M. Granato, M. S. Gilardini Montani, M. Filardi, A. Faggioni M. Cirone, Capsaicin triggers immunogenic PEL cell death, stimulates DCs and reverts PEL-induced immune suppression, *Oncotarget* (2015)

## Legend to figures

**Fig. 1. High glucose containing medium interferes with the differentiation of monocytes into iDCs.** **A)** FACS analysis of CD1a and CD14 expression on iDCs developed in the absence (left panels, CTR) or in the presence (right panels, HG) of high glucose medium (grey histograms). The black histograms represent staining with an isotype-matched control Ab. The percentage of positive cells are indicated in the panels. One representative experiment out of three is shown. The means of fluorescence intensity (MFI) and the standard deviation (SD) from three experiments are reported in the table under the panel A. **B)** FACS analysis of FITC-dextran (1mg/ml) uptake by iDCs differentiated in the absence (CTR) or in the presence (HG) of high glucose medium. Each

experiment was performed by incubating the cells at 37 °C (grey histograms) or at 4 °C as control (black histograms) for 60 min. The percentage of positive cells are indicated in the panels. One representative experiment out of three is shown. The means of fluorescence intensity (MFI) and the SDs from three experiments are reported in the table under the panel B.

**Fig 2. High glucose containing medium affects DC maturation and function.** **A)** FACS analysis of CD86 and CD83 expression on DCs, differentiated in the absence or in the presence of high glucose medium and, stimulated by LPS (100 ng/ml) for 24 hrs. The black empty histograms represent staining with an isotype-matched control Ab, the filled black histograms represent the control DCs and the grey histograms represent the DC differentiated in the presence of high glucose. The percentage of positive cells is indicated in the histograms, and the MFI with SD of three independent experiments is also reported at the bottom of the panel A. One representative experiment out of three is shown. **B)** MLR stimulated by DCs differentiated in the presence of control (CTR) or high glucose medium (HG) both activated by LPS. The mean of proliferating cells and the error bars, representing SD, of three independent experiments are shown. *P* value is shown .

**Fig. 3. Hyperglycemic sera interfere with differentiation and maturation of DCs.** **A)** FACS analysis of CD1a and CD14 expression on iDCs developed in the presence of normoglycemic (upper panels) or hyperglycemic serum (lower panels). The grey histograms represent the percentage of positive cells and the black histograms represent staining with an isotype-matched control Ab. One representative experiment out of three is shown. The means of fluorescence intensity (MFI) and the standard deviations (SD) from three experiments are reported in the table under the panel A. **B)** and **C)** FACS analysis of CD86 and CD83 (**B**) and CD80 (**C**) expression on DCs differentiated in the presence of normo and hyperglycemic serum and stimulated by LPS for 24 hrs. The black empty histograms represent staining with an isotype-matched control Ab, the filled black histograms represent the DCs developed in the presence of normoglycemic serum and the empty grey histograms represent the DCs developed in the presence of hyperglycemic serum.

The percentage of positive cells are indicated on the histograms, and the MFI with SD of three independent experiments are reported in the table. One representative experiment out of three is shown. **D)** MLR stimulated by DCs differentiated in the presence of normo (NG) and hyperglycemic serum (HYG) and activated by LPS. The mean of proliferating cells and the error bars, representing SD, of three independent experiments are shown. *P* value is shown.

**Fig. 4** DCs differentiated in HG condition show reduced maturation in response to bortezomib-induced immunogenic cell death. **A)** FACS analysis of CD86 and CD83 expression on DCs differentiated in the presence of control or HG medium and cocultured with bortezomib-treated BCBL1 PEL cells for 24 hrs. The black empty histograms represent staining with an isotype-matched control Ab, the filled black histograms represent the control DCs and the grey histograms represent the DC differentiated in the presence of HG. The percentage of positive cells are indicated on the histograms and the MFI with SD of three independent experiments are reported in the table. One representative experiment out of three is shown. **B)** FACS analysis of CD86 and CD83 expression on DCs differentiated in the presence of normo and hyperglycemic serum and cocultured with bortezomib-treated BCBL1 cells for 24 hrs. The black empty histograms represent staining with an isotype-matched control Ab, the filled black histograms represent the DCs developed in the presence of normoglycemic serum and the empty grey histograms represent the DCs developed in the presence of hyperglycemic serum. The percentage of positive cells are indicated on the histograms and the MFI with SD of three independent experiments are reported in the table. One representative experiment out of three is shown.

**Fig 5. High glucose increases ROS production and activates p38 MAPK and Wnt/ $\beta$ -catenin pathways .** **A)** FACS analysis of ROS production measured by DCFDA staining (10  $\mu$ M) of

monocytes cultured in CTR or of HG medium. The mean of fluorescence intensity is indicated. One representative experiment out of three is shown. **B)** ROS release quantified by luminol-enhanced luminescence from monocytes after the addition of glucose. The bars represent the means of triplicate with the SD. **C)** Western blot analysis of phosphorylated and total p38MAPK and Wnt/ $\beta$ -catenin in iDCs developed in the presence of control (CTR) or of HG medium or in the presence of normoglycemic (NG) or hyperglycemic serum (HYG) for 6 days. Total p38 MAPK and Actin were included as control. One representative experiment out of three is shown together with the densitometric analysis of the specific proteins on Actin.

**Fig. 6. Quercetin counteracts the DC dysfunction inhibiting p38MAPK and Wnt/ $\beta$ -catenin pathways and ROS production.** **A)** Western blot analysis of Wnt/ $\beta$ -catenin and phosphorylated and total p38MAPK in iDCs developed in normoglycemic serum (sNG) or hyperglycemic serum (HYG) pre-treated or not with 10  $\mu$ M Q. Actin and Tubulin were included as control. One representative experiment out of three is shown together with the densitometric analysis of the specific proteins on Actin/Tubulin. **B)** FACS analysis of ROS production measured by DCFDA staining of iDCs differentiated in normoglycemic serum (black line) or hyperglycemic serum pre-treated (dotted line) or not (grey line) with 10  $\mu$ M Q. One representative experiment out of three is shown. Mean fluorescence intensity are indicated. **C)** CD1a expression on iDCs developed in normoglycemic serum (NG), hyperglycemic serum (HYG) and HYG pretreated with 10  $\mu$ M Q. Histograms represent the mean of CD1a positive iDCs with the SD of three independent experiments. P value is shown. **D)** FACS analysis of CD86 and CD83 expression on DCs differentiated in HYG serum in the absence or in the presence of 10  $\mu$ M Q and stimulated by LPS (100 ng/ml) for 24 hrs. The black empty histograms represent staining with an isotype-matched control Ab, the filled black histograms represent the DC differentiated in HYG serum and the empty grey histograms represent the DC differentiated in HYG serum plus 10  $\mu$ M Q. The percentage of positive cells are indicated on the histograms and the MFI with SD of three independent

experiments are reported in the table. One representative experiment out of three is shown.

Fig.7 SB203580 partially rescues the impairment of DC differentiation and maturation induced by hyperglycemic sera. A) CD1a expression on iDCs developed in normoglycemic serum (NG), hyperglycemic serum (HYG) and HYG pretreated with 20  $\mu$ M SB203580 (SB). Histograms represent the mean of CD1a positive iDCs with the SD of three independent experiments. P values are shown B) Histograms represent the mean of MFI of CD86 and CD83 expression on DCs differentiated in HYG serum in the absence (1 and 3) or in the presence (2 and 4) of 10  $\mu$ M SB203580 and stimulated by LPS (100 ng/ml) for 24 hrs. P values are shown.

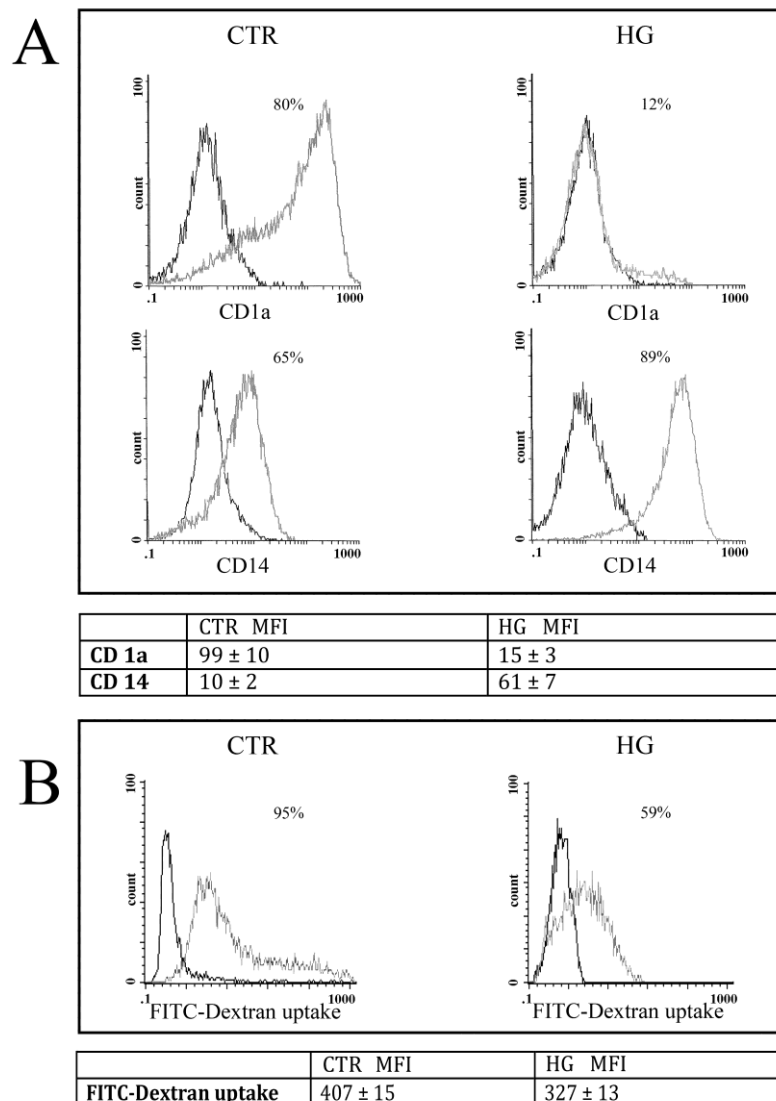


Figure 1

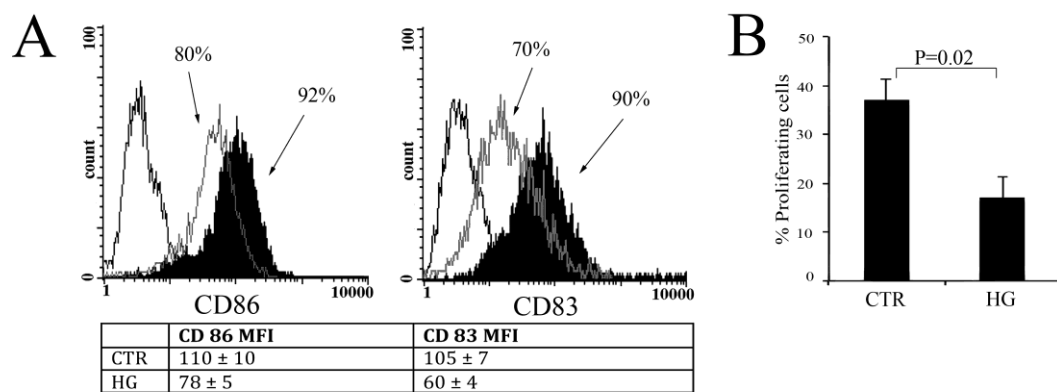


Figure 2



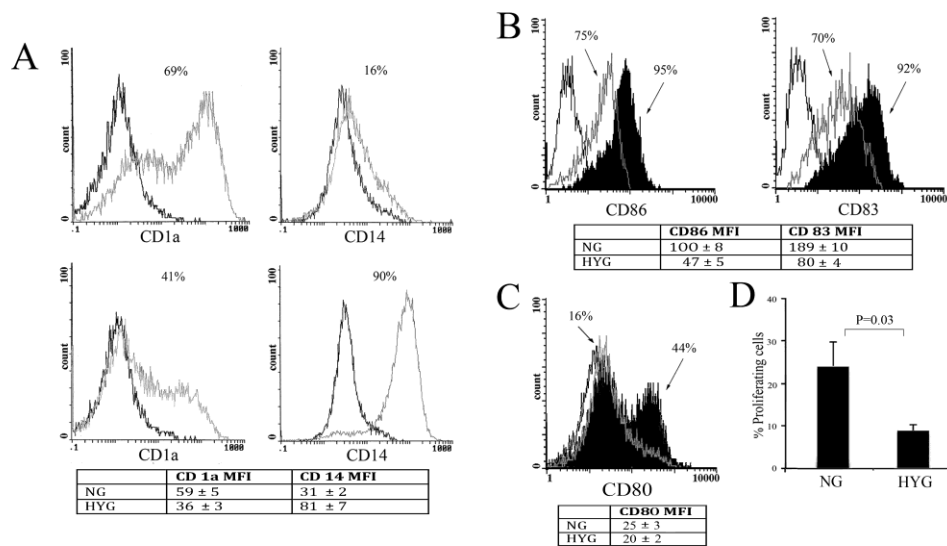


Figure 3

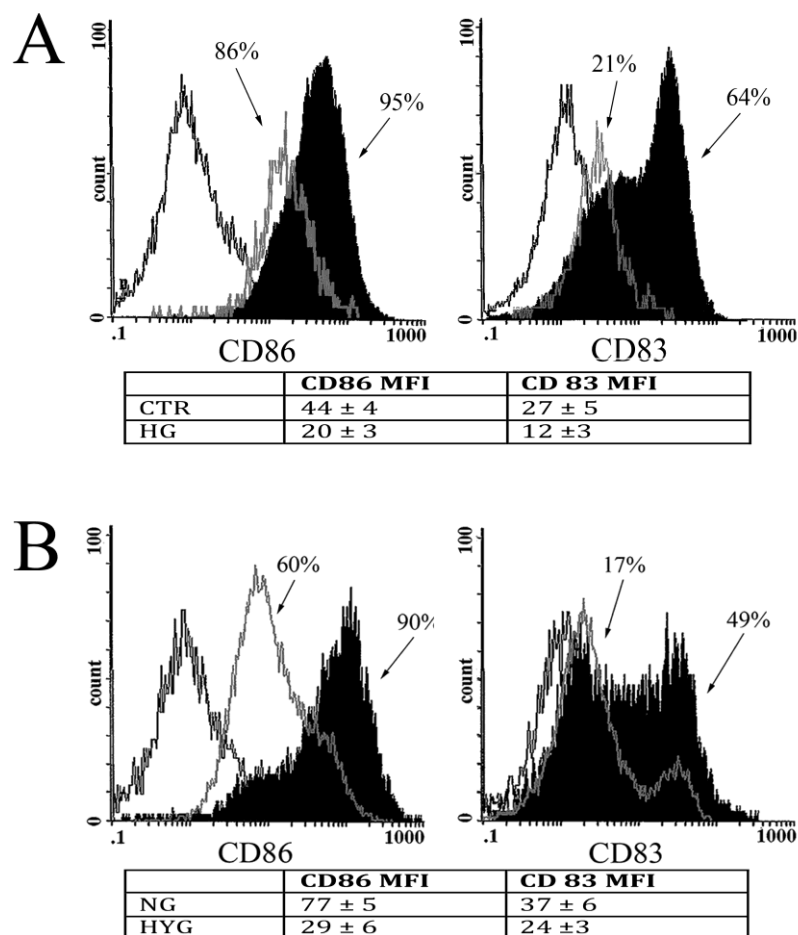


Figure 4

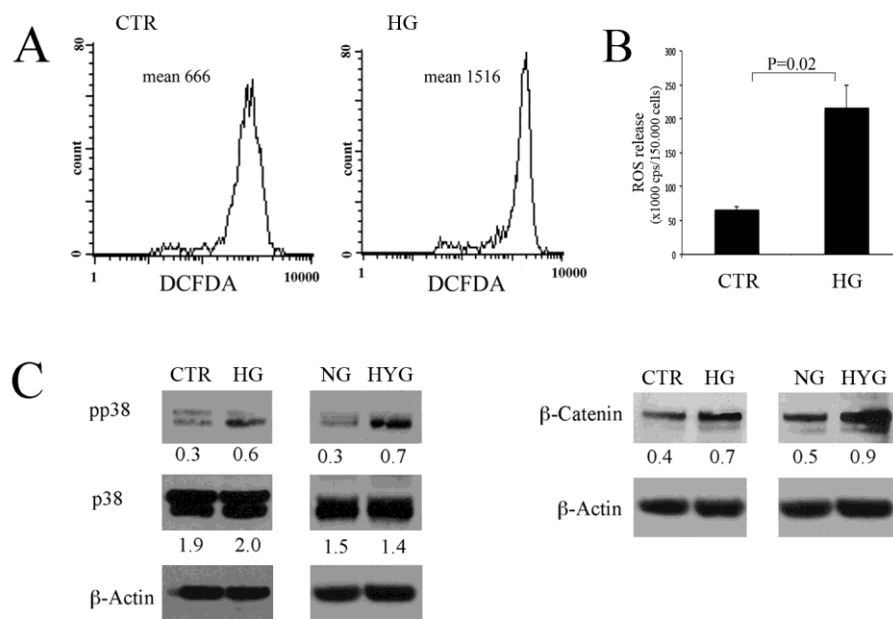


Figure 5

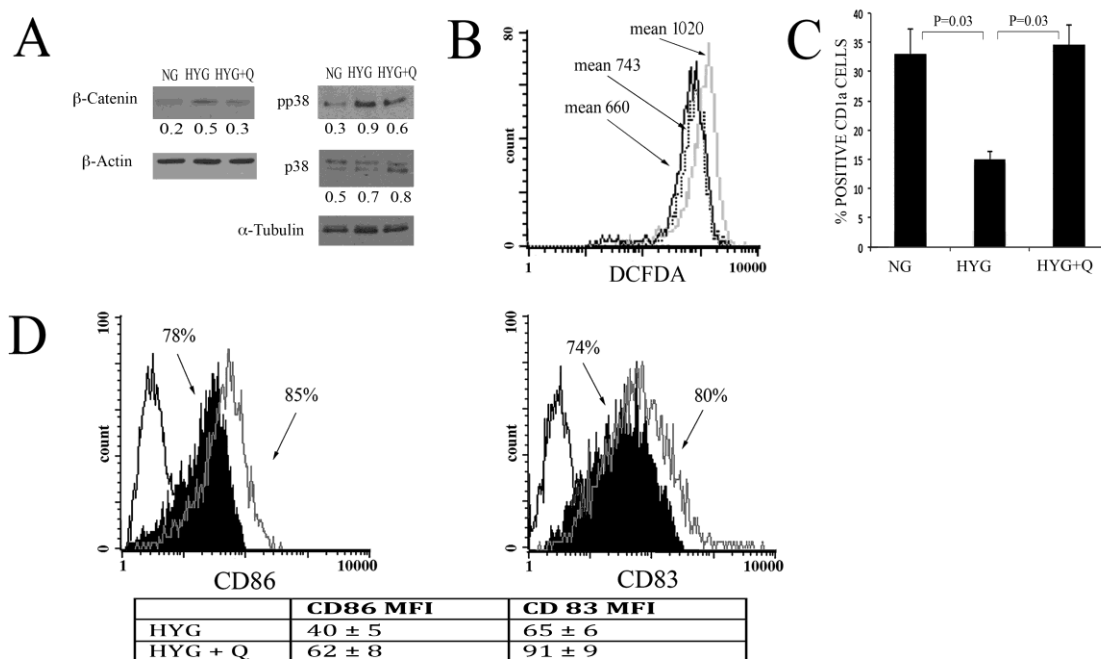


Figure 6

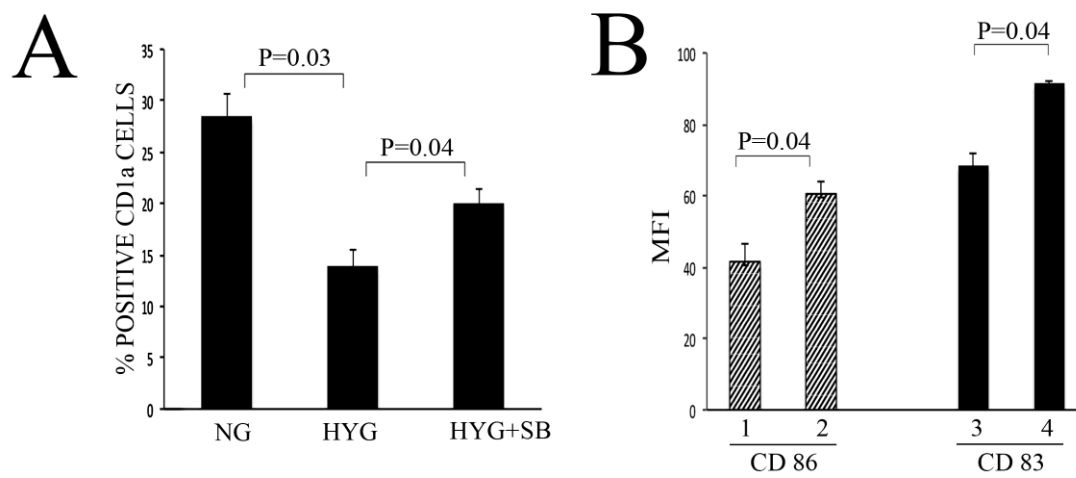
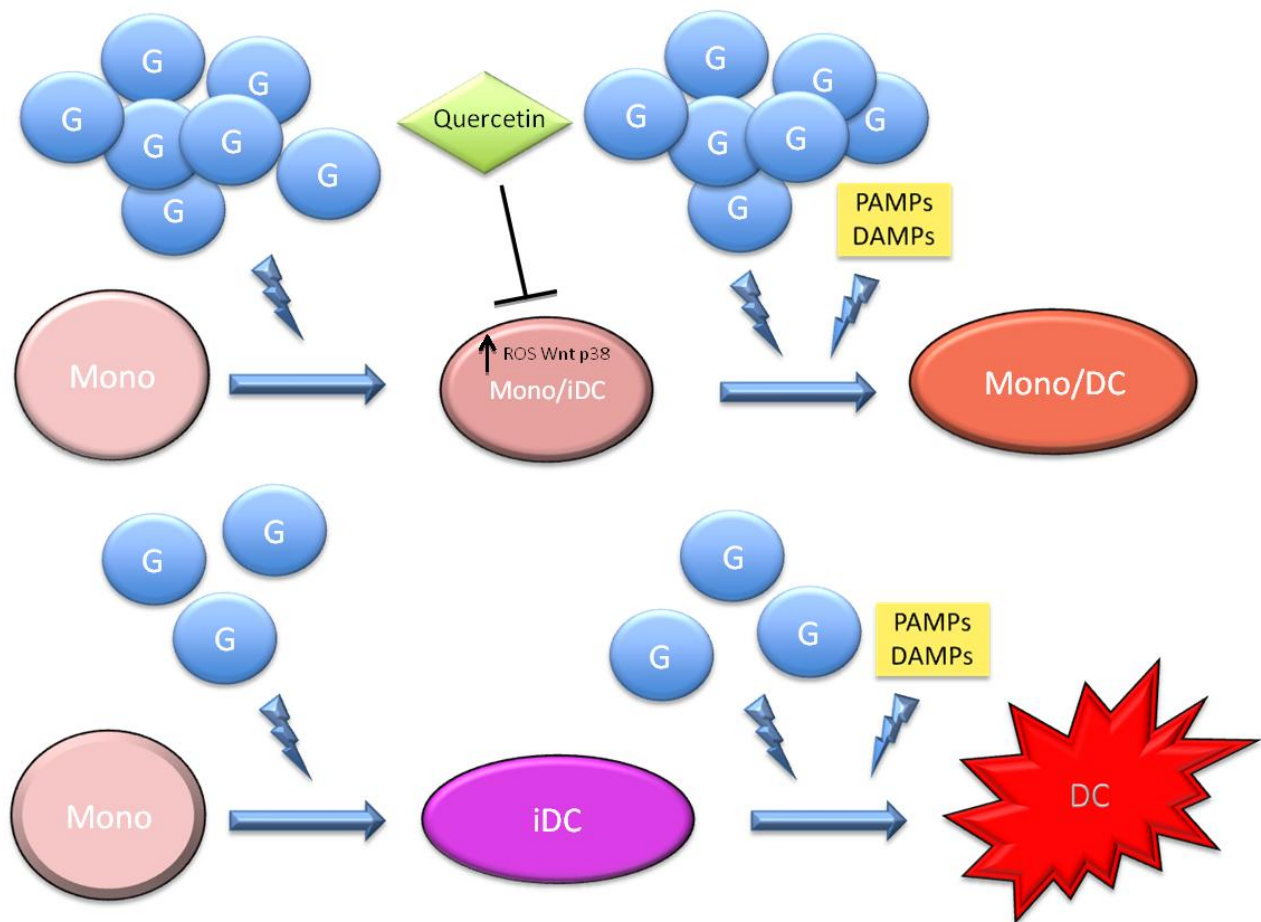


Figure 7



Graphical abstract

**Conflict of Interest Form**

We have no financial or commercial conflicts of interest to declare.

ACCEPTED MANUSCRIPT

**Highlights**

High glucose and hyperglycemic sera from type 2 diabetic patients impair DC differentiation and maturation.

High glucose and hyperglycemic sera increase ROS production and activate Wnt/ $\beta$ -catenin and p38MAPK pathways in dysfunctional iDCs.

Quercetin partially reverts the immune-suppressive effects mediated by high glucose and by hyperglycemic sera.