Porphyromonas gingivalis -Stimulated Hyperglycemic Microenvironment Alters the Immunometabolism of Dendritic Cells

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Abstract

Introduction: Periodontitis in patients with diabetes mellitus results in chronic inflammation, which is the central issue in developing an efficient and consistent treatment plan. Dendritic cells (DC) are antigen presenting cells that initiate the immune inflammatory responses and contribute to the pathogenesis of both diseases. In this study, we investigated the impact of hyperglycemic microenvironment on DC immunometabolism, the cell phenotypes and immunogenic functions. Methodology: Human monocyte differentiated DC and mice bone marrow derived DC were cultured in the presence of 5.5-, 11-, and 25- mM glucose to simulate diabetic microenvironment. Cells were activated with advanced-glycation-end product (AGE) and lipopolysaccharides (LPS) from *Porphyromanas gingivalis* for 24 hours and processed for transcription, metabolic and microscopic analysis. Expression of activation markers (CD80, CD83, CD86, HLA-DR) and proteins involved in glycolysis (HK2, LDHA, GLUT1) in DC were calculated by qRT-PCR. Lactic acid production and OXPHOS assays, including Seahorse metabolic flux analyzer were utilized to determine the effects on metabolism. Impact on the phagocytic capacity was analyzed using fluorescent microspheres uptake. Cytokine expressions for tumor necrosis factor alpha [TNF- α], interleukin [IL]-1 β , IL-6, IL-10, and Interferon gamma [IFN- γ] were evaluated in cell supernatants from DC and DC-T cell coculture. Results: Under simulated hyperglycemic microenvironment an increase in cell dendrite extensions, and activation markers were upregulated in both monocytes differentiated DC and BMDC. There was a significant increase in glycolysis as evident from the gene expression, cell metabolic flux, and lactic acid production. Cell OXPHOS activities was reduced to compensate for the increase in glycolysis. Pro-inflammatory cytokines (TNF- α and IL-1 β) were significantly increased and this increase was directly proportional to the glucose concentrations. Whereas, phagocytic capability of DC, and their ability to activate T cells decreased with hyperglycemia. Conclusions: Hyperglycemic microenvironment resulted in DC changes with increased expressions of activation markers, glycolytic metabolism, and increased pro-inflammatory cytokines, while impairing phagocytosis and adaptive immunity induction. BMDC and human monocyte differentiated-DC exhibit similar responses toward hyperglycemia, AGE, and LPS. This work emphasizes that diabetes mellitus has an inflammatory impact on DC immunometabolism and immunogenic functions.

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Methodology: Human monocyte differentiated DC and mice bone marrow derived DC were cultured in the presence of 5.5-, 11-, and 25- mM glucose to simulate diabetic microenvironment. Cells were activated with advanced-glycation-end product (AGE) and lipopolysaccharides (LPS) from *Porphyromanas gingivalis* for 24 hours and processed for transcription, metabolic and microscopic analysis. Expression of activation markers (CD80, CD83, CD86, HLA-DR) and proteins involved in glycolysis (HK2, LDHA, GLUT1) in DC were calculated by qRT-PCR. Lactic acid production and OXPHOS assays, including Seahorse metabolic flux analyzer were utilized to determine the effects on metabolism. Impact on the phagocytic capacity was analyzed using fluorescent microspheres uptake. Cytokine expressions for tumor necrosis factor alpha [TNF-α], interleukin [IL]-1β, IL-6, IL-10, and Interferon gamma [IFN-γ] were evaluated in cell supernatants from DC and DC-T cell coculture.

Results: Under simulated hyperglycemic microenvironment an increase in cell dendrite extensions, and activation markers were upregulated in both monocytes differentiated DC and BMDC. There was a significant increase in glycolysis as evident from the gene expression, cell metabolic flux, and lactic acid production. Cell OXPHOS activities was reduced to compensate for the increase in glycolysis. Pro-inflammatory cytokines (TNF-α and IL-1β) were significantly increased and this increase was directly proportional to the glucose concentrations. Whereas, phagocytic capability of DC, and their ability to activate T cells decreased with hyperglycemia.

Conclusions: Hyperglycemic microenvironment resulted in DC changes with increased expressions of activation markers, glycolytic metabolism, and increased pro-inflammatory cytokines, while impairing phagocytosis and adaptive immunity induction. BMDC and human monocyte differentiated-DC exhibit similar responses toward hyperglycemia, AGE, and LPS. This work emphasizes that diabetes mellitus has an inflammatory impact on DC immunometabolism and immunogenic functions.

Introduction :

Periodontitis and diabetes mellitus (DM) are both chronic diseases with a high prevalence and have a strong inter-relationship (1). Periodontitis affects approximately 25 – 50% of the adult population worldwide, and is considered as the main cause of tooth loss (2). On the other hand, DM is a multifactorial metabolic disease affecting at least 422 million people globally (3). DM is characterized by hyperglycemia due to impairment in either insulin secretion or its action, or both (4). Previous clinical studies have reported that DM is a

major risk factor for periodontitis and its treatment outcome (2). DM can increase the severity/prevalence of periodontitis by three folds (1). Interestingly, there is a significant likelihood for periodontitis patients to have uncontrolled DM. So it is not surprising that a strict glycemic control and treating periodontitis can improve the treatment outcome of periodontitis and DM (1,5).

Chronic inflammation and impaired immune cell responses are the key linkers between the two diseases (6). Pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, and inflammatory molecules such as advanced glycation end products (AGE), adipokines, RANKL, and reactive oxygen species (ROS) generated by hyperactivated immune cells in each disease condition could exacerbate the other and react with the immune cells to damage their respective immunogenic responses. If this persists for long, it could result in a negative feedback on local tissue inflammation and bone resorption in patients with periodontitis and DM (6–8).

Recent studies have emphasized that metabolic programming of immune cells tightly controls their expression, differentiation, and functions (9–11). A Warburg effect is commonly adopted by both innate and adaptive immune cells to fulfill their respective immunogenic functions, where glycolysis is upregulated ; and this dysregulation could directly promote pathogenesis of both periodontitis and DM (12). Innate immune cells such as neutrophils use glycolysis for adhesion and microbicidal activities, but systemic glucose or infectious microenvironment could reduce this metabolic reprogramming, thereby promote bacterial infections and insulin resistance (13,14). It is possible to determine if adaptive T and B cells will transition to glycolysis or remains on mitochondrial metabolism based on their effector or memory subsets. In DM and periodontitis, the metabolism of T and B cells were altered with attenuated effector functions via mTORC1 and AMPK related pathway (10,15,16). Such metabolic reprograming from mitochondrial respiration to glycolysis is also required for the activation and function of dendritic cell (DC), the most efficient antigen presenting cells (APC) that connect the innate and adaptive immunity (17–19).

Pathogen associated molecular patterns (PAMPs), or danger associated molecular patterns (DAMPs) signals initiates phenotypic and functional changes in DCs upon interacting with its surface receptors. DCs upregulate surface co-stimulatory activation markers upon phagocytosis of the antigens, which upregulates cytokine secretions, and migrations to lymph node for T cell activations (20). For oral immune homeostasis, DCs play a crucial role as their absence or dysfunction directly promotes periodontal inflammation via uncontrolled bacterial infection (21,22). Recent studies have also emphasized on the essential role of DC in DM autoimmune responses and suggested its potential to be good a therapeutic target in DM (23,24). There is enough evidence to point at DCs in the pathogenesis of periodontitis complicated with DM. Conflicting DC phenotypes and functions were reported in these two diseases, with a combination of upregulated and suppressed activation markers CD80 and CD86 and an overall upregulated pro-inflammatory cytokine profile. However, the specific effect on DC immunometabolism in response to periodontal pathogen complicated by diabetic microenvironment is vague. To date, there are no reports on how the underlying immunometabolism may govern these dysregulated phenotypes and immunogenic functions of DC in periodontitis and DM.

In this study we investigated the potential alterations of DC immunometabolism induced by periodontal bacterial LPS, complicated with hyperglycemia. DCs were exposed to an *in vitro* microenvironment simulating periodontal infections and diabetic hyperglycemia by using LPS from oral bacteria *Porphyromonas gingivalis* (*P. gingivalis*), different glucose concentrations, and AGE. We hypothesized that these stressors could dysregulate the DC immunometabolism, specifically the glycolysis pathway and its downstream mitochondrial respiration, therefore impairing the cell phenotypes and functions.

Material & Methods

Monocyte-differentiated DC culture

Human monocyte cell line THP-1 were differentiated into DC using a well-established protocol (25). THP-1 cells were cultured in complete cell culture medium (RPMI-1640, Gibco, with 10% FBS, Sigma-Aldrich, antibiotic-antimycotic solution, Gibco and 2-Mercaptoethanol, Gibco) with 5.5 mM glucose, 11 mM glucose, and 25 mM glucose and supplements of IL-4 (20 ng/mL) and GM-CSF (20 ng/mL) for 5 days to differentiate

into immature DC. Fresh media were replenished every 2 days. On Day 5, suspended cells were collected and cultured in RPMI with 5.5 mM glucose, 11 mM glucose, and 25 mM glucose and supplements of IL-4 (20 ng/mL, LifeSciences), GM-CSF (20 ng/mL, LifeSciences), TNF-α (20 ng/mL, LifeSciences), and ionomycin calcium (20 ng/mL, Sigma-Aldrich) to differentiated into mature DC for 24 hours. Dendrites formation and cell adherences confirms the differentiation. On Day 6, differentiated DC were seeded in a 6 well plate with a density of 1×10^6 cells/2 mL cell culture medium per well with supplements of GM-CSF (20 ng/mL), ionomycin calcium (20 ng/mL),*P. gingivalis* lipopolysaccharides (LPS, 1 μg/mL, Invitrogen) and advanced glycation endproduct (AGE, 2 μg/mL, Sigma-Aldrich) for 24 hours. Cells were cultured at 37 and 5% CO_2.

BMDC culture

Bone marrow cells were extracted from femur and tibia bone of 6 – 12 weeks old C57BL/6 mice and plated on a 100 mm Petri culture dish at a density of 4×10^6 cell/10 mL with GM-CSF (20 ng/mL) in complete cell culture medium RPMI-1640 (Gibco) with glucose concentration of 5.5 mM, 11 mM, and 25 mM. BM cultures were fed on Day 3 and 6 with 10 mL cell culture medium containing fresh GM-CSF (20 ng/mL, Biolegend). On Day 9, BMDC were seeded in a 6 well plate with a density of 1×10^6 cells/2 mL cell culture medium per well. *P.gingivalis* lipopolysaccharides (LPS, 1 μg/mL, Invitrogen) and advanced glycation endproduct (AGE, 2 μg/mL, Sigma-Aldrich) was added for 24 hours. Cells were cultured at 37 and 5% CO_2.

Splenic T cell extraction

Spleens from C57BL/6 mice were minced and passed through a 40 μm strainer prior to T lymphocyte isolation. Mice T cells were purified from splenic cell suspension using EasySep^{TM} Mouse T Cell Isolation Kit (Stemcell Technologies) following the manufacturer's protocol.

DC-T cell coculture

0.5×10^6 splenic T cells were cultured in a 12 well plate with inactivated BMDC cultures at 1:1 ratio supplemented with GM-CSG (20 ng/mL, Biolegend) for three days with or without AGE (2μg/mL, Sigma-Aldrich) and LPS (1 μg/mL, Invitrogen). On Day 3, cell supernatant were collect for ELISA analysis. All cells were cultured at 37 and 5% CO_2.

Cell Morphology Assay

The differentiated mDC were grown in different glucose concentrations with or without activation. The cells were fixed on glass coverslips at a density of 0.8×10^6 and stained with Hoechst dye (Sigma), TRITC-conjugated phalloidin (Sigma), CD83 primary antibody (Invitrogen), and FITC-conjugated secondary antibody (Invitrogen). Cells were imaged using a fluorescent microscope system (Zeiss LSM 800, Germany). Nine random areas were imaged at 40× magnification and the images were processed with Fiji ImageJ software.

Effect on DC Phenotype and Metabolism- Gene Expression Assays

Total RNA of inactivated or activated DC was extracted using RNeasy mini kit (Qiagen) and the cDNA was synthesis using SensiFAST cDNA Synthesis kit (Bioline) with random primers. Quantitative real time RT-PCR was performed using SYBR Green Master Mix Reagent (Applied Biosystems) by CFX Real Time System (Bio-Rad). Each sample was analyzed in triplicates and normalized to control gene β-actin. Relative expression was reported using the $2^{\zeta(\Delta Ct)}$ method. Table 1 and Table 2 presents the primer sequences used in the experiments.

Metabolic assay

The glycolysis activity of monocyte-differentiated DC was measured using Agilent Seahorse XFe24 metabolic flux analyzer with glycolytic stress test kit (Agilent Technologies). DC were seeded at a density of 1.75×10^5 in the 24-well XF Cell Culture Microplate. The extracellular acidification rate (ECAR) of the cells were measured in triplicate following each injection of glucose, oligomycin, and 2-deoxy-glycose. L-lactate from

cell supernatant was quantified following the manufacturers protocol (Cayman Chemical). OXPHOS activity was measured using a commercial kit (Thermofisher).

Cytokine profile

The supernatants from monocyte-differentiated DC culture and BMDC culture were collected on Day 7 and stored at -20 until further use. Cytokine production by the DC were assessed using ELISA (IFN-γ, IL-6, IL-17, and IL-10) (Biolegend, California, United States) following manufacturers protocol.

Phagocytosis assay

Inactivated BMDC were used in phagocytosis assay. Approximately 1×10^6 cells were seeded in a 6 well plate (Corning) with 2 mL complete cell culture medium RPMI-1640 (Gibco) supplemented with GM-CSF (20 ng/mL, Biolegend). Cells were cultured with 5.68×10^7 Fluoresbrite carboxylate microspheres for 3 hours to assess cell phagocytosis. Cells were washed and stained with Hoechst dye (Sigma) and imaged using a fluorescent microscope system (Zeiss LSM 800, Germany). Six random areas were imaged at $10\times$ magnification and the images were processed with Fiji ImageJ software.

Statistical Analysis

The experiments were done in triplicates and repeated minimum of three times. All data were analyzed with GraphPad Prism with unpaired t test and ANOVA test. The difference between experimental groups was set to be statistically significant at $P < 0.05$.

Results

High glucose modulates DC morphology

Impact of hyperglycemia (11- or 25-mM glucose) was examined on inactivated or *P. gingivalis* LPS and AGE-product activated mDC differentiated from human monocytes using immunofluorescence staining. At the glucose concentration of 5.5 mM, inactivated mDCs had a spherical or elliptical morphology with few dendrites (Figure 1A). Without stimulation, or by increasing the glucose concentrations alone increased dendrites on the surface of cells, indicating cell spread and maturation (Figure 1. C, E). However, in presence of LPS and AGE, dendrites formation on activated mDC was stimulated in all three glucose concentrations, yet the cells in 11- and 25-mM glucose had more extended dendrites in comparison to the 5.5 mM glucose culture (Figure 1. B, D, F). Both inactivated and activated mDCs in all three glucose concentrations expressed CD83 (Figure 1. A-F).

DC phenotypes are modulated under hyperglycemia

To assess the impact of glucose on BMDC activation, we next performed RT-qPCR for surface markers CD80, CD86, CD83, and MHC-II complex.The relative mRNA expressions are reported as fold change in comparison to the inactivated cells cultured in 11 mM glucose (Figure 2. A – D). The co-stimulatory markers CD80, CD83, and MHC-II were significantly upregulated in the presence of AGE and LPS in 11 mM glucose cell cultures. These three genes' expression were also increased upon activation in 25 mM glucose but with this difference was not statistically significant except for MHC-II (Figure 2. A, C, D). Elevated CD86 was observed in both 11 mM and 25 mM glucose cultures with not statistical difference (Figure 2. B). Human monocyte differentiated-mDC demonstrated comparable findings, which LPS and AGE significantly upregulated expression of CD80, CD86, and HLA-DR (Figure 2 E – H). Expression level of CD83 was not statistically different (Figure G).

Hyperglycemia upregulates DC glycolysis activity

We then examined the expression of genes involved in glycolysis under different glucose concentrations in BMDC. All gene expressions were adjusted relative to the inactivated cells cultured in 11 mM glucose. A glucose dose-dependent increase was observed in mRNA of all three analyzed genes in inactivated and activated DC (Figure 3. A – C). Cells cultured in 11 mM glucose were significant elevation in HK2 and LDHA but not GLUT1 upon activation by AGE and LPS, while cell activation significantly upregulated

LDHA in 25 mM glucose culture, but not a statistical increase for HK2 and GLUT1 levels, (Figure 3 A – C). Similar increase in the expression of genes associated with glycolysis in response to increasing glucose levels was observed in monocyte-differentiated DC. Furthermore, these glycolysis genes were significantly up in the presence of AGE and LPS (Figure 3 D – F).

To further corroborate the effect on DC glycolysis, we utilized Seahorse Glycolysis Street Test to measure the glycolysis activity under 5.5 mM, 11 mM, and 25 mM glucose with or without LPS and AGE stimulations. In the absence of AGE and LPS, the inactivated mDC previously cultured in higher glucose concentrations exhibited high extracellular acidification rate (ECAR) that was proportional to the relative glycolysis activity when glucose was supplemented (Figure 3G). This increase in ECAR value was observed to be directly dependent on glucose concentrations. Activation of mDC by AGE and LPS further upregulated the ECAR and the cell glycolysis, such that higher ECAR values was observed with the similar glucose dose-dependent increasing trend, except for the activated mDC cultured in 25 mM glucose (Figure 3H). This alteration in cell metabolic flux was also reflected on the glycolysis rate and maximal glycolytic capacity (Figure 3I – J).

Extracellular L-Lactate secreted from the cells also demonstrated a dose-dependent increase with the increasing glucose concentration in both inactivated and activated DC, and the cell activations resulted in a higher L-Lactate release under all glucose conditions (Figure 3K). On the other hand, the DC OXPHOS activity, measured by the relative intensity of the cell membrane potential, gradually decreased with the increasing glucose concentrations, with or without the stimulation of AGE and LPS (Figure 3L).

Elevated glucose concentration attenuates DC phagocytosis

By coculturing the inactivated BMDC with Fluoresbrite carboxylate microspheres and staining the cell nucleus, we next the impact of glucose on DC phagocytosis capability using fluorescent microscopy. A reduction in inactivated BMDC phagocytosed-Fluoresbrite microspheres was observed with the increase in glucose concentration from 5.5 mM to 25 mM (Figure 4. A – C). Quantification of relative fluorescent microsphere to cell nucleus ratio significant decreased from 1.2 to 0.5 according to glucose concentrations (Figure 4. D).

Hyperglycemia boost pro-inflammatory cytokine production in DC

An increase in pro-inflammatory cytokines TNF-α and IL-1β was observed for the inactivated and activated BMDC and human monocyte differentiated DC in presence of high glucose concentrations (Figure 5). Activation of the cells by AGE and LPS significantly upregulated both inflammatory cytokines (Figure 5. A, B, D, E). On the other hand, anti-inflammatory cytokine, IL-10, showed a similar trending but no significant elevation was observed upon activation in BMDC, while it was significantly up in human monocyte differentiated DC (Figure 5. C, F). Stimulation of human monocyte differentiated DC with AGE alone demonstrated limited impact, while the stimulation with LPS alone induced potent upregulation of TNF-α. TNF-α levels under these separate stimulation also increased with increasing glucose concentrations, and AGE and LPS together showed an additive effect on TNF-α secretions with glucose (Supplementary Figure 1).

T cell function is impaired when primed with DC cultured in hyperglycemic conditions

We co-cultured mouse BMDC with mouse splenic T cells to investigate the impact of hyperglycemia on DC priming and T cell functions. T cells incubated with BMDC in the absence of AGE and LPS gradually reduced the levels of pro-inflammatory cytokines, IFN-γ and IL-17, which was inversely relative to the increasing glucose concentration (Figure 6. A, C). AGE and LPS resulted in activation of DC that further activated T cells. In comparison to the unstimulated cells, levels of both cytokines significantly increased in all glucose concentrations upon AGE and LPS-stimulation. Cytokines released from these activated cells showed an inverse correlation with the glucose concentrations. The level of IL-6 from T cells also decreased with increasing glucose concentration in the unstimulated culture. However, upon stimulation, IL6 significantly increased to approximately 1020 pg/mL in 5.5 mM, 11 mM, and 25 mM glucose concentrations. (Figure 6. B).

Discussion

In this study, we have demonstrated that the stimulation of AGE, hyperglycemia, and LPS from oral bacterial *P. gingivalis* can synergistically dysregulate the DC phenotype, immunometabolism, and effector functions. Hyperglycemia and infection-activated immune cells together induce inflammation, which is the key to connect periodontitis and DM, two chronic inflammatory diseases with a bidirectional relationship to each other. The Warburg effect-like transition from mitochondrial oxidative phosphorylation to aerobic glycolysis as the main pathway for ATP generation is one critical step for DC activation upon interaction between antigen and TLR (26). Interaction between LPS and surface TLR on DCs could activate the glycolysis transition via Akt-induced signaling, initiated by TLR-associated TBK1 and IKKε proteins, thus the Akt downstream protein mTORC1 is also determined to be involve in the glucose-sensitive transduction circuit for glycolysis metabolism transition (27,28). PFKFB3, an major driver of glycolysis pathway, is identified as a direct target mTOR under LPS stimulation (29). Our data showed a significant increase in gene expression of enzymes such as HK2, LDHA, and GLUT that catalyzes different steps of glycolysis. In the presence of LPS, levels of L-lactate, which is the final product of glycolysis, increased in DC. The induction of glycolytic activity by LPS has been well reported in other immune cells such as macrophages, monocytes, and neutrophils (30–32). LPS can attenuate mitochondrial OXPHOS activity. In macrophages LPS suppresses isocitrate dehydrogenase and succinate dehydrogenase in the citric acid cycle, and blocks the mitochondrial respiration while promoting glycolysis (33,34). Excessive citrate and succinate, both upregulate inflammatory molecules such as NO and IL-1β (33). On the other hand, cells under hyperglycemia shunt glycolytic intermediates into pentose phosphate pathway as a protective mechanism, which limits the glycolytic activity (35,36). As macrophages and DC are closely inter-related in pro- and anti-inflammatory phenotypes, similar pathways could be involved in DC. Moreover, AGE can also reduce glycolysis and mitochondrial respiration through HIF-1α/PDK4 pathway, thereby reducing ECAR and OCR (37,38). This may explain the observed inhibition of glycolysis pathway in the LPS and AGE-stimulated group under hyperglycemia in the cell metabolic efflux assay. An inhibitory effect of hyperglycemia and AGE on glycolysis could reduce the stimulating effect from LPS. Under diabetic microenvironment, glycolysis is not only disrupted by hyperglycemia, AGE, and LPS, rather other DM-associated metabolites such as ROS, free fatty acid, and cholesterol could also impact cell immunometabolism transitions by regulating different signaling pathways (31).

The relative expression of DC surface markers can reflect the cell activation status. Upregulation of protein CD80, CD86, and MHC-II complex is a key evidence of DC activation. Here we have shown that depending on glucose concentration these three co-stimulatory molecules can polarize the cells toward a hyperactivated status in both inactivated and activated DC. Similar results were reported in DC obtained from various lineages under *in vitro* and *in vivo*stimulations (27,39,40). Thomas *et al* demonstrated that brief exposure of hyperglycemic culture could significantly elevate co-stimulatory surface markers in all immature, mature, and tolerogenic DC (39). BMDC retrieved from streptozotocin induced-diabetic mice showed comparable phenotypes as well (27,39). Such increase in CD80, CD86, CD1α, and MHC-II was also observed when comparing the hyperglycemia treated-human monocyte derived DC to the normoglycemia cell culture (40). Glucose and LPS activated-Akt pathway that initiate glycolysis transition may also upregulate the expression of these DC surface markers. The transcription factor NF-κB directly regulates the receptor genes that are involved in the cell immunogenic functions (41,42). Note that the HIF-1α activated by Akt pathway during DC metabolic reprogramming is also a transcription factor that regulate CD80 and CD86 expression (43,44).

The metabolic reprogramming in innate immune cells is a TLR induced event paired with phagocytosis, which is a critical for rapid infection clearance by APCs. Our data demonstrated that hyperglycemia significantly impairs the phagocytosis by immature DC in a glucose-dose-dependent manner. Similar results have been reported in DC differentiated from human PBMC obtained from T2DM patients, where cells were unable to take up the FITC-dextran fluorescein, or this uptake was significantly reduced in hyperglycemic media (45). Similarly, in other phagocytes such as macrophages and monocytes the phagocytotic abilities were reduced in both *in vitro* and *in vivo* hyperglycemic environment. High glucose locks the cell in their pro-inflammatory phenotype that is characterized by dysregulated activation markers and upregulated pro-inflammatory cytokines (46–48). Similar results were seen in the present study where hyperglycemia induced a pro-inflammatory DC with compromised phagocytic effect. Reduced phagocytosis in these innate

immune cells collectively under hyperglycemia can completely impair the infection clearance; thus leading to the chronic immune-inflammatory responses seen in periodontitis and DM patients (6). Such lowered phagocytotic capability could also result from hyperglycemia induced altered cell surface receptors. Under hyperglycemia and hypoxia conditions, in the expression of genes encoding CD36 and SCARB-1 in human monocyte-differentiated macrophages is reduced. CD36 and SCARB-1 are proteins responsible for phagocytosis (47). Expression of FCγ receptor CD32 and CD64 that mediate phagocytosis with antigen recognition receptor CR3 in monocytes were also reported to be reduced with increasing HbA_{1c} level and body mass index in DM patients (48). Interestingly, no significant differences were observed in phagocytosis protein CD36 on DC from DM patient but MFGE8, another phagocytosis protein was significantly reduced (49). MFGE8 is frequently associated with impaired phagocytosis of various phagocytes generally and under DM conditions (50–52). However, there is limited knowledge to associate the reduced MFGE8 levels with the aerobic glycolysis transition upon DC activation. Either way, correcting the expression levels of this protein can result in profound wound healing potential by promoting DC and macrophages into immune suppressive phenotypes, which favors mitochondrial respiration rather than glycolysis (53,54). The antigen presentation ability beyond phagocytosis mechanism could also be impacted by hyperglycemia. High glucose evidently delays antigen processing and impairs antigen presentation in human monocyte-derived macrophages via repressed Rab and Cathepsin, leading to reduced T cell activation (55). Defect in antigen presentation has been reported in splenic macrophages from NOD mice (56).

Exaggerated cytokine secretion is a characterized functional change upon DC activation. Significant levels of pro-inflammatory cytokine, TNF-α and IL-1β from the activated DC indicates that hyperglycemia, AGE, and LPS could together stress DC to exert a more immunogenic state. Similar cytokine profiles were reported in other studies from human and mice DC as each of the three stressors are known to be pro-inflammatory (40,57,58). Besides their role in interconnecting the chronic inflammatory states in periodontitis and DM, such increase in these pro-cytokines may help in developing other autoimmunity in innate immune cells as well. Recent studies have shown that TNF-α could stimulate the synovial fibroblast cells to increase the glycolysis related-proton efflux and promote rheumatoid arthritis, which is another risk factors associated with both periodontitis and DM (59). Similarly, in autoimmune disease Sjögren syndrome as well the inflammatory response between TNF-α and DC via TLR-7 and TLR-9 has been confirmed (60). IL-1β can induce autocrine responses during bacterial infection and reprogram the immunometabolism through glycolysis (61). If this immunometabolism transition to aerobic glycolysis can play a role in the trained immunity of DC under hyperglycemic stress remains unclear, yet it is an important question worth investigating.

DC have a unique capacity to induce activation and proliferation of antigen specific T cell responses as its APC function in adaptive immunity. The presence of co-stimulatory molecules CD80, CD86, and MHC-II complex play an important role in T-cell activation; wherein, signals from DC synergistically interact with both the T cell receptor and CD28 on T cells for complete activation (62). On the other hand, lack of any one of the co-stimulatory signals can result in immune tolerance or ignorance of activation to prevent aberrant response of T cells. Increase in the expression of DC co-stimulatory markers should lead to hyperactivation of the T cell lymphocytes (63); however, contradictory results were observed in our study. Although DC stimulated with hyperglycemia, AGE, and LPS had high expression of co-stimulatory markers , which increases with increase in glucose levels. T cells cocultured with DC in higher glucose showed attenuated proliferation and Th-1 type cytokine secretions, indicating a lowered T cell priming capability. This was expected as the DC cultured in high glucose concentration were more activated and matured while the phagocytosis ability of immature DC is only optimal (64). Samuel *et al* had also indicated that Th1 is only secondary predominant while Th17 is the predominant pathway in DM mice (65). Such impairment in the DC function could also be due to compromised phagocytosis and antigen presentation capability in hyperglycemia. Antigen presentation on DC is required for the effective physical interaction and T cell activation *in vivo* , whereas unpulsed DC fail to induce lymphocyte responses as shown by Ingulli *et al* (66). Also, a minimum threshold antigen dose is required for a stable DC-T cell priming (67). With attenuated phagocytosis ability and therefore the lowered antigen presentation on DC in hyperglycemia, the T cell activation is weak, and this may lead to a prolonged inflammation in DM and periodontitis. However, the antigen presentation using LPS model is

a non-specific or bystander response from T cells, as presence of LPS could induce inflammatory responses from a wide array of cells. Extension of the adaptive immune responses using OT-II mice may could help in further understand such compromised antigen presentation from DC by pulsing DC and T cells co-culture with specific antigens like ovalbumin (67).

Periodontitis is a chronic disease caused by a multispecies bacterial biofilm, and DM is a systemic autoimmune disease that involves numerous inflammatory molecules and immune cells. Therefore, the simple use of bacterial LPS and hyperglycemia may not comprehensively mimic the complicated disease microenvironment. *In vivo* studies utilizing diabetic mice with induced periodontitis could provide better understanding of the diseased state and immune responses. While we performed the transcription analysis of DC surface markers in this study, it is possible that the hyperglycemia could affect the cell translation mechanism. Furthermore, the intracellular localization of the phagocytosed fluorescent microspheres would be an enticing next step to understand the DC phagocytosis mechanism.

In conclusion, this study highlights that hyperglycemia with AGE and LPS can polarize DC toward a more pro-inflammatory phenotype and cytokine profile by upregulating glycolysis, but its phagocytosis and T cell priming is compromised. Together, this DC function dysregulation may explain the conflicting adaptive immune cell profiles observed in DM and periodontitis.

Data availability statement

The raw data supporting the conclusion of this article will be made availability by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by University of Toronto animal use protocol (AUP) number 20012294.

Author contributions

BY and AS designed the experiments and analyzed data. BY and AS wrote the paper. BY and MG performed the experiments and organized data. AS and DW helped in overall study design and manuscript formation. All authors contribute to the article and approve the submitted version.

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Conflict of interest

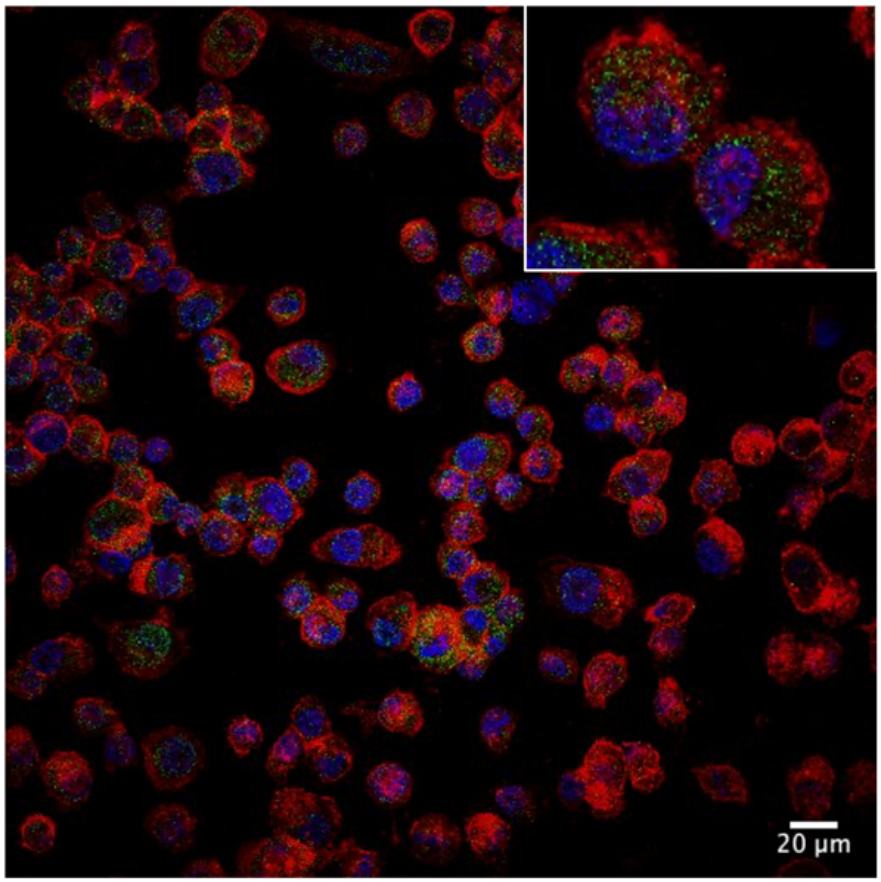
The authors deny any conflicts of interest related to this study.

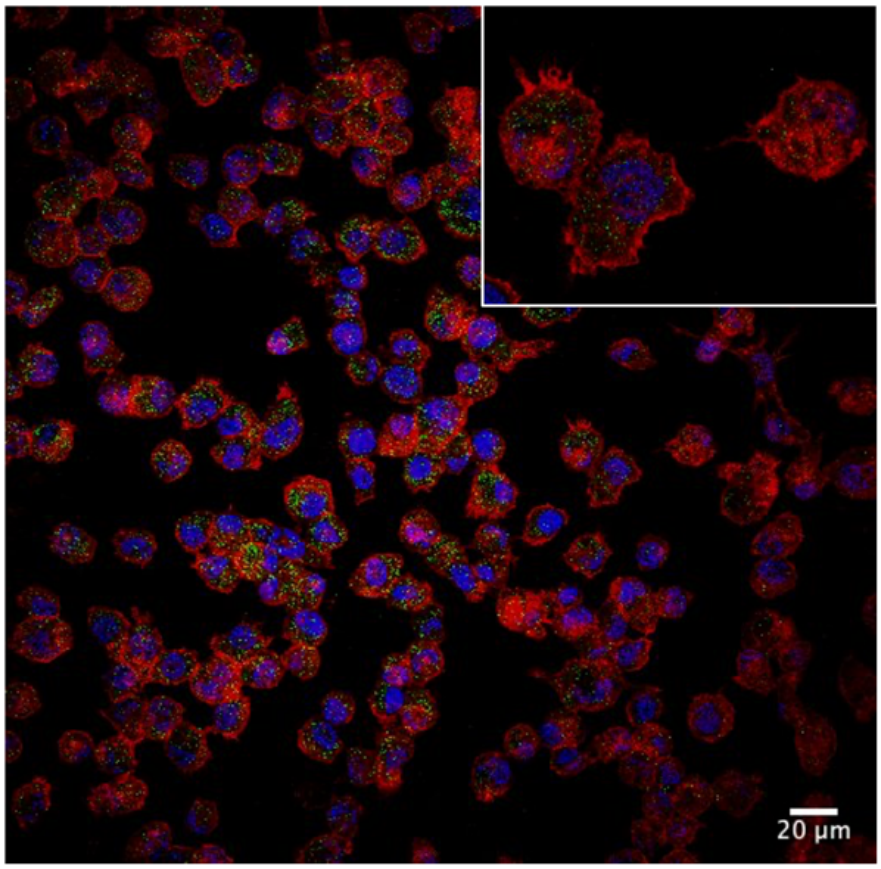
Contribution to the field statement

Presence of hyperglycemia related to diabetes mellitus can negatively impact immune inflammatory responses in chronic periodontitis by influencing the antigen presenting dendritic cells. This study highlighted that hyperglycemia impacted immunometabolism of dendritic cells towards glycolysis and subsequent pro-inflammatory phenotypes. Understanding dendritic cells' dysregulation may explain the conflicting adaptive immune cell profiles observed in diabetes mellitus and chronic periodontitis.

Figure 1.

A B





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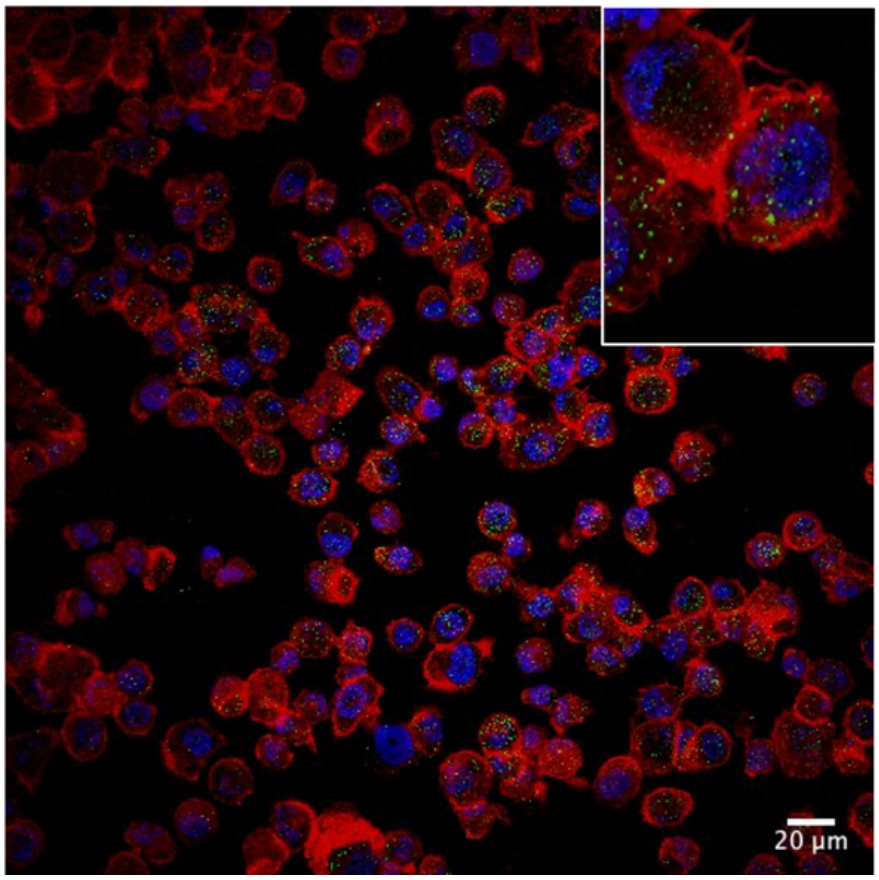
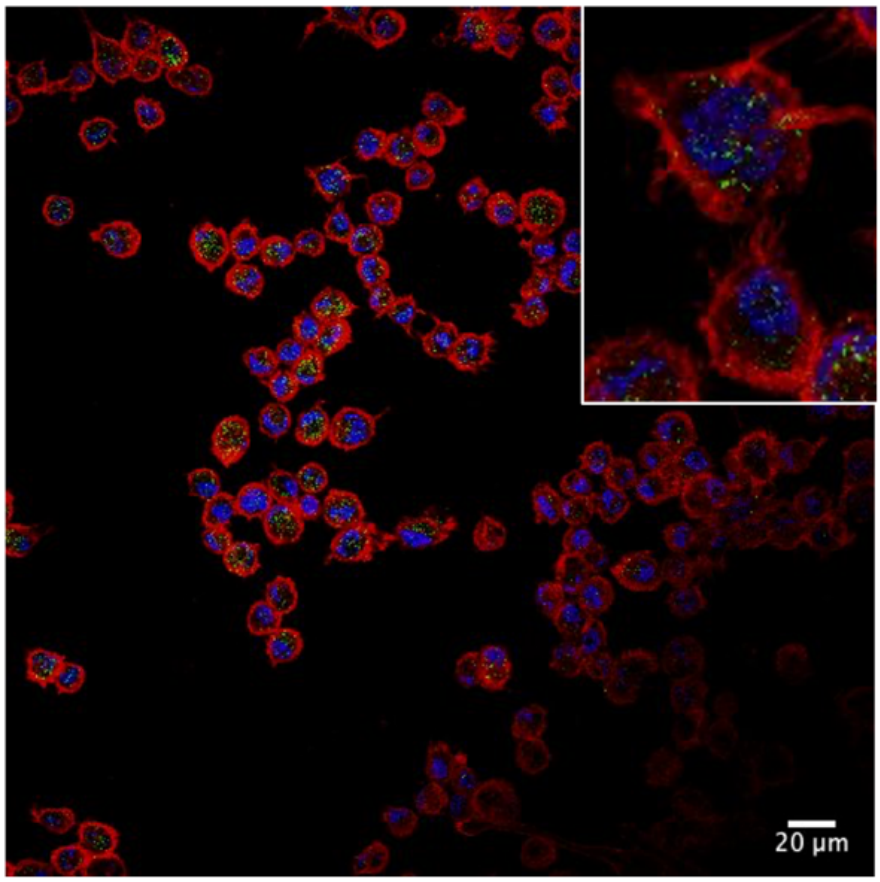
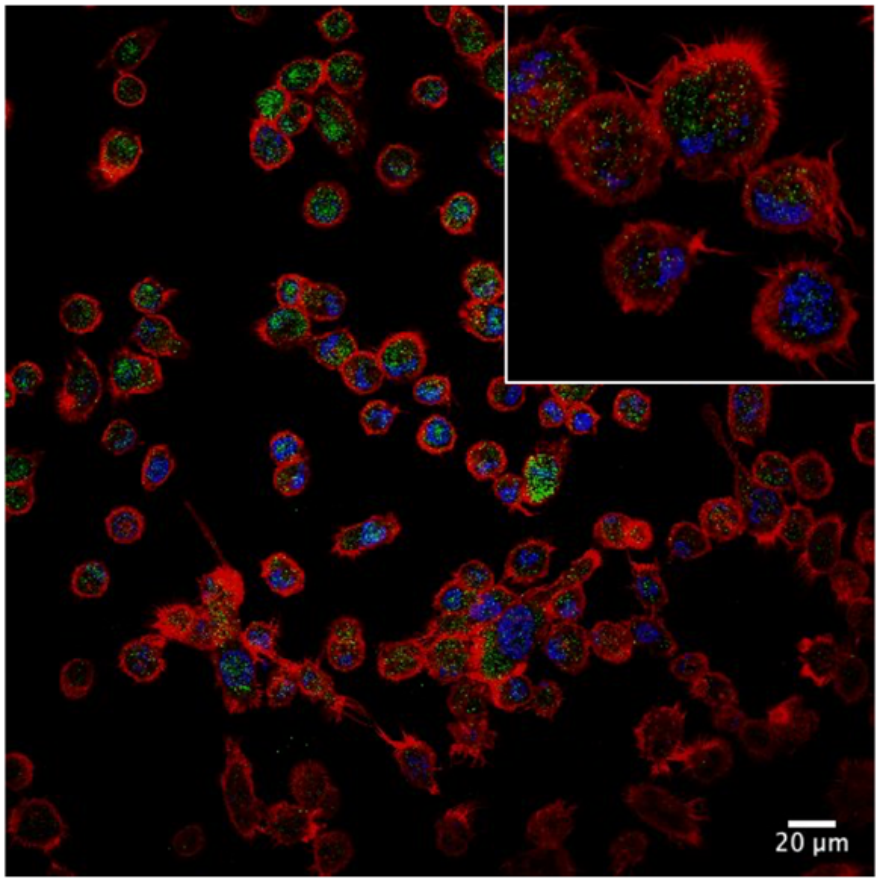




E F



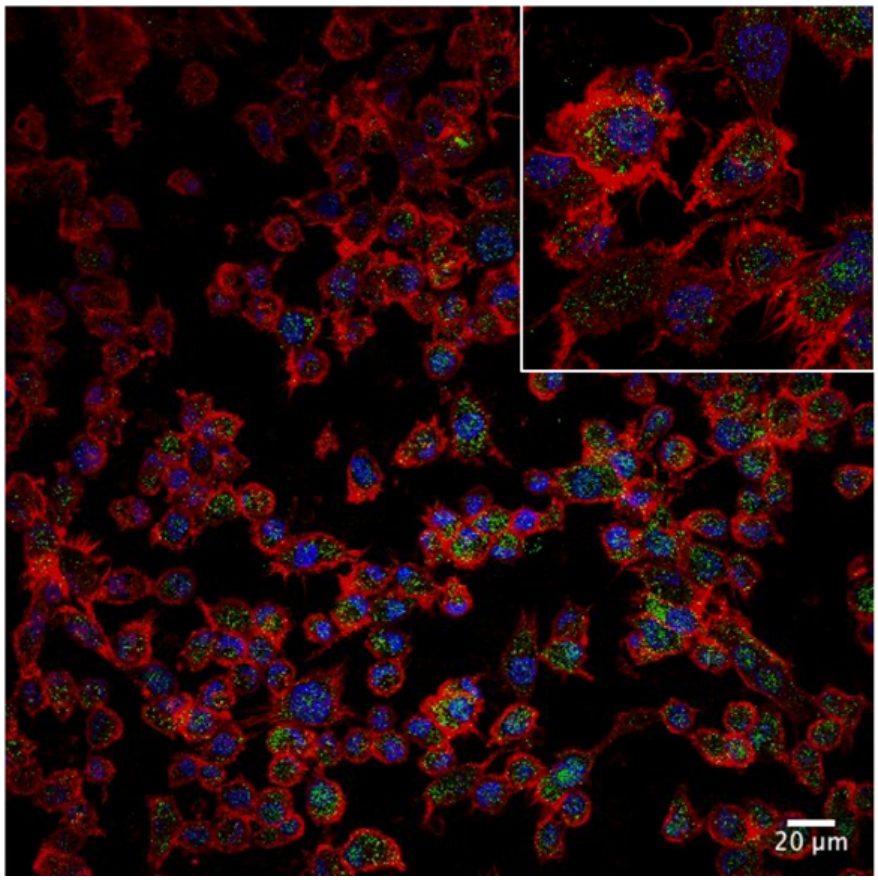


Figure 1. Immunofluorescence image of inactivated mDC in 5.5- (A), 11- (B), and 25 mM glucose (C); and immunofluorescence image of AGE & LPS-activated mDC in 5.5 mM glucose-(B), 11- (D) and 25 mM glucose (F). The cell nucleus was stained with Hoechst dye (blue), the F-actin was stained with TRITC-conjugated phalloidin (red), and the CD83 was stained with FITC-conjugated antibody (green). Images were captured at 40× magnification. Magnified areas from the respective images are displayed on the top right corner to demonstrate cell morphologies and dendrites formation.

Figure 2.

A B C

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Figure 2. Relative gene expression of surface markers CD80, CD86, CD83, and MHC-II in inactivated and AGE & LPS-activated BMDC (A – D) and human monocyte differentiated mDC (E – L) in 11 mM and 25 mM glucose. Values were normalized to mRNA level of inactivated mDC in 11 mM glucose. (ns = not significant, *P<0.05, **P<0.01, ***P<0.001).

Figure 3.

A B C

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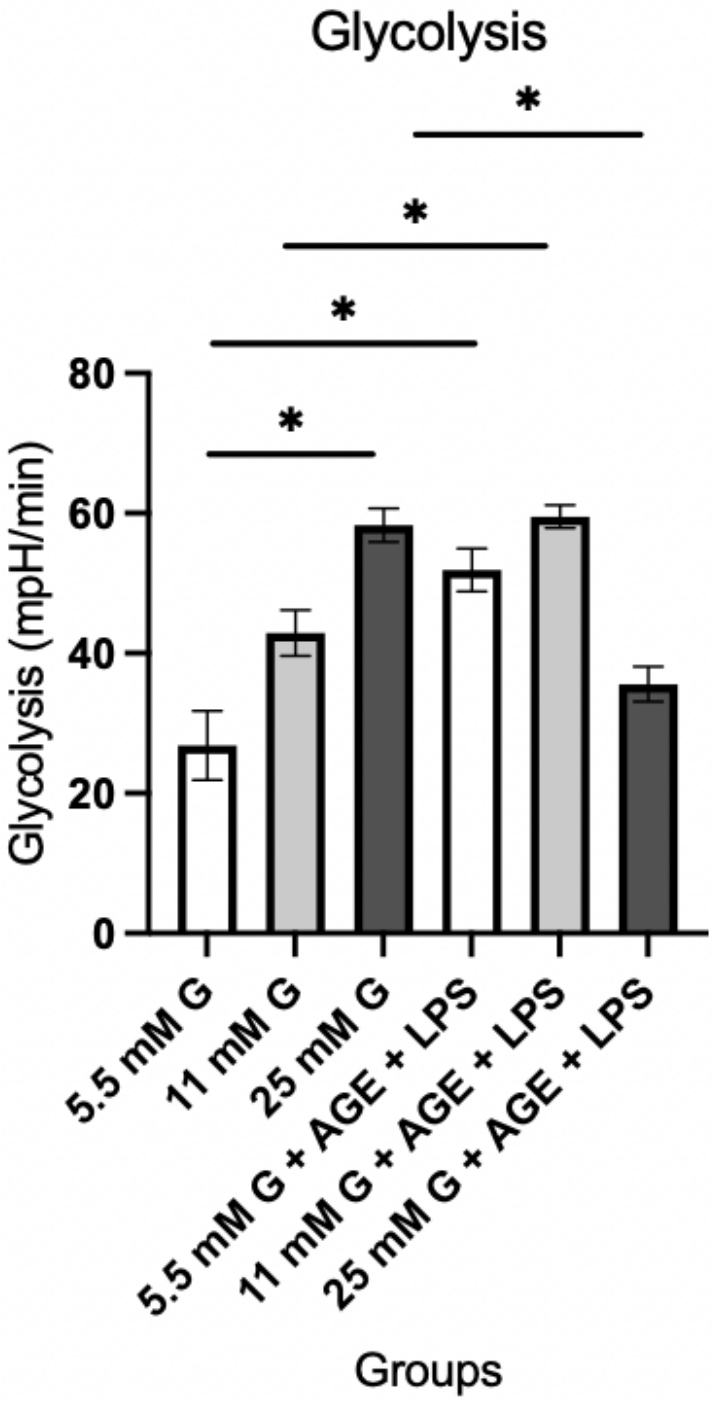
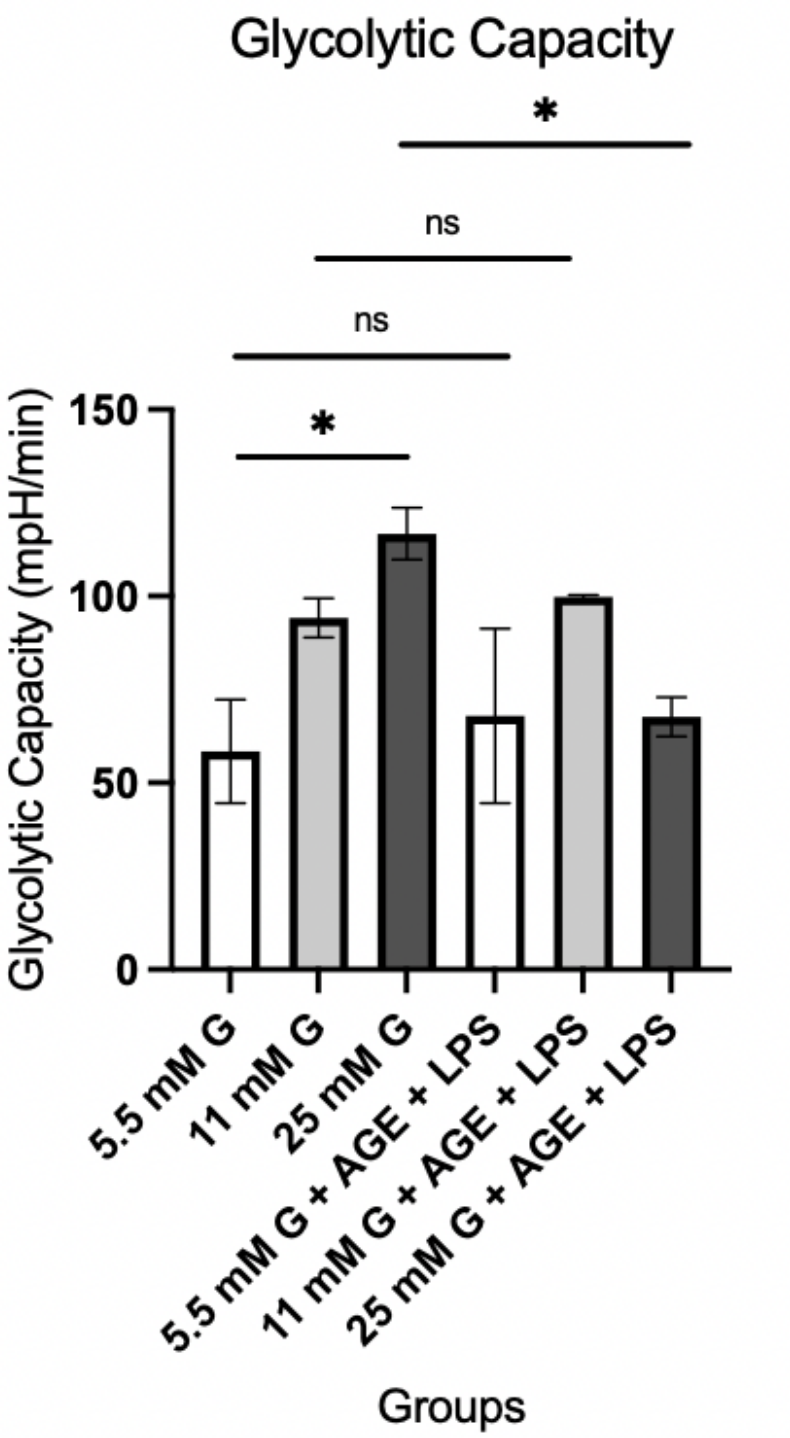
I J





K L

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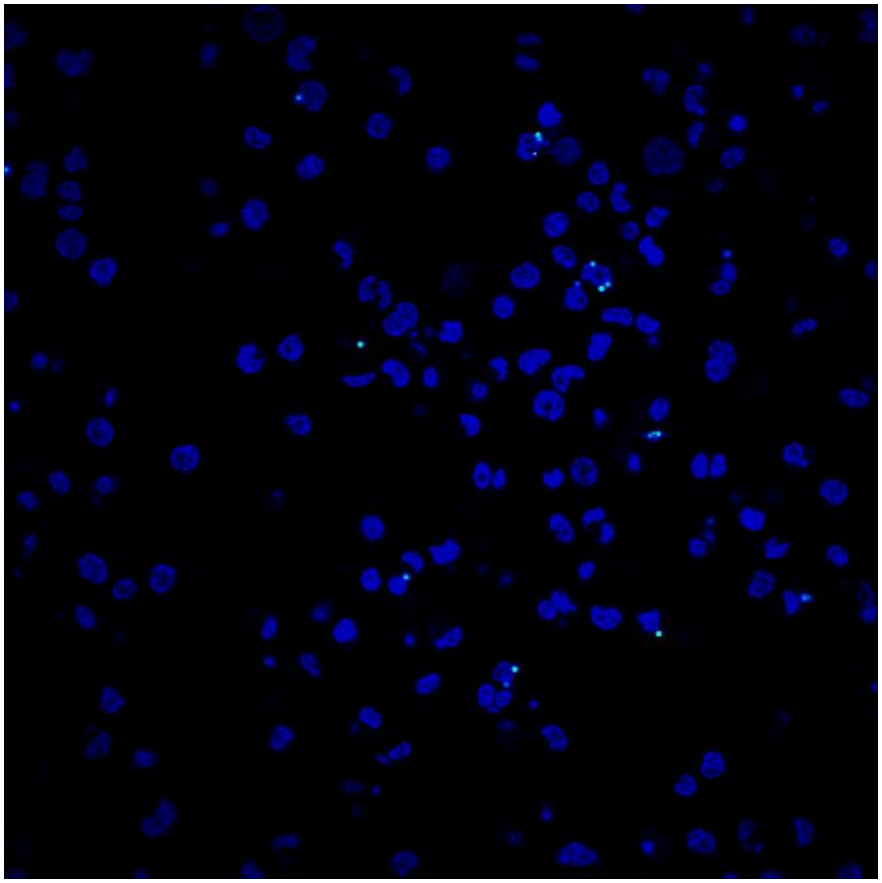
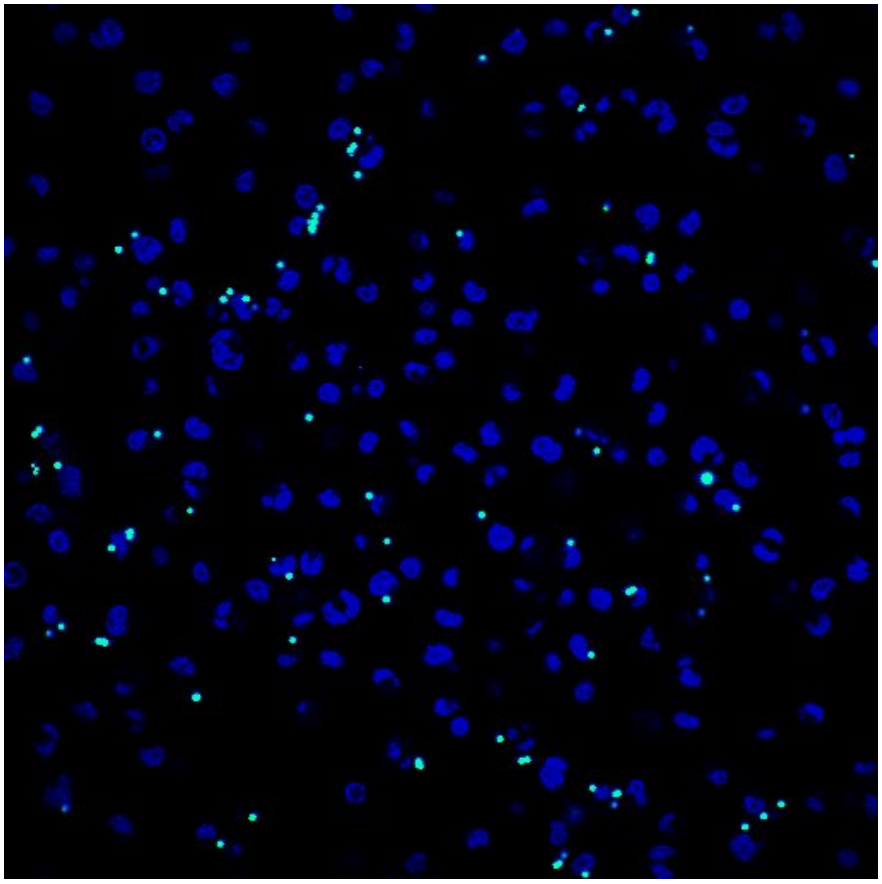
Figure 3. Relative gene expression of glycolysis genes HK2, LDHA, and GLUT1 in inactivated and AGE & LPS-activated BMDC (A – C) and human monocyte differentiated-mDC (D – F) in 5.5-, 11-, and 25-mM glucose. Values were normalized to mRNA level of inactivated mDC in 11 mM glucose. (ns = not significant, *P<0.05, **P<0.01, ***P<0.001). Overall rate curves of metabolic flux of inactivated monocyte differentiated-DC (G) and AGE & LPS-activated monocyte differentiated-DC (H) in 5.5-, 11-, and 25-mM glucose (plated in 75000 cells/well). Glycolysis rate (I) and glycolytic capacity (J) of DC. Data demonstrated in units of extracellular acidification rate (ECAR). (**P<0.01, ***P<0.001). Lactate production (K) and OXPHOS activities (L) of inactivated and AGE & LPS-activated BMDC in 5.5-, 11-, and 25-mM glucose. (ns = not significant, *P<0.05).

Figure 4.

A B C





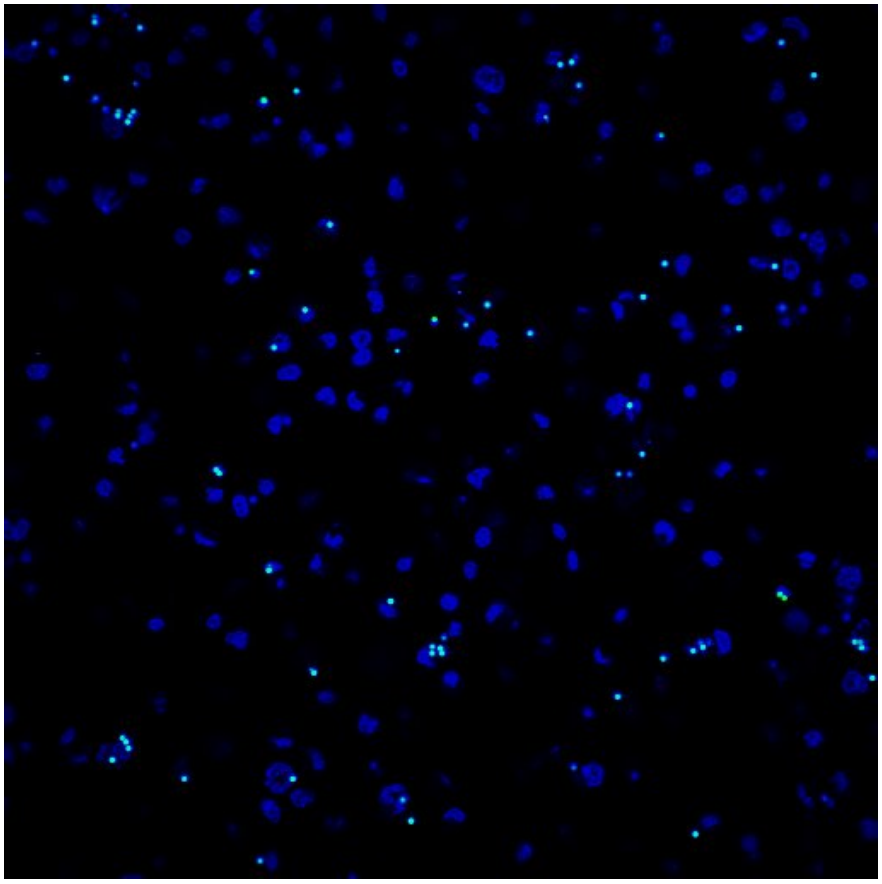


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Figure 4. Immunofluorescence image of phagocytosed Fluoresbrite carboxylate microspheres in inactivated BMDC in 5.5-(A), 11- (B) and 25 mM glucose (C). The cell nucleus was stained with Hoechst dye (blue). Images were captured at 10× magnification. (D) Quantified relative ratio between fluorescent particle and Hoechst stains. Values were normalized to ratio in 11 mM glucose culture. (***P<0.001).

Figure 5.

A B C

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Figure 5. Cytokine profile of pro-inflammatory TNF-α, IL-1β, and anti-inflammatory IL-10 secreted from inactivated and AGE & LPS-activated BMDC (A – C) and human monocyte differentiated-mDC (D – F) in 5.5-, 11-, and 25-mM glucose. Cytokine concentrations were quantified using ELISA. (ns = not significant, *P<0.05, ***P<0.001).

Figure 6.

A B C

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Figure 6. Cytokine profile of IFN-γ (A), IL-6 (B), and IL-17 (C) secreted from splenic T cells primed by inactivated and AGE & LPS-activated BMDC in 5.5-, 11-, and 25-mM glucose. Cytokine concentrations were quantified using ELISA. (ns = not significant, *P<0.05, ***P<0.001).

Table 1. Primers used in RT-PCR experiments for BMDC.

Oligonucleotides

Primers used for qPCR	Primers used for qPCR	Source	Reference
HK2	Forward primer CTTACCGTCTGGCTGACCAACAC Reverse primer CTCCATTTCCACCTTCATCCTTCT	ThermoFisher Scientific	(68)
LDHA	Forward primer TGCGTGCTGGAGCCACT Reverse primer GCGAGGAGAAGCAGCGTG	ThermoFisher Scientific	(69)
GLUT1	Forward primer GGCCTGACTACTGGCTTTGT Reverse primer TGCATTGCCCATGATGGAGT	ThermoFisher Scientific	(70)
β-actin	Forward primer CGTGCGTGACATCAAAGAGAA Reverse primer TGGATGCCACAGGATTCCAT	ThermoFisher Scientific	(71)
Oligonucleotides	Oligonucleotides	Oligonucleotides	Oligonucleotides
Primers used for qPCR	Primers used for qPCR	Source	Reference
CD80	Forward primer ACCCCCAACATAACTGAGTCT Reverse primer TTCCAACCAAGAGAAGCGAGG	ThermoFisher Scientific	(72)
CD83	Forward primer CGCAGCTCTCCTATGCAGTG Reverse primer GTGTTTTGGATCGTCAGGGAATA	ThermoFisher Scientific	(72)
CD86	Forward primer CTGGACTCTACGACTTCACAATG Reverse primer AGTTGGCGATCACTGACAGTT	ThermoFisher Scientific	(72)
MHC-II	Forward primer AGCCCCATCACACTGTGGAGT Reverse primer GATGCCGCTCAACATCTTGC	ThermoFisher Scientific	(73)

Table 2. Primers used in RT-PCR experiments for human monocyte differentiated-DC.

Oligonucleotides

Primers used for qPCR	Primers used for qPCR	Source	Reference
HK2	Forward primer GAGTTTGACCTGGATGTGGTTGC Reverse primer CCTCCATGTAGCAGGCATTGCT	ThermoFisher Scientific	(74)
LDHA	Forward primer ATGGCAACTCTAAAGGATCAGC Reverse primer CCAACCCCAACAACTGTAATCT	ThermoFisher Scientific	(75)
GLUT1	Forward primer CTGCTCATCAACCGCAAC	ThermoFisher Scientific	(76)

	Reverse primer CTTCTTCTCCCGCATCATCT		
β-actin	Forward primer CGGGACCTGACTGACTAC	ThermoFisher Scientific	(77)
	Reverse primer GAAGGAAGGCTGGAAGAG		
Oligonucleotides	Oligonucleotides	Oligonucleotides	Oligonucleotides
Primers used for qPCR	Primers used for qPCR	Source	Reference
CD80	Forward primer CTCTTGGTGCTGGCTGGTCTTT	ThermoFisher Scientific	(78)
	Reverse primer GCCAGTAGATGCGAGTTTGTGC		
CD83	Forward primer TACAGAGCGGAGATTGTCCTGC	ThermoFisher Scientific	(79)
	Reverse primer GCTCGTTCCATGCCAGCTTTAG		
CD86	Forward primer CCATCAGCTTGTCTGTTTCATTCC	ThermoFisher Scientific	(80)
	Reverse primer GCTGTAATCCAAGGAATGTGGTC		
HLA-DR	Forward primer GTCTGGCGGCTTGAAGAATT	ThermoFisher Scientific	(81)
	Reverse primer ACCTTGAGCCTCAAAGCTGG		

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