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Enhancing Immune Tolerance and Insulin Secretion of Mesenchymal Stem Cell-Derived Insulin-Producing cells via Interferon-γ and Tumor Necrosis Factor-α Priming

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Abstract

Background: Mesenchymal stem cells (MSCs) have the potential to treat type 1 diabetes mellitus (T1DM) by differentiating into insulin-producing cells (IPCs). However, various immunological factors were limiting the therapeutic efficacy of transplanted MSC-derived IPCs. Preconditioning MSCs with proinflammatory cytokines was proven to enhance their immunosuppressive capabilities. Yet, it remains unclear how cytokine stimulation influences the immunoregulatory features of theses differentiated IPCs. Therefore, we aimed to investigate the potential role of tumor necrosis factor-alpha (TNF-α) and/or interferon-gamma (IFN-γ)–primed MSC-derived IPCs in inducing and regulating immunological tolerance.

Methods: MSCs were isolated from human adipose tissue **(**hAT-MSCs) and differentiated into IPCs. Subsequently, the cells were assigned into five groups as follows: the control (hAT-MSCs) group, the differentiated (IPCs) group, the differentiated cells primed with IFN-γ (IPCs+ IFN-γ group), the differentiated cells primed with TNF-α (IPCs+ TNF-α group), the differentiated cells primed with both cytokines (IPCs+ Mix group). Flow cytometry, reverse transcription polymerase chain reaction (RT-PCR), and enzyme-linked immunosorbent assay (ELISA) were employed to detect the expression of immunomodulators, insulin/c-peptide, and pancreatic gene expression.

Results: The results indicated that IPCs treated with both cytokines exhibited the highest expression of insulin/c-peptide and pancreatic genes, as well as the immune checkpoints programmed death ligand 1 (PD-L1) and kynurenine (KYN), which are immunosuppressants. Furthermore, IFN-γ priming also enhanced the expression of the immunological regulators: human leukocyte antigen class 1 (HLA-ABC) and class 2 (HLA-DR).

Conclusions: Preconditioning IPCs with IFN-γ/TNF-α enhances their immunomodulatory profile through checkpoint molecules and insulin secretion, suggesting a novel immunological strategy for treating T1DM.

Keywords: Cytokine Priming, Immunotolerance, Insulin-Producing Cells, Interferon-gamma (IFN-γ), Tumor necrosis factor-alpha (TNF-α).

Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disorder that results from a disruption in the equilibrium between inflammatory and regulatory T cells (Tregs). It is characterized by the immunological destruction of insulin-producing β-cells in the

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pancreatic islets of Langerhans, leading to hyperglycemia (1). While exogenous insulin administration can clinically manage hyperglycemia, it does not either prevent acute or chronic complications of the disease. Pancreas or islet transplantation offers an alternative treatment approach but is limited by the scarcity of donor tissue and the need for lifelong immunosuppression (2).

Mesenchymal stem cell (MSC) therapy has emerged as a hopeful approach for treating T1DM by promoting β-cell regeneration via differentiation, immunomodulation, and paracrine signaling. These cells can modulate innate and adaptive immune responses through various mechanisms (3). MSCs secrete anti-inflammatory cytokines and immunomodulatory substances that suppress effector T-cell activity while promoting Treg development. They exhibit low expression of major histocompatibility complex (MHC) class I and lack costimulatory molecules and MHC class II, helping them to elude immune recognition (3). Additionally, they can suppress the activity of dendritic cells (DCs) and natural killer cells (NK), therby protect islet grafts and reduce hyperglycemia in T1DM animal models (3).

Practically, this effective therapy faces numerous challenges, including sensitivity to the immune-mediated disease environment, cell senescence after expansion, low long-term survival post-transplantation, transient glycemic control, immune rejection, and tumorigenic potential (4). Therefore, enhancing MSCs' therapeutic effectiveness and immunomodulation is crucial, and preconditioning MSCs with various stimuli has proven effective in improving their immunomodulatory ability. Preconditioning with cytokines, growth factors, or physical/chemical factors upregulates protective gene expression and protein secretion, improving MSCs' potency (5,6).

Notably, interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) cytokines are commonly used, as they represent early proinflammatory signals in inflammation/autoimmunity, inducing the immunosuppressive activity of MSCs. It was previously documented that IFN-γ can upregulate the secretion of several important immunosuppressive factors in MSCs, such as programmed death-ligand 1 (PD-L1) and indoleamine 2,3-dioxygenase (IDO) (7). PD-L1 is essential for suppressing inflammation by binding to its receptor (PD-1) on stimulated T-cells, leading to suppression of their activation, restoring the subpopulation balance of T cells, and inducing apoptosis (8). Additionally, these cytokines enhance the secretion of IDO, which converts tryptophan to kynurenine (KYN), suppressing different inflammatory responses by stimulating Treg cells and inhibiting T-cell proliferation (9).

Moreover, MSCs' potency is altered by differentiation induction, which downregulates apoptosis genes, shortens telomere length by decreasing the activity of telomerase, modifies MSCs' specific cell surface molecules, and reduces immunomodulatory effectiveness (10). The effect of proinflammatory cytokines on the immunomodulation of differentiated MSCs is unclear, with conflicting reports on whether differentiation induces immunogenicity or preserves immunomodulatory function (10,11). Therefore, our study aimed to examine how preconditioning insulin-producing cells (IPCs), derived from human adipose tissues MSCs (hAT-MSCs), with pro-inflammatory cytokines IFN-γ and/or TNF-α influences their immunomodulatory properties and insulin secretion. Understanding such interplay may provide insights towards developing regenerative medicine strategies for T1DM by protecting islet grafts from immune rejection and supporting clinical translation in the future.

Materials and Methods

Retrieval of human adipose tissue mesenchymal stem cells (hAT-MSCs); isolation and expansion

The necessary approvals for this study were obtained from the institutional research board (IRB) of Mansoura University under the MDP.21.02.57 code. Liposuction aspirates

were obtained from healthy nephrectomy donors for transplantation surgeries at the Urology and Nephrology Center, Mansoura University, Mansoura, Egypt, after they provided written informed consent, excluding of any other patients.

The isolation and culturing method of the collected adipose tissues were been performed according to Gabr *et al.* (12). Adipose tissue samples were digested for 45 minutes at 37 °C with light shaking using 0.075% collagenase type I (Sigma-Aldrich, USA). The digested samples were centrifuged at 200×g for 10 minutes at room temperature (RT). This centrifugation step was repeated twice. The pellets were then resuspended in low-glucose Dulbecco's Modified Eagle Medium (DMEM-LG, Sigma-Aldrich, USA) containing 10% fetal bovine serum (FBS, Sigma-Aldrich, USA) and plated in a 25 cm² tissue culture flask. Finally, the isolated cells were incubated at 37 °C in a 5% CO2 incubator. Every 3 days, the culture medium was changed, and cells were subcultured at 80% confluency using trypsin/EDTA (Sigma Aldrich, USA).

Flow cytometry verification of isolated hAT-MSCs

Following the third passage, approximately 1×10^6 cells were gathered and stained using anti-CD90, anti-CD105, anti-CD14, and anti-CD34 antibodies (BD Bioscience, USA) conjugated with FITC or PE to assess and determine whether the cultured cells were MSCs or not. The cells were analyzed using a FACS Calibur flow cytometer (Becton, Dickinson, USA) (13).

Differentiation of hAT-MSCs into insulin-producing cells (IPCs) and cytokine priming

The three-step differentiation protocol of Gabr *et al*. (14) was followed for the differentiation process. First, the cells were cultured in DMEM-LG containing 55 nM Trichostatin-A (TSA, Sigma-Aldrich, USA) for three days. Then, the cells were cultivated for a further eight days in a differentiation medium that included 10% FBS, 25 mM high glucose medium (DMEM: DMED/F12 at a ratio of 1:1), 10 nM glucagon-like peptide-1 (Sigma-Aldrich, USA), and 10 nM nicotinamide (Sigma-Aldrich, USA).

On the twelfth day of differentiation, the cells were divided into 4 groups (10×10^6 cells each) beside the control group (hAT-MSCs), which was undifferentiated hAT-MSCs as follows:

1) Differentiated cells (IPCs) group: IPCs without any additions.

2) IPCs + IFN-ɣ group: differentiated hAT-MSCs primed with 10 ng/ml recombinant human IFN-γ protein active (Abcam ab9659, UK) (15).

3) IPCs + TNF-α group: differentiated hAT-MSCs primed with 10 ng/ml TNF-α protein (Abcam ab9642, UK) (16).

4) IPCs+mix group: differentiated hAT-MSCs preconditioned with a combination of 10 ng/ml IFN-ɣ and 10 ng/ml TNF-α.

The priming cytokines were added to the differentiation media for 2 hours. Then, cells were washed with phosphate buffered saline (PBS, Sigma-Aldrich, USA) before culturing in the differentiation media. The cytokine pulse stimulation was repeated three times, with PBS washing and 24 hours of resting time in the differentiation medium in a CO2 incubator. Following the final cytokine pulse, cells were grown in the differentiation media provided with 1µl of 100 ml-1 PRDX-6 protein (R&D Systems, USA) and 0.1 nmol of Exendin-4 (Tocris Bioscience, UK) and rested in the incubator for 6 days prior to their assessment.

After finishing the differentiation procedure, immunocytochemistry was used to evaluate the differentiated cells. Also, the percent of cells expressing c-peptide and insulin was measured by flow cytometry, and RT-PCR was utilized to assess the expression levels of the pertinent pancreatic endocrine genes.

Assessment of the primed cell population
RT-qPCR for gene expression

By using the Direct-zol™ RNA miniprep kit (Zymo Research, California, USA), total RNA was extracted from the cells in each group

according to the manufacturer's instructions. The extracted RNA's concentration was quantified by a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). cDNA was synthesized using the RT² First Strand Kit (Qiagen Sciences, Germantown, MD, USA).

Reverse transcription was used to create first-strand cDNA from 3 µg of the collected RNA. The website of the National Centre for Biotechnology Information was used to design primers (Table 1). This investigation assessed the pancreatic endocrine genes' expression. The following genes were analyzed: the pancreatic endocrine hormones insulin, glucagon (GCG), and somatostatin (SST); the glucose transporter member 2 (GLUT-2); and the relevant transcription factors: pancreatic and duodenal homeobox 1 (PDX1), neurogenic differentiation factor 1 (Neurod1), and v-Maf musculoaponeurotic fibrosarcoma oncogene homologue A and B (MAFA & MAFB). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalization/internal control to calculate the relative gene expression (17).

Quantitative real-time PCR (qRT-PCR) was carried out to determine the expression levels of genes. Reactions were set up in 20 µL volumes with 2 µL of reverse and forward primers (5 nmol each), 10 µL of Maxima SYBR Green Master Mix (Thermo Fisher Scientific), 7 µL of nuclease-free water, and 1 µL (100 nmol) of cDNA template. Amplification was done by using the CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA). The thermocycling protocol involved an initial denaturation at 95 °C for 3 minutes, followed by 40 cycles of 15 seconds at 95 °C, 30 seconds at 60 °C, and 30 seconds at 72 °C. To ensure reproducibility, each sample was run in triplicate.The Pfaffl method (18) was used to calculate the relative gene expression, which was then normalized to undifferentiated hAT-MSCs. This approach allows for direct comparison of expression levels between target and reference genes within each sample.

Table 1. RT-qPCR primers specific to human genes.

Primers	Forward	Reverse	Accession no.
Endocrine Genes			
Insulin	GCCTTTGTGAACCAACACCT	CGGGTCTTGGGTGTGTAGAA	NM 000207.3
GCG	ATTTCCCAGAAGAGGTCGCC	TATAAAGTCCCTGGCGGCAA	NM 002054.5
SST	CCAACCAGACGGAGAATGAT	CCATAGCCGGGTTTGAGTTA	NM 001084.4
Glucose Transporter			
GLUT-2	TTGGGCTGAGGAAGAGACTG	CACCAACTGCAAAGCTGGAT	XM 01151388.1
Transcription Factors			
PDX-1	GAGCTGGCTGTCATGTTGAA	CGCTTCTTGTCCTCCTCCTT	NM 000209.4
Neurod1	TACATCTGGGCTCTGTCGGA	CCCTTGCAAAGCGTCTGAAC	NM 002500.4
MafA	AGAGCGAGAAGTGCCAACTC	TTGTACAGGTCCCGCTCTTT	NM201589.4
MafB	GGTATAAACGCGTCCAGCAG	CTGCTTAAGCTGCTCCACCT	NM005461.5
Internal Control			
GAPDH	ACCCAGAAGACTGTGGATGG	GAGGCAGGGATGATGTTCTG	NM 001289746.2

GCG: glucagon; SST: somatostatin; GLUT-2: glucose transporter member 2; PDX1: pancreatic and duodenal homeobox 1; Neurod1: neurogenic differentiation factor 1; Maf.A: v-Maf musculoaponeurotic fibrosarcoma oncogene homologue A; Maf.B: v-Maf musculoaponeurotic fibrosarcoma oncogene homologe B; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Flow cytometry to assess secreted hormones and immunological checkpoint molecules required for IPC-induced cell immunomodulation

At the end of the study, the cells of all groups were collected and washed with PBS. Following the manufacturer's guidelines, cells were fixed and permeabilized for intracellular staining with the BD Cytofix/Cytoperm kit (BD Biosciences, USA). After that, the BD Stemflow hMSC Analysis kit (BD Biosciences, USA) was utilized to stain the cells for 30 minutes with PE- and APC-conjugated anti-C peptide and anti-insulin antibodies.

For surface markers, cells were treated for 30 minutes at 4 °C with FITC-conjugated anti-programmed death-ligand 1 (PD-L1), APC-conjugated anti-human leukocyte antigen Class-I (HLA-ABC), and PerCP-Cy5.5-conjugated anti-human leukocyte antigen class-II (HLA-DR) antibodies from the BD Stemflow hMSC Analysis kit.

Stained cells were acquired on a FACS ARIA III cell sorter (Becton, Dickinson) at a wavelength of 488 nm. 20,000 collected per sample. Negative controls included undifferentiated cells that were stained and unstained, as well as differentiated cells that were treated without the primary antibody. Data were analyzed using FlowJo software (17).

Immunocytochemistry of primed and control MSC-derived IPCs

Cells from each group were cultivated on 2-chamber slides (Nunc, Rochester, NY, USA) and fixed at RT for 10 minutes with 4% paraformaldehyde. They were then permeabilized for 10 minutes with 100% chilled ethanol, blocked for 1 hour at RT with 5% goat serum, (Sigma-Aldrich, USA) and incubated with the primary antibodies overnight at 4 °C. Afterward, they underwent a PBS wash and a 2-hour RT incubation with the secondary antibodies. DAPI was used as a counterstain for nuclei (Cell Signaling Technology, Danvers, MA, USA). The primary antibody was omitted in the negative controls. The Leica TCS SP8 microscope (Leica Microsystems, Mannheim, Germany) was acquired using to take confocal images (17).

Estimation of different cytokine expression by enzyme-linked immunosorbent assay (ELISA)

At the end of the differentiation phase, the supernatant medium from all groups was collected and used to detect specific human cytokines using sandwich ELISA kits in accordance with manufacturer's directions. The cytokines detected included PD-L1 (R&D, USA) and kynurenine (KYN) (Abbexa, USA) (19).

Statistical Analysis

Statistical analysis was carried out using version 20 of SPSS. Results are reported as medians with interquartile ranges (IQR). The Kruskal-Wallis test was used for multiple comparisons because the data were nonparametric. A $p\text{-value} \leq 0.05$ was defined as statistically significant.

Results

The phenotypic characteristics of hAT-MSCs

Following the completion of the expansion process, the adhering cells revealed the typical spindle-shaped morphology of MSCs. The surface markers of MSCs, CD90 (93.31%) and CD105 (80.94%) were positively expressed in the collected cells; in contrast, the markers of hematopoietic stem cells (HSCs), CD14 (0.7%) (Fig. 2C), and CD34 (0.2%), were not expressed.

Functional assessment of differentiated cells Immunofluorescence

Immunocytochemistry results indicated that the differentiated IPCs can release insulin and c-peptide **(**Figs. 1A & B), whereas the undifferentiated hAT-MSCs did not. Furthermore, both C-peptide and insulin were expressed in the same cells (Fig. 1C).

Flow cytometry quantitation of the secreted insulin and C-peptide by IPCs

Insulin was detected in approximately 15.7% of the cells, while C-peptide was found in around 11.2% of the cells (Fig. 2).

***Gene* expression by RT-PCR**

The RT-PCR results of the IPCs group confirmed the expression of essential pancreatic endocrine genes, including transcription factors, hormones, enzymes, and transporters (Fig. 3).

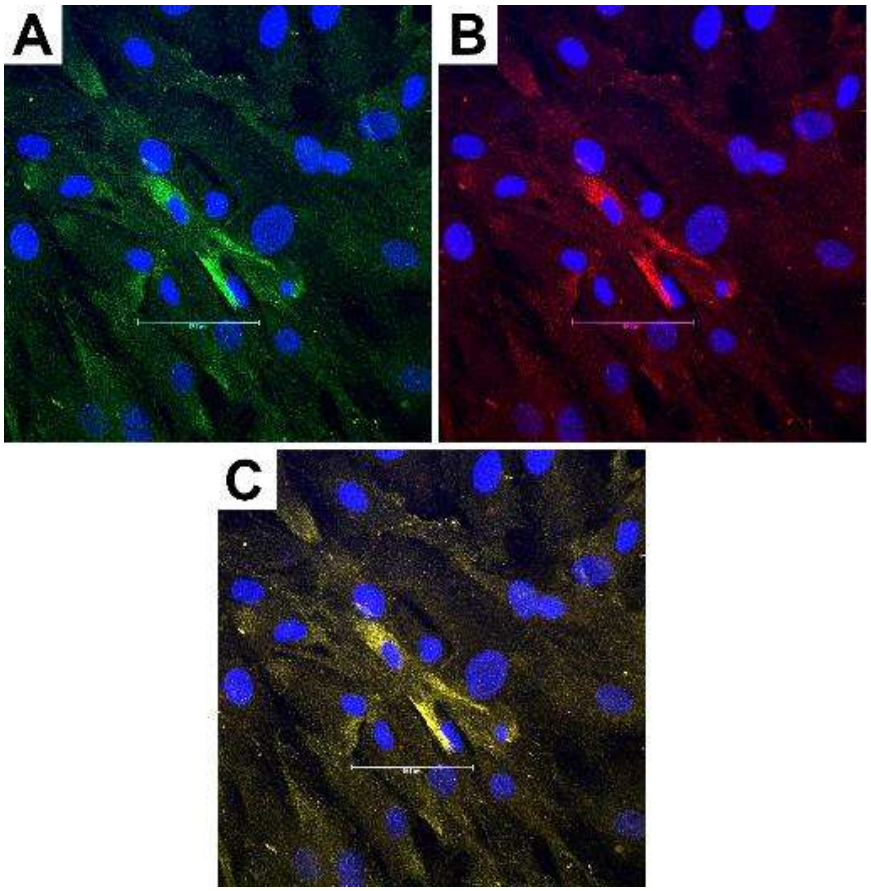


Fig. 1. Immunocytochemistry of IPCs group. (A) Granule-positive cells expressing insulin (green), (B) Granule-positive cells expressing c-peptide (red), (C) simultaneous expression of both hormones in the same cells (yellow). DAPI was used to stain nuclei (blue).

Impact of IFN-ɣ and TNF-α cytokines on immunomodulatory features of IPCs

Flow cytometry

The results revealed both significant and non-significant differences in marker expression between groups. Specifically, while there were no statistical differences between the hAT-MSCs and IPCs groups for any markers measured (P> 0.05), the IPCs group showed a significant increase in insulin, C-peptide levels as well as slight increases in HLA-ABC and HLA-DR expression, with decrease in PD-L1 compared to the hAT-MSCs group (Fig. 2).

In contrast, the IPCs+Mix group exhibited significantly increased expression of all markers relative to the hAT-MSCs group (P< 0.01). Similarly, the IPCs+IFN-ɣ group had significantly higher levels of insulin, C-peptide, HLA-ABC, (P< 0.01) and HLA-DR (P< 0.05) than the hAT-MSCs group, despite nonsignificant increases in PD-L1. Moreover, the IPCs+Mix group showed a marked increase in insulin expression compared to IPCs+TNF-α (P< 0.01). Also, HLA-DR showed significantly greater increase in IPCs+IFN-ɣ group than in IPCs+TNF-α (P< 0.05). (Fig. 2).

Notably, PD-L1 (P< 0.05) and HLA-ABC (P< 0.01) were significantly higher in the IPCs+Mix and IPCs+IFN-ɣ groups versus the IPCs group (Fig. 2). Overall, these results indicate that certain pretreatments may enhance the immunomodulatory profile of differentiated IPCs, with potential implications for improving islet graft survival.

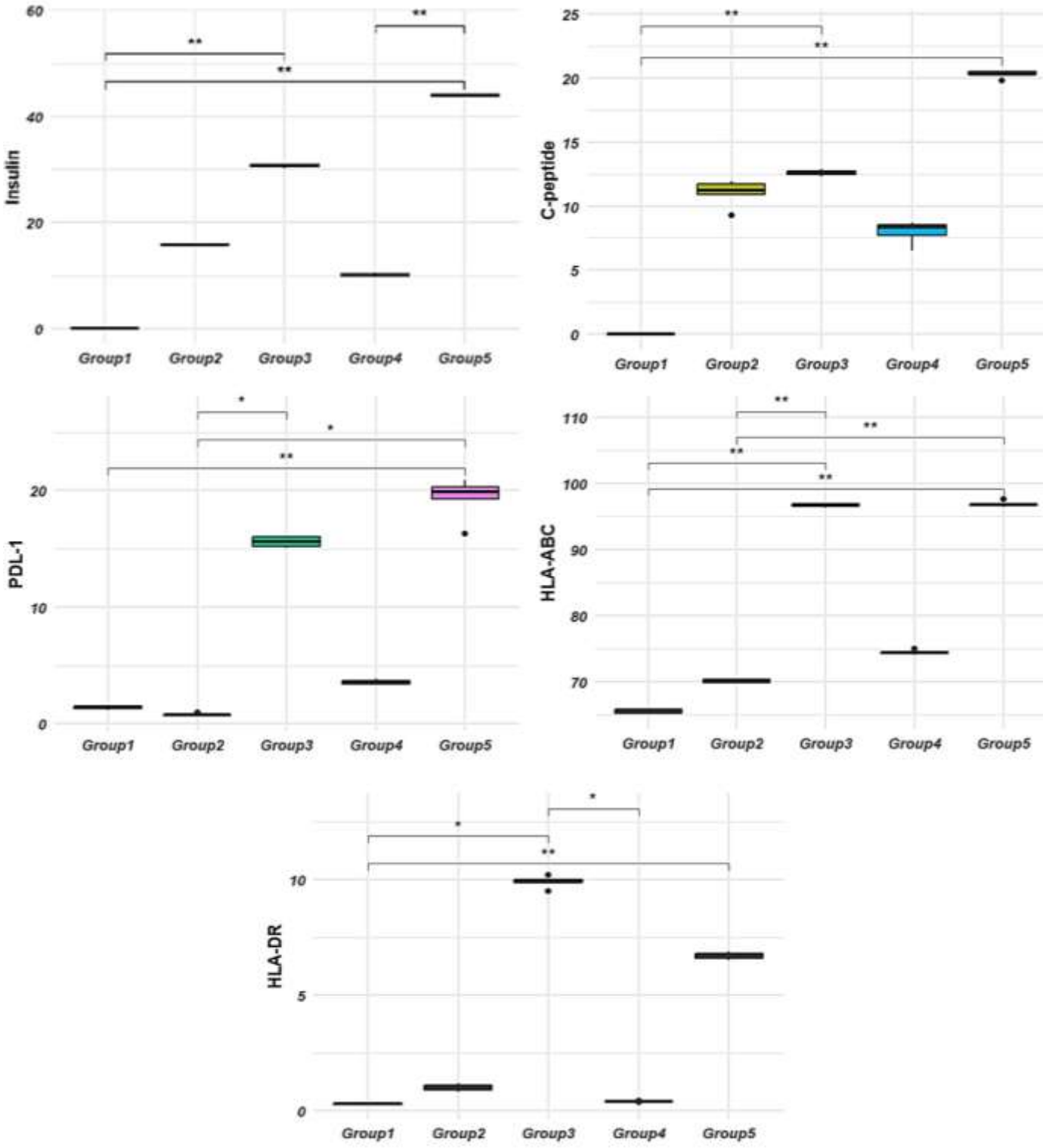
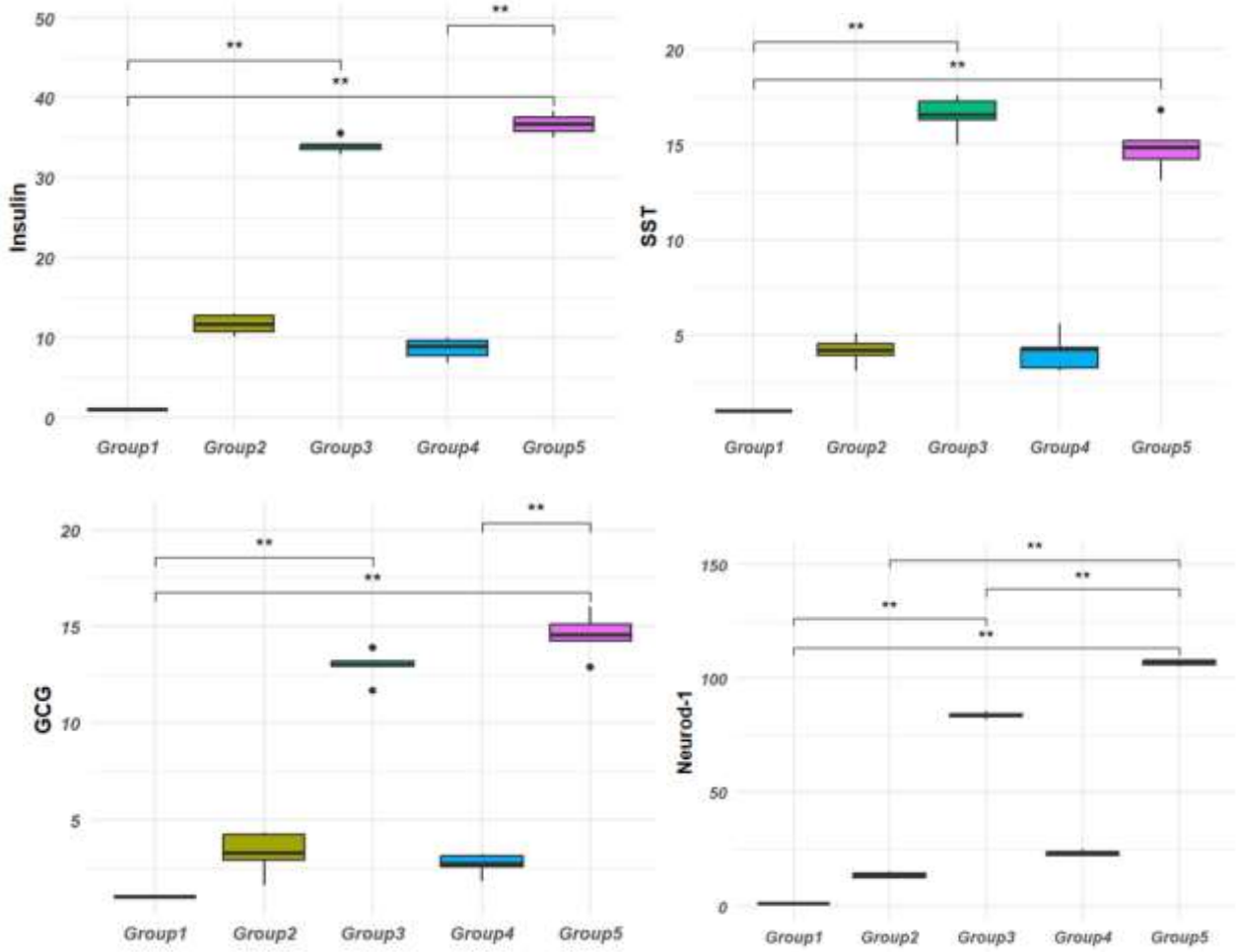


Fig. 2. Boxplots of quantitative expression of immune checkpoint molecules involved in cell immunomodulation and hormone-positive cells by flow cytometry. The results are presented as median (iqr) (n = 6). The *, and ** indicate P< 0.05, P< 0.01, respectively. PD-L1: programmed death ligand-1; HLA-ABC: human leukocyte antigen class 1; HLA-DR: human leukocyte antigen class 2; Group 1: hAT-MSCs group; Group 2: IPCs group; Group 3: IPCs+IFN-ɣ group; Group 4: IPCs+TNF-α group; Group 5: IPCs+Mix group.

To find out how cytokines affected the expression of the pancreatic endocrine genes, RT-PCR was performed at the end of the experiment. The results showed that all primed groups expressed all relevant genes. The IPCs+Mix group and IPCs+IFN-ɣ group showed a marked increase in the expression of all genes compared to the hAT-MSCs group (P< 0.01). All genes in the IPCs+Mix group had statistically significant increases in expression levels compared to the IPCs+TNF-α group (P< 0.01), except for SST and MAFB. Additionally, the IPCs+ IFN-ɣ group showed a marked increase in the expression level of MAFB compared to the IPCs+TNF-α group (P< 0.01) (Fig. 3).

Conversely, there was no statistical significance between the IPCs+IFN-ɣ group and the IPCs group, the IPCs group and the IPCs+TNF-α group, the IPCs+Mix group and the IPCs+IFN-ɣ groups (P> 0.05). With the exception of the SST and Neurod1 genes, all genes showed the lowest expression levels in the IPCs+TNF- α group (Fig. 3).



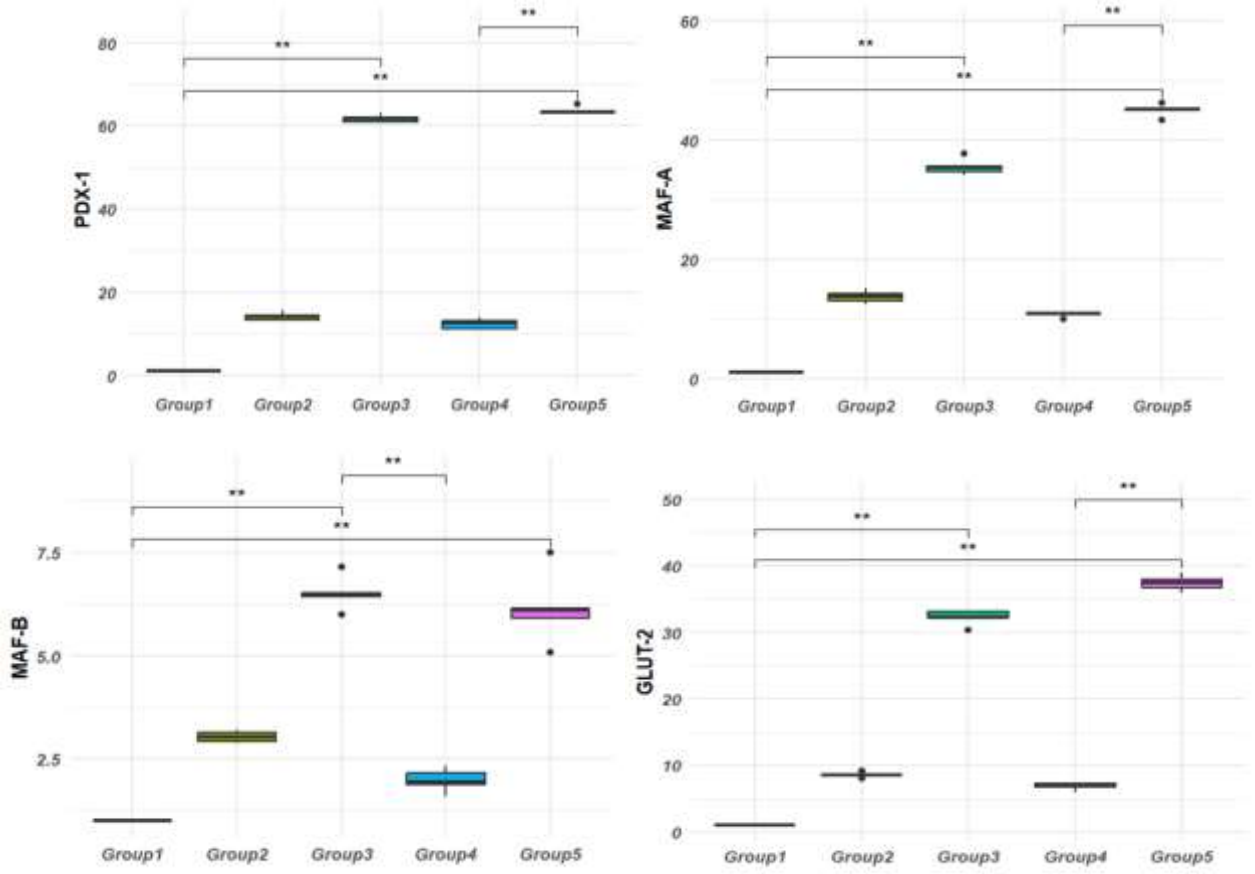


Fig. 3. Boxplots of relative gene expression of the differentiated and primed groups compared to the control group. The assessment of mRNA levels was done using qRT-PCR. GAPDH was used as an internal control (n=6). The results are presented as median (IQR) (n = 6). The *, and **, indicate P< 0.05, P< 0.01, respectively. GCG: glucagon; SST: somatostatin; GLUT-2: glucose transporter member 2; PDX1: pancreatic and duodenal homeobox 1; Neurod1: neurogenic differentiation factor 1; Maf.A: v-Maf musculoaponeurotic fibrosarcoma oncogene homologue A; Maf.B: v-Maf musculoaponeurotic fibrosarcoma oncogene homologue B; Group 1: hAT-MSCs group; Group 2: IPCs group; Group 3: IPCs+IFN-ɣ group; Group 4: IPCs+TNF-α group; Group 5: IPCs+Mix group.

ELISA

ELISA results illustrated that the concentration of PD-L1 sehowed a statistically significant increase in the IPCs+Mix group compared to both the control and IPCs groups (P< 0.01). Likewise, it was significantly higher in the IPCs+IFN-ɣ group than in the IPCs group (P< 0.05) (Fig. 4A). Furthermore, when comparing the IPCs group to the IPCs+Mix and IPCs+IFN-ɣ groups, there was a statistically significant increase in the KYN concentration (P< 0.05) (Fig. 4B).

Effect of IFN-ɣ and TNF-α cytokines on the secretion of insulin and C-peptide by immunocytochemistry

Immunofluorescence results showed that all the differentiated groups demonstrated positivity for insulin and c-peptide. The IPCs+Mix group (Figs. 5A-C) showed the highest number of positive cells, while the IPCs+ TNF-α group (Figs. 5D-F) showed the lowest number of positive cells compared to other cells. Additionally, the number of positive cells was markedly higher in the IPCs+ IFN-γ group (Fig. 10) than in the IPCs+ TNF-α group (Figs. 5G-I) and IPCs group (Fig. 1).

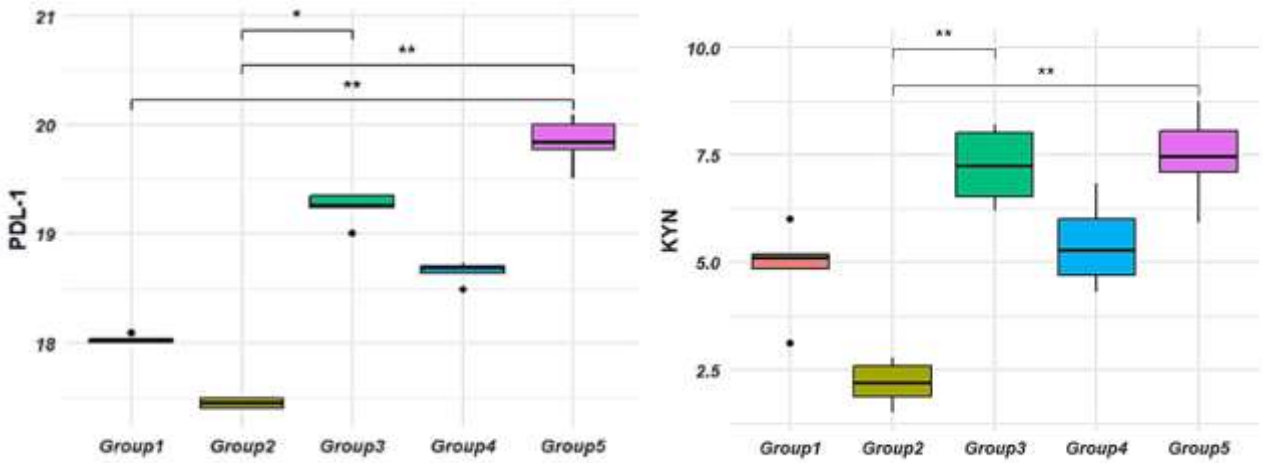


Fig. 4. Boxplot of the quantitative expression of PD-L1 and KYN using ELISA technique. The results are presented as median (iqr) (n = 6). The *, and ** indicate P< 0.05 and P< 0.01, respectively. PD-L1: programmed death ligand-1; KYN: kynurenine; Group 1: hAT-MSCs group; Group 2: IPCs group; Group 3: IPCs+IFN-ɣ group; Group 4: IPCs+TNF-α group; Group 5: IPCs+Mix group.

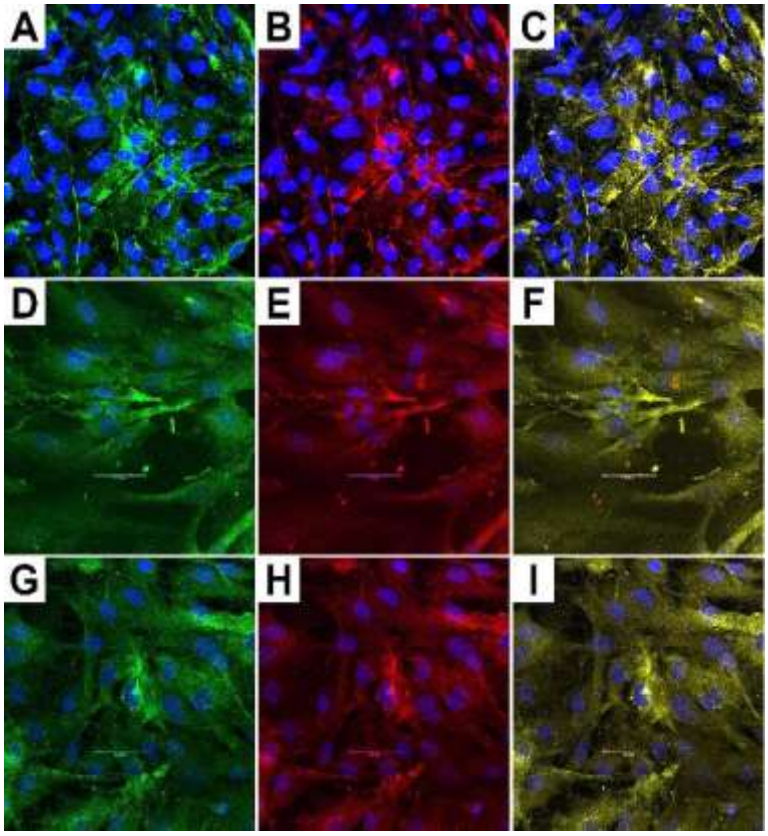


Fig. 5. Immunocytochemical staining of the three differentiated treated group. Immunocytochemistry for the IPCs+Mix group: (A) Granule-positive cells expressing insulin (green), (B) Granule-positive cells expressing c-peptide (red), (C) simultaneous expression of both hormones in the same cells (yellow). Immunocytochemistry for the IPCs+TNFα group: (D) Granule-positive cells expressing insulin (green), (E) Granule-positive cells expressing c-peptide (red), (F) simultaneous expression of both hormones in the same cells (yellow). Immunocytochemistry for the IPCs+INFγ group. (G) Granule-positive cells expressing insulin (green), (H) Granule-positive cells expressing c-peptide (red), (I) simultaneous expression of both hormones in the same cells (yellow). DAPI was used to stain nuclei (blue)**.** IFN-ɣ: Interferon-gamma; TNF-α: Tumor necrosis factor-alpha; IPCs+IFN-ɣ group: differentiated cells primed with IFN-ɣ group; IPCs+TNF-α group: differentiated cells primed with TNF-α group; IPCs+Mix group: differentiated cells primed with both cytokine groups.

Discussion

In this study, we hypothesized that priming IPCs differentiated from hAT-MSCs with IFN-γ and/or TNF-α would enhance their immunoregulatory capabilities by interacting with immune cells and induce insulin/C-peptide expression profiles, which could provide a novel and effective strategy for managing T1DM. Although different studies have documented that both MSCs and IPCs can modulate the immune system and potentially arrest autoimmune attacks in T1DM, the hostile inflammatory microenvironment in T1DM poses challenges to MSC survival and function. Consequently, there is current interest is enhancing the immunomodulatory properties of MSCs and IPCs (20).

Here, we isolated MSCs from human adipose tissue; they are considered an abundant source of MSCs (~5000 cells/ 1 ml aspirate) with high differentiation capacity and availability through surgical procedures (21). Additionally, their strong inhibitory effect on T-cell proliferation is well documented (17). The isolated cells exhibited typical fibroblast-like morphology and expressed MSC surface markers CD90 and CD105, but not HSC markers CD14 and CD34. This confirms their mesenchymal identity, consistent with the criteria set by the International Society for Cellular Therapy for MSCs (22). After that, these cells were successfully differentiated into functional IPCs capable of insulin and C-peptide secretion and expression of other pancreatic endocrine genes, which was validated by Immunocytochemistry, flow cytometry, and RT-qPCR analysis. This differentiation underscores the potential for cell-based therapies for diabetes, bolstered by prior studies that utilized various protocols involving pancreatic transcription factors and nicotinamide. This study supports the validity of the approach taken (23, 24).

Recently, researchers documented that priming MSCs with proinflammatory cytokines like interleukin-1 (IL-1), IFN-γ, and TNF-α enhances their immunomodulatory effects, but it is still unknown how differentiation affects MSC immunomodulation, with conflicting research findings (25).

Undifferentiated MSCs express MHC-I (HLA-ABC), but not MHC-II (HLA-DR) or costimulatory molecules like CD40, CD80, and CD86 that are critical for T-cell activation (26). While MHC-I presents endogenous antigens to CD8+ cytotoxic T cells, MHC-II and co-stimulation are necessary for antigen-presenting cells (APCs) to activate CD4+ T-helper cells (27). Several studies have documented that priming undifferentiated and differentiated MSCs with TNF-α and IFN-γ cytokines induces upregulation of both HLA-ABC and HLA-DR without triggering T-cells responses, preserving immunosuppressive function due to lack of costimulatory molecules (28, 29). Van-Megen, et al. reported that IFN-γ allows MSCs to process antigens and upregulate immunosuppressive checkpoints, transforming them into non-conventional APCs useful for immune modulation (28). IFN-γ promotes multilineage differentiation, whereas TNF-α inhibits it (30). Furthermore, IPCs derived from human umbilical cord MSCs highly express HLA-ABC while maintaining hypoimmunogenicity (11). Consistent with these reports, we observed elevated HLA-ABC and HLA-DR expression in the IPCs+ Mix and IPCs+ IFN-γ groups. However, TNF-α and IFN-γ failed to induce HLA-DR on chondrocytes derived from WJ-MSCs, which is attributed to their higher immunological characteristics compared to AT-MSCs and BM-MSCs (31).

IFN-γ and TNF-α are known inducers for PD-L1 expression in various cell types, including tumor and immune cells. PD-L1 is a cell surface protein that regulates immune responses by binding to its receptor (PD-1) on T cells, resulting in inhibition of T-cell activity and immune evasion, which is important in cancer, autoimmunity, and inflammation (32). Furthermore, PD-L1 has a critical role in MSC immunosuppression by reducing the release of inflammatory cytokines and T-cell activation and enhancing Treg production (25, 33, 34). Although the IPCs derived from MSCs express

PD-L1 and can temporarily protect transplanted IPCs from autoimmune damage, their immune protection is often lost, suggesting a need to understand and enhance their immunoregulatory function (35).

IFN-γ primarily induces PD-L1 expression in MSCs through stimulating the Janus kinase-signal transducer and activator of transcription (JAK-STAT1) signaling pathway and increasing the interferon regulatory factor 1 (IRF1) transcription factor binding to the PD-L1 gene promoter (36). TNF-α alone does not stimulate the expression of PD-L1 but enhances IFN-γ's effects by upregulating IFN-γ receptors via the activation of the nuclear factor kappa-B (NF-κB) signaling pathway, increasing JAK/STAT1/IRF1 activity (36). As a result, different studies have documented that combined IFN-γ and TNF-α stimulation markedly increases PD-L1 expression, promoting the immunosuppressive effect of MSCs (7, 36-38). This mechanism was in harmony with our flow cytometry and ELISA data, which demonstrated a marked elevation of PD-L1 expression in the IPCs group treated with both cytokines, highlighting the potential of modulating PD-L1 to improve the protection of transplanted cells in TIDM.

While TNF-α and IFN-γ are generally known to impair insulin secretion via inducing free radical formation and chronic inflammation that harm the function of β-cells (39), we observed elevated insulin, C-peptide, and pancreatic endocrine gene expression in IPCs treated with IFN-γ alone or in combination with TNF-α. In contrast, TNF-α alone decreased these markers, with the strongest effect observed. These findings may be attributed to the synergistic activation of the JAK/STAT pathway by IFN-γ and TNF-α, which leads to phosphorylation of STAT proteins that enter the nucleus to activate insulin secretion-related genes (40, 41). Activation of STAT1 and STAT3 in differentiated IPCs promotes glucose-stimulated insulin and C-peptide secretion. Beyond regulating the immune response, the JAK/STAT pathway stimulates anti-apoptotic mechanisms, cell cycle progression, and growth factor signaling, collectively enhancing insulin sensitivity and secretion (42). Thus, IFN-γ-driven JAK/STAT activation, alone or in combination with TNF-α optimizes IPCs' insulin secretory phenotype, which is crucial for T1DM.

Depending on the synergistic effect between TNF-α and IFN-γ, we found an increase in the secretion of KYN in the IPCs+Mix and IPCs+IFN-γ groups due to IFN-γ's ability to upregulate IDO expression, the key enzyme that converts tryptophan into KYN in the the KYN pathway. KYN and its downstream metabolites have immunosuppressive effects by suppressing immune cell proliferation and activation, such as T cells (43). IFN-γ stimulates the IDO expression in MSCs via the JAK-STAT1, NF-κB, and activator protein-1 (AP-1) signaling pathways, cooperating with IRF-1 more effectively than TNF-α alone, enhancing tryptophan catabolism and KYN production (7, 44). Our results were aligned with other previous studies showing that IFN-γ pretreatment improves KYN levels and immunomodulation of undifferentiated and differentiated MSCs (10, 45). Thus, IFN-γ, particularly in combination TNF-α, significantly induces the IDO-KYN pathway implicated in MSC-mediated immune modulation.

In conclusion, these findings demonstrated that combining IFN-γ and TNF-α preconditioning enhances the immunomodulation of IPCs derived from hAT-MSCs by upregulating immune checkpoint molecules and insulin/c-peptide expression. This optimized cell product holds promise for future T1DM cell therapy. However, it is crucial to consider the potential limitations and challenges associated with pre-treatment approaches. The optimal cytokine preconditioning concentration, timing, and duration must be carefully calculated to avoid potential adverse effects and maintain the desired immunomodulatory function. Moreover, the complex interplay between multiple signaling pathways and molecular factors involved in the regulation of immunosuppressive molecules, including costimulatory molecules, the JAK/STAT

pathway, and the NF-κB pathway, necessitates further investigation to fully understand the underlying mechanisms, including potential synergistic effects.

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Conflicts of Interest
The authors have no conflicts of interest to declare.

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Ethical Statement
The appropriate approvals for this study were obtained from the institutional research board (IRB) of Mansoura University under the MDP.21.02.57 code. Liposuction aspirates were obtained from healthy donors' nephrectomy during transplantation surgeries at the Urology and Nephrology Center, Mansoura University, Mansoura, Egypt, after they provided written informed consent with exclusion of any other patients.

Authors contribution
Mohga Mohamed Al-issawi: conceptualization, writing of original draft, methodology, investigation, formal analysis. Mahmoud Mohamed Gabr: conceptualization, methodology, data curation, validation, supervision. Sherry Mohamed Khater: methodology, investigation, validation. Abdel-Aziz Fatouh abdel–aziz: writing, review & editing, validation, supervision.

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