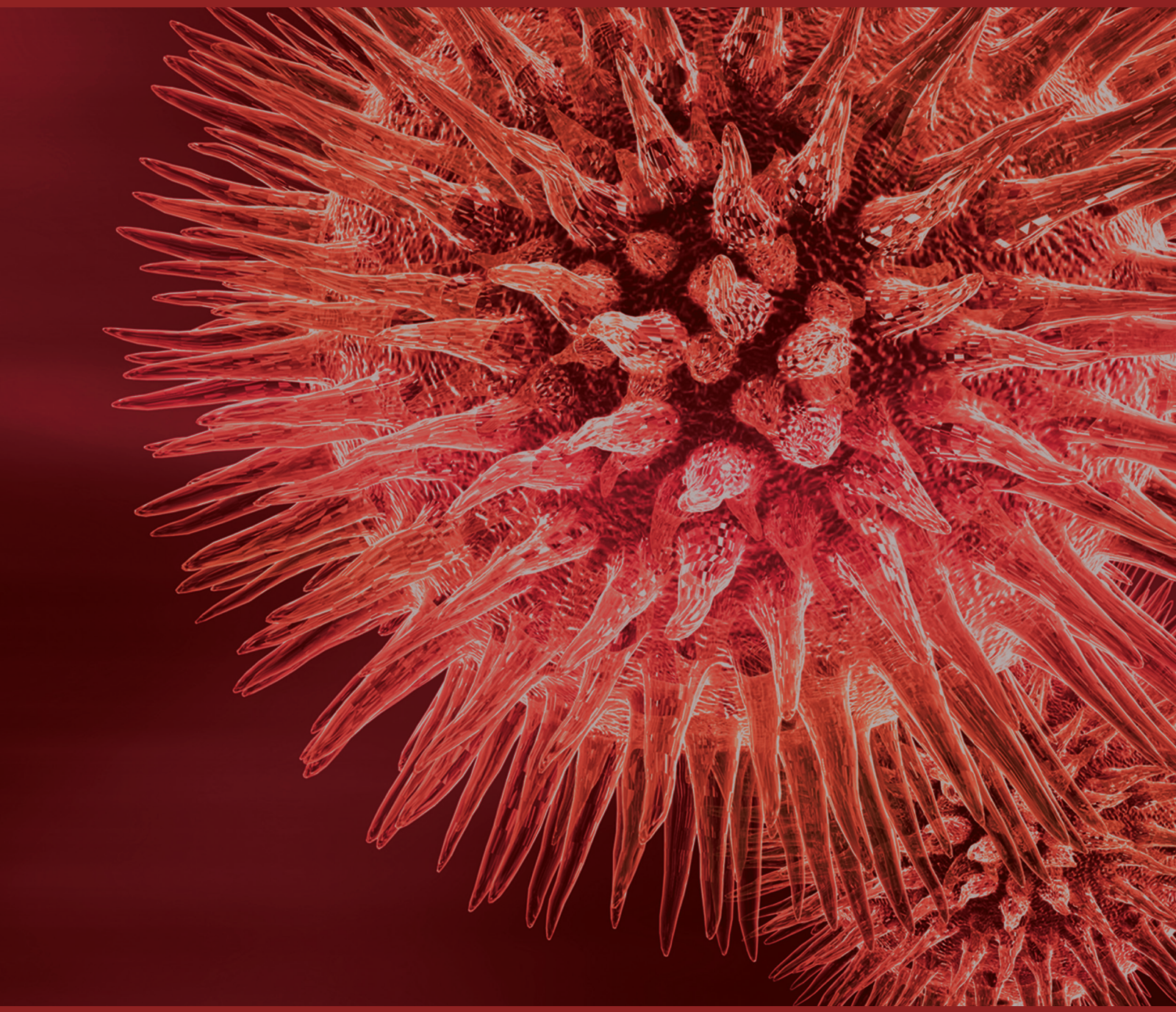
BioMed Research International

Interplay between the Endocrine System and Immune Cells

Guest Editors: Joilson O. Martins, Livia A. Carvalho, Jantje M. Gerdes, Carina Strell, and Graham R. Wallace



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Editorial

Interplay between the Endocrine System and Immune Cells

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Immunoendocrinology is an important field that studies the connection between the immune and endocrine systems. Hormones, including molecules like vitamin D and metabolic components, and neurotransmitters are compounds produced by different cell types that are capable of regulating the cross talk among cells from different tissues. During the last years, it became evident that hormones and neurotransmitters are specific modulators of cells of the immune system by fine-tuning their activation and key functions.

This special issue covers the most recent research elucidating how hormones are able to regulate the recruitment of immune cells to an inflammatory site as well as to explain the hormonal impact on the intracellular signaling cascades that follow after tissue injury. It will cover the mechanisms by which hormones can influence inflammatory process.

Diabetes mellitus is the most common cause of end-stage renal disease and chronic low-grade inflammation is an important factor in the pathogenesis of diabetic complication. Mycophenolate mofetil (MMF) has an anti-inflammatory effect, inhibiting lymphocyte proliferation. J.-W. Seo et al. study the effect of MMF on diabetic nephropathy and investigate its action mechanisms in type 2 diabetic mouse models. They demonstrated that MMF treatment attenuates diabetic nephropathy by decreasing CD4+ T cell infiltration and its related cytokines and chemokines. This anti-inflammatory effect of MMF attenuates podocyte apoptosis independent of glucose control.

MicroRNAs are small noncoding RNA molecules that regulate gene expression in all cell types, which are involved in a wide range of biological processes, exerting functional effects at cell, tissue, and organ levels. G. Ventriglia et al. summarize the most recent data on the potential involvement of microRNAs in autoimmune diabetes and indicate that miRNAs represent major players in the dialogue between beta cells and immune system, regulating several aspects of their function and survival.

Infection with the protozoan parasite *Trypanosoma cruzi* induces a hormonal systemic deregulation. A. F. Nardy et al. illustrated how the sphingosine-1-phosphate (S1P) system regulates both thymic and lymph node T cell egress which is essential for producing and maintaining the recycling T cell repertoire. The presence of this T cell receptor (TCR) bearing cells in the periphery may have potential implications for disease outcome. The authors discuss the possibility that the early egress of undifferentiated CD4−CD8− T cells plays a role in the immunopathologic events in Chagas disease by altering adaptive immune responses.

The current evidence of the role of liver dendritic cells (DC) in the progression from nonalcoholic fatty liver disease (NAFLD) to fibrosis is reviewed by P. Almeda-Valdes et al. Link between lipid metabolism and DC function suggests that immunogenic DC are associated with liver lipid storage, which might represent a possible pathophysiological mechanism in NAFLD.

Interactions between the pineal gland and the immune system have been studied ex vivo. A. P. Herman et al. provide evidence on how interleukin- (IL-) 1β suppresses melatonin secretion and its action seems to be targeted on the reduction of pineal gland arylalkylamine-N-acetyltransferase (AA-NAT) protein expression.

Varicocele is the most common cause of infertility in men and the development of varicocele-related testis damage may be caused by disruption of homeostasis between cell proliferation and cell death. In addition, apoptosis is a physiological process by which a sequence of intracellular events results in the programmed elimination of a cell from its environment. Specifically, alterations in the apoptosis of germ cells may be crucial in varicocele-related human infertility and, as a direct consequence, targeting apoptosis may represent an alternative and rational therapeutic strategy in the treatment of varicocele complications. L. Minutoli et al. studied the effect of Polydeoxyribonucleotide (PDRN), an agonist of adenosine A2A receptor, on testicular Neuronal Apoptosis Inhibitory Protein (NAIP) and survivin expression in an experimental model of varicocele. They found that PDRN may represent a rational therapeutic option for accelerating recovery from depressed testicular function through a strategic modulation of apoptosis in experimental varicocele.

Acknowledgments

We would like to thank the authors and reviewers for their valuable contributions to this special issue.

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Research Article

Mycophenolate Mofetil Ameliorates Diabetic Nephropathy in db/db Mice

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Chronic low-grade inflammation is an important factor in the pathogenesis of diabetic complication. Mycophenolate mofetil (MMF) has an anti-inflammatory effect, inhibiting lymphocyte proliferation. Previous studies showed attenuation of diabetic nephropathy with MMF, but the underlying mechanisms were unclear. This study aimed to identify the effect of MMF on diabetic nephropathy and investigate its action mechanisms in type 2 diabetic mice model. Eight-week-old db/db and control mice (db/m mice) received vehicle or MMF at a dose of 30 mg/kg/day for 12 weeks. MMF-treated diabetic mice showed decreased albuminuria, attenuated mesangial expansion, and profibrotic mRNA expressions despite the high glucose level. The number of infiltrated $CD4^+$ and $CD8^+$ T cells in the kidney was significantly decreased in MMF-treated db/db mice and it resulted in attenuating elevated intrarenal TNF-α and IL-17. The renal chemokines expression and macrophages infiltration were also attenuated by MMF treatment. The decreased expression of glomerular nephrin and WT1 was recovered with MMF treatment. MMF prevented the progression of diabetic nephropathy in db/db mice independent of glycemic control. These results suggest that the effects of MMF in diabetic nephropathy are mediated by $CD4^+$ T cell regulation and related cytokines.

1. Introduction

Diabetes mellitus (DM) is the most common cause of end-stage renal disease and the leading risk factor for cardiovascular disease. Current treatment for diabetes remains conservative care including glycemic and blood pressure control based on the renin-angiotensin system blockade with a low-protein diet and lipid-lowering agents. The therapeutic limitations originate from the incomplete understanding of the pathogenesis of diabetes complications. Recent studies have associated chronic inflammation with the development and aggravation of type 2 DM [1, 2]. In addition, some inflammatory factors such as tumor necrosis factor- (TNF-) α, interleukin- (IL-) 6, and IL-1β have been thought to predict macrovascular complications in type 2 DM [3, 4].

In particular, activation and infiltration of T lymphocytes with monocytes/macrophages in the kidney along with associated cytokines have been investigated in patients with diabetic nephropathy. We previously reported marked interstitial infiltration of $CD4^+$ and $CD8^+$ T cells with increased interferon- (IFN-) γ and TNF-α in streptozotocin- (STZ-) induced diabetic mice, as well as increased infiltration of $CD4^+$, $CD8^+$, and $CD20^+$ cells in the renal interstitium of patients with type 2 diabetic nephropathy [5]. In addition, some results of previous investigations have indicated that mice deficient in intercellular adhesion molecule-1 had defects in leukocyte homing to the kidney, resulting in attenuation of renal injury [6]. Therefore, the recruitment of lymphocytes plays a key role not only in development but also in the progression of diabetic nephropathy.

Recently, mycophenolate mofetil (MMF) in experimental diabetic nephropathy was shown to ameliorate renal injury and prevent the development of nephropathy through various mechanisms [7–10]. Using MMF in diabetic nephropathy reduced various inflammatory cytokines and chemokines and attenuated podocyte apoptosis. Mycophenolic acid, the active form of MMF, inhibits T lymphocyte proliferation by blocking the early phases of the cell cycle and inducing apoptosis in stimulated T lymphocytes [11]. Based on these results, the inhibition of intrarenal T cells could possibly be the effect of MMF. However, there are only a few data regarding the effect of MMF in attenuating increased renal T cell infiltration in diabetic nephropathy. Previous diabetic animal study did not show a reduction in intrarenal T cell after MMF administration [7]. Furthermore, even though previous studies showed a podocyte-protective effect of MMF administration, the underlying mechanism has not been revealed.

The purpose of this study is to identify the role of MMF in diabetic nephropathy, especially in intrarenal T cell recruitment using db/db mice.

2. Materials and Methods

2.1. Animal Model and Experimental Design. Six-week-old male nondiabetic db/m and diabetic db/db mice were purchased from Jackson Laboratory (Sacramento, CA, USA). All mice received a diet of rodent pellets (348 kcal/100 g) containing 5.5% crude fat and tap water *ad libitum*. Mycophenolic acid was incorporated into chow (Dooyeol Biotech, Seoul, Republic of Korea) to reach an oral dose of 30 mg/kg body weight/day at a chow consumption of 0.2 g/g body weight. Various doses of MMF (20–40 mg/kg) were used in previous studies [7, 10, 12, 13]. In the preliminary study, we tested with a dose of 20 mg/kg/day, but there was no significant reduction in albuminuria in db/db group. At 8 weeks of age, mice were divided into three groups of six mice each: the nondiabetic control (db/m), the diabetic group (db/db), and the diabetes with MMF (Roche Pharma AG, Grenzach-Wyhlen, Germany) (db/db + MMF) from 8 to 20 weeks. During experiments, food intake, water intake, urine volume, body weight, fasting plasma glucose level, and glycosylated hemoglobin (HbA1c) were measured monthly. HbA1c was measured by immunoassay (DCA 2000 system; Bayer Diagnostics, Elkhart, IN, USA). To determine urinary albumin excretion, each individual mouse was placed in a metabolic cage and urine was collected for 24 hours. The urinary microalbumin concentration was determined by a competitive enzyme linked immunosorbent assay (ALPCO, NH, USA) and corrected by urinary creatinine (R&D Systems, Minneapolis, MN). All animal experiments were performed in compliance with the guidelines of the Animal Research Ethics Committee of Kyung Hee University, Seoul, Republic of Korea.

2.2. Light Microscopy. For light microscopy, the kidney tissue was fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 3 μm sections, and stained with periodic acid-Schiff (PAS) reagent. The degree of glomerular mesangial matrix expansion for each glomerulus was evaluated semiquantitatively using a score of 0–4: grade 0, no lesion; grade 1, <25%; grade 2, 25–50%; grade 3, 50–75%; and grade 4, <75%. At least 50 glomeruli per section were analyzed in a blinded manner.

2.3. Determination of Cytokines. Kidney tissues at 20 weeks were washed with phosphate-buffered saline and were homogenized on ice. Tissue homogenates were centrifuged to remove tissue residues at 12.000 g for 10 minutes at 4°C. Total protein concentration of the supernatant was measured using Pierce BCA protein assay kit. The cytokines were analyzed in the supernatant separated from tissue residues and plasma by using Luminex-bead array (Mouse Cytokine/Chemokine Magnetic Bead Panel, Millipore, Billerica, MA, USA) for detection of IFN-γ, TNF-α, IL-4, IL-6, and IL-17 according to the manufacturer's specifications. Concentrations of all cytokines measured were expressed as pg/mL.

2.4. Immunohistochemistry and TUNEL Assay. Immunohistochemistry for CD4 and CD8 was carried out using the Bond Polymer Refine Detection system (Vision BioSystems, Australia), according to the manufacturer's instructions with minor modifications. In brief, 4 μm sections of formalin-fixed and paraffin-embedded tissues were deparaffinized by Bond Dewax Solution and an antigen retrieval procedure was carried out using Bond ER solution for 30 min at 100°C. Endogenous peroxidase was quenched by incubation with hydrogen peroxide for 5 min. Sections were incubated with primary polyclonal antibodies for CD4 (1 : 100, Abcam, Cambridge, UK) and CD8 (1 : 100) using the biotin-free polymeric horseradish peroxidase-linked antibody conjugate system in a Bond-maX automatic slide stainer (Vision BioSystems, USA).

Immunohistochemical staining of CD68, for macrophages, and nephrin and Wilms tumor-1, for podocytes, was performed manually. Deparaffinized sections were rehydrated and microwaved in 10 mM citrate buffer (pH 6.0) for 20 minutes. After the retrieval, the sections were incubated in 3% H_2O_2 in methanol for 30 minutes to block peroxidase activity. The sections were then washed in PBST, blocked with 1% BSA/PBS for 30 minutes, and incubated with the primary antibodies: CD68 (1 : 100, AbD Serotec, NC, USA); nephrin (1 : 100, ENZO Life Sciences, NY, USA); and WT1 (1 : 100, Santa Cruz, CA, USA). For visualization the ZytoChem Plus HRP Polymer Kit (Zytomed Systems, Berlin, Germany) and the 3,3′-diaminobenzidine (DAB) substrate (Bethyl Laboratory, TX, USA) were used. Counterstaining was performed with hematoxylin except WT1. Infiltrated lymphocytes and macrophages were counted in 10 interstitial fields and 20 glomeruli per mouse under ×200 and ×400 magnification. Nephrin-positive area was measured in 30 glomeruli per mouse using ImageJ software, version 1.49o (NIH, Bethesda, USA). WT1-positive cells were counted in at least 30 randomly selected glomeruli under ×400 magnification.

For tubular and glomerular apoptotic scores, In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, USA), was used according to the manufacturer's instruction. TUNEL-positive cells were screened, counted, and analyzed

using a fluorescence microscope in 10 interstitial fields and 20 glomeruli per kidney.

2.5. Isolation of Total RNA, RT, and Real-Time PCR. Total RNA was extracted from kidney tissue using the Total RNA Isolation Kit (MACHEREY-NAGEL, Germany). Real-time PCR was performed using SYBR Green PCR Master Mix (FastStart Universal SYBR Green Master, Roche). The real-time PCR reaction was performed with an ABI StepOne real-time PCR system (Applied Biosystems, USA) following the manufacturer's guidelines. The primers for transforming growth factor-β1 (TGF-β1), connective tissue growth factor (CTGF), type I collagen, type IV collagen, CXCL1 (chemokine (C-X-C motif) ligand 1), CXCL2, CXCL9, CCL (chemokine (C-C motif) ligand) 2, CCL3, CCL20, and 18S are used. The sequences of used primers are shown in the supplementary Table 1 in the Supplementary Material available online at http://dx.doi.org/10.1155/2015/301627. Each sample was run in triplicate in separate tubes for reproducibility. The target gene expression levels were normalized to 18S expression.

2.6. Western Blot Analysis. Kidney tissues were washed with phosphate-buffered saline and lysed with ice cold lysis buffer (10 mMTris·HCl, 150 mMNaCl, 1% Triton X-100, 5 mM EDTA pH 8.0) and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The lysate was centrifuged at 4°C for 10 min at 10,000 g and the supernatant was recovered. Equal amounts of total cellular protein were subjected to SDS/PAGE in 12% acrylamide gel and then transferred to a PVDF membrane (Millipore, Madrid, Spain) by electroblotting, and the membrane was blocked with 5% fat-free milk in Tris-buffered saline with 0.5% Tween 20 (TBS-T). The membranes were incubated with primary antibodies against β-actin, NADPH oxidase 4 (Nox4), p67-phox (1 : 1000, Santa Cruz, CA, USA), Bax, Bcl-2 (1 : 1000, Cell Signaling Technology, MA, USA) in TBS-T, and 5% Bovine Serum Albumin (BSA) overnight at 4°C. Then the blots were washed and incubated with secondary antibody in blocking solution (goat anti-rabbit horseradish peroxidase- (HRP-) conjugated and goat anti-mouse HRP-conjugated, 1 : 10000, Santa Cruz, CA, USA) for 2 h at room temperature. The signal was detected by a pico enhanced peroxidase detection (EPD) western blot detection kit (Mbiotech, Seoul, Republic of Korea), and bands were visualized using a G: Box chemi XL (Syngene, Cambridge, UK). β-actin was used as an internal control.

2.7. Statistical Analysis. All values are expressed as means ± SE. Results were analyzed using the Kruskal-Wallis nonparametric test for multiple comparisons. Significant differences in the Kruskal-Wallis test were confirmed by the Wilcoxon rank sum, Mann-Whitney, and Friedman test (used to compare mean differences); p values < 0.05 were considered statistically significant.

3. Results

3.1. MMF Treatment Attenuates Diabetic Nephropathy Independent of Glycemic Control. The 30 mg/kg dose of MPA was well-tolerated, and there were no typical side effects such as diarrhea and weight loss. The various biochemical parameters were determined in three experimental groups of mice, as shown in Table 1. Both db/db and db/db + MMF mice showed higher HbA1c level than db/m mice; in particular, there was no difference in HbA1c between db/db and db/db + MMF mice at 20 weeks (Figure 1(a)). The result of insulin tolerance test also showed no difference between db/db and db/db + MMF mice (Figure 1(b)). To determine the severity of diabetic renal injury in each group, we confirmed the mesangial matrix expansion by PAS staining. As shown in Figures 1(c) and 1(d), MMF treatment improved mesangial matrix expansion in the db/db mouse kidney. In addition, MMF treatment reduced TGF-β1, CTGF, type I collagen, and type IV collagen mRNA expression in the diabetic mice kidney (Figure 1(e)). The increased urinary albumin/creatinine excretion ratio was significantly decreased by MMF treatment (Figure 1(f)). These findings suggest that MMF has renoprotective effect in diabetic nephropathy without glycemic control.

Table 1: The clinical parameters of study groups.

Characteristics	Week	db/m	db/db	db/db + MMF
Body weight (g)	8	27.7 ± 0.25	42.9 ± 1.76*	45.2 ± 0.37*
	20	31.4 ± 0.5	51.0 ± 3.2*	50.1 ± 1.9*
Daily food intake (g/day)	8	3.97 ± 0.01	5.82 ± 0.28*	5.76 ± 0.17*
	20	5.6 ± 0.4	7.6 ± 1.2*	8.8 ± 0.8*
Daily water intake (mL/day)	20	5.0 ± 0.3	13.5 ± 0.6	15.6 ± 1.6
Urine output (mL/day)	20	0.4 ± 0.2	2.4 ± 1.4*	7.5 ± 3*#
Fasting glucose (mg/dL)	20	95.8 ± 14.4	391 ± 40.6*	434.9 ± 40.7*
Kidney/BW (%)	20	1.54 ± 0.04	1.33 ± 0.17	1.05 ± 0.02*
Liver/BW (%)	20	4.85 ± 0.65	5.9 ± 0.33	6.1 ± 0.38
Fat/BW (%)	20	3.2 ± 0.5	4.9 ± 0.17	6.3 ± 0.6*

MMF: mycophenolate mofetil.
The results are expressed as the mean ± S.E.M.
*Significantly different with respect to the db/m mice; #significantly different with respect to db/db mice.
*#$p < 0.05$.

3.2. MMF Decreases T Lymphocyte Infiltration and Its Related Cytokines in the db/db Kidney. As shown in Figure 2, infiltration of $CD4^+$ and $CD8^+$ T cells in tubulointerstitium was significantly increased in db/db mice compared with db/m mice. MMF treatment markedly reduced the number of infiltrated $CD4^+$ and $CD8^+$ T cells in the db/db kidney. To confirm the effect of MMF on regulating intrarenal T cells, we investigated systemic and intrarenal T cell-related cytokines which are known to regulate inflammatory and immune responses to the development of progression of diabetic nephropathy. MMF treatment significantly decreased intrarenal TNF-α and IL-17 levels (Figure 3(a)). The plasma levels of IL-4 and IL-6 were increased in db/db mice and were

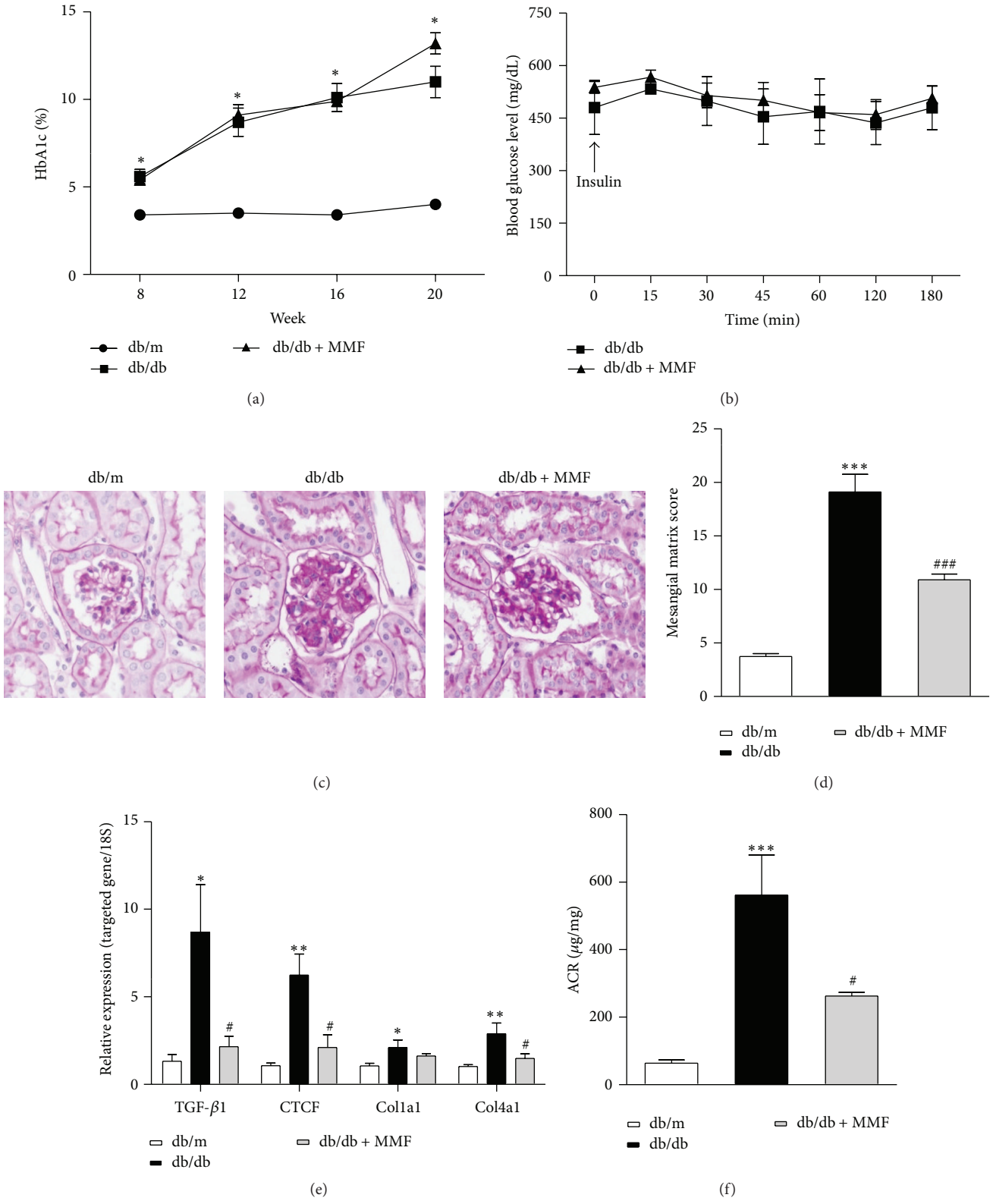

Figure 1: The effect of MMF on glucose control and diabetic nephropathy of db/db mice. HbA1c and insulin tolerance test (a and b). Kidney injury was observed by PAS staining (original magnification ×400) (c), and the glomerular matrix score was quantified (d). Real-time PCR for TGF-β1, CTGF, and type I and type IV collagen (e). Urinary albumin/creatinine excretion (f). The results are expressed as mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus db/m; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus db/db.

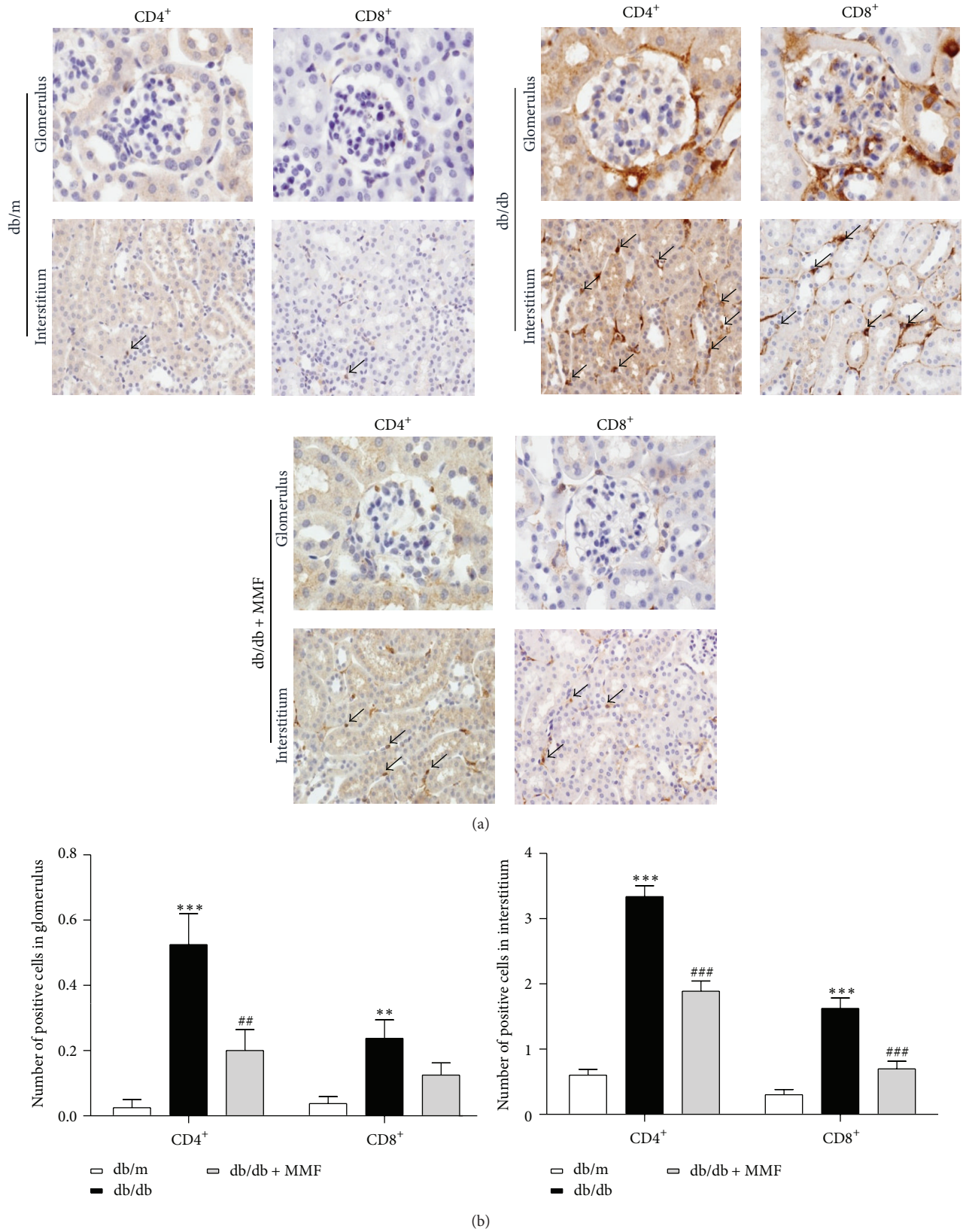

FIGURE 2: MMF attenuates T cells infiltration in diabetic kidney. Infiltration of renal $CD4^+$ and $CD8^+$ cells in glomeruli and tubulointerstitium was observed by immunohistochemical analysis (a) and the infiltration of $CD4^+$ and $CD8^+$ cells was quantitatively scored (b). Original magnification ×400. The results are expressed as the means ± SE. $^{**}p < 0.01$, $^{***}p < 0.001$ versus db/m; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus db/db.

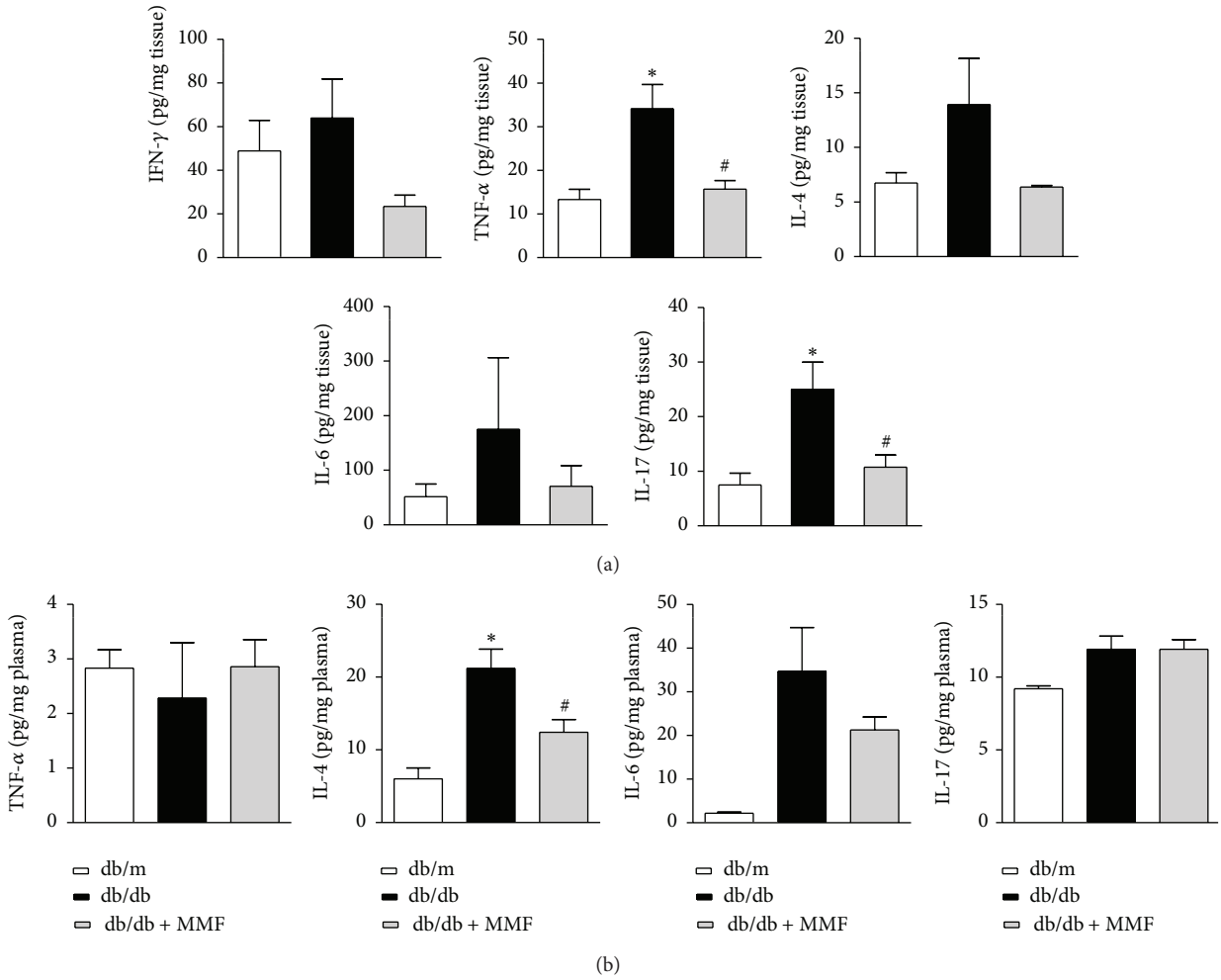

Figure 3: MMF decreases $CD4^+$ T cells related cytokines in the db/db kidney. The cytokines IFN-γ, IL-4, IL-6, IL-17, and TNF-α were detected by a Luminex-bead array in kidney (a) and plasma (b). The results are expressed as mean ± SE. $^*p < 0.05$ versus db/m; $^\#p < 0.05$ versus db/db.

decreased by MMF treatment. The increased levels of TNF-α and IL17a in db/db were reduced due to MMF treatment in the kidneys, but no changes were observed in plasma.

3.3. MMF Reduces Renal Chemokines and Macrophages Recruitment. Next, we examined the expression of intrarenal chemokines associated with CD4+ T cells. The expression of CCL2, CCL3, and CCL20, which are controlled by IL-17, was significantly increased and attenuated by MMF treatment in the db/db mouse kidney (Figure 4(a)). The number of infiltrated CD68+ macrophages in tubulointerstitial area in db/db mice was significantly increased compared with db/m mice but was also markedly reduced by MMF treatment (Figures 4(b) and 4(c)). These results indicate that MMF treatment attenuates diabetic nephropathy by decreasing macrophage infiltration in the kidney through the regulation of IL-17-related chemokines in db/db mice.

3.4. MMF Attenuates Podocyte Injury. We investigated podocyte injury to examine the role of MMF in the diabetic kidney related with improved microalbuminuria. The loss of glomerular nephrin was recovered in MMF-treated db/db mice compared with db/db mice (Figures 5(a) and 5(d)). WT1 was also increased by MMF treatment (Figures 5(b) and 5(e)). TUNEL-positive apoptotic cells in glomeruli were increased in db/db mice and were attenuated by MMF treatment (Figures 5(c) and 5(f)). These findings indicated that MMF attenuates diabetic nephropathy by reducing podocyte apoptosis.

3.5. The Effects of MMF on Apoptosis and ROS in the Diabetic Kidney. We also evaluated the effect of MMF on apoptotic injury in renal tubules of db/db mice. The Bax/Bcl-2 ratio in the diabetic kidney was decreased with MMF treatment (Figures 6(a) and 6(b)). TUNEL-positive apoptotic cells in the tubular area were also attenuated by MMF treatment (Figures 6(c) and 6(d)). In addition, we evaluated the effect of MMF on diabetic ROS injury in the kidney. Increased expression of Nox4 and p67phox in the kidney of db/db mice was decreased by MMF (Figures 6(e) and 6(f)).

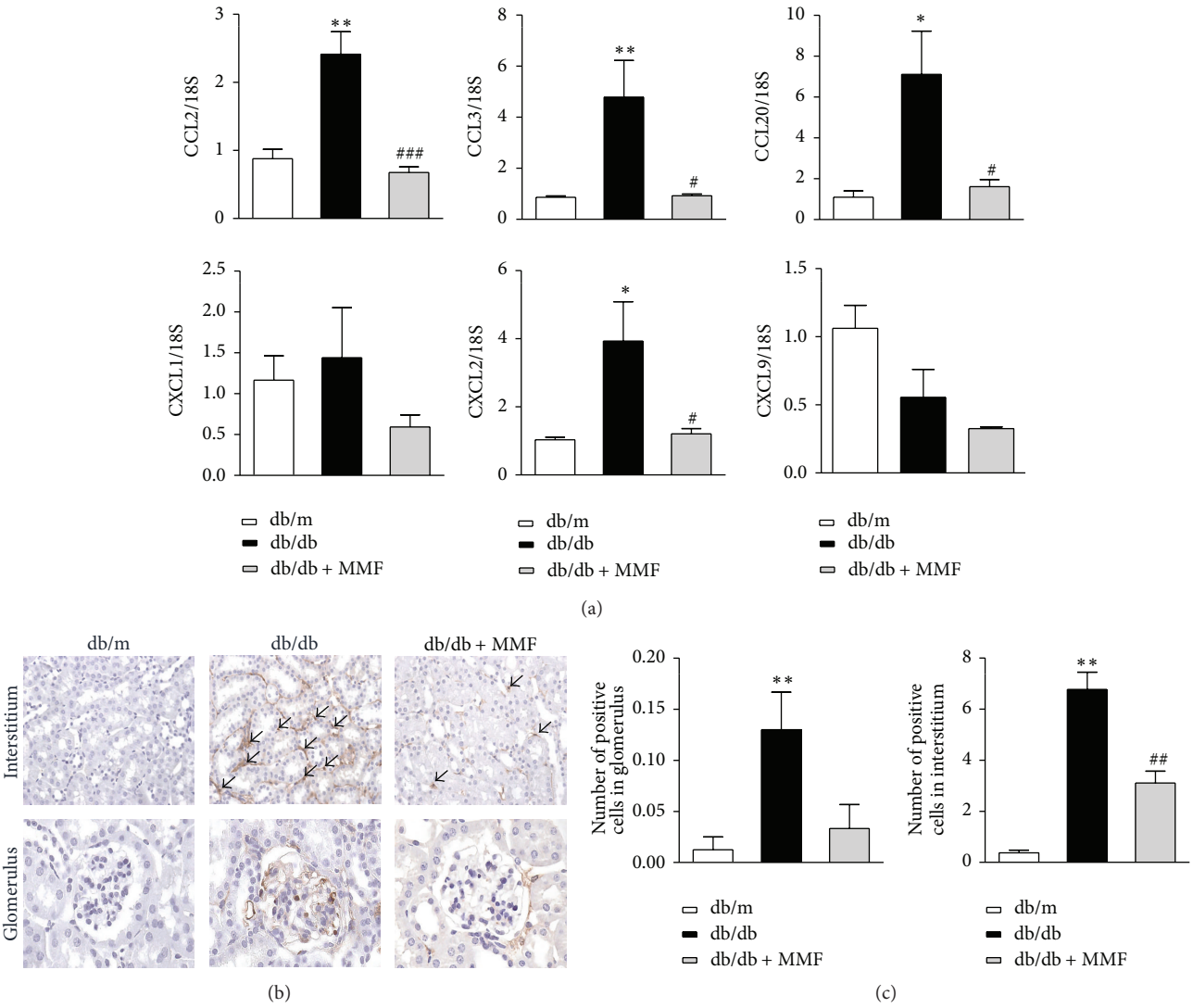

FIGURE 4: MMF reduces renal chemokines and macrophages recruitment. Real-time PCR for CCL2, CCL3, CCL20, CXCL1, CXCL2, CXCL9, and 18S (a). Infiltrating the renal CD68$^+$ cells in glomeruli and tubulointerstitium was observed by immunohistochemical analysis (b) and the infiltration of CD68$^+$ cells was quantitatively scored (c). Each sample was run in triplicate in separate tubes to permit quantification of the target gene expression normalized to 18S expression. The results are expressed as mean ± SE. $^*p < 0.05$, $^{**}p < 0.01$ versus db/m; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ versus db/db.

4. Discussion

Results from this study indicate that MMF treatment ameliorates diabetic nephropathy in db/db mice with a decrease in albuminuria, mesangial proliferation, and podocyte injury regardless of hyperglycemia. This study shows that MMF decreases intrarenal CD4$^+$ and CD8$^+$ T cell recruitment in db/db mice. Previous study showed that MMF administration in STZ-induced diabetic rats did not result in reduction in CD3$^+$ lymphocyte infiltration in the renal cortex [7]. Sequentially, T cell inhibition led to reduced expression of T cell-related cytokines and chemokines. These anti-inflammatory effects of MMF contributed to improvement in diabetic nephropathy.

Diabetic complications, especially diabetic nephropathy, were ameliorated by intensive blood glucose and blood pressure control. Glucose control is very important factor to reduce microvascular complications. Unfortunately, many diabetic nephropathy patients progress to end-stage renal disease despite the use of strict glucose and blood pressure control, and these subgroups of diabetic nephropathy patients may be susceptible to inflammatory injury. Although HbA1c was not reduced, the beneficial effects of MMF on diabetic nephropathy were found in this study. These results indicate that a partial role of immune cells-related inflammatory injury is one of the important therapeutic targets to diabetic nephropathy.

Increased CD4$^+$ and CD8$^+$ T cell infiltration was attenuated in the interstitium after 12 weeks of MMF treatment in db/db mice. When we measured intrarenal Th1- and Th17-associated cytokines, we confirmed that IL-17 and TNF-α

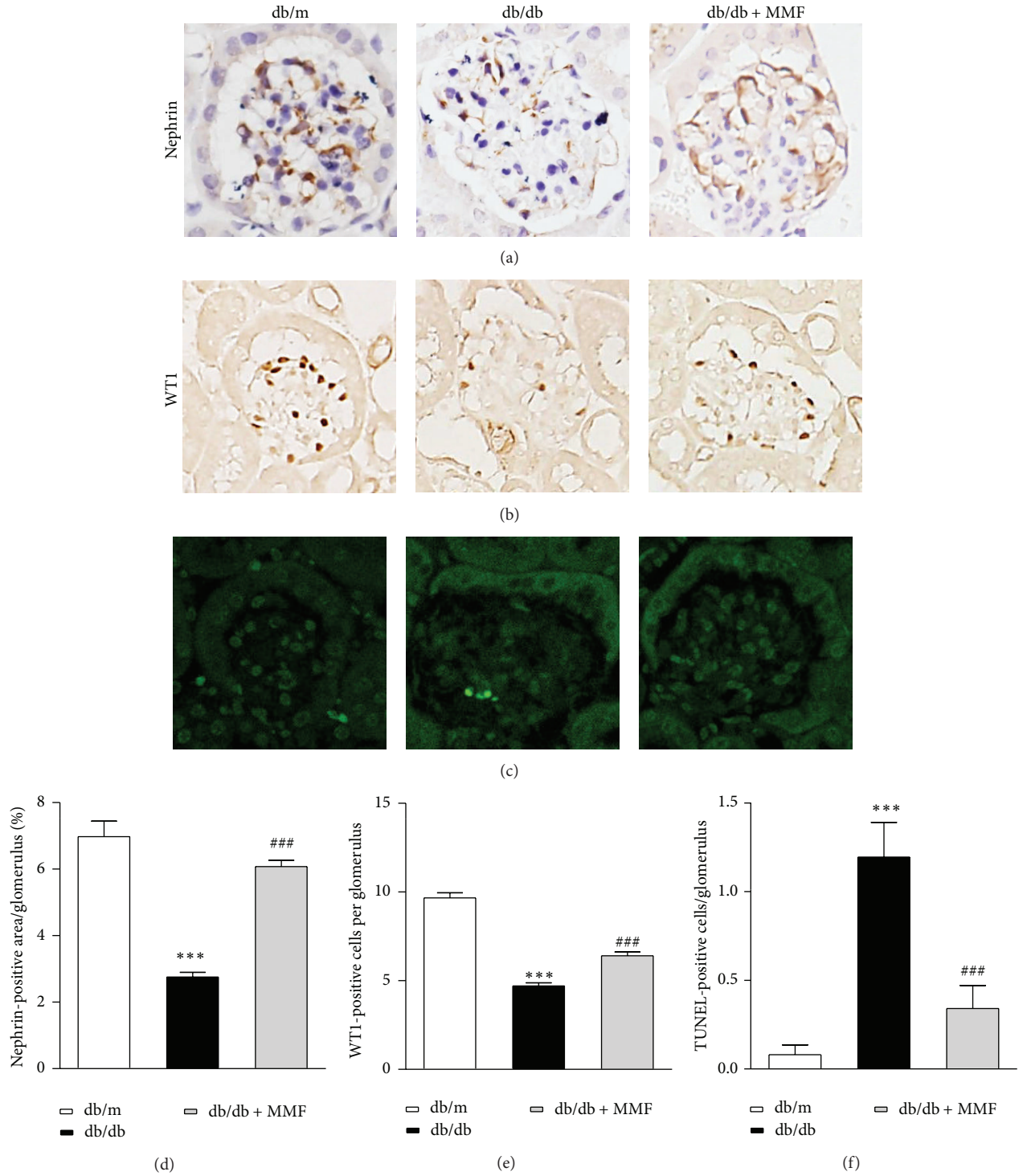

FIGURE 5: The effect of MMF on podocyte injury. Nephrin and WT1 are represented for db/m, db/db, and MMF-treated db/db mice (a, b and d, e, resp.). TUNEL assay for apoptotic injury in glomeruli (c), and TUNEL-positive cells counted for quantitative analysis (f) in three experimental groups. The results are expressed as mean ± SE. $^{***}p < 0.001$ versus db/m; $^{###}p < 0.001$ versus db/db. Original magnification ×400.

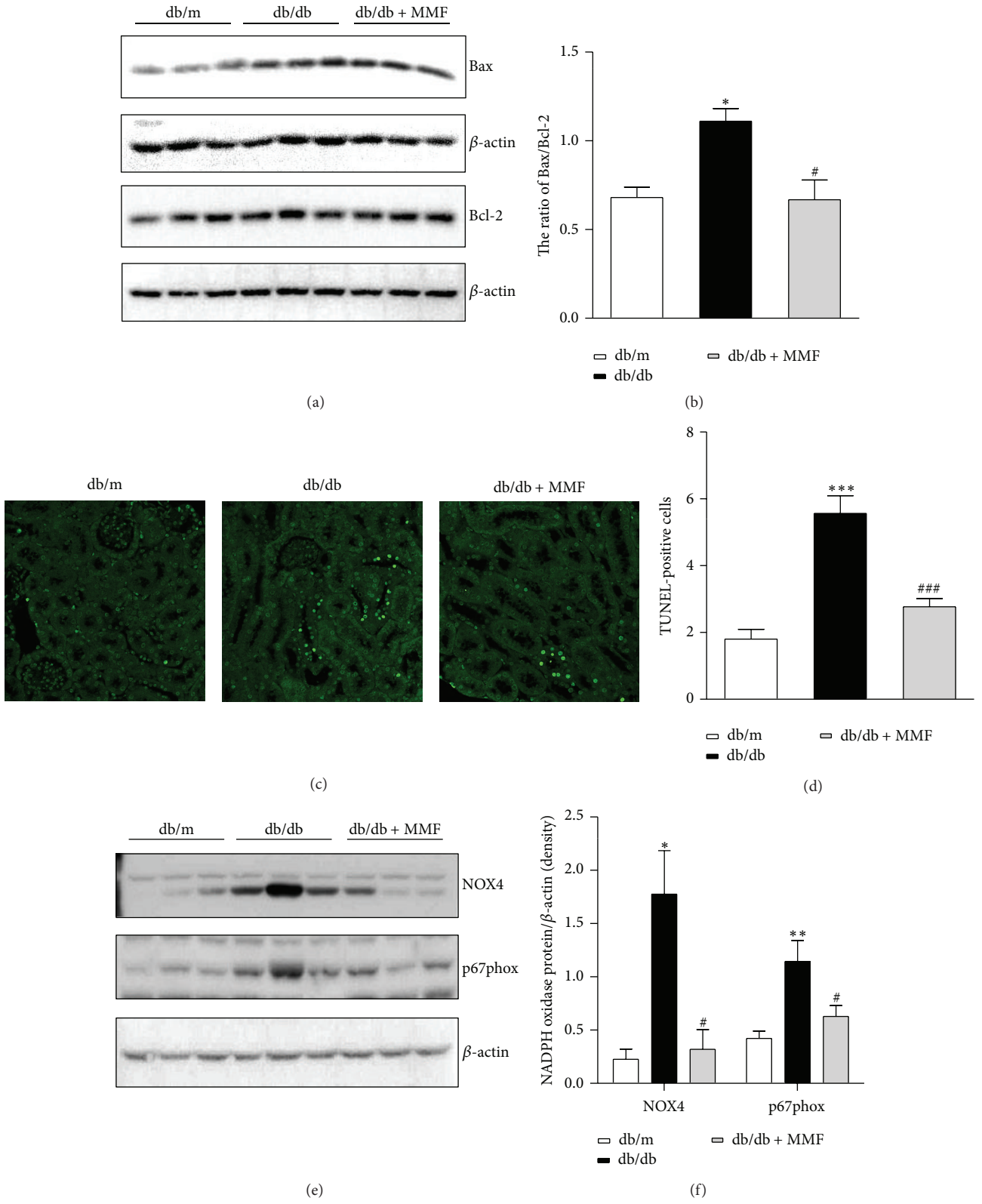

FIGURE 6: The effect of MMF on apoptotic injury and NAD(P)H oxidase. The proapoptotic Bax and antiapoptotic Bcl2 were analyzed by western blotting (a) and quantitative analyses of the Bax/Bcl2 ratio (b). Tubulointerstitial TUNEL staining (c) and TUNEL-positive cells were assessed for quantitative analysis (d) in three experimental groups. Original magnification ×400. Representative western blot images of NADPH oxidase, NOX4, and p67phox are shown (e). The quantification of NADPH oxidase (f). The results are expressed as mean ± SE. $^{*}p < 0.05$, $^{***}p < 0.001$ versus db/m; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ versus db/db.

were decreased by MMF treatment. Particularly, IL-17-associated chemokines such as CCL2, CCL3, and CCL20 [14] were significantly attenuated.

An interesting finding is that the increased T cell-related cytokines in plasma were different to those in the kidney. The plasma levels of IL-4 and IL-6 were increased in db/db mice and were decreased by MMF treatment. The levels of TNF-α and IL-17 were reduced due to MMF treatment in the kidneys, but no changes were observed in plasma. A previous study also reported that MMF treatment reduced T cells in local atherosclerotic inflammatory region, not systemic T cells [13]. Therefore, we carefully conclude that systemic inflammatory activity is different from that in the kidney and that the renal T cells are more susceptible to MMF treatment in db/db mice. These results suggest that the diabetic kidney with albuminuria is more actively responsive to inflammation than systemic circulation.

Th1 response is recognized as accompanying type 1 diabetes [15], and enhanced Th1-related cytokines/chemokines have been correlated with proteinuria and renal function in type 2 diabetes [16]. MMF treatment might contribute to reduced proteinuria and improved pathologic findings by reducing renal $CD4^+$ T cell and aberrantly regulating its cytokines/chemokines. An interesting finding in this study is that MMF more sensitively regulated intrarenal IL-17 which is related to CCL2, CCL3, and CCL20. Increase in the number of Th17 cells is considered as a part of the pathogenesis of diabetes, and it has been supported by findings in type 1 DM murine model and by human data [17, 18]. Although a precise relationship between IL-17 and diabetic nephropathy needs to be confirmed, our results suggest that IL-17 could play proinflammatory role in diabetic nephropathy and that MMF has beneficial effects in reducing intrarenal IL-17 and related chemokine levels. In addition, the levels of CXCL2, which is known to arrest rolling monocytes [19], were attenuated in the kidney of MMF-treated db/db mice. Therefore, the suppression of intrarenal immune cells by MMF might lessen the secretion of proinflammatory cytokines/chemokines, and the downregulation of these cytokine/chemokines could attenuate macrophage recruitment.

Nephrin, a slit diaphragm-associated protein, prevents the development of albuminuria, and WT1, a podocyte-specific marker, reflects podocyte number [20, 21]. In diabetic nephropathy, podocytes are important in regulating glomerular filtration, and the loss of podocytes leads to glomerular damage and causes albuminuria. In the current work, MMF treatment preserved podocytes in db/db mice. Although the exact mechanisms associated with podocyte apoptosis are still unknown, a previous work has shown that TGF-β1 induces apoptosis in cultured podocytes [22]. Another study demonstrated that high glucose induces reactive oxygen species (ROS) generation and leads to apoptosis of podocytes [21]. Treatment with NADPH oxidase inhibitor alleviates podocyte apoptosis and prevents glomerular injury in type 1 and type 2 diabetic animal models [23]. Therefore, the beneficial role of MMF in protecting against the loss of podocytes is thought to arise from its antioxidant and anti-inflammatory effects.

ROS and apoptosis are important mechanisms of diabetic nephropathy which is largely dependent on glycemic control. Upregulated Bax and downregulated Bcl-2 expression were reversed by MMF treatment, as was also confirmed by TUNEL staining. We also found that through NAD(P)H oxidase, a critical generator of ROS [24], MMF could be associated with the attenuation of ROS injury independent of hyperglycemia. In db/db mice, Nox4 induces ROS generation and regulates profibrotic renal injury through p38 mitogen-activated protein kinase [25]. However, our results indicated that MMF attenuated intrarenal Nox4 and p67phox regardless of high blood glucose level. Proinflammatory immune responses promoted by diabetes are associated with augmented ROS generation by NAD(P)H oxidase [26], and inflammatory cytokines contribute to activation of ROS injury through upregulation of NAD(P)H oxidase and inducible NO synthase [27].

In conclusion, we demonstrated that MMF treatment attenuates diabetic nephropathy by decreasing $CD4^+$ T cell infiltration and its related cytokines and chemokines. The anti-inflammatory effect of MMF attenuates podocyte apoptosis independent of glucose control. This result suggests that MMF may have a beneficial role in the treatment of diabetic nephropathy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jung-Woo Seo and Yang Gyun Kim equally contributed to this study as first authors.

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Review Article

Modulation of Intrathymic Sphingosine-1-Phosphate Levels Promotes Escape of Immature Thymocytes to the Periphery with a Potential Proinflammatory Role in Chagas Disease

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The sphingosine-1-phosphate (S1P) system regulates both thymic and lymph nodes T cell egress which is essential for producing and maintaining the recycling T cell repertoire. Infection with the protozoan parasite *Trypanosoma cruzi* induces a hormonal systemic deregulation that has impact in the thymic S1P homeostasis that ultimately promotes the premature exit of immature $CD4^-CD8^-$ T cells expressing TCR and proinflamatory cytokines to peripheral lymphoid organs, where they may interfere with adaptive immune responses. In what follows, we review recent findings revealing escape of these immature T cells exhibiting an activation profile to peripheral compartments of the immune system in both experimental murine and human models of Chagas disease.

1. Introduction

T lymphocytes are key players in acquired immunity and have a lineage commitment characterized by expression of the T cell receptor (TCR), which has a vital role in recognizing pathogen antigens during the development of host resistance to infections [1]. The activation and differentiation of T cells depend on the TCRs being specific for exogenous antigens but not mounting an autoimmune response against self-antigens and generating a collateral response. This quality control of the immune system is performed during the maturation of T cell precursors in the thymus [2, 3].

The thymus is the primary lymphoid organ in which T cell precursors derived from the bone marrow undergo cell differentiation process consisting of the sequential expression of multiple lymphocyte differentiation genes and rearrangement of the T cell receptor (TCR) genes. During thymic maturation, thymocytes express TCRs, some of which interact with peptides presented by molecules of the major histocompatibility complex (MHC) on the surface of the thymic stromal cells. These interactions determine the positive and negative selection events that are crucial components of the program of terminal thymocyte differentiation [4–6].

During intrathymic development, thymocytes begin to express on their membranes the TCR/CD3 complex together with CD4 and CD8 coreceptors, thus becoming double-positive (DP) thymocytes, distributed throughout most of the cortical region of the organ. At this phase of intrathymic maturation of thymocytes, the generation of a highly diverse TCR repertoire produces many T lymphocytes expressing TCRs that recognize "self-antigens." These autoreactive T lymphocytes are negatively selected in the thymus as part of the process called central tolerance. In this process, the self-reactive lymphocytes die by apoptosis, while a small percentage of positively selected cells move to the medulla of the thymus where their differentiation proceeds [6–8].

During the course of their differentiation, thymocytes develop into T cells expressing high densities of TCR/CD3 and they become simple positive (SP) for one or another (but not both) of the coreceptors CD8 or CD4, which recognize,

respectively, peptides complexed with class I and class II MHC molecules. These naïve T cells ultimately leave the thymus to form part of the repertoire of peripheral T cells [1, 9]. They are exported from the thymus under the control of the lipid mediator sphingosine-1-phosphate (S1P) [10–12]. Sphingosine-1-phosphate is a biologically active sphingolipid derivative critical to the signaling pathways involved in the traffic of leukocytes [10, 13, 14].

The tissue concentration of S1P increases in several inflammatory conditions such as asthma and autoimmune diseases and this lipid agonist engages and activates a family of G-protein coupled receptors (S1P1–S1P5) [15–17]. Several groups have demonstrated the importance of S1PRs in the trafficking of leukocytes mediating effector responses in the immune system. Their findings indicate a key role of the S1P-S1PRs axis in the development and maintenance of immunity [18, 19].

2. Fine-Tuned Metabolic Regulation of Sphingosine-1-Phosphate

Sphingolipids are essential lipids rich in cholesterol that are concentrated in microdomains known as "lipid rafts" or "lipid platforms" on the plasma membrane. These lipids can be rapidly metabolized upon activation of an enzymatic cascade that converts sphingolipids such as sphingomyelin and glycosphingolipid complexes to ceramide and subsequently to sphingosine, two sphingosine kinases (SphK1 and SPHK2) and then phosphorylate sphingosine to sphingosine-1-phosphate [17, 20, 21].

Sphingosine-1-phosphate has both cell-extrinsic and intrinsic activities affecting homeostasis and cellular function [22]. Much emphasis has been given to the extrinsic function of S1P in the immune system, which was recognized through studies of the immunosuppressive agent, FTY720, a drug mediator proved capable of binding to and blocking sphingosine-1-phosphate receptors (S1PRs) [23]. FTY720 induces lymphopenia by causing sequestration of lymphocytes in the lymph nodes, thus blocking the egress of mature thymocytes to the periphery [24, 25].

The tissue levels of S1P are determined not only byits rate of biosynthesis but also by its rate of degradation. It is constantly produced by most cells and is irreversibly degraded by S1P lyase or dephosphorylated by S1P phosphatases [26–28]. In most tissues including lymphoid organs, S1P levels are extremely low. Exceptions are the blood and lymph, in which S1P levels are generally high, ranging from submicro to micromolar concentrations, respectively [29, 30]. The S1P levels in serum arise mainly from its production by endothelial cells, while the high levels in plasma are contributed by the erythrocytes [31, 32].

Deletion of the genes encoding both of the kinases, SphK1 and SPHK2, results in embryonic lethality due to the absence of S1P. In addition, conditional deletion of these two genes results in deficiency in circulating S1P. However deletion of either one of the two genes is without effect, showing that these kinases have redundant functions [29, 33].

3. Expression of the Specific G Protein Coupled Receptors for S1P and the Regulation of Cellular Traffic in the Immune System

The discovery of the "orphan receptor"-associated G protein gene originally known as endothelial differentiation gene 1 (EDG1) opened a new frontier in our understanding of the mechanism of action of S1P [34]. Since then, S1P has been shown to be the ligand for five different members of this "orphan receptor" family, S1P1–S1P5 [35]. These receptors mediate several cellular functions through associated heterotrimeric G proteins (αI, αq, or α12/13) and are expressed by most cells of the immune system. However, there is heterogeneity in terms of their pattern of expression among immune cells [36].

Although S1PRs are present in other physiologic systems, S1P3–S1P5 are mainly limited to the immune system. T cells express S1P1 and S1P4 [12, 37–39], while mast cells and macrophages express S1P1 and S1P2 [40–45]. Expression of S1P1 is also found in B lymphocytes and dendritic cells [46–50]. The primary function of most S1PRs is to regulate the migratory responses of cells by inducing proteins with Rac GTPase activity [36, 51]. S1P signaling plays a role both in the migration or homing of immune cells to lymphoid organs and in their egress into the blood and lymph, a topic that has received much attention recently [52–54]. The S1P gradient between lymphoid tissues, which have low levels of S1P, and their vascular compartments, which have high levels of S1P, is a key factor determining the egress of leukocytes from lymph nodes and thymus into the blood [32]. The signaling pathways activated by S1P1 in response to this gradient of S1P control not only the egress of T and B lymphocytes from lymph nodes but also the exit of mature T lymphocytes and natural killer T cells (NKT) from the thymus to vascular compartments [10, 12, 14].

S1P-mediated chemotaxis via S1P1 is dependent on the concentration of SIP: *in vitro* studies have demonstrated that low concentrations promote S1P chemotaxis, while high concentrations tend to inhibit it [54, 55]. It appears that high S1P levels stimulate ubiquitin-dependent lysosomal membrane protein sorting and degradation, which causes breakdown of S1P1 [56]. Interestingly, elevated concentrations of the synthetic agonist of S1PRs, FTY720, are also highly effective in inducing internalization, ubiquitination, and degradation of S1P1 [23, 57–59].

4. Disruption of S1P Homeostasis Promotes Thymocyte Precursor Release and Organ Atrophy in Chagas Disease

In most vertebrates, acute short-term stress signals induced by infectious pathogens are responsible for evoking host innate defense responses [60]. If a pathogen persists chronically the stress signals can cause the host immune response to be suppressed, thus increasing susceptibility to the microorganism; this results at least in part from a shift from T helper 1-mediated cellular immunity to T helper 2-mediated humoral

immunity. These stress signals are the result of release of neurotransmitters, hormones, and cytokines secreted during inflammatory responses [61].

In Chagas disease, infection by *T. cruzi* generates an inflammatory syndrome mediated by TNF-α in the acute phase. This cytokine activates the hypothalamus-pituitary-adrenal (HPA) axis leading to release of stress hormone corticosterone. The primary consequence is atrophy of the thymus leading to severe reduction in thymic cell numbers, followed by a reduction in the thymic output of T cells to the periphery [62, 63].

Although thymic atrophy occurs in infections caused by several pathogens, the impact of this trait on thymic central tolerance has only recently been clarified. We have demonstrated that the alterations in the thymic microenvironment induced by *T. cruzi* infection do not affect the key elements needed for intrathymic negative selection of maturing thymocytes during thymopoiesis [64]. Nevertheless, we have observed that in severe atrophy of the thymus the number and frequency of developing extrathymic thymocytes bearing low TCR levels increase markedly during the acute phase [65].

Moreover, we found that the immature thymocytes released into the periphery (subverting the process of negative selection) acquired similar activated phenotype to that described for activated effector cells and single-positive memory cells, suggesting a possible imbalance in the mechanism controlling thymocyte exit to the periphery [64]. As the signaling pathway mediated by sphingosine-1 phosphate (S1P) through its receptors is responsible for the egress of mature thymocytes, we investigated whether some modulation of the sphingosine-1-phosphate (S1P) pathway was responsible for the early release of thymocytes in thymic atrophy [66].

The gene expression profile of the enzymes involved in the S1P pathway in the *T. cruzi* infected thymus during acute phase in fact showed a reduction of sphingosine-1-phosphate accompanied by reduced expression of the activating kinases SPHK1/2 and upregulation of the inactivating phosphatase SGPL1. However, the S1P levels in the sera of infected and normal mice were similar [66]. These findings indicate that the gradient of S1P level from the thymus to the blood of *T. cruzi* infected mice becomes steeper upon infection and this may promote thymocyte egress. Since high concentrations of S1P are needed to stimulate ubiquitin-dependent lysosomal membrane protein sorting and degradation the breakdown of S1P1 this feedback mechanism should not be active in the infected thymus because of the reduced S1P concentration.

Interestingly, analysis of the expression of the S1P receptor in developing thymocytes indicated a substantial upregulation of S1P1 and S1P3 expression in immature $CD4^-CD8^-$ T cells upon *T. cruzi* infection, suggesting that some S1P-mediated pathway could also contribute to the premature exit of these cells [66]. The upregulation of S1P receptors should increase the sensitivity of thymocytes to S1P-mediated chemotaxis in the atrophic thymus. Using an *ex vivo* Transwell migration assay, we found that the sensitivity of the $CD4^-CD8^-$ cells to S1P-mediated chemotaxis increased during *T. cruzi* infection [66].

These findings together indicate that the export of developing $CD4^-CD8^-$ thymocytes could be favored by a disturbance of the physiological levels of S1P in the thymus during *T. cruzi* infection. In fact, we found that when we blocked the S1P receptors with FTY720 in infected mice we inhibited the escape of these cells to the periphery thus restoring physiological levels of $CD4^-CD8^-$ thymocytes [66].

Furthermore, when we assessed the differentiation status of the $CD4^-CD8^-$ thymocytes released during *T. cruzi* infection, we found that these immature cells (present in peripheral lymph nodes) exhibited a significant increase in the expression of the cytokines IL-17 and TNFα upon polyclonal stimulation [66]. The findings we have described correlate with the presence in patients with the indeterminate or cardiac clinical forms of Chagas disease of increased numbers of circulating $CD4^-CD8^-$ T cells exhibiting an activated phenotype as defined by the expression of activation marker, HLA-DR [64].

5. Concluding Remarks

Overall our studies indicate that infection with *Trypanosoma cruzi* promotes thymic alterations, due in part to the effects of modulation of the S1P-S1P1 receptor axes on intrathymic $CD4^-CD8^-$ T cells. As a result, thymocytes undergoing differentiation become prematurely chemotactically responsive to S1P [66]. These double-negative thymocytes are therefore able to emigrate from the thymus before undergoing the negative selection process necessary for self-tolerance. Moreover these immature T cells that escaped to periphery have an activated phenotype in both experimental murine and human models of Chagas disease [64].

The presence of these cells bearing TCRs in the periphery may have potential implications for disease outcome. In other systems, these cells have been shown to have pathogenic properties: they are able to recognize antigens and signal through their TCR in an MHC-independent manner [67, 68]. Importantly, as demonstrated in patients with systemic lupus erythematous and myasthenia gravis, there is a direct correlation between $CD4^-CD8^-$ T cells and the development of human autoimmune diseases, suggesting a role for these cells in autoimmune responses [69].

It is also possible that the early egress of undifferentiated $CD4^-CD8^-$ T cells plays a role in the immunopathologic events in Chagas disease by altering adaptive immune responses, since they produce proinflammatory cytokines when activated. In addition to these various phenomena, our results indicate a direct link between the changes in the level of the $CD4^-CD8^-$ T cell subset and the severity of myocardial lesions in human Chagas disease, thus identifying a potential clinical marker of disease progression [66].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Role of Inhibitors of Apoptosis Proteins in Testicular Function and Male Fertility: Effects of Polydeoxyribonucleotide Administration in Experimental Varicocele

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Neuronal apoptosis inhibitory protein (NAIP) and survivin might play an important role in testicular function. We investigated the effect of PDRN, an agonist of adenosine A2A receptor, on testicular NAIP and survivin expression in an experimental model of varicocele. After the creation of experimental varicocele (28 days), adolescent male Sprague-Dawley rats were randomized to one of the following treatments lasting 21 days: vehicle, PDRN (8 mg/kg i.p., daily), PDRN + 3,7-dimethyl-propargylxanthine (DMPX, a specific adenosine A2A-receptor antagonist, 0.1 mg/kg i.p., daily), varicocelectomy, and varicocelectomy + PDRN (8 mg/kg i.p., daily). Sham-operated animals were used as controls. Animals were then euthanized and testis expression of NAIP and survivin was evaluated through qRT-PCR, western blot, and immunohistochemical analysis. Spermatogenetic activity was also assessed. NAIP and survivin expressions were significantly reduced following varicocele induction when compared to sham animals whereas PDRN-treated rats showed an increase in NAIP and survivin levels. Immunohistochemistry revealed an enhanced expression of NAIP and survivin with a characteristic pattern of cellular localization following PDRN treatment. Moreover, administration of PDRN significantly restored spermatogenic function in varicocele rats. PDRN may represent a rational therapeutic option for accelerating recovery from depressed testicular function through a strategic modulation of apoptosis in experimental varicocele.

1. Introduction

Varicocele is the most common cause of infertility in men [1] and the exact pathophysiological mechanism by which it impairs fertility in affected men remains unknown [2, 3]. Consequently, the early diagnosis of varicocele is necessary before testicular damage might occur and, as indicated by several clinical studies, the varicocele repair is possible through surgical procedure [4–7]. Although many advances have occurred in the treatment of varicocele, it still represents an important and challenging aspect of basic research (male reproductive physiology and endocrinology, pathophysiology, and pharmacology of reproduction and fertility) and medical practice for urologists, pediatric surgeons, and general physicians, to date [4–7].

The development of varicocele-related testis damage may be caused by disruption of homeostasis between cell proliferation and cell death [8–12]. This phenomenon is also related to different pathophysiological mechanisms (imbalance between reactive oxygen species and seminal antioxidants, lipid peroxidation, DNA fragmentation, and apoptosis) in testis following varicocele induction [13, 14]. Basically, apoptosis is a physiological process by which a sequence of intracellular events results in the programmed elimination of a cell from its environment [15, 16]. Specifically, alterations in the apoptosis of germ cells may be crucial in varicocele-related human infertility [17] and, as a direct consequence, targeting apoptosis may represent an alternative and rational therapeutic strategy in the treatment of varicocele complications [18–21].

Emerging contributors in this context are the inhibitors of apoptosis proteins (IAPs), which halt cell death in response to diverse stimuli [22]. IAPs family influences apoptosis by direct inhibition of caspases and modulation of the transcription factor nuclear factor-kB (NF-kB). Eight mammalian IAPs are known at present: X-chromosome-linked IAP (XIAP), cellular IAP1 and IAP2 (cIAP1 and cIAP2), neuronal apoptosis inhibitory protein (NAIP), survivin, BRUCE, livin, and testis-specific IAP (Ts-IAP).

NAIP was originally identified while searching for a gene on chromosome 5q13 responsible for childhood muscular atrophy and is also associated with spinal muscular dystrophy [23, 24]. Survivin too has a central role in the negative regulation of apoptosis; however the exact mechanism by which survivin controls programmed cell death has not yet been clarified [25].

It has been indicated that NAIP contrasts apoptosis by inhibition of the executioner caspase-3 and caspase-7 [26] while survivin has been shown to modulate the executioner caspase-3 [27]. However, recent findings suggest that IAPs have a much broader spectrum of action than promoting cell survival by caspase regulation; indeed, a crucial function of some IAPs consists in the regulation of inflammatory and innate immune signaling pathways, a function attributed to their E3 Ub-ligase activities [28].

Polydeoxyribonucleotide (PDRN) is the active fraction extracted from trout spermatozoa used for tissue repair [29] and, acting through stimulation of the adenosine A2A receptor (A2AR), is able to induce vascular endothelial growth factor (VEGF) production during pathologic conditions of low tissue perfusion [30]. This evidence prompted us to investigate the effect of PDRN on experimental varicocele; our previous published data indicated that A2AR stimulation could represent an interesting target to positively modulate the harmful pathophysiological signaling which characterizes the experimental varicocele [31, 32]. Indeed, it has been also shown that PDRN improves the innate mechanism of neoangiogenesis, through compensatory oxygen and metabolite supply to testis, thereby enhancing testicular function and restoring spermatogenic function [31, 32].

In light of this background, we explored the effect of PDRN on testis neuronal apoptosis inhibitory protein (NAIP) and survivin expression in an experimental model of varicocele.

2. Materials and Methods

2.1. Animals and Experimental Procedures. The protocol was approved by the Committee of Animal Health and Care of University of Messina and all animal procedures were carried out according to Guide for the Care and Use of Laboratory Animals. A total of 42 male Sprague-Dawley adolescent rats aged 7 weeks and weighing 200 to 225 g were used [33]. During the experiments, the animals were maintained under controlled environmental conditions (12-hour light/dark cycle, temperature approximately 23°C) and provided with standard laboratory food and water *ad libitum*. After induction of anesthesia with an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg), varicocele was induced as previously described elsewhere [11, 31, 32, 34]. Briefly, varicocele was induced by an abdominal midline incision. The left renal vein, inferior vena cava, and left spermatic vein were identified and a clamp was passed behind the left renal vein just distal to the spermatic vein insertion. A 4-zero silk ligature was loosely placed around the left renal vein at this site and a rigid hydrophilic guide wire of 0.64 mm in diameter was placed on the left renal vein. The ligature was tied around the vein over the top of the guide wire. The guide wire was then withdrawn and the vein allowed to expand to the limits of the ligature causing the vein diameter to be decreased to approximately half of its original diameter. The renal vein and spermatic vein in each animal were dilated immediately. The midline incision was closed in 2 layers with 3-zero silk suture. Sham-operated rats underwent the same vertical midline incision, and the suture was also placed, but it was not tied. Animals were housed one per cage only during the first week following surgical procedures in all experimental groups to minimize the impact and time of social isolation [35].

Twenty-eight days after the creation of varicocele, animals were randomized to the following treatment: vehicle (0.9% NaCl solution 1 mL/kg i.p., daily), PDRN (8 mg/kg i.p., daily), PDRN + 3,7-dimethyl-propargylxanthine (DMPX, a specific adenosine A2A-receptor antagonist, 0.1 mg/kg i.p., daily), varicocelectomy, and varicocelectomy + PDRN (8 mg/kg i.p., daily).

Twenty-one days after randomization, all animals were euthanized by sodium pentobarbital overdose, and the left testis was harvested to evaluate NAIP and survivin expression by use of real-time quantitative polymerase chain reaction (qRT-PCR), western blot, and immunohistochemical analysis previously shown elsewhere [29–32]. Other molecular hallmarks of apoptosis (i.e., Bcl-2, BAX) and/or cell proliferation (i.e., VEGF) were previously examined in our research laboratory [31].

2.2. Real-Time Quantitative PCR (qRT-PCR) for NAIP and Survivin. For the gene expression study, the extraction of total mRNA was performed from tissue testis using TRIZOL (Invitrogen, Milan, Italy) under sterile conditions, following the manufacturer's protocol. For each sample, 5 μg of mRNA was reverse-transcribed into cDNA through cDNA Archive High-Capacity Reverse Transcription kit (Life Technologies, Foster City, CA, USA). cDNA was stored at −20°C and it

was used to quantify by TaqMan Gene Expression Assay (Life Technologies) the amount of NAIP (Rn01757809_m1; Life Technologies, Foster City, CA, USA) and survivin (Rn00574012_m1; Life Technologies, Foster City, CA, USA), using the instrument SDS 7300 Real-Time PCR (Applied Biosystems, Foster City, CA, USA). The result was expressed using $2^{-\Delta\Delta CT}$ method (normalized versus varicocele + vehicle). β-actin (Rn00667869_m1; Life Technologies, Foster City, CA, USA) was used as endogenous control.

2.3. Isolation of Proteins and Determination of NAIP and Survivin by Western Blot Analysis. Samples from testis were homogenized in lysis buffer (1% Triton; 20 mM Tris/HCl, pH 8.0; 137 mM NaCl; 10% glycerol; 5 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 1% aprotinin; 15 μg/mL leupeptin) and centrifuged at 15000 rpm for 15 min at 4°C, and the protein content was determined by using the DC protein assay (Biorad, Milan, Italy). Protein samples were denatured in 2X Laemmli sample buffer (Biorad, Milan, Italy) and separated by SDS-PAGE. The separated proteins were transferred onto a nitrocellulose membrane using a transfer buffer (39 mmol/L glycine, 48 mmol/L Tris, pH 8.3, and 20% methanol) at 200 mA for 1 hour. The membranes were stained with Ponceau S (0.005% in 1% acetic acid) to confirm equal amounts of protein and were blocked with 5% nonfat dry milk in TBS-0.1% Tween for 1 hour at room temperature, washed 3 times for 10 minutes each in TBS-0.1% Tween, and incubated with a primary antibody for NAIP (Abcam, Cambridge, MA, USA) and survivin (Cell Signaling, Beverly, MA, USA) in TBS-0.1% Tween overnight at 4°C. After being washed 3 times for 10 minutes each in TBS-0.1% Tween, the membranes were incubated with a secondary antibody peroxidase-conjugated goat anti-rabbit immunoglobulin G (Pierce, Rockford, IL, USA) for 1 hour at room temperature. The protein signal was quantified by scanning densitometry using a bioimage analysis system ECL plus (Thermo Scientific, Waltham, MA, USA). The results were expressed as relative integrated intensity subtracting the respective backgrounds represented by β-actin (Cell Signaling, Beverly, MA, USA) expression.

2.4. Immunohistochemistry. Excised rat testes were longitudinally sectioned, formalin-fixed, and paraffin-embedded. Parallel serial sections (4 μm) were cut and antigen retrieval was performed using 0.01 M sodium citrate buffer heated for 30 minutes in microwave oven. Tissues were overnight incubated at 4°C with polyclonal rabbit anti-NAIP (Abcam, Cambridge, MA, USA) and monoclonal rabbit anti-survivin (Cell Signaling, Beverly, MA, USA) antibodies, diluted 1 : 25 and 1 : 400, respectively.

Secondary antibody was provided by Innovex (Richmond, CA, USA) and the location of the reaction was visualized with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Aldrich, Milan, Italy). Slides were counterstained with hematoxylin and mounted with coverslips.

2.5. Spermatogenic Activity. Spermatogenesis was quantified in fifty tubular cross sections for each animal, using Johnsen's score system. A score of 1–10 was given to each seminiferous tubule, depending on the maturation rate of the germ cells: 10: full spermatogenesis; 9: slightly impaired spermatogenesis, many late spermatids, and disorganized epithelium; 8: less than five spermatozoa per tubule, few late spermatids; 7: no spermatozoa, no late spermatids, and many early spermatids; 6: no spermatozoa, no late spermatids, and few early spermatids; 5: no spermatozoa or spermatids, many spermatocytes; 4: no spermatozoa or spermatids, few spermatocytes; 3: spermatogonia only; 2: no germinal cells, Sertoli cells only; 1: no seminiferous epithelium [36].

2.6. Statistical Analysis. All data are expressed as mean ± standard error of the mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc evaluation. $P < 0.05$ was considered statistically significant.

2.7. Compounds. DMPX and 0.9% NaCl solution were obtained from Sigma (Milan, Italy).

3. Results

3.1. Testis NAIP and Survivin mRNA Levels. Testis from sham animals had measurable levels of NAIP and survivin evaluated as mRNA level (Figures 1(a) and 1(b)). Testis from varicocele-injured rats showed a significant decrease in NAIP and survivin mRNA expression compared to sham animals (Figures 1(a) and 1(b)). The treatment with PDRN markedly increased NAIP and survivin mRNA expression. However, the effect of PDRN was abrogated by administration of DMPX. Varicocelectomy alone did not modify significantly the NAIP and survivin expression compared to sham animals while the concomitant administration of PDRN increased mRNA levels of these IAPs (Figures 1(a) and 1(b)).

3.2. NAIP and Survivin Expression by Western Blot. Varicocele produced a significant reduction in both testicular NAIP and survivin expression in comparison with sham animals (Figures 2(a) and 2(b)). Administration of PDRN markedly augmented both members of IAPs family. The concomitant administration of DMPX plus PDRN abrogated the effects of PDRN on the antiapoptotic proteins expression (Figures 2(a) and 2(b)). Varicocelectomy alone did not modify significantly the NAIP and survivin expression whose levels are similar to sham animals. Varicocelectomy plus PDRN increased the NAIP and survivin expression but at significantly lower levels compared to those revealed in animals treated with PDRN alone (Figures 2(a) and 2(b)).

3.3. NAIP and Survivin Detection by Immunohistochemical Analysis. NAIP and survivin were also evaluated immunohistochemically. Specifically, NAIP immunostaining showed diffuse and strong positivity in spermatogonia, spermatozoa, and Leydig cells of the following groups: sham, varicocele + PDRN, and varicocelectomy + PDRN (Figures 3(a), 3(c), and 3(e)). Differently, just a focal immunoreaction was proved in sperm and Leydig cells of animals subjected to varicocelectomy alone (Figure 3(d)) and no immunostaining

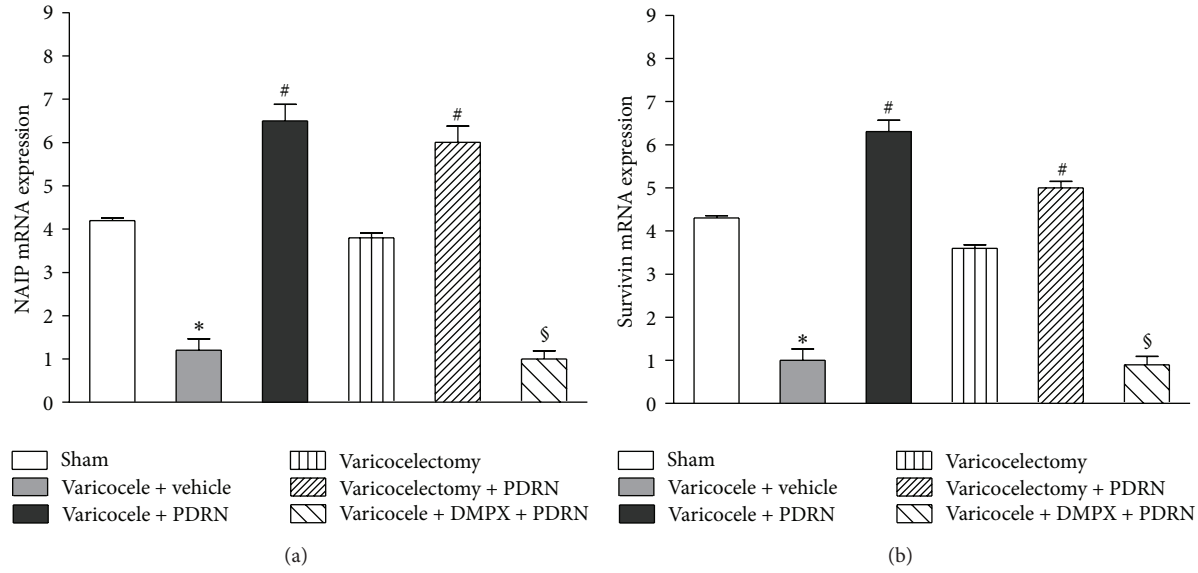


Figure 1: Expression of mRNA for NAIP (a) and survivin (b) (mean ± SEM of seven animals) in specimens of left testis of sham-operated rats and varicocele rats treated with vehicle (1 mL/kg, i.p.), PDRN (8 mg/kg, i.p.), DMPX (0.1 mg/kg, i.p.) plus PDRN (8 mg/kg, i.p.), varicocelectomy alone, or varicocelectomy plus PDRN (8 mg/kg, i.p.). $^{\#}P < 0.001$ versus varicocele + vehicle; $^{*}P < 0.001$ versus sham; $^{\S}P < 0.001$ versus varicocele + PDRN.

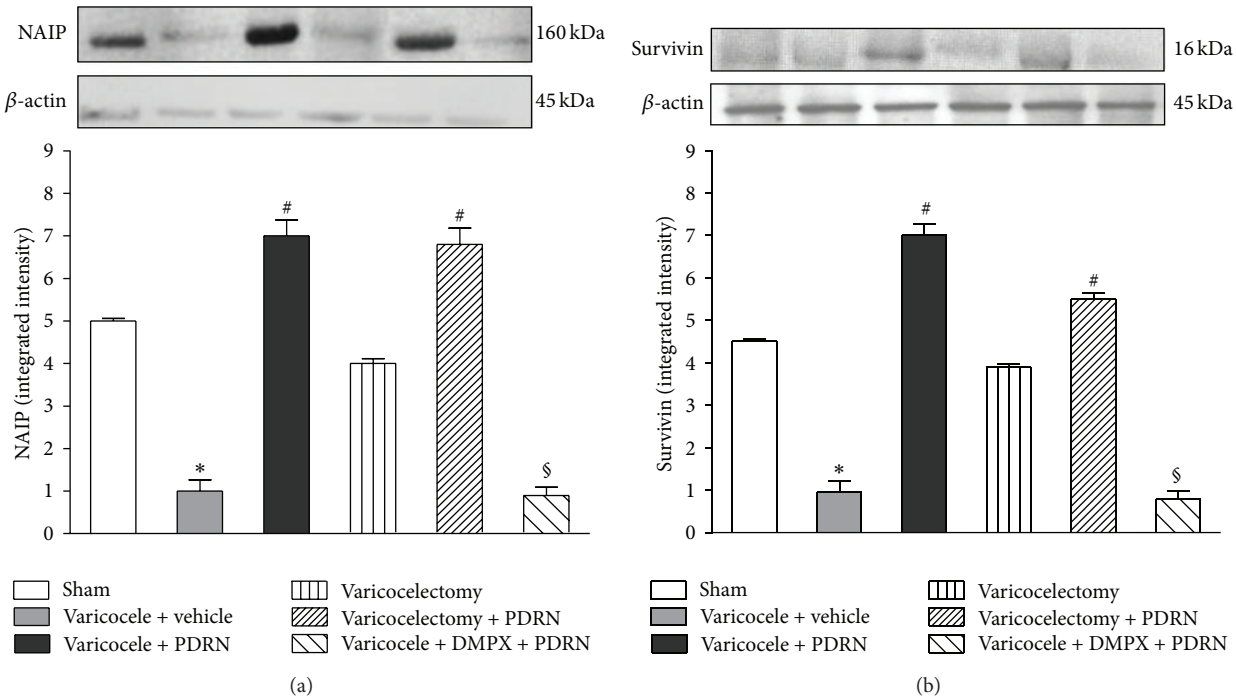
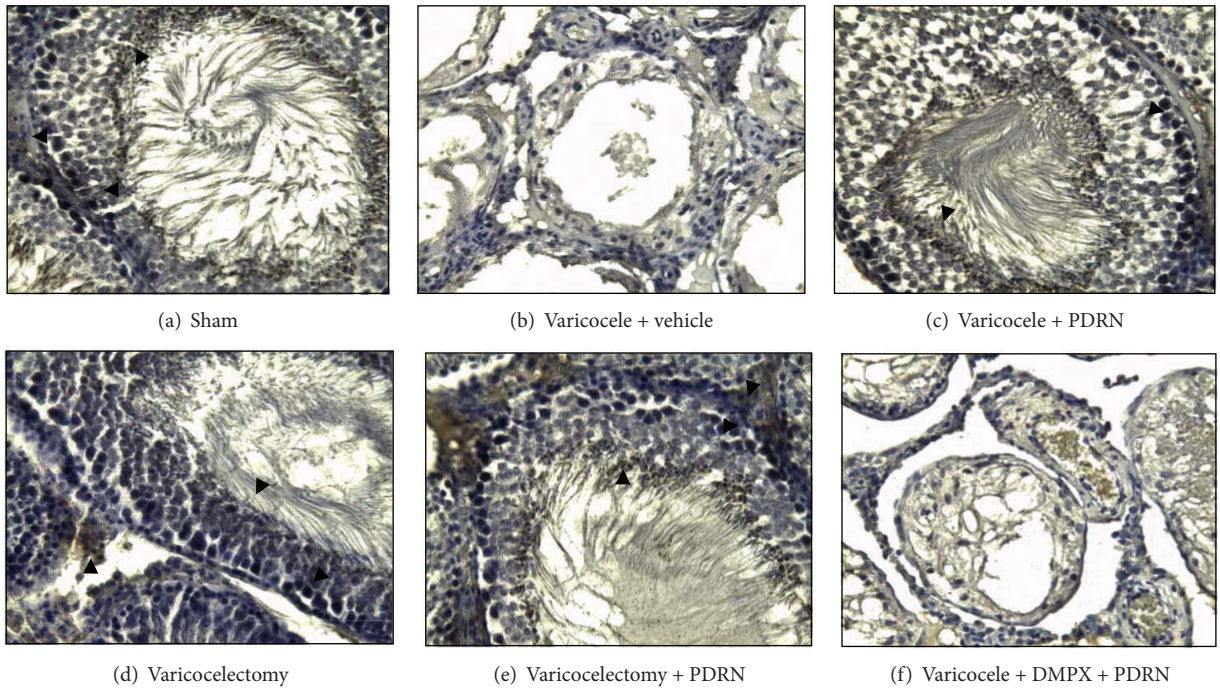


Figure 2: Chemiluminescence image highlighting NAIP (a) and survivin (b) expression and relative quantitative data (mean ± SEM of seven animals) in specimens of left testis of sham-operated rats and varicocele rats treated with vehicle (1 mL/kg, i.p.), PDRN (8 mg/kg, i.p.), DMPX (0.1 mg/kg, i.p.) plus PDRN (8 mg/kg, i.p.), varicocelectomy alone, or varicocelectomy plus PDRN (8 mg/kg, i.p.). $^{\#}P < 0.001$ versus varicocele + vehicle; $^{*}P < 0.001$ versus sham; $^{\S}P < 0.001$ versus varicocele + PDRN.

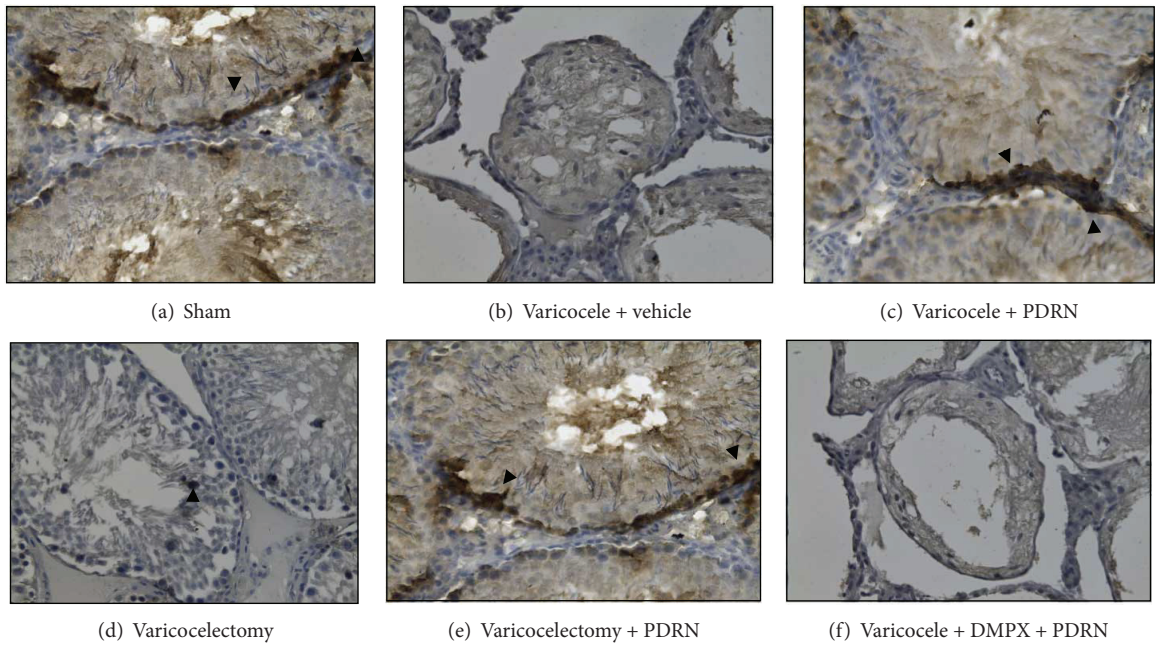
was detected in varicocele group (Figure 3(b)). Survivin immunostaining showed diffuse and strong positivity in spermatogonia of sham, varicocele + PDRN, and varicocelectomy + PDRN groups (Figures 4(a), 4(c), and 4(e)). By contrast, slightly focal immunoreactivity was observed in germ cells of rats subjected to varicocelectomy (Figure 4(d)) and a negative reaction for survivin in varicocele group (Figure 4(b)). DMPX, antagonist of the A2AR, abrogated



(a) Sham (b) Varicocele + vehicle (c) Varicocele + PDRN

(d) Varicocelectomy (e) Varicocelectomy + PDRN (f) Varicocele + DMPX + PDRN

Figure 3: High-power-view light micrograph of a left testis harvested from sham-operated rats and varicocele rats administered with vehicle (1 mL/kg, i.p.), PDRN (8 mg/kg, i.p.), DMPX (0.1 mg/kg, i.p.) plus PDRN (8 mg/kg, i.p.), varicocelectomy alone, or varicocelectomy plus PDRN (8 mg/kg, i.p.) at day 21 showing NAIP immunostaining (original magnification ×200). Arrowheads show positive cells.



(a) Sham (b) Varicocele + vehicle (c) Varicocele + PDRN

(d) Varicocelectomy (e) Varicocelectomy + PDRN (f) Varicocele + DMPX + PDRN

Figure 4: High-power-view light micrograph of a left testis harvested from sham-operated rats and varicocele rats administered with vehicle (1 mL/kg, i.p.), PDRN (8 mg/kg, i.p.), DMPX (0.1 mg/kg, i.p.) plus PDRN (8 mg/kg, i.p.), varicocelectomy alone, or varicocelectomy plus PDRN (8 mg/kg, i.p.) at day 21 showing survivin immunostaining (original magnification ×200). Arrowheads show positive cells.

PDRN effects on both NAIP and survivin expression (Figures 3(f) and 4(f)).

3.4. Spermatogenic Activity. Rats subjected to varicocele and treated with vehicle showed a significant impairment in spermatogenic activity after 21 days (Table 1). In contrast, administration of PDRN significantly increased Johnsen's score while DMPX cotreatment did not improve the altered spermatogenesis, and it abated the beneficial effects of PDRN (Table 1). Varicocelectomy alone significantly reverted the

Table 1: Evaluation of spermatogenesis (Johnsen's score) of left testis in different experimental groups.

Experimental treatment	Johnsen's score (mean ± SEM)
Sham	9.5 ± 0.3
Varicocele + vehicle	2.9 ± 0.4*
Varicocele + PDRN	8.7 ± 0.5#
Varicocele + PDRN + DMPX	2.9 ± 0.4§
Varicocelectomy	8.4 ± 0.4#
Varicocelectomy + PDRN	8.9 ± 0.4#

*$P < 0.001$ versus sham; #$P < 0.001$ versus varicocele + vehicle; §$P < 0.001$ versus varicocele + PDRN.

altered spermatogenic activity while varicocelectomy with PDRN group did not observe any additional significant restorative effect of spermatogenic function (Table 1).

4. Discussion

We have previously shown that PDRN, acting as a selective agonist on adenosine A2AR, was effective in improving the experimental varicocele [31, 32]. Specifically, PDRN succeeded in ameliorating the histologic damage and the poor spermatogenesis in the affected rats.

The present data represent novel and adjunctive findings distinct from those published previously [31, 32]. In fact, PDRN administration resulted in a specific and highly regulated action on apoptosis, particularly through the control of expression of NAIP and survivin. Indeed, it appears useful to point out that varicocele induction and subsequent postsurgical isolation of rats could interfere with our results in this experimental model [35, 37, 38]. Consequently, since social isolation in rodents is associated with a range of behavioural and physiological changes, some of which indicate a stress response, we have minimized the impact and time of social isolation (only one week) in all experimental groups. This approach allowed us to promote adequate healing and postoperative recovery and prevent the spread of infections.

As a first step, our experimental findings suggest that NAIP and survivin could play a pivotal role in the pathogenesis of varicocele. In fact, NAIP and survivin were markedly reduced following varicocele induction while PDRN administration markedly augmented both members of IAPs family; moreover, the concomitant administration of DMPX, a specific adenosine A2AR antagonist, abrogated the effects of PDRN on the antiapoptotic proteins.

Indeed, our sham testis samples had measurable levels of NAIP in accordance with previous data obtained on human tissues [23]; this experimental finding, indicating the presence of NAIP within tissues that are not usually affected a neuron disease, would suggest that NAIP has functional/physiological implications in a broad spectrum of tissues, exerting both antiapoptotic and immunoendocrine modulatory functions.

As a matter of fact, qRT-PCR and western blot analysis revealed an enhanced expression of NAIP in testis samples following PDRN administration compared to those vehicle-treated and/or after varicocelectomy, alone. This leads us to speculate that PDRN may reawake the depressed apoptotic machinery. In agreement with this latter observation, immunostaining for NAIP revealed that PDRN significantly causes diffuse and strong positivity in spermatogonia and spermatozoa, confirming the crucial role of the apoptotic signals in germ cells differentiation and testis endocrine and spermatogenic function [17]. This observation is intriguing in terms of infertility management in mammalian species because a previous work by Maier and coworkers indicated that (i) the expression pattern of NAIP in testis did not necessarily reflect the immunohistochemical data and (ii) the increase of NAIP might be also related to a generic compensatory mechanism involving inflammatory cells as macrophages in target tissues [23].

The difference between our results and those previously described could be easily due to the different species examined (rat versus human), different microenvironment (pathological versus normal condition), and different therapeutic approach targeting specific molecular pathways.

So far, current knowledge indicates that, besides NAIP, survivin is highly expressed in adult human testis, especially in the nuclei of mature spermatocytes, and its downregulation is linked with spermatogenic failure [39–41]. Moreover, recent experimental evidences indicated that survivin could be associated with a direct control of cell survival: it acts as protein inhibitor of apoptosis by inhibiting caspases and antagonizing mitochondria-dependent apoptosis [42].

In our model of experimental varicocele we found an enhanced expression of survivin following PDRN administration in agreement with previous experimental observations by Bayomy and coworkers [43]. They showed that a left-sided varicocele causes bilateral testicular histological abnormalities, angiogenesis, and apoptosis, including a marked increase in the expression of caspase-3 and a marked decrease in the expression of survivin [43]. In the present research, we found that survivin immunostaining shows a more diffuse and strong positivity particularly in spermatogonia and Leydig cells of PDRN-treated rats; as expected, DMPX, blocking A2ARs, abrogated PDRN effects on survivin expression in varicocele-induced rats.

The positive modulation of IAPs molecular pathway obtained by PDRN treatment was also supported in our model by the evidence of a significant restorative effect of spermatogenic function. These effects are comparable to those obtained in rats subjected to varicocelectomy [4–7], suggesting that PDRN could represent a novelty in terms of valuable medical therapy of varicocele-induced infertility.

So far, the present data allow us to better clarify our previous experimental observations about the molecular pathways involved in testis following surgical induction of varicocele in rats. As summarized in Figure 5, the stasis and the consequent hypoxic-ischemic state following varicocele may severely damage the testicular function. Then, the activation of A2AR by polynucleotides (PDRN) induces a further production of VEGF that, in turn, improves the angiogenic response in injured testis. At this stage, it is very likely that a pathophysiological cross-talk occurs between testicular flow,

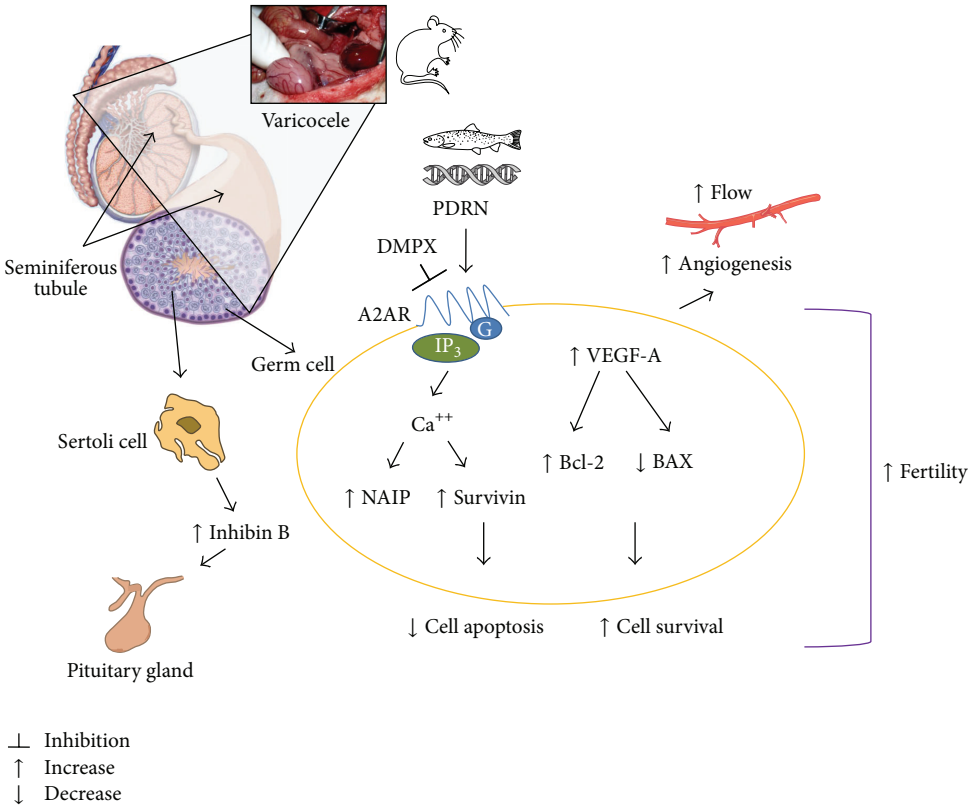


Figure 5: Scheme showing some of the molecular pathways varicocele-induced and their strategical modulation by PDRN.

hormones, and cell homeostasis pathways and a plethora of signaling molecules including IAPs could have a great impact on angiogenesis, fertility, and testicular function [29–32, 44].

Altogether, these findings point out the key role played by NAIP and survivin in the positive regulation of apoptosis during varicocele, also confirming that, in the context of translational medicine, these members of IAPs family could represent an interesting and innovative target in testis homeostasis. Consequently, a specific approach with PDRN, a ligand of A2AR, could represent a valuable therapeutic strategy for varicocele management in subfertile patient; moreover, PDRN may also offer a structural model for the design of new analog compounds helpful for male infertility.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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Review Article

The Role of Dendritic Cells in Fibrosis Progression in Nonalcoholic Fatty Liver Disease

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Nonalcoholic fatty liver disease (NAFLD) is the most frequent cause of chronic liver disease. NAFLD encompasses a wide range of pathologies, from simple steatosis to steatosis with inflammation to fibrosis. The pathogenesis of NAFLD progression has not been completely elucidated, and different liver cells could be implicated. This review focuses on the current evidence of the role of liver dendritic cells (DCs) in the progression from NAFLD to fibrosis. Liver DCs are a heterogeneous population of hepatic antigen-presenting cells; their main function is to induce T-cell mediated immunity by antigen processing and presentation to T cells. During the steady state liver DCs are immature and tolerogenic. However, in an environment of chronic inflammation, DCs are transformed to potent inducers of immune responses. There is evidence about the role of DC in liver fibrosis, but it is not clearly understood. Interestingly, there might be a link between lipid metabolism and DC function, suggesting that immunogenic DCs are associated with liver lipid storage, representing a possible pathophysiological mechanism in NAFLD development. A better understanding of the interaction between inflammatory pathways and the different cell types and the effect on the progression of NAFLD is of great relevance.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most frequent cause of liver disease in the Western world. Its prevalence among liver diseases, and in the general population, has been rising in recent years along with associated conditions, including obesity, insulin resistance, metabolic syndrome, and diabetes. Interestingly, in a recent study, we analyzed the epidemiology of NAFLD in the Americas based on the prevalence of obesity, and we found that the estimated prevalence rates of NAFLD were higher in the United States (29%) and in Mexico (26%), countries that also have a high prevalence of obesity. The prevalence of NAFLD and obesity is directly correlated (Figure 1) [1]. In addition, the prevalence of NAFLD in Europe and the Middle East ranges from 20% to 30% [2–5], whereas the prevalence of NAFLD in Japan and China is similar to that in Europe [6].

NAFLD is characterized by fat deposition in hepatocytes, mainly in the form of triglycerides. In most individuals, hepatic steatosis is present as a benign condition. However, a proportion of patients with NAFLD develops inflammation and necrosis with or without fibrosis and evolves to nonalcoholic steatohepatitis (NASH). The pathogenesis of NASH has not been completely elucidated; multiple inflammatory and noninflammatory factors are implicated. The hepatic lipid deposition induces oxidative stress and hepatic cell injury, with subsequent inflammatory cell infiltration. Lipid accumulation triggers proinflammatory cytokines (CTN) linked to the activation of hepatic cellular subtypes that sustain CTN production (Figure 2) [7]. Interestingly, in recent years, it has

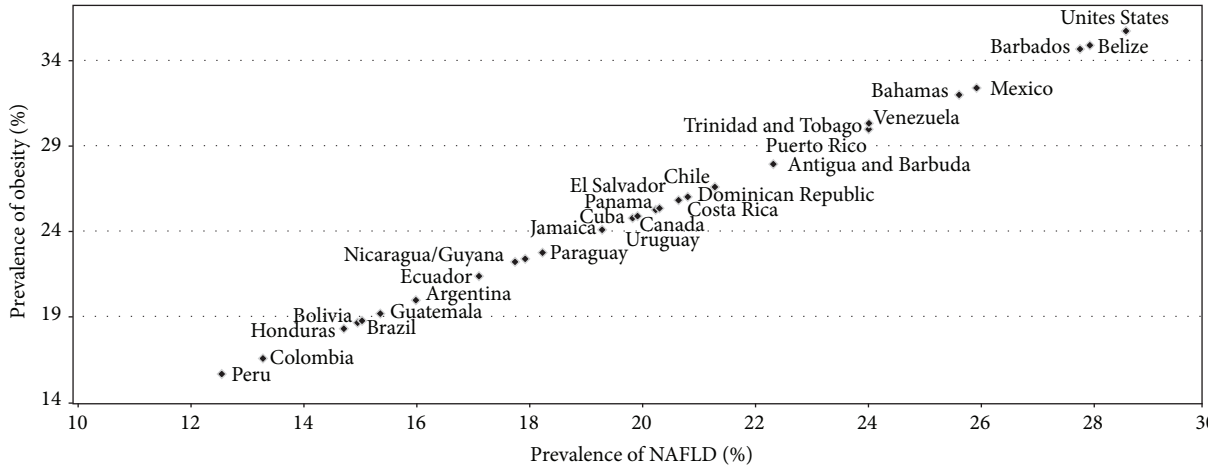

Figure 1: Correlation between the prevalence of obesity and NAFLD in the Americas. The graph was built with data from the prevalence of obesity for each country; NAFLD prevalence was estimated assuming that about 80% of obese patients might develop NAFLD in the Americas countries. Reproduced with permission of the journal [1].

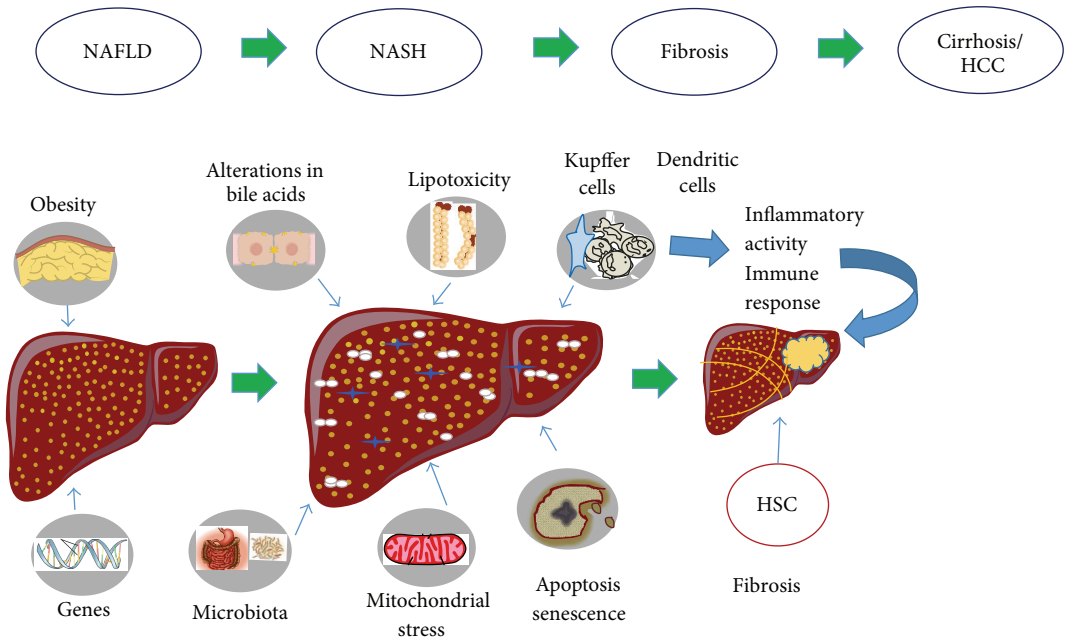

Figure 2: Schematic mechanisms involved in the progression of NAFLD to NASH. Lifestyle factors such as obesity and genetic predispositions contributing to the development of insulin resistance and hepatic steatosis. In the following steps multiple parallel metabolic hits lead to cellular damage, via a process called "lipotoxicity." Injured hepatocytes initiate an inflammatory response, predominantly via toll-like receptors, and activate proinflammatory signaling pathways in the setting of increased adipokine levels. Also the apoptosis and senescence are alternative cell fates that are likely to be of greater importance to disease progression. Direct recruitment of Kupffer cells and other components of the innate immune response such as dendritic cells occurs with activation of the inflammasome and the coordinated release of proinflammatory and profibrogenic cytokines and ligands. Hepatic stellate cells (HSCs) are subsequently activated to produce extracellular matrix leading to progressive fibrosis and cirrhosis and its complications such as hepatocellular carcinoma (HCC).

been reported that liver dendritic cells (DCs) appear to be involved in liver fibrosis in NAFLD [8], although the role of DC in liver disease has not been clearly defined. This review focuses on the current evidence of the role of DC in fibrosis progression in NAFLD.

2. Liver Dendritic Cells

Liver DCs are a heterogeneous population of hepatic sinusoidal antigen-presenting cells, found preferentially in the periportal and pericentral space and constituting less than 1%

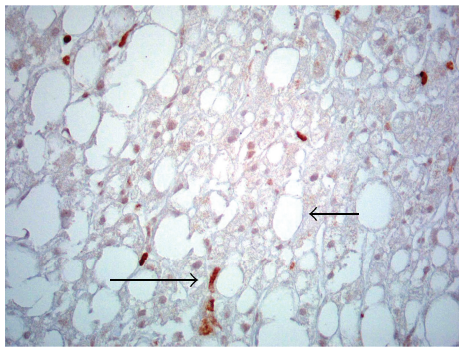

Figure 3: Immunohistochemistry for hepatic dendritic cells. Some dendritic cells are CD11C positive (large arrow) in a patient with nonalcoholic fatty liver disease. The vast majority of hepatocytes contained vacuoles of lipids (small arrow) (grade 3 micro- and macrovesicular steatosis) (×400).

Table 1: Different dendritic cell subtypes.

Subset	Phenotype
Lymphoid	CD8α^+, B220$^-$, CD11b$^-$, and DX5$^-$
Myeloid	CD8α^-, B220$^-$, CD11b$^+$, and DX5$^-$
Plasmacytoid	CD8α^-, B220$^+$, CD11b$^-$, and DX5$^-$
Mixed lymphoid + myeloid	CD8α^-, B220$^-$, CD11b$^-$, and DX5$^-$
NKDC	CD8α^-, B220$^+$, CD11b^{lo}, and DX5$^+$

NKDC: natural killer dendritic cell.
Modified from [9].

of the nonparenchymal cells [9]. They are part of the hepatic reticuloendothelial system, which also includes sinusoidal endothelial cells and Kupffer cells [10]. In particular, DCs have a migratory capacity and a remarkable ability to produce CTN; this feature distinguishes liver DC from Kupffer cells and promotes the adaptive immune system response [11]. Liver DC can be defined in broad terms, such as CD45$^+$ cells with a high expression of major histocompatibility complex class II (MHCII) and the absence of other hematopoietic markers [11], but many markers are needed to identify dendritic cell subsets [12]. In murine models, three subsets of hepatic DC (CD19−, CD11c+) have been characterized: lymphoid (CD8α+, B220−, and CD11b−), myeloid (CD8α−, B220−, and CD11b+), and plasmacytoid (B220+, CD11b−). The first two subtypes are denoted as conventional DC and are located at the periportal region and central veins whereas the plasmacytoid DC subtypes are located in the liver parenchyma. Another liver DC subtype containing mixed features of the myeloid and lymphoid subtypes has been characterized [13]. In addition, a new subset called natural killer dendritic cell which has an intermediate development state has been recognized [14]. On Table 1 the different liver DC subsets with its markers are shown. Figure 3 shows a hepatic DC displaying positivity for CD11c.

Natural killer T cells (NKT) are a group of T lymphocytes that express both NK, such as CD161 and CD94, and T-cell markers. These cells comprise 20 to 35% of mouse liver lymphocytes and 10 to 10% of rat and human liver lymphocytes. NKT recognize lipid antigens and respond to injury stimulating Kupffer cells, hepatocytes, and DC [15]. In animal [16] and human [17] NAFLD models, an inverse correlation between NKT cells and accumulation of hepatic lipid has been reported. NKT cells generate both Th1 and Th2 cytokines; therefore, NKT depletion may result in an increase of Th1 cytokines such TNF-α, IL-2, and IFN-γ [18]. In contrast, some other studies have shown NKT cells accumulation as NAFLD progresses [19]. Liver DCs stimulate the release of proinflammatory CTN release by NKT cells and become activated after the NKT cells population is eliminated [20].

Because of the heterogeneity of DC and our limited understanding of their role in human tissues, the contribution of these cells to the development of fibrosis is unclear at this point [21].

3. Liver Dendritic Cells in Health and Disease

DCs are derived from hematopoietic progenitor cells in the bone marrow. *In vitro* and *in vivo* studies have shown that DCs are potent inducers of immune responses. Their main function is to induce T-cell mediated immunity by antigen processing and presentation to T cells. The features of DC include their ability to capture antigens, the capability of processing and presenting peptides, and migration to lymphoid organs [22]. On the other hand, DC can prevent activation of T cells, playing an important role in preventing autoimmunity and rejection [23].

DCs process and present antigens to T lymphocytes. Fragments of antigens bound to major histocompatibility complex (MHC) molecules are recognized by receptors in the T cells. MHC class I molecules stimulate cytotoxic T cells, and MHCII molecules stimulate T helper cells. Activation of cytotoxic T cells can kill target cells, whereas activation of T helper cells can induce regulatory immune properties. The induction of a T-cell immune response requires peptide recognition by T cells. DCs bring antigens to T cells and express costimulatory molecules, facilitating the induction of the immune response [22].

DCs are present in the skin [24], inner lining of the airways [25], interstitial spaces of diverse organs [26], lymphoid tissues [27], and blood [28]. In humans, after contact with antigens, DCs migrate from the peripheral tissues to the lymphoid organs [29]. Self-antigens captured and presented by DC may induce tolerance [30]. DCs are capable of maintaining tolerance and inducing immunity in different contexts (steady state versus inflammation) [31]. Furthermore, it has been suggested that the role of DC is mainly determined by their state of development. DCs exist in two conditions: immature and mature. Most DCs in the peripheral tissues are in the immature state.

In vitro, immature dendritic cells (IDC) capture antigens, phagocyte particles [32], form pinocytic vesicles, and express receptors to mediate endocytosis, including C-type lectin receptors (such as the macrophage mannose receptor) and DEC-205, as well as Fcγ and Fcε receptors [33]. IDC in mice possess compartments rich in MHCII, where MHCII-peptide complexes are formed [34]. IDC may prompt tolerance by diverse mechanisms such as deletion of T cells and induction

of regulatory T cells. In this state, they express low levels of surface MHC class I and II and costimulatory molecules. They capture self-antigens and innocuous environmental proteins and target them to MHCII in the lysosomes, but they are not used for the formation of MHCII-peptide complexes [35]. IDC in lymphoid tissues perform endocytosis and express low levels of costimulatory molecules such as CD86 and CD40. The ability of IDC to form MHCII-peptide complexes may be important to tolerate T cells [23]. In the thymus, DCs delete self-reactive T-cell clones [36]. In animal models, IDC circulate through tissues, enter afferent lymphatics, and migrate to the T-cell area, where they die [28]. In this way, DCs play a role in developing immune tolerance: presenting self-antigens to T cells, deleting autoreactive lymphocytes, and inducing regulatory T-cell formation, which induces tolerance by suppressing the responses of T cells to stimuli [23].

When DCs are exposed to immune or inflammatory signals, including microbial products and proinflammatory CTN, they undergo maturation and they are directed to the T-cell areas of the lymphoid organs, such as the spleen and lymph nodes. CTN including IL-1, GM-CSF, and TNF-α stimulate human DC maturation, whereas IL-10 blocks it [37]. In addition, DC migration involves adhesion molecules and chemotactic factors [38]. Mature dendritic cells (MDC) have a reduced capacity to take up antigens but can participate in the immune response by stimulating T cells. They process antigens and present them bound to MHC molecules, initiating immunity [36]. Maturation causes changes in the phenotype of human DC. These changes include an increase in the production of MHCII-peptide complexes [35], expression of costimulatory molecules with the ability to bind T cells, and the production of CTN [39].

The changes in DC associated with maturation that makes them immunogenic include the redistribution of MHCII molecules from the intracellular compartments to the plasma membrane [40] and the expression of molecules that interact with receptors in T cells to increase adhesion and signaling. In mice identified molecules include liver fibrosis A-3/CD58, ICAM-1/CD54, and B7-2/CD86 [41]. In addition, human MDC synthetize CTN, chemokines (CHM), and receptors that promote their movement to lymphoid organs [42, 43].

The traffic of DC is a critical step for their immune function. *In vitro* studies have demonstrated that CHM are major regulators of DC migration. Both immature and MDC produce CHM, constitutively and by stimulation, respectively [44]. Inductors of the maturation of DC also change their migratory characteristics. Inflammatory CHM may function as signals for the localization of DC in nonlymphoid organs. This change may have a role in allowing DC to leave peripheral tissues. There is a large heterogeneity among DC in expression and responsiveness to CHM, modulated by their maturation.

CCR1, CCR2, and CCR5 are chemokine receptors responsible for migration of mice IDC to areas of inflammation [9]. In addition, maturation of DC induces a change in the expression of CHM receptors such as CCR7 and increases the levels of the platelet-activating factor (PAF) receptor. MDC are characterized by a high expression of CXCR4 and low levels of CCR4. These changes in the expression of CHM receptors and other ligands appear to be an important mechanism for localization and/or migration of DC in response to stimuli [43].

The increase in surface MHCII molecules is a hallmark of the maturation of DC. *In vitro* studies have shown that in IDC MHCII molecules are collected in endosomes and lysosomes, whereas, in MDC, MHCII molecules gather at the cell surface [45]. Dendritic cell lysosomes can confiscate antigens for long periods and form MHCII-peptide complexes [40]. Lysosomes may provide a mechanism for the regulation of the T-cell receptor ligands [23].

DCs mature by exposure to microbial or viral pathogens. In mice, microbial products enhance the production of CTN by DC, such as IL-12 [46]. Inflammatory CTN stimulate innate (natural killer cells) and acquired (B and T cells) immunity [22]. The toll-like receptor (TLR) and tumor necrosis factor receptors, mainly CD40, have a prominent role in the maturation of DC [31].

In humans, MDC initiate T-cell responses and can also differentiate the immune response to Th1 or Th2 types [47]. DC can affect the immune response in different ways according to the type of T cell that they stimulate. In mice, DC can activate cytotoxic T cells that interact with MHC class I-expressing cells. They can also activate T helper cells that can turn into Th1 cells in the presence of DC and IL-12. Th1-helper cells produce interferon-γ (IFN-γ) that activates macrophages. Macrophages promote differentiation of T cells into killer cells. On the other hand, IL-4 DCs induce T cells to differentiate into Th2 cells, which secrete IL-5 and IL-4. These CTN activate eosinophils and help B cells to produce antibodies [46].

The maturation of DC can occur not only in response to infectious agents but also during transplantation [48], contact allergy [49], and autoimmunity. In these circumstances, the necessary receptors for maturation have yet to be identified. TNF family members on mast cells and platelets and members of the hematopoietin family (such as granulocyte macrophage colony-stimulating factor, IL-4, and IL-13) influence DC development and maturation. These alternative incentives may produce DC with different functions or may be required in concert with TLR signaling for complete dendritic cell activity. In addition, different stimuli may produce different states of maturation [23].

DCs appear to also induce central and peripheral tolerance to autoantigens. In the thymus, T-cell deletion occurs, and, in lymphoid organs, this is caused by the induction of anergy or deletion of mature T cells. On the periphery, DC can capture and present autoantigens to T cells and induce tolerance to proteins that have no access to the thymus [22].

DCs play a role in diseases including infections, tumors, and infection with human immunodeficiency virus (HIV). MDC help in diffusing human HIV to T cells. Some tumors do not provoke a specific T-cell response; this may be because of the absence of functional DC or because of the secretion of factors that reduce the development and function of DC. When DCs exposed to tumor antigens are instilled, immunity develops. This may result in protection against tumors or a decrease in tumor size, which may represent a potential

therapeutic application [50]. DCs are also present in inactivated viruses, which indicates another area for development of vaccines that target them [51].

4. Liver Dendritic Cells in Liver Fibrosis

Liver fibrosis and cirrhosis are the end stages of chronic liver disease. Fibrosis is the result of an imbalance between synthesis and degradation of the extracellular matrix (ECM) [52]. The more extensive investigations about the cells contributing to hepatic fibrogenesis have focused on hepatic stellate cells (HSCs) and myofibroblasts (MFs). HSCs possess the capabilities of fibrillar collagen synthesis, chemotactic and vasoactive factor secretion, contractile activity, and production of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) [53]. MMPs and TIMPs regulate the turnover of ECM proteins [52]. Soluble factors and different cell types, including Kupffer cells, hepatocytes, and endothelium cells, are able to activate HSCs [54, 55]. Fibrosis is regulated by the adaptive and innate immune systems [52]. Myofibroblasts are the other kind of cells implicated in liver fibrogenesis by producing ECM [56]. Fibrosis is linked to chronic inflammation; therefore, the recruitment of immune cells is one of the hallmarks of fibrogenesis [57]. Recently, Rahman and Aloman proposed a potential mechanism for the contribution of DC to fibrogenesis [11]. They stated that DCs regulate the number and activity of cells involved in the development of fibrosis (such as natural killer cells and $CD8^+$ cells), and tissue remodeling, originally attributed to macrophages/monocytes, may be dependent on DC because of their ontogenetic relationship [11].

During the steady state, DCs are immature and tolerogenic. However, in an environment of chronic inflammation, DCs are transformed to a mature proinflammatory subset; interestingly, DCs prevent damage during acute injury [58]. The mature proinflammatory DCs facilitate the deposition of monocytes and activation of HSCs [58]. In addition, in a fibrosis model in mice, it was demonstrated that $CD11c^+$ DCs were elevated induced by the administration of thioacetamide (TAA) and recombinant leptin. In this situation, there was an increase in IL-6 and TNF-α with the activation of the HSCs [59]. Ibrahim et al. [60] have analyzed the link between lipid metabolism and DC function. The experiments were conducted in NASH mice induced with a methionine/choline-deficient diet, a mice liver fibrosis model induced with TAA, bile duct ligation-induced liver injury in rodents, and human cellular isolation from patients undergoing hepatic resection. Human DCs were lin^- $HLA\text{-}DR^+$, and mice DCs were $CD11c^+$ cells. They found two DC populations in both humans and mice defined by high (H-DC) or low (L-DC) lipid content. H-DCs had high concentrations of phospholipids and triglycerides. Cholesterol levels were similar between H-DC and L-DC. The different lipid profiles were associated with the lipid-rich hepatic microenvironment; steatohepatitic human livers showed an increased proportion of H-DC fraction, and mice with NASH also displayed a greater fraction of H-DC. It is noteworthy that H-DCs produced high levels of various proinflammatory CTN and CHM, while L-DCs were virtually nonproductive. The principal CTN found to be elevated were TNF-α, IFN-γ, IL-6, IL-4, and IL-2. H-DCs also showed an exaggerated response to TLR ligation and a strong T-cell proliferation. Furthermore, H-DC seemed to activate natural killer and natural killer T cells. This evidence suggests that immunogenic DCs are associated with liver lipid content [60], and in NAFLD, this could be an important pathophysiological mechanism with the interplay between the endocrine system and immune cells.

On the other hand, it has been suggested that DC could play a role in the regression of liver fibrosis. Jiao et al. [61] induced liver injury in a murine model using carbon tetrachloride (CCl_4) and evaluated fibrosis regression after cessation of insult in an environment depleted of DC. In concordance, expansion of DC accelerates hepatic fibrosis regression. Furthermore, Henning et al. [62], using an animal model, found that DCs ($CD11c^+$ $MHCII^+$ cells) limit $CD8^+$ T-cell expansion and restrict TLR expression and cytokine generation from Kupffer cells, neutrophils, and monocytes in NASH. Furthermore, ablation of DC cells resulted in delayed resolution of inflammation and fibroplasia.

There is some evidence that supports the modulation role of DC in liver fibrosis, but it is not clearly understood. Some evidence indicates that DCs promote liver fibrosis, while another study shows otherwise. Indeed, the markers used in the studies conducted so far do not differentiate the DC from other cells such as macrophages or monocytes; therefore, the current understanding about the role of DC is mostly based on the study of $MHCII^+$ myeloid or $CD11c^+$ cell populations [58]. In the future, efforts will need to concentrate on finding a model capable of differentiating human DC from other myelomonocytic cells to assess their role in liver diseases including NAFLD.

5. Conclusions

NAFLD is a complex disease that encompasses a wide range of pathologies, from steatosis to steatosis with inflammation to fibrosis. It will be very important in the near future to know how the inflammatory pathways interact with different cell types and immune tissues resulting in the progression of NASH. In addition, DCs also appear to play an important role in NAFLD; we have shown in this review that a reduction in DC results in a deterioration of NASH severity, suggesting a regulatory role for DC in NASH.

Glossary

CHM: Chemokines
CTN: Cytokines
ECM: Extracellular matrix
FBR: Fibrogenesis
HIV: Human immunodeficiency virus
HSC: Hepatic stellate cell
IDC: Immature dendritic cell
IL-12: Interleukin-12
DC: Dendritic cells
MDC: Mature dendritic cell
MHC: Major histocompatibility complex

MHCII: Major histocompatibility complex class II
MF: Myofibroblast
MMP: Matrix metalloproteinase
NAFLD: Nonalcoholic fatty liver disease
NASH: Nonalcoholic steatohepatitis
TIMPs: Tissue inhibitors of metalloproteinases
TAA: Administration of thioacetamide
TLR: Toll-like receptor.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Interleukin-1β Modulates Melatonin Secretion in Ovine Pineal Gland: *Ex Vivo* Study

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The study was designed to determine the effect of proinflammatory cytokine, interleukin- (IL-) 1β, on melatonin release and expression enzymes essential for this hormone synthesis: arylalkylamine-N-acetyltransferase (AA-NAT) and hydroxyindole-O-methyltransferase (HIOMT) in ovine pineal gland, taking into account the immune status of animals before sacrificing. Ewes were injected by lipopolysaccharide (LPS; 400 ng/kg) or saline, two hours after sunset during short day period (December). Animals were euthanized three hours after the injection. Next, the pineal glands were collected and divided into four explants. The explants were incubated with (1) medium 199 (control explants), (2) norepinephrine (NE; 10 μM), (3) IL-1β (75 pg/mL), or (4) NE + IL-1β. It was found that IL-1β abolished ($P < 0.05$) NE-induced increase in melatonin release. Treatment with IL-1β also reduced ($P < 0.05$) expression of AA-NAT enzyme compared to NE-treated explants. There was no effect of NE or IL-1β treatment on gene expression of HIOMT; however, the pineal fragments isolated from LPS-treated animals were characterized by elevated ($P < 0.05$) expression of HIOMT mRNA and protein compared to the explants from saline-treated ewes. Our study proves that IL-1β suppresses melatonin secretion and its action seems to be targeted on the reduction of pineal AA-NAT protein expression.

1. Introduction

The daily changes in environmental light conditions have profound impact on all aspects of vertebrate physiology. A chemical messenger of darkness is melatonin (N-acetyl-5-methoxytryptamine), indoleamine mainly synthesized in the pineal gland. Its production is under photoperiodic control via the suprachiasmatic nucleus, hypothalamic structure located in the anterior hypothalamus above the optic chiasm which generates an endogenous "circadian" rhythm with a period very close to 24 hours. [1, 2]. In all mammalian species, melatonin production is regulated by norepinephrine (NE), which is released from sympathetic nerve fibers exclusively at night [2]. Biosynthesis of melatonin is a well-characterized multistep sequence of reactions starting with the hydroxylation of tryptophan to 5-hydroxytryptophan (5-HTP) by an enzyme tryptophan hydroxylase (TPH). Next, aromatic amino acid decarboxylase (DDC) converts 5-HTP to 5-hydroxytryptamine (serotonin). Then, serotonin is transformed to N-acetylserotonin by arylalkylamine-N-acetyltransferase (AA-NAT). Finally, N-acetylserotonin is converted to melatonin by an enzyme hydroxyindole-O-methyltransferase (HIOMT) [3]. In all vertebrates, the key enzyme in melatonin production is AA-NAT, often called the melatonin rhythm enzyme. It is generally accepted that all mechanisms regulating melatonin synthesis converge at the control of AA-NAT enzyme activity [4]. The melatonin is not stored in the pineal parenchyma but is released directly into the general circulation and into the cerebrospinal fluid immediately after its formation [5]. Thus, the circulating levels of the indoleamine faithfully reflect pineal secretory activity [6]. Plasma level of melatonin increases at night, whereas diurnal levels of these hormone are relatively low [7].

Among numerous actions, melatonin plays a role of immunomodulator, regulating the development, differentiation, and function of lymphoid tissues. Moreover, diurnal and seasonal changes in immune function are thought to directly reflect changes in pineal melatonin production which suggests an important role of circulating melatonin in the development and maintenance of immune function [8]. The melatonin mediated photoperiodic effect on the immune system was evidently demonstrated in the studies performed on hamster. It was shown that the artificial shortening of day lengths or exogenous melatonin injection induced an increase in thymus weight and spleen hypertrophy [9, 10]. The results of other studies also showed that melatonin controls diurnal and seasonal rhythms of leukocyte proliferation [11], cytokine production [12], and NK cell activity in mammalian bone marrow cells. [13]. Melatonin regulates inflammatory and immune processes acting as both an activator and inhibitor of these responses. Melatonin demonstrates endocrine, paracrine, and autocrine effects in the leukocyte compartment and differentially modulates proinflammatory enzymes, controls production of inflammatory mediators such as cytokines and leukotrienes, and regulates the lifespan of leukocytes by interfering with apoptotic processes. Melatonin is a potent antioxidant that allows scavenging of oxidative stress in the inflamed tissues. It can affect lipoxygenase activity, which suggests that melatonin might promote early phases of inflammation on the one hand and contribute to its attenuation on the other hand, in order to avoid complications of chronic inflammation [14].

The process of interaction between the immune system and pineal gland seems to be bidirectional. However, the feedback effect of inflammatory response on the pineal gland neuroendocrine functions is poorly understood. It is well known that inflammatory mediators such as cytokines, prostaglandins, and histamine penetrate the region of brain during an immune/inflammatory challenge [15]. The immune mediators have easy access to the pineal gland because it is part of the brain lacking the blood-brain barrier. A few studies showed that the secretory activity of pinealocytes could be modified by the antigenic stimulation [16], histamine [17], cytokines [18, 19], and prostaglandins [20]. Moreover, it was previously described that the rat pineal astrocytes and microglia react to bacterial endotoxin such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA). The pineal gland reacts to LPS by the existence of membrane receptors for LPS, such as toll-like receptor 4 (TLR4) and cluster of differentiation 14 (CD14) [21]. It was described that the systemic inflammation induced by the injection of LPS stimulated the gene expression of potent and pleiotropic proinflammatory cytokine, interleukin- (IL-) 1β, directly in the rat pineal gland [22], which suggests that this cytokine may be involved in the inflammatory-dependent modulation of melatonin secretion.

Therefore, the current study was designed to determine the effect of IL-1β on melatonin release and expression of AA-NAT and HIOMT in ovine pineal gland, taking into account the immune status of animals before sacrificing, because our previous study carried out on the pituitary explants showed that immune status of organ donor may affect the responsiveness of the gland on this cytokine action [23].

2. Materials and Methods

2.1. Animals and Experimental Design. These studies were performed on adult, three-year-old blackface ewes in December during short day period. The animals were maintained indoors in individual pens and were exposed to natural daylight. The ewes were in good condition; that is, their body condition was estimated to be 3 according to a five-point scale [24], and the animals were acclimated to the experimental conditions for one month. The ewes were always within visual contact with other members of the flock to prevent isolation stress. The animals were fed a constant diet of commercial concentrates with hay and water available *ad libitum*. All procedures were performed with the consent of the Local Ethics Committee of Warsaw University of Life Sciences-SGGW.

The animals ($n = 12$) were randomly divided into two groups: control ($n = 6$) and LPS-treated ($n = 6$) ewes. Two hours after sunset the animals were intravenously (i.v.) injected by the appropriate volume of LPS from *E. coli* 055:B5 (400 ng/kg) (Sigma-Aldrich, St. Louis, USA) dissolved in saline (0.9% w/v NaCl) (Baxter, Deerfield, IL, USA) into the jugular vein. The maximum volume of LPS solution (10 mg/L) injected into any animal never exceeded 2.5 mL. The control group received an equivalent volume (based on their body weight) of NaCl. The efficiency of the LPS treatment to induce an inflammatory response in the animal was evaluated through the measurement of the animal's body temperature 1 h before and 3 h after the injection. All procedures of the *in vivo* experiment were performed under the dark using the red light.

2.2. Incubation of Pineal Explants. All animals were euthanized by decapitation three hours after the LPS or saline injection. The brain was immediately removed from the skulls, and the pineal gland was dissected and divided into four fragments (explants). The explants were first preincubated for 1 h in 24-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) with medium 199 (M199; 600 μL). The medium was replaced with fresh medium every 15 min. Next, all explants were incubated for additional 30 min in M199. Finally, the explants from each ewe were treated with (1) M199 only (control explants), (2) NE (10 μM; Sigma-Aldrich, St. Louis, USA) (a positive control), (3) IL-1β (75 pg/mL; Sigma-Aldrich, St. Louis, USA), or (4) NE + IL-1β and incubated for 3 h at 37°C (87% O_2, 5% CO_2). The incubation medium consisted of M199 HEPES with Earle's salts, sodium bicarbonate, and HEPES (25 mM) with penicillin-streptomycin (10 mL/L) (Sigma-Aldrich, St. Louis, USA). After 3-hour incubation, the explants were weighed, immediately frozen in liquid nitrogen, and stored at −80°C until assay.

2.3. Assays

2.3.1. Melatonin Assay. Melatonin was assayed in the experimental media according to the method of Fraser et al., [25]

Table 1: All genes analyzed by real-time PCR are listed with their full name and abbreviation.

GenBank Acc. number	Gene	Amplicon size [bp]	Forward/reverse	Sequence $5' \rightarrow 3'$
NM_001034034	GAPDH: glyceraldehyde-3-phosphate dehydrogenase	134	Forward	AGAAGGCTGGGGCTCACT
			Reverse	GGCATTGCTGACAATCTTGA
U39357	ACTB: beta-actin	168	Forward	CTTCCTTCCTGGGCATGG
			Reverse	GGGCAGTGATCTCTTTCTGC
NM_001076910	PPIC: cyclophilin C	131	Forward	ACGGCCAAGGTCTTCTTTG
			Reverse	TATCCTTTCTCTCCCGTTGC
BC108088.1	HDAC1: histone deacetylase 1	115	Forward	CTGGGGACCTACGGGATATT
			Reverse	GACATGACCGGCTTGAAAAT
NM_001009461	AANAT: arylalkylamine-N-acetyltransferase	154	Forward	CGAGAGGCCTTCATCTCTGT
			Reverse	GTCTCTCCTCATCCCACAGG
KC290950	HIOMT: hydroxyindole-O-methyltransferase	167	Forward	AGCTTCCATGAAGGGGATTT
			Reverse	AGGAGGCTCTCGATGACCAG
X54796.1	IL-1β: interleukin-1 beta	137	Forward	CAGCCGTGCAGTCAGTAAAA
			Reverse	GAAGCTCATGCAGAACACCA
NM_001206735.1	IL-1R1: interleukin 1 receptor, type I	124	Forward	GGGAAGGGTCCACCTGTAAC
			Reverse	ACAATGCTTTCCCCAACGTA

modified in our laboratory, using ovine antimelatonin serum (AB/S/01, Stockgrand Ltd., Surrey, UK). Synthetic melatonin (Sigma-Aldrich, St. Louis, USA) was used as a standard and [O-methyl-3H]-melatonin (Amersham PLC, Amersham, UK) as a tracer. The sensitivity of the assay was 16.8 ± 8.0 pg/mL and the intra- and interassay coefficients of variation were 10.5 and 13.2%, respectively.

2.3.2. Isolation of mRNA and Protein from Pineal Explants. The total RNA and protein from the explants were isolated using the NucleoSpin RNA/Protein Kit (MACHEREY-NAGEL Gmbh & Co., Düren, Germany). All steps of the isolation were performed according to manufacturer's protocol. The purity and concentration of the isolated RNA were quantified spectrophotometrically. The RNA integrity was confirmed by electrophoresis using 1% agarose gel stained with ethidium bromide.

2.3.3. Real-Time PCR Assay. To synthesize cDNA, the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, Waltham, USA) and 2 μg of total RNA were used. Real-Time RT-PCR was performed using the HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) and HPLC-grade oligonucleotide primers (Genomed, Warszawa, Poland). The primer sequences were designed using Primer 3 software [26, 27] (Table 1). One reaction mixture (total volume: 20 μL) contained 4 μL of PCR Master Mix (5x), 14 μL of RNase-free water, 1 μL of primers (0.5 μL each primer, working concentration 0.25 μM), and 1 μL of the cDNA template. The reactions were run on the Rotor-Gene 6000 instrument (Qiagen, Dusseldorf, Germany). The following protocol was used: 95°C for 15 min and 30 cycles of 95°C for 10 s for denaturation, 60°C for 20 s for annealing, and 72°C for 10 s for extension. A final melting curve analysis was performed to confirm the specificity of the amplification.

The relative gene expression was calculated using the comparative quantification option [28] of the Rotor-Gene 6000 software 1.7 (Qiagen). Four housekeeping genes were examined: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), histone deacetylase 1 (HDAC1), and cyclophilin C (PPIC). Endogenous housekeeping gene was chosen based on the result analysis performed with BestKeeper software [29]. ACTB was selected for normalization of the gene expression of enzymes involved in melatonin synthesis and HDAC1 was chosen for IL-1β and IL-1R1. The results are presented in arbitrary units, as the ratio of the target gene expression to the expression of the housekeeping gene.

2.3.4. Poly(A) Tail Length Determination. From the total RNA isolated as described above, mRNAs were isolated using NucleoTrap mRNA (MACHEREY-NAGEL Gmbh & Co., Düren, Germany). The mRNAs were processed using USB Poly(A) Tail Length (Affymetrix, Inc., Cleveland, USA), according to the manufacturer's protocol. In first step, using poly(A) polymerase, a limited number of guanosine and inosine residues were added to the 3′-ends of mRNA. In second step, the tailed-RNAs were converted to cDNA through reverse transcription using the newly added G/I tails as the priming sites. Next, control PCR amplification products were generated using a gene-specific forward and reverse primer set designed upstream of the polyadenylation site (Table 2). The second set of primers uses the gene-specific forward primer and the universal reverse primer provided with the kit to generate a product that includes the poly(A) tails of the gene of interest. The reactions were run on the Tgradient (Biometra GmbH, Goettingen, Germany).

Table 2: Gene-specific primers designed for the poly(A) tail length analysis.

GenBank Acc. number	Gene	Amplicon size [bp]	Forward/reverse	Sequence $5' \rightarrow 3'$
NM_001009461	AANAT: arylalkylamine-N-acetyltransferase	178	Forward	GAACAGTGACCGCTGACTCC
			Reverse	GCCTCCCCACCTTCTCTTTA
KC290950	HIOMT: hydroxyindole-O-methyltransferase	310	Forward	GTCTTGGCCAGAAAGTGAGC
			Reverse	ACCCAGGAGGAACCTCATCT

The following protocol was used: 94°C for 2 min and 30 cycles of 94°C for 10 s for denaturation, 60°C for 1 min for annealing and extension, after the cycles 72°C for 5 min for final extension, and cooling at 4°C for 1 min. After the PCR, the products were separated on 4% agarose gel stained with ethidium bromide (Sigma-Aldrich, St. Louis, USA) in the presence of ready-to-use DNA M50pz ladder (DNA Gdansk, Gdansk, Poland). The length of poly(A) tails was determined by estimation of the distance between the end of "smear" generated by poly(A) containing PCR products and the minimum expected size of AA-NAT and HIOMT, amplified products: 244 bp (209 bp of AA-NAT 3′ end + 35-bp oligo(dT)-anchor) and 373 bp (338 bp of HIOMT 3′ end + 35-bp oligo(dT)-anchor), respectively.

2.3.5. Western-Blot Assay. Before electrophoresis, the protein concentration in the samples isolated using the NucleoSpin RNA/Protein Kit (MACHEREY-NAGEL Gmbh & Co., Düren, Germany) was quantified by Protein Quantification Assay Kit (MACHEREY-NAGEL Gmbh & Co., Düren, Germany). To the appropriate volume of samples containing 50 μg of total protein the appropriate volume of molecular grade water (Sigma-Aldrich, St. Louis, USA) was added to make the total samples volume of 20 μL. In addition to 20 μL of these samples, 19 μL of Laemmli buffer (Sigma-Aldrich, St. Louis, USA) and 1 μL of β-mercaptoethanol (Sigma-Aldrich, St. Louis, USA) were added. The received mixtures were boiled for 3 min. Electrophoresis was performed in the presence of molecular weight marker, Spectra Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific Inc., Rockford, USA). Denatured samples and molecular weight standard were loaded on 4–12% polyacrylamide gels and subjected to electrophoresis in Tris-glycine running buffer in the Protean II xi Cell (Bio-Rad Laboratories, Inc., Hercules, USA), according to the manufacturer's instructions. Next proteins were transferred in Tris-glycine blotting buffer to polyvinylidene difluoride membrane, Immobilon-P (0.45 μm) (Merck KGaA, Darmstadt, Germany), using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Inc., Hercules, USA) for 30 min at 20 V. The membranes were blocked overnight at 4°C in blocking buffer, Tris buffered saline, at pH 7.5 with 0.05% Tween-20 (TBST) (Sigma-Aldrich, St. Louis, USA) containing 3% bovine serum albumin fraction V (Sigma-Aldrich, St. Louis, USA). Next, the membranes were incubated for 1 h with primary rabbit anti-HIOMT polyclonal antibody (cat no. bs-6961R; Bioss Inc., Boston, USA), goat anti-AA-NAT polyclonal antibody (cat no. sc-55612, Santa Cruz Biotechnology Inc., Dallas, USA), and mouse anti-ACTB monoclonal antibody (cat no. sc-47778, Santa Cruz Biotechnology Inc., Dallas, USA) dissolved in blocking buffer in dilution 1–200, 1–200, and 1–1000, respectively. After three-time washing, the membranes were incubated with secondary HRP conjugated antibody: bovine anti-rabbit IgG-HRP (cat no. sc-2379, Santa Cruz Biotechnology Inc., Dallas, USA), donkey anti-goat IgG-HRP (cat no. sc-2304, Santa Cruz Biotechnology Inc., Dallas, USA), and goat anti-mouse IgG1 heavy chain (HRP) (cat no. sc-2304, Abcam, Cambridge, UK) dissolved in blocking buffer in dilution 1–10000. After three-time washing, the visualization of membranes was performed using chromogenic detection by the Pierce 1-Step TMB-Blotting Substrate Solution (Thermo Fisher Scientific, Waltham, USA). After the visualization, the membranes were dried and scanned using EPSON Perfection V370 Photo Scanner (Seiko Epson Corporation, Suwa, Japan). The densitometric analysis of the scanned membrane was performed using ImageJ software (Research Services Branch, National Institute of Mental Health, Bethesda, USA).

2.4. Statistical Analysis. The raw data, after passing the normality test, were subjected to repeated-measures two-way analysis of variance (ANOVA, GraphPad Prism, San Diego, CA, USA) followed by a post hoc Sidak's multiple comparison test. Statistical significance was established at $P < 0.05$. Data are presented as normalized to the control of saline-treated group.

3. Results

3.1. Effect of IL-1β on Melatonin Release from the Pineal Explants. Norepinephrine increased ($P < 0.05$) melatonin release from the pineal explants (Figure 1). This effect of NE on melatonin release was completely abolished by IL-1β. The explants coincubated with both NE and IL-1β did not increase melatonin secretion to the media. There was no influence of animals immune status before sacrificing on the *ex vivo* melatonin secretion.

3.2. Influence of IL-1β on AA-NAT and HIOMT Expression in the Pineal Explants. Norepinephrine administration significantly ($P < 0.05$) stimulated AA-NAT gene expression in the explants from both saline- and LPS-treated ewes (Figure 2(a)). IL-1β did not affect basal and NE-stimulated AA-NAT gene expression in the pineal glands from both groups of ewes. Similarly, NE stimulated ($P < 0.05$) significantly AA-NAT protein expression but only in the explants obtained from saline-treated animals (Figure 2(b)).

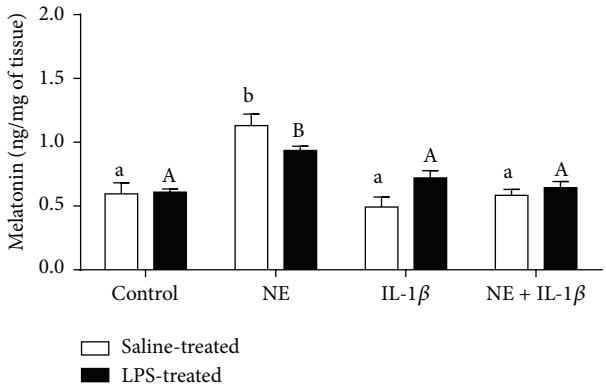

FIGURE 1: The effects of norepinephrine (NE, 10 μM), interleukin- (IL-) 1β (75 pg/mL), and mixture of NE with IL-1β on melatonin concentration (pg/mg of tissue) in the medium of pineal gland explants collected from saline- and lipopolysaccharide- (LPS-) treated ewes. Different lowercase letters indicate significant ($P < 0.05$) differences within the saline-treated group; different capital letters indicate differences within the LPS-treated group; control: no NE/IL-1β treatment.

Treatment with IL-1β did not affect basal protein expression in both groups; however, it significantly reduced NE-stimulated expression of AA-NAT. There was no effect of LPS treatment on the pineal gland explants response to the treatment.

There was no effect of NE or IL-1β treatment on gene expression of HIOMT in the explants from both saline- and LPS-treated ewes. Additionally we observed higher HIOMT mRNA expression in explants collected from LPS-treated than saline-treated animals (Figure 3(a)). Similarly, the pineal explants collected from LPS-treated ewes had significantly ($P < 0.05$) higher expression of HIOMT protein than those from saline-treated ewes. IL-1β significantly ($P < 0.05$) increased HIOMT protein expression in the explants from both saline- and LPS-treated ewes (Figure 3(b)). NE did not affect basal and IL-1β stimulated protein expression of the pineal glands from both groups of ewes.

3.3. Effect of IL-1β on the Gene Expression of IL-1β and IL-1R1. All pineal explants expressed mRNA encoding IL-1β. Moreover, in explants obtained from LPS-treated ewes, the gene expression of IL-1β was downregulated by NE (Figure 4(a)). On the other hand, IL-1β stimulated ($P < 0.05$) the gene expression of its type I receptor only in the explants from saline-treated ewes (Figure 4(b)). However, this stimulatory effect of IL-1β on IL-1R1 gene expression was abolished by NE treatment. No influence of IL-1β on the IL-1R1 mRNA level was found in explants from LPS-treated animals. In these groups of explants NE reduced IL-1R1 gene expression only when acting alone, because in explants coincubated with NE and IL-1β this reducing effect was completely diminished.

3.4. Effect of IL-1β on the Length of Poly(A) Tail of AA-NAT and HIOMT mRNA. No effects of IL-1β, NE, and immune status on the length of poly(A) tail of mRNA encoding enzymes, AA-NAT and HIOMT, were found (Figure 5).

4. Discussion

The results of our study demonstrate that IL-1β may be an important mediator via immune system which regulates the melatonin secretion. The experiment carried out on ovine pineal explants showed that IL-1β suppressed NE-stimulated melatonin secretion. This result fully supports the previously published *in vivo* study displaying the inhibitory action of these cytokines on melatonin secretion in rat [19]. They found that exogenous recombinant human IL-1β decreased the melatonin synthesis at dose-dependent manner. Moreover, this effect of IL-1β was abolished by introduction of the anti-human IL-1 receptor antibody. Our *ex vivo* study also showed that IL-1β-induced decrease in melatonin release generally reflects the changes occurring in AA-NAT protein expression, because the effect of IL-1β on the transcription of this enzyme seems to be more ambiguous. It should be pointed out that the regulatory signals that control AA-NAT and melatonin biosynthesis vary among vertebrate species [2]. In all mammals NE regulates the melatonin synthesis by activation of two subtypes of adrenergic receptors. Activation of β1-adrenergic receptors increases the intracellular concentration of cAMP followed by activation of the cAMP-dependent protein kinase A (PKA). Both elevated cAMP level and PKA activation are indispensable for stimulation of AA-NAT and melatonin synthesis in all mammalian species. In turn, activation of α1-adrenergic receptors increases the intracellular calcium ($[Ca^{2+}]_i$) concentration caused by release of calcium ions from intracellular stores [30]. The NE-dependent activation of the β1-adrenergic/cAMP/PKA and α1-adrenergic/$[Ca^{2+}]_i$ pathways is conserved in mammalian physiology, but the downstream mechanisms that link these signaling cascades with AA-NAT activation and melatonin production exhibit marked species-to-species variations [2]. In rodents, the cAMP/PKA pathway controls transcriptional mechanisms regulating melatonin synthesis. It was demonstrated that stimulation by NE approximately 100-fold increased cAMP level in the rat pinealocytes [31]. Moreover, the day/night rhythm of changes in cAMP concentration in the rat pineal gland are parallel to the pattern of changes in AA-NAT mRNA expression, which increases by approximately 150-fold during the night [4, 32]. In ungulates and primates, melatonin synthesis is controlled by mechanism targeted on the posttranslational regulation of AA-NAT. In these animals, pinealocytes constantly synthesize AA-NAT protein from continually available AA-NAT mRNA. In the absence of noradrenergic stimulation, AA-NAT protein is destroyed by proteasomal proteolysis. Under NE stimuli, elevated cAMP levels cause phosphorylation of AA-NAT by PKA. This post-translational modification leads to interaction of phosphorylated AA-NAT with 14-3-3 proteins protecting AA-NAT from degradation [2, 33]. Our results showing that IL-1β abolished NE-induced increase in AA-NAT protein expression but did not affect this enzyme transcription suggest that the action

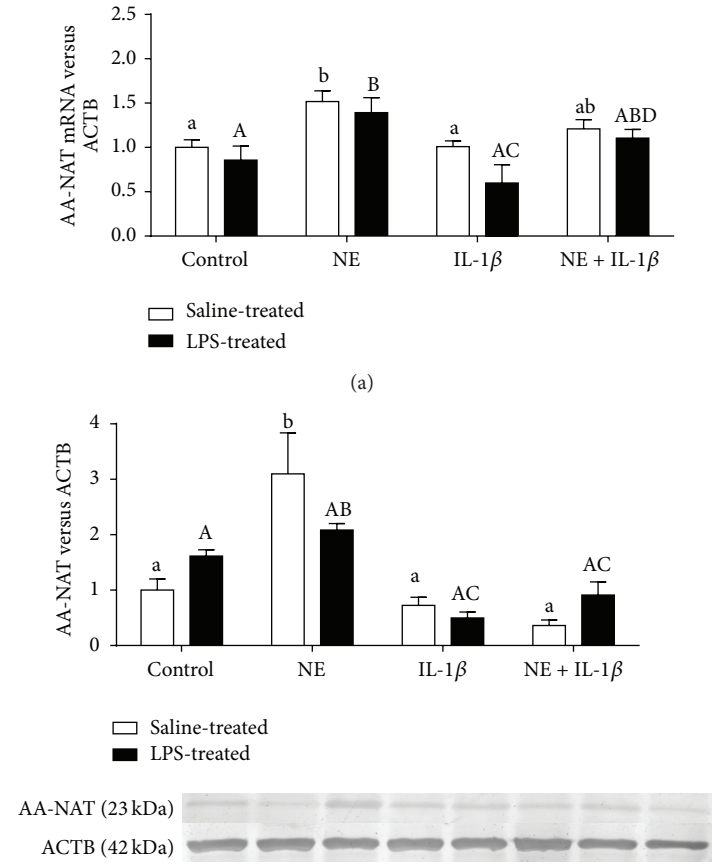

Figure 2: The effects of norepinephrine (NE, 10 μM), interleukin- (IL-) 1β (75 pg/mL), and mixture of NE with IL-1β on arylalkylamine-N-acetyltransferase (AA-NAT) relative gene (a) and protein (b) expression (mean ± SEM) in the pineal gland explants collected from saline- and lipopolysaccharide- (LPS-) treated ewes. Different lowercase letters indicate significant ($P < 0.05$) differences within the saline-treated group; different capital letters indicate differences within the LPS-treated group.

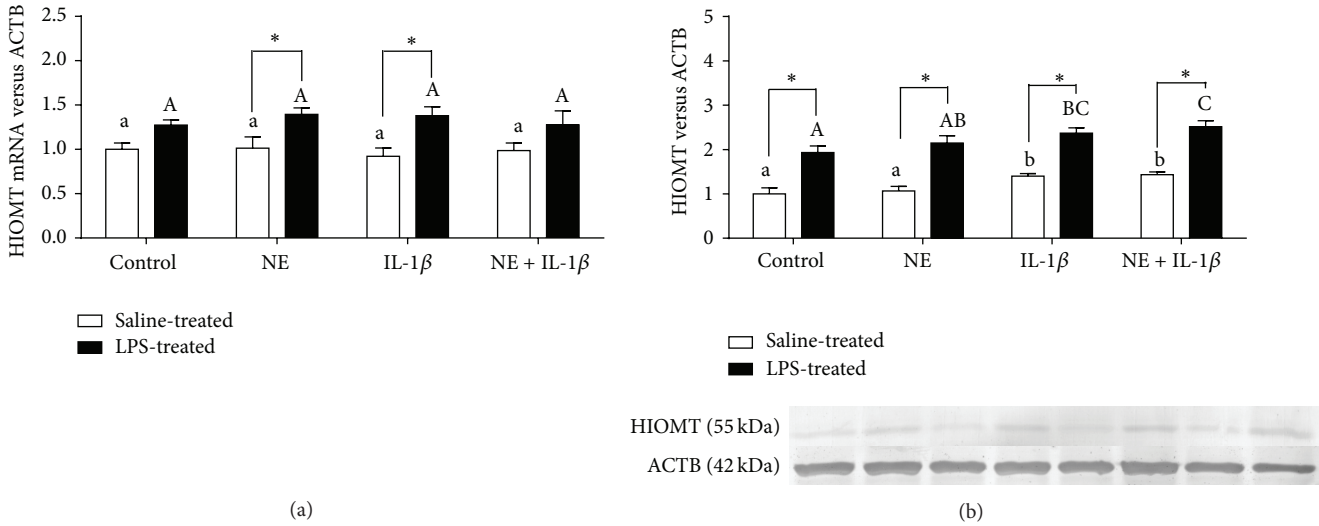

Figure 3: The effects of norepinephrine (NE, 10 μM), interleukin- (IL-) 1β (75 pg/mL), and mixture of NE with IL-1β on hydroxyindole-O-methyltransferase (HIOMT) relative gene (a) and protein (b) expression in the pineal gland explants collected from saline- and lipopolysaccharide- (LPS-) treated ewes. Different lowercase letters indicate significant ($P < 0.05$) differences within the saline-treated group; different capital letters indicate differences within the LPS-treated group; asterisk designates a significant ($P < 0.05$) difference between the saline- and LPS-treated groups; control: no NE/IL-1β treatment.

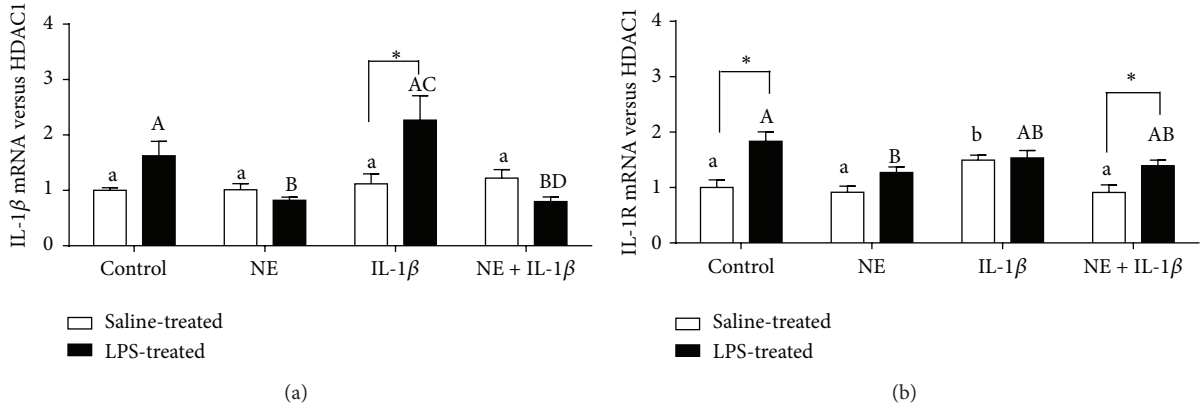

Figure 4: The effects of norepinephrine (NE, 10 μM), interleukin- (IL-) 1β (75 pg/mL), and mixture of NE with IL-1β on the poly(A) tail length of mRNAs encoding arylalkylamine-N-acetyltransferase (AA-NAT) and hydroxyindole-O-methyltransferase (HIOMT) in the pineal gland explants collected from saline- and lipopolysaccharide- (LPS-) treated ewes. M: DNA ladder M50pz, numbers indicate each experimental group: (1) control, (2) NE, (3) IL-1β, and (4) NE + IL-1β. The minimum expected size of AA-NAT and HIOMT, amplified products, was 244 bp (209 bp of AA-NAT 3′ end + 35-bp oligo(dT)-anchor) and 373 bp (338 bp of HIOMT 3′ end + 35-bp oligo(dT)-anchor), respectively.

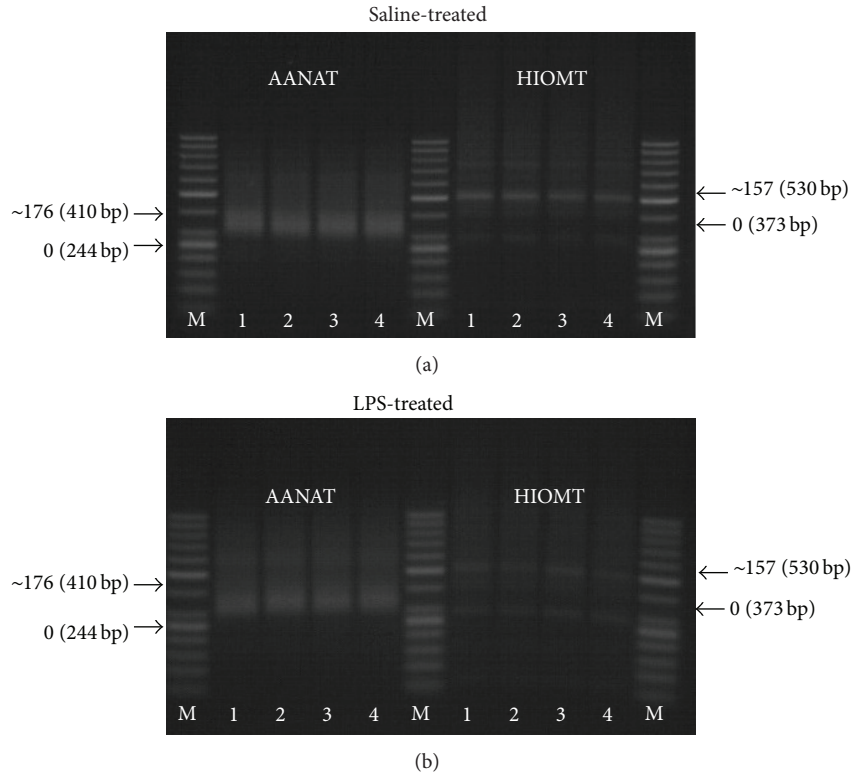

Figure 5: The effects of norepinephrine (NE, 10 μM), interleukin-1β (IL-1β, 75 pg/mL), and mixture of NE with interleukin- (IL-) 1β on IL-1β (a) and its type 1 receptor (IL-1R) (b) relative gene expression (mean ± SEM) in the pineal gland explants collected from saline- and lipopolysaccharide- (LPS-) treated ewes. Different lowercase letters indicate significant ($P < 0.05$) differences within the saline-treated group; different capital letters indicate differences within the LPS-treated group; asterisk designates a significant ($P < 0.05$) difference between the saline- and LPS-treated groups; control: no NE/IL-1β treatment.

of IL-1β is targeted on the reduction of the rate of this enzyme proteolysis in the sheep pinealocytes. It is also worth pointing out that in our experiment mild but significant upregulation of AA-NAT gene expression was found in NE-treated explants. This result is consistent with previously published data which demonstrates that in sheep pineal gland slight day/night fluctuation of AA-NAT mRNA level occurs [34]. They found that nocturnal level of mRNA encoding AA-NAT is about 2 times higher than daily level of this transcript. However, the availability of mRNA to translation

not only depends on the level of transcription but is also affected by this mRNA stability. The poly(A) tail of mRNA has an important influence on the dynamics of gene expression. On the one hand, it promotes enhanced mRNA stability to allow production of the protein, even after inactivation of transcription. On the other hand, shortening of the poly(A) tail, deadenylation, slows down translation of the mRNA or prevents it entirely, by inducing mRNA decay. Thus deadenylation plays a crucial role in the posttranscriptional regulation of gene expression, deciding the fate of individual mRNAs [35]. Our study also showed that neither NE nor IL-1β affects the AA-NAT mRNA stability, because there was no influence of these factors on the length of poly(A) tail of this transcript. This seems to support the thesis that this enzyme expression is mainly regulated at the posttranscriptional level.

Although it is postulated that melatonin secretion is mainly regulated at the AA-NAT level, in our study the expression of HIOMT was also assayed. In contrast to AA-NAT, immune status of animals before sacrificing affected HIOMT expression. The expression of this enzyme was higher in explants collected from LPS- and then saline-treated ewes. However, no effects of experimental treatments were found on HIOMT mRNA poly(A) tail length. The stimulatory effect of inflammatory condition on HIOMT gene expression and HIOMT enzymatic activity was previously described in chicken [3]. Similar to our study, the changes in HIOMT gene expression and activity did not affect melatonin synthesis in these birds, probably due to the simultaneous inhibition of N-acetylserotonin biosynthesis. Due to the lack of data indicating the effect of immune mediators on HIOMT expression and activity based on the studies carried out on rodents, Piesiewicz et al. [3] speculated that these changes could be caused by stress hormones stimulating pineal HIOMT gene expression and activity, which resulted in increased melatonin secretion [36]. However, our findings that IL-1β stimulated the protein expression of HIOMT in pineal explants collected from both LPS- and saline-treated animals seem to shed some light on this issue. It suggests that inflammatory-dependent increase in HIOMT synthesis in the pineal gland may be induced by inflammatory mediators such as IL-1β.

The synthesis of melatonin may be regulated by circulating IL-1β. Our study suggests that IL-1β is locally synthesized in the pineal gland; therefore, this cytokine may also affect the melatonin synthesis in paracrine way. We determined relatively high and constitutive IL-1β mRNA expression in pineal explants collected from saline-treated ewes. Moreover, in the case of pineal tissues from LPS-treated animals, significant suppression of IL-1β transcription was determined under the influence of NE. This result supports previous report about constitutive expression of IL-1β in the rat pineal gland, which has also been shown to correlate with the diurnal melatonin rhythm, since IL-1β mRNA is higher during the day than during darkness [37]. Unexpectedly, they determined that IL-1β expression was upregulated in pineal cultures after treatment with NE. Increased IL-1β expression by NE *ex vivo* and the decline in IL-1β expression at night when NE levels are elevated were explained based on immunocytochemical data showing that astrocytes are the predominant cell type expressing this cytokine *in vivo*, whereas IL-1β-positive cells are predominantly microglia in pineal explants and dispersed cell cultures. They assumed that these two types of IL-1β expressing cells may differently affect the pinealocytes activity. However, our study performed on *ex vivo* model suggests that NE rather inhibits than stimulates IL-1β expression in pineal gland. It suggests that at least in sheep NE regulates pineal IL-1β synthesis via its anti-inflammatory mechanism of action, which was previously reported for other mammalian cells [38, 39].

IL-1β may affect the melatonin secretion thanks to the existence of IL-1R1 in the pineal gland. We found that IL-1β stimulated the gene expression of its type I receptor, but this effect was also diminished by NE. On the other hand, IL-1β did not affect IL-1R1 mRNA expression in the explants collected from LPS-treated ewes but this receptor transcription was also reduced by NE. It is worth mentioning that explants collected from LPS-treated ewes are characterized by higher gene expression of IL-1R1 than those from saline-treated animals. However, without immunohistochemical analysis, it is impossible to judge which type of pineal cells expresses the higher amount of IL-1 receptors. It is postulated that, in addition to direct actions on pinealocytes, cytokines and other immune factors regulate pineal gland functioning by indirect actions on pineal glial cells [8]. It is suggested that the effect of cytokines such as IFN-γ and IL-1β on pinealocytes may be mediated by microglia, when TNFα may exert its biological effects acting directly on pinealocytes [40].

Summarizing, our study supports the thesis that interaction between pineal gland and the immune system is bidirectional. It was found that proinflammatory cytokine, IL-1β, suppresses the process of melatonin synthesis in ovine pinealocytes, mainly affecting AA-NAT protein expression. Moreover, the fact that IL-1β is synthesized in the pineal gland suggests that not only exogenous but also "local" IL-1β may be involved in the regulation of the melatonin synthesis. Our study also shows that immune status of animals affects HIOMT expression in pineal tissue even in *ex vivo* condition, few hours after disappearance of inflammatory stimuli. Although stimulatory influence of both inflammatory condition and IL-1β on HIOMT expression is unambiguous, the explanation of physiological significance of this phenomenon requires future detailed studies.

Conflict of Interests

All of the authors have declared that there is no conflict of interests regarding this work.

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Review Article

MicroRNAs: Novel Players in the Dialogue between Pancreatic Islets and Immune System in Autoimmune Diabetes

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MicroRNAs are small noncoding RNA molecules that regulate gene expression in all cell types. Therefore, these tiny noncoding RNA molecules are involved in a wide range of biological processes, exerting functional effects at cellular, tissue, and organ level. In pancreatic islets of Langerhans, including beta-cells, microRNAs are involved in cell differentiation as well as in insulin secretion, while in immune cells they have been shown to play pivotal roles in development, activation, and response to antigens. Indeed, it is not surprising that microRNA alterations can lead to the development of several diseases, including type 1 diabetes (T1D). Type 1 diabetes is the result of a selective autoimmune destruction of insulin-producing beta-cells, characterized by islet inflammation (insulitis), which leads to chronic hyperglycemia. Given the growing importance of microRNA in the pathophysiology of T1D, the aim of this review is to summarize the most recent data on the potential involvement of microRNAs in autoimmune diabetes. Specifically, we will focus on three different aspects: (i) microRNAs as regulators of immune homeostasis in autoimmune diabetes; (ii) microRNA expression in pancreatic islet inflammation; (iii) microRNAs as players in the dialogue between the immune system and pancreatic endocrine cells.

1. Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease characterized by the selective destruction of insulin-producing beta-cells by the immune system. In early disease stages, islets of Langerhans are characterized by insulitis, an inflammatory process mediated by T cells (both CD8+ and CD4+ lymphocytes), B-lymphocytes, NK cells, and macrophages. The immune infiltrate has a pivotal role in beta-cell demise; however, the exact mechanisms involved in the dialogue between pancreatic islets and immune infiltrating cells are still under investigation [1].

Growing evidence indicates that microRNAs (miRNA), short RNA molecules involved in post-transcriptional repression, play a crucial role both in pancreatic beta-cell biology and in immune cell homeostasis. Indeed, miRNA alterations have been reported in murine beta-cells when settled in a proinflammatory environment, which mimics the *in vivo* inflammatory milieu induced by islet-infiltrating cells [2]. In addition, miRNAs alterations have also been reported in circulating immune cells in type 1 diabetic subjects [3, 4].

miRNAs represent a class of evolutionary conserved small (18–24 nucleotides), endogenous, single stranded, noncoding RNA molecules, which are important regulators of gene expression. miRNAs influence RNA stability and translational efficiency by targeting the 3′ untranslated region (UTR) of messenger RNA, leading to its degradation or to inhibition of protein translation. The human genome encodes 1881 miRNA precursors generating more than 2000 mature miRNAs [5]. Since their first discovery in 1993, miRNAs have now been recognized as key players in a wide range of biological processes such as differentiation, proliferation, ageing, and cell death.

The various stages of miRNAs biogenesis occur both in the nucleus and in the cytoplasm, where their maturation is tightly controlled by several enzymes. Among them, two are of major importance: Drosha-DGCR8 in the nucleus and Dicer in the cytoplasm. Dicer is the final enzyme, which

generates mature miRNAs, which in turn can be loaded into the RISC (RNA Induced Silencing Complex) assembly, thus mediating the functional role of miRNAs. An important aspect of miRNAs biology is represented by the complexity of post-transcriptional gene expression controlling network. Indeed, each miRNA can potentially regulate the expression of several genes, while, on the other hand, a single mRNA can be targeted by numerous miRNAs [6]. Therefore it is not surprising that the presence of miRNAs is strictly necessary for the regulation of gene expression and, consequently, for the homeostasis of whole cellular processes.

In this review we will specifically focus on the role of miRNAs in three important aspects of autoimmune diabetes etiology:

(i) The role of miRNAs in immune homeostasis and the influence of miRNA alterations on the development of autoimmune diabetes.

(ii) miRNAs role in the response of pancreatic islets and/or of beta-cells to immune-mediated stress.

(iii) Secreted miRNAs as a mechanism of islet-immune cells dialogue in autoimmune diabetes.

Importantly, all these three different faces of autoimmune diabetes are strictly linked among each other, thus generating a complex interplay, which is tightly modulated by miRNAs.

2. MicroRNA as Regulators of Immune Homeostasis in Autoimmune Diabetes

Among the broad range of functions in which miRNAs are involved, there is growing evidence that miRNAs are crucial modulators of immune cell functions, thus representing major players in the regulation of immune homeostasis. In particular, miRNAs are associated with many facets of immune responses such as development, activation, and differentiation. In order to uncover the role of miRNAs in murine immune system, several studies depleted miRNAs in specific immune cells by deleting key enzymes involved in miRNA biogenesis. For example, Lck-Cre-mediated ablation of Dicer during thymocyte development compromised the survival of $\alpha\beta$-lineage cells, whereas the number of $\gamma\delta$ expressing thymocytes was not affected [7]. Remarkably, the CD4/CD8 lineage commitment appeared unaltered in the absence of Dicer. Conditional deletion of Dicer in CD4+ T cells at a later time point resulted in reduced cell number due to both increased cell death and impaired proliferation. As for T helper (Th) cell polarization, Dicer-deficient CD4+ T cells failed to differentiate into Th2 phenotype due to their increased polarity to Th1 effector cells and to the inability to block interferon-γ (INF-γ) production [8]. Of note, similar results were obtained upon Drosha or Argonaute deletion in T cells [9, 10]. Given miRNAs multiple roles in T cells, it is not surprising that these short noncoding RNA molecules regulate also humoral immunity by fine tuning B cells differentiation and activation. Thus, ablation of Dicer in early B cell progenitors results in a substantial arrest at the pro-B to pre-B cell transition [11], while ablation of Dicer in antigen activated B cells, that is, at a later time point, results in a defective production of high affinity class-switched antibodies with an impaired formation of germinal centre B cells, long-lived plasma cells, and memory B cells [12]. Taken together, these studies show how global miRNAs deletion has detrimental effects on T and B cells development and function; moreover, mice with miRNA-deficient CD4+ T cells are prone to develop immune pathology as they age, suggesting a role for miRNAs in the maintenance of immune homeostasis [7]. Among immune cells with regulatory activity, CD4+ regulatory T (Treg) cells play an essential role in immune homeostasis and self-tolerance. Treg cells are characterized by high expression of the IL-2Rα chain (CD25) and the transcription factor forkhead box P3 (FOXP3), which serves as a lineage specification factor. The role of Treg in autoimmunity is highlighted by patients with IPEX (Immune-dysregulation Polyendocrinopathy Enteropathy X-linked syndrome), in which absence of Treg cells results in an enhanced susceptibility to autoimmune diseases including type 1 diabetes [13].

Specific ablation of miRNAs in Treg cells secondary to Dicer or Drosha deletion resulted in an early fatal onset of autoimmunity similar to what has been previously observed in FOXP3-deficient mice [9]. Indeed, miRNA-deficient Treg cells show decreased levels of FOXP3 together with altered differentiation and function [14–16]. However, since lack of Dicer and Drosha interferes with the generation, not only of canonical miRNAs, but also of other small RNA species, Jeker et al. generated mice with Treg cells lacking Dgcr8, RNA-binding protein required in the processing of canonical miRNAs. Interestingly, mice lacking canonical miRNAs showed a similar phenotype to that of Dicer-deficient mice, thus demonstrating that miRNAs are essential for normal FOXP3 expression and suppressive Treg function and suggesting a high degree of dependence on miRNAs-mediated regulation for normal Treg development and function [15]. Several studies have also begun to uncover the role of single miRNAs in immune cells. Studies by Cobb et al. in a murine model uncovered a characteristic set of miRNAs, which are enriched in Treg with respect to naïve CD4 T cells; these include miR-223, miR-146, miR-155, miR-21, and miR-24 [17]. Interestingly, miR-155 is of particular interest because of the FOXP3 mediated regulation of the intronic region of B cell integration cluster (Bic), which encodes the gene for miR-155 [18]. miR-155 deficient mice showed reduced number of thymus-derived and peripherally induced Treg cells [19]; in contrast, Treg suppressive function was unaffected both *in vitro* and *in vivo* in these mice [20]. Of note, miR-155 guarantees Treg cell homeostasis by targeting suppressor of cytokine signal 1 (SOCS1), a negative regulator of IL-2 pathway, thereby increasing the sensitivity of these cells to their main growth factor with a crucial role in Treg cells development [19].

In addition to miR-155, miR-146a was identified to be prevalently expressed in Treg cells and important for their function. Lu et al. have shown that miR-146a deficiency, limited to FOXP3+ Treg cells using a mixed bone marrow chimera approach, resulted in IFN-γ- and Th1-mediated disorders, similar to the alterations observed in mice bearing Treg cell-specific Dicer or Drosha deficiency [21]. This

phenomenon can be explained by the direct targeting of miR-146a to the signal transducer and activator of transcription 1 (STAT1), a key transcription factor in IFN-γ response and in Th1 differentiation [21].

It is becoming increasingly evident that miRNAs modulate several pathways involved in immune cells homeostasis and that miRNA alterations can lead to specific dysfunctions that could favor the development of autoimmune diseases including T1D.

Jeker et al. have shown that miR-10a is preferentially expressed in mouse thymus-derived Treg cells, although it does not seem to directly regulate FOXP3 or other factors involved in Treg homeostasis. Interestingly, miR-10a expression is lower in Treg of nonobese diabetic mice (NOD), a spontaneous murine model of autoimmune diabetes, whereas miR-10a levels are higher in autoimmunity-resistant C57BL/6 mouse strain [22]. Moreover, Takahashi et al. showed that miR-10a could be induced by retinoic acid and by TGF-β in inducible Treg cells [23]. Indeed, they showed that miR-10a attenuated phenotypic conversion of inducible Treg cells to follicular helper T cells by simultaneously targeting the transcriptional repressor Bcl-6 and corepressor Ncor2 [23]. Thus, miR-10a is a candidate player involved in the maintenance of Treg cell specific phenotype by targeting factors that could lead to conversion to other cell fates.

As for the potential protective role of miRNAs in autoimmune diabetes, Berry et al. showed a connection between miR-34a and diabetes protection [24], reporting an impaired B cell lymphopoiesis in diabetes-resistant NOD.B10 Idd9.3 mice, congenic for insulin-dependent diabetes (Idd) Idd9.3 locus. Interestingly, they showed that miR-34a was significantly higher in B cell progenitors and in marginal zone B cells from NOD.B10 Idd9.3 mice versus wild type NOD mice. Furthermore, miR-34a expression in these cell populations inversely correlated with levels of Foxp1, an essential regulator of B cell lymphopoiesis, which is directly repressed by miR-34a. Moreover, mature B cells from NOD.B10 Idd9.3 mice were unable to prime islet-specific CD4+ T cells *in vitro*, which may contribute to T1D protection in NOD.B10 Idd9.3 mice [24].

Recently published studies have also reported miRNA specific signatures in peripheral blood immune cells from type 1 diabetic patients. Interestingly, analysis of miRNA performed by Hezova et al. in Tregs from normal and from type 1 diabetic subjects showed an increased expression of miR-510 and a decreased expression of miR-342 and miR-191 [25]. Salas-Pérez and colleagues showed that miR-21a and miR-93 are reduced in peripheral blood mononuclear cells (PBMC) of patients with T1D, likely as a consequence of chronic hyperglycemia since cultures of PBMC from nondiabetic subjects downregulated miR-21a expression levels when cultured in the presence of high glucose [3]. In contrast, glucose levels in culture media did not affect miR-93 expression in PBMC and bioinformatic analysis of miR-93 predicted targets showed STAT3 (a protein with a crucial role in immune responses) as potential target gene [3].

Yang et al. compared miRNA expression profiles of PBMC from newly diagnosed T1D patients versus nondiabetic individuals, identifying miR-146a as the most downregulated miRNA in PBMC of T1D patients; this downregulation was independent of hyperglycemia and of disease duration, while it was associated with high glutamic acid decarboxylase autoantibodies (GADA) titers [26]. The association between miRNAs expression and severity of autoimmune response in T1D was also previously analyzed by our group. Indeed, we analyzed miRNAs expression in PBMC from patients with T1D showing an increased expression of miR-326, which was positively correlated with the presence of diabetes-associated autoantibodies [4]. Interestingly, bioinformatic target genes analysis revealed vitamin D3 receptor (VDR) and ETS1 as putative miR-326 target genes [4]. Both are reported to be involved in the regulation of immune cell homeostasis and thus it is possible to speculate that an alteration of miR-326 may regulate islet autoimmunity. Interestingly, miR-326 has also been found to be upregulated in peripheral blood leukocytes (PBL) of patients with relapsing remitting multiple sclerosis when compared to age-matched controls [27]. Moreover, PBLs from patients with relapsing multiple sclerosis had significantly higher miR-326 expression than those from patients with remitting multiple sclerosis, suggesting an association between miRNAs expression and severity of autoimmune response.

Taken collectively these findings demonstrate how miRNA regulation of lymphocyte biology can be crucial for the maintenance of self-tolerance. On the other hand, there is increasing evidence suggesting a contribution of the innate immune system to the breakdown of tolerance in several autoimmune diseases. The innate immune system ensures a first line of defence against foreign pathogens and is mediated mainly by macrophages, granulocytes, dendritic cells, and natural killer cells. Recently, several studies are shedding light on the role of the innate immune system in T1D development [28–30]. For example, new evidence demonstrated that dendritic cells play a pivotal role in activating naïve T cells with islet-specific antigen autoreactive capacity. Indeed, it has been suggested that diabetic NOD mice dendritic cells have increased potential to activate T cells compared to those derived from nondiabetic mice, mainly through augmented IL-12 production and costimulatory molecule expression [31, 32], thus demonstrating an active role of dendritic cells in the induction or exacerbation of autoimmune responses.

Interestingly, terminally differentiated and short-lived cells such as neutrophils are also likely to play a role in autoimmune diabetes. Valle et al. have observed a reduced number of circulating neutrophils in patients with established diabetes as well as in normoglycemic subjects with high risk for T1D development (nondiabetic first-degree relative of T1D patients) with respect to nondiabetic subjects [33], while an increased infiltration of neutrophils has been observed in the pancreatic exocrine tissue of T1D donors. The authors concluded that neutrophils may play a role in the initiation and progression of the disease.

Additional components of innate immune cells such as $\gamma\delta$ T-lymphocytes have been linked to diabetes. Indeed, Markle et al. using an adaptive transfer model have shown the detrimental role of IL-17-producing $\gamma\delta$ T cells in diabetes development [34].

Another essential element of the innate immune system is the Toll Like Receptor (TLR) system, which consists of a group of transmembrane proteins expressed in immune and in nonimmune cells, whose role is to recognize components of foreign pathogens. TLRs have been associated with T1D development in mouse and in human studies [35, 36], thus underlining the importance of innate immune system in diabetes etiology.

Emerging data have identified miRNAs as important regulators of development and function of innate immune components in addition to the aforementioned adaptive immune cells. For example, Cebpa-Cre driven deletion of Dicer compromises definitive maturation of neutrophils from myeloid precursors [37]. Moreover, studies focusing on the effect of Dicer ablation in skin-draining lymph nodes migratory dendritic cell subset, namely, Langerhans cells, showed a disturbed expression of surface molecules and reduced antigen presentation capacities to CD4+ T cells, increased turnover, reduced half-lives, and increased rates of apoptosis [38].

Interestingly, as already mentioned, Lck-Cre-mediated ablation of Dicer during thymocyte development does not affect $\gamma\delta$ T cell number; in contrast there is a substantial increase of $\gamma\delta$ T cells in the double negative thymic compartment [7]. Lastly, also TLR signalling pathways are deeply regulated by miRNAs [39].

It is becoming increasingly evident how miRNAs modulate immune cell function and how miRNA dysregulation in these cells can lead to immune pathology. However, further studies are needed to fully elucidate the role of single miRNA as well as of groups of specific miRNAs in the regulation of the immune response in health and in disease.

3. MicroRNAs: Active Participants in the Immune-Mediated Beta-Cell Damage

Pancreatic islet inflammation and beta-cell damage represent a hallmark of both type 1 and type 2 diabetes. Although the etiology differs between the two forms of diabetes, immune cell infiltrates have been observed in both of them [40]. Beta-cell dysfunction and damage can be in part attributed to the unfavorable environment mainly characterized by detrimental milieu of proinflammatory cytokines, which represents a common phenomenon occurring in type 1 and type 2 diabetes.

Beta-cells are very sensitive to diabetes-associated inflammatory mediators and in turn activate a series of molecular mechanisms which can lead to (i) beta-cell dysfunction (e.g., reduced insulin secretion or insulin content); (ii) beta-cell apoptosis; (iii) exacerbation of inflammatory phenomena through the secretion of chemokines which lead to an increased immune cell infiltrate. The molecular mechanisms involved in these types of responses are tightly regulated by miRNAs.

One of the first studies aimed at elucidating the effects of an inflammatory environment on beta-cell miRNA expression has been published in 2010 by Roggli and colleagues [2], who analyzed the effects of a combination of cytokines on miRNA expression profiles in beta-cells and their putative link to beta-cell function and/or survival. To this end, authors exposed beta-cell line MIN6 cells (mouse insulinoma beta-cell line 6) for 24 hours to cytokines typically secreted by infiltrating immune cells: IL-1β or a combination of IL-1β, TNF-α, and IFN-γ. The evaluation of miRNA expression profiles through microarray technology revealed three differentially expressed miRNAs. Specifically, miR-21, miR-34a, and miR-146a expression levels were increased both by IL-1β and by the cytokine mix. Of particular interest, IL-1β alone was revealed as the strongest inducer of miR-21 and miR-146a expression, although these miRNAs were also upregulated by TNF-α but not by IFN-γ. The expression of miR-34a was equally stimulated by IL-1β and TNF-α while IFN-γ did not show any effect on its expression levels [2]. Importantly, similar results, characterized by an increased expression of miR-21, miR-146a, and miR-34a, were obtained upon exposure of cultured human islets to IL-1β. The same expression pattern was also clearly observed in pancreatic islets isolated from NOD mice at 8 and 13 weeks of age versus 4-week-old mice. Of note, 4-week-old NOD mouse pancreatic islets did not show any sign of infiltration, indicating the relevance of infiltrating immune cells in the secretion of cytokines, which in turn modulate the expression of these miRNAs. The exposure of MIN6 cells to IL-1β induced a reduction of insulin gene activity and of proinsulin mRNA content; however, overexpression of miR-21 and miR-146a in MIN6 cells did not induce such effects, while a slight reduction of insulin gene activity and proinsulin mRNA was observed upon miR-34a overexpression [2]. Conversely, it was shown that miR-34a and miR-21 had major effects on glucose stimulated insulin secretion (GSIS) through the regulation of VAMP2 expression, a molecule involved in insulin granule exocytosis, and of Rab3a (GTPase) and that this effect can be mimicked by a weak and short exposure to cytokines. In support of this, it has been demonstrated that, in MIN6 cells, overexpression of miR-34a and of miR-21, but not of miR-146a, leads to an impaired GSIS, while the inhibition of their activity during IL-1β exposure improved GSIS.

Whereas short exposure to cytokines leads to an impaired insulin secretory machinery, a prolonged cytokine exposure leads to increased apoptotic cell death. Interestingly, it was shown that this phenomenon might be partially mediated by miRNAs. Indeed, a lower cell death rate was observed by blocking miR-34a or miR-146a activity, while miR-21 knockdown did not show such effect and, conversely, promoted apoptosis. These data indicate a role for these miRNAs in cytokine-mediated cell death secondary to prolonged cytokine exposure and established a link between cytokines and miRNAs in beta-cells.

Although miR-34a and miR-146a have a deleterious effect on beta-cell survival by promoting apoptosis, the role of miR-21 has not yet been clearly elucidated. The function of miR-21 relies on the effect of various cytokines and/or growth factors via NF-κB pathway [41]. A recent study pointed out a specific role for miR-21 strengthening its role in protecting beta-cells from cytokines induced apoptosis. Indeed, in this study the authors characterized the molecular mechanism, which leads to the expression of miR-21 and to protection of beta-cells from apoptosis [42]. They specifically identified

a relationship between miR-21 and PDCD4 (programmed cell death protein 4). PDCD4 has been previously characterized as a pivotal factor in a number of apoptotic pathways and its overexpression in the beta-cell line betaTC1-6 increased susceptibility to cytokine-mediated cell death. Indeed, it was demonstrated that PDCD4 deficiency rendered mouse islets resistant to cytokine-induced apoptosis, thus highlighting a deleterious effect of PDCD4 in beta-cell survival. To elucidate the molecular link between PDCD4 and miR-21, the authors showed that miR-21 targets PDCD4 and that their expression levels are inversely correlated during exposure of betaTC1-6 to proinflammatory cytokines. Moreover, they demonstrated that inhibition of NF-κB decreased the miR-21 expression levels leading to a significant increase of PDCD4 mRNA and to an increased cell death. In light of such data it is possible to hypothesize that NF-κB exerts its antiapoptotic effect through the induction of miR-21 which in turn negatively regulates the expression levels of cell death inducer PDCD4, thus leading to a protective response during cytokines exposure of beta-cells. As a matter of fact, this study is perfectly in line with Roggli et al., who demonstrated that inhibition of miR-21 promoted apoptosis in beta-cells treated with proinflammatory cytokines.

Another example of miRNA contribution to the progression of insulitis has been reported in 2012 by Roggli et al., who examined miRNA expression profiles in pancreatic islets of NOD mice at different disease stages. Specifically, they analyzed pancreatic islets isolated from prediabetic NOD mice at 4 weeks, 8 weeks, and 13 weeks of age. The results showed a differential expression of several miRNAs and, among these, they focused on miR-29 family, composed of miR-29a, miR-29b, and miR-29c [31]. They observed a progressive increase of miR-29 expression levels during insulitis progression in NOD mice with a maximum upregulation at 13 weeks of age. A similar expression pattern was observed in MIN6 cells exposed to a proinflammatory cytokines mix; this effect was observed also in mouse islets and in human islets, thus highlighting a conserved effect on the expression of miR-29 family members following beta-cell exposure to cytokines [43]. Of note, upregulation of miR-29 family members induced a reduction of insulin mRNA content and a decreased GSIS, mainly exerted by miR-29 targeting and negative regulation of Onecut2 (a transcriptional repressor of granuphilin-4, an inhibitor of insulin granules exocytosis). A specific role for miR-29 in the exacerbation of cytokine-induced apoptosis was also identified. This effect was mainly mediated by the downregulation of Mcl1, an antiapoptotic protein, which has been demonstrated to be a specific target of miR-29 family members [43].

The role of miRNAs has also been evaluated in other inflammatory stimuli. A recent study by Bravo-Egana and colleagues evaluated miRNAs expression profiles in rat islets transplanted under the kidney capsule of syngeneic recipients [44]. Indeed, inflammation plays a key role in islet engraftment and survival after transplantation. In the posttransplant period, the graft is exposed to a series of inflammatory stimuli characterized by chemokine secretion, host tissue factor, and/or macrophages activation, which lead to a detrimental graft environment. In this context a pattern of 31 miRNAs with altered expression was identified: 26 of them were upregulated and 5 downregulated. This dataset was compared with miRNA expression profiles of rat islets exposed *in vitro* to a mixture of IL-1β, TNF-α, and IFN-γ; eight miRNAs correlated between the two conditions; specifically, miR-21, miR-98, miR-27a, miR-143, let-7d, miR-126, and miR-22 were upregulated, while miR-129 was downregulated upon inflammatory environment exposure. Bioinformatic analysis of predicted target genes, coupled to a previously published microarray dataset of differentially expressed mRNAs during proinflammatory stimuli of beta-cell [45], revealed that most of the differentially expressed miRNAs putatively regulate the expression of genes involved in the control of insulin signal transduction, islets development and function, and insulin secretion.

The importance of miRNAs in cytokine-mediated beta-cell damage has also been pointed out in a study focused on the evaluation of overall miRNAs contribution in immune-mediated cell distress [46]. In this study the role of miRNAs was analyzed in a mouse model of autoimmune diabetes, which is initially triggered by multiple low doses of streptozotocin (MLDS). Immune-mediated beta-cell death is a hallmark of MLDS. Whereas a single high dose of streptozotocin mainly induces beta-cell death via a direct toxic effect, MLDS induces a major increase in the production of IFN-γ, IL-1β, and TNF-α, which lead to beta-cell death and dysfunction. In this study, the incidence of diabetes in wild type mice versus beta-cell specific Dicer knockout mice (DICER-RIP-Cre KO miRNA-deficient beta-cell) exposed to MLDS has been evaluated. The results showed that the incidence of MLDS-induced diabetes was higher in DICER-RIP-Cre KO versus WT mice, indicating that the lack of miRNAs in beta-cells enhances the susceptibility to MLDS-induced diabetes and accelerates and exacerbates diabetes onset [46]. However, in the explanation of this phenomenon it should be taken into account the fact that the elimination of miRNAs biogenesis machinery can induce severe beta-cell dysfunction and diabetes, per se, through retarded and incomplete beta-cell maturation.

Recently, it has been reported that the differentiation status of beta-cells is a fundamental prerogative for their susceptibility to deleterious effects of proinflammatory cytokines. As a matter of fact, mature beta-cells are sensitized to the effects of IL-1β, while an immature phenotype confers protection [47]. Indeed, miRNAs expression profiles analyzed in INSr$\alpha\beta$ cells (a subclone of INS-1 beta-cell line) exposed to IL-1β, with or without a mature phenotype (conferred by induction of PDX1 expression), revealed a differential expression of miRNAs dependent on the cell maturation status. Specifically, the exposure of mature INSr$\alpha\beta$ cells to IL-1β induced a differential expression of miR-375 and miR-194 (upregulation of miR-375 and downregulation of miR-194), whereas this effect was lost in the immature INSr$\alpha\beta$ cells, highlighting the importance of phenotype in response to stress stimuli [48].

The concept of beta-cell phenotype loss as a mechanism of self-protection from metabolic and/or inflammatory insults has been recently introduced by Domenico Accili's group. Indeed, Talchai and colleagues found strong evidence of

beta-cell dedifferentiation in several murine models of diabetes, describing a regression of beta-cell to an endocrine progenitor-like phenotype, pointing out an advantage to beta-cell in adopting a dedifferentiated fate, which may facilitate their survival [49]. In this context, miRNAs may play major roles in the maintenance of beta-cell phenotype, thus representing candidate regulators of dedifferentiated fate acquisition. Indeed miRNAs are involved in the control of beta-cell phenotype through a tight regulation of those genes necessary for fate transition and/or specification [50]. In consequence of their possible alteration, miRNAs may be responsible for beta-cell phenotype loss as well as sensitization to several cytokines or other types of inflammatory stressors in type 1 or type 2 diabetes.

Altogether, these studies demonstrate that miRNAs are pivotal players in the response of beta-cell during immune-mediated damage. Indeed, miRNAs actively modulate several different pathways in response to cytokines stimuli, as depicted in Figure 1. The presence of infiltrating cells within pancreatic islets generates a cytokines milieu principally composed of IL-1β, TNF-α, and IFN-γ. These cytokines induce detrimental effects in beta-cells also through the modulation of several miRNAs. Therefore, the upregulation of miR-146a, miR-34, and miR-29s induces beta-cell apoptosis through different mechanism, while the upregulation of miR-21 seems to confer a protective effect. miR-34, miR-29s, and miR-21 upregulation induced also the alteration of insulin granules exocytosis through the inhibition of several genes involved in the regulation of glucose stimulated insulin secretion (e.g., VAMP2, OC2). Furthermore, recent lines of evidence reported that cells are able to communicate using secreted miRNAs contained within budding microvesicles. It is not unlikely that both beta-cells and immune infiltrating cells are able to exchange information via microvesicles containing miRNAs and that some of them may also confer detrimental effects both in endocrine and immune cell components.

4. Secreted MicroRNAs: Messengers in the Dialogue between Pancreatic Islets and the Immune System

While the majority of miRNAs have been found inside cells, several miRNAs have also been detected in biological fluids such as plasma, serum, urine, saliva, semen, and breast milk [51, 52] usually complexed with ribonucleoproteins (such as Argonaute 2) or lipoproteins (HDL, LDL) or inside membrane-derived vesicles [53–56]. Such complexes confer protection from RNAse degradation with consequent high degree of stability and measurability. However, the origin of circulating miRNAs is still unclear although two main hypotheses have been raised: the first argues that miRNAs could be passively released into the circulation during tissue injury, remaining stably complexed to ribonucleoproteins in the extracellular environment [57, 58], whereas the second supports the active and regulated secretion of endogenous miRNAs within microvesicles (e.g., exosomes), which are released into the extracellular environment by different cell types [59]. Several reports proposed that regulated secretion of miRNA-containing microvesicles may play an important role in cell-to-cell communication, both in physiological and in pathological conditions. There are three different types of microvesicles containing extracellular miRNAs: apoptotic bodies, shedding vesicles, and exosomes [60]. The latter are vesicles of 40–100 nm diameter, which are formed via the inward budding of plasma membrane into multivesicular bodies within endosomes. In response to extracellular stimuli, in both physiological and pathological conditions, they are released into the extracellular compartment upon fusion of endosomes with the plasma membrane of the recipient cell [61, 62]. Exosomes are produced by a wide variety of cell types including reticulocytes, neurons, and epithelial and tumor cells, as suggested by *in vitro* studies [55, 61, 63–65], and appear to contribute to different biological functions including immune response, antigen presentation, and protein and nucleic acids transport [55, 66–68]. It is also possible to hypothesize that specific miRNAs can be selectively secreted from cells, packaged into appropriate carriers to be transported to target cells (or tissues), and released into recipient cells, for example, through the fusion of exosomes with the plasma membrane or by binding to specific receptors, in order to regulate gene expression [69–71]. It remains unclear whether circulating miRNAs are secreted in a specific and signal-dependent manner [55, 71–74]. Indeed, gaining insight into the relationship between circulating and tissue miRNAs will certainly contribute to the understanding of the origin of circulating miRNAs. To date, many studies in oncology have reported a similar trend of expression between circulating miRNAs and tissue miRNAs [59, 75, 76]. In contrast, several authors have also described opposite differential expression profiles of miRNAs transported in vesicles compared to parent cells, suggesting that some miRNAs may be transcribed only to be exported or that their secretion is tightly regulated [55, 72, 74].

In light of these studies, it is not unlikely that virtually all cells may be able to secrete miRNAs in a specific and controlled fashion, in order to regulate gene expression also in distant tissue sites. Recently, it has been suggested that both islet beta-cells and immune cells are capable of miRNAs secretion and that this phenomenon may represent a mechanism involved in functional defects both at islet and at immune system level. Indeed, a recent study reported the analysis of miRNA content in microvesicles released by several beta-cell lines and primary pancreatic islets and tested the biological relevance of miRNA transfer among beta-cells. Analysis of the global profile of miRNAs in released microvesicles revealed that it did not reflect that of the parent cells. Importantly, some miRNAs poorly expressed in parent cells were abundant in vesicles, suggesting a preferential release of a subset of miRNAs. It was also shown that the expression levels of several miRNAs released into the medium changed upon exposure of beta-cells to cytokines or to palmitate. Indeed, the incubation of naïve beta-cells with miRNAs-containing microvesicles from donor cells treated with cytokines does not affect the secretory functions of recipient cells but results in an increase in apoptosis. Taken together, these data suggest that the cytokine milieu derived from immune cells infiltrating islets can negatively influence

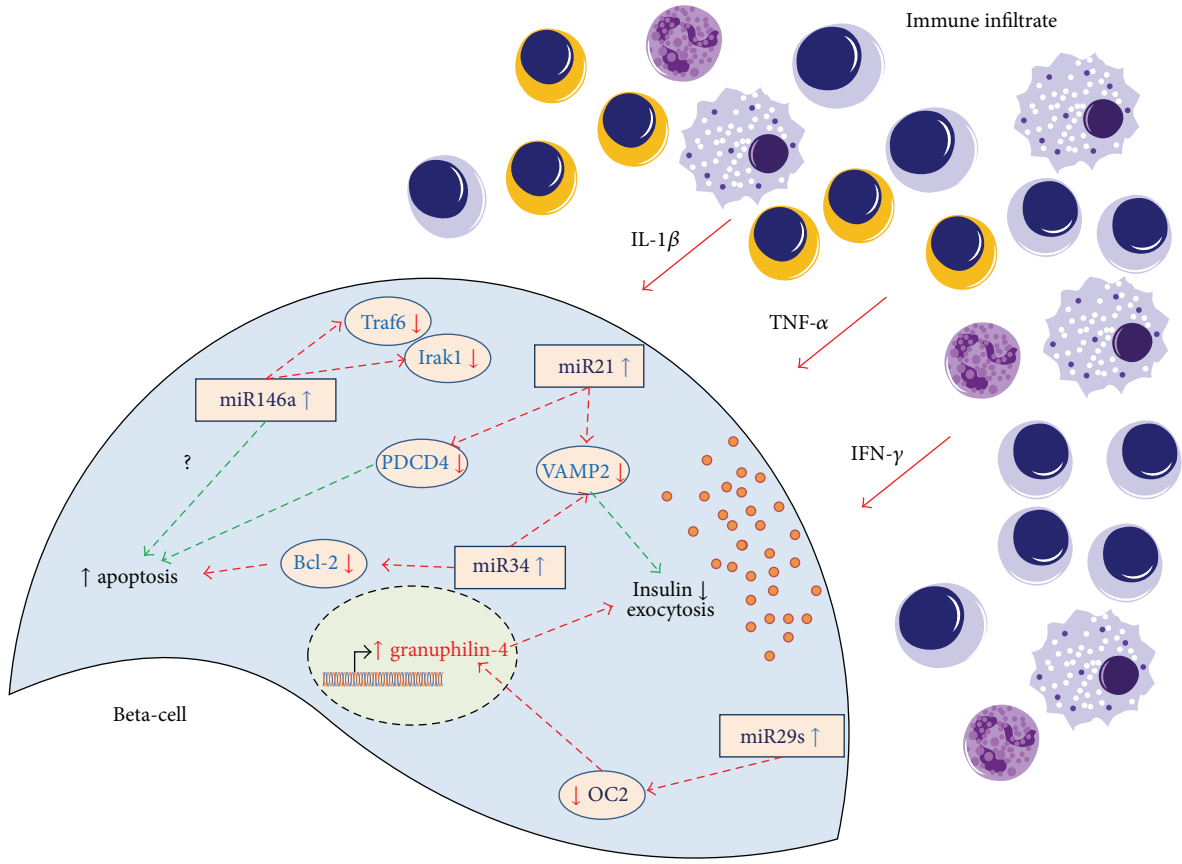

FIGURE 1: MicroRNAs as major players in immune-mediated beta-cell damage in autoimmune diabetes. The figure reports a representing scheme of microRNAs (miRNA) response induced by infiltrating immune cells (mainly CD4+ and CD8+ T-lymphocytes, B-lymphocytes, and macrophages) and secreted cytokines. The secretion of IL-1β, TNF-α, and IFN-γ induces changes in expression of several miRNAs. Specifically, upregulation of miR-146a, miR-34, miR-21, and miR-29s is shown. Each miRNA targeting specific genes (with consequent inhibition of their expression) is reported as a red dotted arrow, while the activation of a specific mechanism is represented as green dotted arrow. Upregulation of miR-146a induces the activation of apoptosis pathway through a not yet understood mechanism, while inhibiting the expression of Traf6 and Irak1 (NF-κB pathway). Upregulation of miR-34 leads to reduction of antiapoptotic gene Bcl-2 and of VAMP2, a molecule involved in the fusion of insulin granules to the plasma membrane. The increased expression of miR-21 leads to a partial protection from apoptosis through the inhibition of cell death inducer PDCD4. It also decreases expression levels of VAMP2, thus blocking insulin secretion. Finally, upregulation of miR-29s (miR-29a-b-c) leads to reduction of OC2 (Onecut2), a transcriptional repressor of granuphilin-4 (inhibitor of insulin secretion), thus leading to its upregulation and subsequently to a reduced insulin exocytosis.

miRNAs secretion from beta-cells, thereby leading to islet dysfunction [77].

In another study, human islets released biological active extracellular vesicles, carrying specific miRNAs (such as miR-27b, miR-126, miR-130, and miR-296) involved in beta-cell function, insulin secretion, and angiogenesis and expressing surface molecules as well as islet-specific proteins (e.g., insulin, C-peptide). Interestingly, microvesicles content could be transferred to endothelial cells, resulting in the induction of insulin mRNA expression and protection from apoptosis and angiogenesis. These data demonstrated that human islets are able to release biological active extracellular vesicles and to transfer beta-cell specific proteins, mRNAs, and miRNAs to endothelial cells, inducing a proangiogenic and antiapoptotic cell phenotype [78].

Given the secretion of miRNA-containing vesicles from parent cells into the extracellular space, it is also possible to speculate that this phenomenon may be mirrored in serum or in plasma. Indeed, several studies have been performed on the evaluation of circulating miRNA expression profiles in serum or plasma of T1D patients, aiming at elucidating the potential role of circulating miRNAs in diabetes.

To this aim, we recently compared the expression profile of circulating miRNAs of 20 T1D patients with that of control subjects showing that 64/206 miRNAs detected were differentially expressed in sera of patients with T1D. Interestingly, these miRNAs are implicated in the regulation of beta-cell function and cell belonging to the immune system [79]. Another study by Nielsen and colleagues compared the expression profile of miRNAs in serum of children with T1D and control subjects and identified 12 miRNAs (including some involved in apoptosis of beta-cells) that were overexpressed in children with T1D (miR-152, miR-30a-5p, miR-181a, miR-24, miR-148a, miR-210, miR-27a, miR-29a, miR-26a, miR-27b, miR-25, and miR200a). Moreover, circulating levels of miR-25 were correlated to the residual beta-cell

function (assessed by C-peptide measurement) and with adequate glycemic control (assessed by the determination of glycated hemoglobin) three months from T1D onset [80].

Experimental lines of evidence associated circulating miRNAs with the development of immune-mediated diseases [81]. Transfer of molecules during immune cells interactions has been widely reported [82] and, to date, the role of exosomes in such phenomenon has been investigated by several authors [83, 84]. Two previous studies clearly demonstrated that transformed Epstein-Barr virus B-lymphocytes and dendritic cells were able to secrete exosomes with the capacity to present MHC-peptide complexes to specific T cells, suggesting a key role for exosomes in intercellular communication in immune system [85, 86]. More recently, several data indicated an exosomes-mediated transfer of miRNA between T cells and antigen presenting cells during antigen recognition and a capacity of transferred miRNAs to regulate gene expression in recipient cells, during immune synapse formation [73, 82].

In a recent study, Mittelbrunn et al. showed that exosomes isolated from T cells, B cells, and dendritic cells presented a unique miRNAs expression profile when compared to their parent cells. In particular, several miRNAs (such as miR-760, miR-632, miR-654-5p, and miR-671-5p) were significantly more expressed in exosomes from all cell types, like miR-335, which were found only in exosomes derived from dendritic cells; conversely, several miRNAs (such as miR-101, miR-32) were found to be more abundant in cells than in exosomes. Interestingly, these exosomes were able to mediate the antigen-driven unidirectional transfer of functional miRNAs (i.e., able to modulate gene expression in recipient cells) from T cells to antigen presenting cells during immune synapse formation. These data suggest that miRNAs are transferred during immune synapse formation, thus mediating several functions in recipient cells [73].

Evidence of the effects of circulating miRNAs in the modulation of immune system in diabetes was reported by Salama and colleagues [87] who showed that pancreatic beta-cell-derived miRNAs induce proinflammatory (TNF-α, IFN-α, IL-12, and IL-6) or suppressive (IL-10) cytokine secretion by mouse dendritic cells. Of note, miR-29b, by interacting with TLR7, stimulates dendritic cells to produce several cytokines and chemokines. Moreover, in a murine model of adoptive transfer of autoimmune diabetes, antigen-specific T cell responses and disease incidence were decreased by the systemic delivery of miR-29b, while *in vitro*, exosomes derived from beta-cells presented specific miRNA expression profiles, including miR-29b. These exosomes were shown to induce cytokine secretion (including TNF-α) from splenocytes isolated from NOD mice, while, in the presence of miR-29b, TNF-α secretion was impaired. On the basis of these results, it was suggested that beta-cell specific miRNAs, such as miR-29b, may modulate autoimmune response by recruiting innate immune cells through receptor-ligand interactions [87].

The hypothesis that miRNAs could mediate intercellular communication is fascinating. However further studies are still needed to fully demonstrate this hypothesis and several questions remain open. For example, it remains to be elucidated how extensively the process of cell-to-cell communication via extracellular miRNAs occurs *in vivo*. Finally, it has not been established in which form miRNAs are present in vesicles (i.e., as mature or pre-miRNAs) [58].

5. Concluding Remarks

An increasing number of studies have uncovered the role of miRNAs in autoimmune diabetes, both at pancreatic islet and at immune system level. Moreover, growing evidence indicates that miRNAs represent major players in the dialogue between beta-cells and immune system, regulating several aspects of their function and survival (Figure 1).

Interestingly, miRNAs have been found to be stably present in the extracellular environment, thus suggesting a possible new emerging role for miRNAs in cell-to-cell communication, in addition to their better-understood intracellular role as negative regulators of gene expression. Given the impact of miRNAs on beta-cells and immune cells in physiologic and disease conditions, secreted miRNAs may represent an important mechanism of islets-immune cell dialogue in autoimmune diabetes. Furthermore, being stable in extracellular fluids such as serum and plasma, miRNAs represent promising biomarkers for disease diagnostics and staging. Further studies aiming at clarifying miRNAs expression profiles in pancreatic islets or more specifically in pancreatic islets and in islet-infiltrating immune cells will shed light on these newly identified phenomena.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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