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CD39 expression on Treg and Th17 cells is associated with metabolic factors in patients with type 2 diabetes

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ABSTRACT

Th17 cells are involved in the pathogenesis of multiple inflammatory diseases such as type two diabetes (T2D). CD39$^+$ Treg cells have been implicated as responsible for suppressing Th17 cells. The aim of this study was to evaluate the number and function of CD4$^+$CD25^{high}CD39$^+$ Treg and Th17 cells in peripheral blood mononuclear cells (PBMC) from T2D patients and healthy control subjects. The Th17 cells were detected in PBMC under culture with human anti-CD3/CD28 and PMA/ionomycin and the levels of IL-17 were assessed by ELISA and qPCR. The T2D patients with obesity showed significantly lower percentages of CD39$^+$ Treg cells. A negative correlation between CD39$^+$ Treg cells and weight, and body mass index was detected. In contrast, the low levels of CD4$^+$IL-17$^+$ cells in overweight and obese T2D patients showed a positive correlation with glucose and HbA1c. Additionally, we found a subpopulation of Th17 cells that express CD39 and were correlated with glucose and HbA1c. Our findings suggest that the expression of CD39 on Treg cells and also in CD4$^+$IL-17$^+$ cells from T2D patients is related to hyperglycemia as well as to overweight and obesity and therefore may participate as a modulator of the effector capacity of Th17 cells.

1. Introduction

T2D represents a group of metabolic conditions characterized by abnormally increased levels of blood glucose due to impaired insulin action and/or insulin secretion. These features have been associated with both genetic and acquired factors [1]. Nowadays, the pathogenesis of T2D is considered linked to both innate and adaptive immune factors as well as an inflammatory process that is recognized as an important etiological component in the development of insulin resistance [2]. Furthermore, low-grade systemic inflammation and immune disorders are linked to the development of diabetic complications [3]. On the other hand, several in vitro functional defects of the immune system have been correlated with metabolic control in diabetic patients, which has been related to an increased susceptibility to frequent and protracted infections in these patients [4,5]. Therefore, the alteration of the immune system in T2D patients and its regulatory mechanisms are relevant factors to be investigated.

The balance between Treg and effector T cell subsets such as Th17 cells is important for immune homeostasis and the immune response. The role of T cell subset imbalance in mouse models of T2D inflammation and the involvement of adipose tissue T cells in T2D patients has been reported [6]. CD8$^+$ and CD4$^+$ T cells, particularly Th1 and CD4$^+$CD25^{high} Treg cells in visceral adipose tissue, play a fundamental role in the regulation of body weight, adipocyte hypertrophy, insulin resistance, glucose tolerance and T2D

Abbreviations: T2D, type 2 diabetes; Treg, regulatory T cells; BMI, body mass index; WHR, waist to hip ratio; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; PBMC, peripheral blood mononuclear cells; ADA, American Diabetes Association; WHO, World Health Organization; OW, overweight; NW, normoweight; Ob, obesity.

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progression in mice and humans [6–8]. In this regard, some studies have suggested that designed strategies for increasing the number of Treg cells might be of therapeutic promise in insulin resistance and T2D [6]. Nevertheless, the newly defined CD39$^+$ Treg subset has emerged as a crucial player in pathogenic Th17 cell suppression and its importance has been demonstrated in diseases like multiple sclerosis and rheumatoid arthritis [9,10,35]. CD39 is an ectonucleotidase that cleaves ATP in a rate limiting step to form AMP, which can then be cleaved by CD73 to form adenosine [11]. Extracellular ATP has multiple proinflammatory effects, and its removal may therefore have a net anti-inflammatory influence. Furthermore, ligation of adenosine to its A2A receptor, which is expressed on T cells, results in elevation of intracellular cAMP and suppression of effector T cell function [12].

The CD39 molecule is as an important modulator of hepatic carbohydrate metabolism. In this regard, mice deficient in CD39 demonstrate impaired glucose tolerance following oral glucose tolerance testing as a consequence of hepatic insulin resistance [13], and also develop an immune diathesis and spontaneous autoimmune alopecia [14]. On the other hand, Th17 cells and their potential role in the pathogenesis of the inflammatory process observed in T2D are not clear. Therefore, we evaluated the number and suppressive function of CD4$^+$CD25^{high}CD39$^+$ and CD4$^+$CD25^{high}CD39$^-$ T cells as well as their ability to suppress Th17 cells in peripheral blood mononuclear cells from T2D patients.

2. Materials and methods

2.1. T2D patients and healthy control subjects

Peripheral blood mononuclear cells (PBMC) were obtained from 30 patients (15 females and 15 males) diagnosed with T2D (mean duration of diabetes = 6.5 years; mean age = 48 years). All the T2D patients were recruited from the Central Hospital of Dr. Ignacio Morones Prieto (S.L.P., México). Subjects with inflammatory disorders, microvascular and macrovascular complications, abnormal liver, renal, or thyroid function, steroid therapy or anti-inflammatory drug use as well as patients with statins and any other hypoglycemic drug different to metformin and glibenclamide therapy were excluded. Control subjects (14 females and 18 males; mean age = 42 years) with normal complete blood count, no family history of diabetes or other chronic diseases and fasting plasma glucose levels of <100 mg/dL were included in the study. Complete blood count, serum lipid profile, fasting plasma glucose (FPG) and HbA1c levels were assayed. Clinical parameters and anthropometric measurements from patients and healthy controls are shown in Table 1. As expected, FPG and HbA1c levels, body mass index (BMI) and waist-to-hip ratio (WHR) were found to be significantly higher in T2D patients compared with the healthy volunteers. Informed consent was obtained from all the subjects, and the study was approved by the Bioethics Committee of the Hospital Dr. Ignacio Morones Prieto (number: 10-11).

2.2. Flow cytometry analysis

PBMC were isolated on a Ficoll–Hypaque gradient and stained with different combinations of monoclonal antibodies to CD4-PerCPCy5.5, CD39-FITC and Foxp3-PE or CD25-PE (eBiosciences, San Diego, CA) for Treg cells. For Th17 analysis, cells were also stained with CD4-PerCPCy5.5 and CD39-FITC (eBioscience). For intracellular staining, cells were treated with Fix/Perm buffer (eBioscience) and stained with anti-FOXP3-PE or anti-IL-17A-PE (eBioscience). Data were acquired with a FACSCalibur flow cytometer (Becton Dickinson, San José, CA) and analyzed using Cell Quest Software. CD4$^+$ T cells were gated on the basis of Foxp3vs CD39 or CD25 vs CD39 for cell sorting on functional assays. The results were expressed as the percentage of positive cells.

2.3. Isolation of CD4$^+$CD25^{high}CD39$^+$ and CD4$^+$CD25+CD39$^-$ Treg cells

CD4$^+$ T cells were isolated from PBMC by negative selection using magnetic cell sorting technology (MACS; Miltenyi Biotech, Germany). Then, CD4$^+$ T cells were separated into CD25$^+$ and CD25$^-$ populations by MACS using anti-CD25 microbeads (Miltenyi Biotech). The CD25^{high}CD39$^+$ and CD39$^-$ T cells were sorted by flow cytometry with a FACSAria II (Becton Dickinson, San José, CA). The purity of the enriched CD4$^+$CD25$^+$CD39$^+$ T cells was approximately 95%, as determined by flow cytometry.

2.4. Immunosuppressive assay

The immunosuppressive capacity of CD4$^+$CD25$^+$CD39$^+$ Treg cells was determined using a classic in vitro Treg and Tresp co-culture system with a carboxyfluorescein succinimidyl ester (CFSE) based T-cell proliferation assay. Briefly, sorted CD4$^+$CD25$^-$ (2×10^4 cells/well) Tresp cells were labeled with 5 μM CFSE (Sigma) for 10 min at 37 °C and quenched by addition of cold FCS and RPMI.Then, labeled cells were co-cultured with autogenic CD4$^+$CD25$^+$CD39$^+$ Treg cells and stimulated with plate-bound anti-CD3 (HIT3a clone) and anti-CD28 (CD28.2 clone) (eBioscience) antibody for at least 1 h at 37 °C. On day 5 of culture, the cells were harvested, fixed and stored at 4 °C until acquisition and analysis by flow cytometry. The percentage of suppression was determined as: 100 − ((percentage of divided cells in the Treg and Tresp cell co-culture/percentage of divided cells of Tresp cell alone)×100). The CD39 ectonucleotidase inhibitor ARL67156 (Sigma–Aldrich, St. Louis, MO) was added to suppression assays at a final concentration of 100 μM.

2.5. Th17 cell detection by flow cytometry

Freshly isolated PBMC (1×10^6 cells/well) were incubated with human anti-CD3/CD28 (eBioscience) at 5 μg/ml for 72 h without polarizing cytokines to optimize the detection of IL-17. PBMC samples were suspended in RPMI media with 50 ng/ml PMA, 0.5 μg/ml ionomycin and 10 μg/ml brefeldin A (Sigma) for 3 h before intracellular staining. Cell-free supernatants were assessed for IL-17 concentration by ELISA.

2.6. Cytokine measurement

IL-17 was determined on supernatants from Th17 cells or co-cultures. PBMC or Treg cells were cultured as described above. After the indicated incubation times, cell-free supernatants were assessed for cytokine concentration by ELISA (Biolegend) according to the instructions provided by the manufacturer.

2.7. RT-PCR

mRNA expression of IL-17 was determined by real-time PCR in PBMC from T2D patients and healthy control subjects previously cultured in the same way as for Th17 cell detection. Total RNA was isolated from 5×10^5 PBMC using TRIzol reagent (Invitrogen, Life Technology, Carlsbad, CA, USA), and the concentrations and integrity of the RNA sample were measured using a spectrophotometer Synergy HT (Biotek). cDNA was generated with 100 ng of total RNA using the High Capacity cDNA ReverseTranscription Kit (Applied Biosystems, Foster, CA, USA) according to the manufacturer's instructions. A total of 100 ng of cDNA was applied to real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems). Amplification of 18s ribosomal RNA was used as a