Original Article

Bacterial Infections Upregulate Cluster of Differentiation 80 on Regulatory B-Cells in Iraqi Children with Type I Diabetes Mellitus

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Abstract

Background: Although type 1 diabetes mellitus (T1DM) is thought to be an autoimmune ailment driven by T-cells, recent research also suggests that B-cells are important in the development of the condition. In order to maintain tolerance, regulatory B-cells restrict ongoing immunological responses and restore immune homeostasis. **Objective:** The current investigation aimed examined the expression and mean fluorescence intensity (MFI) of cluster of differentiation 80 (CD80) molecule on B-regs, and the association between assessing the soluble form of lipopolysaccharide (LPS)-binding protein (LBP) and toll-like receptor 4 (TLR4) in serum for T1DM without and with urinary tract infections in comparison to healthy individuals. **Materials and Methods:** A total of 90 male children, whose ages ranged between 5 and 15 years participated in this study. The frequency of B-cell subsets was measured using flow cytometry and cultivation urine on agar to indicate urinary tract infections (UTIs).. The level of human serum LBP and TLR4 was measured using enzyme-linked immunosorbent assay (ELISA) assay. **Results:** According to the study's findings, T1DM with UTIs significantly increased CD80 expression on B-regs, whereas T1DM without UTIs only slightly decreased it when compared to the control group. Additionally, it demonstrated that in both groups of T1DM patients, the MFI of CD80 expression on B-reg has significantly decreased. The study also showed that T1DM with UTIs had significantly higher serum levels of TLR4 than other two groups. **Conclusions:** This study suggests a significant role for costimulation via CD80 molecule that up-regulates on B-regs surface in T1DM progression and innate immune stimulants and modifiers derived from LPS may have important utility in the future to protect against infectious diseases and provide opportunities for immunotherapy to target it.

Keywords: Bacterial infections, CD19+IL-10+ B-cell, CD80 molecule, regulatory B-cells, type 1 diabetes mellitus

Introduction

Type 1 diabetes mellitus (T1DM), an organ-specific autoimmune disease with a multifactorial etiology, is characterized by the immune-mediated destruction of beta cells in pancreatic islets, resulting in insufficient insulin production.[1] Immune cells that infiltrate the pancreas and attack insulin-producing cells induce and accelerate the development of T1DM by gradually presenting the immune system with islet antigens, which creates an inflammatory environment characteristic of insulitis.[2] Increasing evidence demonstrates a role for B-cells in autoimmune diabetes, B-cells invade the islets and contribute to the start of the autoimmune response that destroys cells.[3]

B-regs is a term that encompasses all B-cells that act to reduce immunological reactions and restore immune homeostasis, which helps to maintain tolerance.[4] Despite interest in these cells, it is still unclear how important they are for human peripheral tolerance maintenance, human cluster of differentiation 19 (CD19)+CD24^{hi}CD38^{hi} B-cells were demonstrated to have regulatory capacity; upon stimulation by the CD40 molecule, CD19+CD24^{hi}CD38^{hi} B-cells inhibited the differentiation of Th1-cells, in part by the administration of interleukin-10 (IL-10), and their suppressive capacity was undone by the addition of the CD80 molecule.[5]

Access this article online

Quick Response Code:

Website: https://journals.lww.com/mjby

DOI: 10.4103/MJBL.MJBL_458_23

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Submission: 21-Apr-2023 **Accepted:** 12-Jun-2023 **Published:** 30-Sep-2025

How to cite this article: Oudah NA, Alsaadi KA, Mohammed Ali HH. Bacterial infections upregulate cluster of differentiation 80 on regulatory B-cells in Iraqi children with type I diabetes mellitus. Med J Babylon 2025;22:704-10.

T1DM inflammatory process depends on T-cell activation, this necessitates T-cell receptor activation in response to the major histocompatibility complex (MHC)-peptide complex on the cell surface of antigen-presenting cells (APC), including B-cells.[6] A second interaction involving a co-stimulatory molecule, CD80, CD86, and PD-L1, which express on the surface of B-reg and belong to the B7 family, is in addition to this antigen-specific stimulation crucial for preserving the equilibrium between a sufficient immune response, immunosuppression, and autoimmune disease.[7,8] Since both CD80 and CD86 interact differently with their ligands, CD28 and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) (CD152), respectively, they each perform distinct roles in immunological regulation, and as a result, their expression is only found on B-reg.[9] A stronger affinity exists between CD80 and CTLA-4 (the negative regulatory) than between CD86 and CTLA-4. Conversely, CD28 has a stronger connection with CD86 (the positive regulatory) than CD80. A co-stimulatory-co-inhibitory system is formed by these interactions, which controls immunological reactions.[10]

In bacterial infections, lipopolysaccharide (LPS) or bacterial endotoxin interacts with LPS-binding protein (LBP), which makes it easier for it to engage with CD14 on the surface of monocytes later.[11] When LPS binds to its specific receptor CD14/toll-like receptor 4 (TLR4), a series of signaling events are triggered, activating genes and causing the production of proteins. In particular, transcription factors and signaling molecules that control the expression of CD80 and CD86 have been identified.[12] In addition to LPS, other stimuli that can cause CD80 expression include IL-4, anti-B-cell receptor antibodies or anti-CD40 antibodies, as well as the stimulation of monocytic cells with LPS and interferon-γ (IFN-γ).[13,14] For T-cell development and immune response activation, CD80 and CD86 are essential. So it stands to reason that immune-regulatory cytokines and mitogens, like LPS, would alter CD80/CD86-mediated signals to improve T-cell inhibition or activation.[15] This study aimed to identify the role of CD80+ B-regs as well as the MFI of this molecule in T1DM patients and determine if urinary tract infections play a role in this consideration.

Materials and Methods

Patient and clinical parameters tests

The practical side of the study was performed at the Al-Husain Teaching Hospital, Karbala. All samples were collected from February 2021 till July 2021. A total of 90 male children participated, whose ages ranged between 5 and 15 years participated in this study, that had 60 patients with T1DM and half of them have urinary tract infections (UTIs), and 30 healthy controls. The criteria based on World Health Organization (WHO) recommendations were used to diagnose T1DM.[16] Fasting plasma glucose (FPG) and hemoglobin A1c (HbA1c) were tested to determine their diabetes, while the glutamic acid decarboxylase antibodies (anti-GAD) and connecting peptide (C-peptide) tests were done to confirm the diagnosis of T1DM, the FPG, HbA1c and C-peptide were investigated by cobas-e-411 analyzer (Roche, Switzerland)[17] but anti-GAD was investigated by enzyme-linked immunosorbent assay (ELISA) from Elabscience, Wuhan, China.[18] All patients were confirmed to be negative for chronic infection disease, allergic, or cancer and did not receive any immunosuppressive medication. Venous blood samples (6 mL) were collected from each person into two types of tubes: 3 mL in ethylenediaminetetraacetic acid (EDTA) tube for flow cytometry and HbA1c, the other one is gel tube was allowed to clot at 37°C for 15–20 min and then centrifuged at 3000×*g* for approximately 12–15 min in deep freezer (–80°C).[19] The collected serum from patients and control were used for the measurements of anti-glutamic acid decarboxylase (GAD) antibodies, C-peptide secretion tests, human TLR4, and human LBP.

Identification of B-regs and CD80 by flow cytometry

Material

The percentage of B-regs was identified by flow cytometry. The following monoclonal antibodies were used: anti-CD19/PE-CY7 MAB (HIB19), anti-IL-10/APC-R700 (cloneJES3-19F1), anti-CD24/FITC (clone ML5), CD27 PE (clone LEU-27), and anti-CD80/APC-H7 (clone L307). The test was conducted at Imam Zain El-Abidine Hospital.

Blood sampling and flow cytometry staining procedures in human

To identify the cell surface markers on B-lymphocytes and intracellular IL-10+ expression by flow cytometry, cells were surface stained with anti-human mAb (all from BD), and the staining was performed in a darkened room. For IL-10 intracellular detection in B-reg, it is necessary to block cytokines' secretion by inhibiting protein transport inside the cell. Therefore, we added BD IntraSure™ kit (intra A and intra B), containing ready-to-use reagents for fixation and permeabilization that enable optimal staining of intracellular markers while maintaining cell surface staining.[20] Samples were analyzed using the gating strategy as shown in Figure 1.

Measurement of lipopolysaccharide-binding protein and toll-like receptor 4

The level of human LBP and TLR4 was measured using ELISA assay. More specifically, the sandwich ELISA for the quantitative determination of human immunological by used Elabscience Kits, China.[21]

Isolation and identification of bacteria

Ten milliliters urine samples were collected from all patients and controls. Patients with UTI were separated according to result of inoculating the sample on

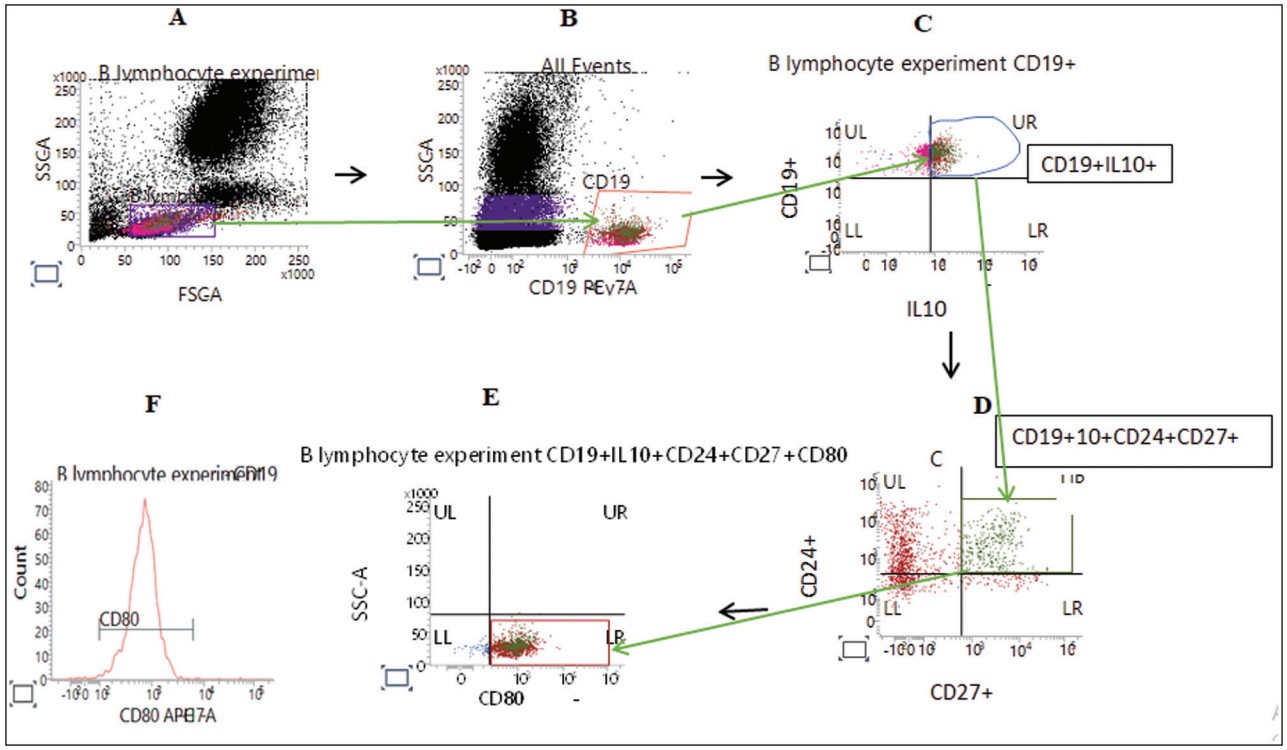

Figure 1: Gating strategy for cluster of differentiation (CD80)+ quantification in B-regs. (A) Within singlets of the examined peripheral blood mononuclear cell (PBMC) population, every lymphocyte was identified based on its forward scatter (FSC) and side scatter (SSC) characteristics. (B) CD19+B-cells gated. (C) IL-10-producing B-cells gated. (D) CD19+IL-10+CD24+CD27+B-regs were calculated. (E) CD19+IL-10+CD24+CD27+ CD80+B-regs were calculated. (F) Mean fluorescence intensity (MFI) of CD19+IL-10+CD24+CD27+CD80+B-regs

MacConkey agar and blood agar base aerobically at 37°C for 24 h. To identify urine samples with substantial bacteriuria, bacterial colonies that developed on the plates following the incubation time were counted. Plates with growth of ≥100 colonies (10^5 cfu/mL) were considered positive bacteriuria and involved in patient group, while ≤100 colonies were excluded.[22,23]

Statistical analysis

The statistical analysis of this study was carried out using the software statistical package for social sciences (SPSS) version 22 (IBM, USA), where the data were expressed as a mean ± standard error of the mean, independent-sample *t* test with their 95% confidence interval, were used to find the association between the categorical variables, $P \leq 0.05$ was considered statistically significant, the significance value was indicated as * between the groups. The level of probability was indicated as *$P \leq 0.05$, **$P \leq 0.01$ *** $P \leq 0.001$ and **** $P \leq 0.0001$.

Ethics approval

The current study was approved by the ethics committee of clinic of Imam Hussein Hospital No. 240-14/2/2022 in Karbala, Iraq. All participants in this study willingly participated, and parents of children were contacted before enrollment to get their informed written consent for sample collection and research.

Results

Demographic and clinical characteristics

Ninety participants were enrolled in the current study, and Table 1 summarizes the general qualities in each group. The T1DM a male patients included 30 with UTIs and 30 without UTIs. The fasting blood glucose levels of both groups of diabetic patients were similar, but the HbA1c was significantly higher than that of healthy controls. Similarly to the levels of serum glucose and blood HbA1c, the mean levels of serum anti-GAD were significantly higher in both T1DM groups compared to control but significant rise in T1DM with bacterial infections which reached 364.49 ± 122.72 U/mL. Of note, the C-peptide for both groups of T1DM was significantly lower than that of healthy controls.

Flow cytometry analysis of B-reg

Level of CD80+B-regs

Figure 2 shows the CD80 expression on B-reg in two groups of diabetic patients and control group. The frequency of CD80-expressing B cells a significant increase for T1DM with bacterial infection compared to control group, if the mean percentage are 92.46 ± 1.25% vs. 89.16 ±1.13% in $P < 0.05$.

Mean fluorescence intensity of CD80 molecule on B-reg

The results of this part of the study revealed the decreased expression of CD80 on the surface of B-reg in T1DM a

Table 1: Study groups' general qualities

Clinical tests	Mean ± SE	Mean ± SE	Mean ± SE
	T1DM with UTIs	T1DM without UTIs	Healthy control
FBG (mg/dL)	250.00 ± 19.76****	245.93 ± 25.98$^{\Delta\Delta\Delta\Delta}$	92.30 ± 0.66
HbA1c (%)	10.29 ± 0.42****	10.43 ± 0.40$^{\Delta\Delta\Delta\Delta}$	4.94 ± 0.06
C-peptide (ng/mL)	0.225 ± 0.12***	0.176 ± 0.07$^{\Delta\Delta\Delta}$	2.458 ± 0.16
GADA (U/mL)	364.49 ± 122.72*	226.94 ± 109.93$^{\Delta\Delta\Delta}$	2.28 ± 0.20

GADA: glutamic acid decarboxylase antibodies, HbA1c: hemoglobin A1C, UTIs: urinary tract infections

The mean ± standard error (SE) for parametric data

*$P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$ mean T1DM with UTIs compared to healthy control

$^{\Delta}P \leq 0.05$, $^{\Delta\Delta}P \leq 0.01$, $^{\Delta\Delta\Delta}P \leq 0.001$, $^{\Delta\Delta\Delta\Delta}P \leq 0.0001$ mean T1DM without UTIs compared to healthy control

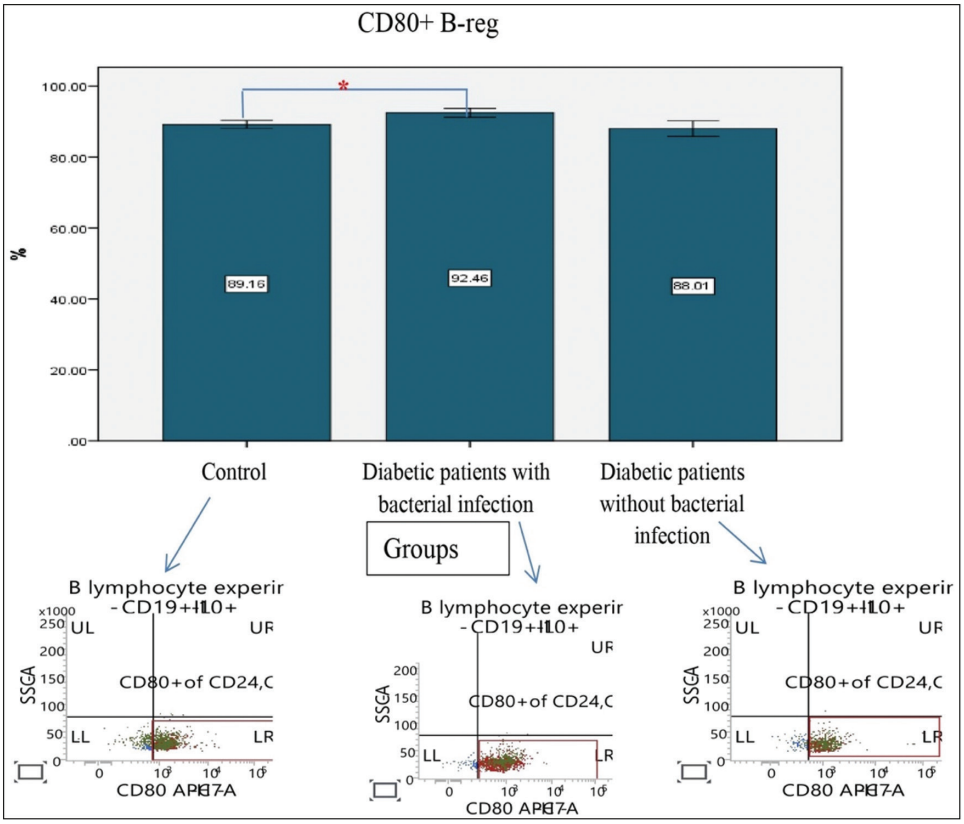

Figure 2: Cluster of differentiation 80 expression on B-reg for T1DM without urinary tract infections (UTIs) T1DM with UTIs and healthy controls

male person for both groups compared to control but the differences did not reach to a significant level [Figure 3].

Lipopolysaccharide-binding protein concentration

Figure 4 shows the serum LBP levels were higher for both patient groups of T1DM compared to control. The results also showed a significant increase in LBP levels for T1DM with bacterial infection and the mean was 3620.80 ± 456.24 ng/mL and T1DM without bacterial infection the mean was 1230.89 ± 138.96 ng/mL at level $P \leq 0.001$ compared to control (540.29 ± 54.89) ng/ml. LBP levels were significantly elevated in diabetic patients with bacterial infections compared with patients without bacterial infection at level $P \leq 0.001$.

Toll-like receptor 4 concentration

The study revealed a significant rising in the serum levels of TLR4 in diabetic patients with bacterial infections and diabetic patients without bacterial infections compared to the control. The results also showed a significant increase in TLR4 levels for T1DM with bacterial infection the mean was 4.584 ± 0.50 ng/mL and for T1DM without bacterial infection the mean was 2.849 ± 0.12 ng/mL on level $P \leq 0.0001$ and $P \leq 0.01$ respectively, compared to control (2.524 ± 0.95) ng/mL. TLR4 levels were significantly elevated in diabetic patients with bacterial infections compared with patients without bacterial infection at level $P \leq 0.05$ [Figure 5].

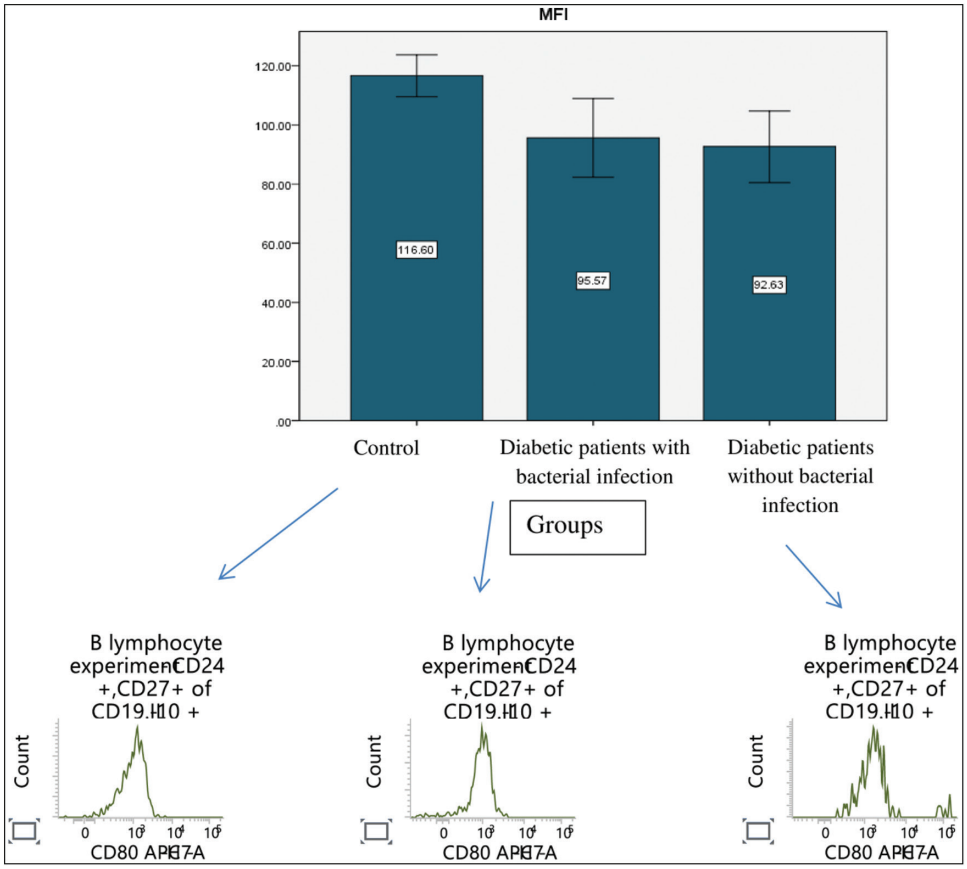

Figure 3: Comparison of cluster of differentiation 80 molecules expression on B-reg between T1DM without urinary tract infections (UTIs) T1DM with UTIs and healthy controls

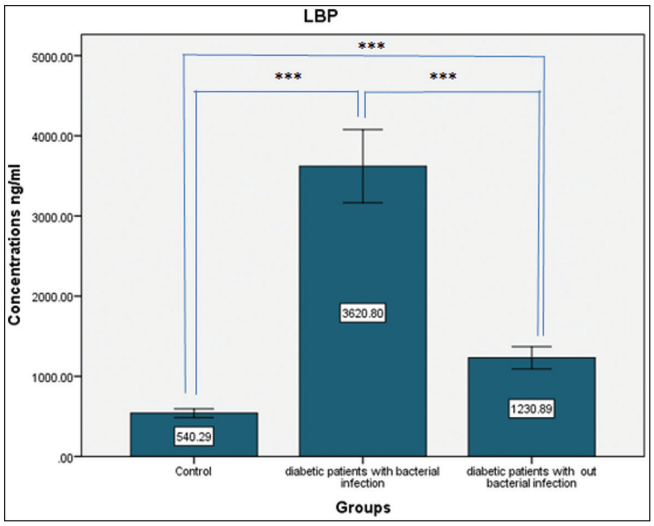

Figure 4: Serum concentrations of lipopolysaccharide-binding protein (LBP) in the groups of the study

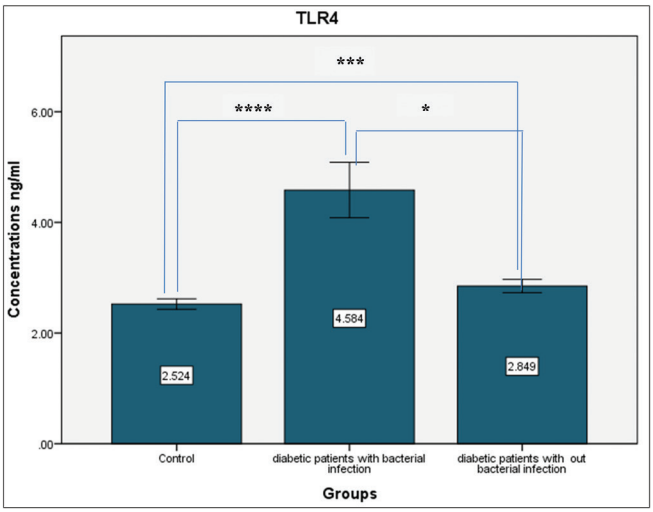

Figure 5: The serum concentrations of toll-like receptor 4 (TLR4)

Discussion

T1DM is a common chronic autoimmune disease characterized by the destruction of insulin-producing pancreatic β-cells by their own immune system, resulting in lifelong insulin deficiency.[1] The diagnosis is made on the basis of the confirmatory screening test, if a patient meets the diabetes criterion of the HbA1c results in >6.5% and FPG ≤125 mg/dL, that person should be considered to have diabetes.[16] After confirming that the children had diabetes, screening tests were conducted to prove that they had type 1 diabetes such as detecting autoantibodies produced by B-cell activation to GAD and the concentration of C-peptide.[24] The autoantibodies multiple because of beta cell failure and undergo

programmed death or degradation by immune cells and may serve as an indication for type 1 diabetes but the low or undetectable levels of plasma connecting peptide can indicate the presence or absence of insulin secretion.[16] The children were chosen to investigated of altered frequency of regulatory B-cells in the target groups in the current study because the disease is in its early stages and provides more accurate information about the changes that occur in the immune system and loss of tolerance of islet antigen-reactive B-reg occurs early in disease.[25]

After the diagnosis, the research groups were identified to investigate expression and MFI value of CD80 molecule on B-reg cell surface and to find the relationship between the soluble form of LPS and TLR4 in serum for T1DM without and with UTIs compared to control. The major findings of the study are 1) a slightly elevated or equal expression of CD80 molecule by B-reg for T1DM with UTIs compared with control, 2) MFI value of CD80 expression on B-reg decreased on the surface of B-reg in T1DM patients for two groups compared to control, 3) in T1DM subjects with/without UTIs had significantly higher levels of LPS and TLR4 compared with control, 4) a significant increase of LPS and TLR4 for T1DM with UTIs patients comparably with T1DM without UTIs and control.

Despite substantial scientific and clinical studies on these molecules, the roles and mechanisms of co-stimulatory molecules in the etiology of diabetes remain poorly understood. Co-stimulatory dyads usually play a paradoxical role in the development of diabetes because of the complicated and distinctive environment of the disease.[26] The following are some theories that could account for the contradictory roles that co-stimulatory dyads play in diabetes mellitus: co-stimulatory molecules like CD80 and CD86 must be expressed on a regular basis to maintain T-reg numbers; during the course of diabetes, a range of inflammatory cytokines may variably regulate several co-stimulatory molecules; CD80 can induce hypoproliferative T-cells that generate both IL-10 and transforming growth factor-β (TGF-β1) and function as adaptive T-regs; different co-stimulatory molecules affect certain cell types differently.[27]

In a mouse model of colitis, groundbreaking investigations revealed that CD80 is important in B-cell inhibition of pathogenic T-cells and in avoiding disease development.[28] These findings were further supported for human IL-10+ B-regs, where IL-10, CD80, and CD86 cooperate to inhibit Th1 responses.[29] Studies in both people and mice have shown that CD80/CD86 and CD28 contact is necessary for peripheral T-reg homeostasis, whereas CTLA-4 engagement is crucial for T-reg-mediated suppression.[30] It was later observed that CD80/CD86-deficient B-cells were unable to induce T-regs.[31] It has been shown that B-cell antigen presentation is necessary for the best effector immune reactions. On the other hand, antigen presentation by B-cells that lack CD80 can induce T-cell anergy or T-regs.[32] Nonspecific immune receptors recognize bacterial-specific motifs. One such receptor/ligand complex is formed between the mammalian cell TLR4 and bacterial LPS.[33] Recent research has revealed that TLR4-mediated responses exhibit substantial phylogenetic and individual variability. Additionally, a number of immunological diseases have been linked to the diversity of LPS structures and the variable identification of these structures by TLR4.[34-36]

It is interesting to find in this study that LPS/TLR4 ligand can induce up-regulation of CD80 molecule along with IL-10 in the B-reg subset while failed in mean fluorescence intensity of CD80. Consequently, the potential use of TLRs as adjuvants to treat T1DM remains of great interest.

Conclusions

Our study contributes to the understanding of how UTIs promote B-reg differentiation in type 1 diabetes. We showed that the infectious risk signaling ligand LPS can drive naive B-cell subsets to produce a sizable amount of CD80+B-reg. These findings underscore the likelihood that B-reg may have been involved in the development of the disease by supporting a function for LPS/TLR4 signaling in the differentiation of B-reg.

Acknowledgments

The authors would like to express gratitude to the parents of children and all the people participating in this study.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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