Published in final edited form as:

Cytokine. 2018 April ; 104: 65–71. doi:10.1016/j.cyto.2017.09.032.

TLR-induced secretion of novel cytokine IL-27 is defective in newly diagnosed type-2 diabetic subjects

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Abstract

Toll-like receptors (TLRs), the innate immune receptors, act as sentinels bridging both innate and adaptive arms of immunity. In the present study, we estimated TLR-induced secretion of IL-27, IL-12, IL-23, IL-8, IP-10, IL-17, IL-6 and TNF- α (by ELISA) and expression of Human Leukocyte Antigen- (Human Leukocyte Antigen - antigen D Related (HLA-DR), CD69, CD80 (also known as B7-1) (by flowcytometry) and Activating Transcription Factor 3(ATF3) (by qRT-PCR) in whole blood cultures of control and type-2 diabetic (both newly diagnosed/NDD and known/KDM) subjects. TLR-induced secretion of IL-27 was significantly reduced in the NDD group compared to the control (Normal Glucose Tolerance (NGT)) and KDM groups. On the other hand, the expression of CD80 was significantly upregulated in both the monocytes and B cells in KDM group. This was associated with increased T cell activation (CD3+CD69+HLA-DR+) with increased IL-17 and reduced TNF- α secretion in this group. Impaired TLR-induced IL-27 secretion and augmented expression of antigen presentation molecules result in chronic T cell activation which may fuel T cell-mediated inflammation in type-2 diabetes.

Keywords

TLR; D80; HLA-DR; IL-27; Type-2 diabetes

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Author's contribution

Conceived and designed the experiments: VA SB VM. Provided samples: VM. Performed the experiments: HM. Analyzed the data: VA and HM. Wrote the paper: HM. All authors have read and approved the final manuscript.

Conflict of interest

None.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cyto.2017.09.032>.

1. Introduction

Chronic inflammation and impaired immunity are two key defects in type-2 diabetes mellitus [1]. Toll-like receptors (TLRs) are innate immune receptors which act as sentinels bridging both innate and adaptive arms of immunity [2]. Out of the 10 TLRs known in humans, TLR2 and 4 have been implicated in T-cell activation wherein they serve as co-stimulatory receptors directly modulating T cell activity [3,4]. On the other hand, TLRs present in professional antigen presenting cells (APCs), when activated, can induce antigen presentation machinery (HLA-DR and CD80/B7-1) and cytokines/chemokine secretion, thereby orchestrating the recruitment and activation of T cells [5]. Aberrant expression of these molecules can result in inappropriate T cell activation leading to inflammation and impaired immunity which has been reported in diabetic nephropathy [6,7]. TLR induced secretion of Th polarizing cytokines especially those belonging to the IL-12 family (IL-12, IL-23 and IL-27) have gained much importance since this marks the transition from innate to adaptive immune responses [8]. Th polarizing cytokines, T cell attracting chemokines and antigen presentation machinery together determine the phenotype of the adaptive immune response [8]. Recently, ATF3 a bZib transcription factor which was previously implicated in the negative regulation of TLR signalling has now been identified as a major hub of adaptive immunity [9].

IL-27, a recently described novel cytokine, belonging to the IL-12 cytokine family, has gained major attention in recent years because of its therapeutic potential in treating diabetes [10]. Compared to other IL-12 family members, the role of IL-27 in inflammation is enigmatic since it has been shown to promote both pro- and anti-inflammatory responses [11]. At one end, it promote IFN- γ secretion from Th1 cells [12] and on the other end it inhibits Th1, Th2, and Th17 differentiation [13]. Even though IL-27 has largely been implicated in autoimmune diseases [11], its role in chronic inflammatory conditions like type-2 diabetes is largely unknown. Serum levels of IL-12 [14], IL-23 [15], IL-27 [16], IL-8 [17] and IL-17 [18] were found to be altered in Type-2 diabetes. However, the effect of type-2 diabetes on TLR-induced secretion of these cytokines is not known. Thus, in the present study TLR-induced secretion of IL-27 along with other Th polarising cytokines (IL-12 and IL-23) and T-cell attracting chemokines (IL-8 and IP-10) in newly diagnosed and known type-2 diabetes (chronic) subjects was estimated and was compared with age and gender matched normoglycemic controls. We also evaluated the expression of TLR-induced expression of antigen presentation molecules (HLA-DR and CD80) and T cell activation (HLA-DR and CD69) in these subjects.

2. Materials and methods

2.1. Study subjects

The participants for this study were selected from the outpatients visiting Dr. Mohan's Diabetes Specialities Centre, Chennai based on the inclusion and exclusion criteria as reported previously [19]. Institutional ethical approval (Ref No-MDRF-EC/SOC/2009//05) was obtained for this study. All the subjects had given their informed consent in writing. As a pilot experiment, controls and age matched diabetic subjects (newly diagnosed and known), 20 per group were included in the study. On the basis of the preliminary results,

with a confidence interval of 95%, an estimated p value < 0.05, and a power of 80%, we derived at a sample size of 30 per group. Furthermore, based on our earlier studies we increased the numbers under each group to accommodate for the wide variation generally seen in clinical studies.

The study subjects include: 1. Healthy subjects with Normal Glucose Tolerance (NGT) (n = 42) who served as control; 2. Subjects with newly diagnosed diabetes and not under anti-diabetic medication (NDD) (n = 34) and 3. Subjects with known diabetes and were under anti-diabetic medication (KDM) (n = 53). The *in vitro* cytokine analysis was performed for all the samples, while the other experiments were done using few samples from NGT (n = 13), NDD (n = 14) and KDM (n = 15) groups which were randomly selected. The study was conducted as per the declaration of Helsinki.

2.2. Diagnosis of type-2 diabetes

Type 2 Diabetes was diagnosed as per WHO guidelines. Subjects were allotted into groups as Control (NGT) and newly diagnosed diabetes based on the Oral Glucose Tolerance Test (OGTT).

2.3. Anthropometric measurements and biochemical parameters

Anthropometric measurements (height, weight and waist circumference) were acquired using standard methods. Serum biochemical parameters including blood glucose, serum cholesterol, serum triglycerides, HDL-cholesterol, urea and creatinine were estimated using a Hitachi-912 Autoanalyser (Hitachi, Mannheim, Germany) [19]. Glycated hemoglobin (HbA1c) was measured using high pressure liquid chromatography (Bio-Rad, Hercules, CA) [19].

2.4. Peripheral blood Leukocyte cultures

Immediately after the withdrawal of the peripheral blood, plasma was separated and the Packed Cell Volume (PCV) was diluted with RPMI medium (1:1 ratio) containing 10% FCS. Cells were either stimulated with TLR2 ligand-PAM3 (100 ng/ml) or TLR4 ligand-LPS (100 ng/ml) or were left unstimulated for 24 h in parallel cultures. Supernatants were collected for cytokine estimation from all samples. The cell pellets were either solubilized in RNeasy lysis reagent or fixed with 4% paraformaldehyde for gene expression and flowcytometry analysis respectively. All the processed samples were stored at -80 °C until analysis.

2.5. Real-time PCR analysis

RNA extraction from cell pellets solubilised in RNeasy lysis reagent was performed using RNeasy Mini Kit (Qiagen). 1 µg of RNA was used for cDNA conversion and real-time PCR was performed using TaqMan probes (Applied Biosystems, US) specific for ATF3 (Assay ID: Hs00231069_m1) and 18 S (control) (Assay ID: Hs99999901_s1).

2.6. Measurement of cytokines by ELISA

The levels of IL-27 (R & D, US), IL-12, IL-17, IL-23, IL-8 and IP-10 in the cell supernatant were estimated using ELISA (Invitrogen, US) as per the protocol given by the manufacturer. The lower detection limits were: IL-12–1.95 pg/ml, IL-17–0.97 pg/ml, IL-23–1.95 pg/ml,

IL-27 19.5 pg/ml, TGF- β 0.97 pg/ml, IL-8 3.91 pg/ml and IP-10 0.97 pg/ml. The coefficient of variance was found to be < 10%.

2.7. Flowcytometry analysis

Cells were stained with flurochrome conjugated monoclonal antibodies specific for CD3, CD14, CD19, CD80, CD69, HLA-DR, IL-6 and TNF- α (BD Biosciences, US) and were analyzed on a FACS Canto (BD Biosciences, US). The monocytes and lymphocytes were first gated on the FSC Vs SSC plot. Monocytes were further gated based on CD14 bright cell population on a CD14 Vs SSC plot (S.Fig. 1). B and T cells were gated on a CD3 Vs CD19 plot and were identified as single positive population (B cells-CD3⁺CD19⁻; T cells-CD3⁺CD19⁺) (S.Figs. 2 and 3). The expression of CD80 and HLA-DR on monocytes and B cells were analysed on CD80/HLA-DR Vs SSC plots on gated monocytes and B cells. The expression of TNF- α and CD69 on T cells were analysed on CD69/IL-6 Vs SSC plots on gated T cells. Both the percentage of cells and mean fluorescence intensity (MFI) were quantified within the gated population of cells. A minimum of 50,000 events were acquired for each tube to arrive at a statistical significant result.

2.8. Statistical analysis

Continuous variables among the groups were compared using Student *t*-test, whereas proportions were compared using χ^2 test or Fisher exact test (as appropriate). Parameters which did not show normal distribution were compared using Kruskal-Wallis test. Holm's correction was performed in multiple comparisons. GraphPad Prism version 5.0 (GraphPad Software, US) software was used to perform all the above mentioned statistical analysis. *p* < 0.05 was considered to be significant.

3. Results

3.1. Clinical characteristics of the study subjects

The clinical and biochemical characteristics of the study subjects have been described previously [19]. The average duration of diabetes in KDM group is approximately two to ten years. None of them had microvascular (diabetic retinopathy, nephropathy and neuropathy) and macrovascular (diabetic cardiomyopathy and peripheral arterial disease) complications. With respect to medication: 35% were on insulin, 41% on metformin, 18% on thiazolidinedione, 41% on sulfonylurea, 18% on medlitide, 18% on alpha-glucosidase inhibitor and 18% on mecobalamin. 20% of subjects were on more than one drug combination.

3.2. TLR-induced secretion of IL-27, IL-12, IL-23, IL-8 and IP-10 in the supernatant of PBL cultures

In the present study, we measured TLR-induced secretion of IL-27, IL-12, IL-23, IL-8 and IP-10 in the supernatant of PBL cultures (Fig. 1). While significantly increased secretion of IL-12 and IL-27 was seen in both the TLR2 and TLR4 stimulated cultures (Fig. 1b and c), moderate levels of IL-23 secretion was seen only in the TLR4 stimulated cultures (Fig. 1d). IL-27 was the only cytokine which was significantly downregulated in the NDD compared to NGT and KDM groups. However, serum IL-27 showed no difference between the groups

(Fig. 1a). With respect to chemokines, while significantly increased IL-8 secretion was seen in both the TLR2 and 4 stimulated cultures (Fig. 1e), IP-10 secretion was seen only in the TLR4 stimulated cultures (Fig. 1f). However no significant difference was seen in the secretion between the groups. Both IL-33 and TGF- β secretion could not be detected in the culture supernatants (data not shown).

3.3. TLR-induced expression of CD80 and HLA-DR in monocytes and B cells

Next, we studied TLR-induced expression of CD80 and HLA-DR in monocytes (Fig. 2a and b) and B cells (Fig. 2c, d, e and f). Both the basal and TLR-induced level of CD80 was significantly upregulated in both the monocytes and B cells in the KDM group compared to NGT and NDD groups (Fig. 2a, c and e). No significant difference was noted in HLA-DR expression in monocytes and B cells under basal level between groups (Fig. 2b, d, f). Following TLR stimulation, upregulation of HLA-DR was seen only in monocytes (but not in B cells) which was comparable between the groups (Fig. 2b and d).

3.4. TLR-induced expression of HLA-DR, CD69, TNF- α and IL-17 in T-cells

Next, we analyzed TLR-induced expression of HLA-DR, CD69, TNF- α and IL-17 in T-cells (Fig. 3). At the basal level, significant upregulation of HLA-DR (Fig. 3a and b), CD69 (Fig. 3c) and secretion of IL-17 (Fig. 3e) was seen in T cells from the KDM group. Upon TLR stimulation strong upregulation of HLA-DR (Fig. 3a) and CD69 (Fig. 3c) was noted. The upregulation of HLA-DR was significantly higher in both the NDD and KDM groups compared to NGT group (Fig. 3b). T cells from the NDD group failed to upregulate CD69 following TLR stimulation (Fig. 3c). CD69 was the only marker which showed significant difference between the NGT and NDD groups. Similarly, T cells from diabetic subjects (both NDD and KDM) failed to upregulate TNF- α following TLR stimulation (Fig. 3d). On the contrary, the basal level secretion of IL-17 was strongly upregulated in the KDM group compared to NDD even in the absence of TLR stimulation (Fig. 3e). No T cell apoptosis (expression of activated caspase-3) or IL-6 secretion were detected (data not shown). Unlike TNF- α , no immunophenotyping was carried out for IL-17 since it is well known that this cytokine is predominantly secreted by T cells in PBL cultures. Finally, ATF-3 showed significantly reduced expression in the KDM group, compared to NDD group (Fig. 3f). Spearman's correlation analysis revealed a negative correlation between TLR2 induced IL-27 secretion and CD3⁺HLA-DR expression and positive correlation between TLR2 induced IL-27 secretion (Fig. 3g) and CD3⁺CD69 expression (Fig. 3h) in the NDD group.

4. Discussion

Even though TLR-induced secretion of cytokines is well studied, comparatively the antigen processing/presentation pathway is less well studied in type-2 diabetic subjects. Monocytes and B cells serve as major APCs which when activated with TLRs express HLA-DR and CD80 molecules and secrete cytokines and chemokines [20,21]. The chemokines secreted by the APCs attract T cells, which then recognize antigen bound MHC and CD80 molecules and get activated/polarized by the Th polarizing cytokines [22]. The cumulative effect of these three signals namely 1. Peptide-MHC (which is recognised by TCR), 2. CD80 (which is recognized by CD28) and 3. Th polarizing cytokines (which is recognized by cytokine

receptors) emanating from APCs determine the activation/polarizing status of the T cells. IL-12 (for Th1), IL-23 (for Th17), IL-33 (for Th2) and TGF- β (for Treg) have now been identified as the major Th polarizing cytokines [23], while IL-8 and IP-10 are the major T cell attracting chemokines, secreted by APCs [24]. IL-27 is a recently identified cytokine which belongs to the IL-12 family. However, unlike its other family members, it does not polarize Th cells, but augments the secretion of IFN- γ from Th1 cells and inhibits other Th cells viz IL-10 secretion [25]. Thus, in the present study we looked at the complete TLR-induced antigen processing/ presentation machinery operating in the transition of innate and adaptive immune responses in type-2 diabetic subjects. The major findings of the study are 1. Out of three major T cell polarizing IL-12 family members, only IL-27 secretion was found to be defective in the newly diagnosed type-2 diabetic subjects, 2. TLR-induced expression of CD80 was found to be significantly upregulated in both the monocytes and B cells in chronic type-2 diabetic subjects and 3. Finally, TLR-induced activated T cells (CD3+HLA-DR+) secreted increased IL-17 and reduced TNF- α in chronic type-2 diabetic subjects.

Out of three major T cell polarizing IL-12 family members, only IL-27 secretion was found to be defective in newly diagnosed type-2 diabetic subjects. However, serum IL-27 levels were not significantly different between the groups studied. The defective secretion of IL-27 was seen only in the NDD group and resembles TLR-induced secretion of pro- and anti-inflammatory cytokines (as we reported previously) [19]. Previous reports have indicated significantly increased levels of IL-27 in subjects with diabetes and coronary artery disease (DM-CAD) [26] and reduced levels in subjects with proliferative diabetic retinopathy (PDR) [16]. The ability of IL-27 to suppress diabetogenic Th17 cells has created renewed interest in using this cytokine for immunotherapy against several autoimmune diseases including diabetes [27]. At least in animal models, neutralization of IL-27 inhibited disease progression in NOD mice [27]. However, IL-27 treatment itself reduced hyperglycemia and pancreatic beta cell loss in streptozotocin induced diabetes, making its role in diabetes highly controversial [28]. The disparity could be due to the ability of IL-27 to suppress IL-22 producing Th17 cells under certain conditions [29] and not under other conditions [30].

Next, we looked at the expression of HLA-DR and CD80 in professional antigen presenting cells (monocytes and B cells). TLR-induced expression of CD80 was found to be significantly upregulated in both the monocytes and B cells in chronic type-2 diabetic subjects. It is important to note that this immunophenotype was seen only in the KDM group and not in NDD group indicating impaired metabolic memory in the chronic activation of these APCs. Previously, we have reported significant upregulation of CD80 in dendritic cells from type-2 diabetic subjects, which was due to the high levels of serum GM-CSF as seen in these subjects [31]. Our results are in agreement with Giulietti et al., who reported increased expression of CD80 in macrophages obtained from type-2 diabetic subjects [32]. Further, type-2 diabetic subjects were found to have aberrant soluble co-stimulatory molecule profile in the serum with increased sCD28 and decreased sCTLA-4 indicating augmented CD80 pathway [6]. To summarise, a chronically activated state with enhanced CD80 expression was noted in both monocytes and B cells in the known diabetic group.

Next, we looked at the activation status of T cells in newly diagnosed and chronic diabetic subjects. TLR mediated activation of T cells is less well studied compared to the activation of APCs. Since IL-27 has a dual effect of both activating and inhibiting T cells, we looked at TLR-induced T cell activation using both early (CD69) and late (HLA-DR) activation markers. Previously, our attempt to activate naïve T cells with TLR ligands was not successful. Hence, in the present work, we used the whole blood cultures to activate T cells. Significant upregulation of CD69 and HLA-DR was seen in T cells even at the basal level in the KDM group which was further upregulated by TLR stimulation. In the present study, we selected CD69 instead of CD25 since the former is an early activation marker and is predominantly expressed in effector T-cells while the latter is expressed in both effector and regulatory T-cells. While CD69 represent recent activation, expression of HLA-DR in T cells represents chronic activation and has been implicated in antigenic cross-presentation of T cells [33]. HLA-DR+ T cells have been reported in type-1 diabetic subjects and serves as a major risk factor for disease progression [34]. However, as far as we know this is the first report documenting HLA-DR+ T cells in type-2 diabetes. Following TLR stimulation, these chronically activated T cells, failed to secrete TNF- α , but constitutively secreted high levels of IL-17. The exact mechanism associated with the defective TLR-induced IL-27 secretion in the NDD group is not known but could be due to the upregulation of ATF3 since this transcription factor is the major negative regulator of TLR signalling [9]. Further, in the NDD group, strong positive correlation between CD69 and IL-27 secretion and negative correlation between HLA-DR and IL-27 secretion indicates that IL-27 might be more associated with chronic T cell activation [12,25]. Unlike TLR2 and 4 other TLRs were poorly expressed in T cells and were not studied in this report. Because of the cross-sectional design, no direct cause and effect relationship could be drawn from this study which is a major limitation. Nevertheless, this study gains importance in that it identifies major defects in an important component of TLR signalling pathway in type-2 diabetic subjects. Impaired TLR-induced IL-27 secretion and augmented expression of antigen presentation machinery results in chronic T cell activation which may fuel T cell-mediated inflammation in type-2 diabetes. Infiltration of these T cells into adipose tissue can worsen insulin resistance. At the same time, defective secretion of TNF- α by these T cells can impair immunity against infections as frequently seen among Type-2 diabetic subjects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

We thank Sajid Bhat and N. Pavankumar for their technical support. The project was funded by Fast Track Scheme for Young Scientists, Department of Science & Technology, New Delhi (SR/FT/LS-105/2009). The Dept of Genetics, University of Madras has received funds for infrastructural support from DST-FIST and UGC-SAP programs.

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Fig. 1. TLR-induced secretion of Th polarizing cytokines and T cell attracting chemokines in PBL cultures of type-2 diabetic subjects as determined by ELISA. Histogram showing serum levels of IL-27 (a). Histogram showing TLR2 and 4 induced secretion of IL-27 (b), IL-12 (c), IL-23 (d), IL-8 (e) and IP-10 (f) in the supernatants of PBL cultures in NGT/control (n = 42), NDD (n = 34) and KDM (n = 53) subjects. Statistical significance was determined by non-parametric Mann-Whitney *U* test and $p < 0.05$ was considered significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. NGT- normal glucose tolerance, NDD- newly diagnosed diabetic, KDM- known diabetic subjects, PBL- Peripheral blood leukocytes.

Fig. 2. TLR-induced expression of CD80 and HLA-DR in monocytes and B cells in PBL cultures of type-2 diabetic subjects as determined by flowcytometry.

Histogram showing the percentage of monocytes, expressing CD80 (a) and HLA-DR (b), and B cells, expressing CD80 (c) and HLA-DR (d), in NGT/control (n = 13), NDD (n = 14) and KDM (n = 15) subjects. Histogram analysis showing the upregulation of CD80 (e) but not HLA-DR (f) in TLR4 stimulated B cells in KDM group (Shaded area-Isotype control; dotted line-NGT; dashed line-NDD and grey line-KDM). Statistical significance was determined by non-parametric Mann-Whitney *U* test and $p < 0.05$ was considered

significant. * $p < 0.05$. NGT- normal glucose tolerance, NDD- newly diagnosed diabetic, KDM- known diabetic subjects.

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Fig. 3. TLR-induced activation of T cells in PBL cultures of type-2 diabetic subjects as determined by flowcytometry and real-time PCR.

Long standing diabetes is characterized by increased TLR-induced HLA-DR expression and decreased TNF- α expression in T cells. Histogram showing the percentage of T cells expressing CD80 (a), HLA-DR (b), CD69 (c) and TNF- α (d). Histogram showing the levels of IL-17 (e) in the supernatants of PBL cultures and the mRNA expression of ATF3 (f) shown as dct following TLR2 and 4 stimulation in NGT/control (n = 42), NDD (n = 34) and KDM (n = 53) subjects. Spearman's correlation analysis showing the association between IL-27 secretion and HLA-DR (g) and CD69 (h) expression in T-cells. Statistical significance

was determined by non-parametric Mann-Whitney *U* test and $p < 0.05$ was considered significant. * $p < 0.05$; ** $p < 0.01$. PBL- Peripheral blood leukocytes, NGT- normal glucose tolerance, NDD- newly diagnosed diabetic, KDM- known diabetic subjects, dct - delta cycle threshold.