






Impact of Low Interferon- γ and IL-10 Levels on TNF- α and IL-6 Production by PHA-Induced PBMCs in Type 2 Diabetes Mellitus

This article was published in the following Dove Press journal:
Journal of Inflammation Research

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Purpose: In this study, we analyzed the production of interferon γ (IFN- γ) and interleukin 10 (IL-10) by peripheral blood mononuclear cells (PBMCs) in patients with type 2 diabetes mellitus (T2DM). We aimed to investigate the capacity of monocytes to produce tumor necrosis factor- α (TNF- α) and interleukin 6 (IL-6) following IFN- γ stimulation and the associated role of IL-10 in TNF- α and IL-6 production.

Patients and Methods: In vitro experiments were conducted on PBMCs obtained from 19 patients with T2DM and 17 healthy participants. PBMCs were isolated from venous blood by density gradient centrifugation, followed by 3-day phytohemagglutinin induction. In vitro production of TNF- α , IL-6, IFN- γ , and IL-10 was measured using the multiplex immunoassay. Statistical analysis was performed using IBM SPSS 23 version.

Results: IFN- γ concentration in the T2DM group was significantly lower than that in control group (T2DM $7,700.86 \pm 3,037.77$ vs control $10,672.69 \pm 5,625.50$ pg/mL; $p = 0.048$). However, TNF- α , IL-6, and IL-10 levels showed no significant difference between the two groups. The TNF- α /IFN- γ and IL-6/IFN- γ ratios were significantly higher in T2DM than in the control group ($p = 0.026$ and $p = 0.048$, respectively). In T2DM, the high TNF- α /IFN- γ ratio was consistent, with the low baseline IL-10 level ($p = 0.022$).

Conclusion: In T2DM, T-cell response is impaired with significantly reduced IFN- γ production, and simultaneously, circulatory monocytes show enhanced cellular responsiveness to inflammatory stimuli. The low baseline IL-10 level likely contributes to such an immune response.

Keywords: type 2 diabetes mellitus, proinflammatory response, cytokines

Introduction

Type 2 diabetes mellitus (T2DM) is considered as a major health concern worldwide.¹ In Indonesia, the prevalence of DM was 10.9% among the population aged >15 years.² Currently, the pathogenesis of T2DM is associated with hyperactivity of innate immune response, which results in insulin resistance.³ Obesity is an essential factor in the development of T2DM because the adipose tissues in patients with obesity produce high levels of inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and interleukin-1 β (IL-1 β). Moreover, it has been established that the M1 macrophages (proinflammatory state) in the adipose tissues produce a high level of proinflammatory cytokines such as TNF- α , IL-1 β , and IL-6 and chemokines such as CCL4 and CCL2. The persistent production of cytokines leads to a chronic low-grade inflammatory

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condition.¹ This condition could disturb insulin signaling by inducing serine phosphorylation of insulin receptor substrate 1 (IRS1) and stimulating the c-Jun N-terminal kinase pathway and I-kappa B kinase β (IKK β)/NF- κ B pathway.⁴

The adaptive or specific immune response is mediated by CD4+ and CD8+ T cells and B cells. In T2DM, CD4+ T cells tend to polarize to T helper 1 (Th1) cells and T helper 17 (Th17) cells in either peripheral blood or adipose tissue.³ Therefore, interferon- γ (IFN- γ) production from those T cells increases.³ Normally, IFN- γ is responsible for combating viral and intracellular pathogenic infections owing to its ability to activate the cellular immune response in macrophages.⁵ However, several studies have revealed that IFN- γ contributes to the pathogenesis of T2DM by increasing the expression of MHC class I and II and adhesion molecules on various types of cells, including β islets of pancreas.⁵ In addition, Bosek et al⁶ reported that the IFN- γ levels between patients with T2DM and healthy subjects were not significantly different. Apart from its high inflammatory activity, cells from patients with T2DM also showed dysregulation of anti-inflammatory responses owing to Th1/Treg cells imbalance and low interleukin-10 (IL-10) production by Treg cells.⁷ IL-10 is a potent anti-inflammatory cytokine that inhibits the production of TNF- α , IL-6, and other cytokines. Moreover, IL-10 could dampen activated macrophages.⁸

Phytohemagglutinin (PHA), a lectin tetravalent, is used for stimulating T cells *in vitro*. The mechanism underlying the activation of T cells by PHA remains controversial; however, some studies have reported that PHA initiates cell signaling via CD2 and CD3 receptor, which leads to Ca²⁺ influx through calcium channels.^{9,10} PHA activated-Th1 cells produce IFN- γ , which subsequently induces other immune cells such as monocytes and macrophages. In monocytes, IFN- γ triggers STAT1 homodimerization and NF- κ B translocation to produce TNF- α , IL-6, and IL-1 β .¹¹

Although many studies have revealed that macrophages and other immune cells from adipose tissue trigger chronic low-grade inflammation, it remains unclear whether chronic hyperglycemia affects the response of the circulatory immune cells to inflammatory stimuli. In the present study, we analyzed IFN- γ production by peripheral blood mononuclear cells (PBMCs) from patients with T2DM. Moreover, we investigated the capacity of monocyte to produce TNF- α and IL-6 following IFN- γ

stimulation and the role of IL-10 in TNF- α and IL-6 production.

Patients and Methods

Subjects

In vitro experiments were conducted on PBMCs obtained from 19 patients with T2DM and 17 healthy participants. Criteria for T2DM group were patients aged 30–50 years, with fasting blood glucose (FBG) > 126 mg/dL, HbA1c > 6.5%, and who have had T2DM for >5 years. The healthy control group included participants with FBG < 100 mg/dL, HbA1c < 5.7%, and who were never diagnosed with T2DM by a physician. Exclusion criteria in both groups were pregnancy, lactation, medication use (steroids, NSAIDs, and antibiotics), and infection during the study. This study was carried out in accordance with the recommendation of Ethics Committee from Faculty of Medicine, Universitas Indonesia with written informed consent from all participants. All participants gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by Faculty of Medicine, Universitas Indonesia with number 19-03-0221.

Fasting venous blood was taken from all participants. The level of FBG, blood glucose at 2 h after meal (BG2PP), and HbA1c were measured in the laboratory of National Institute of Research and Development, Ministry of Health in Bogor. Isolation and culture of PBMCs and cytokine analysis were conducted in Integrated Laboratory, Universitas Indonesia.

PBMC Isolation and Culture

PBMCs were isolated from RPMI 1640-diluted blood by centrifugation through Ficoll-Paque Plus (GE Healthcare Life Sciences, USA). PHA is lectin extract from red kidney beans that could stimulate T cell activation and proliferation, so we used PHA-M (mucoprotein form) for lymphocyte stimulation. After isolating, PBMCs were resuspended in cell culture medium comprising RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 1% streptomycin at a concentration of 10⁶ cells/mL. Additionally, 500 μ L cell suspensions and 500 μ L of 10 mM Gibco phytohemagglutinin-M (Thermo Fisher Scientific, California, USA) were added to the wells of the 24-well culture plate. For determining baseline cytokines level, other PBMC suspensions were cultured without the addition of phytohemagglutinin-M. The culture plates were incubated at 37°C, 5% CO₂ for 3 days.

Cytokine Assays

TNF α , IL-6, IFN- γ , and IL-10 were measured using bead-based multiplex assay (R&D Systems, Minneapolis, USA) following the manufacturer's directions. The detection limits were 0.6 pg/mL for TNF- α , 1.11 pg/mL for IL-6, 1.27 pg/mL for IFN- γ , and 0.3 pg/mL for IL-10.

Data Analysis

Data on patient characteristics and cytokines production were compared between patients with T2DM and healthy control groups using the independent *t*-test or Mann-Whitney *U*-test, as appropriate. A *p* value of <0.05 was considered statistically significant. All analyses were performed using Statistical Package for the Social Sciences (IBM SPSS) version 23 for Windows 10.

Results

Baseline Characteristics of Participants

We compared some metabolic parameters between the healthy control and T2DM groups (Table 1). As expected, the T2DM group had higher BMI ($p < 0.001$), FBG level

($p < 0.001$), BG2PP level ($p < 0.001$), and HbA1c ($p < 0.001$).

Cytokine Production in PHA-Induced PBMCs

Table 2 presents cytokine concentration in PBMCs at baseline and following PHA induction. After 3 days of PHA induction, IFN- γ concentration in the T2DM group was significantly lower than that in the control group ($p = 0.048$). However, the two groups showed no difference in TNF- α , IL-6, and IL-10 levels. In addition, our multivariate analysis showed no significant difference between the two groups based on age and sex (Supplement 1; Supplement 2).

In PHA-induced PBMCs, we expected that IFN- γ from activated T cells would trigger TNF- α and IL-6 production from macrophages and monocytes. To analyze the monocyte and macrophage function after IFN- γ stimulation, we calculated the ratios between TNF- α and IFN- γ and between IL-6 and IFN- γ . As shown in Figure 1, the TNF- α /IFN- γ and IL-6/IFN- γ ratios were significantly

Table 1 Baseline Characteristic of Subjects

Baseline Characteristic of Subjects	Healthy Control Group (n=17)	T2DM Group (n=19)	<i>p</i>
Female sex (n, %)	11 (64.7%)	10 (52.6%)	0.463
Age (mean \pm SD, age)	40.17 \pm 4.5	46.26 \pm 6.6	0.003**
BMI (mean \pm SD, kg/m ²)	22.96 \pm 2.6	27.42 \pm 3.1	<0.001***
FBG (mean \pm SD, mg/dL)	92.34 \pm 13.9	211.61 \pm 100.3	<0.001***
BG2PP (mean \pm SD, mg/dL)	128.78 \pm 30.4	288.42 \pm 136.86	<0.001***
HbA1c (median [min-max], %)	5.03 (3.78–5.49)	8.9 (5.05–13)	<0.001***

Notes: ** $p < 0.01$; *** $p < 0.001$.

Abbreviations: T2DM, type 2 diabetes mellitus; BMI, body mass index; FBG, fasting blood glucose; BG2PP, blood glucose 2 h after meal; HbA1c, glycosylated hemoglobin; SD, standard deviation.

Table 2 Cytokines Concentration from Baseline and PHA-Induced PBMC Cultures

Cytokines	Baseline			PHA		
	Control	T2DM	<i>p</i>	Control	T2DM	<i>p</i>
TNF- α (mean \pm SD)	2.65 \pm 2.08	2.82 \pm 1.86	0.789	6,453.24 \pm 3,504.51	7,176.12 \pm 3,180.06	0.521
IL-6 (median [min-max])	20.72 (3.55–79.3)	20.52 (3.73–110.92)	0.962	393 (36.27–987)	400.93 (190.92–1,336.61)	0.579
IFN- γ (mean \pm SD)	10.47 \pm 3.89	10.67 \pm 6.58	0.919	10,672.64 \pm 5,625.5	7,700.86 \pm 3,037.77	0.048*
IL-10 (median [min-max])	3.62 (1.1–24.09)	2.42 (0.1–16.74)	0.172	115 (37.82–487)	140.67 (28–982.96)	0.763

Note: * $p < 0.05$.

Abbreviations: PHA, phytohemagglutinin; T2DM, type 2 diabetes mellitus; PBMC, peripheral blood mononuclear cells; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; IFN- γ , interferon- γ ; and IL-10, interleukin-10.

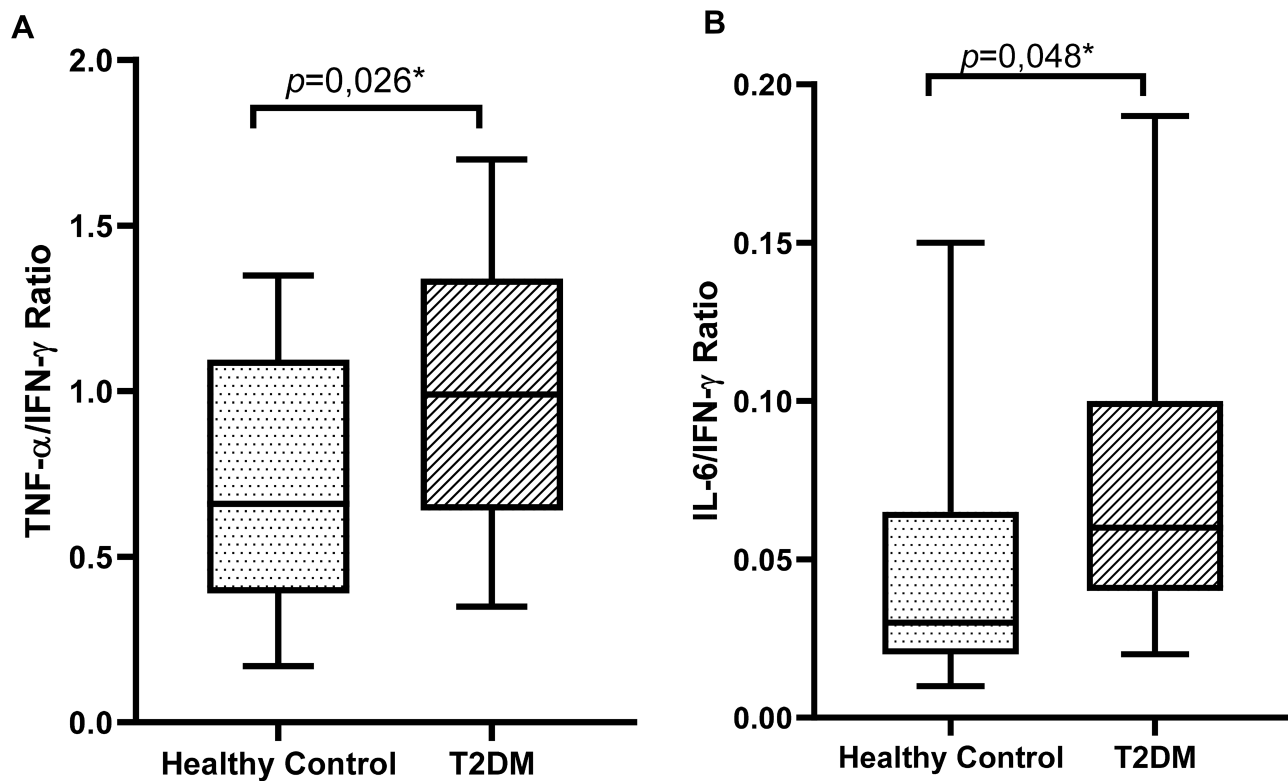


Figure 1 Ratios of (A) TNF- α /IFN- γ and (B) IL-6/IFN- γ in 3-day PHA-induced PBMC cultures in the control and T2DM groups. PHA, phytohemagglutinin; T2DM, type 2 diabetes mellitus; PBMCs; peripheral blood mononuclear cells; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; IFN- γ , interferon- γ . * $p < 0.05$.

higher in the T2DM group than those in the control group ($p = 0.026$ and $p = 0.048$, respectively).

Having observed that the cellular responsiveness in monocytes was higher in T2DM, we attempted to investigate the role of IL-10. We found that the baseline IL-10 level affects monocytes activity. Figure 2 shows that 52.6% of the patients with T2DM had lower baseline IL-10 level and higher PHA-induced TNF- α /IFN- γ ratio, indicating that monocyte activity was not suppressed. In contrast, most of the healthy control subjects (58.8%) had a higher baseline IL-10 level and suppressed monocyte activity ($p = 0.022$). Interestingly, 50% of the patients with T2DM (4 out of 8) who had high baseline IL-10 levels presented with a high PHA-induced TNF- α /IFN- γ ratio. However, only 23% of healthy controls (3 out of 13) had high baseline IL-10 levels and high PHA-induced TNF- α /IFN- γ ratio.

Discussion

T2DM is a chronic hyperglycemic condition. This condition leads to the formation of advanced glycation end products, polyol products, and reactive oxygen species, which activate cellular immune responses in target

tissues such as adipose tissue.¹² However, cellular responses in circulatory immune cells such as monocytes and lymphocytes remain inconclusive. In this study, we found that IFN- γ level was lower in patients with T2DM than that in the control group, indicating that the capacity of T cells to produce IFN- γ is decreased. This finding is consistent with the findings of Foss-Freitas et al.¹³ They found that the plasma level of IFN- γ decreased in patients with T2DM compared with healthy controls and patients with type 1 DM. Stalenhoef et al.¹⁴ also described impaired response of Th1 cells to PHA induction in patients with T2DM. This impaired Th1 cell response was characterized by reduced IFN- γ level, thus contributing to an increased chance of infection. The decrease in IFN- γ production in CD4+ and CD8+ T cells might be due to insulin resistance¹⁵ and genetic factors such as IFN gamma-874*A homozygote (low producer gene).⁵ Interestingly, the levels of TNF- α and IL-6 were similar between patients with T2DM and healthy controls. These findings contradict the conventional notion that proinflammatory cytokines such as TNF- α and IL-6 contribute to insulin resistance.^{16–18}

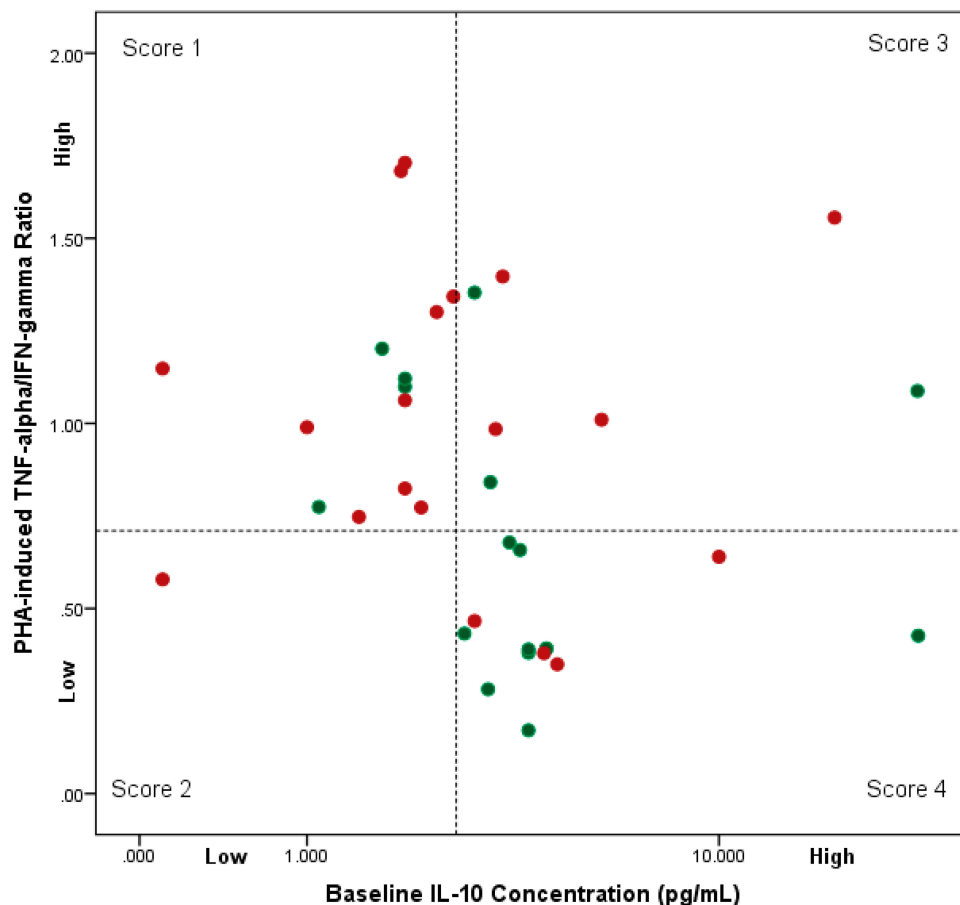


Figure 2 Scatter plots of PHA-induced TNF- α /IFN- γ ratio against the baseline IL-10 level for control subjects (●) and patients with T2DM (●). Cutoff points were determined to categorize the PHA-induced TNF- α /IFN- γ ratio (cutoff point = 0.71, sensitivity = 71.2%, and specificity = 59.1%) and the baseline IL-10 level (cutoff point = 2.71, sensitivity 76.5% and specificity 57.9%). Based on those classifications, 15 participants had low baseline IL-10 level, among which 14 (93.3%) had high TNF- α /IFN- γ ratio. Low baseline IL-10 and high TNF- α /IFN- γ ratio were more prevalent among patients with T2DM (71.4%). In total, 21 participants had high baseline IL-10 level and among them, 10 participants (47.6%) who had both high baseline IL-10 level and low TNF- α /IFN- γ ratio were control subjects. Based on the role of IL-10 in suppressing PHA-induced TNF- α /IFN- γ , we ranked subjects using a scoring system. Score 1 was given to subjects who had low baseline IL-10 with high PHA-induced TNF- α /IFN- γ ratio; score 2 for subjects who had low baseline IL-10 and low TNF- α /IFN- γ ratio, score 3 for subjects who had high baseline IL-10 and high TNF- α /IFN- γ ratio, and score 4 for subjects who had high baseline IL-10 and low TNF- α /IFN- γ ratio. Total scores between control and T2DM groups were analyzed using Mann–Whitney *U*-test. Our result showed that anti-inflammatory activity was higher in the control group with median of 4 (1–4) than in the T2DM group with median of 1 (1–4) ($p = 0.022$). PHA, phytohemagglutinin; T2DM, type 2 diabetes mellitus; TNF- α , tumor necrosis factor- α ; IFN- γ , interferon- γ ; and IL-10, interleukin-10.

In this study, TNF- α and IL-6 production was demonstrated to be induced by IFN- γ from T cells. TNF- α /IFN- γ and IL-6/IFN- γ ratios were higher in patients with T2DM than in healthy controls, indicating that the response of monocytes and macrophages to inflammatory stimuli has been impaired. Circulatory monocytes and macrophages in T2DM were believed to be more sensitive to proinflammatory stimuli; therefore, even at low IFN- γ exposure, they could produce high amount of proinflammatory cytokines. Giulietti et al¹⁹ reported that monocytes from T2DM showed higher levels of TNF- α , IL-6, IL-1, IL-8, and COX2. Fadini et al²⁰ reported an imbalance between M1 and M2 monocytes owing to the low number of M2 monocytes. Moreover, Jagannathan et al²¹ revealed

increased level of proinflammatory monocytes (CD14+/CD16+) in T2DM, and it correlated with HbA1c level.

High TNF- α and IL-6 production in response to IFN- γ in circulatory monocytes may result from higher level of STAT1 phosphorylation induced by IFN- γ in macrophages under high glucose conditions.²² Phosphorylated STAT1 translocates to nucleus and activates several proinflammatory genes such as MMP1.²² It is also found that mRNA and miRNA expression from monocytes is altered, resulting in the upregulation of TNF- α and IL-6 gene expression²³ and downregulation of miR-146a and miR-155 expression, the key regulators of inflammatory process.^{24–26}

IL-10 is an anti-inflammatory cytokine secreted by Treg cells, Th2 cells, B cells, macrophages, and DCs.²⁷ IL-10

downregulates the production of proinflammatory cytokines and chemokines by lymphocytes and macrophages through JAK/STAT3/SOCS3 pathway.²⁷ The binding of IL-10 to IL-10R activates JAK1 and subsequently induces STAT3 phosphorylation. STAT3 activation in turn upregulates SOCS3 expression, which prevents NF- κ B nuclear translocation. Moreover, STAT3 upregulates the expression of anti-inflammatory response genes such as Bcl3, Etv3, and SHIP-1, which suppresses TNF- α expression.²⁸ Our study showed that although the baseline IL-10 level was not different between patients with T2DM and control subjects, following IFN- γ induction, the majority of patients with T2DM having low baseline IL-10 level showed high TNF- α level. Higher circulating IL-10 level helps cells combat the chronic low-grade inflammation in obesity and T2DM.²⁹ In contrast, low IL-10 level indicates that the regulatory mechanism has failed, resulting in the activation of monocytes and lymphocytes.³⁰

Despite the small sample size in this study, we found that 50% of the patients with T2DM having high baseline IL-10 level also had high PHA-induced TNF- α level. This indicates that immune cells in T2DM exhibit IL-10 hyporesponsiveness. This finding is consistent with the findings of Barry et al³¹ who demonstrated that IL-10 was less effective in inhibiting TNF- α production in whole blood culture, and this was not explained by changes in IL-10 receptor or downstream signaling. Our findings might support the theory that IL-10 contributes to hyporesponsiveness in chronic low-grade inflammatory condition in T2DM.

Conclusion

In summary, we have demonstrated that in T2DM, T cell response is impaired owing to markedly reduced IFN- γ production. Moreover, circulatory monocytes showed enhanced cellular responsiveness to inflammatory stimuli such as IFN- γ , and the low baseline IL-10 level contributed to this increased responsiveness.

Acknowledgments

The authors would like to thank the participants, nurses, doctors in a cohort study for non-communicable disease, National Institute of Health Research and Development, Ministry of Health, Indonesia, to all staff in Integrated Laboratory Faculty of Medicine, Universitas Indonesia, and also to all who help this research.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, and analysis as well as interpretation of data; participated in drafting the article or revising it critically for important intellectual content; provided final approval of the version to be published; and agree to be accountable for all aspects of the work.

Funding

This research was supported by the Indexed International Publication for Student Projects (PITTA) grant; World Class Research (WCR) grant from the Ministry of Research, Technology, and Higher Education, Republic of Indonesia number 173/SP2H/LT/DRPM/2019; and LPDP publication grant from the Ministry of Finance, Republic of Indonesia.

Disclosure

Dr Rona Kartika, Dr Dyah Purnamasari, Dr Rahma Ayu Larasati, and Drs Heri Wibowo report grants from Universitas Indonesia, grants from the Ministry of Research, Technology, and Higher Education, Indonesia, grants from the Ministry of Finance, Indonesia, during the conduct of the study. The authors report no conflicts of interest in this work.

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