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REKOMENDASI PERSETUJUAN ETIK

Nomor : 160/UN4.6.4.5.31/ PP36/ 2021

Tanggal: 10 Maret 2021

Dengan ini Menyatakan bahwa Protokol dan Dokumen yang Berhubungan Dengan Protokol berikut ini telah mendapatkan Persetujuan Etik :

No Protokol	UH21030119	No Sponsor Protokol	
Peneliti Utama	**dr. Heidy Agustin, SpP**	Sponsor	
Judul Peneliti	ANALISA EKSPRESI mRNA gen TLR 4,CD4 DAN CD8 PADA MENCIT YANG TERINFEKSI TB MDR DENGAN DIABETES MELITUS		
No Versi Protokol	**1**	Tanggal Versi	**2 Maret 2021**
No Versi PSP		Tanggal Versi	
Tempat Penelitian	Laboratorium Biologi Molekuler dan Imunologi Fakultas Kedokteran Universitas Hasanuddin Makassar		
Jenis Review	☐ Exempted ☒ Expedited ☐ Fullboard Tanggal	Masa Berlaku **10 Maret 2021** sampai **10 Maret 2022**	Frekuensi review lanjutan
Ketua KEP Universitas Hasanuddin	Nama **Prof.Dr.dr. Suryani As'ad, M.Sc.,Sp.GK (K)**	Tanda tangan	
Sekretaris KEP Universitas Hasanuddin	Nama **dr. Agussalim Bukhari, M.Med.,Ph.D.,Sp.GK (K)**	Tanda tangan	

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- Menyerahkan Amandemen Protokol untuk persetujuan sebelum di implementasikan
- Menyerahkan Laporan SAE ke Komisi Etik dalam 24 Jam dan dilengkapi dalam 7 hari dan Lapor SUSAR dalam 72 Jam setelah Peneliti Utama menerima laporan
- Menyerahkan Laporan Kemajuan (progress report) setiap 6 bulan untuk penelitian resiko tinggi dan setiap setahun untuk penelitian resiko rendah
- Menyerahkan laporan akhir setelah Penelitian berakhir
- Melaporkan penyimpangan dari prokol yang disetujui (protocol deviation / violation)
- Mematuhi semua peraturan yang ditentukan

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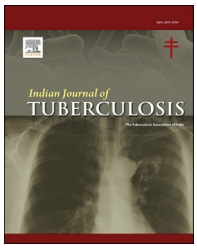

Original article

Correlation expression Toll-like receptor 4 with multidrugs resistant tuberculosis in diabetes mellitus condition

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ABSTRACT

Background: Toll-like receptor (TLR) are ligand homologous protein in the APC cell membrane that has functions as a receptor to triger leukocytes and innate immune responses. When there is a *Microbacterium tuberculosis* (MTB) infection enters from droplets to the lungs, the alveolar macrophages perform a phagocytic function. The interaction between *M. tuberculosis* and the TLR macrophage receptors produces chemokines which induce migration of monocytes and dendrite cells for destruction. Diabetes militus (DM) has become risk factor for developing tuberculosis. DM condition will reduce immunity and the ability of immune cell phagocytes bactery and triger severe infections. The consequences of more severe infection and metabolic disorders that occur make a person more likely to experience Multidrugs resistant MTB. Not much data that reports on the expression of TLR4 as a ligand that triggers an immune response in conditions of MDR and DM. We try to find out correlation between TLR-4 in MDR MTB, diabetes and level of MTB bacteria in experimental animals.

Methods: We conducted an experimental study on 30 experimental mice weighing 25 grams consisting of negative control grub, infected with MTB, infected with MDR MTB, negative control diabetes, MTB DM, MDR MTB DM. DM animals were induced by streptozosin to experience DM, then in the treatment of infection, intraperitoneal MTB and MDR MTB bacterial injections were given. Termination was carried out on day 14. We count number of bacteria level in the lungs and perform evaluation TLR4 from blood sampel.

Results: The negative control group had mean TLR value of 1.47 (± 0.46) while the MTB group showed an increase in TLR 9.22 (± 0.39) followed by MDR MTB 9.50 (± 0.29), DM negative

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control 9, 21 (± 0.24) and more increasing in conditions of DM MTB 13.36 (± 0.32) and DM MDR MTB 13.35 (± 0.34). ANOVA analysis showed a significant difference (P = 0.00). pearson correlation analysis find strong correlation TLR4 in MTB and MDR MTB with diabetes.

Conclusion: there were a significant difference level TLR4 between MTB and MDR TB infection with diabetes. higher TLR4 level higher in DM MTB, DM MDR MTB. TLR 4 strong correlates with an increase in the number of MTB bacteria.

1. Background

Toll-like receptor (TLR) is a homologous protein in the APC cell membrane that has functions as a ligand receptor to activates leukocytes and trigger innate immune responses against pathogens. This protein was first discovered in Drosophila as a Toll protein. These receptors consist of leucine-rich areas in the extracellum and in the cytoplasmic tail region which are receptors for IL-1 and IL-8 and are called Toll/IL-1 receptors (TIR).[1]

Activation of the TLR ligand will trigger pathogenic phagocytosis and inflammatory responses that release phagosome content. Several TLRs, namely TLR2 and TLR4, are capable of assisting the placement of phagosomes, then become as immune system's that earliest contact with potentially damaging microbial antigens. The most important characteristic of TLR activation is the formation of proinflammatory conditions represented by certain cytokines and chemokines, dominated by TNF-α and IL-12 on (NF) -kB and α/β IFN on the IRF-3 TLR ligand marker.[1,2]

When there is an MTB infection that enters from droplets in the lungs, the alveolar macrophages perform a phagocytic function. The interaction between *Mycobacterium tuberculosis* and receptors for macrophages (Toll-like receptors/TLRs) produces chemokines that induce the migration of monocytes and dendrite cells from the bloodstream to the infected lung. The dendritic cells will then include bacteria, then mature and are destroyed by CD4 and CD8 T cells. There were a phenomenon of granuloma formation due to the accumulation of macrophages, T cells, B cells, endothelial cells, dendritic cells, and epithelium, as well as other cells that are useful for limiting infection, but can become a residence for *M. tuberculosis* in a relatively long period of time latent bacteria and reactivated when there is a cytokine imbalance.[3–5]

Diabetes mellitus (DM) is a major risk factor for developing active pulmonary TB. Hyperglycemia in diabetic patients will lead decrease ability of cell phagocytes that has correlation with wider spread of infection, granuloma formation and p reactivation caused by the lagging and missing granulocytic phase of inflammation. In addition, individuals with diabetes has low elastic recoil reserves in the airways accompanied by thinning of the alveolar epithelium and capillary basement membrane, centrilobar emphysema and microangiopathy. These changes will affect low lung defense mechanism.[6,7]

The condition of decreased immune response, delay in diagnosis and reactivation of TB in individuals with diabetes will increase the risk of developing MDR which affects patient outcomes. In this study, we wanted to determine the relationship between TLR-4 in MDR TB and diabetes mellitus and its correlation to the level of MTB bacteria in experimental animals.

2. Methods and materials

The research was carried out at the molecular biology and immunology laboratory Faculty of Medicine Hasanuddin University. This experimental research was carried out on 30 experimental mice weighing 25 grams that divided into 6 groups. Group 1 was a negative control animal, group 2 animals with MTB bacterial infection, group 3 animals with MDR MTB bacterial infection, group 4 animals with negative control animals with diabetes condition, group 5 animals with MTB diabetes conditions and group 6 MDR MTB with diabetes. To trigger diabetes, an intraperitoneal injection of Streptozosin 40mg/kgbw was carried out to damage pancreas then trigger hyperglycemia. 3 days after blood glucose sticks were examined that taken from the tails of the mice. An increase in fasting blood glucose above 126mg/dl was an indicator of diabetes. 3 days after the adaptation of diabetes treatment and negative control, animals treated with MTB infection and MDR MTB were given an injection intraperitoneal of 0.2 ml of normal saline mixture with culture of bacterial MTB and MDR MTB bacterial colonies after 14 days, animal get termination, venous blood sampling was carried out to measure TLR-4 level and examination of MTB bacterial levels from lung tissue. The TLR-4 examination was carried out by isolation of Peripheral Blood Mononuclear Cells (PBMC) from blood and flow cytometry staining buffer. Examination of the number of bacteria is done by counting the number of levels of bacteria per 100 microscope fields of view.

3. TLR expression

Peripheral Blood Mononuclear Cells (PBMC) was isolated from the blood. Then the blood sample was dissolved 1: 1 with PBS in a conical tube. Support the dissolved sample with an amount of Ficoll volume equal to the volume of the original sample. Centrifuge was performed for 20 minutes (1000×*g*) with the brake OFF position. The PBMC is located at the junction between the PBS and Ficoll layers into the newly extracted tube. Fill the tube with PBS to wash the cells. Again centrifugation of the cell suspension 4–5 minutes (300-400×*g*) at 4 0C, discard the supernatant. Next, re-suspend the cell pellets in the flow cytometry staining buffer and perform cell

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counts and viability analysis. Centrifuge the cells as in the previous step, then re-suspend them with an appropriate volume of flow cytometry staining buffer so that the concentration is as needed.

4. Subject characteristics

Table 1 shows the current blood sugar profile and bacterial level per field of view in all groups. The blood glucose value shows that the mean blood sugar levels are increased in the streptozosin induced group as diabetes group. The highest blood glucose level value was obtained in the DM MDR TB group with level 408 (±20.59) followed by the DM MTB group 394.40 (±24.95) and diabetes negative control without MTB bacteria 394.20 (±13.19). Diabetes conditions in experimental animals with MDR TB have a higher blood sugar value than other groups. There was a significant difference in blood glucose level in all treatment groups (P = 0.00).

Table 1 – Characteristic blood glucose in experimental animals.

Animal profile	Mean	SD ±	Min–Max	Median	P
Blood glucose level g/dl					
Control negative	102.40	7.86	95–115	101	0.00
MTB	100.80	10.66	90–116	98	
MDR TB	104.00	10.22	89–114	106	
Control negative DM	394.20	13.19	380–414	390	
DM MTB	394.40	24.95	370–423	403	
DM MDR MTD	408	20.59	379–437	409	

5. The correlation between TLR-4 and MTB conditions, MDR MTB with diabetes mellitus

Correlation analysis was carried out to assess the direction of the relationship between the TLR-4 level and the increase in the number of MTB bacteria in all treatment groups. In Fig. 1, we can see there is a direct relationship with the increase in TLR4 within each group and bacterial level. The increase in TLR will increase more number of bacterial level. Mostly found in the conditions of DM MTB and DM MDR TB. Table 2 shows the correlation between TLR in MTB conditions, MDR MTB and diabetes mellitus. The analysis showed a significant correlation between TLR and the amount of AFB with a strong correlation coefficient of R = 0.744 and P = 0.000.

6. Comparison TLR 4 gene expression on MDR MTB with diabetes militus

In this study, an analysis of TLR 4 gene expression was carried out in the treatment group of diabetic rats and then infected with MTB and MDR MTB bacteria. Evaluation was carried out for each experimental animal group, the TLR4 value can be seen in Fig. 2 and Table 3.

Table 2 – Correlation of TLR to the number of bacteria MTB and MDR MTB with diabetes.

Variable	R	P[a]
TLR4- groups	0.878	0.000
TLR4-bacterial level	0.744	0.000

[a] Pearson Correlation analysis.

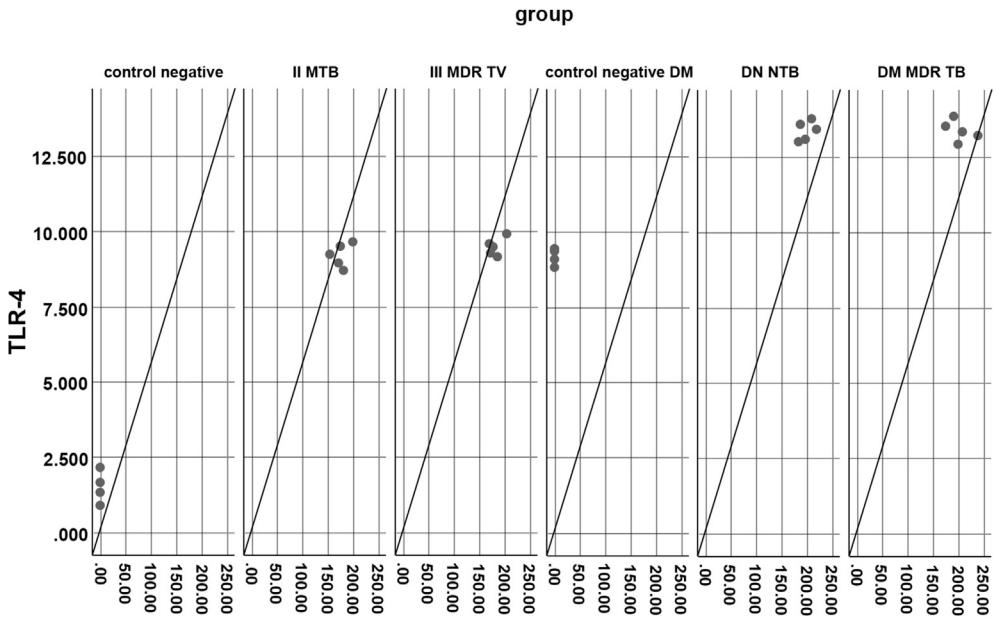

Fig. 1 – Bacterial level and expression of TLR-4 in each treatment group, there is a linear increase in the number of bacterial in MTB, MDR MTB and higher in diabetic condition.

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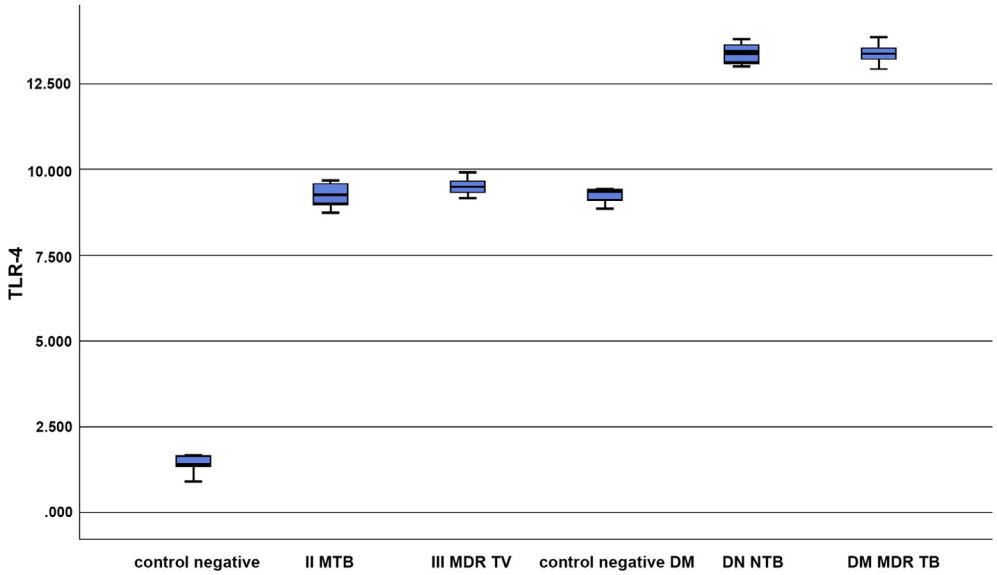

Fig. 2 – Expression of TLR 4 gene in diabetic MTB, MDR MTB and control groups. The highest expression value was obtained in the DM MTB and MDR MTB treatment groups.

TLR expression level in negative control group was 1.47 (±0.46) while the MTB group had higher level TLR 9.22 (±0.39) followed by MDR MTB 9.50 (±0.29), DM negative control 9, 21 (±0.24) and increasing in conditions of DM MTB 13.36 (±0.32) and DM MDR MTB 13.35 (±0.34). Diabetes conditions are able to give the effect of increasing TLR4. In the analysis of variance (ANOVA) there was a significant difference in the mean of TLR expression in the treatment group (P = 0.00) (see Table 4).

Based on the post hoc LSD analysis, there are significant differences in all groups except the MTB vs MDR MTB group, MDR vs negative control and DM MTB vs DM MDR MTB group which did not have a significant difference (P > 0.05). DM MDR MTB and DM MTB were the highest difference value on the expression value of TLR-4 compared to negative control.

7. Discussion

In this study, bacterial level in animals with diabetes has a higher number per field of view. The highest mean bacterial level was in the DM MDR MTB 201 group (±23.52), followed by the DM MTB group 197.60 (±14.31), the MDR MTB group 181.60 (±13.93) and MTB 174 (±15.95). There were significant differences in the number of bacterial level in the DM MTB MDR group compared to other groups. This suggests that diabetes triggers a higher number of bacterial level of MTB. The bacterial level in diabetic patients will also increase due to metabolic and the absorption problems of TB drugs, so patient cannot reach the therapeutic targets to eliminate bacteria. This will lead to an increase in the number of bacteria and the risk of drug resistance. Nijland et al. Reported that TB patients with diabetes had low level serum of rifampin 53% than non-diabetic TB patients. Failure to reach the correct dose will inhibit bacterial elimination and allow the risk of MDR. Antonia et al. Reported a significant relationship between diabetes mellitus and the development of MDR MTB.

Table 3 – TLR 4 expression level.

Treatment groups	Mean	SD ±	Min–max	P[b]
Control negative	1.47	0.46	0.881–2.15	0.00
MTB	9.22	0.39	8.17–8.66	
MDR MTB	9.50	0.29	9.16–9.92	
Control negative DM	9.21	0.24	8.84–9.41	
DM MTB	13.36	0.32	12.99–13.76	
DM MDR MTB	13.35	0.34	12.90–13.84	

[b] ANOVA test.

Table 4 – Comparison between treatment groups. By carrying out a Post-hoc LSD analysis, the differences between experimental animal treatments can be assessed.

Treatment groups	Mean differences	P[c]
Control negative vs MTB	−7.75	0.00
Control negative vs MDR TB	−8.03	0.00
Control negative vs control negative DM	−7.73	0.00
Control negative vs DM MTB	−11.89	0.00
Control negative vs DM MDR TB	−11.88	0.00
Control negative DM vs DM MTB	−4.15	0.00
Control negative DM vs DM MDR MTB	−4.14	0.00
MTB vs MDR MTB	−0.28	0.220
MTB vs control negative DM	−0.012	0.956
MTB vs DM MTB	−4.14	0.00
MTB vs DM MDR TB	−4.13	0.00
MDR TB vs control negative DM	−0.29	0.201
MDR MTB vs DM MTB	−3.86	0.00
MDR MTB vs DM MDR MTB	−3.85	0.00
DM MTB vs DM MDR MTB	0.0084	0.970

[c] Post Hoc LSD test.

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Individuals with tuberculosis and diabetes mellitus have a 6.8 greater risk of developing MDR MTB.[8,9]

TB DM patients were also described as having more severe TB infection, requiring longer treatment and possibly developing MDR-TB than patients with TB alone. Jenn et al reported that TB patients with DM who were compared without DM, also experienced more severe infections, higher levels of mycobacterial load, 17% higher treatment failure rate and longer mycobacterial elimination than TB patients alone.[10]

Increased bacteria and a higher risk of MDR in patients with comorbid TB-DM. It has been reported that poor glucose control is often associated with phagocytic dysfunction, production of reactive oxygen species (ROS), chemotaxis and T-cell reactions in DM patients, thus triggering a stronger rate of bacterial development. On the other hand, a higher mycobacterial burden, changes in the pharmacokinetics of anti-TB drugs and lower adherence to treatment also encourage MDR TB to occur in diabetic patients.[11]

8. The correlation between TLR on MTB bacteria and MDR MTB diabetes conditions

TLRs are known to be homologous proteins on the APC cell membrane that function as functional receptors that activate leukocytes to trigger innate immune responses and responses against pathogens. This protein was first discovered in Drosophila as a Toll protein. These receptors consist of leucine-rich areas in the extracellum and in the cytoplasmic tail region which are receptors for IL-1 and IL-8 and are called Toll/IL-1 receptors (TIR). TLR activation will trigger immunity coding genes that cause changes in the immune response and influence the development of TB disease.[1]

Once activated the TLR ligand will trigger pathogenic phagocytosis and an inflammatory response to the phagosome content. Some of the most dominant TLRs in TB are TLR2 and TLR4 which are capable of assisting phagosome placement, as the immune system's earliest contact with potentially damaging microbial antigens. The most important characteristic of TLR activation is the formation of proinflammatory conditions represented by certain cytokines and chemokines, dominated by TNFα and IL-12 on (NF) -kB and α/β IFN on the IRF-3 TLR ligand marker. TLR4 recognizes mycobacterial lipopolysaccharides (LPS) and can trigger one of two innate immune response pathways, the dependent or independent MyD88 pathway. When there were an MTB infection that enters from droplets in the lungs, alveolar macrophages perform the function of phagocytosis. The interaction between *M. tuberculosis* and the TLR macrophage receptors produces chemokines which induce the migration of monocytes and dendritic cells from the bloodstream to the infected part of the lung. The dendrite cells will include bacteria, mature cell then destroyed by CD4 and CD8 T.[12–14]

In this study, wa can see the value of TLR4 expression in conditions of bacterial infection of MTB, MDR MTB, DM MTB, DM MDR MTB and negative control. When there is infection, the TLR4 value increases following MDR MTB. In conditions of heavier infection accompanied by diabetes status, the TLR4 value is also higher in both DM MTB and DM MDR MTB. then also there was a significant difference in TLR4 values in each group with significant strong correlation. The existence of a continuous increase from this is an indicator that TRL 4 can be a biomarker for TB infection conditions including during MDR MTB and diabetes. Previously has been known that patients with MDR TB will usually have worse clinical pathology than TB infection alone. Soedarsono et al. Reported that TLR was significantly associated with more severe clinical conditions of TB infection and MDR TB. This decrease in signaling ability against TLR4 can greatly affect the susceptibility of TB disease. TLR4 are of the most studied and shown to be associated with TB susceptibility and more severe disease conditions.[13,15]

The results of this study also explain that the conditions of TB and MDR TB infection accompanied by diabetes will have a higher TLR4 value. Previously, TLR4 was also reported to have correlation with diabetes condition and proinflammatory stage. Mohammad et al. Reported an increased expression of TLR2 and TLR4 in nonobese type 1 diabetic rats, then has correlation with increased activation of nuclear factor κB (NFKβ) in response to LPS TLR4 ligands resulting in increased proinflammatory cytokines. Sridevi et al. Also demonstrated the increased expression and activity of TLR2 and TLR4 in monocytes diabetic animals, also explaining that increased TLR4 expression contributes to the pro-inflammatory state of diabetes.[16,17]

9. Conclusion

There is a significant difference between TB and MDR TB infection with diabetes will have a higher TLR4 value. TLR 4 correlates with an increase in the number of MTB bacteria.

Author contribution

Heidy Agustin: first author, literature search, data collector, Muhammad Nasum Massi: supervisor in literature search, Irawati Djaharuddin: methods and study design, Agus D. Susanto: writing and literature search, Andi A. Islam: methods and study design, Mochammad Hatta: methods and study design, Agussalim Bukhari: data analysis, Nur A. Tabri: data analysis, Arif Santoso: data analysis, Erlina Burhan: literature search and statistic interpretation, Fathiyah Isbaniyah: literature search and statistic interpretation, Zulham Effendy: Data collecting and analysis

Conflicts of interest

The authors have none to declare.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ijtb.2022.03.012.

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