MEDICINA UNIVERSITARIA

www.elsevier.com.mx

ORIGINAL ARTICLE

Mycobacterium tuberculosis lipids induce an immunosuppressive environment in diabetic patients

Ana I. Vázquez-Armendáriz, Adrian G. Rosas-Taraco, Mario C. Salinas-Carmona, Alma Y. Arce-Mendoza

Department of Immunology. School of Medicine. Dr. José E. González University Hospital. Universidad Autónoma de Nuevo León. Monterrey, N.L., Mexico.

Abstract

The increased susceptibility of diabetic patients to *Mycobacterium tuberculosis* infection is not clearly understood. Cytokines produced by the host during the infection against the mycobacteria wall components may help to elucidate this relation. For this study, four groups were considered: healthy controls, patients with type 2 diabetes mellitus (DM), patients with pulmonary tuberculosis and patients with type 2 DM infected with *M. tuberculosis*. Mononuclear cells were isolated from peripheral blood and cultured to obtain adherent cells. The selected cells were stimulated with apolar lipids of *M. tuberculosis*. Proinflammatory and anti-inflammatory cytokines production were measured by ELISA. Anti-inflammatory cytokines production, IL-10 and IL-4, were increased in DM patients with tuberculosis. On the other hand, IFNγ production was found to be lower in these patients. In conclusion, apolar lipids of *M. tuberculosis* are inducing an anti-inflammatory environment; which could be associated with type 2 DM patients' susceptibility to *M. tuberculosis* infection.

Corresponding author: Alma Arce-Mendoza. *Universidad Autónoma de Nuevo León.* Gonzalitos 235 Norte, Mitras Centro. Monterrey, N.L., Mexico. Z.P. 64460. *E-mail:* aya_mayola@yahoo.com

Los lípidos de Mycobacterium tuberculosis inducen un ambiente inmunosupresor en los pacientes diabéticos

Resumen

A pesar del extenso conocimiento que se tiene sobre diabetes mellitus (DM) tipo 2 y tuberculosis, aún no se han logrado elucidar los mecanismos involucrados en la incrementada susceptibilidad, que poseen los pacientes diabéticos de contraer tuberculosis. Las citocinas producidas durante la infección contra los componentes de la micobacteria podrían ser la clave para descifrar esta relación. En este estudio se formaron cuatro grupos de estudio: controles sanos, pacientes con tuberculosis pulmonar, pacientes con DM tipo 2 y pacientes con DM tipo 2 con tuberculosis pulmonar. Células mononucleares fueron aisladas de sangre periférica y cultivadas para la obtención de células adherentes. Más tarde, dichas células fueron estimuladas con una concentración determinada de lípidos apolares de M. tuberculosis. La producción de citocinas proinflamatorias y antiinflamatorias fue establecida mediante ELISA. Se observó un aumento significativo en la producción de citocinas antiinflamatorias, IL-10 y IL-4, en los pacientes diabéticos infectados con M. tuberculosis. Por otro lado, los niveles de IFNγ fueron significantemente menores en estos pacientes. En conclusión, los resultados sugieren que los lípidos apolares de M. tuberculosis están induciendo un ambiente antiinflamatorio, que podría estar asociado con el mayor riesgo que poseen los pacientes diabéticos de contraer tuberculosis.

Introduction

Tuberculosis is one of the main causes of illness and death worldwide. In 2006, about 9.2 million cases of people infected with *Mycobacterium tuberculosis* were reported by the World Health Organization and is expected to kill more than 70 million people over the next two decades.[1,2] Diabetes mellitus (DM) is a metabolic disorder that weakens the immune system and is considered an important predisposing factor for tuberculosis. Recently, studies have shown that between 10 to 30% of the patients with tuberculosis suffer from type 2 DM as well.[3] Cohort studies demonstrated that people with type 2 diabetes have 3-fold risk of developing active tuberculosis.[4] Even when these diseases have been widely studied, the connection between them is not clear. Cytokines produced by the host during *M. tuberculosis* infection against mycobacteria, especially wall components, may help to elucidate this link.[5]

During active tuberculosis, interferon gamma (IFNγ) is a key cytokine that participates in macrophage activation by mediating host defenses against *M. tuberculosis*.[6] In contrast, production of immunosuppressive molecules during active tuberculosis contributes to the establishment of chronic mycobacterial infections.[7] Additionally, overproduction of interleukin 10 (IL-10) by T cells has been associated with immunosuppression and it increases susceptibility to mycobacterial infection.[8]

Several studies have shown evidence in support of the relationship between DM and alternated host immunity against *M. tuberculosis*. For instance, studies have shown that chronic diabetic mice have significantly lower production of IFN-γ and Interleukin 12 (IL-12) compared to euglycemic mice.[7] Moreover, IL-6 is a good inducer of acute phase protein, however, Aderka et al. reported that IL-6 inhibits LPS-induced TNF-α production.[9]

In human models, high levels of insulin have been shown to induce a decrease in Th1 immunity on plasma cells.[10] Furthermore, an *in vitro* comparison study of Th1 cytokines determinate that nonspecific IFN-γ levels were significantly lower in people with diabetes compared to non diabetic individuals.[11]

The aim of this study was to elucidate if Th1 cytokine response is being inhibited by *Mycobacterium tuberculosis* lipids in adherent cells of diabetic patients and if it could be associated with the induction of an anti-inflammatory environment.

Materials and methods

Study Population
Sixty subjects between the ages of 18 and 60 were studied. Each one belonged into one of four groups: 15 healthy controls, 15 patients with type 2 DM, 15 patients with pulmonary tuberculosis, and 15 patients with type 2 DM and pulmonary tuberculosis. The Bioethics Committee

from the School of Medicine at the *Universidad Autónoma de Nuevo León* approved the project, ensuring that it was in accordance with Institutional Review Board criteria.

Healthy controls were TST-negative and they did not present type-2 DM or other metabolic disorder. Alternatively, type 2 diabetes patients were selected based on their glycosylated hemoglobin alfa (A1), which needed to be between 6.0 to 8.0%. Pulmonary tuberculosis patients were enrolled during the first three months of treatment and the diagnosis was based on positive sputum smear test, confirmed by a positive culture of tuberculosis. All enrolled subjects were HIV negative and did not present any other concurrent infectious disease.

The sixty individuals studied were mostly between the ages of 35 and 50 (45%), followed by 20 to 35 year old subjects (33%). In addition, 22% were above 50 years and only 2% corresponded to the ages between 18 and 20. The gender distribution was 57% male and 43% female. Detail information about the ages and gender of each study group is shown in **Table 1**.

This scientific approach has been done mostly in mice with larger number of subjects. However, the size of the sample for this study was determinate based in previous studies in humans in which conclusive results were achieved employing less study subjects.

Extraction of mononuclear cells from peripheral blood

Blood with EDTA was drawn from the subjects and mononuclear cells were isolated by Ficoll-diatrizoate gradient centrifugation. The cells were collected from the interphase and thoroughly washed with pyrogen free phosphate buffer (PBS). After the last wash, the cells were resuspended in RPMI 1640 medium with 10% of FBS. This solution was transferred to a culture plaque and remained in incubation for 20 hours at 37°C with 5% of CO_2.

Obtaining monocyte-derived adherent cells and stimulation with *M. tuberculosis* apolar lipids

Using the plate adhesion technique, supernatants were carefully removed from de culture plaque. After two washes with RPMI 1640 medium, the adherent cells were detached with a scrapper and collected in an assay tube. The cells were centrifuged and the cellular pellet was resuspended in RPMI 1640 medium with 10% of FBS. Final cell concentration was adjusted to 1×10^6 cell/mL. Subsequently, the cells were stimulated with 10ug of *Mycobacterium tuberculosis* apolar lipids resuspended in RPMI 1640 medium with 10% of DMSO for 24 hours at 37°C with 5% of CO_2. The mycobacteria lipids were obtained from the *M. tuberculosis* H37Rv biomass using the protocol reported by Arce-Mendoza A et al.[12] Adherent cells were stimulated with RPMI with 10% DMSO as negative controls.

Cytokine quantification

Supernatants were collected from the cell culture and kept at -80°C until analysis. After all samples were complete, IL-4, IL-6, IL-10, IL-12p70, and IFNγ cytokines were screened in duplicate by ELISA (eBioscience ELISAkits®, California). Standard curves for each cytokine was generated using reference cytokine concentrations supplied by the manufacturer. In order to obtain only the cytokine production induced by the apolar lipids, a relation between stimulated and non-stimulated samples was established.

Statistical analysis

For the statistical analysis, results were evaluated with GraphPad Prism version 4 Software with a one-way ANOVA being applied with the Tukey test, a value of $p \leq 0.05$ was considered statistically significant.

Results

Cytokines

Supernatants were divided into two groups to establish cytokine production on each study group. One group received the stimulation of lipids of *M. tuberculosis* and the other group only received culture medium.

For IL-6 production analysis, the group with pulmonary tuberculosis and the group with type 2 DM showed a significant increase with respect to the control. IL-6 production was also high in the supernatants of adherent

Table 1. Age and gender distribution in each study group.

Study groups	Control n=15	Tuberculosis n=15	Diabetes mellitus n=15	Tuberculosis + Diabetes mellitus n=15
Age media (years)	30	38	45	46
Standard deviation	7.8	11.1	9.6	9.0
Age media (years)	28	39	46	43
Female (%)	40	41	51	43
Male (%)	60	59	49	57

cells from patients with DM and pulmonary tuberculosis, but it was not significant (**Figure 1**). IFNγ production presented the opposite behavior to that of IL-6. For IFNγ, all groups exhibited a significant decrease with respect to the control (**Figure 2**). IL-12 was not detected in any study group (data not shown).

In addition, IL-10 production was significantly increased in the groups with pulmonary tuberculosis only and with type 2 DM, with respect to the control (**Figure 3**). The DM with pulmonary tuberculosis group was the only one that exhibited significant increase with respect to the control for the IL-4 production. Nevertheless, a significant increase of IL-4 production was also detected in the DM with pulmonary tuberculosis group with respect to the pulmonary tuberculosis group (**Figure 4**).

Discussion

Diabetic patients have a higher risk of developing active tuberculosis, but the mechanism has not been elucidated yet. Therefore, we hypothesized that the Th1 cytokine response in patients with pulmonary tuberculosis and type 2 DM is inhibited by an anti-inflammatory environment induced through apolar lipids of *M. tuberculosis*. Studies based on animal models indicated that the production of Th1-related cytokines in response to *M. tuberculosis* declines in patients with DM.[13,14]

In order to ensure the statistical significance of the results, we based our sample size on previous human studies that have been done using similar sample sizes. Weng SF et al. presented a study, comparing diabetic and non diabetic patients with extrapulmonar tuberculosis, with

comparable study groups and number of subjects.[15] Furthermore, R J Al-Attiyah et al. showed almost identical study groups and size; and surprisingly also concluded that diabetic patients with tuberculosis had higher Th2 bias.[16]

The results of this study show that IL-10 has the capacity of downregulation IFNg production in patients with tuberculosis alone or with DM. Patients with pulmonary tuberculosis expressed higher amount of IL-10. Furthermore, our results are consistent with other findings reported by De la Barrera S et al. (2004), where IL-10 levels were increased and this increase was associated with the reduction in IFNγ production in tuberculosis patients.[17] Rocha-Ramirez et al. (2008) reported that *M. tuberculosis* apolar lipids can induce IL-10 production, especially the Beijing strain. However, we demonstrate in this study that H37Rv strain can also induce IL-10 production.[18] Moreover, Higgins D et al. reported that the lack of IL-10 could be related with disease progression and a higher inflammatory environment in the lung.[19] In these experiments, IFNγ levels were lower in all the studied patients in comparison to healthy subjects. The high susceptibility of diabetic patients to *M. tuberculosis* could be in part explained by lower IFNγ production. It is well known, that IFNγ is considered as a key cytokine in the cell-mediated immunity response against intracellular bacteria by inducing nitric oxide production and increasing MHC-II molecules.[11] In addition, IL-10 by itself can inhibit IFNg functions, like macrophages activation, antigen presentation, among others.

IL-6 production was significantly increased in pulmonary tuberculosis patients and in patients with diabetic

Figure 1. IL-6 Production. IL-6 levels are presented in base of its relation between stimulated and non stimulated macrophages and data shown are average±SEM. The p value of the IL-6 for the tuberculosis group was *p*<0.01, for the diabetes mellitus group was *p*<0.01.
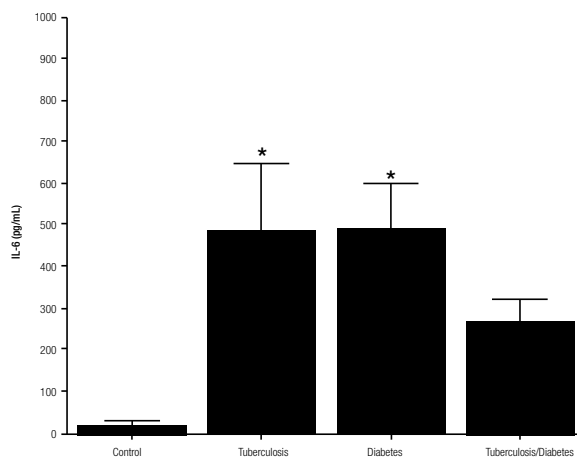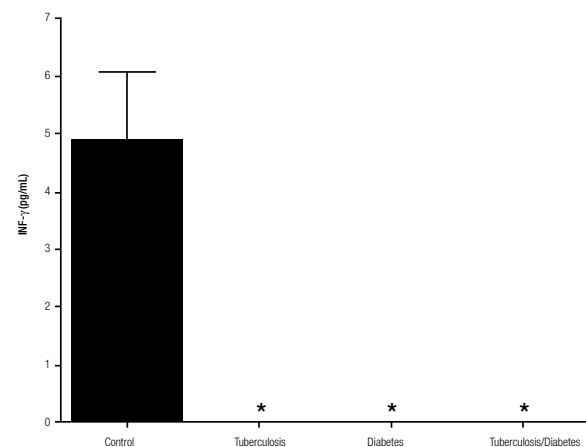
Figure 2. INFγ gamma Production. IFNγ levels are presented in base of its relation between stimulated and non stimulated macrophages and data shown are average±SEM. The p value of the IFNγ for the tuberculosis was *p*<0.05 and *p*<0.01 for the diabetes mellitus group.

Figure 3. IL-10 Production. IL-10 levels are presented in base of its relation between stimulated and non stimulated macrophages and data shown are average±SEM. The p value of the IL-10 production for the tuberculosis group was *p*<0.001, for the diabetes mellitus group was *p*<0.05 and for the diabetes mellitus with tuberculosis was *p*<0.01.

Figure 4. IL-4 Production. IL-4 levels are presented in base of its relation between stimulated and non stimulated macrophages and data shown are average±SEM. The *p* value of the IL-4 production for the diabetes mellitus with tuberculosis group with respect to the control was *p*<0.05 and with respect to tuberculosis group was *p*<0.05.
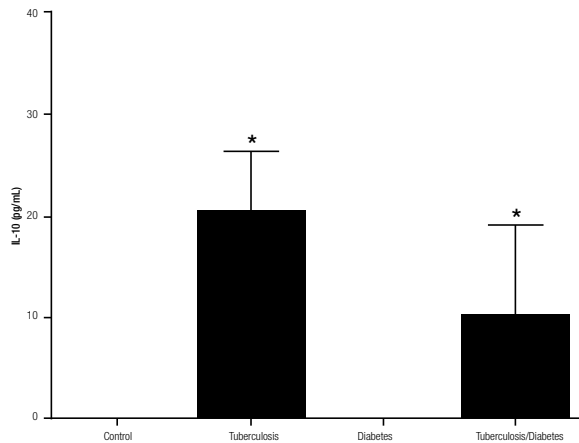
mellitus. Besides, other studies have shown data suggesting that the lipids of *M. tuberculosis* are potent inducers of IL-6 gene regulation or transcription (use one of the two words) in peripheral blood monocytes.[20,21] Previous studies with LPS confirmed an inhibition of TNFα production by IL-6 in cultured human peripheral blood monocytes. [9] Further investigations of these cells will help to clarify if IL-6 has a role in assisting or interfering in the host defense mechanisms.

IL-4 is a cytokine that plays a significant role in the differentiation of the precursor CD4+ T cell towards a Th2 phenotype. The production of IL-4 by the patients with diabetic mellitus and tuberculosis was higher in comparison with the other groups. In addition, this cytokine production was significantly superior in tuberculosis patients with respect to other patient groups. Besides the higher susceptibility of DM patients to develop tuberculosis, studies have demonstrated that the clinical conditions of diabetics with tuberculosis deteriorate faster than regular tuberculosis patients. These studies concluded that diabetic patients with tuberculosis shift towards Th2, which may explain the rise in the levels of IL-4 and thus, the deterioration in health of the patients.[22] These increased levels of IL-4 production on diabetics patients with tuberculosis could explain the lack of significant production of IL-6 in these patients. Studies have showed that both IL-10 and IL-4 inhibit LPS-induced IL-6 mRNA and protein production by inhibiting the transcription rate of the IL-6 gene. However, the inhibitory effect of IL-4 was more pronounced than IL-10.[23] Furthermore, IL-4 levels could be favoring a Th2 response even though, the most important and effective immune mechanism
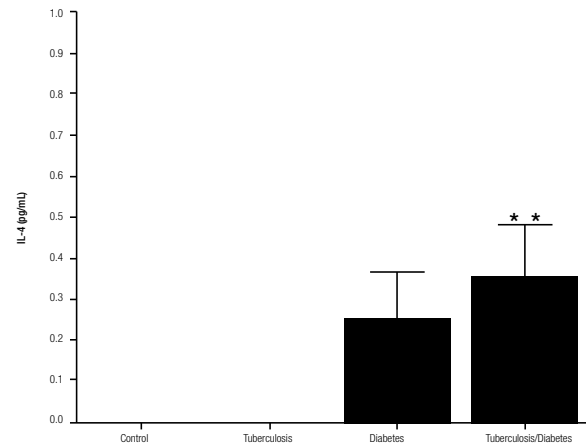
that is activated in the fight against *M. tuberculosis* is the one involving the Th1 cells. Therefore, here we demonstrate that apolar lipids of *M. tuberculosis* are inducing an anti-inflammatory environment by IL-10 production and favoring a Th2 response in adherent cells from type 2 DM and tuberculosis patients.

Acknowledgments

References

1. World Health Organization. Global Tuberculosis Control: Surveillance, Planning, Financing: WHO Report 2008. Geneva, Switzerland. WHO. 2008.
2. Murray CJ, Salomon JA. Modeling impact of global tuberculosis control strategies. Proc Natl Acad Sci USA 1998;95:13881-13886.
3. Tatar D, Senol G, Alptekin S, et al. Tuberculosis in diabetics: features in an endemic area. Jpn J Infect Dis 2009;62:423-427.
4. Christie YJ, Megan BM. Diabetes Mellitus Increases the Risk of Active Tuberculosis: A Systematic Review of 13 Observational Studies. PLoS Med 2008;5:52.
5. Barnes PF, Lu S, Abrams JS, et al. Cytokine production at the site of disease in human tuberculosis. Infect Immun 1993;61:3482-3489.
6. Dlugovizky DA, Torrez-Morales A, Rateni L, et al. Circulating profile of Th1 and Th2 cytokines in tuberculosis patients with different degrees of pulmonary involvement. FEMS Immunol Med Microbiol 1997;18:203-207.

7. Boussiotis VA, Tsai EY, Yunis EJ, et al. IL-10- producing T cells suppress immune responses in anergic tuberculosis patients. J Clin Invest 2000;105:1317-1325.

8. Mosmann TR, Coffman RL. Th1 and Th2 cells. Different patterns of lymphokine secretion lead to different functional properties. Ann Rev Immunol 1989;7:145-173.

9. Aderka D, Le JM, Vilcek J, et al. IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice. J Immunol 1989;143:3517-3523.

10. Viardot A, Grey ST, Mackay F, et al. Potential anti-inflammatory role of insulin via the preferential polarization of effector T cells toward a T helper 2 phenotype. Endocrinology 2007;148:346-353.

11. Stalenhoef JE, Alisjahbana B, Nelwan EJ, et al. The role of interferon-gamma in the increased tuberculosis risk in type 2 diabetes mellitus. Eur J Clin Microbiol Infect Dis 2008;27:97-103.

12. Alma AM, Julieta RI, Mario SC, et al. Production of CD64, CD206, and RAGE in Adherent Cells of Diabetic Patiens Infected with Mycobacterium tuberculosis. Arch Med Res 2008;306-311.

13. Sugawara I, Yamada H, Mizuno S, et al. Pulmonary tuberculosis in spontaneously diabetic goto kakizaki rats. Tohoku J Exp Med 2004;204:135-145.

14. Martens GW, Arikan MC, Lee J, et al. Tuberculosis Susceptibility of Diabetic Mice. Am J Respir Cell Mol Biol. Am J Respir Cell Mol Biol 2007;37:518-524.

15. Weng SF, Hsu CH, Lirn ML, et al. Extrapulmonary tuberculosis: a study comparing diabetic and nondiabetic patients. Exp Clin Endocrinol Diabetes 2009;117(6):305-307.

16. Al-Attiyah RJ, Mustafa AS, et al. Mycobacterial antigen-induced T helper type 1 (Th1) and Th2 reactivity of peripheral blood mononuclear cells from diabetic and non-diabetic tuberculosis patients andMycobacterium bovis bacilli Calmette-Guérin (BCG)-vaccinated healthy subjects. Immunol 2009:158:64-73.

17. De la Barrera S, Aleman M, Musella R, et al. IL-10 down-regulates costimulatory molecules on Mycobacterium tuberculosis-pulsed macrophages and impairs the lytic activity of CD4 and CD8 CTL in tuberculosis patients. Clin Exp Immunol 2004;138:128-138.

18. Rocha-Ramirez LM, Estrada-Garcia I, Lopez-Marin LM, et al. Mycobacterium tuberculosis lipids regulate cytokines, TLR-2/4 and MHC class II expression in human macrophages. Tuberculosis 2008;88:212-220.

19. Higgins DM, Sanchez-Campillo J, Rosas-Taraco AG, et al. Lack of IL-10 alters inflammatory and immune response during pulmonary Mycobacterium tuberculosis infection. Tuberculosis 2009;89:149-157.

20. Zhang Y, Broser M, Rom WN, et al. Activation of the interleukin 6 gene by Mycobacterium tuberculosis or lipopolysaccharide is mediated by nuclear factors NF-IL6 and NF-kappa B. Proc Natl Acad Sci USA 1994;91:2225-2229.

21. Al-Attiyah RJ, Mustafa AS. Mycobacterial antigen-induced T helper type 1 (Th1) and Th2 reactivity of peripheral blood mononuclear cells from diabetic and non-diabetic tuberculosis patients and Mycobacterium bovis bacilli Calmette-Guérin (BCG)-vaccinated healthy subjects. Clin Exp Immunol 2009;158:64-73.

22. Takeshita S, Gage JR, Kishimoto JO, et al. Differential regulation of IL-6 gene transcription and production by IL-4 and IL-10 in human monocytic cell lines. J Immunol 1996;156:2591-2598.

23. Dokter WH, Koopmans SB, Vellenga E, et al. Effects of IL-10 and IL-4 on LPS-induced transcription factors (AP-1, NF-IL6 and NF-kappa B) which are involved in IL-6 regulation. Leukemia 1996;10:1308-1316.