Elevated IL-35 Level and iTr35 Subset Increases the Bacterial Burden and Lung Lesions in Mycobacterium Tuberculosis Infected Mice

Yu F[1]., Li Q[2]., Xu W[3]., Gao Y[4]., Wen Y[5]., Zhang Q[6]. and Dou J[7].
[1,2,3,4]School of Preclinical Medicine Wannan Medical College, Wuhu 241001, PR China [5]School of Public Health, Wannan Medical College, Wuhu 241001, PR China [6]Center of Disease Control and Prevention, Wuhu, Anhui, 241001, PR China [7]Medical School, Southeast University, Nanjing, 210009, PR China

ABSTRACT

Background and Objective: *Mycobacterium tuberculosis (Mtb) harbors immune evasion that impairs immune responses and prevents optimal immunity against Mtb. However, little is known about mechanisms of immune evasion in Mtb infected individual. In this study, the relationship among the IL-35 level, IL-35-producing regulatory T cells (iTr35) subset, the bacterial burden and lung lesions in mice infected with Mtb were investigated to assess the impact of immune evasion on the infected mice.*

Methods: *A total of twenty C57BL/6 male 6-7 weeks old mice, were injected with 1×10^5 colony-forming units (CFU) of attenuated H37Ra strain of Mtb in a volume of 200 µL, to prepare mouse tuberculosis infection models. Colony forming units in left lung and spleen coefficient were determined followed by histopathology, quantitative RT-PCR for mRNA, Western blot analysis, cytokine IL-35 detection and flow cytometry of fork head box protein P3-expressing T cells.*

Results: *Compared with the control mice, the mRNA expressions of the p35 and EBI3 of IL-35 were significantly increased in the spleen of 8-week infected mice, and their protein expressions were not only increased in 8-week but in lungs of 4-week infected mice, accompanied with an elevated level of serum IL-35. In addition, iTr35 subset was increased in the spleen of 8-week infected mice compared with the control mice. Importantly, the high bacterial burden, lung lesions, and low mouse weight were found in the 8-week infected mice.*

Conclusion: *The mice infected with Mtb H37Ra strain resulted in elevated IL-35 levels and iTr35 subset. There were increased bacterial burden and lung lesions, suggesting that IL-35 and iTr35 cells play an immune evasion role in chronic Mtb infected mice.*

KEYWORDS: *Mycobacterium tuberculosis, H37Ra,IL-35,iTr3, Immune evasion.*

INTRODUCTION

Mycobacterium tuberculosis (Mtb) infection leads to human tuberculosis (TB) and remains a major cause of morbidity and mortality worldwide. More than one-third of people in the world are infected with TB, and up to 2 billion people in latent TB infection state, are at a risk of progression to active TB.[1,2] The only TB vaccine approved for use in humans, bacillus calmette guerin (BCG), shares many of the immune evasion proteins but does not completely confer protection against latent TB infection state; however, the role of proteins in T cell response elicited by BCG is poorly understood.[3]

Published studies suggest that in naïve hosts, Mtb grows for several weeks without being restricted by IFNγ-producing immune cells, which eventually accumulate and establish the chronic Mtb infection.[4,5] Serum IL-35 levels are elevated in the patients with active TB, while the regulatory T cells (Treg) are thought to be a key source of IL-35 that is the newest member of IL-12 family. It consists of a dimeric protein including two separate subunits, and has manifested its inhibitory role on immune system via formation of IL-35-producing regulatory T cells (iTr35), which increases Treg development.[6] Epstein-Barr virus-induced gene 3 (EBI3), is a subunit of immunoregulatory cytokine IL-35, and has representatively been assessed and explored in terms of its effects triggered by extracellular EBI3.[7] However, little is known about serum IL-35 levels, iTr35 cell subset, and expressions of EBI3 and p35 (another subunit of IL-35) in chronic Mtb infected individuals.

In the current study, serum IL-35 level, iTr35 cell subset number, both p35 and EBI3 expressions were tested in 4-week and 8-week Mtb infected mice via tail vein injection of Mtb, to evaluate if these immunological molecules and cells, associated with the immune evasion roles, would influence the

Corresponding Author: Jun Dou, Department of Pathogenic Biology and Immunology, School of Medicine, Southeast University, 87 Ding Jiaqiao Rd. Nanjing 210009, PR China. E-mail: njdoujun@seu.edu.cn.

bacterial burden and lung lesions in chronic Mtb infected mice.

METHODS

The ethical approval of study was given by Animal Research Ethics Board of Wannan Medical School, China.

H37Ra

The attenuated H37Ra strain of Mtb was provided by the Centers for Disease Control and Prevention, Jiangsu Province, PR China, and was grown in Sauton medium enriched with 0.5% sodium pyruvate and 0.5% glucose. In the current study, the viable attenuated H37Ra strain of Mtb was used as the challenge study.

Mice

C57BL/6 male 6-7 weeks old mice were supplied as specific-pathogen-free by Animal Center of Qinglong mountain, Jiangning Zone, Nanjing of China, license number SCXK (Jiangsu) 2017-0001. Mice were raised under SPF level animal facility, at the Experimental Animal Center, Wannan Medical School, Wuhu, PR China. All animal experiments were conducted following the guidelines of the Animal Research Ethics Board of Wannan Medical School, China.

Mouse Tuberculosis Infection Model

Twenty male C57BL/6 6-7 weeks old mice were infected by tail vein injection with 1×10^5 colony-forming units (CFU) of attenuated H37Ra strain of Mtb in a volume of 200µL.[8] Ten mice were executed at 4 weeks, and the remaining mice were monitored over a 8-week period. As control, ten mice were infected by tail vein injection with 200µL Phosphate Buffered Saline (PBS). The experiment was repeated twice. Before the chronic infection mouse model was established, the H37Ra strain of Mtb infected mouse model was successfully established, by identifying the bacteria morphology in each mouse lungs.

Colony Forming Units and Spleen Coefficient

CFU in left lung were assessed at 4-week and 8-week by serial dilutions of left-lung homogenates. The number of bacteria in lung homogenate suspensions after lysis with saponin 0.1 % in distilled water was estimated by plating 10-fold dilutions, prepared in distilled water, on Sauton medium enriched with 0.5% sodium pyruvate, 0.5% glucose, 50 µg/ml carbenicillin, and 20 µg/ml trimethoprim. The numbers of CFUs were determined after 21 days of incubation at 37℃ in humidified air. CFUs were counted visually. Spleen coefficient = spleen mass/mouse mass × 100%.[9]

Histopathology

For histopathological study, 5µm sections were stained with hematoxylin and eosin (H&E) and with Alizarin red to detect calcified lesions for routine evaluation in a blinded fashion by 2 investigators. The histopathological parameters were evaluated, which include peribronchiolitis, perivasculitis, alveolitis, and granuloma formation; each semiquantitative scored as absent, minimal, slight, moderate, marked and strong, noted as 0, 1, 2, 3, 4 and 5 respectively. In this score the frequency and severity of the lesions were also incorporated.[10-12]

Quantitative RT-PCR (qRT-PCR)

qRT-PCR analysis was performed on an ABI step one plus real-time system. Total cellular RNA was isolated from each sample. cDNAs were amplified by PCR with primers as follows: EBI3 (sense, 5′-CATTGCCACTTACAGGCTCG-3′; antisense, 5′-GGATGT ACGATTTACAGTGACGT-3′); p35(sense, 5′-CAATCACGCTA CCTC CTCTTTT-3′; antisense, 5′-CTTTGT AATAAGGACGTGACGAC-3′);β-actin (sense, 5′-GGCT GTATTCCCCTCCATCG-3′; antisense, 5′-TGTACCGTAACAATGGTTGACC-3′). The mRNA levels of the genes of interest were expressed as the ratio of each gene of interest to β-actin for each sample and quantitative PCR amplifications were performed. The comparative Ct (ΔΔCt) method was used to determine the expression fold change.[13,14]

Western Blot

The assay was performed as reported in the previous literatutre.[15] The lung suspensions were acquired from the 4-week and 8-week infected mice. The membrane was then incubated with the APC anti-IL-27/35EBI3 (Rat anti-mouse, R&D, IC18341A) and PE anti-IL-12/35p35 (Goat anti-mouse, R&D, IC2191P), respectively for overnight at 4°C. The membrane was rinsed for 5 min with an antibody wash solution for 3 times before adding the goat anti-rat or the rabbit anti-goat fluorescence secondary antibody to it and the immunoreactive bands were detected.

Cytokine IL-35 Detection

Serum isolated from the 4-week and 8-week infected mice were assayed for cytokine IL-35. A double antibody sandwich enzyme linked immunosorbent assay (ELISA) was performed for detecting serum IL-35 (1:1000-fold dilution).[16]

Flow Cytometry (FCM)

The analysis of fork head box protein P3 (FOXP3)-expressing T cells was carried out. The mononuclear cells were stained with surface molecule antibody (Ab) and washed with cold PBS. The cells were resuspended in the fix/perm buffer and incubated at 4˚C 3 h in the dark, and then washed

twice in a permeabilization buffer. Anti-mouse/rat Foxp3-PE was then added. The cells were incubated for 30 min and washed twice in PBS, resuspended in PBS, and then analyzed in FCM. For analysis of the $CD4^+$or $CD25^+$-expressing T cells, the suspensions of splenocytes were co-stained with the fluorescein isothiocyanate (FITC)-conjugated anti-CD4 Ab, phycoerythrin (PE) conjugated anti-p35-PE and EBI3-APC antibodies for FCM detection.[17,18]

STATISTICAL ANALYSIS

Statistical comparisons were performed using the Student's t-test method for any statistically significant differences in the results between the experiment and the control groups. SPSS version 23.0 was used for statistical analysis. A *P-value* below or equal to *0.05* was considered statistically significant.

RESULTS

Increased Spleen Coefficient in Chronic Mtb Infected Mice

In order to establish the Mtb infected mouse model, a microbiological confirmation was carried out in mice infected with the Mtb via tail vein injection with 1×10^5 CFU of H37Ra strain. The viable bacteria in the lungs were determined at 24 hours by plating serial dilutions of whole lung homogenates onto nutrient medium, and acid fast bacilli were identified by Ziehl–Neelsen staining. As shown in Figure 1A, acid fast bacilli were observed under light microscope (× 1000). The results demonstrated that the Mtb infected mouse model was established appropriately for further study of Mtb chronic infection. Figure 1B indicates the spleen coefficient having statistically significant changes in control and experimental groups.

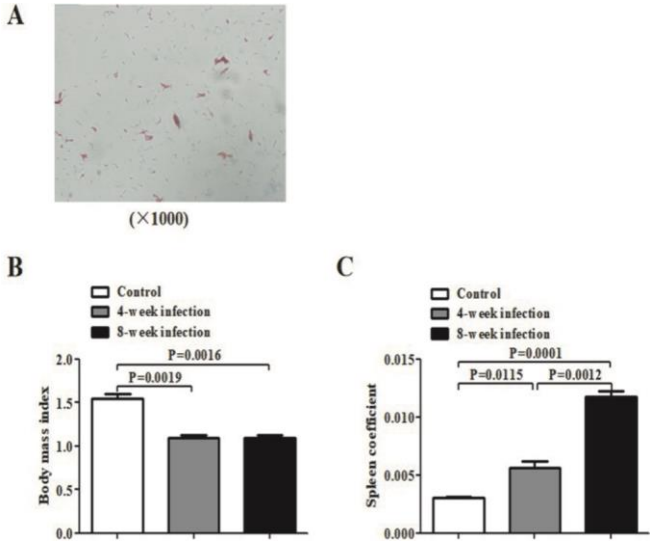

Fig. 1: *Detection of Mtb and spleen coefficient. A: The figure represents the acid fast bacilli under light microscope (Ziehl–Neelsen stain ×1000). B: The body mass index in the different groups. C: The spleen coefficient in the different groups. All the data represent mean ± S.D (n = 10), referring to the differences as shown.*

Pathological Change and Lung Bacterial Burden in Chronic Mtb Infected Mice

Figure 2A gives a representative clinical pictures of different lung tissues fixed in 10% formalin from the mice on week 4 without infection (left), week 4 (middle) and week 8 (right) post-infection of Mtb. It exhibits the gross appearance of lungs from the 4-week and 8-week Mtb infected mice, and no tubercular nodule was found in lung from the control mice. Figure 2B shows the histopathological changes in lung tissue of mice. The control exhibits the normal alveolar architecture without any infection and no obvious pathological changes. However, at 4 and 8 weeks the lung injury was more serious; with formation more pronounced necrotizing granulomas. There was mononuclear inflammatory cell infiltrate and more epithelium-like change in mononuclear cells in the 8-week Mtb infected mice than those (middle one) in the 4-week Mtb infected mice were noticed. There was a statistically significant difference in the total numbers of CFU between the 8-week Mtb infected mice and the 4-week Mtb infected mice as shown in Figure 2C where the histogram represents a set of data for 10 mice.

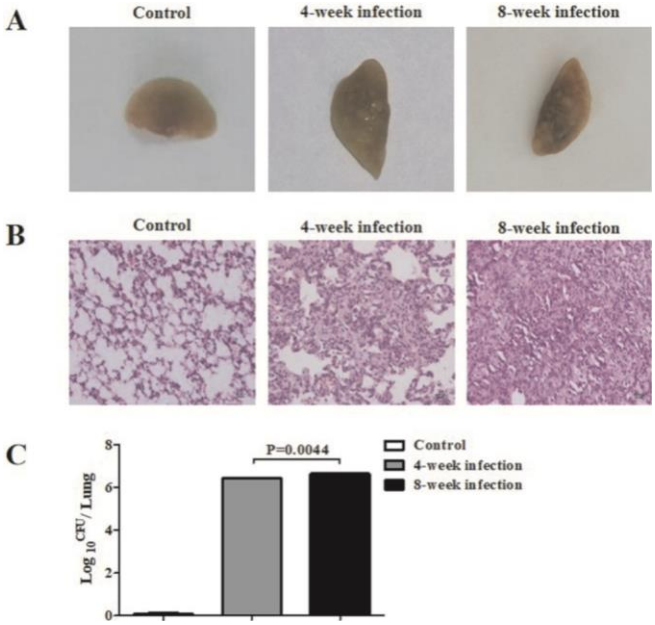

Fig. 2: *Mtb infection causes lung histopathological changes in mice. A: Gross appearance of lung tissue. B: Histopathological changes observed. C: The statistical analysis of total numbers of CFU in the different groups.*

Expressions of EBI3 and p35 and IL-35 Level in Chronic Mtb Infected Mice

To investigate the levels of IL-35 in chronic Mtb

infected mice, first the mRNA expressions of the both subunits (p35 and EBI3) of immunoregulatory cytokine IL-35 were detected. Figures 3A and 3B indicate the mRNA expressions of EBI3 and p35 in spleen, and both EBI3 and p35 expressions in the 8-week Mtb infected mice were more than the 4-week Mtb infected mice, which was statistically significant when compared with the normal mice. Figure 3C exhibits that the protein expressions of p35 and EBI3 in the lung tissues, confirmed by western blotting. The statistically significant increase of EBI3 protein expression is shown in Figure 3D in three groups. Figure 3E shows the statistical analysis of P35 protein expression in 8-week and 4-week Mtb infected mice versus the normal mice. These results were further supported by the data from the serum IL-35 detection (Figure 3F). The serum levels of IL-35 from the mice, with the infection on week 8 post-injection of Mtb, indicated higher level than that in week 4 post-injection of Mtb. It was statistically significant when compared with the 4-week Mtb infected mice ($P < 0.0184$) and the control mice ($P < 0.0007$). The results suggested that the chronic Mtb infection in mice led to increasing both p35 and EBI3 expressions and serum IL-35 levels. Each histogram represents a set of data for 10 mice.

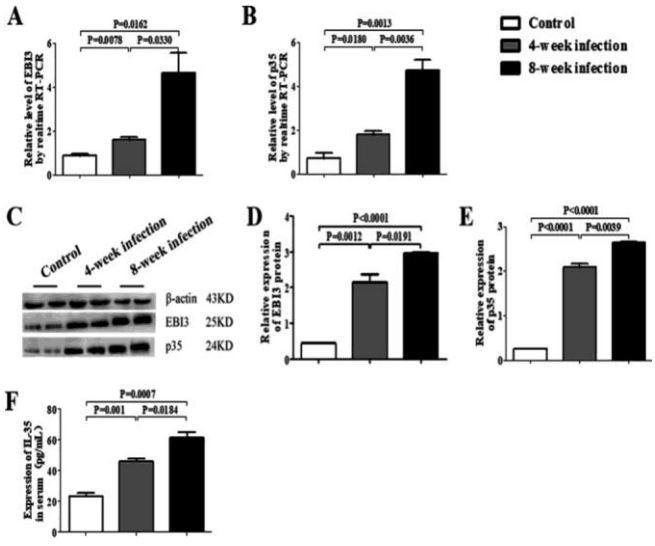

Fig. 3: *Detection of EBI3 and p35 expressions and serum IL-35 level. A: The splenocyte EBI3 mRNA expression detected by qRT-PCR in the week 4 and week 8 postinfection of Mtb. B: The splenocyte p35 mRNA expression detected by qRT-PCR in the week 4 and week 8 postinfection of Mtb. C: The expressions of p35 and EBI3 in lung tissues analyzed by Western blot in the week 4 and week 8 postinfection of Mtb. D: Semi-quantification of EBI3 protein expression. E: Quantification of p35 protein expression. F: The serum IL-35 levels tested by ELISA in the 4-week and 8-week Mtb infected mice.*

iTr35 Cells/CD4$^+$Foxp3$^-$T Cell Proportion in Chronic Mtb Infected Mice

The iTr35 cell subset/CD4$^+$Foxp3$^-$T cell proportion was investigated in chronic Mtb infected mice. Figure 4A gives the results of the iTr35 cell subset analyzed by FCM. It was found that the iTr35 cell subset number in the spleen of the 8-week Mtb infected mice was the highest among the three group mice, and the difference was statistically significant between the 8-week Mtb infected and the control groups but it was not statistically significant among the 8-week and the 4-week Mtb infected groups or between the 4-week Mtb infected and the control groups; shown in Figure 4B. These results indicated that the iTr35 cell subset was increased in the 8-week Mtb infected mice, accompanied with enhancing iTr35 cells/CD4$^+$Foxp3$^-$T cell proportion.

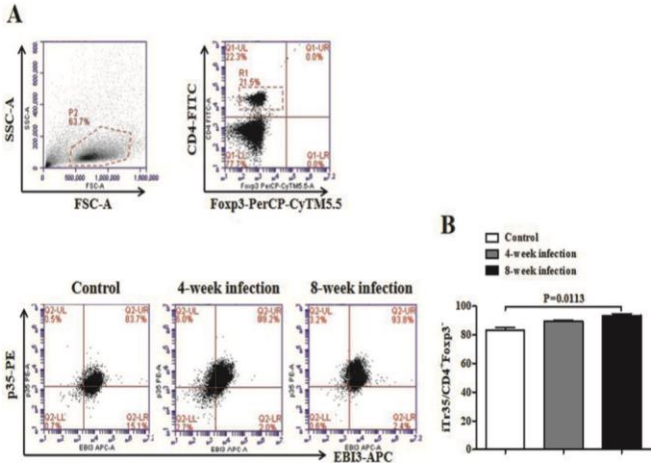

Fig. 4: *FCM analysis of iTr35 cell subset. A: The figure represents the iTr35 cell subset in the spleen cells of the 4-week and 8-week Mtb infected mice. B: The statistical analysis of total numbers of the iTr35 cell subset in the different groups. All the data represent mean ± S.D (n = 10), referring to the differences as shown.*

DISCUSSION

TB caused by Mtb is a highly prevalent infectious disease that remains a worldwide public health problem. Owing to an immune correlate of protection or an effective vaccine having yet to be completely developed, researchers have focused the investigation into the mechanisms of host-pathogen interactions and immune evasion mediated by Mtb chronic infection.[19,20] Cell immune mediated protection against Mtb can serve as biomarkers of ongoing bacterial infection, of which Treg and regulatory B cells (Breg) have critical roles as a negative regulator of immunity, mainly due to the fact that they secrete a high level of interleukin 10 (IL-10), transforming growth factor (TGF-β), and IL-35. From immune evasion mechanisms, the induction of immunosuppression by

Treg and Breg through IL-10, TGF-β, and IL-35 generates most effective, which optimizes the conditions for the survival of pathogens.[21,22] This mechanism is also used by widespread bacteria and viruses that are capable of chronically persisting in the human body like; Mtb, hepatitis viruses, human immunodeficiency virus, and others.[22,23] However, limited studies are available on the role of IL-35 and IL-35-inducible Treg (iTR35) cells on the chronic infectious TB disease.

For this reason, first the chronic infectious mouse model via tail vein injection of Mtb was established. Later sequentially the mouse model was used to observe the change of the levels of IL-35 and IiTR35 cell subset in this study. We adopted the attenuated Mtb strain H37Ra, which is similar to Mtb strain H37Rv but the virulence of Mtb strain H37Ra is weaker than Mtb strain H37Rv, and Mtb strain H37Ra is commonly used to study new and old antituberculous agents. The difference in protein expression or structure due to mutation between Mtb strain H37Ra and Mtb H37Rv may have an impact on the virulence property of Mtb H37Rv. However, Mtb H37Ra has the benefits of lower experimental costs and less administrative barriers such as the requirement of a biosafety Level III environment.[24,25] From the animal experiment results, it is depicted that the Mtb strain H37Ra developed well in C57BL/6 mice 4 weeks after injection with 1×10^5 CFU by tail vein, which was reflected in the spleen coefficient was significantly increased, and lung histopathological changes in the mice was markedly increased. IL-35's subunits p35 and EBI3 expressions, serum IL-35 level as well as IL-35-producing iTr35 cell subset were concurrently increased in the spleen of mice 4 weeks post infection, especially in 8-week infected mice. It is speculated that the change of these molecules and iTr35 cell subset are closely associated with immune evasion, which may result in aggravating the bacterial burden and lung lesions in chronic Mtb infected mice due to immunosuppressive effects on T cell mediated immune response. Accordingly, how to avoid or block immune evasion roles mediated by IL-35 and iTr35 cells is an important strategy for evaluating host-Mtb interactions and efficacy of antibiotics targeting Mtb.

CONCLUSION

The present study demonstrates that establishing chronic infected mouse model via tail vein injection of Mtb caused elevated serum IL-35 level and iTr35 cell subset, accompanied with the increase of bacterial burden and lung lesions in 8-week infected mice. This approach here also supports evidence that the levels of IL-35 and iTr35 cell subset may be associated with the immune evasion role that may play a negative regulatory role during Mtb infected mice.

LIMITATIONS OF STUDY

Despite the above-mentioned findings are promising, a limitation of the present study is that the precise mechanism behind Mtb infection causing increase in the levels of IL-35 and iTr35 cells remain unexplored. Also the analysis of TGF-β and IL-10 expression level could not be carried out for further integration because of financial constraints. Further studies focusing on the mechanism of immune evasion in chronic Mtb infected mice need to be planned in future with more advanced techniques.

ACKNOWLEDGEMENT

We are thankful to Dr. Cheng Bing in Wannan Medical College for providing a technique support for FCM analysis and guiding us how to prepare the cells. We also thank Dr. Zhang Quan in Wannan Medical College who gave suggestions to scientific way of writing introduction and discussion sections.

AUTHOR'S CONTRIBUTION

FY and JD: Conception and design of analysis data, article drafting.
FY, QL, WX, YG, YW, and QZ: Data acquisition.
JD: Critically reviewed manuscript for intellectual content.
All authors read and approved the final manuscript.

CONFLICT OF INTEREST

None to declare.

GRANT SUPPORT AND FINANCIAL DISCLOSURE

Th is study has been supported by Natural Science Research fund of Anhui Education Department (KJ2017A253).

REFERENCES

1. Wallis RS, Pai M, Menzies D, Doherty TM, Walzl G, Perkins MD, et al. Biomarkers and diagnostics for tuberculosis: progress, needs, and translation into practice. Lancet. 2010; 375(9729): 1920-37.
2. Centers for Disease Control and Prevention. 2018. Tuberculosis. Data and statistics. "Available online at https://www.cdc.gov/tb/statistics/default.htm. [Last accessed in February, 2018].
3. Smith CM, Proulx MK, Olive AJ, Laddy D, Mishra BB, Moss C, et al. Tuberculosis susceptibility and vaccine protection are independently controlled by host genotype. mBio. 2016; 7 (5): e01516-16.
4. Fine PE. Variation in protection by BCG: implications of and for heterologous immunity. Lancet. 1995; 346 (8986): 1339–45.
5. Andersen P, Doherty TM. The success and failure of BCG-implications for a novel tuberculosis vaccine. Nat Rev Microbiol. 2005; 3(2): 656–62.
6. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, et al. The inhibitory cytokine IL-35 contributes to

regulatory T-cell function. Nature. 2007; 450 (7169): 566–9.
7. Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Manganiello VC, Beavo JA, et al. Foxp3-dependent programme of regulatory T-cell differentiation. Nature. 2007; 445 (7129): 771–5.
8. Salam N, Gupta S, Sharma S, Pahujani S, Sinha A, Saxena RK, Natarajan K. Protective immunity to Mycobacterium tuberculosis infection by chemokine and cytokine conditioned CFP-10 differentiated dendritic cells. PLoS One. 2008; 3 (8): e2869-74.
9. Cafaro A, Piccaro G, Altavilla G, Gigantino V, Matarese G, Olivieri E, et al. HIV-1 Tat protein vaccination in mice infected with mycobacterium tuberculosis is safe, immunogenic and reduces bacterial lung pathology. BMC Infect Dis. 2016; 16(3): 442-49.
10. Miyazaki E, Miyazaki M, Chen JM, Chaisson RE, Bishai WR. Moxifloxacin (BAY12-8039), a new 8-methoxyquinolone, is active in a mouse model of tuberculosis. Antimicrob Agents Chemother. 1999; 43 (1): 85-9.
11. Dormans J, Burger M, Aguilar D, Hernandez-Pando R, Kremer K, Roholl P, et al. Correlation of virulence, lung pathology, bacterial load and delayed type hypersensitivity responses after infection with different mycobacterium tuberculosis genotypes in a BALB/c mouse model. Clin Exp Immunol. 2004; 137 (3): 460-8.
12. Yu F, Wang J, Dou J, Yang H, He X, Xu W, et al. Nanoparticle-based adjuvant for enhanced protective efficacy of DNA vaccine Ag85A-ESAT-6-IL-21 against Mycobacterium tuberculosis infection. Nanomedicine. 2012; 8 (8): 1337-44.
13. Burk U, Schuebert J, Wellner U, Schmalhofer O, Vincan e, Spadernaet S, al. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep. 2008; 9 (6): 582-9.
14. Chen D, Wang J, Zhang Y, Chen J, Yang C, Cao W, et al. Effect of down-regulated transcriptional repressor ZEB1 on the epithelial-mesenchymal transition of ovarian cancer cells. Int J Gynecol Cancer. 2013; 23 (8): 1357-66.
15. Zhang H, Wang J, Cai K, Jiang L, Zhou D, Yang C, et al. Downregulation of gene MDR1 by shRNA to reverse multidrug- resistance of ovarian cancer A2780 cells. J Cancer Res Ther. 2012; 8(3): 226-31.
16. Rosenberg SA, Sherry RM, Morton KE. Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+T cells in patients with melanoma. J Immunol. 2005; 175(4): 6169–76.
17. Wang X, Zhao F, Shi F, He X, Pan M, Wu D, et al. Reinforcing B16F10/GPI-IL-21 vaccine efficacy against melanoma by injecting mice with shZEB1 plasmid or miR200c agomir. Biomed Pharmacother. 2016; 80(3): 136-44.
18. Yu F, Zhao FS, Cai K, He X, Xu W, Zhang Y, et al. Polyethylenimine modified nanoparticle adjuvant increases therapeutic efficacy of DNA vaccine Ag85A-ESAT-6-IL-21 in mice infected with Mycobacterium tuberculosis. Int J Clin Exp Med. 2017; 10 (8): 12123-31.
19. Dou J, Wang Y, Yu F, Yang H, Wang J, He X, et al. Protection against Mycobacterium tuberculosis challenge in mice by DNA vaccine Ag85A-EAST-6-IL021 priming and BCG boosting. Int J Immunogenet. 2012; 39 (2): 183-90.
20. Harrington-Kandt R, Stylianou E, Eddowes LA, Lim PJ, Stockdale L, Pinpathomrat N, et al. Hepcidin deficiency and iron deficiency do not alter tuberculosis susceptibility in a murine M.tb infection model. PLoS One. 2018; 13 (1): e0191038-45.
21. Dai YC, Wang WD, Zhang JA, Chen C, Luo HL, Xu H, et al. MTB driven B cells producing IL-35 and secreting high level of IL-10 in the patients with active pulmonary tuberculosis. Mol Immunol. 2019; 112(3): 175-81.
22. Garib FY, Rizopulu AP. T-regulatory cells as part of strategy of immune evasion by pathogens. Biochemistry (Mosc). 2015; 80 (8): 957-71.
23. Layhadi JA, Eguiluz-Gracia I, Shamji MH. Role of IL-35 in sublingual allergen immunotherapy. Curr Opin Allergy Clin Immunol. 2019; 19 (1): 12-7.
24. Heinrichs MT, May RJ, Heider F, Reimers T, Sy SK, Peloquin CA, et al. Mycobacterium tuberculosis Strains H37ra and H37rv have equivalent minimum inhibitory concentrations to most antituberculosis drugs. Int J Mycobacteriol. 2018; 7 (2): 156-61.
25. Jena L, Kashikar S, Kumar S, Harinath BC. Comparative proteomic analysis of mycobacterium tuberculosis strain H37Rv versus H37Ra. Int J Mycobacteriol. 2013; 2 (2): 220-6.

- Received for publication: 02-08-2019
- First revision received: 25-09-2019
- Second revision received: 30-10-2019
- Accepted for publication: 07-12-2019