Jurnal Biosains Pascasarjana Vol. 25 (2023) pp

IN VITRO TUBERCULOSIS GRANULOMA MODEL IN *M. TUBERCULOSIS* H37RV

Talita Shofa Adestia[1], Zulfikran Moh. Rizki Azis[2], Theresia Indah Budhy. S[3], Rebekah J. Setiabudi[4]

[1,2]Graduate Student of Immunology, Postgraduate School, Airlangga University

[3]Department of Oral and Maxillofacial Pathology, Faculty of Dental Medicine, Airlangga University

[4]Faculty of Medicine, Airlangga University

[1]e-mail: talitashofa@gmail.com

[2]e-mail: zulfikranzis99@gmail.com

[3]e-mail: terebudhy@gmail.com

[4]e-mail: rebekahsetiabudi@gmail.com

Abstract

M. tuberculosis is a bacterium that has many evasion mechanisms against the immune system, one of them is the formation of granulomas which is beneficial for the bacteria's survival. The granuloma structure is useful for limiting the spread of *M. tuberculosis* and localizing infection, also considered as part of *M. tuberculosis* life cycle that successful fighting the body's immune system. This study aims to look at the formation of an in vitro tuberculous granuloma model. This study used the True Experiment type which began with blood sampling, PBMC isolation, macrophage isolation, MOI 10 making and granulomas making. Granulomas were observed on day 0, 1, 4, 7, 9, 10 and 14. Cells started to aggress on day 1 and giant cells were seen on day 4. The granuloma formed on day 9 and was maintained on day 10, however, the granuloma ruptured on day 14 which caused the cells to re-aggregate.

Keywords: Granuloma, M. tuberculosis, PBMC

Abstrak

M. tuberculosis merupakan bakteri yang memiliki banyak mekanisme penghindaran terhadap sistem imun, salah satunya adalah pembentukan granuloma yang menguntungkan bagi kelangsungan hidup bakteri ini. Struktur granuloma bermanfaat untuk membatasi penyebaran dari M. tuberculosis dan melokalisir infeksi, tetapi juga dianggap sebagai bagian dari siklus hidup M. tuberculosis yang berhasil dalam melawan sistem imun tubuh. Penelitian ini bertujuan untuk melihat pembentukkan model granuloma tuberkulosis in vitro melalui isolasi PBMC darah orang yang sehat. Penelitian ini menggunakan jenis True Experiment yang diawali dengan pengambilan sampel, isolasi PBMC, isolasi makrofag, pembuatan MOI 10 dan pembuatan granuloma. Granuloma in vitro diamati pada hari ke-0,1, 4, 7, 9, 10 dan 14. Sel mulai beragregas di hari ke-1 dan giant cell terlihat pada hari ke-4. Granuloma terbentuk pada hari ke-9 dan granlom atersebut dapat dipertahankan di hari ke-10, namun, granuloma tersebut pecah di hari ke-14 yang menyebabkan sel beragregasi kembali.

Kata kunci: Granuloma, M. tuberculosis, PBMC

1. INTRODUCTION

Mycobacterium tuberculosis (*M. tuberculosis*), the causative agent of tuberculosis (TB), is a highly contagious human pathogen (Mohareer *et al*., 2018). One of the complications in solving TB disease is the formation of *M. tuberculosis* granulomas which can undergo reactivation and reinfection when the body system of someone who has been infected has decreased. People who have recovered from *M. tuberculosis* infection have a 5% chance of experiencing reactivation and reinfection.

DOI 10.20473/jbp.v25i1.2023.66-73

Granuloma is a pathological feature of TB which contains mostly macrophages, epithelioid cells (differentiated macrophages) and multinucleated giant cells and is surrounded by T lymphocytes. survive, modulate the immune response to ensure its long-term survival without damage (Silva Miranda et al., 2012).

Granulomas are organized structures that are dense and rich in immune cells such as macrophages and their differentiation products that surround *M. tuberculosis*, besides that there are other cells such as dendritic cells, neutrophils, Natural Killer (NK) cells, T and B lymphocyte cells (Eley and Beatty 2009). The granuloma structure is useful for limiting the spread of *M. tuberculosis* and localizing infection, but it is also considered as part of the life cycle of *M. tuberculosis* which is successful in fighting the body's immune system. M. tuberculosis can trigger an immune response through the secretion of IFN-γ and TNF-α to form granuloma protective immunity, which is useful in controlling the growth and elimination of *M. tuberculosis.* The granuloma will become homeostatic when the immune system in the granuloma is balanced between pro-inflammatory and anti-inflammatory cytokines, which causes *M. tuberculosis* to become inactive and move towards dormancy by adjusting its metabolism in the granuloma environment. However, when there is an imbalance between pro-inflammatory and anti-inflammatory cytokines, the granuloma will undergo necrosis and cause the development and spread of *M. tuberculosis* (Ehlers and Schaible, 2013).

Granulomas can be a safe place for *M. tuberculosis* to survive, because of *M. tuberculosis*'s ability to evade the immune system, causing *M. tuberculosis* to remain alive in a dormant state and can be active again if the immune system decreases (Redeker and Arens 2016; Setiati et al. ., 2014). Granulomas are also considered as a mechanism for avoiding *M. tuberculosis* from the immune system, in addition to inhibiting the work of macrophages through the virulence factor ESX-1 which prevents the formation of phagolysosomes (Ndlovu and Marakalala, 2016; Chai et al., 2020). *M. tuberculosis* also survives in the granuloma by secreting virulence factors in the form of PtPA and PtPB enzymes which play a role in inhibiting phagosome acidification, inhibiting macrophage apoptosis and suppressing proinflammatory cytokines (Chaurasiya and Srivastava, 2009; Zhou et al., 2010; Fan et al., 2018 ; Chai et al., 2020).

This study aims to look at the formation of an in vitro tuberculous granuloma model by isolating PBMCs from the blood of healthy people.

2. RESEARCH METHOD

2.1 Research Design

This study uses the True Experiment type with the technique of taking the research object, namely random allocation. The inclusion criteria were men with an age range of 22-26 years. Meanwhile, the exclusion criteria in this study were experiencing severe stress, suffering from infectious diseases,

taking steroid drugs, consuming alcohol, being an active smoker and not being willing to be a respondent. The location of this research was the Stem Cell Research and Development Center Lab, Airlangga University and the Tuberculosis Laboratory (Institue of Tropical Disease), Airlangga University.

2.2 Sampling

The sample used was 20 ml of venous blood with Lithium heparin anticoagulant using a vacutainer tube.

2.3 PBMC Isolation

10 ml of blood was taken from the donor using a heparin tube, diluted using sterile PBS (1:1 ratio), then homogenized and put into a 50 ml sterile polystyrene tube which had been filled with 10 ml of Ficoll density gradient solution. The sample was centrifuged at 1200 g for 20 minutes at room temperature and the buffy coat layer formed was taken, then washed 3 times, using 10 ml PBS and centrifuged for 10 minutes at 200 g speed. Red blood cells were lysed using lysis solution after washing, then 10 ml of complete RPMI 1640 was added after the third wash to resuspend the cell precipitate at the bottom of the tube.

2.4 Macrophage Isolation

The PBMC isolated cells were put into a 5 cm petri dish, then incubated in 5% CO_2 at 37ºC for 24 hours, then washed the cells after incubation with RPMI. Cells were harvested after 15 minutes of incubation in trypsin, then scraped using a scaper cell and then put into a 15 ml centrifuge tube and centrifuged 250 g for 10 minutes at 4ºC. After that the supernatant was discarded. Harvested cells were resuspended with 1% Fetal calf serum and 5 ml maturation media containing (full RPMI and 10 ng/ml GM-CSF), and incubated for 5 days. Every 2-3 days the differentiation medium is replaced with a new one. On the 5th day, the cells had differentiated into macrophages and attached to the plastic, so 5 ml of cold PBS was added to release them. The petri dish containing the cells was placed on ice for at least 30 minutes. Cells were scraped using scaper cells, after which the cells were centrifuged 250 g for 5 minutes and resuspended with RPMI 5% FCS as much as 5 ml.

2.5 The Making of MOI 10

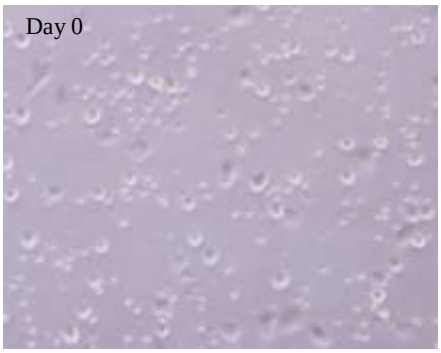
The isolated macrophages were put into Well 12 which had been given a cover slip, then added complete RPMI media, then M. tuberculosis strain H73Rv 10:1 with the number of macrophage cells and incubated at 37ºC CO2 5% for 24 hours. Infected macrophages are used for granuloma creation.

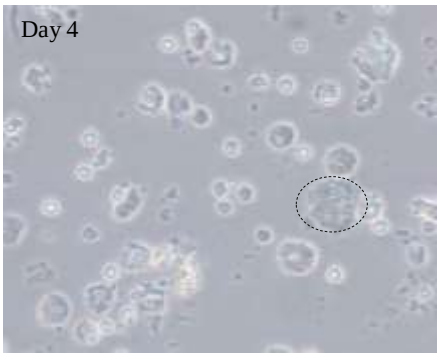
2.6 The Making of Granuloma

PBMC was added to macrophages that had been infected with MOI 10 with a ratio of 1:5 and incubated with 37°C CO2 5% for 45 minutes, then 20% RPMI was added to the well and incubated at 37°C CO2 and the media was replaced once every 3 days. Cells were observed on days 0, 1,

4, 7, 9, 10 and 14 using an inverted microscope.

3. RESULT AND DISCUSSION





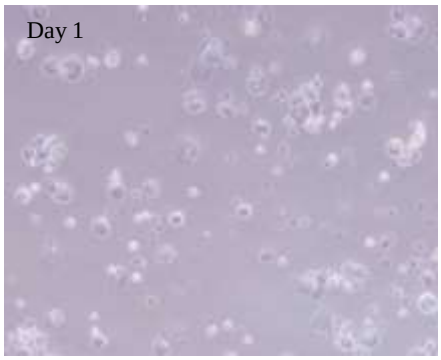


Figure 1. Granuloma in vitro day 0, 1, 4 and 7.

Observation of granuloma in vitro on day 0 shows lymphocytes marked by black arrows and macrophages marked by red arrows. These lymphocytes and macrophages were also seen in the observation of granulomas on day 1, 4

and 7. Aggressive cells on day 1 are indicated by red circles. These aggregated cells will continue to develop into giant cells which begin to appear on observation of granulomas on the 4th day (marked by a black circle). On the 7th day of observation, you can see the number of aggregating cells (marked by a red circle) and giant cells (marked by a black circle).

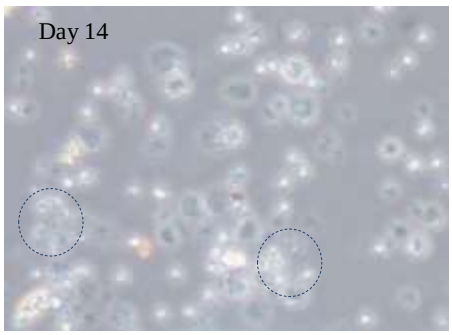


Figure 2. Granuloma in vitro day 9, 10 and 14.

Observation of granuloma in vitro on day 9 showed giant cells which were marked by a black circle, the size was bigger than the observation on day 7 and granulomas were formed on day 10 (marked by a red circle). On the 14th day of observation of granulomas in vitro, the cells burst and reaggregated which were marked by a blue circle.

During the early infection phase, the interaction between macrophages and *M. tuberculosis* has a negative effect on the host, which dampens the immune response and leads to increased *M. tuberculosis* survival (Sholeye *et al*., 2022). On the 0 day of observing granulomas in vitro, the PBMC media which contained lymphocytes and monocytes had not responded to *M. tuberculosis* infection so that granulomas had not formed. Initial aggregation of macrophages occurs in response to a persistent stimulus triggered by a granulomatous reaction in their nuclei. Cells began to aggregate as seen in the formation of granulomas on day 1, and cell aggregation was increasingly seen on day 4 and day 7. These aggregated cells are dominated by macrophages. Macrophages differ in the nucleus of the granuloma. Macrophages undergo a series of distinguishable morphological changes that demonstrate their immunometabolic characteristics, most importantly, differentiation of epithelioid cells (Sholeye *et al*., 2022).

Macrophages are central to granuloma formation. Macrophages comprise the bulk of the granuloma cell population and form the inner lining of the granuloma, serving as the central scaffold where the rest of the cell population is nucleated. While the macrophage population is highly motile under normal physiological conditions or infection, during granuloma formation, these cells undergo a morphological differentiation called 'epithelioid differentiation' where they interlock and aggregate with their neighbors to form granulomas. These epithelioid macrophages are a central characteristic of tuberculous granulomas, but they are joined to other macrophage populations, including conventional macrophages, lipid-laden

foamy macrophages and giant cell multinucleate macrophages. Outside the macrophage population, granulomas are characterized by a more widespread immune response that recruits many other cell types to the granuloma. Cell populations recruited to granulomas include myeloid populations such as neutrophils, dendritic cells, eosinophils, and mast cells, lymphocyte populations including T cells, B cells, NK cells and ILC and nonhematopoietic cells such as fibroblasts, endothelial and epithelial cells (Cronan, 2022).

Furthermore, the polarization of macrophages into M1 and M2 types is finely regulated by the host for the purpose of managing chronic infection, thereby regulating the promotion and formation of granulomas (Sholeye *et al.*, 2022). The pro-inflammatory response, promoted by M1 macrophages, bridges the innate and adaptive immune responses to infection. This functionality is considered the cornerstone of effective host defense. At the same time, M2 macrophages promote anti-inflammatory response, which is critical for immune regulation, also prevents chronic inflammatory states from worsening, and simultaneously promotes maintenance of tissue homeostasis (Abbas *et al*., 2016).

Granulomas have formed on the 9th day and the peak of granuloma formation occurred on the 10th day. Granuloma is a hallmark of *M. tuberculosis* infection and immunopathogenesis occurs in Tb in the early stages of latently infected patients. The granuloma structure is clustered, organized in aggregate cells consisting of various innate immune cells and adaptive immune cells including macrophages, foamy macrophages (derivatives of macrophage cells that proliferate in response to infection with *M. tuberculosis* bacteria), epithelial cells, multinucleate giant cells (cells Langerhans) is surrounded by a ring of lymphocytes (Genoula *et al*., 2018). After the 10th day the granuloma granulates (ruptures). The main function of the granuloma is localization and containment of *M. tuberculosis* and concentrating the protection of the immune response to a limited area infected with TB (Peddireddy *et al*., 2017). In the observation picture of the granuloma on the 14th day the granuloma structure breaks and aggregation occurs again.

4. CONCLUSION

Cells began to aggregation on day 1 and aggregation increased on day 4 and day 7. Granulomas had formed on the 9th day and the peak of the granulomas occurred on the 10th day. The granuloma granulated (ruptured) over the 10th day or in this study the cells re-aggregated again on the 14th day.

ACKNOWLEDGEMENT

A deep gratitude to Stem Cell Research and Development Center Lab, Airlangga University and the Tuberculosis Laboratory (Institue of Tropical Disease), Airlangga

University. Also a great appreciation to all researchers who participated, and thank you to all those who supported the author.

REFERENCES

Abbas, A. K., Lichtman, A. H., dan Pillai, S. 2016. Imunologi Dasar Abbas; Fungsi dan Kelainan Sistem Imun. Edisi 5. Elsevier. Killiney Road. Singapore.

Chai, Q., Wang, Lin., Hua, C. L., and Baoxue, G. 2020. New Insights into the Evasion of Host Innate Immunity by Mycobacterium Tuberculosis. Cellular and Molecular Immunology 17(9):901–13.

Chaurasiya, S.K. and Srivastava, K.K. 2009. 'Downregulation of protein kinase C- αenhances intracellular survival of Mycobacteria: Role of PknG', *BMC Microbiology*, 9, pp. 1–14.

Cronan, Mark. 2022. In the Thick of It: Formation of the Tuberculous Granuloma and Its Effects on Host and Therapeutic Responses. Frontier in Immnulogy Vol. 13.

Ehlers, S., and Schaible, U. E. 2013. The granuloma in tuberculosis: dynamics of a host–pathogen collusion. Frontiers in Immunology. Frontiers in Immunology. 3(411): 1-9.

Eley, B. S., and Beatty, D. W., 2009. Tuberculosis in *The basic immunology of Tuberculosis*. Elsevier Inc. 1:75–86.

Fan, L. *et al.* 2018. 'MptpB promotes mycobacteria survival by inhibiting the expression of inflammatory mediators and cell apoptosis in macrophages', *Frontiers in Cellular and Infection Microbiology*, 1–10.

Genoula, M., Franco, J. L. M., Dupont, M., Kviatcovsky, D., Milillo, A., *et al*. 2018. Formation of foamy macrophages by tuberculous pleural effusions is triggered by the interleukin- 10/signal transducer and activator of transcription 3 axis through ACAT upregulation. Frontiers in Immunology.

Mohareer, K., Suman, A., and Sharmistha, B. 2018. Cell Death at the Cross Roads of Host-Pathogen Interaction in Mycobacterium Tuberculosis Infection. Elsevier. 113(1):99–121.

Ndlovu, H., and Marakalala, M. J. 2016. Granulomas and Inflammation: Host-Directed Therapies for Tuberculosis. Frontiers in Immunology 7(1).

Peddireddy, V., Doddam, S. N., & Ahmed, N. 2017. Mycobacterial dormancy systems and host responses in tuberculosis. Frontiers in Immunology 1–19.

Redeker, A., and Ramon, A. 2016. Granulomas and Inflammation : Host-Directed Therapies for Tuberculosis. Frontiers in Immunology 7:1–11.

Setiati, S., Alwi, I., A. W., Sudoyo, M., Simadibrata, B., Setyohadi, and Syam, A. F. 2014. Buku Ajar Ilmu Penyakit Dalam, Edisi Enam Jilid *2*. 6th ed. Jakarta.

Silva Miranda, M. *et al.* 2012. 'The tuberculous granuloma: An unsuccessful host defence

mechanism providing a safety shelter for the bacteria?', *Clinical and Developmental Immunology*, 2012.

Sholeye, Abisola Regina., Aurelia A. Williams., Du Toit Loots., A. Marceline Tutu van Furth., Martijn van der Kuip., *et al*. 2022. Tuberculous Granuloma: Emerging Insights From Proteomics and Metabolomics. Frontiers in Neurology Vol. 13.

Zhou, B. *et al.* 2010. 'Targeting mycobacterium protein tyrosine phosphatase B for antituberculosis agents', *Proceedings of the National Academy of Sciences of the United States of America*, 107(10), 4573–4578.