Jurnal Riset Kesehatan Poltekkes Depkes Bandung

Vol 18 No 2, Oktober 2026
e-ISSN: 2579-8103
p-ISSN:1979-8253

Effect of macrophage number on tumor necrosis factor-α levels in a tuberculous granuloma model: an in vitro study

Pengaruh Dosis Makrofag terhadap Kadar Tumor Necrosis Factor-α dalam Model Granuloma Tuberkulus: Studi In Vitro

Laila Kamilla[1], Ari Nuswantoro [1,2*], Bagus Muhammad Ihsan[1], Syopingi Syopingi[1]
[1]Department of Medical Laboratory Technology, Poltekkes Kemenkes Pontianak, Pontianak, Indonesia
[2]Doctoral Program of Medical Science, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
*Email: arinuswantoro82@gmail.com

ABSTRACT

Background: *Tumor necrosis factor-alpha (TNF-α) is a key cytokine secreted by macrophages in the formation and integrity of tuberculous granulomas.*
Objective: *This study aimed to analyze the influence of macrophage number variation on TNF-α levels in an in vitro tuberculous granuloma model.*
Methods: *The granuloma model was constructed by co-culturing peripheral blood mononuclear cells (PBMC) from a healthy donor with autologous macrophages at numbers of 1x10⁵, 2x10⁵, and 3x10⁵ cells/well, followed by infection with Mycobacterium tuberculosis H37Rv. Supernatants were collected up to the 5th day of incubation, and TNF-α levels were measured by ELISA. Data were analyzed using statistical tests.*
Results: *TNF-α levels varied between the number groups. The lowest mean TNF-α level was found in the 1x10⁵ cells/well macrophage number group (0.013 pg/mL). Results from the ANOVA and post-hoc tests indicated a significant difference within the groups (p=0.009) and between specific groups: between the 1x10⁵ cells/well macrophage group and the control (p=0.026), the 2x10⁵ cells/well group (p=0.035), and the 3x10⁵ cells/well group (p=0.033) at a 0.05 significance level.*
Conclusion: *Macrophage number affects TNF-α levels in the in vitro tuberculous granuloma model in a non-linear manner, suggesting threshold-dependent immune regulation. These findings are confined to the in vitro context and require further validation.*

Keywords: *granuloma, in vitro model, macrophage, Mycobacterium tuberculosis, TNF-α*

ABSTRAK

Latar Belakang: Tumor necrosis factor-alpha (TNF-α) merupakan sitokin kunci dalam pembentukan dan integritas granuloma tuberkulosis yang disekresi oleh makrofag.
Tujuan: Penelitian ini bertujuan untuk menganalisis pengaruh variasi dosis makrofag terhadap kadar TNF-α dalam model granuloma tuberkulosis in vitro.
Metode: Model granuloma dibangun dengan mengko-kulturkan *peripheral blood mononuclear cells* (PBMC) dari donor sehat dengan makrofag autolog pada dosis 1x10⁵, 2x10⁵, dan 3x10⁵ sel/sumur, kemudian diinfeksi *Mycobacterium tuberculosis* H37Rv. Supernatan diambil pada hari inkubasi ke- 5, serta kadar TNF-α diukur dengan ELISA dan data dianalisis dengan uji statistik.
Hasil: Kadar TNF-α bervariasi antar kelompok dosis. Rerata kadar TNF-α terendah terdapat pada kelompok dosis makrofag 1x10⁵ sel/sumur (0,013 pg/mL). Hasil uji ANOVA dan *post-hoc* menunjukkan adanya perbedaan dalam kelompok (p=0,009) dan antar kelompok, yaitu antara kelompok dosis makrofag 1x10⁵ sel/sumur dengan kontrol (p=0,026), kelompok 2x10⁵ sel/sumur (p=0,035), dan kelompok 3x10⁵ sel/sumur (p=0,033) pada taraf signifikansi 0,05.

https://doi.org/10.34011/juriskesbdg.v18i1.3265

Vol 18 No 2, Oktober 2026
e-ISSN: 2579-8103
p-ISSN:1979-8253

Kesimpulan: Dosis makrofag memengaruhi kadar TNF-α dalam model granuloma tuberkulosis in vitro, namun hubungannya bersifat tidak linear. Hal ini menunjukkan adanya mekanisme regulasi kompleks dan ambang batas dalam aktivasi imun. Temuan ini memperkaya pemahaman tentang dinamika sitokin awal pada patogenesis tuberkulosis.

***Kata kunci**: granuloma, makrofag, model in vitro, Mycobacterium tuberculosis, TNF-a*

INTRODUCTION

Tuberculosis (TB) is a chronic disease caused by *Mycobacterium tuberculosis* (*M. tuberculosis*).[1] It is the second leading cause of death from infectious disease worldwide, after COVID-19. In 2021, there were 10.6 million people with active TB and 1.6 million deaths.[2] Among those infected with *M. tuberculosis*, some will develop active TB and become symptomatic, while others (approximately 23%) develop latent tuberculosis infection, and 5–10% of these latent TB cases will progress to active TB.[3]

The main immune pathogenesis of TB involves the formation of granulomas around the site of infection. These immune cell-containing structures aim to contain *M. tuberculosis* bacteria, preventing them from spreading to broader tissues and worsening the disease.[4–6] One key component of the granuloma is the macrophage.[7,8] Macrophages play a role in maintaining granuloma integrity by internalizing *M. tuberculosis* and recruiting other macrophages through the secretion of tumor necrosis factor-alpha (TNF-α).[9–12]

TNF-α is a pleiotropic pro-inflammatory cytokine that plays an essential role in resistance to *M. tuberculosis* by mediating granuloma integrity through the regulation of cell adhesion proteins, chemokine attraction, preventing granuloma disintegration, and modulating inflammation by regulating T helper cells (CD4+) and cytotoxic T cells (CD8+) that produce interferon. Another mechanism involves promoting apoptosis of mycobacteria-containing macrophages, thereby preventing intercellular bacterial spread.[13–16]

Previous studies have extensively characterized the critical role of TNF-α in granuloma formation and maintenance, demonstrating that TNF-α blockade disrupts granuloma integrity, reduces multinucleated giant cell formation, and downregulates Th1, Th17, and regulatory T cell cytokine responses in in vitro PBMC-based granuloma models.[17,18] Macrophages are known to express a TNF-regulated recombinatorial immune receptor (TCRαβ) implicated in granuloma formation, and TNF produced by these cells is essential for macrophage recruitment and activation at the infection site.[19]

Despite this knowledge, existing studies have largely focused on qualitative aspects of TNF-α function—such as the effects of complete TNF blockade or the presence versus absence of TNF signaling—rather than systematically investigating how quantitative variations in macrophage numbers might influence TNF-α secretion dynamics. Furthermore, no previous research has systematically examined how variations in macrophage number influence TNF-α production in a PBMC-based granuloma system at a defined post-infection time point. The novelty of this study lies in its systematic evaluation of the relationship between macrophage number and TNF-α secretion at day 5 post-infection using a controlled in vitro model that isolates the effect of macrophage number while maintaining other cellular and bacterial parameters constant.

This approach provides a quantitative framework for understanding whether macrophage recruitment operates under threshold-dependent regulatory mechanisms or exhibits linear amplification of pro-inflammatory signals. The scientific contribution of this research is twofold: first, it offers empirical evidence on the efficiency of macrophage-mediated TNF-α secretion in relation to cell density, and second, it provides a foundational reference for optimizing cellular compositions in future in vitro granuloma

https://doi.org/10.34011/juriskesbdg.v18i1.3265

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e-ISSN: 2579-8103
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models, ultimately contributing to the development of more physiologically relevant experimental systems for tuberculosis research. Therefore, this study aimed to evaluate the effect of macrophage number variations on TNF-α secretion dynamics in an in vitro PBMC-based tuberculous granuloma model infected with *M. tuberculosis* H37Rv.

METHODS

Study design

This study employed a true experimental laboratory design with a post-test-only control group. The independent variable was macrophage number, operationalized as varying cell numbers (1×10^5, 2×10^5, and 3×10^5 cells/well) seeded into the granuloma culture system, with a control group receiving no additional macrophages. Group assignment was performed by culture plate layout; no randomization or blinding was performed. The dependent variable was TNF-α level, measured in the culture supernatant following granuloma formation and infection with *M. tuberculosis* H37Rv using enzyme-linked immunosorbent assay (ELISA). This study was conducted from January to February 2026 at the laboratory of the Department of Medical Laboratory Technology, Poltekkes Kemenkes Pontianak.

Data source and sampling procedure

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood of a single healthy donor by density-gradient centrifugation using Ficoll-Paque™ PLUS. Monocytes were then enriched from the PBMC fraction by plastic adherence and differentiated into macrophages by culturing in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and macrophage colony-stimulating factor (M-CSF) for 7 days, with medium replacement every 2–3 days. Adherent macrophages were harvested using a cell scraper, washed, and counted before seeding. The donor was a 25-year-old male, not suffering from active TB, physically and mentally healthy, and willing to become a volunteer.

Variables of the study

This study designed an in vitro granuloma model treated with the addition of macrophages at 1×10^5 (Group 1), 2×10^5 (Group 2), and 3×10^5 (Group 3) cells/well, and TNF-α secretion was observed after 5 days. The number of replications in this study was set at 6 times[20] and 2 times for the control, so the total number of samples including the control was 20 replications. Since this study uses a single healthy donor, the replicates (n=6) represent technical replicates of the same biological condition, rather than independent biological replicates.

Data collection

Data were collected from the measurement of TNF-α levels in the supernatant after 5 days post-infection using the ELISA method.

Measurement and instruments

PBMCs were obtained from donor blood as follows: (1) fresh anticoagulated blood was mixed with 1x phosphate-buffered saline (PBS) (Gibco) at a 1:1 ratio in a 50 mL conical tube and homogenized; (2) Ficoll-paqueTM PLUS (GE Healthcare) was added to the mixture at a 1:2 ratio in another conical tube; (3) the mixture was centrifuged at 3000 rpm for 30 minutes, forming 4 sequential layers from the top: plasma, buffy coat, PBMCs, and erythrocytes; (4) the PBMC supernatant was separated from the pellet and washed 3 times, each time with 1x PBS at a 1:1 ratio, followed by centrifugation at 3000 rpm for 5 minutes; and (5) PBMCs were separated and their concentration was calculated by adding 0.4% trypan blue (Bio-Rad) at a 1:1 ratio and then counting using an improved Neubauer counting chamber.

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Vol 18 No 2, Oktober 2026
e-ISSN: 2579-8103
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Macrophages were obtained by differentiation of monocytes isolated from the PBMC fraction. Briefly: (1) PBMCs were resuspended in RPMI 1640 medium (Gibco) supplemented with 10% FBS and seeded into a petri dish; (2) after incubation at 37°C with 5% CO_2 for 3 hours, non-adherent cells were removed by washing with warm RPMI 1640, leaving monocytes adhered to the dish; (3) adherent monocytes were cultured in RPMI 1640 medium containing 10% FBS and M-CSF for 7 days to induce differentiation into macrophages, with medium replacement every 2–3 days; (4) differentiated macrophages were detached using a cell scraper, transferred to a 15 mL conical tube, and washed three times with RPMI 1640 by centrifugation at 3000 rpm for 5 minutes; and (5) the macrophage concentration was calculated by adding 0.4% trypan blue (Bio-Rad) at a 1:1 ratio and counting using an improved Neubauer counting chamber.

This study used *M. tuberculosis* H37Rv isolate obtained from the Microbiology Laboratory, Institute of Tropical Disease, Universitas Airlangga, Surabaya. The bacteria were incubated at 37°C with 5% CO_2 and prepared in a tube at a concentration of 1×10^5 CFU/mL before being infected into petri dishes containing PBMCs and macrophages.

The procedure for constructing the in vitro granuloma model in this study was as follows: (1) a 24-well plate was prepared; (2) 4 rows of wells were used representing treatment groups: 1×10^5, 2×10^5, and 3×10^5 cells/well, each consisting of 6 replications; (3) 2×10^5 cells/well of PBMCs were added to each well used; (4) macrophages were added to the treatment rows to achieve final seeding densities of 1×10^5 cells/well (Group 1), 2×10^5 cells/well (Group 2), and 3×10^5 cells/well (Group 3), while the control row received no additional macrophages; (5) all wells were infected with *M. tuberculosis* H37Rv at 1×10^5 CFU/mL; (6) the plate was incubated at 37°C with 5% CO_2 for 5 days; (7) the supernatant was separated by centrifugation; and (8) TNF-α levels were measured using the ELISA method. The multiplicity of infection (MOI) ranged from 0.2 to 0.5 across the treatment series, and was 0.5 for the control series, calculated as the ratio of *M. tuberculosis* H37Rv CFU to total immune cells per well.

At 5 days post-infection, the suspension from all wells was harvested and then centrifuged at 10,000 rpm for 5 minutes to obtain the supernatant, which was then analyzed using the ELISA method (Elabscience, catalog number E-EL-H0109) at 450 nm according to the manufacturer's instructions. According to the manufacturer's specifications, the kit has a sensitivity of 4.69 pg/mL and a detection range of 7.81–500 pg/mL.[21] TNF-α concentrations were estimated from the absorbance readings using the calibration curve generated from the supplied standards. The concentration estimates were interpreted in relation to the assay's stated measurement range. Values estimated beyond the calibration range, particularly below its lower limit, cannot be assumed to represent reliably quantified concentrations and require appropriate analytical validation. The distinction between detection sensitivity and the validated quantification range was considered when interpreting the results.

Ethical considerations

This study was conducted at the laboratory of the Department of Medical Laboratory Technology, Poltekkes Kemenkes Pontianak, and obtained ethical approval from the Health Research Ethics Commission of the Poltekkes Kemenkes Pontianak, Number 531/KEPK-PK.PKP/XI/2025 dated November 10th, 2025. The blood donor was fully informed about the purpose and procedures of the study and provided written informed consent prior to blood collection.

Data analysis

Data were analyzed using descriptive and inferential statistical methods. Descriptive statistics, including mean and standard deviation (SD), were calculated for each group.

https://doi.org/10.34011/juriskesbdg.v18i1.3265

Vol 18 No 2, Oktober 2026
e-ISSN: 2579-8103
p-ISSN:1979-8253

The Shapiro-Wilk test was used to assess the normality of data distribution. Homogeneity of variances was evaluated using Levene's test. One-way analysis of variance (ANOVA) was performed to compare TNF-α levels among the four groups, followed by Tukey's post-hoc test for multiple comparisons to identify which specific groups differed significantly. The data analyzed consisted of TNF-α levels derived from the conversion of optical density (OD) values obtained via ELISA. A p-value of less than 0.05 was considered statistically significant.

RESULT

TNF-α levels in the supernatant of in vitro granuloma model cultures infected with *M. tuberculosis* H37Rv were measured after 5 days of incubation. Measurements were performed on treatment groups with varying number of autologous macrophages (1×10^5, 2×10^5, and 3×10^5 cells/well) and a control group (without macrophages). The lowest macrophage number induced a marked reduction in TNF-α secretion, whereas increasing macrophage numbers beyond 2×10^5 cells/well did not further enhance cytokine production (Figure 1).

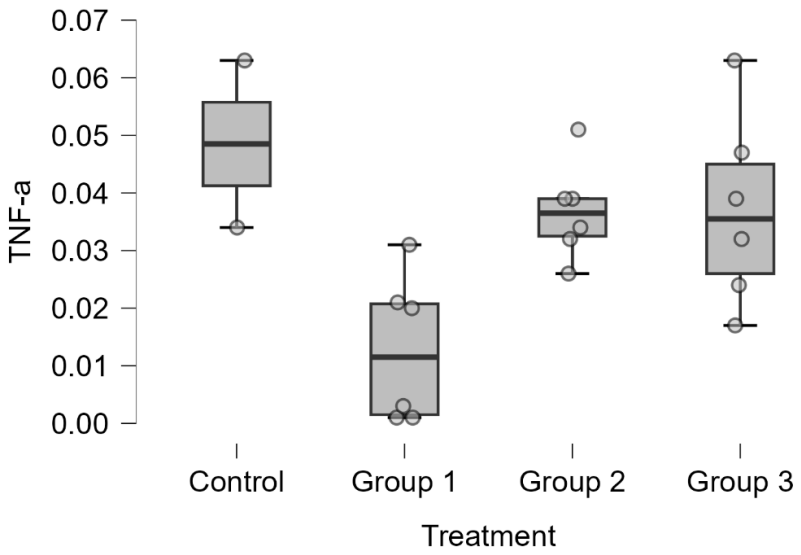


Figure 1. TNF-α Levels (pg/mL) Based on Macrophage number Variations in an In Vitro Tuberculosis Granuloma Model

Table 1 shows that the highest mean TNF-α level was observed in the control group (0.049 ± 0.021 pg/mL), followed by Group 2 (0.037 ± 0.008 pg/mL) and Group 3 (0.037 ± 0.017 pg/mL), while the lowest was in Group 1 (0.013 ± 0.013 pg/mL). The Shapiro-Wilk test indicated normal data distribution for all groups ($p > 0.05$), and Levene's test confirmed homogeneity of variances ($p = 0.249$). One-way ANOVA revealed a significant difference in TNF-α levels among groups ($F = 5.395$, $p = 0.009$). Tukey's post-hoc test showed that Group 1 had significantly lower TNF-α levels compared to the control group ($p = 0.026$), Group 2 ($p = 0.035$), and Group 3 ($p = 0.033$). No significant differences were found between the control group and Group 2 or Group 3, nor between Group 2 and Group 3 ($p > 0.05$).

https://doi.org/10.34011/juriskesbdg.v18i1.3265

Vol 18 No 2, Oktober 2026
e-ISSN: 2579-8103
p-ISSN:1979-8253

Table 1. Descriptive and Inferential Statistical Analysis of TNF-α Levels (pg/mL) Based on Macrophage number Variations

Descriptive Statistics				
	TNF-α Levels (pg/mL)			
	Control	Group 1	Group 2	Group 3
Median	0.049	0.012	0.037	0.036
Mean	0.049	0.013	0.037	0.037
Std. Deviation	0.021	0.013	0.008	0.017
Shapiro-Wilk	-	0.845	0.952	0.978
P-value of Shapiro-Wilk	-	0.144	0.755	0.939
Minimum	0.034	0.001	0.026	0.017
Maximum	0.063	0.031	0.051	0.063

Test for Equality of Variances (Levene's)			
F	df1	df2	p
1,512	3.000	16.00	0.249

ANOVA - TNF-a					
Cases	Sum of Squares	df	Mean Square	F	p
Treatment	0.003	3	0.001	5.395	0,009
Residuals	0.003	16	1.861×10^{-4}		

Post Hoc Comparisons - Treatment (Tukey)				
	Control	Group 1	Group 2	Group 3
Control		0.026	0.725	0.733
Group 1	0.026		0.035	0.033
Group 2	0.725	0.035		1.000
Group 3	0.733	0.033	1.000	

The interpretation of TNF-α measurements requires consideration of the analytical range of the ELISA assay. Because the concentrations reported in the original analysis were below the manufacturer's stated detection range, the reliability of the absolute concentration estimates could not be established from the reported results alone. Accordingly, the observed differences between experimental groups should be interpreted cautiously and cannot independently establish a quantitative relationship between macrophage number and TNF-α concentration.

DISCUSSION

Based on the results of this study, several key findings provide deep insights into the immunopathogenesis of tuberculosis, particularly the role of macrophages and TNF-α in the context of granuloma formation.

Non-Linear Relationship between Macrophage number and TNF-α Levels

The finding that the addition of macrophages at the lowest number (1×10^5 cells/well) resulted in significantly lower TNF-α levels compared to both the control group and groups with higher macrophage numbers indicates the presence of a complex, non-linear

https://doi.org/10.34011/juriskesbdg.v18i1.3265

Vol 18 No 2, Oktober 2026
e-ISSN: 2579-8103
p-ISSN:1979-8253

regulatory mechanism. This phenomenon aligns with the concept of immune efficiency, in which cells infected with a pathogen can modulate cytokine responses to avoid excessive inflammatory damage to host tissues while maintaining a sufficient response to control infection. Zhang et al. (2021) explained how innate immune responses are precisely activated not only to efficiently eliminate pathogens but also to avoid excessive inflammation and tissue damage. Frank et al. (2020) emphasized that an imbalance in NK and T cell responses can lead to two outcomes: excessive inflammation and tissue damage, or inadequate antiviral function and uncontrolled infection. Aguilera et al. (2020) characterized the pulmonary inflammatory response as a double-edged sword: recruitment of immune effectors is essential for eliminating bacteria and virus-infected cells, but inflammatory cytokines drive changes conducive to increased pathogen replication.[22–24] However, further validation using more sensitive kits (e.g., ultra-sensitive ELISA) is required to confirm this non-linear relationship.

Modulation of TNF-α production by *M. tuberculosis*

The finding that TNF-α production did not increase proportionally with macrophage number raises the question of whether *M. tuberculosis* may actively suppress cytokine output in a cell-density-dependent manner. It is well established that *M. tuberculosis* possesses multiple mechanisms to manipulate host immune signaling. For example, the major virulence factor ESAT-6 has been shown to modulate macrophage inflammatory responses through various pathways, including the regulation of TNF-α expression via the lncNEAT1/miR-125b-5p axis,[25] and to drive macrophage polarization from a pro-inflammatory M1 phenotype toward an anti-inflammatory M2 phenotype at later stages of infection.[26] While these findings demonstrate that *M. tuberculosis* can actively influence macrophage function, our study did not directly assess the involvement of ESAT-6 or NF-κB signaling in the observed TNF-α patterns. One possible explanation for our results is that at lower macrophage densities, the bacterium's modulatory effect on each individual cell may be relatively more pronounced—potentially through ESAT-6-mediated pathways, NF-κB inhibition, polarization shifts, or other mechanisms—thereby limiting aggregate TNF-α production despite the presence of effector cells. This interpretation is, however, speculative and will require targeted experiments—such as using ESAT-6 knockout strains or NF-κB inhibitors—to determine whether the observed saturation and density-dependent effects are indeed associated with these specific bacterial virulence mechanisms. Alternatively, the observed pattern may also be explained by cell-cell contact-dependent regulatory mechanisms, limited receptor availability, or feedback inhibition of TNF-α synthesis that becomes more efficient at higher cell densities, independent of bacterial virulence factors. Further studies are needed to elucidate the precise molecular mechanisms underlying these findings.

The importance of balance over absolute cell number

The finding that TNF-α levels in the control group (containing only PBMCs, without exogenous macrophage addition) were comparable to those in the highest macrophage number groups (2×10^5 and 3×10^5 cells/well) is particularly noteworthy. Theoretically, increasing the number of key effector cells such as macrophages should augment cytokine production capacity.[27–29] However, this result—coupled with the significantly lower TNF-α levels observed in Group 1—suggests that granuloma formation and cytokine homeostasis are highly coordinated processes dependent on the balance among immune cell populations,[30–32] rather than solely on the absolute number of a single cell type.

The reduced TNF-α output in Group 1 likely reflects insufficient cellular cooperativity within the granuloma-like structure. TNF-α is essential for macrophage activation, chemokine production, and the recruitment of T cells to the site of infection.[33,34]

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Vol 18 No 2, Oktober 2026
e-ISSN: 2579-8103
p-ISSN:1979-8253

Experimental studies using cell-specific TNF deletion in mice have demonstrated that macrophage-derived TNF is critical for early control of *M. tuberculosis*, whereas T cell-derived TNF becomes essential for sustaining protection during chronic infection.[35] Thus, the diminished TNF-α in Group 1 suggests a suboptimal cellular composition that fails to establish the positive feedback loops between macrophages and T cells required for effective containment. This finding aligns with computational models predicting that multiple TNF-dependent functions—including macrophage activation and cellular recruitment—must act in synergy to achieve optimal granuloma performance.

The equivalence in TNF-α levels between the control group and the highest exogenous macrophage groups has two plausible and non-mutually exclusive explanations, both anchored in the biology of granuloma formation. First, the control group, which contained only PBMCs, still contained monocytes capable of differentiating into macrophages during the incubation period. Critically, monocytes are not passive precursors; their differentiation into macrophages can be actively modulated by *M. tuberculosis*.[36,37] This in situ differentiation, influenced by microenvironmental signals and mycobacterial products, may have generated a functionally sufficient macrophage population to maintain TNF-α levels comparable to those achieved with high-number exogenous addition. This highlights that the quality and activation status of macrophages—influenced by local signals and their differentiation trajectory—may be more determinative of cytokine output than their initial quantity. Second, the plateau in TNF-α at higher macrophage numbers suggests a saturation phenomenon, consistent with observations that TNF-α can suppress its own production and that of other protective cytokines at high concentrations, reflecting a negative feedback mechanism to limit immunopathology.[38] Excessive TNF-α production can lead to macrophage necrosis and tissue damage, thereby promoting bacterial dissemination.[33]

Together, these observations reinforce a model in which TNF-α acts as a key conductor of the cellular orchestra, but its effects are context-dependent. The cytokine response is not a simple linear function of cell number; rather, it reflects a dynamic balance between pro-inflammatory and regulatory signals. Multi-scale modeling studies have predicted that this balance—particularly between TNF-α and the anti-inflammatory cytokine IL-10—is essential for controlling bacterial growth while minimizing host tissue damage.[33] Our findings, though derived from an in vitro system, support this view by demonstrating that both the composition of the cellular inoculum and the potential for in situ differentiation contribute to the final TNF-α output. We acknowledge, however, that extrapolation to the in vivo setting is constrained by the use of a single donor and a limited cytokine panel; future studies incorporating broader immune components and more physiologically relevant models will be essential to validate these observations and explore the regulatory networks that govern this balance.

Threshold-dependent and saturated responses

The statistical results showing significant differences between groups but without a linear number effect lead to the understanding that TNF-α responses in granulomas are threshold-dependent and saturated. This means there is a minimum threshold of macrophage number or stimulation required to trigger adequate TNF-α production (as seen in the difference between Group 1 and the others). However, once this threshold is met, the addition of more macrophages does not necessarily further increase TNF-α production, indicating that the system has reached a saturation point. This concept is supported by cellular signaling theory, in which receptors on cells can undergo desensitization, or intracellular signaling pathways have maximum capacity.[39,40] Additionally, the availability of substrates or cellular energy for cytokine protein synthesis may also be a limiting factor.

https://doi.org/10.34011/juriskesbdg.v18i1.3265

Vol 18 No 2, Oktober 2026
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p-ISSN:1979-8253

Comparison with previous studies

The non-linear relationship observed in our study—where TNF-α levels did not increase proportionally with macrophage number—contrasts with the intuitive expectation that more macrophages would yield higher cytokine secretion and highlights the presence of regulatory mechanisms that may override simple number-dependent amplification. This finding can be contextualized within the broader literature on in vitro granuloma models. Agrawal et al. (2016) employed a human PBMC-based in vitro granuloma model infected with *M. tuberculosis* H37Rv at a low MOI of 0.005 and demonstrated a positive association between TNF-α secretion levels and the extent of granuloma formation, though they were unable to detect any statistically significant relationship between cytokine production and bacterial growth.[41] Similarly, Silva et al. (2018) showed that TNF-α blockade using Infliximab in an in vitro PBMC-based granuloma model significantly impaired granuloma structure and downmodulated Th1, Th17, and regulatory T cell cytokine responses, reinforcing the central role of TNF-α in granuloma integrity.[42] More recently, a study by the eLife group (2025) using a similar in vitro granuloma system demonstrated that CXCL9, granzyme B, and TNF-α secretion were associated with reduced mycobacterial growth across different *M. tuberculosis* complex lineages.[43] In a granuloma model for biomaterial testing, a two-step system based on macrophage seeding followed by fibroblast introduction showed that TNF-α induction resulted in granuloma-like formations, and the incorporation of fibroblasts decreased TNF-α secretion.[44] These studies consistently affirm the importance of TNF-α in granuloma formation and maintenance, yet none have systematically investigated how varying macrophage numbers quantitatively influence TNF-α secretion dynamics in a PBMC-based tuberculous granuloma system. Furthermore, previous research has focused on qualitative TNF-α function—such as the effects of complete TNF blockade[42] or the presence versus absence of TNF signaling—rather than examining number-dependent relationships between macrophage cell numbers and cytokine output. This gap underscores the necessity of our study, which specifically addresses the effect of macrophage number variations on TNF-α levels, an aspect that remains unexplored in the existing literature.

Clinical implications

These findings should be interpreted strictly within the context of an in vitro model. Nevertheless, they may inform future translational hypotheses: if TNF-α output is indeed threshold-dependent rather than linearly proportional to macrophage number, then therapeutic strategies aimed at merely increasing macrophage quantity might be less effective than those modulating macrophage functional quality (e.g., polarization toward a protective M1 phenotype). Such hypotheses would require validation in in vivo and clinical studies before any therapeutic recommendation can be made.

Limitations of the study

This study has several limitations that should be acknowledged. First, the in vitro model used, although valuable, cannot fully replicate the complexity of the in vivo tissue environment involving lung tissue, blood circulation, and interactions with other organ systems. Second, the use of a single donor limits generalizability and may not represent interindividual immune variability. Third, the number of replications was not equal between treatment groups (n=6) and the control group (n=2), which may affect statistical power for the control group.

Fourth, this study did not include an uninfected control group or a blank medium control, which limits the ability to differentiate TNF-α secretion specifically induced by *M. tuberculosis* infection from baseline cytokine production or assay background. While a PBMC-only control (no additional macrophages) was included, it was infected with

https://doi.org/10.34011/juriskesbdg.v18i1.3265

Vol 18 No 2, Oktober 2026
e-ISSN: 2579-8103
p-ISSN:1979-8253

bacteria and thus served as a comparative baseline for macrophage number rather than a true negative control. Additionally, the ELISA kit used has a detection range of 7.81–500 pg/mL, yet our measured values (0.013–0.049 pg/mL) fell below the lower limit of quantification. This may be explained by the low MOI and short culture duration used, or by genuine low TNF-α production in our model. Therefore, absolute concentrations should be reported as below the detection limit (<7.81 pg/mL) rather than as precise numerical values, and the results should be interpreted with caution.

Finally, the measurements focused only on TNF-α, whereas granuloma formation involves a network of other cytokines such as IFN-γ, IL-12, and IL-10 that interact with each other. Simultaneous multiparameter measurements would provide a more holistic picture. Moreover, macrophage isolation based on plastic adhesion may not capture the full diversity of macrophage subpopulations relevant in vivo. Future studies with more complex models, cells from multiple donors, and multi-omics analyses are needed to confirm and extend these findings.

CONCLUSION

This study does not provide sufficient analytical evidence to establish a reliable quantitative relationship between macrophage number and TNF-α concentration in the in vitro tuberculous granuloma model. The concentrations reported in the original analysis were below the manufacturer's stated detection range, limiting the interpretation of absolute concentrations and statistical comparisons. Therefore, the observed differences cannot be considered conclusive evidence of a macrophage-number-dependent TNF-α response. Further studies using an assay with an appropriate validated quantification range and independent biological replicates are required to determine this relationship reliably.

ACKNOWLEDGMENTS

The authors would like to express their sincere gratitude to the Department of Medical Laboratory Technology, Poltekkes Kemenkes Pontianak, for providing the laboratory facilities and support necessary to conduct this research. Special thanks are extended to the Laboratory of Tuberculosis, Institute of Tropical Disease, Universitas Airlangga, Surabaya, for supplying the *Mycobacterium tuberculosis* H37Rv isolate.

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