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Original Article

Sequential co-infection of *Heligmosomoides polygyrus* and *Mycobacterium tuberculosis* determine lung macrophage polarization

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ABSTRACT

Background: Tuberculosis is a chronic infection caused by *Mycobacterium tuberculosis* (M.tb), which needs proper macrophage activation for control. It has been debated whether the co-infection with helminth will affect the immune response to mycobacterial infection.

Objective: To determine the effect of sequential co-infection of *Heligmosomoides polygyrus* (H.pg) nematodes and M.tb on T cell responses, macrophages polarization and lung histopathological changes.

Method: This study used 49 mice divided into 7 treatment groups, with different sequence of infection of M.tb via inhalation and H.pg via oral ingestion for 8 and 16 weeks. T cells response in the lung, intestine, and peripheral blood were determined by flow cytometry. Cytokines (IL-4, IFN-γ, TGB-β1, and IL-10) were measured in peripheral blood using ELISA. Lung macrophage polarization were determined by the expression of iNOS (M1) or Arginase 1 (M2). Mycobacterial count were done in lung tissue. Lung histopathology were measured using Dorman's semiquantitative score assessing peribronchiolitis, perivasculitis, alveolitis, and granuloma formation.

Result: M.tb infection induced Th1 response and M1 macrophage polarization, while H.pg infection induced Th2 and M2 polarization. In sequential co-infection, the final polarization of macrophage was dictated by the sequence of co-infection. However, all groups with M.tb infection showed the same degree of mycobacterial count in lung tissues and lung tissue histopathological changes.

Conclusion: Sequential co-infection of H.pg and M.tb induces different T cell response which leads to different macrophage polarization in lung tissue. Helminth infection induced M2

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lung macrophage polarization, but did not cause different mycobacterial count nor lung histopathological changes.

1. Background

Tuberculosis (TB) is a chronic infection caused by *Mycobacterium tuberculosis* (*M.tb*). According to the World Health Organization (WHO) report in early 2018, it is estimated that 10 million individuals in the world suffer from TB infection, particularly in developing and low-income countries.[1,2] The high incidence of TB in most of those countries was usually associated with the high prevalence of helminth infection and low Bacillus Calmette-Guerin (BCG) vaccination effectiveness.[3,4] There have been debates about the impact of helminth infection on TB infection. Helminth infections has known to cause alteration in the immune response that harms the body's defenses against TB infection.[5–7] The debate about the effect of helminthic infection on the severity of TB needs to be resolved as soon as possible in order to make the right countermeasures. Thus, it will reduce the efforts and costs that have been spent on the prevention and treatment of TB.[1,8]

M.tb is a parasitic facultative intracellular bacillus.[9] The main immune response to eliminate TB is cellular immunity played by macrophages, $CD4^+$ T lymphocytes that secrete IFN-γ, $CD8^+$ T lymphocytes that eliminate mycobacteria-infected macrophages, as well as $\gamma\delta$ T lymphocytes.[10] This response requires a strong Th1 type cytokines. In contrast, helminth infections stimulate the activation of eosinophils, mast cells, basophils, and IgE formation, which are parts of Th2 type immune responses.[11,12] The dominant Th2 type immune response may counteract the Th1 type immune response through suppression by IL-4. Thus, theoretically, helminth infection can suppress the immune response to TB infection,[13] but many previous animal and human studies had yielded contradictory results.[5–7,14] These discrepancies might be due to the difference in mycobacteria strain and helminthes species that were used, the intervals of co-infection, or the duration of the infection.

To solve this problem, it is necessary to conduct a co-infection research of helminthes and tuberculosis sequentially. A study with sequential infection of *M.tb* with the standard model of mice nematode (*Heligmosomoides polygyrus*) was conducted. To ensure the chronicity of nematode infection an interval of at least 8 weeks is required before the mice co-infected with *M.tb*. Chronic nematode infection is recognized to trigger regulatory T cells (Tregs) response.[15,16] Tregs may affect the balance of Th1 and Th2 immune responses. The Th1 – Th2 balance will also affect macrophage function in overcoming the mycobacterial infection.[17,18] If it is proven that chronic infection of nematode stimulates the activity of Tregs that are capable of altering the balance of Th1 – Th2 type immune responses and macrophage functional activity, the debate about the effect of helminth infection on TB infection will be resolved. This study aimed to determine the effect of sequential co-infection of *H. polygyrus* (H.pg) nematodes and M.tb on T cell responses, macrophages activation and lung histopathological changes.

2. Method

2.1. Subjects and infectious agents

The subjects of this study were 8–12 week-old Balb/c male mice (*Mus musculus*) weighted 30–35 grams. All of the mice were purchased from PN Bio Farma (Persero) Bandung and put under pathogen-free environment according the Federation of European Laboratoy Animal Science Associations (FELASA) suggestion. For TB infection we used stock solution of H37Rv strain of M.tb obtained from Bacteriology Laboratory for Tuberculosis Infection, Institute of Tropical Disease, Universitas Airlangga. For nematode infection we used the stage 3 larvae of H.pg obtained from generous donation by Associate Professor Kenji Ishiwata, DVM, PhD, Department of Tropical Medicine, The Jikei University School of Medicine, Tokyo, Japan, with signed material transfer agreement and approved by National Institute of Health Research and Development, Indonesian Ministry of Health with decree No. LB.02.01/I.2/14311/2012.

2.2. Study design, allocation to groups of interventions, and ethics

This experimental study was done in the Department of Clinical Parasitology, Faculty of Medicine, Universitas Brawijaya, Malang, and the Bacteriology Laboratory for Tuberculosis Infection, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia. There were totally 49 mice which were randomly allocated into 7 groups of interventions, i.e.: (1) infected with *M.tb* for 8 weeks (M.tb-8), (2) infected with *M.tb* for 16 weeks (M.tb-16), (3) infected with *H.pg* for 8 weeks (H.pg-8), (4) infected with *H.pg* for 16 weeks (H.pg-16), (5) infected with *H.pg* for the first 8 weeks and then with *M.tb* for the next 8 weeks (H.pg+M.tb), (6) infected with *M.tb* for the first 8 weeks and then with *H.pg* for the next 8 weeks (M.tb+H.pg), and (7) control group without nematode and tuberculosis infection (Control). The ethical clearance for this study was obtained from Animal Care and Use Committee (ACUC) of Veterinary Faculty, Universitas Airlangga, Surabaya, Indonesia No. 151-KE.

2.3. Animal infection procedures

For tuberculosis infection, the mice were exposed to *M.tb* through inhalation using nose-only inhalation system i.e. modified Middlebrook Inhalation Exposure System (Glas-Col,

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Terre Haute, IN). Mice were exposed to 10 mL PBS-Tween 80 solution which contained 10^6 bacilli via aerosol nebulization for 30 minutes done in biosafety level 3 laboratory facility.[19] For nematode infection, the mice were inoculated orally using blunt-tipped gavage needle with 100 μL PBS solution containing 2000 L3/mL stage 3 larvae of *H.pg*.[19] The mice were then evaluated according to the groups of intervention procedure mentioned above. After 16 weeks, all the mice were sacrificed.

2.4. *Determination of T cell responses and lung macrophage activation*

Mice were sacrificed using injection of a mixture of Ketamine (100 mg/kg of body weight) and Xylazine (10 mg/kg of body weight) IM on their thigh muscles. Dissection and blood drainage was conducted from the right heart of the mice. The pulmonary veins were perfused with Saline-EDTA to eliminate all blood cells in the pulmonary intravascular.[20] The lung tissues were cut and minced into small pieces according to a protocol detailed elsewhere.[21] The jejunum and ileum tissue segments were taken and processed for the histopathology and immunohistochemical examination.[22] Th1, Th2, and Tregs responses in the peripheral blood serum, lung and intestinal tissues were analyzed using flow-cytometry technique with FACSCalibur using appropriate monoclonal antibodies (BD-Bioscience, Becton Dickinson, San Jose, CA, USA). Cytokines (IL-4, IFN-γ, TGB-β1, and IL-10) were measured in peripheral blood serum using ELISA according to manufacturer's protocol (Boster Immunoleader, CliniSciences, Nanterre, France). Macrophage activation were determined in lung tissue by the expression of iNOS (Thermo Scientific, Freemont, CA, USA) for classically activated macrophage M1, or by the expression of Arginase 1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for alternatively activated macrophage M2. All procedures were done according to the manufacturer's protocol. Observation and quantification was done by 2 independent observers The quantifications were done according to modification of a technique developed by Soini et al,[23] and Pizem & Cor.[24]

2.5. *Quantification of M. tuberculosis bacilli and lung histopathology assessment*

Slides of lung tissue were prepared and stained with Ziehl Neelsen (Brightfield). The *M.tb* were quantified using the WHO scale criteria.[25] The degree of histopathological changes in lung tissue were assessed semiquantitatively according to Dormans criteria[26] using a scoring system for 4 types of histopathological parameters i.e. peribronchiolitis, perivasculitis, alveolitis, and granuloma formation, each scored as absent, minimal, slight, moderate, marked or strong, noted as 0, 1, 2, 3, 4, and 5, respectively.

2.6. *Statistical analysis*

All variables data were tested for normal distribution using Shapiro–Wilk test. Data with normal distribution and had homogeneity of variance were analyzed with one-way ANOVA. Data with non-homogenous variance were analyzed with Brown–Forsythe test. Analysis for multiple comparation was done using Games–Howell test. The difference in histopathological changes between intervention groups were analyzed with non-parametric statistics Kruskal–Wallis test. The correlation between variables with numerical scale and normal distribution was conducted using Pearson test. For categorical or abnormally distributed variable data, Spearman non-parametric test was used. Path analysis was performed by linear regression test. All statistical calculations were performed using the SPSS-15.0 software for Windows (SPSS Inc., Chicago, IL, USA). The difference was considered significant if the value of p is ≤ 0.05.

3. Results

3.1. *The Th1 lymphocytes response*

The Th1 lymphocytes response were measured as the IFN-γ levels in peripheral blood serum (Table 1). As had been expected, *M.tb* infection stimulated high Th1 lymphocyte response. The highest peripheral blood IFN-γ levels were in the M.tb-8 group (106.48 ± 5.44 pg/mL) compared to the lowest in control group (7.20 ± 2.03 pg/mL) ($p < 0.001$), but it appeared to be somewhat blunted in M.tb-16 group (62.97 ± 7.82 pg/mL). Serum IFN-γ levels were also increased in the groups with *H.pg* and *M.tb* co-infection, but the degree of the increase was dictated by the sequence of the infection (89.92 ± 3.53 pg/mL in H.pg+M.tb group, and 46.16 ± 7.82 pg/mL in M.tb+H.pg group). Group with *M.tb* infection near the end of the study had significantly higher INF-γ levels ($p < 0.001$).

The percentage of $CD4^+$ T lymphocytes that expressed intracellular IFN-γ molecules in lung tissue, intestinal tissue, and peripheral blood was measured by flow cytometry using antibodies to CD4 and INF-γ simultaneously (Fig. 1). The highest expression of Th1 $CD4^+$ lymphocytes in lung tissue was in the M.tb-8 group (4.50 ± 0.94%), compared to the lowest in the control group (0.03 ± 0.01%). The overall results pattern was in parallel with IFN-γ levels pattern in peripheral blood serum. The expression of lung tissue Th1 $CD4^+$ lymphocytes significantly differ in all groups of intervention ($p < 0.001$). In the intestinal tissue, there was no significant difference in Th1 $CD4^+$ expression in all groups of intervention ($p = 0.109$). The expression of Th1 $CD4^+$ in peripheral blood was almost similar as in lung tissue. The highest percentage of Th1 $CD4^+$ expression in peripheral blood serum was in the M.tb-8 group (5.86 ± 0.19%) compared to the lowest values in the control group (0.46 ± 0.18%). There were significant differences of peripheral blood Th1 $CD4^+$ expression in all groups of intervention ($p < 0.001$; Table 1).

3.2. *The Th2 lymphocytes response*

The Th2 lymphocytes response was measured as the IL-4 levels in peripheral blood serum. *M.tb* infection did not stimulate Th2 lymphocytes. In contrast, *H.pg* infection stimulated robust Th2 lymphocytes response as can be seen in Table 1. The highest peripheral blood IL-4 levels were in the H.pg-8 group (93.88 ± 7.27 pg/mL), compared to the lowest in the

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Table 1 – Level of peripheral blood cytokines, T cells response in the blood, intestinal or lung tissues, and lung macrophage activity.

Test	H.pg-8	H.pg-16	H.pg+M.tb	M.tb+H.pg	M.tb-16	M.tb-8	Control	p
Peripheral Blood Serum								
IFN-γ (pg/mL)	8.56 ± 1.41	29.46 ± 6.27	89.92 ± 3.53	46.16 ± 7.82	62.97 ± 7.82	106.48 ± 5.44	7.20 ± 2.03	0.000*
IL-4 (pg/mL)	93.88 ± 7.27	78.96 ± 12.37	20.78 ± 4.04	66.62 ± 13.93	16.96 ± 5.20	14.00 ± 4.41	5.01 ± 1.35	0.000*
IL-10 (pg/mL)	54.22 ± 7.18	20.59 ± 5.36	18.53 ± 3.70	18.23 ± 5.05	20.38 ± 5.99	61.62 ± 7.83	4.65 ± 0.57	0.000*
TGF-β (pg/mL)	61.36 ± 8.58	25.34 ± 4.45	17.92 ± 2.58	16.52 ± 3.01	36.70 ± 4.69	72.74 ± 9.14	8.54 ± 1.92	0.000*
$CD4^+$ + IFN-γ (%)	0.52 ± 0.31	1.90 ± 0.33	4.95 ± 0.23	2.42 ± 0.41	3.64 ± 0.54	5.86 ± 0.19	0.46 ± 0.18	0.000*
$CD4^+$ + IL-4 (%)	5.58 ± 0.32	4.66 ± 0.24	1.19 ± 0.55	3.96 ± 0.30	0.96 ± 0.27	0.84 ± 0.17	0.33 ± 0.15	0.000*
Foxp3 + IL-10 (%)	3.26 ± 0.59	1.26 ± 0.27	1.11 ± 0.26	1.03 ± 0.32	1.28 ± 0.28	3.70 ± 0.42	0.26 ± 0.17	0.000*
Foxp3 + TGF-β (%)	3.69 ± 0.43	1.51 ± 0.30	1.07 ± 0.24	0.96 ± 0.16	2.19 ± 0.25	4.35 ± 0.39	0.50 ± 0.21	0.000*
Intestinal Tissue								
$CD4^+$ + IFN-γ (%)	0.04 ± 0.03	0.06 ± 0.04	0.26 ± 0.16	0.21 ± 0.15	0.14 ± 0.05	0.29 ± 0.28	0.13 ± 0.08	0.109
$CD4^+$ + IL-4 (%)	4.27 ± 0.48	3.94 ± 0.23	1.04 ± 0.35	2.71 ± 0.50	0.12 ± 0.08	0.16 ± 0.10	0.19 ± 0.11	0.000*
Foxp3 + IL-10 (%)	3.21 ± 0.42	1.46 ± 0.35	1.14 ± 0.36	1.04 ± 0.25	0.61 ± 0.33	0.70 ± 0.29	0.11 ± 0.10	0.000*
Foxp3 + TGF-β (%)	3.68 ± 0.29	1.63 ± 0.32	1.27 ± 0.17	1.16 ± 0.37	0.70 ± 0.29	0.90 ± 0.20	0.18 ± 0.13	0.000*
Lung Tissue								
$CD4^+$ + IFN-γ (%)	0.13 ± 0.07	1.10 ± 0.47	3.24 ± 0.51	1.27 ± 0.66	2.05 ± 0.84	4.50 ± 0.94	0.03 ± 0.01	0.000*
$CD4^+$ + IL-4 (%)	0.81 ± 0.12	0.82 ± 0.23	0.79 ± 0.22	0.88 ± 0.30	0.92 ± 0.19	0.69 ± 0.18	0.01 ± 0.01	0.000*
Foxp3 + IL-10 (%)	1.13 ± 0.35	0.78 ± 0.14	0.72 ± 0.23	0.73 ± 0.18	0.80 ± 0.25	1.14 ± 0.24	0.33 ± 0.24	0.000*
Foxp3 + TGF-β (%)	1.21 ± 0.28	0.82 ± 0.26	0.75 ± 0.16	0.69 ± 0.20	0.80 ± 0.23	1.20 ± 0.33	0.15 ± 0.09	0.000*
Mϕ iNOS	10.00 ± 1.73	10.80 ± 1.64	21.00 ± 2.35	12.60 ± 2.30	11.40 ± 1.67	26.40 ± 3.29	4.40 ± 1.82	0.000*
Mϕ Arginase 1	19.20 ± 0.45	23.40 ± 1.14	10.00 ± 1.41	25.00 ± 2.00	15.20 ± 1.64	8.80 ± 0.45	3.80 ± 0.45	0.000*

Note: H.pg = *Heligmosomoides polygyrus* infection; M.tb = *Mycobacterium tuberculosis* infection; 8 and 16: denotes infections for 8 and 16 weeks, respectively; H.pg+M.tb = *Heligmosomoides polygyrus* infection followed by *Mycobacterium tuberculosis* infection; M.tb+H.pg = *Mycobacterium tuberculosis* infection followed by *Heligmosomoides polygyrus* infection; Mϕ = macrophage; *significant $p < 0.001$, multiple comparations between groups, one-way ANOVA and Games–Howell test.

control group (5.01 ± 1.35 pg/mL) ($p < 0.001$). Again, the response seemed to be somewhat blunted in the H.pg-16 group (78.96 ± 12.37 pg/mL). Serum IL-4 levels were also increased in the groups with *H.pg* and *M.tb* co-infection, but again, the degree of the increase was dictated by the sequence of the infection (20.78 ± 4.04 pg/mL in H.pg+M.tb group, and 66.62 ± 13.93 pg/mL in M.tb+H.pg group). Group with *H.pg* infection near the end of the study had significantly higher IL-4 levels ($p < 0.001$). The groups with *M.tb* infection had low IL-4 levels in the peripheral blood (14.00 ± 4.41 pg/mL in M.tb-8 group, and 16.96 ± 5.23 pg/mL in M.tb-16 group; $p = 0.948$ between both of them).

The percentage of $CD4^+$ T lymphocytes that expressed intracellular IL-4 molecules in lung tissue, intestinal tissue, and peripheral blood was measured by flow cytometry using antibodies to CD4 and IL-4 simultaneously (Fig. 1). There were no significant difference of Th2 $CD4^+$ expression in lung tissue lymphocytes between groups of intervention, but each of them significantly differ from the control group ($p < 0.001$). In the intestinal tissues, *H.pg* infection induced the highest increase in the expression of Th2 $CD4^+$ lymphocytes (4.27 ± 0.48% in H.pg-8 group, and 3.94 ± 0.23% in H.pg-16 group, respectively). Groups with *M.tb* infection had very low expression of Th2 $CD4^+$ lymphocyte in the intestine, which were comparable to the control group. In the co-infection groups, *H.pg* infection near the end of the study had significantly higher Th2 $CD4^+$ expression (2.71 ± 0.50%), while *M.tb* infection near the end of the study seemed to dampen the Th2 $CD4^+$ response in the intestine (1.04 ± 0.35%). The expression of Th2 CD4+ lymphocytes in the peripheral blood follow the pattern of peripheral blood IL-4 levels (Table 1).

3.3. *The regulatory T lymphocytes response*

The regulatory T lymphocytes (Tregs) response was measured as the immunoregulatory cytokines levels (IL-10 and TGF-β) in peripheral blood serum. Contrary to our expectation, the groups with longer duration of infection, either with *M.tb* (M.tb-16) or *H.pg* (H.pg-16), do not show the highest levels of IL-10 and TGF-β. Instead, the group infected with *M.tb* or *H.pg* for 8 weeks had the highest levels of IL-10 (61.62 ± 7.83 pg/mL in M.tb-8 group, and 54.22 ± 7.18 pg/mL in H.pg-8 group) and TGF-β (72.74 ± 9.14 pg/mL in M.tb-8 group, and 61.36 ± 8.58 pg/mL in H.pg-8 group), suggesting that the Tregs response wanes with time Table 1.

The percentage of Tregs, defined as $CD4^+$ T lymphocytes that expressed CD25 and Foxp3 molecules and produced IL-10 and/or TGF-β, in lung tissue, intestinal tissue, and peripheral blood was measured by flow cytometry using antibodies to CD25, Foxp3, and IL-10, or TGF-β simultaneously (Fig. 2). Overall, the pattern of those Tregs responses follow that of the IL-10 and TGF-β levels in the peripheral blood serum, except in the intestinal tissue where the infection with *H.pg* for 8 weeks (H.pg-8 group) clearly showed the highest percentage of Tregs response (3.21 ± 0.42% IL-10 producing Tregs, and 3.68 ± 0.29% TGF-β producing Tregs, Table 1). Infection with *H.pg* for 16 weeks (H.pg-16 group) showed a damped Tregs response (1.46 ± 0.35% IL-10 producing Tregs, and 1.63 ± 0.32% TGF-β producing Tregs, Table 1). Interestingly, albeit in low level, infection with *H.pg* for 8 weeks did influence the percentage of

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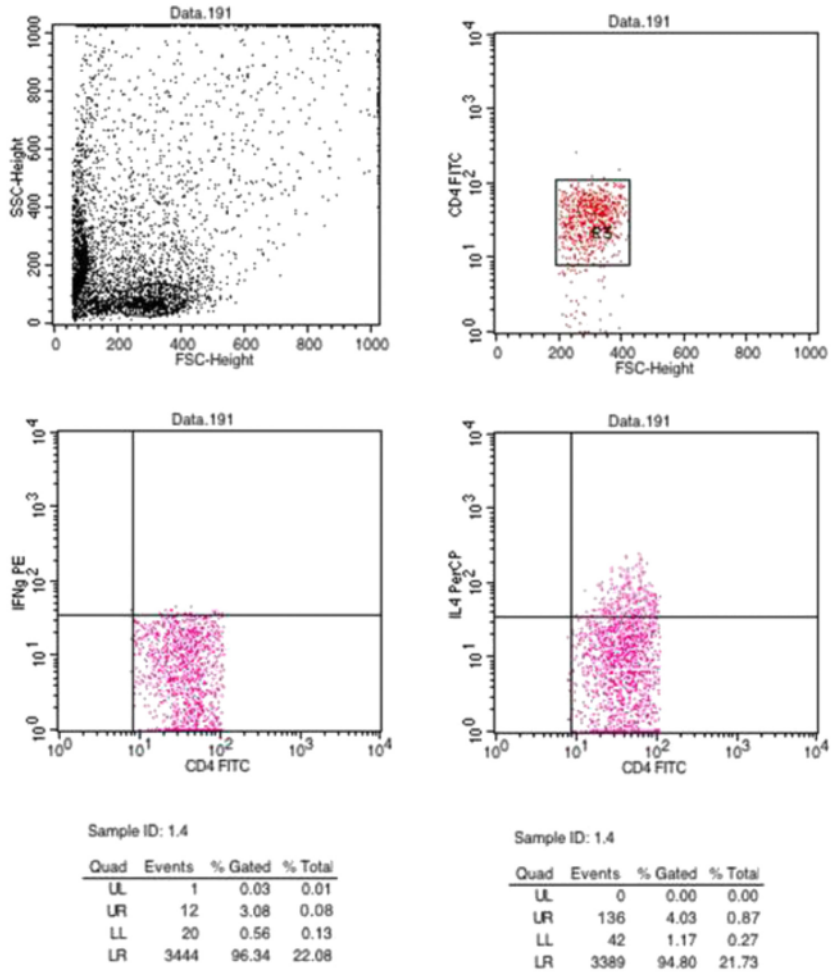

Sample ID: 1.4

Quad	Events	% Gated	% Total
UL	1	0.03	0.01
UR	12	3.08	0.08
LL	20	0.56	0.13
LR	3444	96.34	22.08

Sample ID: 1.4

Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	136	4.03	0.87
LL	42	1.17	0.27
LR	3389	94.80	21.73

Fig. 1 – Flow cytometry of Th1 and Th2 CD4$^+$ T Lymphocytes in Lung Tissue. The CD4$^+$ T lymphocytes were identified by anti-CD4 antibody conjugated with fluorochrome fluorescein isothiocyanate (FITC). The cells were then permeabilized and marked with anti−IFN−γ antibody conjugated with pycoerythrin (PE) for Th1 lymphocytes or with anti-IL-4 antibody conjugated with peridinin chlorophyll protein (perCP) for Th2 lymphocytes. The percentage of each can be read in the accompanying table as the percentage of event in upper right quadrant (UR). The figure represent the data for subject no 1.4.

Tregs in lung tissues which was comparable to infection with *M.tb* for 8 weeks (1.13 ± 0.35% IL-10 producing Tregs, and 1.21 ± 0.28% TGF-β producing Tregs in H.pg-8 group, compared to 1.14 ± 0.24% IL-10 producing Tregs, and 1.20 ± 0.33% TGF-β producing Tregs in M.tb-8 group, $p > 0.05$, Table 1). For groups with co-infections, the Tregs responses were all in the mid-range, and the sequence of infection did not cause any significant difference.

3.4. *The macrophage activity in lung tissue*

Macrophage activity in lung tissues can be that of classically activated macrophage (M1) which expressed iNOS or that of alternatively activated macrophage (M2) which expressed Arginase 1. The quantification of macrophage activation was carried out by two independent observers which showed a consistent results and good correlation ($p = 0.341$ on paired t test, and $p < 0.001$ on Pearson correlation test).

The highest value of iNOS expression was found in the M.tb-8 group (26.74 ± 3.29), and the lowest was in the control group (4.40 ± 1.82) with a significant comparison between groups ($p < 0.001$; Table 1). The highest value of Arginase1 expression was found in the M.tb+H.pg group (25.00 ± 2.00) and followed by the H.pg-16 group (23.40 ± 1.14), which were significantly differ compared to the control group (3.80 ± 0.45; $p < 0.001$). The duration of *M.tb* infection affected the level of iNOS and Arginase1 expression by macrophages in lung tissue ($p < 0.001$). In contrast, *H.pg* infection in the intestine did not profoundly affect the expression of iNOS by macrophages that

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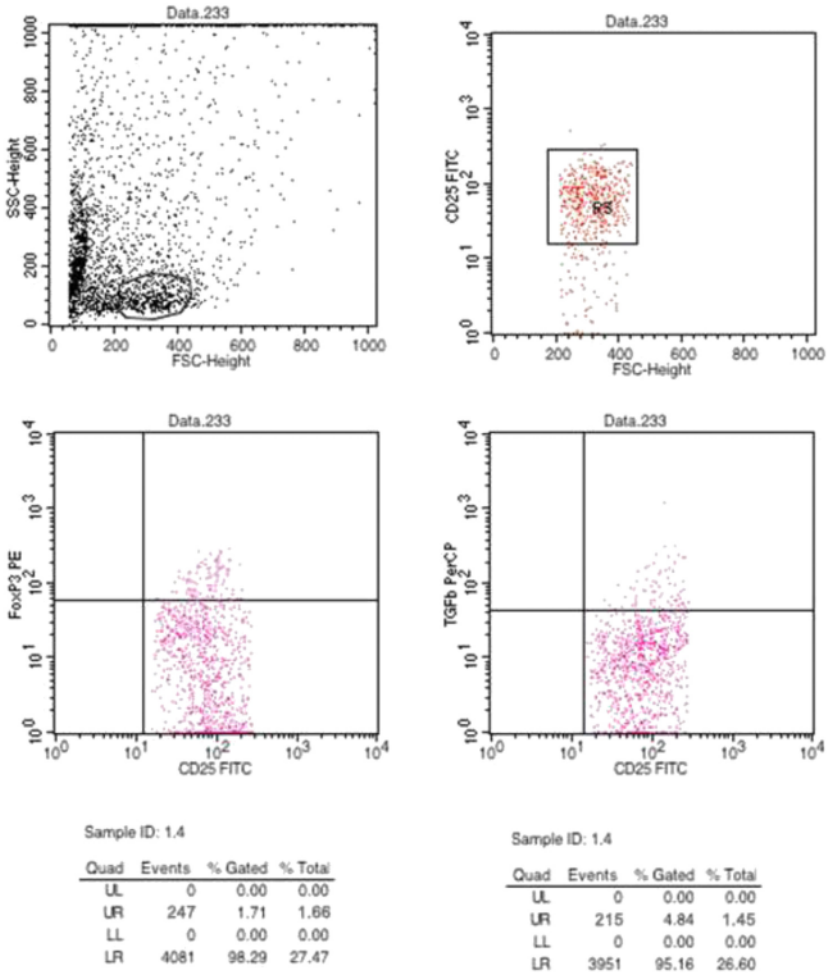

Sample ID: 1.4

Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	247	1.71	1.66
LL	0	0.00	0.00
LR	4081	98.29	27.47

Sample ID: 1.4

Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	215	4.84	1.45
LL	0	0.00	0.00
LR	3951	95.16	26.60

Fig. 2 – Flow cytometry of $CD4^+CD25^+Foxp3^+$ T Lymphocytes in Lung Tissue. The $CD4^+CD25^+Foxp3^+$ T lymphocytes were identified by anti-CD25 antibody conjugated with fluorochrome fluorescein isothiocyanate (FITC). The cells were then permeabilized and marked with anti-Foxp3 antibody conjugated with pycoerythrin (PE) and with anti-TGF-β antibody conjugated with peridinin chlorophyll protein (perCP) for Th2 lymphocytes. The percentage of each can be read in the accompanying table as the percentage of event in upper right quadrant (UR). The figure represent the data for subject no 1.4.

infiltrated lung tissues, but instead induced increased Arginase 1 expression. The expression level of iNOS and Arginase 1 was affected more by the presence of *M.tb* infection in the lung. The expression level of Arginase 1 was also influenced by the presence of co-infection with *H.pg*.

3.5. *Quantification of* M. tuberculosis *bacilli*

The successful infection of *M.tb* using our modification of Middlebrook Inhalation Exposure System is shown in Fig. 3. With Ziehl–Neelsen staining, the lung tissue slides clearly showed groups of bright red colored acid-fast bacteria in mice infected with *M.tb*. It was also clear that in the group infected only with *H.pg* and in the control group no acid-fast bacteria was found in lung tissue. The quantification of the acid-fast bacteria were done using scoring system according to the Guidelines for Mycobacteriology Service in California developed by California Department of Public Health and California Tuberculosis Controllers Association,[25] which is also still recommended by CDC/ATS and WHO. The results were shown in Table 2.

3.6. *Changes in lung tissue histopathology*

The semiquantitative scoring for histopathological changes in lung tissue as assessed by 4 parameters, i.e. peribronchiolitis, perivasculitis, alveolitis, and granuloma formation[25] in each group of interventions can bee seen in Table 3.

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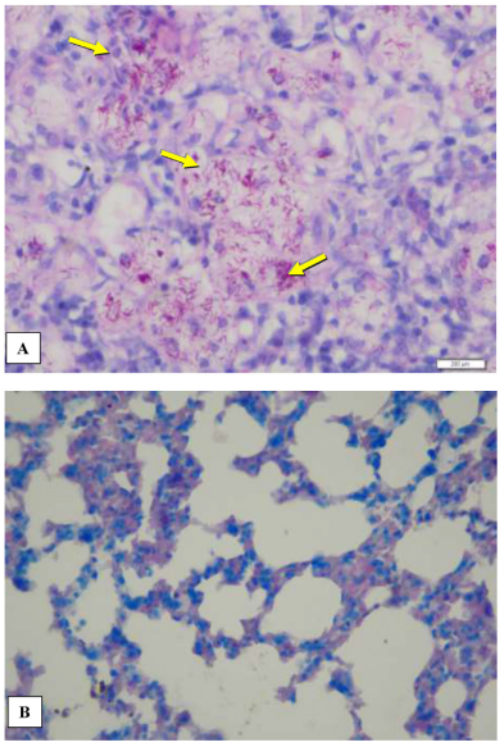

Fig. 3 – Ziehl-Neelsen staining of acid-fast bacteria in lung tissue. The colony of *Mycobacterium tuberculosis* (M.tb) is seen as a group of red colored acid-fast bacteria with Ziehl Neelsen staining on lung tissue slides (yellow arrows) which spread widely on the visual field (A). For comparison, also shown lung tissue slides without M.tb infection (B). The pictures above are seen with light microscopy with 400× power of magnification.

3.7. *Inter variable correlations*

The percentage of Th1 lymphocytes activity in lung tissue was significantly associated with the percentage of Th1 lymphocytes activity in peripheral blood, and intestinal tissue ($p < 0.001$), while the percentage of Th1 lymphocyte activity in peripheral blood was significantly correlated with peripheral blood IFN-γ levels ($p < 0.001$). The same trends were also observed for Th2 lymphocytes and Tregs lymphocytes (Table 4).

Details of the correlation between iNOS or Arginase 1 expression by lung macrophages and Th1 or Th2 lymphocyte activity were presented in Table 5. The correlation between lung macrophage activity and mycobacterial count and lung histopathological changes could be seen in Table 6.

4. Discussion

Immunity to *M.tb* infections clearly needs the host's ability to mount Th1 immune response, in which several Th1 cytokines production such as interferon-γ (IFN-γ), IL-2, and tumor necrosis factor-α (TNF-α) activate the macrophage to kill the mycobacteria.[27,28] Other subsets of T cells such as γδ T cells and CD1-restricted T cells are also stimulated, and together they induce granuloma formation at the site of infection.[29] Immunity to helminthes such as *H.pg*, on the other hand, need a robust Th2 immune response that produce IL-4, and IL-5, which in turn induce accumulation and activation of eosinophils, mast cells, and the production of IgE, all known to promote nematode expulsion from the intestine.[30,31] There have been conflicting results about the influence of helminth infection on immunity to tuberculosis. Some authors stated that helminth infection had negative impact,[4–7] while others found no effects.[14] The dispute can only be solved by studying the lung macrophage polarization during infection with *M.tb*, *H.pg*, or *M.tb* and *H.pg* co-infection.

There were substantial variation in the susceptibility of different mouse strains to infection with virulent *M.tb* H37Rv. Mouse strains can be divided into clusters of susceptible or resistant strains according to their ability to survive an infection with *M.tb* for more than 300 days. We chose BALB/c mouse strains which is categorized as resistant strain and considered it as appropriate. For aerosol infection of *M.tb* we exposed the mice by nebulizing 10 mL of PB-Tween 80 containing 10^6 bacilli/mL (equivalent to $10^{2.7}$ CFU of *M.tb*) as per protocol suggested by the literatures.[32] One literature stated that inoculation by aerosol route showed a faster rate of bacillary growth in the lungs, so it might be the case in our study. Moreover, the use of semiquantitative scoring system

Table 2 – Quantification of *Mycobacterium tuberculosis* in lung tissue (WHO Scale)[a].

WHO Scale Brightfield (1000× magnification)		Groups of intervention						
Number of acid-fast bacilli (AFB) per field	Notation (score) for report	H.pg-8	H.pg-16	H.pg+M.tb	M.tb+H.pg	M.tb-16	M.tb-8	Control
No AFB	Negative	✓	✓					✓
1-2 AFB per 300 fields	Report number observed							
1-9 AFB per 100 fields	Report number observed							
1-9 AFB per 10 fields	1+							
1-9 AFB per field	2+							
10-99 AFB per field	3+			✓	✓	✓	✓	
>99 AFB per field	3+							

[a] Semi-quantitative reporting of acid-fast specimens using Ziehl-Neelsen staining procedure according to Centers for Disease Control/American Thoracid Society (CDC/ATS) and World Health Organization (WHO) guidelines.

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Table 3 – Histopathological changes in lung tissues.

Group of interventions	Score for histopathological changes in lung tissues[a]				
	Peribronchiolitis	Perivasculitis	Alveolitis	Granuloma	Total score
H.pg-8	1	1	1	0	3
H.pg-16	2	1	1	0	4
H.pg+M.tb	4	5	5	4	18
M.tb+H.pg	5	4	5	5	19
M.tb-16	5	5	5	5	20
M.tb-8	5	4	4	5	18
Control	1	1	0	0	2

[a] The score for each group of intervention is expressed as the average score of all members in the particular group. Each parameter of histopathological changes is scored as: absent, minimal, slight, moderate, marked or strong, noted as 0, 1, 2, 3, 4, and 5, respectively, according to Dormans et al[29]

according to the Guidelines for Mycobacteriology Service in California developed by California Department of Public Health and California Tuberculosis Controllers Association to quantify the acid-fast bacilli could be regarded as inappropriate, as this reporting system was originally meant for mycobacterial quantification in the sputum, not in the lung tissues. We should have used the more sophisticated and more accurate methods such as quantitative real-time PCR,[33] nested PCR,[34] or stereological analysis of bacterial load,[35] which we were unaware at the time of our study conception. Dormans criteria for semiquantitative scoring of histopathological changes in lung tissue,[26] was also appeared not sensitive enough to detect the significant difference between the intervention groups. Alternatively, the change in lung histopathology caused by mycobacterial infection is a slowly evolving process, so that 8 or 16 weeks of observation may not be sufficient to detect the evolution of the granuloma and histopathological changes.

Helminth infections induced the appearance of regulatory T lymphocytes ($CD4^+$ $CD25^+$ $Foxp3^+$) in both intestinal tissue, peripheral blood, and lung tissue. Regulatory T cell activity could be assessed by increasing levels of IL-10 and TGF-β cytokines in peripheral blood serum, as well as the percentage of regulatory T lymphocytes in intestinal tissue, lung tissue, and

Table 4 – Association of Th1, Th2, and Tregs lymphocytes activity in various tissues and in peripheral blood.

Inter variable correlations	Pearson's correlation	
	R	p
Th1 type response		
Lung tissue vs. peripheral blood T cells	0.95	<0.001
Lung vs. intestinal tissue T cells	0.55	<0.001
Intestinal tissue vs. peripheral blood T cells	0.52	0.001
Peripheral blood T cells vs. serum IFN-γ level	0.98	<0.001
Th2 type response		
Lung tissue vs. peripheral blood T cells	0.45	<0.01
Lung vs. intestinal tissue T cells	0.40	<0.05
Intestinal tissue vs. peripheral blood T cells	0.96	<0.001
Peripheral blood T cells vs. serum IL-4 level	0.98	<0.001
Tregs response		
Lung tissue vs. peripheral blood Tregs cells	0.82	<0.001
Lung vs. intestinal tissue Tregs cells	0.61	<0.001
Intestinal tissue vs. peripheral blood Tregs cells	0.48	<0.01
Peripheral blood Tregs cells vs. serum IL-10 level	0.97	<0.01
Peripheral blood Tregs cells vs. serum TGF-β level	0.98	<0.001

r: correlation coefficient; p: level of significance.

Table 5 – Association between iNOS and Aginase1 expressions by lung macrophages with Th1 and Th2 lymphocytes activity.

Type of T lymphocyte response	iNOS expression		Arginase 1 expression	
	R	p	r	p
Th1 response				
IFN-γ cytokine levels in peripheral blood serum	0.91	<0.001	0.06	0.714
Percentage of Th1 lymphocyte in peripheral blood	0.90	<0.001	0.03	0.832
Percentage of Th1 lymphocyte in lung tissue	0.90	<0.001	0.09	0.587
Percentage of Th1 lymphocyte in intestinal tissue	0.62	<0.001	−0.07	0.674
Th2 response				
IL-4 cytokine levels in peripheral blood serum	−0.24	0.166	0.60	<0.001
Percentage of Th2 lymphocyte in peripheral blood	−0.26	0.131	0.58	<0.001
Percentage of Th2 lymphocyte in lung tissue	−0.33	0.047	0.71	<0.001
Percentage of Th2 lymphocyte in intestinal tissue	−0.27	0.115	0.35	0.040

r: correlation coefficient; p: level of significance.

Table 6 – Association between lung macrophage activity and mycobacterial count or lung histopathological changes (Dorman's score).

Inter variable correlation	Spearman correlation	
	R	p
iNOS expression vs. mycobacterial count	0.72	<0.001
Arginase 1 expression vs. mycobacterial count	0.23	0.188
iNOS expression vs. Dorman's score	0.52	0.001
Arginase 1 expression vs. Dorman's score	0.31	0.068
Mycobacterial count vs. Dormans score	0.87	<0.001

r: correlation coefficient; p: level of significance.

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peripheral blood. Similar to the findings on the immune responses of Th1 and Th2 cells, the regulatory T cell response was most pronounced in the group of helminth infections for up to 8 weeks and then seemed to subside in longer infection period of 16 weeks. We thought that the emergence of regulatory T cells mainly played a role in reducing the excessive inflammatory process that could have pathological effects on the hosts, as also had been suggested by others,[36,37] and had been reviewed extensively elsewhere.[38]

Apparently, the activation of regulatory T cells was also observed in *M.tb* infection, particularly in shorter duration (8 weeks) of infection, and then relatively subdued in longer duration (16 weeks) of infection. Other studies have also reporting the induction of Tregs in tuberculosis infection.[39] The emergence Tregs could be regarded as detrimental in terms of controlling the pathogen. However, we thought that it was more a manifestation of immunoregulation to control the inflammatory response, which if left unchecked might lead to excessive tissue damage to the host. That is why the Tregs activities were more pronounced in the shorter period of infections (either with *H.pg* or *M.tb*), where the inflammation caused by the pathogens were in its peak, and then somewhat damped in the longer period of infections when the inflammation begun to be reduced. Our study also consistent with the result of Leepiyasakulchai et al,[40] who found that in mouse strain that relatively resistant to tuberculosis infection (such as BALB/c mice) Tregs response were increased compared to susceptible mice strain (such as DBA/2 mice).

Our study also demonstrated the plasticity of T cells and macrophage response to different types of pathogens. H.pg infection (H.pg-8, H.pg-16 groups) clearly induced Th2 type immune response in the intestine and peripheral blood (but not in the lung), and it was associated with M2 lung macrophage polarization. M.tb infection (M.tb-8, M.tb-16 groups) induced Th1 type immune response in the lung and peripheral blood (but not in the intestine), and was associated with M1 lung macrophage polarization. While in the M.tb and H.pg co-infection, the Th1 or the Th2 immune response dictated by the sequence of co-infection. All of those responses were believed to involve the role of antigen presenting cells such as dendritic cells. This plasticity was also observed by Cervi et al[41] Both infection with H.pg or M.tb induce Tregs immune response, which serve more as immunoregulation to control the inflammatory response and prevent excessive tissue damage. Tregs response was higher in shorter duration of H.pg or M.tb infection when the inflammation was in its peak.

5. Conclusion

The sequential co-infection of H.pg and M.tb induces different T lymphocyte immune response which leads to different macrophage polarization in lung tissue as measured by different levels of iNOS and Arginase1 expression. Although helminth infection influence the expression of Arginase 1 (M2 macrophage polarization), it did not cause different mycobacterial count nor different levels of lung histopathological changes as measured by Dorman's score.

Conflicts of interest

The authors have none to declare.

REFERENCES

1. Organization WH. *Global Tuberculosis Report* 2013. World Health Organization; 2013.
2. Erawati M, Andriany M. Prevalence and Demographic Risk Factors for Latent Tuberculosis Infection (LTBI) Among Healthcare Workers in Semarang. *Indonesia. J Multidiscip Healthc*. 2020;13:197–206. https://doi.org/10.2147/JMDH.S241972.
3. Lipner EM, Gopi P, Subramani R, et al. Coincident filarial, intestinal helminth, and mycobacterial infection: helminths fail to influence tuberculin reactivity, but BCG influences hookworm prevalence. *Am J Trop Med Hyg*. 2006;74(5):841–847.
4. Elias D, Britton S, Kassu A, Akuffo H. Chronic helminth infections may negatively influence immunity against tuberculosis and other diseases of public health importance. *Expert Rev Anti-infect Ther*. 2007;5(3):475–484. https://doi.org/10.1586/14787210.5.3.475.
5. Resende Co T, Hirsch CS, Toossi Z, Dietze R, Ribeiro-Rodrigues R. Intestinal helminth co-infection has a negative impact on both anti-Mycobacterium tuberculosis immunity and clinical response to tuberculosis therapy. *Clin Exp Immunol*. 2007;147(1):45–52. https://doi.org/10.1111/j.1365-2249.2006.03247.x.
6. Potian JA, Bhatt K, Liu Z, Gause W, Salgame P. Helminthic infection enhances susceptibility to tuberculosis in a murine coinfection model (43.31). *J Immunol*. 2007;178(1 suppl ment). S42-S42.
7. Bhatt K, Liu Z, Gause WC, Salgame P. Nippostrongylus brasiliensis infection modulates Mycobacterium tuberculosis induced Th1 response (43.45). *J Immunol*. 2007;178(1 suppl ment). S45-S45.
8. Corbett EL, Watt CJ, Walker N, et al. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Arch Intern Med*. 2003;163(9):1009–1021.
9. Todar K. *Mycobacterium tuberculosis and Tuberculosis. Online Text Book of Bacteriology Madison*. 2008. Wisconsin.
10. Hunter RL. The pathogenesis of tuberculosis: the early infiltrate of post-primary (adult pulmonary) tuberculosis: a distinct disease entity. *Front Immunol*. 2018;9. https://doi.org/10.3389/fimmu.2018.02108, 2108-2108.
11. Yazdanbakhsh M, van den Biggelaar A, Maizels RM. Th2 responses without atopy: immunoregulation in chronic helminth infections and reduced allergic disease. *Trends Immunol*. 2001;22(7):372–377.
12. Anthony RM, Rutitzky LI, Urban Jr JF, Stadecker MJ, Gause WC. Protective immune mechanisms in helminth infection. *Nat Rev Immunol*. 2007;7(12):975–987. https://doi.org/10.1038/nri2199.
13. Coffman RL. Origins of the TH 1-TH 2 model: a personal perspective. *Nat Immunol*. 2006;7(6):539.
14. Frantz FG, Rosada RS, Turato WM, et al. The immune response to toxocariasis does not modify susceptibility to Mycobacterium tuberculosis infection in BALB/c mice. *Am J Trop Med Hyg*. 2007;77(4):691–698.
15. Finney CAM, Taylor MD, Wilson MS, Maizels RM. Expansion and activation of CD4(+)CD25(+) regulatory T cells in Heligmosomoides polygyrus infection. *Eur J Immunol*. 2007;37(7):1874–1886. https://doi.org/10.1002/eji.200636751.
16. McSorley HJ, Harcus YM, Murray J, Taylor MD, Maizels RM. Expansion of Foxp3+ regulatory T cells in mice infected with

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the filarial parasite Brugia malayi. *J Immunol*. 2008;181(9):6456–6466. https://doi.org/10.4049/jimmunol.181.9.6456.
17. Stout RD, Suttles J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J Leukoc Biol*. 2004;76(3):509–513. https://doi.org/10.1189/jlb.0504272.
18. Vega M, Corbí A. Human macrophage activation: too many functions and phenotypes for a single cell type. *Immunologia*. 2006;25(4):248–272.
19. Camberis M, Le Gros G, Urban Jr J. Animal model of nippostrongylus brasiliensis and Heligmosomoides polygyrus. *Curr Protoc Im*. 2003;55(1):19.12.11–19.12.27. https://doi.org/10.1002/0471142735.im1912s55.
20. Moerloose KB, Robays LJ, Maes T, Brusselle GG, Tournoy KG, Joos GF. Cigarette smoke exposure facilitates allergic sensitization in mice. *Respir Res*. 2006;7(1). https://doi.org/10.1186/1465-9921-7-49, 49-49.
21. Vermaelen K, Pauwels R. Accurate and simple discrimination of mouse pulmonary dendritic cell and macrophage populations by flow cytometry: methodology and new insights. *Cytometry Part A*. 2004;61A(2):170–177. https://doi.org/10.1002/cyto.a.20064.
22. Herbert DBR, Yang J-Q, Hogan SP, et al. Intestinal epithelial cell secretion of RELM-beta protects against gastrointestinal worm infection. *J Exp Med*. 2009;206(13):2947–2957. https://doi.org/10.1084/jem.20091268.
23. Soini Y, Pääkkö P, Lehto VP. Histopathological evaluation of apoptosis in cancer. *Am J Pathol*. 1998;153(4):1041–1053. https://doi.org/10.1016/S0002-9440(10)65649-0.
24. Pizem J, Coer A. Detection of apoptotic cells in tumour paraffin sections. *Radiol Oncol*. 2003;37(4):225–232.
25. Parmer J, Allen L, Walton W. CE: tuberculosis: A new screening recommendation and an expanded approach to elimination in the United States. *Am J Nurs*. 2017;117(8):24–34. https://doi.org/10.1097/01.NAJ.0000521946.45448.90.
26. Dormans J, Burger M, Aguilar D, 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):460–468. https://doi.org/10.1111/j.1365-2249.2004.02551.x.
27. BoseDasgupta S, Pieters J. Macrophage-microbe interaction: lessons learned from the pathogen Mycobacterium tuberculosis. *Semin Immunopathol*. 2018;40(6):577–591. https://doi.org/10.1007/s00281-018-0710-0.
28. de Martino M, Lodi L, Galli L, Chiappini E. Immune response to Mycobacterium tuberculosis: a narrative review. *Frontiers in Pediatrics*. 2019;7(350). https://doi.org/10.3389/fped.2019.00350.
29. Herbst S, Schaible UE, Schneider BE. Interferon gamma activated macrophages kill mycobacteria by nitric oxide induced apoptosis. *PloS One*. 2011;6(5), e19105. https://doi.org/10.1371/journal.pone.0019105.
30. Huang Z, Luo Q, Guo Y, et al. Mycobacterium tuberculosis-induced polarization of human macrophage orchestrates the formation and development of tuberculous granulomas in vitro. *PloS One*. 2015;10(6). https://doi.org/10.1371/journal.pone.0129744. e0129744-e0129744.
31. Ryan NM, Oghumu S. Role of mast cells in the generation of a T-helper type 2 dominated anti-helminthic immune response. *Biosci Rep*. 2019;39(2).
32. Ordway DJ, Orme IM. Animal models of mycobacteria infection. *Curr Protoc Im*. 2011;94(1):19.15.11–19.15.50. https://doi.org/10.1002/0471142735.im1905s94.
33. Pathak S, Awuh JA, Leversen NA, Flo TH, Åsjø B. Counting mycobacteria in infected human cells and mouse tissue: a comparison between qPCR and CFU. *PloS One*. 2012;7(4), e34931. https://doi.org/10.1371/journal.pone.0034931.
34. Park JS, Kang YA, Kwon SY, et al. Nested PCR in lung tissue for diagnosis of pulmonary tuberculosis. *Eur Respir J*. 2010;35(4):851–857. https://doi.org/10.1183/09031936.00067209.
35. Luciw PA, Oslund KL, Yang X-w, et al. Stereological analysis of bacterial load and lung lesions in nonhuman primates (rhesus macaques) experimentally infected with Mycobacterium tuberculosis. *Am J Physiol Lung Cell Mol Physiol*. 2011;301(5):L731–L738. https://doi.org/10.1152/ajplung.00120.2011.
36. Redpath SA, van der Werf N, Cervera AM, et al. ICOS controls Foxp3(+) regulatory T-cell expansion, maintenance and IL-10 production during helminth infection. *Eur J Immunol*. 2013;43(3):705–715. https://doi.org/10.1002/eji.201242794.
37. Layland LE, Mages J, Loddenkemper C, et al. Pronounced phenotype in activated regulatory T cells during a chronic helminth infection. *J Immunol*. 2010;184(2):713–724. https://doi.org/10.4049/jimmunol.0901435.
38. Taylor MD, van der Werf N, Maizels RM. T cells in helminth infection: the regulators and the regulated. *Trends Immunol*. 2012;33(4):181–189. https://doi.org/10.1016/j.it.2012.01.001.
39. Shafiani S, Dinh C, Ertelt JM, et al. Pathogen-specific Treg cells expand early during mycobacterium tuberculosis infection but are later eliminated in response to Interleukin-12. *Immunity*. 2013;38(6):1261–1270. https://doi.org/10.1016/j.immuni.2013.06.003.
40. Leepiyasakulchai C, Ignatowicz L, Pawlowski A, Källenius G, Sköld M. Failure to recruit anti-inflammatory CD103+ dendritic cells and a diminished CD4+ Foxp3+ regulatory T cell pool in mice that display excessive lung inflammation and increased susceptibility to Mycobacterium tuberculosis. *Infect Immun*. 2012;80(3):1128–1139. https://doi.org/10.1128/iai.05552-11.
41. Cervi L, MacDonald AS, Kane C, Dzierszinski F, Pearce EJ. Cutting edge: dendritic cells copulsed with microbial and helminth antigens undergo modified maturation, segregate the antigens to distinct intracellular compartments, and concurrently induce microbe-specific Th1 and helminth-specific Th2 responses. *J Immunol*. 2004;172(4):2016–2020. https://doi.org/10.4049/jimmunol.172.4.2016.

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