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SHORT REPORT: MODULATION OF *MYCOBACTERIUM TUBERCULOSIS* INFECTION BY *PLASMODIUM* IN THE MURINE MODEL

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Abstract. A large proportion of people with latent tuberculosis live in malaria-endemic areas, so co-infection with these two organisms is likely to be common. To determine whether there might be a biologic interaction between these two pathogens *in vivo*, we infected mice with *Mycobacterium tuberculosis* and then with a non-lethal strain of *Plasmodium yoelii* eight weeks later. Mice chronically infected with *M. tuberculosis* simulate the equilibrium between pathogen and host thought to exist in human latent infection. Co-infected mice were less able to contain growth of *M. tuberculosis* in lung, spleen, and liver (mean ± SEM $\log_{10}$ colony-forming units = 5.50 ± 0.11 versus 5.12 ± 0.08, 4.58 ± 0.07 versus 4.13 ± 0.10, and 2.86 ± 0.10 versus 2.49 ± 0.10, respectively) and had increased mortality. In populations where both diseases are endemic, there may be implications for increased incidence of clinically detectable tuberculosis.

More than one-third of the global population harbors the latent form of tuberculosis infection. During latency, *Mycobacterium tuberculosis* is postulated to exist in a dormant state where the host can effectively contain the pathogen.[1] Some aspects of this equilibrium have been modeled using a strain of mouse C57BL/6 (B6), which is relatively resistant to infection with *M. tuberculosis*. One day after low-dose aerosol infection, 50–100 bacilli can be cultured from the lung of infected mice. The bacilli grow logarithmically in the lung reaching a plateau at 10^5–10^6 colony-forming units (CFU) within four weeks. Cell-mediated immune responses promoted by the release of Th1 cytokines interferon-γ (IFN-γ) and interleukin-12 (IL-12) effect containment of the infection, allowing B6 mice to survive for almost a year.[2] Although there is a plateau in the bacterial growth, the murine host fails to eliminate the pathogen.[3]

B6 mice are highly susceptible to infection with a murine malaria parasite *Plasmodium yoelii*.[4] Intraperitoneal infection using parasitized erythrocytes results in a steady increase in parasitemia, reaching up to 60% of the red blood cells. With a lethal *P. yoelii* strain, mice die within eight days of infection. With the non-lethal *P. yoelii* 17X strain, however, blood stage infection completely resolves and death is rarely seen.[5] Infection with this and other strains of rodent malaria parasites (*P. chabaudi chabaudi*, *P. vinckei vinckei*, and *P. berghei*) is accompanied by initial Th1 type immune responses. A shift from the Th1 to Th2 type is thought to play a critical role in the resolution of infection.[6,7] In this study, we sought to characterize the effect of infection with the non-lethal *P. yoelii* 17X during chronic infection with *M. tuberculosis* in the murine model (Figure 1A).

Chronic infection with *M. tuberculosis* was established in 6–7-week-old female B6 mice (Charles River Laboratories, Wilmington, MA) using low-dose exposure to *M. tuberculosis* CDC1551 strain (five million CFU/mL to give 80–100 organisms in the lung at day 1 after a 30-minute whole body exposure in a Middlebrook chamber (Glas Col, Terre Haute, IN).[8,9] Five mice were killed on day 1 to verify the number of bacteria in the lung as a result of aerosolization. Eight weeks later, the *M. tuberculosis*-infected mice were infected intraperitoneally with 10^6 non-lethal *P. yoelii* 17X–parasitized erythrocytes per mouse using previously described methods.[4] Parasites were maintained by serial passage through mice. Parasitemia was determined by microscopic examination of 100 high-power fields in each Giemsa-stained thin blood smear every 2–4 days post-infection until resolution or death. In parallel, another group of mice was infected with either *M. tuberculosis* or non-lethal *P. yoelii* 17X alone. At 25 days post-infection with non-lethal *P. yoelii* 17X, mice from all groups were killed for bacterial counts and immune assays. Mice were maintained in microisolator cages in biosafety level 3 conditions and fed commercial mouse chow and water *ad libitum*. All animals were maintained in accordance with protocols approved by the Johns Hopkins University Institutional Animal Care and Use Committee.

The CFU from the lung, spleen, and liver were determined after homogenization in phosphate-buffered saline (PBS)/0.05% Tween 80, plating serial dilutions on Middlebrook 7H10 agar supplemented with oleic acid, albumin, dextrose, catalase enrichment media (OADC; Becton Dickinson, Sparks, MD), 0.05% Tween 80 and 5% glycerol, and incubating at 37°C for 3–4 weeks to allow for the enumeration of colonies. Twenty-five days post-infection with non-lethal *P. yoelii* 17X, the bacterial burden of *M. tuberculosis* in co-infected mice was significantly higher in spleen, lung, and liver compared with mice infected with *M. tuberculosis* alone ($P < 0.01$) (Figure 1B). Our data suggest that the host containment mechanisms seen in chronic infection with *M. tuberculosis* were compromised in the co-infected experimental group at the time of sacrifice.

Whole, formalin-fixed infected organs harvested from 2–3 mice per group showed qualitative differences in pigmentation and size. Groups infected with *M. tuberculosis* displayed grossly visible lesions in the lungs. We observed no qualitative difference in size of the tuberculous lesions between groups. The lungs of the co-infected group however, showed darker pigmentation resulting from the accumulation of hemozoin produced by the parasite during digestion of red blood cell hemoglobin[10] (Figure 1C). Pigmentation was more pronounced in the lungs of the co-infected group compared with the malaria-only infected group. These findings were mirrored in the hemozoin pigmentation in the livers of the two groups infected with non-lethal *P. yoelii* 17X. The characteristic splenomegaly of the mice infected with non-lethal *P. yoelii* 17X also appeared to be accentuated in the spleens of the co-infected mice. Mice infected with both pathogens and

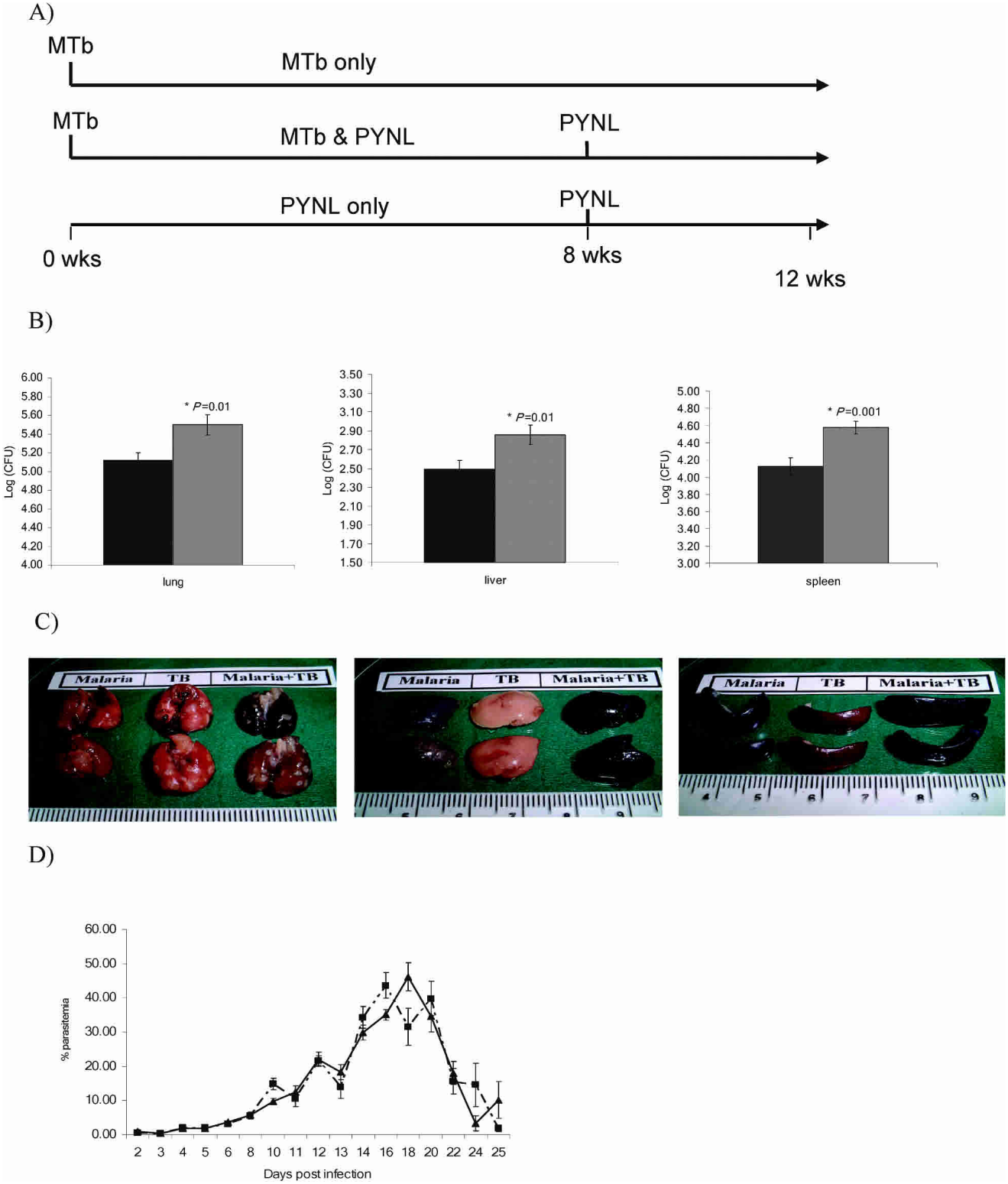

FIGURE 1. **A**, Study design. Two of three groups of 6–7-week-old B6 mice were aerosol-infected with *Mycobacterium tuberculosis* (Mtb). Eight weeks (wks) later, one group was co-infected with non-lethal *Plasmodium yoelii* 17X strain (PYNL) intraperitoneally, while the third group was infected with PYNL only. All mice that did not die during the experiment were sacrificed at 12 weeks. **B**, Increased bacterial load in co-infected mice compared with *M. tuberculosis*-only infected mice. A higher organism burden was measured in mice infected with both *M. tuberculosis* and *P. yoelii* (**grey**) than *M. tuberculosis*-only (**black**) infected mice. Pooled data from two experiments are shown with 8–10 mice per group per time point. Bars represent the mean ± SEM $\log_{10}$ colony-forming units (CFU) in the labeled organ. Statistical differences were calculated by a two-tailed Student's *t*-test. **C**, Gross morphology of the lungs, liver, spleen (**left to right**). Labels indicate the organs of mice infected with *P. yoelii* only (**Malaria**), *M. tuberculosis* only (**TB**), and both *P. yoelii* and *M. tuberculosis* (**Malaria+TB**). Shown are representative organs from each experimental group at the final killing. **D**, Early peak parasitemia levels in co-infected mice. The symbols indicate the average percent parasitemia over time in the blood of mice infected with *P. yoelii* 17X NL only (▲, solid line) or both *M. tuberculosis* and *P. yoelii* 17X NL (■, dashed line). Pooled data from two experiments are shown. Parasitemia percentages were expressed as a mean of results obtained from 10–13 mice per group per time point. Bars represent the mean ± SEM.

killed three months later continued to show evidence of splenomegaly in contrast to the spleens of mice infected with non-lethal *P. yoelii* 17X only, whose spleens reverted to slightly larger than normal size (data not shown).

Parasitemia in mice singularly infected with non-lethal *P. yoelii* 17X peaked at 18 days post-infection at 46% with clearance of parasites by 24 days. The co-infected group displayed a slightly earlier parasitemia peak at 16 days with no significant difference in time to complete clearance (Figure 1C). Five (22%) of 23 mice died in the co-infected group versus only 2 (10%) of 21 in the non-lethal *P. yoelii* 17X group, and no mice singularly infected with *M. tuberculosis* died. At time of death, mice exhibited severe anemia, weight loss, and splenomegaly, which are all consistent with high parasitemias. Figure 1D shows only those mice that survived infection with attrition of those mice that died. This may account for the lack of a significant parasitemia difference between the two groups.

In our studies, mice infected concomitantly with malaria and tuberculosis had significant changes in the course of the tuberculosis infection and increased morbidity. Although only one time point after co-infection was analyzed, co-infected mice had higher burdens of *M. tuberculosis* in both lung and spleen. Similarly, mice chronically infected with *M. tuberculosis* and then treated with monoclonal antibody against tumor necrosis factor alpha had a half-log difference in CFU that persisted. This difference in CFU correlated with a significantly earlier mortality.[11] Another study of major histocompatibility class Ia null mice ($K^{b-/-}D^{b-/-}$) showed a half-log or less increase in CFU after aerosol infection that was also associated with a more rapid time to death.[12] Our findings with *M. tuberculosis* co-infection mirror those reported for mice infected with the facultative intracellular bacterium *Listeria monocytogenes*. The non-lethal *P. yoelii* 17X co-infected mice had more *Listeria* recovered in the liver and spleen compared with the control mice.[13]

We also sought to characterize cellular alterations in the various organs of the three groups of mice (infected with *M. tuberculosis*, non-lethal *P. yoelii* 17X, or both). Four weeks post-infection with non-lethal *P. yoelii* 17X, lung and spleen single cell suspensions were obtained from infected mice by previously described methods.[14] Red blood cells were lysed with NH_4Cl-Tris solution, and cells were washed twice. Cells were stained for cell surface markers using antibodies against CD8 (fluorescein isothiocyanate antibody), CD4 (CyChrome antibody), and T cell receptor γδ (phycoerythrin [PE] antibody) in PBS containing 20% mouse serum, 0.1% bovine serum albumin, and 0.1% sodium azide for 30 minutes at 4°C. All antibodies were used at a concentration of 0.2 mg/10^6 cells (PharMingen, San Diego, CA). Cells were fixed with 4% paraformaldehyde for 4–5 hours, analyzed in a fluorescence-activated cell sorter, and further analyzed using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cells were gated on the lymphocyte population by size. Staining for intracellular cytokines was performed as described previously.[15] Briefly, cells were either stimulated with antibodies to CD3 (0.1 mg/mL) and CD28 (1 mg/mL) (PharMingen) or left unstimulated for 5–6 hours in the presence of 3 mM monensin (Sigma, St. Louis, MO). At the end of the stimulation period, cells were stained for CD4 and CD8, fixed, permeabilized, and stained for intracellular IFN-γ (PE Ab) (PharMingen).

There was a significantly higher percentage of CD4+ T cells in the lung of the co-infected group compared with the other two groups. ($P < 0.05$) Among these CD4+ T cells, a significantly higher percentage was producing IFN-γ in the co-infected group ($P < 0.05$). The percentages of CD4+ and CD8+ T cells in the lung in non-lethal *P. yoelii* 17X–infected mice that were also producing IFN-γ were relatively few compared with the other two groups. Mice infected with non-lethal *P. yoelii* 17X alone had higher percentages of γδ+ T cells in both the lung and the spleen compared with the co-infected group as previously reported[5] ($P < 0.05$). The CD8+ and γδ+ T cell populations in the co-infected group had a mean percentage that was midway between the two singularly infected groups, unlike our findings in the CD4+ T cell population. γδ+ T cell percentages were lowest in the *M. tuberculosis*-only infected group, which was consistent with previous observations of murine infection with *M. tuberculosis*.[16] No significant differences existed in the spleen T cell populations between the three groups (Table 1).

The critical role of CD4+ T cells in the control of both malaria and tuberculosis is well documented, although their exact function is more controversial. Additionally, CD8+ and γδ+ T cells play important roles in the immune responses during infection.[2,6,16–19] Mounting evidence shows that the early stages of malaria are characterized by the production of IL-12 and IFN-γ by Th1 T cells. To aid in clearance of parasite, the response shifts to a predominantly Th2 response characterized by the production of IL-4 and other cytokines that initiate the antibody-dependent mechanisms required for complete clearance or resolution of infection.[7,20] During infection with tuberculosis, a predominantly Th1 cytokine profile persists throughout the course of the infection and plays an important role in containment of bacterial growth. The significance of Th1 type immunity is further exemplified by

TABLE 1
Lymphocyte subsets in lung and spleen of mice infected with *Plasmodium yoelii, Mycobacterium tuberculosis,* or both*

	TB only (%)†	Lung *P. yoelii* and TB (%)	*P. yoelii* only (%)	TB only (%)	Spleen *P. yoelii* and TB (%)	*P. yoelii* only (%)
CD4+ cells	24.6 ± 2.4	37.3 ± 1.7‡§	31.4 ± 2.4	22.3 ± 2.1	19.7 ± 2.0	27.2 ± 4.4
IFN-γ+, CD4+ cells	8.6 ± 1.7	21.1 ± 1.9‡§	6.12 ± 1.4	7.7 ± 1.3	8.9 ± 0.4	9.0 ± 1.4
CD8+ cells	14.2 ± 1.5	18.5 ± 2.1	20.5 ± 1.9	11.5 ± 1.1	8.3 ± 1.2	7.2 ± 1.1
IFN-γ+, CD8+ cells	6.8 ± 1.3	8.7 ± 0.7	2.7 ± 0.6	5.5 ± 1.8	9.0 ± 0.8	5.8 ± 2.1
δγ+ cells	0.9 ± 0.1	1.4 ± 0.7	3.1 ± 0.5§¶	0.6 ± 0.1	1.9 ± 0.2	3.2 ± 0.8§¶

* Values are the mean ± SEM. TB = tuberculosis; IFN-γ = interferon-γ.
† Percent gated on cell size during flow cytometric analysis.
‡ Significant values ($P < 0.05$) based on the two-tailed Student's *t*-test comparing nonlethal *P. yoelii* 17X strain with non-lethal *P. yoelii* 17X strain only.
§ Significant values ($P < 0.05$) comparing non-lethal *P. yoelii* 17X strain and *M. tuberculosis* with *M. tuberculosis* only.
¶ Significant values ($P < 0.05$) comparing non-lethal *P. yoelii* 17X strain with *M. tuberculosis* only.

studies on CD4- and IFN-γ-null mice, which rapidly succumb to infection with tuberculosis.[21,22] The Th1 to Th2 shift observed in malaria infection does not appear to occur in tuberculosis infection.[16,24] Conversely, however, IL-4 and IL-10 knockout mice are not better able to defend themselves against tuberculosis.[25]

In the co-infected mice, our observation of increased numbers of CD4+ T cells that were producing IFN-γ is particularly interesting because the tuberculosis infection was less well-controlled. Because flow cytometry analysis cannot differentiate the antigenic stimulus to which specific cells are elaborated, these cells could represent either an ineffective immune response to tuberculosis or a specific augmentation of the anti-malarial response that prevents clearance of the parasites. In support of the latter hypothesis, animals that died had notable anemia and relatively high parasitemia at the time of death. Therefore, the increased mortality may be related not only to an increased burden of tuberculosis infection, but also to poor malaria control due to a prolonged Th1 response in these mice.

We sought to model the effect of malaria on chronic tuberculosis in mice. Our results suggest that chronic tuberculosis worsens in the presence of an acute malarial infection. The co-infected mice had difficulty containing the bacterial infection as shown by higher bacillary loads in lung, liver and spleen. The equilibrium that allowed the bacillary burden to be contained during chronic tuberculosis in the mouse was compromised by the acute *Plasmodium* infection. If these results can be extrapolated to human latent tuberculosis, *Plasmodium* infection could play a significant role in increasing the incidence of reactivation tuberculosis in adults or primary active tuberculosis in children in areas where the two diseases are endemic. Because of the global importance of both malaria and tuberculosis, further investigation is warranted to study and the Th1/Th2 immunomodulation during the course of co-infection.

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