IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Trehalose 6,6-Dimycolate from *Mycobacterium tuberculosis* Induces Hypercoagulation

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Tuberculosis (TB) remains a global health concern. Trehalose 6'6-dimycolate (TDM) activates innate inflammation and likely also stimulates chronic inflammation observed during disease progression. Noninfectious models using purified TDM oil/water emulsions elicit pathologic findings observed in patients with TB. We introduce a new TDM model that promotes inflammatory lung pathologic findings and vascular occlusion and hemorrhage. C57BL/6 and BALB/c mice were injected with 10 μ g of i.p. TDM in light mineral oil (TDM-IP). At day 7, another injection of 10 μ g of i.v. TDM in oil/water emulsion was given (TDM-IV). The i.p./i.v. TDM (TDM-IVIP) group was compared with mice injected once with i.v. or i.p. TDM. The responses to TDM-IP, TDM-IV, or TDM-IVIP were consistent between mouse strains. Mice that received TDM-IV and TDM-IVIP had inflammatory pathologic findings with increases in inflammatory and T-cell cytokines, and the TDM-IVIP group had further enhancement of IL-10 and granulocyte-macrophage colony-stimulating factor. The TDM-IVIP group had increased CD4⁺ T cells in lung tissue, significantly increased coagulation, decreased clot formation time, and increased maximum clot firmness. Masson's trichrome staining revealed increased deposition of collagen in the occluded vasculature. TDM-IVIP promotes a hypercoagulopathy state, independent of inflammation. This new model argues that TDM is sufficient to generate the hypercoagulopathy observed in patients with TB. (*Am J Pathol* 2016, 186: 1221–1233; <http://dx.doi.org/10.1016/j.ajpath.2015.12.019>)

Tuberculosis (TB) remains a serious health burden to the world's population. The World Health Organization estimates that approximately one-third of the world's populations are infected with *Mycobacterium tuberculosis*, the causative pathogen of TB disease. In 2013, there were 9 million cases of TB disease and 1.5 million deaths (Centers for Disease Control and Prevention, <http://www.cdc.gov/tb/statistics/default.htm>, last accessed September 24, 2015). The pathology of TB disease is a complex interaction between pathogen and host immune responses. Understanding these specific mechanisms will lead to improving current therapeutics and vaccines.

M. tuberculosis possesses several pathogen components that can stimulate the innate and adaptive immune response. Trehalose 6'6-dimycolate (TDM), the major glycolipid on the cell wall of *M. tuberculosis*, is a strong inducer of host immunity.^{1,2} The acute immune response to TDM peaks at day 7 with innate lymphocytes and macrophage activation.³ During the acute TDM response, several inflammatory cytokines are

produced, including tumor necrosis factor (TNF)- α , IL-6, and IL-1 β .^{4,5} Innate cytokines, such as IL-12 and IL-10, produced by activated macrophages are also increased in response to TDM.^{6,7} Recently, IL-17 has also been observed to be increased in lung tissue in TDM-injected mice.⁸ The acute response to TDM resembles the acute phase of *M. tuberculosis* infection, eliciting macrophage/dendritic cell innate responses to promote development of the adaptive T-cell response.

The adaptive response to TDM evokes strong lymphocyte inflammation, promoting large clusters of leukocyte infiltration into lung tissue and high levels of interferon (IFN)- γ . This adaptive response can be transferred to nonsensitized hosts by CD4⁺ T cells,^{9,10} and it requires the presence of CD1.^{10,11} The adaptive response is dominated with high

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levels of IFN- γ , IL-2, and IL-12, eliciting a strong T-cell helper type 1 response.¹² Thus, TDM is capable of generating an adaptive T-cell helper type 1 immunity and the antigen-specific memory that is observed in *M. tuberculosis* infection.

Macrophages, professional antigen-presenting cells, carry surface recognition molecules for *M. tuberculosis* components and TDM that allow phagocytosis of *M. tuberculosis* and TDM particles and generate an innate immune response. The immune mechanism of TDM is mapped to macrophage inducible C-type lectin, dectin-3, and additional C-type lectin receptors to activate NF- κ B.^{13–16} Macrophage activation by TDM induces an inflammatory response, leading to production of TNF- α .⁶ In addition, TDM protects *M. tuberculosis* from macrophage intracellular killing mechanisms,⁷ creating a protective niche for organism growth and antigen production. These unique attributes of TDM, stimulating host inflammation and protecting *M. tuberculosis* against host cell killing, are thought to be major contributors to the presence of foamy macrophages. These highly activated macrophages in TB disease are hypothesized to be a result of continuous stimulation by *M. tuberculosis* antigens, specifically TDM.

The clinical lung pathologic findings of TB disease remain largely unknown. Although no animal model is capable of reproducing all the clinical components, each animal model is capable of reproducing at least one aspect of the disease observed in human patients. In the 1950s, a mouse model using repeated i.p. injections of TDM in oil¹⁷ along with subsequent reports published in 1970s and 1980s revealed that TDM can induce pneumonitis, accompanied with pronounced hemorrhage in the lung tissue.^{18–20} This TDM-induced pneumonitis has been observed in lung tissues of patients who died of active TB disease. This finding suggests that lipid pneumonia is an important part of TB disease.²¹

To incorporate these 2 TDM pathologic findings, immune responses and hemorrhagic pneumonia, into a single model, a new model was developed that incorporated the TDM in oil i.p. with the TDM in oil/water emulsion i.v. This new TDM protocol stimulates immune responses that result in development of both inflammation and pneumonitis in lung tissue, simulating the pathologic findings observed during prolonged *M. tuberculosis* infection. This new model can be used as a noninfectious model to investigate the mechanisms of several pathologic findings observed during *M. tuberculosis* infection.

Materials and Methods

Mouse

Female C57BL/6 mice and female BALB/c mice (both 6 weeks; both from Harlan Laboratories, Indianapolis, IN) weighing approximately 20 g initially were used. All *in vivo* experiments were conducted under approved guidelines of the animal ethics committee at the University of Texas, Health Science Center at Houston (HSC-AWC-13-123).

TDM-Induced Lung Pathologic Findings

M. tuberculosis—derived TDM (cord factor) was purchased from Santa Cruz Biotechnology (Dallas, TX). Drakeol 6VR, light mineral oil, was purchased from Penreco (Dickinson, TX).

TDM (10 μ g per mouse) was solubilized in hexane:ethanol (ratio of 9:1), the appropriate amount was aliquoted, and hexane:ethanol evaporated under stream of air. TDM (10 μ g per mouse) in oil was prepared by homogenizing in Drakeol (100 μ L per mouse) with a glass tube and Teflon pestle for 1 minute. The TDM 10 μ g per mouse dose was chosen because it was the dose that did not induce severe physical distress in the C57BL/6 and BALB/c mice from preliminary studies (animal husbandry observations). The TDM in oil formulation was injected i.p. The TDM oil/water emulsion was prepared as previously described.^{5,22} The evaporated TDM (10 μ g per mouse) was homogenized in Drakeol (2 μ L per mouse) for 1 minute. To the TDM-Drakeol mixture, 48 μ L per mouse of 1 $\times$ Dulbecco's phosphate-buffered solution (DPBS; Cellgro; Mediatech Inc, Manassas, VA) with 0.2% Tween 80 (Mallinckrodt, Hazelwood, MO) was added and homogenized for another 2 minutes. The TDM oil/water emulsions were injected i.v. Controls with no TDM in oil and oil/water formulations were also prepared.

C57BL/6 and BALB/c mice were treated as follows: i) control: naive, no injections; ii) oil i.p.: no TDM, oil only, injected i.p.; iii) oil/water i.v.: no TDM, oil/water emulsion, injected i.v.; iv) TDM i.p. (TDM-IP): 10 μ g per mouse of TDM in Drakeol (100 μ L per mouse) injected i.p.; v) TDM i.v. (TDM-IV): 10 μ g per mouse of TDM in oil/water emulsion (100 μ L per mouse) injected i.v.; and vi) TDM i.p./i.v. (TDM-IVIP: 10 μ g per mouse of TDM in Drakeol (100 μ L per mouse) injected i.p. and, after 7 days, 10 μ g per mouse TDM in Drakeol (100 μ L per mouse) were injected i.v. The second TDM injection was given on day 7, at the height of the primary TDM immune response.

All mice were sacrificed at day 7 after the last TDM or control formulation injections. On sacrifice, lungs were perfused with 1 mmol/L EDTA in DPBS, weighed, and sectioned to evaluate pathologic findings, cytokine production, and immune cell typing by flow cytometry.

Lung weight index (LWI)^{5,9–11,22,23} was calculated as a rough measure of lung inflammation intensity using the following equation:

$$LWI = \frac{\sqrt{\frac{\text{Lung weight (g)} \times 1000}{\text{Mouse weight (g)} / 10}}}{10} \quad (1)$$

Lung Cytokine Analysis

A section of lung tissue was homogenized and placed into 2 mL of Dulbecco's modified Eagle's medium containing 50 mg/L of L-arginine, 50 mg/L of HEPES, 10% fetal

bovine serum, 100 µg/mL of penicillin, and 50 µg/mL of gentamicin (Sigma-Aldrich, St. Louis, MO). Samples were incubated for 4 hours at 37°C with 5% CO₂. The resulting supernatants were analyzed by enzyme-linked immunoassay (ELISA).

Levels of cytokines in cell supernatants were measured by a sandwich ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Absorbance was read at 570 nm with background subtracted at 450 nm on a plate reader (Molecular Devices, Menlo Park, CA). The lower range limit for detection sensitivity was 16 to 32 pg/mL.

Identification of Immune Cells in Lung Tissue

Section of lung tissue is processed into a single-cell suspension. Lung section was cut into tiny pieces (<1 mm) and digested in a solution of 1 mg/mL of collagenase type II and 30 µg/mL DNase (Sigma-Aldrich) in 1× DPBS at 37°C, in a shaking incubator, for 1 hour. After digestion, glass slides were used to breakup tissue clumps. Once the bigger clumps settle to the bottom of the tube, the remaining cell suspension was pelleted at 350 × *g* for 5 minutes. The cell pellet was resuspended in flow staining buffer (1% bovine serum albumin in 1× DPBS).

Identification of surface molecule expression by flow cytometric analysis (mean of 20,000 events per sample) was conducted as previously described.^{24,25} Briefly, samples were blocked by incubating purified anti-CD16/32 (Fc block; BD Bioscience, San Diego, CA) on ice for 15 minutes. Then, samples were stained with CD4 fluorescein isothiocyanate, CD8 phycoerythrin, CD3 allophycocyanin, CD11b–peridinin chlorophyll–Cy5.5, Gr1-Pacific Blue, and NK1.1-Cy7 (eBioscience, San Diego, CA) on ice for approximately 1 hour. Stained samples were fixed with 4% paraformaldehyde on ice for 15 minutes. Stained and fixed samples were stored in 500 µL of staining buffer. Samples were read on a Gallios Flow Cytometer (Beckman-Coulter, Atlanta, GA). Analysis was conducted with Cyflogic version 1.2.1.

Histologic and Immunohistochemical Analysis

The large right lobe of the mouse lung was collected and fixed in 10% buffered formalin. For histologic analysis, the lung was sectioned (5 µm thick) and stained with hematoxylin and eosin (H&E) or Masson's trichrome as per standard procedures. The number of plaques per lung section was counted under the microscope.

Formalin-fixed, paraffin-embedded human lung samples from our collection were sectioned (5 µm thick) and stained with H&E and acid fast. Images were taken under light microscopy and color balanced with Adobe Photoshop.

Thromboelastometry

Whole blood was taken from TDM-IPIV–injected C57BL/6 and BALB/c mice into a tube with 3.2% citrate to achieve a

final dilution of nine parts blood with one part anticoagulant. Thromboelastometry was performed using ROTEM software version 1.6.0 (Pentapharm, GmbH, Munich, Germany) δ analyzers. The instrument monitors changes in the viscoelastic properties of whole blood as the clot is formed by measuring the impedance of a pin attached to a rotating shaft ($\pm 4.75^\circ$) connected to a spring. Clotting in whole blood was initiated via the intrinsic pathway by the addition of reagents supplied by the manufacturer (in-TEM, diluted 1/10 with saline, and 0.2 M CaCl₂; TEM Systems Inc, Durham, NC).

Statistical Analysis

Data obtained were compared across groups and against naive mice or mice challenged with vehicle formulation without TDM, then analyzed by an unpaired *t*-test or one-way analysis of variance with the Tukey post-hoc test; differences in the means were significant at a level of $P \leq 0.05$. Data are presented as a compilation of all experimental repeats (two or three). Each experiment had three to six mice.

Results

Novel TDM Model Induces Inflammation in C57BL/6 and BALB/c Mice

To examine TDM-induced inflammation in lung tissue, C57BL/6 and BALB/c mice were injected with *M. tuberculosis*–derived TDM, comparing among several different methods: i) TDM-IP, ii) TDM-IV, and iii) TDM-IPIV. Controls included naive mice, light mineral oil (no TDM) only i.p., and oil/water emulsion (no TDM) only i.v.

Naive and vehicle (no TDM)–injected mice had no noticeable lung pathologic findings, with normal parenchyma and little to no noticeable cellular infiltrates (Supplemental Figure S1). C57BL/6 mice injected with TDM-IP had minimal lung pathologic findings with very small clusters of leukocytes and thickening of alveoli walls. BALB/c mice injected with TDM-IP also had few to no lung pathologic findings that appeared to be mostly normal with no obvious thickening of alveoli walls. Mice injected with TDM-IV had inflamed lung pathologic findings, similar to published data, and small granuloma clusters mainly composed of myeloid cells and thickened alveoli walls. A more enhanced inflammatory response was observed in C57BL/6 mice compared with BALB/c mice. The difference between the two mouse strains became more prominent with TDM-IPIV. Although BALB/c mice lungs appeared similar in pathologic findings to the TDM-IV–injected mice, C57BL/6 mice had an increase in inflammation, larger leukocyte clusters with widespread alveoli thickening, and accompanying hemorrhage. The severity of lung hemorrhage in C57BL/6 mice was not always consistent across all individuals. However, every C57BL/6 mouse given TDM-IPIV had small hemorrhagic regions, and at least one

Figure 1 Trehalose 6'6-dimycolate (TDM)—induced lung histologic findings. Lungs from C57BL/6 (**A**) and BALB/c (**B**) mice injected with i.p. TDM (TDM-IP), i.v. TDM (TDM-IV), or i.p./i.v. TDM (TDM-IPIV) were processed at day 7, fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin. **A:** TDM-IPIV reveals two individual lungs, the **top panels** reveal a lung with maximum hemorrhage, and the **bottom panels** show a lung with minimal hemorrhage. Original magnification: $\times 40$ (**left column**); $\times 100$ (**middle column**); $\times 200$ (**right column**).

mouse from each experimental repeat manifested widespread hemorrhage across the entire lung section. The other noticeable finding was that the mice given TDM-IV and TDM-IPIV (C57BL/6 and BALB/c) had signs of vascular occlusion (**Figure 1**). Gross lung observations clearly revealed the extensive hemorrhage in C57BL/6 TDM-IPIV group compared with the C57BL/6 TDM-IV, BALB/c TDM-IV, and BALB/c TDM-IPIV groups (**Figure 2**).

Overall lung inflammation was measured as LWI. For both C57BL/6 and BALB/c mice, TDM-IV treatment increased LWI significantly against the TDM-IP and non-TDM groups (**Figure 3**). Interestingly, both clonal strains that received TDM-IPIV had a wider range of LWI that was significantly

decreased compared with TDM-IV in C57BL/6 and BALB/c mice.

Whole-lung cytokine environments were examined for inflammation (TNF- α , IL-6, IL-1 β), T-cell activity (IFN- γ and IL-17), and T-cell helper type 1 differentiation (IL-12p40 and IL-10). In mice that did not receive TDM, no cytokine production of all analytes was found. Only mice given the oil/water vehicle produced any measurable cytokine levels but were significantly lower than the TDM-injected groups (**Supplemental Table S1**).

In general, C57BL/6 mice produced higher levels (approximately 10 $\times$) of cytokines compared with BALB/c mice in response to TDM. In all cytokines measured, the

Figure 2 Gross lung pathologic findings of mice injected with trehalose 6'6-dimycolate (TDM). The left lobe of lung from C57BL/6 and BALB/c mice was collected from mice injected with i.v. TDM (TDM-IV) and i.p./i.v. TDM (TDM-IPIV) at day 7. The lungs were fixed in 10% formalin. Photograph was taken under normal lighting conditions with a white background. One representative lung is shown per group.

mice injected with TDM-IV and TDM-IPIV induced significantly higher levels of cytokines compared with the TDM-IP group. Only IL-6 revealed no difference among the TDM-IP, TDM-IV, and TDM-IPIV groups. The C57BL/6 mice had an increase in IL-1 β in the TDM-IV and TDM-IPIV groups compared with the TDM-IP group (Figure 4A). In the BALB/c mice, the IL-1 β level in the TDM-IPIV group was decreased compared with the TDM-IV group (Figure 4B), with both groups having increased levels compared with TDM-IP.

C57BL/6 and BALB/c mice given TDM-IPIV and TDM-IV induced significantly higher levels of IFN- γ in the lung tissue. Although both C57BL/6 and BALB/c mice given TDM-IV had an increase in lung IL-17 levels compared with TDM-IP, only C57BL/6 mice given TDM-IPIV produced significantly higher IL-17 levels compared with TDM-IV (Figure 4).

Production of IL-12p40 and IL-10 in C57BL/6 mice was increased as follows: TDM-IP < TDM-IV < TDM-IPIV (Figure 4A). In BALB/c mice, IL-12p40 production increased in both the TDM-IV and TDM-IPIV groups compared with the TDM-IP group (Figure 4B). IL-10 production in BALB/c mice was significantly increased in the same trend as in the C57BL/6 mice: TDM-IP < TDM-IV < TDM-IPIV (Figure 4).

Granulocyte-macrophage colony-stimulating factor production in C57BL/6 mice was higher overall compared with BALB/c mice (Figure 4A), but only the BALB/c mice had an increase in GM-CSF from TDM-IPIV mice

compared with the TDM-IP and TDM-IV mice (Figure 4B).

Leukocyte Infiltration into Lung Tissue after TDM Administration

The cytokine levels from C57BL/6 and BALB/c mice indicated that the different TDM injections induced a varied inflamed lung environment. Infiltrating leukocytes drawn by TDM and/or the inflamed environment can contribute to progression and modulation of the immune response. To investigate whether the cytokine environment correlated to the leukocyte population, multicolor flow analysis of immune cell markers was examined.

Naive, oil vehicle i.p., and oil/water emulsion i.v. controls all had relatively low percentages of T cells, macrophage, and neutrophil populations in the lung. Only the oil/water vehicle i.v. induced an increase in macrophage and neutrophils (Supplemental Table S2). With the use of forward and side scatterplot, lymphocytes and myeloid cells were differentiated (confirmed by lack of CD3⁺ in the myeloid population). Both C57BL/6 and BALB/c mice had the same trend in lymphocyte and myeloid percent makeup. Both mouse strains had increase lymphocyte populations: TDM-IV > TDM-IPIV > TDM-IP. Consequently, the increase in lymphocyte population correlated with a decrease in myeloid cell population, as reflected by the changes in the myeloid population: TDM-IP > TDM-IPIV > TDM-IV (Figure 5A).

The lymphocyte population was delineated into CD3⁺ and CD3⁻ populations. The TDM-IP mice (C57BL/6 and BALB/c) had a balanced population of CD3⁺ and CD3⁻ groups. The

Figure 3 Lung weight index (LWI). Lungs from C57BL/6 and BALB/c mice injected with trehalose 6'6-dimycolate (TDM) i.p. (TDM-IP), i.v. (TDM-IV), or i.p./i.v. (TDM-IPIV). Lungs were also isolated from the following control groups: naive (noninjected), oil vehicle i.p. (Drakeol), and oil/water vehicle i.v. (2% Drakeol, 98% 1 $\times$ phosphate-buffered saline with 0.2% Tween 80). At day 7, lungs were collected and weighed and compared against the weight of the mouse. Data were analyzed by one-way analysis of variance and Tukey post-hoc tests. * P < 0.05, *** P < 0.001.

Figure 4 Lung cytokine environment of the C57BL/6 and BALB/c mice injected with trehalose 6'6-dimycolate (TDM). A portion of the lung from i.p. TDM (TDM-IP), i.v. TDM (TDM-IV), and i.p./i.v. TDM (TDM-IPIV) C57BL/6 (**A**) and BALB/c (**B**) mice was collected at day 7. The lungs were homogenized in Dulbecco's modified Eagle's medium with 10% FBS and incubated for 6 hours at 37°C with 5% CO₂. Supernatants were analyzed by enzyme-linked immunosorbent assay. Data were analyzed by one-way analysis of variance and Tukey post-hoc tests. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.

TDM-IV and TDM-IPIV mice had significantly increased CD3⁺ population and decreased CD3⁺ population (Figure 5B). The CD3⁺ population was then split into CD4⁺ and CD8⁺ populations. In both strains of mice, the TDM-IV and TDM-IPIV mice had decreased CD3⁺CD4⁺ cells compared with the TDM-IP group (Figure 5C).

The myeloid population can be divided into macrophages (CD11b⁺GR1⁺) and neutrophils (CD11b⁺GR1⁺). No differences in neutrophil numbers were observed in all TDM C57BL/6 mice; however, the TDM-IPIV group had an

increase in macrophages compared with the TDM-IV and TDM-IP groups. In BALB/c mice, both the TDM-IV and TDM-IPIV groups had increased macrophages in the lungs compared with TDM-IP. In addition, BALB/c mice given TDM-IV and TDM-IPIV had decreased neutrophil numbers compared with the TDM-IP group (Figure 5D).

Little to no cells that expressed NK1.1 were CD3⁺, suggesting little to no natural killer T cells migrated to the lung tissue during the TDM pathologic process. Most cells expressing NK1.1 were CD3⁺; thus, these cells are categorized

Figure 5 Infiltration of leukocyte populations into lung tissues of C57BL/6 and BALB/c mice. A portion of the lung from mice injected with i.p. trehalose 6'6-dimycolate (TDM-IP), i.v. TDM (TDM-IV), and i.p./i.v. (TDM-IPIV) were collected at day 7. Lung tissue was digested in collagen I and DNase in 1× phosphate-buffered saline for 60 minutes at 37°C in a shaking incubator. Single-cell suspensions were collected and stained with CD3, CD4, CD8, CD11b, NK1.1, and Gr1 on ice for 45 to 60 minutes. **A:** Lymphocytes and myeloid cells differentiated by forward and side scatterplot. **B:** Lymphocytes analyzed for expression of CD3. **C:** CD3⁺ cells were analyzed for co-expression of CD4 and CD8. **D:** Myeloid cells were analyzed for expression of CD11b and Gr1. **E:** CD3⁺ cells were analyzed for expression of NK1.1. Data were analyzed using Cyflogic version 1.2.1. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

as innate natural killer cells. The TDM-IP group from both C57BL/6 and BALB/c mouse strains had the highest number of NK1.1⁺ of CD3⁺ cells. The BALB/c TDM-IV group revealed the lowest percentage of NK1.1⁺CD3⁺ cells (Figure 5E).

Collagen Deposition and Vascular Occlusion

Histologic analysis of TDM-IV and TDM-IPIV revealed distinct vascular changes under typical H&E stains (Figure 1). The vascular walls were thickened and obstructed by occlusions composed of protein and circulating

leukocytes. These structures were found attached to the endothelial wall.

Masson's trichrome staining revealed significant collagen deposition (blue) in the occluded blood vessels in both TDM-IV and TDM-IPIV C57BL/6 and BALB/c mice (Figure 6). No pathologic findings, including vascular occlusion, was observed in naive mice (Supplemental Figure S2). Visually, there was a noticeable difference in the number of occlusions between the TDM-IV and TDM-IPIV mice. A manual count of occlusions was conducted using two lung sections per animal. Initial analysis revealed no difference between the mouse

Figure 6 Trichrome stain of lung sections from mice injected with trehalose 6'6-dimycolate (TDM). Lungs from C57BL/6 and BALB/c mice treated with i.p. TDM (TDM-IP), i.v. TDM (TDM-IV), or i.p./i.v. TDM (TDM-IPIV) were processed at day 7, fixed in 10% formalin, sectioned, and stained with Masson's trichrome. Images are at lower magnification for the TDM-IP groups because few pathologic findings are observed and collagen is usually present around blood vessels. Images are at higher magnifications for the TDM-IV and TDM-IPIV groups and focused on occlusion events in blood vessels. Blue indicates collagen. Original magnification: $\times 200$ (top panels); $\times 400$ (middle and bottom panels).

strains; thus, the data were separated only by TDM-IV and TDM-IPIV treatment. The TDM-IPIV groups had a significant increase in number of occlusions compared with the TDM-IV group (Figure 7).

Hypercoagulation in TDM-IPIV Mice

The appearance of vascular occlusion and the experimental observation of blood thickening suggested a change in coagulation status in the TDM-IPIV mice. Using the ROTEM system, mouse (C57BL/6 and BALB/c) whole blood from naive or TDM-IPIV-treated groups was examined for coagulopathy after initiation of clotting via the intrinsic pathway. The thromboelastometric parameters recorded were clotting time (CT), the time from the start of analysis until an initial clot is formed (2-mm amplitude); clot formation time (CFT), the time to reach 20-mm amplitude; and maximum clot firmness (MCF), clot strength and stability. An example of the ROTEM tracing revealed distinct differences between naive and TDM-treated mice (Figure 8A). No significant difference in CT was observed. The TDM-IPIV-treated mice had a significant decrease in CFT and an increase in MCF (Figure 8B), which indicate an increase in coagulation in the TDM-IPIV mice compared with the control group.

TDM-IPIV Model Generates Lung Pathologic Findings Comparable to Human Clinical Disease

Lung tissue samples from patients with TB are difficult to find in the postantibiotic era. Histologic analysis of lung tissue from four autopsy TB individuals was visualized by light microscopy and compared with the TDM-IPIV-induced pathologic findings.

In all four cases analyzed, there was presence of hemorrhage, both interstitial and in the alveolar spaces (Figure 9A). The hemorrhage pattern is comparable to the hemorrhage induced by the TDM-IPIV model in the C57BL/6 mice (Figure 1). In addition, the clinical pathologic features are induced by very few *M. tuberculosis* numbers (Figure 9B), suggesting that perhaps pathologic findings developed in response to *M. tuberculosis* antigens rather than to the high numbers of the *M. tuberculosis* pathogen. In addition, the presence of partial thrombus is evident in two clinical lung samples (Figure 10). These partial thrombi resemble the vessel occlusion pathologic finding observed in the TDM-IV and TDM-IPIV models in both mouse strains.

Discussion

This report describes a new model of TDM-induced pulmonary inflammation pathology. The TDM-IPIV model has many isolated aspects of human TB disease, including influx and aggregation of leukocytes (to form granuloma-like structures), vascular inflammation, and endothelial and alveolar thickening. Experiments administering TDM via the i.v. route reaffirm histologic findings previously identified in the mouse.^{11,12,22,23} Unique features were identified in the TDM-IPIV mice that had distinct patterns of hemorrhage development and hypercoagulation. The TDM-IPIV model produced a more aggressive lung pathologic response compared with the previously published acute TDM

Figure 7 Number of plaque-like formations per lung section. Lungs from C57BL/6 and BALB/c mice treated with i.v. trehalose 6'6-dimycolate (TDM-IV) or i.p./i.v. TDM (TDM-IPIV) were processed at day 7, fixed in 10% formalin, sectioned, and stained with Masson's trichrome. Plaque-like formations were counted manually. Two lung sections from each mouse, with four mice per group, and two repeated experiments were counted for each TDM group. Data were analyzed by the *t*-test ($P = 0.0007$). *** $P < 0.001$.

Figure 8 ROTEM analysis of whole blood in naive and i.p./i.v. trehalose 6'-dimycolate (TDM-IPIV) mice. Whole blood was collected with 3.2% citrate to achieve a final dilution of nine parts blood with one part anticoagulant. Thromboelastometry was performed using ROTEM software version 1.6.0 (Pentapharm, GmbH, Munich, Germany) δ analyzers. Clotting in whole blood was initiated via the intrinsic pathway by the addition of reagents supplied by the manufacturer (in-TEM, diluted 1/10 with saline and 0.2 M CaCl_2 ; TEM Systems Inc, Durham, NC). **A:** ROTEM tracing of one experiment comparing TDM treated (green) and naive/control (red) mice. **B:** ROTEM data from two experimental repeats. Data analyzed by the *t*-test (CFT: $P = 0.046$; MCF: $P = 0.002$). * $P < 0.05$; ** $P < 0.01$. CT, clotting time (time from the start of analysis until an initial clot is formed; 2-mm amplitude); CFT, clot formation time (time to reach 20-mm amplitude); MCF, maximum clot firmness (the length it took to break clot, measures clot strength and stability); TEM, thromboelastometry.

models.^{5,12,22} The pathologic response detailed in this report resembles lung pathologic findings from *M. tuberculosis* infected mice at 19 weeks during the chronic phase of *M. tuberculosis* infection.²⁶

One major difference between *M. tuberculosis*-induced pathologic findings and the model described here is the absence of large clusters of foamy macrophages, which are readily apparent during *M. tuberculosis* infection. The TDM model described in this article generates only small clusters of foamy macrophages. This finding most likely reflects the difference between the innate and adaptive immune response and the host acute and chronic response to *M. tuberculosis* antigens. Foamy macrophages increase in number and foaminess when stimulated by CD4 T cells. In the TDM model, most lymphocytes in the lung tissue are innate lymphocytes, which do not activate macrophages through the major histocompatibility complex mechanism. In essence, the TDM-IPIV model generates an encompassing lung pathologic response in 2 weeks that takes >19 weeks with aerosol challenge infection. Reversing the TDM injection order, TDM-IVIP, does not generate comparable lung pathologic findings and does not induce pulmonary hemorrhage (Supplemental Figure S3). Thus, the TDM-IPIV model is a novel and useful tool to examine the mechanisms of TDM-induced inflammatory pathologic findings and to apply subsequent findings to TB disease in an effort to find novel therapeutics and vaccines.

This TDM-IPIV model is distinctively different compared with the published TDM hypersensitive model. The hypersensitive model sensitization used a 50% oil/50% water emulsion that generates a depot effect for steady antigen release. In addition, the hypersensitive model pushed for the adaptive immune response by incorporating methylated bovine serum albumin and complete Freund's adjuvant in the immunization vehicle. The TDM-IPIV model's sensitization was achieved with 100% oil with no addition of

adjuvants. The hypersensitive model was generated by prior sensitization and immunization of TDM and challenged at 2 weeks at the initiation of the adaptive immune response. The TDM-IPIV model examined the acute response (before 2 weeks). The hypersensitive TDM model generates, as expected, an increase in lymphocytes infiltrating into the lung tissue.^{9–11} The TDM-IPIV model found no increase in the lymphocyte population by histologic analysis, and analysis by flow cytometry revealed a decrease in overall lymphocyte populations. The most important difference was that the TDM-IPIV model induced pulmonary hemorrhage that has not been observed in any other published TDM models.

The route of TDM administration was decided by the pathologic response it would produce in the lung tissue and not to mimic the natural route of infection. In the TDM model, the tail vein injection is effective in delivering the TDM emulsion only to the lung issue because the lung capillary beds are the first to be encountered by TDM when injected through the tail vein.²⁷ This TDM-induced lung pathologic response was not observed in the liver and spleen tissues. In addition, the presence of hemorrhage and vessel occlusion induced by TDM in the lung was not observed elsewhere (Supplemental Figure S4). Thus, this TDM model, although it does not mimic the natural route of *M. tuberculosis* infection, delivers the TDM primarily to the lung tissue and causes pathologic findings only in the lung tissue.

This investigation was conducted in C57BL/6 and BALB/c mice, both commonly used in *M. tuberculosis* infection and TDM models. Several studies have noted significant differences between these strains relative to their resistance to bacterial infection.^{28–32} Our goal was to examine whether these differences in host immunity between C57BL/6 and BALB/c mice would result in lung pathologic variations in response to TDM. Only minor

Figure 9 Human lung samples collected at autopsy from tuberculosis-infected individuals. Four formalin-fixed, paraffin-embedded lung samples collected during routine autopsy was analyzed. **A:** Hematoxylin and eosin stain for lung samples. **B:** Staining for acid fast bacilli of the two bottom lung sample from **A**. Arrows indicate the presence of *Mycobacterium tuberculosis*. Original magnification: $\times 100$ (**A**, top left); $\times 400$ (**A**, top right); $\times 600$ (**A**, bottom row, and **B**).

differences in histologic responses were observed between the two strains of mice. A larger difference was identified in the level of inflammation, as measured by the concentration of cytokines produced in lung tissue. The BALB/c strain responded with 10-fold reduced cytokine levels compared with the C57BL/6 strain. This report indicates that there was little to no difference in the inflammatory trends of TDM-IPIV-induced pathologic findings, but there was a distinct difference in the level of lung cytokine concentration between the two mouse strains. In previously published reports, the TDM concentration used in BALB/c mice is $4\times$ the TDM used in C57BL/6 mice, reaffirming that BALB/c mice has lower immune responses to TDM than C57BL/6 mice.^{3,5,11,22}

Despite the lower inflammation level induced in BALB/c mice, both mouse strains have some similar and some notable differences in the lung cytokine environment. The most significant difference is in the production of IL-1 β . BALB/c mice produced significantly lower levels of IL-1 β in lung tissue as follows: TDM-IP > TDM-IV > TDM-IPIV. This result is potentially explained by the corresponding decrease in neutrophil population in lung tissue. Neutrophils are one of the acute responders against pathogenic infection, and their production of IL-1 β plays essential roles in the initial host protective response.^{33–35} Neutrophils express the receptors for TDM recognition and response via the macrophage inducible

C-type lectin pathway.³⁶ This finding suggests that the main producers of IL-1 β during the acute TDM response are neutrophils, and BALB/c mice experience a decrease in neutrophil presence in lung tissue on application of TDM-IPIV.

The other major difference observed between C57BL/6 and BALB/c lung cytokine environments is in the level of IL-12p40. In conjunction with IL-12p35, IL-12p40 forms the active IL-12p70 cytokine that is critical for differentiation of naive T cells to T-cell helper type 1, which is critical toward protection against *M. tuberculosis* infection and TB disease.³⁷ Most IL-12 production is from macrophages and dendritic cells.³⁸ In the C57BL/6 mice, the increase in IL-12p40 from the TDM-IPIV group reflects the increase in macrophages (CD11b⁺ cells) in the lung tissue. In the BALB/c mice, there was no increase in the level of IL-12p40, and no increase was observed in the macrophage population in the lungs of TDM-IPIV group compared with the TDM-IV group. From these data, the increase in IL-12p40 in lung tissue seems to be directed by the influx of macrophages to the site.

No differences were observed in the percentage population of lymphocyte, myeloid, CD3⁺, CD3[−], CD3⁺CD4⁺, or CD3⁺CD8⁺ cells in lung tissue. Both strains had an increase of CD3[−] cells in lung tissue in the TDM-IV and TDM-IPIV groups, suggesting that most of the lymphocytic cytokines (IFN- γ and IL-17) measured were most likely produced by innate lymphocytes. This observation is in contrast with a recent publication that reported that most of the IL-17-producing lymphocytes were $\gamma\delta$ T cells (by flow cytometry), and their activation is responsible for chemotaxis of neutrophils.⁸ More studies are needed to understand the role of CD3[−] and CD3⁺ lymphocytes during the TDM host response.

The most consistent observations among the TDM-injected mice are vascular inflammation and occlusion. Because no differences were observed between the mouse strains, subsequent analysis was stratified only by TDM treatment. The occlusion structures attached to the blood vessel lumen appeared to be composed of lymphocyte and unactivated monocytes (which without activation appears lymphocyte-like as small round cells with little cytoplasm) and proteins. The trichrome stain revealed extensive collagen deposition in these structures. Remodeling of lung tissue during *M. tuberculosis* infection has been extensively

Figure 10 Presence of thrombus in *Mycobacterium tuberculosis*-infected lungs. Hematoxylin and eosin stain of two formalin-fixed, paraffin-embedded lung samples collected during routine autopsy. Original magnification, $\times 400$.

reported and studied in both clinical cases and animal models.^{39–42} However, no reports have addressed collagen deposition in lung tissue in noninfectious TDM animal models. The data reported in this article suggest TDM in an oil/water emulsion introduced i.v. can induce collagen deposition observed in *M. tuberculosis* infection.

The major difference between the TDM-IV and TDM-IPIV groups is the extent of the vascular inflammation and occlusion. Only the TDM-IPIV group had hemorrhage in the lung. Visually, the TDM-IPIV group had more occluded blood vessels and more occlusion structures in large blood vessels. When actual numbers of these structures were counted per lung section, a significant increase was observed in the TDM-IPIV group compared with the TDM-IV group. The increased number of occlusion structures in the TDM-IPIV group may contribute to potential blood vessel damage that resulted in the observed hemorrhage.

With the observed pathologic changes in blood vessels and the experimental observation of thicker blood, the hypothesis that TDM-IPIV may affect coagulation was proposed. Thromboelastometric analysis (ROTEM) was conducted and compared between mice treated with TDM-IPIV and a naive group. The significant increase in CFT and MCF of the TDM-IPIV group is most likely due to TDM. Hematologic parameters that can affect coagulation in C57BL/6 and BALB/c strains have been previously described.⁴³ Although C57BL/6 mice have higher platelet counts compared with BALB/c mice,⁴³ they both have similar coagulopathy and allow the data from both strains to be treated similarly. Thus, the TDM-induced coagulopathy observed is not due to strain differences in coagulation factors.

Several studies have reported similar coagulation changes in TDM mouse models.⁴⁴ The mechanisms of TDM-induced coagulopathy have only been briefly examined. One proposal suggests that the activity of TDM coated beads requires binding of fibrinogen and the hydrolysis of fibrinogen to fibrin. When TDM beads were exposed to thrombin or to supernatants that contained thrombin-like activity, the TDM-induced pathologic response is decreased.^{45,46} A second proposal suggests that TDM monolayer induces procoagulant activity by acting directly on macrophages and monocytes and inducing production of tissue factor, stimulating activation of the coagulation pathway.^{47,48}

How does the TDM model relate to the clinical disease? The hypothesis is that during the chronic/asymptomatic phase of TB disease, there is a buildup of *M. tuberculosis* antigens, including TDM. The evidence comes from lung tissue collected during autopsy of individuals with untreated or poorly treated TB disease. With the use of immunohistochemistry, the presence of TB antigens is highly positive even in areas with very low numbers of *M. tuberculosis* by acid fast staining. This finding suggests that the clinical lung TB disease can be generated by small numbers of *M. tuberculosis* bacteria producing large quantities of antigen. This finding also suggests that the *M. tuberculosis* antigens may not be readily cleared even as bacterial

numbers decrease. This finding further suggests the possibility that there is a buildup of *M. tuberculosis* antigens in the lung microenvironment. At some stage, this bolus dose of *M. tuberculosis* antigens, including TDM, is exposed to host immunity, setting off an acute response and result in rapid development of pathologic findings.²¹

Currently, no publications address the amount of TDM that can be continuously produced by *M. tuberculosis*. *In vitro* studies on clinical *M. tuberculosis* isolates found that isolates collected from pulmonary sites produce a mean of 5 to 8 µg of TDM per gram of wet weight of *M. tuberculosis* compared with isolates collected from extrapulmonary sites that produce a mean of 1 to 2 µg of TDM per gram of wet weight of *M. tuberculosis* (R. Arora, unpublished data). This finding suggests that the *M. tuberculosis* in the pulmonary site produces high levels of TDM and that the 10-µg TDM concentration used in the TDM-IPIV model is not out of physiologic concentrations possible during clinical infection.

Comparison with lung tissue collected during autopsy of untreated or poorly treated individuals clearly indicates that the TDM-IPIV model—induced pathologic response resembles that observed during clinical TB disease. TB patients have been reported to develop a hypercoagulation state, which increases patient mortality.^{49–51} Analysis of coagulation factors indicates that patients with active TB have increased factors and decreased factors of the anticoagulation pathway, the latter resulting in decreased levels of activated protein C. This overall increase in coagulation also stimulated production of fibrinolytic factors, increasing plasmin levels. In addition, endothelium in patients with TB also revealed an enhanced ability to bind platelets through the increase in von Willebrand factor and a decrease in ADAMST13.⁵² The hypercoagulation state has also been observed in TB animal infection models with similar observational changes.⁵³ Although no mechanism has been proposed for how *M. tuberculosis* infection leads to hypercoagulation, the data presented here suggest that TDM may be responsible. In laboratory settings, TDM is easily removed from the *M. tuberculosis* cell wall without affecting bacteria viability. In addition, *M. tuberculosis* will replenish the removed TDM within 1 week.⁶ It has been hypothesized that during *M. tuberculosis* infection in patients, TDM is readily freed from the cell wall and continuously replenished. The freed TDM is hypothesized to be a major cause of human lung pathologic findings.

These studies present a new noninfectious TDM model that produces extensive lung pathologic findings that are typically observed in chronic *M. tuberculosis* infectious mouse models and in patients with active TB. This model has several limitations. Mainly, this TDM model is a model for the acute response to a *M. tuberculosis* antigen. It is hypothesized that during clinical TB disease, the acute response occurs in isolated points during chronic infection when the *M. tuberculosis* antigens suddenly promote an extreme reaction from host immunity. This acute response during the chronic infection is hypothesized to result in the clinical observations of disease activation, blood in sputum,

and hypercoagulopathy. The TDM-IPIV model revealed an increase in vascular inflammation and occlusion and collagen deposition that may be linked to an increase in coagulation state. There is no correlation with the inflammatory response induced by TDM, suggesting other mechanisms may be involved. Further studies are now ongoing to examine the TDM mechanism to promote coagulation and whether this can lead to tissue remodeling and collagen deposition often observed in patients with TB. This TDM-IPIV model is a useful tool that produces consistent lung pathologic findings within a short time (2 weeks) in which the role of TDM during *M. tuberculosis* infection can be investigated.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.12.019>.

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