Fibrinogen Regulates the Cytotoxicity of Mycobacterial Trehalose Dimycolate but Is Not Required for Cell Recruitment, Cytokine Response, or Control of Mycobacterial Infection\textsuperscript{\textdagger}

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Received 22 April 2009/Returned for modification 19 June 2009/Accepted 25 November 2009

During inflammatory responses and wound healing, the conversion of soluble fibrinogen to fibrin, an insoluble extracellular matrix, long has been assumed to create a scaffold for the migration of leukocytes and fibroblasts. Previous studies concluded that fibrinogen is a necessary cofactor for mycobacterial trehalose 6,6\textsuperscript{-}dimycolate-induced responses, because trehalose dimycolate-coated beads, to which fibrinogen was adsorbed, were more inflammatory than those to which other plasma proteins were adsorbed. Herein, we investigate roles for fibrinogen in an in vivo model of mycobacterial granuloma formation and in infection with \textit{Mycobacterium tuberculosis}, the causative agent of tuberculosis. In wild-type mice, the subcutaneous injection of trehalose dimycolate-coated polystyrene microspheres, suspended within Matrigel, elicited a pyogranulomatous response during the course of 12 days. In fibrinogen-deficient mice, neutrophils were recruited but a more supplicative lesion developed, with the marked degradation and disintegration of the matrix. Compared to that in wild-type mice, the early formation of granulation tissue in fibrinogen-deficient mice was edematous, hypocellular, and disorganized. These deficiencies were complemented by the addition of exogenous fibrinogen. The absence of fibrinogen had no effect on cell recruitment or cytokine production in response to trehalose dimycolate, nor was there a difference in lung histopathology or overall bacterial burden in mice infected with \textit{Mycobacterium tuberculosis}. In this model, fibrinogen was not required for cell recruitment, cytokine response, or response to infection, but it promoted granulation tissue formation and suppressed leukocyte necrosis.

Fibrinogen is an approximately 340-kDa circulating glycoprotein, a heterotrimer of \textit{\alphaA}, \textit{\betaB}, and \textit{\gamma} chains (35). This plasma protein is produced primarily in the liver by hepatocytes, with strong upregulation in response to proinflammatory agents (16, 20, 35). The role of fibrinogen in the coagulation cascade is well characterized, with the cleavage of fibrinogen by thrombin to form fibrin, which then is covalently cross-linked by activated Factor XIII to form the rigid, hemostatic fibrin clot. Less well-known are the other functions of fibrinogen, such as the mediation of platelet spreading, the promotion of angiogenesis, and the stimulation of fibroblast proliferation (3, 7, 14, 22, 34). Fibrinogen also is thought to provide a scaffold along which leukocytes can migrate, and stimulated monocytes and neutrophils express a high-affinity receptor for fibrinogen, the integrin \textit{\alphaM\beta}2 (Mac-1) (1).

\textit{Mycobacterium tuberculosis} is the leading cause of death due to bacterial infection and currently is estimated to infect a third of the world population (10, 12). In the context of tuberculous infection, fibrinogen has been shown to be upregulated during experimental tuberculosis in mouse models and in natural human infections, as occurs in other infectious diseases, resulting in a hypercoagulable state (15, 30). Fibrinogen also has been identified as a cofactor for the pathological effects of mycobacterial trehalose 6,6\textsuperscript{-}dimycolate (TDM). Retzinger et al. demonstrated that TDM adsorbed fibrinogen preferentially to the exclusion of other plasma proteins, which increased the pyroganulomatous response to TDM (25). TDM is a predominant mycobacterial cell wall component and an important virulence factor for \textit{M. tuberculosis}, in that it is granulomagenic, can delay phagosome maturation, and is an active component of complete Freund’s Adjuvant (2, 17, 18, 32). The presentation of TDM as a higher-order polymer, as a monolayer either in oil-water emulsions or on polystyrene particles, is required for maximal bioactivity (13, 26). We use a bead-based delivery model using 90-\textmu m polystyrene microspheres suspended within Matrigel, a commercially available mixture of extracellular matrix components, to study the contributions of individual lipids to the inflammatory response to mycobacteria (13, 27).

The development of a fibrinogen \textit{\alphaA} chain-deficient mouse (Fib knockout [KO]) has facilitated the direct study of the roles of fibrinogen in inflammation (36). The use of this mouse strain has revealed the roles of fibrinogen in the limitation of \textit{Listeria monocytogenes} growth in vivo (21), in the exacerbation of crescentic glomerulonephritis (9), in the organization of wound healing and wound stability (8), in tumor metastasis (23), and in cell adhesion to biomaterials (6). On the other

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\textsuperscript{\textdagger} Published ahead of print on 22 December 2009.
hand, a study of bleomycin-induced pulmonary fibrosis in Fib KO mice showed that fibrosis developed independently of fibrinogen, and the absence of fibrinogen increased the presence of neutrophils (40).

In this study, we use a subcutaneous granuloma model in Fib KO mice to determine whether fibrinogen is necessary for the inflammatory response to TDM. Our results show that while fibrinogen is important for the organized formation of granulation tissue, fibrinogen deficiency has no effect on leukocyte recruitment to TDM-coated beads or proinflammatory cytokine production by the recruited cells. Fib KO mice also show no differences in pulmonary histopathology and only a transient difference in pulmonary bacterial burden in response to intravenous infection with M. tuberculosis. However, Fib KO mice show a suppurative response to TDM, resulting from the exacerbation of neutrophil necrosis and matrix degradation, whereas wild-type (WT) mice develop a granulomatous response with less cell degeneration, necrosis, and matrix degradation. These results show that fibrinogen is required neither as a co-factor for initiating an inflammatory response to TDM nor for controlling infection with M. tuberculosis, but rather suppresses the cytotoxic effects of TDM on recruited neutrophils while promoting the formation of granulation tissue.

MATERIALS AND METHODS

Mice and cell culture. Fib KO mice (36) that were backcrossed seven generations to C57BL/6 mice were generously supplied by Jay Degern (Children's Hospital Research Foundation, Cincinnati, OH) and were bred at the Cornell University Transgenic Mouse Core Facility. Taul snip genotyping was used to identify the Fib KO, heterozygous (HET), and WT mice. Two- to 7-month-old male and female mice were used for all in vivo experiments with the randomization of age and sex of individuals within and between treatment groups. Mice were weighed immediately after injection and every other day thereafter. Animals were housed in a specific-pathogen-free animal facility. The Cornell University Institutional Animal Care and Use Committee reviewed and approved all techniques used in these experiments. Murine bone marrow-derived macrophages (BMMφ) were cultured as described previously (13).

Neutrophils were harvested from WT bone marrow using fluorescein isothiocyanate (FITC)-labeled, anti-mouse Gr-1/Ly-6G antibody (1A8; BD Biosciences, San Jose, CA) and anti-FITC magnetic beads (Miltenyi Biotec, Gladbach, Germany), followed by positive selection using an LS magnetic column in a Midi Flex (Miltenyi Biotec, Auburn, CA). Neutrophils were harvested from WT bone marrow using fluorescein isothiocyanate (FITC)-labeled, anti-mouse Gr-1/Ly-6G antibody (1A8; BD Biosciences, San Jose, CA) and anti-FITC magnetic beads (Miltenyi Biotec, Gladbach, Germany), followed by positive selection using an LS magnetic column in a Midi Flex (Miltenyi Biotec, Auburn, CA). Neutrophils were harvested from WT bone marrow using fluorescein isothiocyanate (FITC)-labeled, anti-mouse Gr-1/Ly-6G antibody (1A8; BD Biosciences, San Jose, CA) and anti-FITC magnetic beads (Miltenyi Biotec, Gladbach, Germany), followed by positive selection using an LS magnetic column in a Midi Flex (Miltenyi Biotec, Auburn, CA).

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Histology, immunohistochemistry, and immunofluorescence. A portion of representative matrices was fixed immediately in 4% paraformaldehyde in PBS for at least 24 h at 4°C, followed by transfer to 70% ethanol for at least 24 h before submission for processing and sectioning by the Cornell University College of Veterinary Medicine Histology Laboratory. Briefly, samples were embedded in paraffin, sectioned at 4 μm thickness, and stained with either hematoxylin and eosin (H&E) or Masson’s trichrome stain. For caspase 3 detection, sections of 7-day-old TDM matrices from Fib KO mice were incubated in 0.1% citrate buffer, pH 6.0, in a microwave for 2-10 min intervals at 800 W for antigen retrieval and then incubated for 2 h at 37°C with rabbit anti-caspase-3 diluted 1:50 in PBS-1% casein. Biotinylated goat anti-rabbit IgG H+L (Vector, Burlingame CA) was applied for 20 min at room temperature, and slides were washed and incubated for 20 min with streptavidin peroxidase (Zymed). AEC substrate was prepared as directed (Zymed kit) and applied for 10 min. Slides were counterstained with Gill’s #2 hematoxylin and mounted with Fluoromount G (both from Fisher Scientific, Fremont, CA). Negative controls were included, substituting normal rabbit IgG (Vector) for the primary antibody at the appropriate dilution equal to the final concentration of primary antibody.

For immunofluorescence, sections of 7-day-old TDM matrices from Fib KO mice were incubated in 1:50 dilutions of rabbit anti-human neutrophil elastase ( Fitzgerald Laboratories, Inc., Concord, MA) and mouse anti-human histone H1 (Acris Antibodies, Herford, Germany) or no primary antibody (negative control)
tools were used on all of the images equally. Photoshop CS2 version 9.0.2 (Adobe Systems Inc., San Jose, CA). Autocontrast SP2 (Carl Zeiss MicroImaging GmbH). Images were processed using Adobe (Carl Zeiss, Jena, Germany). Images were created using LSM 510 version 4.0 (Carl Zeiss, Jena, Germany) immediately before being mounted in 9% neutral buffered formalin overnight at 4°C, and then they were wetted with 1:1,000 Draq5 (Biostatus Ltd., Leicestershire, United Kingdom) immediately before being mounted in 9% Mowiol 4-88 (Calbiochem, La Jolla, CA) in glycerol-Tris (1:4, vol/vol). Confocal microscopy was performed using an AxioImager M1 microscope with a laser-scanning microscope (LSM) 510 Meta confocal head with an EC PLAN Neofluor (×40 magnification, 1.3 numeric aperture) oil lens and Argon2 and HeNe lasers (Carl Zeiss, Jena, Germany). Images were created using LSM 510 version 4.0 SP2 (Carl Zeiss MicroImaging GmbH). Images were processed using Adobe Photoshop CS2 version 9.0.2 (Adobe Systems Inc., San Jose, CA). Autocontrast tools were used on all of the images equally.

*M. tuberculosis* intravenous infection model. Two-month old, female Fib KO and HET mice that were bred and maintained at the Trudeau Institute were used for this experiment. *M. tuberculosis* strain H37Rv was grown as previously described (29). Mice were injected intravenously via the tail vein at a final dose per mouse of 1 × 10^5 CFU in saline. Mice were euthanized by CO₂ narcosis, and samples of lung, spleen, and liver then were individually homogenized in physiological saline, and serial dilutions of the organ homogenate were plated on nutrient 7H11 agar. Bacterial CFU were counted after 3 weeks of incubation at 37°C (29). A caudal lung lobe was perfused with 10% formalin–saline and fixed for 1 week. Tissues were sent to Colorado Histoprep (Fort Collins, CO) for routine paraffin embedding, serial sectioning, and staining using H&E and acid-fast stains.

Statistical methods. All cell number calculations and cytokine measurements were performed for individual mice, and the standard deviations (SD) from the mean values (n = 4 or 5) are presented. The statistical significance of differences in the means between Fib KO and HET or WT mice was calculated using a Student’s t-test.

RESULTS

The response to BCG TDM in the subcutaneous compartment of WT mice is pyogranulomatous, as opposed to suppurative, in Fib KO mice. Previously, we developed a peritoneal granuloma model to study the immunostimulatory activity of different mycobacterial lipids (13). In preliminary experiments, the injection of TDM matrices into the peritoneal cavity of Fib KO mice resulted in fragmented pieces instead of the cohesive, adherent matrices typically observed in our peritoneal granuloma model in WT mice (13 and data not shown). As the increased exposed surface area of TDM-bearing matrix could skew cell recruitment to the matrices, we opted for subcutaneous injection, wherein the matrices would be trapped as a single mass in the subcutis.

By histologic examination, neutrophils were the primary leukocyte recruited to the TDM beads in WT mice at 4 and 7 days, with a shift toward increasing numbers of macrophages beginning at 7 days and then a predominant macrophage response at 12 days, with occasional multinucleated giant cells (Fig. 1A to C). By day 12, scattered foci within, but not limited to, the center of the granulomas also contained eosinophilic ghost cells of recruited neutrophils (red arrows) is prominent in panel F. Original magnification, ×1,000; bar = 50 μm.

FIG. 1. Subcutaneous TDM matrices in WT mice are primarily neutrophilic at early time points but later become pyogranulomatous to granulomatous, while Fib KO matrices become progressively suppurative. WT and Fib KO mice (n = 4 to 5 per time point) were injected subcutaneously in the scruff with 300 μl of TDM-coated 90-μm-diameter microspheres suspended within Matrigel. Matrices were removed for analysis at days 4 (A and E), 7 (B and F), and 12 (C, D, and G). Representative samples were fixed, sectioned, and stained with H&E. Photomicrographs represent leukocytic infiltrates in WT matrices (A to D) and in Fib KO matrices (E to G) typical for the depicted time point and are representative of three separate experiments. Multinucleated giant cells (black arrows) are present in panel C. An area showing neutrophil degeneration and necrosis, as well as peripheral eosinophilic ghost cells, as observed sporadically in 12-day-old TDM matrices from WT mice, is shown in panel D. The nuclear streaming of recruited neutrophils (red arrows) is prominent in panel F. Original magnification, ×1,000; bar = 50 μm.

By histologic examination, neutrophils were the primary leukocyte recruited to the TDM beads in WT mice at 4 and 7 days, with a shift toward increasing numbers of macrophages beginning at 7 days and then a predominant macrophage response at 12 days, with occasional multinucleated giant cells (Fig. 1A to C). By day 12, scattered foci within, but not limited to, the center of the granulomas also contained eosinophilic ghost cells of recruited neutrophils and macrophages, accompanied by the degradation of the matrix, resembling early features of caseous necrosis (Fig. 1D). These histologic features, an early neutrophilic response developing into pyogranulomatous inflammation, are similar to those reported in our peritoneal granuloma model (13), differing only in slower kinetics.

At 4 days, the beads in granulomas from Fib KO mice were cuffed by moderate numbers of neutrophils, similarly to those in the WT and HET mice (Fig. 1E). By day 7, however, severe neutrophilic infiltration, with the degeneration and necrosis of these leukocytes and nuclear streaming, was the predominant feature in these matrices (Fig. 1F). These features were accompanied by the degradation of the Matrigel, characterized by the contraction of the fractured pieces and hypereosinophilic staining. At day 12, the centers of some of these matrices were completely degraded and difficult to examine histologically, as much of the liquefied material was washed away during processing (Fig. 1G). The increased liquefactive necrosis and destruction of the matrix observed in Fib KO mice shows that fibrinogen suppresses the cytotoxic effects of TDM or improves the survival of neutrophils in this subcutaneous model.

Fibrinogen is required for early organized granulation tissue formation, and this deficiency can be complemented by
exogenous fibrinogen. In WT and HET mice, adherent, neovascularized, pale yellow-white, translucent granulomas formed by day 4 and developed a pale pink capsule by day 12 (Fig. 2A to C and data not shown). Matrices injected into Fib KO mice also were pale yellow and adherent to the surrounding tissues; however, at days 4 and 7, they generally were less vascularized than those in WT mice (Fig. 2D and E). By day 12, however, the matrices in Fib KO mice were covered by a pale pink, well-vascularized capsule, grossly indistinguishable from those in the WT or HET mice (Fig. 2F). In contrast to the WT and HET mouse granulomas, the centers of KO granulomas occasionally were white, liquified, and oozed from the cut surface.

The histologic examination of the periphery of 4-day-old TDM matrices in WT or Fib KO mice revealed loosely arranged but parallel fibroblasts interspersed with neutrophils and macrophages (Fig. 3A and D). Well-developed granulation tissue was present around the WT matrices by day 7, with the characteristic parallel sheets of fibroblasts perpendicularly transected by immature blood vessels and interspersed with neutrophils, macrophages, and lymphocytes (Fig. 3B). By day 12, mature collagen could be observed surrounding granulomas from these mice (Fig. 3C). Fib KO mice, on the other hand, did not develop well-organized granulation tissue until day 12. Tissues surrounding 7-day-old matrices instead were edematous, hypocellular, and poorly vascularized (Fig. 3E). This delay in the development of granulation tissue in Fib KO mice at 7 days was complemented by the addition of 500 μg/ml of fibrinogen (Sigma) to the matrix prior to injection (Fig. 3F). This dose of exogenous fibrinogen was selected because it was the highest circulating concentration of fibrinogen reported during experimental Candida peritonitis in mice (28) and was the highest dose that could be added to the matrix without diluting it significantly. These results show that fibrinogen aids the early formation of granulation tissue but is not required for capsule formation. Interestingly, the addition of fibrinogen did not reduce the recruited leukocyte death in Fib KO matrices, as determined qualitatively by histopathology (data not shown).

Fibrinogen protects neutrophils from TDM-induced necrosis in vitro. To determine the mechanism of cell death induced by TDM in Fib KO mice, and because fibrinogen has been reported previously to delay apoptosis (31), sections of 7-day-old TDM matrices from Fib KO mice were stained for activated caspase 3, an effector caspase in the apoptosis pathway. No staining above that of negative controls was observed (data not shown), supporting the histopathologic evidence that apoptosis was not the mechanism by which recruited neutrophils were dying. We therefore proceeded to study this mechanism in vitro, using neutrophils harvested from murine bone marrow. Purified neutrophils were exposed to wells coated with TDM, vehicle control (n-hexane), or 60 μM staurosporine for 1, 2, 5, and 18 h in the presence or absence of 500 μg/ml fibrinogen, and then they were stained with APC-annexin V and propidium iodide (PI) to determine the percentages of cells that were apoptotic or necrotic, respectively, by flow cytometry (Fig. 4 and data not shown). Although induced necrosis overall was low, statistical analysis showed significantly greater necrosis ($P < 0.05$) in response to TDM-coated wells at 2 h in the absence of fibrinogen (Fig. 4A). n-hexane-treated wells did induce a background level of necrosis; however, it was significantly less ($P < 0.05$) than that induced by TDM (Fig. 4A). Fibrinogen also was significantly protective against necrosis in the n-hexane-treated neutrophils. TDM induced negligible neutrophil apoptosis at all of the time points studied (data not shown). Fibrinogen also was protective in decreasing staurosporine-induced apoptosis in neutrophils at 2 h (Fig. 4B), as has been reported previously (31). Fibrinogen, therefore, plays
Neutrophil NETs are induced by TDM in the absence of fibrinogen. The nuclear streaming that was a predominant feature of the 7-day-old TDM matrices in Fib KO mice was histologically similar to reports of neutrophil extracellular traps (NETs), which are extruded neutrophil DNA and histones complexed with neutrophil elastases that together have antibacterial activity (4). Furthermore, *M. tuberculosis* recently has been reported to induce NET formation in vitro (24). To investigate the possibility that this lesion was consistent with neutrophil NETs and that TDM could be a mycobacterial factor inducing this response, we performed immunofluorescence on sections of Fib KO matrices using anti-neutrophil elastase, anti-histone H1 antibodies, and the DNA stain Draq5 and examined them for colocalization by confocal microscopy. Strong Draq5 and anti-histone H1 staining of the streaming material confirmed the presence of DNA and histone H1 (Fig. 5A and E), while elastase was present both associated with this DNA as well as in the cytoplasm of surrounding intact neutrophils (Fig. 5C). These results suggest that TDM is stimulating the formation of NETs, particularly in the absence of fibrinogen, and that fibrinogen plays a role in the regulation of NETs.

**Fibrinogen is not required for cell recruitment or cytokine response to TDM, or for responses to infection with *M. tuberculosis***. The flow-cytometric analysis of cells harvested from granulomas in three separate experiments showed no differences in the number of leukocytes in matrices from WT or HET mice compared to those from Fib KO mice (data not shown). Leukocytes that were analyzed included neutrophils, macrophages, B cells, T cells (CD4+, CD8+, and γδTCR subsets), and eosinophils that were identified by the combinations of surface markers outlined in Materials and Methods (data not shown). Matrices supplemented with exogenous fibrinogen showed no differences in cell recruitment compared to that of unsupplemented matrices from Fib KO mice (data not shown). Levels of proinflammatory cytokines (TNF-α, IL-6, IL-1α, and IL-1β) were measured in supernatants of cells cultured from explanted matrices. ELISA results showed no consistently significant differences in the cytokines analyzed between the WT and HET groups or the Fib KO group at any of the time points (data not shown). These data suggest that the cell recruitment and cytokine responses to TDM are not dependent upon the presence of fibrinogen.

To test the overall importance of fibrinogen in the context of *M. tuberculosis* infection, Fib KO and HET mice were infected by tail vein injection, and tissues were taken at days 1, 8, 17, 30, and 60 for histopathology and bacterial enumeration. No histopathologic differences were observed in the lung, liver, or spleen at any of the time points (data not shown). A single statistically significant (*P* < 0.05) difference in the load of viable bacteria was observed at 17 days in the lung, with lower numbers in the Fib KO mice (3.57 log10 CFU ± 0.05 for Fib KO mice and 3.89 log10 CFU ± 0.19 for HET mice) (Table 1). A low-dose (100 CFU) aerosol infection experiment also was performed as described in Mogues et al., and no significant differences in CFU or histopathology were observed at 50 days postinfection (R. North, personal communication) (19). These results suggest that fibrinogen is required for neither immune...
responses to TDM nor the overall control of infection with *M. tuberculosis*.

**DISCUSSION**

The tuberculosis granuloma represents a complex dance between the bacillus and the host. Mycobacterial components stimulate a robust but organized leukocyte response that serves to contain the mycobacteria while not eradicating them completely, allowing the host and parasite to coexist for decades. However, some granulomas in some individuals progress to necrosis and caseation, facilitating the release of bacteria into the airways and, therefore, transmission. The factors that determine whether this progression occurs currently are unknown. TDM, a strong proinflammatory mycobacterial cell wall component, is postulated to play a dominant role in driving granuloma development and breakdown.

Fibrinogen is a critical component of the acute inflammatory response and forms a scaffold along which leukocytes, endothelial cells, and fibroblasts could migrate in order to begin the repair process. Fibrinogen has been described as a necessary cofactor for the toxic and proinflammatory activities of TDM (25). This would have predicted that Fib KO mice would mount a lesser inflammatory response to TDM. Interestingly, Fib KO mice showed exacerbated immunopathology, no reduction in either cell recruitment or proinflammatory cytokine response to TDM-coated particles, and no clear phenotype in intravenous or aerosol infection with *M. tuberculosis*. The response in Fib KO mice to TDM was more suppurative, which, while not appreciated by FACS analysis, clearly could be observed by histopathology. This discrepancy likely is due to the exclusion of dead (propidium iodide-positive) cells and debris in the FACS analysis. Slight kinetic differences were observed in the granulation tissue and neovascularization response to these matrices, processes that are well documented in the literature to rely on fibrinogen (5, 8, 33). This disorga-
TABLE 1. Absence of fibrinogen results in only a slight, transient decrease in liver bacterial burden on day 17 of infection with M. tuberculosis

<table>
<thead>
<tr>
<th>Day</th>
<th>Group</th>
<th>Lung Log_{10} CFU per organ</th>
<th>Spleen Log_{10} CFU per organ</th>
<th>Liver Log_{10} CFU per organ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HET</td>
<td>1.58 ± 0.27</td>
<td></td>
<td>2.53 ± 0.27</td>
</tr>
<tr>
<td>8</td>
<td>HET</td>
<td>5.84 ± 0.17</td>
<td>5.00 ± 0.14</td>
<td>2.48 ± 0.33</td>
</tr>
<tr>
<td>17</td>
<td>HET</td>
<td>6.03 ± 0.21</td>
<td>4.96 ± 0.19</td>
<td>3.89 ± 0.19</td>
</tr>
<tr>
<td>30</td>
<td>KO</td>
<td>5.93 ± 0.14</td>
<td>4.99 ± 0.16</td>
<td>3.57 ± 0.05b</td>
</tr>
<tr>
<td>60</td>
<td>HET</td>
<td>5.41 ± 0.06</td>
<td>4.3 ± 0.00</td>
<td>4.68 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>5.71 ± 0.18</td>
<td>4.32 ± 0.28</td>
<td>4.83 ± 0.02</td>
</tr>
</tbody>
</table>

a Values are means ± standard deviations (4 mice per group) for M. tuberculosis CFU in organ homogenates from Fib HET or KO mice.

b Significantly different (P < 0.05) from the value for HET mice at 17 days of infection as calculated by Student’s t test.

The leukocyte death that was a predominant feature of the TDM matrices in Fib KO mice also may be the result of reduced neovascularization. In this model, we could only qualitatively assess neovascularization, at necropsy and by histopathology. Whereas 4- and 7-day-old matrices from Fib KO mice seemed less vascularized grossly and histologically, by 12 days no differences were observed between Fib KO and WT mice. It is possible that this delay resulted in a reduced oxygen supply to the center of the Fib KO matrices, causing necrosis secondary to hypoxia. In this case, the cytotoxic effect of TDM in the absence of fibrinogen would be an indirect result of the delay in neovascularization. A direct cytotoxic effect of TDM on neutrophils, however, was shown in vitro (Fig. 4A). Furthermore, necrosis was not limited to the centers of the Fib KO matrices but occasionally was observed surrounding TDM beads at the periphery and also within WT matrices that were well vascularized (Fig. 1D). Matrix degradation in the presence of fibrinogen, as studied by in situ zymography, is due primarily to matrix metalloproteinase 9 activity (K. Sakamoto, unpublished results), and certainly neutrophil elastases may play a role (Fig. 5); however, the mechanism of TDM-induced necrosis in the absence of fibrinogen needs further study.

Fibrinogen also may enhance the survival of recruited leukocytes. Studies have shown that the binding of fibrinogen to the integrin receptor αβ2/Mac-1 is critical for leukocyte function and regulates apoptosis in neutrophils (11, 38, 39). The immunohistochemical staining of TDM matrices from both Fib KO and WT mice, however, showed no activated caspase 3 staining, suggesting that leukocyte death in this case is primarily by necrosis. Furthermore, bone marrow-derived neutrophils stimulated with TDM in vitro showed a significantly higher percentage of necrotic, but not apoptotic, cells in the absence of fibrinogen (Fig. 4A). The histopathologic appearance of dead neutrophils, along with the colocalization of neutrophil histone H1, elastase, and DNA in these lesions, suggests that TDM induces the formation of NETs in the absence of fibrinogen (Fig. 1F and 5). Why greater leukocyte necrosis and NET formation are observed in the absence of fibrinogen, however, still is unclear. The study of TDM effects in mice with a targeted deletion of the αβ2 binding motif within the fibrinogen γ chain may help to answer this question (11).

There are many events that occur in the tuberculosis granuloma, and it is thought that not all are productive for the containment or resolution of the infection. TDM is thought to be a virulence factor because it reproduces several symptoms of tuberculosis, namely, the excessive production of proinflammatory cytokines, cachexia, weight loss, and local necrosis (and in some cases, caseous necrosis in mice) (17). In other reports, we have demonstrated that although TDM triggers granulogenic events, they often are excessive and unregulated compared to the responses elicited by intact bacilli (13). Notably, TDM elicits the excessive production of TNF-α and recruits large numbers of granulocytes, events associated with destructive tissue remodeling and toxicity in mice. The mechanisms that drive these pathological events are unknown. Our current findings indicate that fibrinogen regulates the response to TDM in at least two ways: (i) fibrinogen contributes to limiting
leukocyte necrosis, and (ii) fibrinogen contributes to the formation of granulation tissue that may serve to contain the response.

ACKNOWLEDGMENTS

We thank Jay Degen for helpful feedback and providing us with the F6 KO mice and Patricia Fisher for the immunohistochemical analysis of the matrices for activated caspase 3. We also thank the Cornell University Transgenic Mouse Core Facility staff, in particular Kathy Mott for maintaining our F6 KO colony. At the University of Georgia, we thank Julie Nelson and Jamie Barber for technical help.

This work was supported by U.S. Public Health Service grant HL055936 to D. Russell. K. Sakamoto was funded during this study by a Ruth L. Kirschstein National Research Service Award (F32 GM074462-02). The authors have no conflicting financial interests.

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