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Trehalose 6, 6'dimycolate (cord factor) induced pulmonary granuloma in naive and lymphopenic mice

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In this study, histopathological features of pulmonary granuloma induced by trehalose 6, 6'dimycolate (TDM) of *Mycobacterium tuberculosis* bacilli in naive and lymphopenic mice are documented. Simultaneously, (a) proinflammatory cytokine (tumour necrosis factor alpha -TNF-α) and macrophage chemotactic factor (macrophage inflammatory protein 1 alpha – MIP-1α) concentrations in the lung homogenates were estimated by a sandwich enzyme-linked immunosorbent assay (ELISA), (b) chemotactic response of mouse peritoneal macrophages to TDM, are assayed. Non-caseating histiocytic granulomas in lungs as well as elevated TNF-α and MIP-1α concentrations in the lung homogenates were observed in naive mice. By contrast, lymphopenic mice showed no demonstrable granulomatous response to TDM and there was marked decrease in TNF-α and MIP1-α concentrations in the lung homogenates. The peritoneal macrophages of naive mice demonstrated optimum chemotactic response to TDM *in vitro,* while peritoneal macrophages of lymphopenic mice showed suboptimal chemotactic response to TDM. The results of this experimental study indicated that: (a) the pulmonary granuloma in mouse following TDM administration depends not only upon the production of TNF-α and MIP-1α in optimal amounts by the host, but also their participation during the active stages of granuloma formation; (b) TDM promotes chemotaxis of peritoneal macrophages in naive mice *in vitro* and this is facilitated by the MIP-1α.

Key words: *Mycobacterium tuberculosis*, trehalose 6, 6' dimycolate, cord factor, pulmonary granuloma, tumour necrosis factor alpha, macrophage inflammatory protein 1 alpha, lymphopenia, dexamethasone.

INTRODUCTION

Granulomatous inflammation is the most characteristic histopathological feature in infection caused by acid-fast bacilli *Mycobacterium tuberculosis*. However, granuloma formation in a tuberculous lesion is a complex immune-mediated process that requires not the only the activation of thymus dependent lymphocytes and macrophages, but also their migration as well as their active participation at the site of mycobacterial infection. Chemotactic factors released by C5a fraction of complement as well as by macrophages also contribute to the granuloma formation during active stages of tuberculous infection.

Trehalose 6, 6' dimycolate (TDM – cord factor) is a pleiotrophic molecule and one of the mycoloyl glycolipids of *M. tuberculosis* bacilli. TDM plays an important role in the immunopathogenesis of tuberculosis and is well known to: (i) promote cytokine and chemokine interaction (Algood et al., 2005); (ii) contribute to the innate macrophage response of in the host (Indrigo et al., 2002); (iii) activate the alternative pathway of complement (Ramanathan et al., 1980). By the combination of the aforementioned, TDM contributes to the development of pulmonary granulomas in mice models (Yamagami et al., 2001; Hunter et al., 2006; Guidry et al., 2007). However, it has also been documented that TDM in rabbit model induces atrophy of lymphoid cells in the thymus and spleen (Hamasaki et al., 2000). Thus, immunological mechanisms mediated by TDM differ in mouse and rabbit models for tuberculosis.

In this experimental study, the precise role of TDM for

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Figure 1. Thin layer chromatogram showing cord factor antigen. Lane 1: Cord factor isolated from M tuberculosis H_{37} R_v strain in the laboratory. Lane 2: Reference cord factor antigen supplied by the Colorado State University, USA.

the genesis of pulmonary granuloma in naïve and lymphopenic mice is being investigated. The sequential histopathological features following TDM administration are correlated with tumour necrosis factor alpha (TNF-α) and macrophage inflammatory protein 1 alpha (MIP-1α) concentration in lung homogenates as well as chemotactic response of mouse peritoneal macrophages elicited by TDM *in vitro*. The results of this experimental study and their possible clinical significance in patients with pulmonary tuberculosis are discussed subsequently.

MATERIALS AND METHODS

Isolation of TDM from *M. tuberculosis*

M tuberculosis $H_{37}R_v$ strain was grown in Sauton's medium for 6 to 8 weeks. At optimum growth, the cultures autoclaved and bacillary sediments in pellet form were recovered by centrifugation. The autoclaved pellets were weighed and sonicated in chloroform/ methanol (4:1, vol/vol) for 15 min at +4°C. Deionized distilled water was added (1:20, total volume) and the aqueous phase was sequentially extracted with chloroform/ methanol (3:1 and 2:1, vol/vol), centrifuged at 5000 G for 30 min and the deposit was allowed to dry at room temperature, and was treated with acetone. The insoluble phase containing TDM was separated by repeated centrifugation. The TDM fraction was precipitated by 'drop-wise' addition of methanol at +4°C and then dissolved in tetrahydrofuran and reprecipitated by 'drop-wise' addition of methanol at 4°C to a final ratio of 1: 2 tetrahydrofuran / methanol (vol/vol). The precipitated TDM fraction was then dissolved in chloroform/acetone (8:2, vol/vol) and then loaded onto a column of silica gel, and TDM was eluted with chloroform/methanol (9:1, vol/vol). The elute containing TDM was weighed and the purity was assessed by thin layer chromatography (TLC) using 10 × 10 cm silica plates in comparison with reference cord factor obtained from Colorado State University, USA (Figure 1). TDM at concentrations of 1000 ng/ml were dispensed in *n*-hexane and stored in sterile aliquots at +4°C.

Antibody to TDM

Two adult male albino rabbits were immunized intramuscularly with 300 µg of TDM antigen mixed thoroughly in: (a) 1 ml of 0.1 M phosphate-buffered-saline (PBS) containing 0.2% tween-80; and (b) 1 ml of incomplete freund adjuvant (IFA) (Sigma Chemicals, Saint Louis, USA). The immunization was repeated on the 14th, 21st and 28th days following primary immunization (Anie et al., 2007). Serial sera samples were collected from both rabbits during the course of immunization and an indirect ELISA was performed to measure anti-TDM antibody concentration in immune rabbit sera. Peak antibody titer (1:2400, end-point dilution) in the immune rabbit serum was detected in ELISA following the 4th immunization, and 10 ml of venous blood collected from each rabbit through the ear vein. The sera were separated, dispended in 1 ml sterile aliquots and stored at -70°C until used.

Experimental design

For this study, the Division of Laboratory Animal Services of this Institute supplied Swiss male mice (6 to 8 weeks old, 25 to 30 g - body weight; n = 36). The mice were divided into three groups of 12 each (Groups A, B, C). They were housed in micro-isolator cages (6 per cage) and maintained under aseptic pathogen-free conditions. This experimental study was conducted under the approval of Institutional Animal Ethics Committee (document no: B/26-2009-IX).

Groups A and B

Induction of lymphopenia: Each of the 12 mice in group A and B were administered intramuscularly with 1 mg of dexamethasone phosphate in 0.3 ml of 0.15 M sterile PBS. The injection was

repeated at three-hour interval for three consecutive days. The peripheral blood total leukocyte-count and differential lymphocyte count in each mouse was undertaken prior to dexamethasone phosphate injection as well as during the injection schedule. Prior to the dexamethasone injection, the mean peripheral total leukocyte and differential lymphocyte count were 7200 cells/cmm and 72% respectively. At the end of third day, there was a 50% reduction in the total leukocyte count (range 2500 to 5200; mean 3100 cells/cmm) and differential lymphocyte count in the peripheral blood ranged between 26 to 48% (mean 34.0). By this schedule, lymphopenia could be induced in all the mice in group A and B.

Group C

Each of the 12 (naive) mice was administered intramuscularly with 0.3 ml of 0.15 M PBS at three-hour interval for 3 consecutive days. There was no decrease in the total leukocyte in the peripheral blood (range - 7400 to 8000 cells/cmm; mean - 7100 cells/cmm) and the differential lymphocyte counts ranged 68 to 78% (mean 71%). There were no differences in the total and differential lymphocyte counts before and after the PBS injection in the naive mice.

Administration of TDM

A TDM *water-in-oil* emulsion was prepared by homogenizing 100 µg of TDM in 3.2 µl of incomplete freund's adjuvant (IFA) and 3.2 µl of 0.15 M PBS. Following this, 93.6 µl of normal saline containing 0.2% tween-80 was added to make the final volume of 100 µl. Each mouse in Groups B and C was injected intravenously (through the tail vein) with *water in-oil* emulsion containing 100 µg of TDM. 3 mice from Groups A, B and C were sacrificed at days 3, 5, 7, 14 following TDM administration. Under aseptic conditions, the lungs and the other visceral organs were collected for estimations of TNF-α, MIP-1α, as well as for the histopathological studies.

Histopathology

One (left) lung and visceral organs (myocardium, thymus, spleen liver, kidney) from each one of mice in Groups A, B and C were fixed in 10% buffered-formalin and were processed for histopathological studies using a standard protocol formulated in the histopathology laboratory. Five-micron thick paraffin sections were stained with hematoxylin and eosin (H & E). The salient histopathological features in lungs and visceral organs in Group A, B and C mice were recorded. Immunohistochemical staining for the demonstration of TDM in the paraffin sections of lungs of the mice in Groups A, B and C were performed using the standard protocol (ABC method) developed in the laboratory. The primary antibody used in the immunohistochemical technique was rabbit immune serum at TDM (1:1500 dilution): Both the H &E and immuno-stained sections were viewed under a research binocular microscope DMRB (Leica DMRB, Germany). Relevant photomicrographs were taken, using a digital camera system attached to the microscope.

Estimation of TNF-α and MIP-1 α in lung homogenates

The lung (right) of mice in Group A, B and C were washed several times in isotonic normal saline and subsequently it was snap-frozen in isopentane, pre-chilled in liquid nitrogen. Using a sterile scalpel blade, the frozen lung was serially sliced (at 1 mm thickness). Entire lung slices were homogenized in 2 ml Dulbecco's modified Eagle's medium (Sigma Chemicals, St Louis Mi) containing 0.01% arginine, 10% FCS, 100 µg penicillin and 50 µg gentamycin and were incubated for 4 h at 37°C in 5% CO_2. Following this, the lung homogenate was centrifuged at 10,000 rpm for 30 min. The supernatant was stored at -20°C for TNF-α and MIP-1α estimation.

TNF-α and MIP-1α concentrations in the cell-free supernatant were measured by a Sandwich ELISA, using an immunoassay kit supplied by Ray Biotech Inc, Norcross, GA. The assay was performed in accordance to the protocol supplied by the manufacturer in the kit. Briefly, the capture antibody coated in each well of the microtiter plate was washed several times with PBS-T (0.5% Tween-20 in 0.15 M PBS) and quenched with 1% bovine serum albumin (BSA), 5% sucrose, 0.05% N_aN_3 in PBS-T. Supernatant from lung homogenate (100 µl) from each mouse of Groups A, B and C was added to its respective well and the plate was incubated for 2 h at +4°C. This was followed by the addition of biotin conjugated secondary antibody and streptavidin - horseradish peroxidase. 3,3', 5,5' tetramethyl benzidine (TMB) was used as the substrate. The reaction was stopped after 30 min using 0.5 M H_2SO_4 and the absorbance in each well, was read at 450 nm with an ELISA reader. (Multimode ELISA Reader, Tecan Gmbh, Austria) The mean of duplicate wells of each lung homogenate was measured directly from the standard graph generated for each assay, using manufacturer-supplied recombinant TNF- α and MIP-1α standards in the kit. The ELISA is sensitive to detect TNF-α as low as 60 pg/ml and for MIP-1α, it was found to be 8 pg/ml.

In vitro chemotaxis assay

Collection of peritoneal macrophages

Resident peritoneal macrophages from naive and lymphopenic mice were collected by lavaging their peritoneal cavities with 5 ml of warm normal saline. After 30 min, the peritoneal cell - suspension was collected in sterile conical tubes and centrifuged at 1500 rpm for 30 min at +4°C. The cell sediments were then resuspended in 5 ml of Gey's balanced salt solution containing 10% fetal calf serum (FCS). The cell suspension was then poured onto a sterile disposable plastic Petri dish and incubated for 1 h with 5% CO_2. The non-adherent cells were removed by repeated rinsing the Petri dish with Gey's solution. The adherent cells (peritoneal macrophages) were detached from the Petri dish by incubating them in Gey's solution containing 10% FCS and 0.02% EDTA at +4°C for 2 h. Subsequently, macrophages were washed twice with Gey's solution and then resuspended in 5 ml - RPMI 1640 (9 parts) and Gey's solution (1 part). The macrophages were counted using an automated electronic cell counter (Beckman Coulter Inc, Brea, CA) and the cell count was adjusted to 1×10^6 cells/ml. These macrophages were then used for *in vitro* chemotaxis assay. Prior to the assay, viability of the macrophages were assessed with 0.4% tryphan blue exclusion and their cytological features were confirmed in 1% toludine blue stained cytospin smears (Figure 2a).

In vitro chemotaxis assay this was performed using a Monocyte Cell Migration Assay kit (Calbiochem; EMD Biosciences Inc; La Jolla Ca). The assay was performed in accordance to the protocol supplied in the kit by the manufacturer. The kit has detachable upper and lower microtiter plates containing 96 wells and separated from each other by 5 µ pore-sized polycarbonate membrane. 200-µl peritoneal macrophage cell suspension (1×10^6 cells in Gey's solution) from naive (group C) and lymphopenic mice (Groups A and B) was added to their respective wells in the upper plate. The macrophages were then allowed to pass from upper plate via the polycarbonate membrane to the wells in the lower plate, containing TDM in Gey's medium. The migrated macrophages in the wells of the lower plate were then pipetted and transferred onto the wells of a black colored microtiter plate containing fluorescent dye – Calcein AM. This plate was incubated for 3 h at 37°C. The uptake of the calcein by the migrated macrophages was measured at 485 nm (excitation wave length) and 520 nm (emission wave length) using a multimode ELISA reader (Tecan). The amounts of calcein uptake
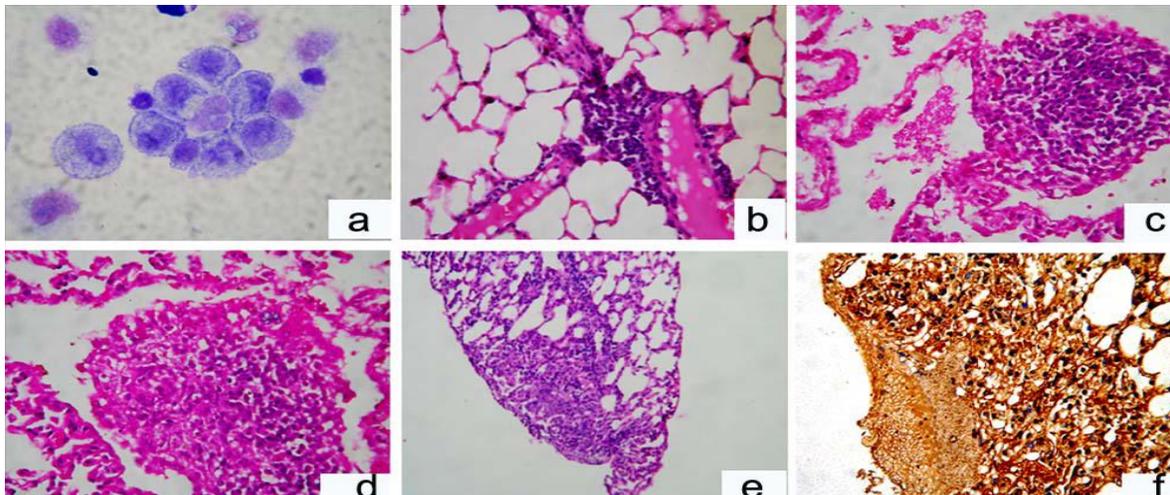
Figure 2. (a) Cytospin smear showing viable peritoneal macrophages (toludine blue stain, ×400); (b) perivascular aggregation of lymphomononuclear cells in the lung (H&E stain, ×250); (c) discrete aggregation of lymphomononuclear cells and few histiocytic cells in the lung (H&E, ×250); (d) showing well-formed cohesive histiocytic granulomas in lung (H&E ×250); (e) showing a granuloma located in the sub-pleural region (H&E ×100); (f) showing TDM positive cells in the lung granuloma (ABC immunostaining, ×250).

by the migrated macrophages are expressed as relative fluorescent unit (RFU). In the assay, TDM was used at different concentration ranging between 25 to 200 μg /ml. Positive control used in the assay was sodium casein (5 mg/ml in Gey's solution). The negative control used in the assay contained Gey's solution alone. The assay was repeated on two different occasions to assess the reproducibility of the assay. The RFU values *in vitro* chemotaxis assay obtained with the peritoneal macrophages of naive and lymphopenic mice were tabulated and were evaluated for statistical significance using Student's 't' test.

RESULTS

Mice

All the 12 naive mice in group C tolerated TDM administration and they survived till the end of the experimental study. They appeared healthy and there was no reduction in their body weight (mean 27 g). Among the 12 lymphopenic mice group B, 5 of them died between 3 to 12 days following TDM administration. In the remaining 7 lymphopenic mice that survived during the experimental study, there was a significant reduction in their body weight (18 to 22 g; mean, 18.0 g). All the 12 lymphopenic mice in Group A survived till the end of the experimental study. There was a reduction in their body weight (mean, 18 g).

Histopathological features

In naive mice

Histopathological features in all the naïve mice were essentially confined to the lungs. At three days following TDM administration, there was a mild degree of inflammatory cellular aggregates in the alveolar and perivascular spaces (Figure 2b). The inflammatory exudates were predominantly composed of lymphomononuclear cells. The lung of mice sacrificed at 5 days following TDM administration showed discrete clusters composed by admixture of lymphomononuclear as well as histiocytes (Figure 2c). By the end of the 7th day, more prominent and cohesive histiocytic granulomas are formed (Figure 2d). The number of granulomas in the lung ranged between 1 and 7 in number and they were scattered within the lung parenchyma with no obvious relation to blood vessels or bronchioles. Occasional granulomas were sub-pleural in location (Figure 2e). Neither caseous necrosis nor Langhan's giant cell reaction were demonstrated in the lungs. Immunohistochemical staining demonstrated the presence of TDM antigen within the cytoplasm of histiocytic cells (Figure 2f). At 14 days following TDM administration, there was a complete resolution of granuloma and microscopically the lung parenchyma appeared within normal limits.

In lymphopenic mice

In contrast to naive mice, lymphopenic mice in Groups B and C showed no histological response to TDM administration, and histiocytic granulomas were conspicuous by their absence in the lungs in these mice. However, the notable feature in the lymphopenic mice was marked depletion of lymphoid cells in thymus and spleen.

Table 1. Sandwich ELISA showing the TNFα, MIP-1α concentrations in lung homogenates.

Days	Naive mice		Lymphopenic mice	
	TNFα (pg/ml)	MIP-1α (pg/ml)	TNF-α (pg/ml)	MIP-1α (pg/ml)
3	90	45	<60	<8
5	230	82	<60	<8
7	475	172	<60	<8
14	70	86	<60	<8

Table 2. Results of *in vitro* chemotaxis assay expressed in relative fluorescent unit.

TDM in µg/ml	Naive mice	Lymphopenic mice
25	70160	12410
50	69525	12410
100	65915	12410
200	66275	12410
Positive control (casein)	52140	12410
Negative control	12140	12140

TNF α and MIP-1α concentrations

The TNF-α and MIP-1α concentration in naive and lymphopenic mice in Groups A and B are shown in Table 1. It is apparent that in naive mice, there is an increase in the TNF-α concentration between 3 to 7 days, following TDM administration and it ranged between 230 and 475 pg/ml (mean 178 ± 7.5 pg/ml). However, the TNF-α concentration tend to decrease by the end of 14th day of the experiment. Similarly, there was a significant increase in the MIP-1α concentration (82 to 172 pg/ml) in the lung homogenates of mice, 3 to 7 days following TDM administration. In lymphopenic mice, there is a marked decrease in the TNF-α concentration and in most of the lung homogenates the TNF-α concentration was less than 60 pg/ml. The TNF-α concentration did not show any significant change at the end of 14th day. There was also marked decrease in the MIP-1α concentrations in the lung homogenates, and were less than 8 pg/ml.

The results of *in vitro* chemotaxis are shown in Table 2. The RFU obtained with positive and negative controls in the assay were 52,137 and 12,410, respectively. TDM at all concentrations (25 to 200 µg/ml) elicited positive chemotactic response of peritoneal macrophages of naive mice. In contrast, peritoneal macrophages from lymphopenic mice showed poor chemotactic response to TDM.

DISCUSSION

The granulomatous response is the most characteristic host immune response in tuberculosis. It is therefore becomes extremely relevant to define those factors that contribute to the development and maintenance of the granuloma in tuberculous lesions (Hunter et al., 2006). Earlier published experimental studies highlighted the granulomatous response elicited by mycobacterial glycolipids of *M. tuberculosis*, TDM in particular; and these studies provided novel insights to define the immunopathopathological mechanisms involved in the genesis of granulomas (Yamagami et al., 2001; Hunter et al., 2006a; Guidry et al., 2007). Accordingly, two types of granulomatous response to TDM administration have been reported in mice: (a) Foreign body type of granuloma in which the lung of mice demonstrated the increased production of proinflammatory cytokines such as TNF-α and this correlated well with development of discrete histiocytic granuloma (Yamagami et al., 2001); (b) hypersensitivity type of granuloma, and this is characterized by development of caseating granulomatous reaction which are often destructive in nature and it also induced a strong lymphocytic response in and around the lesion (Hunter et al., 2006a).

Development and maintenance of granulomatous response in tuberculosis is highly dependent upon the host's ability to launch an effective cell-mediated immune response (Roach et al., 2002). Host with impaired cell - mediated immunological status do not elicit granulomatous response in mycobacterial infection. Welsch (Welsch et al., 2008) evaluated granulomatous responses, induced by TDM in wild-type C57BL/6 mice as well as mice that are genetically deficient in TNF-α, C5a and IL6. In their study, optimal granulomatous response observed in wild-type mice and in addition, lung homogenates of these mice also showed increase in the

TNF-α, MIP-1α and interleukin-6 (IL – 6) concentrations. By contrast, mice that are deficient in TNF-α, IL–6, C5a did not elicit any histological response to TDM, and in them the cytokine production was not increased following TDM administration. The results of their study also suggested that TNF-α is critical for the initiation of granulomatous response while C5a and IL–6 play a significant role in the maintenance of granulomatous response. The data in our study indicated that lymphopenic mice did not generate granulomatous reaction following TDM administration. This could be attributed to the depletion of lymphoid cells in thymus; and as a result, there is a quantitative reduction in thymus dependant lymphocytes and its subsets. Because of this, the lymphopenic mice did not generate optimal TNF-α, following TDM administration, and hence, macrophage did not accumulate to form granuloma in the lung. It has also been reported that thymic atrophy induced by TDM was closely related to granuloma formation in mice (Ozeki et al., 1997). Thymectomised mice did not develop *Mycobacterium bovis* cell wall induced granuloma but they regained their capacity to generate granuloma after the transfer of thymic lymphoid cells in them. On the other hand, naive mice with activated thymus dependant lymphocytes generated optimal production of TNF-α following TDM administration. TNF-α elicited the proliferation of macrophages and along with the release of chemotactic factor-MIP-1 α, granuloma formation in the lungs was facilitated. Besides TNF-α, other host factors also contribute to the granuloma formation in mycobacterial infection. These include C5a fraction of complement, as well as chemotactic factors released by the macrophages. Matsunaga et al. (1996) conducted *in vitro* chemotaxis assay with peritoneal macrophages of ICR mice and TDM. They observed that cell suspensions of activated peritoneal macrophages contained two chemotactic factors namely macrophage inflammatory protein-1 (MIP-1) and monocyte chemoattractant protein (MCP-1). They emphasized that both these chemotactic factors play an important role in granuloma formation. Our study also established an elevated MIP-1α concentration in the lung homogenate of naive mice following TDM administration.

The observations of this study will require confirmation to explore the true response of immune response induced by TDM in patients with human pulmonary tuberculosis because *M. tuberculous* bacilli is composed not only by TDM antigen but also by several immunodominant antigens. These mycobacterial antigens are released from *M. tuberculosis* bacilli during active stages of pulmonary tuberculosis. Hence, it will be inappropriate to compare the granulomatous response generated by TDM in mice model in relation to human pulmonary tuberculosis. Nonetheless, the results of this study would substantiate the concept that TDM does promote the accumulation of macrophages through cytokines released by the thymus dependant lymphocytes, and that it also facilitates the migration of macrophages to the site of mycobacterial infection through the release of MIP-1α from the activated macrophages.

In conclusion, granuloma formation in tuberculosis is essential in localizing the infection and thus prevents dissemination of the tuberculous lesion. High mortality and inadequate granuloma formation observed in the lymphopenic mice following TDM administration in our study might have a clinical significance. Patients with prolonged lymphopenic state or impaired cell-mediated immune status are the candidates who are likely to manifest disseminated tuberculosis and this contributes to higher mortality rates.

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