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## Lactoferrin modulation of mycobacterial cord factor trehalose 6,6'-dimycolate induced granulomatous response

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### Abstract

The immune system responds to tuberculosis (TB) infection by the formation of granulomas. However, subsequent immune-mediated destruction of lung tissue is a cause of significant morbidity and contributes to disease transmission. Lactoferrin, an iron-binding glycoprotein, has demonstrated immunomodulatory properties which decrease tissue destruction and promote T<sub>H</sub>1 immune responses, both of which are essential for controlling TB infection. The cord factor trehalose 6,6'-dimycolate (TDM) model of granuloma formation mimics many aspects of TB infection, with similar histopathology accompanied by proinflammatory cytokine production. C57BL/6 mice were intravenously injected with TDM. A subset of mice was given 1 mg bovine lactoferrin 24 hours post-TDM challenge. Lung tissue was analyzed for histological response and production of proinflammatory mediators. C57BL/6 mice demonstrated granuloma formation that correlated with increased production of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IL-12p40, IFN- $\gamma$ , and IL-10 protein. Mice treated with lactoferrin post-challenge had significantly fewer and smaller granulomas compared to those given TDM alone. Proinflammatory and T<sub>H</sub>1 cytokines essential to the control of mycobacterial infections, such as TNF- $\alpha$  and IFN- $\gamma$ , were not significantly different in mice treated with lactoferrin. Furthermore, the anti-inflammatory cytokines IL-10 and TGF- $\beta$  were increased. A potential mechanism for decreased tissue damage seen in the lactoferrin treated mice is proposed. Because of its influence to modulate immune responses, lactoferrin may be a useful adjunct in the treatment of granulomatous inflammation occurring during mycobacterial infection.

### Keywords

TDM; Cord Factor; Lactoferrin; Granuloma; Inflammation; Lung; Tuberculosis

### Introduction

TB is responsible for approximately 1.8 million deaths each year, making it the leading bacterial cause of death worldwide (1). Additionally, nearly one-third of the world is latently infected

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with *Mycobacterium tuberculosis* (MTB) (1). With the exception of the potential role of the fluoroquinolones, there have been no new anti-TB drugs created since rifampin was introduced in the 1960s (2). Thus, agents with anti-mycobacterial activity or a beneficial effect when used in combination with the current anti-TB drugs are desperately needed.

The immunocompetent host responds to MTB infection by the formation of granulomas, which consist of central infected macrophages that may differentiate into Langhan's giant cells, foamy macrophages, and epithelioid cells (3). A fibrous cuff develops around the infected macrophages, which serves to sequester and prevent dissemination of the bacteria. However, granuloma formation also causes physical partitioning of infected cells from peripheral lymphocytes capable of killing the infected cells (3). Furthermore, it is thought that the TB bacilli enter a non-replicating latency state within the granuloma; such bacilli are resistant to the action of antimicrobials (4,5). The majority of granulomas do not result in active disease. However, certain granulomas develop an increase in foamy macrophages that are hypothesized to cause caseation, the hallmark of active disease (3,6). The result is necrosis and liquification of the granuloma, forming a cavity that places infectious bacilli in the airways. The damage to the lung causes a productive cough, producing infectious aerosols that may infect a new host. Thus, modulation of the granulomatous pathology will likely facilitate improved response to antimicrobials, limit transmission of the disease, and decrease morbidity.

New evidence has identified lactoferrin as a regulator of the immune response to a variety of infectious and injurious stimuli. Lactoferrin is a member of the transferrin family and is found in mucosal secretions as well as in neutrophil granules (7). Receptors for lactoferrin are found on many immune system cells, including dendritic cells, macrophages, and T-cells (8–10). Specific effects of lactoferrin include the activation of macrophages, increasing polymorphonuclear cell phagocytosis, enhancement of killer cell activity, and promotion of B- and T-cell maturation (11–15). Of considerable interest, lactoferrin modulates macrophage production of cytokines in the context of their environment (16–18). For example, lactoferrin added to macrophages that were sub-optimally stimulated with LPS increased proinflammatory cytokine production (19). However, lactoferrin decreased cytokine production in macrophages fully stimulated with LPS. Critical to the studies proposed here, lactoferrin has been shown to protect against immune-mediated tissue damage. Mice treated with lactoferrin had increased survival and decreased gut tissue destruction after LPS injection (7,20). Additionally, lactoferrin added to the BCG vaccine resulted in increased protection against an aerosol TB challenge and decreased lung damage (21).

Cord factor TDM is the most abundant glycolipid produced on the surface of MTB. TDM plays multiple roles in the pathogenesis of MTB (22), including the formation of caseation in the lung post-infection (23). TDM emulsion injected intravenously into mice induces lung immunopathology that mimics many aspects of MTB infection, including granuloma formation and the production of proinflammatory cytokines. The TDM model of granuloma formation has been used to elucidate the immunological factors involved in the granulomatous response (24,25). TDM can induce activated foreign body type granulomas in naïve mice (26,27), and hypersensitivity (immune) granulomas in appropriately sensitized mice (23,28,29). Therefore, this model system is ideal for exploring the potential of immunomodulators to alter granuloma structure, with perhaps specific utility to extrapolate findings to immune related pathology identified during clinical manifestation of tuberculosis disease.

## Methods

### Animals

Four-week old, female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animal studies were approved by the University of Texas Health Science Center

Institutional Review Board (protocol number HSC-AWP 07-093), performed under approved and accepted ethical guidelines. Up to eight mice were used per group, per time points indicated.

### Lactoferrin and MTB

Bovine-derived lactoferrin (>95% purity, <20% iron saturated) was generously provided by PharmaReview Corporation (Houston, TX). Lactoferrin was dissolved in PBS, and stored at  $-80^{\circ}\text{C}$ . The lactoferrin used in experimentation was identical in composition as that used in published investigations into immune mediation of DTH development (19,30,31).

**Mycobacterium tuberculosis**—Erdman (TMC 107, American Type Cell Culture) was grown to log phase in 7H9 broth (Remel, Lenexa, KS) with 10% supplement at  $37^{\circ}\text{C}$  in an orbital shaker. MTB was diluted in  $1 \times \text{PBS}$  to  $3 \times 10^8$  bacteria per ml by using McFarland standards (Sigma, St. Louis, MO). The bacterial concentration was confirmed by plating serial dilutions on 7H11 plates (Remel), and enumerated after incubation for 3–4 weeks at  $37^{\circ}\text{C}$ .

### Isolation of bone marrow-derived macrophages (BMM), challenge with TDM-coated microspheres, and infection with mycobacteria

BMMs from C57BL/6 mice were prepared as per previously described procedures (31). Femurs were flushed with McCoy's medium (Sigma) and  $5 \times 10^5$  cells were added to 24-well tissue culture plates (Corning, Corning, NY). Cells were grown in McCoy's medium containing 10% fetal bovine serum (FBS) (Sigma), antibiotics (50  $\mu\text{g}/\text{ml}$  gentamycin and 100  $\mu\text{g}/\text{ml}$  penicillin G) (Sigma), and 10 ng/ml recombinant murine granulocyte/macrophage colony stimulating factor (GM-CSF) (Cell Sciences, Canton, MA). Cells were incubated at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  for seven days with two media changes containing GM-CSF. The cells were washed with  $1 \times \text{PBS}$  and the media changed to Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 0.01% L-arginine (Sigma), 0.01% HEPES (Sigma) prior to stimulation with TDM-coated beads or infection with MTB.

TDM- and BSA-coated microspheres were prepared as previously described (32). Beads were briefly sonicated before use, and added to cells at a ratio of 10 beads per cell in the presence or absence of 100  $\mu\text{g}/\text{ml}$  lactoferrin. Alternatively, BMM were infected with MTB Erdman at a multiplicity of infection (MOI) of 1:1 with or without lactoferrin (100  $\mu\text{g}/\text{ml}$ ). For all conditions, supernatants were collected after 24 and 72 hours and assessed for cytokine production by ELISA (see below).

### In vivo administration of TDM, tissue processing, and lung histopathology

A water-in-oil emulsion of TDM was prepared as previously described (25,27). Briefly, mice were injected intravenously with 50  $\mu\text{l}$  of the emulsion. The emulsion was prepared by dissolving 25  $\mu\text{g}$  per dose of purified TDM (Sigma) in 9:1 hexane/ethanol followed by evaporation of the solvent. Two percent Drakeol (Penreco, Dickinson, TX) was added and homogenized. Finally, 0.2% Tween 80 (Mallinckrodt, Hazelwood, MO) in PBS was added to give the final volume and admixed. An emulsion control without TDM was also prepared. Subsets of mice were administered either 1 mg of lactoferrin or 1 mg of bovine apo-transferrin (Sigma) in 100  $\mu\text{l}$  of PBS intravenously 24 hours after administration of TDM.

Mice were sacrificed at days 0, 4, and 7 post-TDM challenge. Lung tissues were immediately removed, weighed, and processed for histological studies or cytokine analysis. Approximately 30 mg of lung tissue was homogenized and placed into 2 ml of DMEM and incubated for 4 hours at  $37^{\circ}\text{C}$ . The supernatants were removed and stored at  $-20^{\circ}\text{C}$  for cytokine ELISA analysis. For histological analysis, the left lung was fixed in 10% buffered formalin, sectioned, and stained with hematoxylin and eosin. An entire 5  $\mu\text{m}$  section of at least 6 mice per group

was digitized with a 2× objective. An analysis was performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). Total granulomas were enumerated over the whole section of the lung. The average granuloma size was determined by randomly selecting 10 granulomas in 3 microscopic fields for each mouse.

### ELISA analysis of lung cytokines

The levels of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IL-12p40, IFN- $\gamma$ , IL-10, and TGF- $\beta$  in the lung homogenate supernatants were determined by a sandwich ELISA as per the manufacturer's instructions (R&D Systems, Minneapolis, MN). The mean of duplicate wells was determined based on a standard curve generated for each assay using manufacturer supplied recombinant molecules. The lower limit for detection sensitivity was 15–32 pg/ml, according to the manufacturer.

### Statistics

The data are presented as the mean  $\pm$  1 SD. Two-way ANOVA was used to compare the differences between groups using GraphPad Prism (GraphPad Software, La Jolla, CA). A *p*-value of < 0.05 was considered as being statistically significant.

## Results

### Lactoferrin modulation of inflammatory response to TDM in bone marrow-derived macrophages

TDM is a potent inducer of proinflammatory cytokines from macrophages (3,27,33). The production of TNF- $\alpha$ , IL-6, and IL-12p40 were significantly elevated in BMM stimulated with TDM-coated beads, compared to macrophages administered BSA-coated beads (Figure 1). In contrast, the anti-inflammatory cytokine TGF- $\beta$  decreased with TDM-bead stimulation. Addition of 100  $\mu$ g/ml bovine lactoferrin, either at the same time as TDM bead stimulation or three hours after, resulted in significantly decreased production of the pro-inflammatory cytokines TNF- $\alpha$ , IL-6, and IL-12p40, but increased production of TGF- $\beta$ . Lactoferrin alone increased TGF- $\beta$  when cells were incubated with control BSA-coated beads, as has been previously reported (30).

### Lactoferrin modulation of TDM-induced histopathology

C57BL/6 mice challenged with TDM formed distinct lung granulomas after a single intravenous dose of TDM. Small, focal, monocytic clusters were apparent 4 days post-TDM challenge (Figure 2A). The granulomas increased in size and number by day 7 (Figure 2B), accompanied by additional pathological manifestations, which included lymphocytic perivascular cuffing. Mice administered an emulsion control failed to form granulomas and exhibited normal lung histology with only minor indications of inflammation. Mice challenged with TDM and treated subsequently with 1 mg of intravenous bovine lactoferrin had fewer and smaller granulomas post-TDM administration, with the granulomatous response markedly diminished by day 7. This response was not due to the iron binding capability of lactoferrin; granuloma formation was not altered in mice given transferrin, an iron-binding control, when given after TDM challenge.

### Cytokine protein profiles in TDM-challenged mice treated with lactoferrin

Lung cytokines produced were evaluated in mice challenged with TDM (Figures 3 and 4). Mice administered the TDM emulsion had significantly elevated levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-12p40, and IFN- $\gamma$  on both days 4 and 7 compared to mice given an emulsion control (Figure 3). Mice treated with 1 mg of bovine lactoferrin 24 hours after challenge with TDM had similar levels of most pro-inflammatory cytokines. Of note, the levels of TNF- $\alpha$  and IFN- $\gamma$  were not

significantly different in the lactoferrin treated mice. The mice administered lactoferrin had decreased IL-1 $\beta$  at day 7. In contrast, the levels of the anti-inflammatory cytokines IL-10 and TGF- $\beta$  were increased at day 4 and day 7, respectively, in the lactoferrin treated mice (Figure 4). Mice administered transferrin 24 hours after mice were administered TDM had similar levels of cytokines compared to mice given a TDM emulsion alone (data not shown).

### Relationship of model to mycobacterial infection

Certain cytokines such as TNF- $\alpha$  have been shown to be critical for the control of MTB infection (34–36). A concern involved the potential decrease in TNF- $\alpha$  production from macrophages stimulated with TDM-coated beads and administered lactoferrin might generate conditions favorable for MTB proliferation inside host cells. Therefore, C57BL/6 BMM were infected with MTB at a MOI of 1:1, with or without lactoferrin. Cytokine production in response to MTB infection is shown in Figure 5. TNF- $\alpha$  production was significantly increased in macrophages given lactoferrin at the same time as bacterial seeding or three hours later. IL-6 was significantly decreased, and there was no alteration in production of IL-12p40. Furthermore, the anti-inflammatory cytokine TGF- $\beta$  was significantly increased at 72 hours post-infection. These results suggest that there is a potential for lactoferrin to decrease the cytokines that mediate tissue destruction while maintaining those that essential for development of responses correlating with protection against mycobacteria.

### Discussion

MTB is a highly successful pathogen, accounting for 1.8 million deaths annually and infects one-third of the world's population (1). The incidence of this disease continues to increase, likely because of the increased occurrence of multi-drug resistant strains and co-infection with HIV (37). The treatment of TB is lengthy, complex, and requires drugs with significant side-effects that often result in poor patient compliance. The WHO's directly observed therapy short-course (DOTS) program is highly effective, yet places a significant financial burden on developing countries with a high incidence of TB. Thus, new therapeutics with the potential to shorten treatment are desperately needed. Immunomodulatory agents, such as lactoferrin, may represent a novel approach to the treatment of TB in that they have the ability to overcome the issue of drug resistance. In the case of lactoferrin, its use may also decrease immune-related pathology that may enhance drug penetration into tissue, and increase the development of protective immune response to potentially kill latent bacilli (4,37).

TDM is the most immunostimulatory and granulomagenic lipid produced by MTB. Administration of TDM leads to an extensive recruitment of inflammatory cells accompanied by the cytokines and chemokines similar to those induced during natural TB infection (3,23). Our results demonstrate that mice given lactoferrin after TDM challenge had decreased lung pathology at the peak of the granulomatous response. Modulation of the granuloma has potential implications for the treatment of both latent and active TB. MTB within granulomas adapt by conversion to a dormancy phenotype that includes decreased replication and changes in biochemical pathways (4,5,38). Antibiotics require actively growing bacilli for effectiveness and are thus less capable of eliminating dormant MTB sequestered within granulomas. Furthermore, decreasing immunopathology in the lung may enhance antibiotic penetration within infected tissue, thus promoting faster response to antimycobacterials (37). The combination of immunomodulators with TB antibiotics has yielded promising results in human clinical trials. One clinical trial examined the addition of etanercept, a soluble TNF- $\alpha$  receptor, to antimicrobial chemotherapy in HIV patients with TB and found significantly decreased time to sputum culture conversion with trends toward improved chest x-rays in the patients treated with the combination of etanercept and anti-TB agents (39). A second trial examined the use of high-dose prednisolone with TB antibiotics in patients with HIV and TB (40). The rate of



sputum culture conversion was significantly increased in patients supplemented with prednisolone, compared to anti-TB chemotherapy alone. Lactoferrin as an adjuvant immunomodulator to TB therapy has a distinct advantage over other immunomodulators available because it is not immunosuppressive and has an excellent safety profile in numerous animal models and human clinical trials (21,41–44)

A possible mechanism for the decreased lung pathology observed is the increase production of IL-10 and TGF- $\beta$ , which have demonstrated critical roles in reducing pathology due to cell-mediated immunity (45). It is theoretically possible that T<sub>H</sub>2 cytokines may decrease immunity to TB (4). However, it is noteworthy that the classical cytokines critical for the control of TB, TNF- $\alpha$  and IFN- $\gamma$  (46), were not significantly altered by lactoferrin treatment. IFN- $\gamma$  is essential for controlling mycobacterial burden, the importance of which is highlighted by the severe mycobacterial infections experienced by patients who lack components of IFN- $\gamma$  signaling (47). TNF- $\alpha$  is essential for granuloma formation and maintenance; absence of TNF- $\alpha$  results in severe necrotic pathology and reactivation of latent disease (38,46,48), as has been demonstrated for both infection and lipid models of pathology (25,34,49). The ability of lactoferrin to mount an anti-inflammatory response while maintaining pro-inflammatory cytokine production is not unique to these studies (7). For example, oral administration of lactoferrin to rats in a colitis model increased the anti-inflammatory cytokines IL-10 and IL-4 (50), as well as modulated immune related pathology in murine LPS models of sepsis (20, 51). In contrast, lactoferrin enhanced the delayed-type hypersensitivity response to BCG, ovalbumin, and sheep red blood cells (11,52,53). The ability of lactoferrin to exert anti-inflammatory and pro-inflammatory response makes it a candidate immunomodulator for a variety of infectious and inflammatory diseases.

We found that lactoferrin-treated macrophages infected with MTB had an increase in TNF- $\alpha$  production. This is in contrast to the global decrease in proinflammatory cytokines observed when the bone marrow-derived macrophages were given TDM-coated beads and treated with lactoferrin. This is likely due to the fact that MTB has a number of immunostimulatory molecules in addition to TDM, including ligands for the toll-like receptors, C-type lectins, and NOD-like receptors (54). Of interest, preliminary experiments found no direct effect of lactoferrin to modulate growth of organisms in vitro. Indeed, lactoferrin has an effect on growth of MTB in macrophages, but only if the macrophages are pre-activated or co-activated prior to lactoferrin treatment (unpublished data, Welsh and Actor). The data presented here utilizing infected macrophages highlight the need to examine the impact of lactoferrin in models of MTB infection. Studies in our laboratory are currently underway exploring the effect of lactoferrin on the pathology and bacterial burden in mice infected with MTB, as well as determining the mechanism of lactoferrin modulation of regulatory cytokine production

The TDM model of granuloma formation mimics many aspects of the immunopathology observed during aerosol infections in mice. This model system is therefore ideal for screening immunomodulators that are expected to alter the lung pathology observed in TB. These studies indicate that lactoferrin decreases lung pathology in a model of TB granuloma formation while maintaining a protective cytokine profile. Lactoferrin may therefore have clinical advantages as a useful adjuvant in the therapeutic treatment of TB.

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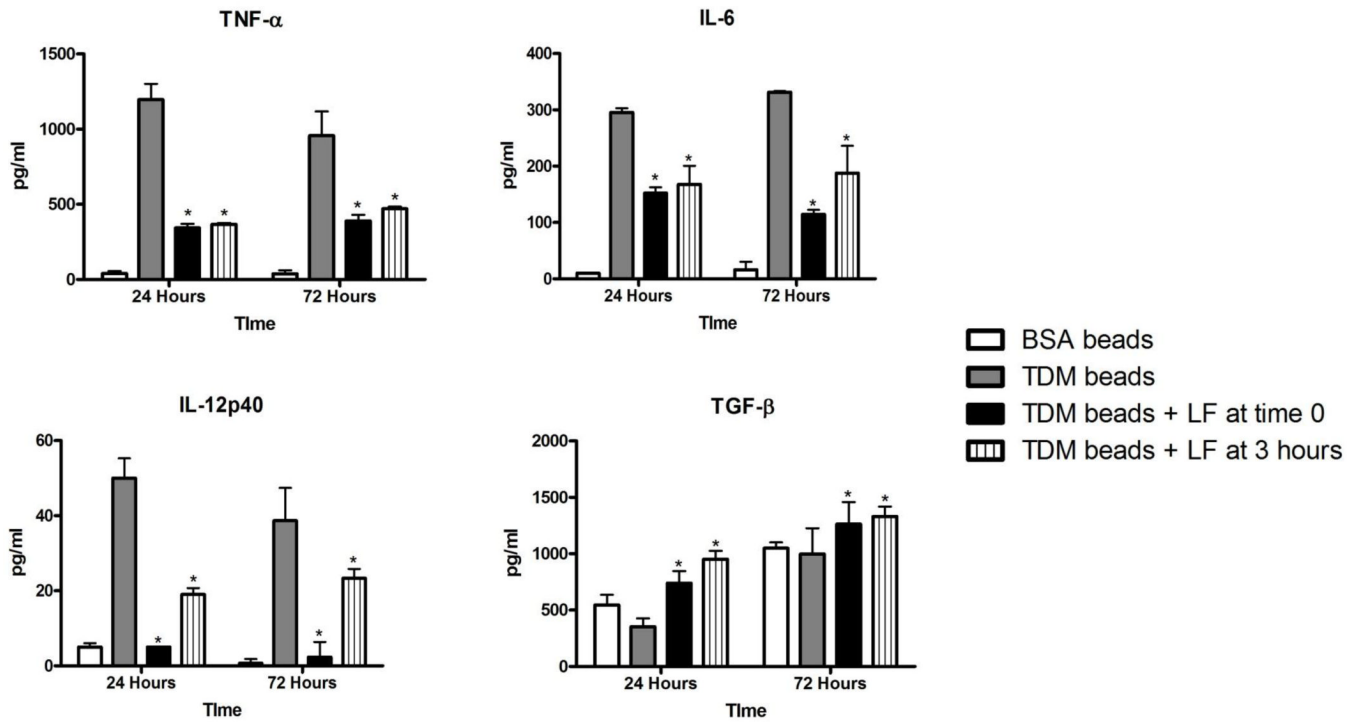
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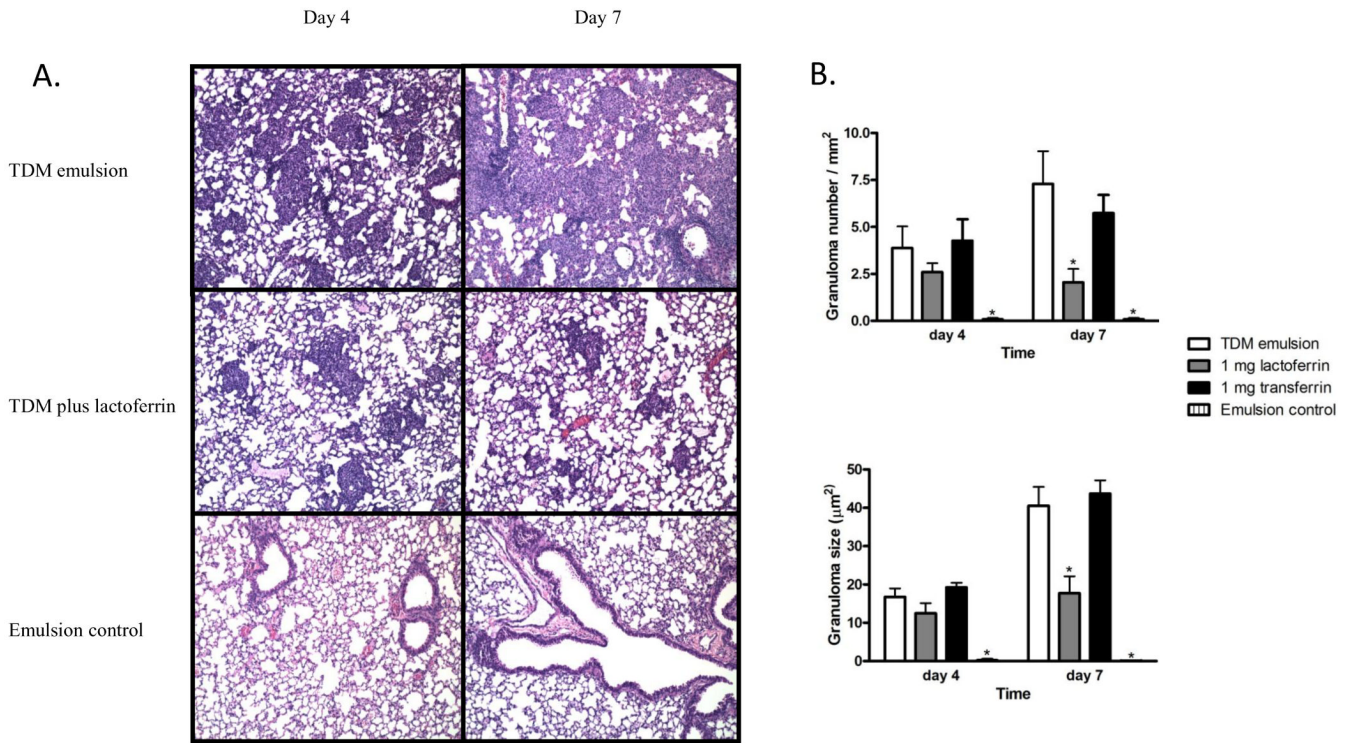
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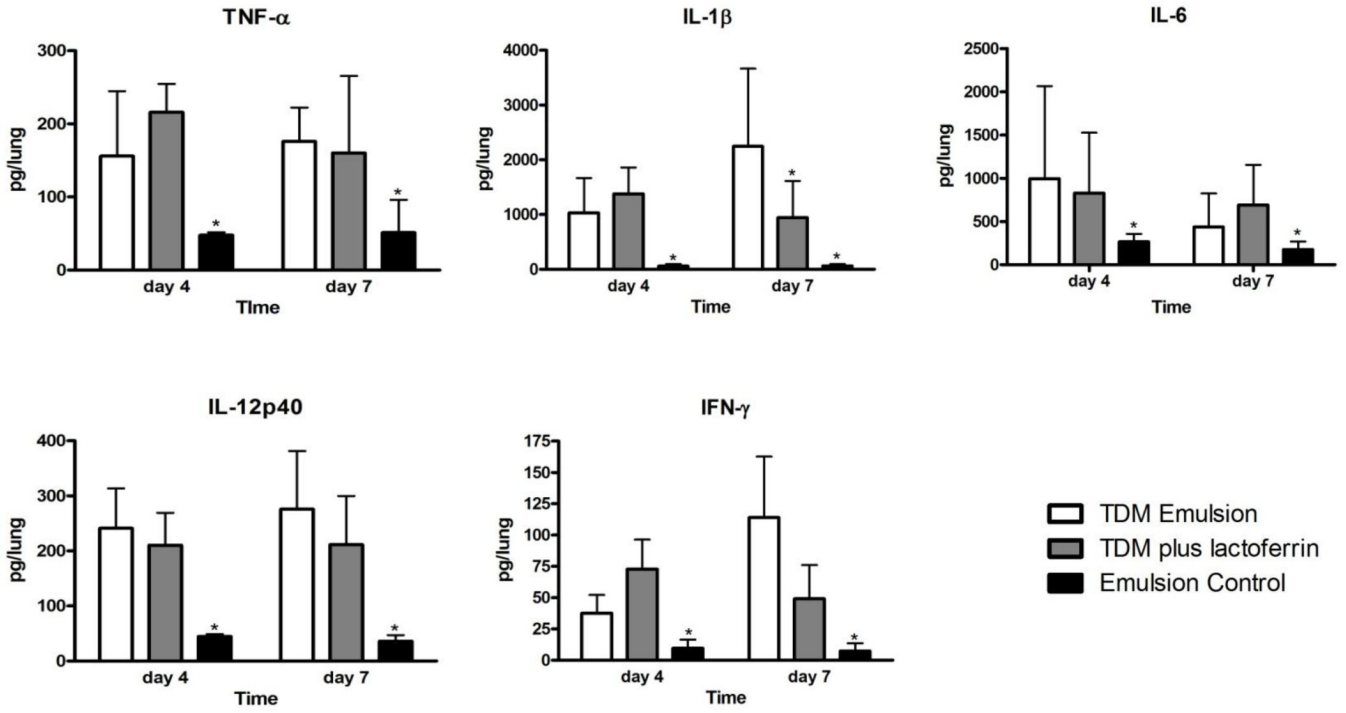
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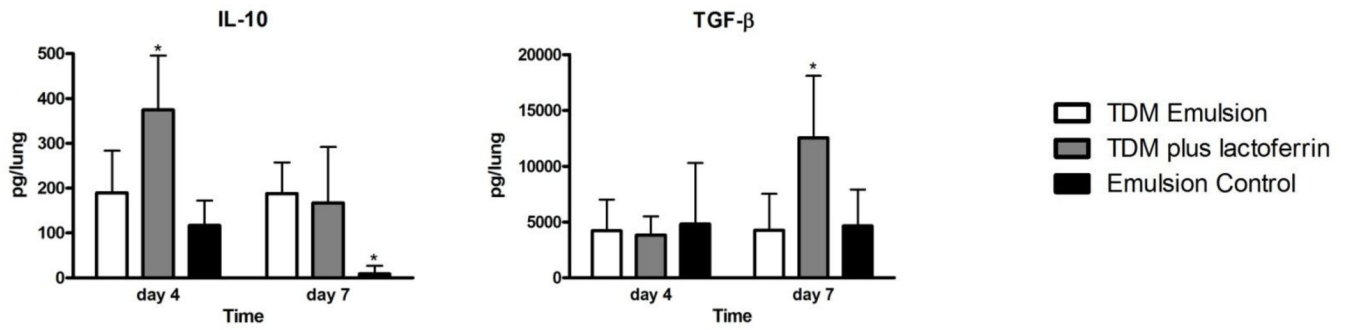
**Figure 1.** Lactoferrin modulation of TNF- $\alpha$ , IL-6, IL-12p40, and TGF- $\beta$  production by BMM in response to TDM-coated beads. Cells were treated with 3  $\mu$ m BSA- or TDM-coated beads alone at a ratio of 10:1, or with 100  $\mu$ g/ml lactoferrin (LF) at the same time as bead stimulation, or with 100  $\mu$ g/ml lactoferrin 3 hours after bead addition. Values were measured by ELISA and expressed as mean pg/ml with standard deviation (SD). \*  $p < 0.05$  compared to treatment with TDM-coated beads alone.



**Figure 2.** Granulomatous response to TDM in lactoferrin treated mice. **A)** Mice were challenged with 25  $\mu\text{g}$  TDM prepared in a water-in-oil emulsion. One mg of lactoferrin or transferrin (not shown) was administered 24 hours post-TDM challenge. Hematoxylin and eosin staining, magnification 100 $\times$ . **B)** Number of lung granulomas per  $\text{mm}^2$  and the size of the granulomas ( $\mu\text{m}^2$ ). Data are expressed as the mean  $\pm$  SD. \*  $p < 0.05$  with comparisons made to the mice administered the TDM emulsion.



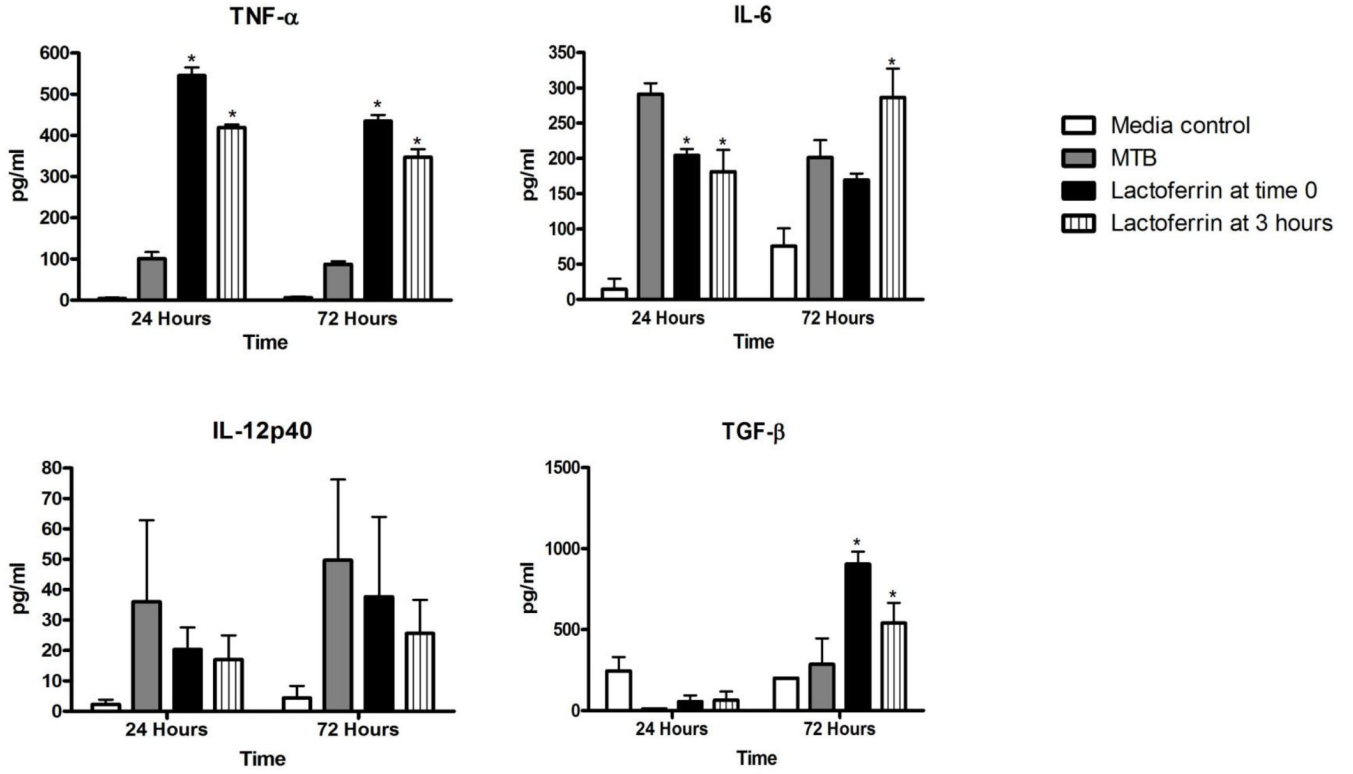
**Figure 3.** Production of pro-inflammatory mediators in mice challenged with TDM and treated with lactoferrin. The levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-12p40, and IFN- $\gamma$  in lung tissue were determined by ELISA. Data are shown as the mean with standard deviation. N = 8 mice per group, per time point. \* $p < 0.05$  with comparison to the TDM emulsion treated mice.



**Figure 4.**

Lactoferrin induced production of anti-inflammatory cytokines in TDM challenged mice. The levels of IL-10 and TGF- $\beta$  were quantified in lung tissue homogenates by ELISA. Data are expressed as the mean with standard deviation. N = 8 mice per group, per time points indicated. \* $p < 0.05$ , comparisons made to the TDM-treated mice.





**Figure 5.** Lactoferrin modulation of BMM cytokine production in response to MTB infection. BMM were infected with MTB Erdman using a MOI of 1:1. 100 μg/ml of bovine lactoferrin was added at the same time as infection or 3 hours after infection. The levels of TNF-α, IL-6, IL-12p40, and TGF-β were measured in the filtered supernatants 24 and 72 hours after infection by ELISA. Data are shown as the mean and SD. \**p*<0.05, comparisons are to cells infected with MTB alone.