Published in final edited form as:

Microbes Infect. 2009 ; 11(6-7): 680–688. doi:10.1016/j.micinf.2009.04.015.

Neutralization of TNF α alters inflammation in guinea pig tuberculous pleuritis

Lan H. Ly^{*}, Amminikutty Jeevan, and David N. McMurray

Department of Microbial and Molecular Pathogenesis, College of Medicine, Texas A&M Health Science Center, College Station, Texas

Abstract

Previously, treatment with anti-gpTNF α antibody enhanced TNF α mRNA expression in pulmonary granulomas microdissected from non-vaccinated guinea pigs, and modified splenic granuloma architecture. In this study, pleural fluid, cells, and granulomatous tissues were collected 3, 5, and 8 days post-pleurisy induction in guinea pigs treated with anti-gpTNF α or normal serum control. Neutralizing TNF α reduced the percentage of macrophages in the pleural exudate while increasing the proportions of neutrophils and lymphocytes. Cell associated mycobacterial loads were increased in guinea pigs treated with anti-gpTNF α antibody. Cells from the pleural exudate in both treatment groups at day 3 expressed predominantly TNF α and IFN γ mRNA. By day 5, treatment with anti-gpTNF α antibody significantly reduced TNF α mRNA and increased TGF β and iNOS mRNA expression, a transition which did not occur in the control group until day 8. TNF α mRNA overwhelmed the cytokine milieu of microdissected pleural granulomas in the control group at day 3 whereas TNF α , IFN γ , and TGF β mRNA dominated the anti-gpTNF α -treated group. At day 8, granulomas from the control group began shifting towards an anti-inflammatory profile with increased levels of TGF β mRNA. Neutralization of TNF α hastened the transition to an anti-inflammatory cytokine response in guinea pig pleural granulomas and exudate cells.

Keywords

TUBERCULOUS PLEURISY; GUINEA PIG; TNF α

1. Introduction

One of the key outcomes of *in vivo* mycobacterial infection is the formation of granulomas. This structure is maintained and stabilized by events mediated by both host and pathogen. It is believed that bacteria can live for prolonged periods of time within the environment of a granuloma while, at the same time, bacterial spreading to other areas of the organism is restricted [1]. In this sense, granulomas represent a fine example of the delicate balance that keeps the infection in equilibrium—an equilibrium that, in most otherwise healthy individuals, does not harm the host but that does not kill the bacterium either. These events lead to the secretion of pro-inflammatory cytokines and chemokines, including TNF α [2]. On the other hand, TNF α also has been implicated in the pathologic response of the host to *M.*

© 2009 Elsevier Masson SAS. All rights reserved.

*corresponding author Email address: lhly@medicine.tamhsc.edu, Telephone: (979) 845-3679, Fax: (979) 845-3479.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

tuberculosis infection and is often cited as a major factor in host-mediated destruction of lung tissue. Tipping the balance of TNF α in the lungs may lead to increased pathology and necrosis.

The importance of TNF α in the control of *Mycobacterium tuberculosis* (*M. tb*) is due to its role as a mediator of macrophage activation. A number of groups have shown that granuloma formation in tuberculosis in the absence of TNF α is disorganized, with fewer activated or epithelioid macrophages [2]. Clearly, TNF α affects cell migration and influences expression of adhesion molecules as well as chemokines and chemokine receptors, and this is certain to affect formation of functional granulomas in infected tissues. Our laboratory has shown that guinea pig macrophages infected with *M. tb* release inflammatory cytokines such as interleukin-1 (IL-1), IL-6, and TNF α [3–5]. Recently, we have demonstrated that resident peritoneal macrophages from BCG-vaccinated guinea pigs stimulated *in vitro* with recombinant guinea pig (rgp) TNF α and/or rgp IFN γ exhibited a significant increase in of H₂O₂ production, MHC class II expression, and IL-12p40 mRNA production [6]. Neutralizing endogenous TNF α in cocultures of T-cells and macrophages from BCG-vaccinated guinea pigs down-regulated the expression of IL-12p40 and IFN γ mRNA while increasing intracellular bacterial growth [7]. *In vivo* neutralization of TNF α in BCG-vaccinated guinea pigs for 3 weeks post aerosol infection with virulent *M. tb* resulted in significant splenomegaly. Furthermore, granulomas microdissected from non-vaccinated guinea pigs infected with *M. tb* were overwhelmed with TNF α mRNA at 3 and 6 weeks post-infection compared to those from BCG-vaccinated guinea pigs in which Type 1 cytokine mRNA (IFN γ , IL-12p40) at 3 weeks post-infection are replaced by TGF β mRNA at 6 weeks [8]. Similarly, lung macrophages from BCG-vaccinated guinea pigs showed increased TNF α mRNA expression in response to antigen-specific and mitogen stimulation [9]. The multiple mechanisms by which TNF α promotes effective granuloma formation, maintenance, and function have yet to be determined, especially in the highly relevant guinea pig model.

The guinea pig model of pleurisy is remarkably similar to that seen in humans as a rapid inflammatory response occurs in the pleural space of animals sensitized by BCG vaccination and injected intra-pleurally with either killed or viable *M.tb*. We have shown that the pleural inflammatory response can be manipulated by treatment with recombinant cytokines or neutralizing antibody to cytokines to provide insight into the local immunoregulatory mechanisms [10–13]. Neutralization of endogenous TGF β throughout the course of disease in guinea pigs induced with pleurisy resulted in a significant reduction in the percentage of lymphocytes and production of IFN γ and CCL5 mRNA in the pleural exudates, while the percentage of neutrophils and CXCL8 mRNA expression were upregulated, indicating that modulation of cytokine function was feasible with this model [12]. In this study, we set out to determine the effect of TNF α neutralization on the inflammatory response and granuloma formation in pleurisy-induced guinea pigs. This model was chosen as it would allow us to inject neutralizing antibody directly into the infected cavity and affect granuloma formation throughout the course of the study. In addition, the focused effect of the neutralizing antibody within a defined anatomical site was expected to mitigate against a dilution of the antibody throughout the entire body.

2. Materials and Methods

2.1 Animals and vaccination

Specific, pathogen-free outbred Hartley strain guinea pigs (250–300 g) from Charles River Breeding Laboratories, Inc. (Willmington, MA) were housed individually and provided commercial chow and tap water *ad libitum*. They were maintained in a temperature- and humidity-controlled environment and exposed to a 12-h light/dark cycle. They were vaccinated intradermally (10³ CFU) with *Mycobacterium bovis* BCG (Danish 1331 strain; Statens Seruminstitut) and allowed to rest for 6 weeks before pleuritis induction. Following virulent

infection, the guinea pigs were housed under ABSL-3 containment. All protocols were approved by the Texas A&M University Laboratory Animal Care Committee.

2.2 Antibody Production

New Zealand white rabbits (2–3 kg) were immunized with recombinant guinea pig (rgp) TNF α by the method previously published by our laboratory [4]. The rgp-TNF α protein was produced according to our established method [4,14]. The serum collected at the end of the immunization protocol was tested by Western blot for reactivity against rgpTNF α as previously described [4]. A dilution of 1:5000 was able to detect as little as 50 ng of rgpTNF α (data not shown). Anti-gpTNF α produced under these conditions has been shown to neutralize gpTNF α in an L929 cell bioassay [14].

2.3 Pleurisy Induction and Antibody Treatment

M. tuberculosis H37Rv (ATCC 27294) was maintained in stock suspensions of known viability and stored at -80°C . BCG vaccinated guinea pigs were anesthetized by intramuscular injection of ketamine (30 mg/kg) and xylazine (2.5 mg/kg), and one milliliter of 2×10^8 CFU/ml bacteria was injected bilaterally into the pleural space using our previously published method [11,15]. The guinea pigs were anesthetized and given daily intrapleural injections of either undiluted rabbit anti-gpTNF α antibody or normal rabbit serum (1 milliliter per guinea pig, per day) beginning on the day of infection. Groups of animals were sacrificed on days 3, 5, and 8 post-induction of pleuritis.

2.4 Necropsy and Exudate Retrieval

Guinea pigs were euthanized by intraperitoneal injection (100 mg/kg) of sodium pentobarbital (Sleepaway; Fort Dodge Laboratories, Inc., Fort Dodge, IA). Using our previously published method [13], the abdominal cavity was opened, a small incision was made at the top of the diaphragm and the accumulated fluid and cells were removed. Samples were spun at $1200 \times g$ for 15 minutes at room temperature. Cell viability was determined by Trypan Blue exclusion. The cell pellets and cell-free supernatants were collected for intracellular and extracellular bacteria analyses, respectively. Samples were plated onto 7H10 agar plates and incubated at 37°C . Colony forming unit (CFU) counts were recorded after 21 days.

2.4 Differential Cell Counts

Fluid retrieved from the pleural cavity was centrifuged onto silanated glass slides (CSA-100; PGC Scientifics, Gaithersburg, MD) at $140 \times g$ for 5 min using a Cytospin 2 (Shandon Southern Instrument, Inc., Sewickley, PA). Cells were stained with Diff-Quik (Dade Behring Inc., Newark, Del) and viewed under a microscope to determine the relative cell composition based on morphology.

2.5 Tissue Processing and Laser Capture Microdissection (LCM)

The diaphragm and surrounding connective tissues containing visible granulomas were excised aseptically. Each tissue was then placed into 10% buffered-formalin for 1 h at 4°C , and transferred into 70% EtOH. Tissue processing and LCM was performed as previously described [8,13].

2.6 Total RNA Isolation and Real-time PCR

Total RNA was isolated from pleural effusion cells and microdissected granulomas as previously published [8,13]. Reverse transcription and real-time PCR, performed by using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), were carried out as previously described using the primer sequences for the guinea pig IL-12p40, TNF α ,

TGF β 1, IFN γ , IL-10, iNOS, and HPRT published previously [6,11,13,14]. Data obtained from pleural exudates were normalized to HPRT mRNA expression of the same sample. Expression values from microdissected granulomas were normalized to HPRT mRNA expression and then to nongranulomatous tissues obtained from the same animal.

2.7 Statistical Analysis

A two-way analysis of variance (ANOVA) was used to examine statistical differences between treatment groups (normal vs. anti-gpTNF α antibody) at the 95% confidence interval.

3. Results

Grossly visible granulomas were not detected in the diaphragm or surrounding tissues in pleurisy-induced guinea pigs until day 8. Granulomas from the control serum group were large, with an intensely necrotic center surrounded by loosely grouped scatterings of epithelioid macrophages and lymphocytes (data not shown). While the cellular concentration in the effusion was not affected by anti-TNF α treatment, the cellular composition was (Table 1). The absence of necrosis in the pleural granulomas of anti-gpTNF α antibody-treated guinea pigs is consistent with the significant reduction in the proportion of macrophages in the effusions. No necrosis was detected in any of the granulomas in antibody treated guinea pigs, suggesting that TNF α may play a central role in the development of necrosis in this disease model.

Fluid was collected from the pleural space of guinea pigs infected with *M. tb* H37Rv at 3, 5, and 8 days post-treatment. The cellular concentration of the accumulated fluid increased gradually over the experimental time course and was similar in guinea pigs treated with either anti-gpTNF α antibody or control serum (Fig. 1A). Cell-associated bacillary loads from both treatment groups remained constant between days 3 & 5 and declined markedly by day 8 (Fig. 1B). Cell-associated bacterial loads in the pleural effusions of guinea pigs treated with the anti-gpTNF α antibody were consistently lower than in the serum control group, although statistical significance was achieved only on day 8 (Fig. 1B). Extracellular bacilli from the same samples were markedly lower compared to cell-associated mycobacteria, and the levels were similar in both treatment groups throughout the experiment (Fig. 1C). The pleural fluid from guinea pigs treated with the control serum was comprised mostly of neutrophils (40–45%), macrophages (~35%), and lymphocytes (20–25%) (Table 1). Treatment with anti-gpTNF α significantly reduced the presence of macrophages while significantly increasing the proportions of neutrophils and lymphocytes. This trend was seen throughout the course of the 8-day study.

Analysis of cytokine mRNA expression of the pleural effusion cells revealed predominant expression of TNF α mRNA and, to a much lesser extent, IFN γ mRNA in both treatment groups at 3 days post-treatment (Fig. 2). The presence of TGF β , IL-12 p40, IL-10, and iNOS mRNA were minimal in both animal groups. Interestingly, TNF α mRNA expression levels were markedly higher (30%) in the group treated with anti-gpTNF α antibody at 3 days post-treatment compared to the control serum group (Fig. 2). However, IFN γ mRNA expression was reduced by 1.5-fold in this same group. By day 5 post-treatment, all cytokine mRNA expression levels in the pleural effusion cells had increased (Fig. 2). The expression of TNF α mRNA continued to prevail over the other cytokines measured in the guinea pigs injected with the control serum. In general, relative expression levels of TGF β and iNOS mRNA and, to a lesser extent, IL-12p40 and IL-10 mRNA increased substantially compared to day 3 post-treatment in the same treatment group. Treatment with anti-gpTNF α antibody at day 5 attenuated TNF α mRNA expression by 3-fold while increasing TGF β , IL-12p40, IL-10, and iNOS mRNA expression levels by 2–6 fold (Fig. 2). Antibody treatment did not affect IFN γ mRNA production, however. At the 8-day interval, the mRNA expression levels of TNF α in the animals treated with anti-gpTNF antibody had subsided dramatically to levels comparable to the control serum group.

(Fig. 2). IFN γ and IL-10 mRNA expression levels remained low and were not affected by antibody treatment at 8 days post-treatment. However, TGF β , IL-12p40, and iNOS mRNA expression levels remained elevated (2–9-fold) in guinea pigs treated with the anti-gpTNF α antibody when compared to the control serum group (Fig. 2).

Comprehensive charts were constructed to illustrate the relative proportions of mRNA transcripts for each of the six cytokines measured in the pleural fluid throughout the course of the study (Fig. 3). These charts clearly depict the dominant presence of the pro-inflammatory cytokine, TNF α mRNA and, to a lesser extent, IFN γ mRNA at day 3 post-treatment in both animal treatment groups. At day 5 post-treatment, cells from the control group continued to produce abundant amounts of TNF α mRNA and it was at this time point when a switch from pro- to anti-inflammatory cytokine mRNA production was seen. TGF β and iNOS mRNA expression levels were approximately 1/4 of the mRNA pool at day 5, and then increased dramatically by day 8 (Fig. 3). At day 5, the mRNA cytokine composition of the anti-gpTNF α antibody treatment group was predominantly comprised of TGF β and iNOS mRNA, which is strikingly similar to the profile seen by the control serum group at day 8 post-treatment.

To determine whether TNF α neutralization affected cytokine mRNA production in the developing granulomas of the pleurisy model, the granulomas from the diaphragm and connective tissues were microdissected by laser capture microdissection (LCM). Granulomas from the control serum group expressed high levels of TNF α mRNA, followed by IFN γ , TGF β , and IL-12p40 mRNA (Fig. 4) up to day 5 post-treatment. Treatment with anti-gpTNF α antibody reduced TNF α and IFN γ mRNA expression levels even further (~700-fold), while significantly increasing IFN γ , TGF β , IL-12p40, and iNOS mRNA expression levels (2–20-fold) (Fig. 4).

The relative proportions of mRNA transcripts are displayed in Figure 5. Granulomas microdissected from the control serum group were overwhelmed by TNF α mRNA up to day 5 post-treatment. By day 8, the presence of TGF β mRNA became apparent. TNF α neutralization altered cytokine mRNA expression levels in granulomas as early as 3 days post-treatment (Fig. 5). At day 3, the granulomas from the anti-gpTNF α antibody-treated guinea pigs were predominantly composed of cells producing TNF α mRNA and to a lesser extent, TGF β and IFN γ mRNA. By day 5, IFN γ mRNA production began to take over the granulomas with a concomitant reduction in TNF α mRNA expression. At day 8 post-treatment, TGF β mRNA production dominated and the presence of IFN γ and TNF α mRNA was greatly attenuated.

4. Discussion

We have previously demonstrated that splenocytes from previously vaccinated guinea pigs stimulated with antigen and rgpTNF α resulted in enhanced lymphoproliferation and IFN γ mRNA production [16]. Also, alveolar and peritoneal macrophages from non-vaccinated animals stimulated *in vitro* with *M. tb* and rgpTNF α upregulated TNF α and IL-12p40 mRNA expression levels [14]. These same cell cultures treated with antibody to neutralize TNF α increased intracellular bacillary growth. Furthermore, neutralizing TNF α antibody decreased the mRNA production of the pro-inflammatory IL-1 β cytokine in cocultures containing alveolar macrophages and neutrophils infected with virulent *M. tb* [17]. It is clear that tipping the balance of TNF α during the immunological response to *M. tb* infection in guinea pigs alters the cytokine milieu and the intracellular fate of virulent mycobacteria.

Our laboratory previously observed that the cytokine milieu of pulmonary granulomas from non-vaccinated guinea pigs following aerosol infection with *M. tb* was overwhelmed by TNF α mRNA [8]. Moreover, cytokine mRNA analysis of splenic granulomas from non-

vaccinated animals showed close resemblance to primary granulomas [13]. These data reflect the importance of TNF α in granuloma formation and maintenance. In the current study, we observed that TNF α neutralization decreased cellularity and necrosis of granulomas in the diaphragm of guinea pigs (data not shown). Similarly, Chakravarty, et al recently demonstrated that TNF α neutralization induced dissolution of lymphoid aggregates in the lungs of mice infected with *M.tb* [18]. We have previously observed that *in vivo* neutralization of TNF α in BCG-vaccinated guinea pigs aerosol-infected with *M.tb* resulted in significant splenomegaly [4]. Taken together these results clearly show a role for TNF α in the immunopathological response of tuberculosis, specifically in the aggregation of lymphocytes and the development of a necrotic center in the granuloma.

We observed the effect of anti-gpTNF α antibody treatment on TNF α and TGF β mRNA expression in the pleural exudates as early as day 3 post-treatment (Fig. 2). Interestingly, TNF α mRNA expression levels were enhanced at this time point which suggests a cellular compensatory mechanism to overcome the drop in TNF α protein by increasing gene transcription. It is known that TNF α shares many similar properties with IL-1 β and, therefore, it is possible that the removal of TNF α protein resulted in the upregulation of IL-1 β protein which, in turn, can stimulate TNF α production in an autocrine manner [19]. Accordingly, a recent study in our laboratory using neutralizing anti-gpTNF α antibody resulted in a transient increase in TNF α mRNA in cocultures infected *in vitro* with live *M.tb* [14]. Interestingly, treatment with anti-gpTNF α antibody decreased IFN γ mRNA expression in the pleural exudates at day 3, which implies a TNF-dependent mechanism (Fig. 2). TNF α and IFN γ are pleiotropic cytokines that have been shown to function either cooperatively or antagonistically [20]. It has also been demonstrated that TNF α may directly or indirectly regulate the expression of IFN γ and vice versa in many *in vitro* models [21]. By day 5 & 8 post-treatment, TGF β , IL-12p40 and iNOS were enhanced by TNF α neutralization demonstrating an anti-inflammatory profile taking shape.

Treatment with anti-gpTNF α antibody had a much more rapid effect on granuloma cytokine mRNA profiles than on the response of pleural effusion cells (Fig. 3). As early as 3 days post-treatment, the cytokine mRNA milieu of granulomas from the control serum and anti-gpTNF α antibody treated guinea pigs was significantly different. The results from this study clearly indicate that TNF α neutralization hastens the resolution of the pleuritis response as the anti-inflammatory TGF β mRNA expression was upregulated at earlier time points in both the pleural exudates and the tissue granulomas from anti-gpTNF α treated guinea pigs (Fig. 3 & 5). A recent study by Chakravarty, et al (2008) demonstrated that neutralization of TNF α in the mouse model of tuberculosis results in disorganization of the tuberculous granuloma and enhanced expression of various proinflammatory cytokines. The discrepancy between their results and ours can be explained by use of different animal models (mouse vs. guinea pig) and different routes of infection (aerosol vs. intrapleural). Our results also contradict previous results from our laboratory which demonstrated that systemic treatment with anti-gpTNF α in guinea pigs aerosol infected with *M.tb* for 3 weeks resulted in significant splenomegaly and disorganization of granulomas in the spleen [4]. Again, this can be explained, in part, by the different infection routes chosen.

TNF α is produced mainly by macrophages and daily treatment with anti-gpTNF α antibody resulted in a decrease in macrophage populations, and an increase in neutrophil and lymphocyte populations throughout the course of the study (Table 1). Although the absolute number of cells between animal groups did not differ (Fig. 1A), the cellular composition and cytokine mRNA profile by day 5 & 8 post-treatment were markedly different. The rapid increase in lymphocyte and neutrophil populations and the elevated TGF β and iNOS mRNA expression counteracted the proinflammatory effects of TNF α and IFN γ cytokine mRNA seen at day 3 (Table 1 & Fig. 2). As mentioned earlier, this resulted in a reduction in cellularity and the

appearance of necrosis (data not shown). During the early phase of antibody treatment (day 3 & 5), the existing macrophages may have responded to the loss of TNF α protein with the overproduction of TNF α mRNA. However, by day 8 post-treatment, the relative loss of macrophages accompanied by increasing populations of neutrophils and lymphocytes helped shift the cytokine milieu to an anti-inflammatory profile in anti-gpTNF α treated guinea pigs.

Induction of pleurisy in the guinea pig requires prior vaccination to develop a rapid local immune response. Cell-associated bacillary loads were significantly reduced by day 8 post-treatment in both animal groups (Fig. 1B), indicating successful vaccination. These data are in contrast with our previous results whereby pulmonary granulomas microdissected from BCG-vaccinated guinea pigs were overwhelmed with TGF β mRNA transcripts at 6 weeks post-infection [8]. TGF β is known to inhibit several events in T-cell and macrophage activation resulting in low production and/or function of antimicrobial mediators pertinent to the host immune response [22]. The discrepancy in these results can be explained, in part, by the route and dose of infection, and the timing of the study.

Neutralizing TNF α decreased cell-associated mycobacterial loads in the pleural exudates while not affecting the levels of extracellular bacilli throughout the course of the study (Fig. 1B & 1C). However, TNF α mRNA expression levels in the pleural effusion cells were increased at day 3 post-treatment and decreased thereafter (Fig. 2), although the relative proportion of TNF α mRNA in the cytokine milieu was still high up to day 5 post-treatment (Fig. 3). It has been shown that TNF α plays an integral role in the host defense against *M. tb* infection by activating macrophages and thus, contributing to induction and regulation of Th1-type cytokine and chemokine expression [23]. By day 5 & 8 post-treatment, the antimicrobial response may have shifted to iNOS. The importance of cytokine-mediated up-regulation of macrophage expression of iNOS in the killing of *M.tb* has been clearly demonstrated in rodent models, but its contribution to the guinea pig model and human macrophages has been controversial [24, 25]. Prior to this study, our laboratory has only been able to detect iNOS mRNA transcripts under very limited circumstances. We detected iNOS mRNA transcripts *in situ* in pulmonary granulomas of non-vaccinated guinea pigs aerogenically challenged with virulent *M. tb*, and essentially none in BCG-vaccinated animals [26]. Thus, there was an inverse relationship between iNOS expression and bacterial control, suggesting that it was the bacterial load in the tissue which drove iNOS mRNA expression. However, we demonstrate here that iNOS was detectable at very high levels in the guinea pig pleurisy model and its expression was associated with bacterial control (Fig. 1B & 2). Interestingly, the relative expression of iNOS transcripts was more prevalent in the pleural exudate than the surrounding tissue granulomas. This may be explained, in part, by the ability of cells and mediators to move more rapidly and freely in the pleural exudate compared to the granulomatous lesions. The contribution and importance of this cytokine to granuloma formation and bacillary control in guinea pigs remains speculative and must be examined more extensively. Studies using iNOS silencer genes in the guinea pig pleurisy model will help delineate the mechanisms by which iNOS contributes to bacterial control and granuloma maintenance.

In summary, neutralization of TNF α in our pleurisy model of TB hastened the progression of an anti-inflammatory response in the pleural exudates and associated granulomas. A concomitant decrease in bacillary loads demonstrated the minimal role for TNF α in bacterial killing in this model. This data are in contrast with previous findings by Chakravarty, et al whereby neutralization of TNF α in the mouse model resulted in enhanced expression of specific pro-inflammatory cytokines [18]. The discrepancy between these findings can be explained, in part, by the route of infection and the animal model used. Our pleurisy model is induced in a previously vaccinated guinea pig. Therefore, our findings can be attributed, in part, to the effect of BCG vaccination as well as the neutralization of TNF α . While TNF α has been reported

as being beneficial to the host and crucial for the control of *M. tb* infection, it is clear that it can also be detrimental when its expression is altered at certain phases of infection.

Acknowledgements

This work was supported in part, by the National Institutes of Health Grant RO1 AI-15495 to Dr. David N. McMurray and P30ES0910607 to the Center of Environmental and Rural Health at Texas A&M University.

References

1. Ulrichs T, Kaufmann SH. New insights into the function of granulomas in human tuberculosis. *J Pathol* 2006;208:261–269. [PubMed: 16362982]
2. Flynn JL, Goldstein MM, Chan J, et al. Tumor necrosis factor- α is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity* 1995;2:561–572. [PubMed: 7540941]
3. Yamamoto T, Lasco TM, Uchida K, et al. *Mycobacterium bovis* BCG vaccination modulates TNF- α production after pulmonary challenge with virulent *Mycobacterium tuberculosis* in guinea pigs. *Tuberculosis (Edinb)* 2007;87:155–165. [PubMed: 17289434]
4. Lasco TM, Cassone L, Kamohara H, Yoshimura T, McMurray DN. Evaluating the role of tumor necrosis factor- α in experimental pulmonary tuberculosis in the guinea pig. *Tuberculosis (Edinb)* 2005;85:245–258. [PubMed: 15958260]
5. Sadek MI, Sada E, Toossi Z, Schwander SK, Rich EA. Chemokines induced by infection of mononuclear phagocytes with mycobacteria and present in lung alveoli during active pulmonary tuberculosis. *Am J Respir Cell Mol Biol* 1998;19:513–521. [PubMed: 9730880]
6. Cho H, de Haas R, Jeevan A, McMurray DN. Differential activation of alveolar and peritoneal macrophages from BCG-vaccinated guinea pigs. *Tuberculosis (Edinb)* 2008;88:307–316. [PubMed: 18165156]
7. Cho H, McMurray DN. Neutralization of tumor necrosis factor α suppresses antigen-specific type 1 cytokine responses and reverses the inhibition of mycobacterial survival in cocultures of immune guinea pig T lymphocytes and infected macrophages. *Infect Immun* 2005;73:8437–8441. [PubMed: 16299346]
8. Ly LH, Russell MI, McMurray DN. Microdissection of the cytokine milieu of pulmonary granulomas from tuberculous guinea pigs. *Cell Microbiol* 2007;9:1127–1136. [PubMed: 17217432]
9. Jeevan A, Majorov K, Sawant K, Cho H, McMurray DN. Lung macrophages from bacille Calmette-Guerin-vaccinated guinea pigs suppress T cell proliferation but restrict intracellular growth of *M. tuberculosis* after recombinant guinea pig interferon- γ activation. *Clin Exp Immunol* 2007;149:387–398. [PubMed: 17565610]
10. Allen SS, Cassone L, Lasco TM, McMurray DN. Effect of neutralizing transforming growth factor β 1 on the immune response against *Mycobacterium tuberculosis* in guinea pigs. *Infect Immun* 2004;72:1358–1363. [PubMed: 14977939]
11. Allen SS, McMurray DN. Coordinate cytokine gene expression *in vivo* following induction of tuberculous pleurisy in guinea pigs. *Infect Immun* 2003;71:4271–4277. [PubMed: 12874302]
12. Allen SS, Mackie JT, Russell K, Jeevan A, Skwor TA, McMurray DN. Altered inflammatory responses following transforming growth factor- β neutralization in experimental guinea pig tuberculous pleurisy. *Tuberculosis (Edinb)* 2008;88:430–436. [PubMed: 18555747]
13. Ly LH, Barhoumi R, Cho SH, Franzblau SG, McMurray DN. Vaccination with Bacille-Calmette Guerin promotes mycobacterial control in guinea pig macrophages infected *in vivo*. *J Infect Dis* 2008;198:768–771. [PubMed: 18627245]
14. Cho H, Lasco TM, Allen SS, Yoshimura T, McMurray DN. Recombinant guinea pig tumor necrosis factor α stimulates the expression of interleukin-12 and the inhibition of *Mycobacterium tuberculosis* growth in macrophages. *Infect Immun* 2005;73:1367–1376. [PubMed: 15731034]
15. Phalen SW, McMurray DN. T-lymphocyte response in a guinea pig model of tuberculous pleuritis. *Infect Immun* 1993;61:142–145. [PubMed: 8418036]

16. Cho H, McMurray DN. Recombinant guinea pig TNF-alpha enhances antigen-specific type 1 T lymphocyte activation in guinea pig splenocytes. *Tuberculosis (Edinb)* 2007;87:87-93. [PubMed: 16899409]
17. Sawant KV, McMurray DN. Guinea pig neutrophils infected with *Mycobacterium tuberculosis* produce cytokines which activate alveolar macrophages in noncontact cultures. *Infect Immun* 2007;75:1870-1877. [PubMed: 17283104]
18. Chakravarty SD, Zhu G, Tsai MC, et al. Tumor necrosis factor blockade in chronic murine tuberculosis enhances granulomatous inflammation and disorganizes granulomas in the lungs. *Infect Immun* 2008;76:916-926. [PubMed: 18212087]
19. Korbel DS, Schneider BE, Schaible UE. Innate immunity in tuberculosis: myths and truth. *Microbes Infect* 2008;10:995-1004. [PubMed: 18762264]
20. Ohmori Y, Schreiber RD, Hamilton TA. Synergy between interferon-gamma and tumor necrosis factor-alpha in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor kappaB. *J Biol Chem* 1997;272:14899-14907. [PubMed: 9169460]
21. Lee JY, Sullivan KE. Gamma interferon and lipopolysaccharide interact at the level of transcription to induce tumor necrosis factor alpha expression. *Infect Immun* 2001;69:2847-2852. [PubMed: 11292697]
22. Toossi Z, Ellner JJ. The role of TGF beta in the pathogenesis of human tuberculosis. *Clin Immunol Immunopathol* 1998;87:107-114. [PubMed: 9614924]
23. Kisich KO, Higgins M, Diamond G, Heifets L. Tumor necrosis factor alpha stimulates killing of *Mycobacterium tuberculosis* by human neutrophils. *Infect Immun* 2002;70:4591-4599. [PubMed: 12117972]
24. Chan ED, Chan J, Schluger NW. What is the role of nitric oxide in murine and human host defense against tuberculosis? Current knowledge. *Am J Respir Cell Mol Biol* 2001;25:606-612. [PubMed: 11713103]
25. Schneemann M, Schoeden G. Macrophage biology and immunology: man is not a mouse. *J Leukoc Biol* 2007;81:579. [PubMed: 17332373]discussion 580
26. Ly LH, Russell MI, McMurray DN. Cytokine profiles in primary and secondary pulmonary granulomas of Guinea pigs with tuberculosis. *Am J Respir Cell Mol Biol* 2008;38:455-462. [PubMed: 18032570]

Figure 1. Cell concentration and bacillary counts

Pleural effusion cells from both control and anti-TNF α treated guinea pigs were analyzed for cell concentration (A), cell-associated (B), and extracellular (C) bacillary loads. Cells were stained with Trypan Blue and counted with a hemocytometer. For cell-associated bacillary counts (B), the pleural exudate was spun down and the pellet was plated onto 7H10 agar plates in duplicate. The remaining supernatant was plated for extracellular bacillary counts (C). Values represent log₁₀ CFU means of each group $\pm$ SEM, n=4 animals per group per time point, performed in one experiment. * p<0.05, ** p<0.01 values were found when comparing the same population of cells between anti-TNF α -treated and control animals at the same time interval.

Figure 2. Kinetics of cytokine mRNA expression in the pleural effusion cells of *M. tuberculosis*-infected guinea pigs treated with either anti-TNF α antibody or pre-immunized rabbit serum Expression of TNF α , IFN γ , TGF β , IL-12p40, IL-10, and iNOS mRNA were measured from the cells of the exudate retrieved from the pleural space of guinea pigs treated with either antibody or normal rabbit serum at 3, 5, or 8 days postinfection. Values for threshold cycle (Ct) were converted to 'expression levels' to allow for fold comparisons between samples, expression level = $2^{(40-Ct)}$. Data were normalized to HPRT mRNA followed by values derived from pleural wash cells obtained from healthy, pleuritis-free guinea pigs and expressed as means $\pm$ SEM, n= 4 guinea pigs per treatment group and time interval, performed in one experiment. * p<0.05, ** p<0.01 values were found when comparing normalized cytokine mRNA expression values between anti-TNF α -treated and control animals at the same time interval.

Figure 3. Comprehensive charts mapping the relative contributions of cytokine mRNA expression by the pleural exudate cells at 3, 5, and 8 days post-treatment

The sum of the relative expression levels of TNF α , IFN γ , TGF β , IL-12p40, IL-10, and iNOS in the pleural effusion cells from *M.tb*-infected guinea pigs treated with either anti-TNF α antibody or normal rabbit serum from Figure 2 was used to calculate the relative percentage of transcripts represented by each cytokine.

Figure 4. Kinetics of cytokine mRNA expression in the granulomas of *M. tuberculosis*-infected guinea pigs treated with either anti-TNF α antibody or pre-immunized rabbit serum
 Expression of TNF α , IFN γ , TGF β , IL-12p40, IL-10, and iNOS mRNA were measured from granulomas microdissected from the diaphragms of guinea pigs treated with either antibody or normal rabbit serum at 3, 5, or 8 days post-infection. Values for threshold cycle (Ct) were converted to 'expression levels' to allow for fold comparisons between samples, expression level = $2^{(40-Ct)}$. Data were normalized to HPRT mRNA followed by values derived from non-granulomatous tissues and expressed as means $\pm$ SEM, n= 4 guinea pigs per treatment group and time interval, performed in one experiment. * p<0.05, ** p<0.01 values were found when comparing normalized cytokine mRNA expression values between anti-TNF α -treated and control animals at the same time interval.

Figure 5. Comprehensive charts mapping the relative contributions of cytokine mRNA expression to granuloma formation at 3, 5, and 8 days post-treatment

The sum of the relative expression levels of TNF α , IFN γ , TGF β , IL-12p40, IL-10, and iNOS in the pleural effusion cells from *M.tb*-infected guinea pigs treated with either anti-TNF α antibody or normal rabbit serum from Figure 4 was used to calculate the relative percentage of transcripts represented by each cytokine.

Table 1**Differential Cell counts**

Pleural effusion cells from both control and anti-TNF α treated guinea pigs were analyzed by staining with Diff-Quik. One hundred cells were counted in each field to calculate percentages. Percentages represent the average of 4 animals $\pm$ SEM, performed in one experiment.

Treatment	interval	Percent of macrophages $\pm$ SEM	Percent of neutrophils $\pm$ SEM	Percent of lymphocytes $\pm$ SEM
serum	3	33.50 $\pm$ 1.19	41.25 $\pm$ 0.85	25.25 $\pm$ 1.65
	5	35.25 $\pm$ 1.49	41.75 $\pm$ 1.44	23 $\pm$ 2.8
	8	33.75 $\pm$ 2.02	46 $\pm$ 0.41	20.25 $\pm$ 2.32
anti-TNF	3	10.25 $\pm$ 0.25**	52.5 $\pm$ 0.87**	37.25 $\pm$ 0.95*
	5	10 $\pm$ 1.47**	57.25 $\pm$ 1.03*	32.75 $\pm$ 1.44**
	8	9.25 $\pm$ 0.75**	57 $\pm$ 0.71*	32.75 $\pm$ 1.45**

*
p<0.05

**
p<0.01 values were found when comparing the same population of cells between anti-TNF α -treated and control animals at the same time interval.