HHS Public Access

Author manuscript

Tuberculosis (Edinb). Author manuscript; available in PMC 2020 May 20.

Published in final edited form as:

Tuberculosis (Edinb). 2011 January ; 91(1): 98–106. doi:10.1016/j.tube.2010.11.004.

Local pulmonary immunotherapy with siRNA targeting TGFβ1 enhances antimicrobial capacity *in Mycobacterium tuberculosis* infected mice

Adrian G. Rosas-Taraco[1,2], **David M. Higgins**[1], **Joaquín Sánchez-Campillo**[1,3], **Eric J. Lee**[1], **Ian M. Orme**[1], **Mercedes González-Juarrero**[1,*]

[1]Mycobacteria Research Laboratories, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523, USA. This work was done at Colorado State University.

[2]Present Address: Dr. Adrian G. Rosas-Taraco, Departamento de Inmunología, Facultad de Medicina. Universidad Autónoma de Nuevo León. Monterrey, Nuevo León. México.

[3]Present Address: Dr. Joaquín Sánchez-Campillo, Departamento de Histología y Anatomía Patológica Comparadas, Universidad de Murcia, 30071 Murcia, Spain.

Abstract

In this study we demonstrate that it is possible to shift the immune system during a chronic infection with *Mycobacterium tuberculosis.* TGFβ and IL10 cytokines inhibit the Th1 response during chronic pulmonary infection with *Mycobacterium tuberculosis.* We show that intrapulmonary delivery of siRNA targeting TGFβ1 is able to reduce the pulmonary bacillary load in mice chronically infected with *Mycobacterium tuberculosis:* an effect that appears to be partly dependent on IL10 expression. To demonstrate this**,** we induced gene silencing of *tgfβ1* in the lungs of wild type and IL10 knockout mice using a non-invasive aerosolized intrapulmonary delivery of siRNA targeting TGFβ1 Five days after the last treatment with siRNA, the levels of *tgfb1* transcripts and TGFβ1 protein were reduced when compared with control groups treated with RNase free water or non-targeting siRNA. Mice treated with siRNA also had increased expression of the antimicrobial mediators (NO and iNOS) which effectively reduced the bacterial load by 0.17 and 0.47 $\log_{10}$ in C57BL/6 and IL10 KO mice respectively when compared with their respective control mice. More importantly, the bacterial load in siRNA treated IL10 KO mice four weeks after the last treatment remained 0.32 $\log_{10}$ lower than in control mice.

Keywords

tuberculosis; TGF; IL-10; siRNA; pulmonary; therapy

*Correspondence: Dr. Mercedes González-Juarrero, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado, 80523-1682. Phone 970-491-7306, Fax 970-491-5129. malba@mail.colostate.edu.

The authors have not conflicting financial interests.

INTRODUCTION

In the last few years, the worldwide struggle against tuberculosis has become even more difficult with the increased incidence of multidrug-resistant (MDR), extensively drug-resistant (XDR) *Mycobacterium tuberculosis (M. tuberculosis)* strains and co-infection with human immunodeficiency virus [1–2]. At the present time the resources to treat these cases are very limited and the necessity for developing new therapies to control the *M. tuberculosis* infection and its spread is imperative.

The only preventive therapy currently available against *M. tuberculosis* infection is vaccination with BCG *M. bovis* and yet this vaccine does not protect from tuberculosis in adults [3]. In mice, vaccination with BCG *M. bovis* prior to aerosol exposure with a virulent strain of *M. tuberculosis* provides a one $\log_{10}$ reduction in the pulmonary bacterial load when compared to unvaccinated mice following the same aerosol infection [4]. On the other hand, post exposure *M. tuberculosis* vaccination with BCG *M. bovis* in mice has no effect on the reduction of pulmonary bacterial load [5]. Thus, the only current treatment for either active or latent tuberculosis is the use of chemotherapy. However, the long term course of treatment required to successfully eliminate the bacteria is a major shortcoming to this therapy. This scenario underscores the necessity for research and development of new therapies that when combined with chemotherapy will overcome the shortcomings of current treatment options.

It is known that the immunoregulatory cytokines TGFβ and IL10 are elevated in patients with tuberculosis [20–21] and high levels of these cytokines are associated with tuberculosis reactivation [22–23]. The main role for these cytokines in the lungs during a chronic pulmonary *M. tuberculosis* infection is to restrain the inflammatory process. These cytokines control inflammation by interfering with Th1 responses and macrophage cell activation; as a result the antimicrobial capacity of these cells is substantially diminished [23–24]. TGFβ in tuberculosis down regulates phagocytic receptors [25], suppresses anti-microbial activity [26–27], induces apoptosis in CD4 T-cells [28], and appears to interfere with the cytotoxic function of CD8 T cells [29].

In this study, using local pulmonary immunotherapy, we regulate the lung environment during chronic infection with *M. tuberculosis* to favor the host response leading to elimination of the mycobacteria. We used small interfering RNA (siRNA) technology. siRNA is a powerful molecular biology tool able to induce gene silencing through nucleotide sequence-specific RNA degradation mechanisms that result in the suppression of gene expression [6]. This technology is being used in a variety of models to study and characterize gene function, as well as in new therapeutic procedures and in drug discovery [7–10]. The major shortcoming for *in vivo* use of siRNA therapy has been the delivery method i.e. local or systemic delivery and the use of siRNA with or without formulation (naked siRNA or siRNA combined with liposomes, peptides, antibodies or polymers) [11–12]. When delivered locally, the use of siRNA therapy for pulmonary diseases has been quite successful [13–14]. Thus, siRNA delivered, with and without formulation, was successfully used in SARS [15–16], RSV [17] and influenza viral infections [18] and bleomycin-induced fibrosis [19].

In light of this, we induced *tgfβ1* gene silencing directly in the lung using aerosolized intrapulmonary delivery of siRNA targeting TGFβ1 in C57BL/6 and IL10 KO mice. *tgfβ1* gene silencing resulted in higher levels of expression of TNF-α, nitric oxide and iNOS in the lungs of siRNA treated mice when compared to mice either treated with RNase-free water or non-targeting siRNA (control mice). Increased expression of these factors in siRNA treated mice was associated with a reduced bacterial load for at least four weeks after treatment. In addition, although the studies presented here used two different approaches to knock down cytokine expression our data demonstrated a synergistic effect at enhancing the antimicrobial activity in the lungs when simultaneously targeting expression of IL10 and TGFβ1.

RESULTS

Gene silencing and protein suppression by aerosolized intrapulmonary delivery of siRNA targeting TGFβ1

The capacity of siRNA therapy to induce *tgfb1* gene silencing was determined by comparing the levels of *tgfb1* mRNA expression in the lungs of mice receiving siRNA with the levels of expression of the same transcript in the lungs of mice receiving either non-targeting siRNA or RNAse-free water (control groups). For this purpose, the levels of expression of *tgfb1* transcripts in the lungs of each mouse in each group (n=5) receiving a total of three doses at 5 day intervals were determined using RT-*q*PCR. The results indicated that 5 days after the last treatment with siRNA, the levels of *tgfb1* transcripts were reduced to 65.5% and 60.6% in C57BL/6 and IL10 KO mice respectively when compared with expression of the same transcript in controls groups (Figure 1a). Additionally, we studied the levels of active TGFβ1 protein by ELISA in the supernatants obtained from lung homogenates from each mouse in each group. These results demonstrated that mice receiving the siRNA showed a reduction in active TGFβ1 protein expression by 27% and 31% in C57BL/6 and IL10 KO mice respectively when compared to levels of protein expression in similar samples obtained from control groups (Figure 1b). We questioned whether siRNA affected the lung tissue distribution for this protein after the treatment. Thus, *in situ* TGFβ expression on lung tissue of each mouse was visualized using immunohistochemistry (IHC) and antibodies to detect TGFβ. The histological findings demonstrated that *in situ* TGFβ expression was found mainly in the core of the granuloma and in macrophages located in the parenchyma wall of all mice in each group. Although there were no differences in the distribution of TGFβ staining between groups, the IHC staining demonstrated that the cluster of cells with positive staining for this cytokine in the center of the granuloma of mice treated with siRNA were less frequent and of lower intensity than the positive staining found in similar clusters of cells from tissue sections of controls groups (Figure 1c).

Changes in the Th1 cytokines after pulmonary siRNA therapy targeting TGFβ1

We questioned whether decreased expression of TGFβ1 could affect the Th1 cytokine response. TNF-α and IFNγ are strong Th1 inflammatory cytokines [24].Thus, we analyzed the levels of TNF-α and IFNγ by ELISA in supernatants obtained from lung homogenates from each mouse after treatment. TNF-α levels were increased in mice treated with siRNA by 64 and 67% in C57BL/6 and IL10KO mice respectively, when compared with control mice

(Figure 2a). Mice treated with siRNA showed a reduction in the total amount of IFNγ protein. This effect was more prominent in IL10 KO mice where the expression of IFNγ protein was 50% less than similar samples obtained from control mice (Figure 2b).

Effect of siRNA therapy targeting TGFβ1 on NO and iNOS production

IFNγ and TNF-α synergize and participate in the control of bacteria growth by activating the enzyme iNOS which in turn produce reactive nitrogen intermediates (RNI) in macrophages [30]. Thus, after treatment with siRNA we compared the levels of expression of two main components of the antimicrobial pathway. These two components were nitric oxide (NO) production determined by the Greiss reaction using supernatants from lung cell cultures stimulated with *M. tuberculosis* and iNOS transcript expression determined by RT-qPCR in total RNA extracted from lung samples collected from each mouse. Our findings demonstrated that the levels of NO increased by 24% and 38% in C57BL/6 and IL10 KO mice respectively when compared to control mice (Figure 3a). Similar results were found when the transcripts for iNOS were analyzed. However, in this instance only the IL10 KO mice had a significant increase of 62–67% in iNOS transcription when compared with similar samples obtained from the control mice (Figure 3b).

Effect of siRNA therapy targeting TGFβ1 on pulmonary bacterial load

The effect of siRNA treatment on the pulmonary bacterial load was determined by comparing the average number of colony forming units (CFU) obtained after plating the lung homogenates from each mouse in each group on 7H11 agar plates. This analysis indicated that the numbers of CFUs was significantly reduced by 0.17 $\log_{10}$ in C57BL/6 and by 0.5 $\log_{10}$ in IL10 KO treated mice compared to the CFUs obtained from their respective control groups (Figure 3c). In view of these results, we decided to determine the long term effect (four weeks after the last treatment) of this therapy in IL10KO mice. The results indicated that the bacterial load was still reduced by 0.32 $\log_{10}$ in treated mice when compared to control groups (Figure 4a). However the levels of TGFβ1 protein expression were increased in siRNA treated mice by 43% when compared to control mice (Figure 4b). Furthermore siRNA treated IL10 KO mice four weeks after the last treatment also showed increased expression of IFNγ (Figure 4c). On the other hand, the expression of iNOS mRNA was not different between groups at this time point (Figure 4d).

Effect of siRNA treatment on pulmonary fibrosis

Fibrosis is a commonly seen process during chronic tuberculosis which is believed to reduce the spread of mycobacteria to others cells and organs; however, excessive fibrosis is also detrimental to the lung physiology. It is believed that TGFβ has a major role in the fibrotic process [31]. For this reason we analyzed whether the siRNA treatment had any effect on fibrosis. We quantified and compared the extent of fibrosis in lung tissue sections from each group of mice using Masson's trichrome staining and computer-assisted image analysis. This analysis demonstrated that the IL10 KO mice treated with siRNA showed up to a 50% reduction in pulmonary fibrosis compared with the control groups when the lungs were analyzed five days after treatment (Figure 5a). However, there were no differences between groups of mice in the percentage of fibrotic tissue four weeks after the last treatment (Figure 5b).

Discussion

The small interference RNA mechanism in cell biology was described ten years ago, and the use of this tool for therapeutic purposes for a variety of diseases has now started to appear [17]. Nonetheless, no studies using a siRNA therapy have been previously reported in the field of tuberculosis. Here we developed a new strategy to control chronic pulmonary *M. tuberculosis* infection, in which we used local pulmonary delivery of aerosolized siRNA to improve the antimicrobial capacity of the host. We specifically targeted the immunosuppressive cytokine TGFβ1 and this in turn decreased the levels of TFGβ1 transcripts and subsequently the production of active TGFβ1 protein. This reduction in the levels of active TGFβ1 protein was associated with an increased efficacy of the Th1 host immune response despite lower expression of total IFNγ in the same groups of mice. Thus, these treated mice had lower expression of TGFβ1 and IFNγ and a reduction in the pulmonary bacterial load when compared to control mice. Moreover, the reduced bacterial load in these mice remained lower than in control groups four weeks after the last treatment.

Furthermore, this study also suggested that there is a synergistic effect between TGFβ1 and the immunosuppressive and anti-inflammatory cytokine IL10. Mice unable to produce IL10 had a significant decrease in the bacterial load and fibrosis when compared to wild type mice after siRNA treatment despite having similar levels of suppression of active TGFβ1 protein. These results indicate that the presence of both TGFβ1 and IL10 are responsible for the diminished antimicrobial capacity observed during the chronic disease.

Similar levels of suppression of active TGFβ1 protein were obtained in both animal models, indicating that the presence of IL-10 does not affect expression of TGFβ1 protein. The levels of suppression achieved with only three doses and one single sequence of siRNA used in this study were capable of reducing the bacterial load of *M. tuberculosis* chronically infected mice between 0.17– 0.5 log. Furthermore, future studies using siRNA therapy will determine whether extended suppression of active TGFβ1 further enhance the antimicrobial activity.

TNF-α is involved in granuloma formation but also participates together with IFNγ to induce iNOS [32]. We evaluated the levels of those cytokines and found that reduction in the expression of active TGFβ1 was associated with higher levels of TNFα (up to 60%) in both C57BL/6 and IL10 KO mice. This is in agreement with other reports indicating that IL10 and TGFβ1 are also potent inhibitors of inflammatory cytokines such as TNF-α [20,33]. Initially, we hypothesized that lower levels of TFGβ1 should enhance the IFNγ expression. However, our data demonstrated that the antimicrobial improvement in the Th1 response observed in mice treated with siRNA did not parallel with increased levels of IFNγ. Unexpectedly, IFNγ levels were 50% lower in mice treated with siRNA targeting TGFβ1. Despite reduction of IFNγ levels and augmentation of the TNFα responses, there was an increase in expression of antimicrobial mediators like iNOS which effectively reduced the bacterial load. Our recent studies [4] demonstrated that mice vaccinated with BCG *M. bovis* prior to pulmonary infection with *M. tuberculosis* were able to reduce by 10 fold the pulmonary bacterial load despite presenting lower expression of pulmonary IFNγ than unvaccinated mice. Furthermore, other reports using anti-tuberculosis chemotherapy also indicated that reduction in pulmonary bacterial load paralleled with a decrease (up to 90%)

in sera levels of IFNγ [21]. Altogether this indicates that, in chronic infections, as the bacterial load decreases so do the levels of IFNγ expressed by the host. Furthermore, reduced bacterial load and IFNγ expression decreases inflammation, size of granuloma lesions and globally improves the host response to infection. Moreover, while a lack of IL10 alone increases IFNγ, a simultaneous decrease in expression of IL10 and TGFB1 reduces expression of the same cytokine.

Additionally, once the bacterial load was reduced during siRNA treatment, four weeks after cessation of treatment the bacterial load in IL10 KO mice remained 0.32 $\log_{10}$ lower than controls. However, at this time this group of mice had higher expression of TGFβ1 and IFNγ than controls and there were no differences in iNOS expression between groups indicating a recovered blockage of antimicrobial activity.

iNOS participates in the production of reactive nitrogen intermediates (RNI) and components which are directly associated with the antimicrobial activity of many intracellular microorganisms including *M. tuberculosis* [24,34]. TGFβ has also been involved in the reduction of the stability and rate of translation of iNOS mRNA and increased iNOS protein degradation [26]. This may also explain our results showing that reduced expression of TGFβ resulted in higher levels of iNOS which was even more pronounced in IL10 KO mice.

Reduction in TGFβ1 expression also reduced fibrosis in IL10 KO mice compared with the control mice, but no differences were found in C57BL/6 mice. Therefore, the percentage of fibrotic tissue was higher in IL10 KO compared with C57BL/6 mice which correlates with similar data obtained by Kitani A *et al,* 2003 in an IL10 deficient model [35]. Previous reports have demonstrated that IL10 inhibits collagen synthesis and prevents fibrosis and many authors have also proposed that IL10 has an anti-fibrotic like activity.

Other groups have tried to control the infection using systemic delivery of a TGFβ antagonist in mice [36] or intrapleural administration of antibodies against TGFβ [37] in guinea pigs. However, blocking the activity of TGFβ did not affect the levels of expression of TGFβ and resulted in increased expression of IFNγ. Thus, our results differed from these studies in that by targeting the levels of expression of TGFβ1, the expression of IFNγ decreased and this reduced the inflammatory component of the Th1 response. Ultimately, by reducing inflammation, we also reduced subsequent inhibition of the natural host antimicrobial activity.

The current challenge in tuberculosis control is reduction of effective duration of drug therapy. Although the great majority of tubercle bacilli in the lungs are killed within two to three weeks by anti tuberculosis drugs using standard regimen of isoniazid, rifampin and pyrazinamide, it is necessary to continue 6 to 8 months of daily treatment of such therapy to be sure that the few remaining bacilli are eliminated. The nature of drug ‘persistors’ is unknown but it is generally assumed that they are in a state of dormancy/latency which renders them functionally resistant to anti tuberculosis agents. Thus, although the decreases in bacteria load obtained in this study are modest, we believe that the capacity of siRNA immunotherapy to enhance the natural host antimicrobial capacity could be exploited if combined with current chemotherapeutic regimens. We suggest, that future studies should

evaluate siRNA immunotherapy or other drugs targeting specific pathways of cytokines to target the drug 'persistor's bacteria population [38] as a new approach to tuberculosis treatment.

In part due to noncompliance with treatment, therapy is now further complicated by the emergence of multidrug drug-resistant strains [38]. Today the incidents of MDR and XDR strains are increasing and new alternative chemotherapies are still long and of high toxicity [38]. As proposed in this investigation, the use of immunotherapy to modulate the lung environment to increase the natural antimicrobial components could be helpful in the control of MDR and XDR infections. We suggest that a therapy containing multiple siRNA specific for TGFβ family members, IL10 and maybe other immunosuppressive cytokines could be an alternative to treat infections with these strains. Furthermore, combined immunotherapy targeting immunosuppressive cytokines and chemotherapy could improve the present course of chemotherapy treatment for tuberculosis.

Materials and Methods

Mice and Experimental infections

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and B6.129P2I1C (IL10 KO) mice were purchased from Charles River Laboratories (Wilmington, MA). The mice were kept in sterile condition in a BSL3 room. Mice were challenged by low-dose aerosol exposure with *M. tuberculosis* Erdman strain using a Glass-Col (Terre Haute) aerosol generator calibrated to deliver 50–100 bacteria into the lungs. All experiments were performed according to CSU institutional ethical guidelines.

siRNA targeting TGFβ1

siRNA targeting TGFβ1 was commercially predesigned from the gene number access for *tgfb-1* (NM_011577) by Qiagen. An siRNA with the sequence [sense] r(GCA ACA ACG CCA UCU AUG A)dTdT and [antisense] r(UCA UAG AUG GCG UUG UUG C)dGdG was chosen. The sequence of the *tgfb1* gene targeted by this siRNA is CCG CAA CAA CGC CAT CTA TGA. The siRNA was commercially produced and purchased from Qiagen (Valencia, CA). AllStars negative control siRNA was included as a negative control and was also obtained from Qiagen (Valencia, CA).

siRNA targeting TGFβ1 intrapulmonary delivery

From previous studies[36] it is well established that mice in the chronic stage of infection express high levels of TGF-pi and so we chose day 60 post-infection in our preliminary studies to validate TGF-pi targeting siRNA. Mice were treated by the intrapulmonary route following a protocol previously described[39]. Mice were anesthetized by intraperitoneal injection of 100mg/kg and 10mg/kg body weight of ketamine and xylazine, respectively. The mice received three doses at 5 days intervals of 10 μg per mouse/dose of siRNA targeting TGFβ1 or AllStars negative control or RNAse-free water using a high pressure microsprayer (Penn-Century, Philadelphia, PA). After intrapulmonary delivery mice were monitored until they recovered from anesthesia.

ELISA assays

Lung sample were homogenized and screened in triplicate by ELISA following manufacturer's protocol for active TGFβ1 (R&D Systems, MN), TNF-α (BD Bioscience, CA) and IFNγ (eBioscience, CA).

RT-qPCR

The lung lobe was homogenized in Trizol and frozen at −80°C immediately. RNA was extracted following the manufacturer's protocol for Trizol (Invitrogen). DNA was digested with RQ1 RNase-free DNase (Promega, Madison,WI) and RNA was re-isolated with Trizol. Finally, the concentration of RNA in each sample was measured by spectrophotometry and the RNA was reverse transcribed with M-MuLV (New England BioLabs, Ipswich, MA) and random hexamers (Roche,Bassel, Switzerland). Real-time PCR was performed using 5 μl of cDNA and Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) in iQ5 thermocycler (Biorad, Hercules, CA) to evaluate relative mRNA expression of TGFβ and iNOS, GAPDH was used to normalize the expression levels. Primers sequences used were:

TGFβ forward primer 5'GACCCTGCCCCTATATTTGGA3' and reverse primer sequence 5'GCCCGGGTTGTGTTGGT3';iNOS forward primer 5'CAGCTGGGCTGTACAAACCTT3' and reverse primer 5'CATTGGAAGTGAAGCGTTTCG3'; and GAPDH forward primer 5'TCACCACCATGGAGAAGGC3' and reverse primer 5'GCTAAGCAGTTGGTGGTGCA3'. Amplification conditions were as follow: 50°C for 2 min, 95°C for 2 min and 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 58°C for 30 s. Specificity was verified by melt-curve analysis. TGFβ and iNOS mRNA levels were normalized with GAPDH levels using ΔΔCt method to calculate relative changes.

Histopathology

The diaphragmatic lobe of the lungs of each mouse was placed into histology cassette and fixed in formalin-free Zn reagent (BD Bioscience, Mountain View, CA). After 48 h, samples were inactivated inside the BSL-3 laboratory and then processed using standard histological protocols. Histopathological evaluation of fibrosis was done by Masson's trichromic stain.

Quantitative Image Analysis of Fibrosis Using Masson's Trichrome Staining

Fibrosis was quantified by Masson's trichrome staining and computer-assisted image analysis as previously reported[40]. Images were taken using an Olympus BX41 microscope and DP70 camera. Image data was analyzed using Adobe Photoshop CS. All images were obtained using a 40x objective Camera settings were under manual control and held constant in order to produce comparable conditions for image collection. For each group (n=5), 25 randomly selected images were captured by a blinded researcher. Positive blue signal, indicating collagen, was selected based on its color ranges and the proportional area in each image was quantified using the histogram tool and expressed as a percentage of the image.

Immunohistochemistry

Paraffin embedded blocks from each group of mice were cut in sections of 5–7 μm. The sections were prepared for IHC using standard protocols. The slides were incubated overnight at 4°C with a primary antibody (TGFβ 1:75 from Santa Cruz Biotechnology, Santa Cruz, CA) and thereafter with another biotinylated secondary antibody during 1h at RT. The specific antibody binding reaction was amplified by incubation with ABC development system (Vector Lab, Burlingame, CA) during 30 min at RT. After the amplification step, the slides were washed and incubated again for 5 min with the chromogen substrate for HRP aminoethycarbazole (AEC) (Vector Lab, Burlingame, CA). Finally the slides were counter stained using Hematoxylin (Vector Lab, Burlingame, CA) and mounted using aqueous mounting media crystal/mount (Biomedia Corp, Foster City, CA)

Nitric oxide quantification

NO quantification was performed using cultures of 1×10^6cells/well and stimulation with 1×10^5 bacilli for 5 days. Thereafter, supernatants were harvested and each supernatant were used in a Greiss reaction (Sigma-Aldrich, St. Louis, MO). The experiments were performed in triplicate.

Viable Count

Serial dilutions of each lung homogenate were plated in 7H11 agar and bacterial load. The CFUs for each sample was determined after 3 weeks incubation at 37°C.

Statistical analysis

The results presented in this publication are representative of three experiments. The data are expressed as the mean± SEM values (n=5) from triplicate assays. One-way analysis of variance and the post-hoc test was Tukey's. Calculations were performed using GraphPad Prism version 4.00 for Windows (San Diego California USA). P-values <0.05 were considered significant.

ACKNOWLEDGMENTS

We thank to Dr. Alan Schenkel for reading the manuscript and their insightful comments. This work was supported by National Institutes of Health (NIH) grant AI-44072.

References

1. Andrews JR, Shah NS, Gandhi N, Moll T & Friedland G Multidrug-resistant and extensively drug-resistant tuberculosis: implications for the HIV epidemic and antiretroviral therapy rollout in South Africa. J Infect Dis 2007;196 Suppl 3, S482–490. [PubMed: 18181698]
2. Zager EM & McNerney R Multidrug-resistant tuberculosis. BMC Infect Dis 2008; 8, 10. [PubMed: 18221534]
3. Orme IM Tuberculosis vaccines: current progress. Drugs 2005;65, 2437–2444. [PubMed: 16296870]
4. Ordway D et al. Enhanced macrophage activity in granulomatous lesions of immune mice challenged with Mycobacterium tuberculosis. J Immunol 2006;176, 4931–4939. [PubMed: 16585589]

5. Moreira AL et al. Mycobacterial Antigens Exacerbate Disease Manifestations in Mycobacterium tuberculosis-infected Mice. Infect. Immun. 2002;70, 2100–2107, doi:10.1128/iai.70.4.2100-2107.2002. [PubMed: 11895976]
6. Shrivastava N & Srivastava A RNA interference: an emerging generation of biologicals. Biotechnol J 2008;3, 339–353. [PubMed: 18320564]
7. de Fougerolles A & Novobrantseva T siRNA and the lung: research tool or therapeutic drug? Current Opinion in Pharmacology 2008;8, 280–285. [PubMed: 18485820]
8. Natt F siRNAs in drug discovery: target validation and beyond. Curr Opin Mol Ther 9, 242–247 (2007). [PubMed: 17608022]
9. iorns E, Lord CJ, Turner N & Ashworth A Utilizing RNA interference to enhance cancer drug discovery. Nat Rev Drug Discov 2007;6, 556–568. [PubMed: 17599085]
10. Hokaiwado N, Takeshita F, Banas A & Ochiya T RNAi-based drug discovery and its application to therapeutics. IDrugs 2008;11, 274–278. [PubMed: 18379962]
11. de Fougerolles A, Vornlocher HP, Maraganore J & Lieberman J Interfering with disease: a progress report on siRNA-based therapeutics. Nat Rev Drug Discov 2007; 6, 443–453. [PubMed: 17541417]
12. de Fougerolles AR Delivery vehicles for small interfering RNA in vivo. Hum Gene Ther 2008; 19, 125–132. [PubMed: 18257677]
13. Birchall J Pulmonary delivery of nucleic acids. Expert Opin Drug Deliv 2007;4, 575–578. [PubMed: 17970661]
14. Bitko V & Barik S Nasal delivery of siRNA. Methods Mol Biol 2008; 442, 75–82. [PubMed: 18369779]
15. Li BJ et al. Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. Nat Med 2005; 11, 944–951. [PubMed: 16116432]
16. Li T et al. siRNA targeting the leader sequence of SARS-CoV inhibits virus replication. Gene Ther 2005; 12, 751–761. [PubMed: 15772689]
17. Bitko V, Musiyenko A, Shulyayeva O & Barik S Inhibition of respiratory viruses by nasally administered siRNA. Nat Med 2005; 11, 50–55. [PubMed: 15619632]
18. Ge Q, Eisen HN & Chen J Use of siRNAs to prevent and treat influenza virus infection. Virus Res 2004; 102, 37–42. [PubMed: 15068878]
19. Matsuyama W et al. Suppression of discoidin domain receptor 1 by RNA interference attenuates lung inflammation. J Immunol 2006;176, 1928–1936. [PubMed: 16424224]
20. Olobo JO et al. Circulating TNF-alpha, TGF-beta, and IL-10 in tuberculosis patients and healthy contacts. Scand J Immunol 2001;53, 85–91. [PubMed: 11169211]
21. Verbon A et al. Serum concentrations of cytokines in patients with active tuberculosis (TB) and after treatment. Clin Exp Immunol 1999;115, 110–113. [PubMed: 9933428]
22. Turner J et al. In vivo IL-10 production reactivates chronic pulmonary tuberculosis in C57BL/6 mice. Journal of Immunology 2002;169, 6343–6351.
23. Bonecini-Almeida MG et al. Down-modulation of lung immune responses by interleukin-10 and transforming growth factor beta (TGF-beta) and analysis of TGF-beta receptors I and II in active tuberculosis. Infect Immun 2004;72, 2628–2634. [PubMed: 15102771]
24. Flynn JL & Chan J Immunology of tuberculosis. Annu Rev Immunol 2001;19, 93–129. [PubMed: 11244032]
25. Tridandapani S et al. TGF-beta 1 suppresses [correction of supresses] myeloid Fc gamma receptor function by regulating the expression and function of the common gamma-subunit. J Immunol 2003;170, 4572–4577. [PubMed: 12707335]
26. Takaki H et al. TGF-betal suppresses IFN-gamma-induced NO production in macrophages by suppressing STAT1 activation and accelerating iNOS protein degradation. Genes Cells 2006; 11, 871–882. [PubMed: 16866871]
27. Tsunawaki S, Sporn M, Ding A & Nathan C Deactivation of macrophages by transforming growth factor-[beta]. Nature 1988;334, 260–262. [PubMed: 3041283]
28. Hernandez-Garay M & Mendez-Samperio P Transforming growth factor-beta decreases survival of Mycobacterium bovis-activated T cells. Arch Med Res 2003;34, 20–25. [PubMed: 12604370]

29. Ranges GE, Figari IS, Espevik T & Palladino MA Jr. Inhibition of cytotoxic T cell development by transforming growth factor beta and reversal by recombinant tumor necrosis factor alpha. J Exp Med 1987; 166, 991–998. [PubMed: 3498791]
30. Ding AH, Nathan CF & Stuehr DJ Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. J Immunol 1988; 141, 2407–2412. [PubMed: 3139757]
31. Branton MH & Kopp JB TGF-beta and fibrosis. Microbes Infect 1999;1, 1349–1365. [PubMed: 10611762]
32. Chakravarty SD 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]
33. Schroder M et al. Different modes of IL-10 and TGF-beta to inhibit cytokine-dependent IFN-gamma production: consequences for reversal of lipopolysaccharide desensitization. J Immunol 2003; 170, 5260–5267. [PubMed: 12734375]
34. MacMicking J, Xie QW & Nathan C Nitric oxide and macrophage function. Annu Rev Immunol 1997; 15, 323–350. [PubMed: 9143691]
35. Kitani A et al. Transforming growth factor (TGF)-beta1-producing regulatory T cells induce Smad-mediated interleukin 10 secretion that facilitates coordinated immunoregulatory activity and amelioration of TGF-beta1-mediated fibrosis. J Exp Med 2003;198, 1179–1188. [PubMed: 14557415]
36. Hernandez-Pando R et al. A combination of a transforming growth factor-beta antagonist and an inhibitor of cyclooxygenase is an effective treatment for murine pulmonary tuberculosis. Clin Exp Immunol 2006; 144, 264–272. [PubMed: 16634800]
37. Allen SS, Cassone L, Lasco TM & McMurray DN Effect of Neutralizing Transforming Growth Factor {beta}1 on the Immune Response against Mycobacterium tuberculosis in Guinea Pigs. Infect. Immun 2004; 72, 1358–1363, [PubMed: 14977939]
38. Lenaerts AJ, Degroote MA & Orme IM Preclinical testing of new drugs for tuberculosis: current challenges. Trends Microbiol 2008; 16(2):48–54. [PubMed: 18182291]
39. Higgins DM et al. Relative levels of M-CSF and GM-CSF influence the specific generation of macrophage populations during infection with Mycobacterium tuberculosis. J Immunol 2008; 180, 4892–4900. [PubMed: 18354213]
40. Lehr HA, van der Loos CM, Teeling P & Gown AM Complete chromogen separation and analysis in double immunohistochemical stains using Photoshop-based image analysis. J Histochem Cytochem 1999;47, 119–126. [PubMed: 9857219]

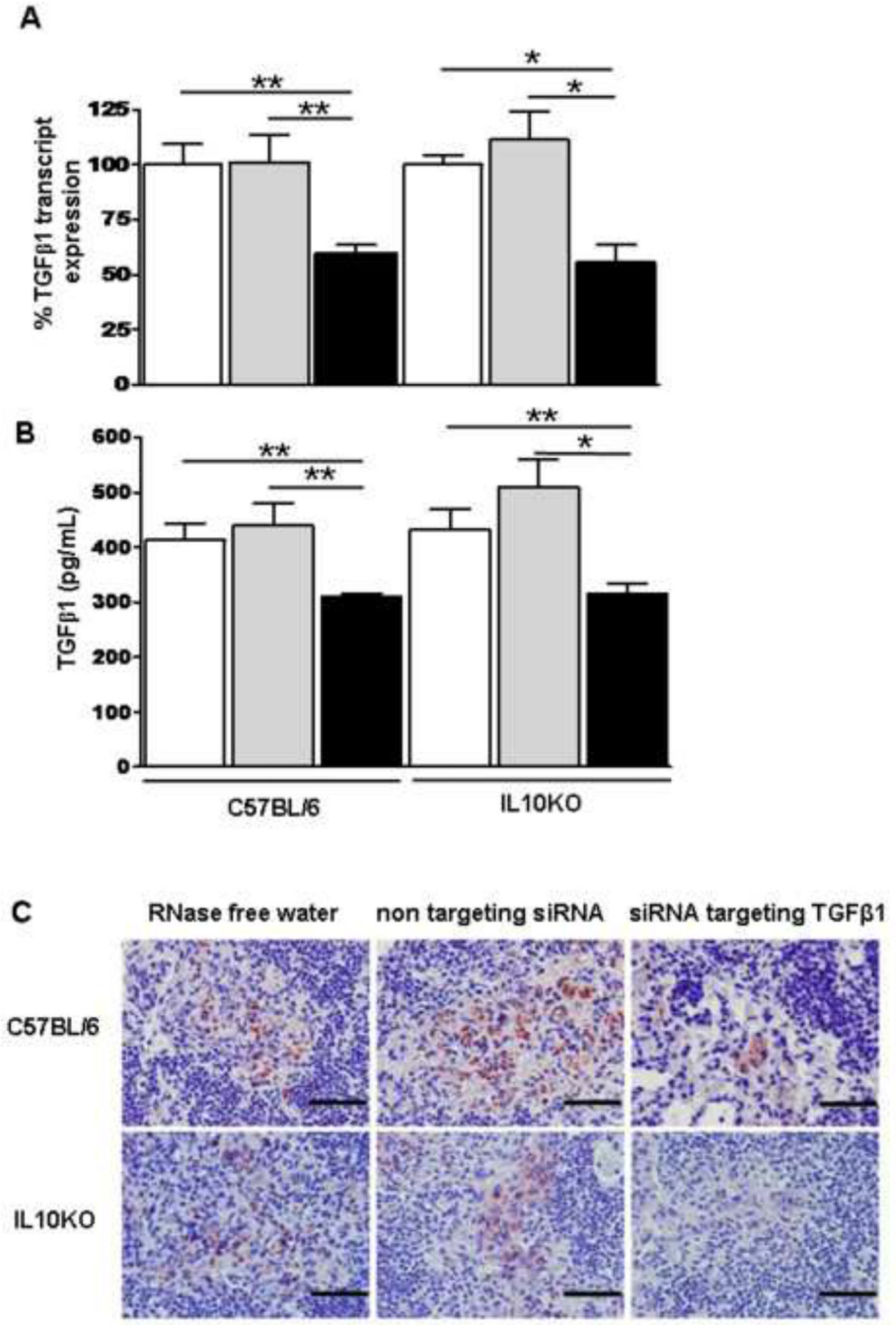

Figure 1. tgfβ1 Gene Silencing and Protein Suppression after treatment.

Groups of C57BL/6 and IL10 KO mice with a 60 days of *M. tuberculosis* infection received by intrapulmonary route three doses (10μg each dose at 5 days intervals) of siRNA targeting TGFβ1 (black bars), control non targeting siRNA (grey bars) or RNase free water (white bars). Five days after the last dose, lung samples were collected and processed for RT-qPCR, ELISA and IHC. **(A) *tgfβ1* gene silencing** represented as the percentage of relative expression of *tgfb1* mRNA by RT-qPCR analysis in lung samples from siRNA targeting TGFβ1 treated mice compared to control groups**. (B) Concentration of total TGFβ1**

protein in supernatants from lung homogenates as determined by ELISA. In A and B data represent the mean of five mice per group ± the SEM. Statistics were done using one-way analysis of variance and the post-hoc test was Tukey's *, $P < 0.01$; **, $P < 0.05$. The data presented in this figure are representative of three experiments of similar design. **(C) TGFβ *in situ* detection**. Photographs are representative images from lung tissue sections from each group of mice when stained by IHC for TGFβ. The staining for TGFβ in these photographs is depicted in red color. Bars 50 μm.

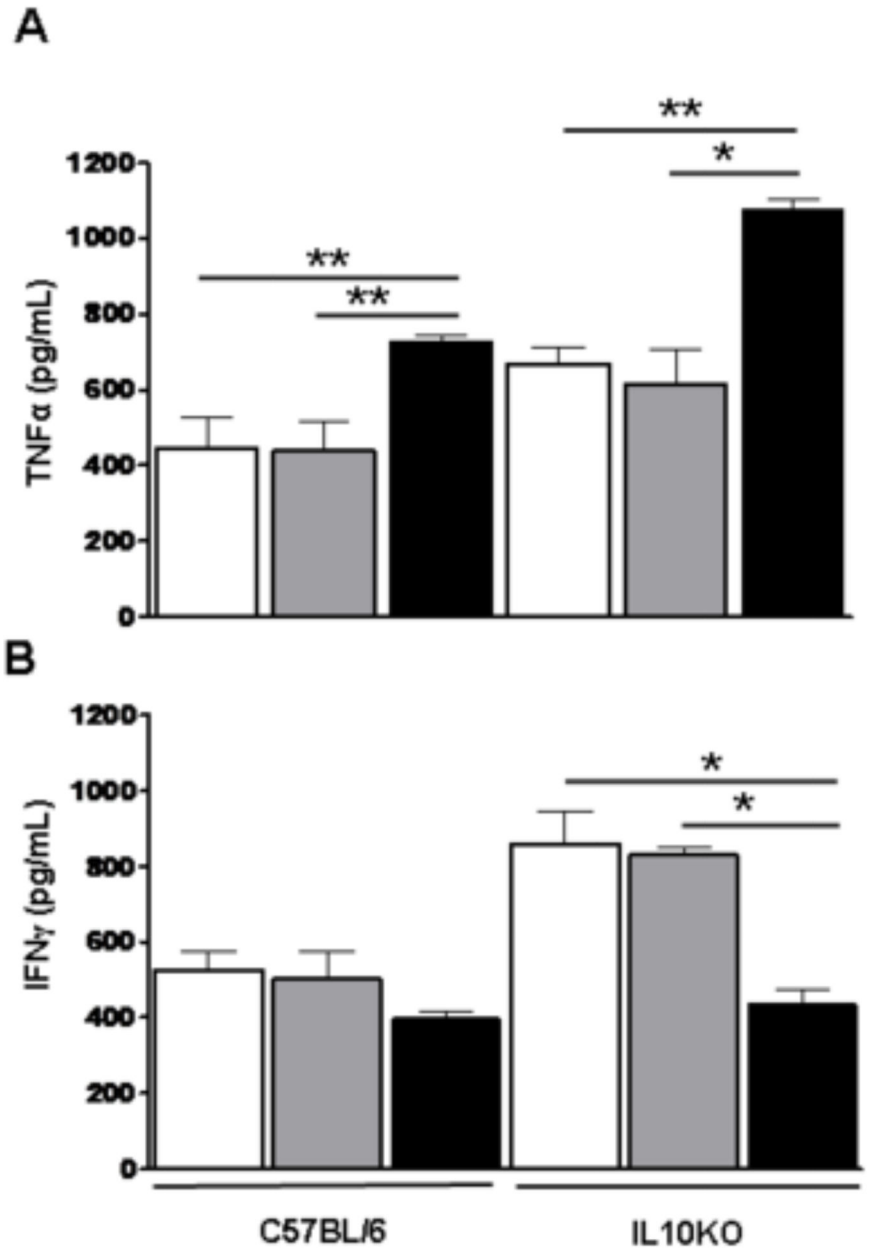

Figure 2. Concentration of Th1 cytokines in the infected lungs five days after treatment.

Following therapy lung samples were collected and processed for ELISA. Graphs show concentration of TNF-α (A) and IFNγ (B) in supernatants from lung homogenates from each group of mice. Data represent the mean of five mice per group ± the SEM. Statistics were done using one-way analysis of variance and the post-hoc test was Tukey's *, $P < 0.01$; **, $P < 0.05$. The data presented in this figure are representative of three experiments of similar design.

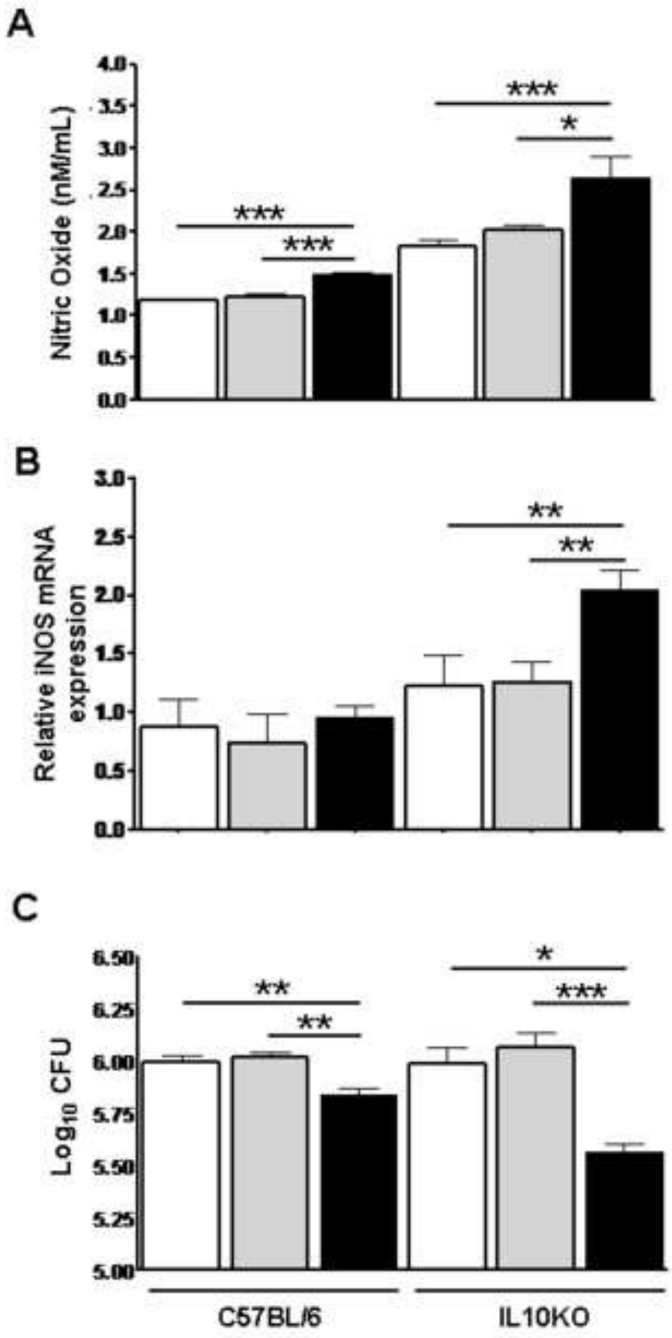

Figure 3. Higher levels of antimicrobial mediators after siRNA targeting TGFβ1 treatment.

Following therapy **l**ung samples from mice were collected (A) as lung cell suspensions to determine the concentration of nitric oxide by the Greiss reaction ; (B) in Trizol for RNA isolation and RT-qPCR assay to determine expression of iNOS mRNA or (C) as lung homogenates to determine the pulmonary bacterial load after therapy. In graph (A) is shown the nitric oxide concentration (nmol/ml) in each group of mice. Data are represented as the mean of five mice per group ± the SEM. Graph in (B) are shown the relative levels of expression of mRNA iNOS. Data are represented as the mean of five mice per group ± the

SEM . Graph in C shows the mean $\log_{10}$ number of colony forming units (CFU) in the lungs from each group of mice at 5 days after the last treatment. The $\log_{10}$ CFU was reduced by 0.17 and 0.5 in the siRNA targeting TGFβ1 C57BL/6 and IL10 KO treated mice respectively when compared to their respective control groups. Statistics were done using one-way analysis of variance and the post-hoc test was Tukey's *, $P < 0.01$; **, $P < 0.05$; *, $P < 0.001$. The data presented in this figure are representative of three experiments of similar design.

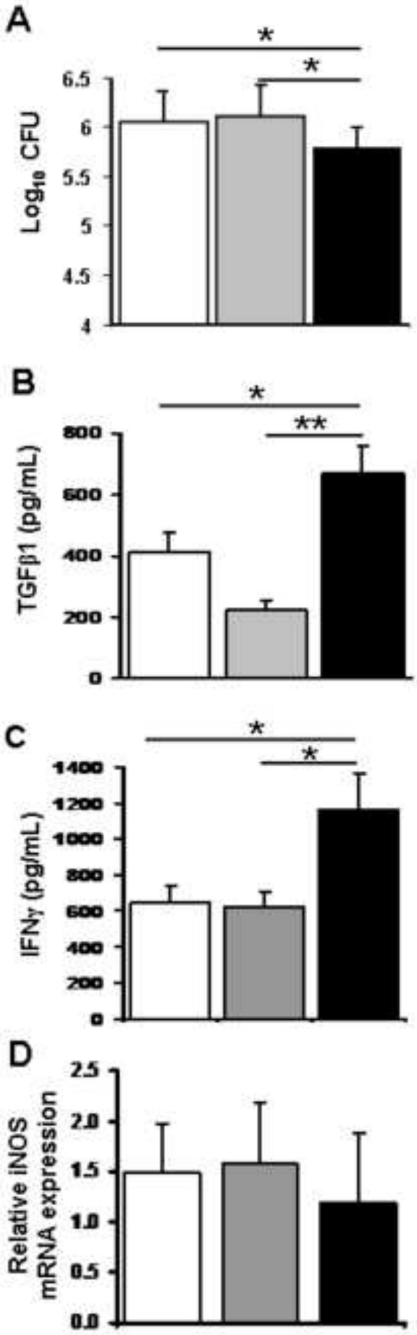

Figure 4. Reduction of bacterial load remained for at least four weeks after the last treatment in siRNA targeting TGFβ1 treated IL10KO mice.

IL10KO mice with a 60 days of *M. tuberculosis* infection received by intrapulmonary route three doses (10μg each dose at 5 days intervals) of siRNA targeting TGFβ1 (black bars), control non targeting siRNA (grey bars) or RNase free water (white bars). Four weeks after the last dose, lung samples were collected and processed for (A) determination of the bacterial load in each lung homogenate, (B) for ELISA using supernatant from lung homogenates to determine the concentration of TGFβ1,(C) of IFNγ and (D) for RNA to determine the levels of expression of iNOS mRNA. In (A) four weeks after the last treatment

IL10KO mice treated with siRNA targeting TGFβ1 have 0.32 $\log_{10}$ reduced bacterial load when compared to control mice. In (D) the relative levels of expression of mRNA for iNOS were not different between groups of IL10KO mice after four weeks of treatment. Data are represented as the mean of five mice per group ± the SEM. Statistics were done using one-way analysis of variance and the post-hoc test was Tukey's *, $P < 0.05$; **, $P < 0.001$. The data presented in this figure are representative of two experiments of similar design

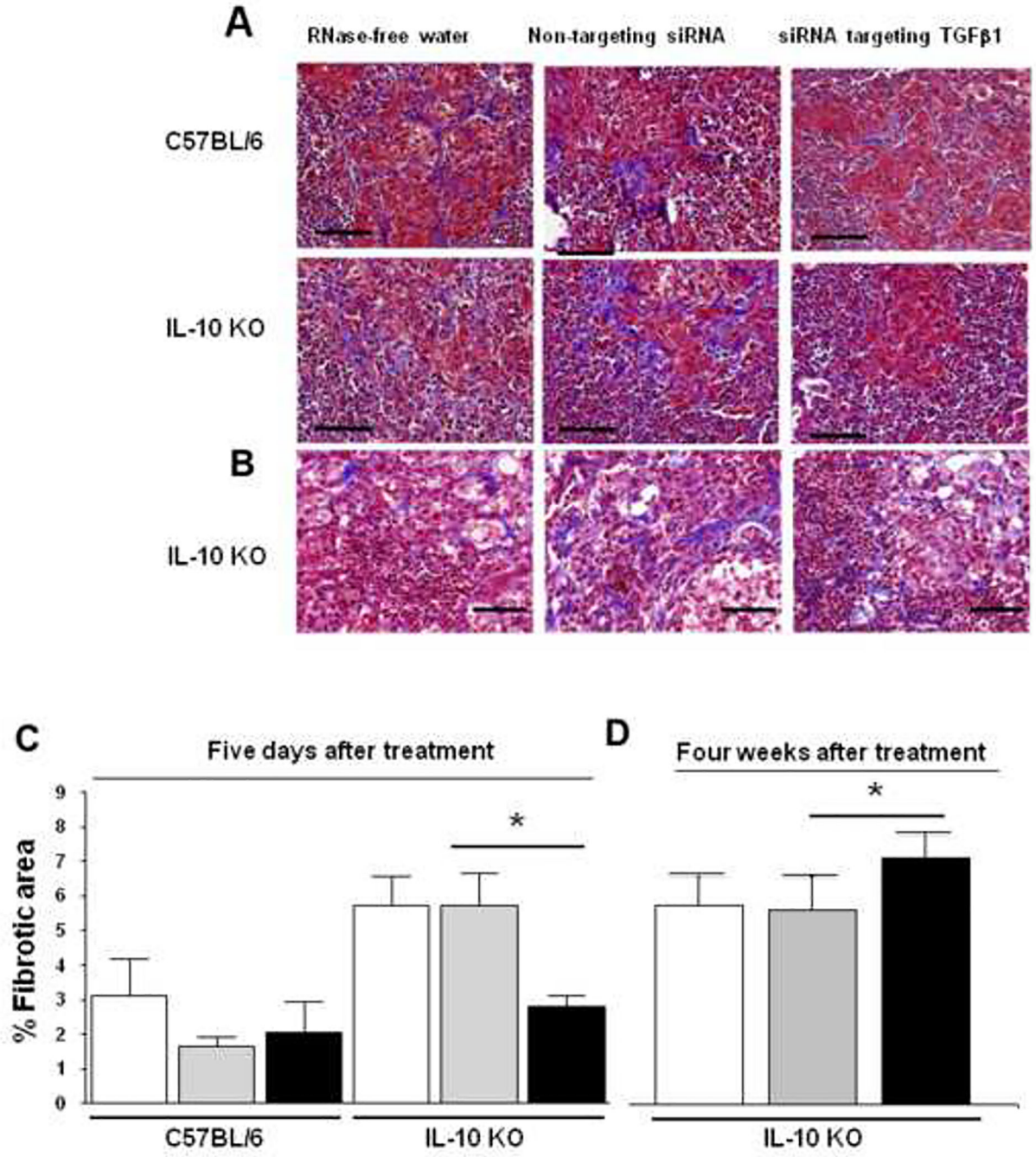

Figure 5. Quantitative image analysis of Fibrosis using Masson's Trichrome Staining.

C57BL/6 and IL10KO mice with a 60 days of *M. tuberculosis* infection received by intrapulmonary route three doses (10μg each dose at 5 days intervals) of siRNA targeting TGFβ1 (black bars), control non targeting siRNA (grey bars) or RNase free water (white bars). (A and D) Five days or (B and D) four weeks after the last dose, lung tissue sections were stained by Masson's trichromic to visualize fibrotic areas. Photos are representative images (40X) from lung tissue sections obtained from each group and stained using the Masson's trichromic protocol. In these photographs the blue color indicates positive staining

for collagen-fibrosis. Graphs in C and D represent the mean ±SEM score of percentage of fibrotic area in each lung section. Score of percentage of fibrotic area of lung section stained were obtained using computer-assisted image analysis. Positive blue signal, indicating collagen, was selected based on its color ranges and the proportional area in each image was quantified using the histogram tool and expressed as a percentage of the image. Five days after receiving the last dose of treatment fibrosis was reduced ~50% in IL10 KO mice treated with siRNA targeting TGFβ1 compared with control mice. However, there were no significant differences in fibrosis between groups after four weeks of the last treatment. Statistics were done using Student *t* test *, $P < 0.05$. Bars 50μm.