**Abstract**

Current tuberculosis (TB) chemotherapy is incapable of rapidly eliminating one hundred percent of the *Mycobacterium tuberculosis* (Mtb) bacilli. Although 99% of the bacteria are eliminated within three weeks of commencing treatment, a small population of drug-persisting bacilli (~ 1%) requires an additional 6-8 months of multidrug treatment to be eliminated. To cure TB faster we need to develop new therapies that effectively and rapidly eliminate drug persistent bacteria. Host targeted therapy has emerged as one choice in achieving these objectives. Previous studies in our laboratory and others have shown that elevated expression of the IL-10 cytokine and subsequent persistent activation of the STAT3 during a chronic infection with *Mtb* obstructs the antimicrobial capacity of the host. In these studies we show that standard TB chemotherapy further enhance pulmonary levels of IL-10 cytokine and the signal transducer and activator of transcription (STAT) 3 activation and reduce expression of IFNg cytokine along with reduced expression of antimicrobial products (NO, NADPH activity) when compared with animal controls chronically infected with Mtb and not receiving standard TB chemotherapy. We also demonstrated that chronically infected C57BL/6 mice after standard chemotherapy followed by intrapulmonary aerosol delivery of siRNA targeting *il-10* or *stat3* had reduced the drug persistent bacterial load by 0.40 log10 compared to controls. Accordingly, the antimicrobial capacity of the host was further enhanced upon treatment with siRNA *il10* and *stat3* as evidenced by higher pulmonary expression of the nitric oxide, NADPH and decreased arginase activity when compared to control groups. Overall, this study demonstrates that a successful targeting of the host anti-inflammatory pathway can modulate the lung host immune response to enhance its own antimicrobial capacity. Our ultimate aim is to develop host targeted therapies that when combined with the current standard TB chemotherapies leads to more rapid elimination of the *Mtb* from the lungs.

**Introduction**

Tuberculosis (TB) represents an enormous challenge to global health because of the inadequacy of currently available drugs and vaccines. The lengthy treatment for TB is the primary cause for the emergence of multidrug resistant tuberculosis, as it frequently results in non-compliance. Accordingly, the failure to comply with the long term chemotherapies for TB are one of the main reasons for the continued emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Mycobacterium tuberculosis* (Mtb) strains which have spread globally. Eradication of TB depends on the development of shorter and more effective treatment regimens, including novel treatment alternatives combined with classical TB therapies. To improve TB control is essential that the length of standard anti-TB chemotherapy regimen is reduced from 6-9 months to 2 months ( ). Host targeted therapies in combination with current therapies for TB has emerged as one approach towards this goal. In this study we show that it is possible to modulate the lung immune environment of the Mtb chronically infected host and increase its own antimicrobial capacity against this bacilli. The end result of this therapy will be increased capacity by the host to eliminate the drug persisting bacilli remaining in the lungs during or after standard chemotherapy.

The host immune response elicited against pulmonary infection with *M.tuberculosis* (Mtb) in most instances is incapable of eliminating hundred percent of the bacilli and thus develops into a chronic stage of infection. The host is able to eliminate the bacilli and control the spread of the infection by producing potent antimicrobial products via activation of the TH1 immune response. The downside of the TH1 response is its associated inflammatory nature which overtime severely compromises the main function of the lungs; inhalation and exhalation of oxygen and carbon dioxide respectively. Therefore, as the chronic stage of Mtb ensues the host (the lungs) has to weight in and balance containment and dissemination of Mtb bacilli with controlling inflammation and healing the damaged lung epithelia. Today we know that the lung overall immune response against Mtb infection controls inflammation by activation of potent anti-inflammatory mechanisms that coexist with the TH1 immune response while the Mtb bacilli is present in the lungs. Chief among the anti -inflammatory mediators is activation of the IL-10 cytokine. The IL-10 cytokine has the remarkable property of blocking the inflammatory properties of TLR and TH1 cytokines, as well as inhibiting nitric oxide production by activated macrophages [ref]. Signal transducer and activator of transcription (STAT) 3 is essential for all known functions of IL-10 [ref]. As it is becoming increasingly clear in the cancer field [ref], containment of inflammation via persistent activation of IL-10 and STAT3 substantially and progressively diminishes the production and efficacy of the host own antimicrobial capacity to eliminate the Mtb bacilli.

The efficacy of antimicrobial products against Mtb has been extensively studied [ref]. Phagocytic cells (macrophages and dendritic cells) are the main niches of Mtb survival during the chronic stage of infection as well as effectors of the antimicrobial capacity in the Mtb infected host. This puzzle is further complicated by the fact that macrophages (and to a lesser extent dendritic cells) are also essential for tissue healing and repair during or after inflammation. Two enzymes: the inducible nitric oxide synthase (iNOS) which produce nitric oxide (NO; potent antimicrobial effector molecule; ref) and arginase 1 (Arg-1; which end product is L-proline an important amino acid used in collagen synthesis during wound healing; ref) are key to antimicrobial and healing-anti inflammatory functions ascribed to macrophages. Both enzymes iNOS and Arg-1 compite with each other for their substrate L-arginine. Furthermore, while TH1 cytokines (IFNΥ, TNF, IL-1) promote expression of iNOS to produce NO, the TH2 cytokines (IL-10, IL-4, GM-CSF and TGFβ1) promote expression of Arg-1. Thus, during a chronic infection with Mtb, the fine-tuned TH1/TH2 cytokine; iNOS/Arg-1 profiles and antimicrobial products are responsible for restraining the bacilli within a granuloma lesion in which inflammation and healing-repair of the tissue is also controlled by persistent activation of the IL-10 signaling pathways. A downside effect in this scenario is reduced expression of NO and subsequent reduction in the microbicidal capacity of the macrophages at the site of the infection. Hence, in our studies we hypothesized that persistent activation of the IL-10 signaling pathways at the Mtb infection site diminishes the macrophage’s own capacity to eliminate both drug susceptible and drug tolerant bacteria.

Furthermore, current anti-TB drugs are slow to eradicate *M. tuberculosis* in the lungs of patients. Most anti-TB target the replicative stage of the bacilli and it is believed that the strong host immune response elicited against this bacillus is capable of slowing down the Mtb replication and thus reduces efficacy of most anti-TB drugs. Thus, animal models of tB infection have shown that the standard TB chemotherapy is capable of rapidly reducing the drug sensitive pulmonary bacterial load (1-2 log10 reduction) during the first two weeks of treatment [ref] but the remaining bacilli left after this period of treatment takes 2-3 additional moths of treatment to be eliminated [ref].

The standard TB chemotherapy during the first two weeks is clearly associated with significant reduction in the inflammatory responses of the lung [ref]. Reduction of inflammation in the lungs after TB chemotherapy has been correlated with reduction in the bacillary load. However the pulmonary profile of TH1/ TH2 cytokines, NOS-2/Arg-1 along with expression of antimicrobial effector molecules after chemotherapy have not been studied. In this study we questioned whether standard chemotherapy synergize or antagonize with the TH1 response and anti-inflammatory scenario presented above and we hypothesized that reduction of the pulmonary bacterial load during chemotherapy correlates with decreased expression of TH1/TH2 cytokines, INOS-2/Arg-1 and antimicrobial effectors in the lungs. For this purpose we first studied the pulmonary of TH1/TH2 cytokine profile along with the levels of expression of antimicrobial mediators (NOS-2, Arg-1, NO, NADPH) after two weeks of standard TB chemotherapy given to chronically Mtb infected C57BL/c mice. Thereafter we hypothesized that successful targeting of the host anti-inflammatory pathway after chemotherapy can modulate the lung immune response and enhance its own antimicrobial capacity to eliminate the drug tolerant bacilli. We delivered via intrapulmonary aerosol siRNA targeting *il-10* and *stat3* and we monitored the pulmonary drug tolerant bacterial load, profile of TH1/TH2 cytokines and expression of antimicrobial effector products (NO, NADPH, NOS-2 and Arg-1) after treatment. In addition, we also monitored changes in the inflammatory course caused by the therapy by recording the histological outcome after chemo- siRNA therapy.

**Material and Methods**

**Mice and Experimental infection.** Six to eight-8 week old C57BL/6 female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were kept in sterile condition in a BSL3 room and they were rested for a week prior to infection. Micewere infected with a low dose aerosol infection using the Glas-Col Inhalation Exposure System calibrated to deliver ~50-100 *Mycobacterium tuberculosis* (Erdman strain, TMC107; ATCC 35801) bacilli per mouse. Bacterial deposition in the lungs after aerosol was determined by sacrificing three mice at day 1 post-infection and the lungs were homogenized and prepared for bacterial load quantification as follow. At different time post infection and post treatment mice were euthanized and the lungs and spleen were homogenized using the Next Advance Bullet Blender (Averill Park, NY). Briefly, the left lobe of the lung or spleen were placed in a 1,5 ml sterile safe lock eppendorf tubes containing 1 ml of sterile saline and 3 x 3.2mm sterile stainless steel beads, thereafter the tubes were placed in the Bullet Blender and 4 min and 8 xxx. The bacterial load was determined using serial dilutions of homogenized organs that were plated on 7H11 agar plates and the colony forming units in each sample was determined after 3 weeks incubation at 37°C as previously described (2). Bacterial load in each animal and organ was expressed as the log10 of colony forming units (CFUs).

**Drug therapy**

On day 45 post-infection mice received drug therapy via oral gavage as reported earlier (3). Briefly, mice treated by oral gavage received isoniazid INH (Sigma Aldrich) and rifampicin (XXX) five times a week at a dose of 25 mg/Kg and (XXX) respectively as previously reported (lennaerts lab). After 2 and 4 weeks of chemotherapy mice were euthanized; the lungs and spleens were homogenized and bacterial load determined as indicated above.

**siRNA targeting *il-10* and *stat-3*. Several small trials were developed to test several sequences predesigned by Dharmacon.** The *stat-3* was selected as reported by XXX et el. and tested in our system in small trial. The commercially predesigned from the gene number access for *tgfb-1* (NM\_011577) and stat-3 (NM\_ XXXXX). 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 for *il-10 form Dharmacon commercial xxx*. The sequence of the *il-10* gene targeted by this siRNA is XXX CAA CAA CGC CAT CTA TGA. The sequence of the *stat-3* gene targeted by this siRNA is CCG CAA CAA CGC CAT CTA TGA. Thereafter the siRNA was commercially produced and purchased from IDT. (XXX, XXX). AllStars negative control siRNA was included as a negative control and was also obtained from Qiagen (Valencia, CA).

**siRNA targeting *Il-10* and *stat3* intrapulmonary aerosol delivery.** Mice were treated with siRNA following a protocol previously described [ref]. Mice received the siRNA by intrapulmonary aerosol delivery using a microsprayer device (Microsprayer, model IA-C; PennCentury, Philadelphia, PA) attached to an FMJ-250 high pressure-syringe device (PennCentury) as described earlier (17, 19-20). Briefly, mice were anaesthetized using isofluorane and oxygen mix (5% isofluorane in oxygen 4L/min; VIP 3000 isofluorane vaporizer] for about 10 minutes until animals were sedated. Each mouse was placed on its abdomen in a perspex support adjusted to 45° angle, the teeth were suspended up with an incisor loop located on top. The mouth was opened and with the help of a cotton tip, the tongue was pulled out. Then, the MicroSprayer™ tip was aimed and introduced into the trachea until reaching the carina and the formulation was sprayed. The mouse was taken off the support and laid on its cage until it awaked from the anesthesia (2-3 minutes). After administration of the anesthetic the animals were monitored for regular breathing and clinical behavior. The mice received four doses at 3 days intervals of (0.75nM) 10 µg per mouse/dose of siRNA targeting *il-10* or *stat3* or AllStars negative control or RNAse-free water.

**ELISA assays.** Lung sample were homogenized and screened in triplicate by ELISA following manufacturer’s protocol for pSTAT3.

**RT-qPCR.** The lung lobe was homogenized in Trizol using Next Advance bullet Blender (Averill Park, NY) 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 analysis.** The diaphragmatic lobe of the lungs of each mouse was placed into histology cassette and fixed in 4% paraformaldehyde. After 48 h, samples were inactivated inside the BSL-3 laboratory and then processed using standard histological protocols.

**Cytometric Bead Array Analysis**

The lung homogenates were analyzed inside the BSL3 laboratory using the CBA kit from BD Biosciences (Franklin Lakes, NJ), which analyzed IL-6, IL-10, IL-12, TNF- IFN-, and MCP-1. Prior to analysis the samples were thawed at 40C and centrifuged to remove all sediments. At the end of the protocol the samples were decontaminated by fixation with 100 ul of 4% PFA per sample and incubation at 4C for at least 24 hrs. Thereafter the samples were read using a FACsCanto II by using BD Biosciences CBA software (Das). The cytokine levels of samples were calculated by extrapolating the mean fluorescence intensity (MFI) by using the standard curves for every cytokine respectively.

**Immunohistochemistry**

Paraffin embedded blocks from each group of mice were cut in sections of 5-7 μm using a microtome and placed onto slides. Thereafter, the paraffin was removed from the tissue sections using EZ-DeWax solution (Biogenex Lab, San Ramon, CA) . Following this step, sections were washed and after blocking the tissue endogenous peroxidise with Peroxidased 1 (Biocare, CA) during 5 min at room temperature. Thereafter the sections were treated with antigen retrieval using the citrate buffer (Dako, CO) and pressure cooker standard procedure. The unspecific binding of antibodieswas blocked by incubating the sections for 5 mintues at room temperature background eraser (Biocare, CA) and then each section was incubated overnight at room temperature with a primary antibody raised against murine F4/80, lysozyme, and IL-10 antigens. After washing the slides three times for 5 min in PBS at RT, the slides were incubated again with the appropriate secondary antibody during 60 min at RT. Thereafter the specific antibody binding reaction was amplified using one of the Tyramide Signal Amplification (TSA) system using a kit purchased from PerkinElmer Life and Analytical Sciences Inc, Boston, MA. This step was done with an adaptation of the manufacturer’s instructions, briefly, when the TSA system was performed, after incubation with the secondary antibody, the sections were incubated with Streptaviding conjugate to Horseradish peroxidise (SA-HRP) at a dilution 1/100 for 30 min at RT followed by a 2 min incubation at RT with the biotinylated TSA reagent. After the amplification step, the slides were washed and incubated again for 5 min with the chromogen substrate for HRP either 3,3’-diaminobenzidine (Impact DAB; VectorLab, MI) or aminoethycarbazole (Impact AEC; VectorLab, MI). Finally the slides were counter stained by immersing the sections for 1 min in Mayer’s Hematoxylin (DakoCytomation, Carpenteria, CA) and mounted for microscopic observation using coverslip and Histomount mounting media (Zymed, San Francisco, CA). As a negative control the procedure described above was also carried out omitting the primary antibody step. Sections were examined using a Olympus X70 microscope.

**Nitric oxide quantification.** NO quantification was performed using lung homogenates and …... The experiments were performed in triplicate. Lung homogenates supernatants spun and f each supernatant were used in a Greiss reaction (Sigma-Aldrich, St. Louis, MO).

**Arginase activity**

**NADPH activity**

**Meyeloperoxidase**

**Superoxide dismutase**

**Statistical analysis.** The results presented in this publication are representative of several small trials and two- three larger trials. 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.

A statistical analysis was performed using one-way analysis of variance followed by and each time point, followed by pairwise contrasts of treatment means using Tukey test

**Results**

**Standard TB Chemotherapy reduced bacterial load but antagonized with host’s own antimicrobial capacity**

The bacterial load in the lungs of chronically Mtb infected mice with (n=5) or without standard chemotherapy (n=5) is shown in Figure 1A. The lungs of chronically Mtb infected mice after two weeks of treatment with INH and RIF had 1.7 log10CFU reduction when compared to the CFU obtained in similar samples from chronically Mtb infected mice without drug treatment [control group]. However as shown in Figure 1B, when RNA lung samples obtained from the same group of mice were tested by RT-PCR for *Il-10,stat3*, iNOS and *arg1* transcripts, mice receiving standard TB chemotherapy had several fold increase in the expression of transcripts for both *il-10* and *stat3* when compared to the control group. The samples demonstrated several fold reduction for iNOS transcript whereas an increased in the *arg1* transcript was observed. Similarly [Figure 1C), the protein expression in the same groups of mice was increased for IL-10 and pSTAT3 (activated form of STAT3) in samples obtained from mice receiving chemotherapy and when compared similar samples of the control group. Furthermore, another two important TH1 immunological biomarkers, IFNg and IL-12 cytokines were studied. Interestingly, IFNg and IL-12 cytokine expression was reduced and increased respectively in the group of mice receiving anti TB chemotherapy when compared with control samples.

The overall antimicrobial capacity in the lung of chronically Mtb infected mice with or without standard chemotherapy is shown in Figure 1D. We assessed the levels of several antimicrobial biomarkers known to affect survival of Mtb in the lungs [ref]. This included expression of transcripts for iNOS, Arg1 and the end products derived from several enzymatic activities (iNOS, NADPH, lysozyme, MPO and SDM) participating in the production of reactive nitrogen intermediaries (RNI) and reactive oxygen intermediaries (ROS). The results obtained demonstrated that lung homogenates samples obtained from chronically Mtb infected mice receiving chemotherapy had lower expression of NO, lysozyme, MPO than similar samples obtained from control groups.

Next we studied the inflammatory outcome after standard TB chemotherapy by comparative examination of the histological preparations of lungs tissues obtained from chronically Mtb infected mice with or without chemotherapy. As shown in Fig 1E tissue sections stained with H&E of mice receiving chemotherapy had lower infiltration of leukocytes and thickening of the lung parenchyma than chronically Mtb infected mice that did not receive standard chemotherapy treatment. However it is important to notice that the MHC class II antigens (an important biomarker for immunological activation of macrophage and dendritic cells) was strongly reduced in the resident macrophages of the lung parenchyma and macrophages and dendritic cells located at the lesion site of mice receiving standard TB chemotherapy when compared to similar samples of chronically Mtb infected mice without chemotherapy (Fig1F). Altogether these results demonstrated that standard TB chemotherapy administration to Mtb chronically infected mice reduced by more than 95% the bacterial load in the lungs but was unable of eliminating a population of bacilli that were slow or drug tolerant. However the results presented above also demonstrated that anti TB chemotherapy also increased the expression in the lungs of anti-inflammatory mediators (IL-10 and STAT3 activation pathway) that paralleled with a reduction in the antimicrobial mediators as well as TH1 response in the host.

**Intrapulmonary administration of siRNA targeting *il-10* or *stat3* reduced the Mtb drug tolerant bacteria load**

We are aiming at identifying therapies that reduce the drug tolerant bacterial load. Thus, mice receiving standard anti TB chemotherapy during two weeks as indicated above were followed with a treatment consisting of intrapulmonary aerosolized siRNA targeting the *il-10* or *stat3* transcripts. After two weeks of chemotherapy mice received four doses of siRNA administered at 3 days intervals during two weeks. Thereafter the mice were euthanized and the lungs were collected and assayed for CFU (Figure 2A), il-10, stat3, nos and arg1 transcripts (Figure 2B), and cytokines IL-10, IFNg, IL-12 (Figure 2C) and pSTAT3 (Figure 2D) as indicated above. The results indicated that mice (n=5) that received either siRNA targeting *il-10* or *stat3* had reduced by 0.4 log 10 CFU when compared to the CFU in the groups of control mice (aka mice not receiving siRNA or mice treated with a sham siRNA) (Figure 2A). The efficacy of the siRNA in reducing the expression of transcripts for *il-10* and *stat3* was demonstrated via RT-PCR. As shown in Figure 2B the expression of transcript for *il-10* was reduced by several folds in mice treated with siRNA targeting *il-10* but not in mice treated with siRNA targeting *stat3* and when compared to the expression of the same transcript in the groups control. However, several folds reduction in *stat3* transcript was found in both groups of mice receiving either the siRNA targeting *stat3* or siRNA targeting *il-10*.

The NADPH oxidase family protein complexes generate superoxide anion and downstream reactive oxygen intermediaries [ROS] . The phagocyte NADPH oxidase is the principal source of ROS generation in activated neutrophils and macrophages. Thus we determined the levels of expression of the NADPH oxidase activity using lung homogenates obtained from chronically Mtb infected mice with or without standard chemotherapy.

Discussion

Our results showed that standard TB chemotherapy reduced the pulmonary bacterial load in mice chronically infected with Mtb but this treatment also antagonized with the host's own bactericidal capacity. Thus, mice with a pulmonary chronic infection of Mtb when treated with standard chemotherapy consisting of INH and Rifampin had a significant reduction of the pulmonary bacterial load. As expected this chemotherapy was unable to eliminate hundred percent of the bacteria present in the lung and thus a population of drug tolerant bacteria was found in the lungs at the end to two weeks of chemotherapy treatment. Standard chemotherapy treatment however also reduced the overall host's own bactericidal capacity. The latter was demonstrated by comparative analysis of the expression of immunological markers associated with anti microbial and anti inflammatory responses in the lung samples from chronically Mtb infected mice receiving standard chemotherapy with similar samples obtained from chronically infected Mtb mice that did not receive standard TB chemotherapy. We observed that mice receiving chemotherapy had reduced IFNg expression and enhanced expression of IL-10 and pSTAT3 which subsequently correlated with reduced expression of antimicrobial effectors (NO,NADPH,Lysozyme) and increased Arg-1 expression. Furthermore, because of reduced bacterial load and more potent expression of anti-inflammatory mediators (IL-10 and Arg-1) the inflammatory response was significantly reduced. Immunohistochemistry staining of tissue sections obtained from mice treated with standard TB chemotherapy also revealed a significant reduction in the expression of the MHC class II antigens (an immunological biomaker for macrophages and dendritic cell activation) in resident macrophages of lung tissue when compared to similar mice that did not receive chemotherapy treatment. Thus, any expectations that the host could eliminate the drug tolerant bacilli remaining after two weeks of chemotherapy are even lower than prior to standard anti TB chemotherapy. Hence it was our approach to reduce the expression of the IL-10 and STAT3 expression after or during chemotherapy to test whether the host’s own antimicrobial capacity was enhanced and whether the drug tolerant bacilli load was decreased.

IL-10 is a feature of human and murine tuberculous granuloma. This cytokine can promote pathogen persistence inside the infected macrophage by contributing to Mtb-phagosome maturation arrest [ref] and by inhibiting iNOS expression [ref] and subsequent reduction of NO [ref]. IL-10 cytokine perform most of its activities via activation of the STAT3 [ref].

The NADPH oxidase family protein complexes generate superoxide anion and downstream reactive oxygen intermediaries [ROS] . The phagocyte NADPH oxidase is the principal source of ROS generation in activated neutrophils and macrophages. Thus we determined the levels of expression of the NADPH oxidase activity using lung homogenates obtained from chronically Mtb infected mice with or without standard chemotherapy.

**The NO by itself or after reacting with O radical, (product of NADPH oxidase metabolism) will generate NOO radical with also very potent antimicrobial activity against Mtb.**

Production of

Both cytokines block the phagocyte immune-derived bactericidal mechanisms [1-2]. TGFβ1 and IL-10 cytokines are elevated in patients with chronic and active TB [including MDR-TB] and in animal models of TB infected with drug susceptible and MDR-TB strains [1, 3-7]. We hypothesize that reducing the expression of TGFβ1 and IL-10 cytokines during the *M. tuberculosis* infection will enhance the immune-derived bactericidal capacity of the host. We first want to demonstrate “as a proof of in vivo concept” that the levels of TGFβ1 and IL-10 cytokines can be transiently reduced by intrapulmonary delivery of host targeted gene therapy and that reduction in the expression of these cytokines is associated with a reduction of the pulmonary bacterial load. Our preliminary data indicate that pulmonary delivery of siRNAs can both inhibit TGFβ1 production and reduce bacterial load in mice chronically infected with *M. tuberculosis*. This effect was enhanced in IL-10 KO mice, suggesting that to achieve maximal enhancement of pulmonary immune-derived bactericidal capacity it is necessary to target both cytokines simultaneously. In the R21 phase of this application we will assess whether simultaneous silencing of the *Tgfβ1* and *Il-10* transcripts in wild type mice chronically infected with *M. tuberculosis* improves the host pulmonary bactericidal activity, including against drug persisting bacilli. Having demonstrated this, we will expand into the R33 arm of this application to study whether combined chemotherapy and immunotherapy eliminates *M. tuberculosis* bacilli more rapidly and to assess whether immunotherapy eliminates multidrug resistant *M. tuberculosis* bacilli. Three specific aims will be pursued to test the following hypotheses:

The broad long-term goal of these studies is to target drug sensitive and persisting bacilli using a two-pronged attack that combines standard anti-TB chemotherapy with therapeutics targeting pulmonary anti-inflammatory pathways. Treatment of TB represents an enormous challenge to global health because of the inadequacy of currently available drugs and vaccines. Although the existing antibiotics are able to kill most strains of *M. tuberculosis* bacilli, the incidence of MDR-TB and XDR-TB is increasing significantly day by day. The long period of TB treatment and the lack of compliance by patients certainly hold a lot of the blame for the emergence of MDR-TB and XDR-TB strains. Adding to this, recent studies reveal that there are great disparities in the outcome of chemotherapy treatment. These disparities are associated with differences in the progression of TB immunity in patients as well as the apparent increased virulence of the *M. tuberculosis* strains circulating around the world [8-9].

The standard regimen for drug-susceptible tuberculosis today requires 6-9 months of multidrug therapy. For TB patients with MDR-TB and XDR-TB infections, treatment lasts for more than two years and are unsuccessful in more than 50% of the cases. Around ten drug candidates are now in clinical development thereby giving us the largest pipeline of early-stage projects and compounds in history. The ultimate goal of the WHO and Gates Foundation is to shorten the duration of TB treatment to 2-4 weeks as this is the only way to reduce the development of resistance against the new chemical entities. Recent efforts by the CPTR program (Critical Path to New TB Regimens) strive to develop a novel short-term combination drug regimen for TB based on 3-4 new drugs with a novel mechanism of action.

Introduction

The primary goal of this proposal is to more rapidly eradicate drug tolerant bacilli using immunotherapeutic approaches. We believe it is possible to enhance the host's own bactericidal response by using immunotherapy to combat pulmonary immunosuppression. Our preliminary studies indicate that delivery of small interfering RNA [siRNA] transcripts targeting the TGFβ1 cytokine, reduces the pulmonary bacterial load of mice chronically infected with *Mycobacterium tuberculosis.* Moreover, this effect is enhanced in the absence of the IL-10 cytokine. Here we will test whether siRNA targeting of TGFβ1 and IL10 can enhance clearance of drug tolerant bacilli and whether this approach is efficacious in chronic MDR-TB infections. The outcomes will be documented using comprehensive bacteriologic, immunologic, pathologic approaches now routine in our laboratory. We are uniquely equipped with state-of-the-art BSL-III research facilities and have assembled a team of highly experienced researchers with expertise in the fields of mycobacteria infection and RNA regulation in eukaryotic cells. Thus, this application uses innovative, cutting-edge research to develop anti-bacterial products directed against NIAID Category C Priority Pathogens.

Current chemotherapy for tuberculosis (TB) is very lengthy due to the small population of persisting bacteria (~ 1%) that resist drug treatment. The protracted treatment for TB is in part responsible for the emergence of multidrug resistant [MDR-TB] and extensively drug resistant [XDR-TB] strains of *Mycobacterium tuberculosis* [*M. tuberculosis*]seen in the last decades. Today at least 52 countries have reported MDR-TB and XDR-TB cases which cannot be cured or contained by current TB therapy. At the latest World Lung Health meeting (Lille, France Oct. 2011) alternative treatment strategies were discussed during several key talks as “lacking in the TB field”, “deserving more attention” and “required to eradicate the 1% drug tolerant bacilli”. Thus there is urgent need to develop alternative therapeutic agents that cure TB faster and more effectively.

**For centuries, the treatment of pulmonary tuberculosis (TB) has been a challenge to global health. The current available treatment regimen for TB is very long (6-9 months) and the emergence of drug-resistant strains has stressed the need for developing new therapies for this disease. To improve TB control is essential that the length of standard anti-TB chemotherapy regimen is reduced from 6-9 months to 2 months.**

The NADPH oxidase family protein complexes generate superoxide anion and downstream ROS. The phagocyte NADPH oxidase is the principal source of ROS generation in activated neutrophils and macrophages.

Myeloperoxidase (MPO), next to the NO synthase2 (NOS2), and NADPH oxidase, is the key enzyme of the oxidative burst responsible for the antimicrobial immunity. Because MPO participates in the eradication of Mycobacterium tuberculosis in the in vitro model and the extracellular enzyme may activate cells to cytokine synthesis, we investigated the changes in the enzyme concentration in serum of patients with active pulmonary tuberculosis (TB) and correlations between MPO and TNF-alpha, IFN-gamma, and IL-12.

In order to replicate and persist in macrophages, Mycobacterium tuberculosis (MTB) must modulate the impact of potentially bactericidal micromolecules, including the reactive oxygen species (ROS) generated by the phagocyte NADPH oxidase (Phox) and the reactive nitrogen species (RNS) generated by the inducible nitric oxide synthase (NOS2) (reviewed by Nathan and Shiloh, 2000). Macrophages are capable of inhibiting MTB replication via NOS2-generated RNS (reviewed by Chan and Flynn, 1999), and NOS2 gene-disrupted mice are highly susceptible o MTB infection (MacMicking

et al., 1997). The role of ROS in host defence against MTB is less clear. MTB is relatively resistant to killing by ROS in vitro

(Chan et al ., 1992), and mice deficient in the p47 (Cooper et al ., 2000) or gp91 (Adamset al ., 1997; Jung et al , 2002) subunit of NADPH oxidase are relatively resistant to MTB infection. in contrast, NADPH oxidase deficiency renders mice exquisitely susceptible to infection by a variety of bacteria, parasites and fungi, including opportunistic pathogens with low pathogenetic potential in immunocompetent hosts. In humans, genetic deficiencies in NADPH oxidase are associated with chronic granulomatous disease CGD) resulting from recurrent life-threatening infections reviewed by Segal et al, 2000a). However, there is little published evidence that the incidence of tuberculosis (TB) is increased among CGD patients (reviewed by Casanova and Abel, 2002). One possible explanation for this paradox is that MTB might possess efficient mechanisms to evade or counter the NADPH oxidase-dependent oxidative burst, thus effectively masking the impact of this antimicrobial mechanism. We therefore asked whether established pathways for ROS detoxification might be important for evasion of the phagocyte NADPH oxidaseby MTB.

Superoxide (O-22 is generated by the single-electron reduction of molecular oxygen during aerobic respiration, and can react with other cellular constituents to generate even more reactive ROS, such as hydroxyl radical (◊OH). Aerobic bacteria, including saprophytic and pathogenic mycobacteria, degrade O 2◊ to water and molecular oxygen by the sequential action of superoxide dismutase (SOD) and catalase. Thus, a lifestyle that includes aerobic metabolism may have ‘preadapted’ certain microbes to resist killing by the phagocyte oxidative burst. Superoxide

In the early stages of infection, ROS production by phagocyte NADPH oxidase is apparently not an essential component of host defence against wild-type MTB, whereas RNS output from NOS2 is critical.

Current anti-TB drugs are slow to eradicate Mycobacterium tuberculosis (Mtb) in patients and have failed to control tuberculosis globally. **Most anti-TB target the replicative stage of the bacilli and it is believed that the host immune response against this infection diminish Mtb replication and thus reduces efficacy of most anti-TB drugs.**.

Existing drugs are slow to eradicate *Mycobacterium tuberculosis* (Mtb) in patients and have failed to control tuberculosis globally. One reason may be that host conditions impair Mtb’s replication, reducing its sensitivity to most antiinfectives.

Some bacterial infections can be cured with a single dose of an antibiotic, and most others can be cured with administration of one drug over several days or weeks. In contrast, routine treatment of drug-sensitive tuberculosis (TB) takes 2 mo of therapy with four drugs and an additional 4 mo with two drugs to reduce the 2-y relapse rate below 5%. The difficulty of completing prolonged treatment is a major reason for emergence of drug resistance. When the infecting strain is resistant to isoniazid and rifampin, the two drugs recommended for all 6 mo of treatment, cure often requires 2 y of daily administration of toxic, expensive, second-line agents that are often unavailable at the point of care. When the causative strain is additionally resistant to a quinolone and an aminoglycoside, the resultant “extensively drug-resistant” TB was fatal to 80% of patients in a leading center (1), although complex multidrug regimens have achieved higher cure rates in populations not previously exposed to the additional drugs (2). In addition to sharing air with someone with TB, leading risk factors for contracting the disease are malnutrition, HIV infection, diabetes, and exposure to smoke from cigarettes or cooking fires (3). These epidemiological challenges exacerbate problems of inadequate diagnostic technology and limited access to drug susceptibility testing and to drugs. Control of the pandemic is not in sight (3).

Am J Respir Cell Mol Biol. 2011 Jul;45(1):172-80. doi: 10.1165/rcmb.2010-0319OC. Epub 2010 Oct 1.

**IL-10 blocks phagosome maturation in mycobacterium tuberculosis-infected human macrophages.**

[O'Leary S](http://www.ncbi.nlm.nih.gov/pubmed?term=O'Leary%20S%5BAuthor%5D&cauthor=true&cauthor_uid=20889800)1, [O'Sullivan MP](http://www.ncbi.nlm.nih.gov/pubmed?term=O'Sullivan%20MP%5BAuthor%5D&cauthor=true&cauthor_uid=20889800), [Keane J](http://www.ncbi.nlm.nih.gov/pubmed?term=Keane%20J%5BAuthor%5D&cauthor=true&cauthor_uid=20889800).

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**Abstract**

Successful phagolysosomal maturation is an important innate immune response to intracellular infection. However, Mycobacterium tuberculosis (Mtb) can manipulate and inhibit this host response to ensure survival within its niche cell. We investigate the role of the anti-inflammatory cytokine IL-10 on Mtb-phagosome maturation. Blocking IL-10, which was secreted from Mtb-infected macrophages, allowed phagosome maturation to proceed. Macrophage cytokine gene expression profiles were not significantly altered by blocking IL-10 3 hours after infection with Mtb. We demonstrate that IL-10 can regulate this protective phenotype in phorbol myristate acetate (PMA)-treated THP-1 cells, monocyte-derived macrophages (MDMs), and human alveolar macrophages (AMs) infected with Mtb. The regulatory effect of endogenous IL-10 was evident in macrophages infected with virulent Mtb H37Rv, as well as in attenuated strains of mycobacteria. Unlike live Mtb, dead bacilli occupy a mature, acidic phagosome. However, the addition of IL-10 to cells infected with killed Mtb successfully inhibited the maturation of this compartment. Importantly, we demonstrate that the addition of IL-10 to MDMs results in enhanced mycobacterial survival and growth. Our results suggest that IL-10 exerts its effects on this early macrophage response in a partly signal transducer and activator of transcription 3 (STAT3)-dependent manner, and independent of mitogen activated protein kinase p38 (MAPKp38) and extracellular regulated kinase 1/2 (ERK1/2) activity. IL-10 is a feature of human tuberculous granuloma, and these new findings support the hypothesis that this cytokine can promote pathogen persistence by contributing to Mtb-phagosome maturation arrest in human macrophages.

IL-10 was reported to have the remarkable property of blocking cytokine, chemokine and nitric oxide production from macrophages activated by bacterial lipopolysaccharide. Signal transducer and activator of transcription (STAT)3 is essential for all known functions of IL-10.

Clin Transl Sci. 2010 Feb;3(1):23-8. doi: 10.1111/j.1752-8062.2010.00180.x.

**Immunoregulation in TB: observations and implications.**

[Ellner JJ](http://www.ncbi.nlm.nih.gov/pubmed?term=Ellner%20JJ%5BAuthor%5D&cauthor=true&cauthor_uid=20443950).

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**Abstract**

Regulation of the immune response during active tuberculosis (TB) has been partly deciphered. In pulmonary TB there is transient systemic immunosuppression due to overexpression of transforming growth factor beta and interleukin-10. This is superimposed on a primary T-cell defect. Locally there is intense inflammation (lung, pleural fluid) with overexpression of immunosuppressive factors (bronchoalveolar lavage) and extensive apoptosis. These observations suggest that immune therapies should be aimed at neutralizing the negative regulatory factors rather than accentuating an already intense immune response. Also a partially effective vaccine carries the potential risk of exacerbating disease.