THE FINAL PROGRESS REPORT (2010-2015 NCE 2017)

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LIST OF TERMS AND ABBREVIATIONS

HP, Hypersensitivity Pneumonitis,
MWF, Metalworking fluid,
NTM, Non-tuberculous mycobacteria,
MI, Mycobacterium immunogenum,
MC, Mycobacterium chelonae,
MA, Mycobacterium abscessus,
2-D Gel, Two-Dimensional Gel Electrophoresis,
ELISA, Enzyme-linked Immunosorbent Assay,
iPCR, Immuno-Polymerase Chain Reaction,
SR, Saccharopolyspora rectivirgula,
PBMCs, Peripheral Blood Mononuclear Cells,
Mab, Monoclonal Antibody,
MOI, Multiplicity of Infection,
BALF, Bronchoalveolar Lavage Fluid
ROC, Receiver Operating Characteristics curve,
AUC, Area Under the Curve,

ABSTRACT

Hypersensitivity pneumonitis (HP) in machine workers, commonly called 'Machine Operator's Lung', is a well-recognized occupational lung disease in machinists. Studies by NIOSH and others have associated this disease with exposure to mycobacteria which frequently colonize modern water-based metalworking fluids (MWFs) used in machining. This is an immune-mediated lung disease caused due to repeated inhalational exposure to microbial antigens; HP is difficult to diagnose due to lack of clinical diagnosis tools and its pathogenesis, and treatment are poorly studied. Considering this, overall goal of this study was to identify and characterize specific antigens from our collection of industrial MWF-isolated mycobacteria [*M. immunogenum* (MI), *M. chelonae* (MC), and *M. abscessus* (MA)] and evaluate their potential for use in HP diagnosis and exposure assessment. In this context, we tested our hypothesis that antigen diversity in MWF mycobacteria species/strains prevalent in commercial MWF is responsible for differentially inducing HP symptoms and the underlying immune response, and that the responsible specific antigens could form the basis for more informative immunodiagnosis and exposure assessment. Relative immunogenicity of twelve MWF-isolated mycobacteria strains belonging to the three species was compared based on HP induction potential and cell-mediated immune response using our optimized mouse models of experimental HP. A repertoire of antigen candidates (both secreted and cell-associated) was identified from different MWF mycobacteria genotypes by differential immunoproteomic profiling. Selected key antigen candidates were recombinantly expressed and purified and evaluated for their potential for HP immunodiagnosis using the developed mouse model and human subjects. Use of human blood and serum samples from machinists and healthy control subjects led to identification of mycobacterial antigens that were selectively seroreactive with the patient samples. Further evaluation of the recombinant antigens for ex-vivo T-cell reactivity using lymphocytes isolated from HP mouse models and HP patients (machinists) led to identification of a pair of potent bivalent antigen candidates (which were both sero-reactive and T cell-reactive). Additional efforts led to optimization of an immune-PCR assay for sensitive detection of mycobacterial antigen in MWF for exposure assessment applications. The specific antigens in MWF mycobacteria identified and characterized in this study will facilitate future epidemiological studies and development of intervention strategies for MWF antigen exposures and incidence of this immune lung disease in machinists. Overall, the resulting information will help NORA's objectives by providing a set of tools for developing applications for differential clinical diagnosis of machinists HP and exposure assessment; these outcomes could also facilitate development of pre-emptive measures and intervention strategies in occupational machining environments in long-term.

SECTION 1 OF THE FINAL PROGRESS REPORT

Nontuberculous Mycobacteria (NTM) colonizing metalworking fluids (MWFs) have been implicated in respiratory symptoms and diseases particularly occupational hypersensitivity pneumonitis (HP) in machine workers exposed to contaminated used machining fluids/cutting fluids. This immune-mediated disease is difficult to diagnose because of lack of our understanding of its causative and selectively immunoreactive antigens. The current study was undertaken with the rationale that characterization of relative immunogenic and HP-inducing potential of MWF-prevalent NTM strains/genotypes using experimental HP mouse model could help identify the differentially immunogenic strains and their critical antigens that have relevance to HP and other respiratory immune disorders in exposed workers. Such antigens could in turn form the basis for development of immunodiagnostic tests both for clinical diagnosis of MWF-linked HP in machinists and for exposure assessment.

a. Significant key findings: This study led to the following key findings:

i). Optimized mouse models of mycobacterial HP, a new acute HP model and an improved chronic HP model, for use in this study and future experimental studies to facilitate development of diagnostic and therapeutic strategies for this immune-mediated disease.

ii). Identified a differentially immunogenic pair of MWF-associated mycobacterial strains, hyperimmunogenic (MI 700506 or MJY10) versus hypoimmunogenic (MJY4), based on screening of 12 MWF-isolated mycobacteria strains (6 of *M. immunogenum*, 4 of *M. chelonae*, and 2 of *M. abscessus*) in the developed HP mouse models.

iii). Identified a repertoire of candidate antigens (both secreted and cell-associated) from different strains/genotypes and based on differential immunoproteomic analysis of the hyperimmunogenic and hypoimmunogenic strains.

iv). Selected antigen candidates were recombinantly expressed and purified and evaluated for their potential for HP immunodiagnosis using the sera from developed mouse models and human subjects. This led to identification of key mycobacterial antigens showing a potential for serodiagnosis of HP in machinists; these were selectively seroreactive with HP patient samples as compared to the healthy control subjects.

v). Further evaluation of the recombinant antigens for ex-vivo T-cell reactivity using lymphocytes isolated from mouse models and HP patients (machinists) led to identification of a pair of potent bivalent antigen candidates (with both sero-reactivity and T cell-reactivity). Such bivalent function antigens stimulating both arms of the immune system could be potentially useful for both serology and cell-mediated immunodiagnosis of HP.

vi). Optimized simulation conditions for mycobacterial colonization of MWF and performed initial optimization of immunoassays (ELISA and Immuno-PCR) for detection of mycobacterial antigen in MWF matrix for exposure assessment.

b&c. Translation of findings and Research outcomes impact: The information generated in this study on antigenic potential of MWF-prevalent NTM strains and their immunoreactive antigens will lead to more informed design and interpretation of future epidemiological studies on machinist HP and related MWF-linked symptoms/disorders. On the other hand, optimization of simulation conditions for mycobacterial colonization of MWF, identification of secreted antigens in laboratory-simulated cultures, and initial optimization of immunodetection assays for MWF matrix (ELISA and Immuno-PCR) accomplished in this study will pave the way for future development of routine fluid monitoring strategies. Future identification of critical epitopes in mycobacterial antigens would help develop the much-required differential clinical diagnosis of MWF-linked HP in machinists. Taken together, these developments would be critical for future development of the exposure assessment, diagnosis, and intervention strategies and applications for MWF-linked respiratory symptoms and disease/disorders in machine workers.

SECTION 2 OF THE FINAL PROGRESS REPORT

SCIENTIFIC REPORT

a. Background and Specific Aims

Occupational HP due to MWF exposures has been listed as one of the top ten occupational pulmonary diseases [1]. NIOSH and several other groups [2-16] have provided increasingly convincing evidence that nontuberculous mycobacteria (NTM) species occurring in the in-use metalworking fluids (MWFs), *Mycobacterium immunogenum and M. chelonae*, are the causal antigens of the immune-mediated respiratory disease hypersensitivity pneumonitis in machine workers exposed to contaminated metalworking fluids ('in-use' or 'used' MWF). Our preceding funding cycles have led to isolation of these two species of MWF mycobacteria and their multiple genotypes/strains (6 independent genotypes of *Mycobacterium immunogenum* and 4 of *M. chelonae*) based on the development and use of a series of DNA-based molecular methods for species-specific detection of mycobacteria in these fluids and for their characterization in terms of biocide resistance and antigenic potential. While the effective MWF monitoring for an early detection and characterization of MWF mycobacteria helps take pre-emptive measures and develop appropriate fluid management practices, equally important is the need for a specific personal exposure assessment to these mycobacteria in the exposed workers and immunodiagnosis in patients (using appropriate causative strain-specific antigen(s)) in order to reach a definitive exposure assessment/diagnosis and intervention strategies in HP environments. In this context, it is worth considering that the extent of mycobacterial colonization reportedly varies from plant to plant and that not all MWF lots with mycobacteria have shown associated HP cases; likewise MWF lots without detectable live mycobacteria have shown HP. We hypothesize that the underlying basis for these observed variations could be the critical antigenic diversity in MWF colonizing mycobacteria or the remaining cell-free causative antigen load (even after biocidal inactivation and/or lysis of mycobacteria). This emphasizes the need for characterization of the specific causal antigens, their prevalence in fluids, and their potential role in immunodiagnosis and pathogenesis of HP and exposure assessment. Despite the recognized role of microbial antigens in HP development, there has been a lack of such studies on specific causal antigens in metalworking fluids.

Only two environmental mycobacteria species of the M. chelonae complex (namely, *Mycobacterium immunogenum* and *M. chelonae*) have been known to dominantly colonize MWF due to their unique inherent ability (unlike other mycobacteria species) to colonize these harsh fluid environments; of these, *M. immunogenum* has been frequently associated with HP-linked fluids and lately shown to induce HP-like symptoms in laboratory animals by us (this study) and others [13-14]. Our subsequent studies expanded this diversity by reporting *M. abscessus* as the third species in industrial MWF [17]. Considering that HP is an immune-mediated disorder and that MWF mycobacteria species can induce such immune pathologies, there is a need to understand the critical antigenic strains of these two MWF-colonizing HP-linked mycobacteria species and the specific causative antigens in there that are associated with HP. Our initial studies have led to *in vitro* identification of antigenic proteins in *M. immunogenum* and *M. chelonae* using immunoproteomic approach [18]. However, it is not yet known which of these antigens are critical in HP development and whether these antigens could serve as a target for immunodiagnosis in exposed workers versus HP patients and in more informative fluid monitoring. Due to the difficulties involved in accessing the relevant MWF mycobacteria-linked HP patients and obtaining HP patient samples (blood/sera) along with the corresponding contaminated metalworking fluid (or the mycobacterial isolates therein), it is not feasible to carry out a controlled study based solely on human cases. Hence, there is a need to use a mouse model of HP that has lately been optimized in our hands (see Preliminary studies).

In view of the foregoing background, **overall objective** of the study in this funding cycle (2010-2015 NCE 2017) was to characterize key antigens of MWF-colonizing mycobacteria relevant to hypersensitivity pneumonitis (HP) and understanding their potential for immunodiagnosis of HP in the exposed machinists and in personal and environmental exposure assessment. Our **specific aims** were: (1). Identify key antigens of

MWF mycobacteria species *M. immunogenum* (MI) and *M. chelonae* (MC) based on their relative interaction with the host immune system in a mouse model of mycobacteria-induced hypersensitivity pneumonitis, (2). Recombinant Production of selected antigens of *M. immunogenum* and *M. chelonae* and generation of their specific antibodies (3). Investigate potential of the specific antigens of MWF mycobacteria for personal exposure assessment and immunodiagnosis of symptomatic (HP) versus asymptomatic (exposed) using the mouse model and human subjects. (4). Identify and evaluate specific cell-free antigen(s) of MWF mycobacteria for environmental exposure assessment (fluid monitoring) using simulated and field samples.

b. Methodology

I. Comparative immunogenic potential of MWF-isolated mycobacterial strains in mouse models of HP

1. **Mycobacterial strains/genotypes from industrial MWF**: Three member species of the *M. chelonae-M. abscessus* group of non-tuberculous myco-bacteria, i.e, *M. immunogenum*, *M. chelonae*, *M. abscessus* originally isolated from field in-use or used metalworking fluids from geographically diverse automotive industrial plants [12, 17] were used in these studies. A total of 12 strains/genotypes belonging to these three species, including six *M. immunogenum* genotypes (MI 700506, MJY3, MJY4, MJY10, MJY13, and MJY14), four *M. chelonae* genotypes (MJY1, MJY2, MJY6, and MJY8), and two *M. abscessus* strains/morphotypes, rough (MJY23) and smooth (MJY25), were included. The mycobacterial species and genotypes/variants were grown in Middlebrook 7H9 medium (BD Diagnostics, Sparks, MD) supplemented with 10% Oleic Albumin Dextrose Catalase (OADC) enrichment (BD Diagnostics, Sparks, MD) unless otherwise indicated. The actinomycete *Saccharopolyspora rectivirgula* used for comparison studies was grown to generate a whole cell lysate, per published procedure [19].

2. Mycobacterial challenge inoculum: For obtaining a monodispersed cell suspension, each strain was grown to a density of 120 Klett units in Sauton's broth containing 0.1% Tween 80 prepared in-house per the following composition: L-asparagine (4 g/L), citric acid (2 g/L), K_2HPO_4 (0.5 g/L), $MgSO_4$ (0.5 g/L), ferric ammonium citrate (0.05 g/L), glycerol (35 ml/L) and Tween-80 (1 ml/L); pH was adjusted to 7.2 using NaOH solution. Using continuous shaking at 200 rpm*, M. immunogenum* 700506 was grown at 37°C and all other mycobacterial strains were grown at 30°C. The cells were washed three times with endotoxin-free PBS, and resuspended in the same medium. The cell suspension was repeatedly passed through a 23-G needle (10 times), and then through a 26-G needle (10 times). The resulting cell suspension was centrifuged 5 min at 100 x g, and the supernatant aspirated off. Bacterial count (in terms of colony-forming units [cfu]/ml) of this monodispersed suspension was determined by plating aliquots of various dilu-tions on Middlebrook 7H10 agar supplemented with 10% OADC enrichment (BD Biosciences).

3. Mycobacterial Antigen preparation: Different test antigen preparations were made per the following protocols. Endotoxin-free water (Sigma) will be used for these procedures.

Each mycobacterial strain/genotype was grown as above (or using Middlebrook 7H9 broth with OADC); the cells were pelleted by centrifugation at 3000 x g, and washed three times with certified endotoxin-free phosphate-buffered saline (PBS, pH 7.4). The resulting cell pellet was resuspended in endotoxin-free PBS for either direct use as test inoculum (as above) or as the starting material to prepare cellular protein fraction as follows.

a). Cellular protein fraction (CF) of the test antigen: The cell suspension prepared above was lysed for use as cellular protein antigen. For whole cell extract, one ml cell suspension was added to a sterile 2-ml tube containing 0.4 g acid-washed glass beads. The tube was shaken in a Mini-Bead beater (Biospec Products, Bartlesville, OK) four times (each for 1 min) with cooling on ice for 2 min in between. The resulting homogenate was decanted from the beads, and the protein concentration then determined using a DC™ protein assay from BioRad (Hercules, CA). Resulting whole cell extracts were frozen at -80°C until further use.

b).Secreted protein fraction (SF) of the test antigen: SF fraction of the test mycobacterial strain was prepared based on established mycobacterial procedure [20] with our optimized modifications.

c). Test antigen for animal studies: Test antigen preparation for mouse challenging (oropharyngeal) for each strain was either the whole cell extract (representing cell-associated antigens) or whole cells themselves.

Positive control antigen from *Saccharopolyspora rectivirgula* (SR) strain ATCC A1313, a known HP-inducing thermoactinomycete, called SR antigen (5 mg/ml) was also prepared as whole cell extracts.
d). Test antigen for splenocyte treatments: Test antigen preparation for splenocyte treatments for each strain was the CF (or a mixture of the CF and SF fractions, mixed in amounts proportional to those present in the intact culture).
4. Protocols for mice experiments: Models and Exposure regimes: Mouse models of HP were optimized using acute exposure regime (one dose daily for 3 consecutive days) and chronic exposure regime (one dose daily for 3 days/week for 3 weeks) and analysis of the response was performed at 4 hours after the last challenge (or for a variable post-exposure intervals). C57BL/6J mice (male, 6-8-wk-old) were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in PIV cages under pathogen-free conditions maintained at 22-24°C with a 40-43% relative humidity and a 12 hr light:dark cycle. All mice had *ad libitum* access to standard rodent chow and filtered water. All experimental protocols used here were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Oropharyngeal aspiration method was used to expose the mice (n = 8/group for the optimization experiments; n = 5/group for viable cell exposure; n = 4-5/group for genotype screen) to bacterial cells or cell extracts. The mice were anesthetized using isoflurane, and suspended on a restraining board tilted backwards 45°. The tongue was gently pulled out with tweezers, 50 µl antigen was added to the base of the tongue, and the nares were immediately blocked to force inhalation through the mouth. Instillations were done using acute or chronic regimes: Acute: One dose daily on three consecutive days; Chronic: one dose daily on 3 alternate days per week for a total of three weeks. With the exception of the time-course study, the mice were then euthanized 4 hr after the final instillation by intraperitoneal injection of Euthasol™. One set of sacrificed animals was lavaged to obtain BAL fluid and lung homogenates. The other set was sacrificed for harvesting the whole lungs for histology.

(i). Lung lavaging: At necropsy, the lungs were lavaged with 2 x 1-ml Ca^{2+}, Mg^{2+}-free PBS; the two aliquots were then combined. The lavaged lungs were then placed in formalin for 24 hr. The bronchoalveolar lavage fluid (BAL fluid) was centrifuged at 4°C for 10 min at 300 x g and the resulting supernatant then recovered and stored at -80°C until further use. The cell pellet was re-suspended in PBS containing 5% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) and the cells then counted in hemocytometer to obtain total numbers in bronchoalveolar lavage fluid (BALF). Differential cell counts were obtained by counting >300 cells on cytospin preparations (Cytospin3; Shandon Scientific, Cheshire, UK) after staining using a Hema 3 kit (Fisher Scientific, Waltham, MA). The supernatant was analyzed for Total protein (BioRad), lactate dehydrogenase/LDH (Cytotox96 kit, Promega), Inflammatory cytokines (as above) and nitric oxide/NO (Griess reagent, Promega).
(ii). Blood and tissue harvesting: Blood collected from the sacrificed mice was separated into serum and the latter stored at -20 C until analysis. The organs/tissues were collected for histopathology (lung) and cell-mediated immune response (spleen) analysis.
(iii). Blood- fractionation and analyses: Blood samples were pooled for each treatment subgroup. The serum was subjected to profiling for antibody response and Th1/Th2 response using standard ELISA procedures. Briefly, the inflammatory cytokines including interleukins and TNFα and IFN-γ were measured, using commercial kits (eBioscience).
(iv). Tissue homogenates preparation and analysis: Lung tissues from the sacrificed mice were homogenized in PBS (2 ml). Supernatant (1500 x g) of the homogenate was frozen (-80^{0}C) for subsequent cytokine analyses.
(v). Histological examination: The isolated lungs, fixed in phosphate-buffered paraformaldehyde (3%) and embedded in paraffin were sectioned and stained with hematoxylin and eosin (general morphological changes), and Masson-Chrome (fibrosis). These services were provided by the Histology Core and data further interpreted by the histopathologist Greg Boivin.
3. Cell-mediated immune response analysis: ***a1.*** *Isolation and culturing of splenic lymphocytes from HP mouse model*: Spleens removed from the challenged mice, washed in HBSS medium and kept in incomplete RPMI medium (serum-free) were homogenized and washed 2x with HBSS. RBC cells were removed from the total splenocytes by osmotic lysis using water for 20 seconds. Again the cells were washed 2x with HBSS and

dissolved in RPMI complete medium. Cells were counted and T-cells sorted by flow cytometry. One million cells/well were seeded in 96 well plates and sensitized with the test mycobacterial antigen (50µg/ml), normal saline, and SR (50 µg/ml), each in triplicate. After 48 h of incubation at 37^0C, the following analysis was performed.

a2. *Human peripheral blood lymphocytes*: Human blood lymphocytes from HP patients or health human subjects were prepared using PBMCs isolated using Ficoll-Paque method. The cells were prepared and challenged with test antigen as described above for mouse splenocytes.

b. *T cell proliferation response*: Lymphocyte proliferation was measured using the cell proliferation assay kit according to manufacturer's instructions. Conconavalin A, a known T-cell mitogen, was used as a positive control for proliferation. CD4/CD8 cell ratio was determined by flow cytometry using specific antibodies.

c. *INF-γ and Th1 cytokine response*: Supernatants from the lymphocyte cultures set up above with the test and control antigens (MI, SR and saline) and incubated for 48 hr were collected by centrifugation and frozen at -70^oC for analysis. Expression of IFN-γ and other cytokines (Th1 versus Th2) was measured as above.

II. Differential immuno-proteomic analysis to identify mycobacterial antigens.

We first optimized extraction and proteomic analysis of the whole cell extracts (cell-associated proteins) and secretome (cell-free secreted proteins) of all genotypes of the member species of M. chelonae complex isolated from metalworking fluids. Proteins in the whole cell extract or supernatant were TCA-precipitated and were fractionated by 2D gel electrophoresis, by optimization of the isoelectric focusing (IEF) conditions. Since most of the proteins were found to be in the acidic range (< pH 7.0), they were focused using pH 4-7 IEF strip. The immunoreactive protein spots were identified using the anti-mycobacterial antibody probe in the Western Blot analysis. Initially, immunoprotein profiles were also compared for the individual genotypes of *M. immunogenum* grown in broth culture, by probing with the anti-rabbit polyclonal antibody as raised against genotype 700506 and using the whole cell protein extract. Likewise, the whole cell protein extracts from *M. chelonae* and *M. abscessus* were probed using this *M. immunogenum* antibody. Putative genotype- & species-specific distribution of MI antigen homologs was thus generated from the test species/genotypes. Subsequently, species-specific anti-rabbit polyclonal antibody raised against a mixture of all MI genotypes or all MC genotypes or all Ma genotypes was used for the probing of 2D-blots for the respective species/genotypes. Identity of the immunoreactive protein bands was determined using mass spectrometry (MALDI-TOF or LC-MS/MS) analysis. This way, a comprehensive set of immunoproteomic proteins (both cell associated and secreted) was identified using a combination of 2D-Gel electrophoresis, immunoblotting, and mass spectrometry.

III. Recombinant expression and evaluation of the identified immunogenic proteins as potential diagnostic antigen(s):

a). Generation of recombinant antigens via cloning and recombinant expression: We cloned the genes of the key 2D-gel separated immunoreactive proteins of *M. immunogenum* for generation of their recombinant protein forms. Based on the mass spectrometry data and corresponding homologs in M. immunogenum and other mycobacterial genomes, we cloned the individual candidate antigen genes using gene-specific PCR. The cloned sequences were reamplified to insert a C-terminal His-tag and expressed in E. coli Rosetta Blue DE3 strain. The recombinant proteins were purified using Ni-NTA method and made free from endotoxin as confirmed by LAL assay. Specific antibodies raised against the His-tag were used for detection and quantification.

b). Screening of antigens based on Antibody response in sera: A panel of individual test antigens were reacted with sera drawn from either mice (different stages of exposure-early vs. late) or using human sera (HP patient sera vs. healthy control sera) using ELISA method. An antigen reacting with the sera drawn from late stages of exposure from symptomatic mice (with HP e.g. week 3) but not with the sera from asymptomatic mice (prior to HP induction e.g. week 1) was considered a potential candidate for HP immuno-diagnosis. Likewise, an antigen reacting with only non-symptomatic sera will be a candidate for sero-differentiation of the exposed-but-

asymptomatic individual from the symptomatic individual. In human sera samples, seroreactivity with patient sera but not with control healthy sera was considered HP-specific seroreactivity.

c). Screening of antigens based on Cell-mediated response: Splenic lymphocyte cultures set up using spleens from animals at different stages of their MI-induced pathology (symptomatic versus asymptomatic) were reacted with individual test antigen. The sensitized T-lymphocyte cell response was measured in terms if IFN-gamma induction and cell proliferation, as described above under mouse models section. For human blood lymphocytes, control and patient blood samples were used to isolate PBMC. Individual antigens were reacted with whole PBMC fraction or purified Dendritic cells (DCs) and T-cells in a DC-T cell assay. The antigens showing T-cell response in terms of IFN-gamma and or T-cell proliferation were considered T-cell reactive antigens.

IV. Development of assays for immunodetection of antigen in MWF (exposure assessment): We optimized two immunoassay formats (ELISA and Immuno-PCR) for antigen monitoring in MWF matrix, using the test antigen (recombinant MI-specific secreted antigen) and its specific antibody. Conventional ELISA approach was suitable for higher antigen levels (ng to µg levels), whereas immuno-PCR (a more sensitive method) was warranted for low antigen levels (in pg and fg amounts).
i). *ELISA-based immunoassay*: Increasing ng amounts of the test antigen were spiked in 5% pristine MWF. The simulated test sample aliquot (100 µl each) was added to microtiter wells in triplicate to adsorb increasing concentrations of the antigen. Appropriate washing to remove MWF matrix components was optimized as these matrix inhibitors may have effect on antibody binding. Specific MAb was reacted and the colorimetric detection (A_{450}) performed using appropriate HRP-conjugated secondary antibody, based on standard ELISA assay protocol. Minimum detection limit was calculated based on the developed dose-response curve.
ii). *Immuno-PCR (iPCR) assay*: Unlike the conventional PCR that targets sample's DNA, iPCR is used to detect antigen in the test sample (by conjugating a standard DNA marker to the antibody specific to the test antigen). We generateed the required antibody-DNA marker conjugate using commercial kit. Subsequently, we preferred to use secondary antibody for the labeling to make the assay broadly applicable and less expensive. Specific primers were used to amplify the DNA marker for real-time detection of the amplicon. The simulated pristine MWF samples generated by spiking increasing fg/pg/ng amounts of the test antigen were used. The antigen in the samples was adsorbed in the microtiter well and reacted with the primary antibody and secondary antibody-DNA marker conjugate, using antigen-antibody hybridization conditions as optimized for ELISA. Quantification signal based on the conjugated DNA marker was generated by real-time PCR using ABI 7500 and standard protocols.

c. Results, and Discussion

Progress on all four aims was made per the originally proposed plan. Furthermore, certain additional but directly related efforts warranted to accomplish the proposed plan more effectively were also pursued as we went along in the study. During this funding period (2010-2015 NCE 2017), the generated data lead to a total of **16** research papers/manuscripts and **9** abstracts/presentations (oral/poster) (see **Publications list at the end**). The results are concisely presented and discussed in the following paragraphs.

Aim 1. Identify key antigens of MWF mycobacteria species *M. immunogenum* (MI) and *M. chelonae* (MC) based on their relative interaction with the host immune system in mouse model of mycobacteria-induced hypersensitivity pneumonitis,

Aim 1A. Compare in vivo immunogenicity (HP-inducing potential and cell-mediated response) of the strains (genotypes) of *M. immunogenum* and *M. chelonae* using mouse models of mycobacterial HP.

In the preceding funding cycles, our laboratory had isolated several genotypes of *M. immunogenum, M. chelonae*, and *M. abscessus* from HP-associated and other field MWF operations. In this study, we generated a short-term (acute) exposure mouse model and a refined chronic exposure mouse model of experimental

mycobacterial-HP and compared the immunogenic and HP-inducing potential of these different genotypes of MWF mycobacteria. These studies have resulted in 3 manuscripts (see the **Publication List**).

I. Optimization/Improvement of mouse models of mycobacterial HP (in terms of Exposure regimen):
We had initially optimized an intranasally challenged mycobacterial-HP murine model (chronic) in our hands using the reference genotype 700506 originally isolated from HP-linked field MWF (with a goal to utilize it for understanding the antigenicity factors in different mycobacterial genotypes). However, our subsequent experimentation with different bacterial genotypes necessitated further optimization of this model in terms of both consistent dosing as well as form of the dose, so we could effectively compare the different strains/genotypes for biological variability by minimizing the experimental variability.

a). Dose delivery method: In order to find a method for consistent and effective dose deposition in the lung in our mouse model, oropharyngeal instillation (a non-invasive alternative to surgery-based intratracheal instillation) was compared with nasal instillation. We used E. coli cell suspension as the test dose (as it is easier and quicker to quantify) and determining the percent recovery of the bacterial cells (CFU analysis on lung homogenates) soon after instillation to understand the net dose deposited in the lung. Oropharyngeal method resulted in 92% (average) delivery of the instilled dose to the lower airways, whereas average delivery after nasal instillation was only 4%. Considering this, we adopted the oropharyngeal instillation route in our mouse model.

b). Dose optimization (form of dose). We performed these optimizations using a chronic exposure dose regimen (dosing 3 days/week for 3 weeks) tested in our initial studies and immunopathological end points (BAL fluid analysis- total protein, immune cell infiltration, IFN-γ and lung tissue histopathology).

i). *Intact cells versus cell lysate*. Overall, the cell lysate form of the dose appeared to be comparable to the intact cell form and thus was considered for further use, albeit after optimization of the method of cell lysis (to achieve more complete cell disruption). Nonetheless, animals instilled with 'intact cells' indicated extensive mycobacterial colonization in the mouth cavity (as revealed by culturing of oral swabs) possibly because of mucosal abrasion during instillation leading to mycobacterial colonization. However, no such side effect was observed with the lysate (sonicate) dosing. Also in subsequent dose-response experiment using optimized chronic exposure regime mouse model and monodispersed bacterial suspension, MI700506-challenged mice were found to be much more sensitive (dyspnea, drop in body temperature, weight loss, lung immune response) to the whole mycobacterial cells as compared to the equivalent MI cell lysates.

ii). *Optimization of cell lysis*. Bead beating (performed in one-minute intervals with 30-s pauses on ice in between for total times of 1, 2, 4, and 6 minutes) resulted in considerably higher protein concentrations at all time points compared with sonication for 4 minutes. Microscopic examination confirmed that practically no intact cells remained after bead beating for 2 minutes or longer.

c). Dose optimization (level of dose). I). Acute dose optimization: A dose-response experiment was performed to determine an appropriate dose of the optimized mycobacterial cell lysate (in terms of protein amount) in a mouse challenge protocol involving a short-term (acute) exposure. Increasing doses (0.2 µg through 60 µg) of MI-700506 lysate were used. Animals were instilled oropharyngeally on three consecutive days, followed by sacrifice 4 h after the last exposure.

Inflammatory response in BALF (total protein, cellularity, cytokines-TNF and IL-6) in BALF showed a gradual increase beginning 0.6 µg MI protein dose and the effect either peaked at 20 or 60 µg. The majority of the increase in total cell count was due to

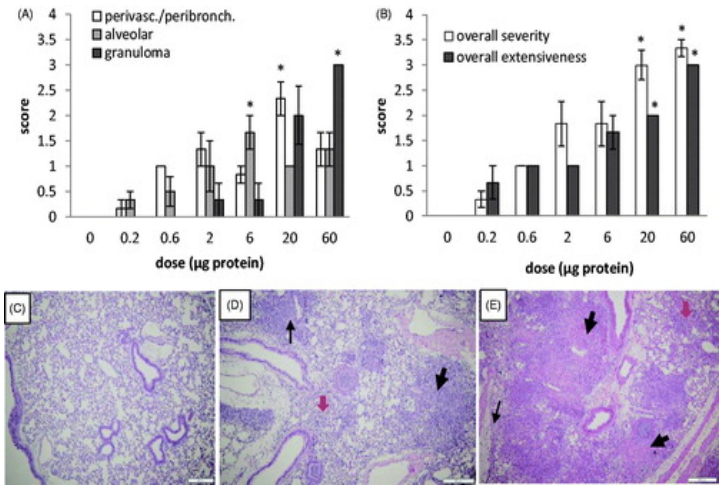

Figure 1. Acute exposure mouse model: Dose-dependent histopathological changes in the mouse lung after oropharyngeal exposure to increasing doses (µg mycobacterial protein/animal) of whole cell lysate of *M. immunogenum* 700506.

an influx of neutrophils. The levels of IL-10 showed a steady increase over the entire 0.6–60 µg dose range. IL-1b levels were mildly increased beginning at 6 µg and showed a steady increase thereafter. In contrast to other cytokines, IFN-γ levels were not significantly increased above background at any
dose (data not shown). The inflammation was primarily restricted to the perivascular and peribronchiolar regions with granulomas in the alveoli and diffuse inflammation in alveolar lumens (**Figure 1**). Granuloma formation was observed beginning with the 2 µg mycobacterial protein dose, with moderate granuloma formation at 20 µg, and severe granuloma formation at 60 µg. Peribronchial and perivascular infiltration was seen at doses of 0.2 µg and above, and was primarily neutrophilic. There was diffuse neutrophilic infiltration into the alveoli separate from the granulomas starting at 2 µg. Both the overall severity and the extensiveness of lung lesions showed a significant increase over the 0.2–60 µg test dose range. Based on this dose-response analysis, either 20 or 60 µg dose appeared to induce the changes consistent with acute HP-like pathology in this 1 week-acute exposure model.

ii). Chronic dose optimization: We optimized both the dose of instilled mycobacterial protein cocktail and the time point of sacrifice after last instillation, using the oropharyngeal route of administration. We used cell extracts of the mycobacterial strain MI 700506 prepared by bead beating (as optimized in the preceding efforts), and used a dose range of 0.075 to 30 µg mycobacterial per animal. Immune cell infiltration in the lung as determined by total cell count in the bronchoalveolar lavage (BAL) fluid increased in a dose-dependent manner beginning with a significant increase at 1.5 µg dose. Protein levels in BAL fluid showed a similar pattern, although there was little change in the BAL protein level in the 0.75-15 µg dose range. TNFα levels increased in a dose-dependent manner, whereas IFN-γ showed greater variation and a less clear dose-dependence. Histopathological analysis on the lavaged lungs showed very-mild to mild granuloma formation at doses up to 4.5 µg, and moderate granuloma formation at doses between 6 and 15 µg. At the highest dose (30 µg) granuloma formation was severe to very severe. Alveolar infiltration followed a similar pattern, while perivascular and peribronchial cuffing showed little correlation with dose. Taken together, these results showed that a reasonable mycobacterial protein dose to obtain a measurable response in this mouse model would be 10 to 20 µg/animal.

II. Screening of the different MWF mycobacteria (*M. immunogenum*, *M. chelonae*, *M. abscessus*) genotypes in the improved Mouse models of mycobacterial HP. Following the improvement of the mouse model based on oropharyngeal instillation, we set out to compare the HP-induction potential of mycobacterial genotypes to that of a positive control [an HP-inducing thermoactinomycete *Saccharopolyspora rectivirgula* from the established farmer's HP mouse model]. In this context, we first profiled the HP-induction potential of the positive control versus the saline (negative control). C57Bl/6j mice (male, 6 weeks old) were exposed oropharyngeally (50ul) with the known HP-inducing thermoactinomycete *S. rectivirgula* (SR) antigen preparation (250µg/50ul) and normal saline (50 µl), respectively using the same dosing regimen for 3 weeks (3 consecutive days each week for 3 weeks followed by sacrificing of all mice 4 hours after the last challenge). All doses were prepared in endotoxin-free water.

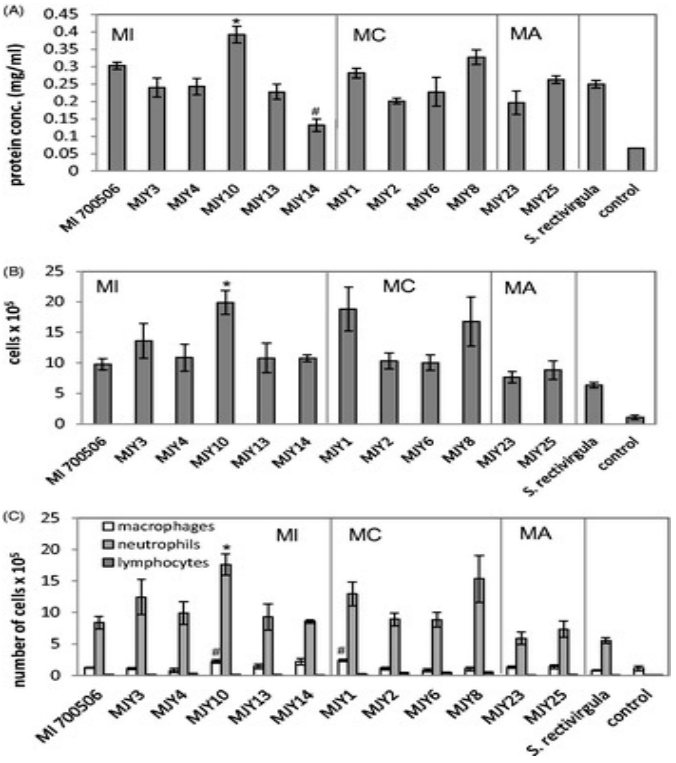

Figure 2. Acute exposure model: Comparison of individual mycobacterial genotypes/ variants (belonging to three different species) for potential to induce cellular changes in BALF of mice after oropharyngeal exposure using whole cell lysates.

i). Using Acute exposure model: For comparison of the mycobacterial genotypes, we first used a 1 week-acute exposure regime (oropharyngeal exposure to mycobacterial protein lysate dose on 3 consecutive days

followed by sacrificing 4 hours after the last instillation) before embarking upon the full 3-week regime (chronic). A total of 11 isolates representing 9 distinct genotypes were compared (**Figure 2**). In general, comparison in terms of cytotoxicity (LDH release), nitric oxide (NO) production, and induction of various pro-inflammatory cytokines showed relatively greater cellular and immunological changes in the lung exposed to MJY1 (*M. chelonae*) and MJY10 (*M. immunogenum*), albeit to a varying degree. In lung histopathological comparisons, there was a relatively greater induction of the HP-like pathology by MJY10 (*M. immunogenum*) and MJY1 (*M. chelonae*) genotypes and the least induction by genotypes such as MJY13/14 (*M. immunogenum*) and MJY2/3/4 (*M. chelonae*). Taken together, pathophysiological and immunological comparison among the mycobacterial genotypes showed a relatively greater HP-induction potential of MJY1 (*M. chelonae*) and MJY10 (*M. immunogenum*). The MJY1-challenged lungs showed changes analogous to but somewhat more intense than those induced by MJY10. However, the only genotype (with two morphotypes) from *M. abscessus* showed no remarkable HP-induction potential.

ii). Using improved chronic exposure model: *Genotype comparisons*: Following the improvement of the chronic HP mouse model based on oropharyngeal instillation of an optimal dose (10 µg/animal) and use of 4 hour post-instillation sacrifice time point, we set out to compare the HP-induction potential of mycobacterial genotypes. The HP-inducing potential of 5 *M. immunogenum* genotypes (MI 700506, MJY3, MJY4, MJY10, and MJY13), 4 *M. chelonae* genotypes (MJY1, MJY2, MJY6, and MJY11), and the *M. abscessus* genotype MJY23 were compared using the chronic exposure model. The tendency of mycobacteria to grow in aggregates and clumps differs between genotypes, and the preparation of monocellular suspensions proved difficult for some genotypes. In order to ensure a consistent dose of each genotype, we therefore used bacterial cell lysates (prepared using bead beating) for dosing, and the measured protein concentration in each extract was the basis for determination of dose. The animals were oropharyngeally instilled with a defined dose (10 µg/animal) for each genotype using the regime as optimized above, and sacrifice for sampling was done at 4 hours after the last instillation as optimized in a previous time course analysis. The animals appeared generally healthy during the course of treatment, except for the animals instilled with MJY10, who appeared to suffer from moderate dyspnea during the last week of exposure regime. No significant differences in body temperature or body weight at time of sacrifice were observed. Further comparisons were made in terms of lung immunological and histopathological responses as well as splenic T-cell responses. Collectively, the intra-species comparison results in this optimized chronic mouse model suggested that the *M. immunogenum* genotypes MJY10 and MI700506 and *M. chelonae* genotypes MJY1 and MJY2 induce the greatest immunogenic response with regard to the endpoints associated with HP, specifically splenocyte T-cell response upon challenge, granuloma formation, and TNFα in BAL fluid. The *M. immunogenum* genotypes MJY13 and MJY4 and the *M. chelonae* genotype MJY11 consistently gave the lowest responses (**Figs 3 & 4**).

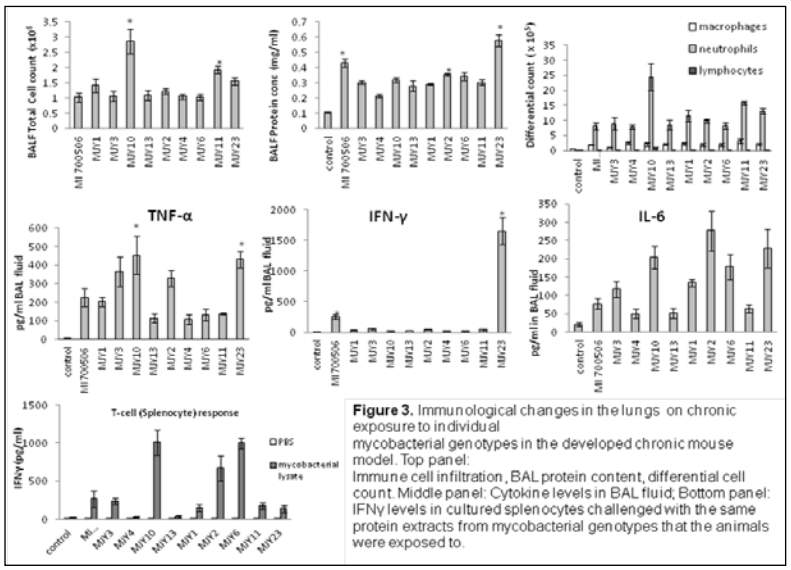

Figure 3. Immunological changes in the lungs on chronic exposure to individual mycobacterial genotypes in the developed chronic mouse model. Top panel: Immune cell infiltration, BAL protein content, differential cell count. Middle panel: Cytokine levels in BAL fluid; Bottom panel: IFNγ levels in cultured splenocytes challenged with the same protein extracts from mycobacterial genotypes that the animals were exposed to.

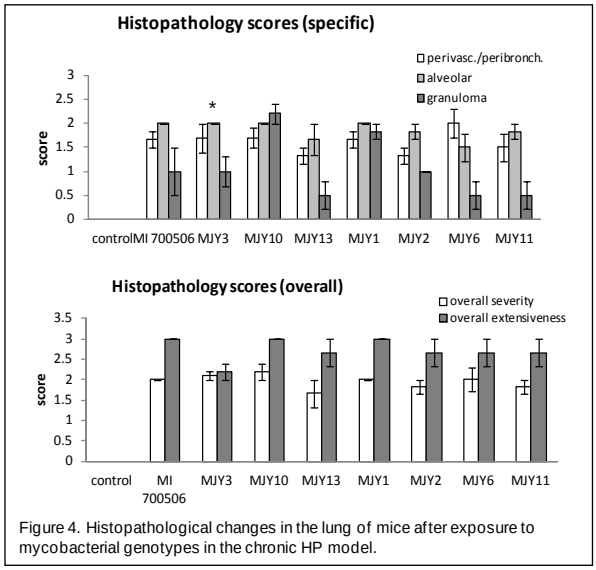

Figure 4. Histopathological changes in the lung of mice after exposure to mycobacterial genotypes in the chronic HP model.

Comparative *In vivo* immunogenicity of MI 700506 versus MJY10 (on DBA/2J versus C57/Bl6J background)*:* The two potent genotypes of *M. immunogenum*. MJY10 and MI700506 selected above were further compared in the developed chronic mouse model of HP. Most models of HP are based on the use of the Th1-biased mouse strain C57BL/6J, which has been shown to be susceptible to the development of HP. We therefore optimized our mouse model of mycobacterial HP using this mouse strain as reported in the preceding paragraphs of this progress report. However, considering that susceptibility of host to mycobacteria may vary with the genetic background, we compared alternative genetic background inbred mice. For example, comparison of the use of DBA/2J (Th2 bias) to C57BL/6J (Th1 bias) using the same (optimized) exposure regime was expected to reveal the role of Th bias in differential HP-inducing potential of the two genotypes. The standard HP antigen *Saccharopolyspora rectivirgula* (commonly used for mouse model of Farmers Lung) was also included for comparison. Briefly, mycobacterial whole cell extracts were prepared from exponentially growing cultures of M. immunogenum 700506 and MJY10 using bead beating. Mice were instilled with 10 µg mycobacterial protein in the form of whole cell extract using the optimized exposure protocol (oropharyngeal instillation on three consecutive days per week for three weeks, with sacrifice 4 h after last instillation).

a). In DBA/2J background, body temperatures at the time of sacrifice were not significantly different in any of the treated groups compared to controls. Body weights were slightly lower than for controls in mice treated with MI 700506 and MJY10, and this trend was significant for the MJY10 group.
b). *The pathoimmunological changes* in DBA/2J mice were very similar to those previously found in C57BL/6J mice in response to mycobacterial exposure. Protein levels in BALF were elevated in all treated groups relative to controls, and this increase was significantly higher in the MI 700506-treated mice compared with the control group. The total number of cells in BAL fluid, on the other hand, was significantly higher in the MJY10 group compared to MI700506-treated mice, and this was mainly due to a large influx of neutrophils, as shown by differential cell counting. IFN-γ levels in BAL fluid were strongly elevated in MI 700506-treated mice, compared with the significant but mild increases in the MJY10 and *S. rectivirgula* groups. By contrast, BAL fluid TNFα levels were significantly higher in MJY10-treated mice than in the MI 700506 group.
c). *Splenocytes* were isolated from all mice and rechallenged with the original antigen for each treatment group. The response to rechallenge was much larger for the MI700506 and MJY10 groups than for mice treated with *S. rectivirgula*. There was, however, no significant difference in response between the MI700506 and MJY10 groups, which was in contrast with the results obtained previously from the C57BL/6J strain, for which splenocytes from MJY10-treated mice gave a significantly greater response on rechallenge than splenocytes from the MI 700506 group.
d). *Histopathological changes*: No granuloma formation was seen in any of the treated groups, which was in contrast with our previous results from C57BL/6J mice, for which all mycobacterial genotypes induced some degree of granuloma formation, with the greatest degree of granuloma formation found in MJY10-treated mice. Very mild to mild lymphocyte and neutrophil perivascular/peribronchial cuffing was found in all treated groups of DBA/2J mice. Neutrophil infiltration of the alveoli was moderate to severe in MI 700605-treated mice, moderate in MJY10-treated mice, and mild in the *S. rectivirgula* group. Collectively, the results showed that the host genetic background could alter the relative in vivo immunogenicity and HP inducing potential of the M. immunogenum genotypes.

Comparative *in vitro* immunogenic potential of *MI genotypes (in antigen presenting cells)**:***
Considering that alveolar macrophages are antigen-presenting cells and play a central role in host defense in the exposed lung, understanding their interaction with MI could provide initial insights into the relative immunogenic potential of individual genotypes and help understand the underlying immunopathogenesis events and mechanisms driven by MI antigens.
Fate of M. immunogenum in AMs. M. immunogenum 700506 was shown to multiply intracellularly within the alveolar macrophages (MH-S cells) in vitro cultures, implying that it may be generating more antigen load within the exposed lung than what it carries with it (as whole cell contents) during MWF exposures.
Optimization of dose and time for M. immunogenum-host interactions. Testing of varying dose (0.001 through 1000 MOI) and time of exposure (3h through 24h) allowed us to select an effective and realistic dose-time combination (100 MOI for 24h) based on induction of cellular and immunological responses in MHS cells.

Comparison of MI genotypes. Using the above-optimized dose (100 MOI) and exposure time (24h) combination, both inflammatory and cellular damage responses were compared for the five different MI genotypes, namely 700506, MJY3, MJY4, MYJ-12 and MJY14. Of these, the 700506 genotype gave the highest cellular response. MJY3 increased LDH release (up to 53.4±5.66 %) to significantly ($p \leq 0.0057$) higher levels as compared to the other genotypes next only to the 700506 genotype (74.8±13.4 %). This cytotoxicity trend paralleled with the observed cell viability loss (MI 700506= 53.63 ± 10.30 % > MJY3 = 42.7 ± 12.84% > other genotypes). The individual genotypes showed significant but differential response in terms of induction of proinflammatory mediators (cytokines/chemokines, NO) in the MH-S cells. In terms of cytokine expression, MJY3, MJY4, and MJY14 showed relatively higher responses than 700506 whereas MJY12 showed the lowest response. For NO induction, MJY3 induced the highest amount of NO (6.4 ± 0.5 µM), next only to 700506, as compared to the other genotypes (MJY14, MJY4, and MJY12).

Collectively, the results imply that MJY3 and 700506 are the most potent genotypes for inducing inflammatory and cellular responses in alveolar macrophages, respectively. MI 700506 was shown to multiply intracellularly (~ 3.5 log increase in CFU within 72 h) within the alveolar macrophages (MH-S cell line) unlike in the mouse lung. Chemical blocking of either p38 or JNK inhibited the induction of proinflammatory mediators (cytokines, NO) by 700506. However, the cellular responses showed a somewhat opposite effect. Though the in vitro results with macrophages do not exactly coincide with the in vivo results in mouse models, these may provide ways to understand mechanisms of interactions of MI or its antigens with the host lung. This work has resulted in 1 publication (see the **Publication list)**.

Aim 1B. Identify key antigens (cell-associated and secreted) in the selected potent mycobacterial genotype of each species using differential immunoproteomic analysis.

The two polar strains of M. immunogenum, MJY10 (most immunogenic) and MJY4 (least immunogenic) , selected based on the immunogenicity screening of all genotypes in chronic mouse model of mycobacterial HP under Aim 1A were subjected to differential immunoproteomic analysis to identify the specific antigens unique (causing potency) in MJY10. Briefly, the whole cell lysates from mid-log cultures (162 Klett reading) of the two genotypes prepared using the optimized bead beating method were resolved on 2D gel electrophoresis, using our previously optimized protocol (Gupta et al 2009, J. Proteome Res.). This was followed by the Western blot analysis step, performed using polyclonal antibodies custom raised in rabbit against a pool of all M. immunogenum genotypes (the pool was prepared by mixing equal amounts of the heat-inactivated individual whole cell suspensions). The differential immmunoreactive spots of interest were picked and identified using mass spectrometry (LC-MS).

Differential proteomic analysis of MJY 4 and MJY 10 gels using Image Master™ 2D Platinum software. Differential analysis of MJY4 and MJY10 gels showed that majority of the MJY 4 proteins were localized towards pH range 4.0-5.4 whereas majority of the MJY10 proteins were found towards pH 5.5- 7.0 as shown in the overlapping image analysis using Image Master™2D Platinum software (**Figure 5**).

Immunoreactive Spot selection on the 2D gel using Western blot as the reference. 2D resolved protein gels of both MJY4 and MJY10 strains were transferred to nitrocellulose membrane and probed with antibodies raised against a pool of MJY genotypes (as described above). The protein hybridization spots appearing on the Western blots (**Figures 6 and 7**) were marked with arrows and their corresponding protein spots on a replica 2D gel were identified and manually picked using spot picker. These immunoreactive spots were then processed for mass spectrometry analysis using LC/MS platform.

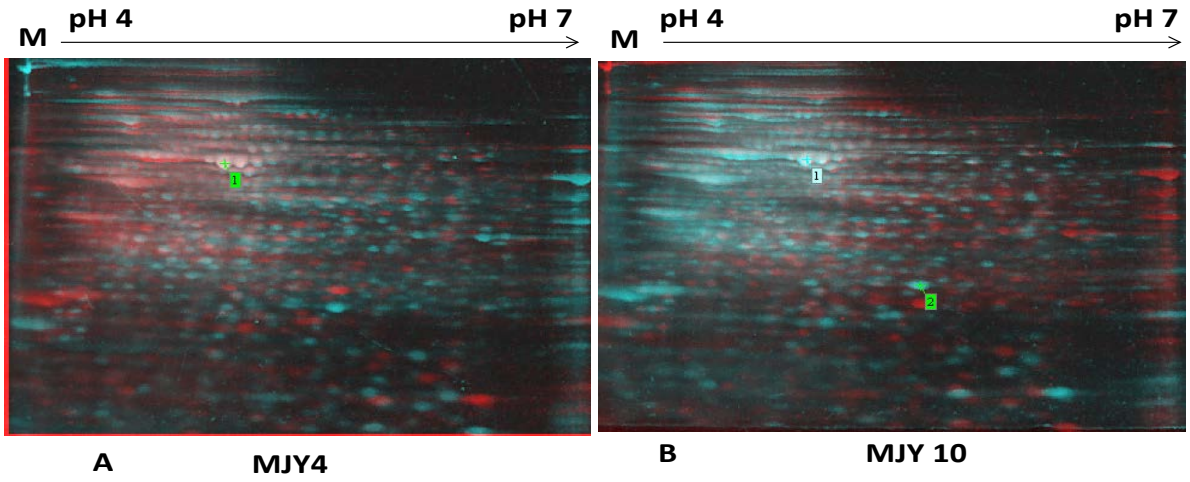

Figure 5: Differential expression analysis of MJY 4 and MJY 10 using Image Master™ 2D Platinum software. Overlapping spots are shown as shades of gray/ white, red spots are specific to MJY 4 in image A and MJY 10 in image B, cyan spots are present only in the sheet reference (MJY10 was taken as sheet reference for MJY4 and vice-versa), Halos of cyan or red around dark spots indicate that the protein is over or under expressed respectively, compared to the sheet reference. Numbers 1 and 2 in the images A and B are reference spots, respectively.

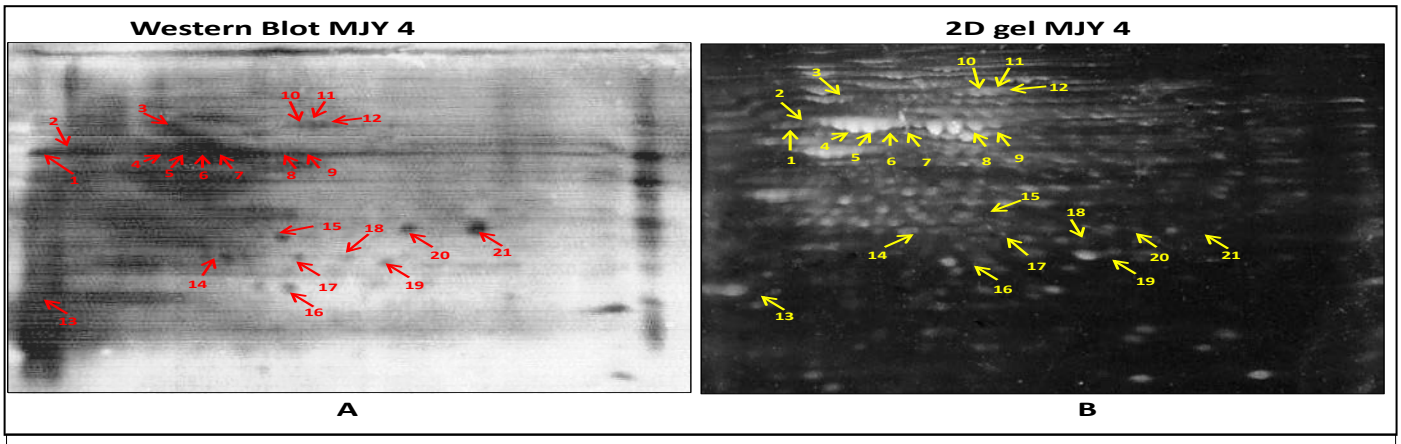

Figure 6: Immunoreactive Spot selection on the 2D gel using western blot as reference. (A) Western profile of 2D resolved proteins of MJY4 strain. Red arrows indicate the corresponding immunoreactive proteins on the 2D gel image (B) 2D gel profile of MJY4 using IEF strip of pH4-7 and 12 % SDS PAGE.

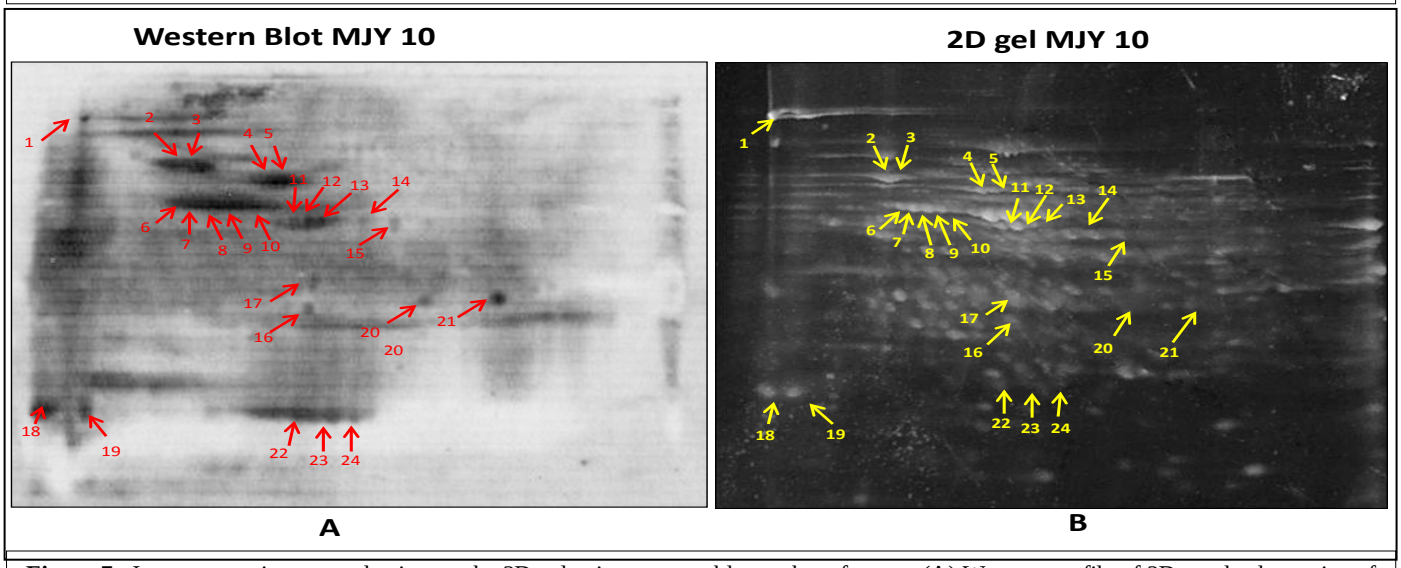

Figure 7: Immunoreactive spot selection on the 2D gel using western blot as the reference. (A) Western profile of 2D resolved proteins of MJY10 strain. Red arrows marked are the corresponding immunoreactive proteins on the 2D gel image (B) 2D gel profile of MJY 10 using IEF strip of pH4-7 and 12 % SDS PAGE.

Identification of differentially expressed proteins: The peptide peaks in the mass spectra were auto analyzed using MASCOT search engine against the closely related mycobacterial protein databases. A total of 47 peptides were identified for MJY10 and 53 for MJY4. In some cases, similar proteins were found in different spots suggesting their multiple isoforms. Further analysis and sorting of the proteins revealed 22 unique peptides for MJY10 and 24 unique peptides for MJY4. Finding of these unique immunoreactive proteins is crucial in terms of the observed HP-induction potential differences in the two genotypes. The MJY10 in our mice model study was found highly immunogenic whereas the MJY4 was found least immunogenic when different genotypes were compared. We have also detected 11 common immunoreactive proteins found in both the strains.

Collectively, the analyses revealed 46 unique immunoreactive proteins, 22 for MJY10 and 24 for MJY4, and 11 common proteins. Finding of these unique antigen candidates is crucial in terms of the observed HP-induction potential differences in the two genotypes. Further efforts focused on generation of recombinant form of these unique immunoreactive proteins and their functional evaluation (Note that one of these expressed proteins (Elongation factor Tu) was fully evaluated- see Aims 2 and 3). These comparative studies have resulted in 1 manuscript (see the **Publication List**).

Aim 2. Recombinant Production of selected antigens of *M. immunogenum* and *M. chelonae* and generation of their specific antibodies:

In our initial efforts on M. immunogenum antigen identification, we had identified 33 immuno-reactive proteins, comprising of 4 secretory, 6 cell wall-associated, 11 membranous, and 12 cytosolic proteins by immunoproteomic profiling of secreted and subcellular protein fractions using a combination of two dimensional electrophoresis (2-DE), immunoblotting, and matrix-assisted laser desorption/ionization-Time of flight (MALDI-TOF) approach. Subsequently we identified additional immunogenic proteins from other genotypes. Overall, more than 100 immunoreactive proteins/antigens from different genotypes of *M. immunogenum* have been identified using 2D-immunoproteomic approach by the end of this funding cycle, leading to 2 manuscripts (see **Publication list**). To understand the immunogenicity of these identified antigens, we undertook cloning and heterologous expression under this aim.

2A and 2B: Cloning, heterologous expression and purification of MI Antigens. We have successfully cloned (via PCR) five of the MI immunoreactive proteins (**Table 1**) using their corresponding genes from the genomic DNA of *M. immunogenum* 700506 in pET30a(+) and expressed them using *E.coli Rosetta Blue (DE3)* strain. The expressed proteins were purified using Ni-NTA column and made free from endotoxin contamination using Triton X114 method.

Table 1: Recombinant antigens generated and evaluated in the study

Antigens	Locus tag	Name	Size	Fractions
Ag A	MAB_0860c	DNA helicase	1.6kb	Cell wall
Ag B	MAB_1310	GTP binding translation Elongation factor	1.974kb	Cell wall
Ag C	MAB_2001	N-acetylmuramoylanyl-D glutamate-2,6-diaminopimelate ligase	1.5 kb	Cell wall
Ag D	MAB_3848c	Elongation factor Tu	1.194kb	Secretory
Ag E	MAB_0578c	Trehalose phosphatase	2.5 kb	Secretory

Additional recombinant antigens: Subsequently, additional 28 antigens (3 antigens of MI 700506, 20 unique antigens of the other potent genotype MJY10, and 5 common antigens between MJY4 and MJY10) have been cloned in the same E. coli expression vector pET30a(+), sequenced, and their protein expression in required quantities was pursued for further evaluation under Aim 3.

Aim 3. Investigate potential of the specific antigens of MWF mycobacteria for personal exposure assessment and immunodiagnosis using cellular or mouse models and human subjects

Aim 3A. Evaluate the identified (recombinant) antigens for immunodiagnostic potential using in vitro, ex-vivo, and in vivo (mouse) models: The 5 purified recombinant antigens strongly induced pro-inflammatory cytokines in a dose-dependent manner in murine alveolar macrophages. Further, these antigens induced T lymphocyte proliferation and production of INF-g release in T-cells in a dose-dependent manner. These results provided first evidence on the immunological potential of secretome proteins in this mycobacterial species and opened the way for understanding the immunogenicity and pathogenesis mechanisms of *M. immunogenum* in HP development and diagnosis. The identified antigens were further evaluated as potential targets for HP diagnosis. The specifics are summarized below.

a). *In vitro/ex-vivo* evaluation for activation of antigen presenting cells (Human dendritic cells and murine alveolar macrophages): The Ni-NTA purified recombinant antigens were tested on mouse alveolar macrophage (AM) cell line MHS for understanding their potential to activate these APCs. The immune response was evaluated in terms of inflammatory cytokines (TNFα, IL-1α and IL-6) and Nitric oxide production in the culture supernatant. All five candidate antigens (Ag A through E) strongly stimulated AMs in a dose-dependent manner to produce these immune mediators albeit to a variable extent (D >C > B > A >E). In human dendritic cell stimulation assays using different antigen doses (5–50 µg/ml), all candidate antigens significantly induced TNF-α. The level peaked at lower concentrations (5 µg/ml for AgA, AgB, AgD, AgE and 10 µg/ml for AgC), following which it decreased significantly at higher concentrations. Treatment with MI whole cell lysate however showed the TNF-α peak at a higher concentration (50 µg/ml) and the induction was dose-independent. The LPS control showed a dose-dependent induction of TNF-α in DCs, with a significant induction at 10 ng/ml but none at 0.2 ng/ml. These studies have resulted in 1 research paper (see the **Publication List**).

b). *In vivo/ex-vivo* evaluation based on the HP mouse model:

i)**. *Serodiagnostic potential using pathostage-specific mouse sera (for differential diagnosis)*:** Of the 5 MI recombinant antigen candidates (AgA through AgE) tested, all reacted strongly with serum from 3 week-challenged mice albeit with differential specificity whereas none showed reactivity with sera from 1 week-challenged mice in the chronic HP model. The order of reactivity was AgC > AgD > AgA > AgB > AgE. The results imply that the five MI antigens could not possibly discriminate between the sera from early exposed versus late exposed (diseased) individuals considering that even MI whole cell lysate did not show reactivity with week 1 sera; this may be possibly because humoral response had not set in within one week. Hence, there is a need to perform systematic future studies to develop more robust mouse model for differential diagnosis of the patients versus the exposed machinists.

ii). *Ex-vivo T cell response to MI antigens*: The five recombinant MI antigens were further tested for their potency to stimulate spleen T cells isolated from control (unexposed) and MI-induced HP mouse model. Briefly, Naïve or MI-challenged (using whole cell lysate from strain 700506) C57BL/6J mice were used to derive spleen T-cells (splenocyctes) for this part of the study. Spleens were removed aseptically and single cell suspensions of spleen T cells were prepared by passing through sterile mesh followed by lysis of erythrocytes. The resulting splenocytes were further purified from the contaminating leukocytes (macrophages) by plating them overnight in complete RPMI medium (Hyclone) supplemented with 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals), 100 units/ml penicillin and 100 µg/ml streptomycin). The resulting non-adhered fraction representing the T-cells (splenocytes) were diluted to a working concentration of 1×10^6/ml of medium. For IFN-g release assay, the spleen T-cells (splenocytes) were stimulated with different concentrations of MI antigens for 48 hours. Cell culture supernatants were analyzed for IFN- gamma release assay by ELISA (eBioscience). Treatment of cells with Concanavalin (5 µg/well) served as positive control. For splenocytes proliferation assay using CellTiter 96® AQueous one reagent (Promega), the splenocytes ($0.2x10^6$ cell/ well/100µl in 96 well plates) were stimulated with various concentrations of individual MI antigens for 48 hours.

Three of the five test antigens were T-cell reactive (AgA, AgB, and AgD); of these, AgA and AgD induced a significant level of IFN-γ and T-cell proliferation in the T splenocytes (Figure 8).

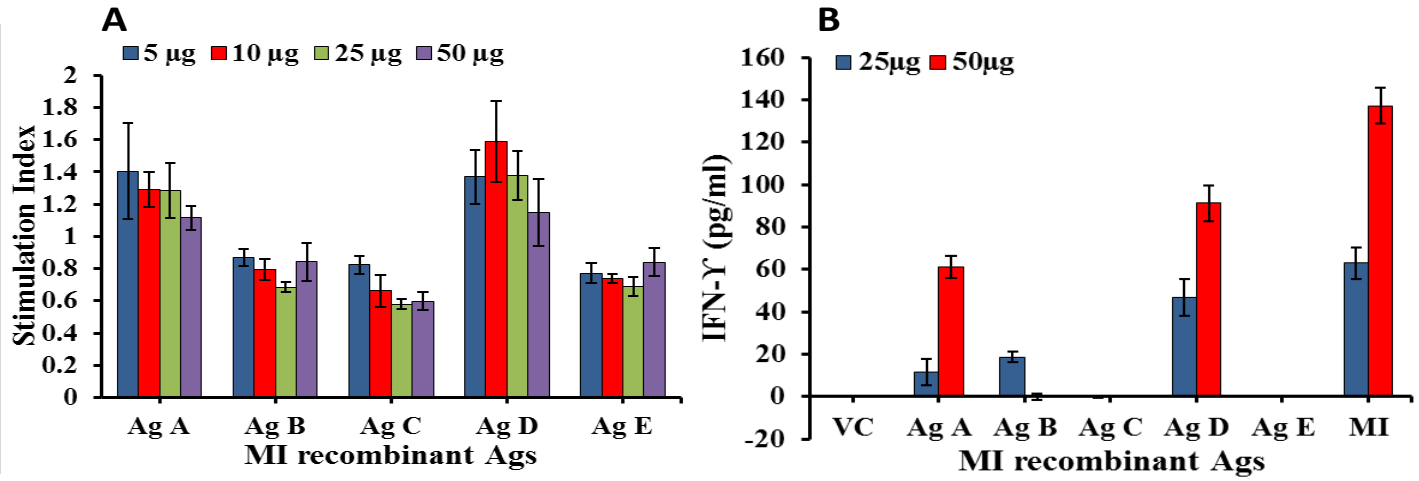

Figure 8: Splenocyte T-cell proliferation (A) and induction of IFN-γ in response (B) to MI recombinant Antigens. Values are presented as means ± standard deviations of triplicates.

The T-cell response for the candidate antigens was further assessed using human naïve CD4+ T cells and primed autologous DCs (DC-T cell assay) using an ELISPOT analysis in terms of IFN-γ secretion. The results indicated that AgD was able to stimulate naïve CD4+T cells significantly at the 5–10 µg/ml dose range whereas AgA and AgB stimulated significantly at higher dose ranges (10–25 µg/ml and10–50 µg/ml, respectively). The stimulation by AgC was not significant at any of the concentrations and AgE stimulated significantly at 5 µg/ml only. The MI whole cell lysate strongly induced the T-cell response in a dose-dependent manner. As a comparison control, the LPS did not induce significant T-cell response at 0.2 ng/ml concentration unlike the higher concentration (10 ng/ml) that significantly induced the response, as compared to the vehicle control.

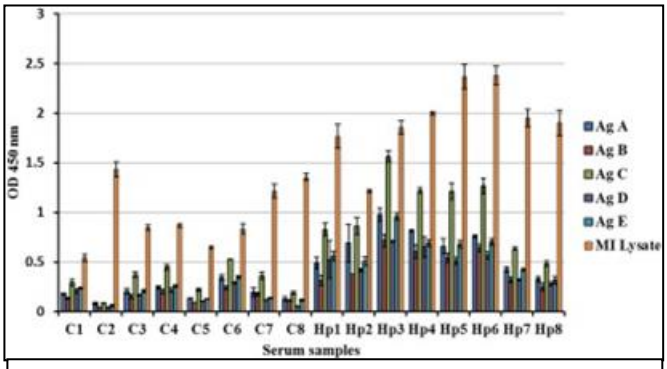

Figure 9. Antibody response of sera from machinist patients with hypersensitivity pneumonitis (Hp1–Hp8) and healthy control subjects (C1–C8) against Mycobacterium immunogenum (MI) recombinant antigens (A-E) and whole cell lysate. Ag, antigen; OD, optical density.

Immunoinformatic analysis for epitope identification: HP is a T cell-mediated immune disorder that is thought to depend on an intricate balance (ratio) between CD4 and CD8 cells. This balance may be governed by the relative abundance of HLA class-specific epitopes in the causative antigen. Therefore, the two above identified promising MI T-cell antigens (AgA and AgD) were further analyzed for epitopes that can bind to HLA-I and HLA-II alleles, using the epitope prediction platforms namely ProPred and ProPred-I. ProPred analysis predicted that AgA and Ag D can bind all (100%) of the class II HLA alleles analyzed as compared to the reference antigens ESAT-6 and CFP-10 which were predicted to bind fewer alleles (84.31% and 88.23% alleles, respectively). This analysis suggested that these candidate MI antigens strongly induce CD4 lymphocytes. ProPred-I analysis predicted AgA and AgD to bind 100% and 91% of the 47 class I alleles as compared to the reference antigens ESAT-6 and CFP-10 that were shown to bind to a much less extent (80.85% and 70.21%, respectively). These results indicate that AgA is capable of inducing more CD8 response than AgD. Collectively these in silico predictions suggest that AgA and AgD might be useful in eliciting both CD4- and CD8- specific immune responses. The combined projected human population coverage (for both class I and class II HLA) for all predicted epitopes were also higher than the reference antigens. AgA and AgD were predicted to have larger population coverage (90.87%

and 88.09%, respectively) as compared to the reference antigens ESAT-6 (82.43%) and CFP-10 (80.21%). Further analysis using IEDB immunogenicity prediction tool showed that AgA and AgD are highly immunogenic as compared to the reference antigens ESAT-6 and CFP-10. Hence, MI antigens AgA and AgD can be useful for inducing both CD4- and CD8-specific T-cell immune responses in a larger human population than the widely studied reference TB antigens ESAT-6 or CFP-10.
This first study on identification of T-cell antigens of M. immunogenum (and their epitopes) opens new avenues for the development of clinical diagnostic tools and therapeutic and/or vaccine targets for machinists HP, a poorly studied and difficult-to-diagnose occupational lung disease. These studies under Aim 3A(b) have resulted in 2 publications (see **Publication List)**.

Aim 3B. Evaluation of the recombinant MI antigens for serodiagnosis potential using sera from human subjects (using ELISA assay):
We used serum samples from HP patients (machinists) that we sampled along with control (unidentified) samples from healthy human subjects. The eight human serum samples drawn from 5 HP patients (Patients 1 through 5; patients 1, 4, and 5 were sampled twice) were analyzed for presence of antibodies to the 5 purified recombinant MI antigens. Specifically, detection of IgG antibody against the recombinant AgA, AgB, Ag C, Ag D and Ag E in HP patient sera was performed by a modified ELISA (**Figure 9**). In all, we used 16 sera from MWF-HP patients (N=8) and healthy subjects (N=8). First, the ELISA conditions were standardized so as to get a zero background without antigen in the controls wells. We tried different blocking agents and conditions and finally we achieved this by using 0.5% gelatin with 6% sorbitol in the blocking buffer, which seems to be the important step in the development of this assay. Mean absorption values OD_{450} nm of ELISA were used to generate Receiver operating characteristics (ROC) and are described as follows:
ROC curves and determination of sensitivity and specificity: ROC curves and Sensitivity vs Specificity plots were generated using XLSTAT software for each antigens A-E. Sensitivity and Specificity for optimal threshold values of individual antigens with 95% Confidence interval are given in **Table 2**. All 5 antigens showed high sensitivity (100%, 95% confidence interval [CI] 0.622-1.00) and specificity (87.5%-100%, 95% CI 0.505-0.0995) for optimal threshold. Ag B showed the best response (sensitivity 100%, 95% CI 0.622-1.00; specificity 100%, 95% CI 0.622-1.00), whereas the MI lysate produced the least response (sensitivity 87.5%, 95% CI 0.505-0.995; specificity 87.5%, 95% CI 0.505-0.995) with a high threshold value (1.434).

Table 2. Evaluation of individual recombinant antigens and whole cell lysate of MI on human sera from patients with hypersensitivity pneumonitis and controls using a serodiagnostic assay based on an enzyme-linked immunosorbent assay

Antigen	Threshold value	Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV	LR^+	LR^-	Accuracy
A	0.329	1.00 (0.622–1.00)	0.875 (0.505–0.995)	0.889	1.00	8.0	0.00	0.938
B	0.249	1.00 (0.622–1.00)	1 (0.622–1)	1	1	+Inf[a]	0.0	1.00
C	0.484	1.00 (0.622–1.00)	0.875 (0.505–0.995)	0.889	1.00	8.0	0.00	0.938
D	0.279	1.00 (0.622–1.00)	0.875 (0.505–0.995)	0.889	1.00	8.0	0.00	0.938
E	0.318	1.00 (0.622–1.00)	0.875 (0.505–0.995)	0.889	1.00	8.0	0.00	0.938
MI lysate	1.434	0.875 (0.505–0.995)	0.875 (0.505–0.995)	0.875	0.875	7.0	0.143	0.875

Abbreviations: CI, confidence interval; LR^-, negative likelihood ratio; LR^+, positive likelihood ratio; MI, *Mycobacterium immunogenum*; NPV, negative predictive value; PPV, positive predictive value.

Comparison of Area-under-the-curve (AUC): AUC values for ROC curves of all antigens were highly significant (P < 0.0001) compared with the cutoff area under the curve value (0.5); values for the different antigens, in descending order, were Ag B (1.00), AgA, Ag C, Ag D, and Ag E (0.984), and MI lysate (0.953).
Interpretation of diagnosis based on optimal threshold for each antigen: Optimal threshold values (decided based on highest sensitivity with specificity >80%) were determined for each antigen and the MI lysate. Values superior or equal to the threshold values of the patients' sera were considered positive for each antigen. Of the 8 sera (Hp1 through Hp8) from the patients with HP, 6 (Hp1 through Hp6) responded to all 5 antigens based on the threshold values, whereas Hp7 and Hp8 responded to only 4 of the 5 antigens. The entire panel of 5 antigens was used to set a global interpretation of likelihood of the disease (HP) on a scale of 1 to 4 (score 1, excellent [≥4 antigens were positive]; score 2, good [2-3 antigens positive]; score 3, fair [1 antigen positive]; score 4, poor [no antigen positive or all 5 antigens negative]).

Collectively, the results demonstrated that the candidate antigens are highly sensitive (100%) and specific (87.5%-100%) for discriminating patients with HP from healthy unexposed controls compared with the crude antigen (MI lysate), which showed lower sensitivity and specificity (87.5% each). This work resulted in 1 publication (see the **Publication List**).

Aim 4. Identify and evaluate specific cell-free antigen(s) of MWF mycobacteria for environmental exposure assessment (fluid monitoring) using simulated and field samples.

Aim 4A. Identify specific cell-free antigens by immunoproteomic analysis: Under this aim, our long-term goal was to identify and characterize cell-free antigens (antigens that are secreted out of the cell) in MWF-colonizing mycobacterial species and genotypes using immunoproteomic approach. In this direction, we have compared six different genotypes of *M. immunogenum* (700506, MJY4, MJY13) and *M. chelonae* (MJY1, MJY2, MJY8) to identify subsets of antigens that are secreted in the aqueous growth media. After several rounds of analysis, we observed consistent differential profiles (variable number and intensities) of the cell-free antigens in all tested genotypes (three each for *M. immunogenum* and *M. chelonae*). The hybridizing protein spots of desirable intensity for each genotype were picked and processed by tryptic digestion and analyzed by mass spectrometry using LC/MS/MS. The analysis of the differential profiles of the individual genotypes led to identification of subsets of specific antigens. Overall, we identified a set of 20 cell-free antigens belonging to the different genotypes. These cell-free antigens have the potential to be secreted in MWF colonizations.

a). MWF colonization by *M. immunogenum*: *Field studies (Isolation of a new genotype).* MWF colonization by mycobacteria was investigated in simulated and field MWF samples. These studies allowed us to understand the growth requirements of mycobacteria in field MWF, such as tramp oil, range of pH, biocide susceptibility, etc. This part of the work has resulted in 3 research papers (see **Publication list**). Lately, we have been able to identify a pair of field MWF samples (with and without mycobacteria) obtained through our industrial collaborator. We isolated and identified a new genotype (MJY27) of *M. immunogenum* from the mycobacteria-positive sample. This sample pair could be used for differential identification of MI antigens prevalent in field MWF (see aim 4B below).

Laboratory simulation studies. *Considering the HP-link and wide prevalence of M. immunogenum* genotype/strain 700506 in HP-linked MWF in industrial setting, we undertook optimization of laboratory culturing of this strain in MWF using a statistical Design of Experiments (DOE) for modelling and optimization of the process (using Response Surface Methodology and Genetic Algorithm). This MWF culture can be compared with the standard broth culture to evaluate the MWF-expressed antigenome of this MWF mycobacterium species. This part has resulted in 1 manuscript (see the **Publication list**)

b). Identify cell-free antigens by immunoproteomic analysis in simulated media and field MWF: In this direction, we have compared six different mycobacterial genotypes, 3 each of MI (700506, MJY4, MJY13) and MC (MJY1, MJY2, MJY8) in aqueous media and identified a set of 20 cell-free antigens from the different genotypes. These cell-free antigens have the potential to be secreted in MWF-grown cultures. Further work in this direction may utilize the MWF-grown culture of MI 700506 optimized above. This is expected to result in at least one manuscript.

In separate efforts, using the above selected pair of field MWF (mycobacteria-positive and mycobacteria-negative), we performed differential 2D-immunoproteomic analysis and shotgun proteomics to identify cell-free MI-specific antigens and other secreted proteins produced in MWF under the field conditions. A total of 8 antigens and 72 secreted proteins (both antigens and non-antigens), respectively were identified using these two approaches. This has resulted in 1 manuscript (see the **Publication list**)

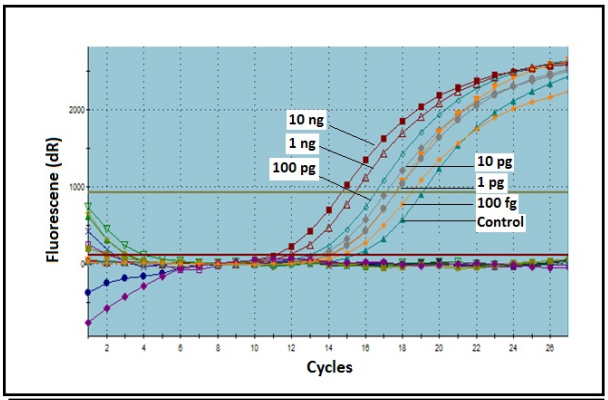

Figure 10: Immuno-PCR assay of different concentrations of test antigens in MWF from *M. immunogenum* using the conditions optimized using RSM coupled with GA.

Aim 4B. Optimization of fluid monitoring assays for mycobacterial antigens (ELISA and Immuno-PCR).

a). ELISA-based immunoassay (for ng level detection): First, the ELISA assay variables were optimized for application to MWF samples. Minimum detection limit for the test MI antigen (AgB) in MWF matrix was found to be 10ng/ml.

b). Immuno-PCR assay for detection of target MI antigen in MWF (at pg to fg levels): In this, an oligo conjugated to secondary antibody in the antigen-Ab complex was used as a probe to qPCR detect/quantify the antigen. This optimized immunoPCR assay (**Figure 10**) showed a minimum detection limit of 0.1 pg antigen, which is 100,000x more sensitive than the ELISA assay adapted for the MWF matrix (synthetic).These fluid monitoring immunoassay development studies have resulted in one manuscript (see the **Publication list**).

d. Conclusions and future direction

Nontuberculous Mycobacteria (NTM) have been implicated in respiratory symptoms/disorders particularly occupational hypersensitivity pneumonitis (HP) in machine workers exposed to metalworking fluids (MWFs). Therefore, characterization of relative immunogenic and HP-inducing potential of MWF-prevalent NTM strains/genotypes using experimental HP mouse model and human samples could help identify the differentially immunogenic strains and their antigens that have relevance to HP and other respiratory immune disorders in exposed workers. Such antigens could in turn form the basis for development of immunodiagnostic tests both for exposure assessment and for clinical diagnosis of MWF-linked HP in machinists. This study led to identification of differentially immunogenic MWF mycobacterial strains (hyperimmunogenic and hypoimmunogenic) using our optimized mouse models of experimental HP and a repertoire of their antigens based on differential immunoproteomics. Ex-vivo evaluation of recombinant form of these antigen candidates for seroreactivity using mouse model and HP patients (machinists) led to identification of a panel of antigens for potential usefulness in differential serodiagnosis of HP as compared to the healthy control subjects. Further screening based on T-cell response also led to identification of a pair of bivalent antigen candidates (both sero-reactive and T cell-reactive) with an expanded immunodiagnostic potential in HP patients. Such bivalent function antigens stimulating both arms of the immune system could potentially be useful for further screening as candidates to confer immunity, which could be a future priority area in mycobacterial HP research. Furthermore, based on the whole antigens and predicted epitopes from this study, future identification of critical functional epitopes could help develop the much required differential clinical diagnosis of MWF-linked HP in machinists (exposure versus disease). The information generated in this study on antigenic potential of MWF-prevalent NTM strains and their seroreactive and bivalent reactive antigens will help in more informed design and interpretation of future epidemiological studies on MWF-linked illnesses. On the other hand, optimization of simulation conditions for mycobacterial colonization of MWF and initial optimization of immunodetection assays for MWF matrix (ELISA and Immuno-PCR) accomplished in this study will pave the way for future development of fluid monitoring strategies such as those based on specific epitopes and sensors. Taken together, these developments would be critical for developing the much needed exposure assessment, diagnosis, and intervention strategies for MWF-linked HP and related respiratory symptoms and disease/disorders in machine workers.

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10. Bhattacharya SS, Yadav JS: [2017] Modeling and Simulation of Colonization of Pristine Water-based Metalworking Fluid by *Mycobacterium immunogenum*. Journal of Hazardous Materials (in peer-review).
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1. Chandra H, Yadav JS: [2013] Immunogenic Potential of Recombinant Protein Antigens of *Mycobacterium Immunonogenum*, an Etiological Agent of Hypersensitivity Pneumonitis, Poster at the 113th Annual General Meeting of the American Society for Microbiology, Denver, CO, May 18-21.

2. Johansson E, Yadav JS: [2013] An Improved Chronic Mouse Model of Hypersensitivity Pneumonitis Induced by *Mycobacterium Immunogenum*. Poster at the 20th Midwest Microbial Pathogenesis Conference (MMPC) 2013, Ohio State University, Columbus, OH, August 23-25.
3. Chandra H, Yadav E, Yadav JS: [2013] Interaction Between Alveolar Macrophages And *Mycobacterium Immunogenum* And Its Antigens, The Etiological Agents Of Hypersensitivity Pneumonitis**.** Poster at the 20th Midwest Microbial Pathogenesis Conference (MMPC) 2013, Ohio State University, Columbus, OH, August 23-25.
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7. Chandra H, Lam YW, Yadav JS: [2015] Antigen Profiling of Field Metalworking Fluids. Poster at the 16th Annual Pilot Research Project (PRP) Symposium of the NIOSH–supported Education and Research Center (ERC) University of Cincinnati, held at Engineering Research Center, University of Cincinnati, Cincinnati OH, October 8-9.
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9. Chandra H, Lam YW, Yadav JS: [2016] Antigen Profiling of Field Metalworking Fluids. Oral Presentation at the 17th Annual Pilot Research Project (PRP) Symposium of the NIOSH–supported Education and Research Center (ERC) University of Cincinnati, held at College of Nursing, Procter Hall, University of Cincinnati, Cincinnati OH, October 13-14.

ADDITIONAL INFORMATION

1. Cumulative Inclusion Enrollment Table

Cumulative Inclusion Enrollment Report

This report format should NOT be used for collecting data from study participants.

Study Title: Mycobacteria in Occupational Hypersensitivity Pneumonitis

Comments: None

Racial Categories	Ethnic Categories									Total
	Not Hispanic or Latino			Hispanic or Latino			Unknown/Not Reported Ethnicity			
	Female	Male	Unknown/ Not Reported	Female	Male	Unknown/ Not Reported	Female	Male	Unknown/ Not Reported	
American Indian/ Alaska										0
Asian										0
Native Hawaiian or Other Pacific Islander										0
Black or African American								1		1
White								2		2
More Than One										0
Unknown or Not Reported								2	8	10
Total	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**13**

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OMB No. 0925-0001/0002

2. Inclusion of gender and minority study subjects

Participants were not be excluded based on gender, ethnicity, race, age, or pregnancy. The unexposed controls were preferably the individuals of similar sex and racial distribution as the patients with HP

3. Inclusion of Children

Children between 18 and 21 years of age will not included as they were not available within the HP patients targeted for study.

4. Material Available for Other Investigators

a. Mycobacterial strains (genotypes) isolated from industrial metalworking fluids
b. Recombinant antigens of MWF mycobacteria (clones)
c. Reprints of publications from this project