Children's Hospital of Pittsburgh

Annual Progress Report: 2006 Formula Grant

Reporting Period

July 1, 2010 – December 31, 2010

Formula Grant Overview

The Children's Hospital of Pittsburgh received $732,956 in formula funds for the grant award period January 1, 2007 through December 31, 2010. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Cell Biology of Cystic Fibrosis Transmembrane Regulator (CFTR) - CFTR is responsible for transporting chloride from the cells lining the airway, digestive tract, biliary tract, pancreatic duct and kidney tubules into the body fluids. Impairment of this function results in the lung disease, pancreatic insufficiency, liver disease, malabsorption and other clinical problems of cystic fibrosis. This project is designed to identify specific factors that determine how CFTR gets to, and stays, at the cell membrane where it needs to be for optimal functionality. The goal is to exploit knowledge of these factors for the design of novel pharmacotherapeutic agents that enhance the functionality of the mutant CFTR in patients with cystic fibrosis.

Duration of Project

1/1/2007 - 12/31/2010

Project Overview

The objectives are to establish a program that will investigate the cellular and biochemical mechanisms by which CFTR is appropriately targeted to the site of its optimal functionality at the cell membrane. The research will provide information upon which to base novel pharmacological strategies to treat the clinical problems of cystic fibrosis.

Specific Aim 1: Identify a network of proteins that regulate CFTR endocytosis.
Specific Aim 2: Examine the specific roles of c-Cbl and Rab4 in CFTR endocytosis.
Specific Aim 3: Examine the mechanism by which sorting within the endocytic pathway determines the half-life of CFTR on the plasma membrane.

These objectives will be achieved by a combination of molecular, biochemical, functional, proteomic and morphological techniques in model cell line systems. This will include studies of protein-protein interactions, perturbations introduced by transfected specific genes and siRNA

molecules, confocal immunofluorescent imaging, immunoelectron microscopy, cell surface labeling, transepithelial conductance analysis and proteomic analysis.

Principal Investigator

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

Through this basic research we will discover new methods for ameliorating the devastating clinical consequences of cystic fibrosis.

Summary of Research Completed

During the last six months of funding we have completed several studies started earlier during the project period. Results obtained specifically during the last six months are as follows: (a) the interaction between CFTR and c-Cbl is regulated by phosphorylation and c-Cbl targets the CFTR chloride channels that are not PKA-activated (Figure 1); (b) Dab2 facilitates signal transduction in the TGF-ß signaling cascade and mediates the TGF-ß dependent downregulation of CFTR gene and protein expression in human airway epithelial cells (Figures 2-4).

The PI participated in the following activities at international scientific symposia:

Invited to present: "c-Cbl regulates CFTR traffic and function in human airway epithelial cells" at the Experimental Biology Meeting in Washington, DC, 2011.

The PI became a member of the following editorial boards:

- Member of the Editorial Board of the Physiological Frontiers since July, 2010
- Member of the Editorial Board of the *American Journal of Physiology – Renal Physiology* since July, 2010.

Figure 1.

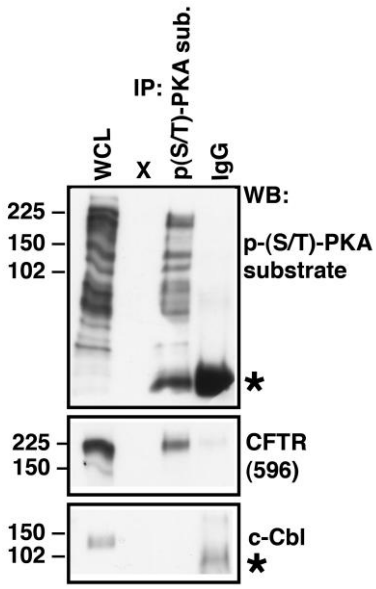
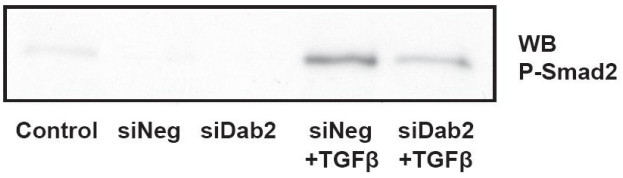


Figure 1. Immunoprecipitation experiment demonstrating that CFTR is phosphorylated on Serine/Threonine (S/T) residues in the PKA consensus sites. PKA substrates phosphorylated on the S/T residues in the PKA consensus sites were immunoprecipitated with an anti—(S/T)-PKA substrate antibody or the IgG control and the immunoprecipitated protein complexes were blotted with anti-CFTR and anti-c-Cbl antibodies. CFTR but not c-Cbl was present among the (S/T)-PKA substrates. These data indicate that CFTR but not c-Cbl is a PKA substrate and that c-Cbl does not form a complex with p-(S/T)-PKA substrates. Asterisk indicates heavy chains of the immunoprecipitating antibody. Experiment was repeated three times from separate cultures with similar results.

Figure 2.

A.



B.

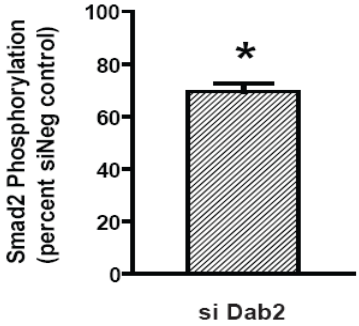


Figure 2. Western blot experiments demonstrating that silencing Dab2 expression inhibits TGFβ-mediated Smad2 phosphorylation. Parental CFBE41o- cells were transfected with siRNA against human Dab2 gene (siDab2) or the non-silencing siRNA control (siNeg) on plastic tissue culture plates and cells were treated with TGFβ (15ng/ml) for 1 hour. Phosphorylated Smad2 was detected in an anti-phospho-Smad2 specific antibody. (**A**) Representative Western blot and (**B**) Summary of data. Asterisks indicate $P<0.05$ *versus* siNeg control. 3 experiments/group.

Figure 3.

A. B.

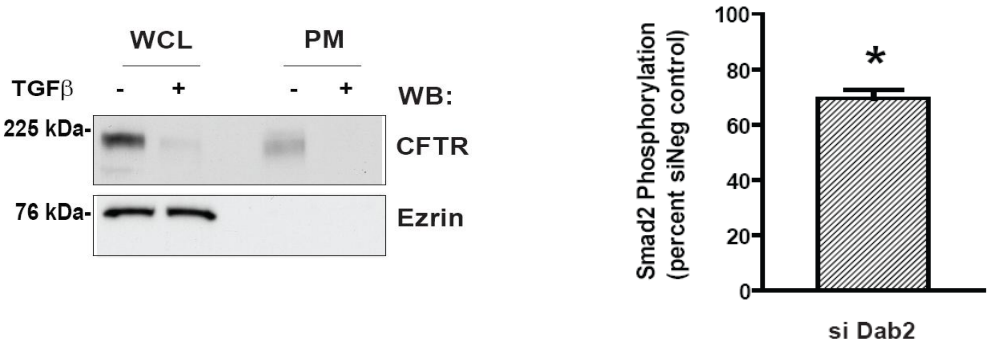


Figure 3. Western blot experiments demonstrating that TGFβ inhibits CFTR protein abundance in whole cell lysates (WCL) and in the plasma membrane (PM) of primary human bronchial epithelial (HBE) cells. HBE cells were treated with TGFβ 15ng/ml for 24 hours and plasma membrane proteins were isolated by selective apical membrane biotinylation. Representative Western blot (**A**) and summary of data (**B**). Ezrin expression in WCL was used as an internal loading control. The absence of ezril signal in the biotinylates samples (PM) confirms that the HBE cell monolayers were intact. Asterisks indicate P<0.05 *versus* siNeg control. 3 experiments/group.

Figure 4.

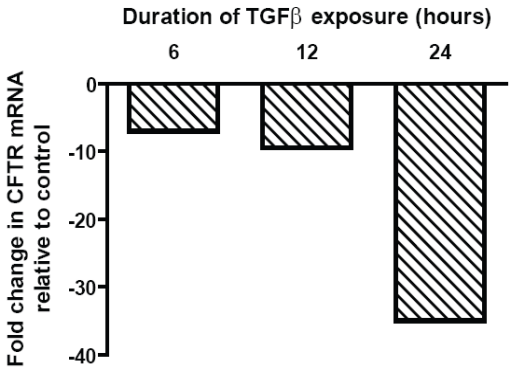


Figure 4. Experiment demonstrating that TGFβ inhibits CFTR gene expression in HBE cells. HBE cells were treated with 15ng/ml of TGFβ for the various periods of time, as indicated in the graph. Total RNA was isolated for quantitative real-time qRT-PCR. Change in CFTR gene transcription is shown as fold change relative to control after normalization to the internal housekeeping gene GAPDH.

Research Project 2: Project Title and Purpose

Inhibition of Chloride Secretion by Cigarette Smoke - The project uses laboratory techniques to study the effect of cigarette smoke on chloride secretion in cultured human bronchial epithelial (HBE) cells. Changes in chloride secretion by airway cells, changes in airway surface liquid height, and changes in mucociliary clearance will be measured with the hypothesis that decreases in chloride secretion will result in decreased periciliary fluid height and decreased mucociliary clearance.

Duration of Project

1/1/2007 - 6/30/2007

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at http://www.health.state.pa.us/cure.

Research Project 3: Project Title and Purpose

IL-23: Actions and Regulation in Pseudomonas aeruginosa Pulmonary Infection – The purpose of this project is to understand how the immune system responds to *P. aeruginosa*, a bacterium that causes severe pneumonias and lower respiratory tract infections. Understanding the immune response is key to the development of new therapies and treatments that are more effective, have fewer side effects and do not contribute to antibiotic resistance. The development of these new therapies is critical because pneumonia is among the top 10 causes of death according to the CDC National Vital Statistics Report.

Duration of Project

1/1/2007 - 12/31/2010

Project Overview

P. aeruginosa is a Gram-negative respiratory pathogen that causes significant morbidity and mortality through actions of the pathogen and the host inflammatory response. Because the pathogen is highly adaptable, anti-pseudomonal therapies quickly become ineffective. Thus, immunomodulatory therapies could provide an effective alternative, with fewer side effects and without the development of resistance. However, their development has been impeded by an inadequate mechanistic understanding of the host's response to *P. aeruginosa*. This project focuses on one mediator of the immune response, interleukin-23 (IL-23). IL-23 is best known for stimulating the production of IL-17 by memory T cells and recruiting neutrophils to the site of infection. In this role, IL-23 bridges the innate and adaptive immune responses. This project focuses on defining the role of IL-23 in mediating the innate response only. Specifically, it determines the role and actions of IL-23, independent of IL-17. It tests the prediction that IL-23

regulates production of IL-1α and IL-1β in the lung, independent of IL-17, and that the IL-23/IL-1 pathway elicits neutrophilic inflammation and matrix metalloproteinases (MMP) production in response to *P. aeruginosa* pulmonary infection. Through the use of recombinant cytokines, neutralizing antibodies and comparison of effects in wild type, IL-23 KO, IL-17KO and IL-23/IL-17 double KO mice the interrelationships between IL-23 and IL-1α and IL-1β will be demonstrated. Zymography will be used to quantify MMP activity.

Principal Investigator

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Other Participating Researchers

Jay K. Kolls, MD - employed by Children's Hospital of Pittsburgh
Anuradha Ray, PhD, Joanne Flynn, PhD - employed by University of Pittsburgh

Expected Research Outcomes and Benefits

This project will help immunology and microbiology researchers better understand how bacteria and the immune system interact to cause pneumonia and other lower respiratory infections and why these infections do not always resolve. By providing a mechanistic understanding of the immune response, this work will lay the foundations for the future development of immunomodulatory therapies to *P. aeruginosa* pulmonary infection. The development of immunotherapy is critical because, in many cases of *P. aeruginosa* infection, the *P. aeruginosa* cannot be adequately cleared from the respiratory tract and results in significant airway damage, scarring and loss of lung function. The findings of this project will have applicability to *P. aeruginosa* pulmonary infection in cystic fibrosis, HIV disease and ventilator associated pneumonia (VAP).

Summary of Research Completed

During this period, the PI has further clarified mechanisms of neutrophil recruitment during the innate inflammatory response to PA that are mediated by IL-23 and IL-1β. She is also further clarifying the importance of IL-17 during the innate immune response and the role of γδ T cells in producing this pro-inflammatory cytokine.

We have previously shown that the co-administration of IL-23 and IL-1β results in significant neutrophil recruitment as measured by percent neutrophils in the BAL fluid and is independent of IL-17. We have also shown that IL-23-deficient mice have a lower percent of neutrophils in BAL fluid when compared to infected C57BL/6 control (WT) mice. During this research period, we have conducted additional studies to evaluate the significance of IL-23 on inflammation. These studies demonstrate that there are significantly higher levels of neutrophils in WT lung tissue compared to IL-23p19-/- lung as assessed by neutrophils/high powered field ($p<0.05$). We

also demonstrate IL-23 dependence of key neutrophil chemoattractants and growth factors including G-CSF, GM-CSF, KC, IL-6 and IL-1β as measured in the BAL fluid ($p<0.001$). We also demonstrate that while IL-17A, IL-17F and IL-22 are not produced at early time points (T< 6 hours post-infection), we see significant induction of all three Th17 cytokines as early as 3 hours post-infection when measured by real time PCR and represented as fold change ($p<0.001$ when compared to control.)

We have previously demonstrated that IL-23 alone is sufficient to induce the production of Th17 cytokines by γδT cells *in vitro* and demonstrated that IL-23 and IL-1β synergistically induce these cytokines. During this research period, we have definitively demonstrated that γδ T cells are the source of these Th17 cytokines during the innate immune response to *P. aeruginosa*. Using FACS to sort lung cells into $CD3^+ \gamma\delta^+$ and $CD3^+ \gamma\delta^-$ populations, we were able to demonstrate that the $CD3^+ \gamma\delta^+$ population from infected mice was induced for these cytokines when compared to uninfected control cells by real time PCR. The $CD3^+ \gamma\delta^-$ population derived from infected mice did not amplify for IL-17A, IL-17F or IL-22 (run to 40 cycles) let alone demonstrate induction.

Importantly, our data show that the production of IL-17A and IL-17F at six hours post-infection does not cause the concurrent inflammation seen. When WT and IL-17 receptor knockout mice are infected with *P. aeruginosa*, there is no difference in the neutrophil recruitment, cytokine production or bacterial load or dissemination at 6 hours. These findings correspond with our prior work which demonstrated that IL-23/IL-1β-induced similar inflammatory phenotypes in WT and IL-17R-/- mice. Taken together, these data demonstrate that while Th17 cytokines are produced during the innate immune response, they are not responsible for the concomitant neutrophilic inflammation observed.

The lack of an immediate inflammatory effect of IL-17A and IL-17F production prompts the question: What is the importance of IL-17A and IL-17F production during the innate immune response if it does not directly result in inflammation? We believe that the production of IL-17A and IL-17F by γδ T cells is critical to promoting ongoing inflammation and promoting the transition to the Th17 phenotype we have previously observed during the later stages of chronic infection. We hypothesize that the IL-17 production results in significant production of IL-6, an inflammatory cytokine that polarizes Th0 cells toward a Th17 versus Th1 or Th2 phenotype. In addition, the production of IL-1β and IL-23 may also promote Th17 differentiation as well as further IL-17 production. We are currently conducting further studies using δTCR-/- (γδ knockout) mice to determine if Th17 differentiation is impaired in the absence of functional γδ T cells.

While we have tried to study the impact of IL-23 receptor (IL-23R) expression on these inflammatory processes, we have had limited success. Real time PCR studies conducted on whole lung do not demonstrate induction of the receptor with inflammation; however, individual cell populations may demonstrate induction. Further, increased expression of protein may be promoted through increased surface expression of previously produced protein. In order to study protein expression, we have initiated preliminary studies testing a panel of antibodies for use in flow analysis of specific cell populations. One of these antibodies demonstrates promise for use by flow cytometry, demonstrating what appears to be specific and reproducible staining;

however, none are verified for this use. We were able to obtain IL-23R knockout mice as a generous gift (Dr. Vijay Kuchroo, Harvard Medical School) and with the support of CURE funds, have recently rederived the line for importation to our vivarium. While we will use these mice in a variety of studies supporting this research, we will use the IL-23R knockout mice as a negative control to validate this IL-23R antibody for flow.

During this funding period, the PI has further clarified the mechanism for neutrophil recruitment during early/innate pulmonary infection with PA. She has also demonstrated that while IL-17 is produced by γδ T cells during PA pulmonary infection, the effects of this IL-17 production are not immediately responsible for neutrophil recruitment. Rather, this early IL-17 production is likely to be relevant to promoting further neutrophil recruitment and the development of a Th17 phenotype. She is also conducting ongoing studies of the role and regulation in IL-23R expression in mediating this IL-23-driven inflammation.

The following manuscript that has resulted from this supported work is currently in review.

> Dubin PJ, Martz A, Eisenstatt JR, Fox MD, Logar A, Kolls JK. IL-23 and IL-1β Synergistically Mediate Inflammation in *Pseudomonas aeruginosa* Pulmonary Infection. American Journal of Respiratory and Critical Care Medicine.

Research Project 4: Project Title and Purpose

Novel IL-17 Producing Memory Cells are Key to Vaccine-based Immunity in the Lung - The project uses laboratory techniques to study the role of a novel T-cell population, Th17 cells and how they control vaccine responses to tuberculosis (TB). This work is critical to develop a more effective vaccine against TB, which remains a global health problem.

Duration of Project

4/1/2008 – 12/31/2010

Project Overview

Tuberculosis is a leading cause of morbidity and mortality in the world as a result of a single infectious agent. Despite the use of Bacille Calmette-Guerin (BCG, an attenuated vaccine strain) for the past 80 years, TB remains a global epidemic with one-third of the world population being infected, an annual rate of 8 million new cases and 2-2.5 million deaths. The development of a new and more effective TB vaccine remains an urgent research priority. Although several candidate vaccines are now in different phases of clinical trials, the protection afforded by these vaccines in animal models is similar to that provided by BCG. Understanding what constitutes a protective memory response to challenge with M. tuberculosis (Mtb) is required in order to improve the design of effective vaccines against TB. Under this research project, we will determine the types of T-cells that are critical for an effective TB vaccine.

Principal Investigator

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

The completion of the proposed studies will advance our understanding of TB and the types of T-cell responses that are critical for vaccine efficacy.

Summary of Research Completed

Specific Aim One: To determine the factors that are involved in survival and maintenance of IL-17- producing cells in the lung.

Much of the work described under this Aim was reported in previous annual reports.

Specific Aim Two: To determine the factors involved in persistence of vaccine-induced IL-17-producing memory cells in the lung.

In Aim two, we had proposed to determine the specific location of IL-17-memory cell population in vivo and the factors that impact the establishment of IL-17-producing CD4 population in the lung. Consistent with this, we now show new data that adoptive transfer of Mtb-specific ESAT6 Th17 cells into B6 mice was able to confer protection in mice at levels similar to that seen in vaccinated mice **(**Figure 1**).** These data support our hypothesis that the persistence of Th17 cells are sufficient to confer protection with pulmonary challenge with Mtb.

The significance of the work reported here is that the data generated has been important in increasing our understanding of the basic requirements for induction of vaccine-induced immunity in the lung against TB. Furthermore, our work shows a critical role for IL-17 and Th17 cells in mediating vaccine-induced immunity and offers new options for manipulating future vaccine strategies against TB.

During this period The PI has received a NIH R01 grant entitled "Role of Interleukin-17 in protective vaccine-induced immune responses against tuberculosis."

The PI was invited to give the following presentations at National meetings or Seminar series:

- September 2010. European Respiratory Society Annual Congress. Hot topics: Interleukin-17 cytokines and Th17 cells in human lung disease, Intracellular pathogens in lungs. Barcelona, Spain. Invited Speaker
- October 2010. IL-17 in immunity to intracellular pathogens. Cytokine 2010. Chicago. Oral Presentation.
- November 2010. Novel insights into the role of Th1 and Th17 pathways in immunity to intracellular pulmonary pathogens. Department of Microbiology and Immunology Seminar Series. Vanderbilt University. Invited Speaker.

The following abstract from the PI's lab was presented at a National meeting:

- R. Gopal, Y. Lin, SA. Khader. 2010 IL-17 in immunity to intracellular pulmonary pathogens. Cytokine 2010. Chicago, Oral. Received International Cytokine Society, Young Investigator Award for this abstract.

The following manuscript related to this project has been published

- S.A.Khader* and R. Gopal. Interleukin-17 in immunity to intracellular pathogens. Virulence. 2010 Sep;1(5):12862. *Corresponding author

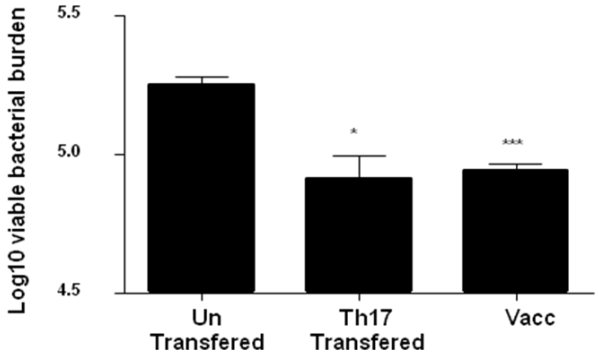

Figure 1: *Adoptive transfer of Th17 Mtb specific cells confers protection in wild type mice.* Th17 cells were generated by culturing naïve ESAT-6 TCR Tg cells *in vitro* in the presence of irradiated APCs, Ag and cytokine cocktails (IL-23, TGFβ, IL-6, anti-IL-4 and anti-IFNγ antibodies) and $2x10^6$ Th17 cells were transferred intra-tracheally into B6 congenic mice and mice were challenged with Mtb H37RV. B6 mice previously vaccinated with ESAT6 $_{1-20}$ peptide in adjuvant and unvaccinated B6 mice were also included as relevant controls. All groups of mice were challenged with Mtb H37Rv by the aerosol route and 30 days following infection lung bacterial levels were determined by plating on agar plates (n=4, Student's *t*-test *** <p.0.0005, * <p.0.05).