Children's Hospital of Pittsburgh

Annual Progress Report: 2007 Formula Grant

Reporting Period

July 1, 2009 – June 30, 2010

Formula Grant Overview

The Children's Hospital of Pittsburgh received $879,637 in formula funds for the grant award period January 1, 2008 through December 31, 2010. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Immunopathogenesis of Chlamydial Genital Tract Disease - Chlamydia trachomatis is the leading cause of bacterial sexually transmitted diseases worldwide. In women, infection may ascend from the cervix into the upper genital tract and lead to the sequelae of ectopic pregnancy, premature delivery, and infertility. The mechanisms by which chlamydiae induce disease are not clear. *Chlamydia* do not make potent toxins or tissue-damaging products; instead, it appears to induce an immune response that in the process of clearing away the bacteria results in collateral damage with inadvertent tissue destruction beyond what is needed to kill the pathogen. This project is designed to determine mechanisms responsible for oviduct pathology associated with *Chlamydial* infection. An understanding of the immunopathogenic mechanisms will promote rational design of a vaccine.

Anticipated Duration of Project

1/1/2008 - 12/31/2010

Project Overview

The objective is to establish a program that will investigate and determine the immune-mediated mechanisms that promote oviduct tissue damage during chlamydial infection of the female genital tract. Toll-like receptors (TLRs) are a family of surveillance proteins that recognize characteristic molecules that are expressed by pathogens. As such, they are important components of the innate immune response, which is initiated as soon as infection has been detected. When stimulated, these receptors drive signaling pathways that lead to activation of immune cells and production of pro-inflammatory proteins called cytokines. Chlamydiae make several cell wall and outer membrane components that are recognized by TLRs. Our research has revealed that Toll-like receptor 2 (TLR2) plays an essential role in controlling the development of pathology. TLR2-deficient mice failed to develop oviduct damage when infected with the mouse strain of *C. trachomatis*, known as *C. muridarum*, although all other aspects of their infection were similar to that seen in normal mice. Importantly, the mice were

still able to mount normal anti-chlamydial antibody and cell-mediated adaptive responses even though the signaling pathway was not operating. Thus, it seems clear that this innate immune signaling pathway drives the tissue-damaging processes that result in chlamydial disease but is not needed for development of a protective response.

In this project, flow cytometric, histological, immunologic, and molecular methods will be used to examine immunobiological responses of immunologically normal, wild-type mice and mice genetically deficient in the innate immune receptor TLR2 after genital infection to accomplish our overall goal. Our specific aims are to:

Specific Aim 1: Determine the responses produced by dendritic cells and epithelial cells secondary to chlamydial engagement of TLR2 that promote effector mechanisms that lead to oviduct pathology including the role of CD4 T cells, neutrophils and host cell death pathways.

Specific Aim 2: Define downstream effector mechanisms that lead to oviduct pathology including the role of CD4 T cells and host cell death pathways.

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Expected Research Outcomes and Benefits

One of the great obstacles to the development of a successful vaccine against Chlamydia is the fact that while the host response may effectively eradicate the organism, it also promotes tissue damage and disease. To this point in time, it has not been possible to elicit a protective response to *Chlamydia* without inducing some pathology, and breakthrough infections that occur with incomplete immunity may induce responses that result in even greater tissue damage. The availability of multiple pathogen recognition receptors and adaptor molecules allows the innate immune system to tailor responses to pathogens with exquisite refinement. The demonstration that mice genetically deficient in the TLR2 receptor, or TLR2 knock-out (TLR2 KO) mice resolve infection with minimal oviduct pathology suggests a chlamydial vaccine can indeed be designed that induces protection without risk of pathology. A comparison of responses in TLR2 KO mice with those of wild-type (WT) mice will directly identify immune correlates of pathogenesis. An ultimate goal will be to discriminate chlamydial ligands that stimulate the deleterious responses defined by these studies from those that induce desired responses necessary for protection. Thus, completion of this project will greatly advance the goal to develop a safe and effective vaccine against *Chlamydia*.

Summary of Research Completed

Specific Aim 1: Determine the responses produced by dendritic cells and epithelial cells secondary to chlamydial engagement of TLR2 that promote effector mechanisms that lead to oviduct pathology including the role of CD4 T cells, neutrophils and host cell death pathways.

Chlamydia-induced TLR2 stimulation promotes neutrophil activation leading to increased cytokine and MMP production and delayed apoptosis. These effects are potentiated by monocytes.

Due to the difficulty of obtaining large numbers of neutrophils from mice, in vitro experiments were conducted primarily with human neutrophils infected with human *C. trachomatis* D/UW-3/Cx and plasmid-deficient CTD153 that fails to activate TLR2. We determined that TLR2 activation of polymorphonuclear neutrophils (PMNs) delays their spontaneous apoptosis and promotes activation, but the presence of a small percentage of monocytes was crucial for this response. Infection of gradient purified PMNs containing < 1% CD14+ monocytes with *C.trachomatis* D/UW-3/Cx or CTD153, revealed that D/UW-3/Cx significantly enhanced upregulation of CD11b and CD66b at 3 and 6 hrs (Fig. 1), and increased TNF, IL-6, G-CSF, IL-8, and MMP9 at 20hrs (data not shown). Additionally, D/UW-3/Cx significantly delayed apoptosis at 4, 8, and 20 hrs compared to CTD153.

The role of monocytes in these preparations was determined using highly purified PMNs depleted of CD14+ cells. In the absence of monocytes, infection with D/UW-3/Cx resulted in enhanced activation marker expression and delayed apoptosis but the difference from CTD153 was not significant. However, the difference in cytokine production between D/UW-3/Cx and CTD153 remained. Accordingly, increasing the ratio of monocytes to PMNs in culture resulted in increased percentages of PMNs producing TNF, e.g. 6 ± 2.0% positive with 1% CD14+ cells versus 66 ± 6 % positive with 30% CD14+ cells. This trend was mirrored for IL-6 (Fig. 2). In these mixed populations, intracellular cytokine staining revealed that the PMNs produced significantly lower levels of cytokines than the monocytes in response to infection with D/UW-3/Cx (PMNs: MFI = 37.7± 0.7 for TNF and 47 ± 6.4 for IL-6; monocytes: MFI = 491 ± 121 for TNF and 238 ± 8 for IL-6 ($p=0.034$ and $p=0.001$ respectively). Comparing highly purified PMNs to peripheral blood mononuclear cells (PBMCs) revealed that TNF, IL-6, IL-8, and IL-10 were primarily produced by PBMCs, whereas MMP9 was mainly produced by PMNs. These effects are potentiated by PBMCs.

Although direct activation of neutrophils via TLR2 stimulation may be involved in the development of pathology, these data imply that monocytes may be critical (directly or indirectly) via their effects on neutrophils.

Neutrophils do not play a primary role in induction of oviduct pathology after chlamydial infection, but monocytes are essential for protection.

We have depleted neutrophils from mice infected with wild-type Nigg by administering anti-1A8 antibody that is specific for the neutrophil surface marker, Ly6/G. Flow cytometric analysis of

cellular infiltrates in the oviducts of mice after infection revealed a > 95% reduction in PMNs in the mice given anti-1A8, but high numbers of neutrophils in the oviducts of mice given control IgG. Interestingly, preliminary experiments have revealed bacterial burden and pathology are not altered by neutrophil depletion.

A subsequent experiment was conducted in which the monocyte population was depleted by diphtheria toxin treatment of CD11b/DTR mice. Flow cytometric analysis on days 3 and 5 post-infection revealed >90% depletion of CD11b+ cells from the peripheral blood. The DT-treated mice succumbed on day 7 post-infection. A control group of DT-treated mice that were uninfected remained viable. These data are significant because they indicate the monocyte/macrophage population is essential for host defense against *Chlamydia*. However, the neutrophil population is completely dispensable. These in vivo data correlate with in vitro data that revealed neutrophils contribute minimally to the overall cytokine response in the absence of monocytes.

Specific Aim 2: Define downstream effector mechanisms that lead to oviduct pathology including the role of CD4 T cells and host cell death pathways.

During pulmonary infection of mice with the mouse-specific strain of *C. trachomatis*, called *Chlamydia muridarum*, IL-17 is required for neutrophil recruitment and dendritic cell production of IL-12p70 to drive Th1 immunity. Our previous studies documented that mice deficient for IFN-γ developed extremely high Th17 responses that were associated with massive infiltration of neutrophils into the infected genital tract. We had also documented decreased numbers of Th17 cells in TLR2-/- mice associated with significantly decreased pathology.

We have examined Th17 and Th1 responses in immunologically normal mice vaginally infected with *C. muridarum*. Similar numbers of *Chlamydia*-specific Th17 and Th1 cells were detected in iliac nodes, the draining nodes for the genital tract, early in infection, but Th1 cells predominated later (Fig. 3). We infected mice genetically deficient for the IL-17 receptor and examined the course of infection and pathological outcome. Interleukin-17 receptor deficiency did not result in an alteration in the course of infection (Fig. 4) or pathology (data not shown). However, neutrophil recruitment was reduced and IFNγ production by NK cells (data not shown) and ILN $CD4^+$ T cells (Fig. 5) was also significantly reduced in the IL-17 receptor deficient mice. Although IL-12p70 production was compromised in *il17ra-/-* mice (Fig. 6), it was produced in sufficient levels to drive a Th1 response that was sufficient to lead to a normal resolution of infection, and no increase in pathology. These data contrast with previous studies of *C. muridarum* pulmonary infection in IL-17-depleted mice that showed overwhelming infection and absence of Th1 immunity.

Interleukin 23 is a cytokine released from dendritic cells that drives IL-17 production from CD4 T cells. We have examined the course and outcome of chlamydial infection in mice deficient for IL-23. We found that the course of infection is significantly prolonged in these mice, but pathological outcome was not altered. Since *il17ra*$^{-/-}$ mice resolve infection normally, but *il23*$^{-/-}$ mice have a significantly prolonged infection, this indicates that an additional mechanism downstream of IL23 is important in host defense against chlamydiae. Since IL-23 also induces IL-22, which has been shown to play a role in mucosal host defense in pulmonary infection

models, we investigated the role of IL-22 in our model. Vaginal inoculation of mice genetically deficient for IL-22 resulted in an infection course similar to that observed in wild-type mice. Thus, alternative mechanisms must be involved in the IL-23-mediated chlamydial inhibition. We have examined multiple proinflammatory cytokine responses in the genital tract secretions of *il23*$^{-/-}$ mice and have not determined any decrease in their secretions. IL-1β, a molecule that has been shown to be important in host defense against chlamydiae, and also to be enhanced by the presence of IL23, was produced in equal levels in *il23-/-* and wild-type mice after infection. This cytokine was also secreted normally in *il23-/-* mice infected with chlamydiae. Ongoing flow cytometric analyses are being used to analyze Th17 and Th1 cells in these mice in parallel with infected *IL17r*$^{-/-}$ mice to determine if absence of IL23 leads to greater inhibition of the Th1 response, rendering the mice more susceptible to infection.

Significance of Data

Our data indicate that Chlamydia-induced TLR2 signaling leads to a hyper-activation and increased longevity of neutrophils in vitro. However, in vitro studies revealed neutrophils contribute little to the cytokine response during in vitro infection. A small percentage of monocytes produce the bulk of inflammatory cytokines, and these cells enhance cytokine production by neutrophils. These data corroborate subsequently conducted in vivo experiments that have revealed neutrophils are not major mediators of tissue damage or induction of oviduct pathology after chlamydial infection, since depletion of this inflammatory cell type did not have any effect on pathology. Additionally, we have determined a key role for monocytes in host defense against chlamydiae in the genital tract. Mice depleted of monocytes succumbed one week post-infection. These data are made more interesting by the fact that *C. muridarum* can grow and replicate in monocytes in vitro. Activation of these cells in vivo must allow them to be effective mediators of chlamydial killing. We are currently performing flow sort experiments to document activated monocytes in vivo are chlamydiacidal.

Our data in the genital tract model using il17-/- mice has revealed that the importance of this cytokine in Th1 development is dependent on the mucosal site of infection. Mice infected in the lung, in the absence of IL17 signaling, develop overwhelming infection that has been demonstrated to be due to a lack of induction of IL-12p70 and subsequent decreased Th1 immunity. However, our data reveal that in the genital tract redundant mechanisms exist for induction of IL12p70 and Th1 responses. Although lack of IL17 signaling resulted in a decreased level of Th1 responses, the responses were nevertheless effective in eradicating infection. Recent data have revealed an importance for IL23 in host defense against chlamydiae. The mechanism for IL23-mediated protection is not due to IL22 or IL1β.

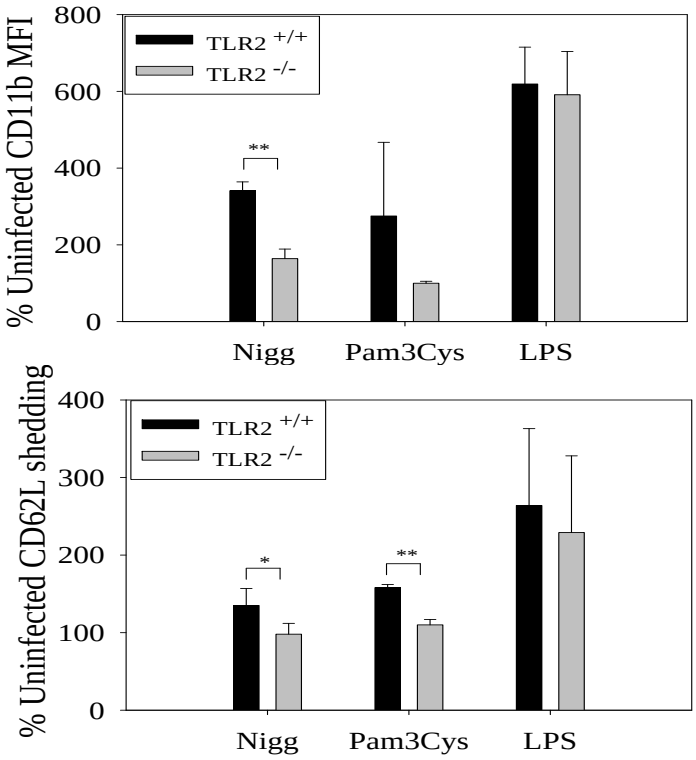

Figure 1. Chlamydial TLR2 signaling significantly enhances murine neutrophil expression of CD11b (A) and shedding of CD62L (B) after 4 hrs.

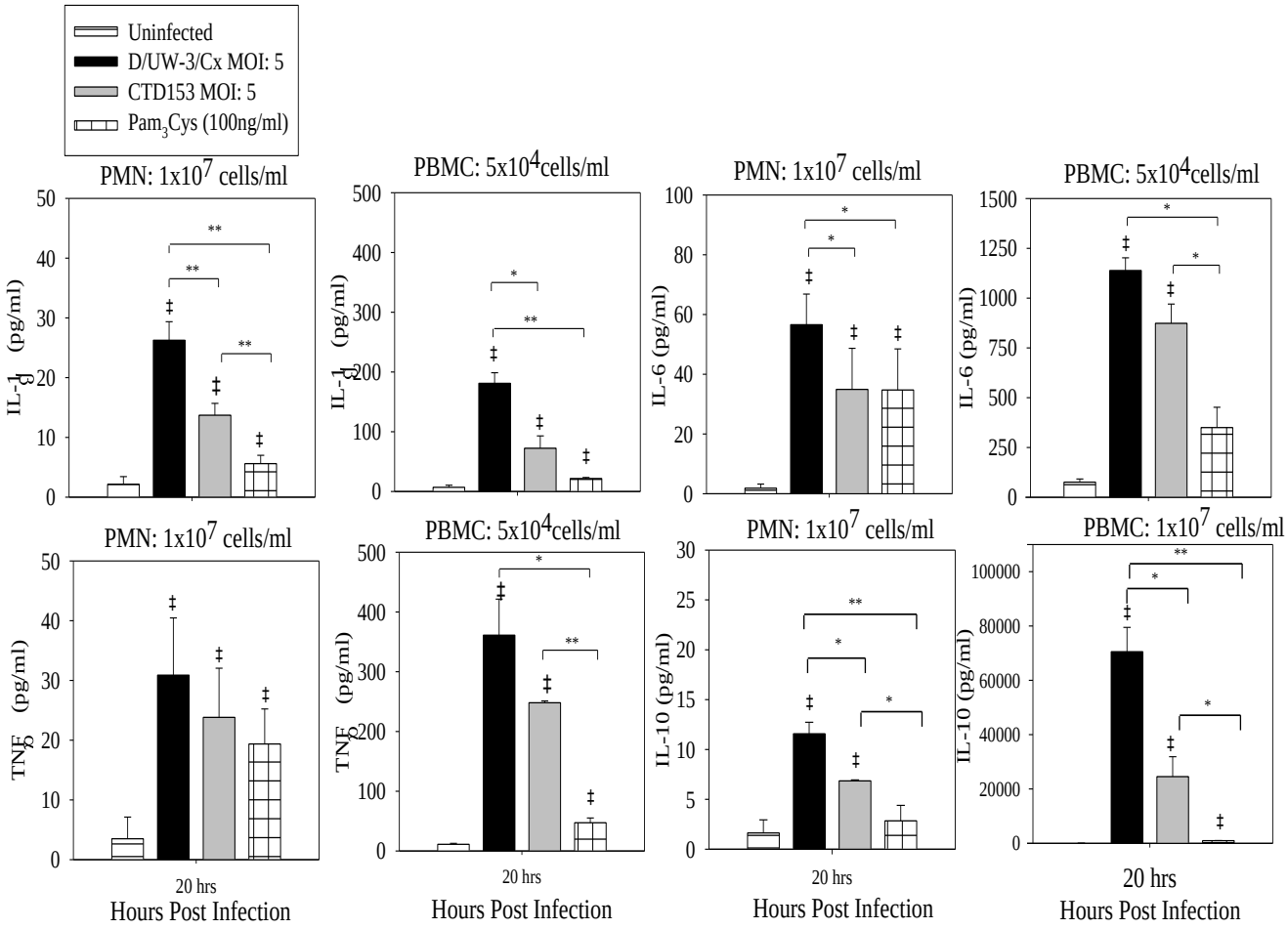

Figure 2. Highly purified PMNS and PBMCs produce significantly more cytokines in response to D/UW-3/Cx than CTD153 at 20 hrs. Cytokine production by PBMCs is significantly higher than that of PMNs for all cytokines tested.

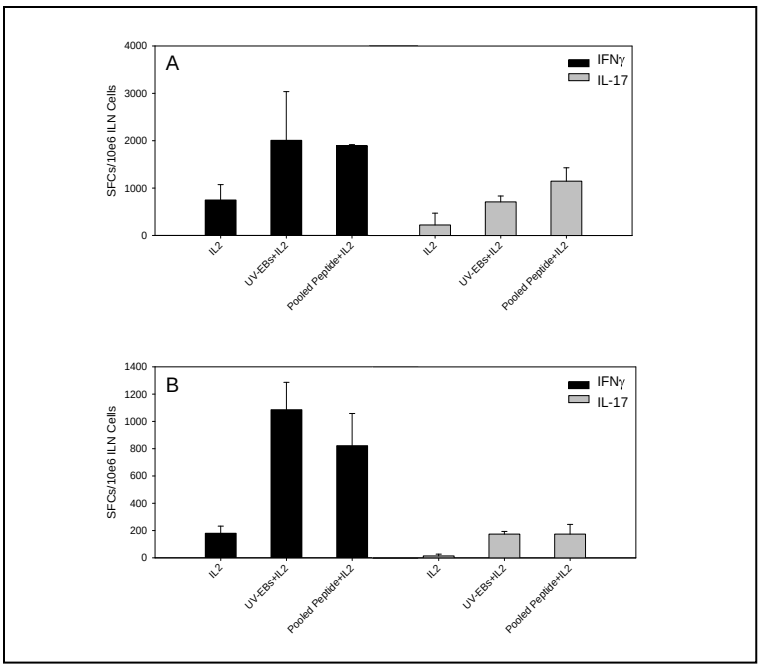

Figure 3. *C. muridarum* antigen-specific Th1 and Th17 cells are detected in similar numbers in the iliac nodes of C57BL/6 mice on day 7 post-infection, but Th1 cells predominate on day 20. Numbers of antigen-specific IFNγ (black bars) and IL-17 (gray bars) producing $CD4^+$ T cells in ILN of C57BL/6 mice were quantified by ELISPOT on days 7 *(A)* and 20 *(B)* post-infection.

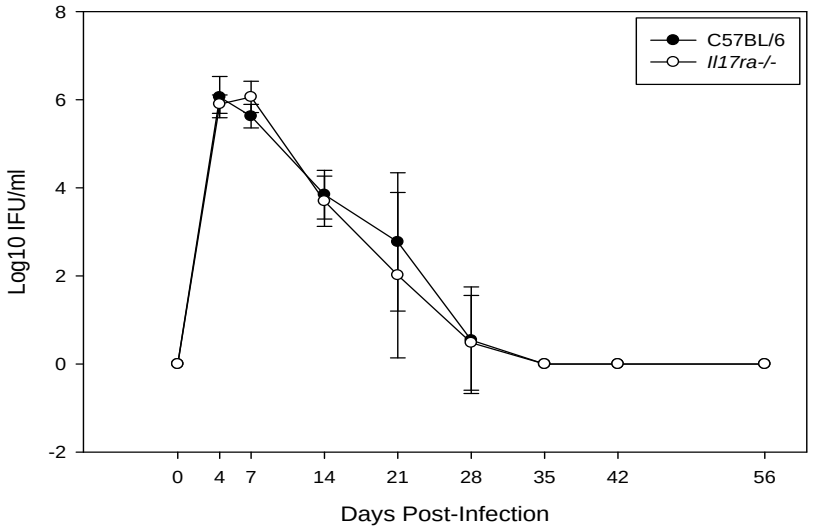

Figure 4. Course of lower genital tract infection in wild-type and IL-17RA knock-out mice.

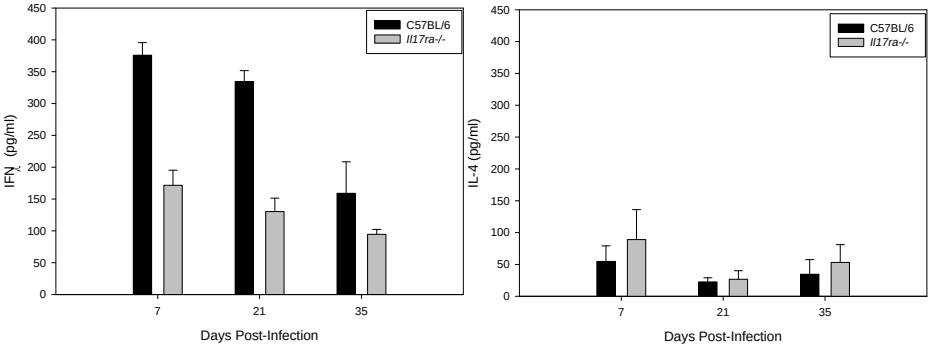

Figure 5. The Th1 response is decreased, but remains intact in IL-17-receptor-deficient mice. The Th2 response is not enhanced in the absence of IL-17 signaling.

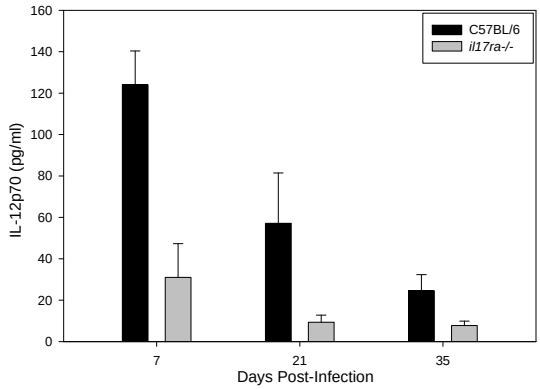

Figure 6. IL-12 p70 is decreased in il17ra-/- mice but is sufficient to induce a Th1 response.