National Institute of Allergy and Infectious Diseases

# NIAID Biodefense Workshop Summary

## DEVELOPMENT OF REAGENTS FOR TLR AND OTHER INNATE IMMUNE RECEPTORS: PRESENT CHALLENGES - FUTURE DIRECTIONS

**JUNE 28-29, 2007**

**Marriott Suites**
**Bethesda, Maryland, 20817**


## Abstract

On June 28-29, 2007, the National Institute of Allergy and Infectious Diseases (NIAID) convened a workshop on the "Development of Reagents for Toll-Like Receptors (TLRs) and other Innate Immune Receptors". A panel of researchers* was assembled to present research findings and to discuss existing and developing experimental methodologies, reagents, resources, and other research tools which facilitate studies of innate immune receptors and a better understanding of the innate immune system.

Topics included:

- Pathogen Recognition by Innate Immune Receptors
- Engineering Small Molecules and Antibodies as TLRs Agonists/Antagonists
- Genetic Approaches to Study Innate Immune Receptors and Their Functions

The participants recognized a clear need for more classical biochemistry studies for structural/functional characterization of the innate immune receptors including support for the propagation and distribution of validated and standardized monoclonal antibodies specific for TLRs and other innate immune receptors, potentially through academia/industry partnerships. The panel also expressed the need for genetic standardization of existing mouse resources and the development of new conditional single or multiple knock out mice which would facilitate the research. The participants suggested that development of a website dedicated to innate immunology would benefit the community at large to share information and available resources.


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## Introduction

The innate immune response is the first line of defense against microbial infections. The targets of innate immune recognition are conserved pathogen-associated molecular patterns (PAMPS), and the innate immune receptors are known as Pattern Recognition Receptors (PRRs). Among PRRs, toll-like receptors (TLRs) are a key family of cell surface and intracellular recognition structures, capable of sensing organisms, such as bacteria, fungi, protozoa, and viruses. Other recently characterized PPR recognize microbial components in the cytosol, for example: members of the NACHT family (characterized by a domain present in NAIP, CIITA, HET-E, and TP1-LRR, Leucine-rich repeat); the NLR family, which includes two nucleotide-binding oligomerization domain (NOD) proteins; NACHT-LRR; and pyrin-domain-containing proteins (NALPs). Furthermore, the RNA helicase, retinoic acid inducible gene-I (RIG-I), was identified as a cytosolic receptor for intracellular double stranded RNA (dsRNA) and induces type I interferons in response to dsRNA viruses in a TLR-independent manner. Understanding the roles of all PRRs in innate immunity and their influence on adaptive and memory immune responses is crucial to advance development of novel adjuvants, vaccines, and therapeutics for biodefense. However, the biological functions of these receptors are not well understood due, in part to the absence of clear biochemical and structural characterizations. Biochemical studies are lagging behind because production of the recombinant receptors in sufficient quantity and purity has proven to be difficult. In addition, many existing antibody reagents are either unreliable or not useful for biochemical analysis. To facilitate further biochemical and structural studies of the innate immune receptors and to generate the required reagents, the panel agreed that collaborations between experts in protein chemistry, crystallography, and immunology from academia and industry are needed.

## Pathogen Recognition by Innate Immune Receptors

The Toll-like receptors (TLR) are conserved structures in both invertebrate and vertebrate organisms capable of recognizing a variety of endogenous and exogenous ligands. Despite the evolutionarily conserved features of TLRs, they have some species specificity in ligand recognitions. Dr. Smith from Washington University presented his findings about TLR5 recognition of bacterial flagellin in human and mouse, and demonstrated that the flagellin recognition site maps to 228 amino acids of the extracellular domain and the concavity region of the modeled TLR5 structure. Mouse TLR5 detects flagellin better than human TLR5, and a naturally occurring amino acid variation in TLR5 residue 268 was shown to be responsible for the species specificity. It is predicted that flagellin binding to TLR5 promotes TLR5 dimerization and signal transduction in a species and cell type specific manner which determines species susceptibility or resistance to a particular infection. The biological significance of differential flagellin recognition in human and other species is unclear and further biochemical analysis of TLR 5 ligand recognition is needed to solve this puzzle.

Lipopolysaccharide (LPS) is usually a potent stimulator of TLR4 and inducer of proinflammatory cytokines. However, to escape host defense mechanisms, *Yersinia pestis* synthesizes a LPS with poor TLR4 stimulating activity at 37$^{o}$C. Dr. Lien (University of Massachusetts) modified *Y. pestis* to produce a potent TLR4 stimulating LPS with a different lipid-A structure. The modified pathogen was shown to be avirulent when injected subcutaneously even at high challenge doses, suggesting that it might be an effective vaccine. This study showed that resistance to infection required TLR4, the adaptor protein, MYD88, and the coreceptor, MD-2, and was enhanced by CD14 and the adaptor Mal.

TLR1, 2, and CD14 all participate in the control of *Borrelia hermsii* infection, and adaptive immunity also contributes to the complete elimination of the bacteria. Dr. Lien's laboratory developed an antibody specific for human TLR2 and a fluorescent tagged TLR3 to detect surface expression and intracellular movement of these receptors.

Dr. Pulendran (Emory University) focused on harnessing innate immunity to augment immunogenicity of HIV vaccines. Some dendritic cells (DCs) in the intestine and other mucosal tissues produce IL-10 and TGF$\beta$ which promote the differentiation of naïve CD4+ T cells to regulatory T cells. Other DCs in these tissues produce IL-6, IL-23, and TGF$\beta$ which induce the development of Th17 cells. For successful vaccine response these pathways need to be regulated or blocked. TLRs play a regulatory role in the function of DCs at mucosal surfaces. Preliminary findings suggest that immune responses to a SIV DNA vaccine candidate are enhanced in nonhuman primates when administered in conjunction with TLR 7, 8, and 9 ligands. In contrast, TLR2 deficient mice were shown to exhibit an enhanced immune response to vaccination. To develop vaccines that induce robust and protective immune responses we need to learn how to harness TLR and non-TLRs receptors to enhance the immune response; identify innate immune signatures that predict the immunogenicity and protective capacity of vaccines; develop novel adjuvants that stimulate non-TLRs; and develop systems to permit oral and intranasal delivery of these adjuvants.

Dr. Montgomery from Yale University spoke about modified TLR responsiveness in immune suppressed or aged individuals that contributes to impaired vaccine responsiveness and/or disease susceptibility. The data suggested that expression of TLR1 and TLR4, but not TLR2, is decreased on peripheral blood mononuclear cells (PBMC) of individuals over 65 years of age, and this decrease correlates with a decrease in cytokine production. The elderly also fail to down-regulate TLR3 in response to West Nile virus infection, possibly enhancing susceptibility. Currently, antibody reagents are available for detection of human TLR1, 2, and 3, but no reliable antibodies are available for surface detection or biochemical analysis of human TLR 5, 6, 7, and 8, and that is a serious deficiency.

The mechanisms by which the recognition of TLR ligands leads to host immunity remained poorly defined. For an effective immune response, microorganisms might stimulate a complex set of PRRs both within and outside of the TLR family. The combined activation of these different receptors can result in complementary, synergistic or antagonistic effects that modulate innate and adaptive immunity. Dr. Trinchieri from the National Cancer Institute talked about production of pro-inflammatory cytokines through the cooperation of multiple innate immune receptors activation. For example, optimum production of IL-12 P70 by human and mouse DCs requires cooperative stimulation of TLR 3, 4, and TLR 7, 8, 9, and 11, whereas, TLR2 has no effect on its own

or in combination with other TLRs. Interestingly, IL-12 production is induced by co-activation of TLR 7, 8, and Dectin-1 receptors which mimic zymosan stimulation, and is inhibited by TLR 2 signals. On the other hand, Dectin-1 and TLR2 costimulation enhances the production of IL-23 in these cells. Expression of TLRs 2, 3, and 9 is required for the control of MCMV infection, and deficiency of any of these three receptors differentially affects the patterns of cytokine secretion and decreases resistance to infection. Many pathogens express a variety of stimulants that are recognized by different PPRs. For example, *Mycobacterium tuberculosis* induces IL-12 and IL-23 production in DCs by stimulating TLR 2, 4, 9, NOD2, and Dectin-1. In the presence of IFN$\gamma$, production of IL-12 by *M. tuberculosis* is amplified by costimulation of TLRs 7, 8, and NOD2 in human and by TLR 9 and NOD2 in mouse DCs. The evolutionary advantage of the cooperative interactions of PRRs is obvious in protection of the host against potential mutations in the pathogen or in the host's receptors.

Dr. Sher from the National Institute of Allergy and Infectious Diseases focused on the role of TLRs in host resistance to *Trypanosoma cruzi.* TLR2 and 9 were shown to cooperatively play a role in host resistance to this pathogen. Cooperation by other PRRs is also implicated. To resolve this degree of complexity *in vivo,* conditional MyD88 and other TLR family knock out mice would be a valuable system to bypass embryonic lethal mutations and to study the role of TLRs in a physiological setting. Although a repository exists to obtain breeding stock of certain PRR mutant strains, it is not known or used by most investigators, or many strains are not readily accessible because of intellectual property issues. Animal resources to produce genetically standardized and fully backcrossed mice are needed to avoid an enormous waste of funds in duplicating colonies at multiple institutions. This would be best achieved by a major commercial breeding facility such as the Jackson Laboratory or Taconic Farms. The facility should handle import, backcrossing, health monitoring, genetic standardization, and cryopreservation of strains and should be able to expand production of the heavily used strains in response to investigator demands.

Although TLRs are considered as the main players in pathogen recognition and initiation of innate immunity, the non-TLRs (NTLRs) also play an important role in these processes.

The innate immune receptor, Dectin-1, also known as the beta-glucan receptor in human, is important in the recognition of pathogenic fungi by macrophages and dendritic cells. Based on sequence analysis and recently resolved crystal structure, the extracellular region of Dectin-1contains a C-type lectin-like domain that binds fungal beta-glucans. Dectin-1 signals are sufficient to trigger phagocytosis and Src-Syk-mediated induction of antimicrobial reactive oxygen species. Dectin-1 collaboration with TLR2 signaling enhances NF-kappaB activation and regulates cytokine production. Dr. Underhill, from Cedars-Sinai Medical Center, demonstrated that Dectin-1 signaling can also directly modulate gene expression through activation of NFAT. Dectin-1 ligation by zymosan particles or live *Candida albicans* yeast triggers NFAT activation which in turn induces early growth response transcription factors (Egr-1, Egr-2, and Egr-3) and regulates IL-2, IL-10, and IL-12 P70 production by zymosan-stimulated DCs. The NFAT activation in myeloid cells is a novel mechanism of regulation of the innate antimicrobial response. Dectin-1 may play a role in inflammatory responses to pulmonary fungal pathogens, but the *in vivo* role of this receptor has not yet fully elucidated.

Dr. Sellati from Albany Medical College presented his laboratory's effort in elucidating the innate immune responses against *Francisella tularensis* in a murine model of infection. His data demonstrated that CD14 and TLR1, 2 and 6 receptors as well as the intracellular receptors NOD2 and NALP3 are important in pathogen recognition and initiation of the immune response, and mice deficient in any of these receptors are susceptible to infection. Furthermore, *F. tularensis* induces the transcription of NOD2 and NALP3 in a TLR-2 dependent fashion. Dr. Sellati listed some of the reagents currently available to study innate immune receptors. For TLRs, some synthetic lipoproteins, Gram- and Gram+ bacteria peptidoglycans, and small molecule ligands, as well as a number of human and mouse TLR genes cloned in various vectors are available. However, very few reagents are available to study NTLRs. There is a need for labeled TLR and NTLR ligands for tracking the extra- intra- and inter-cellular distribution of these molecules. In addition, single, double, and multiple TLR and/or NTLR deficient mice on a variety of backgrounds will facilitate research. Furthermore, access to databases comprised of transcriptome, proteome, metabolome information related to variety of microbial infections is most helpful to the investigators. Repositories that cover everything from molecules to mice will accelerate the research in this field.

T cells expressing a V$\gamma$2V$\delta$2 receptor exist in humans and nonhuman primates and constitute 60-95% of total circulating gamma/delta T cells. These cells recognize non-peptide phosphoantigens found on a wide variety of pathogenic organisms. One example is HMBPP, [(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate], from BCG and *M. tuberculosis.* After ligand exposure, V$\gamma$2V$\delta$2 T cells proliferate rapidly and secrete large amounts of proinflammatory cytokines and chemokines that contribute to the innate immune response to the pathogens. The ubiquitous nature of the antigens converts essentially all activated V$\gamma$2V$\delta$2 T cells to memory cells. Dr. Chen, from the University of Illinois, demonstrated that *B. anthracis* and *Y. pestis* and a number of other biodefense pathogens carry genes capable of synthesizing non-peptide phosphoantigens recognized by V$\gamma$2V$\delta$2 T cells. The treatment of macaques with HMBPP and IL-2 induces prolonged expansion of V$\gamma$2V$\delta$2 T cells and coincides with increases in the number of $\alpha\beta$ CD4 and CD8 T cells. The expanded V$\gamma$2V$\delta$2 T cells preferentially express CCR5 and migrate to the lungs. When re-stimulated with the phosphoantigen these cells produce IFN$\gamma$ and exhibit cytotoxic function. At present, antigen presentation to and recognition mechanisms used by human V$\gamma$2V$\delta$2 T cells are poorly understood.

**Engineering Small Molecules and Antibodies as TLRs Agonists/Antagonists**

Drs. Weiss and Gioannini from the University of Iowa studied how TLR4 recognizes LPS and the roles of CD14 and the adaptor proteins LBP and MD-2 in this process. Their data showed that LPS forms a remarkably stable and potently bioactive monomeric complex with MD-2 that is the ligand for TLR4. Moreover, they demonstrated that discrete structural modifications of either LPS or MD-2 can lead to formation of LPS/MD-2 complexes that still bind with pM affinity to TLR4 but do not efficiently activate TLR4. Such complexes might be very potent TLR4 antagonists. It was demonstrated that nasal instillation of wild type LPS/MD-2 is nearly 10-fold more potent in inducing airway inflammation than the equivalent amount of LPS applied as purified aggregates. The goal is to understand how engagement of TLR4 by LPS/MD-2 leads to receptor activation; to study the structural correlates of TLR4 agonist, antagonist, and the properties of wild-type and variant LPS/MD-2 complexes. This knowledge will be used

to develop novel TLR4-directed immunomodulators acting as agonists to prime host defenses when needed or as antagonists to blunt pathogenic LPS-driven inflammation.

Dr. Carson's laboratory at the University of California, San Diego developed a TLR7 agonist to be used as an immune therapeutic and a vaccine adjuvant. TLR 7 recognizes single stranded RNA and certain small molecules. Imiquimod (R837) is a commercially available TLR7 ligand but is not a good adjuvant. Therefore, small molecules were synthesized by chemical modification of the R837 structure and were screened for TLR7 binding and activation. One molecule (1V150) was found to bind and activate TLR7, and when conjugated to mouse serum albumin it retained its TLR agonist activity and exhibits greater potency and reduced toxicity. It also induced local activation of innate immunity, as measured by production of IFN$\gamma$, IL-12, and IL-6. Therefore, this molecule would be a good candidate for a novel vaccine adjuvant. A liposome-conjugated 1V150 was shown to enter cells and when administered intranasally induces local rather than systemic cytokine release which is harmful to the host. Liposome-conjugated 1V150 is currently being evaluated for adjuvant activity with influenza and anthrax vaccines.

Dr. Lipford from the Coley Pharmaceutical Group presented their research program to identify small molecule ligands for TLR7, 8, and 9 to be used as adjuvants with vaccines, or as antagonists for the treatment of asthma/allergy and infectious or autoimmune diseases. TLR7, 8, and 9 are a subfamily of interacellular TLRs. Using limited mutational analysis they identified potential ligand binding sites on TLR7, 8, and 9 and modeled interactions with their ligands. The data suggest that TLR7 is stimulated by GU-rich and TLR8 by AU-rich nucleic acid fragments. However, the efforts to study crystal structure and to conduct reliable protein binding assays are hampered by the difficulties in production of the TLR proteins. Other problems include loss of ligand recognition by recombinant TLR7 and 8. Furthermore, different formulations yield different activation profiles *in vitro* and *in vivo*. In general, low expression levels, trafficking, and endosomal/lysosomal localization of these TLRs present a challenge in expressing and purifying the proteins. The lack of stability of the RNA ligands is another problem. Additionally, there are variations among species in cellular distribution and responsiveness to ligands.

Engineered antibody fragments as designer modulators of TLRs were presented by Dr. Kenan from Duke University. The goal was to design modulator antibodies either as antagonists to compete for ligand binding or as agonists to induce receptor dimerization, multimerization, or clustering leading to intracellular signaling. The original approach was to purify recombinant ectodomains of human TLR2 and 4, immunize the mice, and construct scFv phage display libraries. However, the scFv antibodies that were selected on purified ectodomain fragments did not recognize the intact TLR proteins on the cell surface. A revised approach used TLR transfected cells for immunization and for the screening and selection of the specific scFv antibodies. This approach resulted in several human TLR2 specific antibody fragments that are currently being expressed in larger quantities and purified for biochemical analysis. These antibodies were shown to cross react with mouse TLR2 but the clones analyzed so far had no significant functional activities.

Dr. Teyton, from the Scripps Research Institute talked about the missing pieces of the puzzle in understanding of innate immune receptors. Dr. Teyton's laboratory started a project in 2003 to produce TLR recombinant proteins, develop specific antibodies for these receptors, characterize the antibodies, and finally distribute them. To produce

recombinant TLR proteins a variety of expression systems were evaluated including bacteria, fly, baculovirus, CHO/L293 and wheat germ. The main obstacle was the inherent inability of TLR proteins to express as recombinant forms. Other problems were the access to full length cDNAs, associated costs, and the required personnel. The antibodies were initially screened by FACS or ELISA, and then characterized by Western blot, immunoprecipitation (IP) and other biochemical methods. Developmental costs were estimated at $6,000 per monoclonal antibody. But characterization is time consuming and requires production in large quantities and a biological readout. Through this effort, mAbs specific for human TLR3, TLR7, and MD-2 were produced, and the anti-TLR3 and 7 mAbs are being distributed through ATCC. The production of mAbs for human TLR4-MD2, and murine TLR2, 4, and 6 is in progress. To characterize a mAb collaboration among several laboratories is required to evaluate its specificity and utility using immunocytochemistry (fixed tissues), biochemistry (IP), functional studies (blocking assays), and animal studies (isotypes). A single laboratory can not do everything, and must have a network of collaborators to achieve this goal. To gain insight into the structure and function of the innate immune receptors, the highest priority is biochemistry and currently it is the missing piece of the puzzle. To fill this gap a coordinated effort is needed from the entire community.

**Genetic Approaches to Study Innate Immune Receptors and Their Functions**

How the body senses an infection, what receptors are triggered, and what events are set in motion to cause inflammation are fascinating questions. Inflammation has evolved to manage infection, but are there endogenous drivers of innate immune receptor activation that are responsible for sterile inflammation, i.e., inflammation in the absence of infection? If so, how can we find them? To address these questions, Dr. Beutler's laboratory at the Scripps Research Institute initiated a "forward genetic" program using N-ethyl-N-nitrosourea (ENU) mutagenesis in mice. To date this project has yielded a total of 98,035 mutants and more than 120 phenotypes. Approximately 70% of all genes have been mutated to give rise to phenovariance. Virtually all phenotypes emanate from coding changes. Among the 90 phenotypes that have been mapped, 72 molecular defects were identified, and among them there were 50 immunological phenotypes. To screen for TLR signaling defects, mice were injected with thioglycolate and exposed to ENU. Three days late, the peritoneal macrophages were harvested and stimulated *in vitro* with various TLRs ligands. TNF$\alpha$ production served as the assay read-out. It is known that individual cytokines produced in response to TLR activation such as TNF$\alpha$, have a non-redundant role in chronic sterile inflammatory diseases. The source of these cytokines and what triggers their synthesis are not known. It is also not clear if endogenous drivers of TLR activation exist and if so, why such drivers operate only in certain people. There is some evidence that TLR activation can mediate chronic inflammatory diseases. The hypothesis is that TLR and the proteome are both constrained by the imperative to avoid self-reactivity. Some changes in the proteome are lethal because they may cause activation of the TLRs. However, some mutations might be viable, but cause sterile inflammatory disease. Alternatively, since TLRs are permissive in their binding, many molecules may act as TLR ligands.

Dr. Makarov from Attagene Inc. spoke about a novel technology (FACTORIAL$^{TM}$) developed by Attagene to assess the activities of the transcription factors (TFs) *in vitro* as well as *in vivo.* An estimated 2,000 mammalian transcription factors exist and this gene regulatory network plays an important role in the control of inflammation, cell proliferation and growth, oxidative stress responses, and cell differentiation. Because

there are many fewer regulatory pathways than genes, TFomics data are easier to interpret than gene expression data. Since TF activity is controlled by modification of existing proteins (by phosphorylation, methylation, etc.) the activity cannot be easily evaluated by measuring protein levels; therefore, functional assays are needed. Traditionally, *in vitro* DNA binding and *in vivo* reporter gene expression assays are used to evaluate TF activities, but there are no reliable conventional assays to evaluate multiplex transcription-based reporter activities. FACTORIAL$^{TM}$ technology is a homogeneous multiplex reporter library and analysis software package. The library can be transferred into the evaluating cells/tissues and the TF activities can be assessed in various tissues for example, in quiescent *versus* activated cells, or under normal *versus* diseased conditions. This system showed that the TF activity profile is not affected by variation in assay conditions such as DNA input, RNA integrity, or amplification. The technology can be used to determine a molecular signature for a disease, cellular responses to a drug or infection, and annotation of gene function.

**Panel Recommendations**

The participants outlined the following priorities for future research.

- Standardized and validated antibody resources, and an open forum such as an innate immunity website at which users can share positive/negative information about the antibodies and other reagents.
- Classical biochemistry
- Expansion of existing animal model resources such as MMRRC www.mmrrc.org ,the Jackson Laboratory www.informatics.jax.edu and Scripps mutagenetix.scripps.edu for studying innate immunity
- Genetic standardization of mouse models
- Conditional, single or multiple knockout mice which are not currently availabl
- New reagents for NTLRs, e.g. carbohydrate ligands
- Definition of the top 200 target reagents to be developed in the next five years through combined efforts of academia and industry.