National Institute of Allergy and Infectious Diseases

BIODEFENSE WORKSHOP SUMMARY
INNATE IMMUNITY TO PATHOGEN-ASSOCIATED MOLECULAR PATTERNS OF NIAID CATEGORY B PROTOZOA

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Abstract

The National Institute of Allergy and Infectious Diseases (NIAID) convened a workshop on March 24, 2005, on innate immunity to Pathogen-Associated Molecular Patterns (PAMPs) of NIAID category B protozoa. A panel was assembled*, including researchers from the fields of innate immunity, mucosal immunity, and parasite molecular biology/biochemistry. The topics presented and discussed included:

- Intestinal mucosal environment and how pathogenic microbes are recognized
- Regulation of the innate and adaptive immune response to protozoa
- Innate immune receptors involved in recognition of protozoan PAMPs
- Effector cells and molecules utilized in response to protozoa

Introduction

In the last decade, there was significant progress in defining the innate immune response to prokaryotic pathogens. Innate immune receptors, such as Toll-like receptors and CD-1, are able to distinguish different classes of pathogens, and evoke a response that provides innate immunological protection and immunological memory. Innate immune activity forms the basis for the development of new vaccine adjuvants tailored to specific pathogens or classes of pathogens.

However, there are still gaps in our knowledge of the innate immune response to eukaryotes, especially the molecular mechanisms by which the innate immune system recognizes PAMPs on eukaryotic protozoa, including *Cryptosporidium parvum, Cyclospora cayetanensis, Giardia lamblia, Entamoeba histolytica, Toxoplasma* and *Microsporidia*. These pathogens can infect humans and have potential to transmit food/water-borne diseases. In an introduction to the workshop, Theodore Nash (NIAID) discussed several general hurdles that researchers and clinicians face with protozoa:

- Most of these organisms are difficult to handle under laboratory conditions because they grow poorly under monocultural conditions.
- few specific reagents are available.
- inocula are poorly defined and vary over time with the same sample.
- some species cannot withstand cryopreservation.

However, it is simple and easy to obtain massive doses of these pathogens in the field from infected feces or animal corpses. Environmentally resistant cysts from *Giardia* are excreted from infected animals at 10^7/gram of feces, and infected calves can produce 10^{10} *Cryptosporidium* cysts or $>10^7$ *Toxoplasma* cysts per day. Typically, the infectious dose is extremely low: 1 cyst for *Toxoplasma*; and less than 10 cysts for *Giardia* and some *Cryptosporidium*. Epidemiological studies suggest similar infective doses for *Cyclospora.* These cysts are resistant to many of the treatments commonly used in public water supplies, making them applicable to the NIAID Category B Priority Pathogens list.

This group of pathogens are a evolutionarily and functionally diverse group, with differences in life cycles, definitive host, intracellular vs. luminal target environment, intracellular site of infection, and obvious pathogenicity. As members of this workshop emphasized in their presentations, the innate immune response to each pathogen also varies. Overcoming these challenges will require coordination between research groups and communication between scientific disciplines.

Intestinal mucosal environment and pathogenic microbes

Marian Neutra (Harvard University) described how the epithelium of the intestine acts as an interactive barrier to pathogens, and can be divided into the villus epithelium and the follicle-associated epithelium. The villus epithelium erects defenses through secretion of mucus and IgA as well as digestive enzymes and defensive anti-microbial proteins. The follicle-associated epithelium provides reduced defenses: lower enzyme levels; no mucus-secreting goblet cells; and no IgA secretion. In addition to the protective components in secretions, the villus epithelial cells have a thick brush border covered with glycocalyx, composed of transmembrane mucin glycoproteins that make it difficult for pathogens to gain access to the epithelial cell membrane. In the normal gut, very small amounts of food antigens and normal flora pass through the villus epithelium. TLR signaling through the villus epithelial cells and underlying macrophages leads to suppression of inflammation, promotion of a homeostatic state of gut biota and the host immune system, and promotes maintenance of the epithelium itself.

In contrast, the follicle-associated epithelial cells and M cells have little or no brush border or glycocalyx, allowing for contact between pathogens and endocytic domains of the cell surface. This greater exposure to the follicle-associated epithelial (FAE) cell promotes uptake across M cells into the organized mucosal lymphoid tissues in which immune recognition and responses are initiated. FAE cells may recognize microbes and toxins through a variety of pathogen-recognition receptors. In addition to the expression of Toll-like receptors on their surfaces, M cells express receptors for IgA, which may provide antigen-specific feedback on the composition of lumenal flora. M cells in mice and rabbits have specific carbohydrate structures exposed on their surfaces that act as receptors for reovirus and possibly other classes of pathogens. Contact between pathogens and the FAE cells results in the release of chemokines that guide dendritic cells to the site. The M cells within the FAE take up lumenal antigens and pathogens and transport them to the intra-epithelial and sub-epithelial DCs, which may then endocytose them.

Regulation of the innate and adaptive immune response to protozoa

Regulatory T (Treg) cells are one component of the regulatory response that determines when the homeostatic balance between the commensal biota and the quiescent immune system should be disturbed. Yasmin Belkaid (NIAID) described how the balance of dendritic cells, Treg cells, and T_H cells changes over the course of an immune response to a pathogen. When the mucosal immune system of the gut is at rest, local dendritic cells and Treg cells are also at rest or in a low state of activation. Upon stimulation by PAMPs, in conjunction with local tissue damage caused by pathogenic agents, the dendritic cells become activated and mature. Subsequently, they co-stimulate both antigen-specific effector T_H cells as well as Treg cells. The expansion of the T_H cells and dendritic cells leads to a protective immune response and inflammation. As the Treg cells expand, activation through their TLRs lead to local suppression of IL-1, IL-2, IL-12, IFNγ and TGFβ, and the down-regulation of inflammation and the T_H response. As the response wanes, a balanced ratio of Treg and T_H cells restores and maintains the homeostasis while holding memory T cells in reserve. Some pathogens may recruit excess Treg cells to the site of infection to disrupt an ongoing protective response and provide an environment for chronic infection.

In addition to the Treg cell pathway, it is becoming apparent that TLR signaling pathways form a complex set of interactions between TLR-activated cells and the rest of innate immune system. In the gut, TLR signaling in Treg cells can lead to a cross-priming-like suppression of local innate immune cells, and TLR signaling in villus epithelium leads to suppression of inflammation and immune responses. In addition, chronic priming with one TLR agonist may lead to innate immune effector cell tolerization to subsequent TLR agonist stimulation. Tolerance may prevent overstimulation and excessive inflammation during infection, but may also be an escape mechanism for some pathogens. The dynamics of these complex interactions are poorly understood and are one of the promising areas for further study.

Innate immune receptors that recognize protozoan PAMPs

Several TLRs have already been directly or indirectly implicated in recognition of protozoan PAMPs. Using *Babesia bovis* as a model organism, Wendy Brown (Washington State University) demonstrated that extracts from the pathogen can induce proliferation of lymphocytes and blood mononuclear cells from non-exposed cattle. This stimulation is due, at least in part, to parasite DNA, as shown by the loss of stimulation after treatment of the extract with DNase. Her group examined DNA from *Babesia bovis*, *Trypanosoma brucei*, and *Toxoplasma cruzi* and found that the DNA is largely unmethylated compared with more complex eukaryotes, and induces a dose-dependent proliferation of bovine B cells. CpG motifs from *B. bovis* had similar effects, and enhanced IgG secretion as well as increased T cell production of IFN-γ and macrophage production of IL-12, TNF-α, type I IFN, and NO. Lipids from *B. bovis*, on the other hand, stimulated production of NO, but not cytokines. This work offers proof that protozoan DNA can stimulate a strong mammalian immune response. Since mouse models in general may be of limited relevance to human immunology because of its small size and

short lifespan, also it will be important to develop large animal models of the interactions between the innate immune system and protozoan parasites.

Many protozoan pathogens express large numbers of surface proteins attached through glycophosphatidylinisotol (GPI) anchors. There is increasing evidence that these GPI anchors offer targets for TLR binding and activation. Reports have shown that GPI anchors of *Plasmodium, Trypanosoma,* and *Toxoplasma* species are immunostimulatory. Ricardo Gazzinelli (Rene Rachou Research Center) presented data to suggest that unsaturated fatty acids from the lipid portion of the anchors bind through both murine TLR-2 and TLR-4, and that the MyD88 pathway is required for optimal signaling. Furthermore, the activation through TLR-2 may suppress excessive inflammation from continued stimulation through TLRs.

The Apicomplexian protists are a complex group of protozoa (>3000 species) that includes the genera *Plasmodium, Toxoplasma, Eimeria, Theileria,* and *Cryptosporidiu*m. They share a common attachment and invasion mechanism at the apical end of the cell. Alan Sher (NIAID) and Felix Yarovinksy (NIAID) fractionated cell lysates from *Toxoplasma gondii* cells to isolate immunostimulatory molecules and found that the protein profilin, shared by many Apicomplexian protists, binds through TLR-11 on dendritic cells in mice and leads to partial protective immunity. If this protection is cross-reactive with other Apicomplexian protists, it may provide broad spectrum protection against malaria, *Toxoplasma*, and *Cryptosporidium* infections. These investigators also identified another protein, cyclophilin-18, that stimulates the production of IL-12, but through a different type of receptor. Cyclophilin-18 apparently binds to CCR5 on dendritic cells and signals through a G-protein-coupled pathway. Little is known about how this pathway may function in innate immune recognition and activation.

CD1 molecules present lipid antigens, and possibly carbohydrates, to subsets of NKT cells. Lawrence Johnson (Trudeau Institute) reported a unique interaction between CD1d and CD4 T cells during *Toxoplasma gondii* infection. Normal mice survive acute infection after an initial period of weight loss. CD1d knockout mice, on the other hand, die within 8-12 days of infection. Histological studies showed a more severe pathology in the small intestine in these animals that was accompanied by elevated levels of CD4 T cells in mesenteric lymph nodes, and increased levels of INF-γ. Deleting the CD4 T cells in the CD1d deficient mice increased their survival time, while deleting Treg cells further decreased survival time. Dr. Johnson concluded that a CD1d-dependent mechanism plays an important role in protection against acute infection by *T. gondii*, and that at least part of this protection is due to regulation of CD4 T cells, which may otherwise cause severe intestinal pathology.

Effector cells and molecules utilized in response to protozoa

Because of the biological and pathological diversity of the NIAID Category B protozoa, the protective immune responses to them vary substantially. Still, there are some similarities that may be useful to the design of adjuvants, vaccines, and therapeutics. The Apicomplexian organisms *Toxoplasma gondii* and *Cryptosporidium parvum* share a requirement for IFN-γ to control acute infection. Alan Sher provided evidence that during

T. gondii infection, IFN-γ is induced through IL-12 stimulation of T cells and NK cells. The cell type responsible for protection is still unknown. In a similar manner, Vincent McDonald (Barts and the London School of Medicine and Dentistry) presented evidence that controlling acute *C. parvum* infection also requires IL-12 stimulated IFN-γ production. His group and others demonstrated that protection depends upon CD4 T cells and probably NK cells, but does not require CD8 T cells, γδ T cells, or B cells. The innate immune response to *C. parvum* also induces the production of IL-8, GRO-α, RANTES, and β-defensin, although it is not yet known if any of these molecules is required for protection. It is speculated that similar results may be found in the innate immune response to *T. gondii*.

How do these results relate to Lawrence Johnson's work on *T. gondii*? He found that increased levels of CD4 T cells and NK cells were correlated with increased intestinal pathology, and deleting the CD4 T cells actually improved survival time in *T. gondii*-infected mice. Does CD1d modulate the response to both *T. gondii* and *C. parvum*, so that appropriate levels of CD4 T cells/NK cells are activated and secreting IFN-γ? Do TLR agonists bind directly to Treg cells in these two infection models and help suppress the overstimulation? These questions are still under study.

While IFN-γ is essential to protection against acute infection by the two Apicomplexian protists, it is not required for protection against some other protozoa. William Petri (University of Virginia Health Systems) presented evidence to suggest that the protective immune response to *Entameoba histolytica* may be very narrowly based on IL-10-dependent and neutrophil pathways. In C57 Bl/6 mice, innate immune resistance persists in the absence of neutrophils, NADPH-oxidase, iNOS, IL-12, IFN-γ, B cells, T cells, or MyD88. Only IL-10 depletion decreased resistance to *E. histolytica*. In some less resistant mouse strains, such as CBA/J, neutrophils also played a protective role.

Protection against *Giardia lamblia* also does not require IFN-γ. Steven Singer (Georgetown University) reported that rapid elimination of *Giardia* from the gut requires αβ CD4 T cells, mast cells, TNF-α, NOS1, and IL-6, but does not require B cells, γδ T cells, IFN-γ, NOS2, IL-4, IL-13, or STAT6. He predicts that *Giardia* PAMPs induce DC to produce IL-6, leading to downstream activation of mast cells. The intestinal biota also play a key role in protection against *Giardia*. Genetically identical mice obtained from two separate breeding colonies show very different levels of susceptibility to infection, and the more susceptible strain gains resistance after being housed with the second strain or after inoculation with gut flora from the second strain. The gut is a complex milieu of host and bacterial populations that interact constantly to produce a balance environment. Discovery of therapeutics or broad spectrum agents against pathogens must be balanced against the effects they will have on the whole microbial community of the gut.

Summary

With the recent increased threat of the use of bioweapons, we are faced with the potential of intentional adulteration of our food and water supplies and emphasizes the importance of encouraging research into the interactions between protozoan pathogens and the host immune response.

In recent years, our understanding of the fields of mucosal immunity of the gut and innate immunity has grown dramatically, as has our appreciation of their convergence. There is a greater understanding of the role of the villus epithelium as a protected area that allows communication between commensal biota and the host immune system and of the role of the follicle-associated epithelium as the area responsible for recognition of many of the pathogenic species that invade the gut. There have also been recent breakthroughs in understanding of the homeostatic balance of the gut biota and the host immune system, and how that balance is maintained by local dendritic cells and Treg cells.

While research on the innate immune response to protozoa has lagged behind research on the innate immune response to prokaryotes, there is evidence to suggest that mammalian innate immune receptors recognize protozoan PAMPs, and that they are important in the stimulation of protective immunity. The complexity and diversity of the protozoan pathogens is highlighted, though, by the divergence of the requirements of protective immunity against different families of parasites. It will be essential to expand our understanding of how to induce protective responses against this variety of pathogens in order to meet the current and future threats against our food and water supplies.

*Alan Sher, NIAID; Theodore Nash, NIAID; Wendy C. Brown, Washington State University; William Petri, University of Virginia Health Systems; Vincent McDonald, Barts and The London School of Medicine and Dentistry; Steve Singer, Georgetown University; Eric Y. Denkers, Cornell University College of Veterinary Medicine; Lawrence L. Johnson, Trudeau Institute; Vernon B. Carruthers, Johns Hopkins University Bloomberg School of Public Health; Ricardo Gazzinelli, René Rachou Research Center, FIOCRUZ; Yasmine Belkaid, NIAID; Brian Kelsall, NIAID; Marian Neutra, Harvard University; David Winter, NIAID.