Meeting report

Complex responses to a diverse environment

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A report on the Keystone Symposium 'Innate Immunity: Signaling Mechanisms', Keystone, USA, 24-29 February, 2008.

The complexity of innate immunity was the message of a recent Keystone meeting on signaling mechanisms in innate immunity. Multiple receptors, signaling pathways and mechanisms have now been defined, greatly expanding on the initial findings more than a decade ago that the *Drosophila* Toll receptor had effects on the fly's immunity to fungal infections. Three major gene families have been identified as encoding distinct innate functions, and additional pathways are emerging. We can now see the outline of the signaling pathways leading from these receptors. Mechanistic and structural analyses have given us a picture of the Toll-like receptor (TLR) complexes binding to their ligands and bringing intracellular domains together to initiate signaling. New modifiers of signaling, and in particular negative regulators that limit the inflammatory response, were prominent at the meeting. Human mutations influencing susceptibility to infections and autoimmune disease, and the targeting of the innate immune system by pathogens confirms the significance of these signaling molecules in innate immunity and human disease.

Surface and intracellular receptors for pathogen components

TLRs are transmembrane proteins with conserved extracellular leucine-rich repeats (LRRs) and intracellular Toll/interleukin 1 receptor (TIR) domains. They are expressed either in endocytic compartments or on cell surfaces, and recognize a variety of activation ligands derived from infectious organisms, environmental triggers or endogenous stress signals. TLR extracellular domains have a curved arch-like structure based on their characteristic LRRs. Interestingly, different ligands bind to different sites on the arch and result in different types of receptor-ligand complexes. Crystal structures of TLR4 and its associated protein MD2 and their ligands were described by Jie-Oh Lee (Korea Advanced Institute of Science and Technology, Daejeon, Korea), revealing that MD2 binds directly to TLR4 on the internal curve of the LRR arch. Eritoran, an antagonist of bacterial lipopolysaccharide (LPS), binds only to MD2, and not to TLR4. Lee demonstrated, using gel-filtration techniques, that the agonist LPS, but not Eritoran, induces the formation of a heterotetrameric complex (LPS-MD2-TLR4 bound to LPS-MD2-TLR4). A different binding mode was seen for TLR1 and TLR2 in complex with the lipopeptide Pam3CSK4. The lipid components of the lipopeptide bind to pockets in TLR1 and TLR2, inducing an M-shaped heterodimeric complex, which is stabilized by further interactions between TLR1 and TLR2. The formation of this complex may activate the receptor by moving the TIR domains of the TLRs closer, inducing TIR dimerization and initiating signaling.

The structure of TLR3, an endosomal receptor for double-stranded RNA (dsRNA), bound to its dsRNA ligand was described by two collaborating scientists from the NIH. Josh Leonard (National Cancer Institute, NIH, Bethesda, USA) found that TLR3 ligand binding was optimal at low pH, as expected from its endosomal location, and that the affinity of TLR ectodomain binding was dependent on the length of the dsRNA ligand. The crystal structure, as described by Lin Liu (National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, USA), revealed that a 48-bp dsRNA ligand bound in two sites to the non-gycosylated face of two TLR3 arches. The rod-like dsRNA holds the two TLR3 molecules in position, inducing an active M-shaped formation, similar to the TLR1:TLR2 complex, that brings their TIR domains into close proximity for signaling.

TLR4 signals through two independent signaling mechanisms as a result of TIR dimer interactions with the adaptor proteins MAL (TIRAP) or TRAM. Ruslan Medzhitov (Yale University, New Haven, USA) showed that the differential

localization of TLR4 signaling complexes with TRAM or MAL controls downstream signaling. The adaptors both occur at the plasma membrane, but whereas MAL recruits MYD88 to the membrane upon LPS binding to the TLR4 complex, TRAM recruits TRIF and moves into the early endosome, where it activates the adaptor TRAF3. This localization is dependent on both myristoylation of TRAM and its binding to phosphatidylinositol at the endosomal face of the membrane. Medzhitov postulated that the endosomal localization of TRAF3 is the reason that TLR pathways that induce type 1 interferon signal through the early endosome. Indeed, he showed that if TRAF3 is forced by mutation to relocate to the plasma membrane, TLR receptors that are exclusively at the plasma membrane (such as TLR1:TLR2) can then induce type 1 interferon. These results show that type 1 interferon-inducing pathways have in common the utilization of TRAF3 in early endosomes.

The RIG-I like receptors RIG-I, MDA5 and LGP2, known collectively as the RLRs, initiate antiviral responses by sensing cytoplasmic viral RNA. RIG-I (retinoic acid-inducible gene I) is composed of an amino-terminal signaling CARD domain, an ATP-binding RNA helicase linker region and a carboxy-terminal repression domain (CTD). Activation of RIG-I by viral RNA results in production of interferon. Takashi Fujita (Kyoto University, Japan) presented a structural model for the sensing mechanism of this pathway, based on mutagenesis and NMR studies. In this model, the helicase linker region, which interacts with the CTD in the absence of ligand, represses activation of RIG-I. Activation occurs when the CTD binds to either dsRNA or 5'ppp-ssRNA, resulting in a conformational change, which is dependent on ATP binding by the helicase domain. This conformational change can then open up the CARD domain, promoting its oligomerization and interactions with downstream signaling components.

The nucleotide-binding oligomerization domain proteins NOD1 and NOD2 are the founding members of the family of intracellular NOD-like receptors (NLRs), currently numbering 22 in the human genome. NLRs contain distinct amino-terminal effector domains, a NOD domain, and carboxy-terminal LRR domains. Gabriel Nunez (University of Michigan, Ann Arbor, USA) presented new results showing that NOD1 and NOD2 can provide a critical second line of defense against invading pathogens. He showed that mice deficient in NOD1 and NOD2 can clear a *Listeria* infection, but that pretreatment of these mice with LPS, known to 'tolerize' cells to TLR4 signals, results in a failure to clear the infection. The interplay between NLRs and TLRs may be critical either when a first line of defense is insufficient or under conditions of secondary infections.

The inflammasome is an NLR-containing intracellular protein complex whose activation leads to the activation of the cytokine interleukin (IL)-1β. Although it was well known that viral RNA or other danger signals can activate inflammasome-dependent IL-1β, it is now clear that there are also sensors for cytoplasmic microbial and host DNA. Jurg Tschopp (University of Lausanne, Switzerland) has identified responses to intracellular DNA that are mediated by the inflammasome. He reported that the response induced by internalized adenovirus appears to depend on the inflammasome components NALP3 (an NLR) and ASC, whereas that mediated by transfected cytosolic bacterial, viral and host DNA appears to depend on ASC, but not NALP3, suggesting that there may be more than one complex that senses intracellular DNA. These findings implicate inflammasomes in host defense against DNA virus infection, and raise the possibility that inflammasome function could be associated with the development of nucleic-acid-dependent autoimmune disease. Indeed, one form of systemic lupus erythematosus and rheumatoid arthritis was recently found by other workers to be associated with mutations in the *NALP1* gene.

New modifiers for signaling in innate immunity

Luke O'Neill (Trinity College Dublin, Ireland) identified a new protein, KIAA644, which contains LRRs and a possible TIR domain. KIAA644 binds to LPS and enhances LPS-mediated signaling. This protein is postulated to be a co-receptor for TLR4 and is abundantly expressed in brain.

The complexity of LPS-mediated signaling was underscored by Sankar Ghosh (Yale University, New Haven, USA). It now appears that some members of the IκB family (initially defined as inhibitors of the transcription factor NF-κB) can positively regulate transcription by acting as transcriptional co-activators. IκBβ-deficient mice are surprisingly more resistant to LPS-induced septic shock and have reduced levels of the LPS-induced cytokine tumor necrosis factor α (TNFα). Gosh proposed that cytoplasmic IκBβ can act as an inhibitor by associating with NF-κB, whereas nuclear IκBβ can be recruited to the TNFα gene promoter and act as a co-activator with NF-κB to positively regulate transcription of the TNFα gene.

Searching for new modifiers in the RIG-I-mediated antiviral type I interferon response, Hiroyuki Oshiumi (Hokkaido University, Japan) reported the identification of Riplet, a novel RING finger domain of E3 ubiquitin ligase that interacts with RIG-I. Overexpression of Riplet enhanced RIG-I function, whereas a dominant-negative mutant or RNA interference of Riplet repressed RIG-I function. His evidence suggests that Riplet forms a complex with RIG-I and positively regulates its function in promoting type 1 interferon production during RNA virus infection.

As signaling pathways for innate immune receptors become more detailed, negative regulators of these responses are being uncovered. Uncoupling of negative regulators from the normal activation signals can result in prolonged or patho-

genic immune responses. MicroRNAs (miRNAs) have recently emerged as a novel class of negative regulators of gene expression in the immune response. TLR activation has been shown to induce miR-155, which negatively regulates c-Maf, a trasncription factor involved in immune function, and miR-146, which negatively regulates the expression of IRAK1 and TRAF6, components of the signaling pathways from multiple TLRs. O'Neill presented results on the expression profile of 150 miRNAs following TLR stimulation with LPS, PamCys or poly(IC) in dendritic cells. In addition to miR-155 and miR-146, which are hyperinduced by all the above ligands, miR-21 was also induced, albeit after a delay. It has previously been shown that miR-21 targets the genes for tropomyosin and PTEN, both of which are tumor suppressors. Negative regulation of tropomyosin expression by miR-21 may lead to a decrease in dendritic cell motility and stability of the actin cytoskeleton.

Carla Rothlin (Salk Institute, La Jolla, USA) has identified TAM (Tyro3, Axl and Mer) receptor tyrosine kinases as pleiotropic inhibitors of TLR signaling. She has shown that mice deficient in all three receptors (TKO) exhibit lymphoproliferation and systemic autoimmunity and that dendritic cells from TKO mice are hyper-responsive to TLR ligands. The TLR3 ligand poly(IC) induces production of type 1 interferon, which then upregulates expression of AXL or other TAMS. AXL then 'hijacks' interferon-mediated STAT1 signaling to enhance expression of SOCS1 (suppressor of cytokine signaling 1) and SOCS3, which are negative regulators of cytokine signaling. TKO mice do not upregulate SOCS expression in response to interferon. Thus, TAMs provide intrinsic feedback inhibition for both TLR- and cytokine-driven immune responses.

Chris Moore and Jenny Ting (University of North Carolina, Chapel, USA) have identified NLRX1, a novel member of the NLR family. Expression of NLRX1 inhibited RIG-like helicase (RLH)-mediated interferon-β promoter activity. Conversely, knockdown of NLRX1 enhanced virus-induced type I interferon production and attenuated the replication of Sindbis virus. Their talks suggested that NLRX1 may negatively regulate RLH-mediated mitochondrial antiviral signaling.

Innate immunity and human disease

Critical players in the human innate immune response to pathogens have been revealed by genetic studies. Jean-Laurent Casanova (Necker Medical School, Paris, France) has identified human patients with mutations of IRAK4 and MYD88 in the TLR and IL-1 receptor signaling pathways. Patients with IRAK4 or MYD88 deficiencies exhibited a childhood susceptibility to *Streptococcus pneumoniae* and *Staphylococcus aureus*. Deficiencies in TLR3 or UNC93, both of which impair the TLR3-to-interferon signaling pathway, were associated with susceptibility to herpes simplex virus encephalitis.

Loss-of-function mutations in NOD2, which recognizes bacterial muramyl dipeptide (MDP), have been identified in 10-15% of patients with Crohn's disease. Why should loss of this function lead to an inflammatory disease? Warren Strober (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, USA) reported that production of the pro-inflammatory cytokine IL-12 by antigen-presenting cells is reduced by NOD2 ligands. Pretreatment of wild-type mice, but not NOD2-deficient mice, with MDP had a protective role in models of colitis. In addition, transgenic expression in mice of the wild-type human NOD2 gene, but not the mutant NOD2 gene from Crohn's disease patients, exerted this protective role. Strober suggested that treatment with MDP to downregulate inflammatory responses should be considered in Crohn's disease patients with normal NOD2 responses.

Rowan Higgs (Royal College of Surgeons, Ireland) presented evidence suggesting that the E3 ubiquitin ligase Ro52 negatively regulates interferon-β gene transcription by initiating proteasomal degradation of the transcription factor IRF3. Interestingly, autoantibodies against Ro52 can be found in Sjogren's syndrome and systemic lupus erythematosus.

Therapies targeting IL-1β are being examined for inflammasome-mediated diseases. Tschopp reported that human *NALP3* mutations that activate the inflammasome and result in high IL-1β production are associated with a number of specific inflammatory diseases. Patients with these diseases, such as the Muckle-Wells syndrome, respond to drugs targeting the IL-1β pathway. NALP3 also mediates activation of the inflammasome by reactive oxygen species, which are generated via the NADP oxidase system after endocytosis of monosodium urate crystals in gout patients or of other irritants such as asbestos and silica. Recent studies suggest that inhibition of IL-1β signaling may be efficacious in gout, underscoring the role of innate responses in this disease.

We are now beginning to understand and expand on the early studies of Elie Metchnikov exploring phagocytosis by macrophages in the late nineteenth century. What was once viewed as simple ingestion has now come to be appreciated as a much more complex response, resulting in the production of cellular mediators and responses that ultimately clear pathogens and drive adaptive immune responses. The variety of infectious and environmental stimuli has been matched by an equally complex set of host responses. Susceptibility to particular pathogens in patients with mutations in distinct pathways underscores the need for diverse responses to distinct pathogens. Genetic associations with chronic inflammatory disease indicate that selection of these pathways in populations is dependent on the immediate danger of infectious disease. Our next step is to harness our knowledge of these pathways to reduce infectious disease and to alleviate chronic inflammation.