

'Toll2008' outgrows its name

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On 24–27 September 2008, members of the scientific community gathered to discuss advances in innate immunity at the 'Toll meeting' in Cascais, Portugal. Before long, attendees noticed that 'Toll2008' might be a misnomer.

First held in 2004 as an opportunity for groups working on innate immunity to share their progress in the exploding field of Toll-like receptor (TLR) research, the third biannual Toll meeting reflected the rapid evolution of innate immunology. It is certainly not just Tolls anymore! Here we will provide a brief summary of some key themes from this diverse and comprehensive meeting for those not able to join us in Portugal.

The party is in the cytosol

Much attention is now focused on several families of innate immune receptors that, unlike transmembrane TLRs, are located in the cytosol. In mammalian systems, the DEAD-box RNA-binding helicases RIG-I, Mda5 and LGP2 constitute the RIG-I-like helicase (RLH) family that is essential to the recognition of replicating RNA viruses. The ancient origins of helicase-mediated immune responses have now been demonstrated through work on antiviral immunity in flies by Jean-Luc Imler (Strasbourg, France), who presented work characterizing the induction of the drosophila gene *vago* by the DExD/H-box helicase Dicer-2, a distant relative of the mammalian RLHs¹. Regulation of RLH responses has been the subject of much discussion. Identification of the cellular inhibitor Dublin (also called MULAN) of mamma-

lian RLHs was discussed by Ashley Mansell (Melbourne, Australia). Dublin localizes to mitochondria and interacts directly with the RLH adaptor protein MAVS to target activated RLH complexes for deubiquitination. In addition to the RLHs, another member of the DEAD-box helicases, DDX3X, has been linked to the induction of type I interferon. DDX3X interacts directly with the kinase TBK1, an 'upstream' regulator of the induction of type I interferon that acts through the interferon-regulatory factor (IRF) family of transcription factors² (Tilman Bückstümmer, Vienna). This same helicase is targeted by the K7 protein of vaccinia virus to prevent induction of the interferon- β (IFN- β) promoter and thus evade the host antiviral response (Andrew Bowie, Dublin)³.

As with RNA, it has long been recognized that immune responses to exogenous DNA can originate from the cytosol. However, identifying the cytosolic DNA receptor has been something of a search for the 'Holy Grail'. It now seems that recognition of cytosolic DNA may originate from several distinct receptors that activate either interferons or proinflammatory cytokines such as interleukin 1 β (IL-1 β). Tadatsugu Taniguchi (Tokyo) demonstrated mechanisms by which the cytosolic DNA-dependent activator of interferon DAI activates the production of type I interferon. He further showed that the requirement for DAI in interferon induction may depend on the cell type studied, as functional redundancy seems to exist⁴. Ruslan Medzhitov (New Haven, Connecticut, USA) presented the exciting discovery that an exonuclease generally involved in DNA repair is also able to modulate the induction of type I interferon. The 3' repair exonuclease Trex 1 can recognize endogenous single-stranded DNA retroelements and seems to downregulate the

interferon-dependent pathology of autoimmune conditions such as chilblain lupus and Aicardi-Goutieres syndrome⁵. Intracellular double-stranded DNA also triggers production of IL-1 β which suggests the existence of a 'DNA-recognizing' inflammasome. New findings show that after DNA binds to its HIN200 domain, the PYHIN family member AIM2 interacts through its pyrin domain with the adaptor ASC to form a caspase-1-activating complex (Veit Hornung, Worcester, Massachusetts, USA). The functional relevance of many cytosolic DNA-sensing receptors for host defense against microbes and for possible immune responses to self DNA remains to be fully elucidated.

A nod to autophagy

One of the processes most recently recognized as a fundamental phenomenon in immunity is the bulk degradation mechanism known as 'autophagy'. This process, whereby cytoplasmic contents are sequestered in a characteristic double-membrane 'autophagosome' and delivered to the lysosome for degradation, has long been understood to be important for the recycling of organelles and as a form of programmed cell death. The involvement of autophagy in the control of intracellular pathogens is a much newer idea, and its importance as an immune response mechanism was emphasized at the meeting in several contexts. In the fly *Drosophila melanogaster*, induction of autophagy by diaminopimelic acid-type peptidoglycan is essential for the prevention of intracellular growth of the bacterium *Listeria monocytogenes* and for host survival during infection with this pathogen⁶ (Shoichiro Kurata, Sendai, Japan). Vesicular stomatitis virus seems to downregulate autophagy in the drosophila host cell by repressing the serine-threonine kinase Akt (Sara Cherry,

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Philadelphia, Pennsylvania, USA), which indicates involvement of autophagy in antiviral as well as antibacterial immunity. In mammals, a key advance has been made in understanding the mechanistic function of the autophagosome in innate immune function. The mammalian autophagy-related protein Atg16L1 mediates formation of the autophagosome, and loss of Atg16L1 results in more release of mature IL-1 β after challenge with lipopolysaccharide by a mechanism dependent on the adaptor TRIF. Notably, Atg16L1-chimeric mice have more chemical-induced colitis and lower survival, which suggests the involvement of autophagy in the pathogenesis of inflammatory bowel disease⁷ (Shizuo Akira, Osaka, Japan). As several important cytosolic immune pathways have been defined, autophagy might be related to more than just IL-1 production. Indeed, Miguel Sanjuan (Memphis, Tennessee, USA) discussed ways in which TLR signaling links the autophagy and phagocytosis pathways in macrophages⁸, and Dana Philpott (Toronto) alluded to functions for the signaling-associated proteins Nod1 and Nod2 in the autophagic process. Although many new findings have been reported, it is now clear that defining the place of autophagy in immunity has become a key question of interest for the immunology community.

Are Tolls still 'toll'?

The German word '*toll*' was originally applied to a gene whose deletion confers an unusual morphology on drosophila embryos; it roughly translates to 'cool' or 'wild'. Mammalian TLRs have occupied the spotlight of innate immunity since their description. However, with so much newfound focus on cytosolic immune receptors, membrane-localized TLRs might seem to have fallen out of fashion. That is far from being true, however, as work on mammalian TLRs continues to be of active interest. Luke O'Neill (Dublin) opened the Toll 2008 meeting with what could be called a 'state of the signaling in TLRs' discussion, during which he reviewed the increasingly complex web of innate immune signaling pathways and set the stage for the conversations to come. An exciting development reported by O'Neill's group is that TLR activation leads to induction of the microRNA molecules miR146, miR155 and miR21; the last targets both PDCD4, a tumor suppressor required for function of the transcription factor NF- κ B, and the cytoskeletal protein tropomyosin. Thus, miRNAs induced by TLRs may act in a negative feedback loop to limit NF- κ B activity in general and cell migratory potential in some cancers.

Although understanding of signaling cascades has become sophisticated, knowl-



The Toll2008 venue in Cascais, near Lisbon, Portugal. Photo: William L Marshall

edge of how receptors, adaptors and kinases interact on the molecular level is still basic. New insights into the structural biology of signaling complexes were discussed by Nicholas Gay (Cambridge). The interaction of the adaptor MyD88 with the kinase IRAK4 occurs as an undecameric complex formed between the death domains of the two proteins, a complex that may be able to drive higher-order formation of oligomers of signaling components. The finding that this undecameric complex differs considerably from the signaling complexes formed by elements of the drosophila Toll pathway⁹ may help to explain the greater capacity for diverse interactions in vertebrate TLR signaling pathways compared with the relative simplicity of the fly signaling cascade.

General knowledge of signaling regulation was also brought to bear in the specific context of innate immunity. An important function for ubiquitin as a regulator of many cell signaling pathways was elegantly explained by Vishva Dixit (San Francisco, USA). So-called 'ubiquitin editing' involves modification of the kinase-activating polyubiquitination of lysine 63 to the proteasome-directing polyubiquitination of lysine 48 by the ubiquitin-editing protein A20 and is an essential regulatory mechanism for such immune-signaling kinases as RIP1 and IRAK1 'upstream' of NF- κ B^{10,11}. Paula Pitha-Rowe (Baltimore, USA) discussed how ubiquitination of lysine 63 is also important for activation of the transcription factor IRF5 and that this ubiquitination event is mediated by the E3 ubiquitin ligase TRAF6. In drosophila, polyubiquitination as well as cleavage of the

adaptor protein IMD is essential for activation of the NF- κ B precursor protein Relish (Neal Silverman, Worcester, Massachusetts, USA). These findings collectively show that polyubiquitination is a conserved regulatory mechanism for controlling innate signaling pathways in invertebrates and mammals.

Many advances have been made in terms of the biology of specific members of the TLR family. TLR4, the most extensively studied of all mammalian TLRs, interacts with a complex variety of coreceptors and signaling adaptor molecules. Jerrold Weiss (Iowa City, Iowa, USA) demonstrated that airway epithelial cells, which express TLR4 without its coreceptor MD-2, can be activated by purified complexes of lipopolysaccharide and MD-2 but not by lipopolysaccharide alone. This complex can in fact be exploited to provide immunoprophylaxis against otherwise lethal airway infection with the bacterium *Yersinia pestis*. TLR4's 'circle of friends' has grown to include an entirely new kind of coreceptor: the proteinase-activated G protein-coupled receptor PAR2. TLR4 directly interacts with PAR2, and knockout of either receptor decreases the expression of proinflammatory genes and proteins in response to the reciprocal receptor's ligand (Stefanie Vogel, Baltimore, USA). These findings suggest an as-yet-unappreciated function for endogenous proteases in tailoring the immune response to specific pathogens.

Signaling from TLR2 is indirectly antagonized by activation of the adenosine 3 receptor, which has high expression in newborn humans and mice. Signaling through the adenosine 3 receptor leads to copious produc-

tion of IL-6 by human cord blood monocytes and considerable release of IL-6 into mouse plasma after challenge with a TLR2 agonist. The anti-inflammatory and T helper type 2-skewing effects of IL-6 seem to mitigate the production of proinflammatory tumor necrosis factor and IFN- γ 'downstream' of TLR2 (Ofar Levy, Boston, USA).

One of the more mysterious TLRs on the cell surface is TLR11. Findings suggest that TLR11 works in concert with TLR5 during innate immune responses in the mouse gut. During infection with *Salmonella typhimurium*, activation of lamina propria dendritic cells by TLR5 and TLR11 is essential for the control of infection and host survival. Moreover, the greater importance of TLR11 relative to that of TLR5 during this infection is shown by the finding that upregulation of TLR11 can compensate for loss of TLR5, which allows resistance to *S. typhimurium* in TLR5-deficient mice, whereas TLR11-deficient mice are highly susceptible to this pathogen (Sankar Ghosh, New Haven, USA).

Although canonical unmethylated CpG motif-containing DNA has been recognized as a TLR9 ligand for some time, the diversity and modulatory functions of TLR9 ligands continue to be explored. Hermann Wagner (Munich) highlighted the essential and different functions of natural phosphodiester and synthetic phosphorothioate backbones in TLR9 oligonucleotide ligands¹². Ann Marshak-Rothstein (Boston, USA) suggested that responses to TLR9 ligands generated naturally during autoimmune disease may be a more highly controlled phenomenon. B cell activation through TLR9 seems to have a strict requirement for the presence of a CpG motif. However, this requirement can be circumvented after IFN- α priming or when non-DNA immune complexes induce an independent B cell antigen receptor signal in the presence of TLR9 agonists. TLRs involved in the recognition of nucleic acids are not found on the cell surface but instead are expressed in endolysosomal compartments, and their proper localization is essential for controlled ligand recognition and signaling. Functional cloning in a CpG DNA-unresponsive cell line has identified previously unrecognized control mechanisms for the activation of endosomal TLRs. Expression of the cysteine proteases cathepsin B and cathepsin L confers CpG DNA responsiveness on an otherwise unresponsive B cell line, which suggests that a proteolytic cleavage event is important for TLR9 activation (Kensuke Miyake, Tokyo). Hidde Ploegh (Cambridge, Massachusetts, USA) suggested that a cleavage event occurs in the TLR9 protein itself and that cleavage of TLR9 is required for signaling¹³.

Keep it clean

Great advances have also been made in the understanding of 'sterile inflammation', which is the activation of inflammatory signaling cascades and pathology in the absence of a pathogen-derived stimulus. IL-1 signaling seems to be a key component of sterile inflammation.

The production of mature IL-1 β by leukocytes is dependent on the activation of caspase-1 by any of several cytosolic multiprotein complexes called 'inflammasomes'. There are several hypotheses about NLRP3 inflammasome activation. Rupture of endolysosomes and release of proteolytic enzymes from these compartments was presented as a newly identified mechanism 'upstream' of activation of the NLRP3 inflammasome (Eicke Latz and Douglas Golenbock, Worcester, Massachusetts, USA). Phagosomal destabilization was suggested to be a key event for activation of the NLRP3 inflammasome by amyloid- β fibrils¹⁴ and crystalline forms of silica, alum¹⁵ and uric acid (Kenneth Rock, Worcester, Massachusetts, USA). Pharmacological disruption of lysosomes could also activate the NLRP3 inflammasome, which suggests that this pathway may serve as a common recognition system for complex damage-associated molecular patterns. Alternatively or additionally, the generation of reactive oxygen intermediates by the phagosomal NADPH oxidase system may also be involved 'upstream' in the activation of NLRP3 (Jürg Tschopp, Lausanne, Switzerland).

Inflammasome-independent IL-1 signaling also seems to be involved in sterile inflammatory processes. In support of published work showing the importance of IL-1 α in the induction of inflammation in response to sterile cell death¹⁶, Gabriel Núñez (Ann Arbor, Michigan, USA) presented data suggesting that IL-1 α released from necrotic cells can trigger secretion of the chemokine CXCL1. Expression of the IL-1 receptor and MyD88, but not IL-1 α itself, TLR2 and TLR4, TRIF or inflammasome components, is required for CXCL1 production in mesothelial cells.

Interesting work reported by Kathryn Moore (Boston, USA) indicates that a previously unknown TLR heterodimer can also mediate sterile inflammation in response to endogenous ligands. The TLR4-TLR6 heterodimer recognizes amyloid- β fibrils and oxidized low-density lipoprotein when these are in complex with the scavenger receptor CD36. This may represent a damage-associated molecular pattern-sensing function for TLRs beyond their well understood function in the recognition of exogenous pathogens.

Dominique Ferrandon (Strasbourg, France) showed that sterile immunity is not specific to mammals and that a similar damage-induced



View of the Ponte 25 de Abril from the Padrao dos Descobrimentos (Monument to the Discoveries), Lisbon, Portugal. Photo: Douglas T Golenbock

immune phenomenon exists in the fly. 'Clean' injury to the fly gut induces the expression of antimicrobial peptides. In the presence of Gram-negative bacteria, such as when injury is induced by the escape of bacteria across the gut wall, the action of these antimicrobial peptides can lead to bacterial damage and release of diaminopimelic acid-type peptidoglycan, which further promotes immune responses through the IMD pathway, resulting in an immune amplification loop.

The implications for the recognition of self-derived 'danger' signals in conjunction with exogenous pathogen-associated signals may be far reaching. Work by the group of Miguel Soares (Oeiras, Portugal)¹⁷ has shed light on the function of free heme (Fe protoporphyrin IX) released from damaged red blood cells in the host during plasmodium infection. Free heme seems to serve as a proinflammatory 'danger' signal that potentially induces immune activation and mediates pathology in severe cerebral malaria. Experimental overexpression of the gene encoding heme oxygenase 1 serves to increase the catabolic breakdown of free heme and protects against cerebral pathology in mice.

Immunity's toll on the 'First World'

As discussed above, the pathological consequences of immune responses during infectious and autoimmune diseases are becoming increasingly well defined. The mechanistic details of inflammation are also being identified in multifactorial chronic diseases such as atherosclerosis and diabetes, which are particu-

larly prevalent in populations in industrialized nations. Peter Libby (Boston, USA) elegantly introduced and summarized the many facets of inflammation in atherosclerosis and set the stage for the discussion of various aspects of immune activation in atherosclerotic lesions. Moshe Arditi (Los Angeles, USA) explained a mechanism whereby TLR-MyD88 activation by the bacterium *Chlamydia pneumoniae* contributes to accelerated atherosclerosis in apolipoprotein E-deficient, hypercholesterolemic mice. Notably, when these mice are also deficient in the nuclear receptor protein liver receptor X- α , markers of atherosclerotic acceleration increase, which suggests involvement of this protein in antagonizing TLR signaling¹⁸. Peter Tobias (La Jolla, California, USA) reported that TLR2, an important contributor to the pathogenesis of atherosclerosis, is expressed in endothelial cells mainly in areas of disturbed blood flow. Stunning imaging studies of chimeric mice containing green fluorescent protein-positive bone marrow-derived cells showed that TLR2 drives local recruitment of leukocytes to atherosclerotic vessel walls¹⁹.

Extensive mRNA analysis of samples of human atherosclerotic plaques and animal plaque models has not only confirmed a proinflammatory gene expression profile in plaque tissue but as also identified the heterogeneous nature of such gene expression. Proinflammatory gene induction varies according to the symptomatic versus asymptomatic state of the patient, the anatomical location of the plaque and even throughout different regions of a single plaque. Specific targeting of the inflammatory 'tone' of plaques may represent a new therapeutic option for atherosclerosis (Samuel Wright, Rahway, New Jersey, USA). The excessive intake of fatty foods that predisposes people to develop atherosclerosis is also a risk factor in the development of type II diabetes. Christopher Karp (Cincinnati, USA) commented on the function of TLR signaling in weight regulation

and development of the metabolic sequelae of obesity in mice fed a high-fat diet.

Thinking beyond the bench

For many years, innate immunity took a back seat to adaptive immunity in the field of immunology. Even as recently as this year's presidential election in the United States, the relevance of studying the fruit fly was called into question. If there is anyone out there who still doubts the importance of innate immunity, that uncertainty should be quashed by Toll2008. Most known immune-stimulating agents now have an assigned signaling receptor, and more details about the immune recognition of 'self' in disease conditions have rapidly materialized. Innate immunity is not simply 'nonspecific' immunity any more but is an elaborate system of signaling receptor families and multidimensional, interconnected signaling pathways.

The innate immunity field has matured to a stage at which the fruitful work in basic research can be translated into new therapies. An important issue arising from the collective work in this field in the past decade is whether innate immune receptors are amenable to pharmacological interference in acute or chronic inflammatory or autoimmune diseases. Toll2008 provided a glimpse of the first few approaches in pharmacological interference with innate signaling. Small-molecule TLR9 inhibitors are being developed (Sally Ishizaka, Andover, Massachusetts, USA) and the nucleic acid-based modulators of the endosomal TLRs are progressing to clinical testing (Sudhir Agrawal, Cambridge, Massachusetts, USA, and Robert Coffmann, Berkeley, California, USA). In addition, potent antagonist antibodies that specifically inhibit the binding of IFN- α to its receptor show promise in the treatment of cutaneous lupus and may have therapeutic value in the treatment of scleroderma and other interferon-mediated disorders (Anthony Coyle, Gaithersburg, Maryland, USA). This

meeting showed that there is great progress in the development of new therapies that, by relying on interference with innate immune receptors, have the potential to be much more specific than the broad-spectrum immunosuppressive agents now in use.

Although much has been learned about innate immune activation at the molecular level, what actually triggers innate immunity remains more than mysterious in most diseases. To be able to make the best use of the many potential 'magic bullets' being developed, ways to detect the innate immune stimulant in a given pathology must be established. Only when the compromised innate immune pathway can be diagnosed can specific new pharmacological agents be delivered without guessing. So, everyone back to work, and we will see you at 'Toll 2011' in Barcelona.

ACKNOWLEDGMENTS

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