The three Rs of innate immune recognition: Toll like receptors (TLRs), RIG-like receptors (RLRs) and Nod-like receptors (NLRs)

The Penridge Suite, London, N11 1NL, UK **:** Friday, 23 September 2011

The function of the innate immune system is thought to be the recognition of invading pathogens, the activation of inflammation to control the pathogen, and the subsequent activation of the acquired immune response. The innate immune system utilises a set of germline-encoded receptors, called PRRs, in order to recognise specific molecular patterns or motifs called PAMPs (Pathogen-Associated Molecular Patterns) on invading pathogens (Medzhitov & Janeway 1998). There are three families of PRRs, the Toll-lke receptor (TLR) family, the RIG-like receptor (RLR) family and the NOD-like receptor family. **The TLR family** of proteins is an integral part of the mammalian innate immune system. TLRs are ancient pattern recognition receptors highly conserved from *Drosophila* to humans, that are expressed on immune cells and are able to distinguish a great variety of microbial ligands (Takeda, Kaisho, & Akira 2003). To date there are at least ten different TLRs that can recognise a wide variety of microbial conserved patterns. Most TLRs (TLR1, TLR2, TLR4, TLR5, TLR6) seem to activate cells by engaging their ligands on the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 seem to trigger signalling intracellularly. These TLRs have been shown to reside in the ER and to recognise their ligands once they have been endocytosed (Heil et al. 2004). **RLRs** have been recently discovered and play a key role in sensing RNA virus invasion. They recognise viral RNA independently of TLRs and unlike TLRs, which are found either on the cell surface or endosomes RLRs are found in the cytoplasm where cellular RNA is also present (Takeuchi & Akira 2009). The two main RNA helicases are RIG-I (retinoic acid inducible protein I) and MDA5 (melanoma differentiation associated gene 5). It has been shown that RIG-I and MDA5 recognise different viruses and different viral RNAs (Kato H et al. 2006;Loo et al. 2008) (Fig. 1). RIG-I recognizes single-stranded RNA (ssRNA) containing a terminal 5 -triphosphate (ppp)(Pichlmair A et al. 2007), as well as linear dsRNA no longer than 23 nucleotides (Kato et al. 2008). MDA5 recognises long strands of dsRNA but the mechanism by which this occurs is less clear (Kato, Takeuchi, Mikamo-Satoh, Hirai, Kawai, Matsushita, Hiiragi, Dermody, Fujita, & Akira 2008). **The nucleotide-binding domain, leucine rich containing** (NLR) protein family is a recent addition to the members of innate immunity effector molecules. NLRs play key roles in the cytoplasmic recognition of whole bacteria or their products. NLR proteins comprise a diverse protein family (over 20 in humans), indicating that NLRs have evolved to acquire specificity to various pathogenic microorganisms, thereby controlling host-pathogen interactions.

The aim of this meeting is to provide an overview of these three families of receptors and provide the most recent advances in the area of innate immune pattern recognition

Meeting chairs: **:** Dr Martha Triantafilou/Professor Kathy Triantafilou, Cardiff University School of Medicine, UK

This event has CPD accreditation and will have a troubleshooting panel session.
On registration you will be able to submit your questions to the panel that will be asked by the chair on the day of the event

8:45 – 9:15 **Registration**

9:15 – 9:30 **Introduction by the Chairs:** Dr Martha Triantafilou/Professor Kathy Triantafilou, Cardiff University School of Medicine, UK

9:30 – 10:00 **NLR activation - what we do and don't know**
Dr Tom Monie, University of Cambridge, UK
This talk will focus on the mechanisms of NLR activation, particularly in the NLRC subfamily. It will introduce our current understanding of how these receptors are activated; how they are regulated; and how they signal within a cell. Recent advances in the areas of ligand recognition and protein:protein interactions will be introduced

10:00– 10:30 **Intracellular DNA recognition by the innate immune system**
Professor Veit Hornung, Universitätsklinikum Bonn¸Germany
A central function of our innate immune system is to sense microbial pathogens by the presence of their nucleic acid genomes or their transcriptional or replicative activity. In mammals, a receptor-based system is mainly responsible for the detection of these "non-self" nucleic acids. Tremendous progress has been made in the past years to identify host constituents that are required for this intricate task. With regard to the sensing of RNA genome based pathogens by our innate immune system, a picture is emerging that includes certain families of the toll-like receptor family and the RIG-I like helicases. At the same time, intracellular DNA can also trigger potent innate immune responses, yet the players in this field are less clear. In this talk an update is given on our latest progress on intracellular DNA sensing by the innate immune system.

10:30 – 11:00 **Mid-morning break and Poster Viewing**

11:00– 11:30 **Assembly and Regulation of TIR/Death Domain Complexes in IL-1 and TLR Signalling**

Dr Nicholas Gay, University of Cambridge, UK

An important theme to emerge from molecular studies in the last two years is that the MyD88 dependent Toll pathways are dependent on initial stimulus induced dimerization of the receptors TIR domains and the assembly of TIR domain containing adaptor proteins, notably MyD88 but also TRAM, TRIF and MAL/TIRAP. This leads to the formation of highly oligomeric, membrane associated complexes that act as a scaffold for numerous signaling and regulatory molecules that act in these pathways. Most remarkable has been the characterization and structural analysis of the Myddosome, a complex formed by the death domains of the MyD88 and IRAK signaling adaptors. Thus, TLR and IL1 signal transduction depends on the cooperative assembly of macro-complexes that act as a scaffold for the complex signaling responses induced.

11:30 – 12:00 **Understanding innate immunity to rhinovirus infection in asthma**

Dr Michael Edwards, National Heart Lung Institute, London, UK

Asthma exacerbations are frequently caused by rhinovirus infection. Asthmatic individuals suffer exaggerated inflammatory responses and deficient innate anti-viral responses. Both harmful, pro-inflammatory responses and beneficial anti--viral reponses are initiated by infection of the bronchial epithelium, naturally infected by rhinovirus in vivo. Understanding the signalling pathways involved in rhinovirus infection that evoke both pro-inflammatory cytokines and beneficial type I and type III intefreons may identify novel therapeutic targets that can alleviate inflammation or boost natural interferon production. This presentation will discuss the role of RLH and TLR signalling in inflammation and host defense in response to rhinovirus infection in asthma.

12:00 –13:00 **Lunch and Poster Viewing**

13:00 - 14:00 **Question and Answer Session and Speakers photo**

Delegates will be asked to submit questions to a panel of experts. Questions can be submitted before the event or on the day

14:00 – 14:15 **Toll-like receptor recognition of hepatitis a virus**

Joanna Hayward, Brighton & Sussex Medical School, UK

Our experiments established that HAV is recognized primarily by TLR-7 and TLR-8 via its viral ssRNA. Thus leading to IFN-γ and IL-6 secretion which cause massive inflammation and induce secretion of the acute phase proteins, explaining the high aminotransferase levels seen in HAV infection. This information may help identify possible targets for the development of TLR-based drug therapy to treat symptoms of HAV in people whose hepatitis is more severe and vulnerable patients with pre-existing liver disease.

14:15 – 14:45 **Inflammasome activation in atherosclerosis**

Professor Eicke Latz, University of Bonn, Germany

We demonstrate that crystals activate the NLRP3 inflammasome in a process that requires phagocytosis and we found that crystal uptake leads to lysosomal damage and rupture. Furthermore, sterile lysosomal damage is also sufficient to induce NLRP3 activation and inhibition of phagosomal acidification or inhibition or lack of cathepsins impairs NLRP3 activation. These results indicate that the NLRP3 inflammasome can sense lysosomal damage as an endogenous danger signal. Our results demonstrate a novel strategy of immune cells to recognize different classes of stimuli by a common, indirect mechanism. We have recently demonstrated that cholesterol crystals can be recognized by the NLRP3 inflammasomes and contribute to inflammation in atherosclerotic plaques. We are currently developing novel therapeutic approaches for atherosclerotic disease that are based on the solubilization of cholesterol crystals.

14:45 – 15:15 **TLR dependent cytokine networks regulating human inflammation**

Professor Ian Sabroe, University of Sheffield¸UK

TLRs initiate inflammation by induction of cooperative responses between cells and between cytokines. This talk will illustrate the networks that TLRs activate to induce inflammation, and show data from models of LPS responses, viral inflammation, and human models of LPS challenge

15:15 – 15:45 **Afternoon Tea/Coffee and Last Poster Viewing**

15:45 – 16:15 **Bacterial Recognition by Pattern Recognition Receptors**

Dr Clare Bryant, University of Cambridge, UK

Bacteria are recognized by many PRRs. LPS produced by Gram negative bacteria activates TLR4/MD2. Other bacterial ligands, such as the protein toxin pneumolysin from Streptococcus pneumoniae, activate TLR4/MD2, but the mechanisms by which this occurs are poorly understood. Salmonellae are recognized by TLRs and NLRs.

NLRP3 and NLRC4 drive IL1b production, but only NLRC4 causes cell death and regulates intracellular bacterial number in response to salmonellae. Species differences in the number and cohort of NLRs between mammals and birds suggest it is unclear how cytosolic recognition of salmonellae occurs in many species where NLRC4 is either truncated or absent.

16:15 – 16:45 **The inflammatory contribution of toll-like receptors in rheumatoid arthritis**
Dr Sandra Sacre, Brighton and Sussex Medical School, UK
Rheumatoid arthritis (RA) is a chronic inflammatory disease of the joints. A potential role for Toll-like receptors (TLRs) in the pathogenesis of RA has been proposed by many groups, and has become particularly relevant with the discovery that TLRs can recognise both pathogens and endogenous molecules that can be found at sites of inflammation and tissue damage. Using a human tissue model of RA, we have shown a link between TLR signalling and cytokine production and gone on to identify a potential role for the endosomal TLRs (TLR 3, 7, 8 and 9) and in particular TLR8.

16:45 - 17:00 **Chairman's summing up**

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Information about the chairs

Over the past few years the Triantafilou group has been focusing on unravelling the molecular mechanisms behind the innate recognition of bacterial as well as viral pathogens. In particular, we have focused on the involvement of the Toll-like receptor (TLR) family of proteins, a recently identified family of pattern recognition receptors (PRRs), in the innate immune sensing. We have the expertise and the research tools for investigating receptor interactions using bio-imaging techniques, such a Fluorescence Resonance Energy Transfer (FRET), Fluorescence Recovery after Photobleaching (FRAP), Single Particle Imaging (SPFI), Single Particle Tracking (SPT), Fluorescent Loss in Photobleaching (FLIP) as well as live cell imaging. Using combinations of these techniques, our group has discovered novel concepts in innate immune recognition of microbial ligands by TLRs and co-operating PRRs. We have been one of the first to demonstrate that the single-receptor concept of innate immune recognition is an oversimplified one and that different combinational associations of receptors determine the innate immune response to different microbial pathogens, using a range of non-invasive biophysical techniques. We performed several studies investigating associations of PRRs in response to bacterial products from Helicobacter pylori,Neisseria meningitidis, and bacterial lipopeptides. Furthermore, we demonstrated that membrane microdomains, or "lipid rafts" play an important role in this receptor cluster formation by providing a microenvironment for these interactions to take place. This was the first ever publication demonstrating that TLRs exist and signal within lipid rafts (making this paper one of the most cited papers in the field). We provided the first dynamic picture of TLR engagement by their ligand by determining the lateral diffusion of receptors involved in the innate immune response before and after stimulation by bacterial products . It has helped us understand the organisation, lateral mobility and confinement of PRRs involved in the innate immune response on the plasma membrane. In addition, using fluorescent imaging, we have revealed that TLR2 exists as a heterodimer prior to ligand engagement, as well as its intracellular trafficking and targeting in response to Gram-positive bacterial products. More recently, we have demonstrated that CXCR4 acts as a negative regulator for TLR2 and its significance in the innate recognition of Porphyromonas gingivalis (Hajishengallis et al. 2008). This was the first study demonstrating that TLR2-CXCR4 association can impair innate immune responses. Finally, we have shown that TLR4, TLR7 and TLR8 are involved in sensing viral products. These were the first studies to reveal how enteroviruses are recognised by the innate immune system.

About the Speakers

Tom Monie is a Wellcome Trust Career Development Fellow who initially began researching viruses in order to understand how they successfully take over cellular function. He moved into the innate immune field working on structural and functional studies of TLR4. In 2008 he started his own group studying the NLR proteins NOD1 and NOD2. He is particularly interested in investigating how these proteins recognise their ligands and how their interaction with other cellular proteins impacts on the response to infection and development of disease

Clare Bryant -1985 BSc (Hons) Biochemistry and Physiology, University of Southampton, 1989 BVetMed, University of London,1992 PhD, University of London. 1992-1995 Wellcome Trust Veterinary Research Training Fellowship, Royal Veterinary College, University of London, 1995-1996 Research Scientist, William Harvey Research Institute, London, 1996-2000 Wellcome Trust Research Career Development Fellow and 2000-2003 Wellcome Trust Research Advanced Fellow, Department of Clinical Veterinary Medicine, The University of Cambridge, 2003-University Lecturer and Senior Lecturer in Clinical Pharmacology, Department of Veterinary Medicine, The University of Cambridge. Research Interests: Role of Pattern Recognition Receptors (PRRs) in bacterial infection; species specificity in PRR activation.

Michael R. Edwards Professional Qualifications: BSc (Hons), 1995, PhD 2001. Joined the laboratory of S.L. Johnston in 2001, became an Asthma UK Research Fellow, 2007. Research Interests & Goals are to understand the molecular and cellular basis of virus-induced asthma exacerbations, focusing on innate host responses involved in inflammation and host defence. Ultimately, to identify novel therapeutic targets for virus-induced asthma exacerbations

Dr Sandra Sacre completed her PhD exmaining the role of annexins in cardiovasular disease in 2000 at University College London (UCL). She then spent one year as a postdoc at the Royal Free Hospital (UCL) focusing on ApoE receptor signalling before moving to the Kennedy Institute of Rheumatology (part of Imperial College London), to work on toll-like receptors in rheumatoid arthritis. In November 2009, Sandra moved to Brighton and Sussex Medical School at the University of Sussex to set up her own laboratory where she continues to work on TLRs in rheumatic diseases.

Veit Hornung received his M.D. from the University of Munich (Germany) in 2003, and his postdoctoral training from the University of Munich and the University of Massachusetts Medical School in Worcester (USA). In 2008 he then joined the University of Bonn (Germany), where he is currently a full professor in the Institute of Clinical Chemistry and Pharmacology. Veit Hornung's research focuses on the recognition of nucleic acids by the innate immune system.

Ian Sabroe is a practicing respiratory physician with interests in asthma, COPD and pulmonary hypertension, and is Professor of Inflammation Biology at the University of Sheffield. His work has focused on identifying expression of TLRs and their functions in innate immune cells, exploring how innate immune cells and tissue cells cooperate to mediate the inflammatory response, and how these responses regulate responses to bacteria and viruses. Recent work has examined new models of inflammation in the human to allow probing of TLR signalling.

NIck Gay worked with Prof. John Walker (Nobel Prize for Chemistry 1997) on the F1Fo ATPase for his PhD and then spent a postdoctoral period at UC San Francisco with Tom Kornberg. Since returning to the UK in 1987 the lab has worked on Toll signalling initially as a developmental system in Drosophila and more recently as key regulators of innate immunity.

Eicke Latz graduated from the Free University and Humboldt University in Berlin, Germany. He received clinical training in the field of Intensive Care Medicine at the Charité University Medical in Berlin and has worked on the molecular recognition of bacterial lipopolysaccharides in the Laboratory of Molecular Sepsis Research at the Humboldt University in Berlin. He has received postdoctoral research training in the pharmaceutical industry and at Boston University and the University of Massachusetts. He is currently appointed as an Assistant Professor at the Department of Infectious Diseases and Immunology at the University of Massachusetts and he is the Director or the Institute of Innate Immunity at the University of Bonn in Germany.

TOLL-LIKE RECEPTOR RECOGNITION OF HEPATITIS A VIRUS

J Hayward, K Triantafilou, M Triantafilou.
Stone Lodge, High Street, Ninfield, Battle, East Sussex,

Background: Hepatitis A is an acute infectious disease of the liver caused by the hepatitis A virus (HAV), which is by ingestion of contaminated food or water or through direct contact with an infectious person. Despite an effective vaccine for HAV, there is still poor uptake in some areas and epidemics of HAV are still occurring across the world. HAV can cause severe disease in adults and may in some cases be fatal. Previous studies have found some success with TLR-antagonists in treating certain inflammatory diseases. A further knowledge of the way HAV interacts with TLR's may pave the way for the development of a successful symptomatic treatment of HAV. To determine which TLR's were activated hepatocytes were infected with live HAV or viral ssRNA HAV as well as UV inactivated HAV, and TLR expression was determined by indirect immunofluorescence and flow cytometry. Live HAV as well as ssRNA elicited an increase in the expression of TLR-7 and TLR-8, but did not activate TLR-2 or TLR-4. UV-inactivated HAV did not activate any of the TLR's tested for.

Infection of hepatocytes with viral ssRNA and live HAV caused secretion of IFN-γ and IL-6.
Furthermore phospho-κB was detected in HEK TLR-7 and HEK TLR-8 cells after incubation with viral ssRNA or live HAV. To verify the involvement of TLR-7 and TLR- 8 in HAV recognition, TLR-7 and TLR8 were silenced using psiRNA and stimulated with HAV or viral ssRNA. The data showed a significant reduction in IFN-γ expression.

Confocal microscopy confirmed significant overlay of TLR-7 and TLR-8 with HAV.

Our experiments established that HAV is recognized primarily by TLR-7 and TLR-8 via its viral ssRNA. Thus leading to IFN-γ and IL-6 secretion which cause massive inflammation and induce secretion of the acute phase proteins, explaining the high aminotransferase levels seen in HAV infection. This information may help identify possible targets for the development of TLR-based drug therapy to treat symptoms of HAV in people whose hepatitis is more severe and vulnerable patients with pre-existing liver disease.

THE RECOGNITION OF HERPES SIMPLEX VIRUS BY CYTOSOLIC INNATE IMMUNE SENSORS

Callum Harmer[a] , Professor Kathy Triantafilou[b]

[a]*4th Year Medical Student, Brighton and Sussex Medical School, The Audrey Emerton Building, The Royal Sussex County Hospital, Eastern Road, Brighton, East Sussex, BN2 5BE United Kingdom, c.harmer1@uni.bsms.ac.uk.*
[b]*Professor of Paediatric Immunology, Department of Child Health, Department of Child Health, Cardiff University School of Medicine, UHW Main Building, Heath Park, Cardiff, United Kingdom.*

Background: Since the discovery of Toll-like receptors (TLRs) by Medzhitov and Janeway there has been increasing scientific interest in the innate arm of the immune system. Most recently there is accumulating evidence that DNA-dependent Activator of Interferon-regulatory factor (DAI) and Absent in Melanoma 2 (AIM2) also act as cytoplasmic recognition receptors for the innate immune system, mediating host immune responses to DNA viruses such as the Herpes Simplex Virus 2 (HSV2). HSV2 has a high prevalence and represents a large economic burden. The aim of the current study was to establish the role of these potential cytoplasmic pattern recognition receptors in the innate immune response to HSV2.

Methods: Indirect immunofluorescence was initially used to investigate levels of the potential Pattern Recognition Receptors (PPRs) of interest in a HeLa epithelium cell line population after stimulation with HSV2, and confocal microscopy used to visualise the location of the prospective sensors. Cytometric bead array with flow cytometry and SDS-PAGE with western blotting were then used to determine whether any increase in receptor number seen after stimulation with the virus led to a meaningful activation of the immune response, an upregulation of cytokine expression and downstream intracellular components. Finally, knockout using RNAi was used to confirm any activation of the innate immune response was due to the initial activation of the suspected cytoplasmic PPRs.

Results: Increases in DAI receptor expression levels and cytokine expression after HSV DNA stimulation were shown to by DAI-dependent, whereas AIM2 failed to increase in expression or mediate a cytokine response, suggesting that DAI and not AIM2 may have a role in the recognition of HSV2 during infection, which is mediated by DNA binding of the receptor. An increase in mean fluorescence after stimulation with the whole virus compared to HSV2 DNA may suggest that another viral component is recognised and upregulates DAI expression, and is therefore also involved in the initial immune response to HSV2 infection.

THE INNATE RECOGNITION OF HUMAN RHINOVIRUS 16 BY RNA HELICASES MDA-5 AND RIG-I IN HUMAN AIRWAY EPITHELIAL CELLS.

Heena Mistry,[1] Dr Kathy Triantafilou and Dr Martha Triantafilou,[2].
[1] Brighton and Sussex Medical School, Falmer, Brighton UK; [2] Department of Child Health, Cardiff University School of Medicine, UHW, Heath Park, Cardiff UK.

Background: Human Rhinovirus-16 is a member of the family of picornaviruses. These viruses are the most frequent cause of acute upper respiratory tract infections in humans, most commonly known as the “common cold”. Symptoms include runny nose and blocked nasal passages, accompanied by sneezing, strep throat, coughing, chills and fatigue. The virus replicates in human airway epithelial cells. Infections can make the upper respiratory tract less resistant to bacterial infections, which can result in lower respiratory tract infections such as Pneumonia and Chronic Bronchitis. This study focuses on the innate recognition of Human Rhinovirus-16 by RNA-helicase receptors RIG-I and MDA5, which are present in the cytoplasm of human airway epithelial cells. Our aim was to determine which specific components of HRV-16 are recognised by the RNA Helicases MDA-5 and RIG-I, and in turn, determine which cytokines are released when activation of these receptors triggers an inflammatory response.

Methods: Using Indirect Immunofluorescence Cell Surface, SDS-PAGE, Immunoblotting, Cytometric Bead Array (CBA) technology, RNA interference using silencing plasmids and Confocal microscopy, we would like to determine which RNA helicases are involved in recognising viral components of HRV-16 and the expression levels of specific cytokines IL-2, IL-4, IL-6, IL-10, TNFα, IFNy and IFNβ released when specific components of HRV-16 are recognised by MDA-5 and RIG-I.

Results: HRV-16 and HRV-16 dsRNA is recognised by MDA5, which results in the activation of an inflammatory response that involves the secretion of IFNβ. These results are in addition to previous studies that identified TLR2, TLR7/8 which recognise two different components of Human Rhinovirus-16, these are HRV-16 capsid and HRV-16 ssRNA respectively. TLR activation results in an inflammatory response which leads to the secretion of cytokines IL-6 and IL-4.

Conclusions: In conclusion, the complex recognition of human rhinovirus involves sensing of different viral components by TLR2, TLR7, TLR8 and MDA5. Activation of these receptors leads to a powerful innate immune anti-viral inflammatory response that produces in particular IL-6 and IFNβ with the aim of eradicating the virus from the body. Using this knowledge about virus recognition by host cells, there is a potential for manipulating components of the overall signalling mechanism in the pathway to develop therapies to hinder the inflammatory responses and over all symptoms of the common cold.

Keywords: Innate Immunity, DNA sensing, Inflammasome, Type I interferon, RLHs, TLRs, Interferon, rhinovirus, asthma, TLR4, rhinovirus, LPS, TLR, signal transduction, scaffold complexes, NLR, PRR, LPS, Salmonella, Innate Immunity, atherosclerosis, NLRP3, Inflammasome, IL-1