

TLRs (Toll-like receptors), NLRs (Nod-like proteins) and RLRs (RIG-like receptors), pathogens sensors of innate immunity

The BioPark Hertfordshire, Welwyn Garden City, AL7 3AX: Friday, October 09, 2009

"Significant advances in our understanding of the innate immune recognition have been made in the last decade following the identification of three families of pattern recognition receptors: Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). TLRs are pattern recognition receptors that detect motifs or signatures from bacteria, viruses, protozoa and fungi. NLRs detect mainly intracellular bacteria and RLRs detect viral genome. These three families of pattern recognition receptors comprise the front line of defence that the host possesses against microbial pathogens. 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". Chairs: Dr Martha Triantafilou/Dr Kathy Triantafilou, University of Sussex, UK

This meeting has CPD accreditation

- 9:00 – 9:45 Registration
- 9:45 – 10:00 Introduction by the Chairs: Dr Martha Triantafilou/Dr Kathy Triantafilou, University of Sussex, UK
- 10:00 – 10:30 Immune recognition of fungal pathogens
Professor Neil A.R. Gow, University of Aberdeen, UK
Candida albicans is the most common agent of life-threatening human disease due to a fungus. We have constructed a series of mutant strains with alterations in *C. albicans* cell wall biosynthesis and used these to explore the role of the glycans on fungal pathogenesis. Cytokine production by mononuclear cells or dendritic cells results from the detection of multiple wall components, singly and in combination. Other cell wall components block or shield the fungus from immune recognition by TLRs and lectin receptors. Therefore fungal recognition by the immune system is a complex and dynamic process triggered by multiple signals and multiple receptor complexes.
[Reference: Netea *et al* (2008) Nat Rev Microbiol 6, 67-78
- 10:30 – 11:00 Structure-function relationship of Toll-like receptor domains in different species and their potential impact on vaccine design
Professor Dirk Werling, Royal Veterinary College, UK
Toll-like receptors (TLRs) are a family of pattern recognition receptors that are an important link between innate and adaptive immunity. Many vaccines incorporate ligands for TLRs as an adjuvant and are developed in rodent models, with the resulting data transferred to other species. Vaccine features can be improved markedly by emphasizing the biological relevance when evaluating other animal models for host-pathogen interaction and by taking greater advantage of the unique experimental opportunities that are offered by large animal, non-rodent models. In the present talk, I will summarize our current knowledge of species-specific TLR responses and briefly discuss that vaccine efficacy in relevant host species might be improved by considering the species-specific TLR responses
- 11:00- 11:10 Speakers photo
11:10 – 11:30 Mid-morning break
- 11:30 – 12:00 RIG-like helicases and viral antagonists
Professor Steve Goodbourn, University of London, UK
The RNA helicases, RIG-I and mda-5, recognise non-self RNA molecules generated in the cytoplasm, and signal through a common downstream adaptor to activate the transcription factors IRF-3 and NF-kappaB. These in turn signal the activation of an innate anti-viral program, including the production of type I interferon. In order to replicate efficiently, viruses must counter this system. This talk will focus on the mechanism of activation of RIG-I and mda-5 by viral RNAs, and their specific antagonism by viral proteins such as the paramyxovirus V protein and the influenza A virus NS1 protein.
- 12:00 – 12:15 Bacterial TIR-like proteins: evading the host innate immune response
Rohini Rana, Imperial College London UK
The intracellular Toll/Interleukin-1 (TIR) domain of Toll-like receptors, recruits downstream adapters initiating signaling cascades, which results in proinflammatory responses. TIR-like proteins (TLPs) from pathogenic bacteria are suggested to facilitate pathogen evasion of the immune response by interfering with host TIR signaling. This

project aims to characterize YpTIR from the plague causing bacterium, *Yersinia pestis*. Interaction between YpTIR and human MyD88, a major adaptor protein, has been detected using pull-down assays. 1D-HSQC-NMR spectra for YpTIR indicates the presence of structured domains and that the protein may be multimeric. Currently YpTIR crystals are being optimized.

- 12:15 – 12:30 **Innate immune signalling in response to francisella tularensis**
Richard Saint, Biomedical Sciences, Dstl Porton Down, Salisbury, Wiltshire, SP4 0JQ, UK
Toll-like receptor (TLR) signalling, in response to infection by *Francisella tularensis*, was examined both in vitro and in vivo. The activation of key factors, by phosphorylation, was monitored using western blotting with the results being quantified by densitometry. Using the activation profiles of each factor it is possible to build a picture of the TLR-initiated immune response to *F. tularensis* throughout the course of the infection.
- 12:30–13:30 **Lunch and Poster Viewing**
- 13:30 – 14:00 **Pattern recognition receptors and the host detection of bacterial infection**
Dr Clare Bryant, University of Cambridge, UK
Bacterial infection continues to cause major disease problems despite the availability of antibiotics. We work on determining which Pattern Recognition Receptors (PRRs) detect important bacterial pathogens (specifically *Salmonella enterica* serovar Typhimurium and *Streptococcus pneumoniae*). Lipopolysaccharide is a component of the Gram-negative bacterial cell wall and its detection by TLR4 and MD-2 drives protective immunity in the host. We work on how TLR-4 and MD-2 detects lipid A structures and our comparative cross species analysis correctly predicted how the active TLR4/MD-2 signaling complex was formed. This talk will focus on PRR recognition of Gram positive and Gram-negative bacteria.
- 14:00 – 14:30 **Pathogen pattern recognition by Toll-like receptors – 20 years on**
Dr Nick Gay, University of Cambridge, UK
In my talk I will describe the molecular mechanisms by which these conserved pathogen associated molecules are recognized by the TLRs with particular reference to lipo polysaccharide and single stranded viral RNAs. I will also present new results which show how receptor activation is coupled to downstream signal transduction and in particular the role played by oligomeric signaling platforms assembled from adaptors and other signaling molecules involved in the pathway. I will discuss the potential for structural analysis to be used in the rational design of new drugs.
- 14:30 – 14:45 **An Epithelial Innate Response mechanism that distinguishes between commensal and pathogenic states of *C. albicans***
Dr David Moyes, Department of Oral Immunology, King's College London, London, UK
Host mechanisms enabling discrimination between commensal and pathogenic organisms are critical in mucosal immune defense and homeostasis. The polymorphic human fungal pathogen *Candida albicans* can act as a commensal or pathogen and as such can be used as a model organism to identify how epithelial cells can discriminate between commensal and pathogenic organisms. We will describe the intracellular mechanisms that enable oral epithelial cells to discriminate between the avirulent yeast and virulent hyphal forms of *Candida albicans*.
- 14:45 – 15:00 **A modified adenine chemically coupled to allergen molecules redirects allergen-specific T_H2 cells.**
Lucia Fili, Laboratory of Immunology, Viale Pieraccini 6, 50134, Firenze, Italy
Several immune response modifiers, such as CpG-ODNs, have been proposed and used as novel adjuvants controlling the overexpression of TH2-related cytokines and chemokines and providing clinical benefit. In this study a modified adenine coupled with *Dermatophagoides pteronyssinus* or natural Der p2 induced innate cells to produce type I interferons and IL-12 at similar levels than other TLR ligands as R-848 or LPS by inducing NF- κ B translocation. Consequently, both DP- and Der p2-conjugates reverted TH2-prone allergen T-cell lines into IFN- γ -producing cells (TH1/TH0 phenotype). The maintenance of modulatory effects of these complexes suggest new opportunities for the development of novel vaccine strategies.
- 15:00 – 15:30 **Afternoon Tea/Coffee and Last Poster Viewing**
- 15:30 – 16:00 **ITAM-coupled receptor signaling and the Nalp3-inflammasome in anti-fungal immunity**
Dr Olaf Gross, University of Lausanne, Switzerland
Fungal infections represent a serious health threat, especially in immunocompromised patients. However, our knowledge about the activation of innate immune responses by fungi is quite limited. IL-1 β is a key cytokine in the activation and orchestration of innate and adaptive immunity. C-type lectin-like pattern recognition receptors bind to

different fungal molecular patterns and activate dendritic cells and macrophages via a signalling pathway including the proteins Card9, Bcl10, and Malt1. Through the transcription factor NF-kappaB, this pathway controls the expression of pro-inflammatory cytokines including pro-IL-1beta. In a second step, an inflammasome complex containing the proteins Nlrp3, Asc, and Caspase-1 cleaves pro-IL-1beta into its secreted and bioactive mature form. Both processes, pro-IL-1beta production and -cleavage depend on the receptor-associated kinase Syk.

16:00 – 16:30 **CARD tricks - more than just a magic show**

Dr Tom Monie, Wellcome Trust Career Development Fellow, Cambridge

Homotypic interactions between the effector domains of NLR proteins are essential for the propagation of signal transduction and the activation of the inflammasome. This talk will discuss the mechanisms by which these interactions are mediated with a particular focus on the caspase activation and recruitment domain (CARD):CARD interactions of NOD1 and its signalling partner the serine/threonine kinase RIP2.

17:00 **Chairman's summing up.**

This meeting was organised by Euroscicon (www.euroscicon.com), a team of dedicated professionals working for the continuous improvement of technical knowledge transfer to all scientists. Euroscicon believe that they can make a positive difference to the quality of science by providing cutting edge information on new technological advancements to the scientific community. This is provided via our exceptional services to individual scientists, research institutions and industry. The event was hosted by 'BioPark' (www.biopark.co.uk), a research and development centre in Welwyn Garden City providing specialist facilities and support for bioscience and health technology businesses to grow, and to develop new products and technologies

About the chairs

The research focus of the Infection and Immunity group at the University of Sussex is host-pathogen interactions and, in particular, the innate recognition of bacteria and viruses by the immune system. Recognition of bacterial products by the innate immune system elicits strong pro-inflammatory responses that can eventually cause fatal sepsis syndrome in humans. Our long-term aims are to unravel the mechanisms involved in the innate recognition of bacteria, and to identify potential therapeutic targets for bacterial sepsis. Over the past few years our group has focused on identifying cellular receptors that recognise bacterial cell wall components such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA). Utilising a combination of proteomics and fluorescent imaging techniques we have identified a cluster of receptors that are capable to bind and recognise bacterial LPS (Triantafyllou, et al. 2001 Nat. Immunol.). Furthermore we have found that membrane microdomains, or "lipid rafts" play an important role in this cluster formation by providing a microenvironment for these interactions to take place (Triantafyllou et al. 2002 J. Cell Science, Triantafyllou et al. 2003 J. Endotoxin Res). Currently we are postulating that different combinational associations of receptors determine the innate immune response to different microbial pathogens (Triantafyllou and Triantafyllou, 2002 Trends Immunol). In addition we are investigating receptors that are utilised by enteroviruses. We have previously identified receptors for Coxsackievirus A9 (Triantafyllou et al. 1999, 2002) and Human Parechovirus 1 (Triantafyllou et al. 2001). Currently we are investigating the innate recognition of viruses by Toll-like receptors as well as RNA helicases.

About the Speakers

Professor Neil A.R. Gow is a former BMS President and current Vice-President of ISHAM and editor in Chief of Fungal Genetics and Biology. His research is focussed on the growth, morphogenesis and pathogenesis of the human fungal pathogen *Candida albicans* and other medically important fungal species. In recent years he has focussed mainly on the genetics of glycosylation and the fungus-host interaction, and on chitin synthesis and hyphal orientation responses.

Professor Steve Goodbourn, University of London, UK, 1979 BA (Hons) Biochemistry, University of Oxford, 1983 D.Phil Clinical Medicine, University of Oxford, 1983-1987, Post-doctoral fellow, Harvard University, 1987-1994, Head of Gene Expression Group, ICRF, 1994- present Senior Lecturer, Reader and the Professor at St. George's, University of London. Research Interests; Innate immunity and Viral evasion strategies

Dr David. Moyes graduated from Birmingham University and went to work at Hammersmith Hospital on Innate Immunity and Septicaemia. He then went to Harefield Hospital for a PhD investigating interferon-gamma signalling in endothelial cells. After this, he went to The Kennedy Institute, Imperial College, working on the role of retroviruses and microbial protein modifying enzymes in autoimmune aetiology. He now works for Dr. Julian Naglik at the Dental Institute, King's College London, investigating the role of epithelial cells in host responses to microbial pathogens – in particular *Candida albicans* and HIV.

Rohini Rana moved from Kathmandu to Rai University, New Delhi to start her undergraduate studies. She completed her BSc(Hons)Biotechnology degree with a first class from Northumbria University, Newcastle-upon-Tyne. She worked under Prof. Gary Black on a putative exoarabinase from *Streptomyces coelicolor* at Northumbria University, and the isolation of human chondrocytes from arthritic bone tissues at Aeirtec, as part of her industrial placement. Her final year project was on the bioinformatics analysis of lipoproteins encoded in the *Thermus thermophilus* genome. She is currently pursuing her PhD on characterization of bacterial TIR-like proteins at Imperial College London under Dr. Bernadette Byrne's supervision.

Lucia Fili was born in Florence in 1977. Graduated in Biological Sciences at University of Florence with full marks and honours in 2003. Since 2001 attended the department of Immunology and Cell Therapies (head Prof. S. Romagnani) devoted to study and cure immune-mediated diseases, allergies and immunodeficiencies. In 2003 winner of a research fellowship for 3 years sponsored by the European Community. In 2009 achieved PhD degree in Clinical and Experimental Medicine at University of Florence discussing the thesis "In vitro effects of natural and synthetic TLR ligands on human immune cells".

Dr Nicholas 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.

Dr Tom Monie did his PhD in the Molecular Virology of Retroviruses at Cambridge and then moved to Imperial College to study the structure and function of translation initiation factors. He returned to Cambridge, changing fields once again to work with Dr Nick Gay on the role of TLRs in innate immunity. Since October 2008 he has held a Wellcome Career Development Fellowship to study the mechanisms of ligand recognition and signal transduction in the NLRs NOD1 and NOD2.

Richard Saint completed his degree in Biomedical Sciences, at The University of Edinburgh, in 2007. Since then he has been working at Dstl as a research microbiologist and is also studying for a PhD in Veterinary Medicine from The University of Cambridge.

Dr 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.

Professor Dirk Werling graduated from the University of Veterinary Medicine in Hannover (Germany) in 1991, and received a PhD in Virology at the University of Zuerich This was followed by an EU-Marie Curie Research Fellowship in the lab of Chris Howard at the Institute for Animal Health (IAH Compton, UK). During this period, they described the functional characterisation of bovine monocyte derived dendritic cells. He then moved back to the Swiss Federal Institute of Technology (ETH) Zuerich as a group leader and senior scientist, continuing the work started on bovine innate immune cells. In 2001, he was appointed as assistant professor (tenure track) at the Immunology Division at the Institute of Veterinary Virology (University of Bern), before moving to the RVC in 2003. He was appointed Chair of Molecular Immunology in 2007.

Dr Olaf Gross studied biology at the Technical University of Munich. In his doctoral studies in the group of Jürgen Ruland in Munich, he investigated the role of the protein Card9 in anti-fungal immunity. Later on, he extended his work on fungal immunity into the field of inflammasome-mediated IL-1 β release. On the basis of an EMBO fellowship, he now works as a postdoc in the group of Jürg Tschopp in Lausanne.

POSTERS

EPITHELIAL INNATE RESPONSE MECHANISM THAT DISTINGUISHES BETWEEN COMMENSAL AND PATHOGENIC STATES OF THE HUMAN FUNGAL PATHOGEN *CANDIDA ALBICANS*

D. Moyes, M. Runglall and J.R. Naglik

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Innate immunity plays the vital role of 'gatekeeper' of the mucosal epithelia and is required to maintain homeostatic function and coordinate immunological reactions against commensal and pathogenic microbes. The specialised and complex interaction between microbes, epithelial cells and local immune cells results in either a degree of mutualism (commensalism) or a breach of the mucosal barrier and subsequent cell injury (pathogenicity). Immune responsiveness begins with pathogen recognition, usually through surface pattern recognition receptors, which activates signalling pathways and ultimately leads to an 'effector response' through the induction of a specific set of cytokines, chemokines and other immune mediators. Of particular interest are 'opportunistic' microbes such as *Candida albicans*, which is a polymorphic fungus that can cause mucosal disease in a significant proportion of immunocompromised individuals. *C. albicans* is the most common fungal pathogen of humans and the causative agent of oral, gastrointestinal and vaginal candidiasis. In this study we set out to determine how oral epithelium 'senses' and 'responds' to this important mucosal pathogen.

The innate immune response of oral (TR146) epithelial monolayers and organotypic reconstituted human epithelium to different *Candida* species and *C. albicans* yeast and hyphal forms was assessed. Gene expression was determined using real-time RT-PCR. Cytokine and chemokine levels were measured by multiplex micro-bead assay. Activation of signalling pathways was assessed by Western blotting and transcription factor activity by the TransAM system. Epithelial cell damage was evaluated by the release of lactate dehydrogenase. Functional analysis was verified using small chemical inhibitors, blocking antibodies and RNA interference.

Of the different *Candida* species, *C. albicans* had the most marked effect on oral epithelial cells. *C. albicans* induced greater tissue damage and a strong proinflammatory cytokine and chemotactic response (IL-1 α , GM-CSF, G-CSF, IL-6, IL-8, CCL20). However, oral epithelium was generally unresponsive to most TLR1-9 agonists, with the exception of TLR3 (Poly I:C) and TLR2 (PAM3CYSK4) agonists. Interestingly, most of the TLR genes were downregulated by *C. albicans*, with the exception of TLR2, which was upregulated. Of the fungal agonists tested (zymosan, curdlan, pustulan, glucan phosphate, mannan), only zymosan induced any noticeable cytokine response, albeit weak. *C. albicans* wild-type cells activated both the NF- κ B (I κ B) and MAPK (ERK1/2, JNK, p38) signalling pathways. For NF- κ B activation, both I κ B phosphorylation and p65 DNA binding activity increased with linear kinetics over time. However, the MAPK pathways (particularly ERK1/2 and JNK) were activated in a bi-phasic pattern with an early weak phase and a late strong phase. The early phase peaked at about 15 min post-stimulation and was associated with a temporary increase in c-Jun DNA binding activity, whilst the late phase peaked at 2 h and was associated with an increase in c-Fos DNA binding activity. This latter, second MAPK phase was also associated with phosphorylation of the MAPK-regulating phosphatase MKP-1. Stimulation of oral epithelial cells with non-filamentous or hyperfilamentous mutants or with pre-induced *C. albicans* hyphae indicated that NF- κ B activation and the early MAPK response was independent of morphology. However, the second MAPK phase constituting MKP-1 and c-Fos activation was hyphal dependent, but in a dose-dependent manner ($10^{4/5}$ cells or above) – doses typically found during oral infections in humans. A comparison of two *C. albicans* strains that either did or did not colonise a murine mucosal model indicated that the colonising strain did not produce hyphae in the presence of epithelial cells. The colonising strain also failed to induce cytokines, cause damage, or activate the second phase of the MAPK bi-phasic response.

In conclusion, only hyphal forms of *C. albicans* cause damage and activate the bi-phasic MAPK response, which constitutes MKP-1 and c-Fos activation. This results in the induction of a pro-inflammatory response that appears to lead to a protective host phenotype in vivo. The yeast phase appears to subvert the bi-phasic MAPK/MKP-1/c-Fos response resulting in the absence of inflammatory mediators, thus permitting the fungus to colonise mucosal surfaces without host challenge. Thus, we propose a mechanism enabling epithelial cells to distinguish between the commensal (yeast) and pathogenic (hyphal) states of the mucosal opportunistic pathogen *C. albicans* through the selective activation of MAPK signalling, MAPK phosphatases and transcription factors.

A MODIFIED ADENINE CHEMICALLY COUPLED TO ALLERGEN MOLECULES REDIRECTS ALLERGEN-SPECIFIC T_H2 CELLS.

L. Fili[§], P. Fanti[§], E. Cardilicchia, L. Maggi, C. Manuelli, A. Vultaggio, F. Nencini, S. Pratesi, F. Fassio, E. Occhiato[§], F. Annunziato, A. Guarna[§], E. Maggi, S. Romagnani and P. Parronchi.

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Objective: Allergen-specific immunotherapy (SIT) is the only treatment able to cure allergic diseases but exhibits side effects and limited efficacy. Although traditional SIT utilizes alum, several other immune response modifiers, such as CpG-containing oligodeoxynucleotides, have been proposed and used as novel adjuvants controlling the overexpression of T_H2-related cytokines and chemokines and providing clinical benefit. We have recently found that the 8-hydroxy substitutes of adenine can revert human allergen-specific T_H2 responses *in vitro*, and *in vivo* downregulate airway inflammation in OVA-sensitized mice via TLR7 triggering. In the present study we assessed a new compound able to be coupled with allergenic molecule(s) as a possible new adjuvant for vaccination protocols where T_H1 responses are hopeful.

Design & Method: 8-OH-substituted adenine SA-26E was chemically coupled with the allergen extract of *Dermatophagoides pteronyssinus* (DP-conj) or low endotoxin natural purified Der p2 (Der p2-conj). NF- κ B induction by both conjugates (or mock allergens) was evaluated in purified CD14⁺ cells as related to TLR triggering. Enzyme (cathepsin D)-digested conjugates were also assessed on TLR-transfected HEK293 cells to verify activation of endosomal expressed receptors. Cytokine and chemokine production by conjugated BDCA4⁺ and CD14⁺ cells was tested by specific ELISAs. Th-skewing effects by DP and Der p2-conjugates were investigated in allergen-specific human T cell lines, allergen-specific T-cell clones or CRTH2 highly purified T cells in comparison with mock allergens. IgE triggering was also assessed by basophil activation test by the use of mock or conjugated allergens. Finally, AHR, total and OVA-specific IgE were measured in C57BL/6 mice sensitized with OVA mock, OVA-conj or PBS (5 mice per group) and challenged with mock OVA.

Results: The chemical derivative of 9-benzyl-2-butoxy-8-hydroxyadenine (SA-2) with critical substitution in position 2 (SA-26E, ester) coupled with *Dermatophagoides pteronyssinus* (DP-conj) or natural Der p2 (Der p2-conj) induced innate cells to produce type I interferons (IFN) and IL-12 at similar levels than the dispersible TLR ligands such as R-848 or LPS. A lower induction of inflammatory IL-6 and TNF- α than R-848 was seen. Relevant IL-10 and CXCL10 production was also observed. As a consequence, both DP- and Der p2-conjugates reverted T_H2-prone allergen T-cell lines into IFN- γ -producing cells (T_H1/T_H0 phenotype) as assessed by cytokine production in the supernatants, intracellular cytokine expression, T_H-related transcription factor expression (GATA-3 and T-bet) and expression of T_H-related cytokines by real-time PCR. Non up-regulation of Tregs or Treg-related factors (IL-10, TGF β , Foxp3) or potentially harmful Th17-related cytokines was seen. Th-skewing effects of allergen-conjugates were confirmed at clonal level and potent down-regulation of IL-4 (together with modest up-regulation of IFN- γ) production was also seen in CRTH2-purified cells. Conjugation of allergen to modified adenine SA-26E also significantly reduced basophil activation mediated by surface IgE monitored by CD63 expression by cytofluorimetric analysis. Finally, OVA-conj sensitized mice were almost protected by AHR after intratracheal mock OVA-challenge and exhibited low circulating levels of both total and OVA-specific IgE.

Conclusion: 8-hydroxy-adenine(s) represent new compounds inducing NF- κ B translocation and exhibiting T_H1-inducing activity *in vitro* and *in vivo*. A system of chemical conjugation to relevant proteins as allergens has been set up with maintenance of modulatory effects of these complexes thus allowing the development of new vaccine strategies.

BACTERIAL TIR-LIKE PROTEINS: EVADING THE HOST INNATE IMMUNE RESPONSE

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Recognition of highly specific pathogen-associated molecular patterns by Toll-like receptors (TLRs) is the first step in host innate immune responses. The intracellular Toll/ Interleukin-1 (TIR) domain of TLRs, is responsible for recruitment of downstream adapter proteins through homotypic TIR-TIR interactions initiating signaling cascade, which results in proinflammatory responses. Recent work has indicated that TIR-like proteins (TLPs) from pathogenic bacteria interfere with host TIR signaling. It has been suggested that by binding to the TIR domains of the TLR receptors and/or the adaptor proteins, these bacterial proteins inhibit the signaling cascade and thereby facilitate pathogen evasion of the host immune response. This project aims to characterize the TIR domain of the TLP from the plague causing bacterium, *Yersinia pestis* (YpTIR). Interaction between His-tagged YpTIR and GST-tagged human MyD88, a major adaptor protein, has been detected using pull-down assays revealing MyD88 as a target of the bacterial TIR protein. One-dimensional HSQC NMR spectra for the highly pure YpTIR protein indicates the presence of globular and structured domains; however, broad peaks suggest the protein may be multimeric, possibly a dimer as seen in the recent structures of the TIR domain of TLR10 and of the TIR domain of the TLP from *Paracoccus denitrificans*. Native crystals of YpTIR have been obtained which diffract to 3 Å and currently crystals of seleno-methionine labeled YpTIR are being optimized. It is anticipated that the crystal structure of YpTIR should provide an insight into the molecular basis of TIR signaling and evidence of evolutionary conservation among TIR domains.

Acknowledgements: ¹Hu Nien-Jen, ³Monie Tom (¹Imperial College London UK, ³Cambridge University UK)



Natural Standard

SPECIES VARIATION IN TLR5 INTERACTIONS WITH SALMONELLA-DERIVED FLAGELLIN

H. J. Metcalfe^{*}, C. Bowman^{*}, A. Best[†], R. M. La Ragonet[†] & D. Werling^{*}

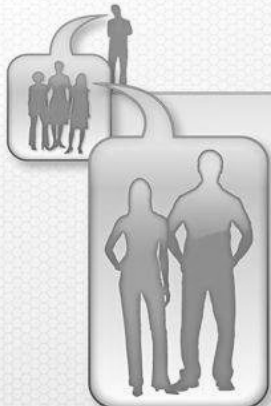
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Salmonella enterica (*Se*) is a world-wide, serologically diverse pathogen with the potential of causing zoonotic diseases. Present research aims to understand the mechanisms involved in species-specific immunopathology which could lead to more targeted immune therapies and disease control. *Se* can secrete flagellin which is the ligand for Toll-like receptor 5 (TLR5). To understand the importance of this interaction, we examined the role of flagellin during *Se* infection of macrophages (MΦ) from different species; and aimed to develop a method for detecting species-specific TLR5 responses. MΦ were challenged with *Se*-derived flagellin or infected with wild-type/flagellin-mutant *Se* isolates *in vitro*. Pro-inflammatory cytokine secretions, intracellular signalling and internalisation of bacteria were monitored. TLR5 from different species are being expressed in HEK293 cells to compare protein functionality using luciferase reporter assays and IL-8 secretion. Present work suggests that different flagellin-preparations induce different IL-8 responses and may interact differently with TLR5 from different species. Furthermore, internalisation of *Se* differs between MΦ from different species. The data from these primary MΦ studies will be considered in combination with the HEK293 cell system to extrapolate TLR5-flagellin responses independent of other TLRs and individual immune-regulation/restriction.

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INNATE IMMUNE SIGNALLING IN RESPONSE TO *FRANCISELLA TULARENSIS*

R. Saint, C. Bryant and H. Atkins

Biomedical Sciences, Dstl Porton Down, Salisbury, Wiltshire, SP4 0JQ, UK.

Aims:

To examine the activation of the key signalling factors involved in Toll-like receptor (TLR) signalling evoked by *F. tularensis* and to investigate possible targets for immune modulation to optimise the immune response to infection.

Methods:

TLRs are activated by a broad range of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide, flagellin and peptidoglycan. The activation of TLRs activates downstream signalling factors via modification of specific residues in their structure. This process was studied by monitoring the phosphorylation states of the host signalling factors, *in vitro* and *in vivo*, following infection with *F. tularensis* live vaccine strain (LVS). Phosphorylation was examined by western blotting with the results being quantified by densitometry.

Results:

Expression profiles for eleven key signalling factors were created. For example, *in vitro* assays demonstrated two peaks of increased phosphorylation (1.75 h and 4 h) of the mitogen-activated protein kinases (MAPKs), ERK-1 and ERK-2, after infection with *F. tularensis* LVS. Another MAPK, p38, showed rapid phosphorylation at 5 min post-infection followed by a rapid decrease in expression by 1 h. The other signalling factors investigated have exhibited a wide range of activation patterns.

Conclusions:

Using the activation profiles of each factor it is possible to build a picture of the TLR-initiated immune response to *F. tularensis*. The activation of the immune system is a key part of understanding the interaction between the host and *F. tularensis* and this work provides the foundation to unravelling this initial contact. In addition, this overall picture can be used to select suitable targets for modulation of the immune response to try and improve the outcome of infection.

GENETICAL ANALYSIS OF TOLL-LIKE RECEPTOR GENES IN GERMAN SHEPHERD DOGS REVEALS POTENTIAL POLYMORPHISM ASSOCIATED WITH INFLAMMATORY BOWEL DISEASE

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The identification of associations between specific polymorphisms in pattern recognition receptors such as Toll-like receptors (TLR) and human Inflammatory Bowel Disease (IBD) has provided data that demonstrates the importance of these genes and their role in innate immune function in the pathogenesis of this disease. To date no studies have been performed to evaluate polymorphisms in pattern recognition receptors in canine IBD. The aim of this study was to investigate whether polymorphisms in canine Toll-like receptor (TLR) 2, 4 and 5 genes are associated with IBD in German Shepherd dogs (GSDs).

Mutational analysis of TLR2, TLR4 and TLR5 was performed in 10 GSDs with IBD. Genomic DNA was extracted from blood stored in EDTA and the coding region of TLR2, TLR4 and TLR5 amplified using a polymerase chain reaction (PCR). PCR products were sequenced (Geneservice, Cambridge, UK) and full-length sequences obtained from each dog. Sequence data was compared to the canine genome (www.ensembl.org/Canis_familiaris). No non-synonymous single nucleotide polymorphisms (SNPs) were identified in the canine TLR2 gene. Four non-synonymous SNPs (T23C, G1039A, A1572T and G1807A) were identified in the TLR4 gene and three non-synonymous SNPs (G22A, C100T and T1844C) were identified in the TLR5 gene.

The four non-synonymous SNPs in TLR4 and three non-synonymous SNPs in TLR5 were evaluated further in a case-control study using a SNaPSHOT multiplex reaction. Sequencing information from 27 GSDs with IBD from the UK were compared to two control groups consisting of 77 healthy GSDs recruited from the USA and 47 GSDs from patients treated for non-inflammatory disease at the Queen Mother Hospital for Animals referral hospital (UK). All the SNPs in both the TLR4 and TLR5 gene were found to be in Hardy-Weinberg equilibrium. When the case population was compared to the healthy controls from the USA the G22A SNP was found to be significantly associated with IBD ($p=0.0044$). When the case population was compared to the GSDs with non-inflammatory disease from the UK the G22A SNP was found to be tending towards significance ($p=0.0523$).

Our study suggests that the TLR5 SNP G22A could play a role in the pathogenesis of IBD in GSDs. Further studies are required to confirm the functional importance of this polymorphism in the pathogenesis of this disease.

HIV-1 RECOGNITION IN CD14+ MONOCYTES & CD4+ T-CELLS

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Toll-like receptors (TLRs) are pathogen recognition receptors (PRRs) implicated in the immune response to multiple classes of pathogens. Whilst interactions between HIV-1 nucleic acids and TLRs have already been characterised, less is known about TLR-mediated recognition of HIV-1 envelope glycoproteins. The interaction between HIV-1 and human CD14⁺ monocytes and CD4⁺ T-cells represent two arms of the immune system linked by PRR-induced cytokine secretion. The early stages of HIV-1 invasion represent attractive therapeutic targets for inhibition. Investigation of the relationships existing between HIV-1 and TLR2, TLR4, CCR5 and CXCR4 will enable further modelling of the response to HIV-1 infection in humans. Using indirect immunofluorescence labelling and subsequent analytical flow cytometry, we demonstrate dose-dependent and glycoprotein-dependent expression of TLR2, TLR4, CCR5 and CXCR4 expression in CD14⁺ monocytes and CD4⁺ T-cells after HIV-1-IIIB gp120/gp140 stimulation \pm prior chemokine receptor inhibition. For these stimulation subsets, via cytometric bead assay and flow cytometry, we demonstrate modulation of cytokines IL-6, IL-4, IL-2, IFN γ , TNF α and IL-10. Our findings point toward collaboration between extracellular TLRs and HIV-1 chemokine co-receptors in the immune response to HIV-1.

MOLECULAR CLONING AND CHARACTERISATION OF THE GRIFFON VULTURE (*GYPS FULVUS*) TOLL-LIKE RECEPTOR-1

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Vultures may have one of the strongest immune systems of all vertebrates because they are unique vertebrates able to efficiently utilise carcass from other animals as a food resource. These carrion birds are in permanent contact with numerous pathogens and toxins found in its food. In addition, vultures tend to feed in large groups, because carcasses are patchy in space and time, and feeding often incurs fighting and wounding, exposing vultures to the penetration of microorganisms present in the carrion. Therefore, vultures were predicted to have evolved immune mechanisms to cope with a high risk of infection with virulent parasites.

Despite the potential interest in carrion bird immune system, little is known about the molecular mechanisms involved in the regulation of this process in vultures. The central challenge faced by all immune systems is the discrimination of foreign non-self from self. A key feature of the innate immune response concerns the recognition of microbe-derived molecules by toll-like receptors (TLRs).

The toll-like receptor (TLR) family is an ancient pattern recognition receptor family, conserved from insects to mammals. Members of the TLR family are vital to immune function through the sensing of pathogenic agents and initiation of an appropriate immune response. We have undertaken the identification of TLRs in the griffon vulture because carrion birds are in permanent contact with pathogens.

In this study, we cloned a cDNA encoding for a griffon vulture (*Gyps fulvus*) orthologue of mammalian TLR1 (Gf-TLR1). The predicted 650 amino acid sequence comprised an extracellular domain with five leucine-rich repeats (LRR) and a LRR-C-terminal (LRR-CT) motif, followed by a 23 amino acid transmembrane segment, and a 190 amino acid intracytoplasmic region containing the Toll/IL-1R (TIR) domain. Vulture TLR1 and TIR domain showed 64% and 86% amino acid sequence similarity with chicken sequences. Despite the similarities in the overall structure and expression pattern of vulture TLR1 with other vertebrate TLRs, Gf-TLR1 exhibits some structural features that could influence its functional role as pathogen receptor. For example, it is possible that the smaller size of Gf-TLR1, the lower number of *N*-glycosylation sites and the grouping of its LRRs in the proximal half of its ectodomain have functional implications. The set of Toll proteins for humans and insects each contain widely divergent LRR regions, and this is viewed as providing the potential to discriminate between different ligands. Perhaps these features provide Gf-TLR1 some advantages on pathogen recognition.

The tissue and cell expression pattern of vulture TLR1 were analysed by real time-PCR (RT-PCR) and correlated with the ability to respond to various pathogenic challenges.

In leukocytes, the expression of Gf-TLR1 was increased after *in vitro* treatment with LPS, as assessed by flow cytometry and real-time PCR.

The TLR gene reported here expands our understanding of the immune regulatory pathways present in carrion birds. These results have implications for the understanding of the evolution of pathogen-host interactions.

The knowledge of homologues of TLRs in other species, particularly in those showing a strong immune system, would contribute to our understanding of how these receptors have evolved and the importance of different homologues to resistance to different pathogens.