



**Schedule of events**

**Speaker abstracts**

**Late breaking abstracts**



## Thursday September 25

### 08.30-10.30 **Comparative Immunology**

Moderator: Alan Ezekowitz

- 08:30 Alan Ezekowitz: *Moderator's introduction*
- 08:40 Sankar Ghosh: *Recognition of bacterial and parasite ligands by Toll-like receptors*
- 09:15 Mihai Netea: *Differential roles of the inflammasome for the activation of IL-1 $\beta$*
- 09:40 Marc Dionne: *New transcriptional regulators of Drosophila immunity*
- 10:05 Zeev Pancer: *Origins of vertebrate adaptive immunity*

### 10.30-10.55 **Coffee and tea**

### 10.55-12:45 **Structure and Function**

Moderator: Jerry Weiss

- 10.55 Vishva Dixit: *Role of Ubiquitin in Editing in Innate Immunity*
- 11.30 Jerry Weiss: *Regulation of TLR4 activity in the airway*
- 11.55 Nick Gay: *A 3-dimensional view of innate immune signalling by the TLRs*
- 12.20 Hermann Wagner: *The DNA sugar backbone as well as mTOR control TLR9 activation*

### 12.45-14.00 **LUNCH**

### 14.00-16.15 **Signal Transduction**

Moderator: Jules Hoffmann

- 14.00 Shizuo Akira: *Atg16L1, an autophagy protein, controls endotoxin-induced inflammasome activation*
- 14.35 Neal Silverman: *Inside and out: Microbial recognition and Signaling in the Drosophila IMD pathway*
- 15.00 Ruslan Medzhitov: *TLR-induced transcriptional response*
- 15.25 Felix Randow: *Somatic cell genetics for the study of signalling in innate immunity*
- 15.50 Anthony DeFranco: *Role of dendritic cells in the in vivo responses to TLR ligands*

### 16.15-16.35 **Coffee and tea, snacks**

### 16.35-18.50 **Intracellular Receptors**

Moderator: Kenneth Rock

- 16.35 Jurg Tschopp: *Activation of the NALP3 inflammasome*
- 17.10 Daniel Portnoy: *How the innate immune system distinguishes between pathogenic and non-pathogenic microbes*
- 17.35 Eicke Latz: *NALP3 inflammasome activation by lysosomal damage*
- 18.00 Kenneth Rock: *Cell death, uric acid, and the sterile inflammatory response*
- 18:25 Douglas Golenbock: *Beta amyloid activates the NLRP3 inflammasome: potential cytopathic effect in Alzheimer's Disease*

### 19.00-21.00 **POSTER SESSION** including cocktails and Hors d'oeuvres, Sala III

## Friday September 26

### 09.00-10.50 Gram-negative recognition

Moderator: Lee Wetzler

- 09.00 Stefanie Vogel: *Interactions between Toll-like Receptor 4 (TLR4) Protease Activated Receptor 2 (PAR<sub>2</sub>)*
- 09.35 Egil Lien: *Evasion of LPS-TLR4 signaling and the evolution of a highly virulent pathogen*
- 10.00 Kensuke Miyake: *Molecules coordinating Toll-like receptor responses*
- 10.25 Bruno Lemaitre: *The gut immune response of Drosophila*

### 10.50-11.20 Coffee and tea

### 11.20-13.35 Co-receptors & Regulators – Parallel session, Sala I

Moderator: Chris Karp

- 11.20 Chris Karp: *TLR Regulation of the Metabolic Sequelae of High Fat Diets*
- 11.55 Lynda Stuart: *The Role of the Phagosome in Sensing of Gram-positive Microbes*
- 12.20 Ashley Mansell: *DUBLIN is a novel negative regulator of RIG-Like Helicase Signaling*
- 12.45 Kathryn Moore: *Endogenous danger signals trigger sterile inflammation via a heterotrimeric complex of CD36, TLR4, TLR6*
- 13.10 Sam Wright: *Atherosclerosis and the Regulation of Inflammation*

### 11.20-13.35 Intracellular Pathogens – Parallel session, Sala II

Moderator: Caetano Reis e Sousa

- 11.20 Gabriel Nunez: *Role of Inflammasome and IL-1R in Microbial and Sterile Inflammation*
- 11.55 Sara Cherry: *Antiviral immunity: Intrinsic mechanisms at work*
- 12.20 Shoichiro Kurata: *Autophagic control of Listeria through intracellular innate immune recognition in Drosophila*
- 12.45 Mary O’Riordan: *Immunomodulation by the X-linked Inhibitor of Apoptosis Protein*
- 13.10 Akiko Iwasaki: *Role of inflammasomes in antiviral immunity*

### 13.35 FREE AFTERNOON

**19.00 Portuguese Wine Tasting and Reception** at Palacio do Marques de Pombal, adjacent to Instituto Gulbenkian de Ciencia (registration required). Buses depart 19.00 from Hotel Cascais Miragem.

## Saturday September 27

### 08.30-10.15 Selected Abstracts 1 – Parallel session, Sala I

Moderator: Veit Hornung

- 08.30 Satoshi Uematsu, Abstract 253: *Regulation Of Humoral And Cellular Gut Immunity By Lamina Propria Dendritic Cells Expressing Toll-Like Receptor 5*
- 08.45 Julien Royet, Abstract 51: *The Drosophila membrane-associated protein PGRP-LF prevents IMD/JNK pathways triggering by blocking PGRP-LC activation*
- 09.00 Hendrik Poeck, Abstract 191: *5'-triphosphate-siRNA: turning gene silencing and RIG-I activation against melanoma*
- 09.15 Jacobien Hoogerwerf, Abstract 144: *Sepsis-Induced Suppression Of Lung Host Defense Is Mediated By St2*
- 09.30 Tilmann Bürckstümmer, Abstract 72: *The DEAD-box helicase DDX3X is a critical component of the TANK-binding kinase 1-dependent innate immune response*
- 09.45 Lynn Hajjar, Abstract 199: *Human-like responses to LPS in a mouse model*
- 10.00 Veit Hornung, Abstract 96: *PISA (PYHIN protein stimulating ASC) triggers inflammasome activation in response to cytosolic B-DNA*

### 08.30-10.15 Selected Abstracts 2 – Parallel session, Sala II

Moderator: Fabio Re

- 08.30 Harald Husebye, Abstract 89: *Role of Rab11 in signaling and intracellular trafficking of Toll-like receptor 4*
- 08.45 Miguel Sanjuan, Abstract 70: *Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis*
- 09.00 Andriy Kubarenko, Abstract 201: *Structural modeling of Toll-like receptors: science or fiction?*
- 09.15 Fredric Sheedy, Abstract 217: *Modulation of microRNA by Toll-like receptors*
- 09.30 Rudi Beyaert, Abstract 80: *Stimulation of TLR3 and TLR4 induces Caspase-1 independent IL-1 $\beta$  maturation*
- 09.45 Paula Pitha, Abstract 39: *The Role of IRF-5 in the antiviral and inflammatory response*
- 10.00 Fabio Re, Abstract 15: *Inflammasome activation by Alum and Alum's adjuvant effect are mediated by NLRP3*

### 10.15-10.40 Coffee and tea, snacks

**10.40-12.55 Inflammatory disease/atherosclerosis** – Parallel session, Sala I

Moderator: Peter Libby

- 10.40 Peter Libby: *An Overview of Inflammation in Atherothrombosis*
- 11.15 Ann Marshak-Rothstein: *Modulation of B cell Responses to Autoantigens by IFN $\alpha$  and BCR Engagement*
- 11.40 Peter Tobias: *TLR2 in the early stages of murine atherosclerosis*
- 12.05 Caroline Genco: *How a Pathogen Exploits TLR2-Mediated Inflammatory Pathways at Sites Distant from Infection*
- 12.30 Moshe Arditi: *Toll-Like Receptors and Chlamydia pneumoniae-mediated acceleration of Atherosclerosis*

**10.40-12.55 Parasites and Fungi** – Parallel session, Sala II

Moderator: Miguel Soares

- 10.40 Miguel Soares: *A central role for free heme in the pathogenesis of severe malaria*
- 11.15 Gordon Brown: *Innate recognition of fungi*
- 11.40 Amy Hise: *Role of TLRs, CLRs and NLRs in host defense against Candida albicans*
- 12.05 Ricardo Gazzinelli: *TLRs in Malaria*
- 12.30 Elena Levashina: *Regulation of anti-Plasmodium responses in the malaria vector Anopheles gambiae*

**12.55-14.10 LUNCH**

**14.10-16.25 Gram-positive bacteria, TB and beyond** – Parallel session, Sala I

Moderator: Dan Portnoy

- 14.10 Alan Sher: *Role of MyD88/IL-1R signaling in the stimulation of Th1 and Th17 responses by Complete Freund's Adjuvant*
- 14.45 Dominique Ferrandon: *Sensing of Gram-negative bacteria in Drosophila: relative roles of peptidoglycan fragment release, injury, and phagocytosis in initiating IMD pathway activation in distinct models of infection.*
- 15.10 Marie Charrel-Dennis: *TLR-independent type I interferon induction in response to an extracellular bacterium*
- 15.35 Gio Teti: *Signaling pathways involved in bacteria-induced IFN- $\alpha$ - $\beta$  production*
- 16.00 Dana Philpott: *Nod1 and Nod2 in innate and adaptive immunity*

**14.10-16.25 Drug development I** – Parallel session, Sala II

Moderator: Ofer Levy

- 14.10 Peter Morley/GSK: *High-throughput screening strategies for the discovery of novel TLR agonists*
- 14.45 Sally Ishizaka/Eisai: *Development and in vivo assessment of small molecule TLR9 inhibitors*
- 15.10 Robert Coffman/Dynavax: *Bifunctional TLR7 and TLR9 Inhibitors: Development for Autoimmune Disease*
- 15.35 Gerald Dubois/Novartis: *Effects of a novel synthetic TLR9 agonist on repeated allergen challenge in allergic monkeys*
- 16.00 Yannis Morel/Innate Pharma: *Preclinical proof of concept for TLR3 targeting in*

*oncology*

**16.25-16.50 Coffee, tea and snacks**

**16.50-19.05 Regulation of Signaling** – Parallel session, Sala I

Moderator: Luke O'Neill

16.50 Hidde Ploegh: *Lysosomal cleavage of TLR9 is a prerequisite for its function*

17.25 Xiaoxia Li: *Regulation of TLR-IL-1R signaling*

17.50 Andrew Bowie: *Viral antagonism of TLR and RLR signaling reveals a role for DDX3 in innate immunity*

18.15 Ahiao Ding: *Distinctive role of MyD88-5 (SARM) in neurodegeneration and host defense*

18.40 Ofer Levy: *The adenosine system differentially regulates TLR-mediated activation of monocytes in an age-specific manner*

**16.50-19.05 Drug Development II** – Parallel session, Sala II

Moderator: Anthony Coyle

16.50 Anthony Coyle/Medimmune: *Interferons, TLRs and Danger signals in autoimmune disease*

17.25 Sudhir Agrawal/Idera: *Synthetic DNA-based antagonists of TLRs.*

17.50 Art Krieg/Pfizer: *Oligonucleotide drugs as intentional and unintentional immune activators*

18.15 Lynette Fouser/Wyeth: *IL-22 is produced by immune cells, including Th17 lymphocytes, and acts on non-immune cells to regulate local tissue inflammation*

18.40 Robert Hershberg/VentiRx: *Development of Novel Small Molecule TLR8 Agonists in Oncology and Allergy*

**20.00 BANQUET** at Museu de Marinha (registration required). Buses depart from Hotel Cascais Miragem at 20.00.

# **SPEAKER**

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## SYNTHETIC DNA-BASED ANTAGONISTS OF TLRs

Sudhir Agrawal

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Through extensive structure-activity relationship studies of synthetic oligonucleotides, we have identified novel compositions which act as antagonists of intracellular TLRs. Two critical components that confer TLR antagonist activity to an oligonucleotide sequence are an immune stimulatory motif and an immune regulatory motif. Immune stimulatory motifs include unmethylated CpG and certain synthetic dinucleotides. Examples of immune regulatory motifs include site-specific incorporation of 2'-O-methylnucleosides, 3'-O-methylnucleosides, and methylphosphonate internucleotide linkages. TLR antagonist oligonucleotides have been shown to inhibit agonist-induced activation of immune responses through TLR9 and TLR7 in vitro and in vivo in mice, and through TLR7, 8, and 9 in human cell-based assays. The observed suppression of immune response is dependent on the dose of antagonist and agonist used. These antagonist candidates have shown encouraging reductions in disease symptoms in various preclinical models of autoimmune diseases, including lupus, psoriasis, multiple sclerosis, and collagen-induced arthritis. A lead TLR antagonist candidate, IMO-3100, has been selected for preclinical development.

## **ATG16L1, AN AUTOPHAGY PROTEIN, CONTROLS ENDOTOXIN-INDUCED INFLAMMASOME ACTIVATION**

Shizuo Akira and Tatsuya Saito

Laboratory of Host Defense, WPI Immunology Frontier Research Center, Osaka University, Japan

Systems for protein degradation are essentially required for tight control of the inflammatory immune response. Autophagy, a bulk degradation system that delivers cytoplasmic constituents into autolysosomes, controls degradation of long-lived proteins, insoluble protein aggregates and invading microbes, and is suggested to be involved in the regulation of inflammation. However, the mechanism underlying the regulation of inflammatory response by autophagy is poorly understood. We demonstrate that Atg16-like 1 (Atg16L1), which is implicated in Crohn's disease, is indispensable for the formation of autophagosomes as well as the control of the endotoxin-induced inflammatory immune response. Atg16L1 deficiency disrupts formation of autophagosome as well as degradation of long-lived proteins. Atg16L1-deficient macrophages produce high amounts of the inflammatory cytokines IL-1b and IL-18 in response to lipopolysaccharide (LPS). Mice lacking Atg16L1 in hematopoietic cells are highly susceptible to dextran sulfate sodium (DSS)-induced acute colitis, which is alleviated by injection of anti-IL-1b and IL-18 antibodies, indicating the importance of autophagy in suppression of inflammatory responses in vivo. These results demonstrate that Atg16L1 is an essential component of autophagic machinery responsible for the control of the endotoxin-mediated immune response; its dysfunction may be associated with inflammatory diseases such as colitis.

## TLR/MYD88 AND LXR $\alpha$ SIGNALING PATHWAYS RECIPROCALLY CONTROL *CHLAMYDIA PNEUMONIAE*-INDUCED ACCELERATION OF ATHEROSCLEROSIS

Moshe Arditi, M.D.

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Experimental and clinical studies link *Chlamydia pneumoniae* infection to atherogenesis and athero-thrombotic events, but the underlying mechanisms are unclear. We tested the hypothesis that *C. pneumoniae*-induced acceleration of atherosclerosis in ApoE<sup>-/-</sup> mice is reciprocally modulated by activation of TLR-mediated innate immune or LXR $\alpha$  signaling pathways. We infected ApoE<sup>-/-</sup> mice and ApoE<sup>-/-</sup> mice that also lacked TLR2 or TLR4 or MyD88 or LXR $\alpha$  intranasally with *C. pneumoniae* followed by high-fat diet feeding for 4 months. Mock infected littermates served as controls. Atherosclerosis was assessed in aortic sinuses and in *en face* preparation of whole aorta. The numbers of activated dendritic cells (DCs) within plaques, and serum levels of cholesterol and proinflammatory cytokines were also measured. *C. pneumoniae* infection markedly accelerated atherosclerosis in ApoE deficient mice that was associated with increased numbers of activated DCs in aortic sinus plaques and higher circulating levels of MCP-1, IL-12p40, IL-6 and TNF- $\alpha$ . In contrast, *C. pneumoniae* infection had only a minimal effect on atherosclerosis, accumulation of activated DCs in the sinus plaques, or circulating cytokine increases in ApoE<sup>-/-</sup> mice that were also deficient in either TLR2, TLR4, or MyD88. However, *C. pneumoniae*-induced acceleration of atherosclerosis in ApoE<sup>-/-</sup> mice was further enhanced in ApoE<sup>-/-</sup>/LXR $\alpha$ <sup>-/-</sup> double knockout mice, and was accompanied by higher serum levels of IL-6 and TNF- $\alpha$ . We conclude that *C. pneumoniae*-infection accelerates atherosclerosis in hypercholesterolemic mice predominantly through a TLR/MyD88-dependent mechanism, and that LXR $\alpha$  appears to reciprocally modulate and reduce the pro-atherogenic effects of *C. pneumoniae* infection.

## **VIRAL ANTAGONISM OF TLR AND RLR SIGNALLING REVEALS A ROLE FOR DDX3 IN INNATE IMMUNITY**

Andrew G. Bowie

School of Biochemistry and Immunology, Trinity College Dublin, Dublin 2, Ireland.

Although many new pattern recognition receptors (PRRs) have been discovered over the past 10 years, the molecular mechanisms whereby they signal are still a matter of debate. One way that viruses evade and manipulate the immune response is by targeting key and sometimes undiscovered innate signalling proteins. Thus we have been exploring mechanisms whereby vaccinia virus (VACV) antagonises host innate signalling, in order to more fully understand the host response pathways. For example, we showed that the VACV protein A52 inhibits Toll-like receptor (TLR) signaling by interacting with IRAK-2, which revealed a critical role for IRAK-2 in TLR signaling to NFkappaB which had been previously unappreciated.

Here we show that the VACV protein K7 can inhibit both TLR- and RIG-I like receptor (RLR)-mediated Ifnb (IFN-beta) promoter induction. K7 prevented TBK1/IKK-epsilon-mediated IRF activation. The target of K7 which accounted for the inhibitory effect on IFN-beta was found to be DEAD-box protein 3 (DDX3). Expression of DDX3 enhanced Ifnb promoter induction by sendai virus (SeV), while siRNA targeted against DDX3 inhibited SeV or dsRNA-induced IRF3 activation. Dominant-negative DDX3 inhibited virus-, dsRNA- and dsDNA-stimulated Ccl5 (RANTES) promoter induction, which is also TBK1/IKK-epsilon-dependent. Furthermore, SeV infection of cells induced an association between DDX3 and IKK-epsilon. Thus analysis of the mechanism whereby K7 inhibits innate immune signaling has revealed a novel role for a DEAD-box helicase in TBK1/IKK-epsilon-mediated IRF activation and Ifnb promoter induction.

Supported by Science Foundation Ireland Principal Investigator Programme 7/IN1/B934

## **TLR-INDEPENDENT TYPE I INTERFERON INDUCTION IN RESPONSE TO AN EXTRACELLULAR BACTERIA IS BASED ON AN INTRACELLULAR RECEPTOR**

Charrel-Dennis M , Latz E., Halmen K., Trieu-Cuot P, Fitzgerald KA, Kasper DL and Golenbock DG

University of Massachusetts, Worcester MA USA

We have identified and characterized the pathway leading to type I interferon (IFN) production by macrophages infected with group B streptococcus (GBS). IFN $\beta$  was produced by macrophages upon stimulation with both heat-killed and live GBS. Exposure of macrophages to heat-killed GBS activated a Toll-like receptor (TLR)-dependent pathway, whereas exposure to live GBS activated a TLR/NOD/Riglike receptor (RLR)-independent pathway. This latter pathway requires bacterial phagocytosis, proteolytic degradation and destruction of the phagolysosomal membrane by GBS pore-forming toxins, leading to the release of bacterial DNA into the cytosol. GBS DNA then triggers IFN $\beta$  by interacting with a cytosolic receptor, with consequent activation of the serine-threonine kinase TRAF-associated NF $\kappa$ B activator (TANK)-binding kinase 1 (TBK1) and phosphorylation of IFN regulatory factor 3 (IRF3). Thus, activation of IFN $\alpha/\beta$  production during infection with GBS, commonly thought of as an extracellular pathogen, appears to result from the intracellular interaction of GBS DNA with a DNAbinding receptor.

## **ROLE OF DENDRITIC CELLS IN THE INNATE AND ADAPTIVE IMMUNE RESPONSES TO TLR LIGANDS**

Baidong Hou<sup>1</sup>, Boris Reizis<sup>2</sup> and Anthony L. DeFranco<sup>1</sup>

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and <sup>2</sup>Dept. of Microbiology, Columbia University Medical Center, New York City, NY, USA 10032

We have generated mice in which the gene encoding MyD88 is selectively deleted in conventional (97%) and plasmacytoid (80%) dendritic cells (DCs). We have used these mice to evaluate the role of DCs in the rapid innate cytokine production that occurs following TLR stimulation and also in evaluating the role of DCs in stimulating adaptive immune responses using TLR ligands as adjuvants. These experiments reveal a predominant role of DCs in most circumstances tested, but the responses to aggregated TLR ligands, such as LPS or CpG oligonucleotides complexed with the cationic lipid DOTAP, were less attenuated in these mice than were responses to uncomplexed CpG oligonucleotides, to lipopeptides, or to a synthetic TLR7 agonist. Monocytes appeared to be the additional cell type in the spleen that responded to aggregated TLR ligands administered i.v. Interestingly, CpG oligonucleotides complexed with DOTAP induced strong production of type I interferons, primarily by plasmacytoid DCs. This type I interferon was only partially decreased in the DC-specific MyD88- knockout mice due to incomplete deletion of *myd88* in plasmacytoid DCs. The residual type I interferon promoted interferon-gamma production by NK cells, and likely was responsible for the CD4 T cell expansion, survival, and Th1 differentiation seen in these mice, which was comparable to control mice. In contrast, use of non-complexed CpG oligonucleotides as adjuvant resulted in good NK and CD4 T cell responses in wild type mice but very poor responses in the mutant mice.

## **DISTINCTIVE ROLE OF MYD88-5 (SARM) IN NEURODEGENERATION AND HOST DEFENSE**

Kim Y. S., Thomas B., Beal M. F., Nathan C. F. and Ding A.

Department of Microbiology and Immunology, Neurology and Neuroscience, Weill Medical College of Cornell University, New York, NY, USA

MyD88's were discovered as cytosolic signaling adaptor proteins for Toll-like receptors (TLRs). Mice with genes disrupted for any of the first four members of MyD88 family (MyD88, Mal, TRIF or TRAM) were severely compromised in their ability to mount innate responses to microbial pathogens. MyD88-5, also called SARM, is the last MyD88 family member detected in the genome. In wild type mice and mice overexpressing MyD88-5-GFP fusion protein under the native control elements, we found that MyD88-5 is expressed in neurons but undetectable in myeloid cells. We generated MyD88-5 knockout mice and found that their macrophages produced TNF and MCP-1 in amounts comparable to wild-type macrophages in response to the TLR agonists LPS, polyIC, Pam3-cys or CpG. Thus, SARM appears not to play a role in TLR signaling in macrophages. MyD88-5 expression was highest in the brain, where it co-localizes in part with mitochondria and a neural stress-induced kinase, JNK3. Mitochondrial dysfunction with over-activated JNK activity contributes to the pathogenesis of several neurodegenerative disorders including Parkinson's disease. MyD88-5 knockout mice were more resistant than wild type controls to the Parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), as evident by attenuations in toxin-induced loss of dopaminergic neurons and striatal dopamine levels. MPTP-induced phosphorylation of c-jun (a JNK3 substrate) and its subsequent nuclear translocation in midbrain dopamine neurons were also significantly reduced in the absence of MyD88-5. Thus MyD88-5 appears unique among MyD88's in functioning to mediate stress-induced neuronal toxicity rather than pathogen-induced responses in myeloid cells. However, MyD88-5 transcripts were also detectable in T cells. A possible role of MyD88-5 in adaptive immunity is under investigation.

## **REGULATION OF THE RESPONSE TO MYCOBACTERIA IN DROSOPHILA**

Marc S Dionne, Martina Yildirim, Rebecca Clark

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London, London, United Kingdom

The systemic transcriptional response to pathogenic mycobacterial infection in *Drosophila* is highly complex. At late stages of disease, it includes markers and effectors of pathogenesis, immune effectors, metabolic regulators, and many uncharacterized activities. Understanding the complex regulation underlying this response will help us understand the pathogen-detection and regulatory mechanisms that operate in this infection. We have taken a paired bioinformatic and genetic approach to this problem, starting with a large set of microarray data generated by us and others. This has allowed us to define a set of transcription factors that apparently regulate the disease process. These include some expected activities, such as NF- $\kappa$ B, AP-1, and gata factors and the forkhead-box protein Foxo; they also include several surprises, including other forkhead-box proteins. In addition to revealing novel aspects of the regulation of later pathogenesis, this analysis has been informative with regard to the regulation of the immediate systemic immune response in the fly. Our technique, and the prospective roles of the identified factors, will be discussed.

## **EFFECTS OF A NOVEL SYNTHETIC TLR9 AGONIST ON REPEATED ALLERGEN CHALLENGE IN ALLERGIC MONKEYS**

Dubois G., Simmons J., Martin E., Willard L., Spooner G., Hanley M., Sullivan T., Kandimalla, E., Agrawal S., Walker C.

- 1) Novartis Institutes for BioMedical Research, Horsham, UK
- 2) Charles River Laboratories, Worcester, US
- 3) Idera Pharmaceuticals, Cambridge, US

The objective of this study was to determine the effects of local administration of a novel synthetic TLR9 agonist in a cynomolgus monkey model of asthma. Study animals were selected on the basis of historical responses to *Ascaris suum* (*A. suum*). Fifteen animals underwent control antigen challenges in Session I prior to assignment to treatment groups to characterize their normal (untreated) response to antigen challenge. Following this control session, animals were assigned to dose groups on the basis of the eosinophil influx to the lung, as well as the magnitude of the acute pulmonary function changes in response to aerosol administration of *A. suum*. During Session II, all animals were challenged with *A. suum* on Days 1, 15 and 29 and received either the vehicle (Group 1) or TLR9 agonist at 0.3 (Group 2) or 1.5 mg/kg (Group 3) by aerosol inhalation on Days 0, 7, 14, and 21. Pulmonary function was recorded throughout each aerosol antigen challenge. BAL samples were collected at baseline (Day -4) and Day 2 (~24 hours post-challenge) during Session I and on Days 14 (pre-challenge), 16 (24 hrs post challenge), 28 (pre-challenge), and 30 (24 hrs post-challenge) during Session II. BAL samples were processed for evaluation of total and differential cell numbers and morphology in order to assess the degree of pulmonary inflammation. BAL supernatant and cell pellet samples were also analyzed for Th2 cytokines and IFN-inducible genes, respectively. Plasma samples were collected and analyzed for IL-6, IFN $\alpha$  and IP-10 levels. Body weights were recorded weekly and clinical signs were recorded daily. There were no adverse clinical signs or changes in body weights directly attributed to test article treatment. As a consequence of the repeated allergen challenge, animals in the vehicle treated group showed a marked increase in both airway resistance and inflammation over time. Treatment with our synthetic TLR9 agonist effectively prevented both the increase in airway resistance and inflammation. In addition, the TLR9 agonist inhibited IL-13 levels in BAL fluid after the allergen challenge. TLR9 agonist treatment increased IP-10 levels in plasma and induced expression of several IFN-dependent genes in BAL cells, both indicative for an effective induction of a Th1 response.

In summary, we showed that once weekly local administration of a novel synthetic TLR9 agonist effectively attenuated inflammatory responses in the lung of cynomolgus monkeys after repeated allergen challenges. These data demonstrate the potential therapeutic use of these novel synthetic TLR9 agonists for the treatment of allergic diseases.

## **THE DETECTION OF GRAM-NEGATIVE BACTERIA IN DROSOPHILA MELANOGASTER**

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The detection of infections in *Drosophila melanogaster* is mediated either through Pattern recognition Receptors (PRRs) or through the detection of the enzymatic activity of microbial virulence factors. The detection step ultimately leads to the activation of either of two NF-kappaB regulatory pathways that control the expression of potent antimicrobial peptides (AMPs) in the fat body, hemocytes or barrier epithelia. As regards Gram-negative bacteria, the detection of DAP-type peptidoglycan (PGN) is mediated by two PRRs of the Peptidoglycan Recognition Protein family. PGRP-LC is the membrane-bound receptor of the Immune deficiency (IMD) pathway, whereas PGRP-LE is thought to function either as a secreted PRR enhancing PGN detection in conjunction with PGRP-LC, or as an intracellular receptor that can also activate the IMD pathway.

DAP-PGN is present within the periplasmic space of Gram(-) bacteria and is thus not directly accessible to PRRs. One model with some experimental support in larvae postulates that hemocytes process Gram-negative bacteria through phagocytosis and activate the IMD pathway either by releasing small PGN fragments, or indirectly by sending a signal to the fat body. Another model proposes that small PGN fragments are secreted by bacteria and detected by the PGRP-LC/LE system. Here, we report our findings on the way Gram(-) bacteria are sensed by adult flies.

**IL-22 IS PRODUCED BY IMMUNE CELLS, INCLUDING TH17  
LYMPHOCYTES, AND ACTS ON NON-IMMUNE CELLS TO REGULATE  
LOCAL TISSUE INFLAMMATION**

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TLR-dependent activation of tissue resident dendritic cells is required for their maturation to an antigen-loaded and cytokine-producing state and subsequent trafficking to draining lymph nodes. Thus, TLR signaling is essential to the initiation of an adaptive immune response. The resultant effector cytokines produced by activated helper T cells (e.g., Th1, Th2, Th17) and Tregs (e.g., IL-10) are critical for the regulation of the innate and adaptive immune responses, be it acute in response to an infection or chronic as in autoimmune pathology. IL-23 derived from TLR ligand-stimulated dendritic cells is essential for the expansion of Th17 cells and production of IL-22, as well as other cytokines. While it is made by activated Th17 cells and other immune cells, IL-22 regulates tissue inflammation via its unique IL-22R/IL-10R2 receptor on only epithelial cells and some fibroblasts. IL-22, either alone or in synergy with IL-17A, induces the expression of chemokines, cytokines, MMPs, anti-microbial peptides and acute phase reactants. IL-22 and IL-22R gene expression is induced in diseased mouse tissue in the collagen-induced arthritis model and a psoriasis-like skin inflammation model, with concomitant detection of IL-22 in the blood. A human antibody that has high affinity for, and potentially neutralizes the activity of, primate and rodent IL-22 blocks the progression of disease by both gross and histological scoring in these animal models of autoimmune disease. We propose that IL-22, via its direct effects on non-immune cells in arthritic joints and lesional skin, is acting locally to modulate disease progression in these tissues. With the knowledge that IL-22 expression is also associated with human autoimmune pathology, we propose that IL-22 antagonism represents a novel therapeutic treatment paradigm with the potential to suppress local tissue inflammation without overtly affecting systemic adaptive immunity.

## **ROLE OF OLIGOMERIC ASSEMBLIES IN INNATE IMMUNE SIGNALLING BY THE TLRs**

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Although most of the components involved in signal transduction by the Toll-like receptors have now been identified, the mechanisms by which they are assembled into functional signalling complexes remain poorly understood. Recent structural studies have established that stimulus induced dimerization of toll-like receptors by a diversity of different microbial ligands is the key event that initiates signalling. Dimerization of the leucine-rich repeats is transmitted to the cytoplasmic TIR domains of the receptor leading to the formation of a new scaffold for the recruitment of adaptor molecules and downstream signal transducers. Recent results in our lab show that the MyD88 adaptor death domain spontaneously assembles with that of the IRAK4 kinase to form an undecameric complex, a process that may drive higher order oligomerisation and is likely to be important for TLR mediated signalling and regulation. By contrast the death domains of *Drosophila* MyD88, the tube adaptor and the pelle kinase form simpler heterotrimeric complexes suggesting that the higher order structures formed in vertebrates are required to service the complex regulation observed in vertebrate TLR signalling.

## INTERFERON-GAMMA IS A CENTRAL MEDIATOR OF GENE EXPRESSION, HYPER-RESPONSIVENESS OF TOLL-LIKE RECEPTORS AND PRO-INFLAMMATORY PRIMING DURING MALARIAL SEPSIS

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Infection with malaria parasites, affect more than 300 million people of all ages, and kill over 1 million children, every year. Pathogenesis of malaria is a multifactorial syndrome, which is a consequence of three main aspects: (i) red blood cell destruction; (ii) adhesion of infected erythrocytes to the capillary veins; and (iii) excessive pro-inflammatory response. The excessive pro-inflammatory response is responsible for septic shock-like symptoms, such as: shivering, headache, chills, spiking fever, sweating, vasodilatation and hypoglycemia. However, the mechanism underlying the burst of pro-inflammatory cytokines during *Plasmodium* infection is not fully understood. One of the main interests of my lab is to understand the role of innate immune receptors on pathogenesis of malaria. Here we show that cells from innate immune system from febrile patients during natural infection with *P. falciparum* malaria are primed and become hyperresponsive to Toll-Like Receptor (TLR) agonists. Microarray analyses demonstrated that an extraordinary percentage of the up-regulated genes, including genes of TLR signaling pathway, had sites for IFN-inducible transcription factors. To further define the mechanism involved in malaria-mediated "priming," we infected mice with *P. chabaudi*. The human data was remarkably predictive of what we observed in the rodent malaria model. Malaria-induced priming of TLR responses correlated with increased expression of TLR mRNA in a TLR9, MyD88- and IFN $\gamma$ -dependent manner. Acutely infected wild-type mice were highly susceptible to LPS-induced lethality while TLR9<sup>-/-</sup>, and to a greater extent, IFN $\gamma$ <sup>-/-</sup> mice were protected. Finally, we show that CD4<sup>+</sup> T cells are the main IFN $\gamma$  source during early stages of infection, and that blockade of T cell-DC interactions or RAG<sup>-/-</sup> mice show ameliorated symptoms associated with cytokinemia during rodent malaria. Our data provide unprecedented evidence that TLR9 and MyD88 are essential to initiate IFN $\gamma$  responses by T cells and favor host hyper-responsiveness to TLR agonists resulting in overproduction of pro-inflammatory cytokines and the sepsis-like symptoms of acute malaria.

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## **INNATE RECEPTORS IN HOST DEFENSE TO FUNGAL PATHOGENS; ROLE OF TLRs, CLRS AND NLRs**

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Oropharyngeal candidiasis (OPC) and denture stomatitis result from an overgrowth of *Candida* species, often in an immunocompromised host. While acquired immunity has been shown to be important in defense against candidiasis, the role of innate pathogen recognition receptors is not clear. We have developed a murine model in which the animals develop the characteristic white patches and epithelial ulceration of oral thrush, allowing the study of mucosal colonization as well as systemic dissemination without the use of immunosuppressive agents. Using our model, we show that TLR2 but not TLR4 or other TLRs is important in resistance to dissemination and mortality from oral *C. albicans* infection. We also define the role of dectin-1, a C-type lectin like receptor shown to be important in recognition of fungal cell wall components in our model of OPC. Finally, we show a critical role for IL-1 $\beta$  in host protection from dissemination and death in OPC. The synthesis, processing and release of IL-1 $\beta$  are tightly regulated and require at least two distinct stimuli. An inflammatory stimulus causes accumulation of intracellular stores of the 31-kDa pro-IL-1 $\beta$  (step 1); a second stimulus activates a multiprotein complex, commonly referred to as the "inflammasome" which controls the activation of caspase-1 and cleavage of the pro-IL-1 $\beta$  (step 2), followed by release of the active mature 17-kDa IL-1 $\beta$  (step 3). This 'three-step' model may guarantee that initiation of innate immune defense requires multiple signals, thereby ensuring that innate immunity is not accidentally triggered, e.g. by commensal bacteria engaging cell surface TLRs. Using in-vitro studies and our murine model, we define the molecular regulation and processing of IL-1 $\beta$  in the context of anti-fungal immunity.

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## ANTIVIRAL INNATE IMMUNITY IN DROSOPHILA

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Insects have been known for more than a century to be strongly resistant to microbial infections, and *Drosophila* has proved to be a good model to unravel innate mechanisms of host-defence. We are using three RNA viruses, the Dicistrovirus *Drosophila* C virus (DCV), the Nodavirus Flock house virus (FHV) and the Alphavirus Sindbis virus (SINV) to study antiviral responses in flies. Our data indicate that resistance to viral infection involves two types of mechanisms: on one hand, infection triggers a transcriptional response that depends in part on the JAK-STAT pathway, and leads to the production of antiviral molecules that remain to be identified; on the other hand, viral double stranded RNAs are recognized by the RNaseIII enzyme Dicer-2, and trigger RNA interference.

We have undertaken the characterization of the gene *Vago*, which is induced by infection with DCV in a JAK-STAT independent manner. *Vago* is induced in the fat body, which serves as a major replication site for DCV, FHV and SINV. Expression of *Vago* is also upregulated following infection by SINV, but not by FHV. The FHV viral suppressor of RNAi B2, which binds to double-stranded RNA, interferes with the upregulation of *Vago* in DCV infected flies, thus explaining why FHV does not induce this gene. We furthermore show that Dicer-2 is involved in the induction of *Vago*, and note that Dicer-2 belongs to the same DExD/H-box helicase family as RIG-I like receptors. We propose that this family represents an evolutionary conserved set of sensors, which detects viral nucleic acids and directs antiviral responses.

## **DEVELOPMENT OF ORALLY AVAILABLE INHIBITORS OF TLR9 ACTIVATION**

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TLR9 has been linked to systemic lupus erythematosus in part by its expression on cell types that are central to disease: B cells and plasmacytoid dendritic cells (PDCs). DNA-containing immune complexes, generated in vitro or derived from SLE patient serum, activate these cell types, further suggesting a connection with disease etiology. Finally, TLR9 activation of PDC results in expression of alpha-interferon, which is responsible for a gene signature associated with active disease. Knockout data on SLE pathology in TLR9-deficient animals has given equivocal results regarding the impact of this receptor in development of spontaneous disease, but knockout systems differ from drug-based inhibition due to the absence of receptor during ontogeny and immune system expansion.

We have investigated the potential for an orally available small molecule inhibitor of TLR9 to suppress disease in spontaneous mouse models. An inhibitor was identified with nanomolar activity in a HEK/TLR9 reporter assay, and optimized for potency in primary cells (mouse spleen and human PBMC), oral bioavailability and in vivo efficacy against CpG oligo challenge. The compound accumulates rapidly in intracellular compartments, and completely blocks TLR9 signaling in PDC as analyzed by microarray. Binding of stimulatory DNA sequences to TLR9 was inhibited by the compound in an in vitro Alphascreen-based binding system. Further data on mechanism of action and in vivo efficacy in spontaneous lupus models will be discussed.

## **TLR REGULATION OF THE METABOLIC SEQUELAE OF HIGH FAT DIETS**

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Obesity is a primary risk factor for type 2 diabetes mellitus (T2DM). Both obesity and T2DM have reached epidemic proportions in the developed world in recent years. Recent studies have shown that inflammation provides a critical link between these twinned metabolic syndromes. High fat diets and obesity are associated with excess production of pro-inflammatory cytokines. In turn, such cytokines are thought to drive insulin resistance through antagonism of insulin receptor signaling. While early studies suggested that adipocytes were the relevant sources of these pro-inflammatory cytokines, it is now clear that macrophages recruited to fat tissue in obesity are a major source of this maladaptive cytokine production. Our data indicate that: (a) TLRs are critical regulators of high fat diet-induced weight gain, adiposity, T2DM, hepatic steatosis and non-alcoholic steatohepatitis; and (b) the published literature on these issues is flawed and misleading, likely due the use of non-isogenic strains of mice.

## **AUTOPHAGIC CONTROL OF LISTERIA THROUGH INTRACELLULAR INNATE IMMUNE RECOGNITION IN DROSOPHILA**

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Autophagy, an evolutionally conserved homeostatic process for catabolizing cytoplasmic components, is implicated in the elimination of intracellular pathogens in the mammalian innate immune response. The mechanisms underlying cytoplasmic infection-induced autophagy and whether autophagy contributes to host survival against these infectious agents, however, are unknown. Here we report that in *Drosophila*, PGRP-LE recognition of diaminopimelic acid-type peptidoglycans is crucial for inducing autophagy to prevent the intracellular growth of *L. monocytogenes*, which is essential for host survival against this infection. The induction of autophagy occurs independently of the NF- $\kappa$ B-inducing IMD pathway. These findings define a clear pathway that leads from the detection of microbes by intracellular pattern recognition receptors to the induction of autophagy and the protection of the host from infection.

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## GENOME WIDE ANALYSIS OF THE GUT IMMUNE RESPONSE OF *DROSOPHILA*

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Since the gut is in constant contact with large numbers of microorganisms, this epithelia must be armed with efficient systems for microbe recognition and control. This is especially true for insects such as *Drosophila*, which live on decaying matter and feed on fermenting medium. However, in spite of growing interest in gut mucosal immunity, very little is known about the immune response of the *Drosophila* gut in comparison to our knowledge of the systemic immune response. For instance, we know very little about the repertoire of immune genes induced in the gut upon infection and how they are regulated. In this paper, we have analyzed how gut cells respond to infection by *Erwinia carotovora 15* at the transcriptome level and have defined some of the regulatory networks controlling the gut immune response. We show that the antimicrobial response in the gut is regulated by the Imd and JAK-STAT pathways but not by the Toll pathway. Our study reveals that oral ingestion of bacteria has a dramatic impact on the physiology of the gut that goes far beyond the activation of the immune response. A major consequence of bacterial persistence is the induction of a stress response and epithelial repair of the gut. Our study reveals that the *Drosophila* gut provides a powerful system to dissect epithelial responses used to limit and repair damage caused by bacterial infections and to analyze the integration between immune response and developmental pathways.

## THE ADENOSINE SYSTEM REGULATES TLR2 FUNCTION IN VITRO AND IN VIVO

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Newborns are susceptible to infection and mount weak vaccine responses, yet the underlying mechanisms are incompletely defined. Neonatal cord blood mononuclear cells (CBMCs) demonstrate impaired TLR2-mediated production of the pro-inflammatory/Th1-polarizing cytokine TNF, but high production of IL-6, a cytokine with anti-inflammatory and Th2-polarizing properties. This skew is mediated by increased CBMC sensitivity to adenosine, an endogenous purine metabolite that acts via the adenosine A3 receptor (A3R) to increase cellular concentrations of cAMP (Levy et al. *J Immunol* 177(3): 1956). To further characterize adenosine regulation of the TLR2 pathway, we studied human cord blood monocytes (CBMo) in vitro and a neonatal mouse model in vivo. Human newborn monocytes exhibit >2fold higher basal expression of A3R mRNA than adult peripheral monocytes (qRT-PCR; n = 3, p < 0.05), corresponding to greater basal A3R protein expression (western blot). To assess TLR2-mediated responses in vivo, newborn (< 24 hours) C57/BL6 mice were injected either intraperitoneal (IP) or intravenous (IV) with the diacylated fibroblast-stimulating lipopeptide (FSL; TLR 2/6). A3R-deficient neonatal mice demonstrated: a) enhanced IP FSL-induced plasma TNF and b) after IV FSL, diminished plasma IL-6 and enhanced liver IFN-g mRNA. Thus, neonatal CBMo express relatively high amounts of adenosine A3R that plays tissue-specific roles in selectively regulating TLR2-mediated cytokine production in vivo, reducing TNF and IFN-g, while increasing IL-6, a pattern that potentially protects the fetus and newborn from harmful inflammation but may also contribute to susceptibility to infection.

## EVASION OF INNATE IMMUNITY AND THE EVOLUTION OF A HIGHLY VIRULENT PATHOGEN

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The Gram-negative pathogen *Yersinia pestis* is the causative agent of plague. The lipopolysaccharide (LPS)/lipid A of *Y. pestis* is mainly tetra-acylated at mammalian body temperature (37° C). This form of lipid A is a poor agonist for the Toll-like receptor 4 (TLR4) - MD-2 complex. We have found that the resulting evasion of anti-bacterial defenses by innate immunity is necessary for the virulence of *Y. pestis*. This was studied by engineering a *Y. pestis* strain that expressed LpxL, an *E. coli* enzyme in the lipid A biosynthesis that *Y. pestis* normally lacks. The *Y. pestis*-pLpxL strain expressed a novel hexa-acylated lipid A and induced strong TLR4-mediated signaling, and importantly, *Y. pestis*-pLpxL was avirulent in mice via the peripheral route, in a TLR4-MD-2-MyD88-dependent manner. Furthermore, IL-1 release and signaling appears to play a central role in the resistance to *Y. pestis*-pLpxL, suggesting that this pathway is effective in control of *Y. pestis* infections. *Y. pestis* is a recently emerged clone of *Y. pseudotuberculosis*, still these human pathogens cause very different diseases. Infection with the plague bacillus can cause a full-blown sepsis and often lethal disease, whereas *Y. pseudotuberculosis* typically produces a limited gastroenteritis. Interestingly, LpxL is expressed in *Y. pseudotuberculosis* and hence this gene has been lost in the evolution of *Y. pestis* from *Y. pseudotuberculosis*. We hypothesize that the loss of LpxL, the production of a tetra-acylated LPS and the subsequent evasion of innate immune signaling was a necessary step in the evolution of *Y. pestis* as a highly virulent pathogen.

## **DUBLIN IS A NOVEL NEGATIVE REGULATOR OF RIG-LIKE HELICASE SIGNALING**

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The RIG-like helicase (RLH) family of cytosolic viral nucleic acid receptors undergo ligand-induced polyubiquitination and signal via the mitochondrial localized adapter protein MAVS (also known as VISA/Cardif/IPS-1). RLH interaction with MAVS initiates a potent antiviral response via activation of NF-kappaB and expression of Type I interferon. While the mitochondrial localized protein NLRX-1 has been proposed as a negative regulator of MAVS signaling by disrupting this complex, little is known of the mechanism of RLH regulation.

Here we describe a novel gene we have termed DUB-Like INhibitor (Dublin) which contains a putative CARD domain and localizes to the outer mitochondrial membrane. Overexpression of Dublin inhibits RLH-induced NF-kappaB- and IFNbeta-promoter activity, while depletion of Dublin by siRNA produces potentiated RLH-mediated inflammatory responses. Crucially, Dublin appears to mediate this negative regulation by promoting specific deubiquitination of RIG-I and MDA-5, yet has no effect on MAVS. Importantly, NLRX-1 does not induce this effect.

Interestingly, Dublin directly interacts with MAVS, but not RIG-I, Mda-5 or NLRX-1 suggesting the mitochondrial localization of Dublin and association with MAVS is designed to specifically target the activated RLH complex once localized to the mitochondria to facilitate negative regulation.

## **MODULATION OF B CELL RESPONSES TO AUTOANTIGENS BY IFN $\alpha$ AND BCR ENGAGEMENT**

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B cells expressing the AM14 B cell receptor, specific for autologous IgG2a, have proven to be a useful prototype for evaluating the role of the BCR and TLR7/TLR9 in the activation of autoreactive B cells. In vitro studies, using immune complexes that incorporate either CpG-rich or CpG-poor regions of the mammalian genome, have clearly shown that TLR9 is activated much more effectively by CpG-rich dsDNA than by CpG-poor dsDNA; stimulatory regions include the CpG islands located in the hypomethylated 45S pre-rRNA gene complex. The response to autoantigen-containing immune complexes can be markedly enhanced by IFN $\alpha$ , both by effects on TLR7 expression and also by effects on the BCR signaling cascade. Importantly, in the case of TLR9-dependent activation, IFN $\alpha$  primes the BCR signaling cascade and thereby reduces the stringency of the B cell response for DNA CpG content. BCR engagement per se can also modulate the response to autoantigens. Protein ICs do not induce cell cycle entry of AM14 B cells, however protein ICs do promote BCR-independent uptake and TLR9 delivery of uncomplexed autoantigens. Autoreactive B cell activation therefore depends on a balance of signals delivered by the BCR and the appropriate TLR - a stronger BCR signal in combination with a weaker TLR signal can be sufficient to reach an activation threshold.

## **ENDOGENOUS DANGER SIGNALS TRIGGER STERILE INFLAMMATION VIA A HETEROTRIMERIC COMPLEX OF CD36, TLR4, TLR6**

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In atherosclerosis and Alzheimer's disease, the deposition of the altered-self components oxidized LDL and  $\beta$ -amyloid triggers a protracted sterile inflammatory response that promotes the formation of plaques. This has been attributed to chronic activation of the innate immune system, however the molecular mechanisms of such activation remain unclear. We have identified a novel heterodimer of the Toll-like receptor family, TLR4/TLR6 that initiates signaling in response to atherogenic lipids and  $\beta$ -amyloid peptide and promotes inflammatory responses characteristic of both diseases. Importantly, assembly and activation of this heterodimer is regulated by the scavenger receptor CD36, a common binding receptor for these disparate ligands. Our results identify CD36/TLR4/TLR6 activation as the molecular mechanism by which these endogenous danger signals stimulate sterile inflammation and highlight the important role of co-receptor interactions in triggering TLR signaling.

## **HIGH-THROUGHPUT SCREENING STRATEGIES FOR THE IDENTIFICATION OF NOVEL TOLL-LIKE RECEPTOR AGONISTS**

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Viral infection of mammalian cells leads to an innate immune response characterised by the secretion of Type I interferons (IFN  $\alpha$ , IFN  $\beta$ ) which may be via Toll-like receptor (TLR) recognition of conserved microbial structures. These interferons are key mediators of the innate anti-viral response, leading to direct antiviral activity within cells. Synthetic compounds such as imidazoquinolines have been shown to stimulate interferon induction in a TLR-dependent manner, however, these immune response modifiers were identified prior to the discovery of the TLRs. There now exists an opportunity to rationally screen for TLR modulators which may have enhanced pharmacological properties, including defined specificity for TLR-dependent pathways.

We have investigated a number of cell-based systems for the identification of small-molecule TLR agonists. These include recombinant assays using cell lines transfected or transduced with human TLRs and in which signalling is quantified by either reporter activity or by nuclear translocation of transcription factors. As an alternative approach, we have investigated primary cell systems in which endogenous TLR expression drives interferon induction. These two approaches have been used within GlaxoSmithKline to screen large compound libraries to identify novel TLR agonists.

We will describe our experience of these two approaches, including the configuration of screening assays and the triage process to confirm activity and mechanism of action of hits. These studies provide evidence that a rational screening strategy can be used to identify novel small-molecule TLR modulators.

## **CANDIDA ALBICANS STIMULATION OF IL-1BETA BYPASSES INFLAMMASOME ACTIVATION IN HUMAN PRIMARY MONOCYTES**

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The release of IL-1beta has been postulated to depend on a protein complex called the inflammasome that activates caspase-1. Controversy has surrounded the capacity of fungal pathogens to induce production of IL-1beta, as no inflammasome activators are expressed by fungi. In the present study we demonstrate that human monocytes respond with release of IL-1beta after stimulation with TLR ligands or the fungal pathogen *C. albicans*. IL-1beta stimulation by *C. albicans* is modulated at the transcription level, through interaction with mannose receptor and dectin-1/TLR2 pathways. Westernblots of demonstrate both the constitutive activation of caspase-1 in monocytes, and the spontaneous release of ATP necessary for IL-1beta secretion. No activation of the inflammasome by fungal PAMPs are necessary for the stimulation of IL-1beta. siRNA experiments demonstrated that the constitutive activation of caspase-1 depends on ASC and NALP3. In contrast, caspase-1 is not active in macrophages, and macrophages respond with IL-1beta release only after two-hit stimulations with TLR ligands and inflammasome activators such as ATP. This dichotomy of IL-1beta processing and release underlines the functional differences between primary human monocytes and macrophages, two cell types that are present in different body compartments and have different functional specializations.

## **TOLL-LIKE RECEPTOR SIGNALING: NOVEL COMPONENTS AND REGULATION**

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For investigators interested in signal transduction, the area of TLRs has proved very fruitful in terms of the discovery of new signalling pathways and processes. We now have a good understanding of the major components activated by TLRs, notably the TIR domain- containing adapters that initiate signalling following recruitment to TIR domains within the TLRs themselves, the IRAK family of protein kinases that are then recruited, and a series of ubiquitination and phosphorylation reactions that ultimately lead to the activation of transcription factors such as NF-kappaB and IRF family members. The structural basis for signalling is still poorly understood however, and we have no appreciation of the kinetics involved in the pathways. Additional components and regulatory cross-talk from multiple signals also continue to be discovered. Genetic variation in signalling components such as in IRAK4, Mal and Unc93b however highlight the importance of these pathways in human health and disease. I will discuss our recent findings of a novel component in TLR4 signaling, what genetic variation in Mal tells us about the evolution of host defence to pathogens and also the molecular basis to how Mal signals, and the emerging role of miRNAs as key regulators of TLR signalling events.

## ORIGINS OF VERTEBRATE ADAPTIVE IMMUNITY

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Instead of immunoglobulin genes, jawless vertebrates such as lamprey and hagfish rearrange antigen receptors that consist of diverse leucine-rich repeat (LRR) modules. These variable lymphocyte receptors (VLR) are thus structurally related to the cardinal innate microbial recognition receptors of animals and plants, including the Toll and Toll-like receptors, the cytoplasmic NBS-LRR proteins and many of the plant disease resistance proteins.

Diverse repertoires of VLRs are assembled in lamprey lymphocytes by recombinatorial insertion of LRR modules, selected from arrays of hundreds of LRR-cassettes that flank the two germline *VLR* genes. An AID/APOBEC member of the family of DNA cytosine deaminases may mediate the gene conversion-like process. Each lymphocyte express a uniquely rearranged *VLR* gene in monoallelic fashion, resulting in a potential repertoire of over  $10^{14}$  antigen receptors. Lamprey immunized with various antigens, including particulate anthrax spores and soluble hen egg lysozyme (HEL), respond by production of circulating antigen-specific VLR, thereby indicating their role in acquired immunity. Hence, at the dawn of vertebrate radiation two completely different types of antigen receptors evolved, the immunoglobulin-based antibodies and T-cell receptors of jawed vertebrates and the LRR-based VLR of jawless fish, which form a link between our innate and adaptive immune systems.

## **SOMATIC CELL GENETICS FOR THE STUDY OF SIGNALLING IN INNATE IMMUNITY**

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To gain further insight into IRF and NF- $\kappa$ B signalling we have isolated mutants from mammalian somatic cell lines. For the IRF pathway we used homologous recombination in human cells to knock out candidate genes. For the NF- $\kappa$ B pathway we isolated mutants after random genome-wide mutagenesis based on their unresponsiveness to TLR agonists. From these mutants we have identified one novel gene, gp96, which is specifically required for the maturation of TLRs in the endoplasmic reticulum. We have also isolated clones deficient in Tlr9, Unc93b1, Myd88, Irak1, IKKbeta, Nemo and RelA.

I will describe insights we have obtained, using our collection of mutant cells, into how the NF- $\kappa$ B and IRF pathways are organized. Emphasis will be given a) to the TBK1 complex, where at least three adaptors (TANK, NAP1 and SINTBAD) compete for binding, and b) to Nemo, for which we have isolated a gain of function allele. This Nemo mutant, even when disabled to bind ubiquitin chains, constitutively activated the IKK complex. This finding suggests that Nemo is not a mere ubiquitin binding adaptor but rather harbours latent activation potential and signal processing ability. The implication of this finding for our understanding of NF- $\kappa$ B activation and potential parallels in IRF signalling will be discussed.

## **INNATE ACTIVATION OF DENDRITIC CELLS**

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Direct sensing of pathogen components is a major trigger of dendritic cell (DC) activation leading to adaptive immunity. We have been studying multiple pattern-recognition pathways that mediate this process. One pathway for sensing infection by RNA viruses involves recognition of viral genomes or virally-infected cells in endosomal compartments and utilises members of the toll-like receptor (TLRs) family, including TLR9, 7, or 3. Viral genomes can additionally be recognised in the cytosol by DExD/H-box helicases such as RIG-I, which are activated by RNAs bearing 5' phosphates. Finally, a distinct pathway involves cell surface and phagosomal recognition of yeasts by a C-type lectin, dectin-1, which signals via Syk kinase. These studies help build a global picture of the receptors and signalling pathways that regulate DC activation and have applications in immunotherapy of cancer and infectious diseases.

## **ROLE OF MYD88/IL-1R SIGNALING IN THE STIMULATION OF TH1 AND TH17 RESPONSES BY COMPLETE FREUNDS ADJUVANT**

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Mycobacteria strongly trigger TLR/ IL-1R signaling pathways and this is thought to play a major role in the induction of the adaptive immune response that controls infection in most individuals. Complete Freund's adjuvant (CFA), a suspension of heat killed *M. tuberculosis* in mineral oil, potently stimulates Th1 CD4+ T cell and humoral responses by mechanisms believed to involve similar innate stimulatory pathways. Of recent interest has been the discovery that the mycobacteria in CFA also promote Th17 development. Indeed, CFA immunization forms the basis of many Th17-dependent autoimmune disease models. We have investigated the specific role played by TLR/IL-1R signaling pathways in the stimulation of Ag specific Th17 versus Th1 responses following immunization with OVA in CFA. As predicted, we observed a major requirement for MyD88 signaling in the induction of OVA specific CD4+ T cell IFN- $\gamma$  and IL-17 responses. Mice doubly deficient in TLR2 and TLR9, two TLR stimulated by live *M. tuberculosis*, displayed only partial defects in these CFA induced Th1 and Th17 responses. In contrast, IL-1R<sup>-/-</sup> mice showed a near complete loss in Th17 and a major reduction in Th1 responses. Preliminary studies also implicate caspase-1 and ASC in CFA induced Th17 polarization. These findings suggest that TLR signaling plays a limited role in CFA triggered Th17 polarization and only partially explains its effects on Th1 induction. Instead the MyD88 dependence of these responses appears to rely primarily on IL-1R signaling triggered through an inflammasome based cascade. While providing information concerning the innate recognition pathways mycobacteria use to promote adaptive CD4+ T responses, these findings establish an easily manipulated in vivo model for dissecting the unique signals involved in Th17 polarization.

This work was supported in part by the NIAID and NCI intramural programs.

## A CENTRAL ROLE FOR FREE HEME IN THE PATHOGENESIS OF SEVERE MALARIA

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Under homeostatic conditions, heme (Fe protoporphyrin IX) exists essentially as a prosthetic group in a large number of hemoproteins. Under inflammatory conditions however, non-covalently bound heme can be released from hemoproteins, generating highly cytotoxic “free heme”. When pre-exposed to low concentrations of free heme, most cell types become resistant to heme toxicity. This acquired resistance occurs via a mechanism mediated by the induction of heme oxygenase-1 (HO-1; encoded by *Hmox1*), a stress responsive enzyme that catabolizes free heme into biliverdin, free iron and the gas carbon monoxide (CO). We found that this mechanism of protection against heme impacts in a very significant manner the pathogenesis of severe malaria, the disease caused by *Plasmodium* infection and accounting for more than one million deaths per year worldwide. The blood stage of *Plasmodium* infection is associated with lysis of red blood cell (RBC) and with release of hemoglobin (Hb) into the circulation. When detached from cell-free Hb during the course of *Plasmodium* infection, free heme can trigger the pathogenesis of severe malaria in mice. However, when mice are exposed to low levels of free heme before *Plasmodium* infection they become protected against the development of severe malaria. This acquired resistance mimics in many ways the protective effect of sickle cell anemia (SCA) against severe malaria. SCA is a hemolytic disease caused by a single amino acid substitution at the sixth position of the  $\alpha$ -chain of hemoglobin (Hb). As it develops, SCA leads to i) Hb release from RBC, ii) generation of circulating free heme and iii) systemic induction of HO-1 expression. When infected with *Plasmodium*, transgenic SCA mice do not succumb to severe malaria, a protective effect lost when the *Hmox1* locus is deleted by homologous recombination or when HO enzymatic activity is inhibited pharmacologically. Taken together, these observations reveal that the heme/HO-1 system plays a critical role in modulating the pathogenesis of severe malaria and unveil a novel mechanism underlying the protective effects of SCA against malaria.

## **THE ROLE OF THE PHAGOSOME IN SENSING OF GRAM-POSITIVE MICROBES**

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Phagocytosis is the process by which particles bound to the cell surface are internalized into a membrane-limited intracellular organelle. After internalization the phagosome matures and particles are delivered to mature phagolysosomes where the acidic and hydrolytic environment limits pathogen replication. The association of certain Toll-like receptors with this organelle led to the suggestion that TLRs might sample the luminal contents of these vacuoles. It has subsequently emerged that TLRs survey these and many other intracellular compartments including endosomes, golgi and the endoplasmic reticulum. However, the molecular details of the contribution of the phagosome to sensing of microbes has not been fully defined. Here we will discuss our progress in understanding the role of these organelles in ligand delivery and initiation of the host response to different pathogens.

## INDUCTION OF IFN- $\alpha/\beta$ BY BACTERIA: SIGNAL TRANSDUCTION AND FUNCTIONAL SIGNIFICANCE

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It has been established since a long time that the type I IFN family, comprising a single IFN- $\beta$  and multiple IFN- $\alpha$  subtypes, has a fundamental role in host resistance against viral infections. Whether endogenous production of these cytokines plays a protective role during infections by non-viral pathogens is controversial. Data obtained in my laboratory indicate that the role of IFN- $\alpha/\beta$  is pathogen-specific. For example, it is detrimental in experimental infections induced by *Listeria monocytogenes*, but it is protective in infections by extracellular pathogens, such as group B streptococci. Therefore, it was of interest to study the mechanisms underlying IFN responses to different species of bacteria. Intact organisms, as opposed to purified bacterial DNA or synthetic oligonucleotides, could not initiate TLR-dependent IFN induction in pDC. However robust responses were measured in other cell-types. Our data indicate that, as observed with viruses, parallel, cell type-specific pathways are involved in IFN responses to a single bacterial agent. Moreover, the mechanisms of IFN induction differ according to the pathogen's intracellular lifestyle. I will discuss the relevance of these findings in relationship to the *in vivo* role played by IFN- $\alpha/\beta$  during bacterial infections.

## **MAMMALIAN TARGET OF RAPAMYCIN (MTOR) ORCHESTRATES THE DEFENSE PROGRAM OF INNATE IMMUNE CELLS**

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The mammalian target of rapamycin (mTOR) can be viewed as cellular master complex scoring cellular vitality and stress. Whether mTOR controls also innate immunedefenses is currently unknown. Here we demonstrate that Toll-like receptors activate mTOR via PI3K/Akt. mTOR physically associated with the MyD88 scaffold protein to allow activation of interferon (IFN) regulatory factor (IRF)-5 and IRF-7, known as master transcription factors for pro-inflammatory cytokine- and type I IFN-genes. Unexpectedly, inactivation of mTOR did not prevent but increased lethality of endotoxin-mediated shock, which correlated with increased levels of IL-1b. Mechanistically, mTOR suppresses caspase-1 activation, thus inhibits release of bioactive IL-1b. We have identified mTOR as indispensable component of pattern recognition receptor signal-pathways that orchestrates the defense-program of innate immune cells.

## **INTRA-NASAL INSTILLATION OF MONOMERIC ENDOTOXIN:HUMAN MD-2 COMPLEX PROTECTS MICE FROM PNEUMONIC PLAGUE**

Athmane Teghanemt<sup>1</sup>, Theresa Gioannini<sup>1</sup>, Suzana Hadina<sup>2</sup>, DeSheng Zhang<sup>1</sup>, Peter Thorne<sup>2</sup>, Ashok Chopra<sup>3</sup>, and Jerrold Weiss<sup>1</sup>

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Virulent *Yersinia pestis* is the cause of pneumonic plague. Evasion of Toll-like receptor (TLR) 4 signaling is a major determinant of *Y. pestis* virulence. We therefore tested if activation of airway TLR4 activity by intranasal (i.n.) instillation of a potent TLR4 agonist could protect against lethal airway *Y. pestis* infection. Monomeric endotoxin (E):MD-2 complexes are unique in their ability to activate, at pM concentrations, cells expressing mCD14/MD-2/TLR4 (e.g., alveolar macrophages) or TLR4 alone (e.g., airway epithelial cells). E:MD-2 induced dose-dependent, TLR4-dependent acute airway inflammatory responses including recruitment of neutrophils and mobilization of chemokines and cytokines into the airway lumen. Maximum responses were induced within 4-24 h by 15 ng of E:MD-2. A complex of E:MD-2F126A that activates macrophages but not airway epithelial cells induced ca. 30% less airway inflammation, suggesting that effects of instilled wt E:MD-2 were due to TLR4-dependent activation of macrophages and epithelial cells. I.n. administration of 15 ng (E) of wild-type E:MD-2 48 h and 24 h before i.n. administration of virulent *Y. pestis* CO92 (10x LD<sub>50</sub> dose) resulted in survival of 90% (9/10) of the infected mice, whereas only 1/30 mice survived infection when E:MD-2 was administered at either the time of infection, 24 h after or not at all. These findings suggest that E:MD-2 could provide a novel approach to immunoprophylaxis in outbreaks of pneumonic plague as could occur following a bio-terrorist attack.

## MACROPHAGES, INFLAMMATION AND ATHEROSCLEROSIS

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Walls of the major arteries are susceptible to pathologic changes that shorten the lives of nearly half of individuals in developed societies. In response to high circulating LDL cholesterol, aging and other risk factors, macrophages accumulate in the artery walls and secrete a variety of chemokines, cytokines, proteases and procoagulant factors. Atherosclerotic plaques (concentrations of accumulated macrophages and lipids) may rupture giving rise to an occlusive and often fatal thrombus. We have used mRNA profiling to examine a large number of plaques taken from arteries in the neck and legs of human subjects. We find heterogeneity, with some plaques characterized by a large number of proinflammatory genes including nearly all those involved in Toll signaling. This profile appears enriched in carotid plaques taken from patients experiencing symptoms vs those from asymptomatic patients. Further work has shown heterogeneity along the length of carotid plaques, with areas of high expression of inflammatory markers coinciding with areas marked by histologic evidence of inflammation. We further profiled aortas taken from atherosclerosis-prone animals during the course of atherogenesis. We observed the progressive expression of a set of proinflammatory genes with extremely high overlap with the "hot" profile of human plaque. In a mated set of studies, we also compared the expression pattern in plaques taken from animals that had been treated with a novel anti-atherosclerotic therapy: an inhibitor of the enzyme 11-beta-hydroxysteroid dehydrogenase (HSD). We found a remarkable overlap between the genes suppressed by HSD inhibitors and the genes induced in atherogenesis or in human hot plaque. The changes appeared not just in the number of macrophages but also in the phenotype of the cells in the lesions. These data suggest that proinflammatory tone may be not only a hallmark but a causal feature of atherogenesis. Modulation of this tone may provide a novel therapy for atherosclerotic disease.

**LATE**

**BREAKING**

**ABSTRACTS**

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## PRO- AND ANTI-INFLAMMATORY LIPIDS MODULATION OF GENE EXPRESSION ON MYCOBACTERIA-INFECTED MACROPHAGES

Paulo Bettencourt<sup>1, 2</sup>, Daniela Fabrino<sup>2</sup>, Jonathon Blake<sup>3</sup>, Mark Kuehnel<sup>4</sup>, Vladimir Benes<sup>3</sup>, Gareth Griffiths<sup>2</sup>, and Elsa Anes<sup>1\*</sup>

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*Mycobacterium tuberculosis* survive within specialized membrane-enclosed organelles, phagosomes, in macrophages. In contrast, the non-pathogenic *Mycobacterium smegmatis* is killed within macrophage phago-lysosomes. Here, phagosomes fuse with lysosomes resulting in the full acidification of the vesicle, the acquisition of acid hydrolases and reactive nitrogen intermediates. The presence of *M. tuberculosis* in the phagosomes inhibits all these killing processes. When activated by gamma interferon or pro-inflammatory lipids such as ceramide (Cer) and arachidonic acid (AA), macrophages can kill *M. tuberculosis*. Conversely, anti-inflammatory lipids, such as eicosapentaenoic acid (EPA) have the opposite effect and facilitate pathogen growth. To better understand the early events that mediate an increased survival/killing of mycobacteria, J774 macrophages treated with either the anti-inflammatory EPA or with the pro-inflammatory Cer, were infected with *M. smegmatis*. The expression profile after 1 hour of infection was dissected by microarray analysis. We found 21 genes strongly down-regulated in infected macrophages treated with EPA. In infected macrophages treated with Cer the list of down-regulated genes increased to 34. Surprisingly, 10 of these genes were common to both lipid treatments and, no genes were found to be over-expressed. Among these genes, we found potential candidates with a role in innate immune responses.

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## NEONATAL INNATE IMMUNE RESPONSES TO BCG VARYING BETWEEN POPULATIONS INFLUENCE THE DEVELOPMENT OF PROTECTIVE TH1 RESPONSES

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**Background** *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) is given to millions of newborns, but its efficacy varies between populations. We studied the role of neonatal innate function herein.

**Methods** Cord blood mononuclear cells (CBMC) collected from deliveries in Papua New Guinea (PNG) and Western Australia (WA) were compared for *in vitro* cytokine responses to BCG (TNF- $\alpha$ , IFN- $\gamma$ , type-I interferon, IL-6, IL-10, IL-12 and IL-23). IFN- $\gamma$  was added to cultures to compare responsiveness under optimal priming conditions. For children in PNG longitudinal associations between innate immune responses at birth and T helper (Th) responses at 3 months were studied.

**Results** BCG-induced production of IL-6 ( $p = 0.010$ ) and IL-10 ( $p < 0.001$ ) was significantly enhanced in PNG compared to WA newborns. IFN- $\gamma$  priming significantly enhanced BCG-induced IFN- $\gamma$ , TNF- $\alpha$ , IL-12 and type-I interferon responses in both groups, but to a significantly higher extent in WA newborns. This was most apparent for IFN- $\gamma$ , which was boosted 150-fold in WA newborns but only 8-fold in PNG newborns ( $p < 0.001$ ). Neonatal innate IL-6 and IL-10 responses were inversely associated with Th1 responses to PPD in 3 month old PNG infants. An opposite trend was seen for Th2.

**Conclusions** In human newborns the innate immune system is characterized by high IL-6, IL-10 and IL-23, and low IFN- $\gamma$ , TNF- $\alpha$ , type-I interferon and IL-12 production. We here show that the skewing of this typical neonatal innate response varies between populations and influences the subsequent development of protective Th1 responses following neonatal BCG vaccination.

## **TOLL-LIKE RECEPTOR EXPRESSION IS NOT ASSOCIATED WITH CLINICAL OUTCOME DURING ACUTE RENAL ALLOGRAFT REJECTION**

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Toll-like receptors (TLRs) are a family of known conserved pattern recognition receptors of which several are expressed in the kidney. Earlier studies have shown that TLRs contribute to ischemia-reperfusion induced injury in the kidney in mice. Knowledge about the contribution of TLRs in renal transplantation in human is limited. Therefore we determined mRNA expression of TLR1-10 in renal tissue in thirty-six patients during acute renal allograft rejection and correlate these data with cell recruitment, renal transplant function and response to conventional anti-rejection therapy. Response to therapy was defined as a decrease in serum creatinine level of maximally 125% of the value prior to the clinically diagnosed episode of allograft rejection. Late graft outcome was defined as return to dialysis and creatine clearance between 6 and 12 months after transplantation. As control, 14 transplant patients who showed no clinical or histopathological evidence of acute or chronic allograft rejection were selected. All patients received similar immunosuppressive therapy. mRNA levels of TLR1-4, TLR7 and TLR8 was increased in biopsies obtained from patients with acute renal allograft rejection compared to control biopsies. Response to conventional anti-rejection therapy and late renal outcome was not correlated to expression of a specific TLR. Except for TLR3, all TLR mRNA levels were correlated to inflammatory infiltrate. In conclusion, TLR profiles in biopsies from acute allograft rejection are not directly correlated to clinical outcome.

## **“SYNTHETIC PATHOGENS FOR INTEGRATED BIOPHYSICAL AND GENETIC DISSECTION OF ANTIGEN PRESENTATION”**

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The study of host-pathogen interactions can have a dramatic impact on the understanding of the outcome of immune responses and has the potential to lead the way for the design of novel effective therapeutic strategies. As a novel approach to understand how the physical and biochemical nature of particulate antigens influences their uptake and fate in Antigen Presenting Cells (APCs), we are studying the internalization, traffic and processing of 'synthetic pathogens'-model particles with distinct, well-defined physical and biochemical properties. TLR (Toll-Like receptors) emerges as the key receptors to distinguish between self/non-self, by recognition a broad range of pathogen components (PAMPs). We are making use of particles with Ovalbumin as model antigen and ligands for TLR, attached by covalently chemistry. We are comparing how signals from pathogen structure/composition itself modulate phagocytosis and subsequent immunity in the context of a single well-defined particle platform. We are determining the signaling events that regulate this process using a lentiviral RNAi library to evaluate the role of known and novel genes in this model system, and 3D time-lapse imaging to characterize each step in detail. These studies will have a major impact by elucidating how pathogen structure and chemistry dictates signaling, intracellular traffic, antigen processing, immune responses and pathogen survival or elimination.

## **A POXVIRAL HOMOLOG OF PELLINO INHIBITS TOLL AND TOLL-LIKE RECEPTOR SIGNALLING**

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Toll-like receptor (TLR) signaling pathways constitute an evolutionarily conserved component of the host immune response to infection. These pathways are subject to subversion by viral immunoevasins, as part of a strategy to evade eradication and to aid pathogen dissemination. *Melanoplus sanguinipes* entomopoxvirus encodes an ORF, the conceptual translation of which encodes a homolog of the Pellino protein. Viral Pellino can inhibit Toll- and TLR-mediated activation of downstream Rel family transcription factors. It attenuates drosomycin promoter activation by Spätzle in *Drosophila melanogaster* cells, and blocks the activation of NF-kappaB by TLR signaling components and by the TLR4 ligand, LPS, in human cells. Viral Pellino can associate with proteins containing the TIR domain and with the kinase IRAK-1. It also functionally antagonises the activity of human Pellino3S. Thus, our findings identify potential immunoevasive capabilities possessed by a poxviral homolog of the Pellino protein and support a crucial role for Pellino proteins in Toll and TLR signaling.

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## ACTIVATION OF DCs BY TLR7 AND TLR9 LIGANDS INHIBITS THE GENERATION OF REGULATORY T CELLS

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In systemic lupus erythematosus (SLE) activated autoreactive T and B cells produce pathogenic antinuclear antibodies. Usually this is prevented by tolerance mechanisms including regulatory T cells (Treg). Endogenous TLR7 and 9 ligands present within these nuclear autoantigens are critically involved in the systemic autoimmune response in SLE. We therefore asked the question if TLR7 and TLR9 ligands in the presence or absence of dendritic cells (DC) inhibit the generation and function of Tregs.

To study this question we cultured naive CD4<sup>+</sup> T cells isolated from the spleen of C57BL/6 mice *in vitro* under conditions ideal for Treg cell generation (TGF- $\beta$ , anti-CD3, anti-CD28 and IL-2) in the presence or absence of CD11c<sup>+</sup> splenic DCs. To investigate the specific effect of TLR7 and TLR9 ligation on Treg cell generation *in vitro* Imiquimod (TLR7) or CpG (TLR9) were added to the culture. The amount of generated Treg cells was assessed by fluorescent staining of CD4, CD25 and Foxp3 after 4 days. The concentration of the cytokines IL-6, IL-17A und IFN- $\gamma$  was measured in the supernatants of the culture and after restimulation.

The number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells generated in the absence of DCs was not influenced by TLR7/9-ligands. Similarly coculture with DCs did not affect Treg cell generation in the absence of TLR ligands. However the number of Tregs generated decreased significantly when TLR7/9-ligands were added to the T cell/DC coculture, whereas TLR4 ligand LPS had no significant effect. This result suggests that DCs activated through TLR7 and 9 inhibit the *de novo* generation of Treg cells from naive T cells *in vitro*. IL-6 and IL-17 production in the coculture was induced by both TLR7 and TLR9 ligation, but did not directly correlate with the extent of inhibition of Treg cell generation, suggesting that other mechanisms including cell-contact-dependent factors exist, which reduce the efficiency of Treg cell generation in the presence of DCs and TLR7/9 ligands.

## **BACTERIAL MEMBRANE VESICLES ENTER HOST CELLS TO MEDIATE NOD1 IMMUNE RESPONSES.**

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The cytosolic host protein, NOD1, acts as an intracellular sensor of bacterial pathogens through its recognition of Gram-negative peptidoglycan (PG). Although epithelial cells are refractory to external stimulation with PG, these cells respond in a NOD1-dependent manner to infection with bacteria that either invade or that can secrete effector molecules into the cytoplasm of host cells. Nevertheless, the mechanisms by which these bacteria are able to release or deliver their PG, respectively, within the cytoplasm have yet to be elucidated. Here we show that outer membrane vesicles (OMVs), which are shed by Gram-negative bacteria during normal growth, act as a delivery system for PG to NOD1 within host cells. External stimulation of epithelial cells and fibroblasts with OMVs from different Gram-negative pathogens was shown to induce NOD1-dependent pro-inflammatory responses in cells. We demonstrated that bacterial OMVs contain PG and that their cellular entry via lipid raft domains is essential for the induction of NOD1 responses. Moreover, orally administered OMVs induced NOD1-dependent innate and adaptive immune responses in mice. Collectively, our data identify OMVs as a novel virulence mechanism by which Gram-negative pathogens may transport their products to innate immune molecules within the cytoplasm of host cells. The presence of OMVs in infected tissues *in vivo* suggests that OMVs may drive the inflammation associated with infection. Furthermore, it is likely that OMV-mediated innate immune signalling contributes to the protective immunity induced by OMV-based vaccines.

## GENETIC DISSECTION OF ANTIGEN CROSS-PRESENTATION PATHWAYS

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Effective immune responses against tumor antigens that are not endogenously expressed by dendritic cells (DCs) and against virus that do not infect antigen presenting cells (APCs) require extracellular antigens to stimulate CD8<sup>+</sup> T cells via the MHC I pathway through a process known as cross-presentation.

The molecular mechanisms that direct endocytosed antigens from the classical MHC II-restricted presentation pathway to the MHC I pathway are for the most part not understood. We have recently completed a primary screen for genes with a role in this process using a subset of an shRNA lentiviral library enriched for mouse kinases and phosphatases. In this screen we measured the proliferation of CD8<sup>+</sup> T (OT-I) cells in response to the stimulation of bone marrow derived DCs after incubation with ova-expressing *S. cerevisiae*. After testing over 1000 genes in duplicate and two rounds of phenotypic validation, we have chosen ~60 genes that reproducibly caused increased (more than 1.5 SDEV) or reduced (less than 1.2 SDEV) rates of CD8<sup>+</sup> T proliferation.

We have now tested these 60 genes in a similar assay but using B3Z as the responder cell line. We have found that 31 genes caused a significant change in the levels of IL-2 production. Because IL-2 production by this cell line requires presentation of the SIINFEKL peptide by MHC I but it is co-stimulator independent, it is likely that this group of genes regulates presentation while the other group regulates co-stimulator induction.

We are currently performing qPCR validation of the knockdowns and additional secondary screens on this group of 31 genes so that we are able to identify those that are specifically required for cross-presentation. These genes will be selected for further mechanistic studies. In the future, we plan to expand the screen using the shRNA lentiviral virus to other functional gene families.

## **EVIDENCE FOR AN INTERFERON-INDEPENDENT HIV-1 ANTAGONIST THAT IS REGULATED BY LPS**

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Macrophages are an important reservoir for primate lentivirus replication and persistence. Most of our understanding regarding factors that regulate virus-host cell interplay have been derived from studies with lymphocytes. We undertook the following study in order to identify factors that regulate interplay between immunodeficiency viruses and macrophages. Lipopolysaccharide (LPS), a major component of Gram-negative bacterial cell walls, activates macrophages through engagement of Toll-like receptor 4 (TLR4) and the CD14 receptor. Exposure to LPS has previously been shown to protect macrophages from HIV-1 infection through different mechanisms, including chemokine release, down-regulation of chemokine receptors and cytokine production. Here, we present evidence for an HIV-1 antagonist that is regulated by LPS and that acts at a step in the viral life cycle prior to or at reverse transcription. Surprisingly, neutralization of LPS conditioned macrophage supernatant with polymyxin B did not rescue the infection. Furthermore, neutralization of type I interferon with B18R, a soluble virus-encoded type I IFN ligand, did not significantly diminish the antiviral activity of LPS stimulated macrophage supernatants. This indicates that the LPS-regulated antagonist is not a secondary result of LPS carryover and Interferon induction. The ability of the antagonist to inhibit pseudotyped HIV-1 infection, which is chemokine receptor independent, further indicates that the antagonist is not a chemokine. Collectively, our data provides evidence for a novel HIV-1 antagonist that is induced from macrophages by LPS.

## SYSTEMATIC IDENTIFICATION OF SPLICING FACTORS WITH A ROLE IN IL-1 $\beta$ SECRETION BY SHRNA SCREENING

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The large percentage of immune relevant genes that are alternatively spliced and the connections between splicing and disease, strongly indicate that alternative splicing (AS) plays a central role in the regulation and fine-tuning of physiological immune responses.

IL-1 $\beta$  is an important proinflammatory cytokine produced by activated macrophages and monocytes. It functions in the generation of systemic and local responses to infection, injury, and is the primary cause of chronic and acute inflammation. IL-1 $\beta$  is produced as an inactive cytoplasmic precursor that is proteolytic processed by the inflammatory caspase-1 to generate the mature secreted active form. Caspase-1 is also synthesized as an inactive form that requires processing by the inflammasome to become active.

We have used a subset of the TRC lentiviral human library to generate loss-of-function phenotypes for most of the splicing factors and regulators of splicing. With this tool we were able to silence the expression of 425 genes involved in splicing with an average 5-fold coverage.

After the primary screen and several rounds of phenotypic validation, we have identified 30 genes that significantly affect the production of IL-1 $\beta$  by THP-1 cells after a 24h challenge with PFA-fixed E. coli, as measured by ELISA in the conditioned media. The levels of the knockdown were analyzed by qRT-PCR for the most significant candidates to validate the phenotypes observed. 20 of these genes were required for IL-1 $\beta$  secretion, while 10 were negative regulators of this process. Silencing of 5 of these negative regulators caused no change in the amount of IL-8 secreted by THP-1 cells in the same conditions. Because IL-8, contrary to IL-1 $\beta$ , is produced in its mature form, it is likely that 5 of those genes are involved in the regulation of the inflammasome.

## **MYD88 CRYSTAL STRUCTURE LENDS INSIGHTS INTO TIR ADAPTOR SIGNALING**

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The innate immune system represents a first line of defense in detection, response and clearance of pathogens. Pattern recognition receptors (PRRs) of the innate immune system detect pathogen-associated molecular patterns (PAMPs) via recognition of pathogen specific structural features. The toll-like receptors (TLRs) are PRRs that bind pathogen derived lipopeptides, dsRNA, ssRNA, LPS and flagellin. Signaling downstream of the TLRs involves cytoplasmic adaptors that contain Toll-IL-1R homology (TIR) domains. Recent structural reports have shed light on TLR-mediated ligand recognition. However, the structural basis of TIR domain mediated signaling remains unclear despite a wealth of functional data and modeling reports. In an effort to better understand the mechanisms of specific TIR domain binding and signaling, we now report the 1.8 Å resolution X-ray crystal structure of MyD88. Studies using dominant negative mutants and knockout mice have clearly shown that MyD88 residues at the BB loop, DD loop, as well as the Poc Ile179 are critical for signaling and appropriate responses to pathogen stimuli. A crystallographic dimer of two MyD88 TIR domains reveals potential interactions between the BB-DD loop and BB-EE loops. Additionally, the BB loop of MyD88 TIR domain adopts a drastically different conformation in comparison with other TIR domain structures, implicating a unique structural feature of MyD88 relevant to its signaling specificity. Furthermore, the Poc site Ile179 is juxtaposed near Pro200 and may affect MyD88 signaling through modulation of the BB loop. These structural insights we hope will aid our understanding of the molecular mechanisms by which TLRs and their adaptors interact and signal.