Abstracts of oral presentations 1 to 46

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Evolutionary biology of endotoxin: from genomics to physiology

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While it is convenient to represent the lipopolysaccharide (LPS) molecule as a static structure, it was apparent from the early structural analyses that there is extensive heterogeneity in the LPS molecular species isolated from a culture of a given organism. The development of refined analytical methods has provided critical detailed insight into the molecular architecture and structural diversity of LPS. At the same time, the identification of genes involved in LPS biosynthesis, and the analysis of LPS structures resulting from specific mutations, has led to an understanding of the biochemistry underlying assembly of the LPS molecule. Much of the initial information has come from model systems involving *Escherichia coli* and *Salmonella* spp. but it is clear that many of the features and concepts also apply to other bacteria. In this presentation, I will use *E. coli* and *Salmonella* to illustrate the contributions of genetic diversity to variations in LPS structure from related strains. I will also discuss some of the biosynthetic reactions and environmentally-regulated modifications that result in structural heterogeneity within a given strain. All of this information is providing a better insight into the importance of specific LPS structural elements in the biology of Gram-negative bacteria.

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Endotoxin signaling

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Remodeling of *Helicobacter pylori* LPS

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The major lipid A species of *Helicobacter pylori* shows several differences to that of *Escherichia coli*. It has fewer acyl chains, a reduced number of phosphate groups, much lower immunobiological activity, and only a single Kdo sugar can be found linked to the lipid A disaccharide. However, *H. pylori* synthesizes a minor lipid A species resembling that of *E. coli*, which is both *bis*-phosphorylated and hexa-acylated suggesting that the major species results from the action of specific modifying enzymes. Using an *in vitro* assay system with defined lipid A substrates, we have demonstrated enzymatic activities for two separate *H. pylori* lipid A phosphatases, and a pEtN transferase. Furthermore, we have discovered a novel Kdo-hydrolase that requires prior removal of the 1-phosphate group for enzymatic activity. Modification of the inner core-lipid A region of *H. pylori* LPS may reduce the endotoxicity of the molecule helping to prolong infection. Since modification of the lipid A domain of LPS has been reported to contribute to the virulence and pathogenesis of various Gram-negative bacteria, investigation of defined *H. pylori* mutants will help unravel the importance of these modifications in *H. pylori* pathogenesis.

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Structure and function of PagP

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Lipid A palmitoylation in enterobacteria is determined by a PhoP/PhoQ-activated gene *pagP*, which encodes an unusual outer membrane enzyme of lipid A biosynthesis. PagP structure and dynamics have now been elucidated by both NMR spectroscopy and X-ray crystallography. PagP is an 8-stranded antiparallel β-barrel preceded by an N-terminal amphipathic α-helix. The PagP barrel axis is uniquely tilted by 25° with respect to the membrane normal. An interior hydrophobic pocket in the upper half of the molecule functions as a hydrocarbon ruler, which allows the enzyme to distinguish palmitate from other acyl chains found in phospholipids. Internalization of a phospholipid palmitoyl group within the barrel appears to occur by lateral diffusion from the outer leaflet through non-hydrogen bonded regions between β-strands. The MsbA-dependent trafficking of lipids from the inner membrane to the outer membrane is necessary for lipid A palmitoylation *in vivo*. Disruption of lipid asymmetry directly activates PagP in outer membranes presumably by promoting the migration of phospholipids into the outer leaflet.

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Lipid A deacylation by PagL: regulation of TLR4 signaling

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Toll-like receptor (TLR) 4-mediated responses, which are induced by the lipid A portion of lipopolysaccharide, are important for host defense against Salmonellae infection. A variety of different data indicate that the acylation state of lipid A can alter TLR4-mediated responses. The *S. typhimurium* virulence genes PhoP/PhoQ sense host micro-environments to regulate the expression of a lipid A 3-*O*-deacylase, PagL, and a lipid A palmitoyltransferase, PagP. We demonstrate that 3-*O*-deacylation and palmitoylation of lipid A decreases its ability to induce TLR4-mediated signaling. De-acylated lipid A, de-acylated and palmitoylated lipid A, palmitoylated lipid A, and unmodified lipid A species were purified from *Escherichia coli* heterologously expressing PagL and/or PagP. The activity of purified lipid A species was examined using human and mouse cell lines that express recombinant TLR4. Compared with unmodified lipid A, the modified lipid A species are 30–100-fold less active in the ability to induce NF-κB-dependent reporter activation. These results suggest that the lipid A modifications reduce TLR4-signaling as part of Salmonellae adaptation to host environments. In addition, we demonstrate that PagL-dependent lipid A de-acylation is usually inhibited by another PhoP/PhoQ-induced membrane modification in *S. typhimurium*.

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Biological relevance of lipid A variation in *Yersinia* spp.

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Important pathogens in the genus *Yersinia* include the plague bacillus *Yersinia pestis* and two enteropathogenic species, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*. A shift in growth temperature induced changes in the number and type of acyl groups on the lipid A of all three species. At a temperature that mimics growth with the flea (21°C), *Y. pestis* synthesized a hexa-acylated lipid A molecule that was modified by a C16:1 fatty acid acyl group. In contrast, after growth at mammalian body temperatures (37°C) *Y. pestis* produced predominately tetra- and penta-acylated lipid A structures. Specifically, *Y. pestis* lipid A consisted of lipid IV_A (a disaccharide precursor of lipid A) and smaller amounts of penta-acylated lipid A structures modified by the addition of either C10:0 or C12:0 fatty acid acyl groups. In contrast, *Y. pseudotuberculosis* primarily synthesized lipid IV_A modified with C16:0, and *Y. enterocolitica* produced a unique tetra-acylated lipid A. The acyltransferase genes (*htrB* and *lpxP*) required for increased acylation at 21°C have been

determined. The more complex lipid A made by the three species at 21°C stimulated human monocytes to secrete TNF-α, but lipid A synthesized at 37°C did not. Finally, the *Y. pestis phoP* gene was required for aminoarabinose modification of lipid A but not for the temperature-dependent acylation changes. Production of a less immunostimulatory form of LPS upon entry into the mammalian host appears to be a conserved pathogenesis mechanism in the genus *Yersinia*, and species-specific lipid A forms may be important for life cycle and pathogenicity differences.

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Lipid-mediated resistance of Gram-negative bacteria against various pore-forming antimicrobial peptides (AMPs)

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Lipopolysaccharides (LPSs) play a dual role as target and as effector molecules. The knowledge of the LPS-induced activation of human immune cells is increasing; however, surprisingly much less effort seems to be directed towards the understanding of the mechanisms leading to the killing of the bacterial organisms, which possibly results in the release of LPS from the bacterial surface into the blood circulation.

In this contribution, mechanisms of interaction of peptides of the innate immune system such as defensins and cathelicidins as well as of externally administered antibiotics such as polymyxin B will be demonstrated. The main focus will be directed on data derived from electrical measurements on a reconstitution system of the outer membrane as an asymmetric bilayer composed on one side of LPS and on the other of phospholipids. These results will be complemented by data obtained with atomic force microscopy.

All three membrane active peptides induce the permeabilization of the reconstituted membranes by the formation of lesions. We found that differences in the antimicrobial activity of the peptides against various sensitive and resistant Gram-negative bacteria can be explained solely by variations in the chemical structure of LPS. A reduction of the net negative charge of LPS is responsible for a reduced interaction with the AMPs and thus for resistance. A most important side effect of polycationic AMPs is the neutralization of the anionic LPS released from the bacterial surface as a consequence of AMP-induced killing.

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Molecules from eukaryotic algae possess LPS-like features but inhibit innate responses of mouse macrophages

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Some eukaryotic algae live either as hereditary endocellular symbionts in invertebrates (*Chlorella*) or as opportunistic pathogens of humans and livestock (*Chlorella, Prototheca*). For successful entry and persistence, the algae must employ means to avoid detection and reduce or block the host's ability to mount a successful defence. We discovered that elicited mouse macrophages avidly phagocytosed cells of a symbiotic strain of *Chlorella* (NC64) and *Prototheca* cells (UTEX289) cultured from isolates obtained from a human infection. In both cases, the phagocytosed algae were sequestered in individual vacuoles and escaped digestion. Moreover, macrophages containing the algal cells were essentially completely refractory to stimulation by Gram-negative endotoxin (lipopolysaccharide, LPS). Molecules extracted from the algae were effective LPS antagonists yet evidenced biological activity and chemical features reminiscent of Gram-negative endotoxin (positive LAL inhibited by polymyxin B; CD14 binding; presence of KDO, hexoses, glucosamine, fatty acids; r-form PAGE). Our data suggest that the algal molecules are immunomodulators that potentiate entrance into the hosts and evasion of the innate defenders allowing long-term survival in host tissues.

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Endotoxin: physical requirements for cell activation

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Starting from the surprising observation that natural isolates of lipid A from *Escherichia coli* express a higher endotoxic activity than similar amounts of the synthetic *E. coli*-like hexa-acylated lipid A (compound 506), we looked

more closely at the chemical composition of natural isolates, in particular at their acylation pattern. In these isolates, we found not only a hexa-acylated fraction but also significant amounts of penta- and tetra-acylated lipid A part structures. As pure compounds, these part structures antagonize the induction of cytokines in human mononuclear cells when administered prior to stimulation with biologically active endotoxin. To mimic the natural isolates with aggregates containing the hexa-acylated as well as the under-acylated endotoxins, we prepared mixed aggregates containing compounds 506 and 406 (tetra-acylated precursor IVa) in different molar ratios by dissolving both compounds in chloroform, mixing them and, after evaporation of the chloroform, forming aggregates in buffer. Surprisingly, the mixtures showed higher endotoxic activity than the pure compound 506 up to an admixture of 20% of compound 406. Similar results were obtained by the admixture of various phospholipids instead of compound 406. These observations can only be understood by assuming that the active unit of endotoxins is the aggregate.

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Innate immune recognition of LPS by TLR4/MD-2

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Lipopolysaccharide (LPS) is recognized by the receptor complex consisting of Toll-like receptor 4 (TLR4) and MD-2. We previously showed the LPS/TLR4/MD-2 complex on the cell surface. The ligand/receptor complex then homotypically interacted with each other and LPS signal is triggered. Lipid IVa, an antagonist on human TLR4/MD-2, bound to TLR4/MD-2 but did not induce subsequent homotypic interaction. TLR4/MD-2 discriminated between lipid A and lipid IVa not in ligand binding but in subsequent homotypic interaction. LPS recognition by TLR4/MD-2 can trigger endotoxin shock. TLR4/MD-2 is a target molecule for therapeutic intervention in endotoxin shock. We studied the effect of two antibodies to mouse TLR4/MD-2 on experimentally induced endotoxin shock by LPS/D-galactosamine. One of the two antibodies was found to be protective against lethal hepatic failure induced by LPS/D-galactosamine. The mechanism underlying the protection will be discussed in this presentation.

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Molecular basis of action of endotoxin agonists and antagonists

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Potent TLR4-dependent cell activation by Gram-negative bacterial endotoxins depends on sequential endotoxin–protein and protein–protein interactions with LBP, CD14, MD-2 and TLR4. Previous studies have suggested that reduced agonist potency of under-acylated endotoxins (*e.g.* tetra- or penta- versus hexa-acylated) is determined by interactions after CD14. To better define the molecular basis of the differences in agonist potency of endotoxins differing in fatty acid acylation, we compared wild-type (hexa-acylated) and *msbB* (penta-acylated) meningococcal lipo-oligosaccharides (LOSs) as well as wild-type treated with acyloxyacylhydrolase, AOAH (tetra-acylated). To facilitate assay of LOS–protein and LOS–cell interactions, LOS was purified after metabolic labeling with [^{3}H]- or [^{14}C]-acetate. All LOS species tested formed monomeric complexes with MD-2. However, cell activation by *msbB* LOS:MD-2 and AOAH treated was < 10% of that of wild-type LOS:MD-2. Moreover, addition of excess *msbB* LOS:MD-2 inhibited cell activation by wild-type LOS:MD-2. These findings suggest that decreased acylation of endotoxin agonists decrease TLR4-dependent cell activation by reducing receptor activation normally conferred by interaction of endotoxin:MD-2 with TLR4.

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Structural interactions of LPS with MD-2 and neutralizing peptides

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Knowledge of structural details on interaction between LPS and proteins and peptides is important for understanding the mechanism of signal initiation as well as for design of LPS-neutralizing compounds as potential drugs. Interaction between TLR4-bound MD-2 and LPS is the last step before the initiation of intracellular signal transduction across the cell membrane. Based on the sequential similarity and fold recognition, we have prepared a structural model of MD-2 based on the ML family, particularly Der p2 and GM2-AP. Disulfide connectivity in the model is in agreement with significance of particular cysteine residues, particularly Cys95–Cys105 loop and Cys133 as the single non-paired residue. Mutagenesis of basic residues confirmed the structural model and importance of electrostatic interactions for recognition of LPS. Presence of the hydrophobic binding site in MD-2 was also observed. This dual electrostatic–hydrophobic nature of interaction was also observed in the NMR structure of the complex between LPS and a peptide fragment of lactoferrin. Despite the absence of persistent structure of this peptide in solution, a defined tertiary structure is induced upon binding to LPS and in anionic and zwitterionic micelles. Structure of the peptide complex with LPS, determined using the transferred NOESY effect, reveals a well-formed hydrophobic core induced upon binding to LPS. Docking of LF11 to LPS and fluorescence quenching data suggest that tryptophan residue lies at the interface between the polar and hydrophobic region of the lipid layer, phenylalanine side chain contacts the alkyl chain region while the distance between clusters of basic residues matches the distance between the phosphate groups of the lipid A moiety of LPS. Similarities between the mode of binding between LF11 and iron uptake receptor FhuA indicate that the pattern of LPS–protein/peptide interaction is conserved.

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Structural determinants of Toll signalling: Spatzle and MD-2

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Lipopolysaccharide (LPS) from the outer cell wall of Gram-negative bacteria is a potent stimulator of the mammalian innate immune system. The Toll-like receptor TLR4 pathway triggers the inflammatory responses induced by LPS in a process that requires the interaction of LPS-bound MD-2 with TLR4. I will review the evidence available about the structural basis of LPS recognition and suggest two possible mechanisms for signalling that take into account the structural information available to date for TLR4 and MD-2, and the determinants of endotoxicity, that is the acylation and phosphorylation patterns of LPS. In the first model, LPS induces association of two TLR4/MD-2 heterodimers by binding of the lipid A acyl chains to two molecules of MD-2. Alternatively, binding to a single TLR4/MD-2 complex might allow recruitment of a second receptor/MD-2 heterodimer. These models contrast with the activation of *Drosophila* Toll which involves crosslinking of the receptor by a dimeric protein ligand, Spatzle. Taken together, these findings suggest that the conservation of leucine-rich repeat structures in Toll receptors reflects the ability of these motifs to rapidly evolve different protein binding specificities, on the one hand for Spatzle and on the other for MD-2.

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TRIF-related adapter molecule (TRAM) – a central adapter in LPS signaling

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TRIF-related adapter molecule (TRAM) is the fourth TIR domain-containing adapter molecule to be described that participates in Toll-like receptor signaling. RNA interference and gene-targeted deletion studies have demonstrated that TRAM functions exclusively in the TLR4 signaling pathway and not in signaling by other TLRs or IL1R family members. TRAM regulates the activation of interferon regulatory factor (IRF)-3 and the induction of interferon-β and interferon-stimulated genes. TRAM signaling to IRF-3 is dependent on TRIF and the IKK-related kinase, TANK-binding kinase (TBK)-1. Recruitment of TRIF to the TLR4 complex is facilitated by TRAM. Confocal microscopy studies with a cyan fluorescent protein (CFP) tagged TRAM reveal that TRAM is localized to both the plasma membrane and Golgi apparatus, where it co-localizes with TLR4. This localization of TRAM appears to be the result of

myristoylation. In fact, mutation of the predicted myristoylation site (TRAM-G2A) results in the dissociation of TRAM from the membrane and its relocation to the cytosol. TRAM, but not TRAM-G2A, can be radiolabeled with [^{3}H]-myristic acid. Furthermore, TRAM-G2A fails to induce IRF3 or NF-κB activation and TRAM-G2A can inhibit TLR4 signaling. MyD88, a universal TLR adapter, normally resides in the cytosol. Replacing the first 7 amino acids of TRAM with those from MyD88, redistributes MyD88 from the cytosol to the plasma membrane. Taken together, these observations suggest that TRAM is myristoylated and targeted to the membrane where it is critical for TLR4 signal transduction.

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Identification of a cytosolic complex involving MyD88 and the transcription factor IRF-7 that triggers Toll-like receptor-induced IFN-α/β gene expression

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The analysis of the interferon (IFN)-α/β system over the last two decades revealed the critical roles of the interferon regulatory factor (IRF) family of transcription factors in the regulation of immunity and oncogenesis. IFN-α/β have been the new focus of attention in the context of linking the innate and adaptive immunities via Toll-like receptor (TLR) signalling in antigen presenting cells. A high-level induction of IFN-α/β is achieved by plasmacytoid dendritic cells (pDCs; also called IFN-producing cells) when infected by viruses or stimulated by unmethylated DNA (CpG DNA) and single-stranded RNA (ss-RNA); CpG DNA and ss-RNA mediate the induction via the activation of the TLR9 subfamily members TLR9 and TLR7, respectively. These TLR family members require the adapter MyD88 for the induction of IFN-α/β genes, but it is unknown which transcription factor is required in the TLR-mediated, MyD88-dependent IFN induction pathway and how pDCs but not conventional DCs (cDCs) can achieve this induction. We recently found that IRF-7 interacts with MyD88 to form a vesicular complex in the cytoplasm. We also provide evidence that this complex also involve IRAK4 and TRAF6 and that this complex provides the foundation for the TLR9-dependent activation of the IFN genes. The complex found in this study is a novel example of how coupling of the signaling adapter and effector kinase molecules together with the transcription factor contribute to process an extracellular signal for the activation of its downstream transcriptional events in the cell. Thus, we propose that this complex functions as a novel cytosolic transductional-transcriptional processor (CTTP). Finally, definitive evidence for the critical role of IRF-7 in IFN-α/β gene induction was also obtained by generating mice deficient in the IRF-7 gene.

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Detoxifying endotoxin: time, place and person

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Having sensitive mechanisms for recognizing Gram-negative bacterial LPS is essential for effective innate immunity. An animal's overall response to LPS is determined not only by these recognition mechanisms, however, but also by numerous phenomena that inactivate LPS or modulate the body's inflammatory reactions to it. The inactivation mechanisms include: (i) proteins that facilitate LPS sequestration by plasma lipoproteins, prevent interactions between the bioactive lipid A moiety and MD-2/TLR4, or promote cellular uptake via non-signaling pathway(s); (ii) enzymes that de-acylate or dephosphorylate lipid A; and (iii) adaptations that modulate LPS-induced mediator production by responsive cells. Endotoxin recognition occurs principally in extravascular tissues. Within sites of microbial invasion, endotoxin inactivation follows activation and is enhanced by elements of the local inflammatory response. In contrast, several endotoxin detoxification mechanisms are constitutively active in the circulating blood. In the systemic compartment, responses to endotoxin occur principally in the splanchnic organs, not in circulating cells; endotoxin-induced production of

mediators by the liver and spleen is modulated by both the sympathetic and parasympathetic nervous systems. Most endotoxin inactivation mechanisms are enhanced during the body's responses to infection, injury and many other stresses. In general, inactivation mechanisms help compartmentalize the body's responses to endotoxin by confining inflammation to the local sites where it is needed to fight microbial invasion. Endotoxin activation and inactivation are both essential for successful host defense.

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Apolipoproteins modulate inflammatory responses to LPS in rodents and humans: implications for sepsis

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Apolipoproteins play a pivotal role in lipoprotein metabolism and atherogenesis. ApoE and apoCI are expressed by a 48 kb gene cluster on chromosome 19 and have opposing effects on the hepatic clearance of lipoproteins. In the past decade, lipoproteins have been shown to bind LPS. We have found that both apoE and apoCI, but not protein-free emulsions, avidly bound to LPS. The monomerisation of LPS, as induced by mouse plasma, strongly depended on the levels of apoE and apoCI. Interestingly, apoE and apoCI had profound and opposing effects on the *in vivo* response to LPS in mice. ApoE increased the uptake of LPS by hepatocytes, resulting in a strongly reduced induction of pro-inflammatory cytokines. In contrast, apoCI markedly prolonged the residence time of LPS in the circulation, resulting in a highly increased inflammatory response. Also, apoCI was positively correlated with TNF-α levels in human endotoxemia resulting from cardiac surgery with cardiopulmonary bypass. Interestingly, plasma apoCI improved survival from sepsis resulting from a pulmonal *Klebsiella pneumoniae* infection in mice, by timely improving the antibacterial attack. These data underscore the close relationship between lipid metabolism and inflammation, and suggest that apoE and apoCI play opposing roles in bacterial infection and sepsis.

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Endogenous apolipoprotein E (apoE) and ATP-binding cassette transporter A1 (ABCA1) promote LPS efflux from macrophages

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We previously showed that in the presence of whole serum, LPS binds transiently to monocytes and is transferred by soluble CD14 to plasma lipoproteins (*J Clin Invest* 2001; **108**: 485). The release or 'efflux' of LPS from monocytes requires the presence of lipoproteins, is accelerated by sCD14, and results in decreased cell activation. We have found that the differentiation of monocytes into macrophages changes the mechanism of LPS efflux. In contrast to monocytes, macrophages expressed high levels of apoE and rapidly released LPS into serum-free medium without a requirement for a lipoprotein acceptor or LPS transfer protein. Macrophage LPS efflux was inhibited by the ABC transporter inhibitors, glyburide and DIDS, and by antibodies to apoE. Activation of ABCA1 expression in murine peritoneal macrophages increased LPS efflux in wild-type macrophages but not in ABCA1-deficient or apoE-deficient macrophages. Low concentrations of exogenously added apoE also increased LPS efflux from THP-1 macrophages. Our data suggest that in tissues where plasma components are scarce, macrophages can efflux LPS into endogenously synthesized apoE. Thus, ABC transporters and apolipoproteins may remove bacterial lipids from macrophages by mechanisms that are similar to those that remove host phospholipids and cholesterol during reverse cholesterol transport.

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Interactions between LPS and lung surfactant proteins

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After penetration into the lower airways, bacterial lipopolysaccharide (LPS) interacts with alveolar cells in a fluid environment consisting of pulmonary surfactant, a lipid–protein complex which prevents alveolar collapsing and participates in lung defense. The two hydrophilic surfactant components SP-A and SP-D are proteins with collagen-like and lectin domains (collectins) able to interact with carbohydrate-containing ligands present on microbial membranes, and with defined regions of LPS. This explains their capacity to damage the bacterial envelope and induce an antimicrobial effect. In addition, they modulate LPS-induced production of pro-inflammatory mediators in leukocytes by interaction with LPS or with leukocyte receptors. A third surfactant component, SP-C, is a small high-hydrophobic lipopeptide which interacts with lipid A and reduces LPS-induced effects in macrophages and splenocyte cultures. The interaction of the different SPs with CD14 might explain their ability to modulate some LPS responses. Although the alveolar fluid contains other anti-LPS and anti-microbial agents, SPs are the most abundant proteins which might contribute to protect the lung epithelium and reduce the incidence of LPS-induced lung injury. The presence of the surfactant collectins SP-A and SP-D in non-pulmonary tissues, such as the female genital tract, extends their field of action to other mucosal surfaces.

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Toll-like receptor-mediated recognition by intestinal epithelial cells

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The family of Toll-like receptors (TLRs) represents key molecules of innate immune recognition. TLRs detect the presence of microbial organisms by the recognition of conserved essential structures that occur in pathogenic, but also in most commensal microbial organisms. Although professional immune cells express the broadest spectrum of TLRs, also epithelial cells at many sites of the body were shown to harbor various TLRs and can be stimulated by low ligand concentrations. TLR stimulation results in cellular activation and the initiation of a pro-inflammatory response. Whereas microbial ligands presented to tissue macrophages undoubtedly represent an adequate signal for immune activation, the situation on epithelial surfaces is more complicated. The presence of a dense and highly dynamic microbial flora on most body surfaces demands control mechanisms that prevent unintended immune stimulation. In fact, the importance of the normal microflora for the disease activity and the recently identified loss of function mutations within the NOD2 gene in patients suffering from inflammatory bowel disease point towards the possibility of microbial ligand-mediated immune stimulation as part of the pathogenesis of this disease. Our work focuses on possible mechanisms involved in the protection from unintended stimulation by TLR ligands within the intestinal epithelial layer.

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Negative regulation of TLR4 signaling by RP105

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Activation of Toll-like receptor (TLR) signaling by conserved microbial signatures promotes the induction of innate and adaptive immune responses. Such responses are tightly regulated. Delay or insufficient vigor can lead to a failure to control infection. On the other hand, excessive or inappropriate inflammation is itself deleterious. RP105 is a TLR homolog, thought to be largely B-cell specific, which lacks a Toll/IL-1 resistance (TIR) signaling domain. Here, we demonstrate that RP105 expression is wider, directly mirroring that of TLR4 on antigen-presenting cell populations in mice and humans. We further demonstrate that RP105 is a specific inhibitor of TLR4 signaling in HEK293 cells, as well as a physiological regulator of TLR4 signaling in dendritic cells and of endotoxicity *in vivo*.

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Taming the humoral immune response to Gram-negative bacteria

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We describe a previously unappreciated mechanism for limiting humoral immune responses to Gram-negative bacterial lipopolysaccharide (LPS). Acyloxyacyl hydrolase (AOAH), an enzyme found in macrophages and dendritic cells, selectively removes the fatty acyl chains that allow LPSs to stimulate host cells via Toll-like receptor 4. When mice that cannot produce AOAH are immunized with LPS or infected with Gram-negative bacteria, they develop significantly greater antibody titers than do normal controls. Both polyclonal (a diverse array of 'natural' antibodies, including autoantibodies) and specific (anti-LPS) antibody responses are affected, yet the adjuvanticity of the LPS is not. The enhanced polyclonal antibody response to LPS seen in AOAH-null mice includes several kinds of anti-nuclear antibodies. AOAH may prevent the production of potentially harmful antibodies when animals are infected with Gram-negative bacteria.

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Non-LPS actions and targets of LPS binding protein (LBP)

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LBP was discovered approximately 20 years ago because of its ability to bind to bacterial lipopolysaccharide (LPS). We have shown that in addition to its complex function of transferring LPS to its cellular receptor, into the cell, or into lipoproteins, LBP also binds to other bacterial compounds and can modulate their ability to stimulate the host's innate immune system. The majority of compounds found to also interact with LBP are lipid-containing molecules such as glycolipids or lipoproteins. Lipoteichoic acid (LTA) of different Gram-positive bacteria is also recognized by LBP and both its complexation with CD14 and biological activity towards immune cells is modulated by LBP. LTA-like glycolipids isolated from spirochetes are recognized by LBP and initiate signaling in the presence of LBP. Lipopeptides present in numerous Gram-positive and Gram-negative bacteria, as well as in spirochetes and mycoplasms are also recognized by LBP. Together with its growing number of related proteins of the BPI-PLUNC family, LBP apparently as soluble mediator has the important ability to recognize a variety of bacterial pathogens before cellular contact has been established. The different sources of LBP in tissues such as lung and intestine further support its role as important defense molecule.

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Evolution and integration of innate immune recognition systems: the Toll-like receptors

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The mammalian immune system, composed of innate and adaptive immunity, senses invading micro-organisms by discriminating between self and non-self. In adaptive immunity, T and B lymphocytes recognize foreign antigen by virtue of antigen receptors. In innate immunity, Toll-like receptors (TLRs) have been shown to play an essential role in the recognition of components of micro-organisms including bacteria, fungi, and viruses. Recognition of microbial components by TLRs triggers activation of the innate immune system, and further instructs the development of antigen-specific adaptive immunity, especially Th1-dependent immune responses. Thus, TLRs have been established to be key molecules for triggering innate and adaptive immune responses. TLR signaling pathways originate from a

cytoplasmic Toll/IL-1 receptor (TIR) domain. The essential role of TIR domain-containing adapters, such as MyD88, TIRAP, TRIF, and TRAM, in TLR signaling pathways have been revealed mainly through analysis of knockout mice. Thus, molecular mechanisms by which innate immune response is initiated are now increasingly being understood.

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Peptidoglycan recognition and innate immunity

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Peptidoglycan (PGN) is a polymer of β(1-4)-linked GlcNAc and MurNAc, crosslinked by short peptides, uniquely present cell walls of virtually all bacteria. Mammals have several PGN recognition molecules, including three pattern recognition receptors with leucine-rich repeats: cell-surface CD14 and Toll-like receptor 2 (TLR2), and intracellular nucleotide-binding oligomerization domain (NOD)-containing proteins, NOD1 and NOD2. CD14 and TLR2 recognize extracellular polymeric PGN, and NOD1 and NOD2 recognize intracellular PGN fragments. Interaction of PGN with CD14, TLR2, and NOD has highly pro-inflammatory effects due to induction of secretion of numerous chemokines and cytokines and other mediators of inflammation.

PGN is also recognized by the PGN recognition protein (PGRP) family, conserved from insects to mammals. *Drosophila*, mosquito, and mammals have families of 13, 7, and 4 PGRP genes, respectively, and some of them are alternatively spliced. PGRPs are differentially expressed in various cells and tissues, their expression is often up-regulated by bacteria, and they mediate host responses to bacterial infections. Insect PGRPs have four functions unique for insects: activation of prophenoloxidase cascade; activation of Toll receptor; activation of Imd pathway; and induction of phagocytosis. One function, enzymatic N-acetylmuramoyl-L-alanine amidase activity (hydrolysis of PGN), is shared by some insect PGRPs and mammalian PGRP-L. Mammalian PGRP-S has antibacterial activity in polymorphonuclear leukocytes, and PGRP-S-deficient mice have defective phagocytic killing of bacteria and increased susceptibility to some bacterial infections. Thus, in insects, PGRPs function as cell-activating receptors, activators of humoral defense mechanisms, or PGN scavengers, whereas, in mammals, PGRPs function primarily as anti-bacterial proteins or PGN scavengers.

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Bacterial cell wall peptidoglycan: structure, function and metabolism

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Peptidoglycan (murein) is a major essential and specific constituent of the bacterial cell wall. Its main function is to protect cells against the internal osmotic pressure and to maintain the characteristic cell shape. It also serves as a platform for the anchoring of specific proteins and other cell wall components. This giant macromolecule is composed of long glycan chains of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues cross-linked by short peptides. Any alteration of the disaccharide-peptide basic unit results in a global change of peptidoglycan structure and properties. Such global variations are encountered in nature as conserved variations along phyletic lines but have sometimes been acquired as a result of mutations or as a mechanism of resistance against cell-wall targeted antibiotics. During bacterial cell growth and division, the peptidoglycan mesh is constantly broken down by a set of highly specific hydrolases (lytic transglycosylases, amidases, endopeptidases, D,D- and L,D-carboxypeptidases) in a maturation process allowing insertion of newly synthesized units in the pre-existing polymerized material. Depending on the bacterial species considered, degradation fragments are either released in the growth medium or efficiently re-utilized for synthesis of new murein in a sequence of events termed the recycling pathway. Peptidoglycan is one of the main pathogen-associated molecular patterns recognized by the host innate immune system. Variations of the structure and metabolism of this cell wall component have been exploited by host defense mechanisms for detection/identification of invading bacterial species. Modification of the peptidoglycan structure could also represent a mechanism allowing bacteria to escape these host defense systems.

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Crystal structure of PGRP domains (± ligand)

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Nod1 and Nod2 are pattern-recognition molecules involved in peptidoglycan detection

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Toll-like receptors (TLRs) represent the most studied family of pattern-recognition molecules (PRMs) involved in the detection of highly conserved microbial motifs. TLRs are membrane-anchored molecules which are located either at the plasma membrane or found intracellularly associated with structures such as the Golgi apparatus. However, it is generally assumed that the detection of microbial motifs by TLRs is achieved at the plasma membrane. A new class of mammalian PRMs is represented by Nod proteins, among which Nod1 and Nod2 remain the most studied members. In contrast to TLRs, Nod1 and Nod2 are cytosolic molecules which achieve microbial detection within this cellular compartment. Nod1 and Nod2 both detect peptidoglycan, a macromolecule found in bacterial cell walls. Within the peptidoglycan polymer, Nod1 detects (GlcNAc)-MurNAc-L-Ala-D-Glu-*meso*DAP, a motif found principally in Gram-negative bacteria, while Nod2 detects (GlcNAc)-MurNAc-L-Ala-D-Glu, the minimal peptidoglycan signature found in all bacteria. In addition to Nod1 and Nod2, TLR2 has been shown to detect peptidoglycan. However, we now provide evidence that TLR2 is not a sensor of highly purified peptidoglycan, and instead efficiently detects bacterial contaminants often co-purified with peptidoglycan, such as lipoproteins or lipoteichoic acids. Therefore, Nods and TLRs are complementary, rather than overlapping, families of microbial PRMs.

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Structural and molecular determinants of pro-inflammatory properties of lipoteichoic acids

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Gram-positive bacteria induce similar inflammatory reactions as Gram-negative bacteria. However, while lipopolysaccharides (LPSs) represent the well-established Gram-negative endotoxin, no such structure of their Gram-positive counterparts has been identified unequivocally. Lipoteichoic acids (LTAs) had been suspected as a putative structural and functional equivalent, but a series of papers about one decade ago demonstrated that the more purified the material was, the less active it was in inducing cytokine release and other inflammatory responses.

Our group has re-addressed this issue following the hypothesis, that the isolation procedure adapted from LPS was not suitable and might lead to decomposition and inactivation of LTA. Indeed, employing a more gentle isolation procedure, bioactive highly-purified LTA was obtained from more than 12 Gram-positive species. Their structures were elucidated by NMR. In fact, a loss of D-alanine substituents was shown to be responsible for the inactivation of classically prepared LTA. The active structure was confirmed by chemical synthesis and employing a series of synthetic derivatives, a structure/function-relationship was established.

Many activities of commercial LTA could be traced back to LPS contamination. LTA represents an endotoxin in its own right. It is toxic in galactosamine-sensitised mice and was able to induce (cross-)tolerance to itself or LPS. All LTAs studied so far employ TLR2 and not TLR4 as well as CD14. LBP appears to play a minor role at least in human monocytes. LTA induces a cytokine pattern characterised by essentially no IL-12 and IFN-γ release but much more prominent formation of chemokines like IL-8 and the hematopoietic growth factor G-CSF. The latter findings might be linked to the clinically well-established pus formation induced by many Gram-positive bacteria. LTA was shown to activate complement via L-ficolin.

Taken together, these findings indicate that LTA represents a highly-conserved Gram-positive endotoxin playing a crucial role in bacterial recognition by the innate immune system.

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CD36 as a sensor of di-acylglycerides acting in conjunction with TLR2 and TLR6

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The mammalian Toll-like receptors (TLRs) activate cells of the innate immune system when stimulated by diverse ligands of microbial origin. In some instances, these ligands are directly engaged by the TLRs; however, this is not necessarily true in all cases. TLR2 recognizes multiple, structurally disparate microbial ligands, consistent with a requirement for co-receptors in ligand binding. Using *N*-ethyl-*N*-nitrosourea (ENU), we generated the recessive immunodeficiency phenotype *Oblivious*, in which macrophages show diminished awareness of the S-enantiomer of the di-acylated bacterial lipopeptide MALP-2 and lipoteichoic acid (LTA), together with an increased susceptibility to *Staphylococcus aureus* infection. *Oblivious* macrophages readily detect the tri-acylated bacterial lipopeptide PAM_3CSK_4 as well as zymosan, revealing that some TLR2 ligands are activated via an *Oblivious*-independent pathway. The gene responsible for the *oblivious* phenotype has been positionally cloned and was proven to result from a nonsense mutation in *Cd36*, which encodes a double-spanning cell-surface protein expressed by murine myeloid cells. A receptor for endogenous molecules including fatty acids, thrombospondin, oxidized LDL, and β-amyloid, CD36 is also a selective and non-redundant sensor of microbial di-acylglycerides, and makes an important contribution to antibacterial defense. We propose that CD36 may initiate TLR2-mediated inflammatory responses through recognition of both endogenous and exogenous ligands.

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Structure–activity relationships of bacterial lipopeptides with TLR2 heterodimers

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The lipopeptide FSL-1 (Pam_2CGDPKHPKSF) activates fibroblasts and macrophages. The level of macrophage-stimulating activity of FSL-1 was higher than that of the lipopeptide MALP-2 (Pam_2CGNNDESNISFKEK). The diacylglyceryl and peptide portions of FSL-1 did not reveal both the macrophage-stimulation and the NF-κB reporter activity in HEK 293 cells transfected with TLR2 and TLR6 (TLR2/6). A single amino acid substitution from Phe to Arg and a fatty acid substitution from palmitic to stearic acid drastically reduced both activities. These results suggest that both portions of FSL-1 are involved in the recognition by TLR2/6. We have found that all 8 leucine-rich repeats (LRRs) of the extracellular domain of TLR2 obtained by the SMART analysis with a LRR consensus sequence (LxxLxLxxNxLxxL) affected the recognition of FSL-1 by TLR2/6; TLR2/1 recognized FSL-1 more strongly than Pam_3CSK_4; Pam_3CSK_4 was recognized by both TLR2/1 and TLR2/6 and the recognition level by the former was only slightly higher than that of the latter; co-transfection of the CD14 gene and recombinant soluble CD14 enhanced drastically the recognition of Pam_3CSSNA by TLR2/1.

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Evolution and integration of innate immune systems from fruit flies to man: lessons and questions

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Drosophila mounts a potent host defence when challenged by various micro-organisms. Molecular and genetic analyses of this defence have now provided a global picture of the mechanisms by which this insect senses infection, discriminates between various classes of micro-organisms and induces the production of effector molecules, among which antimicrobial peptides are prominent. A major result in these studies was the discovery that most of the genes involved in the *Drosophila* host defence are similar to genes implicated in the mammalian innate immune response. Recent progress in research on *Drosophila* immune defence and the newly discovered similarities or differences between *Drosophila* immune responses and mammalian innate immunity will be discussed.

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Monomeric and polymeric Gram-negative peptidoglycan, but not purified LPS, stimulate the *Drosophila* IMD pathway

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Insects depend solely upon innate immune responses to survive infection. In *Drosophila*, the IMD pathway is required for antimicrobial gene expression in response to Gram-negative bacteria. The exact molecular component(s) from these bacteria that activate the IMD pathway remain controversial. We found that highly purified LPS did not stimulate the IMD pathway. However, lipid A, the active portion of LPS in mammals, activated melanization in the silkworm *Bombyx morii*, via an unidentified receptor. On the other hand, the IMD pathway was remarkably sensitive to polymeric and monomeric Gram-negative peptidoglycan. Recognition of peptidoglycan required the stem-peptide sequence specific to Gram-negative peptidoglycan and the receptor PGRP-LC. In *Drosophila* cells, recognition of monomeric and polymeric peptidoglycan required different PGRP-LC splice-isoforms. In flies, recognition of polymeric Gram-negative peptidoglycan also required PGRP-LC, however, recognition of monomeric Gram-negative peptidoglycan required either PGRP-LC or PGRP-LE. Unlike PGRP-LC, PGRP-LE does not encode a transmembrane protein and may function as an intracellular peptidoglycan receptor.

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Pattern recognition and lectin-complement pathway

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Immunity to infection is mediated by two general systems, acquired (or adaptive) and innate (or natural). Innate immunity was formerly thought to be a non-specific immune response characterized by phagocytosis. However, innate immunity has considerable specificity and is capable of discriminating between pathogens and self. Recognition of pathogens is mediated by a set of pattern recognition molecules which recognize conserved pathogen-associated molecular patterns (PAMPs) shared by broad classes of micro-organisms, thereby successfully defending invertebrates and vertebrates against infection. Mannose-binding lectin (MBL) and ficolins, characterised by the presence of a collagen-like domain and a carbohydrate-binding domain, are recognition molecules of the lectin complement pathway which has been thought to play a pivotal role in innate immunity during the lag period before the onset of adaptive immunity. To elucidate the structure and function of the primitive complement system, we have isolated ascidian lectins similar to mammalian MBL and ficolins, and ascidian C3. The ascidian complement system, consisting of the lectin–proteases complex and C3, functions in an opsonic manner. These results indicate that the complement system has played a pivotal role in innate immunity by recognizing pathogen and enhancing phagocytosis since before the establishment of acquired immunity.

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Positive and negative regulation of Toll-like receptor signal transduction

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Signalling by Toll-like receptors (TLRs) is initiated by the Toll-lL-1R_Resistance (TIR) domain, present on the cytosolic face of each TLR. It is thought to recruit via homotypic interactions, adapter proteins that contain TIR domains. Five such adapters have been discovered to date including MyD88, Mal (also known as TIRAP), Trif, Tram and SARM. Signals activated include the transcription factor NF-κB and MAP kinases, which lead to induction of gene expression. Differences are emerging between TLRs in terms of which adapter is recruited by which TLR. A key question concerns specific roles for each adapter protein. MyD88 and Mal engage with IL-1 receptor-associated

kinases (IRAKs), leading to Traf6 engagement. Mal can also engage directly with Traf6, as can Trif. In addition Trif can activate the kinase TBK-1 which leads to activation of the transcription factor IRF3, promoting production of type I interferons. The activation of Mal requires that it be processed by caspase-1, providing an additional role for this caspase in inflammation. In addition to these positive signals, a number of negative processes have been found, including receptors such as SIGIRR and ST2, splice variants of MyD88 and IRAK2 and inhibitory proteins such as Triad3, IRAK-M and RIP3. Signalling by TLRs will therefore be a balance between positive and negative signals. Manipulation of these processes presents a number of opportunities for enhancing host defence or disease resistance, or strategies to limit inflammation.

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SOCS1 and SOCS3 are central regulators for macrophage and dendritic cell activation

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Cytokines activate Janus tyrosine kinases (JAKs) and signal transducer and activator of transcription (STAT) as well as Ras-MAP kinase pathway are two major signaling pathways which are downstream of JAKs. The strength of cytokine signals is regulated, in part, by a family of endogenous JAK kinase inhibitor proteins referred to as suppressors of cytokine signaling (SOCS) and cytokine-inducible SH2 proteins (CIS). Among these, SOCS1 is induced by interferons, and SOCS3 is strongly induced by a variety of cytokines and other stimulations including IL-6, IL-10, G-CSF, leptin as well as lipopolysaccharide (LPS). Both SOCS1 and SOCS3 have an N-terminal kinase inhibitory region and inhibit JAK kinase activity. SOCS1 directly binds to JAKs, while SOCS3 inhibits JAKs through binding to cytokine receptor tyrosine residues. Interaction of SOCS3 with cytokine receptors through its SH2 domain with high affinity probably ensures relatively specific inhibition of a particular cytokine signaling, including IL-6, IL-12, LIF, G-CSF and leptin.

By using gene-disrupted mice, we have defined the physiological roles of SOCS1 and SOCS3, and found that SOCS1 and SOCS3 play an important regulatory roles in activation of macrophages and dendritic cells (DCs). SOCS1 can directly inhibit the Toll-like receptor (TLR) mediated NF-κB activation by an unknown mechanism. On the other hand, in macrophages lacking the SOCS3 gene or carrying a mutation of the SOCS3 binding site (Y759F) in gp130 not only IL-10 but also IL-6 suppressed LPS-induced TNF production. SOCS3 protein was strongly induced by both IL-6 and IL-10 in the presence of LPS, but selectively inhibited IL-6 signaling. These data indicate that SOCS3 selectively blocks IL-6 signaling, interfering with its ability to inhibit LPS signaling. In conclusion, SOCS1 and SOCS3 are negative and positive regulators of innate immunity, respectively.

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Reprogramming of circulatory cells in sepsis and SIRS

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Immune status is altered in patients with systemic inflammatory response syndrome (SIRS) or sepsis. Reduced *ex vivo* TNF production has been regularly reported with endotoxin–activated monocytes. We show in sepsis that the responsiveness to Pam_3CysSK_4, a specific TLR2 ligand, was also reduced as compared to healthy controls. However, responsiveness to heat-killed *Staphylococcus aureus*, heat-killed *Escherichia coli* and muramyl dipeptide (MDP) was similar to that obtained with cells from healthy donors. MDP is sensed by Nod2 intracellular receptor. We found that Nod2 mRNA expression was unchanged in sepsis while that of Nod1 was significantly reduced. Production of IL-10 in response to LPS and Pam_3CysSK_4 was enhanced but that induced by heat-killed bacteria was unchanged. We investigated the expression of various intracellular molecules involved in the negative regulation of TLR signaling. We showed by RT-PCR that in monocytes of sepsis patients, mRNA expression of Tollip and SOCS1 was similar to that of healthy controls, while MyD88 and SIGIRR mRNA expression was significantly enhanced. Analysis of non-infectious SIRS patients will be also presented. Thus, leukocyte hyporesponsiveness to microbial agonists is not a generalized phenomenon in sepsis and SIRS, and the terms ‘leukocyte reprogramming’ better define the phenomenon than anergy, immunodepression, or immunoparalysis.

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TLRs and their adaptors in adjuvant immunotherapy for cancer

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Microbial components that activate the host immune system have been designated as adjuvants. Adjuvants have often been used for immunotherapy for cancer since they potentially induce antibody (Ab) production, cytotoxic T cells (CTL) and NK cell activation. Myeloid dendritic cells (DCs), representative antigen-presenting cells, are targets for most of the adjuvants. Recently, Toll-like receptors (TLRs) were found to serve as receptors for a variety of adjuvants. TLRs recognize microbial patterns by the extracellular leucine-rich repeat domain and deliver signaling through the cytoplasmic domain, named Toll-IL-1 receptor homology domain, TIR. Since each TLR recruits distinct sets of adapter molecules that in turn activate specific downstream signaling molecules, each adjuvant confers unique immune response on DCs. So far, ten TLRs and four adapters have been identified, and selection of TLRs and adapters appear to determine the particular TLR signaling pathway leading to the activation of specific transcription factors such as NF-□B, c-Jun (AP-1) or IRF-3. MyD88 is a pivotal adapter that activates NF-□B to induce cytokines, TNF-□, IL-6, IL-8 and IL-12, whereas activation of IRF-3 and the IFN-□ promoter largely depends on TICAM-1/TRIF, a recently identified adapter. We have used the *Mycobacterium bovis* bacillus Calmette-Guérin cell-wall skeleton (BCG-CWS) for adjuvant immunotherapy for patients with cancer. BCG-CWS activates Toll-like receptor (TLR) 2 and TLR4 on myeloid DCs to produce the NF-□B-inducible cytokines, but barely induces type 1 interferon (IFN-□/□). On the other hand, poly(I:C), a representative of double-stranded RNA, effectively induces IFN-□/□ by acting as a ligand for TLR3 on DCs. Here, we investigated the adjuvant potential of BCG-CWS and poly(I:C) to elicit antitumor immunity using a syngeneic mouse tumor model (B16 melanoma in C57BL/6). BCG-CWS mainly raised CTL response via MyD88-mediated signaling in DCs and poly(I:C) engaged NK activation via TICAM-1-mediated signaling in DCs. NK and CTL induced secondary to DC maturation eliminated MHC-negative and MHC-positive tumor cells, respectively, as their targets. In summary, DCs mature in distinct ways by a given adjuvant species that selectively activates adapters of TLRs, leading to CTL and/or NK activation. These results provide insight into the mechanism of the adjuvant-based antitumor potential, which may be useful for testing DC-mediated immunotherapy for patients with cancer.

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Genetic bases of host responses to endotoxin and resistance to infection

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Most of us think of the C3H/HeJ mouse strain in the context of failed LPS signaling. However, there are literally thousands of genes apart from TLR4 that are necessary for optimal LPS signaling. In this overview, I will highlight specific genes and how their products directly or indirectly modulate TLR signaling and how such mutations impact resistance to infection.

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Nod2: from human genetics to physiopathology of Crohn's disease

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Crohn's disease (CD) is a chronic and debilitating disease affecting more than 500,000 individuals in the US and millions world-wide. Both environmental (particularly enteric bacteria) and genetic factors are known to play a critical

role in the development of CD. Recent human genetics studies have revealed that genetic variations in *Card15/Nod2* gene are associated with susceptibility to CD and Blau syndrome (BS). NOD2 is an intracellular protein that recognizes muramyl dipeptide (MDP), a conserved motif in peptidoglycan (PGN), and activates a host defense pathway against bacteria. NODs including NOD1 and NOD2 are members of a family of cytosolic proteins with homology to plant disease resistance (R) gene products. NODs contain variable N-terminal effector domains, a centrally located nucleotide-binding oligomerization domain (NOD) and C-terminal leucine-rich repeats (LRRs). NOD1 recognize a conserved but distinct structural motif in bacterial peptidoglycan. Activation of NF-κB through NOD1 and NOD2 is mediated through RICK, a serine/threonine kinase that interacts with the IκB kinase (IKK) complex, suggesting potential complementation of deficient NOD2 function via NOD1 signaling. Homozygosity or compound heterozygosity for NOD2 mutations increases the risk 20–40-fold, while heterozygosity leads to minimal increase in risk, indicating that a genetic dosing effect is important for disease development. Consistent with the genetic observations, all three CD-associated NOD2 mutations result in proteins that are deficient in inducing NF-κB activation in response to bacterial components including PGN and MDP. In contrast, NOD2 mutations associated with BS function as activating mutations. Systematic mutational analyses revealed a general mechanism for recognition of pathogens by the LRRs of NOD1 and NOD2. Our data indicate that NOD1 and NOD2 function as intracellular receptors for specific microbial components leading to the activation of a cellular response against the pathogen. The ability of NOD2 to activate NF-κB in response to bacteria suggests that a defective sensing of intestinal bacteria somehow promotes susceptibility to disease. However, many challenging questions remain unanswered, including the mechanism by which NOD2 mutations cause CD and why NOD2 mutations are associated with ileal disease.

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Toll-like receptor 4 discriminates pathogens from commensals through pathogen specific recognition receptors

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Mucosal surfaces form powerful barriers that protect internal tissues from pathogens while allowing the normal flora to persist in the lumen. This functional dichotomy is unique for the mucosal compartment. The systemic antimicrobial defense, in contrast, is equipped to eliminate invading microbes, by chasing down and killing the prey at any cost. The mucosal host defense must be able to discriminate between potential pathogens and commensals, and selectively recruit defense effector mechanisms that eliminate the pathogens. We have investigated the molecular mechanisms of pathogen recognition and TLR4 activation, using urinary tract infection (UTI) as the model. UTIs are among the most common bacterial infections in man, and an important cause of acute morbidity and chronic renal disease. Uropathogenic *Escherichia coli* attack epithelial cells from the lumen and, as a result, the epithelial cells may be activated to produce inflammatory mediators that direct the recruitment of inflammatory cells, and the clearance of infection. In asymptomatic carriers, this response is not activated, however. The mucosa is refractory to LPS as the epithelial cells lack membrane bound CD14, and we have shown that this is an essential mechanism to allow asymptomatic carriage. The pathogenic bacteria differ from the carrier strains in the capacity to attach to uro-epithelial cells. Attachment does not only enhance bacterial persistence but is essential for epithelial cell activation and the adherence factors of the pathogens differ from those of the commensals. Virulent UPEC strains express P fimbriae that bind to glycosphingolipid (GSLs) receptors in the human urinary tract mucosa through the papG adhesin at the fimbrial tip. The mucosal response to P fimbriated *E. coli* is controlled by *tlr4 in vitro* and *in vivo* but adherence is independent of TLR4. The GSL receptors thus offer a mechanism of pathogen recognition as a discriminating step in TLR activation at mucosal surfaces. Specific adherence to epithelial cell receptors was also found to influence the adapter proteins involved in downstream signalling. P fimbriae were needed to trigger a TLR4 dependent *in vivo* response to *E. coli* urinary tract infection and TRIF/Lps-2, TRAM rather than the LPS related MyD88/TIRAP adaptors were involved. TRIF/TRAM KO mice developed transient symptomatic infections but MyD88 knockout mice became asymptomatic carriers. The results offer a new mechanism to discriminate pathogen sensing by TLR4 at mucosal surfaces, where most infectious challenges occur, and where the early host defence is crucial. Pathogen recognition receptors for other bacterial ligands may work in a similar manner and help solve the need for specificity with the convergence on a limited number of mucosal TLRs.

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Inherited human IRAK-4 deficiency and infectious diseases

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Members of the Toll-like receptor (TLR) and interleukin-1 receptor (IL-1R) superfamily share an intracytoplasmic Toll-IL-1 receptor (TIR) domain, mediating recruitment of the IL-1R-associated kinase (IRAK) complex via TIR-containing adapter molecules. We have described three unrelated children with inherited IRAK-4 deficiency. A fourth patient was subsequently reported by another group. This disease is caused by recessive mutations in the *IRAK4* gene. Clinically, these patients display no developmental abnormalities such as anhidrotic ectodermal dysplasia. Febrile and inflammatory responses were minimal or delayed and the patients developed transient neutropenia during infectious episodes. They presented multiple life-threatening infections, mostly caused by pyogenic Gram-positive bacteria. *S. pneumoniae* and *S. aureus* were the most frequently found. The infections began early in life, and for some patients became less frequent with age. The human TIR-IRAK signaling pathway is crucial for protective immunity against pyogenic bacteria, whereas it is redundant against most other micro-organisms. The absence of overt susceptibility to most other micro-organisms in these patients suggests that IRAK-4 independent and/or TIR-independent pathways are important in innate host defense. We have previously shown that the first 3 patients did not respond to IL-1β, IL-18, or any of the TLR1–6 or TLR9 agonists tested, assessed by NF-κB and p38MAPK activation, and IL-1β, IL-6, IL-12, TNF-α and IFN-γ induction. In an attempt to understand the narrow clinical phenotype of IRAK-4 deficiency, we have more recently investigated the patient's cell activation to other TLR agonists (TLR7 and TLR8), and the induction of type I IFN in response to TLR3–4 and TLR7–9 stimulation.

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Short and long-term suppression of atopic responses in the airways by TLR-4 agonists

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Background: As vaccine adjuvants, TLR-4 agonists are able to promote the production of Th1 cytokines, even a background alum-induced Th2 responses. TLR-4 agonists are active in the airways as mucosal adjuvants, and can induce non-specific resistance to airway infectious challenge.
Hypothesis: Administration of TLR-4 agonists to the airways with subsequent allergen exposure results in suppression of allergen sensitivity by reprogramming the allergen-specific T cell response away from a Th2-biased repertoire.
Results: Cohorts of ragweed allergic beagles were pretreated intranasally with various formulations of TLR4 agonist CRX-675, followed by ragweed challenge 24 h later and at 5-week intervals thereafter. Results were monitored by acoustic rhinometry, cytology, and measurement of mast-cell derived effector molecules. Allergen desensitization was observed at the 24-h challenge time-point, with concomitant reductions in leukotrienes and histamine in nasal secretions. Interestingly, half of the life-long allergic dogs remained desensitized at 5 weeks after a single dose of CRX-675; all of the remaining dogs showed a long-term response after a second dose. No increase in neutrophils was observed in nasal lavage samples, even at the highest CRX-675 dose of 100 μg. All dogs regained allergen sensitivity at approximately 6–9 months post CRX-675 exposure. Concomitant mouse studies of TLR4 agonists provide corroborating evidence of anti-allergic effects, including induction of Th1 cytokines and tryptophan catabolism, as well as effects on recruitment of regulatory T cell subsets.
Conclusion: Direct delivery of TLR4 agonist activity to the airways is a novel and highly promising approach to allergen desensitization. A clinical dose escalation trial of CRX-675 is currently underway in ragweed sensitive subjects.

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The role and regulation of apoptosis in sepsis

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Today, sepsis continues to be a growing problem in the critically ill patient population. A number of laboratories have been interested in understanding how changes in immune cell apoptosis during sepsis appear to contribute to septic morbidity. Consistently, it has been found that immune cell apoptosis is altered in a variety of tissue sites and cell populations. While divergent mediators, such as steroids and TNF, contribute to some of these apoptotic changes, their effects are tissue and cell population selective. Alternatively, inhibition of FasL-Fas signaling (by either FasL gene deficiency, *in vivo* gene silencing [siRNA] or with FasL binding protein) protects septic mice from the onset of marked apoptosis and the morbidity/mortality seen in sepsis. This extrinsic apoptosis response can utilize aspects of the caspase-8 mediated caspase cascade and/or Bid-induced mitochondrial pathway depending on the tissue site. This is in keeping with the findings that pan-specific caspase inhibition or the over-expression of Bcl-2 also protect these animals from the sequella of sepsis.

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A systems approach to study of sepsis and innate immunity

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