



Pattern recognition receptors and ligands

P143

Single molecule analysis of the RIG-I-like receptor, LGP2

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Introduction. The RIG-I-like receptor (RLR) proteins, RIG-I, MDA5, and LGP2 are cytoplasmic DExD/H box proteins that function to specifically recognize molecular features of RNA species that discriminate the pathogen from the host. Both RIG-I and MDA5 are able to activate downstream antiviral signal transduction, while LGP2 has been linked to diverse effector functions both upstream and downstream of RIG-I and MDA5, leaving the overall function of LGP2 largely unresolved. All of the RLRs bind dsRNA with varying affinities and exhibit RNA-enhanced ATP hydrolysis activity. The RNA binding ability of these proteins is critical for antiviral responses, but the importance of ATP hydrolysis is poorly understood.

Methods. We have utilized biochemical methods and single molecule TIRF microscopy-based imaging to investigate the enzymatic and RNA binding activities of LGP2 and mutant variants.

Results. LGP2 was found to have a uniquely high level of basal ATP hydrolysis activity in the absence of dsRNA, in addition to the dsRNA-enhanced ATP hydrolysis activity. These two enzymatic activities are separable by specific point mutations to LGP2 that create defects in basal, but not dsRNA-stimulated, ATP hydrolysis. Single molecule analysis of LGP2-dsRNA interaction revealed no evidence for ATP-driven LGP2 translocation on dsRNA, as was reported for RIG-I. Instead, we observe that ATP hydrolysis activity enhances the ability of LGP2 to engage dsRNA. This ATP-enhancement of dsRNA binding requires basal ATP hydrolysis activity, and enables LGP2 to form more interactions with diverse dsRNA ligands that are intrinsically poor substrates.

Conclusion. These findings indicate LGP2 uses basal ATP hydrolysis to enable recognition of a broader range of target dsRNA ligands.

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P144

Pathogen recognition by the long pentraxin PTX3: Roles of glycosylation and interplay with complement

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Introduction. The long pentraxin PTX3 is a soluble Pattern Recognition Molecule (sPRM) with non-redundant roles in resistance to selected pathogens, such as the opportunistic fungus *Aspergillus fumigatus*. PTX3 facilitates recognition and phagocytosis of fungal conidia mainly by polymorphonuclear neutrophils (PMNs) via Fcγ receptors (FcγRs) and alternative complement pathway (ACP) mechanisms. The human PTX3 protein contains a single N-glycosylation site with complex type sialylated oligosaccharides that has been involved in a number of PTX3 functions [1]. The present study was designed to characterize the role of this post translational modification in the PTX3-dependent recognition and phagocytosis of *A. fumigatus*. This microorganism has developed several strategies to evade the immune response, such as attracting onto the conidial wall inhibitors of the ACP, like factor H (FH, a ligand of PTX3). Here we present preliminary evidences on the effect of PTX3 on FH engagement by *A. fumigatus*.

Methods. Recombinant as well as natural human PTX3 (i.e., from PMNs) was purified by immunoaffinity chromatography and the attached oligosaccharides were analyzed using glycosidases, lectin blotting and mass spectrometry. A range of PTX3

glycoforms were made *in vitro* by enzymatic hydrolysis of terminal monosaccharides of the recombinant protein. The opsonic and pro-phagocytic activities of these variants were characterized in buffer solutions and whole human peripheral blood, respectively, by flow cytometry. The influence of PTX3 on FH binding to conidia was studied in competition assays using microtiter plates.

Results. Complex-type sugars were found on PTX3 of both natural and recombinant origins. These showed heterogeneity with regard to the relative content of terminal sialic acid (SA) and bi-, tri- and tetraantennary glycans, depending on the cellular source. Enzymatic removal of SA caused ~50% reduction of PTX3 binding to *A. fumigatus* conidia, with no further change due to additional deglycosylation. Conversely, saccharide hydrolysis had no effect on PTX3 binding to PMNs, where FcγRs have been proposed as putative receptors of this pentraxin [1]. However, consistent with binding data from conidia, desialylated PTX3 showed reduced potentiation of conidia phagocytosis by PMNs as compared to the unmodified protein. Also, we observed that PTX3 inhibits the binding of FH to *A. fumigatus* conidia, even when these were pre-opsonised with the long pentraxin and FH was applied at physiologically relevant concentrations.

Conclusion. Different pathogens express neuraminidases on their surface during infection and natural immunity cells, such as PMNs, mobilize intracellular neuraminidases to the plasma membrane upon inflammation. Here, we report that the glycosylation status of the sPRM PTX3 changes, with respect to SA, depending on cellular source and perhaps inducing stimuli. We describe that SA modulates the PTX3-dependent recognition and phagocytosis of *A. fumigatus*, which points to glycosylation as a strategy to tune PTX3 functions in innate immunity. Furthermore, we provided evidences that PTX3 impairs the interaction of FH with *A. fumigatus*, where this might link to a new mechanism to counterbalance the complement evasion strategy exploited by this pathogen.

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Reference

[1] Inforzato A et al. Tissue Antigens 2011;77:271–82.

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P145

Innate immune activation through ITAM-Syk-CARD9 signaling coupling to inflammasome is essential for the development of contact allergy to organic chemical allergens

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Introduction. Allergic contact dermatitis (ACD) is a major cause of occupational skin diseases and is caused by T cells sensitized previously to contact allergens. One of the clinically most important categories of contact allergens are small organic molecule that are chemically reactive (chemical sensitizers). Although the sensitization process of T cells requires activation of skin dendritic cells (DCs) by chemical sensitizers, it is unclear how the sensitizers activate the innate immune system. CARD9 is an essential adaptor protein regulating NF-κB activation and inflammatory cytokine production through tyrosine-based activation motif (ITAM)-coupled receptors and the tyrosine kinase Syk on myeloid cells, and is known as a crucial molecule in the innate activation pathway for antifungal defense. However its role in allergic diseases is unknown.

Methods. Contact hypersensitivity (CHS) to trinitrochlorobenzene (TNCB) was sensitized by painting TNCB on skin in mice lacking MyD88 or ITAM-associated signaling molecules, or by injection of TNBS (a water-soluble form of TNCB)-modified bone marrow-derived dendritic cells (BMDCs) from the knockout mice. Elicitation and

evaluation of CHS response (ear swelling, histology and the development of TNCB-specific T cells) was performed on day 5. In addition, cytokine productions and signaling activation in response to various contact sensitizers in BMDCs from the knockout mice were examined

Results. Expression of CARD9 in DCs and that of MyD88 in T cells are both required for sensitization of CHS to TNCB. IL-1R-MyD88-mediated signaling in T cells was required for sensitizer-activated DCs to generate TNCB-reactive T cells producing IL-17. On the other hand, activation of the signaling pathway through the ITAM-containing adaptor DAP12 and Syk was essential for ROS generation and NLRP3-dependent Caspase-1 activation induced by TNBS stimulation in DCs. Although the CARD9/BCL10 signaling was dispensable for the inflammasome activation, it was essential for contact sensitizer-induced pro-IL-1 β synthesis in DCs through NF- κ B activation. In addition, we found that all contact sensitizers tested (FITC, Oxazolone and DNFB) could induce Syk activation, and that the ROS production, Caspase-1 activation and IL-1 β production induced by these sensitizers were all dependent on Syk and the pro-IL-1 β synthesis through NF- κ B activation was all dependent on CARD9.

Conclusion. The ability to induce the Syk-mediated ROS production coupling to NLRP3 inflammasome activation and the CARD9-mediated NF- κ B activation leading to pro-IL-1 β synthesis may be a common characteristic of chemical contact sensitizers that is required for skin DCs to sensitize T cells through the IL-1 β -IL-1R pathway.

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P146

TLR2-ligands induce NF- κ B activation exclusively from endosomal compartments
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Introduction: Antiphospholipid antibody syndrome (APS) is an auto-immune disease associated with arterial or venous thrombosis and/or recurrent fetal loss and is caused by pathogenic antiphospholipid antibodies (aPLA). We recently demonstrated TLR2 as a crucial factor in recognizing aPLA by monocytes. While TLR4 is known to transmit signals from both the plasma membrane and endosomal compartments, less is known about the function of endosomal trafficking on TLR2 signaling.

Methods. Primary human monocytes and HEK-Blue2TM cells were treated with TLR2 ligands, i.e. LTA and Pam₃CSK₄, or with aPLA, in the presence of endocytosis inhibitors. Expression of TNF and tissue factor (TF) as well as NF- κ B activation were assessed. In order to evaluate the importance of receptor internalization, clathrin expression was reduced by siRNA and NF- κ B activation assessed after treatment with TLR2 ligands and aPLA. Endocytosis of biotinylated LTA and Pam₃CSK₄ or aPLA was evaluated by flow cytometry.

Results. We demonstrate that blocking antibodies to TLR2, TLR1 or TLR6, as well as antibodies to the TLR co-receptor CD14, significantly decreased TNF and TF responses to LTA, Pam₃CSK₄ and aPLA. TLR2 ligands internalization is observed in primary human monocytes and HEK-Blue2TM cells and we show that NF- κ B-dependent cell activation is associated to ligand internalization. Pharmacological blockade and siRNA approaches revealed the importance of the clathrin/dynamin-dependent endocytic pathway in ligand-induced NF- κ B activation. In addition, LTA-, Pam₃CSK₄- and aPLA-beads, which could not be internalized, are unable to activate NF- κ B while soluble ligands and LPS-beads (a TLR4 ligand) are competent to trigger NF- κ B activation. Furthermore, in a similar manner as TLR4, we demonstrate that TLR2 ligands uptake is regulated by CD14. Unlike LPS, TLR2 ligands do not induce phosphorylation of IRF-3 or IFN β expression. Finally, CD14-dependent endocytosis is shown to regulate the activation of NF- κ B induced by LTA, Pam₃CSK₄ and aPLA in HEK-Blue2TM cells or primary human monocytes.

Conclusion. Our results reveal that TLR2-ligand complexes are internalized, via clathrin- and CD14-dependent endocytosis into primary human monocytes and HEK-Blue2TM cells; endocytosis of these complexes is required for NF- κ B activation. The evidence that TLR1 and TLR6 play a part in the pathogenicity of aPLA contributes to a better understanding of the antiphospholipid syndrome and provides insight for the development of novel therapies. In conclusion, our study reveals a new important aspect of the regulation of TLR2-dependent signaling. Although present at the cell surface, TLR2-ligands induce NF- κ B activation exclusively from endosomal compartment.

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P147

The physiological role of PAI-2 in endotoxin-induced sepsis

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Introduction: Sepsis caused by uncontrolled inflammation is the leading cause of death in intensive care units today, but no effective therapeutic medication is available currently. During microbial infections, pathogen-associated molecular patterns (PAMPs) are recognized by the host innate immune system, which then mounts a defensive response. Myeloid cells play central roles in PAMP recognition and in the initiation and amplification of inflammatory cascades. Recent studies indicate that a novel system termed the inflammasome carries out processing of cytoplasmic stored pro-IL-1 β , and pro-IL-18. Both IL-1 β and IL-18 are highly potent proinflammatory cytokines. In addition, inflammasome activation has also been linked to an increase of host-cell death in certain cell types. Therefore the regulation of activation of the inflammasome is very important, but its mechanism is unclear. Plasminogen activator inhibitor 2 (PAI-2), a member of a large group of proteins that inhibit serine proteases, is regulated by IKK β -dependent activation of the transcription factor NF- κ B. Previous studies have shown that mice with *Ikk β* deletion in myeloid cells are hypersusceptible to endotoxin (LPS)-induced septic shock, and increased LPS susceptibility is associated with elevated plasma IL-1 β . Reconstitution of *Ikk β* -deficient macrophages with PAI-2 blocks apoptosis and IL-1 β release. These results suggest that PAI-2 modulates endotoxin-induced IL-1 β production and apoptosis in macrophages.

Methods. To determine the physiological role of PAI-2 *in vivo*, we generated transgenic mice that specifically express PAI-2 in macrophages and neutrophils, and crossed this transgenic mice with mutant mice with *Ikk β* deletion in myeloid cells.

Results. We are currently examining if myeloid deletion *Ikk β* mice restored PAI-2 expression can resist LPS-induced septic shock. In addition, we are also investigating the underlying mechanism by which PAI-2 regulates LPS-induced IL-1 β production.

Conclusion. Our data would reveal the functional role of myeloid-specific expression of PAI-2 in LPS-induced septic shock *in vivo*.

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P148

TLR-2 and TLR-4 mediated responses in monocytes from preterm and term newborns are distinct from those of adults

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Introduction: The neonatal immune system, particularly in very-preterm newborns, has been considered functionally immature and recent studies suggest that this susceptibility to infections may be due to functional alterations in antigen-presenting cells that can lead to secondary deficiencies in adaptive responses. The aim is to evaluate the activation and response of monocytes derived from umbilical cord blood of healthy adequate-for-gestational-age term and preterm newborns, after *ex vivo* Toll-like receptor 2 and 4 (TLR-2 and TLR-4) stimulation.

Methods. Cord blood mononuclear cells (CBMC) from healthy preterm <34 weeks of gestation (Group 1), ≥ 34 and <37 weeks of gestation (Group 2) and term (Group 3) newborns, and peripheral blood mononuclear cells (PBMC) from healthy adults were isolated by density gradient centrifugation. Adherent cells (monocytes) were prepared by culturing CBMC or PBMC (2.0×10^6 /ml) in RPMI 1640 with 10% fetal calf serum in a 12-well plate at 37 °C and 5% CO₂ for 2 h. The nonadherent cells were removed by wash with medium. Purified monocytes were stimulated with TLR-2 and TLR-4 agonists, Pam3CSK4 and LPS, respectively, for 24 h at 37 °C. The functional outcome of the stimulation was determined by pro- and anti-inflammatory cytokine secretion in the supernatants (measured by cytometric bead array) and the expression profile of activation markers on monocytes exhibiting normal forward- and side-scatter properties by flow cytometry and staining normally for CD14 and HLA-DR.

Results. After LPS or Pam3 stimulation, interleukin (IL)-8, IL-6, TNF- α and IL-1 β production by monocytes was substantially higher in term and both preterm groups when compared with adults, but inversely, IL-10 production was lower in the 3 groups of newborns compared to adults. Regarding the expression profile of activation markers on monocytes after stimulation with Pam3 or LPS, very-preterm newborns from Group 1 showed reduced expression of HLA-DR, CD80 and CD86 when compared with the other groups.

Conclusion. So far, our data suggest that neonates present greater capacity of developing an inflammatory response, which gets even more accentuated by the apparent lower production of IL-10. These results indicate an imbalance in cytokine production in the neonatal period which can increase the susceptibility to develop sepsis.

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