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ABSTRACT E-BOOK

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ORAL ABSTRACTS
IN
SELECTED ABSTRACT SESSIONS

O-001
General Research » Antiviral Defense

AN EVOLUTIONARILY CONSERVED MECHANISM OF STING-MEDIATED NF-κB ACTIVATION INVOLVING THE C-TERMINAL α-HELIX OF STING AND THE IKK-COMPLEX

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OBJECTIVES:The stimulator of interferon genes (STING) pathway plays an important and conserved role in antiviral immunity. Human STING (hSTING) mediates antiviral immunity by activating the transcription factors interferon regulatory factor 3 (IRF3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Activation of IRF3 by hSTING involves the C-terminal tail (CTT) of hSTING and TANK-binding kinase 1 (TBK1) and is well-described. In contrast, the molecular mechanisms underlying NF-κB activation remain unclear. To uncover these mechanisms, we exploited the conserved Drosophila melanogaster STING (dSTING) pathway. Similar to the human pathway, dSTING confers antiviral immunity. However, dSTING has no CTT and drives the expression of antiviral genes via the NF-κB homologue Relish. Interestingly, the mechanisms by which dSTING and hSTING activate NF-κB signaling appear quite similar.

METHODS:Proximity labeling, CRISPR-Cas9 knock out, siRNA knock down, luciferase reporter assay, proteomics, co-immunoprecipitation, confocal laser-scanning microscopy, site-directed mutagenesis, AlphaFold structure and complex prediction, IP/MS.

RESULTS:We have designed a proximity labeling approach that can specifically detect activity dependent STING interactors. We show that dIKKγ is recruited by dSTING upon activation and that the recruitment of dIKKγ is essential for dSTING-mediated activation of Relish. We furthermore identify a region in the C-terminal α-helix of dSTING in which mutations abolish recruitment of dIKKγ and activation of Relish. Interestingly, we find that mutations in the same region of hSTING also abolish NF-κB but not IRF3 activation. Corroborating these findings, we show that the CTT of hSTING as well as TBK1 are dispensable for hSTING-mediated activation of NF-κB.

CONCLUSIONS:Our findings suggest an evolutionarily conserved mechanism of STING-mediated NF-κB activation involving the C-terminal α-helix of STING and the IKK-complex. This mechanism is independent of TBK1 and the CTT of hSTING and is thus distinct from the mechanism of IRF3 activation.

Keywords: NF-κB, STING, signaling mechanism, antiviral immunity, proximity labeling.

O-002
General Research » Antiviral Defense

ACE2-DEPENDENT AND -INDEPENDENT SARS-COV-2 ENTRIES DICTATE VIRAL REPLICATION AND INFLAMMATORY RESPONSE IN CELLS

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OBJECTIVES:Excessive inflammation is the primary cause of mortality in severe coronavirus disease 2019 (COVID-19) patients, yet the underlying mechanisms remain poorly understood.

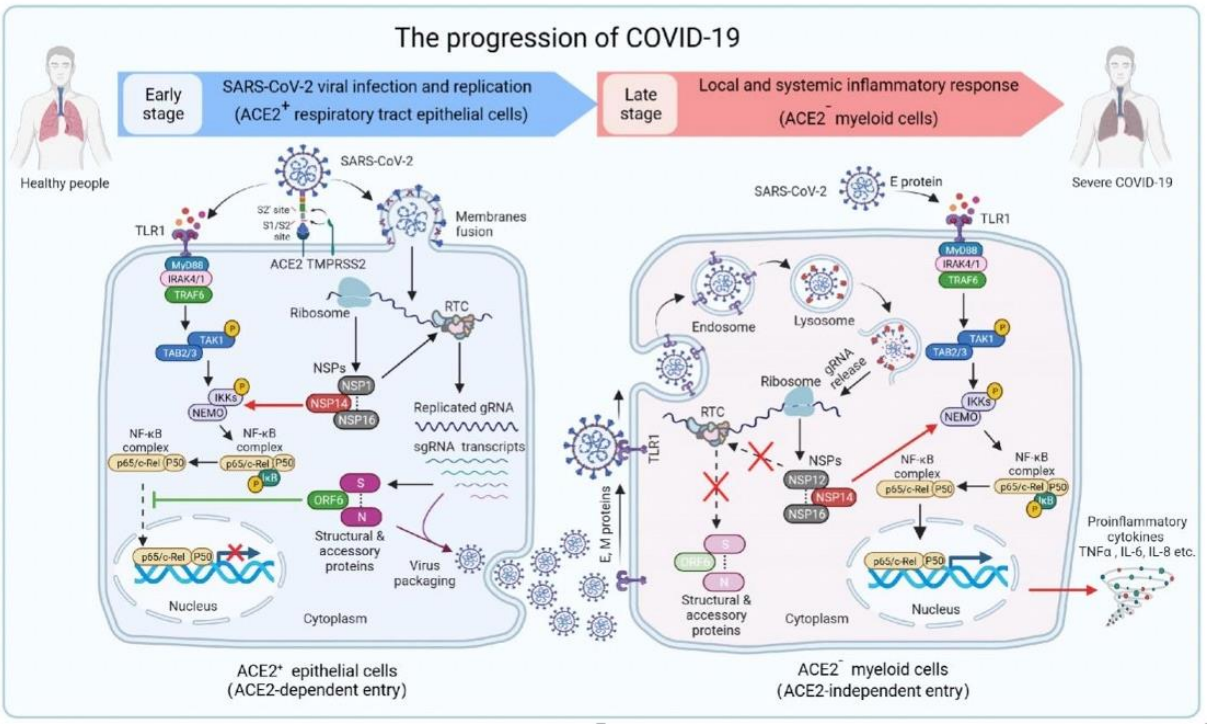
METHODS:We generated various cell lines with or without expression of ACE-2 using CRISPR/Cas9 and ectropic expression of ACE-2 in vitro and vivo to determine viral infection/entry, replication and inflammatory responses.

RESULTS:To understand how these regulators operate in different cells and contribute to excessive inflammation, we find that the ACE2-independent entry of SARS-CoV-2 in myeloid cells triggers a hyperinflammatory response but abrogates productive viral replication. Mechanistically, ACE2-dependent SARS-CoV-2 entry into epithelial cells enables viral subgenomic RNA transcription and causes all viral proteins to be expressed to support productive viral replication, but the translated ORF6 markedly inhibits the inflammatory response by shutting down the NF-kB signaling. In contrast, ACE2-independent entry of SARS-CoV-2 into myeloid cells does not allow translation of ORF6 or other viral structural proteins due to low-efficient transcription of subgenomic RNA, but NSP14 could be directly translated from genomic RNA, resulting in an abortive replication and hyperactivation of the NF-kB signaling pathway for proinflammatory cytokine production. Finally, we identified TLR1 as a critical entry factor responsible for SARS-CoV-2 entry and virus-triggered inflammatory response through interaction with viral E and M proteins.

CONCLUSIONS:Collectively, our findings provide molecular insights into the mechanisms by which strong viral replication but scarce inflammatory response are observed during the early (ACE2-dependent) infection stage, followed by low viral replication and potent inflammatory response in the late (ACE2-independent) infection stage, which contributes to the progression of COVID-19. TLR1 regulates SARS-CoV-2 entry and virus-triggered inflammatory response through regulation of ORF6 expression, which inhibits NF-kB signaling pathway.

Keywords: COVID-19, Viral entry, inflammatory response, Toll-like receptor, NF-kB, ACE2

ACE2-dependent and independent infection and immune response



ACE2-dependent viral infection and -independent viral entry through TLR6 dictate subsequent inflammatory responses via ORF6-mediated inhibition of NF-kB signaling pathway.

O-003
General Research » Antiviral Defense

DUAL ANTIVIRAL MECHANISMS OF OLIGOADENYLATE SYNTHETASE 1 IN DRIVING TRANSLATIONAL SHUTDOWN AND PROTECTING INTERFERON PRODUCTION AND ITS CONSEQUENCES ON ANTIMICROBIAL INNATE IMMUNITY.

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OBJECTIVES: In response to viral infection, how cells balance induction of antiviral components, like interferons (IFNs) and translational shutdown to limit viral replication is not well understood. Moreover, how distinct isoforms of IFN-induced Oligoadenylate Synthetase 1 (OAS1) contribute to this antiviral response also requires further elucidation.

METHODS: Using human OAS1-deficient cells in vitro and mouse Oas1b-knock in mice in vivo, we report that that human, but not mouse, OAS1 inhibits SARS-CoV-2 replication through its canonical enzyme activity via RNase L. In contrast, both mouse Oas1b and human OAS1 protect against West Nile virus infection by a mechanism distinct from canonical RNase L activation.

RESULTS: Unbiased RIPseq showed that OAS1 binds to multiple cellular mRNAs, including IFNβ We mapped the AU-rich element (ARE) in the 3'UTR of IFNβ mRNA as responsible for OAS1 binding. This binding leads to the sequestration of IFNβ mRNA to the endomembrane regions resulting in prolonged half-life and continued translation. The essential role of IFNβ in mediating the antiviral activity of OAS1 is further confirmed by in vivo and in vitro disruption of IFN signaling.
Beyond antiviral activity, the same ARE-binding property of OAS1 also confers in vivo protection from cytosolic bacterial infection such as *Francisella novicida* and *Listeria monocytogenes*. At the cellular level, the bactericidal activity of IFNγ is severely compromised in OAS1-deficient macrophages. The antibacterial effects of OAS1 are not exerted through its canonical enzymatic activity. OAS1 binds and localizes IRF1 mRNA to the rough ER-Golgi endomembranes, which licenses effective translation of IRF1 without affecting its transcription or decay. OAS1-dependent translation of IRF1 leads to the upregulation of antibacterial effectors, such as GBPs, which restrict intracellular bacteria.

CONCLUSIONS: We propose that OAS1 has a broader non-canonical function in modulating protein expression during microbial infection.

Keywords: Oligoadenylate synthetase, West Nile virus, SARS-CoV-2, cytosolic bacteria, Interferon-stimulated genes

O-004
General Research » Inflammasomes

MOLECULAR MECHANISMS OF EFFECTOR RECOGNITION BY THE NLRP1B INFLAMMASOME

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OBJECTIVES:Inflammasomes are cytosolic immune complexes that recognize pathogen-associated stimuli to initiate a potent inflammatory response. While some inflammasomes directly recognize pathogen-associated molecules, others, such as the NLRP1B inflammasome, respond to pathogen-associated activities. In particular, NLRP1B senses the enzymatic activity of pathogen-secreted proteases and E3 ligases through a mechanism of 'functional degradation' – those effectors that promote the proteasomal degradation of NLRP1B induce activation of this inflammasome. However, why pathogens would target NLRP1B for degradation and thusly induce a robust immune response is unclear. We propose that NLRP1B acts as a molecular mimic of other host proteins antagonized by pathogens; in particular, NLRP1B encodes sequences and/or features chemically similar to the other substrates of pathogen secreted enzymes. As such, pathogen effectors cannot distinguish between those factors that they seek to target for degradation and NLRP1B, thusly promoting one immune response while antagonizing another. Here, we investigate the molecular mechanism through which NLRP1B recognizes the *Shigella flexneri* E3 ligase IpaH7.8.

METHODS:To uncover how the NLRP1B inflammasome recognizes that activity of IpaH7.8 we have generated a series of mutants to identify the surfaces on NLRP1B and on IpaH7.8 that mediate their interactions and examined their ability to promote inflammasome activation.

RESULTS:We find that mutations in IpaH7.8 that disrupt activation of NLRP1B also disrupt the degradation of another IpaH7.8 target, GSDMB. We further find that IpaH7.8 recognizes both GSDMB and NLRP1B through a conserved interface. We have additionally identified the surface on NLRP1B that mediates the interaction with IpaH7.8.

CONCLUSIONS:Our results demonstrate that IpaH7.8 recognizes both GSDMB and NLRP1B through a conserved interface, consistent with our model that NLRP1B 'mimics' the GSDMs.

Keywords: NLRP1, Inflammasome, Molecular Mimicry

O-005
General Research » Inflammasomes

ACUTE SUPPRESSION OF MITOCHONDRIAL ATP PRODUCTION PREVENTS APOPTOSIS AND PROVIDES AN ESSENTIAL SIGNAL FOR NLRP3 INFLAMMASOME ACTIVATION

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OBJECTIVES:The protective function of NLRP3 that justifies its evolutionary conservation despite its penchant to instigate pathological inflammation is unclear. Although countless studies associated its disease-promoting activity to mitochondrial stress, a common mitochondrial effect of all its structurally diverse activators could not be defined. How mitochondria reconcile their roles in NLRP3 inflammasome activation and subsequent pyroptosis, and in the intrinsic apoptosis pathway remains elusive. We set out to study cell death decision-making at mitochondria.

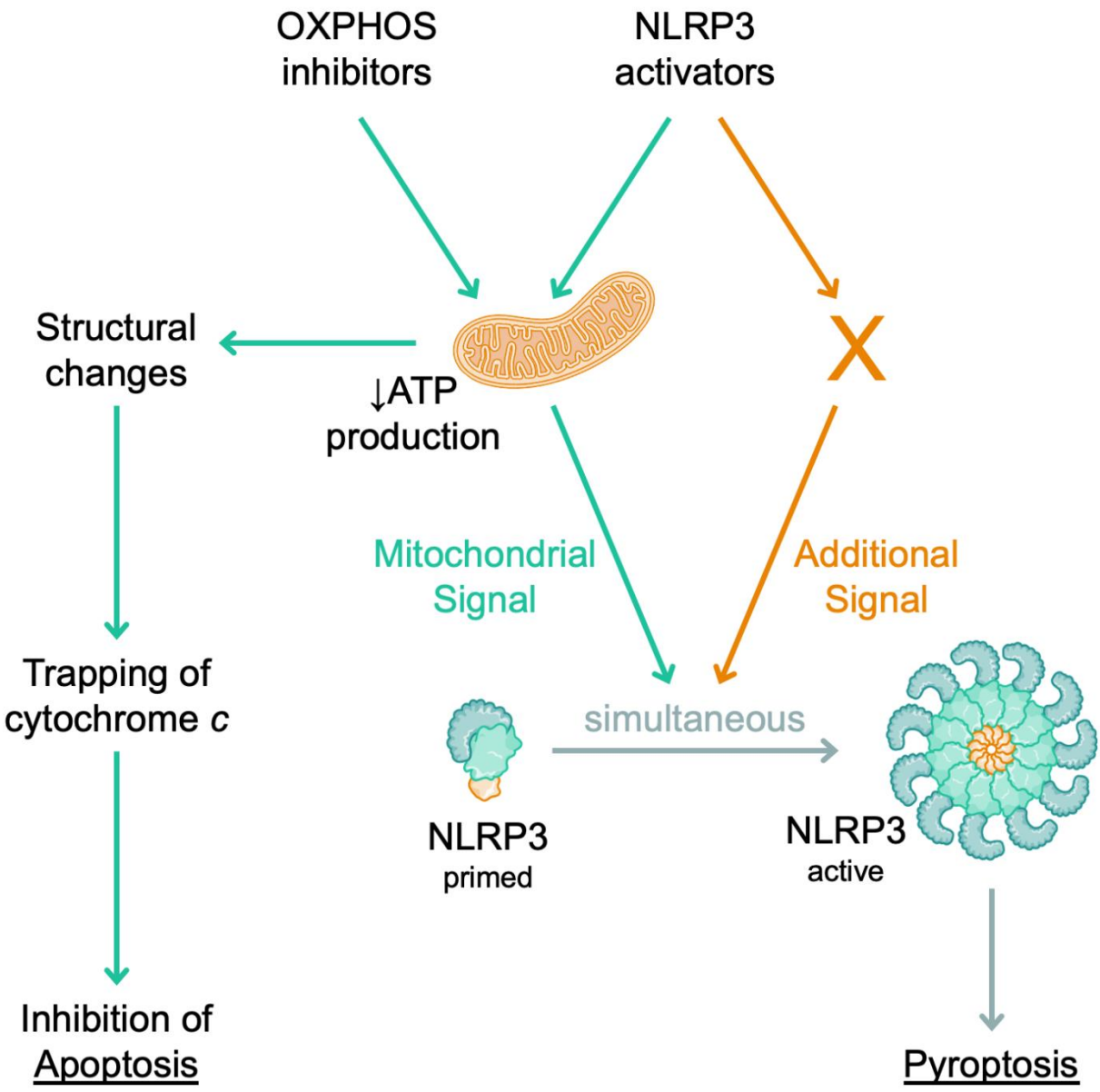
METHODS:We used 15 different NLRP3 activators including ATP and SARS-CoV-2, 8 distinct inhibitors of mitochondrial ATP production (OXPHOS), and 5 specific apoptosis inducers. Effects on mitochondria, apoptosis, and NLRP3 were determined by immunoblot, ELISA, enzymatic assays, FACS, microscopy, TEM, extracellular flux analysis, and untargeted metabolomics, using different cell lines and macrophages form various knockout mouse strains. The electrons shunts idebenone and pyocyanin were used for rescue experiments.

RESULTS:We find that, when cells are challenged simultaneously, apoptosis is inhibited and inflammasome activation prevails. Apoptosis inhibition was not a consequence of inflammasome activation but rather of direct effects of NLRP3 activators on mitochondria. NLRP3 activators turned out to commonly inhibit OXPHOS through distinct mechanisms. OXPHOS inhibition by NLRP3 activators as well as conventional ETC inhibitors or mitochondrial uncouplers disrupted mitochondrial cristae architecture, leading to trapping of cytochrome c. This effect was alone sufficient for apoptosis inhibition but not for NLRP3 activation, yet OXPHOS inhibitors acquired the ability to trigger NLRP3 when combined with a second signal.

CONCLUSIONS:The common effects of NLRP3 activators and other OXPHOS inhibitors on mitochondria facilitate stringency in cell death decisions. We propose a model in which NLRP3 activation requires two simultaneous signals: one mitochondrial involving OXPHOS suppression, and one additional cellular signal. Finally, our work points towards a host-protective function of NLRP3 as a guard of mitochondrial function and a sensor for pathogens suppressing apoptosis.

Keywords: NLRP3, mitochondria, apoptosis, OXPHOS, ATP, cytochrome c

Graphical Abstract



O-006
General Research » Inflammasomes

NLRP3 IS A THERMOSENSOR THAT IS NEGATIVELY REGULATED BY HIGH TEMPERATURE

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OBJECTIVES:Inflammation is an essential response to infection and injury, but unregulated inflammation is damaging and must be limited by negative feedback signalling. Inflammasome activation is highly inflammatory, driving both local inflammation and systemic responses like fever. The NLRP3 inflammasome is activated by a vast number of stimuli and senses perturbations of cytoplasmic homeostasis. As temperature is a fundamental environmental stressor, we hypothesised that NLRP3 inflammasome signalling would be sensitive to changes in temperature.

METHODS:We investigated the effects of high temperatures on NLRP3 in mouse and human macrophages.

RESULTS:Short-term incubation at high fever range temperatures significantly inhibits NLRP3 activation, while secretion of the inflammasome-independent cytokines TNF and IL-6 are much less affected. High temperature blocks NLRP3 inflammasome formation in a transcription-independent manner, and the effects on NLRP3 are specific as NLRC4, AIM2, and NLRP1 inflammasomes are not inhibited.

Using cellular and in silico assays we show that the effect of high temperature on NLRP3 is protein intrinsic. The activation of NLRP3 is associated with a decrease in the thermal stability of the protein and molecular dynamics simulations show that high temperature decreases the stability and increases the intrinsic flexibility of NLRP3. Molecular dynamics simulations further identify a peptide in the C-terminal of the FISNA domain (COFI) that undergoes a significant conformational shift at high temperature. Cellular assays demonstrate that the COFI regulates NLRP3 stability and is required for activation.

Finally, in vivo experiments demonstrate that elevation of mouse body temperature negatively regulates LPS-induced inflammatory cytokine production.

CONCLUSIONS:Our studies reveal that high temperatures associated with fever limit NLRP3 activity in a classical negative feedback mechanism and identify a novel role for NLRP3 as a protein thermosensor.

Keywords: Inflammasome, NLRP3, fever, NLR, inflammation, temperature

O-007
General Research » Intracellular sensing of nucleic acids and interferon signaling

DYSREGULATION OF TREG HOMEOSTASIS BY ADAR1 DEFICIENCY AND CHRONIC MDA5 SIGNALING

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OBJECTIVES:Type I interferonopathies including Aicardi–Goutières syndrome (AGS), are rare monogenic autoinflammatory diseases characterized by continuous production of type I interferons (IFN-I). AGS-related genes encode for proteins involved in nucleic acid metabolism or sensing, such as loss-of-function mutations in adenosine deaminase acting on RNA 1 (ADAR1) and gain-of-function mutations in melanoma differentiation-associated protein 5 (MDA5). ADAR1 deficiency activates several downstream sensors, including MDA5 – resulting in production of IFN-I; and protein kinase R (PKR) – resulting in eIF-2α-mediated translational

shut-down and cell death. Some patients with AGS develop symptoms of systemic lupus erythematosus, a paradigm autoimmune disease, likewise associated with perturbed IFN-I production. Naturally occurring regulatory T cells (Tregs) are indispensable for maintaining immune tolerance, and Treg loss or dysfunction results in severe or fatal autoimmune diseases, in humans and mice.

METHODS:We investigated the Treg population in patients with AGS, carrying mutations in ADAR1 or MDA5 and found a significant reduction of effector Tregs. We analysed the underlying mechanisms using murine models.

RESULTS:We found that Treg-specific expression of an MDA5 G821S gain-of-function mutant caused peripheral Treg loss, severe autoimmunity and lethality. Moreover, Treg-specific deletion of *Adar1* (*Foxp3ΔAdar1*), similarly caused Treg loss and a severer, earlier-onset scurfy-like lethal autoimmune phenotype. Abrogation of MDA5 signaling by mitochondrial antiviral-signaling protein (MAVS) knockout (*Mavs−/−Foxp3ΔAdar1*), did not prevent Treg loss but improved the survival of *Foxp3ΔAdar1* mice. Both *Foxp3ΔAdar1* and *Mavs−/−Foxp3ΔAdar1* Tregs exhibited high phosphorylation of eIF-2α, indicating the involvement of PKR. *Pkr−/−Foxp3ΔAdar1* mice similarly showed improved phenotype and survival but also retained a larger fraction of Tregs.

CONCLUSIONS:Altogether, we show that constitutive MDA5 signaling, and ADAR1 deficiency causing aberrant downstream activation of MDA5 and PKR, disturb Treg homeostasis in mice. These results indicate a potential contribution of Tregs in the variety of symptoms in patients with type I interferonopathies.

Keywords: regulatory T cells, RNA sensing pathways

O-008
General Research » Intracellular sensing of nucleic acids and interferon signaling

ADA2 IS A LYSOSOMAL ADENOSINE DEAMINASE ACTING ON DNA (LADAD) INVOLVED IN REGULATING TLR9-MEDIATED IMMUNE SENSING OF DNA

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OBJECTIVES:Identified over 50 years ago, ADA2 has traditionally been regarded as an extracellular adenosine deaminase; however, a body of experimental evidence casts doubt on the physiological relevance of this activity. Here we investigated its role in nucleic acid sensing (NAS).

METHODS:Microscopy, Recombinant ADA2 WT and mutants production and purification, Electrophoretic mobility shift assay, DNA deaminase assay, Inosine content in DNA by mass spectrometry, TLR9 activation via HEK TLR9-SEAP reporter cells.

RESULTS:Our study reveals that ADA2 localises within the lysosomes, where it is targeted through modifications of its glycan structures. We go on to show that ADA2 interacts with DNA molecules, altering their sequences by converting adenosine bases to inosine. We also characterise its DNA substrate preferences and provide data supporting the hypothesis that DNA is its natural substrate. Finally, we demonstrate that adenosine deamination of DNA molecules and ADA2 regulate lysosomal immune sensing of nucleic acids (NAs) by modulating TLR9 activation.

CONCLUSIONS:Our results identify a novel contributor to the intricate processes linking NA metabolism with cell-intrinsic immune responses.

Keywords: ADA2, Nucleic Acid Sensing, LADAD, TLR9

O-009
General Research » Intracellular sensing of nucleic acids and interferon signaling

RNA SENSOR-MEDIATED SIGNALING PATHWAY REGULATES THE EXPRESSION OF TYPE I INTERFERON AND CHEMOKINES IN VAGINAL EPITHELIAL CELLS AGAINST GENITAL HERPES

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OBJECTIVES:Genital herpes, caused by herpes simplex virus (HSV), is a common sexually transmitted infection, for which currently no cure exists. As HSV is a dsDNA virus, the DNA-sensing cGAS-STING signaling pathway has been widely studied. During HSV infection, dsRNA is also produced due to genomic stress, mitochondria stress, and viral replication. However, the role of RNA sensors like RIG-I/MDA5 during HSV infection is not well studied.

METHODS:A murine vaginal and HSV-2 infection model was established to study the symptoms of C57BL/6 (WT), Mavs-/-, and cGas-/- mice. Mouse vaginal organoids and bone marrow-derived macrophages were established to study the innate responses of different cell types. The crosstalk between epithelial and immune cell types was analyzed by imaging mass cytometry.

RESULTS:Both Mavs-/- and cGas-/- mice are more susceptible to HSV-2 vaginal infection than WT. Both KO mice also had significantly higher viral load in the vaginal washes, but not in the spinal cord at day 5 post infection, as compared with those of WT mice at day 2 post infection. The vaginal infection model was mimicked in an in vitro organoid system, where we observed Mavs- and cGAS-dependent type I interferon and chemokine production after HSV-2 infection. Interestingly, MAVS-KO macrophages infected with HSV-2 did not show any decrease in type I interferon and chemokines compared to the wild type.

CONCLUSIONS:In genital herpes HSV-2 attacks epithelial cells, in which both DNA and RNA sensors contribute to triggering innate immune responses. A lack of MAVS or cGAS leads to impaired antiviral activity and cell-cell interaction with immune cells, in which only cGAS plays a role in triggering innate immune responses. The role of MAVS during HSV-2 infection is cell type-specific, and we found MAVS to have a role specifically in the antiviral defense in epithelial cells.

Keywords: MAVS, HSV, genital herpes

O-010
General Research » Inflammatory cell death

REGULATION OF CELL DEATH BY THE METABOLITE FUMARATE

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OBJECTIVES:Fumarate is a Kreb's cycle metabolite with a wide variety of functions. Changes in cellular metabolism result in fumarate accumulation whereby fumarate can react with thiol groups on cysteine residues by a process known as succination. Succination impacts protein function and cellular processes. Recently, we identified a novel role for fumarate in regulating pyroptotic cell death via the succination and inactivation of GSDMD. However, how fumarate regulates other forms of cell death and innate immune responses is unclear. We have identified that the neuroprotective protein PARK7 is subjected to succination by fumarate. PARK7 is an important neuroprotective redox sensor that responds to oxidative stress in dopaminergic neurons and initiates anti-apoptotic responses to limit neurodegeneration and inflammatory responses.

METHODS:Mass spectrometry, metabolomics, western blotting, RNA-seq, iPSC derived neuron cell culture, immunohistochemistry, neurotoxicity assays.

RESULTS:Here we discuss the functional characterization of PARK7 succination in neuron cell death and how PARK7 succination impacts the progression of Parkinson's Disease. Deletion of fumarate hydratase conditionally in dopaminergic neurons results in fumarate accumulation and suppression of PARK7 function. Mechanistically, fumarate competes with PARK7-Cys106 oxidation to prevent PARK7 activation and enhance neuron cell death. In addition, fumarate hydratase deficient neurons develop an interferon stimulated gene (ISG) expression signature as a result of PARK7 succination and loss of its neuroprotective function.

CONCLUSIONS:Our studies define a novel signaling crosstalk mechanism between cell metabolism and neuron cell death and unravel the signaling events that orchestrate these events. This work defines how the fumarate PARK7 signaling axis regulates neurotoxicity and neuroinflammation and provides a mechanistic understanding of how this pathway influences innate immune driven progression of Parkinson's disease.

Keywords: Cell death, Neuroinflammation, ISGs, Fumarate, Succination, Parkinson's disease

O-011
General Research » Inflammatory cell death

MAP3K5 REGULATION OF GSDMD POST-TRANSLATIONAL MODIFICATIONS DICTATES PYROPTOTIC PROGRESSION

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OBJECTIVES:Pyroptotic release of IL-1 family cytokines requires the coordinated assembly of multiple supramolecular complexes. While regulation of proteolytic activation of IL-1 pyrogens via inflammasomes provides a major pharmacologic target for treatment of inflammatory disease, mechanisms regulating mobilization and activation of the pyroptotic effector gasdermin D (GSDMD) represent another attractive therapeutic target. We therefore sought to detail the temporal requirements for mitogen activated protein kinases (MAPKs) necessary for pyroptotic release of IL-1b following NLRP3 inflammasome triggering.

METHODS:By employing dynamic MAPK translocation biosensors and acute pharmacologic inhibition in the NLRP3 inflammasome trigger step, we assessed temporal activation and utilization of the p38, ERK, and JNK signaling axes preceding pyroptotic IL-1 release. We further employed mass spectrometry to identify phosphorylated GSDMD peptides downstream of MAP3K activation.

RESULTS:We find that both JNK and p38 kinase signaling axes exhibit biphasic activation during the inflammasome trigger step where JNK supports, and p38 limits, core inflammasome formation and downstream pyroptosis. We define two distinct JNK signaling branches where MAP3K7:JNK1:mitochondrial ROS supports core inflammasome formation while a distinct xanthine oxidase/ROS:MAP3K5:JNK2 axis regulates mobilization of GSDMD. Furthermore, MAP3K5 supports dynamic phosphorylation within a sub-pool of GSDMD to engage both the N terminal pore-forming and the C-terminal autoregulatory domains in coordinated progression to the pyroptotic pore-transition event required to release active IL-1b.

CONCLUSIONS:In this work, we have uncovered a novel MAP3K5:JNK2 signaling cascade that specifically leads to GSDMD phosphorylation to support pyroptosis without impacting formation of the core NLRP3 inflammasome complex. The post-translational modifications of GSDMD that we identify here constitute pharmacologically targetable regulatory nodes for therapeutic intervention and further highlight the therapeutic potential of developing inhibitors of GSDMD function to limit IL-1 mediated inflammatory disorders.

Supported by the Intramural Research Program of NIAID, NIH

Keywords: Inflammasome, Interleukin-1, Gasdermin, Pyroptosis, Biosensor, MAPK Signaling

O-012
General Research » Inflammatory cell death

MLKL'S EXECUTIONER DOMAIN IS RELEASED DURING NECROPTOSIS PRIOR TO ITS RIPK3-MEDIATED PHOSPHORYLATION

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OBJECTIVES:Necroptosis is required to maintain tissue homeostasis. While this form of cell death helps clear pathogens and cancer cells, when dysregulated, can also cause devastating tissue damage, especially in ischemia-reperfusion and chemical-induced injuries. A detailed biochemical mechanism of necroptotic execution is necessary to identify the best targets for pharmaceutical intervention to prevent necroptotic tissue damage.

Biochemically, necroptosis is tightly regulated by multiple phosphorylation events, leading to the final phosphorylation of the executioner protein MLKL by RIPK3. The exact purpose of this phosphorylation event is not well-defined but is assumed to promote MLKL oligomerization and translocation to internal membranes, leading to cellular demise. However, this phosphorylation event alone is not sufficient to cause cell death. This is best demonstrated by the fact that phosphomimetic MLKL cannot cause cell death, as well as the observation that certain MLKL mutants are phosphorylated in the absence of cell death. These results suggest that, in addition to RIPK3-mediated phosphorylation, there are further requirements for MLKL activation and subsequent necroptotic cell death.

METHODS:To elucidate these additional requirements, we performed mutational analysis of MLKL and investigated its effect on MLKL phosphorylation, oligomerization, and cell death.

RESULTS:Surprisingly, we observed that mutations in the MLKL N-terminus were able to prevent phosphorylation of the C-terminus. Our results are consistent with a model where the N-terminal executioner domain of MLKL is released from the brace prior to phosphorylation by RIPK3. We further tested this model using pharmacologic inhibitors and neutralizing monobodies against MLKL.

CONCLUSIONS:Given these findings, we present a new biochemical activation model that is consistent not only with our data but also with the observation that phosphomimetic MLKL cannot cause cell death.

Keywords: necroptosis, cell death, RIPK3, MLKL

O-013
General Research » Inflammatory cell death

THE ROLE OF NINJ1 IN *MYCOBACTERIUM TUBERCULOSIS*-INFECTED MACROPHAGES

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OBJECTIVES:Tuberculosis is a global health problem, killing approximately 1,5 million people every year. The disease is caused by infection with *Mycobacterium tuberculosis* (Mtb). Upon entering the human lung, Mtb bacteria are phagocytosed by alveolar macrophages, their first host cells. These Mtb-infected macrophages go through several cell death pathways during infection; for instance, we have previously published a story showing that Mtb can induce pyroptosis through damaging the plasma membrane of their macrophage host cell. However, there is still an incomplete understanding of the roles of the various cell death pathways in this context, and how Mtb can induce them. Ninjurin 1 (NINJ1) was recently found to be essential for lysis in several modes of cell death, and we have later shown that the oligomerization of NINJ1 can be inhibited by the amino acid glycine. Putting these elements together, we examine the role of NINJ1 in Mtb-infected human macrophages.

METHODS:Fluorescence microscopy, LDH assays, native-PAGE
RESULTS:We have found that NINJ1 oligomerizes and is important for lysis in Mtb-infected human macrophages. Furthermore, we have studied the role of NINJ1 in pyroptosis, post-apoptosis lysis, ferroptosis and necroptosis, in order to pick apart the components of total lytic cell death in this context.

CONCLUSIONS:Our results show that NINJ1 oligomerizes in Mtb-infected macrophages, but also that even if oligomerized, NINJ1 is not always essential for lysis.

Keywords: Mtb, Mycobacterium tuberculosis, NINJ1, Ninjurin 1, lytic cell death, macrophages

O-014
General Research » Innate immunity to parasites

IL-1α PROMOTES INTESTINAL INTEGRITY DURING MURINE VISCERAL LEISHMANIASIS TO PREVENT MICROBIOTA TRANSLOCATION AND DAMPPENING CHRONIC TH1-INFLAMMATION

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OBJECTIVES:Interleukin-1 is a pleiotropic cytokine important in inflammatory and infectious diseases, among during Visceral Leishmaniasis.

METHODS:We aimed to determine the role of IL-1 cytokines in murine L. infantum-induced Th1-inflammatory process.

RESULTS:We showed that IL-1R1 deficiency enhanced the Th1-inflammation and restricted parasites in an IL-1α-dependent way into the target organs. Bone-marrow chimera transplantation demonstrated that hematopoietic cells are the primary source of IL-1α during VL. The infection induces the expression of IL-1α in the duodenum, which is important for intestinal integrity through the induction of tight junction molecules. The absence of IL-1α leads to intestinal permeability and microbiota translocation, which consequently exacerbates the Th1 immune response and is reverted by intestinal microbiota depletion. In addition, IL-1α is mainly expressed by myeloid cells in the mesenteric lymph node (MLN) and is important to Th17 response generation.

CONCLUSIONS:Our results demonstrate that IL- 1α promotes intestinal integrity during visceral leishmaniasis, preventing microbiota translocation avoiding Th1-inflammation.

Keywords: Interleukin-1; gut inflammation; intestinal integrity, visceral leishmaniasis, Th1-inflammation

O-015
General Research » Responses to intracellular pathogens

NAIP—NLRC4 INFLAMMASOME ACTIVATION IN TUFT CELLS ACTIVATES A PGD2-ILC3 SIGNALING CIRCUIT THAT CAN PROTECT AGAINST ENTERIC INFECTION

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OBJECTIVES:Epithelial cells are the first cells encountered by invading pathogens in the intestine. It is not clear whether specialized subtypes of these cells all respond the same way to infection. Inflammasomes are sensors of the innate immune system in the cytosol, and the NAIP-NLRC4 inflammasome specifically detects bacterial ligands from type-3 secretion systems and flagellin. When NAIP-NLRC4 is activated in epithelial cells during gastrointestinal bacterial infection, they are expelled, and IL-18 and eicosanoids are released. Tuft cells are a rare subtype of epithelial cell important for restriction of parasitic infections. We found recently, that tuft cells uniquely release the eicosanoid prostaglandin D2 (PGD2) after NAIP-NLRC4 inflammasome activation. The objective of the presented study is to understand the signaling of tuft cell released PGD2 and its consequences.

METHODS:We use mouse models of infection and inflammasome activation and cell type specific expression of inflammasomes.

RESULTS:We show that tuft cell released PGD2 triggers an antibacterial response via the receptor CRTH2. After tuft cell inflammasome activation, IL-22 protein and antimicrobial proteins Reg3γ and Reg3β in the small intestine are increased. Interestingly, IL-18 is unchanged after tuft cell inflammasome activation. CCR6 negative type 3 innate lymphoid cells (ILC3) express CRTH2, and the numbers of IL22 expressing ILC3 increase after tuft cell inflammasome activation. Chemical inhibition of CRTH2 prevented the increase of IL-22 and antimicrobial proteins. In a mouse model of NAIP-NLRC4 activation exclusively in tuft cells, a gastrointestinal bacterial pathogen is cleared faster from the small intestine than in inflammasome deficient controls, confirming sufficiency of this response.

CONCLUSIONS:Overall, our study expands on the role of tuft cells as mediators of the intestinal immune response, showing them capable of activating an antibacterial response in addition to their established roles in parasitic infections. Our results suggest that tuft cells are important in sensing all classes of pathogens.

Keywords: Inflammasome, intestine, epithelial cell, ILC3, eicosanoids, Salmonella

O-016
General Research » Model systems of innate immunity

PEROXISOME LIPID METABOLISM ORCHESTRATES INFLAMMATORY PATHWAYS FROM FLY TO HUMANS

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OBJECTIVES:Amyloid fibrils have long been associated with disease. It is now evident that there are amyloids, termed "functional amyloids," that have normal biological activities. These functional amyloids serve as a signaling platform to execute downstream signaling in multiple immune and inflammatory pathways, including the NLRP3-ASC-mediated inflammasome signaling, and the RIPK1/RIPK3 signaling in the TNF pathway. How the polymerization of the amyloid-forming proteins is regulated is still unknown. Recent studies suggested the initiation of functional amyloids is controlled by immunometabolism.
Our groups use the humble fruitfully, Drosophila melanogaster, as model organism to untangle complex immune signaling. Our groups established that in the Drosophila Immunodeficiency (IMD) pathway that is the ortholog of the mammalian Tumor necrosis factor(TNF)-pathway, the adaptor protein IMD forms amyloids to initiate the inflammatory cascade, similarly to what reported for RIPk1/3 in the TNF pathway. We then found that peroxisomes, essential lipid metabolic organelles, are required for the the activation of IMD signaling.
We hypothesized that: 1) peroxisomal metabolism regulates IMD-amyloid formation, 2) peroxisomes regulate also other amyloid-dependent inflammatory pathway.

METHODS:We used Crispr/Cas9 technology, lipidomics, genetics and cell biology approaches to study peroxisomal immunemetabolism and amyloid-formation in Drosophila macrophages and human peripheral blood mononuclear cells.

RESULTS:Our data demonstrated that peroxisomes supply specific diacylgicrerides that are required for the processing of IMD protein and the assembly of amyloids to activate the signaling in response to microbial challenge.
We found that the requirement for peroxisomal lipids is conserved in human monocytes and it regulates various amyloid-forming proteins in humans such as the NLRP3-ASC in the inflammasome. Indeed, peroxisomal dysfunction is a feature of inflammasopathies

CONCLUSIONS:Our study elucidates the specific role of peroxisomal immunometabolism in regulating functional amyloids in Drosophila immunity and translated the result into humans. Our study open a novel line of research in immunity with physiological and pathological relevance.

Keywords: peroxisomes, funcional amyloids, IMD pathway, TNF pathway, inflammasome, inflammasopathies

O-017
General Research » Responses to intracellular pathogens

CARDIOLIPIN AS CASPASE-4/11 INHIBITOR: BACTERIA IMMUNE ESCAPE OR HOST ALLY IN SEPSIS?

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OBJECTIVES:Bacterial infection is sensed by the immune receptors caspase(casp)-4 (casp-11 in mice), which detect bacterial lipopolysaccharide (LPS), and induce inflammatory cell death (pyroptosis). Casp-4/11 activation is vital for bacterial clearance, while exacerbated signaling causes lethal sepsis.
Thus, there is a need for safe and specific casp-4/11 inhibitors.
We have previously shown that cardiolipin (CL), a lipid present in both mitochondria and bacteria, inhibits the extracellular receptor of LPS. Therefore, our objective was to investigate whether CL inhibits also the intracellular LPS receptors, casp-4/5/11.

METHODS:To investigate the ability of CL to specifically inhibit casp-4/11 in vitro and in vivo, we have:
1. Evaluated the ability of CL to prevent LPS binding to casp-4/11 by co-immunoprecipitation of casp-4 and 11 by biotinylated LPS with or without CL.
2. Treated primary murine and human macrophages with the casp-4/11 activator iLPS or the casp-1 activator nigericin with or without CL and quantified cytokine secretion and pyroptosis as a measure of caspase signaling.
3. Tested the ability of CL to inhibit casp-11 in vivo by measuring cytokines in the serum of mice intraperitoneal injected with LPS with or without CL.

RESULTS:1. CL prevents LPS binding to casp-4/11.
2. CL inhibits iLPS-induced casp-4/11 activation but not nigericin-induced casp-1 activation in murine and human primary macrophages.
3. Intraperitoneal administration of CL to mice decreases iLPS-induced casp-11-dependent inflammatory cytokine in the serum.

CONCLUSIONS:We demonstrate CL as a specific casp-4/11 inhibitor we propose as a sepsis treatment.
Notably, dysfunctional mitochondrial CL in disease is associated with susceptibility to sepsis, while bacterial CL is required for the virulence of intracellular pathogens. Our data suggest that mitochondrial cardiolipin is necessary for fine-tuning casp-4/11 activation during bacterial clearance, while bacterial CL may be used by bacteria to escape casp-4/11 detection. Both hypotheses are under investigation and might open the way for new understanding and treatment of infectious diseases.

Keywords: caspase-4, caspase-11, non-canonical inflammasome, inhibitor, pyroptosis, sepsis

O-018
General Research » Model systems of innate immunity

FADD INTEGRATES INDEPENDENT STING AND IMD SIGNALING TO CONTROL CASPASE-MEDIATED NF-κB ACTIVATION IN DROSOPHILA MELANOGASTER

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OBJECTIVES:In *Drosophila*, the STING pathway responds to viral infection whereas the functionally distinct IMD pathway responds to bacterial infection, yet both pathways use the NF-κB-like transcription factor Relish. How signals originating from distinct pattern recognition receptors are mechanistically integrated by shared signaling proteins or/and transcription factors continues to be a subject of substantial interest in immunology. Here, we address how STING and IMD can both activate Relish.

METHODS:Alphafold, Cell culture, Co-immunoprecipitation, Proximity labeling, Cas9 editing, mutant fly lines.

RESULTS:STING signaling directly activates Relish through proteolytic cleavage, similar to the IMD pathway. We demonstrate that this activation is crucial for *in vivo* antiviral immunity. The STING and IMD proteins independently interact with FADD, which recruits the caspase 8-like protein DREDD, responsible for cleaving Relish. A crucial aspect of STING activation involves the formation of a multimer, constructed by successive STING dimers. Our *in silico* model reveals that FADD binds STING at the interface of two STING dimers, explaining why FADD selectively binds activated STING. This finding is experimentally supported by FADD mutants, allowing the decoupling of IMD and STING signaling.

CONCLUSIONS:Our results place FADD as a novel component in the *Drosophila* STING pathway and provide detailed 3D structural information showing how FADD interacts with activated STING and initiates signaling. Additionally, we provide 3D structural information on how FADD interacts with IMD and how FADD recruits DREDD to mediate cleavage of the NF-κB transcription factor Relish.

Keywords: STING, NF-κB, FADD, Caspase, Signaling, Structure

O-019
General Research » TLR Signaling

DIFFERENTIAL SIGNALING BETWEEN TLR7 AND TLR9 THAT CONTROLS LUPUS IS GENETICALLY MAPPED TO THEIR RESPECTIVE TIR DOMAINS

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OBJECTIVES:Toll like receptor (TLR) 7 and 9, endosomal sensors for RNA and DNA, are key mediators of lupus autoreactivity. Although generally considered homologous, they have opposing effects on lupus: TLR7 exacerbates disease while TLR9 protects from disease, with these differential effect largely mediated by B cells. We have been studying how these two TLRs mediate opposing effects in lupus. We recently published data (Leibler et al, Nat. Imm. 2022) suggesting that TLR9 has a MyD88-independent regulatory signaling pathway. We therefore hypothesized that differences in signaling qualities of the two TIR domains, which share only 45% homology, could be responsible for the opposite outcome on disease.

METHODS:To test this, we introduced the hypothetically protective TIR of TLR9 into the endogenous TLR7 locus of TLR9-deficient (TLR9-/-) lupus-prone MRL/lpr mice, creating a chimeric molecule termed TLR779.

RESULTS:Stimulation with TLR7 agonists induced MyD88-driven NF-kB translocation in TLR779 B cells, that was decreased compared to TLR7WT B cells. Even though TLR779 had pro-inflammatory signaling capacities, TLR779 mice had a very mild disease, similar to MRL/lpr mice completely lacking both TLR7 and TLR9. Further, we reciprocally created a chimeric TLR9 molecule (TLR997) by replacing the TLR9 TIR domain with the hypothetically pro-inflammatory TLR7 TIR and introduced it in the endogenous TLR9 locus of TLR7-/- MRL/lpr mice. Strikingly, lupus was exacerbated in TLR997 compared to the native TLR9 mice (all TLR7-/-), establishing that TLR7 and TLR9 TIR domains indeed have different properties and mapping the differences in lupus disease promotion to these TIR domains, as these are the only differences between mutant and control mice.

CONCLUSIONS:The finding that TLR9 and TLR7 TIR domains signal differently is novel, and opens exciting avenues to develop therapeutic targets in lupus. It also has major implications for understanding fundamental TLR and innate immune biology.

Keywords: TLR7, TLR9, TIR domain, B cell, Lupus

O-020
General Research » Model systems of innate immunity

TOLL REGULATES EPITHELIAL RENEWAL IN THE "*"DROSOPHILA"*" GUT

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OBJECTIVES:The regulatory mechanisms by which Toll-Like Receptors (TLRs) may control intestinal stem cells (ISC) in normal conditions and in response to injury or infection are still unexplored. This is where the use of the vinegar fly "*"Drosophila melanogaster"*" can lead to faster progress. We are studying the role of Toll in the fly intestinal epithelium and the role of Toll pathway activation in maintaining homeostasis as well as sustaining the density and diversity of commensal bacteria.

METHODS:"*"Drosophila"*" ISCs will divide to renew themselves and (depending on the signalling microenvironment) produce an enteroblast (EB, 95% of cases) or an enteroendocrine precursor (EEP, 5% of cases). EBs and EEPs will not divide but differentiate to enterocytes (ECs) and enteroendocrine cells (EEs) respectively. We are using the powerful genetic toolbox of the fly to identify cell types and manipulate Toll signalling in these cells separately, to analyse the role of Toll in the gut.

RESULTS:We have found that, following infection, Toll is required in ISCs for their mitosis. In this context, the Toll ligand Spätzle, acts as a mitogen. Toll integrates with the Notch and JNK pathways to regulate ISC mitosis as well as the density of the gut bacteriome. Our results are consistent with the hypothesis that, following enteric infection, Toll activation increases ISC mitosis to provide more ECs and EEs and thus renew the epithelium while at the same time increasing commensal gut bacterial density (without harming diversity). Increasing the numbers of gut bacteria functions as a barrier to prevent intestinal colonisation by non-commensal bacteria.

CONCLUSIONS:Our experiments place Toll at the centre of the immunological regulation of ISC mitosis both following infection as well as in homeostatic conditions. Moreover, Toll functions as a "leash" to control the ecosystem of intestinal bacteria, using them as an additional barrier to avoid pathogen establishment.

Keywords: Toll, intestinal stem cells, gut bacteria, Drosophila

O-021
General Research » TLR Signaling

SLAMF1-DERIVED PEPTIDE INHIBITS SIGNALING INITIATED BY ENDOSOMAL TOLL-LIKE RECEPTORS

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OBJECTIVES:Inflammation plays an important role in the development and progression of many diseases and is often driven by overstimulation and signaling downstream pattern recognition receptors (PRRs). One of the approaches to control PRRs' signaling is to block protein-protein interactions at the signaling crossroads. Recently we found that signaling lymphocyte activation molecule family 1 (SLAMF1) has a critical role in regulation of IFNβ expression and secretion downstream toll-like receptor 4 (TLR4). Based on the insights from this study, we further developed a SLAMF1-derived peptide P7 and linked it to the cell-penetrating peptide penetratin (Pen) for intracellular delivery (P7-Pen). In addition to striking inhibitory activity of P7-Pen towards TLR4-mediated signaling, P7-Pen peptide was tested for the ability to inhibit signaling by endosomal TLRs.

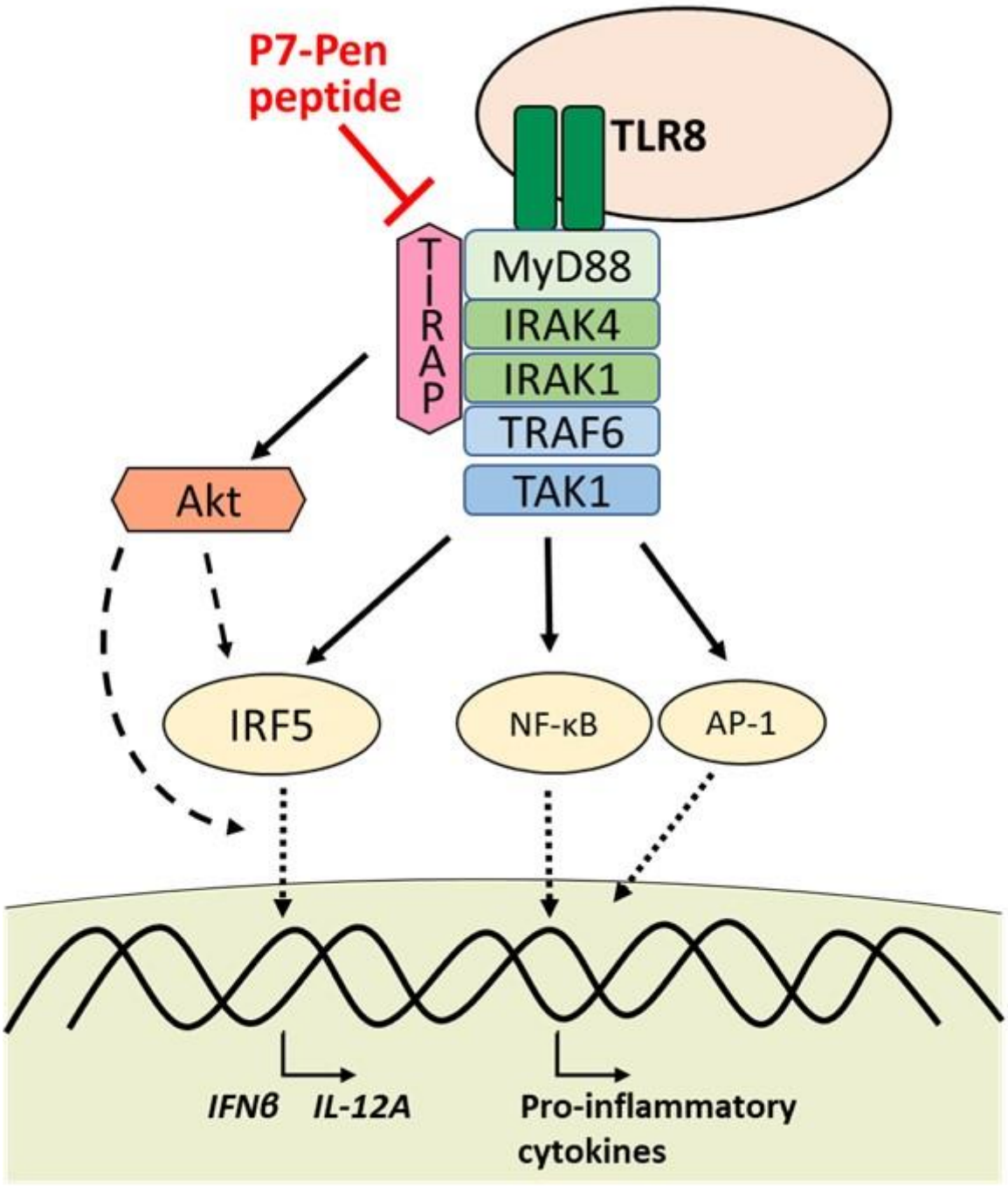
METHODS:Several model systems were used: in vitro cell cultures, primary human monocytes and ex vivo whole-blood assays.

RESULTS:We found that P7 inhibited IFNβ expression and secretion downstream TLR7, 8 and 9, IL-12p70 secretion downstream TLR8, and proinflammatory cytokines expression (TNF, IL-1β, IL-6) downstream TLR9. We have previously shown that the recruitment of TIRAP to TLR8 signaling complex promotes the expression and secretion of the IRF5-dependent cytokines IFNβ and IL-12p70 via TIRAP-dependent positive regulation of IRF5 nuclear translocation and activation of Akt kinase. Indeed, the pretreatment of monocytes by peptide blocked TIRAP recruitment to TLR8-MyD88 complex, inhibited Akt activation and nuclear translocation of IRF5.

CONCLUSIONS:we suggest that P7-Pen inhibits signaling pathways downstream endosomal TLRs by interfering with TIRAP-MyD88 protein complex formation. Overall, P7-Pen peptide has unique mode of action, demonstrates high efficacy in vitro and in vivo. It could be further chemically optimized and tested for the treatment of TLRs-driven inflammatory diseases, where blocking of high levels of IFNβ induced by endosomal TLRs shown to be beneficial for the patients.

Keywords: Endosomal TLRs, IFNβ, SLAMF1, peptide

Summary



Graphical abstract

O-022
General Research » Model systems of innate immunity

BACTERIAL ADP-HEPTOSE INITIATES A REVIVAL STEM CELL PROGRAM IN THE INTESTINAL EPITHELIUM

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OBJECTIVES:The intestinal epithelium has an exceptional capacity to repair following injury, and recent evidence has suggested that YAP-dependent signaling was crucial for the expansion of *Clu*$^{+}$ revival stem cells (revSCs) with fetal-like characteristics, which are essential for epithelial regeneration. However, neither the mechanism underlying where these revSCs emerge from nor the nature of the physiological cues that induce this revSC program, are clearly identified.

METHODS:Methods include immunoblot, immunofluorescence, and single-cell RNA-sequencing on intestinal organoids.

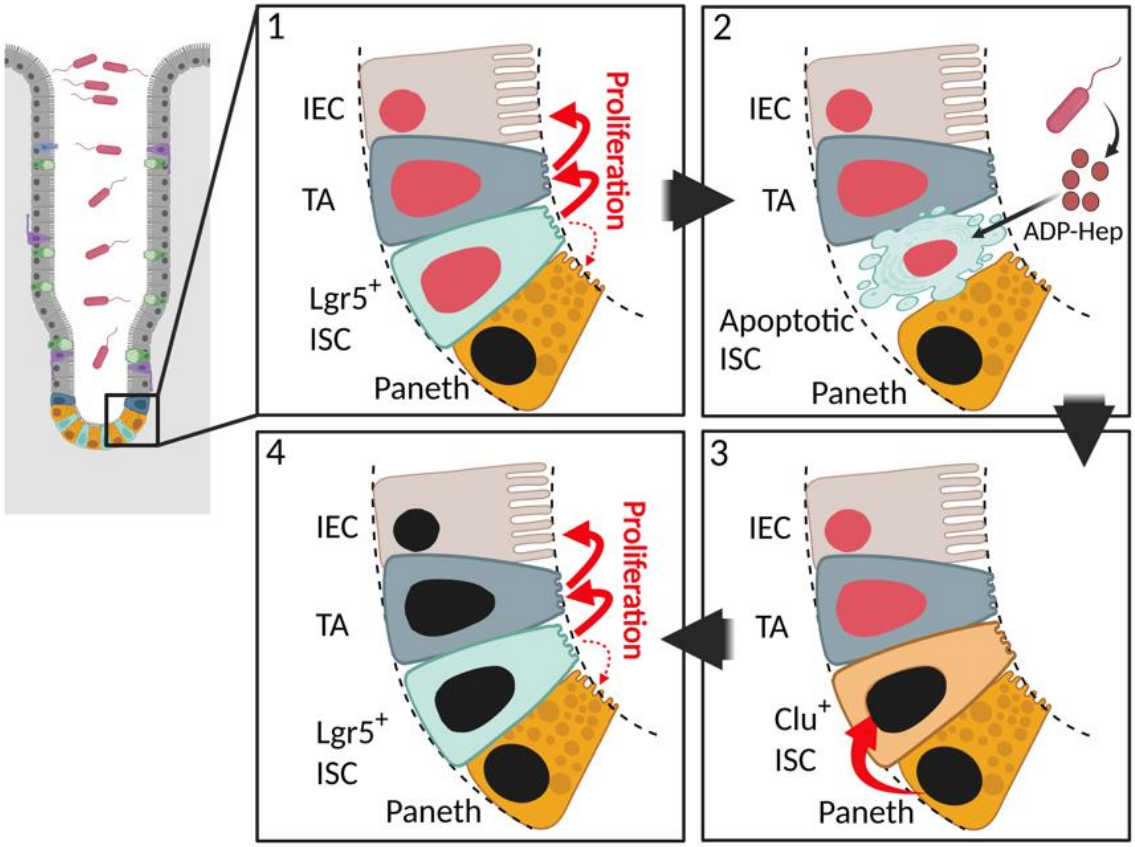
RESULTS:Here, we first demonstrate that *Alpk1* and *Tifa*, which encode the proteins essential for the detection of the bacterial metabolite ADP-heptose (ADP-Hep), were expressed by the stem cell pool in the intestinal epithelium. Treatment of intestinal organoids with ADP-Hep not only induced acute NF-κB pro-inflammatory signaling but also TNF-dependent apoptosis within the crypt, causing blunted proliferation and acute disruption of the crypt architecture, while also triggering induction of a revSC program. To identify the molecular underpinnings of this process, we performed single-cell RNA-seq analysis of ADP-Hep-treated organoids as well as lineage-tracing experiments. Our data reveal that ADP-Hep induced the specific ablation of the homeostatic intestinal stem cell (ISC) pool. Removal of ADP-Hep resulted in the rapid recovery of ISCs through dedifferentiation of Paneth cells, which transiently acquired revSC features and expressed nuclear YAP. Moreover, lineage tracing from *Lyz1*$^{+}$ Paneth cells showed that ADP-Hep triggered Paneth cell de-differentiation towards pluripotent and proliferative cells in organoids. *In vivo*, revSC emergence in response to irradiation-induced injury was severely blunted in *Tifa*-deficient mice, suggesting that efficient epithelial regeneration in this model required detection of microbiota-derived ADP-Hep by the ALPK1-TIFA pathway.

CONCLUSIONS:Together, our work reveals that Paneth cells can serve as the cell of origin for revSC induction in the physiological context of microbial stimulation, and that the transient loss

of *Alpk1*-expressing ISCs is the initiating event for this regenerative process.

Keywords: Alpk1, Tifa, ADP-Hep, intestinal stem cells, organoids

Graphical Summary



O-023
General Research » TLR Signaling

HIGH THROUGHOUT IDENTIFICATION AND CHARACTERIZATION OF FUNCTIONAL LNCRNAS IN INNATE IMMUNITY

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OBJECTIVES:Advances in deep sequencing technologies have revealed that the majority of the human genome is actively transcribed into RNA. Our lab is focused on characterizing the largest group of RNA produced from the genome named long noncoding RNA (lncRNAs) and their associated protein binding partners.

METHODS:To date only 3% of lncRNAs have been functionally validated. Here we performed two independent high-throughput CRISPRi screens to understand the role of lncRNAs in monocyte function and differentiation. The first was a reporter-based screen to identify lncRNAs that regulate TLR4-NFkB signaling in human monocytes and the second screen identified lncRNAs involved in monocyte to macrophage differentiation.

RESULTS:We successfully identified numerous novel non-coding and protein-coding genes that can positively or negatively regulate inflammation and differentiation. To understand the functional roles of lncRNAs in both processes, we chose to further study the lncRNA LOUP (lncRNA originating from upstream regulatory element of SPI1 [also known as PU.1]), as it emerged as a top hit in both screens. Not only does LOUP regulate its neighboring gene, the myeloid fate determining factor SPI1, thereby affecting monocyte to macrophage differentiation, but knockdown of LOUP leads to a broad upregulation of NFkB-targeted genes at baseline and upon TLR4-NFkB activation. LOUP also harbors three small open reading frames (sORFs) capable of being translated and are responsible for LOUP's ability to negatively regulate TLR4/NFkB signaling.

CONCLUSIONS:This work emphasizes the value of high-throughput screening to rapidly identify functional lncRNAs in the innate immune system.

Keywords: NFkB, Innate immunity, long noncoding RNAs, CRISPR, Pooled screens, TADs

POSTER ABSTRACTS

P-001

General Research » Adjuvants and vaccine development

IMMUNOMODULATION WITH CHEMICAL PRECISION: NOVEL SYNTHETIC PICOMOLAR TLR4 AGONISTS AS POTENTIAL IMMUNOTHERAPEUTICS AND ADJUVANTS

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OBJECTIVES:Pro-inflammatory signaling mediated by the TLR4/MD-2 complex plays a critical role in immediate protection against infectious challenge. Activation of TLR4 also enhances adaptive immunity, which is being exploited in the development of vaccine adjuvants through the use of minimally toxic TLR4 activating ligands. Although tremendous progress has been made in understanding the molecular basis of LPS-induced TLR4-mediated signaling, tailored TLR4 activation by molecularly defined homogeneous ligands remains underexplored.

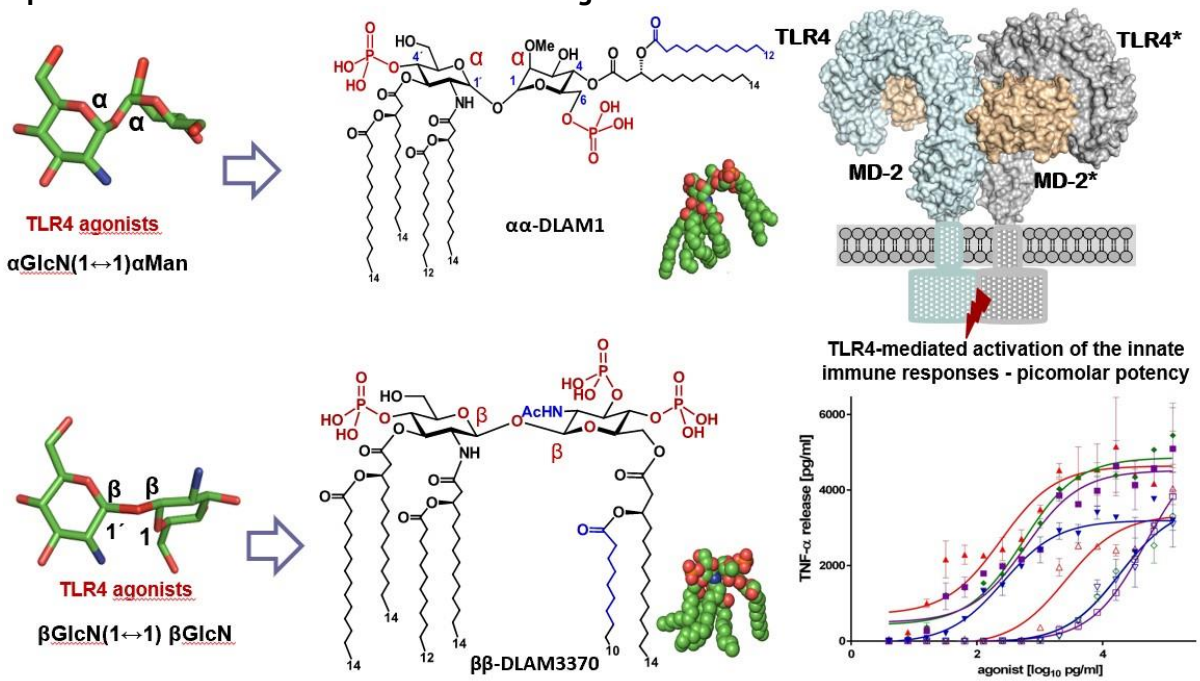
METHODS:glycochemistry, glycobiology, chemical synthesis, in-vitro screening consisting of: NFκB activation and cytokine production by reporter cell lines, cytokine production by primary human and murine immune cells, upregulation of surface costimulatory markers by primary monocyte-derived dendritic cells.

RESULTS:We report on the tailored modulation of cellular pro-inflammatory responses through the application of novel fully synthetic glycolipids - lipid A mimetics derived from an artificial sugar scaffold (DLAMs) with picomolar affinity for TLR4/MD-2. Using crystal structure-inspired design, we developed endotoxin mimetics in which the inherently flexible β(1→6)-linked diglucosamine backbone of lipid A is replaced by a conformationally restricted (1↔1)-linked disaccharide backbone with deviating tertiary structure. While the 3D-moleculer shape of the disaccharide backbone of DLAMs determines the biological activity (TLR4 agonist or antagonist), synthetic manipulation of the attachment sites of the phosphate groups and branched lipid chains to the sugar backbone governs the potency. The ability of DLAMs to potently activate human/mouse-TLR4-mediated responses in a species-independent manner was confirmed in TLR4/MD-2-transfected HEK293 cells, human airway epithelial cells, primary immune cells (hMNC, hDCs, mBMDM, etc.).

CONCLUSIONS:We developed a new class of fully synthetic (free of any biological impurities) TLR4-specific lipid A mimetics capable of inducing innate immune responses with pico-molar potency similar to that of ""*E.coli*"" LPS. Our library of lipid A mimetics derived from the artificial sugar scaffolds allows for tailor-made activation of TLR4-mediated signaling pathways, making DLAMs perfect candidates as vaccine adjuvants and immunotherapeutics.

Keywords: TLR4 signaling, lipopolysaccharide, glycolipid adjuvant, immunomodulation, lipid A mimetics

Lipid A mimetics derived from the nonreducing disaccharide scaffolds



P-003
General Research » Adjuvants and vaccine development

INDUCED LOSS OF TREX1 IN HOST CELLS INFLAMES THE TUMOR MICRO-MILIEU AND INVIGORATES CTL-MEDIATED CANCER CONTROL

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OBJECTIVES:Activation of local innate immunity in tumor tissue, aiming at induction of adaptive responses, is a major concept in cancer immunotherapy. The cGAS/STING pathway triggers type I IFN and pro-inflammatory cytokine responses upon detection of cytosolic DNA and can robustly enhance anti-tumor immunity. Identification of optimal strategies for therapeutic activation of cGAS/STING signalling is currently an important area of research. The DNase 3' repair exonuclease 1 (TREX1) is a central component of cellular DNA disposal preventing accumulation of endogenous DNA in the cytosol, activation of cGAS and cell-intrinsic innate immune responses. While constitutive TREX1-deficiency causes severe mendelian autoimmunity, temporary pharmacological inhibition of the enzyme may represent an efficient means to activate anti-tumor immunity.

METHODS:We inducibly inactivated the Trex1 gene in Trex1FL/FL R26 CreERT2 mice ('TREX1 iKO') bearing syngeneic tumors. Tumor growth was monitored and the intratumoral immune response was investigated by FACS and single cell RNA sequencing. We also combined TREX1 iKO with PD-1 blocking antibody.

RESULTS:Induced loss of TREX1 resulted in improved immune control of established syngeneic tumors that was type I IFN- and CD8+ T cell-dependent. This was associated with a dramatic increase of immune cell infiltration of tumor tissue while other (non-tumor) tissues were unaffected or showed only marginal inflammation. TREX1 iKO triggered an inflammatory switch of intra-tumoral monocyte, macrophage and dendritic cell populations associated with enhanced antigen presentation capacity. Invigoration of CD8+ T cell responses was reflected in robust increases of cells differentiating along the exhaustion trajectory but expressing significant signatures of effector function. Combination of TREX1 iKO with immune checkpoint inhibition synergistically promoted tumor control.

CONCLUSIONS:We conclude that activation of STING in host cells by pharmacological TREX1 inhibition is a promising therapeutic approach to enhance anti-tumor immunity.

Keywords: STING, cancer, adjuvant, TREX1

P-004
General Research » Adjuvants and vaccine development

A CHEMICALLY STABLE ADJUVANT HYPERACTIVATES DENDRITIC CELLS TO INITIATE DURABLE ADAPTIVE IMMUNITY

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OBJECTIVES:Adjuvants are key to any vaccination strategy. They stimulate dendritic cells (DCs) to initiate the adaptive immune system to mount antigen-specific immune responses. For effective adaptive immunity, immunostimulatory DCs must complete the following functions: 1) capture, process, and present antigens, 2) migrate to lymph nodes, 3) express co-stimulatory molecules required for T cell activation, 4) secrete inflammatory cytokines to promote effector responses, and 5) secrete interleukin-1beta (IL-1β) to promote memory formation. State of the art adjuvants induce some but not all these features effectively. CRN-1001, a combination of resiquimod and a stable, proprietary lipid developed for clinical use, was evaluated for inducing these five activities in DCs.

METHODS:Human monocyte-derived DCs and bone marrow-derived murine DCs were utilized to study the mechanistic effects of CRN-1001 on DC cell state and function in vitro. The inhibitor MCC950 was utilized to determine which phenotypes were dependent upon NLRP3 inflammasome activation. In vivo murine studies were subsequently carried out by subcutaneously injecting CRN-1001 with protein antigens. Cellular and humoral responses were then quantified.

RESULTS:CRN-1001 engages the TLR7/8 signaling pathway and the NLRP3 inflammasome in living DCs, hallmarks of a cell state known as hyperactivation. Hyperactivated DCs remain viable while secreting IL-1β. Unlike pyroptosis, hyperactivation enables IL-1β secretion and other immunostimulatory signals simultaneously, which is otherwise elusive. Additionally, CRN-1001-treatment enhances migratory behavior in vitro and in vivo. Mouse vaccination studies demonstrate that CRN-1001 primes both B cell and T cell responses to targeted antigens, an effect poorly elicited by other adjuvant strategies. In an influenza challenge, CRN-1001-vaccinated mice are protected from lethality.

CONCLUSIONS:CRN-1001 hyperactivates DCs, enabling them to carry out all five functions needed to prime durable B cell and T cell responses. CRN-1001 has the potential to be the first therapeutic to mechanistically target DC hyperactivation as an adjuvanting strategy.

Keywords: inflammasome, hyperactivation, dendritic cells, adjuvant, vaccine

P-005
General Research » Adjuvants and vaccine development

DESIGN AND PREPARATION OF TLR7 AGONIST ANTIBODY-CONJUGATES FOR ONCOLOGY AND IMMUNOLOGY APPLICATIONS

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OBJECTIVES:Our goal is to design antibody-drug conjugates (ADCs) that deliver potent and selective TLR7 agonists to antigen expressing cells. While the early focus of our work in oncology applications, we believe that this technology may be applicable to various immunological disorders.

METHODS:Building on a previous publication from our team, we systematically prepared a series of imidazoloquinoline molecules as potential TLR7 agonists. Lead agonists were screened in a variety of reporter cell assays (TLR7, TLR8, Ramos, THP1) and functional assays in primary immune cells (cytokine release). Several lead agonists were conjugated to B-cell targeting antibodies and tumor targeting antibodies. The resulting conjugates were tested in various mono-culture and co-culture assays intended to prove antigen-specific activation of TLR7. Repeat dose PK studies were performed in SD mice, using a standard ELISA quantitation.

RESULTS:Using HEK reporter cells, we demonstrate that our lead agonists have low nM activity against TLR7 while exhibiting >1000-fold lower activity against TLR8. Anti-CD38 conjugates of these agonists activate the NFκB pathway in B-cells and induce IFN-alpha release in PBMCs, while the corresponding isotype control ADCs (anti-HER2) do not. Further, we show that anti-HER2 TLR7 agonist ADCs activate mouse macrophages when co-culture with high-HER2 expressing cell lines, while the corresponding isotype control ADCs do not. The ADCs induce cytokine (TNF, IL6) release in PBMCs only when co-cultured with high-HER2 expressing cells. Finally, we assessed the PK of a set of anti-HER2 TLR7 agonist ADCs and found that the initial PK exposure varied depending on the nature of the TLR7 agonist chemistry. Interestingly, however, we found a rapid drop in exposure after the 2nd dose – indicative of an adaptive immune response.

CONCLUSIONS:We have designed an antibody-mediated delivery system for a series of potent TLR7 agonists. We believe that this technology has potential application in infectious disease, immunology, and oncology applications.

Keywords: TLR7, antibody conjugate, ADC, adjuvent

P-006
General Research » Adjuvants and vaccine development

ANTI-TUMOR AND NEOADJUVANT ACTIVITIES OF A NEW CLASS C TLR9 AGONIST IN MICE

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OBJECTIVES:We have recently shown that peritumoral (p.t.) administration of hydrogel encapsulated TLR9 agonists (including C-3) results in sustained release and tumor growth inhibition in multiple mouse models (Kandimalla et al, AACR Annual Meeting, 2021; China patent CN114099535B). The encapsulation of TLR9 agonist in hydrogels may permit less frequent dosing in patients and can be expanded to other therapeutic modalities of oligonucleotides. In the current study, we have tested the efficacy of C-3, our lead TLR9 agonist delivered p.t. with other hydrogels, and intratumorally (i.t.) as LNP, or as solution (unformulated).

METHODS:C-3 was tested for anti-tumor activity in the CT26 and MC38 models. It was also tested for its neoadjuvant activity in the highly metastatic 4T1-luc breast cancer orthotopic model.

RESULTS:C-3 induced high levels of IFN-α, IFN-γ, IL-12p70 and other Th1 cytokines in vivo in mice. In a CT26 tumor study, administration of a single injection of C-3 (250 μg) was sufficient to induce a complete response in a number of animals (33%) and long-term memory resulting in rejection of tumors implanted subsequently at a distant site (100%). In the abscopal MC38 study, C-3 delivered i.t. as LNP or in solution on days 1, 5 and 10 in the right flank resulted in tumor growth inhibition of injected as well as distant tumors. In the neoadjuvant 4T1 luc model, C-3 (250 μg) i.t. administration 4 days before resection of orthotopically implanted tumor resulted in strong anti-tumor and neoadjuvant activity as seen by decreases in mean flux values and tumor volumes on day 49. TLR9 target engagement was confirmed by cytokine induction 6 hours post dose.

CONCLUSIONS:Our TLR9 agonist C-3 can be used for immunooncology and its strong activity in multiple formulated systems makes it an ideal candidate for use as an adjuvant for prophylactic and therapeutic vaccines.

Keywords: TLR9, IFN-α, adjuvant, anti-tumor

P-008
General Research » Adjuvants and vaccine development

BACTERICIDAL/PERMEABILITY-INCREASING PROTEIN INSTRUCTS DENDRITIC CELLS TO ELICIT TH22 CELL RESPONSE

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OBJECTIVES:Neutrophil-derived bactericidal/permeability-increasing protein (BPI) is known to be bactericidal against Gram-negative bacteria. The elevation of BPI in bacterial, fungal and viral infections and its association with human monocyte-derived dendritic cells (DCs) indicate an unknown immune function.

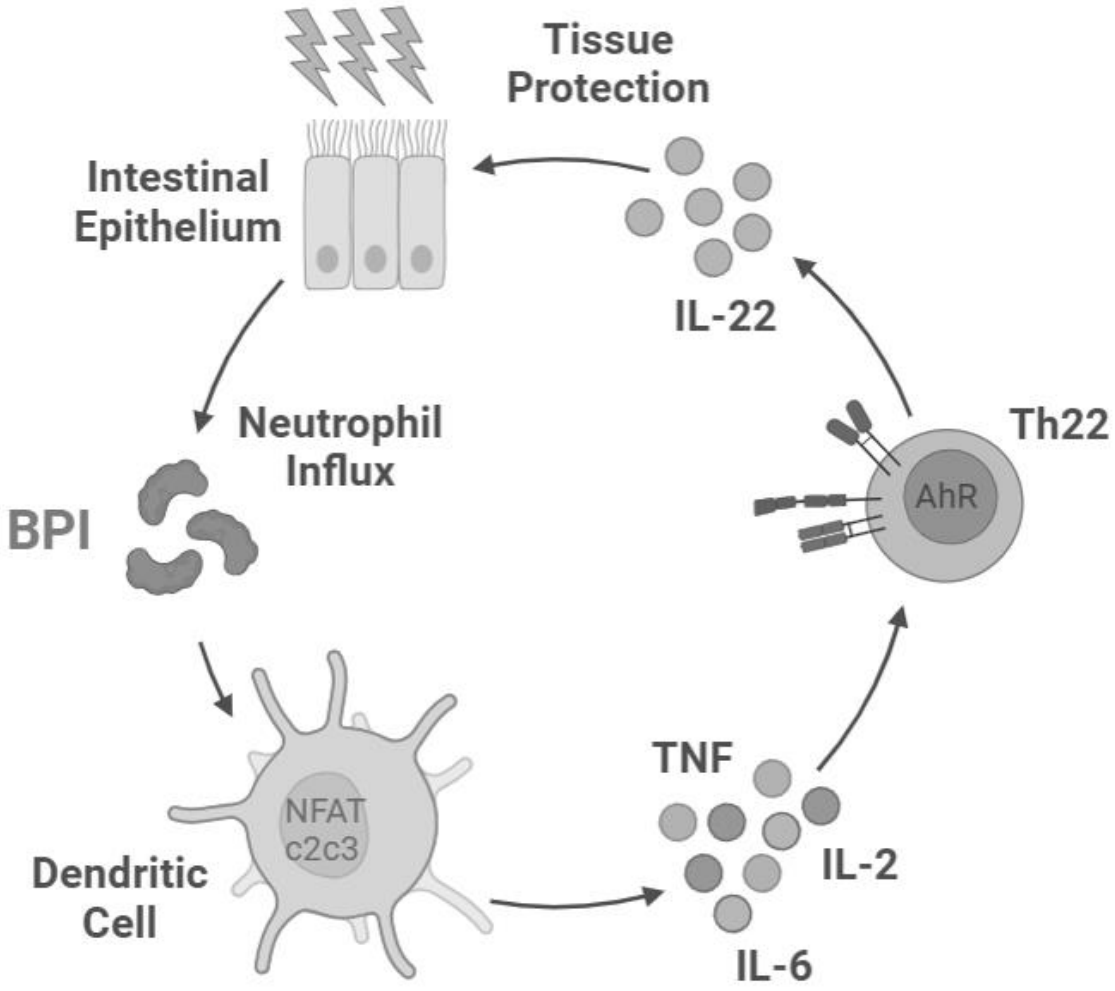
METHODS:GM-CSF-cultured bone marrow derived cells (BMDCs), conventional DCs and macrophages were stimulated with recombinant BPI. BPI-stimulated BMDCs were used to activate and differentiate CD4+ T cells. In vivo experiments were performed using BPI-deficient mice in a dextran sodium sulfate (DSS)-induced colitis model.

RESULTS:Here we define BPI as a selective activator of murine DCs. While conventional DCs respond to BPI with cytokine secretion, M-CSF cultivated or peritoneal lavage macrophages do not. BPI induces secretion of interleukin IL-2, IL-6 and TNF in BMDCs, consecutively activating CD4+ T cells to predominantly secrete IL-22 and, when naïve, to differentiate into Th22 cells. Congruent with the tissue protective properties of IL-22 and along with impaired IL-22 induction, disease severity is significantly increased during DSS-induced colitis in BPI-deficient mice.

CONCLUSIONS:In conclusion, BPI is a potential adjuvant to trigger Th22 cell differentiation with impact on intestinal homeostasis.

Keywords: bactericidal/permeability-increasing protein, dendritic cells, T cells, Th22, interleukin-2, interleukin-22

BPI – DC – Th22 Axis



Graphical Abstract

P-009

General Research » Adjuvants and vaccine development

AN INTRAOPERATIVE TLR3 LIGAND-RELEASING HYDROGEL PREVENTS CANCER RECURRENCE

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OBJECTIVES:Surgery remains the main treatment option for most solid cancers. However, recurrences are common and associated with a poor prognosis. Adjuvant systemic immunotherapy with checkpoint inhibitors has provided improvements in survival, but only in selected cancers and in a proportion of patients.

METHODS:We developed a surgically optimised hyaluronic acid-based hydrogel for sustained local delivery of innate immune agonists and mapped its in-vivo degradation kinetics. We assessed the safety, dose, and scheduling of low dose, local innate immune agonists and demonstrated the efficacy of hydrogel-loaded immunotherapy using mouse models of incomplete tumour resection. The underlying immunological mechanisms were characterised using flow cytometry, RNA sequencing and cytokine blocking studies. Finally, we assessed the safety and feasibility of the hydrogel in a veterinary clinical trial in canine patients undergoing surgical removal of soft tissue sarcoma.

RESULTS:The surgically optimised hydrogel could be easily applied in the wound bed after cancer surgery. We identified TLR3 agonist poly(I:C) as the optimal local immunotherapy, resulting in local and systemic anti-tumour immunity, when released over 2-3 weeks by the hydrogel, and improving surgical wound healing. The poly(I:C) hydrogel prevented tumour recurrence in multiple mouse models. Mechanistically, poly(I:C) induced a transient IFNα response that reshaped the tumour microenvironment (TME), attracting inflammatory monocytes and depleting Tregs from the TME. In addition, RNAseq analysis showed that a pre-existing IFN gene signature predicted response to the hydrogel, and we demonstrated that the hydrogel sensitised tumours to anti-PD-1 or anti-CTLA4. Finally, the hydrogel proved safe in canine cancer patients and was easy to use for the surgeon. Using KLH as a systemically tractable biomarker, we found the hydrogel induced a measurable antigen-specific systemic immune response.

CONCLUSIONS:The poly(I:C) hydrogel provides a safe and effective approach to prevent recurrence of solid tumours following surgery.

Keywords: TLR3, cancer immunotherapy, hydrogel, intraoperative delivery

P-0198

General Research » Adjuvants and vaccine development

ALPHA-KINASE 1 (ALPK1) AGONISTS STIMULATE POTENT ANTITUMOR IMMUNITY

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OBJECTIVES:Targeting innate immune pathways to stimulate inflammatory responses is an emerging strategy currently under clinical development for novel cancer immunotherapy. We previously identified a novel cytosolic innate immune receptor alpha-kinase 1 (ALPK1) that directly recognizes a bacterial metabolite ADP-heptose essential for LPS biosynthesis. ADP-heptose-activated ALPK1 phosphorylates TRAF-interacting protein with forkhead-associated domain (TIFA), thereby stimulating the NF-κB signaling and proinflammatory cytokine production. This study aimed to investigate the effect of ALPK1 agonists on cancer therapy and to unravel the molecular mechanisms driving their therapeutic effects.

METHODS:In vitro cell culture models and in vivo animal studies were uesd. ALPK1 agonists were administered alone and in combination with established cancer therapies. Kinase assay, luciferase assay, animal tumor growth studies and immune cell profiling were performed to evaluate the therapeutic potential and mechanisms of ALPK1 agonists.

RESULTS:Using the cellular assay system, we tested a series of synthetic ADP-heptose analogs, and identified UDSP-heptose as a more potent and stable ALPK1 agonist. In several commonly used syngeneic mouse models, administration of UDSP-heptose resulted in evident tumor growth inhibition or complete tumor clearance through induction of effective antitumor immune responses. Features of the antitumor immunity were investigated by scRNA-seq analyses, followed by profiling the cytokines and immune cell population changes in the tumor microenvironment in UDSP-heptose-treated mice. In certain “cold” tumor models, ALPK1 agonist exhibited marked synergistic effects with immune checkpoint inhibitors, such as PD-L1 or CTLA4-blocking antibodies. The antitumor immunity triggered by the ALPK1 agonist genetically required Alpk1 and was dependent upon antigen presentation as well as a key cytokine MCP-1 downstream of the canonical NF-κB signaling.

CONCLUSIONS:The ALPK1 innate immune signaling has a functional role of in tumor immunity. ALPK1 agonists represent a promising strategy for developing the next-generation cancer immunotherapy or improving the efficacy of the existing checkpoint inhibitor therapy.

Keywords: ALPK1, TIFA, ADP-heptose, NF-kB pathway, tumor immunotherapy, immunoadjuvants

P-010
General Research » Antiviral Defense

THE IMMUNE ADAPTOR TASL IS ESSENTIAL FOR TLR7/9-DEPENDENT IMMUNE RESPONSES *IN VIVO*

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OBJECTIVES:Endosomal nucleic acid sensing by TLR7-9 play a central role in antimicrobial immunity and in several autoimmune conditions such as systemic lupus erythematosus (SLE). We recently identified a novel innate adaptor, TASL, which is critically involved in TLR7/9-induced responses. Mechanistically, TASL forms a complex with the endosomal solute carrier SLC15A4 and, upon stimulation, mediates the activation of the transcription factor IRF5 and the downstream production of type I IFN and proinflammatory cytokines. Notably, all the components of this signaling axis have been associated to SLE in genome wide association studies. Here, we address the pathophysiological role of TASL *in vivo*.

METHODS:Newly generated *Tasl* knockout mice were characterized side-by-side with *Slc15a4*-deficient (*feeble*) mice. Responses to TLR7/9 stimulation were assessed in primary cells *ex vivo* as well as upon agonist challenge *in vivo*. The impact of *Slc15a4* and *Tasl* deficiency on antiviral and autoimmune responses was investigated upon chronic LCMV infection and in the pristane-induced SLE model respectively.

RESULTS:Here we show that IRF5 activation and cytokine production upon TLR7/9 triggering are impaired in primary immune cells from both *Slc15a4*-deficient and *Tasl* knockout mice. *In vivo*, *Slc15a4* and *Tasl* knockouts show a profound defect in type I IFN and proinflammatory cytokine production upon stimulation with endosomal TLR agonists. Accordingly, *Slc15a4*- and *Tasl*-deficient mice displayed reduced antiviral responses upon LCMV infection. Importantly, knockout mice were protected from disease development in the pristane-induced SLE model.

CONCLUSIONS:Altogether, this study demonstrates the critical pathophysiological role of SLC15A4 and TASL for TLR7 and TLR9-driven inflammatory responses *in vivo*, further supporting the therapeutic potential of targeting this complex in SLE and related diseases.

Keywords: Endosomal TLR, SLC15A4, TASL, IRF5, SLE

P-011
General Research » Antiviral Defense

EVASION OF HUMAN ANTIVIRAL INNATE IMMUNITY BY MONKEYPOX VIRUS

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OBJECTIVES:Monkeypox virus (MPXV) is a rodent zoonotic orthopoxvirus (OPXV) that has recently emerged with unprecedented human-to-human transmission. Global MPXV (lineage B) is phylogenetically linked to West African endemic strains with much lower transmissibility (lineage A), suggesting increased human adaptation of lineage B. Innate immunity exerts strong selective pressure during viral transmission. We hypothesised that distinctive features in the modulation of human innate immunity define MPXV (relative to other OPXV) as well as endemic and globally circulating lineages, arguably influencing their pandemic potential.

METHODS:We used a comparative virology approach involving signal transduction, RNA sequencing and viral replication assays to measure the ability of MPXV lineages and related OPXV to modulate human innate defences.

RESULTS:We show that both endemic and global MPXV effectively suppressed human antiviral innate immunity, with a major role for the viral 2'3'-cGAMP nuclease (poxin) in preventing IRF3 and IFN activation. Transcriptome analysis revealed a unique inflammatory response in MPXV-infected fibroblasts that was not observed with other OPXV nor in THP-1-differentiated macrophages. Notably, MPXV lineages triggered differential expression of selected cytokines including CCL5 and IFNβ, although this was not accompanied by global IFN-stimulated gene (ISG) induction, further demonstrating innate evasion. Despite its rodent origin and in contrast to rodent-restricted OPXV, both MPXV lineages showed significant capacity to replicate in IFN-treated human cells, although they were restricted to certain extent, offering a window for therapeutic intervention. In line with this, treatment with diABZI, a non-cyclic dinucleotide STING agonist impervious to poxin activity, completely suppressed MPXV replication in a STING- and IFN-dependent manner.

CONCLUSIONS:Our work (i) demonstrates conserved and unique features in innate immune evasion by circulating MPXV lineages, highlighting the potential of endemic MPXV lineages to irrupt globally in the absence of universal anti-OPXV herd immunity; and (ii) identifies strategies for novel host-directed therapeutics against Mpox.

Keywords: Monkeypox virus, immune evasion, inflammation, interferon, STING, diABZI.

P-012
General Research » Antiviral Defense

LNCRNA *IRF1-AS1* REGULATES EXPRESSION AND FUNCTION OF INNATE TRANSCRIPTION FACTOR IRF1 IN HUMAN AND MURINE MACROPHAGES

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OBJECTIVES:Effective viral clearance requires tight control of transcriptional programs directing the expression of antiviral effector genes. IRF1 is a key transcriptional regulator of antiviral immunity through coordinated regulation of interferon-stimulated genes. Here, we describe the proximal antisense lncRNA *IRF1-AS1* as an additional layer of regulation of IRF1 and its downstream gene program.

METHODS:To identify cis-regulatory lncRNAs that control expression of proximal coding genes induced by viral infection, we implemented an unbiased approach to identify candidate lncRNA–mRNA pairs (localized within a genomic distance of 5 kb) that are differentially expressed in the circulation of infected individuals versus healthy controls. Using loss of function approaches we tested whether the loss of the expression of *IRF1-AS1*, or its mouse ortholog *Gm12216*, alters IRF1/Irf1 expression and its transcriptional activity. To test whether *Gm12216* plays a role in antiviral immunity, we used CRISPR/Cas9 technology to generate a *Gm12216*-knockout mouse, and infected *Gm12216*−/− and WT mice with a sublethal dose of influenza A/PR8/34 virus via intranasal inoculation.

RESULTS:*IRF1-AS1* and *IRF1* are upregulated in the circulation of patients infected with human metapneumovirus, influenza A virus or SARS-CoV2. In macrophages, expression of *IRF1-AS1* and *Gm12216* is increased in response to viral infection or TLR3 agonist treatment. Depletion of *IRF1-AS1/Gm12216* reduced expression of its antisense gene *IRF1/Irf1*, as well as IRF1 target genes, resulting in increased viral susceptibility. Consistently, *Gm12216*-deficient mice exhibit a reduced antiviral immune response upon sublethal challenge with influenza A virus.

CONCLUSIONS:Together, these findings identify a role for the conserved lncRNA *IRF1-AS1* in regulating antiviral immunity by coordinating the function of its antisense gene *IRF1*.

Keywords: Long noncoding RNA, interferon stimulated genes, antiviral immunity, innate immune signaling.

P-013
General Research » Antiviral Defense

METABOLITES INVOKE SUBPROGRAMS OF THE INTERFERON RESPONSE TO ADAPT ANTIVIRAL DEFENSES

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OBJECTIVES:Variation in infection outcomes within and between individuals has been associated with the activation of innate immunity but the mechanisms remain ill-defined. Interferon (IFN)-stimulated genes (ISGs) are upregulated early in infection to activate intrinsic and cell-mediated defenses.

METHODS:Unexpectedly, we find widespread heterogeneity in tonic ISG levels across human tissues and individuals independent of IFN and IFN receptor expression. Using a cell culture model to explore the basis of ISG heterogeneity including functional relevance, we uncover that certain induced ISGs like IRF1 and IFITM3 are differentially regulated by metabolites such as glucose at the protein level in type I and type II IFN-primed human cells.

RESULTS:Mechanistically, metabolites trigger selective protein degradation of IRF1 in media conditions that enhance mitochondrial activity; namely, galactose supplementation. Notably, glucose-rich conditions unlock IFN-g efficacy to attenuate herpes- and poxvirus by one hundred-fold relative to cells in untreated glucose conditions. In contrast, cells in galactose media display no differences between untreated and IFN-primed conditions in herpes- and poxvirus replication. However, overall herpes- and poxvirus replication is decreased in untreated cells grown in galactose media relative to glucose-rich media. Deletion and rescue experiments show that IRF1 is an essential vaccinia virus restriction factor in glucose/IFN-g conditions. Furthermore, we demonstrate that glucose media enhancement of IFN-g induced IRF1 protein levels is conserved for nearly one hundred million years of evolution.

CONCLUSIONS:These data reveal unappreciated ISG subprograms that, in principle, could rapidly adapt immune responses by sensing changing metabolite levels consumed during viral replication and cell proliferation.

Keywords: adaptation, ISG subprograms, IRF1, vaccinia, antiviral, glucose

P-014
General Research » Antiviral Defense

IFI44: LINKING INTERFERON SIGNALLING AND RED BLOOD CELL HOMEOSTASIS?

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OBJECTIVES:IFI44 is a prototypical interferon-stimulated gene (ISG), conserved from molluscs to humans. IFI44 is highly expressed in most cell types in response to pathogen infection, in patients with type I interferonopathies and is used as a biomarker for systemic lupus erythematosus. Despite this, its molecular function remains elusive. We aimed to uncover the precise role that IFI44 plays in innate immunity.

METHODS:Ifi44 knock-out mice were generated. Poly(A) RNA-sequencing of murine spleens identified differential gene expression between Ifi44-/- and Ifi44+/+ mice, which was validated using RT-qPCR. To investigate splenic red pulp macrophage (RPM) populations, splenocyte suspensions were generated by enzymatic digestion of the spleen and cell surface markers were analysed by flow cytometry. To determine the presence of abnormalities in erythrocytes, haematological analysis of the blood was performed using a Pentra ES 60 machine. Abundance of erythroid precursors in the bone marrow were investigated by flow cytometry.

RESULTS:In Ifi44-/- splenocytes, genes involved in the heme biosynthetic pathway (e.g. Urod, Uros and Sptb) and genes expressed in RPMs (e.g. Hmox1, CD163 and Slc40a1) were downregulated. RPM populations were less abundant in Ifi44-/- mice than Ifi44+/+ mice, and the RPMs present in Ifi44-/- mice expressed less of the heme scavenger receptor CD163. Ifi44-/- mice displayed a mild but reproducible microcytic anaemia; however, their erythroid precursor populations remained indistinguishable from those of Ifi44+/+ mice.

CONCLUSIONS:We showed in vivo that mice lacking IFI44 displayed a mild microcytic anaemia, coupled with a reduction in RPM populations, which had significantly less CD163 on their cell surface. As CD163 is a heme scavenger, this indicated that these RPMs were less efficient at recycling iron. We postulate that IFI44 is important for RPM maintenance and function, and when IFI44 is not present, this may lead to a dysregulation of iron recycling, and subsequent microcytic anaemia.

Keywords: Interferon, Interferon Stimulated Gene, ISG, Macrophages, Heme, Iron

P-015
General Research » Antiviral Defense

ANTIVIRAL DEFENSE RESPONSES OF THE OLFACTORY BULB ARE PRIMED BY TIME OF DAY OF INTRANASAL POLY(I:C)

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OBJECTIVES:Brain antiviral defense mechanisms are critical for survival, yet incompletely understood. Since time of day alters severity of neurotropic (brain-targeting) virus infection, we used time of day as a tool to uncover brain antiviral defense mechanisms. We have shown that the olfactory bulb (OB), a site of neurotropic virus entry into the brain, rhythmically expresses neuroinflammation-related transcripts enriched in genes associated with functional aspects of microglia, which are necessary for the defense against neurotropic virus infections. We also find that antiviral-related transcripts are upregulated at active phase onset, a time of enhanced survival following neurotropic virus infection. Thus, we hypothesized that time of day primes the OB to differentially respond to an intranasal virus-like challenge. Here, our objective was to determine how time of day of intranasal poly(I:C) influences antiviral responses of the OB at both tissue and cellular levels.

METHODS:We intranasally challenged mice at resting phase onset (ZT0) or active phase onset (ZT12) with vehicle or poly(I:C) and collected tissues at 0-, 3-, 12-, and 24-hours post-inoculation. OB transcriptional responses were measured using NanoString technology. We next intranasally challenged mice with vehicle or poly(I:C) at ZT0 or ZT12. We then isolated OB microglia at 24 hours post-inoculation and used imaging flow cytometry to analyze a population of cells characteristic of microglia.

RESULTS:We found intranasal poly(I:C) induced antiviral responses in the OB that unfolded more rapidly in mice challenged at ZT12 compared to ZT0. Surprisingly, we also observed a high proportion of microglia that contained intrinsically fluorescent puncta, whose numbers were reduced following intranasal poly(I:C) at ZT12 but not affected following intranasal poly(I:C) at ZT0.

CONCLUSIONS:Time of day primes the OB to mount differential antiviral and microglial responses to intranasal virus-like stimuli, which may provide an antiviral gating mechanism underlying differential susceptibility to neurotropic virus exposure via the nasal route.

Keywords: microglia, neuroimmune, neuroinflammation, circadian, interferon signaling

P-016
General Research » Antiviral Defense

IFI16 SUPPRESSES SARS-COV-2 REPLICATION AND IS TARGETED BY THE VIRUS FOR DEGRADATION

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OBJECTIVES:The innate immune response is crucial for defence against viral infections, with human PYHIN proteins, including IFI16, PYHIN1, AIM2, and MNDA, playing key but as yet not fully understood roles. IFI16 and AIM2 are known to be pathogen DNA sensors in the cytosol. This study aimed to investigate whether PYHIN proteins, particularly IFI16, impacted on SARS-CoV-2 replication and if so to understand the mechanisms involved.

METHODS:Ectopic expression of PYHIN proteins was examined for its impact on SARS-CoV-2 replication using plaque assays and western blot analysis of viral nucleoprotein. Further investigations focused on the effect of SARS-CoV-2 infection on IFI16 protein levels in epithelial cells. The role of viral proteins in modulating IFI16 expression was explored, and the impact of ORF8 on various PYHIN proteins was assessed. Mechanistic insights into ORF8-mediated reduction of IFI16 were obtained through cycloheximide chase experiments and treatment with the proteasome inhibitor MG132.

RESULTS:Ectopic expression of IFI16 was found to suppress SARS-CoV-2 replication, suggesting a direct viral restriction role for IFI16. Interestingly, during SARS-CoV-2 infection of epithelial cells, IFI16 protein levels decreased, suggesting a targeting of IFI16 by the virus. ORF8 expression alone exhibited a concentration-dependent reduction in IFI16 levels. ORF8 targeted IFI16 and mouse PYHIN p204, but not other tested proteins. ORF8 reduced the half-life of IFI16 and degraded IFI16 in a proteasomal-dependent manner.

CONCLUSIONS:This study suggests that IFI16 is a restriction factor for SARS-CoV-2, as has been shown for other RNA viruses, further extending its role in anti-viral defence beyond DNA sensing. The study also unveils a novel immune evasion strategy employed by SARS-CoV-2 through ORF8 protein- mediated degradation of IFI16. The findings emphasize the intricate interplay between viral proteins and host defence mechanisms, providing valuable insights for potential therapeutic interventions against viral infections.

Keywords: Viral restriction, Viral immune evasion, PYHIN proteins, IFI16, SARS-CoV-2, ORF8

P-017
General Research » Antiviral Defense

INDEL MUTATIONS ALLOW THE EVOLUTION OF NEW ANTIVIRAL FUNCTIONS NOT ACCESSIBLE BY MISSENSE MUTATION

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OBJECTIVES:Unlike most of the vertebrate genome, antiviral proteins evolve rapidly by accumulating a variety of mutations in their virus-recognition surfaces. Missense mutations have been demonstrated to dramatically alter the specificity of virus recognition in numerous antiviral proteins. Here, we investigate the unknown adaptive benefit of in-frame indel mutations, which have been recurrently sampled during the natural evolution of antiviral proteins.

METHODS:We apply a high-throughput pooled screening approach to test the antiviral function of many variants of human TRIM5alpha, an antiviral protein that inhibits retroviruses. This approach relies on the stable expression of variant libraries in cells, challenging these cells with a GFP-marked retrovirus, and sorting of GFP-negative cells to identify potently antiviral TRIM5 variants by deep sequencing.

RESULTS:We identify a retrovirus, a simian immunodeficiency virus infecting African green monkeys (SIVsab), which cannot be inhibited by any single missense variant of human TRIM5alpha. Indeed, we identify a minimal evolutionary path that requires at least 5 missense mutations in TRIM5alpha for SIVsab restriction. In contrast, we find that an indel mutations are sufficient to confer this novel antiviral function. Most notably, duplication of a single amino acid residue (F339) confers human TRIM5alpha with the strongest SIVsab restriction we can identify. We also demonstrate that naturally occurring amino acid insertions are both necessary and sufficient for TRIM5alpha to acquire new antiviral functions.

CONCLUSIONS:Our work demonstrates that indel mutations are a critical and under-appreciated source of evolutionary innovation. Such mutations can allow an antiviral protein to acquire new antivirial functions, which would otherwise require multiple missense mutations that are not individually adaptive, in a single evolutionary leap.

Keywords: TRIM5, lentivirus, restriction, evolution

P-018
General Research » Antiviral Defense

THE NLRP1 INFLAMMASOME – A NOVEL PLAYER IN THE PHLEBOVIRUS-HOST ARMS RACE

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OBJECTIVES:We and others found that arthropod-borne alphaviruses activate the NLRP1 inflammasome in human keratinocytes and consequently trigger an inflammatory response in the skin (Bauernfried et al. 2020, Jenster et al. 2023). Phleboviruses (order Bunyavirales), a distinct group of RNA viruses, are globally emerging arboviruses that cause a wide spectrum of disease. Rift Valley fever virus (RVFV), for example, causes fever, hepatitis, encephalitis, or hemorrhagic fever, i.e. pathologies associated with a strong inflammatory response. However, little is known about the local anti-phleboviral immune response in the skin.

METHODS:We thus infected human keratinocytes with different strains of RVFV and examined the innate inflammasome and interferon responses.

RESULTS:Upon infection with a recombinant RVFV strain expressing the fluorescent reporter Katushka instead of the virulence factor NSs (RVFV-Katushka), keratinocytes assembled inflammasomes and released the downstream pro-inflammatory cytokine IL-1β. Both depended on NLRP1 and its downstream adapter protein ASC, and additionally required active viral replication. RVFV-infected cells displayed robust p38 MAP kinase activation and characteristics of the ribotoxic stress response (RSR), with NLRP1 activation being driven partially by the RSR kinase ZAKα and dependent on p38. Of note, recombinant RVFV-Katushka lacks the virulence factor NSs, a strong inhibitor of the interferon system. Infection with NSs-expressing RVFV, in contrast, not only failed to induce inflammasome assembly, but also inhibited NLRP1 activation with anisomycin. Our data suggests that RVFV NSs additionally evolved as potent and specific NLRP1 antagonist.

CONCLUSIONS:In summary, RVFV infection drives p38-dependent NLRP1 inflammasome activation in human keratinocytes. The subsequent release of mediators like IL-1β may thus shape the inflammatory environment – and thereby the immune response at the initial site of RVFV transmission. Consistently, the virulence factor NSs inhibits not only the interferon response but also NLRP1 inflammasome assembly, supporting an anti-phleboviral role of this innate immune pathway.

Keywords: NLRP1, inflammasome, antiviral immunity, host-pathogen interaction

P-019
General Research » Antiviral Defense

THE EFFECT OF BCG VACCINATION AND LATENT INFECTIONS ON CLINICAL PROGRESSION OF COVID-19

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OBJECTIVES:The *Bacillus Calmette–Guérin* (BCG) vaccine is known for its ability to provide off-target heterologous protection against non-mycobacterial infections. These effects are proposed to be mediated by BCG-induced immunomodulation, including functional reprogramming of the innate immune system, termed "trained immunity". Similarly, latent infections such as *Toxoplasma gondii* and cytomegalovirus (CMV) have proven to improve the response of innate immune cells to unspecific pathogens. Here we study whether previous BCG vaccination or latent infections can provide protection against severe bacterial or SARS-CoV-2 pneumonia.

METHODS:To demonstrate the possible association between previous BCG vaccination/latent infections and pneumonia severity, we performed a retrospective study on individuals who survived SARS-CoV-2 infections. Serum titers of anti-*Toxoplasma*/BCG /CMV IgG in plasma of fully recovered patients that have a history of mild, severe and critical pneumonia caused by SARS-CoV-2 were assessed by ELISA, and correlated to the severity of COVID-19. Furthermore, we tested the response of peripheral blood monocytes isolated from COVID-19 convalescent patients to re-stimulation with SARS-CoV-2 and LPS using gene expression analysis and

cytokine detection (LEGENDplex) in order to determine monocyte response to secondary challenge.

RESULTS:We observed higher CMV- and *Toxoplasma*- positivity and lower anti-BCG IgG levels in group patients recovered from severe and critical COVID-19.

CONCLUSIONS:Correlation of the outcome of bacterial and SARS-CoV-2 pneumonia severity with the titers of IgG against CMV, BCG and *T. gondii* and monocyte activation status may provide and simple tool to stratify the population at risk.

Keywords: BCG, pneumonia, SARS-CoV-2, latent infection, monocytes

P-020
General Research » Antiviral Defense

HOW HUMAN MACROPHAGES SENSE NEIGHBOURING CELL DANGER DURING VIRAL INFECTION TO DRIVE PRO-INFLAMMATORY AND ANTI-VIRAL RESPONSES

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OBJECTIVES:Macrophages are key cells of the innate immune system that sense and respond to signs of infection and danger. Macrophages can directly sense an invading micro-organism, such as influenza A virus (IAV), to switch on pro-inflammatory (e.g., interleukin 6; *Il6*) and anti-viral (e.g., interferon; *Ifnb1*) gene expression. As sentinel innate immune cells, macrophages can also detect neighbouring cell infection and damage. IAV infection triggers robust, lytic epithelial cell death, accompanied by the release of immunostimulatory molecules derived from either the virus or the host into the extracellular space.

METHODS:Here, we investigated how human macrophages sense these IAV-infected, dying human bronchial epithelial cells and how these immunostimulatory molecules shape the ensuing inflammatory response using supernatant transfer and co-culture models.

RESULTS:We observed that macrophages only triggered anti-viral *Ifnb1* expression when they were directly infected with replicating IAV; as an IAV replication inhibitor, baloxavir marboxil blocked *Ifnb1* and interferon stimulated gene induction. In contrast, IL-1β released from dying, IAV-infected epithelial supernatants induced macrophage pro-inflammatory *Il6* and *Il1b* gene expression, as the IL-1 antagonist anakinra completely ablated macrophage *Il6* and *Il1b* mRNA induction. In agreement with this, we observe that IL-1 released from epithelial cells activated with the NLRP1 activator talabostat also induces macrophage *Il6* and *Il1b* mRNA induction.

CONCLUSIONS:Our results suggest that IL-1β is the critical amplifier of inflammation during IAV infection and inflammasome-dependent epithelial cell death, rather than other epithelial derived PAMPs or DAMPs. Further, our data indicates that epithelial but non-macrophage tropic viruses may selectively trigger detrimental macrophage-driven inflammation without concomitant protective anti-viral responses.

Keywords: macrophage, influenza, IL-1, cell death, epithelial

P-021
General Research » Antiviral Defense

IMPAIRED EARLY INTERFERON RESPONSE ENABLES HYPER-ACTIVATION OF NF-KB IN MICROGLIA TO DRIVE IMMUNOPATHOLOGY IN HERPES SIMPLEX ENCEPHALITIS

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OBJECTIVES:Herpes Simplex encephalitis (HSE) is a devastating disease caused by HSV-1, with a high level of mortality and morbidity despite antiviral treatment with acyclovir. In humans, defects in the type I interferon (IFN) pathway predisposes to HSE, including the interferon regulatory factor 3 (IRF3) R285Q mutation. However, the molecular and cellular events leading to development of HSE in susceptible individuals remain unknown.

METHODS:To uncover central mechanisms in the pathogenesis of HSE we generated a transgenic mouse model carrying a R278Q mutation, orthologous to the human R285Q mutation, and compared key data to iPSC-derived brain cells from a pediatric HSE patient heterozygous for the IRF3 R285Q mutation.

RESULTS:Microglia, astrocytes, and bone marrow-derived macrophages from IRF3 R278Q mice exhibited impaired IFN-I response to HSV-1 infection, similar to iPSC-derived microglia generated from the HSE patient. Importantly, both heterozygous and homozygous IRF3 R278Q mice showed accelerated HSE-like disease development upon HSV-1 infection, suggesting that the mice phenocopy the patient. Compared to wildtypes, the IRF3 R278Q mice show impaired IRF3-mediated antiviral control in the periphery leading to higher viral inoculum into the CNS, where viral replication was further enhanced. This led to elevated expression of NF-κB-driven inflammatory chemokines and cytokines in the CNS, and subsequently directed infiltration of inflammatory monocytes, as well as development of hyperinflammatory subsets in the CNS. Blocking NF-κB activation or the downstream chemoattractant CCL2, alleviated disease severity, and reduced risk of death. Collectively, we show that impaired early IFN-response leads to elevated viral load in the CNS, thus inducing hyper-activation of NF-κB in microglia to drive immunopathology in HSE.

CONCLUSIONS:Our work provides significant new understanding of the mechanisms driving HSE. Importantly, it highlights that curbing the pathological inflammatory mechanisms, while leaving the antiviral mechanisms untouched, represents a promising treatment of HSE in combination with antiviral therapy

Keywords: Herpes Simplex Encephalitis, Interferon response, Model systems of innate immunity, Mouse model, Antiviral defense

P-022
General Research » Antiviral Defense

DEUBIQUITINATING ACTIVITY OF SARS-COV-2 PAPAIN-LIKE PROTEASE DOES NOT INFLUENCE VIRUS REPLICATION OR INNATE IMMUNE RESPONSES *IN VIVO*

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OBJECTIVES:The coronavirus papain-like protease (PLpro) is crucial for viral replicase polyprotein processing. Additionally, PLpro can subvert host defense mechanisms by its deubiquitinating (DUB) and deISGylating activities. Our aim was to elucidate the role of these activities during SARS-CoV-2 infection.

METHODS:To separate the DUB and deISGylating activities from viral replicase polyprotein processing, we introduced mutations that disrupt binding of PLpro to ubiquitin or ISG15. Thereby, the DUB and deISGylating activities can specifically removed without affecting the protease activity that is essential for virus replication.

RESULTS:Using *in vitro* assays, we identified several mutations that strongly reduced DUB activity of PLpro, without affecting viral polyprotein processing. In contrast, mutations that abrogated deISGylating activity also hampered viral polyprotein processing and when introduced into the virus these mutants were not viable. SARS-CoV-2 mutants exhibiting reduced DUB activity elicited a stronger interferon response in human lung cells. In a mouse model of severe disease, disruption of PLpro DUB activity did not affect lethality, virus replication, or innate immune responses in the lungs. This suggests that the DUB activity of SARS-CoV-2 PLpro is dispensable for virus replication and does not affect innate immune responses in vivo. Interestingly, the DUB mutant of SARS-CoV replicated to slightly lower titers in mice and elicited a diminished immune response early in infection, although lethality was unaffected. We previously showed that a MERS-CoV mutant deficient in DUB and deISGylating activity was strongly attenuated in mice.

CONCLUSIONS:Here, we demonstrate that the role of PLpro DUB activity during infection can vary considerably between highly pathogenic coronaviruses. Therefore, careful considerations should be taken when developing pan-coronavirus antiviral strategies targeting PLpro.

Keywords: COVID-19, SARS-CoV-2, PLpro, ubiquitin, ISG15, antiviral innate immune response

P-023
General Research » Antiviral Defense

IDENTIFICATION OF MURINE 2‘-5‘-OLIGOADENYLATE SYNTHETASE-LIKE PROTEIN 2 (MOASL2) AS A NOVEL RESTRICTION FACTOR OF MURINE GAMMAHERPESVIRUS-68 (MHV68)

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OBJECTIVES:The interferon-stimulated gene products murine 2‘-5‘-oligoadenylate synthetase-like proteins 1 (mOASL1) and mOASL2 belong, together with cGAS, to the OAS protein family. During DNA virus infections, mOASL2 can exhibit two different roles: like human OASL, it inhibits cGAS and the downstream type I IFN response, thus acting proviral. However, mOASL2 also acts antiviral by producing the second messenger 2’- 5’-oligoadenylate upon binding to dsRNA, which binds to and activates RNase L, leading to the degradation of host and viral RNA.

METHODS:Due to these multifaceted functions, we investigated the role of mOASL2 during infection with the DNA virus murine herpesvirus-68 (MHV68), which is a frequently used mouse model system to study Epstein-Barr virus pathogenesis, in vitro and in vivo.

RESULTS:We can show that mOASL2 acts antiviral in the infected host, with mOASL2 knockout (KO) mice exhibiting significantly higher MHV68 titers in lungs and spleens 3 days post infection than wildtype mice. By using immortalized and primary bone marrow-derived mOASL2 KO macrophages (BMDM), we further show that mOASL2-mediated restriction is only partially dependent on its catalytic activity required for RNase L activation. Additionally, we will present newly identified cellular interaction partners of mOASL2 in MHV68-infected macrophages, which may reveal novel insights into its mechanism of action.

CONCLUSIONS:Altogether, mOASL2, albeit its reported inhibitory role for the cGAS-mediated type I IFN response, acts antiviral in the context of MHV68 infection, which is partially independent of its ability to activate RNase L. This suggests that it exerts its antiviral function in a manner distinct from its known functions.

Keywords: viral infection, immune response, murine herpesvirus 68, ISG, IFN, OASL

P-024
General Research » Antiviral Defense

NONSENSE-MEDIATED DECAY CONTROLS REACTIVATION OF THE ONCOGENIC HUMAN HERPESVIRUSES EBV AND KSHV

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OBJECTIVES:The two human oncogenic gamma-herpesviruses Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) establish lifelong latent infections in large proportions of the adult world population and are the causative agents of various human malignancies. A prominent feature of herpesviruses including EBV and KSHV is the biphasic life cycle consisting of latent and lytic stages of infection. Both latency and lytic reactivation are important contributors to virus-associated disease, yet the molecular details of the (host) factors that control viral reactivation remain incompletely understood. Nonsense-mediated decay (NMD) is an evolutionarily highly conserved cellular RNA degradation pathway that targets mRNA transcripts displaying specific features (e.g. premature termination codons or long 3'-UTRs) for degradation. While these features are abundantly present throughout the herpesvirus genomes, the role of NMD in targeting viral transcripts and regulating the (herpes)virus lifecycle remains largely unknown. In this study, we determined the role of NMD in controlling latency and reactivation of the oncogenic herpesviruses EBV and KSHV.

METHODS:We used various classical virological, biochemical, and cell-biological methods in combination with an RNA-immunoprecipitation and RNAseq (RIPseq) approach and a small-molecule inhibitor to characterize the herpesvirus-derived transcripts that are targeted for degradation by NMD and to assess the contribution of NMD to controlling herpesvirus latency and reactivation in a variety of cell types.

RESULTS:We found that NMD controls reactivation of EBV and KSHV. Mechanistically, the NMD machinery induces the degradation of the viral polycistronic Rta transactivator transcripts following recognition of specific features in their 3'-UTRs. RNAi-mediated interference with NMD activity or treatment with small-molecule NMD inhibitors resulted in potent viral reactivation in a variety of EBV- or KSHV-infected cell types.

CONCLUSIONS:Our results identify NMD as an important host process that regulates reactivation of oncogenic human herpesviruses, presenting potential therapeutic targets for the induction of viral reactivation and the eradication of virus-infected tumor cells.

Keywords: herpesvirus, nonsense-mediated decay, RNA degradation, intrinsic immunity, latency, reactivation

P-025
General Research » Antiviral Defense

MELATONIN SHOWS POTENTIAL AS AN ANTIVIRAL AGENT THROUGH MODULATION OF THE IFN-III PATHWAY IN HUMAN INTESTINAL ORGANOIDS

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OBJECTIVES:Our study aimed to evaluate possible antiviral properties of melatonin when used as treatment vs pretreatment in Poly(I:C)-stimulated gastrointestinal organoids.

METHODS:The human intestinal epithelial Caco-2 cells (passages 10-20) were used for an *in vitro* infection model. Cultured in DMEM at 37°C 5% CO2 for 21 days, they formed organoids. The monolayer was pretreated with melatonin (1, 10, 50, or 100 μM) for 24 hours, followed by TLR3 stimulation with Poly(I:C) for another 24 hours. Alternatively, cells were treated with melatonin after stimulation. Proliferation was measured using CCK-8. IFNλ1, IFNβ were tested using ELISA. Apoptosis, interferon lambda receptor 1 (IFNLR1) and STAT1-3 expression were analyzed using flow cytometry. Statistical analysis was performed with SPSS 29.0.

RESULTS:Caco-2 cells displayed direct correlation between IFNλ1 expression and higher Poly(I:C) concentrations, while IFNβ expression was absent. IFNλ1 levels were highest in melatonin-pretreated cells infected with high Poly(I:C) concentrations, while melatonin treatment reduced IFNλ1 expression. Melatonin reduced IFNLR1 expression in both treated and pretreated cells compared to stimulated controls. Interestingly, it induced IFNLR1 expression in non-infected cells but did not stimulate IFNλ1 production. In pretreated cells, IFNλ1 levels moderately correlated with STAT1-3 expression (highest with STAT2, r=0.509), but did not correlate with IFNLR1. In treated cells, there was a reverse weak or no correlation between STAT1-3 and IFNλ1, while STAT2 showed the strongest correlation with IFNLR1 (r=0.318). Melatonin pretreatment reduced apoptosis, while melatonin treatment on infected cells did not protect them from apoptosis despite decreasing levels of IFNλ1, IFNLR1 and STAT1-3.

CONCLUSIONS:Melatonin demonstrated a potential antiviral effect via III IFN pathway by reduction of IFNλ1 expression in treated cells and induction of IFNLR1 expression in non-infected cells. Interestingly, its impact on STAT1-3 and IFNλ1 correlation varied between pretreated and treated cells, highlighting the intricate interplay in the modulation of antiviral response.

Keywords: gastrointestinal organoids, melatonin, antiviral, TLR3 stimulation

P-026
General Research » Antiviral Defense

MONO-ADP-RIBOSYLATION IN HOST-PATHOGEN INTERACTIONS

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OBJECTIVES:Mono-ADP-ribosylation (MARylation) is a post-translational protein modification initially identified among bacterial toxins MARylating host factors, thereby contributing to pathogenicity. Close homologues to the diphtheria toxin are responsible for intracellular MARylation in eukaryotic cells, the ADP-ribosyltransferases diphtheria toxin-like, also known as PARP enzymes. They consume NAD^+ to transfer a single ADP-ribose unit to a substrate protein with release of nicotinamide. The genes encoding several of these MARylating enzymes have been identified as interferon-stimulated genes, indicating a potential function in the antiviral innate immune response. So-called macrodomains are closely linked to ADP-ribosylation, with some capable of reversing MARylation. Macrodomains are highly conserved and found in all domains of life. Also, some positive single-stranded RNA viruses, among them the alphavirus Chikungunya virus (CHIKV), encode for macrodomains.
We could attribute de-MARylation activity to the CHIKV macrodomain and found that this activity is essential for viral replication. Further, we identified PARP10 as restriction factor for CHIKV replication supporting a functional role of MARylation in antiviral defense. However, the exact mechanisms of how PARP10 restricts CHIKV replication or the macrodomain contributes to viral replication remain elusive, largely because no substrates are known.
We hypothesized that part of the antiviral immune response is mediated by MARylation of host factors, which is counteracted by the viral macrodomain.

METHODS:In this study, we aimed at the identification of host factors potentially being MARylated/de-MARylated during antiviral defense. Therefore, we employed proximity-dependent biotinylation using BirA-fusions of either PARP10 or CHIKV-nsP3 to map transient interactions and thus potential substrates of both proteins using mass spectrometry.
RESULTS:By comparison of the interactomes of the individual proteins we could define an intersection, which we expected to encompass potential substrates. Indeed, we were able to confirm some common interactors as substrates of PARP10 and the CHIKV macrodomain.
CONCLUSIONS:Comparative interactome analysis deems appropriate for substrate identification.

Keywords: Mono-ADP-ribosylation, viral macrodomains, PARPs, innate immunity, antiviral defense

P-027
General Research » Antiviral Defense

ANTI-INTERFERON ARMAMENTARIUM OF SARS-COV-2: ORF6 IN FOCUS

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OBJECTIVES:The study aimed to profile all SARS-CoV2 proteins for their role in type I Interferon antagonism. There were two objectives
1. To screen all SARS-CoBV-2 proteins using reporter systems to test their ability to antagonize Interferon induction and downstream antiviral signaling.
2. Subsequently, we examined the SARS-CoV-2 protein ORF6 in detail for its role in interferon antagonism and underlying molecular mechanisms.

METHODS:We utilized the IFNb-Luc and ISRE-Luc system to assess the majority of SARS-CoV-2 proteins and determine their ranking based on their capacity to hinder Type-I IFN induction and subsequent signaling. Our investigation focused on ORF6, examining its impact on the steps of IFN induction and its interaction with signaling components. To delineate the protein domain and specific amino acids of ORF6 involved in IFN-antagonism, we generated truncated constructs and mutants. Additionally, we explored the influence of ORF6 on TRIM25 stability, its interaction, and the associated K48 and K63 linked ubiquitination of RIG-I.

RESULTS:We observed that numerous SARS-CoV-2 proteins exhibit antagonistic effects on IFN induction and signaling, with ORF6 displaying the most prominent inhibitory impact. Our findings indicate that ORF6 directly interacts with RIG-I, impeding downstream Type-I IFN induction and signaling by blocking K63-linked ubiquitination of RIG-I through the E3 Ligase TRIM25. This inhibition involves the ORF6-mediated targeting of TRIM25 for proteasomal degradation, a phenomenon also observed in the context of SARS-CoV-2 infection. The specific type-I IFN antagonistic activity of ORF6 was identified in its C-terminal cytoplasmic tail, particularly within amino acid residues 52-61.

CONCLUSIONS:Our study sheds light on the armament of IFN-antagonistic proteins of SARS-CoV-2 and the distinct mechanisms employed by the ORF6 protein to inhibit Type-I IFN induction and signaling.

Keywords: SARS-CoV-2, Type-I IFN, ORF6, RIG-I, TRIM25, Ubiquitination

IFN Antagonism by SARS-CoV-2 ORF6

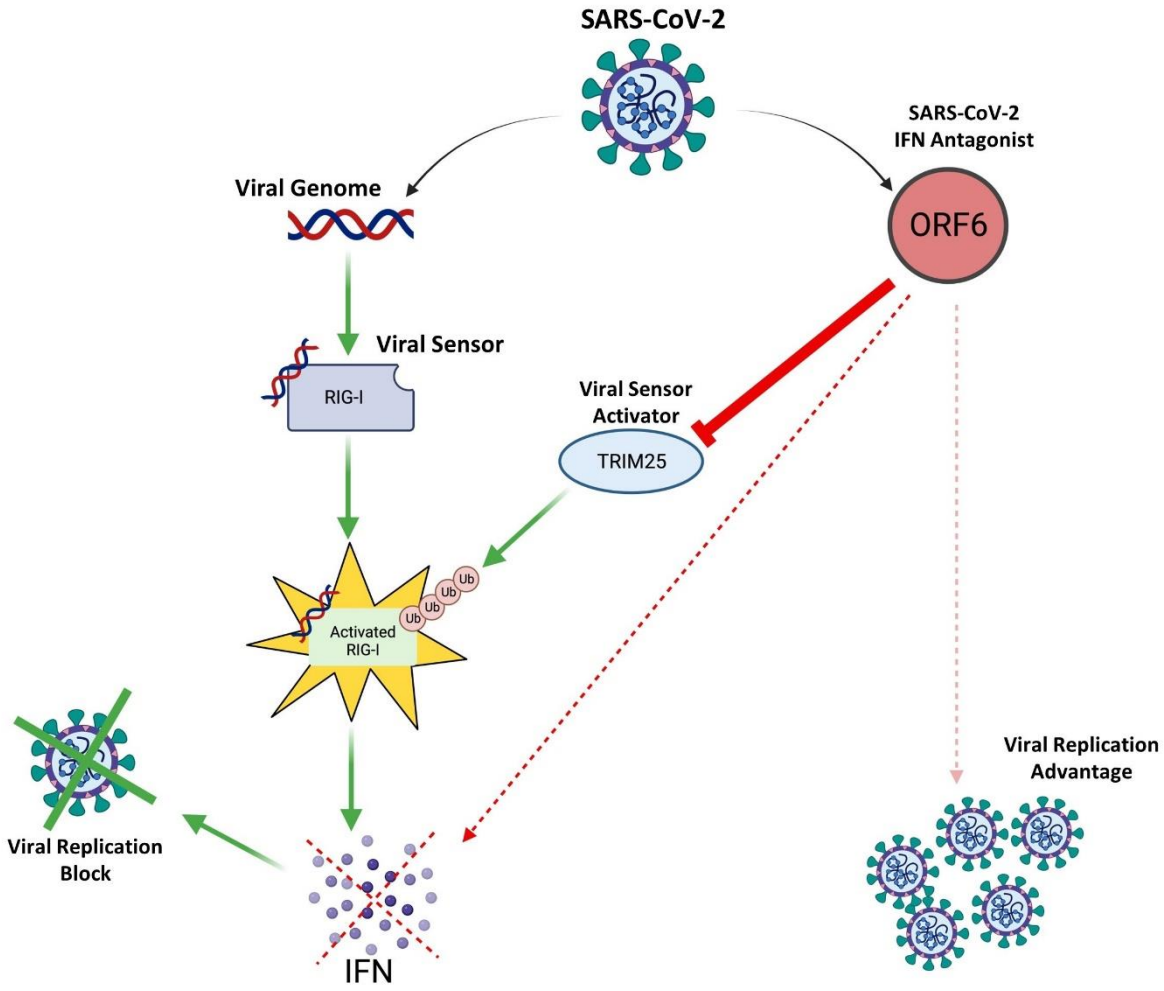

Image shows a novel mechanism of IFN-anatgonism by SARS-CoV-2 ORF6, where it targets TRIM25 for proteasomal degradation to inhibit RIG-I activation in SARS-CoV-2 infected cells.

P-028
General Research » Antiviral Defense

RLR-DEPENDENT TYPE I IFN PRODUCTION REGULATES ANTIGEN DOSE AND ACTIVATION IN YF-17D-INFECTED DCS

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OBJECTIVES:The yellow fever vaccination is a paradigm for an effective vaccine. The live-attenuated YF17D vaccine virus induces a robust innate immune response followed by rapid development of protective neutralizing antibodies and specific T cells, which provide long-lasting immune protection against yellow fever. YF17D triggers multiple receptors including TLRs and RLRs to activate antigen presenting cells (APCs) and induce type I Interferon (IFN).
METHODS:The contribution of different APC subpopulations and viral sensing pathways to this response was investigated by infecting isolated human primary DC and monocyte subpopulations as well as cells deficient for various pattern recognition receptors with a YF17D fluorescent reporter virus in vitro.
RESULTS:YF17D infection could be detected in all DC and monocyte subsets and frequency of infected DCs and monocytes was greatly increased by blocking the IFN-α/-β receptor (IFNAR) demonstrating that YF17D is highly restricted by type I IFN in these cell types. YF17D induced the upregulation of costimulatory molecules and HLADR in infected DCs and monocytes and to a lower degree in bystander cells. Loss of function experiments in murine and human DCs and cell lines demonstrated a dominant role of the RIG-I-like receptor (RLR) and the mitochondrial antiviral signaling protein (MAVS) pathway for type I IFN induction and the restriction of YF17D. The type I IFN response in infected cells was mediated by 5' phosphate dsRNA intermediates formed during YF17D infection. In vivo proximity labelling (IPL) of RIG-I and next-generation sequencing confirmed interaction between RIG-I with and YF17D dsRNA in infected cells.
CONCLUSIONS:Thus, YF17D induces type I IFN production in infected APCs preferentially via the RLR pathway. YF17D thereby seems to strike the right balance between production of viral antigens, costimulation and limitation of viral replication to form a safe but highly effective vaccine.

Keywords: Yellow fever, Vaccine, RLR, Interferon, dsRNA

P-029
General Research » Antiviral Defense

GAS7 COORDINATES DEGRADATIVE MACROPINOCYTOSIS TO ENSURE EFFICIENT DESTRUCTION OF VIRAL PATHOGENS BY HUMAN MACROPHAGES.

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OBJECTIVES:Macrophages constitutively sample the environment through macropinocytosis thus continuously surveying the surrounding tissues. This process also raises the risk of macrophage infection due to increased pathogen load in intracellular compartments. It remains unclear how macrophages cope with the need to sample their environment without becoming a vehicle for viral propagation.

METHODS:In this work, we uncovered the role of a cytoskeleton associated protein, Growth Arrest-Specific-7 (GAS7), as a critical factor allowing macrophages to perform continuous surveillance of the surrounding media, while also promoting the efficient degradation of viral pathogens.

RESULTS:Using primary human monocyte-derived macrophages (MDMs), we found that GAS7 is essential for the control of the replication of multiple viral pathogens that are representative of the most common viral groups. Such restriction was independent of viral sensing and type I Interferon signalling. Further experiments revealed that the expression of GAS7 allows macrophages to block viral propagation at an early stage after exposure.
GAS7 localizes to the macrophage plasma membrane, being particularly enriched in regions of membrane ruffling. Silencing GAS7 in MDMs decreases the uptake of a fluid phase marker. Also, the acidification of macropinocytosed material was severely impaired in macrophages silenced for GAS7. Brief exposure to viral pathogens revealed that GAS7 boosts the capacity of macrophages to capture viral particles but limits the occurrence of productive infection. Mechanistically, GAS7 sustains the activation of Rac1 to drive actin polymerization and promote the uptake of extracellular media, while also promoting Rac-1-dependent ROS production in macropinosomes. Specific inhibition of NOX2, led to a complete loss of the antiviral effect of GAS7. Our current work aims to understand how the GAS7-Rac1-NOX2 axis promotes degradation of viral particles in macrophages.

CONCLUSIONS:In conclusion, GAS7 coordinates the remodelling of the actin cytoskeleton with the degradative pathway to ensure constitutive uptake of viral pathogens and their efficient destruction.

Keywords: GAS7, virus, macropinocytosis, intracellular degradation, antiviral response

P-030
General Research » Antiviral Defense

A NEW NOVEL DYNEIN ADAPTOR COMPLEX PROMOTES THE INFLUENZA A VIRUS CELL ENTRY AT LAMP1-POSITIVE STRUCTURES

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OBJECTIVES:Influenza A virus (IAV) enters host cells via endocytosis. The viral ribonucleoproteins (vRNPs) can carry the unanchored ubiquitin chains, recognized as misfolded proteins by the histone deacetylase 6 (HDAC6), facilitating the disassembly of viral particles (VPs) during/upon fusion at endosomes. The interaction between HDAC6 and dynein further mediates the transport of these misfolded proteins along the microtubule to aggresomes, leading to viral uncoating. However, the impairment of IAV infection under HDAC6-deficient conditions is notably less significant than dynein deficiency, suggesting the involvement of an alternative pathway in dynein-mediated viral cytoplasmic release. In this study, our objective is to explore a new novel dynein adaptor complex promotes the Influenza A virus cell entry.

METHODS:We used protein mass spectrometry to discover dynein adaptor proteins that involved in IAV infection and further employed IAV cell entry assays and electron microscopy approach to validate the candidate that mediated IAV infection. Finally using immunoprecipitation and co-immunofluorescence approaches to determine protein-protein interaction.

RESULTS:In this study, we discovered that PCNT, a large protein that organizes microtubules in the centrosome, associates with LC3 proteins (LC3s), the hallmark of autophagy, which are crucial for IAV infection. Our data revealed that LC3s are recruited to vRNPs, forming a vRNP-LC3 complex at LAMP1-endosomes and PCNT serves as a dynein adaptor responsible for transporting the vRNP-LC3 complex along microtubules and this role of LC3s is autophagy independent. We further confirmed that HDAC6 is not involved in the formation of the vRNP-LC3s-PCNT-DYNC1I1 complex. The collaborative silencing of HDAC6 and PCNT, rather than their independent silencing, restricts IAV infection to the same extent as dynein deficiency, indicating that HDAC6 and PCNT-LC3 represent two independent mechanisms during IAV cell entry.

CONCLUSIONS:In conclusion, our data identifies a novel dynein adaptor, PCNT, which interacts with vRNP-LC3s to promote IAV cytoplasmic release from the LAMP1-endosomal membrane via microtubule.

Keywords: Influenza A virus, IAV, microtubule, cell entry, dynein, PCNT

P-031

General Research » Epigenetic regulation of innate immune responses

MACROPHAGE TRAINING CAN REACTIVATE LATENT HIV-1 FROM HAART-TREATED PATIENT PBMCS

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OBJECTIVES:HIV virus can persist in a latent but activatable chronic state, predominantly in resting CD4+ T-cells but also to a lesser extent in tissue-resident macrophages. This necessitates lifelong combination antiretroviral therapy (cART) for HIV+ patients. HIV-1 latency in macrophages is associated with increased chromatin condensation induced by histone methylation at viral integration sites. We hypothesized that training of macrophages could enhance viral reactivation by chromatin opening.

METHODS:We used the established innate training stimuli MDP and β-glucan, along with a Syk kinase inhibitor recently reported by our group, to train the THP89GFP cell line, which is an experimental monocyte model for latent HIV-1. To determine reactivation by trained immunity in HIV infected patients, we used a quantitative viral outgrowth assay in macrophages isolated from low viremia HIV patients on HAART. 3 out of 17 patients had detectable counts of HIV in macrophage reservoir populations and demonstrated reactivation upon training with Syk inhibitor, MDP and beta-glucan. To assess epigenetic changes in the pro-viral HIV-1 DNA associated with training, we performed ChIP-qPCR for the histone mark, H3K27Ac.

RESULTS:All 3 trained immunity stimuli activated transcription of HIV-1 genes in these cells. We observed that multiple regions of the HIV-1 LTR had increased deposition of H3K27Ac in the trained THP89GFP macrophages. We also found that co-culturing the trained macrophages with a T-cell latent HIV reservoir cell line (2D10 Jurkat) reactivated HIV from the T-cells. Though training of macrophages with beta-glucan and MDP did not reactivate ERVs, training through Syk inhibition led to substantial endogenous retroviruses (ERV) reactivation.

CONCLUSIONS:Our studies thus show that induction of trained immunity is a viable approach to the reactivation of latently infected HIV-1 from both T-cells and macrophages, and this strategy could be developed as a novel therapeutic approach to facilitate elimination of latent HIV reservoirs.

Keywords: Trained immunity, macrophages, HIV, latency, reactivation, beta-glucan

P-033
General Research » Epigenetic regulation of innate immune responses

TLR-MEDIATED SIGNALING REGULATES EPIGENETIC FACTOR HMGA1 FOR MACROPHAGE ACTIVITY

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OBJECTIVES:High mobility group A 1 (HMGA1) is a protein that binds to DNA and can modify chromatin structure. It has been previously implicated in regulating the transcription of genes involved in inflammation across various cell types. However, its role in macrophage activity remains ambiguous. Our objective is to elucidate the role of HMGA1 in macrophages in initiating the innate immune response.

METHODS:To determine the function of HMGA1 in macrophages, we utilize Bone Marrow-Derived Macrophages (BMDMs) along with genetically engineered mice lacking HMGA1 in macrophages. We employ cutting-edge technologies such as next-generation sequencing and proteomics, as well as in vivo and in vitro assays, for the functional and mechanistic understanding.

RESULTS:Using RT-qPCR, we determined that the expression of Hmga1 in BMDMs increased upon exposure to TLR 3 and TLR 4 agonists, while remaining constant with other TLR agonists. This pattern was also observed in RAW 264.7 cells, where treatment with a TLR 4 agonist (LPS 100 ng/ml) led to elevated Hmga1 expression.
To evaluate the effects of HMGA1 depletion, TLR4 agonist was applied to stimulate BMDMs from both *Hmga1fl/fl x Csf1rCre+ve* and *Hmga1fl/fl x Csf1rCre-ve* macrophages. Results indicated a significant increase in the expression of proinflammatory genes such as *Tnf*, *Cox2*, *Il1b*, and *Il6* in response to HMGA1 depletion.
Furthermore, we investigated the impact of Hmga1 depletion on macrophage function using macrophage killing assays with *E. coli* O157:H7 in vitro. The findings revealed that *Hmga1 fl/fl x*

Csf1rCre+ve macrophages exhibited diminished killing ability compared to *Hmga1fl/fl x Csf1rCre –ve* macrophages. Additionally, flow cytometry analysis demonstrated that both *Hmga1fl/fl x Csf1rCre –ve* and *Hmga1fl/fl x Csf1rCre +ve* macrophages exhibited similar phagocytic capacities.

CONCLUSIONS:We conclude that TLR 3 and 4 mediated signaling upregulates the expression of Hmga1 in macrophages. In addition, HMGA1 is required for the macrophage-mediated elimination of the gram-negative bacteria *E.coli*.

Keywords: Macrophages, TLR signaling, HMGA1

P-034
General Research » Inflammasomes

INVESTIGATING EPITHELIAL-INTRINSIC MECHANISMS THAT COORDINATE INFLAMMATION AND INTESTINAL EPITHELIAL BARRIER INTEGRITY FOLLOWING INFLAMMASOME ACTIVATION

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OBJECTIVES:Intestinal epithelial inflammasomes rapidly initiate inflammation in response to infection. This highly inflammatory innate immune pathway prevents bacterial replication and dissemination by triggering cellular extrusion and pyroptosis. To counteract inflammation and cell death, regenerative mechanisms in the epithelium are crucial to avoid epithelial barrier collapse. Our work seeks to identify the epithelial-intrinsic mechanisms that coordinate inflammation and epithelial barrier integrity following inflammasome activation.

METHODS:Epithelial-intrinsic responses were assessed using murine ileal organoids stimulated with flagellin or FlaTox. Inflammasome activation was assessed by Western blot and LDH release. WT and *Nlrc4-/-* organoids were stimulated with flagellin for 4 h prior to analysis by bulk RNA-sequencing. Follow-up gene expression analysis was done with RT-qPCR.

RESULTS:We are the first to report that intestinal epithelial cells (IECs) internalize exogenous bacterial flagellin in a TLR5-independent manner which triggers activation of a Caspase-1 and -8 inflammasome that is dependent on NAIP-NLRC4. Bulk RNA-seq analysis of flagellin treated WT and *Nlrc4-/-* organoids revealed an inflammasome activation dependent transcriptional program. While common inflammatory genes were upregulated in both genotypes, WT IECs had specific upregulation of genes involved in EGF receptor signalling, tight junction organization, and cell-cell adhesion. Analysis of *Gsdmd-/-* and *Il18-/-* organoids revealed that this transcriptional program was dependent on pore formation but not IL-18. The excessive activation of inflammasomes by the potent activator FlaTox, resulted in the loss of Lgr5+ stem cells which led to the induction of a fetal-like transcriptional signature.

CONCLUSIONS:We reveal that IECs can rapidly internalize exogenous bacterial flagellin and activate the NAIP-NLRC4 inflammasome. Inflammasome activation in IECs induces a unique transcriptional program that suggests a coordinated upregulation of genes involved in epithelial proliferation and barrier reinforcement. The induction of a fetal-like transcriptional signature in response to extreme inflammasome mediated damage suggests a fundamental regenerative pathway used by IECs to sustain function after injury.

Keywords: Naip, Nlrc4, regeneration, inflammasome, organoids, RNA-sequencing

P-035
General Research » Inflammasomes

NLRP6 RECOGNIZES CYTOSOLIC INFECTION OF *L. MONOCYTOGENES* VIA ITS NACHT-LRR DOMAINS

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OBJECTIVES:Canonical inflammasomes are formed by members of the Pyrin, Aim2 or NOD-like receptor family (NLRs) and activate caspase-1 to induce cytokine release and pyroptosis upon recognition of endogenous or pathogen-derived danger signals. NLRP6 plays a role in regulating the susceptibility to gastrointestinal infections and has been proposed to form an inflammasome in response to lipoteichoic acid (LTA) from Gram-positive bacteria or dsRNA. However, mechanistic insights into its activation mechanism remain lacking.

METHODS:To investigate the activation mechanism of NLRP6, we developed an inflammasome reconstitution assay using FACS-based analysis of ASC-specks as a readout for complex formation. We tested several bacterial pathogens and ligands for their potential to activate NLRP6 and determined the sensing domain of the receptor using NLRP6 mutants and NLRP6-NLRP3 chimeras.

RESULTS:We found that *Listeria monocytogenes* activated NLRP6 dependent on the virulence factor Listeriolysin O (LLO), indicating that cytosolic entry was required. However, neither LLO itself, nor any of the previously published ligands were sufficient to activate NLRP6 in our assay. In contrast, activation required ActA, a virulence factor mediating intracellular bacterial spread via actin-based motility. Moreover, activation of NLRP6 required ATP binding of the receptor, as well as a conserved motif in the FISNA-like domain. By testing chimeras between NLRP6 and the closely related NLRP3, we found that the NACHT-LRR domains of NLRP6 recognize *L. monocytogenes* infection. Interestingly, recognition of *L. monocytogenes* by NLRP3 was mediated by the NACHT domain in a potassium efflux independent manner. In contrast, NLRP3 activation by nigericin was blocked by extracellular potassium and required the linker-FISNA domain.

CONCLUSIONS:Our data suggests that NLRP6 can recognize the cytosolic infection of *L. monocytogenes* via its NACHT-LRR. In contrast, *L. monocytogenes* sensing by NLRP3 seems to be potassium independent and mainly driven by the NACHT domain, suggesting a different activation mechanism to potassium-dependent NLRP3 activation.

Keywords: NLRP6, NLRP3, inflammasome, infection

P-036
General Research » Inflammasomes

ROLE OF IL-1β AND IL-18 IN OSTEOCLASTOGENESIS OF BONE MARROW MACROPHAGES STIMULATED WITH CHOLESTEROL CRYSTALS IN THE PRESENCE AND ABSENCE OF LPS

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OBJECTIVES:Cholesterol crystals (CC) are frequently observed in the lesion of refractory apical periodontitis. CC has been reported to induce IL-1β and IL-18 production via the NLRP3 inflammasome in myeloid cells. IL-1β promotes osteoclastogenesis, and IL-18 has both osteoclastogenic and anti-osteoclastogenic effects. However, the role of CC in apical periodontitis has not been clarified. This study aimed to investigate the role of CC particles in osteoclastogenesis.

METHODS:Bone marrow-derived macrophages (BMMs) were pre-treated with 10 ng/ml RANKL and 50 ng/ml M-CSF for 2 days. Cells were then stimulated with CC (0, 7.5, 15, 30 μg/ml) in the presence or absence of LPS, for another 2 days. TRAP staining was then performed, and TRAP-positive cells with three or more nuclei were counted as osteoclasts. For inhibition assays, MCC950 (NLRP3 inflammasome inhibitor), Z-YVAD-fmk (caspase-1 inhibitor), IL-1 receptor antagonist (IL-1ra) or IL-18 binding protein (IL-18 BP) were added prior to stimulation. Levels of IL-1β and IL-18 were analyzed via qRT-PCR and ELISA.

RESULTS:CC upregulated osteoclastogenesis in RANKL-treated BMMs in the presence and absence of LPS. CC upregulated IL-1β expression in the presence of LPS, but not in its absence. Osteoclastogenesis was significantly suppressed by IL-1ra in the presence of LPS, suggesting that CC upregulates osteoclastogenesis via IL-1β production in the presence of LPS. Interestingly, CC increased IL-18 expression in RANKL-treated BMMs. Furthermore, IL-18 BP inhibited osteoclastogenesis, indicating that CC-upregulates osteoclastogenesis by IL-18 production in the absence of LPS. MCC950 and Z-YVAD-fmk suppressed CC induced osteoclastogenesis both in the presence and absence of LPS, suggesting that CC activated the NLRP3 inflammasome and promoted IL-1β and IL-18 maturation.

CONCLUSIONS:CC promoted osteoclastogenesis in RANKL-treated BMMs via IL-1β production in the presence of LPS and via IL-18 production in the absence of LPS. CC may play an important role in bone resorption in apical periodontitis.

Keywords: Cholesterol crystal, NLRP3 inflammasome, osteoclastogenesis, IL-1β, IL-18

P-037
General Research » Inflammasomes

THE SEPTIN MODIFIER, FORCHLORFENURON, ACTIVATES NLRP3 VIA A POTASSIUM-INDEPENDENT MITOCHONDRIAL AXIS

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OBJECTIVES:The NLRP3 inflammasome initiates potent inflammatory responses and tissue damage, and is causally associated with a myriad of inflammatory diseases. Potassium efflux from the cell is identified as unifying signal triggered by almost all NLRP3-activating stimuli, except for the imidazoquinolines imiquimod and resiquimod. Potassium-independent NLRP3 activation is cryptic and largely unresolved, however it is known to be linked to mitochondrial fitness. Here, we stumbled upon a novel potassium-independent NLRP3 activator that is linked to the septin cytoskeleton. We then further characterised the cell biology of this enigmatic niche in inflammasome biology.

METHODS:We began by treating primary mouse and human macrophages with the septin-modifying drug forchlorfenuron (FCF). We also utilised the flow cytometric analysis of time of flight inflammasome evaluation (TOFIE). FCF triggers the rearrangement of septins; key cytoskeletal proteins that participate in mitochondrial dynamics. We monitored septin rearragement and mitochondrial dynamics by Airyscan confocal microscopy. Following this, we performed Seahorse metabolic assays to more accurately sample mitochondrial respiration and distress under FCF treatment.

RESULTS:Here, we identify forchlorfenuron (FCF) as a novel inflammasome activator that triggers human and mouse NLRP3 signalling independently of potassium efflux. We report that FCF triggers the rearrangement of SEPT2 into tubular aggregates, and induces concomitant mitochondrial fragmentation and collapse of the mitochondrial membrane potential. FCF-induced mitochondrial collapse resembled mitochondrial poisoning by imiquimod, and yet FCF itself is structurally distinct from imidazoquinolines.

CONCLUSIONS:FCF thereby joins the imidazoquinolines as a second, structurally-distinct class of molecules that triggers NLRP3 inflammasome signalling by inducing mitochondrial damage. These data implicate dysregulations in cellular septin architecture as inducers of both mitochondrial damage and NLRP3 inflammasome-driven inflammation and tissue damage. Intriguingly, dysregulated septin biology is linked to several neurodegenerative diseases in which NLRP3 exerts pathophysiological functions. These data also uncover a new facet of the cryptic potassium-independent pathway for NLRP3 activation.

Keywords: Inflammasomes, NLRP3, macrophages, septins, pyroptosis, IL-1

P-038
General Research » Inflammasomes

MODULATION OF HOST INFLAMMASOMES DURING LATENT KAPOSI'S SARCOMA HERPESVIRUS INFECTION

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OBJECTIVES:Inflammasomes are innate immune sensors that initiate inflammatory signaling and cell death upon pathogen detection. While the role of inflammasomes during host defense and pathogenesis has been well-characterized, its significance during DNA virus latent infection remains understudied. Kaposi's sarcoma (KS) herpesvirus (KSHV) is the causative agent of KS, one of the most prevalent skin cancers in people living with HIV. Current treatments target lytic replication, but KS tumors predominantly contain latent KSHV genomes. This emphasizes the need for developing therapeutics targeting KSHV reservoirs. The anti-apoptotic protein B cell lymphoma extra-large (Bcl-xL) was identified as an essential host factor for the survival of latent KSHV-infected endothelial cells. Bcl-xL also antagonizes the NLRP1 inflammasome. We hypothesized that the requirement for Bcl-xL to inhibit virus-induced apoptosis disrupts its suppression of inflammasome activation, sensitizing latent KSHV infected endothelial cells to inflammasome-mediated clearance.

METHODS:The capacity of anti-apoptotic proteins (Bcl-xL and Bcl-2) to negatively regulate inflammasome signaling was tested in HEK293T cells, where inflammasome signaling components and anti-apoptotic proteins were transiently transfected and IL-1β processing was measured as a proxy for inflammasome activation. To determine if the dependency of KSHV latency on Bcl-xL renders infected cells uniquely susceptible to inflammasome activation, mock or KSHV latently infected endothelial cells were treated with drugs that inhibit Bcl-xL and modulate the NLRP1 and CARD8 inflammasomes.

RESULTS:We found that Bcl-xL and Bcl-2 antagonize both the NLRP1 and CARD8 inflammasomes. In endothelial cells, I observed that drug-induced inhibition of Bcl-xL and inflammasome activation enhanced cell death in latent KSHV infected cells but not uninfected cells.

CONCLUSIONS:My findings expand on the regulatory function of anti-apoptotic proteins across different programmed cell death pathways. We speculate that such regulatory crosstalk provides a therapeutic opportunity to selectively eliminate infected cells via targeting of host dependency factors of KSHV latency in combination with drug-induced inflammasome activation.

Keywords: herpesviruses, KSHV, inflammasomes, endothelia, NLRP1, CARD8

P-039
General Research » Inflammasomes

IL-1β MEDIATES LUNG PATHOLOGY CAUSED BY MYCOBACTERIUM ABSCESSUS INFECTION THROUGH THE REGULATION OF IL-17 PRODUCTION

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OBJECTIVES:In this study, we aimed to elucidate the roles of NLRP3 and IL-1β in host defense against MAB infection.

METHODS:For intranasal inoculation of MAB, the mice were inoculated with 2 × 107 CFU (in 40μl of D-PBS) of MAB intranasally. After 5 days, mice were euthanized, and lung tissues were collected under sterilized condition. The rest of the lobes except the left lobe were homogenized with 500 μl of D-PBS and the lysate were used for CFU measurements. The left lung lobe was used for the histological examination.

RESULTS:MAB-induced production of IL-1β was completely abolished in macrophages deficient in NLRP3, but not NLRC4. The adaptor protein ASC and caspase-1 were also found to be essential for the production of IL-1β in response to MAB. Western blot analysis showed that cleavage of caspase-1 and IL-1β in response to MAB was absent in macrophages deficient in NLRP3, ASC, and caspase-1. The deficiency of NLRP3 and IL-1β did not affect the intracellular growth of MAB in macrophages, and the bacterial burdens in the lungs of NLRP3- and IL-1β-deficient mice were also comparable to those in wild-type (WT) mice. In contrast, deficiency of IL-1β ameliorated the lung pathology in MAB-infected mice. Notably, in the lung homogenates of IL-1β-deficient mice, there was a reduction in the production of IL-17, but not of IFN-γ and IL-4, compared to their WT counterparts. Further investigations using an in vitro co-culture system demonstrated that IL-1β signaling was essential for IL-17 production in response to MAB. Lastly, we confirmed that the administration of an anti-IL-17 antibody effectively mitigated the lung pathology induced by MAB.

CONCLUSIONS:These findings highlight the potential role of IL-1β in contributing to lung pathology induced by MAB infection through the elevation of IL-17 production.

Keywords: Mycobacterium abscessus; NLRP3 Inflammasome; IL-1β; IL-17; Pulmonary Inflammation

P-041
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A NOVEL REPORTER FOR INFLAMMASOME ACTIVATION AND CASPASE-1 RECRUITMENT

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OBJECTIVES:Inflammasomes coordinate inflammation by inducing rapid cytokine secretion and protect against invading pathogens by initiating death of infected cells. In order to further our understanding of inflammasome biology, we aimed to develop a novel reporter that fulfils the following criteria: 1) it should not require staining; 2) it should identify inflammasome activation in living or fixed cells; 3) it should be suitable for primary cells and tissues; 4) it should offer single cell resolution; 5) it should lend itself to quantification by microscopy and flow cytometry; 6) it should have low background; 7) and it should report on the recruitment of caspase-1.

METHODS:We developed novel reporters for inflammasome activation composed of the caspase-1 CARD fused to fluorescent proteins, which recapitulate the recruitment of caspase-1 to assembled inflammasomes. We tested and characterized the reporter in different cell types, including primary cells and organoids. Using structure-guided mutants of the CARD, we investigated the recruitment mechanism of caspase-1 and its regulation by CARD17.

RESULTS:The new reporter allowed robust identification, localization, and quantification of inflammasomes by microscopy or flow cytometry without the need for staining. It was functional in cell lines as well as primary macrophages, keratinocytes, T cells, and intestinal organoids. Downstream signaling was not impaired. The recruitment of the reporter and its mutants provided strong evidence that caspase-1 is recruited to the inflammasome via the formation of CARD filaments. These can be terminated by CARD17 to reduce cytokine secretion.

CONCLUSIONS:We present a novel reporter to study inflammasome biology. It visualizes inflammasomes in real-time and enables readouts by microscopy and flow cytometry. Its capacity to mimic caspase-1 has already led to new insights in inflammasome assembly and regulation, *i.e.* the formation of caspase-1 filaments and their regulation by CARD17.

Keywords: Inflammasome assembly, cellular reporter, organoids, caspase-1

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HUMAN NLRC4 CAN ACT AS A DIRECT SENSOR FOR CYTOSOLIC FLAGELLIN

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OBJECTIVES:Bacterial flagellin is directly recognized by the receptors NAIP5/6 (NLR family apoptosis inhibitory protein 5/6) to trigger conformation-dependent activation of the NLRC4 (NLR family CARD containing protein 4) inflammasome in mouse immune cells. Human NAIP clearly recognizes non-flagellin patterns associated with bacterial type 3 secretion systems. But whereas some results point to a potential role of NAIP in cytosolic flagellin sensing, other reports show human NAIP to not recognize cytosolic flagellin directly. We therefore explored other possibilities, namely that NLRC4 might be a direct cytosolic sensor of flagellin in the human immune system.

METHODS:We used immunoprecipitation to detect the binding of NAIP or NLRC4 to Legionella FlaA and other flagellins and ELISA to detect the release of IL-1β (interleukin-1β) in a cellular NLRC4 inflammasome HEK293T (Human embryonic kidney 293 cell) reconstitution system. Oligomers of NLRC4 were detected by native PAGE and fluorescence microscopy. Knockout human leukemic monocytic THP-1 cell lines were generated via CRISPR-Cas9. We also generated an inducible FlaA-expressing stable THP-1 cells to study NAIP independence and NLRC4 dependence of flagellin sensing in human macrophage-like cells by genetic means.

RESULTS:Immunoprecipitation experiments showed that human NLRC4, and not human NAIP, bound to different flagellins, most strongly Legionella FlaA. Ectopic FlaA expression also induced NLRC4 oligomerization in the absence of NAIP. Unexpectedly, the presence of NAIP diminished the binding of NLRC4 with flagellins and also subsequent IL-1β release. Interestingly, in resting THP-1 cells a stable interaction between NAIP and NLRC4 was detectable. Moreover, in the absence of NAIP, THP-1 responses were increased but completely

NLRC4-dependent. Structural modeling supported the notion of a direct FlaA-active human NLRC4 interaction with certain flagellins.

CONCLUSIONS:Our data suggest NLRC4 to act as a putative direct sensor for certain cytosolic flagellins but raise the intriguing possibility of NAIP as a negative regulator of flagellin sensing in humans.

Keywords: NAIP, NLRC4, inflammasome, flagellin

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DISSECTING THE MECHANISM OF DSRNA-MEDIATED NLRP1 ACTIVATION

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OBJECTIVES:NACHT, LRR and PYD domains-containing protein 1 (NLRP1) is an inflammasome sensor that upon activation mediates pyroptosis, a lytic type of cell death, and inflammatory cytokine release. NLRP1 is predominantly expressed in barrier tissues, such as skin and respiratory epithelium, and its activating signals differ between species. Human NLRP1 can be activated by various triggers including viral protease activity, double-stranded RNA (dsRNA), and the ribotoxic stress response. We have previously shown that long dsRNA generated in the life cycle of positive-stranded RNA viruses, such as Semliki Forest virus, activates NLRP1 by directly binding to its N-terminal domains. In contrast, ribotoxic stress signaling has been shown to trigger a MAPK signaling cascade through the sensor kinase ZAKα, leading to the activation of p38 kinases. Both ZAKα and p38 kinases were described to phosphorylate NLRP1 in an N-terminal disordered linker region (DR). However, the mechanism leading to inflammasome activation downstream of NLRP1 phosphorylation remains elusive. We aim to dissect the molecular mechanisms that lead to the formation of an active NLRP1 inflammasome.

METHODS:We used CRISPR/Cas9 to generate genetic knock-outs in an immortalized epithelial cell line and performed stimulation assays. We measured release of the proinflammatory cytokine IL-1β using ELISA and visualized key (phospho)proteins by Western blot.

RESULTS:Mutation of important phosphorylation sites in the DR abrogates the inflammasome response to both dsRNA and ribotoxic stress. However, strong activation of p38 kinases in response to different stimuli does not necessarily lead to activation of NLRP1.

CONCLUSIONS:Taken together, our results show that NLRP1 phosphorylation is necessary, but not sufficient to activate the inflammasome. We suggest that similar to NLRP3, phosphorylation plays a priming role that sensitizes NLRP1 to subsequent activation. The current progress on these studies will be presented here.

Keywords: NLRP1, dsRNA, ZAKα, p38, ribotoxic stress response

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MULTI-OMICS REVEAL NOVEL REGULATORS THAT CONTROL GASDERMIN B EXPRESSION

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OBJECTIVES:We leveraged the power of multi-omics to identify novel regulators that control gasdermin B (GSDMB) isoform expression and/or function.

METHODS:We conducted a comprehensive analysis of genome-wide association studies (GWAS) to identify key disease-associated GSDMB genetic variants. We subsequently evaluated potential implications on protein structure/function through structural modeling. In addition, we characterized GSDMB isoform-specific transcription via transcriptomic analysis of human tissue samples.

RESULTS:Multiple intragenic GSDMB genetic variants are associated with chronic inflammatory disorders, such as asthma, inflammatory bowel disease, rheumatoid arthritis, and type 1 diabetes. Their distribution across the gene implies that differential regulatory mechanisms may alter gene expression, as evidenced by a novel “junction” that modulates GSDMB expression. Moreover, transcriptomic analysis reveals tissue-specific and disease-dependent GSDMB transcript expression profiles. Finally, computational modeling highlights how GSDMB genetic variants may confer changes in GSDMB structure and function.

CONCLUSIONS:Given the emerging complexity of GSDMB biology, there remains significant gaps in the current understanding of: 1) how disease-associated genetic variants impact protein function, and 2) which regulatory mechanisms govern isoform expression. Our genomic and transcriptomic analyses highlight novel regulators that may impact GSDMB expression in the setting of human health and disease. Moreover, our computational modeling underscores a critical need to determine how disease-associated genetic variants affect downstream GSDMB function.

Keywords: gasdermin, genomics, transcriptomics

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GASDERMIN-D REGULATES CASPASE-4 CLEAVAGE TO LIMIT IL-18 PROCESSING.

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OBJECTIVES:Emerging evidence suggests inflammasome activation is highly regulated and cells fine-tune the balance between cytokine secretion and cell death. Little is known about how this is occurs. In epithelial barriers, inflammasome activation must be tightly controlled to facilitate cytokine secretion, while limiting death to maintain an effective barrier. We aimed to investigate how caspase-4 modulates IL-18 production and pyroptosis in intestinal epithelial cells.

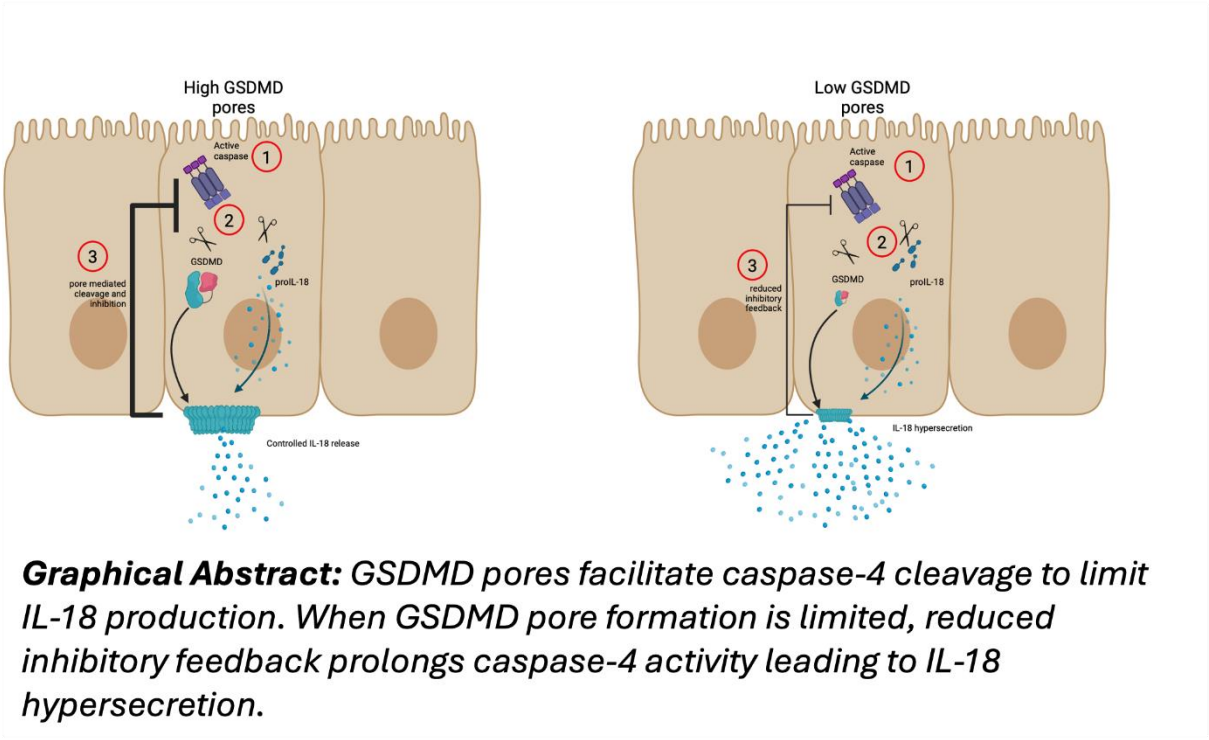
METHODS:Wild Type (WT) and GasderminD (GSDMD) knockout epithelial cells were electroporated with LPS. WT and GSDMD knock down (KD) human enteroid monolayers were infected with *Salmonella* or *Shigella*. GSDMD pore formation was visualized by propidium iodide uptake with timelapse microscopy. IL-18 was measured by ELISA. Cell death was measured by cytotoxicity assay. Caspase-4 cleavage was measured by western blot of lysates and supernatant. Cell lysis was blocked by treatment with glycine. GSDMD pore formation was blocked by treatment with dimethylfumerate (DMF). WT or cleavage-mutant caspase-4 were co-expressed with IL-18 in HEK cells.

RESULTS:In WT cells, caspase-4 activation resulted in IL-18 secretion and appearance of a caspase-4 cleavage fragment. In *GSDMD-/-* cells, caspase-4 activation led to significantly more caspase-4 -dependent IL-18 production, without caspase-4 cleavage. During infection, WT and GSDMD KD human enteroid monolayers died equivalently, however GSDMD KD cells produced dramatically more IL-18 with no evidence of caspase-4 cleavage. In WT cells, limiting GSDMD pore formation but not cell lysis, prevented caspase-4 cleavage and resulted in increased IL-18 production. Overexpression of cleavage-mutant caspase-4 resulted in increased IL-18 processing compared to WT caspase-4.

CONCLUSIONS:Our results demonstrate that GSDMD pore formation facilitates caspase-4 cleavage to limit IL-18 production. They suggest that independently from pyroptosis, inflammasomes encode feedback loops to modulate caspase activity and fine-tune cytokine production. This feedback loop is likely important in epithelial tissues to facilitate cytokine release to initiate a broader immune response, while maintaining barrier integrity.

Keywords: Gasdermin, Caspase, Inflammasome, IL-18, Cell Death, Epithelium

Graphical Abstract



Graphical Abstract: *GSDMD pores facilitate caspase-4 cleavage to limit IL-18 production. When GSDMD pore formation is limited, reduced inhibitory feedback prolongs caspase-4 activity leading to IL-18 hypersecretion.*

Graphical Abstract

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CARD8 INFLAMMASOME ACTIVATION UPON HIV CELL-CELL TRANSMISSION

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OBJECTIVES:Inflammasomes are cytosolic innate immune complexes that assemble upon detection of diverse pathogen-associated cues and play a critical role in host defense and inflammatory pathogenesis. Our previous work demonstrated that CARD8 detects HIV infection by sensing the enzymatic activity of the HIV protease, resulting in CARD8-dependent inflammasome activation and cell death. Here, we sought to understand CARD8 responses to HIV when the virus is transmitted from infected cells to target cells via a viral synapse (cell-cell), a physiologic and important mode of HIV spread.

METHODS:To assess HIV-dependent inflammasome responses during cell-cell transmission of HIV-1, we developed HIV coculture systems with a panel of immortalized and primary cells, including human monocyte-derived macrophages. Using this physiologic infection system, we also used genetic knockouts and sensor-specific inhibitors to distinguish the relative contributions of CARD8 versus other inflammasomes in response to cell-cell HIV infection. To evaluate the viral determinants of CARD8 sensing, we tested a panel of HIV protease inhibitor resistant clones to establish how variation in HIV protease affects CARD8 activation.

RESULTS:We observed that cell-cell transmission of HIV induces CARD8 inflammasome activation in immortalized and primary human monocyte-derived macrophages. Genetic knockout of the adaptor protein ASC and treatment with the NLRP3 inhibitor MCC950 suggest that cell death associated with HIV-dependent inflammasome activation is primarily CARD8-dependent whereas cytokine release may be amplified through secondary modulation by the NLRP3 inflammasome. Additionally, we identified mutant HIV-1 proteases from a panel of protease inhibitor resistant HIV-1 strains that differentially cleave and activate CARD8 compared to wildtype HIV-1.

CONCLUSIONS:These data implicate CARD8 activation as a major contributor of pyroptosis by multiple modes of HIV infection, thus providing further physiological relevance for the possible role CARD8 inflammasome activation in HIV pathogenesis.

Keywords: CARD8, HIV, protease, cell death, inflammasome

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P2X7 RECEPTOR INHIBITION REDUCES NLRP3-INDEPENDENT IL-1β RELEASE IN HUMAN MACROPHAGES

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OBJECTIVES:Interleukin (IL)-1β is a highly potent pro-inflammatory cytokine and released following activation of the inflammasomes. The P2X7 receptor plays a key role in the activation of the NLRP3 inflammasome. However, the underlying mechanisms are poorly understood in human cells. Here, we aimed to characterize the role of P2X7 receptor and NLRP3 inflammasome activation on IL-1β secretion by human macrophages.

METHODS:THP-1 differentiated macrophages were used to characterize IL-1β secretion. Cells were primed with the Toll-like receptor (TLR)2/1 agonist Pam3CSK4 and stimulated with established P2X7 receptor agonists ATP and BzATP, or novel P2X receptor modulator and synthetic anti-LPS peptide Pep19-2.5 to facilitate IL-1β secretion. The potassium ionophore nigericin was used as control. Pharmacological inhibitors and cells deficient in NLRP3 were used to assess the involvement of the NLRP3 inflammasome. IL-1β secretion and protein expression were quantified by ELISA and Western blot, respectively.

RESULTS:Stimulation of Pam3CSK4-primed macrophages with P2X7 receptor agonists or the P2X7 modulator Pep19-2.5 facilitated NLRP3-independent IL-1β release while nigericin induced IL-1β secretion proved to be fully NLRP3 dependent. P2X7 receptor inhibitors fully blocked NLRP3-independent IL-1β release by P2X7 receptor agonists and modulators. Furthermore, P2X receptor modulation by Pep19-2.5 reduced nigericin-induced IL-1β secretion.

CONCLUSIONS:Our findings underline the potential of P2X7 receptor inhibitors in the treatment IL-1β associated diseases. Mechanistically we highlight NLRP3-independent IL-1β release in TLR-primed human macrophages.

Keywords: NLRP3, Inflammasomes, Interleukin (IL)-1β, P2X7

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UNRAVELING THE ROLE OF NLRP1 INFLAMMASOME IN MYELOPOIESIS: INSIGHTS FROM ZEBRAFISH MODELS AND THERAPEUTIC IMPLICATIONS

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OBJECTIVES:Chronic inflammatory and infectious diseases often trigger inflammatory events linked to hematopoietic lineage imbalance, resulting in excessive myeloid cell production. Our previous work highlighted the pivotal role of the NLRP1 inflammasome, activated through the ZAKα/P38 kinase axis, in this process by inactivating the master erythroid transcription factor GATA1 in hematopoietic stem and progenitor cells (HSPCs). Furthermore, we identified negative regulation of the NLRP1 inflammasome in HSPCs by LRRFIP1/FLII, independently of DPP9. This study employs the zebrafish model to deepen our understanding of the Nlrp1 inflammasome role in regulating myelopoiesis during infectious and sterile inflammatory insults.

METHODS:We utilized CRISPR-Cas9 technology to inactivate nlrp1, zaka, flii, and lrrfip1a/b in zebrafish reporter embryos. Manipulations included forced expression of human NLRP1 and zebrafish csf3a mRNAs and treatments with nilotinib (tyrosine kinase inhibitor), VX-765 (CASP1 inhibitor), or anisomycin (protein synthesis inhibitor). Infection with Salmonella enterica serovar Typhimurium (ST) was performed, followed by imaging and quantification of neutrophils, macrophages, and erythrocytes in larvae.

RESULTS:Genetic Nlrp1 inhibition in zebrafish larvae led to decreased neutrophils and macrophages, alongside increased erythrocyte counts. Zaka inhibition, both genetically and pharmacologically, mimicked Nlrp1 deficiency, while Lrrfip1 or Flii deficiency resulted in neutropenia/monocytopenia and erythrocytosis. Zaka inhibition alleviated neutrophilia in relevant models, while Lrrfip1/Flii deficiencies exacerbated neutrophilia. Either Zaka or Nlrp1 inhibition increased larval susceptibility to ST infection, which was partially rescued by Csf3a overexpression. Remarkably, Lrrfip1- and Flii-deficient larvae displayed increased resistance to ST infection, suggesting the Nlrp1 inflammasome regulates resistance to ST through regulating myelopoiesis rather than bacterial clearance mechanisms of myeloid cells.

CONCLUSIONS:Our findings underscore the crucial role of the Nlrp1 inflammasome in regulating myelopoiesis during inflammatory and infectious diseases. Since nilotinib (Tasigna) is

a FDA/EMA-approved drug for the treatment of chronic myeloid leukemia, it is attractive for repurposing in the treatment of hematopoietic alterations associated with these conditions.

Keywords: Nlrp1 inflammasome, HSPC, neutrophils, macrophages, erythrocyte, hematopoiesis

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NLRP3 SELECTIVELY DRIVES IL-1β SECRETION IN NEUTROPHILS INFECTED WITH EXOS EXPRESSING *PSEUDOMONAS AERUGINOSA* AND REGULATES DISEASE SEVERITY

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OBJECTIVES:The NLRC4 and NLRP3 inflammasomes are multi-protein complexes that are required for activation and secretion of pro-inflammatory cytokine IL-1β, resulting in pyroptotic cell death in macrophages, but not neutrophils. In macrophages, gram-negative bacteria such as *Pseudomonas aeruginosa* activate the NLRC4 inflammasome by detection of bacterial flagellin or structural proteins that are part of the Type III secretion system (T3SS), an important virulence factor. The objective of this study is to examine mediators of IL-1β secretion in *P. aeruginosa*-infected neutrophils *in vitro* and *in vivo*.

METHODS:*In vitro*: bone marrow neutrophils (BMN) and bone marrow-derived macrophages (BMDM) from C57BL/6, NLRC4, or NLRP3 knockout mice were primed for 3h with LPS then infected for 1h with *P. aeruginosa* strains PAO1 (expressing ExoS and ExoT) and mutant strains lacking the T3SS needle (ΔpscD), exotoxins S and T (ΔexoST), or ExoS ADP ribosyl transferase (ADPRT) or GTPase-activating protein (GAP) activity. *In vivo*, we used a well-established murine model of *P. aeruginosa* corneal infection where neutrophils comprise >80% cells recruited to the corneal stroma.

RESULTS:We found that neutrophil IL-1β secretion was dependent on expression of *P. aeruginosa* T3SS and neutrophil NLRP3, which contrasts with NLRC4 usage by macrophages. We found that activation of NLRP3 in neutrophils is mediated by ExoS ADPRT activity. Importantly, we used gene knockout mice and pharmacological inhibition (MCC950) to demonstrate that NLRP3 and not NLRC4 is required to limit bacterial growth in *P. aeruginosa* infected corneas and thereby modulate disease severity.

CONCLUSIONS:Overall, these findings reveal a novel role for ExoS ADPRT in regulating inflammasome usage in IL-1β secretion by neutrophils compared with macrophages, and an unexpected role for NLRP3 in the pathogenesis of *P. aeruginosa* infection.

Keywords: inflammasome, NLRP3, Pseudomonas aeruginosa, exotoxin, neutrophils, cornea

Figure 1 - Working Model

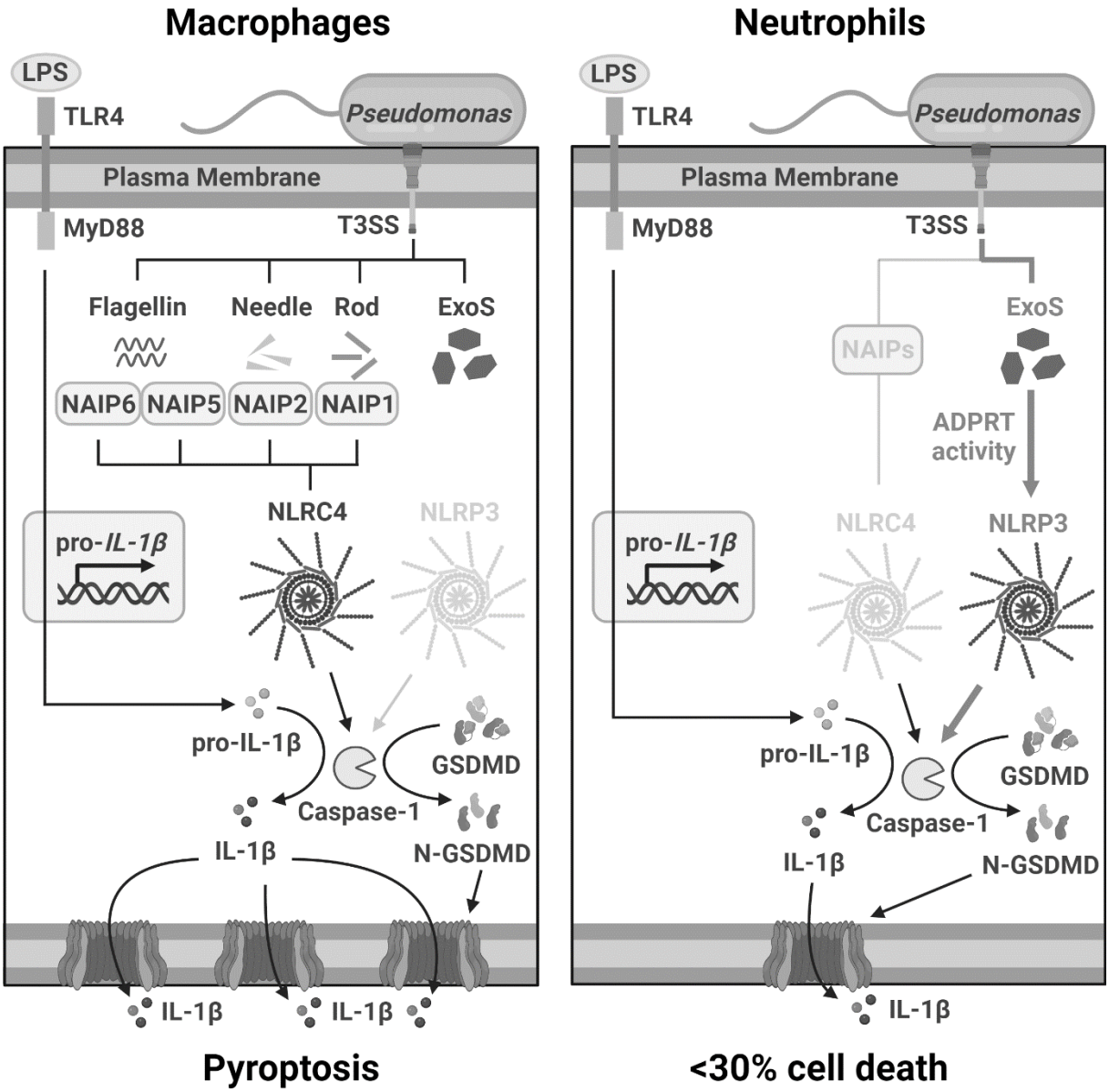

Figure 1 - Predicted sequence of events in *P. aeruginosa* induced IL-1β secretion by macrophages and neutrophils. In contrast to macrophages, neutrophils infected with ExoS expressing *P. aeruginosa* selectively activate the NLRP3 inflammasome. Created with BioRender.com.

Predicted sequence of events in P. aeruginosa induced IL-1b secretion by macrophages and neutrophils

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NON-DECAMERIC NLRP3 FORMS AN MTOC-INDEPENDENT INFLAMMASOME

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OBJECTIVES:NLRP3 is an inflammasome-forming protein that plays a key role in inflammatory conditions ranging from infections to Alzheimer's disease. NLRP3 is activated by many potassium (K+)-dependent or -independent stimuli. Molecularly, human NLRP3 assembles into a decameric 'cage' and its interaction with both the trans-Golgi network (TGN) and the microtubule organization centre (MTOC) has been proposed as critical for NLRP3 activation. Our study's objective was to dissect the complex interactions between the NLRP3 inflammasome's various oligomeric states and key organelles to gain a deeper understanding of NLRP3's activation and regulation in response to both potassium-dependent and independent stimuli.

METHODS:We employed advanced live cell imaging to observe NLRP3 inflammasome activation in real time. This approach, complemented by structural information, molecular biology methods, cellular assays and RNA editing, allowed for a comprehensive analysis of NLRP3's behavior in human macrophages. We focused on the spatial and temporal dynamics of NLRP3 interaction with cellular organelles under varying stimuli.

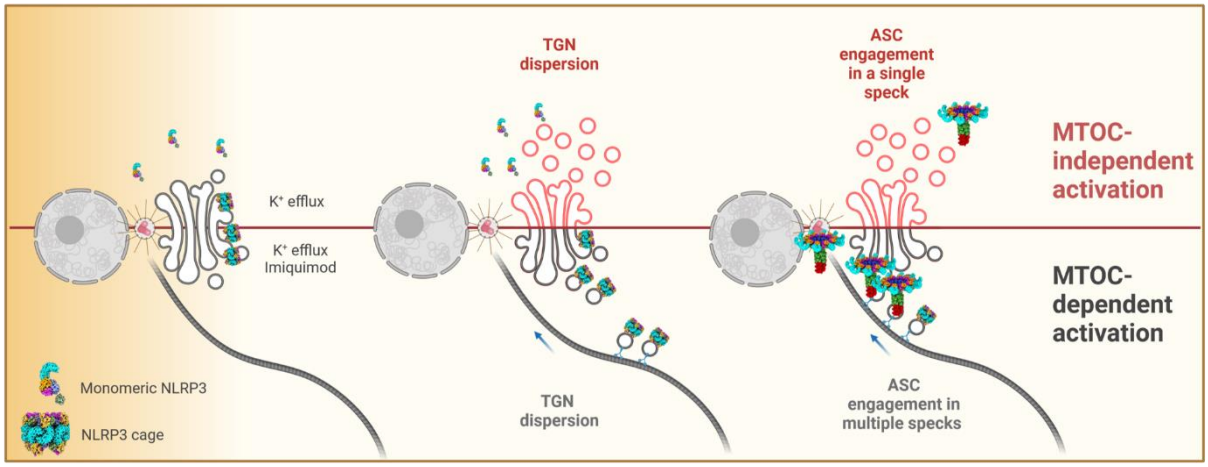
RESULTS:Our results show that NLRP3 stimulation triggers two distinct activation pathways. The first is the aforementioned decameric cage-dependent pathway, essential for responding to the potassium-independent stimulus, imiquimod. The second pathway is independent of both the decameric cage structure and the involvement of the TGN/MTOC. This pathway remains functional in an engineered NLRP3 protein variant designed not to form decamers and interact with membrane lipids. Morever, primary neutrophil appear to exclusively employ this MTOC-independent pathway. These findings delineate two parallel yet biologically discrete pathways for NLRP3 activation.

CONCLUSIONS:The identification of two activation pathways for NLRP3 advances our knowledge of inflammasome regulation and accommodates previous, at times conflicting data, in a unified framework. This opens avenues for further research into the molecular mechanisms governing inflammatory responses and may aid the development of targeted treatments for

NLRP3-related diseases.

Keywords: NLRP3, inflammasome, macrophage, Trans-Golgi Network, MTOC, microtubules

2 parallel NLRP3 activation pathways



K+-dependent NLRP3 stimulation triggers two distinct activation pathways: (i) the decameric cage-dependent pathway, which was also exclusively required for the K+-independent stimulus, imiquimod; and (ii) a cage- and TGN/MTOC-independent pathway that was fully functional in an NLRP3 protein engineered to not form decamers and unable to interact with membrane lipids.

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ADENOVIRUS INFECTION ANTAGONIZES INFLAMMASOME SIGNALING IN HUMAN EPITHELIA

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OBJECTIVES:Cytosolic innate immune sensors that form inflammasomes detect pathogenic or stress-induced stimuli, which promotes the induction of pro-inflammatory cytokines (IL-1β/IL-18) and pyroptosis (lytic cell death). NLRP1 is one of the predominant inflammasome sensors in human epithelia where it has been implicated in sterile inflammatory disease and shown to sense pathogenic activities of RNA viruses and bacterial pathogens. Here we sought to determine if NLRP1 can sense the human adenoviruses (HAdVs), an important class of human pathogenic dsDNA viruses that cause inflammatory disease in the epithelia of the eyes, airway, and gut.

METHODS:Given their long-term coevolution with humans, HAdVs may both activate and evade inflammasomes. To test both possibilities, wild type and *NLRP1* knockout ocular (corneal, conjunctival) epithelial cells were infected with HAdVs in the absence or presence of Val-boroPro (VbP), an NLRP1-specific activator, to assess for HAdV-driven activation or suppression of the inflammasome, respectively. IL-1β secretion and cell death were measured as readouts of inflammasome function.

RESULTS:We found that HAdV-B16 infection alone modestly induced NLRP1-dependent cell death, however, this response was atypical, and did not result in secreted IL-1β. Interestingly, HAdV-B16- and HAdV-C5-infection of VbP-treated cells suppressed VbP-induced cytokines in an MOI-dependent manner. Infections with early (E) gene deletion viruses also suppressed cytokines, indicating that suppression is likely mediated by a virion-associated factor and does not require viral gene transcription.

CONCLUSIONS:These findings suggest that while NLRP1 senses HAdV-B16 infection, HAdV-B16 suppresses NLRP1 signaling via a virion-associated antagonist. The ability of multiple HAdVs to antagonize NLRP1 implies that inflammasome activation is an important selective pressure on HAdV fitness, and therefore may play a role in host barrier defense and HAdV pathogenesis. Furthermore, our observation that HAdV-C5 E1/E3 viruses retain the ability to antagonize inflammasomes is a consideration for the design and use of HAdV vectors in therapeutics and vaccines.

Keywords: adenovirus, inflammasomes, NLRP1, epithelia

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DIPEPTIDYL PEPTIDASE 9 (DPP9) DEPLETION FROM HEPATOCYTES IN MICE RETARDS TUMOUR GROWTH, INCREASES CASPASE-1 ACTIVATION AND BECLIN-1 AND ALTERS METABOLIC MARKERS

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OBJECTIVES:Dipeptidyl peptidase 9 (DPP9) is a ubiquitous intracellular peptidase that supresses NLRP1 inflammasome activation, autophagy and BRCA2 activity. These actions of DPP9 suggest potential for this protease to influence tumourigenesis and tumour growth. Global DPP9 gene inactivation is lethal in mice and humans. Therefore, to study liver cancer, we produced and studied mice in which DPP9 expression is depleted only from hepatocytes.

METHODS:A hepatocyte-specific DPP9 knockout mouse (Alb-DPP9-KO) was achieved by floxing exons 5 to 7 of full-length DPP9 and crossing this DPP9fl/fl mouse strain with a mouse strain that expresses Cre recombinase under an albumin promoter. Primary liver cancer was induced in mice by treating with diethylnitrosamine (DEN; once), then thioacetamide (TAA) and an atherogenic high fat diet (HFD) until 28 weeks of age. Measurements included tumour burden, and total liver qPCR of immunological and autophagy markers.

RESULTS:Alb-DPP9-KO mice were healthy at all ages. Compared to littermate controls, Alb-DPP9-KO mice had reduced liver mass and subcutaneous adipose tissue mass and had lower fasting plasma glucose. Total numbers of macroscopic liver nodules and tumours, inflammation score and steatosis score did not differ between the two genotypes. However, the Alb-DPP9-KO mice had fewer small macroscopic liver nodules (<3 mm diameter) compared to littermate controls. Alb-DPP9-KO livers had increased levels of active caspase-1 protein, indicative of increased inflammasome activity, and increased levels of Nfkbib, Cxcl10 and Ccl5. However, the number of tumour infiltrating CD8+ T cells was not greater in Alb-DPP9-KO mice. The Alb-DPP9-KO mice showed increased protein levels of Beclin1, which implicates autophagy and/or the tumour suppressor p53.

CONCLUSIONS:Lifelong DPP9 depletion in hepatocytes led to reduced tumour sizes. Mechanisms of tumour growth retardation might include increased caspase-1 activation following NLRP1 activation, increased autophagy or p53 and altered energy metabolism in epithelial cells.

Keywords: Protease, steatosis, innate immunity, inflammasome, tumour, metabolism

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PALMITOYLATION INFLUENCES NLRP3 SIGNALING

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OBJECTIVES:Nod-like receptors (NLRs) detect danger signals to form multiprotein complexes named inflammasomes, which serve as platforms that initiate innate immune responses through pyroptosis and cytokine secretion. Abnormal NLRP3 signaling is associated with a large number of chronic and degenerative inflammatory diseases, such as atherosclerosis, type 2 diabetes and Alzheimer's disease. Our aim is to shed light on the molecular mechanisms triggering NLRP3 activation since a unifying model is still lacking despite intense research efforts.

METHODS:We used mass spectrometry, pharmacological and genetic approaches.

RESULTS:S-palmitoylation consists in the covalent attachment of a 16-carbon fatty acid to cysteine residues, regulating protein trafficking and enrichment at membranes. We show that NLRP3 is S-palmitoylated and that inhibition or deficiency of S-acyltransferases abrogates NLRP3 interaction with membranes and inflammasome signaling.

CONCLUSIONS:Our findings identify a post-translational regulation mechanism of NLRP3 inflammasome signaling, with potential implications for inflammation-related diseases.

Keywords: NLRP3, inflammasome, palmitoylation.

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INFLAMMASOME ACTIVATION DRIVES PARALLEL SIGNALING PATHWAY INACTIVATION

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OBJECTIVES:Inflammasomes trigger in response to both endogenous and exogenous danger signals (DAMPs and PAMPs.) The resulting pyroptotic cell death drives both the release of cytokines and the recruitment of immune cells. While the pathways that lead to inflammasome assembly and gasdermin cleavage have been well studied, little is known about the effect of inflammasome activation on concurrent signaling pathways and these pathways' effects on pyroptosis-independent gene expression.

METHODS:Pan-phosphoproteomics identified previously unknown pathways affected by NLRP3 inflammasome activation. Western blot and cell death assays were coupled with RNA seq analysis to determine inflammasome-directed signaling effects on gene expression.

RESULTS:Rapid ERK1/2 inactivation occurred within minutes of inflammasome stimulation. Remarkably, most other signaling pathways were unaffected, and this effect was independent of cell death. This ERK inactivation had a broad effect on the cell, as RNA seq analysis revealed that rather than a global loss of RNA, subsets of expressed mRNA, including cytokine- and chemokine-encoding genes, were resistant to degradation. Additionally, biochemical analysis suggests that ERK inactivation primes the cell to undergo apoptosis should pyroptosis fail.

CONCLUSIONS:ERK activity is specifically downregulated in response to inflammasome activation. This has widespread cell biological consequences, including effects on mRNA stability and apoptotic priming, setting the stage for an alternative death method in the face of failed pyroptosis.

Keywords: inflammasome, signaling, ERK

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NLRP6 CONTROLS SENTINEL GOBLET CELLS IN RESPONSE TO PROTOZOAN INFECTION THROUGH DETECTION OF *BACTEROIDES*-DERIVED SPHINGOLIPIDS

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OBJECTIVES:The inflammasome sensor NLRP6 has been shown to protect against colorectal cancer. In an attempt to replicate these findings in the Apcmin model, we noticed that this protection only occurred if mice were colonized with *Tritrichomonas*, a common protozoan parasite in animal facilities, suggesting that NLRP6 may sense protozoan infection.

METHODS:We developed a novel protist infection model utilizing *Tritrichomonas* species. Protists were harvested and FACS-purified from the cecum of infected, specific pathogen free mice and delivered by oral gavage into recipient animals.

RESULTS:*Tritrichomonas* colonization induced in vivo thickening of the colonic mucus in an NLRP6-, ASC- and Caspase-11-dependent manner. Ex vivo analysis of mucus growth from colonic explants confirmed NLRP6 dependency, consistent with the activation of sentinel goblet cells. Surprisingly, mucus growth was recapitulated with cecal extracts from *Tritrichomonas*-infected mice but not purified parasites, suggesting that NLRP6 may indirectly respond to *Tritrichomonas* infection through detection of parasite-induced microbial dysbiosis. In agreement, *Tritrichomonas* infection caused a profound shift in the microbiota with expansion of *Bacteroides* and *Prevotella*, and untargeted metabolomics revealed a dramatic increase in several classes of metabolites, including sphingolipids. Finally, using gnotobiotic mice, we identified that wild-type but not sphingolipid-deficient *B. thetaiotaomicron* was sufficient to induce NLRP6-dependent sentinel goblet cell function during protozoan infection.

CONCLUSIONS:We propose that NLRP6 is a sensor of intestinal parasitic infection through monitoring microbial sphingolipids.

Keywords: inflammasome, goblet cells, parasitic infection, NLRP6

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IRAK4 PHOSPHORYLATION CONTROLS INFLAMMATORY SIGNALING BY ACTIVATING IRAK OLIGOMERIZATION

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OBJECTIVES:The controlled oligomerization of signaling proteins is an essential feature of many inflammatory signaling pathways. An example is IL-1 receptor signaling, which relies on the oligomerization of the Death Domain (DD)-containing proteins MyD88 and IRAK family kinases, to form a complex called the Myddosome. Disrupting this complex assembly holds potential for anti-inflammatory treatments. Apart from the DD assembly, IRAKs' signaling activity is also regulated by auto-/trans-phosphorylation of their Kinase Domain, and it is unclear if these processes operate at or downstream of Myddosome assembly. This project elucidates the contribution of IRAK4 kinase domain and activity in myddosomal organization.

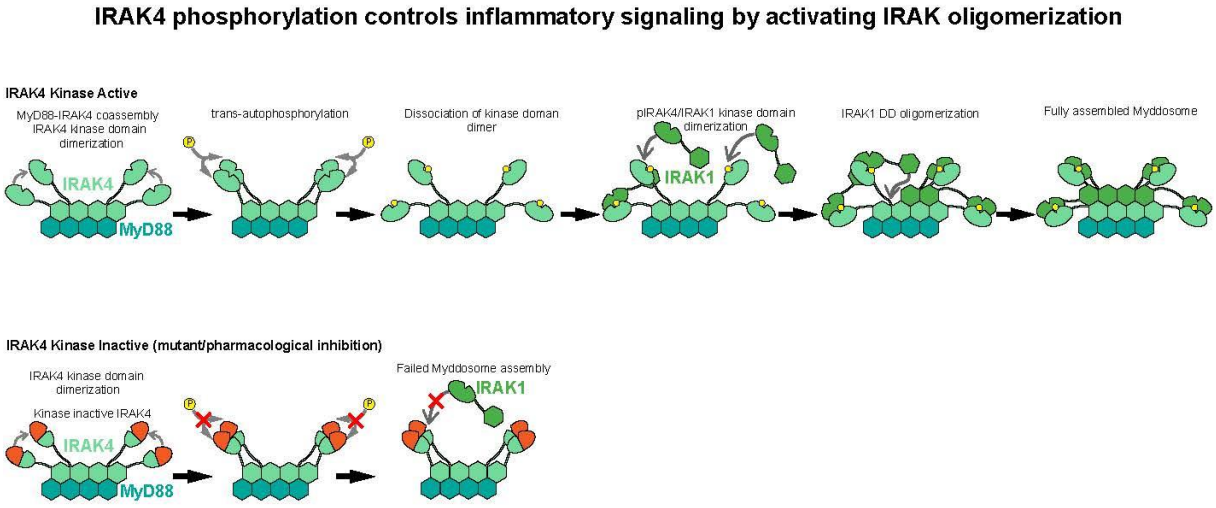
METHODS:Live cell Microscopy on Supported Lipid Bilayers, Fixed Cell Microscopy, Video Image Analysis, ELISA, Co-immunoprecipitation

RESULTS:We find that the initial stage of Myddosome assembly is solely controlled by MyD88:IRAK4 DD interactions. In later stages, IRAK4 auto-phosphorylation serves as a switch, regulating the active incorporation of IRAK1/2/3 DD into the MyD88:IRAK4 core complex.

CONCLUSIONS:Our data reveals IRAK4 auto-phosphorylation as an energy-dependent switch activating the heterotypic assembly of IRAKs' DDs and downstream signalling. This highlights how a signaling cascade integrates phosphorylation and protein oligomerization steps.

Keywords: Myddosome, Inflammasome, phosphorylation, IRAK, Kinase Domain

IRAK4 kinase domain autophosphorylation is essential for co-assembly with IRAK1



Cartoon demonstrating how IRAK4 autophosphorylation behaves as a switch that activates IRAK1 death domain assembly and incorporation into Myddosomes. IRAK4 kinase domain autophosphorylation disrupts the homo-dimer of IRAK4 kinase domain, and allows it to hetero-dimerize with the IRAK1 kinase domain. This interaction tethers IRAK1 at assembling Myddosomes thereby facilitating IRAK1 Death Domain oligomerization. This triggers the oligomerization of IRAK1 death domain at nascent Myddosomes.

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UNDERSTANDING THE ROLE OF THE NLRP3 INFLAMMASOME IN HUMAN KERATINOCYTES

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OBJECTIVES:Inflammasomes play a pivotal role in innate immunity and inflammation, resulting in the maturation and release of pro-inflammatory cytokines. While extensively studied in hematopoietic cells, the significance of NLRP3 in human epithelial cells remains controversial. Therefore,the objective of this study is to elucidate the dynamics and functional consequences of NLRP3 inflammasome activation in human keratinocytes. We aim to characterize the stimuli, signalling pathways, and regulatory mechanisms involved in NLRP3 activation within keratinocytes. Additionally, we seek to understand the impact of NLRP3 activation on the inflammatory response and immune signaling in the context of skin health and disease.

METHODS:We performed a combination of molecular biology techniques including western blots to look at key inflammasome readouts and quantification of secreted IL-1b by ELISA. In addition, we performed a variety of cellular assays and live-cell imaging.

RESULTS:We demonstrate that primary human skin keratinocytes express functional NLRP3 under specific inflammatory conditions. We also also show that various pore-forming toxins, including alpha-hemolysin (Hla), a potent virulent factor produced by Staphylococcus aureus, triggers NLRP3-dependent inflammasome activation in keratinocytes.

CONCLUSIONS:This study points to a potential cooperation among multiple NLR sensors in antibacterial defense of the human skin. In addition, the insights gained from this research are anticipated to contribute to our understanding of the role of NLRP3 inflammasomes in cutaneous biology, providing a foundation for the development of targeted therapies for inflammatory skin disorders.

Keywords: Inflammasomes, NLRP3, Keratinocytes

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DECODING THE INTRACELLULAR COMPLEMENT SYSTEM IN HUMAN MACROPHAGES

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OBJECTIVES:The cell-autonomous and intracellularly active complement system is an important regulator of basic metabolic pathways underlying the normal function of immune cells. We have previously demonstrated that intracellular complement, particularly the C5 system, contributes to inflammasome activation upon sensing of danger signals in monocytes and macrophages. However, the exact mechanisms by which intracellular complement regulates this process have not been established. Furthermore, the role of intracellular complement in bacteria-induced killing of macrophages, e.g. by Mycobacterium tuberculosis, is unclear.

METHODS:We have used confocal microscopy and immunostaining to map the sub-cellular location of C3 split products in human monocyte-derived macrophages (MDMs) following inflammasome activation by LPS and nigericin stimulation. The role of C3 in inflammasome activation was further investigated through treatment of human iPSC-derived macrophages (iPSC-DMs) with siRNA targeting C3. Moreover, CRISPR-Cas9 gene edited THP-1 cells lacking C5 and C5a receptor 1 (C5aR1) were used to explore the role of the intracellular C5 system.

RESULTS:We have identified intracellular stores of C3a, C3b and C3c in MDMs. Interestingly, we observed co-localisation of anti-C3bc and ASC-specks upon inflammasome activation. The C3bc antibody detects a neoepitope which appears on the C3c part of C3b upon C3 cleavage, and which is preserved on the C3c fragment following cleavage of C3b. Supporting a role of C3 in inflammasome activation, preliminary results from experiments in iPSC-DMs show reduced cell death and IL-1B production upon LPS and nigericin treatment of C3 siRNA-treated cells. Furthermore, preliminary results from THP-1 C5 and C5aR1 KO cells show decreased IL-1B production and cell death following inflammasome activation in KO cells compared to the wild-type. Infection of the KO cells with M. tuberculosis also caused lower levels of cell death and IL-1B release compared to wild-type cells.

CONCLUSIONS:These data demonstrate an important role for intracellular complement in mediating inflammatory reactions in human macrophages.

Keywords: Intracellular complement, inflammasomes, Mycobacterium tuberculosis

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NEK7 ACCELERATES NLRP3 INFLAMMASOME ACTIVATION

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OBJECTIVES:Inflammasomes regulate proinflammatory IL-1 family cytokines and induce pyroptosis. Unlike other inflammasomes activated by pathogens, NLRP3 responds to sterile danger signals, playing a crucial role in pathogenic inflammation across diseases. Urgently needed are specific NLRP3 inhibitors preserving IL-1 and other inflammasomes' protective effects. Understanding NLRP3's elusive activation is crucial for designing rational inhibitors. NEK7 is identified as essential for NLRP3 activation through direct interaction.

METHODS:Assessing NEK7 necessity in NEK7-deficient human THP-1 cells and murine bone marrow-derived macrophages (BMDMs) via stimulation with diverse NLRP3 activators. ELISA measures cytokine production, and a colorimetric assay gauges pyroptosis. Western Blot is used to analyze downstream activation. Mathematical modeling, combining time-course and dose-response, unveils inflammasome activation velocity in NEK7-deficient and wild-type BMDMs. Microscopy explores single-cell kinetics, while ATAC sequencing identifies cytokine pre-stimulation roles in NEK7-deficient and wild-type BMDMs.

RESULTS:Combined time-course and dose-response data revealed that the velocity for inflammasome activation is reduced in the absence of NEK7, but that the maximal amplitude of IL-1β release and pyroptosis is unaltered. This leads to a pronounced NEK7-dependency only at early time-points or under suboptimal stimulation conditions. Single-cell analysis suggests that onset of inflammasome formation is unaltered, but the rate of activation in additional cells is delayed without NEK7. Consequently, cytokines such as (T cell-derived) GM-CSF that can accelerate NLRP3 inflammasome activation further reduce NEK7 dependency. Moreover, ATAC-seq. reveals that the cytokine GM-CSF induces changes in chromatin accessibility.

CONCLUSIONS:Data suggest that the absence of NEK7 delays but cannot prevent NLRP3 activation in murine and human cells. Our data indicate that NEK7 lowers the threshold for NLRP3-specific activation. Furthermore, NEK7 dependency can be modulated by the time and dose of stimulation in vitro. Therefore, therapeutic targeting of the NEK7/NLRP3 interaction is attractive to tune NLRP3 activation and achieve a balance between dampening of pathogenic inflammation and functional host protection

Keywords: Inflammasome, NLRP3, NEK7, Activation

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COLD AFFECTS NLRP12 INFLAMMASOME ACTIVITY

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OBJECTIVES:Nucleotide-binding leucine-rich repeat-containing proteins (NLRs) are a group of intracellular
proteins that act as molecular pattern recognition receptors (PRRs) and confer regulation of immune pathways. NLRP12 was reported to have both anti-inflammatory and pro-inflammatory properties and can, for example, negatively regulate monocyte migration by affecting chemokine signalling pathways. Some polymorphisms in NLRP12 are associated with familial cold autoinflammatory syndrome type 2 (FCAS2), where patients suffer from inflammatory flares upon cold exposure.
The cellular and molecular details of how the activation threshold of NLRP12 is affected by cold remain elusive and are addressed in this work.

METHODS:Using HeLa cells with stable expression of NLRP12 and HEK293T iGLuc luciferase reporter assays we analysed the molecular function and protein-protein interactions upon cold exposure. The monocytic cell line THP1 with CRISPR-Cas9-mediated deletion of NLRP12 was used as a physiologically relevant model to study NLRP12 functions.

RESULTS:We show that exposure of NLRP12-expressing HeLa cells to 28°C increased ASC-speck formation and IL-18 release, accordingly NLRP12-induced caspase-1 processing in reporter assays was also significantly enhanced by cold. Mechanistically, FCAS2-associated NLRP12 R352C showed increased interaction with the heat shock protein HSC70 compared to WT NLRP12 and this interaction was greatly reduced upon expose to cold. In myeloid cells, cold exposure also decreased expression of the chemokine receptor CCR2 in a NLRP12-dependent manner. Our data suggest that regulation of CCR2 by NLRP12 involves TRPM8 and TRPA1 ion channels.

CONCLUSIONS:Overall, our data revealed mechanistic insights of the interaction between cold and the NLRP12 inflammasome. Differential binding of HSC70 to FCAS-associated NLRP12 variants might affect NLRP12 inflammasome activity and NLRP12 regulates monocyte responsiveness to chemokines during cold exposure. This provides a first framework for understanding the still enigmatic role of cold exposure and NLRP12 inflammasome activation in FACS2 patients.

Keywords: FCAS2, temperature, inflammasome, autoinflammatory syndrome, heat shock protein

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MATURATION OF THE CYTOKINE INTERLEUKIN-1β BY AN *STAPHYLOCOCCUS AUREUS* PROTEASE

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OBJECTIVES:*Staphylococcus aureus* (*S. aureus*) is a Gram-positive bacterium and a pathogen commonly found in the human microbiota, often colonizing the upper respiratory tract and the skin. Apart from its role in the microbiota, *S. aureus* is accountable for the majority of skin and soft tissue infections in humans, such as cellulitis, impetigo, and infected ulcers and wounds. Furthermore, *S. aureus* can cause invasive and life-threatening infections, including abscesses, pneumonia, osteomyelitis, endocarditis, and sepsis. The innate immune system is the first line of defense against pathogens, consisting of genetically encoded pattern recognition receptors. *S. aureus* can, for instance, activate the NLRP3 inflammasome through its pore-forming hemolysins, which assemble into the membrane of mammalian cells and thus lead to potassium efflux and subsequent NLRP3 activation. The skin is passively and actively participating in the innate immune response of an organism, with keratinocytes being the first cell type to come in contact with pathogens. In this study, we investigated whether there is inflammasome activation in keratinocytes upon *S. aureus* infection.

METHODS:We used an *S. aureus* infection model with the primary-like, immortalized keratinocyte cell line N/TERT-1. Interleukin-1β (IL-1β) release was measured by ELISA and immunoblotting of cleaved IL-1β was performed. In addition, genetic perturbation studies were conducted using CRISPR/Cas9-based ribonucleoprotein particles to knock out inflammasome components.

RESULTS:We showed that infecting keratinocytes with *S. aureus* leads to a robust IL-1β response, and this response is independent of inflammasome components. Stimulating keratinocytes with the bacteria-free supernatant of an *S. aureus* culture was sufficient to trigger IL-1β release, suggesting that pro-IL-1β is cleaved by a secreted factor of *S. aureus*. Ultimately, we identified an *S. aureus* protease able to cleave pro-IL-1β.

CONCLUSIONS:In conclusion, these results demonstrate that *S. aureus* secretes a protease which processes pro-IL-1β into bioactive IL-1β without the requirement for the inflammasome.

Keywords: Staphylococcus aureus, keratinocytes, inflammasomes, interleukin-1β, protease

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DECIPHERING NLRP3 PRIMING USING A RANDOM MUTAGENESIS APPROACH

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OBJECTIVES:NLR family pyrin domain containing 3 (NLRP3) serves as an inflammasome sensor that causes pyroptotic cell death and proinflammatory cytokine release. Activation of NLRP3 is a two-step process: first, priming brings NLRP3 into a preactivated state, which can then respond to a second activating stimulus that induces inflammasome formation. While post-translational modifications of NLRP3 are known to contribute to NLRP3 priming, their significance in the priming and subsequent activation mechanism remains unclear. In this study, we aim to employ a random mutagenesis screen to identify amino acid residues crucial for NLRP3 priming regulation.

METHODS:We used the human BLaER1 monocyte model to generate a NLRP3-/- screening cell line. We optimized error-prone PCR (epPCR) to achieve a mutation frequency averagely corresponding to a single amino acid substitution per NLRP3 gene. EpPCR products were expressed in NEK7-/- x NLRP3-/- BLaER1 cells by transduction followed by FACS selection for a fluorescent marker on the expression plasmid. As a control, wild-type NLRP3 was expressed from the same screening vector.

RESULTS:We selected the BLaER1 NLRP3 knockout single clone with the most pronounced abrogation of cell death in response to stimuli, which is crucial for a successful screening process. With single amino acid substitution per gene on average, we conducted a pilot screen for NLRP3 mutants focusing on the region around the FISNA domain, which was shown to be critical for recruitment of NLRP3 to the trans-Golgi network and subsequent activation. Leveraging the unconstrained search space of cell death screens, we analyzed a multitude of NLRP3 mutants concurrently.

CONCLUSIONS:We successfully optimized pivotal steps in the NLRP3 mutagenesis screening strategy and performed a pilot screen to demonstrate the validity of our approach. Future work involves scaling up our screen and analyzing the identified mutations to unravel their functional significance in the NLRP3 priming and activation mechanisms.

Keywords: NLRP3, inflammasome, random mutagenesis screen

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A SESQUITERPENE LACTONE ESTAFIATIN INHIBITS THE ACTIVATION OF NLRP3 INFLAMMASOME AND ALLEVIATES COLLAGEN-INDUCED ARTHRITIS IN MICE

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OBJECTIVES:Estafiatin (EST), a new sesquiterpene lactone, is known to exert anti-inflammatory properties. In the present study, we evaluated whether EST inhibits the activation of NLRP3 inflammasome which is a crucial component of the innate immune system that mediates IL-1β activation in response to microbial infection and cellular damage.

METHODS:For NLRP3 inflammasome activation Murine bone marrow-derived macrophages (BMDMs) were primed with LPS (100 ng/ml) for 6 h followed by EST added with indicated concentrations for 30 min and then ATP (1 mM, 30 min), nigericin (5 μM, 30 min) and MSU (200 μg/ml, 4 h) were added and incubated.
In CIA model, Eight weeks old male wild-type mice on DBA/1 background were purchased from DBL. The single immunization mouse CIA model was established by using bovine type Ⅱ collagen (Chondrex, Woodinvile, WA, USA) and complete Freund's adjuvant containing 2 mg/ml of Mycobacterium tuberculosis H-37 RA (CFA; Chondrex)

RESULTS:We found that ATP-, nigericin- and MSU-induced release and cleavage of IL-1β was concentration-dependently suppressed by EST pretreatment in LPS-primed BMDMs, but not TNF-α. Moreover, EST significantly reduced the ATP-, nigericin- and MSU-induced ASC oligomerization. In mice model of collagen-induced arthritis (CIA), EST treatment improved the paw swelling and clinical score of arthritis. The levels of IL-1β in serum and homogenates of paw and spleen were lower in EST-treated mice than in control mice. Histopathological analysis revealed that EST treatment ameliorates joint inflammation and cartilage destruction in mice

CONCLUSIONS:Our results suggest that EST may alleviate rheumatoid arthritis by suppressing NLRP3 inflammasome activation.

Keywords: NLRP3 inflammasome, Estafiatin, Collagen-induced arthritis

P-065
General Research » Inflammasomes

AIM2 INHIBITING NANOBODIES DEFINE CELL-TYPE-SPECIFIC INFLAMMASOME RESPONSE TO POXVIRUS IN HUMAN PRIMARY CELLS

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OBJECTIVES:1. To evaluate inflammasome responses to poxvirus infection in human primary cells.
2. Generate nanobody based molecular tools to study inflammasomes.

METHODS:To visualize and quantify inflammasome responses, we established a recombinant vaccina virus (VACV) strain encoding an inflammasome reporter caspase-1CARD-EGFP (C1C-EGFP) and infected primary human macrophages, monocytes and keratinocytes. To identify the activated inflammasomes in primary cells, we utilized the NLRP3 inflammasome inhibitor drug CRID3 and generated AIM2-specific inhibitory nanobodies, i.e. single domain antibody fragments derived from camelid heavy chain-only antibodies as there were no commercially available AIM2 inhibitors. Recombinant VACV strains encoding antagonistic AIM2 nanobodies and C1C-EGFP were then engineered and used to infect primary cells followed by analysis at a single cell resolution by flow cytometry.

RESULTS:We identified several nanobodies that bind to AIM2PYD in the cytosol and interfere with its oligomerization, effectively inhibiting filament formation and subsequent activation of the AIM2 inflammasome. VACV strains encoding antagonistic AIM2 nanobodies revealed that VACV infection activates inflammasomes in most primary cell types: AIM2 inflammasome in GM-CSF macrophages and keratinocytes, but NLRP3 inflammasome in human monocytes. Moreover, we show that incoming genomes are detected, and that AIM2 activation requires prior cytokine priming of cells. Viral uncoating and early gene expression were critical for all inflammasome responses, while viral DNA replication was dispensable.

CONCLUSIONS:In conclusion, our findings highlight the intricate cell-type-specific regulation of inflammasomes activation which warrants further investigation to identify potential regulatory factors involved. The specific AIM2 inhibitory nanobodies we generated are valuable added tools in studying both inflammasome-dependent and -independent roles of AIM2.

Keywords: Inflammasomes, Poxvirus, Nanobodies, AIM2, NLRP3, Innate Immunity

P-066
General Research » Inflammatory cell death

NARNA-LL37 COMPOSITE DAMPS DEFINE STERILE NETS AS INTENTIONAL SELF-LIMITING DRIVERS OF INFLAMMATION

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OBJECTIVES:Neutrophil extracellular traps (NETs) are a key antimicrobial feature of cellular innate immunity mediated by polymorphonuclear neutrophils (PMNs). NETs trap and kill microbes but have also been linked to inflammation in atherosclerosis, arthritis, or psoriasis by unknown mechanisms. We here sought to fully characterize RNA released during NET formation as a novel NET component and putative DAMP

METHODS:Confocal microscopy, electron microscopy, ELISA and RT-qPCR were used to characterize NET-associated RNA (naRNA) and its effects on primary human neutrophils, primary human keratinocytes, stem-cell derived neutrophils, other cell types, organotypic human skin models and experimental psoriasis in vivo models. RNAseq was used to determine the composition of naRNA.

RESULTS:We here report that NET-associated RNA (naRNA) stimulated further NET formation in naïve PMNs via a unique TLR8-NLRP3-caspase-1-gasdermin D-dependent inflammasome pathway. Keratinocytes also responded to naRNA with expression of psoriasis-related genes (e.g. IL17, IL36) via atypical NOD2-RIPK signaling. In vivo naRNA drove skin inflammation, which was drastically ameliorated by genetic ablation of RNA sensing. The naRNA-LL37

‘composite DAMP’ was pre-stored in resting neutrophil granules, defining sterile NETs as intentionally inflammatory webs that amplify neutrophil activation. However, the activity of the naRNA-LL37 DAMP was transient and hence supposedly self-limiting under physiological conditions.

CONCLUSIONS:We identfied naRNA-LL37 as a novel composite DAMP intended to elicit transient inflammation in the wake of NET formation and TLR-NLRP3-mediated naRNA sensing as both potential cause of disease and new intervention target.

Keywords: Neutrophil extracellular trap, RNA, Toll-like receptors, NLRP3 inflammasome, DAMP

P-067

General Research » Inflammatory cell death

GASDERMIN ACTIVATION AND CELL DEATH TRIGGER STEROID-UNRESPONSIVE INFLAMMATION IN HUMAN MACROPHAGES EXPOSED TO CIGARETTE SMOKE AND LIPOPOLYSACCHARIDE

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OBJECTIVES:Lytic cell death is a trigger of chronic inflammation. Cigarette smoke (CS) is a major cause of lung diseases and corticosteroids are poorly effective in treating inflammation in smokers. CS inhibits macrophage TLR4-dependent pro-inflammatory transcriptional program while activating the TLR4-TRIF-Caspase-8 axis leading to gasdermin D/E activation and cell death. Consistently, cleaved GSDMD is increased in smokers alveolar macrophages. The present work aimed at evaluating the impact of corticosteroids on gasdermin activation, cell death and consequent inflammatory reactions in lipopolysaccharide (LPS)/CS extract (CSE)-treated macrophages.

METHODS:Human monocyte-derived macrophages (hMDMs) were exposed to CSE and LPS. After 24h, cell death (LDH release), caspase-8, -1, -3/7 activation (enzymatic assay and western blot, WB), gasdermin D/E cleavage (WB), IL-6, TNF and S100A8/9 release (ELISA) were evaluated. The impact of fluticasone propionate (FP) and dexamethasone (Dex) and cell death inhibition (zVAD/necrostatin 1) on these endpoints was evaluated. NF-κB-regulated genes and damage associated molecular patterns (DAMPs) expression was evaluated in alveolar macrophages from smokers (N=39) vs nonsmokers (N=49) (public datasets).

RESULTS:Alveolar macrophages from smokers displayed a general inhibition of NF-κB-regulated genes and DAMP upregulation, including HSPs, Galectin-3 and S100A9. In CSE-exposed hMDMs, LPS failed to induce TNF and IL-6 while it promoted caspase-8 activation, with consequent cleavage of caspase-1 and -3/7 and gasdermin D and E. This led to enhanced release of the DAMP S100A8/9 and cell death. Treatment with steroids failed to inhibit cell death as both FP and Dex were not able to inhibit caspase activation, gasdermin cleavage and S100A8/9 release. On the contrary, zVAD/Nec1 treatment inhibited caspase and gasdermin cleavage blocking the release of S100A8/9.

CONCLUSIONS:LPS/CSE-induced gasdermin activation, cell death and S100A8/9 release were not inhibited by steroids. This suggests to consider cell death and gasdermins as novel targets for treating steroid-resistant inflammation in smokers.

Keywords: macrophage; damage-associated molecular patterns; cigarette smoke; corticosteroids; chronic inflammation

P-068
General Research » Inflammatory cell death

UNRAVELING INTERCELLULAR CROSSTALK OF PYROPTOTIC AND BYSTANDER CELLS WITH PROTEOMICS

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OBJECTIVES:Inflammatory cell death orchestrates tissue homeostasis in response to infection and tissue malfunction. However, unresolved cell death is detrimental and leads to chronic inflammatory conditions such as metabolic disorders, neurodegenerative diseases, and cancer. A special type of programmed cell death, termed pyroptosis, triggers inflammation in a cell-autonomous and non-cell-autonomous fashion by releasing signals that alert bystander cells and the whole organism. These signals comprise cytokines such as interleukin (IL)-1beta and danger-associated molecular patterns (DAMPs). The immunogenicity of cell death is believed to correlate with their release. While the pro-inflammatory capacity of pyroptosis can be attributed to the release of IL-1 family proteins in many settings, sterile pathology independent of major cytokines has raised excitement. These observations suggest that yet undescribed endogenous intercellular signals contribute to sterile inflammatory conditions.

METHODS:Here we set out to explore the hypothesis that unexplored protein-mediated intercellular signaling circuits regulate cell death-mediated inflammation. We combine mass spectrometry (MS)-based proteomics methods, such as stable isotope labelling with amino acids in cell culture (SILAC) and cell-type-selective proteomics using engineered methionyl-tRNA-synthetaseL274G (MetRS*) to differentiate cell autonomous and non-cell autonomous inflammatory mechanisms of dying cells and their interplay with bystander cells

RESULTS:We further use biochemical and pharmacological strategies to overcome the challenge of identifying released proteins, such as the evolutionary conserved leaderless proteins IL-1 or HMGB1, which cannot be inferred computationally, among the plethora of released proteins. Systems biology approaches support us with interpretation of unexplored communication circuits between cell types contributing to sterile inflammation.

CONCLUSIONS:With these enabling tools, we dissect the complexity of simultaneously released cytokines and cell death-derived proteins during pyroptosis and the related proinflammatory cell death form necroptosis. Our study contributes to the understanding of immunogenicity differences between cell death modalities and may reveal the molecular origin of paracrine activities in bystander cells independent of known inflammatory signals.

Keywords: pyroptosis, inflammation, proteomics, intercellular signalling

P-069
General Research » Inflammatory cell death

NINJURIN 2 ACTS AS AN ENDOGENOUS REGULATOR OF NINJ1-MEDIATED CELL LYSIS

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OBJECTIVES:Ninjurin 1 (Ninj1) is a plasma membrane protein shown to execute the lytic rupture of cells following induction of necrotic cell death. During programmed cell death, plasma membrane Ninj1 oligomerizes to form amphipathic filaments that are capable of capping membrane edges and forming membrane lesions that release danger signals and drive inflammation. Ninjurin 2 (Ninj2) is a highly homologous protein that has been shown to form filaments in vitro that share similar architecture to Ninj1 filaments. However, it is unknown if Ninj2 interacts with Ninj1 or if it has the potential to lyse cells.

METHODS:We combine biochemistry, live cell imaging, super resolution microscopy and the manipulation of primary human and immortalized mouse cells to determine a role for Ninj2.

RESULTS:We show that Ninj2 can form filaments in the plasma membrane on its own and that is capable of permeabilizing cells, albeit with slower kinetics than Ninj1. Moreover, we observe Ninj2 biochemically interacting with Ninj1 and colocalizing by microscopy. In doing so, Ninj2 slows down Ninj1-mediated permeabilization as quantified by cell permeabilization during live cell imaging. We compare the filaments formed by Ninj2 alone to the heteromeric filaments of Ninj1 and Ninj2 by super resolution microscopy and describe the relative contribution of Ninj1 and Ninj2 to lysis in primary and immortalized cells.

CONCLUSIONS:In conclusion, we demonstrate the ability of Ninj2 to permeabilize cells and show that it plays a regulatory role for Ninj1-mediated lysis.

Keywords: cell death, inflammation

P-070
General Research » Inflammatory cell death

THE RAB11-FAMILY INTERACTING PROTEIN 2 IS A KEY REGULATOR OF THE NLRP3 INFLAMMASOME

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OBJECTIVES:The Rab11-family interacting protein 2 (FIP2) is an important regulator of endocytic trafficking. Pyroptosis is a type of cell death that upon a high level of inflammation is triggered by various pathological stimuli causing cellular damage. Here we show that FIP2 is a key regulator of NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome activation and subsequent macrophage cell death. Pyroptotic death is mediated by inflammasome assembly, which is accompanied by GSDMD cleavage and IL-1β release. The NLRP3 inflammasome is primed by LPS resulting in up-regulation of NLRP3 and pro-IL-1β (pro-interleukin-1β). During inflammasome activation, ASC (Apoptosis-associated speck-like protein containing a CARD -specks) assembles into a complex containing NLRP3, Caspase-1 and Pro-IL-1β. This large multimeric complex is termed the “ASC-speck", and serves as a platform for caspase-1 mediated cleavage of pro-IL-1β into IL-1β. Following ASC-speck formation GSDMD is cleaved by caspase-1 to form GSDMD-pores to allow IL-1β release before cell swelling and pyroptotic cell death. We have here investigated the role of FIP2 in NLRP3 inflammasome acitivation

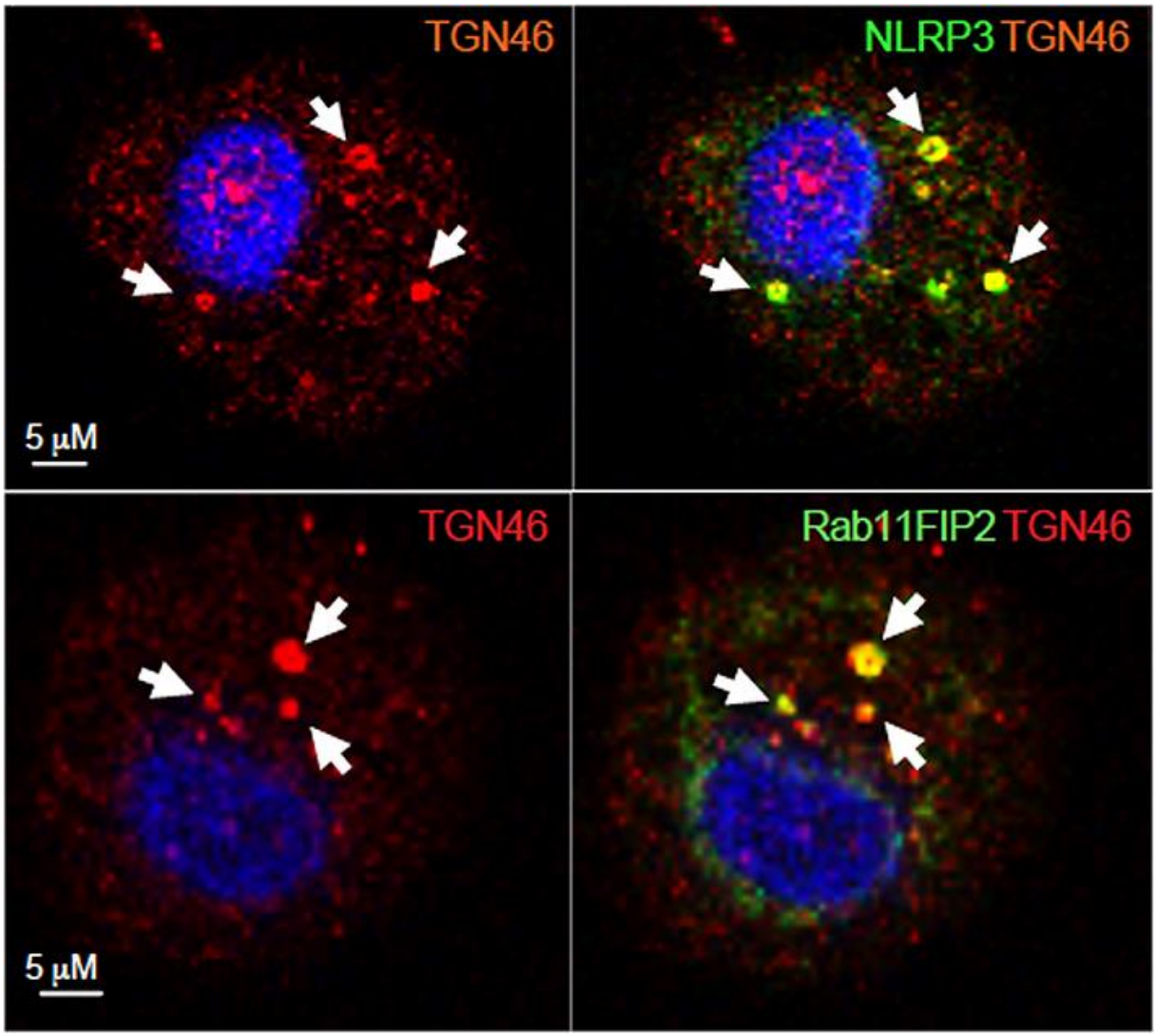
METHODS:Cell culture, immunostaining, confocal microscopy, co-immunoprecipitation, Western blotting, ELISA, LDH-assay, CRISPR-Cas9 gene editing and lentiviral co-expression of FIP2.

RESULTS:We found that FIP2 localizes to the ASC-specks and FIP2-depleted cells showed defective LDH and IL-1β release, GSDMD cleavage, IKKβ-activation and NLRP3 translocation to trans-Golgi. Furthermore, we show that FIP2 controls stability of NLRP3 and pro-IL-1β, and that FIP2 interacts with NLRP3 but not ASC. The FIP2 binding site in NLRP3 was found to be identical to its KMKK motif (KKKK in mouse) binding phosphatidylinositol-4-phosphate (PtdIns4P). FIP2 is also a PtdIns-binding protein, that we found it to bind NLRP3 through its n-terminal PtdIns- binding C2-domain.

CONCLUSIONS:We here demonstrate that FIP2 controls NLRP3 inflammasome assembly and activation by a direct interaction with NLRP3.

Keywords: Rab11-FIP2, NLRP3 inflammasome, trans-Golgi, ASC-speck, pyroptotic cell death

NLRP3 and Rab11FIP2 on dTGN



NLRP3 or Rab11-FIP2 location on TGN46 positive endosomes/dTGN following NLRP3 inflammasome activation

P-071
General Research » Inflammatory cell death

INHIBITING MEMBRANE RUPTURE WITH NINJ1 ANTIBODIES LIMITS TISSUE INJURY

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OBJECTIVES: The cell-surface protein NINJ1 plays an integral role in plasma membrane rupture (PMR), a phenomenon observed in cells undergoing pyroptosis or apoptosis. Although not essential for cell death, PMR contributes to the inflammatory response by releasing damage-associated molecular patterns (DAMPs) and activating immune cells. Reports suggest Ninj1 deficiency reduces the severity of pulmonary fibrosis and multiple sclerosis in mice. Genome wide association studies have also linked Ninj1 with reduced serum levels of two clinically important biomarkers of hepatocellular injury (liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST)). Therefore, we hypothesized that NINJ1 might play a role in hepatocellular PMR associated with liver injury.

METHODS:The role of NINJ1 in PMR following apoptotic stimuli was thoroughly studied in ex vivo experiments in BMDMs. Ninj1fl/fl Rosa26-creERT2 mice were generated to examine the role of NINJ1 in apoptosis-associated PMR in vivo. This enabled tamoxifen-inducible systemic Ninj1 deletion in adult animals, circumventing the developmental hydrocephalus observed in a substantial number of Ninj1-/- newborns. Following tamoxifen treatment, these mice were examined in several in vivo murine hepatocellular injury models.

RESULTS:Following exposure to either TNF combined with the transcriptional inhibitor D-galactosamine (D-Gal), or Concanavalin A (ConA), Rosa26-creERT2 control mice demonstrated extensive hepatocellular PMR, as evidenced by elevated serum ALT, AST, and LDH. Ninj1 deficiency eased hepatocellular PMR instigated by the same challenge. Correspondingly, significant reductions in serum levels of LDH, ALT, AST, and DAMPs including IL-18 and HMGB1 were observed.

CONCLUSIONS:Our studies have identified a genetically encoded regulator of plasma membrane rupture- NINJ1. NINJ1-mediated plasma membrane rupture is not limited to pyroptosis, but also occurs following other cell death stimuli, including apoptosis. Ninj1 deficiency limits PMR and proinflammatory DAMP release not only ex vivo, but also in vivo. Inhibiting NINJ1 may therefore be beneficial in managing diseases caused by excessive cell death.

Keywords: Pyroptosis, PMR

P-072
General Research » Inflammatory cell death

ANTAGONISTIC NANOBODIES REVEAL MECHANISM OF GSDMD PORE FORMATION AND UNEXPECTED THERAPEUTIC POTENTIAL

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OBJECTIVES:Inflammasome activation results in cleavage of the pore-forming protein gasdermin D (GSDMD) by pro-inflammatory caspases. As methods to study pore formation in living cells are limited, we raised nanobodies (VHHs) against human GSDMD. Nanobodies are single-domain antibodies derived from heavy-chain only antibodies present in camelids. Due to their small and specific nature, we also aimed to show their promise as a therapeutic.

METHODS:Nanobodies were selected using phage display and their intracellular binding to GSDMD was confirmed by LUMIER assays. Nanobodies were either expressed in macrophages or added recombinantly to the culture medium, upon which their molecular mechanism was further clarified using a biochemical and structural approach. To visualize GSDMD and the effect of the nanobodies on pore formation, we performed live cell confocal microscopy using nanobody-expressing macrophages or fluorescently-labelled recombinant nanobodies.

RESULTS:Cytosolic expression of VHH-1 and VHH-2 prevented oligomerization of GSDMD-NT, the release of IL-1β, and pyroptosis in human macrophages. These results are further corroborated by the structure of GSDMD in complex with VHH-2. The nanobody-stabilized GSDMD-NT monomers partitioned predominantly into the plasma membrane. Inhibition of GSDMD pore formation switched cell death from pyroptosis to apoptosis, driven by the enhanced caspase-1 activity observed in the absence of pores. Interestingly, recombinant nanobodies added to the medium also prevented cell death by pyroptosis. Using live cell microscopy, we could show that nanobodies enter cells through initial GSDMD pores, thereby curtailing the assembly of additional pores.

CONCLUSIONS:We generated two pyroptosis-inhibiting nanobodies and show that membrane insertion of GSDMD-NT does not require oligomerization or prepore formation. This revealed that the plasma membrane was the first target of GSDMD pore formation. Furthermore, we discovered a novel layer of regulation of caspase-1 activity by GSDMD pores. GSDMD nanobodies may also be suitable to treat the ever-growing list of diseases caused by activation of (non-)canonical inflammasomes.

Keywords: GSDMD, pyroptosis, nanobodies, apoptosis, inflammasome, cell death

P-073
General Research » Inflammatory cell death

VIRAL DOUBLE-STRANDED RNA MIMETIC POLY(I:C) INDUCES LYTIC CELL DEATH BY A NLRP3-INDEPENDENT PATHWAY IN HUMAN MACROPHAGES

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OBJECTIVES:RNA viruses can cause massive inflammatory reactions that can impair host's functions. One of the common Pathogen-Associated Molecular Patterns (PAMPs) of these viruses is double-stranded RNA (dsRNA). Type I IFNs release and cellular death are the main responses mounted by human macrophages against viral dsRNA and uncontrolled responses of this type may lead to excessive inflammation and tissue damage. However, the mechanisms underlying RNA virus-induced cell death remain poorly undefined. The main objective of this study is to define the role of pro-apoptotic and/or pro-pyroptotic factors (such as NLRP3, Caspase-1 and Gasdermins) in cell death caused by viral dsRNA.

METHODS:Human Monocyte-derived Macrophages (hMDMs) were treated with the dsRNA mimetic Polyinosinic-polycytidylic acid (Poly (I:C)) complexed or not with lipofectamine 3000. IFN-β and IL-1β expression and release were evaluated by RTqPCR and ELISA assay, respectively. Lytic cell death was assessed by LDH assay, and the active forms of apoptotic and pyroptotic caspases (Caspase-8, -3 and -1) as well as GSDME and IL-1β cleavage were detected by Western Blot. NRLP3 gene expression was evaluated by RTqPCR. ASC-Speck formation, nuclear morphology and mitochondrial membrane potential were evaluated by fluorescence microscopy.

RESULTS:Intracellular delivery of Poly(I:C) was required to induce IFN-β expression and release. Cytosolic Poly (I:C) induced apoptotic features such as nuclear fragmentation/condensation and mitochondrial membrane depolarization and led to lytic cell death. We found that cytosolic Poly(I:C) activated Caspase-8,-3, GSDME-axis, an alternative pathway leading to inflammasome-independent pyroptosis secondary to apoptosis. Consistently, intracellular Poly (I:C) did not induce NLRP3 inflammasome activation, as demonstrated by the lack of NLRP3 expression, ASC-Speck formation, Caspase-1 activation and IL-1β release.

CONCLUSIONS:These findings describe a pathway of caspase-dependent pro-inflammatory death in macrophages, in response to Poly(I:C) stimulation, which does not involve activation of the canonical NLRP3 inflammasome. Future studies will identify the viral dsRNA-binding receptors involved in triggering this pathway.

Keywords: Poly (I:C); Human Macrophages; Caspases; Gasdermins; Pyroptosis.

P-074
General Research » Inflammatory cell death

STING AGONIST INDUCED-MONOCYTE DEATH COMBINES APOPTOSIS, PYROPTOSIS AND CASPASE 8 ACTIVATION

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OBJECTIVES:The cyclic-GMP-AMP synthase – stimulator of interferon genes (cGAS-STING) pathway is important for recognition of double-stranded DNA, a marker of infection or cell damage. The cGAS-STING pathway activation leads to production of proinflammatory cytokines by immune cells that then modulate subsequent immune responses with antiviral and antitumoral properties. Consequently, compounds that activate the cGAS-STING pathway are of therapeutic interest.
We recently described that STING agonists also trigger cell death of monocytes. Therefore, we characterized monocyte cell death in terms of apoptosis, pyroptosis and necroptosis.

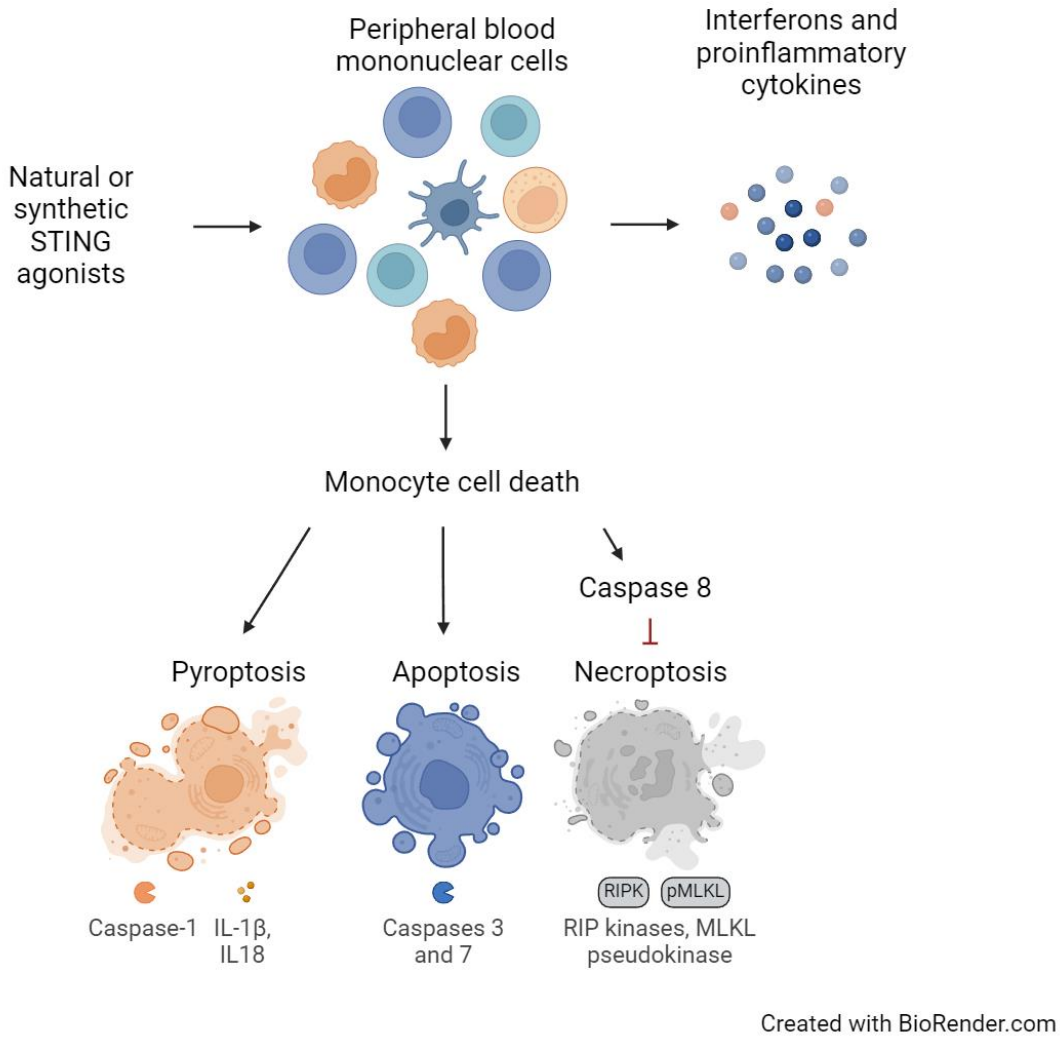
METHODS:Monocyte cell death was characterized by monitoring the activation of caspases 3, 7, 1 and 8, along with kinases RIP1, RIP3 and MLKL. The induction was assayed either in peripheral blood mononuclear cells (PBMCs) using multiparametric flow cytometry-based immunophenotyping with FAM-FLICA staining of active caspases, or phospho-flow, or in enriched monocytes (to demonstrate the direct effect of STING agonists) using reporter-based assays and western blot. The secretion of cytokines was analyzed using multiplex assay with Luminex technology.

RESULTS:STING agonists trigger secretion of a broad cytokine portfolio in PBMCs. STING agonists induced activation of apoptotic caspases 3 and 7, and of pyroptotic caspase 1 already 4 hours after treatment. However, necroptotic cascade was not involved as no phosphorylation of RIP kinases or MLKL pseudokinase was detected. We suggest that necroptosis was blocked by the activation of caspase 8, as we detected cleavage of RIP1 kinase upon STING agonist treatment.

CONCLUSIONS:The cGAS-STING pathway activation in PBMCs leads to proinflammatory cytokine secretion accompanied by a rapid monocyte cell death. The monocytes undergo regulated cell death featuring activation of both apoptosis and pyroptosis, while necroptosis is likely inhibited by activation of caspase 8. We propose that such immunogenic cell death may be an important immunoregulatory feedback loop for rapid inhibition of proinflammatory cytokine secretion and activation of subsequent immune processes.

Keywords: apoptosis, pyroptosis, necroptosis, STING, cytokines, monocytes

STING agonist-induced cell death of monocytes



The cGAS-STING pathway activation in PBMCs leads to proinflammatory cytokine secretion accompanied by a rapid monocyte cell death. The monocytes undergo regulated cell death featuring activation of both apoptosis and pyroptosis, while necroptosis is likely inhibited by activation of caspase 8.

P-075
General Research » Inflammatory cell death

CARD9 CONTROLS THE MICROGLIA RESPONSE TO NEURONAL DAMAGE

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OBJECTIVES:Our objective was to study which pattern recognition receptor (PRR) families and signaling pathways play a role in the primary microglial response to localized neuronal damage.

METHODS:We performed a facial nerve axotomy (FNA) on wild-type (WT), Card9, and ASC (Pycard)-deficient mice after which neurons in the facial motor nucleus degenerate. In response, microglia get activated locally. We studied the immune response in the tissue using immunohistochemistry. In vitro, we used primary macrophages, microglia, and cortical neurons to study molecular pathways of interest more closely.

RESULTS:1) There was no difference in microglial activation between WT and mice and mice deficient for the central inflammasome component ASC 7 days post-axotomy.
2) There was lower microglial activation in Card9-deficient mice compared to WT 7 dpa.
3) There was no difference in microglial proliferation in Card9-deficient mice compared to WT after FNA.
4) Treating primary macrophages or microglia with damaged primary neurons *in vitro* without additional intervention did not lead to inflammatory activation of macrophages/microglia.

CONCLUSIONS:Card9 controls the microglial activation in response to neurodegeneration, indicating ITAM-coupled receptors such as C-type lectins are PRRs of interest in this context. The lack of response to damaged neurons by macrophages and microglia *in vitro* could indicate that tissue context is important. Given that *in vivo* there was no difference in microglia proliferation with Card9-deficiency and given the dynamics in microglia numbers over time we observed after FNA, we hypothesize that Card9 plays a role in the survival of microglia in the inflammatory context. We are exploring this hypothesis further with immunohistochemistry and single nucleus RNAseq after FNA as well as in ongoing *in vitro* experiments.

Keywords: Card9, pattern recognition receptor (PRR), microglia, facial nerve axotomy (FNA), neurodegeneration

Figure 1

Fig 1 – Facial motor nucleus after FNA over time. Green signal shows NeuN (neuronal marker), purple signal shows Iba-1 (microglial marker). (dpa = days post-axotomy)

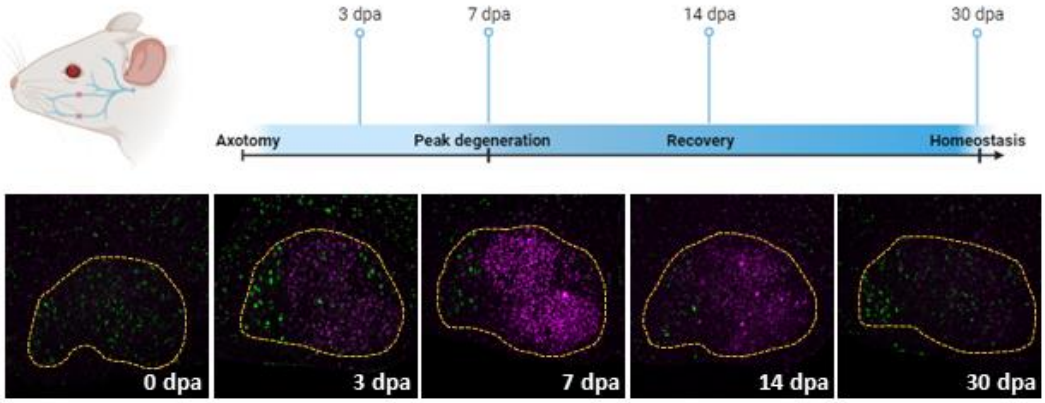
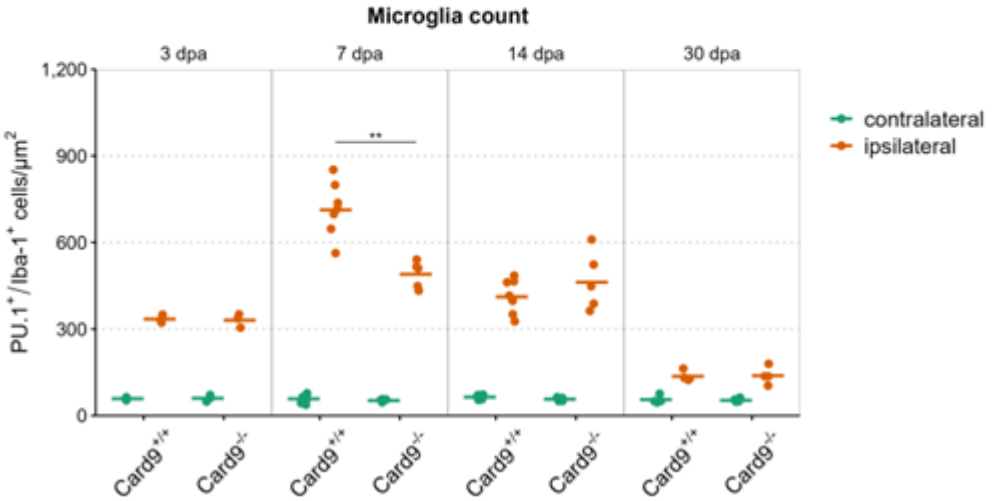


Figure 2

Fig 2 – Microglia count after FNA in Card9-deficient mice. After FNA, mouse brains were stained for NeuN, PU.1 and Iba-1 and counterstained with HOECHST. Microglia within the facial motor nucleus (based on NeuN staining) with a healthy-looking nucleus were counted when positive for PU.1 and Iba-1. (** = p-value < 0.01, dpa = days post-axotomy)



P-076
General Research » Inflammatory cell death

EXTRACELLULAR CALCIUM TRIGGERS THE RELEASE OF EXTRACELLULAR DNA TRAPS IN PARALLEL WITH INFLAMMASOME ACTIVATION IN HUMAN MONOCYTE DERIVED MACROPHAGES

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OBJECTIVES:Extracellular DNA traps (ET's) are long DNA filaments that have evolved to kill microbes. The ET composition and the mechanism of its release are best characterised in neutrophils. In comparison to neutrophils, ET release in macrophages have received limited attention and their essential characteristics are not clearly defined. We investigated the capacity of human macrophages to release ETs in response to extracellular calcium and lipopolysaccharide (LPS).

METHODS:Human monocyte derived macrophages (hMDM) were primed with LPS and activated with extracellular calcium in the presence or absence of the inhibitors. ET release and inflammasome activation was investigated via the confocal microscopy. ET release was quantified by pico-green assay and matrix metalloproteinase12 (MMP12) protein expression and histone3 citrullination was analysed via immunoblotting.

RESULTS:We show that extracellular calcium and LPS enables hMDM to release extracellular DNA decorated with citrullinated histone, myeloperoxidase (MPO) and MMP12. Importantly, extracellular DNA release was initiated alongside with ASC aggregation and IL-1ß maturation, indicating inflammasome activation. In contrast to neutrophils, stimulation with the microbial TLR4 agonist LPS alone did not induce extracellular DNA release in hMDM. The ability of macrophages differentiated with either gm-CSF or m-CSF to release ET's was unaffected, however, only gm-CSF differentiated MDM's expressed MMP12 and the ET's released by the latter had more bactericidal activity towards E. coli. Mechanistically, inhibition of phospholipaseC and PAD2 suppress ET release. Interestingly, ET release was reduced along with histone3 citrullination by inhibiting the NLRP3 inflammasome. Finally, GSDMD mediated pore formation was dispensable for ET release but was instead completely blocked by plasma membrane stabilization.

CONCLUSIONS:Altogether, we demonstrate that extracellular calcium activated hMDM extrude DNA, containing citrullinated histones, MPO, MMP12, ASC specks and released ET's kill bacteria. We believe that calcium-activated hMDM add a physiologically relevant condition to cell death triggered by calcium ionophores that may be important in autoimmunity.

Keywords: ASC: apoptosis-associated speck-like protein containing a caspase recruitment domain, ET's: extracellular DNA traps, GSDMD: gasdermin D, gm-CSF: granulocyte-macrophage

colony-stimulating factor, LPS: lipolysaccharide, ROS: reactive oxygen species

P-077
General Research » Inflammatory cell death

KUPFFER CELL DEATH IS IMPLICATED IN THE PATHOGENESIS OF HIGHLY PATHOGENIC TICK-BORNE BUNYAVIRUSES

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OBJECTIVES:Bunyaviruses include highly pathogenic agents for humans, such as Crimean-Congo hemorrhagic fever (CCHF) virus. Strong inflammatory responses designated cytokine storm has been implicated in the CCHF pathogenesis. The aim of our study is to clarify the mechanism of the CCHF pathogenesis in a mouse model and provide potential treatments for CCHF.

METHODS:We performed *in vivo* infection with Hazara virus as a model pathogen of CCHF virus and CCHF virus itself. We mainly utilized type I interferon α/β receptor 1 (IFNAR1) knockout (KO) mice as a lethal animal model of CCHF. We also analyzed cell-type specific IFNAR1 KO mice to further investigate the mechanistic basis of the pathogenesis.

RESULTS:During RNA virus infections, the mitochondrial antiviral signaling protein (MAVS) is a crucial molecule for inducing type I interferons and inflammatory cytokines. Hazara virus-infected IFNAR1 KO mice showed weight loss, MAVS-dependent cytokine storm and lethality associated with liver injury. Interestingly, the Hazara virus-induced liver disease onset in IFNAR1 KO mice was significantly delayed by additional MAVS deficiency, but the lethality was not cancelled. These findings suggest that liver dysfunction contributes to the lethality of Hazara virus infection and that cytokine storm facilitates liver dysfunction but is not essential for its initiation. Hepatocytes and Kupffer cells have been reported as CCHF virus-antigen-positive cells in patients and a mouse model. Notably, hepatocyte-specific IFNAR1 KO mice were resistant to the infection with both CCHF virus and Hazara virus, while macrophage-specific IFNAR1 KO mice showed susceptibility and liver injury along with Kupffer cell death, indicating that Kupffer cell death leads to liver dysfunction and determines the lethality.

CONCLUSIONS:Our study suggests that Kupffer cell death is a potential target of treatments for CCHF. We currently investigate the mechanism of CCHF virus-mediated Kupffer cell death and are exploring treatments targeting the cell death for CCHF in a mouse model.

Keywords: Virus, Inflammation, Cytokine storm, Cell death, Kupffer cell

P-078
General Research » Inflammatory cell death

RAPTINAL INDUCES WIDESPREAD MEMBRANE DAMAGE AND CELL DEATH ASSOCIATED INFLAMMATION

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OBJECTIVES:Raptinal is a small molecule rapidly inducing intrinsic apoptosis, independently of BAX, BAK and BOK, suggesting a novel mode of mitochondrial outer membrane permeabilization (MOMP). However, whether other cell death pathways may be involved in raptinal-induced cytotoxicity remains uncharacterized. Here, we investigated additional cell death modalities potentially induced by raptinal.

METHODS:Targeting apoptosis-associated and autophagy-related genes using CRISPR/Cas9 technology in combination with various compounds, we investigated raptinal-induced cell death by microscopy, immunoblotting and cell death readouts. Moreover, we explored raptinal-induced cell-death associated inflammation in differentiated monocytes using interleukin 6 (IL-6) ELISA.

RESULTS:Raptinal induces rapid MOMP, independently of the BAX/BAK, CASP8/BID or Cathepsin-BID axis, and does not require mitochondrial calcium overload to induce apoptosis. In parallel to apoptosis, raptinal also induces cytoplasmic vacuolization and a paraptotic cell death, which is particularly pronounced in CASP9-deficient cells, likely due to strong perturbation of the endoplasmic reticulum membrane structure. In addition, raptinal induces V-ATPase-dependent unconventional autophagy likely due to lysosomal permeabilization. Furthermore, we observe strong IL-6 release upon raptinal stimulation in differentiated monocytes.

CONCLUSIONS:Due to its unique amphipathic property, raptinal may cause widespread membrane damage and multiple independent cell death modalities, including apoptosis, paraptosis, and lysosomal cell death. The non-apoptotic cell death induced by raptinal may cause an inflammatory response in monocytes.

Keywords: cell death, apoptosis, paraptosis, inflammatory cell death, raptinal, lysosomal cell death

P-079
General Research » Inflammatory cell death

HIJACKING OF HOST CELL DEATH SIGNALING BY SALMONELLA TYPHIMURIUM REVEALS NOVEL THERAPEUTIC OPPORTUNITIES TO FIGHT INTRACELLULAR BACTERIAL PATHOGENS

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OBJECTIVES:We show the different types of cell deaths involved in the homeostasis of neutrophils and macrophages in basal and upon Salmonella enterica serovar Typhimurium (ST) infection, and the contribution of each cell death pathway to bacterial host susceptibility.

METHODS:Nlrp3, Gsdme and Ripk3 were inactivated using CRISPR-Cas9 technology. The inflammasome of either neutrophil or macrophages were specifically activated or inhibited by wt and dominant negative (DN) forms of Gbp4 and Asc. Embryos were also treated by bath immersion with DMSO, Z-DEVD-FMK (CASP3 inhibitor) or Necrostatin 2 racemate (RIPK1 inhibitor). Total number of cells was counted in each larva. The number of dead larvae were analyzed from 1-5 days post-infection.

RESULTS:In homeostatic conditions pyroptosis, apoptosis and necroptosis concomitantly operated to regulate the clearance of innate immune cells. Although ST infection initially induced emergency myelopoiesis, it was able to kill neutrophils and macrophages via T3SS. Genetic and pharmacological inhibition of Nlrp3, Gsdme, Ripk1/3 and Casp3 revealed that while ST promoted pyroptotic and apoptotic cell death of neutrophils, it killed macrophages through necroptosis. Inhibition of either apoptosis or pyroptosis rescued ST-induced neutrophil cell death. Although neutrophils from Nlrp3- and Gsdme-deficient larvae, and larvae with inactivated neutrophil inflammasome, were resistant to ST-mediated killing, they were hypersusceptible to infection. Activation of neutrophil or macrophage inflammasome by overexpressing wt Gbp4 robustly increased larval resistance to ST and neutrophil number, but failed to prevent neutrophil killing by ST. Furthermore, the increased resistance to ST of larvae overexpressing Gbp4 in neutrophils and macrophages was dependent on Nlrp3/Gsdme inflammasome. Finally, activation of neutrophil inflammasome in Gsdme-deficient larvae led to neutrophil cell death through apoptosis and necroptosis.

CONCLUSIONS:Our results revealed the complexity of the cell death mechanisms in innate immune cells, and their hijacking by intracellular bacterial pathogens and suggest the potential benefits of combination therapy targeting cell death signaling pathways.

Keywords: Inflammasome, Cell death, Zebrafish

P-080
General Research » Inflammatory cell death

S-PALMITOYLATION OF GASDERMINS REGULATE THEIR MEMBRANE LOCALIZATION AND PORE FORMATION

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OBJECTIVES:Gasdermin D (GSDMD)-mediated pyroptotic cell death drives inflammatory cytokine release and downstream immune responses upon inflammasome activation, which play important roles in host defense and inflammatory disorders. Upon activation by proteases, the GSDMD N-terminal domain (NTD) undergoes oligomerization and membrane translocation in the presence of lipids to assemble pores. Currently, the regulatory mechanisms for GSDMD pore-formation remain poorly understood.

METHODS:HEK293T and THP-1 cell lines were our chosen models for assessing pore formation by gasdermins. Acyl biotin exchange (ABE) assay was used to analyze S-acylation of gasdermins, in conjunction with inhibitors for palmitoyl transferases to investigate potential palmitoylation events. Pyroptotic cell death was measured by lactate dehydrogenase (LDH) cytotoxicity assay and membrane permeabilization by propidium iodide uptake. IL-1β release was assessed by ELISA and gasdermin protein localization was investigated by fluorescence imaging and subcellular fractionation.

RESULTS:Here we report that a conserved residue Cys191 in human GSDMD was S-palmitoylated, which promoted GSDMD-mediated pyroptosis and cytokine release. Mutation of Cys191 or treatment with palmitoyl transferase inhibitors suppressed GSDMD localization to the membrane and dampened pyroptosis or IL-1β secretion. By contrast, introduction of exogenous palmitoylation motifs restored pyroptosis to the C191A mutant. Furthermore, other gasdermins such as GSDMA are also S-palmitoylated, which regulates its localization to different subcellular organelles.

CONCLUSIONS:Our data suggest that S-palmitoylation of gasdermins is a shared regulatory mechanism for membrane translocation by this family of pore-forming proteins.

Keywords: Gasdermin, Pyroptosis, S-palmitoylation, Membrane localization

P-081
General Research » Inflammatory cell death

THE RIPK1 DEATH DOMAIN RESTRAINS ZBP1- AND TRIF-MEDIATED CELL DEATH AND INFLAMMATION

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OBJECTIVES:RIPK1 is a multi-functional kinase that regulates cell death and inflammation and has been implicated in the pathogenesis of inflammatory diseases. Comprising a kinase domain, an intermediate domain harboring a RIP homotypic interaction motif (RHIM), and a death domain (DD), RIPK1 promotes necroptosis and apoptosis in a kinase-dependent manner, whereas its RHIM regulates ZBP1-mediated necroptosis. How the RIPK1 DD regulates cell death and inflammation remains poorly understood. Here we studied the function of the RIPK1 DD *in vivo* using genetically engineered mouse models.

METHODS:We generated knock-in mice expressing RIPK1 with a mutation (R588E) that disrupts DD-dependent interactions and used them to study the function of the RIPK1 DD on apoptosis, necroptosis and inflammation. We employed genetic, immunohistological, flow cytometric, molecular and biochemical approaches to investigate the effects of DD mutation *in vivo*. In addition, we used biochemical, cell biological and molecular methodologies to study the role of the RIPK1 DD in primary cells and the underlying mechanisms.

RESULTS:Genetic studies showed that Ripk1R588E/R588E mice developed perinatal lethality that was induced by ZBP1-mediated necroptosis and postnatal inflammatory pathology that was mediated by necroptosis-independent TNFR1, TRADD and TRIF signaling. Biochemical studies revealed a critical role of DD-dependent interactions in regulating the function of RIPK1 downstream of TNFR1, ZBP1 and TRIF, and elucidated the underlying mechanisms.

CONCLUSIONS:Our results revealed a critical physiological role of DD-dependent RIPK1 signaling in controlling necroptosis and apoptosis that is important for the regulation of tissue

homeostasis and inflammation.

Keywords: RIPK1, Death domain, ZBP1, TRIF, Necroptosis

P-082
General Research » Inflammatory cell death

TARGETING RIPK1 CAN DRIVE STRONG IMMUNOGENIC CELL DEATH IN CANCER

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OBJECTIVES:Inducing a robust immunogenic response against tumours is crucial for achieving effective treatment. A key step in this direction involves activating innate immune pathways. Toll-like receptor 3 (TLR3) belongs to the family of toll-like receptors, which are pattern recognition receptors (PRRs) associated with innate immunity. TLR3 recognises double-stranded RNA (dsRNA) from viruses or endogenous retroviral elements (ERVs), triggering a strong immune response upon activation. Furthermore, its activation can initiate cell death, enhancing the potential for a potent immunogenic response.
Using small Protac molecules that target RIPK1, we demonstrate the key role of this kinase scaffold in regulating TLR3 pathway. Moreover, cancer cells can exploit RIPK1 to evade cell death mechanisms, promote tumour growth and drive immunosuppressive chemokine signalling. Therefore, activating TLR3 pathway in the absence of RIPK1 can trigger immunogenic cell death in cancer.

METHODS:In vitro: primary and cancer cell lines, western blot, immunoprecipitation, cell death assays, RT-PCR, proteomics
In vivo: E0771 breast cancer model

RESULTS:TLR3 drives necroptosis in absence of RIPK1 in macrophages and cancer cells. Targeting cancer with TLR3 agonists and RIPK1 degraders can induce potent immunogenic response.

CONCLUSIONS:We find that targeting RIPK1 for degradation combines well with TLR3 activation that drives the induction of TNF and IFNs, which sensitise cancer cells to non-canonical activation of RIPK3.

Keywords: RIPK1, TLR3, dsRNA, necroptosis, cell death

P-146
General Research » Inflammatory cell death

BLOCKING GDF-15: A THERAPEUTIC INTERVENTION IN PANCREATIC AND BRAIN CANCER?

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OBJECTIVES:GDF-15, a TGF-β family cytokine is known to be involved in several homeostatic and pathological conditions. Amongst pathological conditions, GDF-15 has been found to be highly expressed in various cancers. Prostaglandin E2 (PGE2), Cyclooxygenase 2 metabolite has been widely known for its cardinal role in cancer cell proliferation, migration, angiogenesis, metastasis, disease progression through the involvement of PI3K/AKT/GSK-3β/β-catenin signalling pathways. Tumor associated macrophages, neutrophils (TAN), fibroblasts are known players which confer immunosuppressive milieu and impart anti-tumor activities through PGE2. Interestingly, GDF-15 also activates the PI3K/AKT/GSK-3β/β-catenin signalling pathways. Pancreatic and brain cancers are the deadliest of all cancers with high GDF15 expression. Infact, GDF-15 showed a strong correlation with immune checkpoint inhibitors in glioblastoma patients and inhibiting GDF-15 bettered anti-PD-1 immunotherapy with concomitant enhanced cytotoxic T cell infiltration in pancreatic cancer mouse model. Glioblastoma patients are also noted to have reduced ROS producing TAN population (with enhanced longevity), and high expression of PD-L1, ensuing in ferroptosis inhibition with secretion of TNFα / Cerruloplasmin (ferroptosis inhibitor). Notably, GDF-15 is known to inhibit ferroptosis by inducing NRF2 pathway.
The neural innervation has played another important role in the progression of these cancers with the productions of various neurotrophins and neurotransmitters. β-adrenergic receptor signalling has been known to contribute to accelerated tumorigenesis with enhanced CD8T cell exhaustion. Also, GDF-15 is known to induce β- adrenergic receptor signalling.
Cancer associated thrombosis (CAT) has been heavily linked with pancreatic cancer and brain cancer, leading to poor prognosis. GDF-15 has been positively related to thromboembolic events across various cancer patients.

METHODS:as per published literature

RESULTS:Laboratory experiments and further clinical trials need to performed to know the outcome

CONCLUSIONS:Summing up, blocking GDF-15 might be an attractive cancer therapy by inhibiting PGE2, neural innervation and CAT, while promoting ROS production (ferroptosis and pyroptosis induction).

Keywords: GDF-15, Cancer, PGE2, Ferroptosis, Pyroptosis, CAT

P-083

General Research » Innate immunity to parasites

DEVELOPMENTAL ENDOTHELIAL LOCUS 1 (DEL-1) PROMOTES AN IMMUNOPROTECTIVE PHENOTYPE MEDIATED BY MACROPHAGES IN VISCERAL LEISHMANIASIS

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OBJECTIVES:Identification of tissue-derived homeostatic molecules regulating immune plasticity in different organs is seminal for understanding the potential of macrophages in contributing to immune responses to intraphagosomal pathogens.
The intraphagosomal parasite Leishmania spp. causes the neglected tropical disease leishmaniasis that affects millions of people. Leishmaniasis manifests in various clinical forms ranging from self-healing cutaneous lesions to potentially fatal visceral infections. Leishmania parasites have developed ways of immune evasion allowing them to avoid destruction and instead they establish a replicative niche within phagocytes.
Developmental endothelial locus-1 (DEL-1) is a functionally versatile homeostatic factor with anti-inflammatory and pro-resolving properties, but its potential role in immune responses to infections has not been previously addressed.
Here, we establish DEL-1 as a novel immune modulator to intraphagosomal pathogens using an in vivo model of visceral leishmaniasis induced by L. donovani.

METHODS:In vivo infection was performed by injecting intravenously parasites. Parasitic burden was assessed using in vivo imaging and histology, while immune cell activation was characterised using flow cytometry.

RESULTS:A two-fold increase in parasitic burden in Del1-KO mice was demonstrated at the peak of infection with in vivo imaging and histology. Consistently, Del1-KO mice had a significantly greater proportion of mature granulomas (defined by area) compared to WT mice. Lack of DEL-1 resulted in enhanced inflammatory response in infected liver as demonstrated by the increased levels of CD4+IFNg+ T cells and inflammatory Ly6Chigh monocyte/macrophage population that produces iNOS, MHC-II and TNF. Of note, parasite burden and formation of large granulomas was decreased in transgenic mice overexpressing DEL-1 in macrophages, but not in those overexpressing DEL-1 in endothelial cells.

CONCLUSIONS:Our findings reveal a hitherto unknown role of DEL-1 in immune response to intraphagosomal pathogens and therefore DEL-1 may represent a novel approach to mitigate immunopathology to leishmaniasis.

Keywords: DEL-1, macrophages, intracellular parasites, intraphagosomal pathogen, granuloma

P-084
General Research » Innate immunity to parasites

DEFICIENCY IN IFN-G, BUT NOT IFN I, SIGNAL ALTERS NK CELL AND MACROPHAGE ACTIVATION, LEADING TO LETHAL SCRUB TYPHUS IN MICE WITH SKIN ESCHAR LESIONS

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OBJECTIVES:Scrub typhus is an acute febrile disease due to Orientia tsutsugamushi (Ot) infection and can be life-threatening with organ failure, hemorrhage, and fatality. Yet, little is known as to how the host reacts to Ot bacteria at early stages of infection; no reports have addressed the functional roles of type I versus type II interferon (IFN) responses in scrub typhus.

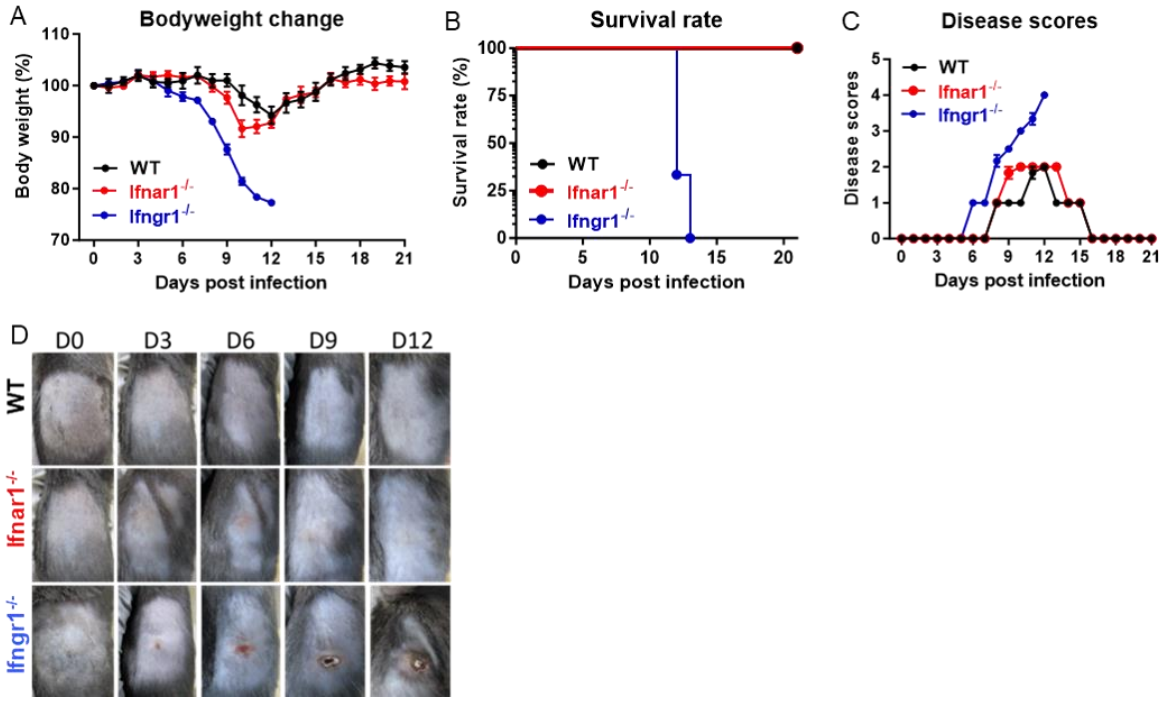
METHODS:In this study, we used comprehensive intradermal (i.d.) inoculation models and two clinically predominant Ot strains (Karp and Gilliam) to uncover early immune events.

RESULTS:Karp infection induced sequential expression of Ifnb and Ifng in inflamed skin and draining lymph nodes at days 1 and 3 post-infection. Using Ifnar1-Ifngr1 double knockout and Stat1-/- mice, we found that deficiency in IFN/STAT1 signaling resulted in lethal infection with profound pathology and skin eschar lesions, that resembled to human scrub typhus. Further analyses demonstrated that deficiency in IFN-γ, but not IFN-I, resulted in impaired NK cell and macrophage activation and uncontrolled bacterial growth and dissemination, leading to metabolic dysregulation, excessive inflammatory cell infiltration, and exacerbated tissue damage. NK cells were found to be the major cellular source of early IFN-γ, contributing to the initial Ot control. In vitro studies with dendritic cell cultures revealed a superior antibacterial effect offered by IFN-γ than IFN-b.

CONCLUSIONS:Comparative in vivo studies with Karp- and Gilliam-infection revealed a crucial role of IFN-γ signaling in protection against progression of eschar lesions and Ot infection lethality. Additionally, our i.d. mouse models of lethal infection with eschar lesions are promising tools for immunological study and vaccine development for scrub typhus.

Keywords: Type I and II IFNs, NK cell, Macrophage, Intradermal inoculation model, Scrub typhus, Eschar formation.

Soong _id models for single KO



Functorial IFN-g, but not IFN-I, responses are required for host control of skin-inoculated Orientia infection.

P-086
General Research » Innate immunity to parasites

TOXOPLASMA GONDII EXPOSURE PROMOTES IL12B TRANSCRIPTION VIA IRAK1-IRF5 IN DENDRITIC CELLS

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OBJECTIVES:Primary resistance to *Toxoplasma gondii* infection is mediated by interleukin (IL)-12, produced by conventional dendritic cells (cDCs), inflammatory monocytes (iMOs) and monocyte-derived dendritic cells (MODCs). These cells recognize parasite structures via endosomal Toll-like receptors (TLRs), and signal transduction is mediated by the myddosome, a complex containing myeloid differentiation primary response 88 (MyD88), Interleukin-1 receptor associated kinase (IRAK)-4, all of which are essential in the resistance against the parasite, in addition to IRAK1 or IRAK2. However, many of the signaling events linking endosomal TLR activation and IL-12 production upon exposure to *T. gondii* remains poorly understood, including which transcription factors drive *Il12b* transcription and whether signaling is mediated by IRAK1 or IRAK2.

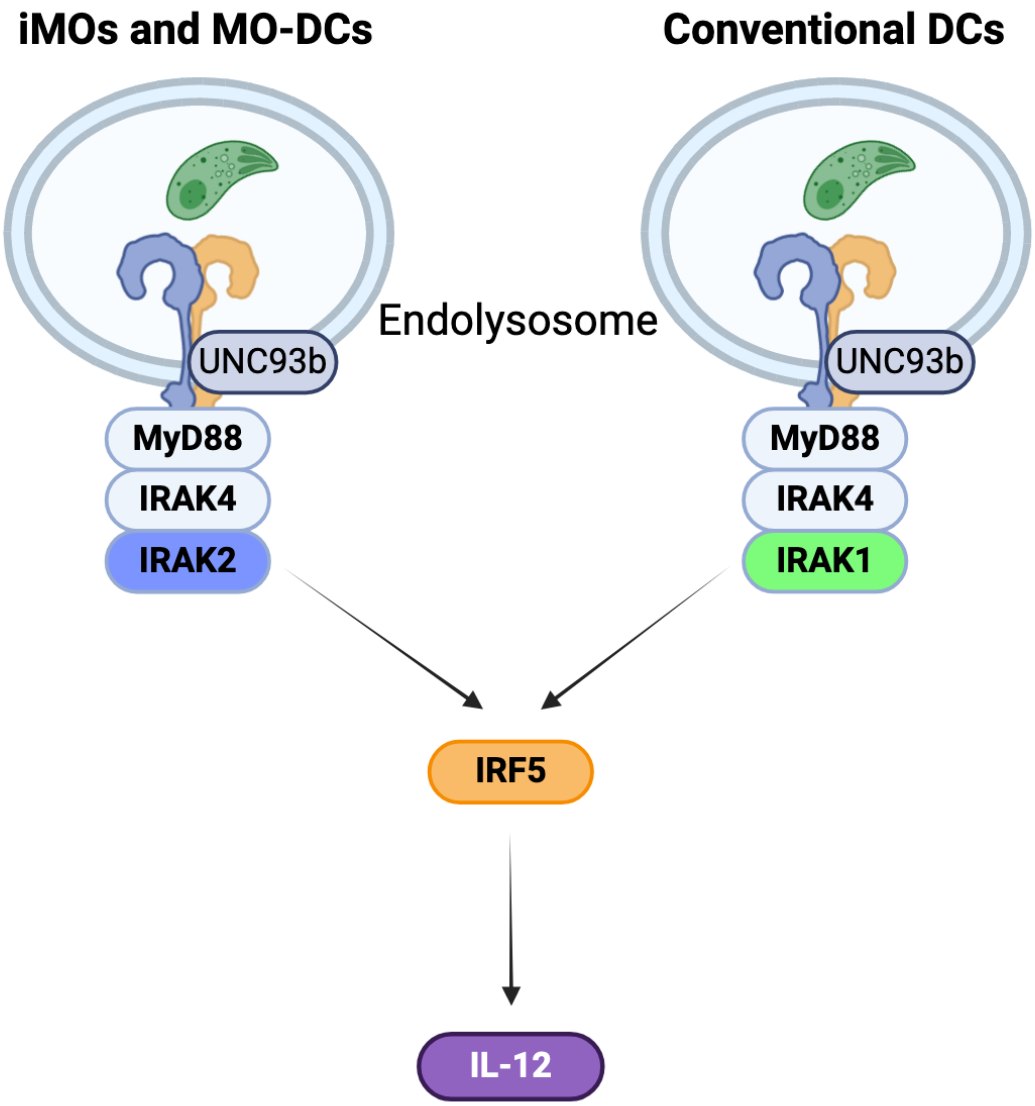
METHODS:Here, we studied IL-12 production in response to *T. gondii* infection in vivo and in vitro in cDCs, iMOs and MODCs isolated from various IRAK-deficient mice.

RESULTS:We report that IL-12 production in *T. gondii*-exposed cells is mediated by the transcription factor interferon regulatory factor 5 (IRF5), which directly binds to the *Il12b* promoter. IRF5 activation is mediated by IRAK1 in cDCs, while iMOs and MODCs required IRAK2. *In vivo*, IRAK1/IRAK2 double knockout (KO), IRAK4 KO and IRF5 KO mice show a profound defect on IL-12 production and were highly susceptible to infection with *T. gondii*. Either IRAK1 or IRAK2 single KO mice showed small to no deficiency in IL-12 production, consistent with a redundant role of DCs and iMOs/MODCs.

CONCLUSIONS:Our findings demonstrate that different cell populations employ the IRAK1/IRF5 or IRAK2/IRF5 axis to drive IL-12 production in response to *T. gondii* exposure. This highlights that IRF5 is central in controlling parasitic infections via production of IL-12.

Keywords: TLR, Toxoplasma gondii, Innate immunity, Dendritic Cell, IRAK

Study conclusions



IL-12 production in response to T. gondii is mediated by IRAK1/IRF5 or IRAK2/IRF5 depending on cell type.

P-087

General Research » Intracellular sensing of nucleic acids and interferon signaling

EMERGING ROLE OF ADENOSINE DEAMINASE 2 IN NUCLEIC ACID SENSING

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OBJECTIVES:Adenosine deaminase 2 (ADA2), primarily expressed by innate immune cells, is an enzyme converting adenosine into inosine. Loss-of-function mutations in the ADA2 gene cause Deficiency of ADA2 (DADA2), a severe and potentially life-threatening condition characterized by a constellation of inflammatory, hematological, and immunological complications. DADA2 is associated with an exaggerated inflammatory response accompanied by an abnormal polarization of macrophages toward the pro-inflammatory M1 subtype. However, the mechanisms underlying excessive inflammation in DADA2 patients are unknown. Understanding how ADA2 deficiency perturbs the inflammatory response holds critical implications for potential therapeutic interventions and the management of DADA2-related complications.

METHODS:We used macrophages of patients with DADA2 and an ADA2-deficient U937 macrophage cell line.

RESULTS:Like previous studies, our research revealed that patients' PBMCs showed a heightened type I interferon (IFN-I) signature, as reflected in an increased IFN score. This pattern was also confirmed in ADA2-deficient macrophages. Moreover, our investigation revealed elevated levels of cell-free DNA in the plasma of DADA2 patients and an accumulation of mitochondrial DNA (mtDNA) in the cytoplasm of ADA2-deficient macrophages compared to ADA2-sufficient cells. When ADA2-deficient macrophages were exposed to synthetic double-stranded DNA, the IFN-I signature was amplified and dampened by inhibiting the cGAS-STING pathway and the mtDNA release into the cytosol. These findings indicate that the heightened recognition of mtDNA by cGAS-STING might underlie the elevated IFN-I signature in ADA2-deficient macrophages. To explore this further, we examined whether mitochondrial dysfunction could be driving the increased cytosolic mtDNA levels. In ADA2-deficient macrophages compared to ADA2-sufficient cells, we observed enlarged mitochondrial dimensions, elevated mtROS production, increased membrane potential, and an overall decline in mitochondrial fitness.

CONCLUSIONS:Our findings indicate that mitochondrial dysfunction, leading to an excessive accumulation of mtDNA in the cytosol, may play a pivotal role in amplifying the cGAS-STING-mediated IFN-I signature.

Keywords: Adenosine deaminase 2, IFN, cGAS-STING, mitochondria, inflammatory response

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General Research » Intracellular sensing of nucleic acids and interferon signaling

GAIN-OF-FUNCTION HUMAN UNC93B1 VARIANTS AS A CAUSE OF TYPE I INTERFERONOPATHY VIA ENHANCED TLR7 AND TLR8 SIGNALLING

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OBJECTIVES:UNC93B1 is a highly conserved protein expressed in the endoplasmic reticulum, that acts as a chaperone for nucleic-acid sensing Toll-like receptors (TLRs). UNC93B1 deficiency

in humans causes susceptibility to herpes simplex encephalitis. In mice, specific Unc93b1 missense mutations result in either an abolition of TLR3, 7 and 9 signaling ("3d" strain), or an upregulation of TLR7 signaling leading to severe autoimmunity via distinct mechanisms: imbalance between TLR7 and TLR9 signaling (D34A mutation), or loss of interaction with regulatory proteins, syntenin-1 and Arl8b. Two recent publications described human mutations in UNC93B1 leading to enhanced TLR7 signalling in three families with systemic lupus erythematosus (Wolf et al. 2024, Mishra et al. 2024).
We have identified five unrelated patients harbouring distinct rare missense substitutions in UNC93B1 in the heterozygous or homozygous state, displaying either systemic lupus or chilblain lupus (two and three probands respectively). A persistent upregulation of interferon stimulated gene expression was present in the blood of all patients sampled. We therefore set out to study the link between these mutations in UNC93B1 and enhanced type I interferon signalling.

METHODS:We analyzed responses to stimulation with TLR ligands in patient peripheral blood cells, a HEK293T reporter system expressing UNC93B1 variants and TLRs, and THP-1 cells expressing mutant UNC93B1. We also studied UNC93B1-TLR interactions using proximity ligation assay (PLA).

RESULTS:In vitro testing of disease-associated putative mutations demonstrates a differential gain of TLR7/8 activity according to genotype, with these data confirmed in patient derived cells. PLA analysis suggests enhanced interaction between UNC93B1 mutants and TLR8 at baseline or upon stimulation, which could explain the gain in TLR8 activation.

CONCLUSIONS:Our work highlights and expands the phenotype of an emerging monogenic cause of a type I interferonopathy state encompassing lupus and chilblain lupus that informs the understanding of the role of UNC93B1 in nucleic-acid immunity.

Keywords: UNC93B1, TLRs, Interferon

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HUWE1-CATALYZED UBIQUITINATION PROMOTES RNA SENSING BY RIG-I-LIKE RECEPTORS AND THE ANTIVIRAL TYPE I INTERFERON RESPONSE

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OBJECTIVES:RIG-I-like receptors (RLRs) play an integral role in antiviral innate immunity by functioning as cytoplasmic RNA sensors that promote type I interferon (IFN) production in response to viral RNA ligands or certain endogenous RNA ligands. The RLR signaling pathway is tightly regulated by dynamic post-translational modifications, including ubiquitination. Huwe1 is a HECT domain-containing giant E3 ubiquitin ligase that has not yet been implicated in the RLR pathway. Here, we investigated whether Huwe1's E3 ligase activity affects RLR signaling.

METHODS:We demonstrate that Huwe1 broadly impacts on type I IFN induction following RLR activation by viral ligands or unedited, endogenous RNA. We analyzed the effect of functional Huwe1 depletion (through genetic manipulation or small molecule-mediated inhibition) on the RLR-induced type I IFN response in human cells. We identified proteins that are modified by ubiquitin in a Huwe1-dependent manner upon RLR activation by diGly proteomics. Finally, co-immunoprecipitation and knockdown assays were performed to validate the identified target substrates at a functional level.

RESULTS:Loss of Huwe1 severely attenuates the expression of IFNβ and IFN-stimulated genes (ISGs) in cells transfected with the virus mimetics poly(I:C) or 5'ppp-hpRNA. Huwe1 also promotes the type I IFN response in the context of ADAR1 deficiency, in which unedited self RNA accumulates and inadvertently triggers sterile RLR-dependent inflammation. Depletion of Huwe1 did not reduce ISG expression upon activation of the type I IFN receptor (IFNAR), indicating that Huwe1 promotes RLR signaling upstream of the IFNAR. Putative Huwe1 substrates were discovered by diGly proteomics, including key components of the RLR pathway (MAVS, TRAFs). We demonstrate that several putative substrates interact with Huwe1 and are required to promote RLR-induced IFN-I responses.

CONCLUSIONS:Collectively, our results identify Huwe1 as an essential E3 ubiquitin ligase in the RLR signaling pathway and provide new insights in ubiquitin-dependent regulation of the antiviral immune response.

Keywords: Antiviral immunity, RIG-I-like receptors, Type I interferon, ADAR1 deficiency, Ubiquitination, E3 ligase

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STING ENGAGES IRF3 VIA PHOSPHORYLATED SERINE 358 TO ACTIVATE NF-KB AT LATE ENDOLYSOSOMAL COMPARTMENTS

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OBJECTIVES:The transcription factor nuclear factor (NF)-κB is central for activation of immunological and stress-related responses, but it also contributes to survival and repair responses. Cytosolic DNA activates the cGAS-STING pathway to induce type I interferons (IFN)s and signaling through NF-κB, thus instigating antiviral and antitumor as well as pathological inflammation. However, the mechanism of STING-induced NF-κB activation remains unknown.

METHODS:Using the Confocal microscope, Gene editing, Co-immunoprecipitation, Organelles pulldown, SPR binding studies, Western Blot, and qPCR etc

RESULTS:we report that STING activates NF-κB with delayed kinetics relative to the IFN response, and that this occurs following exit of STING from the Golgi to endolysosomal compartments. The activation of NF-κB depends on the IFN-inducing transcription factor IRF3, but is independent of IFN signaling. This activation pattern is evolutionary conserved in tetrapods. Mechanistically, monomer IRF3 is recruited to serine 358 of STING upon its phosphorylation, occurring with delayed kinetics relative to the recruitment of IRF3 to phorphorylated serine 366 of STING, which promotes IFN responses. IRF3 engagement to pS358 STING enables STING exit from the Golgi, trafficking to late endolysosomal compartments, enabling recruitment of TRAF6, phosphorylation of IκBα, and activation of NF-κB. We uncover a novel TRAF6 binding motif in IRF3 that works in synergy with IRF3 serine396, facilitating the recruitment of TRAF6.

CONCLUSIONS:Our work defines a novel signaling surface on STING, and reveals a function for IRF3 in immune signaling as adaptor protein. This suggests that the STING pathway allows NF-κB activation only within a short window between exit from Golgi and lysosomal degradation. This may be favorable to limit inflammation under homeostatic and danger-sensing conditions.

Keywords: Innate immunology, NF-kB, DNA sensing, cGAS-STING pathway

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PML IS A TARGETABLE IFN-RESPONSIVE FACTOR PROMOTING MICROGLIAL ACTIVATION AND ISG SIGNATURE IN A TYPE-I INTERFERONOPATHY MOUSE MODEL

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OBJECTIVES:Promyelocytic leukemia – nuclear bodies (PML-NBs) are nuclear structures that are formed via oligomerization of the PML protein and can associate with more than 40 other proteins. These structures are disrupted in acute promyelocytic leukemia (APL) via a chromosomal translocation, generating an oncogenic PML chimeric protein. PML-NBs have diverse functions, and many of them are related to stress response.

METHODS:We found in an MDA5-R822Q (MDA5-RQ) gain-of-function (GOF) mouse model of type I interferonopathy that constitutive activation of MDA5 leads to IFN-I upregulation with microgliosis and microglial activation. Given that PML is a downstream target of IFN-I in immune cells, we investigated its potential involvement in the context of type I interferonopathies.

RESULTS:We found that microglia usually do not have PML-NBs formed in their nuclei at steady state, however microglia of MDA5-RQ mice had significantly higher numbers of PML-NBs compared to control animals. To assess the role of PML and PML-NBs in this context, we generated MDA5-RQ mice with microglia-specific Pml deletion (MDA5-RQ x PML-KO). MDA5-RQ x PML-KO mice displayed lower levels of the macrophage activation marker CD68 and reduced number of microglia, accompanied by significant reduction in the expression of Ifnß and interferon stimulated genes. Patients with APL are treated with arsenic trioxide (ATO), an ancient drug targeting PML chimeric oncoprotein. MDA5-RQ mice treated with ATO displayed PML downregulation correlating with reduced microglial activation markers, similarly to what was observed in MDA5-RQ x PML-KO mice.

CONCLUSIONS:Altogether our findings highlight the significance of PML-NBs in context of type-I interferonopathies and their potential as therapeutic target.

Keywords: Promyelocytic Leukemia (PML), Nucleic Acid Sensing, MDA5 (melanoma differentiation-associated protein 5), Type I Interferonopathy, Interferon Signalling

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SPATA5, A PROTEIN LINKED TO NEURODEVELOPMENTAL DISORDERS, MEDIATES NON-CANONICAL AUTOPHAGY-DEPENDENT CLEARANCE OF STING

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OBJECTIVES:Autophagy-related-16-like 1 protein (ATG16L1) is the key player in regulating canonical and non-canonical autophagy. The WD40 C-terminal repeat domain (CTD) of ATG16L1 is dispensable for canonical autophagy but is essential for driving the less explored process of non-canonical autophagy. We sought to identify binding partners of ATG16L1 specific to the WD40 CTD and found spermatogenesis-associated protein 5 (SPATA5), an AAA-type ATPase protein, as a direct interactor. Gene variants of SPATA5 are associated with neurodevelopmental disorders that feature epilepsy, spastic cerebral palsy and sensorineural hearing loss. We hypothesized that SPATA5 interaction with ATG16L1 may play a role in non-canonical autophagy.

METHODS:A yeast-2-hybrid screen was employed to discover binding partners of the WD40 CTD domain of ATG16L1 and SPATA5 was identified. We employed lentiviral knock-down of SPATA5 in different cell lines; knock-out cells were non-viable. Cell biology and biochemical approaches were used to discover the function of SPATA5. Cells from patients with SPATA5 gene variants validated the in vitro findings.

RESULTS:SPATA5 deficiency did not impact canonical autophagy but was found to stall non-canonical autophagy involving the conjugation of ATG8 (yeast LC3 ortholog) to single membranes (CASM) and prompted a Stimulator of Interferon Gene (STING)-dependent tonic type-I interferon (IFN) response. Mechanistically, we demonstrate that SPATA5 is recruited downstream of vacuolar-type H+-adenosine triphosphatase (V-ATPase)-ATG16L1 interaction during CASM and mediated lysosomal turnover of phosphorylated STING at steady-state. Consequently, SPATA5 knock-down cells exhibited enhanced type 1 IFN and ISG expression. Cells from SPATA5 patients also exhibited aberrant IFN-driven gene signatures.

CONCLUSIONS:Taken together, our study reveals an unprecedented role of SPATA5 in mediating non-canonical autophagy, thereby mitigating aberrant STING signalling, which may underlie a rare human congenital disease associated with SPATA5 variants.

Keywords: SPATA5, STING, non-canonical autophagy, neurodevelopmental disorders

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MICRORNAS RELEASED BY UV-TREATED KERATINOCYTES ACTIVATE PDCS VIA TLR7: A MODEL MECHANISM OF TYPE I INTERFERON TRIGGERING IN PSORIASIS

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OBJECTIVES:Excessive production of type I interferons (IFNs) by plasmacytoid dendritic cells (pDCs) is the crucial event responsible for autoreactive T cell activation and tissue damage in a subgroup of autoimmune conditions known as “type I IFN-mediated diseases”, including systemic lupus erythematosus (SLE) and psoriasis. We previously showed that circulating small extracellular vesicles (sEVs) from SLE patients activate pDCs via TLR7 triggering, suggesting a role for GU-rich microRNAs (miRNAs) as TLR7 endogenous ligands. Here, we used psoriasis as a model condition to investigate if deregulated miRNA secretion may represent a new pathogenic mechanism activating pDCs in type I IFN-mediated diseases.

METHODS:Inflamed and UV-treated keratinocytes were used as in-vitro models of psoriatic skin to collect sEVs. Small RNAs contained in sEVs were sequenced and analyzed in terms of upregulated and TLR7-binding miRNAs. sEVs were used to stimulate primary human pDC activation, which was assessed in terms of type I IFN secretion and allogeneic CD8+ T cell activation. The involvement of TLR7 was assessed by using specific inhibitors. An anti-BDCA-2 antibody and inhibitors of sEV production were also used. Psoriatic skin biopsies were collected and used to extract and quantify miRNAs by RT-PCR.

RESULTS:TLR7-activating GU-miRNAs were selectively upregulated in sEVs derived from UV-treated keratinocytes as well as in psoriatic skin lesions. sEVs from UV-treated keratinocytes stimulated TLR7-dependent production of type I IFN and activation of cytotoxic CD8+ T cells by pDCs. This activation was blocked upon triggering of the pDC-inhibitory receptor BDCA-2.

CONCLUSIONS:Our results identify miRNAs released by damaged keratinocytes as novel pathogenic mediators of pDC activation in the onset of psoriasis, setting the bases for the identification of new therapeutic targets and options in psoriasis and potentially in other type I IFN-mediated diseases.

Keywords: exosomes, miRNAs, TLR7, pDCs, interferonopathies, psoriasis

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A NOVEL TYPE I INTERFERON REGULATORY NETWORK CONSISTING OF HEPARIN, MEMBRANE-BOUND AND SOLUBLE BDCA-2 MODULATES TLR9-DRIVEN IFN-α PRODUCTION IN PLASMACYTOID DENDRITIC CELLS

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OBJECTIVES:Plasmacytoid dendritic cells (pDCs) produce type I interferons (IFNs) after sensing viral/bacterial RNA or DNA by toll-like receptor (TLR) 7 or TLR9, respectively. However, aberrant pDCs activation can cause adverse effects on the host and contributes to the pathogenesis of type I IFN-related autoimmune diseases. This work aimed to identify the natural ligand of BDCA-2 a human specific regulatory receptor of type I IFNs in pDCs, and gain new insights into the regulation of type I IFNs by BDCA-2.

METHODS:Co-stimulations of pDCs, BDCA2-deficient and BDCA2-reconstituted CAL-1 cells (human pDC cell line) with CpG-ODN 2216 and heparin were performed, and IFN-α was measure by ELISA. A modified ELISA, immunoprecipitation and microscale thermophoresis were used to study BDCA-2-heparin interaction. Identification of soluble BDCA-2 (solBDCA-2) in plasma and evaluation of solBDCA-2 levels in patients with scrub typhus, bullous pemphigoid and psoriasis were measured by ELISA.

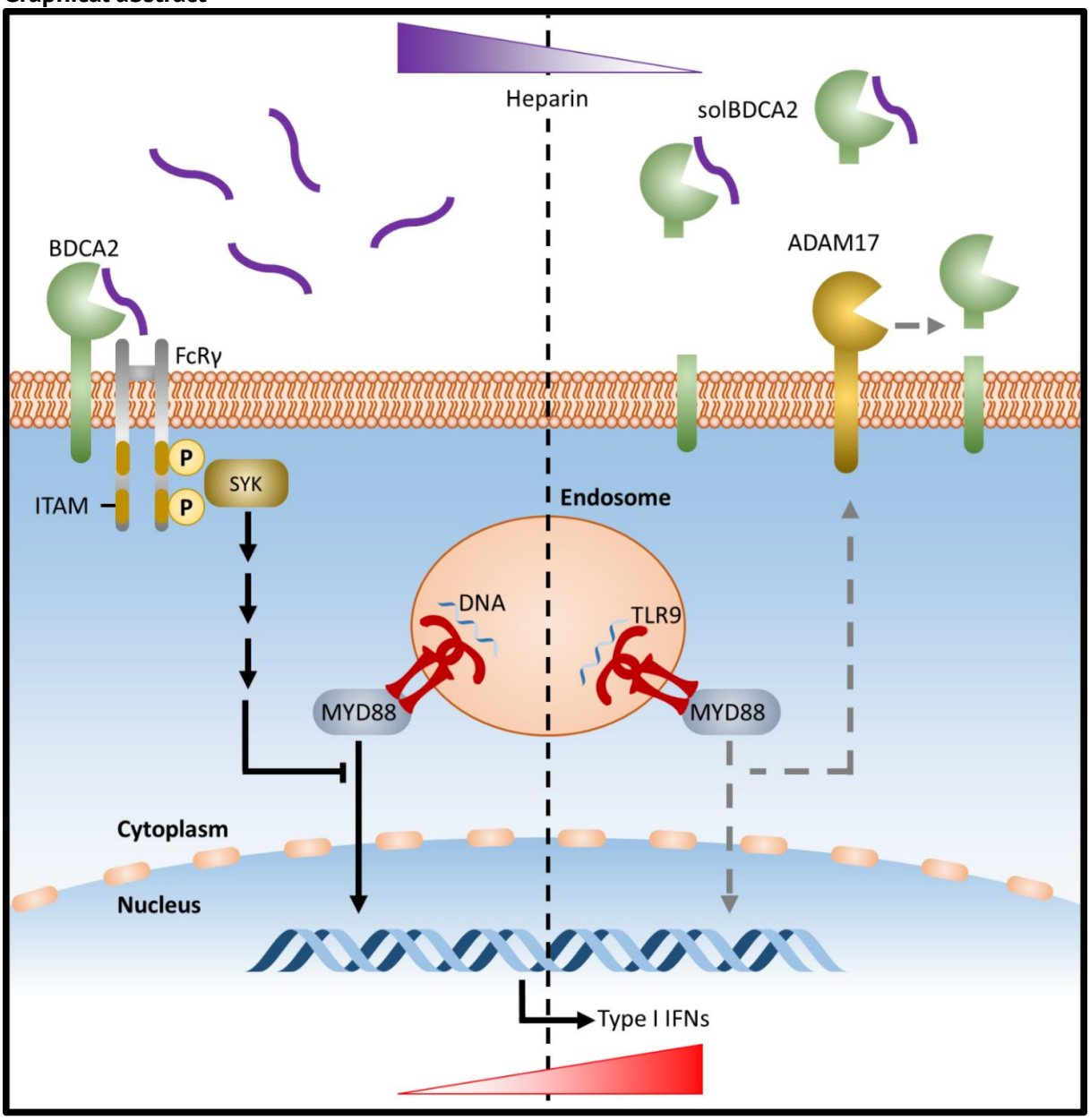
RESULTS:We show that heparin interacts with the human pDCs specific receptor BDCA-2. This interaction depends on heparin sulfation and receptor glycosylation resulting in inhibition of TLR9-driven type I IFN production in primary pDCs and pDC-like cell line CAL-1. This inhibition is mediated by medicinal heparin, as well as endogenous heparin from plasma, suggesting that local blood environment controls the production of IFN-α in pDCs. Additionally, we identified an activation-dependent generation of solBDCA-2 in human plasma that functions as heparin antagonist and thereby increases TLR9-driven IFN-α production in pDCs. Of importance, solBDCA-2 levels were increased in patients with scrub typhus compared to healthy controls and correlated with anti-dsDNA antibodies. In contrast, solBDCA-2 levels from patients with bullous pemphigoid or psoriasis were reduced.

CONCLUSIONS:We identify a regulatory network consisting of heparin, membrane-bound and solBDCA-2 modulating IFN-α production in pDCs. Importantly, this observation generates new insight into pDCs function/regulation with implications for the treatment of pDCs-related

autoimmune diseases.

Keywords: Heparin, Plasmacytoid dendritic cells, Glycosaminoglycans, Type I interferons, BDCA-2, Autoimmune disease

Graphical abstract



Graphical abstract. Graphical representation of the IFN regulatory network based on BDCA-2, solBDCA-2 and heparin.

pDCs in circulation are in tight control on type I interferon production due to the presence of heparin and, this control is lifted once the cells leave the circulation and move to tissues where the concentration of heparin is lower and the activation of pDCs triggers the release of solBDCA-2. FcRγ: Fc receptor gamma-chain; an adapter protein containing immunoreceptor tyrosine-based activation motif (ITAM). SYK: Tyrosine-protein kinase SYK. MYD88: Myeloid differentiation primary response protein MyD88; an adapter protein involved in the signaling of TLRs.

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ARCHAEA-INDUCED TLR8-DEPENDENT SIGNALING IN HUMAN IMMUNE CELLS AND ITS ASSOCIATION WITH CHILDHOOD ASTHMA

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OBJECTIVES:Early childhood exposure to a diverse microbial environment is inversely related to the development of asthma and allergies. Recently, the presence of archaea as integral parts of the human microbiome has been increasingly appreciated. In particular, the methanogens M. smithii (Msm) and M. stadtmanae (Mst) have been studied, and the latter one was found to be associated with a reduced odds ratio of allergic diseases in children. We could demonstrate that both archaeal strains activate cells via their RNA and that recognition through TLR8 is essential for cellular activation. However, they possess a strikingly different capacity to induce inflammatory cytokine secretion from human immune cells. To get a more complete picture of their stimulatory capacity and the molecular mechanisms behind the asthma association, we extended our study on CRISPR/Cas9-generated KO cells lacking key innate signaling adapters and performed an in-depth analysis of mRNA induction and cytokine release by both Mst and Msm in PBMCs from healthy donors.

METHODS:We used CRISPR/Cas9-generated knockouts in human BLaER1 cells and RNAseq/multiplex Mesoscale ELISA analysis of Archaea-stimulated PBMCs from healthy donors.

RESULTS:We found that archaea-induced TLR8-dependent signaling also requires IRAK4 and IKK1 but is independent of IKK2. IRF5-deficiency reduces Mst-induced proinflammatory cytokine release of IL-1β, IL-6 and TNFα, but completely blocks induction by Msm. Stimulation of human PBMCs with Mst leads to much higher activity than with Msm, drives Th1 polarization in naïve T-cells and elicits a strong Th1 and IL-10 effector response. Furthermore, the number of genes exclusively differentially expressed upon stimulation with Mst is considerably higher. However, a set of specific cytokines such as IFN-beta, MCP-1 or IP-10 are induced by Msm in comparable strength.

CONCLUSIONS:Taken together, our results indicate that the immune response-inducing capacity of archaea is more complex than originally thought and apparently not dictated by their TLR8-dependent RNA recognition alone.

Keywords: innate immunity, TLR8, RNA, archaea

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IMPACT OF TYPE I INTERFERONS ON THE FUNCTIONALITY OF CAR-T CELLS IN A PRECLINICAL PANCREATIC CANCER MODEL

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OBJECTIVES:Previous studies have demonstrated that type I interferon (IFN) signaling in T cells can have both enhancing and inhibitory effects, depending on concentration as well as timing of the signal relative to T cell receptor activation. We aim to investigate T cell-intrinsic effects of type I IFNs in therapeutic T cells to boost the effectiveness of CAR-T cell therapy in solid tumors.

METHODS:Human T cells equipped with a tumor antigen-specific CAR construct were co-incubated with the human PDAC cell line SUIT-2 upon (1) 24-hour pre-stimulation or (2) concomitant stimulation with different concentrations of IFN-beta. CAR-T cell functionality was assessed using diverse activation and exhaustion markers via flow cytometry (FACS). Additionally, we determined CAR T cell killing capacity through an LDH release assay in vitro. Furthermore, we investigated the type I IFN effect using CAR-T cells in a repetitive antigen stimulation experiment.

RESULTS:Time-dependent application of IFN-beta in the context of CAR activation resulted in equal cancer cell killing capacity by CAR-T cells in a short-term killing assay over 72 hours. CAR-T cell-specific exhaustion markers were slightly reduced, accompanied by increased expression of CD107a, independent of IFN-beta timing. However, prestimulated CAR-T cells were more susceptible to senescence, since their proliferative capacity was decreased by 50%, whereas concomitantly treated CAR-T cells showed no proliferative impairment. In the context of repetitive antigen stimulation, IFN-beta-co-treated CAR-T cells demonstrated improved functionality regarding cancer cell killing and activation/exhaustion as well as proliferation.

CONCLUSIONS:We identified type I IFNs as potential mediators of enhanced CAR-T cell functionality in a physiological repetitive antigen stimulation setting. Combining type I IFNs or type I IFN-inducing agents with CAR-T cells might represent a promising approach for optimizing adoptive T cell therapy in solid tumors. Further investigations are required to determine the therapeutic applicability of this effect.

Keywords: Type I Interferons, type I interferon (IFN) signaling, CAR-T Cells, Pancreatic ductal adenocarcinoma, cell-based tumor therapies

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MODULATION OF CELL-FATE DECISION UPON 3P-RNA SENSING BY RIG-I AND VIRAL RESTRICTION FACTORS

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OBJECTIVES:In this study we describe the individual contributions of RIG-I and the antiviral restriction factors OAS and PKR on cell-fate and immunological cues like IFN-expression upon cytoplasmic nucleic acid sensing. Activation of RIG-I by 5'-triphosphate-dsRNA triggers an antiviral response, characterized by the induction of type I interferons, proinflammatory cytokines, and apoptosis. We have shown earlier that 3p-RNA-induced apoptosis involves a two-step process: RIG-I–dependent priming followed by an OAS/RNase L–dependent effector phase. By modification of the 3p-RNA characteristics we aimed to differentially influence the RIG-I-mediated cytokine axis and RNase-L-mediated cell death axis and thereby decipher ligand preferences of both receptor systems and their influence on cell-fate.

METHODS:A set of defined in vitro-transcribed 3p-RNA ligands were analyzed in different knockout tumor cell lines activating either RIG-I alone, RIG-I and OAS, or PKR to understand the functional role of each axis and to separate cytokine response from translational inhibition and cell death.

RESULTS:Our findings revealed a clear length-dependent effect of 3p-RNA on cytokine and cell death induction. Initially, IFN levels increased with 3p-RNA length until a maximum was reached, beyond which they declined rapidly. In contrast, cell viability showed a steady decline with increasing 3p-RNA length. This aligns with a more potent OAS/RNase L and PKR-mediated translational arrest. Hence, a more efficient activation of OAS and PKR by long 3p-RNA negatively regulated IFN levels due to early translational shutdown but increased tumor cell death and with this potentially antigen availability.

CONCLUSIONS:In conclusion, we were able to generate cell death-optimized 3p-RNA and elucidate individual ligand preferences of RIG-I and the antiviral restrictions factors OAS and PKR. Through precise manipulation of both receptor systems, we further aim to carefully balance cytokine and cell death induction and investigate the impact of the RNA receptor signal integration on the induction of a potent systemic immune response.

Keywords: 3p-RNA, RIG-I, OAS/RNaseL, PKR, IFN, Cell death

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ARIES DOMAINS USE DIVERSE MECHANISMS TO DRIVE INTERFERON AND METABOLIC CELLULAR ACTIVITIES

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OBJECTIVES:Type I interferon (IFN) responses mediate host protection against infection through the secretion of IFN-β and the upregulation of antiviral interferon stimulated genes (ISGs). The multimerization of key signaling adaptors links sensing by Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and cGAS to downstream IFN responses. Such receptor-proximal adaptor proteins in each of these pathways contain a conserved pLxIS motif, however the mechanisms by which pLxIS-containing domains stimulate IFN responses in different contexts are unclear.

METHODS:Using a novel synthetic biology-based approach, we engineered modular protein-based signaling platforms that are activated by small molecules to drive oligomerization-induced IFN responses via pLxIS motifs.

RESULTS:This platform enabled us to screen orphan pLxIS motif-containing proteins to identify two new IFN-inducing proteins, which act independently of the TLR, RLR, and cGAS pathways to promote IFN responses. Mechanistic studies of all IFN signaling proteins revealed a diversity of pLxIS signaling mechanisms. The pLxIS motif represents one component of a multi-motif signaling entity—the ARIES domain—which has diverse functions in activating IRF3, the TRAF6 ubiquitin ligase, IκB kinases, mitogen-activated protein kinases, and glycolysis. This diversification endowed select ARIES domains with potent antiviral immunity in human cells.

CONCLUSIONS:These data collectively illustrate the modular nature of ARIES domain activities and highlight the value of synthetic biology as a tool to discover novel regulators of innate immunity.

Keywords: interferon, TLR, RLR, STING, TBK1, IRF3

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STALLED, REVERSED REPLICATION FORKS ARE DETECTED AS DAMAGE-ASSOCIATED MOLECULAR PATTERN DURING REPLICATION STRESS

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OBJECTIVES:DNA damage can cause an innate immune response, for instance when DNA from micronuclei is detected in the cytosol by the DNA sensor cGAS, which occurs several days after recovery from double strand DNA breaks. We found that human epithelial cells can also initiate an earlier response to DNA damage, within the first hours of treatment with DNA damaging chemotherapy agents. This acute response to DNA damage involved the predominantly nuclear DNA binding protein IFI16, which activated STING in a cGAS-independent manner. However, the molecular DNA features that are detected as damage-associated molecular patterns (DAMPs) in this context were unknown.

METHODS:We treated human epithelial cells and epithelial-derived cancer cells with DNA damaging chemotherapy agents, replication stress inducers or ionizing radiation. Activation of innate immune signalling was monitored by confocal microscopy, qRT-PCR and ELISA, and the involvement of signalling factors was probed using CRISPR gene targeting, RNA interference and small molecule inhibitors. Proteins binding to replication forks were identified using iPOND.

RESULTS:We found that replication stress acts as crucial priming signal for the innate immune system, and that IFI16 binds to stalled replication forks in the nucleus. The remodelling/reversal of stalled replication forks was required for the detection of replication stress by IFI16, and for the induction of pro-inflammatory cytokines following treatment with chemotherapy agents that cause replication stress-associated DNA damage. This pathway of cGAS-independent STING activation is also functional in cancer cell lines that have lost the ability to sense cytosolic DNA.

CONCLUSIONS:We identify stalled, reversed replication forks as a DAMP which can be sensed by the innate immune system in epithelial cells and epithelial-derived cancers. This alternative, more inflammatory STING signalling pathway may contribute to the remodelling of the immune microenvironment during chemotherapy treatment.

Keywords: STING, IFI16, DNA sensing, replication stress, chemotherapy

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THE ROLE OF METHYLATION AND RNA GLYCOSYLATION IN SELF/NON-SELF DISCRIMINATION OF RNA

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OBJECTIVES:RNA modifications prevent erroneous self-recognition. Recently, RNA glycosylation was introduced as a novel type of RNA modification, but evidence for “glycoRNA” is ambiguous and its interaction with Toll-like receptors (TLRs) is unknown. Described targets of glycosylation are also expected to be 2’O-methylated (e.g. tRNA methylation by the methyltransferase TAR-binding protein 1, TARBP1). We were interested in dissecting the roles of glycosylation and methylation in the interplay of small RNAs with TLRs.

METHODS:“GlycoRNA” was visualized by metabolic labelling and click chemistry. The glycan:RNA linkage was investigated by enzymatic treatments and mobility shift assays. Human PBMCs were stimulated with glycan-rich RNA with or without the transfection reagent DOTAP, and were co-stimulated with RNA40. IFN-∝ secretion was measured by ELISA. TARBP1-deficient THP-1 cells were assessed for expression of interferon-stimulated genes (ISGs) by qPCR. RNA from these cells was used to stimulate PBMCs.

RESULTS:We identified glycans within extracts of small cellular RNAs in line with previous publications. However, in our hands “glycoRNA” was insensitive towards nuclease treatment, and did not co-migrate with distinct RNA bands. Treatment with Proteinase K abolished the signal. Stimulation of PBMCs with glycan-rich small RNA preparations antagonized TLR7 activation by RNA40 dose-dependently both via intra- or extracellular administration. No such effect was observed with RNA from TARBP1-deficient THP-1 cells, supporting a dominant role of 2’O-methylation in antagonizing TLR7. In line with this observation, TARBP1-deficient THP-1 cells endogenously expressed higher levels of ISGs.

CONCLUSIONS:We herein provide evidence that protein contamination may be part of a “glycoRNA” complex. Glycan-rich RNA preparations potently blocked TLR7 activation by an intra- and extracellular mode of action. This effect depended on the methylation status, as no TLR7 antagonism was observed for glycan-rich RNA from TARBP1-deficient THP-1 cells. We found TARBP1 to be involved in labelling RNA as “self” and that loss of TARBP1 promotes autoimmunity.

Keywords: Toll-like receptor 7, self-recognition, RNA methylation, glycoRNA, Interferon alpha, TAR-binding protein 1

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INNATE IMMUNITY IS SUPPRESSED IN DIVIDING CELLS BY CYCLIN DEPENDENT KINASES 4 AND 6

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OBJECTIVES:Growing evidence demonstrates that host sensors such as cGAS and the RIG-I-like receptors bind nucleic acids of both pathogenic and cellular origin and their activity must be tightly regulated to avoid aberrant self-recognition and uncontrolled IFN production. We hypothesised this would be particularly important during cell division, where nuclear envelope breakdown and complex organelle rearrangements such a mitochondrial fission and fusion could expose self-nucleic acids to host sensors.

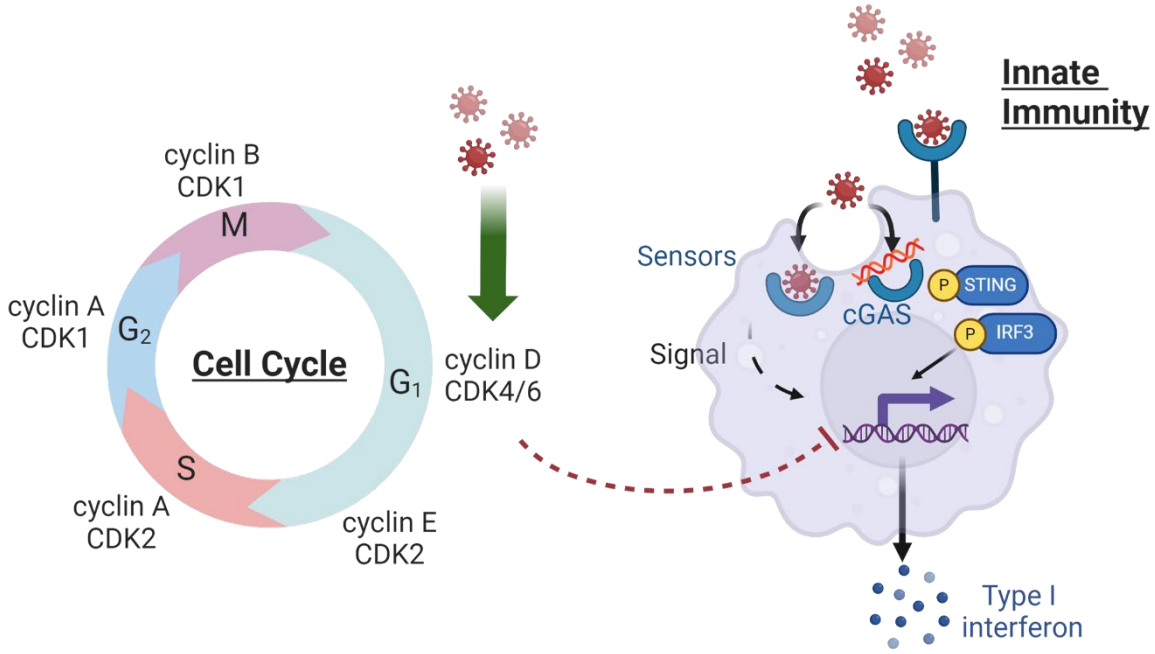
METHODS:Using a combination of genetic and chemical approaches we manipulated cell division in THP-1 monocytes and other cells and assessed their ability to mount an innate response to nucleic acids.

RESULTS:We observed that innate immunity to nucleic acids is universally suppressed in dividing cells and discovered a novel role for cell cycle regulatory proteins cyclin dependent kinases (CDK) 4/6 in dampening IFN. Blocking CDK4/6 activity in dividing cells using shRNA-mediated depletion, chemical inhibition, or overexpression of cellular inhibitor p16INK4a greatly enhanced DNA- and RNA-induced IFN-stimulated gene (ISG) expression. Mechanistically, CDK4/6 had no effect on cytoplasmic signalling such as STING and IRF3 phosphorylation, or IRF3 nuclear translocation, but inhibited IFNβ mRNA expression suggesting regulation at the promoter. CDK4/6-mediated inhibition of IFN was universal, occurring in both primary and transformed human and murine cells. Several viruses have evolved to activate or mimic CDK4/6 and given our novel findings we hypothesised that this would dampen host antiviral responses. Indeed, we found that HTLV-1 Tax inhibits IFNβ expression dependent on its ability to bind and activate CDK4/6.

CONCLUSIONS:Together, we have uncovered a novel role for CDK4/6, which are active through much of the cell cycle, in dampening IFN responses in dividing cells. We hypothesise this is necessary to allow cells to safely divide without aberrant sensing of self but may be hijacked by viruses as a novel means of innate immune evasion.

Keywords: interferon, nucleic acid sensing, cell cycle, CDK4/6

Innate immunity is suppressed in dividing cells by CDK4/6



CDK4/6 activity in dividing cells suppresses innate immunity downstream of nucleic acid sensors such as cGAS, allowing cells to divide without aberrant recognition of self. CDK4/6 suppress innate immunity by dampening type I interferon expression at the transcription level. Viruses express proteins that specifically upregulate CDK4/6 activity e.g. HTLV-1 Tat, which subsequently suppresses IFN expression and contributes to viral innate immune evasion.

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STING ACTIVATION DEPENDS ON ACBD3 AND OTHER PHOSPHATIDYLINOSITOL 4-PHOSPHATE-REGULATING PROTEINS

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OBJECTIVES:STING induces transcription of type I interferons and other pro-inflammatory genes upon activation at the Golgi apparatus. Many of the regulators involved in STING activation are unknown. We investigated the role of ACBD3 and other phosphatidylinositol 4-phosohate (PIP4) regulating proteins in STING activation.

METHODS:We used a reporter-based genome-wide CRISPRi screen to identify factors that promote or antagonize STING activation. We investigated the effect of identified host factors on reporter activation, STING localization and activation, and downstream immune responses.

RESULTS:We show that proper STING localization and activation at the Golgi depended on ACBD3 and PI4KB expression. Furthermore, depleting PIP4 by inactivating PI4KB or overexpressing Sac1 diminished STING activation. STING signalling was also regulated by the lipid-shuttling protein OSBP, which removes PIP4 from the Golgi. OSBP inhibition by the FDA-approved antifungal itraconazole and other OSBP inhibitors greatly enhanced STING activation by increasing the levels of STING-activating phospholipids. Itraconazole-enhanced STING activation strongly increased expression of interferon-beta and other cytokines. Furthermore, a PIP4-binding mutant was defective in stimulation-induced trafficking to the Golgi and subsequent activation. Finally, forced relocalization of STING to PIP4-enriched areas provoked STING activation, even in the absence of stimulation.

CONCLUSIONS:In conclusion, the phospholipid PIP4 is critical for STING activation and manipulating PIP4 levels is a promising therapeutic strategy to alter the STING immune response.

Keywords: STING, innate immune responses, intracellular trafficking, phospholipids

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DNA FROM BK POLYOMAVIRUS IS SENSED DURING INFECTION OF RESERVOIR CELLS VIA CGAS-STING PATHWAY

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OBJECTIVES:Human polyomaviruses (HPyVs) include 14 species that cause primary asymptomatic infections after which the virus persists in the host by a mechanism that is not well understood. Reactivation of the infection in immunosuppressed patients leads to severe disease. Recently, it has been suggested that moderate innate immune responses in reservoir cells contribute to viral persistence. For BK polyomavirus (BKPyV), cells from the urinary tract are thought to act as viral reservoirs.
Here, we investigated the potential role of the cGAS-STING signalling pathway in the interferon (IFN) responses launched by primary microvascular endothelial cells from bladder (HMVECs bd) in response to BKPyV infection.

METHODS:We followed the kinetics of viral proteins and virion production using confocal and infectivity assays. IFN responses were analysed by measuring IFN β, ISG 56, CXCL10 and CCL20 by RT-qPCR. The role of the cGAS sensor was evaluated by examining its binding to viral DNA by fluorescent in situ hybridization and by measuring 2-3 cGAMP levels by ELISA. Then, the role of STING was investigated by detecting its phosphorylation at serine 366 by Western blotting.

RESULTS:First massive production of viral LT and VP1 proteins was detected after 36hpi and 48hpi, respectively. Virions were detected in the medium from 48hpi. Next, we showed that HMVECs bd elicit a moderate IFN response at a late time point after infection (around 72hpi). In the cytosol, we observed colocalization of cGAS with both incoming viral DNA (24hpi) and viral DNA leaked to the cytosol (62hpi). However, cGAMP production and activation of STING were detected only after 62hpi.

CONCLUSIONS:Our data show that BKPyV infection in reservoir cells leads to cGAS-STING activation at late time post-infection. Interestingly, the observed non-productive binding of cGAS to incoming virus could indicate a non-IFN-related function of cGAS during infection.

Keywords: BKPyV, cGAS, STING, INTERFERON

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FROM HUMAN TO YEAST: HARNESSING *SACCHAROMYCES CEREVISIAE* FOR A BETTER UNDERSTANDING OF THE HUMAN CGAS-STING PATHWAY

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OBJECTIVES:The GMP-AMP cyclic synthase (cGAS) is an intracellular receptor that senses cytosolic DNA associated with viral infection or cell damage. When DNA is detected, cGAS generates a cyclic dinucleotide second messenger, cGAMP, which stimulates the STING effector to activate the TBK1 protein kinase, necessary for the downstream events that lead to the eventual secretion of interferon and other inflammatory mediators.
The objective of this work is to reconstitute the cGAS-STING pathway in yeast and study the effects of STING mutations associated to vasculopathy with onset in infancy (STING V155M and STING V155R) using the model yeast *Saccharomyces cerevisiae*.

METHODS:The proteins cGAS, STING and TBK1 were expressed under the control of the inducible GAL1 promoter and tagged with fluorescent proteins to assess their subcellular localization. The expression and phosphorylation of TBK1, STING and their variants was verified by immunoblotting with specific antibodies. Non-phosphorylatable STING (S366A) and TBK1 (S172A) as well as kinase-dead TBK1 (K38A) were constructed.

RESULTS:In yeast cells, cGAS-GFP was localized to cytosolic spots co-localizing with Mdm34, a component of the ERMES complex, a molecular tether between mitochondria and the endoplasmic reticulum. By studying truncated versions, we observed that its C-terminal domain is necessary to form such cytosolic aggregates whereas the N-terminal region rather prevents the nuclear localization of cGAS. TBK1, but not its kinase-dead versions, was toxic for the yeast cell. Both wild type and mutant STING versions co-localized with TBK1 at the endoplasmic reticulum and were phosphorylated by this kinase independently of the presence of cGAS.

CONCLUSIONS:These results suggest that the yeast model is useful to study diverse molecular of this important cell signaling pathway.

Acknowledgment: Projects PID2019-105342GB-100, PID2022-138591NB-I00 and FPI grant PRE2020-093244 funded by MCIN/AEI/ 10.13039/501100011033.

Keywords: yeast, cGAS-STING pathway, interferon, cytosolic DNA, inflammatory mediators.

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IDENTIFICATION OF ENDOGENOUS NON-CODING RNA TARGETED BY RIG-I DISEASE VARIANTS

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OBJECTIVES:RIG-I gain-of-function (GOF) mutations can cause monogenic autoimmune diseases such as Singleton–Merten Syndrome and Lupus. In this study, we aim to address the mechanisms of the disease onset, focusing on self-RNA sensing of an RIG-I disease variant E373A.

METHODS:We utilized transgenic mice expressing hRIG-I E373A to monitor the development of autoimmune disorders. RIG-I E373A-bound RNA was isolated by immunoprecipitating RIG-I E373A protein:RNA complex. The obtained RNA was further separated into different fractions and used for stimulation of the RIG-I KO cells expressing hRIG-I E373A. Immunostimulatory RNA fraction was further analyzed by RNA-seq.

RESULTS:The mice expressing hRIG-I E373A spontaneously developed lupus-like nephritis with interstitial inflammation. This renal pathology recapitulates clinical features observed also in human individuals with a RIG-I GOF mutation. Isolation of RIG-I E373A protein:RNA complex from the human cells identified two types of non-coding (nc) RNAs designated Small Nuclear ILF3/NF90-Associated RNA (snaR) and Ro60-associated Y RNA (Y-RNA), as a potential endogenous RNA ligand. Of note, RIG-I immunoprecipitation using the kidney lysate from the RIG-I mutant mouse also enriched murine homologs of the ncRNA. These ncRNAs not only bound to, but also activated RIG-I E373A, as well as other RIG-I GOF variants in cellulo. Depletion of the identified ncRNA significantly downregulated transcription of ISGs in RIG-I E373A-expressing cells. These indicate a potential role of the identified endogenous ncRNA in the renal disease development.

CONCLUSIONS:We identified immunogenic ncRNA that activates RIG-I disease variants. The identified ncRNA is one of the main contributors to the IFN signature in RIG-I-related autoimmune diseases.

Keywords: RIG-I, Nucleic acid sensing, Autoimmunity, Nephritis

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EVALUATING THE DETERMINANTS OF RNA RECOGNITION BY INNATE IMMUNE SENSORS USING THE RNA SENSING REPORTER CELL LINES

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OBJECTIVES:Viral RNA induces the innate immune system through its recognition by various pattern recognition receptors (PRR), including RIG-I like receptors (RIG-I, MDA5), Toll like receptors (TLR3, TLR7, TLR8) and emerging sensors such as NLRP1. Activation of these PRRs triggers an antiviral response mediated by type IFNs and proinflammatory cytokines. These receptors possess distinct RNA sensing and signalling properties, enabling them to recognize a wide range of viruses and orchestrate distinct functions leading to their elimination. RIG-I and MDA5 are the main double strand RNA (dsRNA) sensors and can act cooperatively or distinctly to detect viruses. RIG-I preferentially binds to short dsRNAs that have blunt ends with 5'triphosphate or diphosphate moiety to discriminate between host and viral RNA. Some viral RNA structures are also required for RIG-I activation like the panhandle motif derived from the influenza virus or the poly(U/UC) tract from the 3' UTR of the hepatitis C virus. The RNA determinants recognized by the other dsRNA sensors (MDA5, TLR3 and NLRP1) are much less understood.

METHODS:Viral 5'UTR and 3'UTR are highly structured regions which may contain specific RNA structures or sequences recognized by RNA sensors. To identify the viral RNA determinants in these regions, we have screened the 5'UTR and 3'UTR from 20 positive or negative strand viruses using RNA sensor reporter cells.

RESULTS:We have developed a series of HEK293- or THP-1-derived cell lines engineered to specifically report the activation of RIG-I, MDA5, TLR3 or NLRP1, or the single strand RNA sensors, TLR7 or TLR8. All these cell lines express a dual reporter system allowing the simultaneous monitoring of the NF-kB (proinflammatory) and IRF (interferon) pathways. They present distinct RNA response profiles and were used for the screening of the viral 5'or 3'UTRs.

CONCLUSIONS:Our results should facilitate the development of specific RNA ligands for improved vaccination or therapeutic strategies.

Keywords: RNA, RIG-I, MDA5, TLR3, NLRP1, virus

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A RIBOSOMAL PRECURSOR DERIVED RNA FRAGMENT WITH 2-,3-CYCLIC PHOSPHATE AND GTP-BINDING ACTIVITY ACTIVATES RIG-I IN RESPONSE TO VIRAL INFECTION

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OBJECTIVES:RIG-I plays a key role in innate immunity to viral infections, mainly recognizing viral RNA with double-stranded structures and 5' phosphate groups. However, cellular RNA fragments were reported to exhibit immunostimulatory properties after digestion with RNase L. Although these RNA fragments bear a 5'-hydroxyl group and are therefore different from the classical RIG-I ligand, they activate RIG-I. Our goal was to characterize these RNA fragments and determine the structures essential for their immunostimulatory properties.

METHODS:We extracted cellular RNA, digested it with RNases, and used it for immunostimulation. Next generation sequencing was used to identify immunostimulatory structures. The immunostimulatory properties of specific RNA sequences and structures were studied using vitro transcription, various protocols for RNA modification, reporter systems, conformational switch assay and binding assays. Furthermore, RNA was isolated from Influenza A-infected cells or virions, modified and also used for immunostimulation. Intracellular generation of RIG-I stimulatory RNA fragments in response to RNase A transfection, RNase L activation, or viral infection was quantified by Northern blot or a specific 3-primer PCR strategy.

RESULTS:We demonstrated that the endogenous RIG-I ligand (eRL) originates from the internal transcribed spacer 2 region of 45S rRNA. For this reason, RNA from proliferating cells shows a higher potential to generate eRLs than RNA from resting cells. The immunostimulatory properties of eRL depend on its ability to bind GTP by guanosine quadruplex structures, and on cyclic 2'3' phosphate moiety which is generated by RNase digestion. The eRL can be generated in cellulo in response to RNase A transfection or activation of RNase L by 2’ 5’ Oligoadenylate. Additionally, we demonstrated that the eRL is induced by IAV infection and also contributes to immunostimulation by IAV-RNA.

CONCLUSIONS:We identified and characterized an endogenous RIG-I ligand that is produced intracellularly during viral infection and amplifies the innate immune response.

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NON-CANONICAL ACTIVATION OF STING BY SARS-COV-2 SPIKE-MEDIATED CELL-CELL FUSION CONTRIBUTES TO VIRAL PATHOGENESIS.

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OBJECTIVES:As an RNA virus, SARS-CoV2 explicitly modulates even canonical DNA sensing cGAS-STING pathway of Interferon-β (IFN) production. Multiple proteins inhibit this pathway but some studies report a sustained elevated Interferon level at advanced stages of the disease.

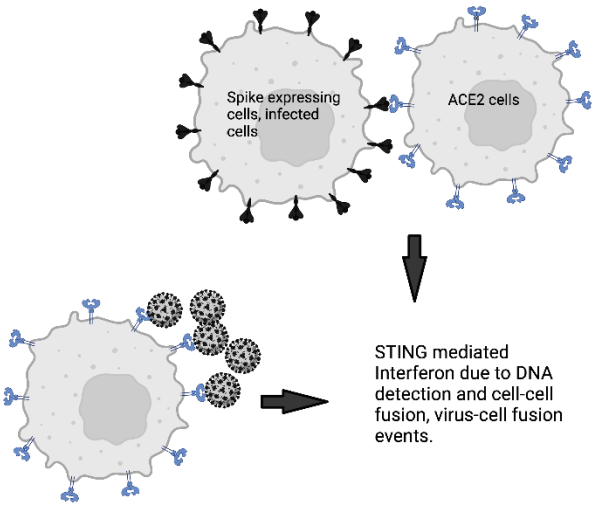
METHODS:1. IFNβ Dual Luciferase assay, to investigate the effect of SARS-CoV2 proteins on cGAS-STING pathway.
2. Co-culture method where-in Donor cells expressing Spike were co-cultured with Acceptor cells expressing the receptor ACE2 and innate immunity proteins like cGAS/STING. Techniques like RT-PCRs, Dual Luciferase reporter assay and western were employed.

RESULTS:1. Multiple SARS-CoV2 proteins inhibit cGAS-STING pathway.
2. In HEKAce2 cells, fusion was observed upon expressing Spike, with an increase in IFN production.
3. Co-culture lead to profuse cell-cell fusion and STING protein lead to IFN induction even in absence of cGAS co-expression. Fusion-mutant Spike was incorporated as a control where no IFN induction was observed. None of the other innate immunity proteins screened, exhibited an increase in their activity upon cell-cell fusion. Using the STING R169A mutant, the fusion-mediated STING activity was found to be lower. The variants of SARS-CoV2 vary widely in their fusogenicity and this may explain the differences in severity of disease with Delta (B.1.617) being highly fusogenic and manifesting a severe disease outcome.

CONCLUSIONS:Our studies on SARS-CoV2 protein shows a non-canonical activation of STING protein. *In-vitro* studies using SARS-CoV2 Spike show cell-cell fusion and this fusion process lead to IFN production in a STING-dependent manner without co-expression of cGAS. Such non-canonical fusion-dependent STING activity went down upon using the STING R169A mutant. We further aim to use this for small molecule drug-therapy based on our experimental findings of hyper-activation of STING under fusion conditions.

Keywords: SARS-CoV2 Spike, ACE2 (Angiotensin Converting Enzyme), IFN (Interferon), STING (Stimulator of Interferon Genes), cGAS (cyclic GMP-AMP synthase), fusion.

Fusion mediated Interferon



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DISPARATE STING SIGNALLING PATHWAYS AS A LINK BETWEEN BLADDER CANCER CELL INNATE IMMUNE RESPONSE AND THE IMMUNOSTIMULATORY STATE OF THE TUMOUR MICROENVIRONMENT FOLLOWING GENOTOXIC THERAPIES

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OBJECTIVES:A T cell-inflamed immunostimulatory tumour microenvironment (TME) is required for successful cancer cell clearance, and for checkpoint inhibitor immunotherapies, whilst a suppressive TME correlates with resistance and poor prognosis. Chemotherapy (CT) or radiotherapy (RT) can direct both immunostimulatory and immunosuppressive states. DNA damage can elicit cytosolic micronuclei which are detected by the DNA sensor cGAS and its adaptor STING. Nuclear DNA damage can also be detected via cGAS-independent STING activity which presents a distinct pro-inflammatory cytokine profile. We are investigating the role of DNA damage-driven innate immune responses in muscle-invasive bladder cancer. Bladder cancer presents with significant recurrence and mortality following standard-of-care cystectomy. Its high mutational load offers a favourable model system to study disparate STING pathway signalling following CT/RT and correlation with the TME immunoreactive state.

METHODS:We are analysing a panel of muscle-invasive bladder cancer cell lines for their mechanistic innate immune responses following treatment with CT agents and IR which mirror UK NICE bladder cancer management guidelines. Micronuclei burden and activation states of cGAS-dependent and cGAS-independent STING signalling were determined through quantitative confocal microscopy, qRT-PCR and ELISA.

RESULTS:Bladder cancer cells display a range of cytotoxic and cytostatic responses following CT agent treatment. We find that sub-lethal CT doses cause an innate immune response in cancer cells, and that the cells in the panel differ in their ability to activate cGAS-dependent and independent STING signalling and the resulting spectrum of type I interferon and cytokine profiles.

CONCLUSIONS:Bladder cancer cytotoxic and innate immune STING activity states are highly relevant to the mechanistic understanding of responses to CT/RT. Ongoing work will integrate innate immune signatures with analysis of patient tissue. This offers relevance for triaging patients who could benefit from bladder-sparing CT/RT and immunotherapy eligibility.

Keywords: STING Signalling, Innate Immune Response, Bladder Cancer, DNA Damage Response, Genotoxic Therapy, Tumour Microenvironment

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MODULATION OF THE INNATE IMMUNE RESPONSE BY SHORT SINGLE STRANDED OLIGONUCLEOTIDES

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OBJECTIVES:The innate immune system is the first line of defence against pathogens, recognising common molecular patterns to detect infections, such as nucleic acids in the cytoplasm. In some cases, overactivation of these systems can lead to more damage than is caused by the infection itself. Overactivation of innate immune signalling cascades can also lead to sterile inflammation in the absence of infection, for instance in autoinflammatory conditions

METHODS:We have found that innate immune nucleic acid sensing systems can be modulated by the presence of single-stranded nucleic acids, which by themselves do not cause an innate immune response in human cells. We find that some oligonucleotides can prevent activation of nucleic acid sensors, reducing the expression of interferons and chemokines upon nucleic acid detection, as observed by qRT-PCR and ELISA in human THP-1 monocytes.

RESULTS:Analysis of signalling factor activation by confocal microscopy shows that inhibition occurs at the level of the sensor proteins and/or their adaptors. Other innate immune sensing pathways, such as those involved in the detection of LPS, are not affected – showing a selective activity of inhibitory oligonucleotides. This work reveals that there are additional layers of regulation which have the capacity to fine-tune innate immune responses in circumstances where multiple PAMPs or regulatory nucleic acids act in concert to determine signalling outputs.

CONCLUSIONS:The balance between synergistic and antagonistic effects on innate immune signalling cascades may be of relevance during the response to infection and during auto-inflammation.

Keywords: Oligonucleotides, cGAS-STING, DNA sensing

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REGULATORS OF PERVASIVE TRANSCRIPTION CONTROL PRODUCTION OF RIG-I ACTIVATING SELF-PAMPS

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OBJECTIVES:The induction of type I and III interferons (IFNs) is controlled by pattern recognition receptors (PRRs) that recognize highly conserved pathogen-associated molecular patterns (PAMPs). However, we now know that cells themselves can generate these PAMPs. To resolve this conundrum, cells spatially separate PRRs and their cellular PAMPs and express enzymes that metabolize mislocalized cellular PAMPs. Yet, how the cell limits the production of these PAMPs remains largely unclear.

METHODS:To identify proteins that regulate the production of cellular self-PAMPs, we performed a targeted CRISPR screen of proteins involved in transcriptional control using an A549 cell line that expresses Gaussia Luciferase from the genomic locus of MX1, a specific and highly inducible IFN-stimulated gene. Follow-up validation and characterization work on the top hits involved overexpression studies, mutational and complementation analyses, RNA-seq, qPCR, WB, and CRISPR KO.

RESULTS:Individual knockout of 3 proteins (DR1, DRAP1, BTAF1) with a shared function in controlling pervasive transcription by RNA Polymerases II and III led to robust IFN activation. Reconstitution of KOs with mutants that disrupt known interactions which control pervasive transcription cannot restore suppression of the IFN response. Loss of any of these 3 genes increased dsRNA inside the cell. Overexpression of the protein DUSP11, which cleaves exposed 5' tri- and diphosphates on RNA, reduced activation of the IFN response in the absence of these proteins. IFN activation is entirely dependent on RIG-I, confirming the deleterious accumulation of RIG-I ligands during pervasive transcription.

CONCLUSIONS:I propose a model where these proteins suppress the production of cellular RIG-I ligands through their suppression of pervasive transcription. On-going work will continue to characterize the molecular requirements of DR1, DRAP1, and BTAF1 necessary for suppressing these self-PAMPs; define the ligands regulated by DR1, DRAP1, and BTAF1 that activate RIG-I; and identify the RNA Polymerase(s) responsible for producing these RIG-I ligands.

Keywords: RIG-I, interferon, PAMPs, self vs non-self

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STIMULATOR OF INTERFERON GENES (STING) PROMOTES PARTIAL EPITHELIAL-TO-MESENCHYMAL TRANSITION VIA REGULATING MITOCHONDRIAL FUNCTION IN RENAL FIBROSIS

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OBJECTIVES:Stimulator of interferon genes (STING), part of the innate immune system, is known to induce the type I interferon signaling in response to cellular DNA damage. Recent investigations suggested that STING is activated in renal tubular epithelial cells by mitochondrial DNA (mtDNA) released upon cisplatin treatment. Global STING deletion shows protection against acute kidney injury and renal fibrosis, in preclinical studies. Here in our study, we aimed at elucidating the cell-type specific role of STING in renal fibrosis.

METHODS:Expression of STING were evaluated in human biopsies of hydronephrosis by immunohistochemistry and the downstream signaling is further investigated in murine kidneys after unilateral ureteral obstruction (UUO) by western blotting. In vitro, to elucidate the role of STING in tubules, we treated immortalized proximal tubular epithelial cells (IM-PTECs) with transforming growth factor-β (TGFβ) to induce partial epithelial mesenchymal transition (pEMT). Expression of STING and the downstream signaling were determined by western blotting and qPCR. The effect of STING activation on pEMT was investigated using STING inhibitor H-151 and was further explored through RNA sequencing analysis.

RESULTS:STING activation and co-localization with α-smooth muscle actin (αSMA) were observed in human biopsies of hydronephrosis and in murine kidneys after UUO by immunohistochemistry and western blotting. In vitro, TGFβ-stimulated IM-PTECs presented increased expression of STING and upregulated TBK1 phosphorylation. Moreover, STING inhibition rescued TGFβ-induced partial EMT in vitro and attenuated early hallmarks of fibrosis in vivo, as determined by reduced expression of αSMA and vimentin in UUO kidneys. RNA sequencing analysis revealed that the H151-mediated rescue of pEMT in IM-PTECs resulted from ameliorated mitochondrial functions demonstrated by relieved mtDNA stress signaling, together with upregulated mtDNA content validated by qPCR.

CONCLUSIONS:STING plays a crucial role in proximal tubular dedifferentiation via regulating mitochondrial function and can be considered a therapeutic target for renal fibrosis.

Keywords: STING, pEMT, mitochondrial dysfunction

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KEY INNATE IMMUNITY GENES REGULATED DURING *VIBRIO PARAHAEMOLYTICUS* O3:K6 INFECTION IN THE CNIDARIAN MODEL *EXAIPTASIA PALLIDA*

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OBJECTIVES:Environmental disturbances driven by climate change and anthropogenic pollution, such as warming and contamination of nearshore waters worldwide, pose an immediate threat to human health.

METHODS:We used a pandemic *Vibrio parahaemolyticus* (Vp) serotype - the major cause of seafood-borne gastroenteritis in humans - and the sea anemone *Exaiptasia pallida* (Ep) – an emerging model to study invertebrate innate immunity - to examine functional conservation over 550 million years of evolution.

RESULTS:Ep's genome and transcriptome have been sequenced, allowing transcriptomic analysis of the innate immune response in host-pathogen interactions. We focused our interest on the anemone's genes homologous to vertebrate innate immunity genes. Regulation of Ep innate immunity homologs was monitored during infection by qRT-PCR and their enzymatic activity was investigated by photospectrometry.

CONCLUSIONS:Clonal anemones (strain CC7) were incubated with Vp O3:K6 (strain RIMD2210633) at 10^6, 10^7 and 108 cfu/mL for 10 days. Our results show that duration and concentration of Vp exposures influenced the regulation of Ep-caspases and validated the trend observed in Seneca *et al.* (2020).

Keywords: Innate immunity, Host-pathogen interactions, {Vibrio parahaemolyticus}, Sea anemone

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INTERACTIONS OF MODEL BACTERIA CAMPYLOBACTER JEJUNI AND HELICOBACTER PYLORI WITH HUMAN INNATE LECTIN RECEPTORS TO MODULATE CELL-AUTONOMOUS RESPONSES

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OBJECTIVES:Epsilon proteobacteria of the Campylobacterota family have a high variety and diversity of glycan moieties on their surface, comprising glycoproteins and LPS glycans. Most Campylobacterota species influence cellular innate immune responses mainly by TLR2 or the ALPK1-TIFA axis. It has already been shown that Campylobacter and Helicobacter species engage a number of lectins of the human cell surface (1,2). Lectins, in particular C-type lectins (CTL), can influence cell-autonomous innate intracellular signaling, using ITIM or ITAM signaling motifs. Helicobacter pylori interacts, for instance, with MINCLE and DC-SIGN receptors. Campylobacter jejuni was reported to interact, among others, with MGL and Dectin1.

METHODS:We have overexpressed a panel of various CTLs and other lectins in human cells as expression system. We have tested the cell-autonomous response of the overexpressing cells to various H. pylori strains which we used as a model system of immune evasive human pathogens. We have also used binding assays of lectin-overexpressing cells to complete H. pylori cells and to H. pylori adhesion proteins for quantitative comparative measurements.

RESULTS:Some of the tested lectins mediated reduced responses towards H. pylori, but not to C. jejuni. The expression of CTL influenced both the binding of H. pylori as well as the binding of purified adhesins. Preincubation with certain glycans reconstituted the inhibitory effect of the CTLs on cell-autonomous activation by whole bacteria.

CONCLUSIONS:We have developed novel test systems for assessing the modulation of cell autonomous innate responses with our model infections of Campylobacterota bacteria and observed effects of human CTL and other lectin expression on bacteria-cell binding and cell-autonomous proinflammatory activation.

1) Nagata M., et al., J. Exp. Med. 2020;218:e20200815. doi: 10.1084/jem.20200815.
2) Mayer S., et al., Front. Immunol. 2018 Feb 13;9:213. doi: 10.3389/fimmu.2018.00213.

Keywords: cell-autonomous immune response, C-type lectins, Helicobacter pylori, Campylobacter jejuni, innate immune activation, bacterial infection

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EXPERIMENTAL INVESTIGATIONS OF CANNABIDIOL IN AN ASTROCYTE-MICROGLIA CO-CULTURE MODEL OF INFLAMMATION

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OBJECTIVES:Glial cells including astrocytes and microglia are key players in central nervous system (CNS) health and disease. Microglia and astrocytes are the main cell types of the innate immune system in the CNS undergoing complex reactive remodelling in response to various brain pathologies. An in vitro astrocyte-microglia co-culture model of inflammation was developed by Faustmann et al., 2003 and allows to study the endogenous inflammatory reaction under drugs in a differentiated manner. Cannabidiol (CBD) is the major non-intoxicating, non-psychoactive phytocannabinoid derived from cannabis. CBD has a wide range of potential therapeutic uses in the treatment of CNS disorders with therapeutic effects including neuroprotective, anti-inflammatory, anti-epileptic, anxiolytic, anti-depressant, and even anti-addictive effects.
Thus, we aim to investigate the effects of CBD in an astrocyte-microglia co-culture model of inflammation.

METHODS:Primary rat astrocytes co-cultures containing 5-10% (M5, "physiological" conditions) or 30-40% (M30, "pathological inflammatory" conditions) of microglia are treated with different concentrations of CBD (50, 500, 1000 ng) for one and 24 h. Glial cell viability is measured by MTT assay. Microglial activation states are analyzed by immunocytochemistry and astroglial connexin 43 (Cx43) expression by Western blot analysis and immunocytochemistry. Gap-junctional coupling is studied via Scrape Loading.

RESULTS:The glial viability was not significantly changed after incubation with different concentrations of CBD, suggesting no toxic effects. Results with regard to microglial phenotypes, Cx43 expression and gap-junctional coupling are in progress.

CONCLUSIONS:The astrocyte-microglia co-culture model can provide novel perspectives for understanding CBD effects on glia-mediated inflammation.

Keywords: astrocytes, microglia, cannabidiol.

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CELL TYPE-AGNOSTIC OPTICAL PERTURBATION SCREENING USING NUCLEAR IN-SITU SEQUENCING (NIS-SEQ)

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OBJECTIVES:Genome-scale perturbation screening is widely used to identify disease-relevant cellular proteins serving as potential drug targets. However, most biological processes are not compatible with commonly employed perturbation screening methods, which rely on FACS- or growth-based enrichment of cells. We set out to develop a broadly applicable perturbation screening method that allows to study cellular signaling pathways in innate immunity.

METHODS:Optical pooled screening uses fluorescence microscopy to determine the phenotype in single cells, and subsequently to identify individual perturbagens in the same cells. Published methods rely on cytosolic detection of endogenously expressed barcoded transcripts, which limits application to large, transcriptionally active cell types, and often relies on local clusters of clonal cells for unequivocal barcode assignment, thus precluding genome-scale screening for many biological processes. Nuclear In-Situ Sequencing (NIS-Seq) solves these shortcomings by creating bright sequencing signals directly from nuclear genomic DNA, enabling screening any nucleus-containing cell type at high density and high library complexity.

RESULTS:We benchmark NIS-Seq by performing three genome-scale optical perturbation screens in live cells, identifying key players of inflammation-related cellular pathways.

CONCLUSIONS:NIS-Seq significantly expands the applications of CRISPR screening by enabling optical phenotyping in living cells of any nucleated type tested. It is compatible with high cell density, high library complexity and highly dynamic phenotypes. Any phenotype observable by microscopy can be studied, including sub-cellular protein transport, cell migration, oscillatory and stochastic behavior, RNA splicing, and RNA localization.

Keywords: CRISPR screening, Pooled Optical Screening, Inflammasome

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DEVELOPMENT OF HUMAN COMPLEMENT CHALLENGE MODELS FOR CLINICAL EVALUATION OF FUTURE COMPLEMENT-TARGETING DRUGS

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OBJECTIVES:innate challenge models can be a valuable tool in the evaluation of the pharmacological activity of novel compounds. At CHDR, Imiquimod (IMQ), UV-B and Lipopolysaccharide (LPS) are used to induce controlled inflammatory responses in healthy volunteers. LPS provokes an innate response via activation of toll-like receptor (TLR)-4 and is widely used preclinically as an initiator of the alternative complement pathway. We aim to use LPS to develop blood-based ex vivo complement models as well as in vivo, human challenge models, with the goal to apply these models for future clinical evaluation of complement-targeted drugs.

METHODS:Ex vivo complement activation by LPS: healthy volunteer serum or whole blood was incubated at 37°C with 2.5-100 µg/mL LPS. SC5b-9 levels were analyzed by ELISA. In vivo complement activation by LPS: to explore the role of complement in the response, 12 healthy volunteers were challenged with 5 ng of LPS intradermally in December 2023. Skin biopsies were taken predose and at 1, 3, 6, 9 and 24h post LPS challenge. Endpoints included non-invasive imaging, immunofluorescence for complement factors and RNA sequencing and protein-based assessments for local biomarkers.

RESULTS:Incubation with LPS caused a dose-dependent increase in SC5b-9 levels in serum and whole blood. Previous clinical studies where LPS was administered intradermally showed an increase erythema and skin perfusion. This was accompanied by general cell infiltration and influx of neutrophils. LPS has also been shown to evoke dendritic cell and monocyte influx, accompanied by pro-inflammatory cytokine production upon intradermal administration in men. The role of complement in the LPS-driven response remains to be elucidated.

CONCLUSIONS:The upcoming results aim to elucidate the role of complement in the intradermal LPS driven response and to clarify its potential for further development as a complement challenge model.

Keywords: Complement, Challenge model, LPS, TLR4

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STING GAIN-OF-FUNCTION MUTATION IN NON-HEMATOPOIETIC CELLS ACTIVATES AUTOREACTIVE LYMPHOCYTES

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OBJECTIVES:STING-associated vasculopathy with onset in infancy (SAVI) is an autoinflammatory disease caused by gain-of-function (GOF) mutations in the dsDNA sensing adaptor protein STING. Nearly all human SAVI patients develop interstitial lung disease (ILD) and produce anti-nuclear antibodies (ANAs). Mice with the STING GOF mutation V154M (VM) also develop ILD but are B cell lymphopenic and do not produce ANAs. Importantly, murine B cells express STING while human B cells do not. The overall goal is to determine how VM expression in hematopoietic and non-hematopoietic cells contributes to the activation of autoreactive lymphocytes and whether endosomal TLRs are involved.

METHODS:To explore the role of B and T cell repertoires in lung recruitment and activation of autoreactive lymphocytes, we generated mixed radiation chimeras by reconstituting VM mice with a mixture of WT and either non-self-reactive BCR transgenic (MD4) or non-self-reactive TCR transgenic (OTI and OTII) bone marrow (BM) stem cells. We also generated chimeras by reconstituting lethally irradiated VM mice with either WT BM stem cells (WT->VM chimera) or UNC93B-deficient BM stem cells (UNC93BKO->VM chimera). The extent of donor cell engraftment and lymphocyte recruitment and activation was determined 8-weeks post-reconstitution by histology, flow cytometry, and serum autoantibody measurements.

RESULTS:In the mixed chimeras, WT lymphocytes were preferentially recruited to the lungs compared to BCR or TCR transgenic lymphocytes, and only WT lymphocytes were activated and contributed to germinal center (GC) formation. WT->VM chimeras, but not VM mice or UNC93BKO->VM chimeras, produced ANAs.

CONCLUSIONS:Our findings demonstrate that lymphocyte activation in VM lungs requires both a diverse B cell and a diverse T cell repertoire, indicating that lymphocyte activation is driven by autoantigens. The expression of the STING GOF mutation in non-hematopoietic radioresistant cells potentiates the development of lung autoimmunity.

Keywords: Autoinflammation, autoimmunity, STING, SAVI, TLRs

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ANTI-SST2 ANTIBODY AS A NEW THERAPEUTIC APPROACH FOR IMMUNOREGULATION IN ULCERATIVE COLITIS

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OBJECTIVES:Ulcerative colitis (UC) is an inflammatory bowel disease characterized by a sustained inflammatory environment and intestinal mucosa injury, with remission episodes. UC has an unknown etiology; however, the immune system has taken a central focus, leading to the discovery of new biomarkers and therapeutic strategies. In UC, the IL-33 “alarmin” decoy receptor sST2 is up-regulated, having a crucial role inhibiting beneficial IL-33/ST2L axis effects. IL-33 is a pleiotropic cytokine sensing danger signals, eliciting mainly type 2 immune responses, therefore expanding ILC2/M2/Tregs depending on the pathophysiological context. IL-33 also stimulates IgA production from B cells, crucial for maintaining microbial homeostasis in the gut. We developed an innovative therapeutic approach with a fully humanised antibody targeting sST2 (AcHu-αsST2), aiming to decrease inflammatory molecules, thus re-establishing homeostasis in UC patients.

METHODS:We selected a scFv (single-chain fragment variable) anti-sST2 from a phage library to develop AcHu-αsST2. Furthermore, we evaluated scFv specificity for sST2 by Flow Cytometry in HMC1 cells, and affinity (KD) through surface plasmon resonance (BiacoreTM). By transcriptomic scRNA-seq, we characterized mucosa cells expressing the main alarmin axis components. Finally, detecting inflammatory cytokine levels by Cytometric Bead Array (CBA) we evaluated AcHu-αsST2 function by treating mucosa explants from UC patients.

RESULTS:scFv anti-ST2 presented a high affinity (KD=10 nM) and recognized sST2 in HMC1 cells (88.9% relative to control). scRNA-seq revealed cells involved in altered IL-33/ST2 gene expression affecting immunoregulatory responses in UC. AcHu-αST2 decreased TNF-α ($P<0.05$) and increased IL-10 levels ($P<0.01$) in cultured UC mucosa explants.

CONCLUSIONS:AcHu-αsST2 is potentially an innovative therapeutic candidate in UC, pointing to an immunoregulatory response in intestinal mucosa and therefore promoting tissue repair.

Keywords: IL-33, ST2, antibody, ulcerative colitis, intestinal mucosa

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PLASMA MEMBRANE RUPTURE PROTEIN NINJURIN A CONTROLS SUSCEPTIBILITY OF DROSOPHILA MELANOGASTER TO INVERTEBRATE IRIDESCENT VIRUS 6 (IIV6) INFECTION IN A TURANDOT-INDEPENDENT MANNER.

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OBJECTIVES:Invertebrate Iridescent Virus 6 (IIV6) has been shown to suppress the Toll and Imd pathways as well as the antiviral RNAi pathway in *Drosophila melanogaster*. Despite this widespread immunosuppression, *Drosophila* mount a response to IIV6 infection through the induction of unpaired (UPD) cytokines which in turn drive *Turandot (Tot)* gene expression. The *Tot* family of genes has been implicated in the *Drosophila* response to a wide variety of cellular stresses, indicating this cascade may have a more general stimulus, rather than recognizing a specific virus or pathogen signature. We aim to further illuminate this pathway by determining the importance of *Ninjurin A (NijA)*, encoding a protein linked to plasma membrane rupture in both *Drosophila* and mammals, to the host defense against IIV6.

METHODS:To evaluate the contribution of *NijA* to this response, Tot expression in NijA null mutant (NijAD3) *Drosophila* was measured via qRTPCR and the amount of virus in these animals was quantified by qPCR. The susceptibility of NijAD3 Drosophila to IIV6 infection was assessed via survival assay.

RESULTS:While NijAD3 null mutants did not alter the induction of *Tot* genes or the amount of virus in infected insects, these animals were significantly more susceptible to IIV6 infection.

CONCLUSIONS:These findings present interesting questions regarding the role of *NijA* and plasma membrane rupture in anti-viral host defense, as well as on the molecular mechanisms triggered by viral infection to induce *Tot* genes.

Keywords: Drosophila, Ninjurin, Plasma Membrane Rupture

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NEW MODEL OF IMMUNE-MEDIATED PREECLAMPSIA

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OBJECTIVES:Preeclampsia (PE) is a leading cause of maternal and perinatal mortality. Animal models of PE are needed for research. Based on the well-known model of miscarriage ♀CBA×♂DBA/2, we developed a model of immunologically mediated preeclampsia. The bacterial component muramyl dipeptide (C7MDP) binds to the NOD2 receptor, triggering the NF-κB signaling pathway and stimulating the production of proinflammatory cytokines by Th1 and macrophages. We hypothesized that C7MDP could be used to model immune-mediated PE.

METHODS:The combination of mouse strains ♀CBA×♂DBA/2 was used. On gestation days (GD) 5 and 7, C7MDP was administered intraperitoneally at a dose 1 mg/kg. Morphofunctional studies of the thymus and spleen were carried out on 8 and 14 gestation days(GD). The spleen was collected to assess the level of lymphocyte proliferation on the 9 GD, and the placenta on the 14 GD.

RESULTS:In our PE model embryo resorption was up to 50.0%, involutional changes in the thymus were observed already on the 8 GD; the formation of multiple large cyst-like Hassal's corpuscles was noted. In the placenta, thinning and disorganization of the layer of giant cells, and in the spongiotrophoblast numerous large cavities were observed. In the labyrinth the volumetric density of the fetal vessels decreased due to an increase in trophoblast; pronounced plethora, stasis and sludge of erythrocytes appeared in the maternal lacunae and fetal vessels. On 8 GD the production of IL-1α, IL-2, IL-6, IL-17 by splenocytes increases and the production of IFN-γ, necessary for decidualization and remodeling of spiral arteries in mice, decreases. At 14 GD the production of proinflammatory cytokines remains high.

CONCLUSIONS:The experimental data obtained on the morphofunctional changes in the organs of the immune system and the placenta make a significant contribution to the understanding of the mechanisms of immunologically mediated preeclampsia.

Keywords: Preeclampsia, model, NOD2

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ENFORCED INTERFERON ALPHA SECRETION FROM DENDRITIC CELLS INSTIGATES LETHAL SYSTEMIC AUTOIMMUNITY INCLUDING IMMUNOLOGICAL FEATURES OF PRIMARY SJOGREN SYNDROME

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OBJECTIVES:Systemic autoimmunity describes a group of detrimental conditions that is characterized by loss of immunologic self-tolerance. In autoimmune patients, lymphocytes receive inappropriate signals from innate immune cells leading to their activation in response to detection of self-antigens. Research on rare monogenic conditions implicated type I interferons (IFN) in the aberrant immune activation. Arriving at definitive conclusions about the contribution of IFNs in the pathogenesis of polygenic autoimmune conditions, such as Primary Sjorgen Syndrome (PSS), remained challenging due to the complex feedback-regulation within the IFN-pathway.

METHODS:In order to connect IFN-signaling with specific immunologic alterations, we developed a new mouse model that allows for conditional expression of mouse Ifnα4. We immunophenotyped Ifnα4 expressing mice and a cohort of 177 PSS patients.

RESULTS:Enforced secretion of Ifnα4 by conventional dendritic cells results in lethal systemic autoimmunity in mice characterized by lymphopenia, multi-organ inflammation, hypergammaglobulinemia and the production of autoantibodies. Furthermore, we found increased abundance of Trim21 protein, the signature autoantigen in PSS, which coincided with the development of autoantibodies against Trim21. Most importantly, lymphopenia, hypergammaglobulinemia and autoantibody levels positively correlated with IFNα serum levels in PSS patients.

CONCLUSIONS:Our study establishes IFNα as a driver of clinically relevant and disease-specific immunological features in polygenic autoimmune conditions like PSS. Hence, we establish the type I IFN pathway as therapeutic target in PSS with high relevance to clinical trial stratification and biomarker design.

Keywords: type I interferon, autoimmunity, primary sjogren syndrome

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ACTIVATION OF INTESTINAL IMMUNITY BY PATHOGEN EFFECTOR-TRIGGERED AGGREGATION OF LYSOSOMAL TIR-1/SARM1

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OBJECTIVES:TIR-domain proteins with enzymatic activity are essential for immunity in plants, animals, and bacteria. However, it is not known how these proteins function in pathogen sensing in animals. We discovered that a TIR-domain protein (TIR-1/SARM1) is strategically expressed on the membranes of a lysosomal sub-compartment, which enables intestinal epithelial cells in the nematode *C. elegans* to survey for pathogen effector-triggered host damage. We showed that a redox active virulence effector secreted by the bacterial pathogen *Pseudomonas aeruginosa* alkalinized and condensed a specific subset of lysosomes by inducing intracellular oxidative stress. Concentration of TIR-1/SARM1 on the surface of these organelles triggered its multimerization, which engages its intrinsic NADase activity, to activate the p38 innate immune pathway and protect the host against microbial intoxication. Thus, lysosomal TIR-1/SARM1 is a sensor for oxidative stress induced by pathogenic bacteria to activate metazoan intestinal immunity.

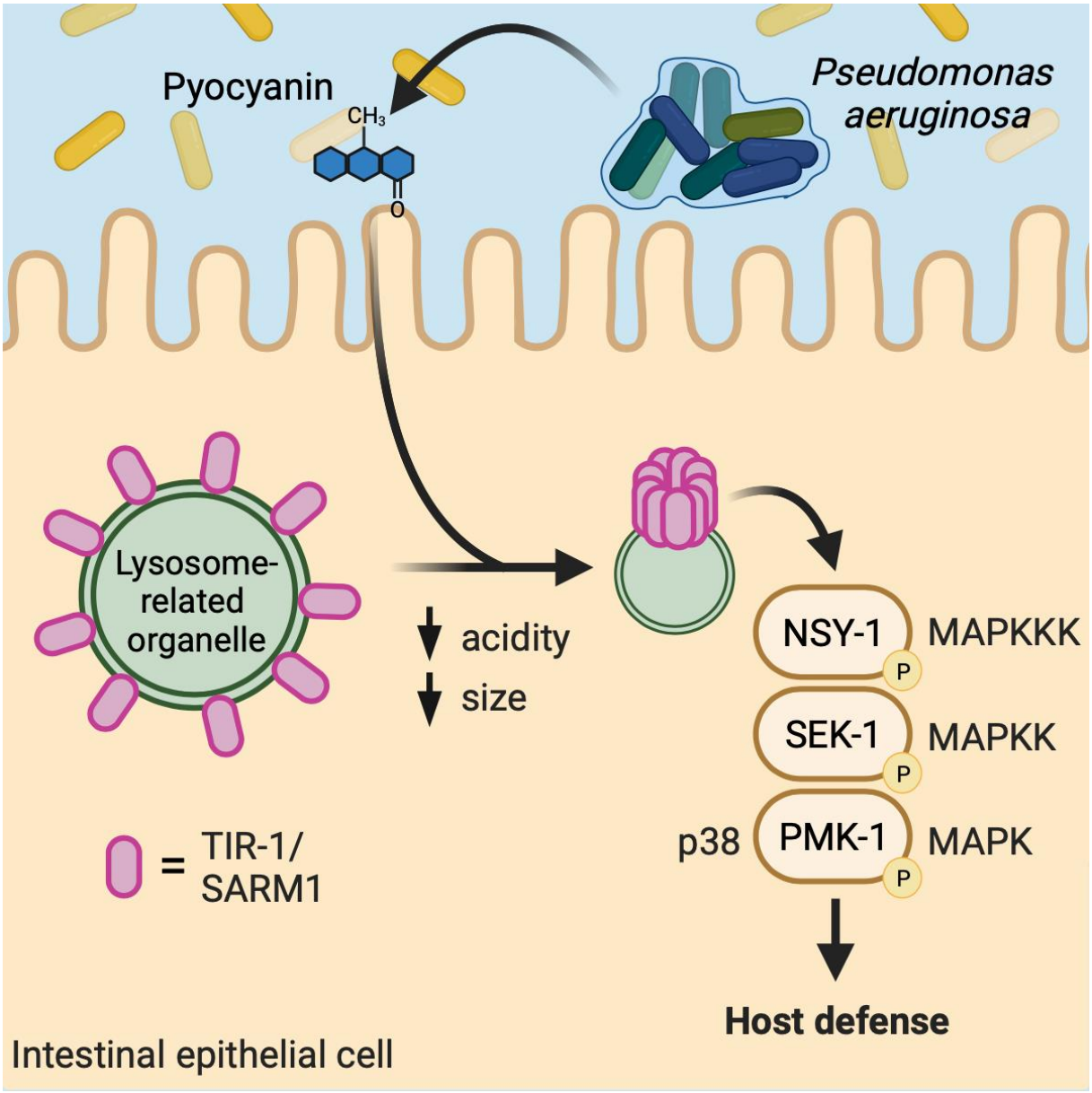
METHODS:See above.

RESULTS:See above.

CONCLUSIONS:See above.

Keywords: effector-triggered immunity, lysosome-related organelles, intestinal immunity, TIR-1/SARM1, Pseudomonas aeruginosa, Caenorhabditis elegans

Graphical Abstract


Pyocyanin
CH3
O
Pseudomonas aeruginosa
Lysosome-related organelle
acidity
size
NSY-1
MAPKKK
P
SEK-1
MAPKK
P
p38
PMK-1
MAPK
P
= TIR-1/ SARM1
Host defense
Intestinal epithelial cell

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NON-CANONICAL PATTERN RECOGNITION IDENTIFIES VIRULENT BACTERIA IN C. ELEGANS

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OBJECTIVES:Distinguishing infectious pathogens from harmless and beneficial microorganisms is essential for animal health. Here, we characterize a non-canonical pattern recognition system in the nematode *Caenorhabditis elegans* that assesses the relative threat of virulent *P. aeruginosa* to activate innate immunity. We discover that the innate immune response in *C. elegans* is triggered by phenazine-1-carboxamide (PCN), a toxic metabolite produced by pathogenic strains of *Pseudomonas aeruginosa*. We identify nuclear hormone receptor NHR-86/HNF4 as the PCN sensor and validate that PCN binds to the ligand-binding domain of NHR-86/HNF4. Activation of NHR-86/HNF4 by PCN directly engages a transcriptional program in intestinal epithelial cells that provides protection against *P. aeruginosa*. Taken together, this study identifies the first pattern recognition receptor in *C. elegans* and reveals that a bacterial metabolite is a pattern of pathogenesis surveilled by *C. elegans* to identify a pathogen among its bacterial diet.

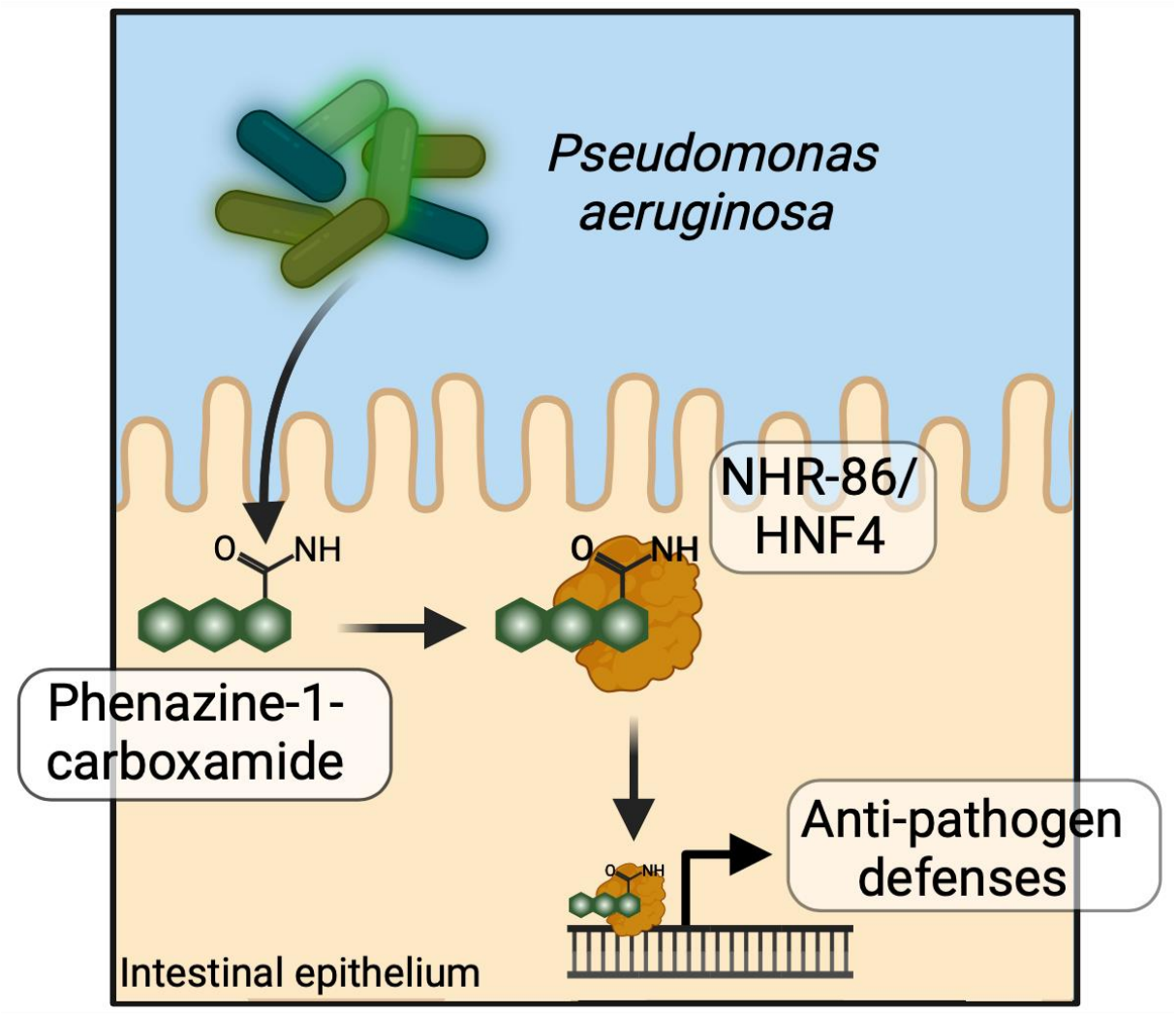
METHODS:See above.

RESULTS:See above.

CONCLUSIONS:See above.

Keywords: Pattern recognition receptor; nuclear hormone receptor; phenazines; Pseudomonas aeruginosa, Caenorhabditis elegans

Graphical Abstract



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A HUMORAL STRESS RESPONSE PROTECTS DROSOPHILA TISSUES FROM ANTIMICROBIAL PEPTIDES

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OBJECTIVES:An efficient immune system must provide protection against a broad range of pathogens without causing excessive collateral tissue damage. While immune effectors have been well characterized, we know less about the resilience mechanisms protecting the host from its own immune response. Antimicrobial peptides (AMPs) are small, cationic peptides that contribute to innate defenses by targeting negatively charged membranes of microbes. While protective against pathogens, AMPs can be cytotoxic to host cells.

METHODS:To identify factors protecting host tissues from AMPs, we harnessed the power of *Drosophila* genetics combined with electrophysiology, structural approaches and molecular dynamics simulations.

RESULTS:Here, we reveal that a family of stress-induced proteins, the Turandots, protect the *Drosophila* respiratory system from AMPs, increasing resilience to stress. Flies lacking *Turandot* genes are susceptible to environmental stresses due to AMP-induced tracheal apoptosis. Turandot proteins bind to host cell membranes and mask negatively charged phospholipids, protecting them from cationic pore-forming AMPs.

CONCLUSIONS:Collectively, these data demonstrate that Turandot stress proteins mitigate AMP cytotoxicity to host tissues and therefore improve their efficacy.

Keywords: Antimicrobial peptides, immunopathology, resilience, stress response

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A NEW HUMAN MONOCYTE TRANSDIFFERENTIATION CELL LINE

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OBJECTIVES:Some B-lineage cells transdifferentiate into macrophages upon overexpression of the myeloid transcription factor CCAAT/enhancer-binding protein alpha (C/EBPα). The BLaER1 cell line originates from the human B cell precursor leukemia cell line RCH-ACV and is engineered to inducibly activate C/EBPα. BLaER1 cells can be transdifferentiated into monocytes and are widely used to model human innate immune signaling. However, a contamination of active squirrel monkey retrovirus (SMRV) has been identified in this cell line, which limits the broader laboratory usage of these cells. This study focuses on reproducing and refining a SMRV-free human monocyte transdifferentiation model system.

METHODS:We stably integrated a fusion construct of C/EBPα and the ligand-binding domain of the estrogen receptor (C/EBPα-ESR1LBD) into RCH-ACV cells using the piggy bac transposon system. Controlled nuclear translocation of the C/EBPα-ESR1LBD transgene, induced by β-estradiol in the presence of interleukin-3 and macrophage colony-stimulating factor, induced the conversion of B-lineage cells to monocytes/macrophages while ensuring cell viability. FACS and ELISA analysis were performed to assess the transdifferentiation efficiency and innate immune responses.

RESULTS:The new cell line exhibits comparable transdifferentiation efficiency and a more sensitive immune response upon activation of various pattern recognition receptors. The functional validation ensures the absence of viral contamination. Further improvements include generation of additional cell lines devoid of fluorescence markers and common antibiotic resistance, enabling broader applications for bioimaging and multiplexed genetic screening.
CONCLUSIONS:In conclusion, this study successfully develops and functionally validates a SMRV-free and functional human monocyte transdifferentiation model system, providing a useful toolbox for the establishment of additional human monocyte transdifferentiation cell lines.

Keywords: model system, cell line, human monocyte cell line, macrophages/monocytes, transdifferentiation

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A CO-CULTURE MODEL FROM INDUCED IPSC-DERIVED MACROPHAGES AND ALVEOLAR EPITHELIAL CELLS TYPE 2

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OBJECTIVES:Cell culture systems permit mechanistic and dynamic investigations that are not possible in patients and challenging in animal models. However, in vitro often only one cell type is used, which disables us to mimic the full arsenal of the host. Thus, there is a growing need to develop more tractable models that recapitulate core features of human infection and permit dissection of relevant basic biological processes. Macrophages are the first cells infected by Mycobacterium tuberculosis (Mtb) when the pathogen is inhaled into the human lung and remain an important niche for the bacterium throughout the course of infection. However, in the alveolar space, Mtb encounters gas-exchanging alveolar epithelial cells type 1 (AT1s) and surfactant producing AT2s in addition to macrophages.

METHODS:We have just established a tractable alveolus-mimetic co-culture model from induced pluripotent stem cell (iPSC)-derived macrophages and AT2s, which we will use to study the cellular crosstalk during infection with Mtb or respiratory virus.

RESULTS:I will present initial data from our ongoing characterization of the model.

CONCLUSIONS:We have established an air-lifted human macrophage- alveolar epithelium co-culture model that enables studies of cellular crosstalk.

Keywords: lung, macrophage, epithelial, co-culture, mycobacterium tuberculosis, inflammation

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SINGLE-CELL RNA SEQUENCING REVEALS THE BRAIN TRANSCRIPTIONAL SIGNATURE OF HERPES SIMPLEX ENCEPHALITIS-LIKE DISEASE IN MICE

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OBJECTIVES:Herpes Simplex Virus 1(HSV-1) is a common human neurotropic virus with the majority of adults harboring latent-recurrent infections. In rare cases, HSV-1 infection can access the central nervous system through the neuronal route and develop into a devastating encephalitis if not controlled by the immune system. Current available antiviral therapy includes acyclovir. Despite the importance of the immune response in control of HSV-1 central nervous system infection, the nature and dynamics of the cellular host response remains unknown.

METHODS:In this work, we use a mouse model for HSV-1 infection to describe the transcriptomic profile of the infected brain stem at the single-cell level and with temporal resolution.

RESULTS:Among resident brain cells, we find microglia to increase during the course of infection, while astrocytes and endothelial cell levels decrease. At the levels of peripheral immune cells we found notably monocytes to strongly influxed the infected brain stem. We identified dynamic changes in the proportion and state of subpopulations following virus infection, revealing the pivotal role of certain cell subpopulations and gene functions in antiviral activity and maintainance of homeostasis. For instance, we found one subpopulation of microglia with strong upregulation of chemokine pathways being significantly increased during infection process. This population was also enriched for viral transcripts, suggesting it to be localized at foci of infection, and orchestrating recruitment of other immune cells. Finally, when investigating the transcriptome post infection, we observed a decrease in the cellular communication strength of brain resident cells, while the communication strength of immune cells showed an increase.

CONCLUSIONS:Our work thus reveals the complex nature of the cellular response in the virus-infected brain, which seeks to eliminate infection and can also prime for pathological changes.

Keywords: single-cell transcriptome, virus-infected brain

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CIRCULATING PROTEINASE 3, MYELOPEROXIDASE AND THEIR COMPLEX AS INDICATORS OF MISSED ABORTION

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OBJECTIVES:A missed abortion in 1-trimester is the common complications of pregnancy. Role of activated neutrophils in early losses pathogenesis is unknown. Serum levels of neutrophil proteinase 3(PR3), myeloperoxidase(MPO) and their complex (PR3/MPO) are indicators of neutrophil activation. Content of PR3/MPO-complex in blood can serve as a quantitative characteristic of neutrophil extracellular traps (NETs).

METHODS:The study included 80 consecutive consenting pregnant women (18-40years old, 5-13weeks of gestation):60 patients had a missed abortion, and 20 women were with uncomplicated pregnancy. Levels of PR3, MPO, PR3/MPO-complex in blood serum were revealed by ELISA. Statistical analysis was performed using Spearman's rank correlations(r), ROC-analysis, Mann-Whitney test with critical value of $p<0.05$. Data are presented as median with interquartile range(Me;Q1-Q3).

RESULTS:Patients with missed abortion had higher PR3 (343ng/ml; 109-455)($p<0.0001$), MPO(707ng/ml; 465-869)($p<0.0001$) and PR3/MPO-complex(10u/ml; 5.9-15)($p<0.0001$), than healthy women PR3 (21ng/ml; 18-22), MPO(217ng/ml; 146-236)and PR3/MPO-complex (5.0u/ml; 4.0-5.4). We revealed a strong positive correlation in both groups between PR3 and MPO (healthy:r=0.717; abortion:r=0.796), and only for abortion between PR3/MPO-complex and PR3(r=0.546), MPO(r=0.396). In group with missed pregnancy there was a negative correlation between MPO and a2-antiplasmin activity(r=-0.503), but MPO had a positive correlation with leukocytes (r=0.410) and neutrophils (r=0.424). PR3 correlated with leukocytes (r=0.395), neutrophils (r=0.499) and ratio of neutrophil/lymphocyte (r=0.400). PR3/MPO-complex showed a modest correlation only with platelets (r=0.430). ROC analysis was applied to estimate the sensitivity(SN)and specificity(SP)of PR3 (SN=97%;SP=100%,AUC=0.995), MPO(SN=87%;SP=95%,AUC=0.941) and PR3/MPO complex (SN=77%;SP=95%,AUC=0.888) to differ women with missed abortion from healthy pregnant women.

CONCLUSIONS:PR3, MPO and their complex in serum can serve as potential markers of missed abortion and indicate neutrophil activation. PR3 increases more strongly. Significant increase of circulating PR3/MPO-complex indicates a possible role of NETs in the missed pregnancy pathogenesis.

Keywords: missed abortion, myeloperoxidase, neutrophil proteinase 3, neutrophil extracellular traps, pregnancy

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IMPACT OF EXOGENOUS TNFR2 ACTIVATION ON MICROGLIAL FUNCTION

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OBJECTIVES:TNFR2 (tumor necrosis factor receptor 2) is crucial in the regulation of inflammation in the central nervous system. Compared to peripheral immune cells, the role of TNFR2 signaling for microglia function is less well characterized. Microglia are local immune cells that play a critical role in maintaining brain homeostasis and are the first responders to injury or infection. Microglia can be polarized into two distinct phenotypes: M1 and M2. The pro-inflammatory M1 phenotype is involved in the initial immune response to injury or infection, while M2 cells are anti-inflammatory and are involved in tissue repair and regeneration. Depending on the specific context and timing of activation, microglial TNFR2 signaling can have a beneficial or detrimental impact on brain function, by promoting either pro- or anti-inflammatory responses. The objective of this work therefore was to gain a deeper understanding of the role of TNFR2 for microglial polarization and function.

METHODS:Using the microglial cell line BV-2, we first analyzed the regulation of TNFR2 expression during polarization by quantitative gene expression and flow cytometry. Then, to gain a deeper understanding of the pro-and anti-inflammatory roles of microglia-TNFR2, we developed a TNFR2 specific agonist. Using this biologic with determined the impact of exogenous TNFR2 stimulation on pro- and anti-inflammatory differentiation and cell survival.

RESULTS:Our results indicate that TNFR2 expression is regulated during pro- but not anti-inflammatory differentiation of BV-2 cells. Further, exogenous TNFR2 activation promoted expression of pro-inflammatory and anti-inflammatory genes and rendered M1 polarized microglia more susceptible for cell death induction.

CONCLUSIONS:Our data indicate that TNFR2 plays an important role for microglia polarization and activity. Exogenous activation of microglial TNFR2 has been shown to promote therapeutic effects in disease model. However, our data highlight that microglial TNFR2 activity may impact both pro- and anti-inflammatory responses and needs to be observed during therapy studies.

Keywords: microglia, TNFR2

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THE IMMUNOSUPPRESSIVE MYELOID COMPARTMENT IN TUMORS IS SHAPED BY TUMOR-INTRINSIC FACTOR

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OBJECTIVES:Myeloid cells have been long recognized as important modulators in health in disease. We investigate functional properties of myeloid cells in the context of pancreatic cancer (PDAC). In cancer patients, myeloid cells expand, are recruited by the tumor and differentiate into immunosuppressive subsets. The immunosuppressive properties of intratumoral myeloid cells are utilized by the cancer to develop an immunosuppressive tumor microenvironment (TME). This study delved into understanding the factors triggering the recruitment and enrichment of neutrophils as well as monocytes in the TME. Additionally, we compare the functional properties of tumor-primed and naïve myeloid cells, analyzing their migratory capacity towards tumor-secreted factors.

METHODS:Over 80 murine PDAC cell lines (KPC model, provided by Prof. Saur, TUM) were characterized for their chemokine secretion using multiplex immunoassays. Ex vivo chemotaxis assays with isolated myeloid cells from tumor-bearing and naive mice were employed to study the chemotactic effects of various secretomes. Multivariate regression models were utilized to identify chemokines with the highest impact on neutrophils and monocyte migration. To explore the impact of PDAC heterogeneity on the myeloid compartment in vivo, a syngeneic orthotopic PDAC mouse model was employed. Flow cytometry was used to characterize myeloid cell expansion and infiltration in the blood, spleen, and tumors. Further, functional properties of tumor-primed and naïve cells myeloid cells were investigated in vitro.

RESULTS:The analysis revealed that myeloid cells are recruited by tumor-intrinsic chemokines. Cluster analysis unveiled correlations between distinct secretion profiles and tumor cell morphologies (epithelial vs. mesenchymal), resulting in either neutrophil-dominant or monocyte/macrophage-dominant TMEs. Intratumoral myeloid cells display significantly different chemokine receptor expression than peripheral myeloid cells and develop immunosuppressive function.

CONCLUSIONS:Myeloid cells are recruited in the TME by tumor cells-intrinsic chemokines. While both Monocyte-dominant and Neutrophil-dominant TMEs exhibit functional redundancy in suppressing antitumoral immune responses, individual therapeutic strategies may be necessary to overcome immunosuppression.

Keywords: immunosuppressive myeloid cells, chemotaxis, chemokine receptors, cancer

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NEUTROPHIL EXPRESSION AND FUNCTION OF LOX-1 DURING THE INNATE IMMUNE RESPONSE TO PNEUMONIA

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OBJECTIVES:Pneumonia is a worldwide public health concern, demanding a better understanding of the host response to pulmonary pathogens. We recently reported that lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is enriched in a subset of airspace neutrophils, where its expression may prevent inflammatory injury during pneumonia. Here, our goal was to distinguish the effects of LOX-1 activity on neutrophils, addressing the unique role of this receptor in the lung.

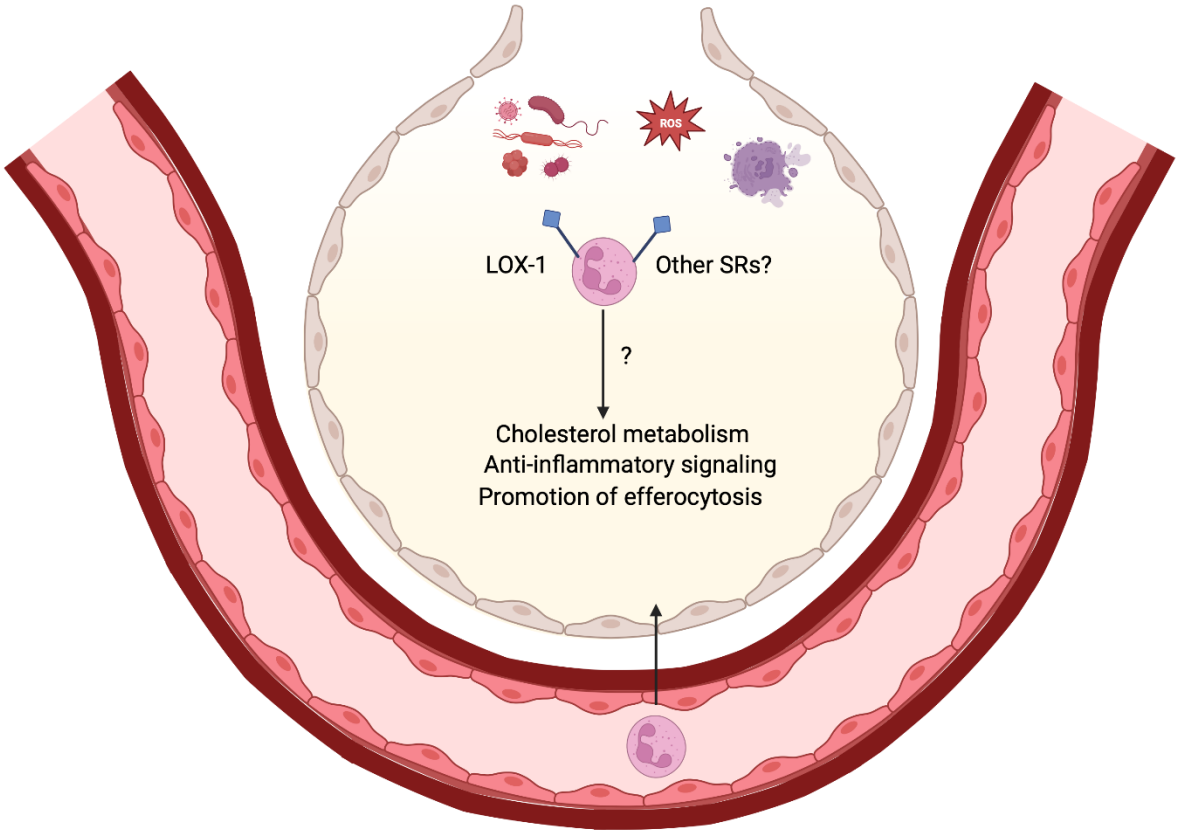
METHODS:The cellular distribution of lung LOX-1 was examined using single-cell sequencing resources. Wild-type mice, LOX-1 KO mice, and mice with a deletion of LOX-1 on neutrophils (LOX-1ΔMrp8) were infected with S. pneumoniae or E. coli for 12-48h. Spectral flow cytometry was used to determine neutrophil responsiveness and phenotype.

RESULTS:We previously observed that intratracheal LOX-1 blockade increases pneumonia-induced injury. A comparison of single-cell sequencing datasets showed substantial enrichment of LOX-1 (Olr1) mRNA in airspace neutrophils during pneumonia; however, LOX-1 is virtually absent in blood and bone marrow. Interestingly, LOX-1 is inducible on bone marrow neutrophils when exposed to bronchoalveolar lavage fluid ex vivo, indicating airway fluid is sufficient to activate its expression. Intriguingly, LOX-1 is only expressed on a subset of neutrophils during lung infection, and LOX-1+ neutrophils have a markedly altered phenotype. We previously showed that LOX-1+ neutrophils exhibit changes in cholesterol metabolism, insinuating PPAR-γ and RXR/LXR activation. Moreover, LOX-1+ neutrophils have elevated CXCR3/4, MARCO, and CD200R expression, which, taken together, suggests these cells are a subtype of pro-resolving neutrophils. In line with our hypothesis, LOX-1ΔMrp8 mice that lack LOX-1 on neutrophils exhibit elevated lung injury, cellular recruitment, and complement receptor expression during S. pneumoniae infection.

CONCLUSIONS:Our data implicate a unique, immunoregulatory role for LOX-1 on recruited neutrophils, possibly taming their responses to infection. These results provide a framework for future research studying the mechanisms connecting neutrophil LOX-1 to pneumonia outcomes.

Keywords: Pneumonia, Inflammation, Neutrophil, LOX-1

LOX-1+ neutrophils limit inflammatory lung injury



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SENSORY NEURONS IMPRINT LOCAL MACROPHAGE IDENTITY VIA TGF-β

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OBJECTIVES:Macrophages play integral roles in maintaining homeostasis and function in their tissues of residence. In the skin, prenatally seeded and highly specialized macrophages physically interact with sensory nerves and contribute to their regeneration after injury. However, mechanisms underlying the development and maintenance of this potentially lifelong commitment of macrophages to nociceptors remain largely elusive.

METHODS:Here, we used fate-mapping, transcriptomic profiling and confocal whole mount imaging both in vitro and in vivo to resolve their origin and tissue-specific cues underlying their functional adaptation to peripheral nerves.

RESULTS:We demonstrate that bone marrow-derived myeloid progenitors were recruited to sensory nerves upon nerve fiber sprouting. Macrophages in close proximity to nerves rapidly adopted a neuro-supportive profile, both in vitro and in the tissue. Hence, interaction with the microenvironment rather than the origin dictated their identity. Functional analysis revealed a crucial role for the transforming growth factor (TGF)-β in the transformation process, which was produced by sensory neurons. Mechanistically, the effect of TGF-β on macrophages was facilitated by their physical association with sensory nerves and integrins, which mediated local activation of latent TGF-β. Conditional depletion of Tgfbr2 in nerve-associated macrophages in the skin resulted in a loss of their identity and self-maintenance in steady state. Upon tissue injury, TGF-β signaling was essential for the adaptation of monocyte-derived macrophages to neurons and local nerve regeneration.

CONCLUSIONS:Collectively, we identify TGF-β as a central mediator governing local imprinting of skin-resident macrophages, and provide novel insights into the bidirectional communication between sensory neurons and macrophages.

Keywords: macrophages, monocytes, skin, neurons, TGF-β

Image 1

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TOLL-LIKE RECEPTOR 3 ACTIVATION IN COLON CANCER CELLS INDUCES NEUTROPHIL RECRUITMENT AND IMPAIRS CXCR2 AND CXCR4 EXPRESSION

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OBJECTIVES:Colorectal cancer (CRC) is the second leading cause of cancer-related deaths. Here we investigated inflammation markers in CRC patient tumor and adjacent healthy, revealing elevated expression of chemokines, including CXCL10, IL8, and neutrophil markers, indicative of neutrophil influx. We further assessed protein expression of Toll-Like Receptor 3 in CRC tissue and investigated how TLR3 activation in colon cancer cells affects neutrophil recruitment and activation.

METHODS:Gene expression in tumor tissue from CRC patients (N=30), and in TLR3 activated colon cancer cells (SW620) was assessed by nCounter gene expression analysis. Neutrophils in tumors exhibit diverse activation states between the extremes N1 and N2, with N1 considered anti-tumorigenic and N2 pro-tumorigenic. Interferon (IFN) β supports N1 neutrophils. Neutrophils from healthy donors were incubated with conditioned medium (CM) from TLR3 activated colon cancer cells, or cytokines, to study how this affected neutrophil recruitment and

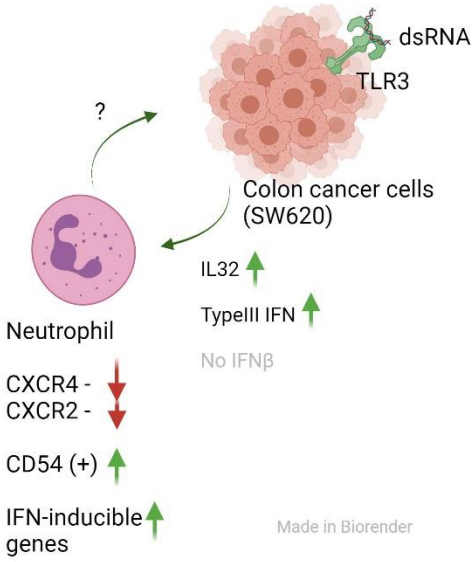
activation. CM-treated neutrophils were assayed for surface expression of activation markers by flow cytometry.

RESULTS:CRC tumors expressed TLR3, CXLCL10, IL8 and neutrophil markers. TLR3 activation in colon cancer cells triggers CXCL10 and IL8 production, but not IFNβ. However, CM from TLR3 activated colon cancer cells, triggered neutrophil migration and upregulation of IFN-inducible genes in these cells. These neutrophils displayed modest expression of activation markers associated with N1 phenotype, such as CD54, although surface expression of the chemokine receptors CXCR2 and CXCR4 was impaired. Investigation into other IFNs released by TLR3-activated colon cancer cells identified Type III IFN production. Interestingly, Type III IFNs did not influence CXCR2, CXCR4 or CD54 surface expression. Conversely, the IFN-inducible cytokine IL32 down-regulated CXCR2 and up-regulated CD54 on neutrophils, suggesting it may encourage an anti-tumorigenic state.

CONCLUSIONS:Our results suggest that IL32 production in TLR3-activated colon cancer cells modulates the expression of surface CXCR2 and CD54 on neutrophils, potentially promoting an anti-tumorigenic phenotype.

Keywords: Toll-like receptors (TLR), interferon (IFN), neutrophil, IL32, colon cancer, chemokine

Toll-like receptor 3 activation in colon cancer cells induces neutrophil recruitment and impairs CXCR2 and CXCR4 expression



Activation of TLR3 in colon cancer cells induces the release of CXCL10, IL8, IL32 and Type III IFNs, but not IFNb. Conditioned medium from TLR3-activated colon cancer cells induces neutrophil recruitment upregulation of activation marker CD54, and impairs CXCR2 and CXCR4.

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IRF5 IS REQUIRED FOR INFLAMMATORY HEMOPHAGOCYTE DIFFERENTIATION IN TLR7-MEDIATED MACROPHAGE ACTIVATION SYNDROME

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OBJECTIVES:Macrophage Activation Syndrome (MAS) is a serious complication of rheumatic disease. MAS is characterized by the development of cytopenias, including anemia and thrombocytopenia, and hemophagocyte accumulation— activated macrophages that phagocytose red blood cells. We previously identified a mouse model of systemic lupus erythematosus that develops spontaneous MAS-like disease, TLR7.1 mice. In this model, overexpression and constitutive active signaling of the endosomal ssRNA sensor TLR7 drives disease. TLR7.1 mice develop thrombocytopenia, anemia, and a novel population of hemophagocytes, inflammatory hemophagocytes (iHPCs), which spontaneously differentiate from Ly6CHI monocytes. iHPCs contribute to anemia indicating that iHPC hemophagocytosis may drive disease in TLR7.1 mice. In humans, SNPs in the gene encoding the transcription factor IRF5 have been associated with increased risk of MAS. Additionally, IRF5 signaling is critical for inflammatory macrophage development in several inflammatory disease models. Whether IRF5 expression in monocytes is required for MAS and iHPC differentiation and function is unknown.

METHODS:We used IRF5-deficient mice, mixed bone marrow chimeras, and mice with conditional deletion of Irf5 in myeloid cells in the TLR7.1 MAS model to examine the requirement of IRF5 for disease development and the differentiation and function of iHPCs. We also used an acute MAS-like model where mice are treated topically with the TLR7 agonist R848.

RESULTS:In the TLR7.1 MAS model, IRF5 signaling was required for iHPC development and hemophagocytosis in a cell-intrinsic manner. We also found that IRF5 expression in myeloid cells is required for iHPC differentiation and some aspects of MAS-like disease in TLR7.1 mice and after topical R848 treatment. We are continuing to investigate the contribution of IRF5 signaling in Ly6CHI monocytes to iHPC differentiation and disease development in spontaneous TLR7-diven MAS.

CONCLUSIONS:These studies show a critical role of IRF5 in TLR7-driven MAS and in the differentiation and function of disease-associated hemophagocytes.

Keywords: TLR7, IRF5, Monocyte differentiation, Macrophage Activation Syndrome

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General Research » Myeloid cell physiology and development

DYNAMIC ROLE OF MONOCYTES AND MENINGEAL MACROPHAGES IN STREPTOCOCCAL MENINGOENCEPHALITIS

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OBJECTIVES:Meningeal macrophages are established to substantially contribute to brain immunity, however their site-specific origin and function in meningitis are incompletely understood.

METHODS:We established a physiological meningoencephalitis model is which the disease results from haematogenous bacterial spread, and applied confocal imaging, single-cell transcriptomics and fate-mapping strategies to understand the intersectionality of myeloid fates in the infection with a focus on localisation and differentiation trajectories.

RESULTS:In a natural model of streptococcal meningitis, where bacteria systemically spread via the blood stream, we found streptococci to mostly localize to the meninges, leading to activation of meningeal macrophages and largely sparing parenchymal microglia in the early disease phase. This coincided with a loss of specific meningeal macrophage subsets, and rapid engraftment of highly activated monocytes in the dura mater. In addition, monocyte progenitors in the skull marrow underwent drastic changes and acquired a more immature phenotype likely due to emergency myelopoiesis. Parenchymal invasion of monocytes, but not granulocytes, correlated to disease severity. Notably, in steady state, dural monocytes were derived from adjacent skull marrow in a CCR2-independent fashion, while the high demand for dural monocytes in streptococcal meningitis required intact CCR2 signaling, indicating heterogeneity in monocyte recruitment. Furthermore, meningitis increased monocyte progeny from MDPs compared to the homeostatic, GMP-dominated origin.

CONCLUSIONS:Monocytes in the dura, recruited from distinct reservoirs depending on disease-inherent needs, are intertwined with the disease course and may thus offer opportunities for therapeutic interventions.

Keywords: Macrophages, Monocytes, Infection, CNS

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General Research » Myeloid cell physiology and development

MONONUCLEAR PHAGOCYTE DYSREGULATION AS A CAUSE OF INTESTINAL INFLAMMATION IN CHRONIC GRANULOMATOUS DISEASE

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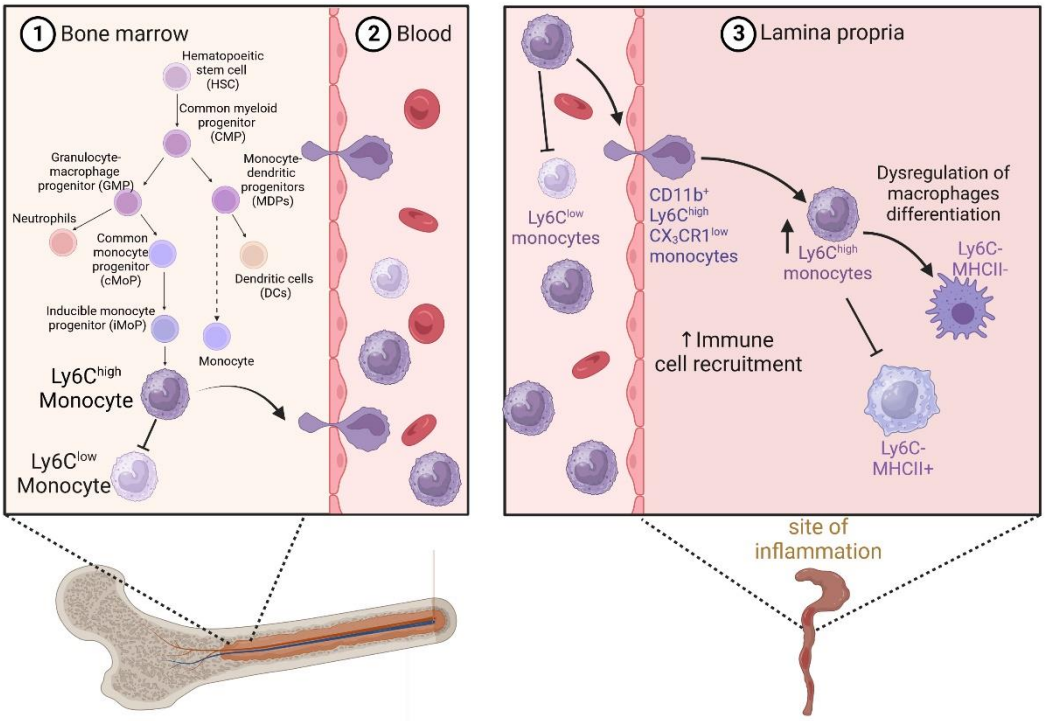
OBJECTIVES:Chronic Granulomatous Disease (CGD) represents a primary immunodeficiency disorder carrying substantial immunopathological consequences. It is caused by mutations in the NADPH oxidase NOX2, which catalyzes the production of reactive oxygen species (ROS) in phagocytes. More than half of CGD patients experience severe intestinal inflammation akin to inflammatory bowel disease. However, it is not understood how gut homeostasis is affected by the absence of ROS production in phagocytes and which cell types contribute to the initiation of colon inflammation.

METHODS:In our study, we utilized imaging and sequencing approaches to scrutinize transgenic CGD mouse models under SPF and wildling conditions and in chemically induced colitis.
RESULTS:We found mice deficient in NADPH oxidase subunits p47phox and gp91phox to exhibit similar baseline disturbances in macrophage differentiation in the lamina propria. In contrast, they showed markedly distinct responses in chemically induced colitis. The severe colitis phenotype in p47phox-deficient mice was only partially rescued by bone marrow transplantation, indicating an interplay of myeloid cells and the microbiota, which was confirmed by microbiota sequencing analysis. However, cross fostering between p47phox and gp91phox-deficient mice only partially modified their discrepant colitis phenotypes and microbiota composition, pointing at very early microbiota fixation under SPF conditions. In contrast, the presence of a complex wildling microbiota triggered spontaneous colitis, granuloma formation and secondary sepsis from intestinal pathogens in both NOX2-deficient mouse lines.

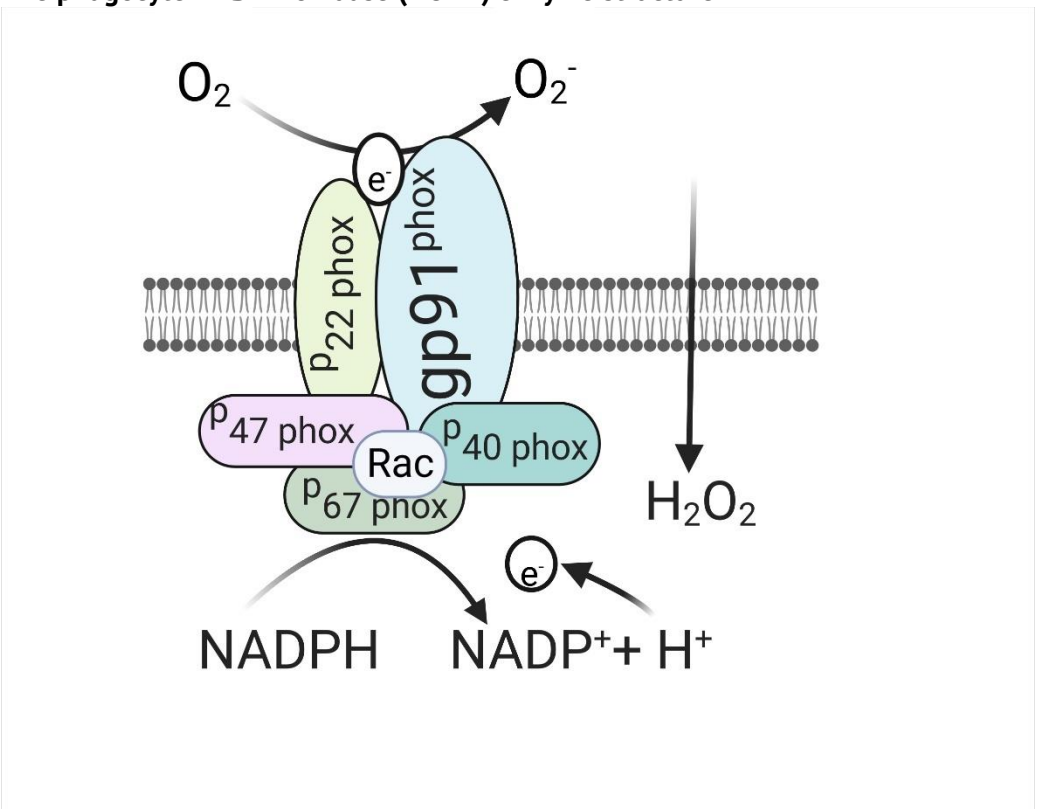
CONCLUSIONS:In summary, these findings provide compelling evidence that the immunopathogenic effects in CGD are intricately and immediately postnatally intertwined with the microbiota to cause intestinal pathology. Moreover, addition of a complex wildling microbiota substantially advances the CGD mouse model with respect to its immunopathological features.

Keywords: Intestinal macrophages, monocyte-to-macrophage differentiation, phagocyte NADPH oxidase, chronic granulomatous disease, colitis.

Monocyte emigration from bone marrow during intestinal inflammation



The phagocyte NADPH oxidase (NOX2) enzyme structure



P-085
General Research » Responses to intracellular pathogens

THE MINCLE/TNFA AXIS CONTRIBUTES TO M1/TH1-SKEWED IMMUNE RESPONSES DURING SEVERE ORIENTIA INFECTION

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OBJECTIVES:Orientia tsutsugamushi (Ot) is a poorly studied, Gram- and LPS-negative bacterium that replicates within the cytosol of endothelial cells, dendritic cells, and macrophages. Human scrub typhus can be lethal, due to excessive inflammation, vasculitis, acute lung injury, and multiorgan failure. The lack of genetic tools for Ot manipulation and suitable animal models has hampered in-depth research for this emerging pathogen. This study is focused on host cellular responses, especially the innate sensors and regulatory mechanisms for severe disease outcomes.

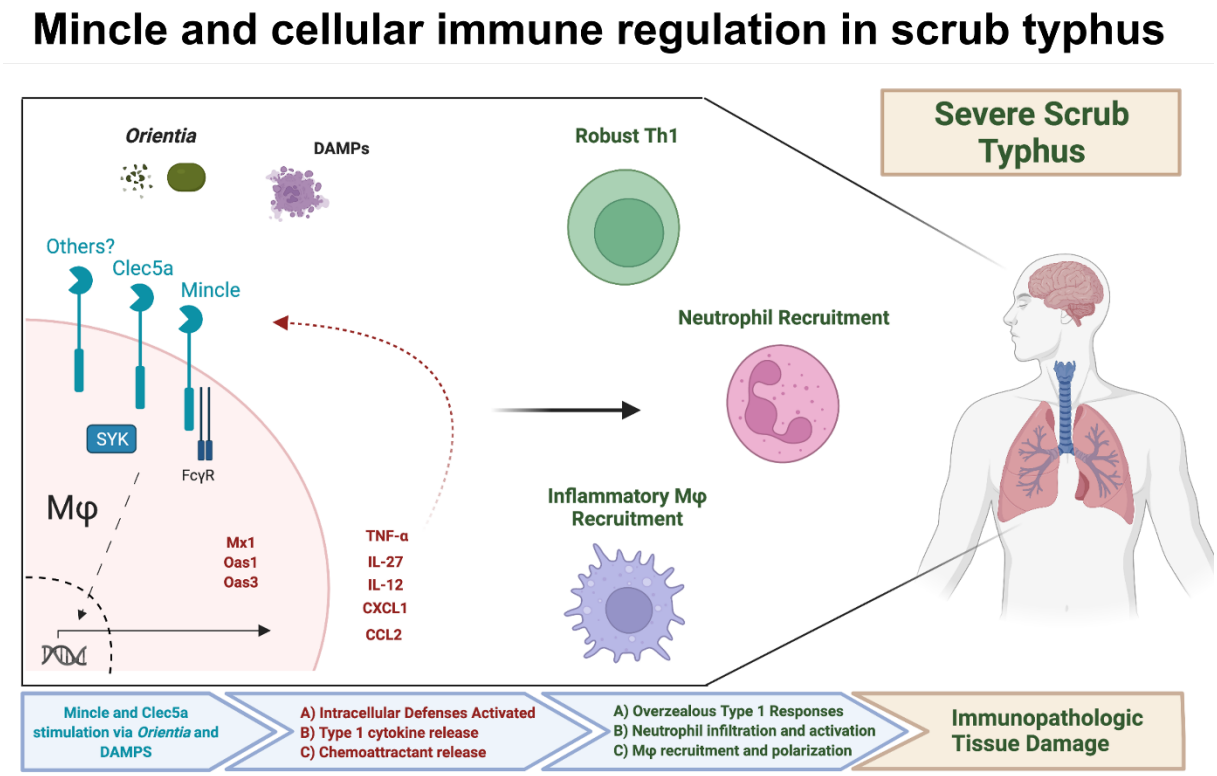
METHODS:Using newly established inbred and outbred mouse models and two clinical prevalent Ot Karp and Gilliam strains (with lethal or self-limiting infection, respectively), we performed organ- and cell-based gene profiling via NanoString or RNAseq approaches. We then validated key host factors by using of gene-targeted knockout mice and their primary macrophage cultures, with the addition of recombinant cytokines or drug inhibitors.

RESULTS:Mincle/Clec4e, Tnf, Cxcr3, Ccr5 and Il27 were among the top 20 highly induced genes in multiple organs, correlating with the severity of disease scores and host lethality. Mincle, but not other examined C-type lectin receptors (CLRs) or TLRs, showed infection dose- and time-dependent expression profiles, as well as novel protein localization patterns, suggesting the specific role of Mincle as host sensors for Ot recognition. In vitro studies with exogenous TNFa or Mincle/CLR-targeted inhibitors revealed a positive regulation between Mincle and TNFa expression, which collectively led to elevated IL-27 and chemokine expression, as well as M1 macrophage and Th1 cell activation. A down-regulation of Th2-promoting CLRs during severe infection was perceived for further investigation.

CONCLUSIONS:This study provides the first evidence for Mincle-based recognition of Ot bacteria and functional roles of Mincle/TNFa/IL-27-related pathways in host responses and pathogenesis. A better understanding of Ot immunology helps design or optimize therapeutics to mitigate pathogenesis responsible for severe scrub typhus.

Keywords: Mincle, C-type lectin receptor, Host immune sensor, Orientia tsutsugamushi, Scrub typhus, Mouse model

Soong_ Mincle and cellular response in scrub typhus



Mincle-based recognition of Orientia bacteria and functional roles of Mincle/TNFa/IL-27-related pathways in host responses and pathogenesis

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General Research » Responses to intracellular pathogens

RV18XX ENHANCES INTRACELLULAR MYCOBACTERIAL ERADICATION BY ELEVATING INTRACELLULAR HEPCIDIN LEVELS IN MACROPHAGES

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OBJECTIVES:This study aimed to investigate the antimicrobial activity induced by Rv18xx, a "Mycobacterium tuberculosis" (Mtb) antigen, and its impact on immune cell activation. With the increasing global threat of Tuberculosis (TB), understanding the potential of novel therapeutic agents is crucial. In particular, we focused on the role of antimicrobial peptides (AMPs), given their significance in innate immunity, and explored the effect of Rv18xx on host defense mechanisms against Mtb.

METHODS:To assess the immune response triggered by Rv18xx, we utilized bone marrow-derived macrophages (BMDMs) stimulated with the Mtb antigen. We measured inflammatory cytokine levels and surface molecular expression, with a focus on Toll-like receptor 4 (TLR4) pathways. Co-culturing Mtb-infected BMDMs, activated by Rv18xx, with T cells allowed us to analyze colony-forming unit numbers, cytokine production, and intracellular hepcidin levels at different co-culture stages. Additionally, Nanostring analysis was employed to identify factors influencing intracellular Mtb survival.

RESULTS:Recombinant Rv18xx, obtained from "Escherichia coli", induced a robust secretion of inflammatory cytokines and surface molecule expression in BMDMs via TLR4 pathways. "In

vitro" analysis of Mtb-infected BMDMs revealed increased proinflammatory cytokine production and reduced intracellular bacterial survival when co-cultured with T cells following Rv18xx stimulation. This enhanced bacterial clearance correlated with the upregulation of hepcidin, known for its antimicrobial properties, and its subsequent production. In the presence of Rv18xx treatment under co-culture conditions with T cells, the inracellular location of hepcidin in cells was observed to adfacent to the phagosome.

CONCLUSIONS:Co-culturing Rv18xx-stimulated macrophages with T cells demonstrated a significant reduction in Mtb growth through the combined effects of inflammatory cytokine production and hepcidin expression. These findings highlight the potential of Rv18xx as a therapeutic target against TB and emphasize the intricate interplay between immune responses and AMPs in combating Mtb infections.

Keywords: Mycobacterium tuberculosis, Mycobacterial antigen, Immune cell activation, Antimicrobial activity, Hepcidin

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General Research » Responses to intracellular pathogens

MOLECULAR MECHANISM OF GBP1-DRIVEN ACTIVATION OF NON-CANONICAL INFLAMMASOME

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OBJECTIVES:The non-canonical inflammasome pathway serves as the main detection mechanism for lipopolysaccharide (LPS) in the host cell cytosol. Caspase-4, the non-canonical inflammasome effector, is activated by direct binding to LPS and processes GSDMD to induce pyroptosis.

Activation of this pathway critically depends on a group of interferon-induced large GTPases called guanylate-binding proteins (GBPs), which act as pattern recognition receptors for the cytosolic pathogens, such as Salmonella or Shigella. GBPs, being present in multiple paralogs, form heteromeric complexes on the surface of bacteria once they escape into the host cytosol. In human, GBP1 initiates the inflammasome assembly via binding to inner core of LPS, promoting the recruitment and activation of Caspase-4 on the surface of cytosolic bacteria by yet-to-be-identified mechanism.

Here, we ask which steps of GBP1 activity cycle contribute to LPS recognition and analyze the nature of GBP1 biochemical activity towards the bacterial surface, leading to Caspase-4 activation.

METHODS:To understand the essence of LPS-binding activity of GBP1, we reconstituted the complex using purified recombinant components. We analyzed the molecular basis and sequence of steps involved in the LPS-GBP1 complex formation and GBP1 contribution to Caspase-4 activation, using size-exclusion chromatography, electron microscopy, reporter assays and photoconversion confocal microscopy.

RESULTS:We find that GBP coat permanently associates with bacterial surface and induces outer membrane stress. Moreover, we find that coat formation depends on GTPase activity of GBP1, while outer membrane stress is GDPase activity dependent. We validate these findings in vitro in LPS binding assays and identify additional residues required for coat formation and inflammasome activation.

CONCLUSIONS:GBP1 catalytic activity induces formation of stable coat on the surface of Gram-negative bacteria, via interaction with lipopolysaccharide. The GBP1-LPS interaction induces the outer membrane stress. Our results support a model in which GBP-induced outer membrane stress or damage promotes caspase-4 recruitment to LPS and its activation.

Keywords: host-pathogen interaction, lipopolysaccharide, Guanylate-binding protein, Caspase-4

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General Research » Responses to intracellular pathogens

CHARACTERIZATION OF ALVEOLAR MACROPHAGE CELL DEATH DURING RESPIRATORY INFLUENZA A VIRUS INFECTION

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OBJECTIVES:Alveolar macrophages (AMs) are lung-resident macrophages located in the alveolar space and are important for the maintenance of lung homeostasis. AMs act as sentinel immune cells and are crucial for initiating proper immunological responses to respiratory pathogens. Absence or dysfunction of AMs typically results in accumulation of dead epithelial cells and proteins resulting in impaired gas exchange and lung failure. During respiratory infections, such as infection with influenza A virus (IAV), a phenomenon known as the 'macrophage disappearance reaction' occurs, leading to a depletion of resident AMs and contributing to disease pathogenesis.

METHODS:Using *ex vivo* culturing systems of primary AMs harvested from the BAL of mice and influenza A virus infection mouse models *in vivo*, we aimed to comprehensively characterize the mechanisms underlying AM disappearance. Additionally, using CRISPR-Cas9 to genetically modify primary AMs *ex vivo*, we were able to study knockouts of key cell death proteins specifically in the AM niche *in vitro* and, *in vivo* after adoptive transfer of gene-edited cells into AM-deficient recipients.

RESULTS:We showed that AMs undergo regulated cell death indicated by activation of caspases, mitochondrial fragmentation, and release of inflammatory cytokines. Genetical or pharmacological inactivation of single cell death pathways targeting key components of either apoptosis, necroptosis, pyroptosis, or ferroptosis did not prevent AM death *in vitro* or *in vivo*. However, simultaneous inactivation of pyroptosis, apoptosis, and necroptosis by targeting of an intracellular nucleic acid sensor restored AM survival and ameliorates morbidity during IAV infection *in vivo*.

CONCLUSIONS:Based on our findings we propose that AM loss during IAV infection is driven by a key nucleic acid sensor at the interface of pyroptosis, apoptosis, and necroptosis.

Keywords: Cell death, Alveolar Macrophages, Influenza A virus

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General Research » Responses to intracellular pathogens

DECIPHERING THE IMMUNOMODULATORY EFFECTS OF 4-HYDROXYNONENAL IN HUMAN MONOCYTE-DERIVED MACROPHAGES: SIZE DOES NOT MATTER IF THE IMPACT IS RADICAL

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OBJECTIVES:Sepsis is a life-threatening syndrome and is a main cause of death worldwide. Treatment and diagnostic options are limited, and morbidity and mortality remain high. Macrophages are important in sepsis. During inflammation, macrophages produce increased levels of pro-inflammatory cytokines and reactive oxygen species (ROS). ROS are essential in clearing pathogens and are highly reactive.
Lipid oxidation by ROS produces 4-hydroxynonenal (4-HNE). 4-HNE interacts with biomolecules, impacting crucial cellular processes. Considering the 4-HNE increase in inflammatory diseases, it raises the question of how 4-HNE affects the innate immune system to regulate inflammation. My project aims to identify modulatory properties of 4-HNE on human monocyte-derived macrophages (hMDM), hypothesizing that high concentrations of 4-HNE propagate inflammation as modified protein and biomolecules are expected to accumulate progressively.

METHODS:Monocytes were isolated from the blood of healthy volunteers and differentiated into macrophages. Naïve macrophages were pre-treated with or without 4-HNE before adding lipopolysaccharide (LPS). RNA sequencing data were validated using reverse transcription-quantitative polymerase chain reaction. Cytokine production was measured by ELISA, and 4-HNE protein adducts and signaling were analyzed using flow cytometry and Western blot.

RESULTS:LPS treatment significantly increased 4-HNE production. RNA sequencing revealed that 4-HNE treatment provokes a pronounced transcriptional reorganization of hMDM, including a decrease in Interleukin (IL)-10 and Interleukin-1 receptor antagonist production. Moreover, 4-HNE increased pro-inflammatory p38 signaling and blocked anti-inflammatory STAT3 activation. However, exhibited no impact on tumor necrosis factor-α and IL-6 production.

CONCLUSIONS:Thus, our data suggest that 4-HNE is an important immune system modulator that can stimulate inflammation by suppressing the IL-10 pathway. Understanding the immunomodulatory mechanisms of 4-HNE aids in understanding its role in ROS-related diseases, like sepsis, and identifying targets to restore damage by oxidative stress.

Keywords: 4-hydroxynonenal, reactive oxygen species, human monocyte-derived macrophages, inflammation

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General Research » Responses to intracellular pathogens

COULD 4-OCTYL- ITACONATE COUPLED WITH PAXLOVID, QUELL THE LONG COVID INFERNO?

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Freelance Researcher

OBJECTIVES:Long Covid, an umbrella term used to classify the pathological state experienced by SARS-CoV-2 patients, post 3 months infection. It is a constellation of varying symptoms (from extreme debilitating to less severe). Long Covid – the aftermath of infections has been plaguing more 65 million people worldwide. Its etiology, pathogenesis and biomarkers are ill-defined and poorly understood. Hence its treatment has been primarily hypotheses driven. The presence of remnant viral reservoir has been one of the most compelling hypotheses posited, substantiated with detection of viral particles in different tissues. The antiviral Paxlovid has been widely tried to manage Long Covid on the basis of it.
Itaconate, a mitochondrial metabolite had been shown to be lowered in severe COVID-19 patients. Supportively, cell permeable 4-Octyl-Itacoante (4-OI) was found to inhibit SARS-CoV-2 (and herpes virus) replication and inflammation by inducing antiviral/antioxidant NRF2 pathway. NRF2 activation is known to inhibit ferroptosis. Interestingly, reactivation of herpesvirus has been noted in Long Covid patients along with Th2 (IL-4) expression. IL-4 has also been known to reactivate herpesvirus latency with 4-OI known to suppress IL-4 driven JAK1 activation. 4-OI, as well is known to inhibit type 1 IFN signaling, and subsequent tissue factor/ thrombin (coagulation cascade) generation and pyroptosis. Notably, similar- inhibiting cell death, antiviral, antiinflammatory and anticoagulant actions are known for dimethyl fumarate (DMF) too.
Long Covid patients have high type 1 IFN signature which can account to observed enhanced immunothrombosis phenomena (microthrombii), lower serotonin, BDNF and higher C4B (leading to cognitive impairment with brain pathology) and neuropathic pains. Interestingly, poor responders to vaccines had high plasma IFN-β.

METHODS:as per published literature

RESULTS:Experiments and randomised clinical trials need to perform to know the outcome

CONCLUSIONS:Hence molecules like 4-OI /DMF having diverse capacities like: inhibiting cell death, antiviral, anticoagulant and antiinflammatory can be thought to bolster Paxlovid

Keywords: Long Covid, Paxlovid, 4-octyl-Itaconate, antiviral, cell death, anticoagulant

P-002
General Research » TLR Signaling

STRUCTURAL BASIS FOR THE RECOGNITION OF PICOMOLAR TLR4 AGONISTS – SYNTHETIC LIPID A MIMETICS - BY HUMAN AND MOUSE TLR4/MD-2 COMPLEXES

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OBJECTIVES:The development of novel synthetic Endotoxin mimetics with a well-defined molecular structure, predictable TLR4-mediated activity and reduced toxicity opens promising opportunities to harness the benefits of TLR4-induced immunity for therapeutic application. A library of synthetic lipid A mimetics based on a conformationally restricted α,α-1,1-linked disaccharide backbone αGlcN(1↔1)αMan (αα-DLAMs) has recently been shown to activate human and mouse TLR4 in vitro with varying potency (pico- to nanomolar) and to induce cytokine production in primary immune cells. To gain structural insights into the molecular basis of TLR4 activation by the DLAMs, we determined atomic cryo-EM structures of five TLR4/MD-2/DLAM complexes.

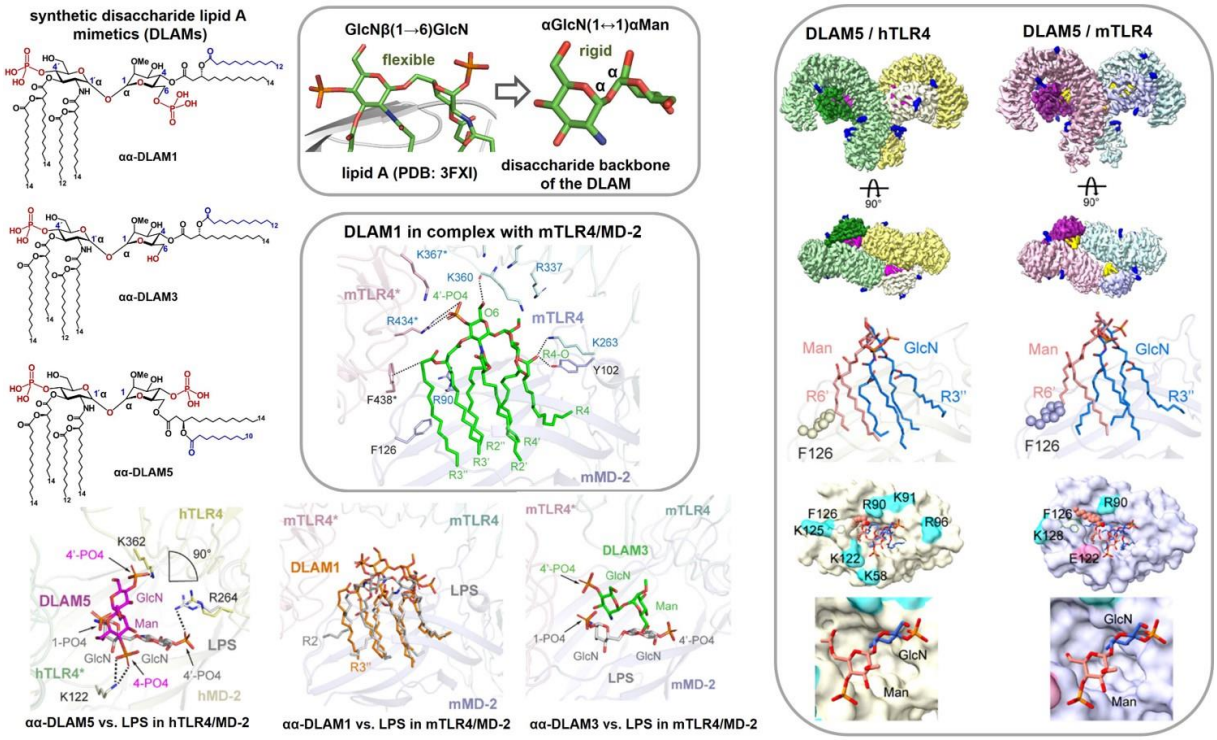
METHODS:Expression and purification of human/mouse TLR4/MD-2 complexes, cryogenic electron microscopy (cryo-EM), image processing, model building, glycochemistry, glycobiology, chemical synthesis.

RESULTS:We generated five cryo-EM atomic structures (2.2 – 2.9Å resolution) of human and murine TLR4/MD-2 in complex with structurally different αα-DLAMs. Analysis of the binding orientation of DLAMs and the interactions at the secondary dimerisation interface of [TLR4/MD-2/DLAM]2 complexes revealed the molecular basis for the TLR4-specific activity. The most potent picomolar TLR4 agonist, bisphosphate DLAM5, binds in the hydrophobic pocket of human and mouse MD-2 with the disaccharide backbone aligned along the secondary dimerisation interface (90°-rotated orientation with respect to ""*E.coli*"" LPS, PDB code: 3FXI/3VQ2) with one lipid chain exposed on the surface of MD-2 and interacting with F126. In contrast, the monophosphoryl endotoxin mimetic DLAM3 showed a species-specific binding, as it was found to bind to mTLR4/MD-2 in a 180° reversed orientation compared to hTLR4/MD-2.

CONCLUSIONS:Our structural study reveals the molecular basis for the potent TLR4-mediated activity of DLAMs and provides in-depth insight into the recognition of structurally different DLAMs by the human and mouse TLR4/MD-2 complexes, including species-specific sensing and correlation with biological activity. Our structural data open new horizons for the atomic-structure-based design and development of novel efficient vaccine adjuvants and immunotherapeutics.

Keywords: TLR4/MD-2/ligand complex, Cryo-EM, atomic structure, synthetic TLR4 agonist, immunomodulation, adjuvant

Cryo-EM structures of DLAMs in complex with human and mouse TLR4/MD-2



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General Research » TLR Signaling

PECULIARITIES IN THE EXPRESSION OF THE INNATE IMMUNITY RECEPTORS NOD-1, TLR8, ZBP-1 AND RIG-1 IN THE MYOMETRIUM IN PREECLAMPSIA

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OBJECTIVES:Preeclampsia (PE) develops during pregnancy and is characterized by increased blood pressure and proteinuria. It is associated with excessive activation of the immune system. However, the main attention of researchers are focused to the pathology of the placenta, while the myometrium remains unclear. The aim of this study was to analyze the expression of TLR8, NOD-1, RIG-1 and ZBP-1 in uterine wall: in myocytes, endothelium and mesenchymal fibroblast-like cells (MFBC) in cases of PE.

METHODS:We examined myometrial samples from 22 women of reproductive age (18-43 years), 27-39 weeks pregnant, 12 of whom were diagnosed with PE (6 patients with severe PE, 6 patients with moderate PE); the control group consisted of 10 women with uncomplicated full-term pregnancies.

RESULTS:The patients with uncomplicated pregnancy showed homogeneous expression of all tested PRPs in myocytes, vascular endothelium and FBC. In PE, pronounced uneven staining of the myometrium was found. A significant increase in ZBP1 and TLR8 receptor expression in vascular endothelium and ZBP1 expression in FBC was observed, mainly in severe PE. We found decreased RIG1 expression in myocytes, FBCs and vascular endothelium, increased NOD1 expression in endothelium and a trend towards increased NOD1 expression in myocytes in PE.

CONCLUSIONS:Immunohistochemical features of innate immunity receptor expression in the myometrium are significantly altered in PE, providing new insights into the pathogenesis of PE, showing that impaired myometrial reception may be associated with poor trophoblast invasion into uterine wall.

Keywords: placenta, preeclampsia, fibroblast-like cells, inflammation, myometrium, pattern recognition receptors

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General Research » TLR Signaling

A PUTATIVE ANTIBACTERIAL GLYCOLIPID FOR TREATING GRAM-NEGATIVE INFECTIONS

Rahul Gupta
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OBJECTIVES:The present proposal is envisioned to study the efficacy of a putative small water soluble bacteriocidal molecule (less than 3 Kd) induced by the chemotherapeutic agent, Mitomycin C. The potential anti-bacterial molecule is produced by Pseudomonas aeroginosa (ATCC 27853), but not by E coli (BW25113). HPLC has shown non-bacteriocidal 10% Mitomycin C carryover in the supernatant, reconfirming the observed bacteriocidal effect to be due to an inducible molecule.

METHODS:Overnight Pseudomonas aeroginosa (ATCC 27853) cultures in LB were diluted 1:10 next morning, to be grown to 0.8 OD. Mitomycin C (Zydus Cadila) reconstituted in water, was added to 0.8 OD cultures at a final concentration 1µg/ml for 1 hr in shaking conditions in 100rpm (in dark). After Mitomycin C incubation, the cultures were centrifuged at 3000rpm/ 15 mins and the supernatant collected for testing antibacterial activity with different pathogens. Supernatant treated with DNase/RNase/Proteinase K (Qiagen)/Lipase/Amylase (SRL) at final concentration of 200 µg/ml for 1.5hr at 370 C. HPLC was performed with C18 column, water/acetonitrile mobile phase (85:15v/v) at 365nm UV absorption maxima for checking Mitomycin C concentrations

RESULTS:Preliminary biochemical characterization of the molecule revealed that the anti-bacterial activity of the supernatant containing the molecule can be fully rescued by lipase treatment and partially rescued by amylase treatment establishing its nature to be glycolipid. More so, HPLC experiments showed the same peak to be diminished by Lipase and Amylase treatment confirming, the carbohydrate moiety to be part of the same lipid molecule. Heat-killed lipase/BSA couldn't rescue the anti-microbial activity of the molecule. The supernatant containing the molecule is potently bacteriocidal against Acinetobacter baumannii (ATCC 19606), Klebsiella pneumonia (ATCC 13883) and E coli (ATCC 27853).

CONCLUSIONS:Hence, if studied in detail, the molecule can be helpful in sepsis management and can be tested for other gram negative pathogens too.

Keywords: Antibacterial, Glycolipid, Gram-negatives, Mitomycin, Sepsis

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General Research » TLR Signaling

IDENTIFICATION OF KEY INTERMEDIATES FOR THE SPATIO-TEMPORAL REGULATION OF TLR2 & TLR3 SIGNALLING

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OBJECTIVES:Toll-like receptors (TLRs) are the first line of host defence and one of the most potent triggers of immune responses. They play a pivotal role in a multitude of pathologies including cancer, chronic inflammatory diseases, and infection, with subcellular localisation of TLRs a strong indicator of their inflammatory profile. However, while duality has emerged for the role of cell surface Vs endosomal TLRs pathogenically, how TLR trafficking modulates spatio-temporal signalling remains largely unknown, hindering its biological and therapeutic manipulation.

METHODS:We used trafficking assays and advanced microscopy approaches, including LSM Confocal, Airyscan and Total internal reflection fluorescence (TIRF) microscopy in both endogenously expressing TLR cell lines (E.G. RPE1), in addtion to overexpression models (E.G. HEK293-Blue™-hTLR2 cells), complemented with functional readouts (E.G. ELISA), immunoblotting and flow cytometry.

RESULTS:We provide novel insight on a common endocytic route mediated by Clathrin, shared by TLR2 ($P < 0.05$) and TLR3 ($P < 0.05$) following activation, including identification of a candidate targeting loci. Our results further identify a common endosomal sorting machinery that promotes TLR2 signalling, while terminating TLR3 signalling by inducing its degradation, signifying a signalling divergence from a common machinery. These observations were mirrored in overexpression assays in addition to endogenous and biologically relevant models such as human derived blood macrophages, validating and strengthening models. Furthermore, through a culmination of trafficking and signalling approaches, we go on to reveal a second endosomal complex holds capability to protect TLR3, through process of ubiquitination.

CONCLUSIONS:Together, our results shine a light on a currently poorly understood duality, and will help pave the way for future targeting of the molecular machineries regulating TLR-driven immunity, utilising differential TLR regulation in bacterial and viral pathways to help define new targeted therapeutics.

Keywords: TLR2, TLR3, Signalling, Trafficking, Endocytic machinery

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General Research » TLR Signaling

HARNESSING TLR3 TO TRIGGER IMMUNOGENIC CELL DEATH IN CANCER

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OBJECTIVES:The way in which a cell dies dictates how it is perceived by the immune system. Apoptosis, a caspase-dependent form of cell death, tilts death towards an immunological silent outcome. In contrast, necroptosis is an explosive form of cell death that causes the release of 'alarmins', which alert the immune system of danger.

Our objective is to identify and target the molecular pathways of cell death, with the goal of directing cancer treatments toward inducing immunogenic cell death (ICD). We aim to achieve this with the help of novel therapeutic agents and combination strategies that exploit the principle of 'pathogen mimicry'. Such drugs simulate the presence of an imaginary pathogen, while collaterally triggering an attack on the tumour (by causing lytic tumour cell death such as necroptosis) and an immunisation against tumour antigens.

Necroptosis can be triggered by death receptors (TNFR1), Toll-like receptors (TLR3/4) and ZBP-1, a Z-RNA binding protein. While TNFR1-induced necroptosis is best studied, other receptors, such as TLR3, may be better suited to pharmacologically trigger a long-lasting anti-tumour immune response, particularly as its adaptor TRIF can directly engage RIPK3, the key necroptotic kinase. Although engagement of necroptosis is a promising new anti-tumour strategy, harnessing necroptosis successfully requires a profound understanding of the mechanisms that control TLR3 signalling.

METHODS:To gain a better understanding of the checkpoints of TLR3-mediated transcriptional responses and cell death, we used a mass spectrometry approach to identify proteins that bind to TLR3 signalling components. The functional role of such proteins was subsequently evaluated using a high-throughput siRNA screen.

RESULTS:We will present our latest findings and discuss how newly identified signalling components might influence TLR3-mediated transcriptional responses and cell death.

CONCLUSIONS:A better understanding of TLR3 signalling may lead to novel therapeutic protocols, enhancing anti-tumour immunity across a broad patient spectrum.

Keywords: TLR3, regulation, necroptosis, cell death

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SYNTAXIN 17 LIMITS INFLAMMATORY RESPONSES IN TLR AND IL-1 RECEPTOR SIGNALING

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OBJECTIVES:The versatile SNARE (soluble N-ethylmaleimide-sensitive-factor attachment receptor) protein syntaxin 17 (Stx17) participates in diverse vesicular processes within intracellular transport pathways. While Stx17 has been linked to autophagosome-lysosome fusion, suggesting a potential role in immunity and inflammation, its specific involvement in inflammatory responses remains unexplored.

METHODS:THP-1 macrophages were activated with Toll-like receptor (TLR) ligands or interleukin (IL)-1ß in the presence of the reversible Stx17 inhibitor EACC (Ethyl (2-(5-nitrothiophene-2-carboxamido) thiophene-3-carbonyl) carbamate). HEK293 cells lacking Stx17 were generated using CRISPR/Cas9-based genome editing and were transfected with an NF-κB-dependent luciferase reporter construct along with IL-1R or TLR4/MD2/CD14. Protein levels of cytokines were detected by ELISA. Gene expression was evaluated by quantitative real-time RT-PCR. Western blot analysis and Immunofluorescence was performed to detect autophagy-related markers SQSTM1/p62 and LC3B.

RESULTS:In TLR- and IL-1ß-stimulated THP-1 macrophages, EACC concentration-dependently induced IL-8 gene and protein expression. Functional characterization of Stx17-Knockout HEK293 cells confirmed decreased autophagic degradation compared to wild-type cells. In Stx17-deficient HEK293 cells, stimulation with IL-1ß or LPS significantly increases transcriptional activity of NF-κB and IL-8 release indicating anti-inflammatory effects of Stx17 in sterile and non-sterile inflammatory conditions.

CONCLUSIONS:Our findings establish a previously unrecognized link between Stx17 and inflammatory responses.

Keywords: Syntaxin 17, autophagy, inflammation, TLRs, IL-1ß

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INTERFERENCE WITH CD14 TRAFFICKING VIA FLOTILLIN-2 DEPLETION INHIBITS PRO-INFLAMMATORY SIGNALING OF TLR4

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OBJECTIVES:CD14 is a GPI-anchored protein found predominantly in nanodomains of the plasma membrane. CD14 serves as a transporter of bacterial lipopolysaccharide (LPS) and regulator of Toll-like receptor (TLR) 4 activity and endocytosis determining the magnitude of the pro-inflammatory response to LPS in macrophages. This fuels our interest in the regulation of CD14 localization, endocytosis and trafficking via flotillin-2 (Flot-2), a scaffolding protein of nanodomains.

METHODS:We studied the role of Flot-2 in CD14 trafficking using Raw264 macrophage-like cells depleted with Flot-2 using shRNA. In these cells we examined the total, surface, and endocytosed levels of CD14 using immunoblotting, on-cell ELISA, and biotinylation-based assay. We analyzed TLR4 endocytosis by FACS and the intracellular distribution of CD14 using confocal microscopy. To determine the impact of the Flot-2 depletion on the inflammatory response of the cells, we analyzed the expression of selected cytokines by qPCR, and their secretion by cytokine array.

RESULTS:In Flot-2-deficient cells the surface and total levels of CD14 were decreased whereas the pool of CD14 in early endosomes was upregulated. In contrast, the surface TLR4 level was not affected. We found that depletion of Flot-2 diminished CD14-dependent processes, like LPS-induced endocytosis of TLR4 and expression of cytokines induced by endosomal TRIF-dependent signaling of TLR4. This also led to attenuation of expression of selected cytokines in cells stimulated with Pam3CSK4, a TLR2/1 ligand but not with Poly(I:C) a ligand of CD14-independent TLR3.

CONCLUSIONS:We found that Flot-2 is involved in maintaining CD14 in the plasma membrane of macrophages. Flot-2 deficiency interferes with CD14 trafficking leading to downregulation of CD14 on the cell surface. As a consequence, the endocytosis of TLR4, and signaling of TLR4 and also TLR2/1 are attenuated. These results indicate that Flot-2 participates in CD14 trafficking and in this way regulates the inflammatory responses of macrophages.

Keywords: CD14, flotillin-2, lipopolysaccharide, protein trafficking, TLR4

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TLR4 ENDOCYTOSIS IS DISSOCIABLE FROM TYPE I IFN EXPRESSION, BUT REQUIRES TLR4 ACTIVITY AND UBIQUITINATION MACHINERY

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OBJECTIVES:Toll-like receptor 4 (TLR4) drives inflammation and antimicrobial responses through recognition of pathogen-derived and endogenous ligands. The current paradigm holds that TLR4 signalling occurs sequentially: cell surface-expressed TLR4 drives pro-inflammatory signalling, which is curbed by TLR4 endocytosis that subsequently enables internalised TLR4 to signal from endosomes, resulting in type I interferon (IFN) expression. Molecular regulators external to TLR4, such as CD14, are proposed to control TLR4 endocytosis, yet CD14-independent TLR4 endocytosis has been reported. Thus, there are significant gaps in our understanding of the mechanisms that govern TLR4 functions, compelling us to elucidate the molecular drivers and regulators of TLR4 endocytosis and signaling outputs.

METHODS:Using pharmacological and mutational approaches in murine primary macrophages and macrophage cell lines, we defined the molecular drivers of TLR4 endocytosis, and assessed how they interlink with TLR4 signalling outcomes.

RESULTS:Our data show that TLR4-induced type I IFN expression does not require TLR4 endocytosis. This challenges the current viewpoint that these processes are inextricably linked. We demonstrate that the presence and functionality of the TLR4 intracellular signalling domain are indispensable for TLR4 endocytosis. Our data further identify that TLR4 endocytosis requires ubiquitin ligase activity, whereas components of canonical TLR signalling pathways are dispensable. This reveals a thus far unrecognised mode of TLR4 signalling that governs activation-induced TLR4 endocytosis.

CONCLUSIONS:Collectively, our data suggest that activation of cell surface-expressed TLR4 results in distinct, TLR4-intrinsic signalling modes that independently control pro-inflammatory signalling and receptor endocytosis. Significantly, the latter is disconnected from endosomal TLR4 signalling output. Therefore, our findings revise the current understanding of TLR4 signalling, reorganising it into functionally-distinct, non-sequential pathways. These novel insights might be harnessed for selective, disease context-specific amplification or restriction of TLR4 functions for beneficial outcomes in infection, chronic inflammation, and cancer.

Keywords: TLR4, endocytosis, type I IFN, signalling

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DECIPHERING A TOLL-LIKE RECEPTOR-MEDIATED INTESTINAL STEM CELLS MICROBIOTA CROSS-TALK

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OBJECTIVES:The gut is composed of a single layer of intestinal epithelial cells (IECs) lines between the external environment and the immune compartment. The epithelial monolayer physically impedes the penetration of microorganisms, but it can also interact with them to maintain homeostasis. Under homeostasis, the IEC secrete anti-microbial peptides (AMP) to limit the bacterial load mainly by Paneth cells. The mechanism for orchestrating AMP secretion from epithelial cells remains unclear. In our study, we aim to illuminate the AMP secretion mechanism by IEC and how it is regulated by Toll like receptor (TLR)-dependent sensing of the gut lumen.

METHODS:We employed TLR-deficient in-vivo models to follow AMP expression and secretion. Single cell RNA-sequencing (scRNA seq) was used to analyze epithelial populations gene expression profiles. Our methodology also involved microbiome manipulations to study the interactions of the epithelium with the environment. Additionally, we used a reductional approach of two-dimensional and three-dimensional in-vitro systems to control the cellular environment and dissect the contribution of the different compartments.

RESULTS:We observed that specific intestinal stem cells (ISC) TLR depletion led to changes in Paneth cell differentiation and a marked reduction in AMP profiles in the two AMP-enriched epithelial cells, enterocytes and Paneth cells. This reduction was also accompanied by decreased gene expression related to innate immunity and cellular response to microbial stimuli in the epithelium. In-vitro experiments revealed a polarized nature of TLR response in ISC. Gram-specific microbiome manipulation further highlighted its impact on epithelial cells innate responses and AMP expression. Our results demonstrate how the epithelial cells of the gut can sense the microbes via TLR-mediated responses to elevate AMP secretion in IEC.

CONCLUSIONS:These findings suggest a symbiotic relationship of the ISC with microbiota mediated by TLRs, with potential implications for gut pathologies such as bacterial infections and Inflammatory bowel diseases (IBD).

Keywords: TLR, AMP, ISC, PRR, SI, Microbiome

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TOLLO/ TOLL8 SIGNALING IN NEURONS MEDIATES LIPID METABOLISM IN *DROSOPHILA*

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OBJECTIVES:*Drosophila* Toll signaling, a conserved NF-κB-mediated immune pathway, extends beyond host defense, impacting metabolism. Notably, Toll pathways are expressed in the nervous system. This study investigates how Toll signaling within specific neural circuits directly regulates metabolism in flies.
Our specific objectives are:
1.Identify specific neuronal Toll receptor that influences fly metabolism.
2. Pinpoint the specific neuronal population harboring these Toll receptors that alter distinct metabolic aspects.
3. Decipher the downstream neural circuitry responsible for Toll-mediated metabolic regulation..

METHODS:1.We leveraged *Drosophila* genetics to perform a targeted screen of neuronal immune receptors. We manipulated the expression levels of various toll receptors within the entire neuronal population and subsequently evaluated their influence on starvation resistance, a proxy for metabolic fitness. This approach aimed to identify a specific Toll receptor critically involved in modulating fly metabolism.
2.Following this screen, we investigated the identified Toll receptor by manipulating its expression within distinct neuronal populations. We then assessed its influence on various metabolic parameters, including triacylglyceride levels, glycogen content, and circulating sugar levels.
3.Finally, we employed fly genetics to dissect the downstream neural circuitry harboring the identified Toll receptor responsible for modulating metabolism.

RESULTS:Our study reveals a discrete neuro-immune circuit involving Toll8/Tollo signaling specifically within leucokinin (LK) neurons that modulates fat metabolism. Selective upregulation of Toll8/Tollo in LK neurons significantly enhances starvation resistance and elevates whole-body lipid storage, while leaving both glycogen content and food intake unaffected. Furthermore, we demonstrate that LK neurons act as modulators of insulin-producing cells, thereby impacting lipid levels.

CONCLUSIONS:This study unveils a dedicated neural circuit mediated by Toll8/Tollo signaling in LK neurons that directly regulate fat metabolism in *Drosophila*. This immune-to-neuronal pathway offers insights into how energy reserves are potentially prioritized during infection and holds potential implications for understanding the neuroimmune axis in various health and disease contexts.

Keywords: Tollo, Toll8, Lipid metabolism, Drosophila melanogaster, nervous system

Fig1_Bhavna_Pydah

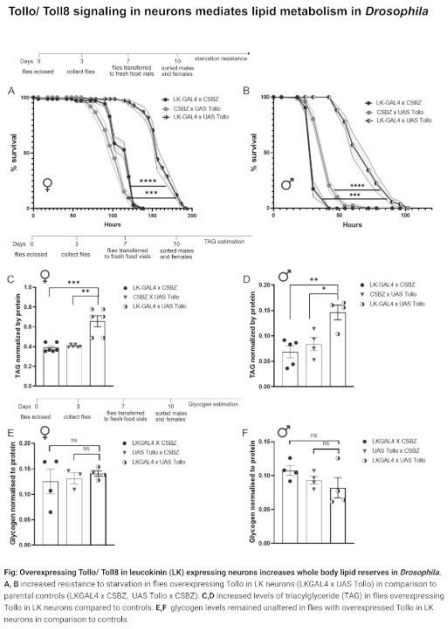

Fig: Overexpressing Tollo/ Toll8 in leucokinin (LK) expressing neurons increases whole body lipid reserves in *Drosophila*. **A, B** increased resistance to starvation in flies overexpressing Tollo in LK neurons (LKGAL4 x UAS Tollo) in comparison to parental controls (LKGAL4 x CSBZ, UAS Tollo x CSBZ). **C,D** increased levels of triacylglyceride (TAG) in flies overexpressing Tollo in LK neurons compared to controls. **E,F** glycogen levels remained unaltered in flies with overexpressed Tollo in LK neurons in comparison to controls.

Fig: Overexpressing Tollo/ Toll8 in leucokinin (LK) expressing neurons increases whole body lipid reserves in Drosophila. A, B increased resistance to starvation in flies overexpressing Tollo in LK neurons (LKGAL4 x UAS Tollo) in comparison to parental controls (LKGAL4 x CSBZ, UAS Tollo x CSBZ). C,D increased levels of triacylglyceride (TAG) in flies overexpressing Tollo in LK neurons compared to controls. E,F glycogen levels remained unaltered in flies with overexpressed Tollo in LK neurons in comparison to controls.

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FREE CHOLESTEROL PRIMES IFN-I RESPONSE BY FACILITATING THE RELEASE OF TLR9 FROM UNC93B1

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OBJECTIVES:Cholesterol accumulation in myeloid cells promotes inflammatory responses in both infections and autoimmunity. While cholesterol regulates multiple convergent pathways, the direct role of membrane-bound free cholesterol in innate immunity is less understood. We therefore aimed to study the regulatory function of free cholesterol in the lysosomal membrane in regulating IFN-I response in plasmacytoid dendritic cells (pDCs) and macrophages, its role in autoimmunity, and the underlying mechanisms.

METHODS:The function of a cholesterol transporter NPC1 in regulating TLR responses and IFN-I production in pDCs and macrophages was investigated using conditional knockout mice, NPC1-deficient cell lines, and an NPC1 inhibitor (U18666A). NPC1 CKO mice on B6.Sle1yaa background were studied for the development of autoimmunity. The regulatory role of cholesterol in TLR9 response was explored through mass spectrometry screening of cholesterol-interacting proteins, click chemistry, and TLR trafficking analyses. Serum samples and PBMCs from healthy volunteers and 11 SLE patients were analyzed for cholesterol and cytokine levels, and TLR9 responses with NPC1 inhibitors.

RESULTS:NPC1 was highly expressed in pDCs, and conditional deletion of Npc1 in dendritic cells impaired IFNα production in TLR9-stimulated pDCs. Npc1ΔDC mice had weakened IFNα response to HSV-1 infection, and ameliorated anti-DNA autoantibodies and nephritis when crossed to B6.Sle1yaa lupus model. Mechanistically, we found that TLR chaperone UNC93B1 could bind to free cholesterol. Juxtamembrane cholesterol gradient, mediated by NPC1, promoted TLR9 release from UNC93B1. Forced delivery of free cholesterol to the plasma membrane could stimulate excessive IFN-I and TNF production from TLR9-activated pDCs in mice and humans.

CONCLUSIONS:We found that NPC1 promotes IFNα response in TLR9-activated pDCs in vitro and in vivo, and facilitates systemic autoimmunity in a lupus murine model. Mechanistically, intracellular TLR chaperone UNC93B1 can bind to free cholesterol, and this interaction promotes TLR9 release and subsequent cytokine response. Our findings may lead to potential therapeutic strategies for autoimmune diseases.

Keywords: TLR9, Autoimmunity, UNC93B1, Cholesterol, IFN-I

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RNASET2 RESTRICTS TLR13-MEDIATED AUTOINFLAMMATION IN VIVO

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OBJECTIVES:RNA-sensing Toll-like receptors reside in the endolysosomal compartment, shielded from endogenous nucleic acids. Inside the endolysosome, the endoribonuclease RNase T2 processes long-structured RNA into short oligoribonucleotides, a step critically required for human TLR8 activation. In contrast to its pro-inflammatory role in TLR8 signalling, RNaseT2-deficient patients display a neuroinflammatory phenotype, indicative of aberrant activation of nucleic acid sensing pathways. In the murine system, RNaseT2 deficiency has been reported to result in an elevated interferon signature in various tissues, as well as dysregulated haematopoiesis and autoinflammation. Of note, TLR8 is not functional in mice, but TLR13 acts as its functional orthologue, recognising single-stranded RNA in a sequence-specific manner. The objectives of this research is to understand how RNaseT2 regulates inflammation.

METHODS:We use genetically modified mice to study the in vivo role of RNaseT2 in instigating inflammation. To characterize the immune cell composition of the spleen of mice, flow cytometry was employed. To study the neuroinflammatory phenotype, immunofluorescence was used. In vitro studies, (ORN stimulation and S.aureus infections) were performed using primary cells derived from mice.

RESULTS:By studying Rnaset2-/- mice, we observed a predominantly inflammatory phenotype characterised by peripheral leukocytosis, as well as by overt splenomegaly dominated by an increase in myeloid cells. Interestingly, these phenotypes were completely attenuated by ablation of the TLR adapter protein MyD88. Further genetic studies revealed that a TLR13-MyD88 dependent signalling axis induces autoinflammation in Rnaset2-/- mice. Considering normal myelopoiesis in Rnaset2-/- mice, we suspect that a TLR13 ligand engages peripheral myeloid cell activation to instigate inflammation. In this regard we hypothesize that RNaseT2 deficiency leads to the accumulation of RNA molecules from either endogenous or bacterial sources.

CONCLUSIONS:Altogether, these results suggest that RNase T2 - in addition to its proinflammatory role related to TLR8 signalling - also plays an important role in limiting erroneous TLR13 activation.

Keywords: Inflammation, TLR signaling, nucleic acid sensing, Ribonuclease

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UNC93B1 VARIANTS UNDERLIE TLR7-DEPENDENT AUTOIMMUNITY

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OBJECTIVES:To investigate the genetic cause and the molecular pathology of early-onset SLE in four patients from two families.

METHODS:Clinical, genetic, and functional analyses in patient cells as well as in HEK293 cells and mouse macrophages.

RESULTS:All four patients developed SLE in the first years of life with hematologic, cutaneous and renal disease. Interferon signatures and proinflammatory cytokines in blood were strongly increased. Genetic analysis revealed a homozygous variant in UNC93B1 (E92G) in the two affected siblings of family 1, and a heterozygous UNC93B1 variant (R336L) in the affected father and son of family 2. Both variants affect highly conserved amino acid residues and were predicted to be deleterious. As UNC93B1 is required for maturation and trafficking of endosomal nucleic acid-sensing TLRs (TLR3/7/8/9), we explored the consequences of the identified UNC93B1 mutations on nucleic acid-sensing TLR function.
Immunophenotyping and scRNAseq of patient PBMCs revealed signs of B cell activation and transcriptional changes typically seen in common SLE. Whole blood stimulation assays using specific agonists revealed hyperactivation of ssRNA-sensing TLR7/8 but not TLR3 or TLR9. This was confirmed in HEK293 cells stably expressing individual TLRs and citrine-tagged UNC93B1 variants as well as in Unc93b1−/− mouse RAW264.7 macrophages re-constituted with Unc93b1 variants. UNC93B1E92G was found to be unstable and to interact less with TLR7, resulting in increased ligand binding site accessibility.

CONCLUSIONS:This study identifies UNC93B1 variants as the cause of monogenic SLE. The UNC93B1 mutations confer a gain-of-function, leading to selective TLR7 hyperactivation with type I interferon activation due to erroneous recognition of self-RNA. These findings delineate a pivotal role for UNC93B1 in preventing autoimmunity by restraining uncontrolled TLR7 activation. Given that TLR7 hyperactivation is a common finding in complex SLE, our findings are expected to accelerate further development of TLR7 antagonists for patients with SLE.

Keywords: systemic lupus erythematosus, UNC93B1, TLR7, genetics, type I interferon

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STIMULATOR OF INTERFERON GENES IS REQUIRED FOR TOLL-LIKE RECEPTOR-8 INDUCED INTERFERON RESPONSE

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OBJECTIVES:The innate immune system is equipped with multiple receptors to detect microbial nucleic acids and induce type I interferon (IFN) secretion to restrict viral replication. When dysregulated, these receptor pathways induce inflammation in response to host nucleic acids and promote development and persistence of autoimmune diseases like Systemic Lupus Erythematosus (SLE). IFN production is regulated by Interferon Regulatory Factors (IRFs), a transcription factor family of proteins that function downstream of several innate immune receptors such as Toll-like receptors (TLRs) and Stimulator of Interferon Genes (STING). Although both TLRs and STING share downstream molecules, the TLR and STING signaling pathways are considered to be independent. Here we show that STING plays a previously undescribed role in human TLR8 signaling to induce IFN.

METHODS:We investigated TLR8-induced signaling and IFN secretion using primary human monocytes and the THP-1 monocytic cell line with dual stable reporters for IRF and NF-kB activity.

RESULTS:Stimulation with TLR8 ligands induced IFN secretion in primary human monocytes and inhibition of STING reduced IFN secretion from primary monocytes from 8 healthy donors. TLR8-induced IRF activity, and IRF7 phosphorylation, was reduced by STING inhibitors. This was independent of a TLR8-IFN feedback loop. Moreover, TLR8-induced IRF activity was blocked by inhibition or loss of the kinase IkB kinase ε (IKKε), but not TANK-binding kinase 1 (TBK1). TLR8 signaling induced rapid phosphorylation of STING, which was dependent on MyD88, connecting the two signaling pathways. Bulk RNA transcriptomic analysis supported a model where TLR8 induces transcriptional responses associated with SLE that can be downregulated by inhibition of STING.

CONCLUSIONS:STING is required for full TLR8-to-IRF signaling and provide evidence for a new framework of crosstalk between cytosolic and endosomal innate immune receptors, which could be leveraged to treat IFN-driven autoimmune diseases.

Keywords: TLR signaling, STING, NF-kB, IRF, Autoimmunity

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MOLECULAR DEFINITION OF THE ENDOGENOUS TOLL-LIKE RECEPTOR SIGNAL TRANSDUCTION PATHWAY

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OBJECTIVES:Toll-like Receptors (TLRs) are key mediators of the immune response to infection. Upon detection of microbes, these prototypical pattern recognition receptors activate inflammatory signal transduction pathways that involve IκB kinases (IKKs), mitogen activated protein kinases (MAPKs), ubiquitin ligases (e.g. TRAF6) and other adaptor and signaling proteins (e.g. TBK1). Current models suggest these signaling proteins operate within functionally distinct multiprotein complexes, which are activated by a receptor-linked adaptor complex known as the myddosome. The mechanisms that connect the protein complexes in the TLR pathways are undefined.

METHODS:To delineate TLR pathway activities, we genetically engineered human and mouse macrophages to add a novel epitope tag to the endogenous myddosome constituent MyD88 which enabled live cell imaging, super-resolution imaging and proteomic analysis.

RESULTS:We found that myddosomes are dynamic in size, number, and composition over the course of 24 hours post TLR activation. Super-resolution microscopy revealed that MyD88 forms a barrel-like structure serving as a scaffold for effector protein recruitment. Proteomics demonstrated that myddosomes contain proteins that act at all stages and regulate all effector responses of the TLR signaling pathways, and genetics defined the epistatic relationship between these effector modules.

CONCLUSIONS:A refined model of TLR signaling pathway operation will be proposed.

Keywords: TLR, MyD88, myddosome

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DISCOVERY OF A NOVEL ACTOR OF THE TLR4 PATHWAY USING COMPARATIVE IMMUNOLOGY ACROSS DOMAINS OF LIFE

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OBJECTIVES:Upon sensing of bacterial lipopolysaccharides by the Toll-Like Receptor 4 pathway, macrophages trigger inflammatory and antimicrobial responses via the activation of transcription factors such as NF-κB. One essential step of signal transduction is the calcium efflux from the endoplasmic reticulum mediated by the channel TRPM2. Calcium efflux is triggered by secondary messengers (ADPR/cADPR) and is known to be essential for efficient NF kB translocation. The link between signal transduction downstream of TLR4 and calcium efflux is still unclear, as the identity of the protein generating the secondary messenger remains disputed.

METHODS:To illuminate this question, we used an approach of comparative immunology across domains of life. Various eukaryotic actors of immunity are considered to have evolved from prokaryotic antiphage proteins, which protect bacteria from viral infections. Using phylogeny-based bioinformatics, we identified a novel key player of the TLR4 pathway through its amino acid sequence similarity with bacterial antiphage Thoeris.

RESULTS:We demonstrate that this protein is essential for signal transduction in macrophages in response to bacterial lipopolysaccharide, via an NAD+-degrading enzymatic activity that activates TRPM2. Such activity is involved in the intracellular restriction of the gram-negative bacteria Salmonella enterica.

CONCLUSIONS:Our results identify a novel cornerstone actor of the TLR4 signaling pathway, potentially addressing the long-lasting controversy of the means by which TLR4 signaling leads to TRPM2-dependent inflammation.

Keywords: TLR4, LPS, comparative immunology, signal transduction, Salmonella enterica

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LPS CHEMOTYPES SHOW DISTINCT KINETICS IN MYD88 ACTIVATION

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OBJECTIVES:Toll-like receptor 4 (TLR4) plays a crucial role in host-pathogen interactions within innate immunity. The activation of the receptor by lipopolysaccharides (LPS) initiates MyD88-dependent and TRIF-dependent signaling pathways. Nevertheless, the precise mechanisms governing the early dynamics of TLR4 activation and signaling remain poorly understood. In this study, we aimed to develop a label-free method for studying the initial events in TLR4 signaling within a native cellular environment.

METHODS:The label-free EPIC system was used to measure the dynamic mass redistribution (DMR) of HEK293-TLR4 reporter cells in response to different LPS chemotypes and in the presence of the MyD88 inhibitor ST2825. RNA-Seq was used to determine differentially expressed genes by LPS chemotypes. To investigate the recruitment of MyD88 by LPS, HEK293-TLR4 cells were transfected with Venus-tagged MyD88, and the cellular localization of MyD88 was evaluated through immunofluorescence analysis.

RESULTS:LPS derived from *Escherichia coli* or *Salmonella minnesota* showed distinct concentration-dependent DMR signal fingerprints at early time points. RNA-Seq analysis revealed no significant differences in gene expression between the two LPS chemotypes. Pharmacological inhibition of MyD88 resulted in a differential attenuation of DMR signals. In the presence of ST2825, LPS from *S. minnesota* induced delayed signals, while the response to *E. coli* LPS was diminished throughout the entire recording period. Cellular localization experiments demonstrated that the reassembly of MyD88 from condensed forms to puncta initiated earlier after stimulation with *E. coli* LPS compared to *S. minnesota* LPS.

CONCLUSIONS:We show that different LPS chemotypes clearly diverge in DMR responses indicating biased signaling downstream of TLR4. Furthermore, we were able to uncover kinetic differences of LPS chemotypes in MyD88 activation using optical biosensor technology.

Keywords: Toll-like receptor 4, lipopolysaccharides, optical biosensor

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THE ROLE OF ADAPTOR PROTEIN MAL/TIRAP IN TRANSDUCING TLR SIGNALING

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OBJECTIVES:Precisely understanding the stoichiometry and cellular dynamics of Toll-Like Receptor (TLR) signaling has been a challenge within the Toll Field. Previous methods to understand TLR signaling such as overexpression models have been limited as proteins at higher than physiological concentrations do not have the same association stoichiometry and kinetics as they do at physiological concentrations thus creating artefacts which have limited our understanding. Purification models have the same limitations and are also missing the regulatory complexity of the immune cell environment and thus also prone to artefacts. The field has further been challenged by the lack of reliable antibodies.

METHODS:This research uses CRISPR/Cas9 gene editing and single molecule imaging to visualise, for the first time, endogenous proteins in the TLR Pathway in macrophages during stimulation. CRISPR/Cas9 homology directed repair was used to endogenously Halo tag Mal/TIRAP, the first intracellular component of the TLR 4 pathway, in immortalised murine bone marrow derived macrophages (iBMMs).

RESULTS:By applying a HaloTag ligand we have been able to visualise fluorescent Mal/TIRAP in iBMMs during LPS stimulation using single molecule imaging techniques to determine the dynamics, expression levels, cellular distribution, stoichiometry and relationship of Mal/TIRAP with TLR4.

CONCLUSIONS:Critically this analysis has shown that Mal/TIRAP is expressed at low levels endogenously suggesting that its expression levels are an important means of regulating TIR signaling.

Keywords: TLR4, Mal/TIRAP, Single Molecule Imaging, Macrophage

Figure 1: Single Molecule Imaging Snapshot of Mal-Halo iBMM live cell

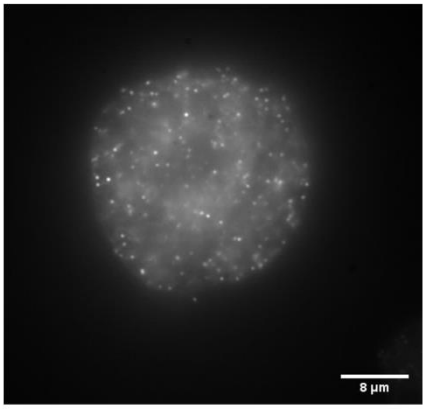

Figure 1: Single Molecule Imaging Snapshot of Mal-Halo iBMM live cell
Unstimulated Mal-Halo iBMM cell stained with JN646 in DMEM media. HiLo microscopy image acquired at 100x magnification, ex 638nm, 160nm pixel size.

Unstimulated Mal-Halo iBMM cell stained with JN646 in DMEM media. HiLo microscopy image acquired at 100x magnification, ex 638nm, 160nm pixel size.

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OPTICAL BIOSENSOR ASSAY DECIPHERS DIFFERENT DYNAMICS OF TOLL-LIKE RECEPTOR SIGNALING

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OBJECTIVES:Toll-like receptors (TLRs), serving as the frontline defenders in the innate immune response, hold significant therapeutic promise for addressing infections, inflammation, and cancer. Activated by dimerization, TLRs initiate intracellular pathways that influence a variety of cellular responses. The current understanding of these pathways is derived primarily from genetic analysis and studies involving small-molecule ligands, focusing on a limited subset of downstream signaling pathways and effector proteins, such as cytokines. Despite extensive efforts to unravel the precise signaling mechanisms of TLRs, considerable knowledge gaps still exist, particularly in understanding TLR function and signaling directly after receptor activation. Here, we present a label-free optical biosensor assay as a powerful tool to decode TLR activation in a native and label-free environment and to delineate the real-time dynamics of TLR pathway activation. This method provides the possibility to bypass the use of further molecular tools that might disturb intracellular processes.

METHODS:Changes in dynamic mass redistribution induced by various TLR agonists and antagonists were recorded by measuring changes in dynamic mass redistribution using an Epic reader. Real-time recordings were performed in HEK293 cells overexpressing different TLR subtypes, myeloid and epidermal cell lines, as well as primary immune cells.

RESULTS:We demonstrate that label-free detection captures real time receptor activation and signaling of TLRs. Our findings indicate that LPS of different origins lead to different signaling traces, suggesting different pathway activation. Furthermore, we reveal different signaling patterns of TLR2 heterodimers and present initial evidence of biased signaling.

CONCLUSIONS:We propose that adopting optical biosensor technology represents a promising experimental approach capable of revolutionizing the investigation of TLR signaling response. This method has the potential to reveal previously unrecognized mechanisms of TLR pathway activation and biased signaling, offering a novel perspective on TLR signal transduction and development of new modulators.

Keywords: Toll-like receptors, pharmacology, optical biosensor, biased signaling, label free

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INVESTIGATING THE ROLE OF NMES1 AS A MODULATOR OF INNATE IMMUNE RESPONSES

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OBJECTIVES:LPS is one of the most potent pro-inflammatory stimuli for macrophages. In addition to activating pro-inflammatory gene expression programs, LPS stimulation leads to metabolic changes, resulting in higher rates of glycolysis and lower rates of oxygen consumption. NMES1 is among the most strongly up-regulated proteins in LPS-stimulated human monocytes and has recently been identified as a novel component of complex IV (CIV) of the electron transport chain (ETC). The exact function of NMES1, however, remains unknown. NMES1 exhibits structural homology to NDUFA4, a component of CIV. Hence, we hypothesised that NMES1 is a NF-κB-inducible negative regulator of the ETC.

METHODS:To investigate the function of NMES1, we used a THP-1 based overexpression model. We performed blue-native PAGE and extracellular flux analyses to assess the effect of NMES1 on the ETC. Furthermore, we measured cytokine release using ELISA to analyze the effect of NMES1 on pro-inflammatory cytokine release.

RESULTS:Studying human monocyte-derived macrophages (MDMs), we found that NMES is indeed highly inducible upon pattern recognition receptor engagement and that NF-κB is the main driver of its expression. Furthermore, in accordance with recently published literature, we confirmed that NMES1 is a component of CIV. However, CIV activity was not affected in a THP-1-based overexpression model. In accordance with the literature, we found that CIV activity was decreased in NDUFA4-deficient THP-1 cells. Overexpression of either NDUFA4 or NMES1 in NDUFA4-deficient THP-1 cells restored CIV activity, indicating that the two proteins are redundant with regard to ETC activity. Interestingly, both NMES1-deficient MDMs and THP-1 cells overexpressing NMES1 showed distinct alterations in cytokine release compared to controls upon stimulation with several TLR ligands.

CONCLUSIONS:Altogether, these results suggests that NMES1 functions as a positive regulator of innate immune responses.

Keywords: innate immunity, electron transport chain, C15orf48

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NOVEL ANTIBODIES TARGETING IL1RAP AFFECTS MARKERS OF VASCULAR INFLAMMATION AND ADHESION IN ENDOTHELIAL CELLS

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OBJECTIVES:Inflammation is central in atherosclerosis and the IL-1 family of cytokines are important in the development of cardiovascular diseases. Previous studies showed that inhibition of IL-1α or IL-1β ameliorated atherosclerosis, while delivery of IL-33 reduced atherosclerosis and IL-36γ increased atherosclerosis in ApoE-/- mice. In humans, the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) previously showed beneficial effects of targeting IL-1β in patients with previous myocardial infarction. The aim of the present study is to evaluate the role of inhibition of the coreceptor IL1RAP with novel antibodies, developed by the Swedish Biotech company Cantargia AB, in vascular cells.

METHODS:Endothelial cells were used to investigate the effects of IL1RAP-blockade with novel antibodies after IL-1α, IL-1β, IL-33 or IL-36γ treatment on inflammatory markers and markers of adhesion in HUVECs via ELISA, real time PCR and Olink® proteomics. The role of IL1RAP blockade on neutrophil adhesion were functionally tested *in vitro*. Correlation between IL1RAP and markers of inflammation and adhesion were studied on 32 atherosclerotic lesions in the GSE43292 dataset.

RESULTS:The IL-6 and IL-8 secretion and mRNA expression were induced by IL-1α, IL-1β or IL-33, but not by IL-36γ, and IL1RAP antibodies blocked this induction. The expression of additional cytokines and chemokines was reduced by IL1RAP blockade after IL-1α or IL-1β treatment, like OPG and CCL4. Expression of the adhesion markers *VCAM* and *SELE* were reduced by IL1RAP-blockade after IL-1α, IL-1β, IL-33 or IL-36γ treatment. In line with this, neutrophil adhesion could be induced by IL-1β and IL-33, and IL1RAP antibodies reduced the number of neutrophils adhering to the HUVEC cells. In human atherosclerotic plaques, IL1RAP expression correlated with markers of inflammation and adhesion like *IL6*, *IL8*, *MCP1* and *ICAM*, shown by microarray.

CONCLUSIONS:IL1RAP targeting antibodies can reduce the expression of chemokine induced inflammation and markers of adhesion in endothelial cells.

Keywords: IL1RAP, IL-1, IL-33, IL-36γ, endothelial cells, adhesion

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GENOME-WIDE CRISPR/CAS9 SCREEN REVEALS NEW REGULATORS OF TOLL-LIKE RECEPTOR SIGNALING

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OBJECTIVES:Sensing of nucleic acids by endosomal Toll-like receptors (TLRs) plays an important role in initiating host defense responses. TLR7-9 activation results in the recruitment of adaptor proteins followed by the engagement of the NF-κB, MAPK and IRF pathways leading to the production of pro-inflammatory cytokines and type I IFN. While it is well appreciated that dysregulation of TLR signaling leading to the transcription factor IRF5 activation can contribute to inflammatory and autoimmune diseases, the detailed regulatory network controlling TLR responses is still not completely understood. Therefore, the purpose of this study is to identify new molecular mechanisms underlying specific TLR signaling.

METHODS:We developed a reporter system triggering cell death upon activation of IRF5 by TLR7 stimulation. A genome-wide CRISPR/Cas9 loss-of-function screen was then performed to identify genes conferring resistance to IRF5 induced cell death. A subset of candidate genes was validated, and follow-up assays were carried out to decipher their molecular function.

RESULTS:The screen revealed genes conferring resistance to cell death when knocked out, indicating their potential role in TLR7 signaling leading to IRF5 activation. Multiple genes already known to be involved in the TLR7 pathway were identified, validating our reporter system, as well as potential new regulators. Among them, we identified and investigated a previously uncharacterized protein required for IRF5 phosphorylation after TLR7-9 engagement. We further demonstrated its presence in the endoplasmic reticulum (ER), acting as a new interactor of multiple TLRs and required for their proper folding, trafficking and/or stability. Consequently, its deletion in multiple human cell lines and immortalized murine macrophages impaired TLR-induced NF-κB, MAPK and IRF5 activation and the downstream production of proinflammatory cytokines.

CONCLUSIONS:Together, our results reveal new regulators of TLR trafficking and signaling, providing a better molecular understanding of innate immune responses.

Keywords: TLR, IRF5, CRISPR/Cas9 screen

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A MICROSCOPY-BASED ASSAY TO MONITOR IRF5 ACTIVATION STATUS IN PRIMARY HUMAN CELLS

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OBJECTIVES:Human genetics and mouse experimentation implicates the transcription factor IRF5 as driver in multiple autoimmune diseases such as systemic lupus erythematosus, where elevated nuclear IRF5 levels have been reported. IRF5 activation occurs upon engagement of endosomal Toll-like receptors (TLR7/8/9) via the SLC15A4/TASL adapter complex, its quantitative assessment is however challenging. The goal of this study is to establish a microscopy-based translocation assay that allows the cell type-specific assessment of IRF5 and NF-κB activation status in peripheral blood mononuclear cell (PBMC) subsets. The assay serves as a tool to profile (I) pathway-specific innate immune stimuli, (II) small molecule inhibitors and (III) IRF5 activation status in different rheumatic disease entities.

METHODS:Cell lines or primary human PBMCs were pre-treated and activated with reference stimuli and immuno-labelled with IRF5- and NF-κB/p65-specific fluorescent antibodies. In addition, PBMCs were pre-stained for immune cell subset-specific surface markers and confocal microscopy was used for image acquisition. A computational image analysis pipeline was developed to quantify cell compartment-specific transcription factor abundances as well as immune cell identification based on surface markers.

RESULTS:Endosomal TLR-induced IRF5 and NF-κB nuclear translocation showed distinct temporal dynamics and knockout of SLC15A4 or TASL in pDC lines confirmed the requirement of the SLC15A4/TASL adapter complex for IRF5, but not NF-κB activation. Differential nuclear translocation of IRF5 and NF-κB upon stimulation was confirmed in relevant immune cell subsets, including monocytes, B cells and T cells. Profiling of a comprehensive set of innate immune stimuli in human PBMCs allowed to determine a cell-type-specific response pattern and confirmed a central role for endosomal TLRs in IRF5 activation.

CONCLUSIONS:This assay serves for the specific quantitative assessment of IRF5 activation in an immune cell subset-specific context. Therefore, the method represents an important step forward to mechanistically characterize autoimmune diseases regarding the involvement of the SLC15A4/TASL/IRF5 pathway.

Keywords: SLE, endosomal TLRs, IRF5, SLC15A4, TASL

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TOLL-LIKE RECEPTOR ADAPTOR PROTEIN TIRAP HAS SPECIALIZED ROLES IN SIGNALING, METABOLIC CONTROL AND LEUKOCYTE MIGRATION UPON WOUNDING IN ZEBRAFISH (*DANIO RERIO*)

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OBJECTIVES:The Toll-interleukin-1 Receptor (TIR) domain-containing adaptor protein (TIRAP), also known as MyD88-associated ligand (MAL), is an essential adaptor protein in Toll-like receptor 2 (TLR2) signaling which links TLR2 and TLR4 to the other adaptor protein myeloid differentiation protein 88 (MyD88). Previously TLR2 and MyD88 have been indicated to be involved in responses to wounding by regulating leukocyte migration, but the role of TIRAP in regulating leukocyte migration has still not been investigated. In this study, the specific roles of TIRAP in leukocyte migration to tissue wounding were identified.

METHODS:A tail-wounded zebrafish larval model and cell tracking analysis were used.

RESULTS:More neutrophils were recruited to the wounded region in *tirap* mutant larvae compared to the wild type controls. In contrast, *tlr2* and *myd88* mutants recruited fewer neutrophils and macrophages to the wounds. We demonstrated that TIRAP controls neutrophil migration speed, but not directional persistence upon tail wounding. We also observed that neutrophils in *tirap* mutant larvae were predominantly staying in the wounded tail region, which suggests that reverse migration of neutrophils is affected by *tirap* deficiency. To explain the characteristics of TIRAP compared to TLR2 and MyD88, we investigated the transcriptomic and metabolomic profiles of zebrafish larvae from a *tirap* mutant and the wild type control under unchallenged normal developmental conditions. By comparison of the three mutants, *tlr2*, *myd88* and *tirap*, it was found that the *tirap* mutant displayed a much higher number of differentially expressed genes (DEGs) than the other two mutants compared to the wild type. In contrast a lower number of significantly altered metabolite levels were observed than in the *tlr2* mutant as compared to the wild type.

CONCLUSIONS:Our findings demonstrate that TIRAP plays specialized roles distinct from TLR2 and MyD88 in signaling, metabolic control, and in regulating neutrophil migration speed and affecting their reverse migration upon wounding.

Keywords: TIRAP, neutrophils, reverse migration, zebrafish, tail wounding

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""*CANDIDA ALBICANS*"" SYSTEMIC INFECTION: THE CRUCIAL ROLE OF MYD88.

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OBJECTIVES:The fungus "" *Candida albicans* "" typically is a benign member of the mycobiome of healthy individuals. However, not only does it occasionally cause mucosal diseases with substantial morbidity, but it also causes life-threatening bloodstream infections in susceptible hosts. As the first line of defense, innate immunity plays a crucial role in recognizing and responding to "" *C. albicans* "", and Toll-like receptors (TLRs) are one of the essential components. MyD88 (Myeloid Differentiation Primary Response 88) appears as a key adaptor protein in TLRs and interleukin-1 receptor (IL-1R) signaling pathways, and its deletion causes a considerable collapse of immune responses to pathogens. Here, we show that in the lack of MyD88, the immune response against "" *C. albicans* "" encountered a failure that affected the host survival rate noticeably.

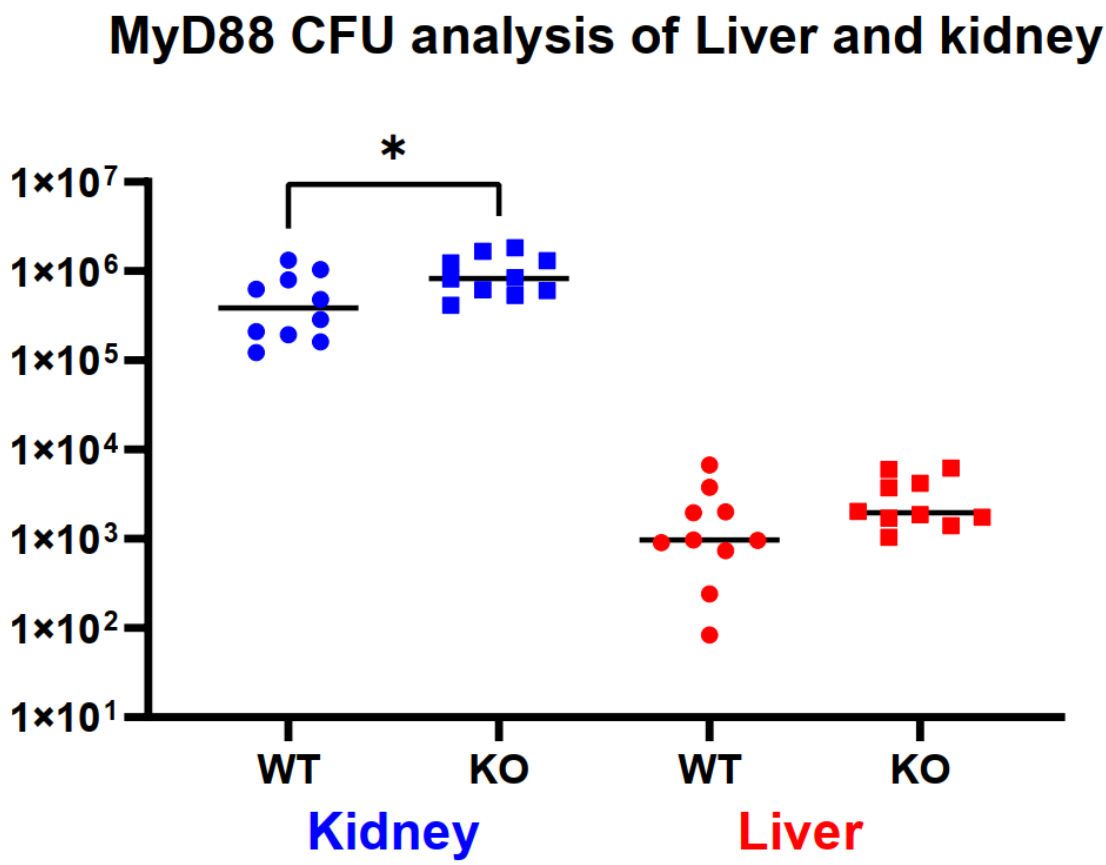
METHODS:To examine systemic candidiasis, "" *C. albicans* "" SC5314-RFP inoculation was performed intravenously on female C57BL6/J MyD88 knockout and wild-type mice. Infection progression was monitored daily, and after 2 weeks or reaching the humane endpoint, mice were sacrificed. Postmortem, heart blood, spleen, liver, and kidney were harvested for FACS analysis and fungal burden quantification.

RESULTS:The survival rate of knockout mice was significantly decreased, and they showed heightened susceptibility to candidiasis. Furthermore, the CFU analysis revealed that knockout mice exhibited significantly higher "" *C. albicans* "" levels than their wild-type counterparts. Interestingly, the FACS analysis demonstrated a marked elevation in the population of activated neutrophils and T cells in the blood of knockout mice compared to wild-type. On the other hand, a notable alteration in the subtypes of B cells within the spleen was observed between knockout and wild-type mice.

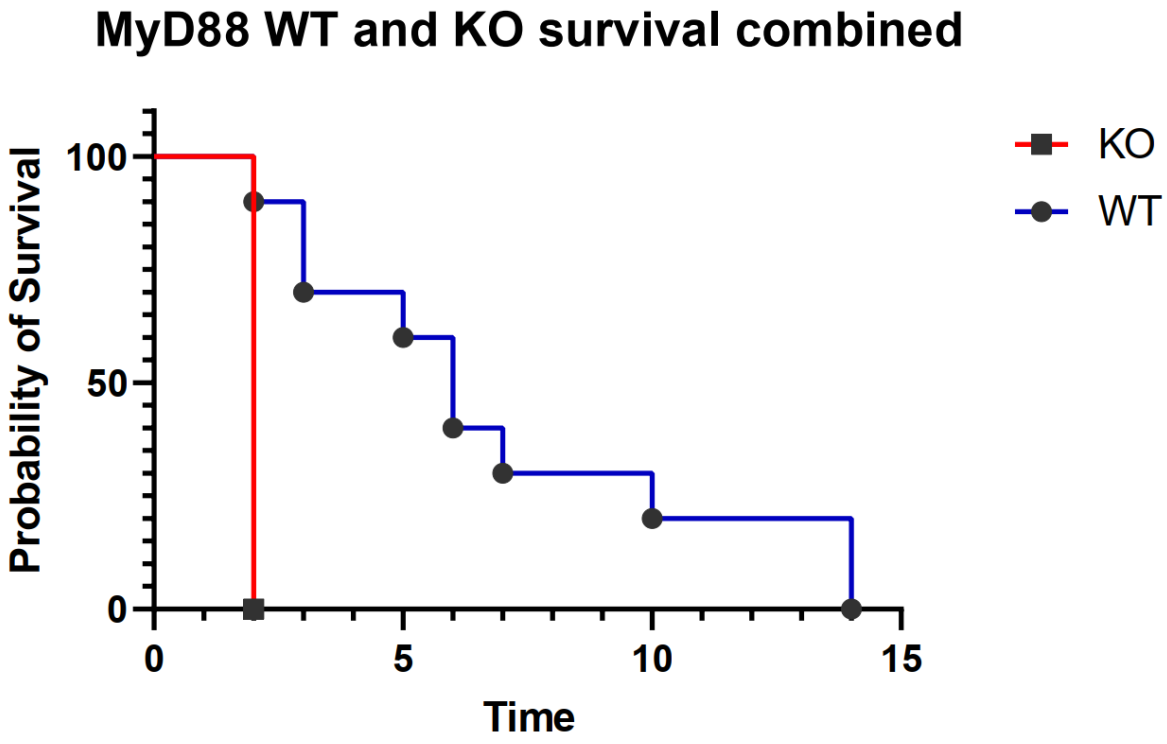
CONCLUSIONS:Together, this work highlights the crucial role of MyD88 in immunity against "" *C. albicans* "" and elucidates noticeable alterations in immune components that require further investigation.

Keywords: Candida albicans, TLRs, MyD88, Systemic infection

CFU analysis



Survival rate of MyD88 knockout and wild-type mice



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THE PYRIN DOMAIN OF THE PYHIN FAMILY MEMBERS SPECIFICALLY DRIVES PROINFLAMMATORY ACTION VIA TLR4/MD2

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OBJECTIVES:IFI16 is a nuclear protein belonging to the PYHIN family (together with AIM2, MNDA, and IFIX), involved in a variety of biological activities including cell cycle regulation, tumor suppression, and virus sensing. Emerging evidence indicates that IFI16 is released in the extracellular milieu under injury or stress conditions, especially in autoinflammatory diseases. Once released, IFI16 acts like a damage-associated molecular pattern (DAMP), propagating danger signals and amplifying IL-6 and IL-8 secretion via TLR4 activation. Here, we expanded on those observations by systematically dissecting the IFI16 sequence elements involved in the interaction with TLR4, and aimed to verify whether this pro-inflammatory activity could be extended to the other human and mouse PYHIN family members.

METHODS:We used antibodies directed against either the N- or the C-terminal domains of IFI16 along with a panel of IFI16 recombinant domains that span the PYRIN, HINA, or HINB domains and truncated proteins that lack either the PYRIN or HINB domains (IFI16ΔPYRIN or IFI16ΔHINB, respectively), in both functional assays and SPR experiments. Moreover, based on in silico prediction, we performed IFI16-PYRIN site directed mutagenesis to inhibit IFI16/TLR4 binding.

RESULTS:We demonstrated that the PYRIN domain of IFI16 is sufficient to induce inflammation when added to human macrophages. In addition, IFI16-PYRIN/TLR4 interaction has been confirmed using IP and SPR analysis. We also showed that point mutations in the PYRIN domain that involve highly conserved amino acids across the different PYHIN family members inhibited the IFI16-induced inflammatory activity. Finally, in silico analysis revealed that these amino acids are not conserved in other PYRIN-carrying proteins not belonging to the PYHIN family, which were indeed unable to induce TLR4-dependent inflammation.

CONCLUSIONS:Collectively, our findings reveal an unprecedented inflammatory activity of the IFI16-PYRIN domain, which is conserved among the different PYHIN family members, as a DAMP able to trigger TLR4-mediated inflammation in macrophages.

Keywords: IFI16; PYRIN; DAMP; inflammation

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CHIMERIC MYD88S REVEAL THE EMERGENT PROPERTIES THAT OLIGOMERIC SCAFFOLDS REQUIRE FOR INFLAMMATORY SIGNALING

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OBJECTIVES:The compartmentalization of biochemical reactions into cellular organelles, termed signalosomes, is a recurring theme in signal transduction. Innate immune signaling provides a striking example of signalosome assembly: the triggered assembly of oligomeric filaments that function as scaffolds to localize and activate signaling effectors. In many cases, the oligomeric filaments at the core of these signaling scaffolds are composed of death domain (DD) superfamily proteins, suggesting a common mechanism of signal transduction and a shared evolutionary origin. However, the critical biophysical properties that these signaling scaffolds require for signal transduction are not clearly defined.

METHODS:To determine the unifying design principles spanning all innate immune signaling scaffolds, a bottom-up approach is required – building a simplified signaling system that can be used to reconstruct the behavior of signaling scaffolds de novo.

RESULTS:We designed a system restricted to the common features of innate immune signaling complexes: the ability to read out information from receptors, the presence of death domains, and TRAF6 binding motifs. We refer to this system as Chimeric Higher-order Assemblies for Receptor-Mediated Signaling (CHARMS). CHARMS, solely constructed from full-length MyD88 fused to the TRAF6 binding motifs, can fully restore IL-1R/TLR sensing in MyD88/IRAK4/IRAK1 triple KO cells. We find that functional CHARMS can be constructed from diverse kinetically stable protein oligomers. This includes bacterial Death domain homologs, suggesting a wider conservation across the death domain superfamily, and synthetic computationally designed helical-forming domains.

CONCLUSIONS:We conclude that the individual domains of proteins that multimerize into functional signaling scaffolds are interchangeable, as long as together they have two emergent properties: multimerization and high kinetic stability. In summary, this work reveals design principles for how innate immune signaling complexes are constructed and opens up avenues for using molecular engineering to reprogram the signaling output of TLRs/IL-1Rs and innate immune signaling pathways in general.

Keywords: Signal transduction, synthetic biology, cellular engineering, innate immune signaling

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MOLECULAR MECHANISM OF PSEUDOURIDINE RNA EVADING TOLL-LIKE RECEPTOR 8 DETECTION

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OBJECTIVES:Toll-like receptor 8 (TLR8) is a key pattern recognition receptor of the innate immune system that senses degradation products of non-self single-stranded (ss)RNA. Fragments needed for binding and activation are processed by the endonuclease RNase T2. RNA modifications, such as pseudouridine, that are present in self-RNA have been shown to impede the immune-stimulatory potential of endogenous RNA. However, the molecular mechanism of this immune evasion has remained elusive. Therefore, we aim to identify how pseudouridine-containing RNAs evade detection by Toll-like receptor 8.

METHODS:Using high-resolution mass spectrometry, we analyze the cleavage products of pseudouridine-modified ssRNA digested by either RNase T2 or a representative member of the RNase A family. Further, we compare the stimulatory effect of uridine, pseudouridine and the therapeutically used N1-methylpseudouridine by studying TLR8 activation in cells. Additionally, we analyze ligand-induced receptor dimerization of recombinantly purified TLR8 using mass photometry.

RESULTS:Our data indicate that pseudouridine-containing RNAs are not properly processed by RNase T2, a critical upstream requirement for TLR8 activation. In contrast to uridine, pseudouridine only induced marginal TLR8 dimerization in vitro at high concentrations. N1-methylpseudouridine (m1Ψ), which is used in the COVID-19 mRNA vaccine, also evades proper processing by RNase T2. Nevertheless, surprisingly, m1Ψ still activates TLR8 in cellulo and also induces TLR8 dimerization in vitro comparable to uridine.

CONCLUSIONS:In conclusion, our data provide a molecular mechanism how pseudouridine, the most abundant RNA modification in endogenous RNA, evades detection by the immune receptor TLR8.

Keywords: TLR8, RNase T2, pseudouridine

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DESTABILIZATION OF MYD88 PROTECTS MICE FROM DSS-INDUCED COLITIS, BUT PROMOTES APOPTOSIS OF IMMUNE CELLS

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OBJECTIVES:Various signaling pathways coordinate to assure appropriate innate immune response and maintain the homeostasis. In a paper Avbelj et al (Front Immun, 2021) we showed that MyD88 contains a caspase-1 recognition site, presenting an additional negative regulation through inflammasome activation. Mutation of hMyD88D148E and mMyD88D162E prevented a decrease in NF-κB activation in presence of increased amount of caspase-1. To evaluate the physiological role of caspase-1 inhibition on MyD88 signaling, we generated single point mutation MyD88D162E mice.

METHODS:Phenotypic characterization of newly established MyD88D162E mice was conducted. DSS-induced colitis was performed and evaluated. Cytokine expression, NLRP3 inflammasome activation were determined in BMDMs. Protein expression, ROS, mitochondrial functionality, apoptosis were determined using flow cytometry, quantitative microscopy, qPCR, WB, TEM.

RESULTS:Approximately 25% of MyD88D162E mice died until sexual maturity and some pups exhibited head malformation and alopecia. MyD88D162E mutation led to reduced stability and lower amount of MyD88D162E. Surprisingly, this reduction in MyD88 and less inflammation during DSS-induced colitis did not translate into decreased cytokine expression in BMDMs. However, it did attenuate the secretion of Il-1β following NLRP3 inflammasome activation. We observed an increase in BMDMs experiencing oxidative stress and displaying dysfunctional mitochondria compared to MyD88wt counterparts. Additionally, reduced expression of the anti-apoptotic protein Bcl-xL and the lymphoma-promoting kinase BTK was noted, resulting in preterm apoptosis under steady state conditions. MyD88D162E BMDMs were also more susceptible to ibrutinib-induced apoptosis.

CONCLUSIONS:We highlight that a decreased MyD88 level does not profoundly impact proinflammatory signaling but significantly influences immune cell survival. The results suggest that MyD88 provides sufficient level of NF-kB activity at steady state conditions to enable protection against apoptosis induction. These findings may hold significant implications for the treatment of MyD88-driven lymphomas, suggesting that interventions targeting the quantity of MyD88, rather than immune signaling pathways, could be a promising therapeutic approach.

Keywords: MyD88-signaling, oxidative stress, mitochondria, apoptosis

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INNATE IMMUNE STIMULATION REGULATES GLYCOLYSIS BY PFKL PHOSPHORYLATION

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OBJECTIVES:It is well documented that innate immune responses are linked to core metabolic pathways. To this end, glycolysis is rapidly upregulated in macrophages in response to innate immune stimuli such as LPS, and this metabolic switch is known to positively regulate pro-inflammatory functions. However, the underlying mechanisms of this interconnection remain unclear. We hypothesized that TLR signaling might induce a post-translational modification in one of the flux-controlling enzymes of the glycolytic pathway that acts as an allosteric switch, resulting in increased early glycolytic flux.

METHODS:To explore this hypothesis, we analyzed a previously published phosphoproteome dataset of LPS-stimulated bone marrow-derived macrophages (BMDM). Here, we found that phosphofructokinase 1, live type (PFKL) was among the top ten proteins showing increased phosphorylation at Ser775 in response to LPS. We generated antibodies that specifically detect Ser775 phosphorylated PFKL or total PFKL and a genetically engineered mouse model incapable of undergoing PFKL S775 phosphorylation (PfklS775A/S775A). We performed Western blots, biochemical assays, seahorse assays, isotope tracing assay, bacterial killing assay and qPCR of BMDM in WT and PfklS775A/S775A mice.

RESULTS:Our results showed that 1) Innate immune stimulation induces PFKL phosphorylation at Ser775 in primary macrophages; 2) Phosphorylation of Ser775 enhances PFKL catalytic activity; 3) PfklS775A/S775A macrophages display a blunted glycolytic response following innate immune stimulation; 4) PFKL Ser775 phosphorylation regulates ROS production; 5) PFKL Ser775 phosphorylation is required for LPS-induced HIF1a and IL1b production.

CONCLUSIONS:Our research establishes a molecular connection between innate immune activation and early glycolysis by uncovering PFKL Ser775 phosphorylation downstream of innate immune signaling.

Keywords: TLR signaling, PFKL phosphorylation, glycolysis, inflammatory response

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TLR4-MEDIATED CHRONIC NEUROINFLAMMATION REDUCED TAU PHOSPHORYLATION BUT HAD NO EFFECT ON TANGLE PATHOLOGY IN A TAUOPATHY MOUSE MODEL

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OBJECTIVES:Alzheimer's disease and related tauopathies are characterized by a stereotypical accumulation of fibrillary aggregates primarily composed of pathological tau protein. Although neuroinflammation is believed to contribute to the pathogenesis of tauopathies, current evidence is insufficient to establish a causal relationship between neuroinflammation and tau tangle formation.
METHODS:In this study, we investigated whether chronic systemic inflammation, triggered by activating the Toll-like receptor 4 (TLR4) signalling pathway with a high dose (5 mg/kg) of lipopolysaccharide (LPS), could modulate neurofibrillary pathology in a tau-transgenic R3m4 mouse model expressing human truncated 151-391/3R tau.
RESULTS:We observed significant microglial proliferation following a period of chronic neuroinflammation in the LPS group compared with the sham group ($p < 0.0001$). In addition, microglia exhibited a reactive yet exhaustive phenotype, as evidenced by a significant decrease in the cell area ($p < 0.0001$), without any discernible changes in other morphometric parameters (perimeter, circumference, circularity, solidity, aspect ratio, or arborization degree). Despite the significant increase in reactive microglia in LPS-treated animals, compared with those in sham animals, microglial phagocytic and clearance potential proved to be inefficient in reducing the number of tangles positive for the antibodies AT8 ($p = 0.599$) or DC217 ($p = 0.893$). Interestingly, contrary to the findings of previous studies, our study revealed reduced levels of hyperphosphorylated tau in the hippocampus ($p < 0.0001$).
CONCLUSIONS:Taken together, these findings suggest that chronic TLR4 stimulation mediated by a high dose of LPS in tau transgenic R3m4 mice does not affect the formation of tau tangles, despite sustained microgliosis and a reduction in tau hyperphosphorylation.

Keywords: Neuroinflammation, tau, lipopolysaccharide, phosphorylation, microglia.

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NOVEL TLR8 C.1552G>A MUTATION IS ASSOCIATED WITH TLR8-MEDIATED IMMUNE CELL DYSREGULATION IN MALE SIBLINGS

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OBJECTIVES:Toll-Like Receptor 8 (TLR8) is an endosomal located receptor that upon activation with ssRNA ligands initiates a pro-inflammatory response via the activation of NF-kB signaling pathway. Single nucleotide polymorphisms (SNPs) in the TLR8 gene can cause an autoimmune and auto-inflammatory disorder to the homozygous carriers, classified as Immunodeficiency 98 with Auto-inflammation, X-Linked. Our study presents a novel TLR8 c.1552G>A (p.A518T) mutation in a family case with male siblings, who suffer from severe hyper-inflammation and immunodeficiency.

METHODS:Genetic investigation was performed using whole blood DNA. Immunophenotyping of peripheral blood mononuclear cells (PBMCs) was conducting using multi-spectral flow cytometry based assay. Serum cytokine analysis was performed. Computational structure modeling was used and functional assays were conducted in vitro using reporter cell lines to further characterize the TLR8 variant.

RESULTS:The siblings inherited the X-chromosome with the TLR8 variant from their heterozygous mother. Immune phenotyping showed a pro-inflammatory phenotype with inverse CD4:CD8 ratio, high frequencies of classical monocytes and strong B-cell dysregulation. Serum analysis revealed elevated pro-inflammatory cytokines and chemokines, in particular high levels

of interferon α and γ in the two male TLR8 gene variant carriers compared to age-matched control individuals and associated with upregulation in the expression levels of interferon-stimulated genes (ISGs) in immune cells from the siblings. Structure modeling of the TLR8 c.1552G>A mutation revealed that the p.A518T variant is located within the homodimer interface, stabilizing the dimer in the activated form. Functional experiments showed that upon stimulation with TLR8 agonists, the p.A518T variant induced NF-kB activity comparable to WT, but the mutant TLR8A518T protein was degraded more rapidly compared to TLR8WT.

CONCLUSIONS:Together, these findings identify significant immune cells dysregulations associated with a TLR8 c.1552G>A mutation in the TLR8 gene.

Keywords: TLR8, genetic variant, structure modeling, immune cells dysregulation

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HBV-INDUCED MICROENVIRONMENT REDUCES PDC RESPONSE TO TLR9 STIMULATION

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OBJECTIVES:Hepatitis B virus (HBV) is a non-cytopathogenic virus with DNA genome. It causes an acute hepatitis that may develop into chronic hepatitis B, liver cirrhosis and hepatocellular carcinoma. HBV is described as “stealth virus” as it possesses various mechanisms of interference with immune recognition.
We investigated the effect of HBV on activation of plasmacytoid dendritic cells (pDCs), as they are a main producer of interferon α (IFNα). IFNα secretion is induced by toll-like receptor 9 (TLR9) activation, yet this pathway is strictly regulated. One of the TLR9 pathway negative modulators is miRNA146a, which silences the TLR signalling proteins. Importantly, miRNA146a is active in the RNA-induced silencing complex (RISC), which binds the target mRNA and silences it. Mature miRNA-RISC can be sorted into extracellular vesicles (EVs) and transported to surrounding cells.
Therefore, we analysed, whether HBV infection affects the quantity of miRNA-146a secreted from hepatocytes, and whether the secreted miRNA146a affects the pDC function.

METHODS:Levels of HBV antigens and IFNα were measured using ELISA. Quantity of intracellular and extracellular miRNA-146a was analyzed by qPCR and digital droplet PCR, respectively. Transfection of miRNA inhibitors was performed with XMAN nanoparticles.

RESULTS:HBV-producing hepatocytes, used as a model of chronic infection, expressed higher levels of intracellular and extracellular miRNA-146a than non-infected controls. The supernatant from the HBV-producing hepatocytes did not activate pDC cell line Gen2.2.. However, Gen2.2. cells exposed to the supernatant from HBV-producing cells secreted lower amounts of IFNα upon TLR9 agonist CpG-A treatment. Importantly, inhibition of miRNA-146a in HBV-producing hepatocytes, restored the IFNα production in Gen2.2. cells.

CONCLUSIONS:High amount of miRNA146a produced by the HBV-producing hepatocytes is transported likely via the EVs to pDC-derived cells, where it negatively regulates TLR9 signalling. Therefore, miRNA146a is an important factor in HBV-mediated immunomodulation, and could be a promising target for the chronic hepatitis B therapy.

Keywords: Chronic HBV, miRNA-146a, pDC,TLR9 signaling, extracellular vesicles

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DISRUPTED DEGRADATIVE SORTING OF TLR7 IS ASSOCIATED WITH HUMAN LUPUS

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OBJECTIVES:Hyperactive Toll-like receptor (TLR) 7 signaling has long been appreciated as a driver of autoimmune disease in mouse models. Recently, mutations in TLR7 or its associated regulator UNC93B1, were identified as monogenic causes of human lupus; the unifying feature of these mutations being TLR7 gain-of-function. TLR7 is an intracellular transmembrane receptor, sensing RNA breakdown products within late endosomes. Hence, its function depends on intricate transport mechanisms and membrane interactions within the endomembrane network. Whether perturbations of any of these endosome-related processes can give rise to TLR7 gain-of-function and facilitate self-reactivity has not been investigated.

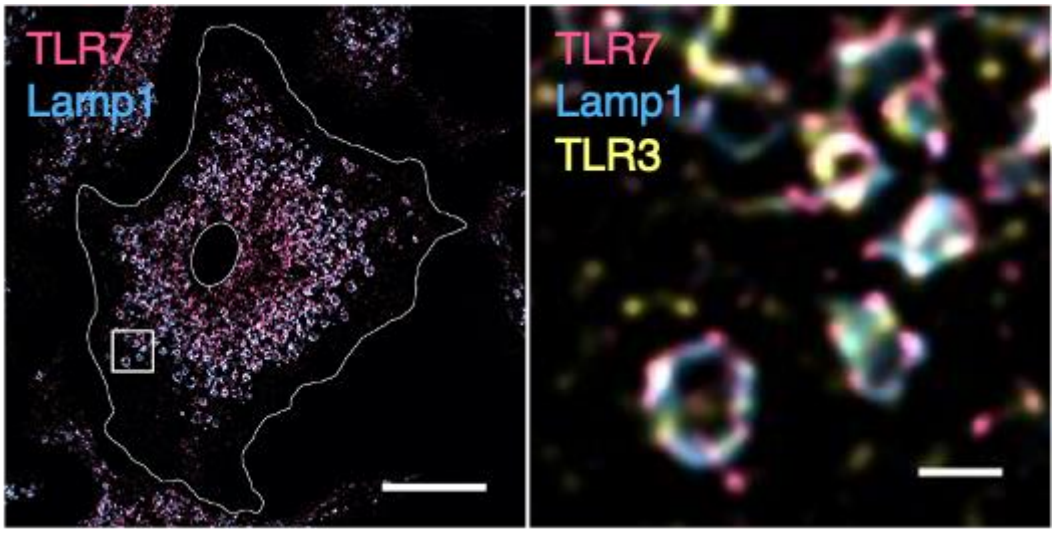
METHODS:To approach this question we combined diverse methods, such as genetic engineering, cell biological, biochemical, immunological and microscopy techniques.

RESULTS:We show that a dysregulated endosomal compartment leads to unrestricted TLR7 signaling and human lupus. The late endosomal BLOC-1-related protein complex (BORC) together with the small Arf1-like GTPase Arl8b controls TLR7 protein levels, and a direct interaction between Arl8b and Unc93b1 is required to regulate TLR7 turnover. We identified an amino acid insertion in UNC93B1 in a patient with childhood-onset lupus, which reduces the interaction with the BORC-Arl8b complex and leads to endosomal TLR7 accumulation. Therefore, a failure to control the proper progression of TLR7 through its endocytic life cycle is sufficient to break immunological tolerance to nucleic acids in humans.

CONCLUSIONS:Our results highlight the importance of an intact endomembrane system to prevent autoimmune disease. As the cellular mechanisms restricting TLR7 signaling can be manifold, identifying and stratifying lupus patients based on a TLR7-driven pathogenesis could be a viable strategy towards a targeted therapy.

Keywords: TLR7, Unc93b1, BORC, Endosome, Lupus

TLR visualization



Super-resolved visualization of endosomal TLR3 and 7 in primary mouse macrophages.

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3-BASE OLIGONUCLEOTIDES MIMIC NATURAL TLR7-ANTAGONISTS TO TREAT INFLAMMATION IN VIVO

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OBJECTIVES:We have made the ground-breaking discovery that select RNA fragments of 3 bases with a 5′-end 2′-O-methyl modified guanosine are *bona fide* natural antagonists of human and mouse TLR7. Based on this finding and systematic structural analyses of synthetic 3-base oligonucleotides, our aim was to develop a new class of TLR7 inhibitors and confirm their therapeutic activity *in vivo* using two distinct models of TLR7-driven inflammation – one topical and one systemic.

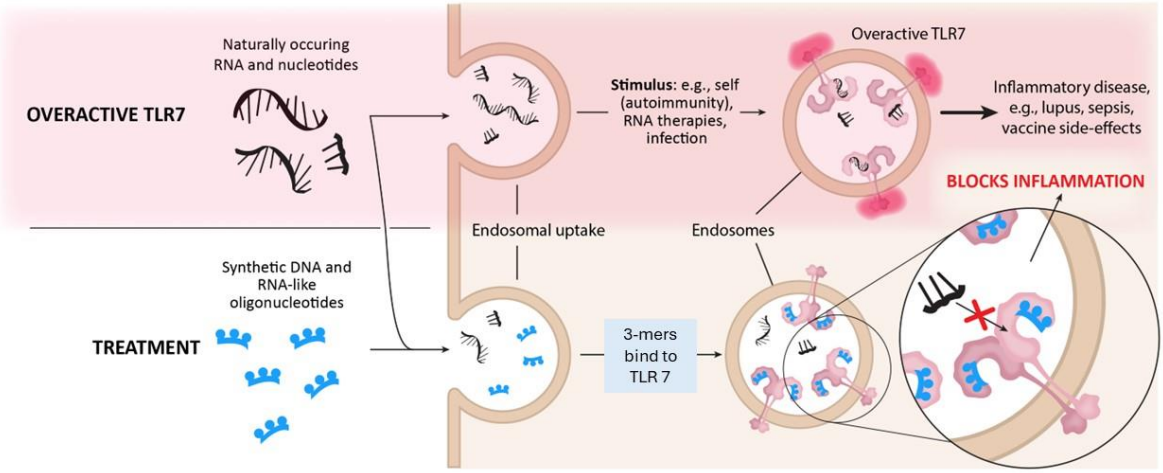
METHODS:To induce skin inflammation, the TLR7 agonist imiquimod was applied daily to the back and ear of mice immediately following, or not, topical application of our lead TLR7-inhibitory oligonucleotide formulated in F127 Pluronic gel. Mice were scored for the appearance and severity of skin inflammation, and inflammatory gene signatures were measured in the skin by RT-qPCR.
To induce systemic TLR7-driven inflammation, mice were injected i.v. with unmodified luciferase mRNA encapsulated in lipid nanoparticles (LNPs) with, or without, our lead TLR7-inhibitory oligonucleotide. Luciferase mRNA expression was quantified by imaging and serum cytokines analysed by multiplex flow cytometry.

RESULTS:Pre-treatment with our 3-base TLR7-inhibitory oligonucleotide greatly ameliorated the severity of skin inflammation concurrent with a significant reduction in both NF-κB-dependent and Type I interferon-stimulated genes in the skin.
LNP-mediated co-delivery of our 3-base TLR7-inhibitory oligonucleotide halved the serum levels of several key mRNA-driven pro-inflammatory cytokines, thereby dampening the reactogenicity of unmodified mRNA without compromising luciferase expression.

CONCLUSIONS:Our work establishes that ultra-short immunomodulatory oligonucleotides mimicking natural TLR7 inhibitors have significant protective activity against topical and systemic TLR7-driven inflammation *in vivo*. This novel technology not only shows excellent potential for further clinical development as a unique class of TLR7 inhibitors, but also opens the door to a new approach in the treatment of inflammatory diseases with ultra-short oligonucleotides.

Keywords: Oligonucleotides, TLR 7 antagonists, Inflammation, therapeutics, Reactogenicity, RNA

TLR7 inibition using 3-base oligonucleotides



Ultra-short immunomodulatory oligonucleotides mimicking natural TLR7 inhibitors can specifically and strongly bind to the receptor's antogonistic pocket, offering a potential avenue toward treating TLR7-driven inflammatory conditions

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SINGLE MOLECULE IMAGING TECHNIQUES ALLOW FOR VISUALISATION OF MYDDOSOME FORMATION FOLLOWING LIPOPOLYSACCHARIDE AND AMYLOID-β STIMULATION

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OBJECTIVES:A key process in the innate immune response is the formation of a signalling complex known as the Myddosome, which consists of several proteins including MyD88 and IRAKs, ultimately resulting in the production of pro-inflammatory cytokines. We aim to understand the differences in Myddosome signalling between the canonical TLR4 agonist Lipopolysaccharide, and amyloid-β fibrils, through real-time imaging of the formation of these complexes, and quantification of the structures formed at fixed timepoints after stimulation. Further work has looked into endogenous labelling of MyD88 using intracellular fluorescent nanobodies - this approach was validated using fluorescence microscopy.

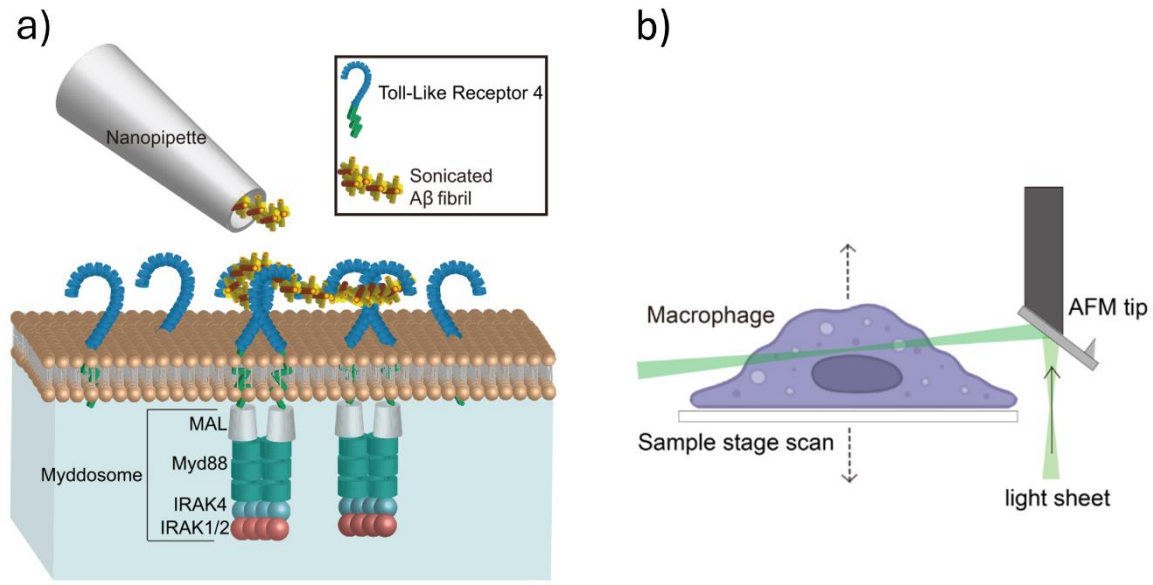
METHODS:Myddosome formation was investigated using a combination of light sheet and superresolution microscopy (dSTORM), in mouse macrophages expressing MyD88-YFP. Live cell imaging allowed for the spatial visualisation and formation kinetics of new complexes, whilst triggered cells were fixed and immunolabelled for high resolution imaging of individual complexes.

RESULTS:Live cell imaging revealed differences in the time taken for Myddosomes to form following stimulation, and the lifetime of the complexes prior to disassembly; amyloid-β triggered Myddosomes took longer to form and longer to disassemble following stimulation compared to LPS. In both cases a range of Myddosome of different sizes (50-500 nm) were formed. In particular, small round Myddosomes around 100 nm in size formed at early time points, then reduced in proportion over time.

CONCLUSIONS:Our data suggests that compared to LPS the multivalency of Aβ fibrils leads to the formation of larger Myddosomes which form more slowly and, due to their size, take longer to disassemble. This may explain why sonicated Aβ fibrils result in less efficient triggering of TLR4 signalling and may be a general property of protein aggregates. Following these results, we are looking to further image protein aggregate mediated TLR4 activation to test this hypothesis.

Keywords: TLR4, Neuroinflammation, Fluorescence Microscopy

Method of agonist delivery and live cell imaging



Nanopipette delivery (a) allows for delivery of amyloid-β fibrils directly to the cell membrane, which combined with light sheet microscopy (b) enables real time imaging of Myddosome complexes following TLR4 activation.

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SPHINGOMYELIN 14:0 – A DRIVER OF NEUROINFLAMMATION DURING OBESITY

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OBJECTIVES:Consumption of Western-type diet (WD), triggers sterile inflammation and long-term epigenetic reprogramming of myeloid cells, altering their immune response (Christ et al., 2018). However, the precise molecular trigger mediating these changes was unknown. We recently identified that during WD, there is an increase in circulating sphingomyelin 14:0 (S14), which was associated with atherosclerotic disease. As obesity, WD and atherosclerosis are all risk factors for Alzheimer's disease (AD) and dementia, we asked whether S14 was the signaling factor linking these conditions with AD.

METHODS:Serum samples were obtained from obese patients for lipidomic analysis. Mice were fed a high-fat diet mimicking WD for 8 weeks, before samples were obtained for lipidomic analysis. Microglia were prepared from wildtype, TLR4-/-, Trif-/- and MyD88-/- mice to assess whether these cells recognize S14, and the induced molecular pathways. Pharmacological inhibition of TLR4 signalling was used as a control. S14 treated cells were prepared for RNAseq, and the genes/pathways were confirmed at a protein level by western blot, and at a functional level using real-time metabolic and phagocytic assays.

RESULTS:Lipidomic analysis confirmed that obese patients have increased circulating levels of S14, which were associated with age and cardiovascular disease. S14 also increased with age in mice. In vitro, S14 triggered an extensive increase in pro-inflammatory genes and pathways. We confirmed S14 binds TLR4, triggering NFkB and JNK activation with downstream TNFα production in microglia. S14 also induced glycolysis and reduced microglial phagocytic capacity.

CONCLUSIONS:Together this shows that S14, a lipid increased during age, obesity and WD conditions, is a TLR4 agonist that activates microglia inducing sterile inflammation. Critically, S14 affected canonical microglia signaling pathways including phagocytosis. Together, our data shows for the first time that S14 is a neuroimmune stimulus, capable of triggering the innate immune responses and inflammation associated with dementia progression.

Keywords: Alzheimer's disease, neuroinflammation, obesity, metaflammation, western diet, microglia

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TLR-SPECIFIC MAPK ACTIVATION PROPERTIES INFLUENCE THE OUTCOMES OF TLR LIGATION.

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OBJECTIVES:Activation of the mitogen activated protein kinases (MAPKs) by Toll-like receptors (TLRs) is a key event in the activation of innate immunity. MAPKs promote gene transcription through the activation of transcription factors such as AP-1, but are also essential for promoting cytokine synthesis through post-transcriptional mechanisms that regulate mRNA stability, processing, and translation. TLR4-induced MAPK activation is switch-like and occurs over a narrow range of ligand concentrations. This allows cells to filter noise and avoid inflammatory responses to low levels of stimulus originating from distant sites of infection and injury. The objective of this study was to establish if this is a general feature of all TLRs.

METHODS:We used high content image analysis for the measurement of multiple activated MAPKs in mouse bone marrow derived macrophages stimulated with ligands for different TLRs.

RESULTS:Our data reveals that switch-like activation of MAPKs is not a universal feature of all TLRs but rather a property of specific TLRs. Furthermore, we demonstrate that maximal activation of the ERK, p38 and JNK pathways may occur at different ligand concentrations for individual TLRs. Analysis of events downstream of ERK activation shows that differences in the duration of TLR-induced ERK activity may be an important factor in shaping TLR-specific transcriptional responses. Finally, we demonstrate that co-stimulation of macrophages with TLR ligand and cytokines such as GM-CSF can lower the MAPK activation threshold and thereby levels of TLR ligand required to induce TNFα secretion.

CONCLUSIONS:Together, our data demonstrate that the dynamics and activation properties of the MAPK pathway can shape the inflammatory outcomes of TLR ligation. These aspects of TLR signalling can be modified, emphasising the importance of context in the regulation of innate immune responses. The finding that some TLRs are less able to filter noise than others may also be relevant to chronic inflammatory disease.

Keywords: MAPKs, activation threshold, ERK

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PROLONGED IMIQUIMOD EXPOSURE IN HEALTHY VOLUNTEERS AS AN INNATE IMMUNE CHALLENGE MODEL FOR EARLY CLINICAL DEVELOPMENT

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OBJECTIVES:Imiquimod (IMQ) is a registered topical agent that has been proven to induce local inflammation via the Toll-like receptor 7 (TLR7) pathway. The purpose of this study was to characterize TLR7-mediated inflammation following 168h of topical IMQ exposure in healthy volunteers, and to compare the effects of short exposure (48-72h) with prolonged exposure (120-168h).

METHODS:IMQ (5mg) was applied under occlusion to five tape stripped treatment sites on the back of ten healthy participants for a maximum of seven consecutive days. Erythema and skin perfusion were measured by multispectral imaging and Laser Speckle Contrast Imaging, respectively, daily up to 168h. Biopsies for immunohistochemically staining and RNA sequencing were collected at 48h, 72h, 120h and 168h post IMQ application.

RESULTS:A total of ten subjects were included. IMQ triggered an inflammatory response starting from 48h after application in terms of erythema of the skin, perfusion of the skin and at the transcriptomic level, with induced TLR7 signalling, IRF involvement and activation of TNF signalling via NF-κB. Furthermore, an enhanced inflammatory response at cellular level was observed after prolonged IMQ exposure, with cellular infiltration of dendritic cells, macrophages and T cells which was also confirmed by RNA sequencing. No difference was found on the erythema and perfusion response after 168h of IMQ exposure compared to 72h (estimated difference: -12.8%, 95% CI [-29.7%, 8.1%], p= 0.2093), erythema (estimated difference: 1.07, 95% CI [-3.67, 5.80], p=0.6567).

CONCLUSIONS:Our study provides a comprehensive understanding of the cutaneous response to both short (48 to 72h) and prolonged (120 to 168h) IMQ exposure in healthy volunteers by using a multimodal approach. Prolonged IMQ exposure revealed enhanced cellular responses, along with more enriched pathways, and can therefore be of interest as model for investigational compounds targeting innate and adaptive immune response.

Keywords: TLR 7, imiquimod, prolonged exposure

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USING BIOLUMINESCENCE RESONANCE ENERGY TRANSFER (BRET) TO INVESTIGATE CONSEQUENCES OF TLR4 ANTAGONISTS ON RECEPTOR CONFORMATION

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OBJECTIVES:Toll-like receptor 4 (TLR4) is an innate immune receptor implicated in a number of traditionally "non-immune" pathologies including pain and addiction. Membrane TLR4 detects endogenous and exogenous danger signals. Once bound, these agonists result in dimerisation and conformational change of TLR4 dimers. Our lab has developed a technique using Bioluminescence Resonance Energy Transfer (BRET) which can visualise real-time activation at the receptor level. It has been reported that TLR4 antagonist TAK-242 does not impact TLR4 conformational change and dimerisation. The aim of this study was to use this highly sensitive BRET assay to confirm this previous assertion by comparing it to LPS-RS, which does prevent TLR4 receptor dimerisation.

METHODS:To achieve a BRET signal, we transfected TLR4 tagged with NanoLuciferase or Venus into Human Embryonic Kidney 293 (HEK293) cells. Antagonists or vehicle were applied 2 hr prior to agonist (LPS 1000 EU/mL) plus antagonist/vehicle (2 hr). BRET signal was obtained by adding substrate (Nano-Glo®) following treatment and reading luminescence on a BMG CLarioStar plate reader (475-30 and 535-30nm). The ratio of these emissions was then calculated to give a BRET ratio for each treatment. Interleukin-8 (IL-8) output was analysed by ELISA using the same transfected cells and treatments.

RESULTS:We observed that both TAK-242 and LPS-RS attenuate the TLR4 BRET signal. However, LPS-RS completely attenuates the signal, while TAK-242 only partially attenuates at high concentrations. In contrast, both antagonists were able fully attenuate IL-8 responses at high concentrations.

CONCLUSIONS:Both antagonists blocked IL-8 release and attenuated BRET interactions between TLR4 monomers. This is in contrast to established literature which states TAK-242 does not impact conformational change and dimerisation. However, we only observed partial attenuation at high TAK-242 concentrations. Therefore, the TLR4 BRET assay is highly sensitive, distinguishing the mechanism of action between TLR4 antagonists TAK-242 and LPS-RS.

Keywords: Signalling, TLR4, BRET

TAK-242 and LPR-RS Attenuate TLR4 BRET Signal Following LPS

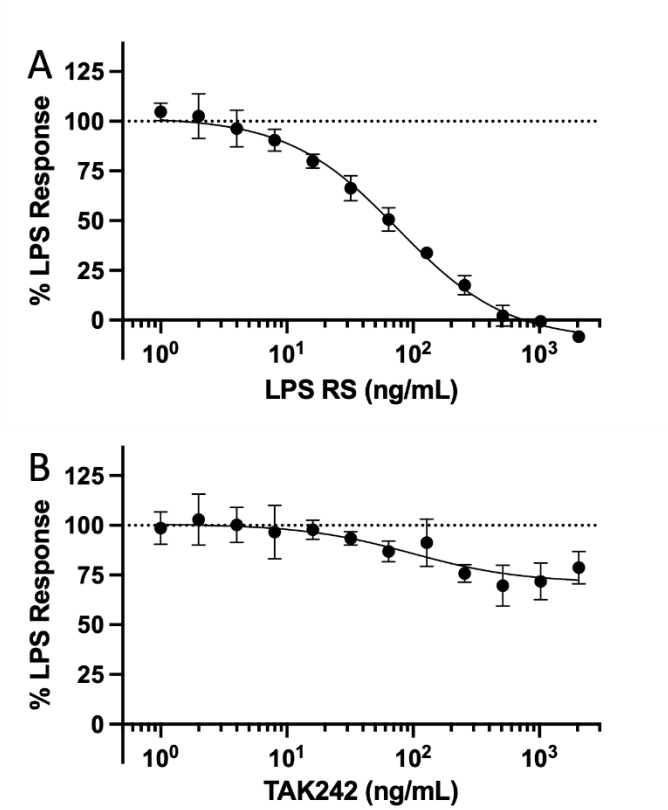

Figure #: TLR4-Venus and TLR4-NLuc were transiently transfected in HEK293 cells. Cells were pre-treated with either 1 ug/mL LPS-RS (A) or 1 ug/mL TAK242 (B) for 2 h followed by the addition of LPS (1000 EU/mL) for 2 h. Following incubation, Nano-Glo® was added and 5 measurements taken to determine BRET ratios. LPS-RS, but not TAK242, was able to completely attenuate the LPS induced BRET signal, while both caused significant concentration dependent decreases ($p < 0.0001$ and $p = 0.038$ respectively). n = 3 separate transfections for all data points, error bars represent SEM.

This is a key figure which shows both antagonists attenuate the BRET signal which results from addition of TLR4 agonist LPS.

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UNRAVELING THE ROLE OF ALTERNATIVE SPLICING IN TLR-ACTIVATED MACROPHAGES: INSIGHTS FROM COMPREHENSIVE TRANSCRIPTOMICS

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OBJECTIVES:To investigate the role of alternative splicing in TLR signaling and inflammatory macrophage responses, particularly in the context of myelodysplastic syndromes (MDS). This study investigates the hypothesis that, in addition to altered gene expression, changes in isoform ratios due to alternative splicing may also play a significant role in modulating immune responses.

METHODS:We developed a novel pipeline integrating Ensembl's reference transcriptome with in-house long-read PacBio sequencing data to capture a comprehensive transcriptome profile. Additionally, we devised a biostatistics test to determine the significance of isoform ratio changes. Macrophages treated with lipopolysaccharide (LPS) were sequenced to investigate changes under inflammatory stimulation. Additionally, macrophages from MDS patients with spliceosome mutations were analyzed to elucidate the impact of splicing aberrations on their transcript profile.

RESULTS:In LPS-stimulated macrophages, a substantial number of genes exhibited a significant shift in their isoform ratios with no substantial change in their overall expression level. This indicates an underappreciated layer of gene regulation through alternative splicing. In MDS patients, defective splicing patterns could contribute to aberrant immune responses due to an inability to modify isoform ratios during macrophage activation.

CONCLUSIONS:This study highlights a broader role for alternative splicing in modulating TLR signaling and inflammatory responses. It proposes an additional layer to our understanding of inflammatory gene regulation, emphasizing the importance of isoform ratio variations irrespective of overall gene expression change. This insight opens new avenues for therapeutic strategies targeting splicing mechanisms in immune-related disorders.

Keywords: Innate immunity, inflammation, alternative splicing, RNA-seq, PacBio, bioinformatics

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TLR4 UNVEILED: REAL-TIME MONITORING OF TLR4 DIMERISATION AND CONFORMATIONAL DYNAMICS USING BIOLUMINESCENCE RESONANCE ENERGY TRANSFER

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OBJECTIVES:Toll-like receptors (TLRs) are innate immune receptors which have critical influences beyond immunity. However, despite extensive study of TLR4, a key TLR family member, currently there are no tools that allow the measurement of ligand-induced TLR4 activation at the earliest points in the TLR4 signaling cascade. The earliest molecular evidence of TLR activation is the stable formation of TLR4 extracellular and cytoplasmic domain dimerisation. Current methods rely on downstream readouts such as translocation of transcription factors or secretion of cytokines. Although these readouts provide biological insight, they are influenced by pathways beyond TLR4's direct impact. This has prevented characterisation of TLR4 specific ligands. This study seeks to address this gap by developing an innovative in vitro assay for monitoring the early-stage dimerisation and conformational dynamics of TLR4 in real-time.

METHODS:We co-expressed differentially tagged TLR4 (NanoLuciferase or Venus) with TLR4 accessory proteins MD2 and CD14 in Human Embryonic Kidney 293 cells. Transfected cells, following the addition of Nano-Glo® substrate, were measured on a BMG CLARIOstar plate reader (475-30 and 535-30nm) in the presence of an activator (LPS) and selective antagonist (LPS-RS). The ratio of these emissions was then calculated to obtain a BRET ratio.

RESULTS:Real-time activation of TLR4 was identified following LPS treatment (EC50 490 EU/mL) and blocked by LPS-RS (IC50 75 ng/mL, in presence of 1000 EU/mL LPS) (Figure 1).

CONCLUSIONS:This assay, based on the principles of BRET to detect protein-protein interactions, represents the first-time ligand-induced TLR4 activation has been measured in real-time in living cells following LPS exposure. This assay will allow the precise investigation of TLR4 activation. The translation of knowledge generated by this novel assay, coupled with its potential as a tool for compound screening, has the potential to rapidly and economically advance new treatments for various pathologies where TLR4 is implicated.

Keywords: TLR4 signalling, real-time, assay

Figure 1

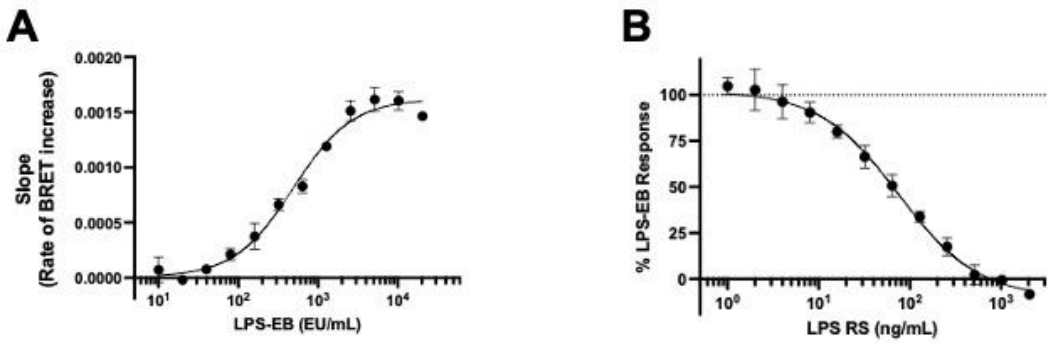

Figure 1: In a BRET assay, (A) a LPS-EB (TLR4 agonist) and (B) LPS-RS (TLR4 antagonist) concentration response curves were generated in HEK293 cells expressing HA-TLR4-Venus, HA-TLR4-Nluc and TLR4 accessory proteins MD2 and CD14. (A) A BRET signal was detected in a LPS-EB concentration dependent manner (EC50 490 EU/mL). (B) LPS-RS treatment prevented an LPS-EB-induced (1000 EU/mL) BRET signal in a concentration dependent manner (IC50 75 ng/mL).

In a BRET assay, (A) a LPS-EB (TLR4 agonist) and (B) LPS-RS (TLR4 antagonist) concentration response curves were generated in HEK293 cells expressing HA-TLR4-Venus, HA-TLR4-Nluc and TLR4 accessory proteins MD2 and CD14. (A) A BRET signal was detected in a LPS-EB concentration dependent manner (EC50 490 EU/mL). (B) LPS-RS treatment prevented an LPS-EB-induced (1000 EU/mL) BRET signal in a concentration dependent manner (IC50 75 ng/mL).

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"LACTOBACILLUS REUTERI" NCHBL-005 PROMOTES WOUND HEALING VIA FIBROBLAST PROLIFERATION THROUGH MAPK SIGNALING

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OBJECTIVES:The skin, the body's largest tissue, harbors a diverse community of microorganisms known as the skin microbiome. The skin microbiome induces interactions with host cells, triggering immune responses that protects the skin. Lactic acid bacteria, including lactobacilli, are members of the skin microbiome that contribute to maintaining the health of the skin through various mechanisms. Based on these mechanisms, lactobacilli have various applications in the field of dermatologic disorders.

METHODS:We induced wounds using a pipette tip on mouse-derived fibroblast (L929 cells) and treated with a "L. reuteri" NCHBL-005 strain, which promotes the migration and proliferation ability of the cell line to evaluate the degree of wound healing. To validate the TLR2-dependent response, MEFs (mouse embryonic fibroblast) were isolated from wild-type (WT) and TLR2 knockout (KO) mice. Additionally, we induced wounds on the skin of mice using 4 mm skin biopsy punch and directly applied the "L. reuteri" NCHBL-005 strain to the wounds, to assess its impact on wound healing.

RESULTS:Our results demonstrated that among the three lactic acid bacteria strains, isolated from honeybees, "L. reuteri" NCHBL-005 exhibited the highest abilities of cell migration and differentiation. Notably, among these strains, it exhibited the most significant activation of MAPK, involved in cell division and migration. Conversely, the treatment of MAPK inhibitors led to the suppression of cell migration and differentiation. In TLR2 KO MEF, we observed a reduction in wound healing and MAPK activation compared to WT MEF. Moreover, when applied to actual wounds on mice, treatment with "L. reuteri" NCHBL-005 notably promote wound healing.

CONCLUSIONS:"L. reuteri" NCHBL-005 can promote wound healing via fibroblast proliferation through MAPK signaling. Our findings suggest that "L. reuteri" NCHBL-005 may be a potential therapeutic agent for wound healing.

Keywords: Lactobacillus, Wound healing, TLR2, fibroblast, MAPK signaling

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SPHINGOMYELIN D18:1/14:0 AS ENDOGENOUS LIGAND OF TOLL-LIKE RECEPTOR 4

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OBJECTIVES:Sphingomyelin 18:1/14:0 (S14) has been shown to be increased in obesity and positively correlated to atherosclerotic plaque burden. We have now identified it as an endogenous ligand of toll-like receptor 4 (TLR4). Currently, we are looking into the details of the TLR4-activation mechanism. S14 levels could serve as a risk marker for atherosclerosis and influencing them might prevent patients from developing cardiovascular diseases.

METHODS:Cytokine release has been tested by ELISA or HTRF on supernatants of various cell lines. NFκB and IRF activation has been confirmed by using reporter THP-1 cells. A TLR4 dimerization assay and molecular dynamics simulation have been performed to support our findings. Assays with CD14 deficient cells have been performed to investigate the role of CD14 and LBP in S14-signaling.

RESULTS:Upon application, S14 binds to TLR4 and leads to its dimerization and internalization. NFkB and IRF are involved in the signaling process. Both bone-marrow derived macrophages and immortalized macrophages show robust TNFa and IL-6 secretion. The specific TLR4-inhibitor TAK-242 was able to abolish the response to S14. In contrast to LPS, S14 does not need CD14 and LBP to signal via TLR4.

CONCLUSIONS:We could show that S14 triggers a pro-inflammatory response via TLR4-activation and that NFkB as well as IRF are engaged. The roles of MD-2 and CD14 have been assessed. With S14 being enriched in obesity it contributes to metaflammation. TLR4 could therefore be a potential target to lower the burden of metaflammation and decrease the risk to develop atherosclerosis in overweight patients.

Keywords: TLR4, Metaflammation, Atherosclerosis, Sphingomyelin, Obesity

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EXPLORATION AND BIOLOGICAL EVALUATION OF NOVEL ISOXAZOLE-BASED SMALL-MOLECULE TOLL-LIKE RECEPTOR 8 ANTAGONISTS

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OBJECTIVES:Toll-like receptor 8 (TLR8) is an endosomal sensor of single-stranded RNA and initiates early inflammatory responses. Overactivation of TLR8 has been implicated in inflammatory and autoimmune diseases driving the need for the development of novel TLR8 antagonists. Here, we biologically characterized and validated a new chemotype of TLR8 antagonists which has been identified by computational modeling.

METHODS:In silico screening was performed to identify potential TLR8 modulators based on their predicted ability to bind in the binding pocket of human TLR8. HEK293T cells stably overexpressing human TLR8 were used to characterize the virtual screening hits for their ability to inhibit TLR8-mediated responses. THP-1 macrophages and human peripheral blood mononuclear cells (PBMCs) were used for signaling pathway interference and selectivity studies.

RESULTS:Five out of 15 selected compounds showed concentration-dependent inhibitory activity, representing a hit rate of 33%. None of the compounds appeared to have agonistic activity on TLR8. The two most potent TLR8 antagonists demonstrated an IC50 value in the nanomolar range and neither caused cellular toxicity nor inhibition of responses induced by other TLR agonists. Both compounds concentration-dependently inhibited TLR8-mediated NF-κB activation and cytokine responses in THP-1 macrophages and PBMCs.

CONCLUSIONS:We experimentally confirmed novel, potent, and selective TLR8 antagonists with low cytotoxicity that are relevant candidates for lead optimization and further mechanistic studies.

Keywords: Toll-like receptors, TLR8, Antagonists, Immunomodulation, Autoimune disorders

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SULFATIDES ARE ENDOGENOUS INHIBITORS OF THE GRAM-POSITIVE BACTERIA SENSOR TLR2

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OBJECTIVES:The objective of the current research is to explore the activity of sulfatides as endogenous antagonists of toll-like receptor 2 (TLR2). TLR2 senses pathogen-associated molecular pattern (PAMP) molecules derived from Gram-positive bacteria, e.g. lipopeptides, and heterodimerizes with either TLR1 or TLR6, to initiate inflammation. In addition, TLR2 is also thought to recognize endogenous danger-associated molecular pattern (DAMP) molecules released from stressed or dying cells, leading to autoinflammation and autoimmunity. Yet, the identity of such endogenous TLR2 ligands is still poorly understood. We recently reported that three molecules of the endogenous lipid C16-sulfatide (3-O-sulfogalactosylceramide) can activate mouse TLR4/MD-2 by mimicking lipopolysaccharide (LPS), its known ligand – a PAMP derived from Gram-negative bacteria. In contrast, C16-sulfatide inhibits LPS activity in human macrophages. In the current research, we examined the activity of sulfatides toward TLR2.

METHODS:Human THP-1 macrophages, mouse BMDM and RAW264.7 macrophages, and HEK293T cells stably overexpressing TLR2, were stimulated with Pam2CSK4, a TLR2/6 agonist, in the presence or absence of sulfatides with fatty acid chains of C16-C24. Pro-inflammatory activities were measured: TNFα secretion, NFκB reporter activity, TNFα promoter reporter activity, and MAP kinases phosphorylation. Modeling of sulfatides in TLR2 was done using human and mouse receptor crystal structures.

RESULTS:Short (C16) and long (C24) fatty acid chain sulfatides competitively inhibit TLR2 activation in human and mouse macrophages. The inhibitory activity of C16-sulfatide towards hTLR2 occurs at concentrations that are an order of magnitude lower than those required for hTLR4 inhibition (apparent Ki for TNFα secretion - 0.15 and 1.25 μM, respectively). Molecular docking simulation has shown that sulfatide fits into the hydrophobic pocket of TLR2, similarly to the lipopeptide.

CONCLUSIONS:Sulfatides, natural membrane glycosphingolipids in mammals, can structurally mimic TLR2 agonists and act as potent endogenous antagonists. Thus, sulfatides may suppress inflammation induced by PAMPs and DAMPs acting at TLR2.

Keywords: TLR2, Sulfatide, DAMP, Endogenous Antagonist

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A NOVEL CLASS OF CYCLIC STRUCTURED OLIGONUCLEOTIDE THERAPEUTICS

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OBJECTIVES:Several synthetic oligonucleotide-based drugs have been approved. These classes of drugs involve multiple mechanisms of action, including targeting disease-associated RNA and acting as PAMPs. To improve the drug-like properties of oligonucleotides, we introduce a novel cyclic-structured oligonucleotide (CSO) to create potent drug candidates.

METHODS:We have designed CSOs composed of modified DNA, RNA, or both, having two 5′ or 3′ ends. These CSOs generally have functional and cyclizing domains linked by 3’-3’ or 5’-5’ linkage. The cyclizing domain is complementary to the end of the functional domain, creating a transient cyclic structure. The functional domain could be antisense to a target RNA or an oligonucleotide sequence that acts as a PAMP. These compounds have been evaluated in various cell-based models

RESULTS:We have studied and evaluated these novel CSOs in which the functional domain is antisense or acts as a TLR9 agonist. In antisense CSOs, the antisense domain is linked to the cyclizing domain via a 5’-5’ linkage. Antisense CSOs show significantly improved potency than gapmer antisense for multiple RNA targets studied. Importantly, antisense CSOs show minimal immune stimulatory responses. In TLR9 agonist CSOs, the immune stimulatory domain is linked to cyclizing domains via 3’-3’ linkage. Depending on the length of each of these domains and the number of CG dinucleotides, TLR9 agonist CSOs induce diverse immune response profiles in rodent and human cells and override species selectivity. We have created a library of TLR9 agonists and profiled these candidates for the immune responses. Detailed data will be presented.

CONCLUSIONS:These novel cyclic-structured oligonucleotides provide a platform to create optimized oligonucleotide-based drug candidates. We refer to these as AntiClastic molecules.

Keywords: AntiClastic, Antisense, TLR9, Agonists, Oligonucleotides, Immunotherapy

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PLASMA EXTRACELLULAR VESICLES IN SEPSIS PROMOTE BRAIN INFLAMMATION VIA MIRNA CARGO AND TLR7 SIGNALING

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OBJECTIVES:Sepsis is a serious clinical condition with life-threatening organ dysfunction caused by dysregulated host response to infection. Many sepsis survivors develop neurocognitive dysfunction, a condition termed sepsis-associated encephalopathy (SAE). While inflammation has been implicated, the molecular mechanisms of SAE are poorly understood. Plasma RNAseq demonstrates predominant presence of miRNAs that are altered in sepsis. Importantly, certain extracellular (ex-) miRNAs are capable of activating innate immune signaling via TLR7 in the brain, lung, and heart. Extracellular vehicles (EVs) are plasma miRNA carriers. Here, we test the hypothesis that septic plasma EVs contribute to brain inflammation via its cargo miRNAs and TLR signaling.

METHODS:Murine sepsis was created by cecal ligation and puncture. Plasma EVs were isolated by ultracentrifugation. Brain immune cells and genes were measured by flow cytometry and qRT-PCR, respectively, following intra-cerebroventricular (ICV) injection of plasma EVs ($8.2x10^8$ particles). Anti-miRNA combo (anti-miR-146a, -122, -34a, -145) and KO mice were employed to test the role of EV-miRNAs and TLR signaling.

RESULTS:ICV injection of septic plasma EVs led to a marked increase in immune cell infiltration as compared to sham EVs (neutrophils: 9.5±2.3 vs. 20.1±4.5 x 10^3, monocytes: 7.8±2.6 vs. 15±3 x 10^4, $p<0.01$). CXCL2, TNFα, IL-6, IL-1β genes were significantly upregulated in the cerebral cortex following single ICV injection of septic EVs. In vitro, septic, but not sham, EVs induced a robust dose-dependent cytokine response in microglia via TLR7, but weak responses in neurons or astrocytes. Pretreatment of EVs with anti-miRNAs led to a marked reduction in septic EV-induced CXCL2 production compared to that of control oligos. Finally, septic EVs-induced brain inflammation via ICV injection was significantly reduced in mice deficient of MyD88, but not TLR7.

CONCLUSIONS:Septic plasma EVs cause marked brain inflammation that is in part attributed to their cargo miRNAs and mediated via MyD88 signaling.

Keywords: TLR, MyD88, miRNA, sepsis, brain inflammation, extracellular vesicles

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MECHANISTIC ROLE OF TLR-MEDIATED INNATE IMMUNITY IN THE PATHOBIOLOGY OF CALCIFIC AORTIC VALVE DISEASE

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OBJECTIVES:Calcific aortic valve disease (CAVD) is a leading cardiovascular disease in the elderly and may result in heart failure. The pathobiology of CAVD includes chronic inflammation and progressive calcification of the aortic valve leaflets. Currently, pharmacological prevention of CAVD progression is unavailable. Aortic valve interstitial cells (AVICs) are actively involved in valvular calcification. Previous studies by our group demonstrate that human AVICs express pro-inflammatory mediators in response to stimulation of Toll-like receptor (TLR) 2 or 4. Further, the TLR-mediated innate immune response in human AVICs leads to cellular pro-osteogenic reprogramming characterized by the expression of osteoblastic biomarkers Runx2 and alkaline phosphatase, and formation of calcium deposits. The objective of this study was to determine the role of TLR-mediated innate immunity in CAVD pathobiology.

METHODS:Cultured human AVICs are stimulated by multiple damage-associated molecular patterns (DAMPs), and cellular innate immunity and osteogenic activity were assessed. The role of TLR in mediating AVIC osteogenic activity were determined using knockout and knockdown approaches, and the role of NF-κB and ERK1/2 pathways was evaluated using specific blockade.

RESULTS:This study uncovered a novel mechanistic role of the AVIC innate immunity in aortic valve calcification. Several endogenous factors can elicit the osteogenic response in human AVICs through TLR2/4, including oxidized low-density lipoprotein and soluble matrilin 2. These endogenous factors function as DAMPs to up-regulate the osteogenic activity in human AVICs mainly through the NF-κB and ERK1/2 pathways.

CONCLUSIONS:Our findings demonstrate that DAMPs are capable of inducing osteogenic response in human AVICs and that the innate immune responses mediated by TLRs have a novel role in modulating the osteogenic activity in human aortic valve cells. These findings suggest that AVIC TLRs and associated signaling pathways play an important role in the molecular mechanism underlying CAVD pathobiology and could be therapeutic targets for suppression of CAVD progression.

Keywords: Toll-like receptors, innate immunity, heart valve, calcific disease

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DIFFERENTIAL TLR RECOGNITION BY VVOMPU, OUTER-MEMBRANE PROTEIN OMPU OF *VIBRIO VULNIFICUS* FOR INDUCTION OF ROS-DEPENDENT AND INDEPENDENT PRO-INFLAMMATORY RESPONSES IN MACROPHAGES

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OBJECTIVES:Our objective was to explore the role of VvOmpU, an outer membrane protein of deadly human pathogen *Vibrio vulnificus* in host immunomodulation with special focus on understanding of the mechanism of pro-inflammatory responses.

METHODS:VvOmpU was purified from the outer membrane of *V. vulnificus*. Purified OmpU was used to treat RAW 264.7 murine macrophage cell line as well as bone marrow differentiated macrophages (BMDMs) obtained from wild type and different TLR/receptor transgenic mice. The immune activation of macrophages with VvOmpU was done in absence and presence of different chemical inhibitors of signalling molecules. Following treatment supernatants were analysed for pro-inflammatory cytokines using ELISA and cells were used for flow-cytometry analysis and microscopy. HEK 293 heterologous system and luciferase assay were used to study receptor co-ordination.

RESULTS:We observed that VvOmpU can induce production of pro-inflammatory mediators IL-6, TNFα and ROS upon macrophage activation. ROS production is dependent on NADPH oxidase activation. TLR2 is involved in IL-6 and TNFα production but not in ROS. However, MyD88 is involved in VvOmpU-induced ROS generation. Further-investigation revealed that TLR4 is majorly involved in VvOmpU-mediated ROS generation in macrophages. Furthermore, we observed that CD36 scavenger receptor also is partially involved in VvOmpU triggered ROS. Our subsequent observation revealed that dynamin-dependent internalization of TLR4 is crucial for VvOmpU-mediated ROS generation and it is independent of CD36, suggesting TLR4 and CD36 probably inducing independent signalling towards ROS production. We further observed that MAPK p38, SRC kinase and PI3K play an important role in the VvOmpU-mediated ROS production, whereas, MAPK JNK and ERK are not responsible for ROS but for pro-inflammatory cytokine production. Further work helped us in dissecting out the receptor-mediated signalling pathways in ROS-dependent and independent pro-inflammatory responses.

CONCLUSIONS:Our study established that VvOmpU modulates host-inflammatory responses by engaging TLR2 and TLR4 and CD36 and triggering distinct signalling pathways.

Keywords: Vibrio vulnificus, TLR4, TLR2, ROS, PI3K, OmpU

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THE COMMENSAL GUT BACTERIA-DERIVED PEPTIDOGLYCAN FRAGMENT, GLCNAC-MURNAC DISACCHARIDE, CONFERS IMMUNO-MODULATORY AND PROTECTIVE EFFECTS VIA TLR4 SIGNALING

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OBJECTIVES:The gut microbiota profoundly impacts the host's health. The resident gut bacteria bestow a rich source of microbial-derived ligands, known as microbe-associated molecular patterns (MAMPs), which are recognized by pattern recognition receptors (PRRs) of the host innate immune system. The gut microbiota-derived peptidoglycan fragments (PGNs) represent an emerging class of effector molecules mediating microbiota-host crosstalk. However, despite the increasing recognition of the importance of gut microbiota-derived PGNs in maintaining the host's steady state, the fundamental question of the structures of natural PGN subtypes in hosts has not been thoroughly addressed due to the lack of appropriate tools.

METHODS:In this work, we developed a robust LC-HRMS/MS platform for the analysis for gut microbiota-derived PGNs. Unlike existing PGN detection assays that rely on protein recognition of certain PGN motifs, our LC-HRMS/MS-based PGN analysis is unbiased and directly reports PGN structures, which not only enables global quantification of gut microbiota-derived PGNs but also unveils their natural subtypes for the first time. Elucidating the scope of gut microbiota-derived PGNs in hosts opens the door to further biological studies.

RESULTS:Intriguingly, we identified an abundance of saccharide-only PGNs in the host gut at steady-state conditions, which are structurally distinct from the classic NOD1/2 agonists. Focusing on the disaccharide GlcNAc-MurNAc motif, we first developed the synthetic route to access the molecules in large quantities, followed by establishing its immunological activity in host immune cells via NOD-independent mechanisms, leading to the discovery of TLR4 as the putative receptor.

CONCLUSIONS:Importantly, we demonstrated that the GlcNAc-MurNAc disaccharide confers protective effects in DSS-induced colitis in mice via TLR4 signaling, highlighting the physiological functions of such natural gut microbiota-derived PGNs in hosts.

Keywords: Gut microbiota, peptidoglycan fragments, immuno-modulatory, TLR4, intestinal inflammation

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TLR4-CONTAINING LIPID RAFTS AS A NEW THERAPEUTIC TARGET IN TREATMENT OF PAIN

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OBJECTIVES:Cholesterol-rich lipid rafts serve as an organizing platform for the assembly of functional receptor complexes. TLR4 is a resident lipid raft protein, highly expressed in spinal microglia and dorsal root ganglia (DRG) nociceptive neurons. ApoA-I binding protein (AIBP) facilitates cholesterol depletion from lipid rafts and binds to TLR4. The goals of this study were to identify the mechanisms of TLR4-raft mediated nociception and to test whether selective depletion of TLR4-rafts by AIBP can alleviate neuropathic pain.

METHODS:Spinal microglia and DRG neurons were isolated from mice that received a chemotherapeutic to induce polyneuropathy and tactile allodynia, followed by injections of AIBP, an AIBP variant that does not bind TLR4 [AIBP(ΔTLR4)], or vehicle. TLR4-lipid rafts were characterized by flow cytometry and proximity ligation assay (PLA) to detect lipid rafts (CTxB binding), TLR4 localization to lipid rafts, TLR4 homodimers, and TLR4-TRPV1 complexes. In addition, TLR4-CTxB PLA in TRPV1 neurons was analyzed in human DRGs from painful and non-painful dermatomes.

RESULTS:Chemotherapy-induced polyneuropathy and tactile allodynia were associated with persistent expression (3 weeks) of TLR4-rafts in the spinal cord and DRG. While in spinal microglia TLR4 formed homodimers, which initiate inflammatory signaling, in DRG neurons TLR4 predominantly associated with TRPV1, a nociceptive receptor. An injection of AIBP, but not AIBP(ΔTLR4), reversed chemotherapy-induced tactile allodynia, with a therapeutic effect of the single injection lasting for over 2 months. Human DRG isolated from painful dermatomes displayed a significantly increased expression of TLR4-rafts.

CONCLUSIONS:These studies suggest the pivotal role of TLR4-lipid rafts in DRG neurons and microglia/macrophages in the development of neuropathic pain. AIBP selectively targets TLR4-rafts in these cell types, affecting multiple pathways involved in spinal neuroinflammation and pain processing, which provides an opportunity for polypharmacology vs. single pain target approaches. The AIBP selectivity toward TLR4-rafts ensures minimal effects on physiological lipid rafts.

Keywords: pain, microglia, neuron, TLR4, lipid rafts, cholesterol

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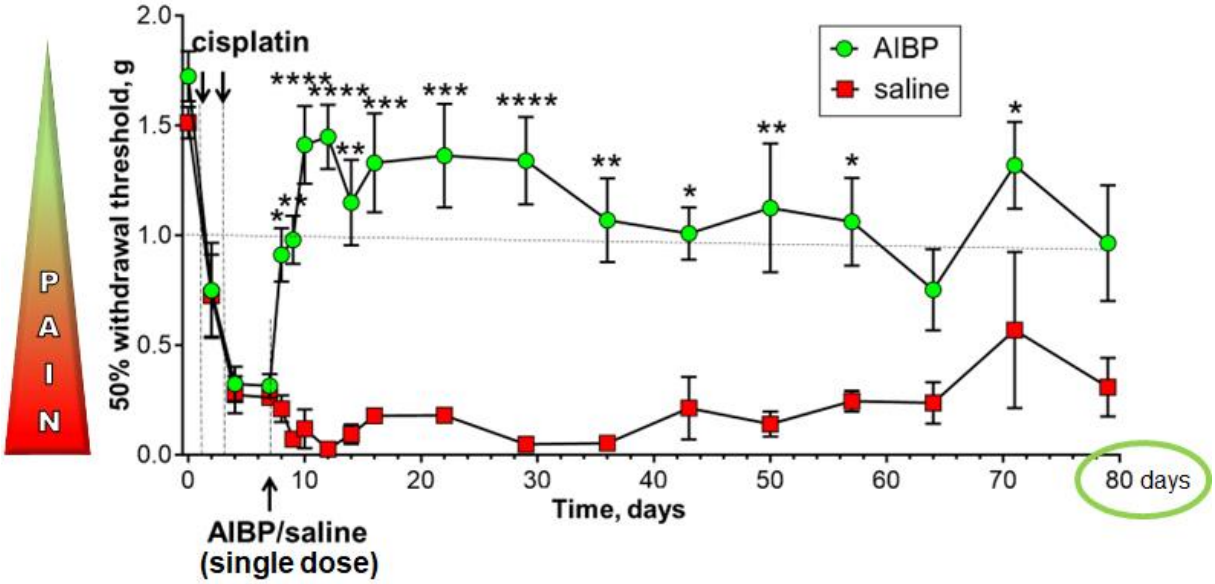

AIBP reverses established tactile allodynia (painful response to normally non-painful, light touch) in a mouse model of chemotherapy-induced peripheral neuropathy