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RIG-I mediates recognition of Salmonella RNA in non-phagocytic cells and contributes to early bacterial replication in vivo

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Introduction. Cytosolic pattern recognition receptors (PRR) have been shown to detect nucleic acids from RNA and DNA viruses in order to launch a type I interferon mediated antiviral state [1]. Here we show that RNA of the facultative intracellular bacterium Salmonella typhimurium is a ligand for RIG-I, triggers production of type-I interferons and contributes to pathogenicity *in vivo*.

Methods. We use RIG-I−/− MEFs [2] or human epithelial cells depleted of RIG-I by lentiviral shRNA transduction for transfection and infection experiments. IFNbeta production was measured by 293T IFNbeta- reporter cells or qPCR. Intracellular bacterial titers were determined by gentamycin protection assay. In vivo infection experiments were performed as described [3] using SL1344 or SL1344 aroA deficient Salmonella strains in RIG-I+/+ and RIG-I−/− mice [4].

Results. Infection of fibroblasts and epithelial cells with S. typhimurium, but not with non-invasive E. coli, triggers IFNbeta transcription, suggesting that intracellular replication is required. RIG-I dependent recognition of bacterial RNA is dominant in non-phagocytic cells, as shown in RIG-I knockout and knockdown model cells. Despite presence of other pathogen-associated patterns (PAMP), like LPS or flagellin, no IFNbeta was produced in fibroblasts and epithelial cells lacking RIG-I upon S. typhimurium infection. In contrast, in macrophages TLR dependent recognition of bacterial PAMPs through TRIF/Myd88 overcomes RIG-I deficiency and leads to robust induction of type I interferon. We observed higher bacterial titers at early time points after infection in the cecum of S. typhimurium infected RIG-I−/− mice, when compared to wild type animals. However, at later stages of infection, S. typhimurium overcomes the innate response leading to similar weight loss and mortality in wild-type and RIG-I−/− mice.

Conclusion. In summary, our data implicate a role of RIG-I mediated innate immune recognition of bacterial RNA in early control of bacterial replication, most likely mediated by non-phagocytic intestinal epithelial cells targeted by S. typhimurium.

Disclosure of interest: None declared.

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IL28B associated polymorphism, RS12979860, controls the activity of liver lymphocytes

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Introduction. The single nucleotide polymorphisms (rs12979860), near the IL28B gene, is correlated with a sustained virological response (SVR) in Hepatitis C Virus (HCV) infected patients treated with Pegylated Interferon-α combined with Ribavirin (1,2). However, the association of rs12979860 polymorphism with immune function of the liver, the site of HCV production, and the mechanism of SVR remain still undefined.

Methods. Patients chronically HCV-infected patients were genotyped for rs12979860 defining C/T polymorphism. C allele is associated to SVR. Liver samples were collected from the needle biopsy achieved for the diagnosis prior any treatment. Single cell suspensions were prepared by mechanical disruption. Liver lymphocytes (T, Treg, NK and NKT) were identified by flow cytometry for the expression of CD45, CD3, CD4, CD8, CD56 and FoxP3 as markers and CD107a for degranulation activity. Expression of CD8β, FoxP3, IL10 and HPRT genes was measured by PCR. Immunohistochemistry were performed on in paraffin sections for the detection of CD8 and FoxP3. Statistical analysis wwas done with Mann–Whitney *U* test and Wilcoxon matched-*t* test.

Results. Lymphocytes from 52 fresh liver biopsies displayed similar distributions of T (CD3), NKT (CD3, CD56) and NK (CD56) among CD45 cells by flow cytometry multi parametric analysis, whatever the IL28B genotype of the patients. Strikingly, higher degranulation activity, revealed by CD107a surface expression, was observed in T ($p = 0.000$), NKT ($p = 0.002$) and NK cells ($p = 0.015$) of patients with CC genotype ($n = 17$) compared to patients with CT or TT genotypes ($n = 35$); patients with CC genotype displayed two fold higher degranulation activity than patients with CT genotype in T ($p = 0.001$), NKT ($p = 0.002$) and NK cells ($p = 0.011$); no significant difference was observed between patients with CT and TT genotypes. Sections of liver from 19 patients showed the frequency of CD4-FoxP3 lymphocytes two fold higher ($p = 0.004$) in patients with CC genotype ($n = 11$) as compared to patients with CT genotype ($n = 8$) supporting the presence of Treg. Previous study demonstrated that the ratio between the number of CD8 cells and FoxP3 cells in parenchymatous necro-inflammatory areas is maintained in the early stage of the chronic hepatitis (3). This is found only in patients with CC genotype, whereas this ratio is reduced in patients with CT genotype due to lower number of FoxP3 cells. Transcriptional analyses confirmed these data and further showed two strong correlations between: one between FoxP3 and CD8β, another between FoxP3 and IL-10 expressions in patients with CC genotype.

Conclusion. Collectively these data provide new insights into the role of IL28B polymorphism related to SVR in treatment of HCV infected patients. CC genotype, which is linked to good response, is associated to higher efficiency of effector lymphocytes (T, NK and NKT) of the liver. The liver immune response appears tightly regulated as suggested by the links between CD8 cells and CD4-FoxP3 cells, and between IL10 and FoxP3 gene expression.

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P135 Defining the intracellular innate immune synapse

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Introduction: . Innate immunity to RNA virus infection is triggered when the cytosolic pathogen recognition receptor RIG-I engages viral RNA in infected cells. RIG-I pathway signaling is transmitted by the RIG-I adaptor protein MAVS, which resides on mitochondria, peroxisomes, and the mitochondrial-associated membrane (MAM), a distinct membrane that links ER to mitochondria. During RNA virus infection, RIG-I is recruited into the MAM where it binds MAVS and drives the actions of a signalosome that mediates downstream induction of antiviral, proinflammatory, and immunomodulatory genes that impart control of infection and immunity. MAM-tethering to mitochondria and peroxisomes coordinates MAVS localization to form a signaling synapse between membranes. The importance of the MAM within this "innate immune synapse" is highlighted by the fact that the hepatitis C virus (HCV) NS3/4A protease cleaves MAVS on the MAM, but not the mitochondria, to evade immunity.

Methods. To identify the components that regulate formation and function of the innate immune synapse and the MAVS signalosome, we characterized the proteome of MAM, ER, and cytosol subcellular fractions from uninfected cells and from cells with either chronic (HCV) or acute (Sendai) RNA virus infections.

Results. Comparative analysis of protein trafficking dynamics during both chronic and acute infection reveals differential protein profiles in the MAM compartment under RIG-I pathway activation. We also identified molecules recruited to the MAM in both chronic and acute RNA viral infections representing proteins that drive immunity and/or regulate viral replication.

Conclusion. Our proteomic analysis reveals dynamic cross-talk between subcellular compartments during both acute and chronic RNA virus infection, and demonstrates the importance of the MAM as a central platform that coordinates innate immune signaling to initiate immunity against RNA virus infection.

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Combined action of type I and type III IFN restricts initial replication of SARS-coronavirus in the lung but fails to inhibit systemic virus spread

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Introduction. STAT1-deficient mice are more susceptible to infection with SARS-Coronavirus (SARS-CoV) than type I IFN receptor-deficient mice. The increased susceptibility of STAT1-deficient mice is potentially due to the lack of functional type III IFN (IFN-λ) signalling.

Methods. We used mice lacking functional receptors for both type I and type III IFN (dKO) to evaluate the possibility that type III IFN plays a decisive role in SARS-CoV protection.

Results. We found that viral peak titres in lungs of dKO and STAT1-deficient mice were similar, although significantly higher than in wild-type mice. The kinetics of viral clearance from the lung was also comparable in dKO and STAT1-deficient mice. Surprisingly, however, infected dKO mice remained healthy, whereas infected STAT1-deficient mice developed liver pathology and eventually succumbed to neurological disease.

Conclusion. Our data suggest that the failure of STAT1-deficient mice to efficiently control initial SARS-CoV replication in the lung is due to impaired type I and type III IFN signaling, whereas the failure to control subsequent systemic viral spread is due to unrelated defects in STAT1-deficient mice.

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Type III interferon impairs bacterial clearance through PDCD4 regulated inflammatory cytokine production

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Introduction . The balance between pro and anti-inflammatory signaling in innate immune responses to bacterial infection is especially critical in the lung. Airway epithelial cells, in addition to resident and recruited cells of immune origin, participate in what must be coordinated proinfammatory signaling in response to inhaled pathogens. The type III interferons are especially important in pulmonary infection, produced in response to viral infection and bacterial PAMPs. IFN-λ is activated by and induces NF-κB signaling and promotes expression of Th1 cytokines. We postulated that common respiratory pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, would stimulate an IFN-λ response and that this would have an important effect on bacterial clearance from the airway.

Methods. To establish that bacterial components can stimulate IFN-λ, we measured the induction of IFN-λ by RT-PCR in murine BMDCs. Biological significance of IFN-λ induction was determined by comparing the ability of wt C57Bl/6 and IL-28R−/− mice (lacking the IFN-λ receptor) to handle an intranasal inoculation of 10^7cfu of either PAK or USA300.

Results. In response to either *P. aeruginosa* PAK or USA300 MRSA on BMDCs there was a 10-fold increase in IFN-λ transcript by 4 h post infection which persisted for up to 8 h; in contrast to the 1000-fold induction of IFN-β by both organisms. The IL-28R−/− mice had significantly improved clearance of either pathogen from the airway and lung tissue ($P < 0.05$ for each). Consistent with the expected participation of IFN-λ in NF-κB signaling, we measured decreased expression of KC, TNF and GM-CSF and increased IL-10 in the IL-28R−/− BAL ($P < 0.05$), although there were no significant differences in the populations of immune cells (neutrophils, DC, macrophages) recruited to the airways of the wild type or IL-28R−/− mice. Deleterious effects of IFN-λ on bacterial clearance were confirmed by exogenous treatment of wt mice with IFN-λ that significantly diminished clearance of both organisms. To address how IFN-λ signaling interferes with bacterial clearance we examined the role of PDCD4 (programmed cell death protein 4) in this model system. PDCD4, which is negatively regulated by miR-21, has been shown to divergently regulate NF-κB and IL-10 signaling, consistent with the observed effects of IFN-λ. At baseline there was significantly more PDCD4 expression in the wt murine lung as compared with the IL-28R−/− mutant and levels of expression were not significantly different at 18 h post USA300 or PAK infection, in contrast to the significant increase in PDCD4 expression in the IL-28R−/− mice following exposure to either bacteria. To determine if PDCD4 in human airway epithelial cells is similarly regulated by IFN-λ, we monitored the kinetics of PDCD4 expression in 16HBE cells noting a 10 fold induction in response to IFN-λwhich was back to baseline by 8 h. The expression of miR-21 was induced by 16-fold at 2 h post IFN-λ and decreased even more briskly back to baseline levels at 4 h.

Conclusion. These results indicate that like IFN-β, bacterial PAMPs also activate IFN-λ signaling which may contribute to airway inflammation without augmenting pathogen clearance. Further dissection of the components of IFN-λ regulation may provide targets to diminish the pathology associated with airway inflammation without compromising the ability to clear pathogens.

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Crosstalk between ITAM and IFN-gamma signaling mediated by GSK3

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Introduction. Ligation of immunoreceptor tyrosine-based activation motif (ITAM)-associated receptors in macrophages can initiate potent induction of negative regulators, including anti-inflammatory cytokine IL-10, signaling inhibitors SOCS3, ABIN3, A20 and transcriptional repressor Hes1 [1]. However under inflammatory conditions, the strong inhibitory pathway initiated by ITAM-bearing receptors was altered. In macrophages isolated from rheumatoid arthritis patients, as well as in blood monocytes/macrophages primed with IFN-γ, ITAM-mediated induction of IL-10 and other inhibitory molecules was markedly attenuated. Here, we investigated mechanisms underlying the suppression of IFN-γ on ITAM-mediated inhibitory pathway.

Methods. We utilized primary human macrophages in this study, and compared gene expression and signaling in IFN-γ-primed versus non-primed cells, by q-PCR and western blotting respectively. Fibrinogen (Fb) was used to ligate ITAM-bearing $\beta_2$intergrin [2]. A combination of biochemical and genetic approaches was conducted to investigate the role of GSK3, including pharmacological inhibitors of GSK3 kinase and RNA interference of GSK3α/β genes. We further analyzed the subcellular localization of GSK3, and its potential substrates, including β-catenin. Microarray experiment was performed to determine the target genes of β-catenin.

Results. We found that IFN-γ markedly increased ITAM-regulated GSK3 kinase activity and nuclear accumulation. Inhibition of GSK3 using pharmacological inhibitors or RNA interference reversed IFN-γ suppression of *IL10* and *HES1*, suggesting that GSK3 mediated the downregulation of inhibitory gene expression by IFN-γ. β-catenin, a major substrate of GSK3, is a transcription factor recently implicated in induction of anti-inflammatory mediator IL-10 in murine dendritic cells [3]. However, effective knockdown of β-catenin by siRNAs had little effect on ITAM-mediated gene expression, as assessed by genome-wide microarray analysis. In contrast, we found that the expression of AP-1, another target of GSK3 [4], as well as its nuclear accumulation was significantly suppressed by IFN-γ.

Conclusion. We provided several lines of evidence that GSK3 was the major mediator in the crosstalk between ITAM and IFN-γ Signaling. AP-1 transcription factor, but not β-catenin, was the major target of GSK3 in this scenario. These findings yield insight into mechanisms of crosstalk between ITAM-associated receptors and IFN-γ that are important for the orchestration of cytokine production and inflammation.

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Continuous exposure to PEG-IFN-Alpha only transiently activates JAK-stat signalling in human liver

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Introduction. IFN-α signals through the Jak-STAT pathway to induce expression of IFN-stimulated genes (ISGs) with antiviral functions. USP18 is an IFN-inducible negative regulator of the Jak-STAT pathway. Upregulation of USP18 results in a long-lasting desensitization of IFN-α signalling. As a result of this IFN-induced refractoriness, ISG levels decrease back to baseline despite continuous presence of the cytokine. Pegylated forms of IFN-α (pegIFN-α) are currently in clinical use for treatment of chronic hepatitis C virus infection. PegIFN-αs show increased anti-hepatitis C virus efficacy compared to nonpegylated IFN-α. This has been attributed to the significantly longer plasma half-life of the pegylated form. However, the underlying assumption that persistently high plasma levels obtained with pegIFN-α therapy result in ongoing stimulation of ISGs in the liver has never been tested. In the present study we there-