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Open Sesame: New Keys to Unlocking the Gate to Norovirus Infection

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Three recent papers highlight the impact of an entry receptor on murine norovirus pathogenesis. CD300lf was shown to be the first proteinaceous entry receptor for a norovirus, specialized intestinal epithelial cells constitute the reservoir for persistence, and a new link between the receptor and a lipid biosynthetic enzyme was revealed.

Human noroviruses (HuNoVs), single-stranded, positive-sense RNA viruses in the *Caliciviridae* family, are a major cause of acute gastroenteritis world-wide and a substantial burden to public health. Studying the biology of HuNoV infection has been hampered by a fairly strict species tropism and the lack of robust and physiologically relevant cell culture models.

Murine noroviruses (MNVs) have emerged as a model to unravel basic mechanisms of norovirus (NoV) biology and pathogenesis because they recapitulate many aspects of HuNoV infection and can be efficiently cultivated *in vitro* and studied in their natural small animal host. Identifying murine macrophages and dendritic cells as a primary source for MNV replication was a paradigm shift, as it was assumed that all enteric viruses, including HuNoVs, primarily target epithelial cells.

Recent studies are beginning to elucidate the HuNoV cell tropism. Novel platforms to study HuNoV infection in a small animal model (Taube et al., 2013), a B cell model (Jones et al., 2014), and a three-dimensional intestinal enteroid model (HIE) (Ettayebi et al., 2016) are being developed and now reveal a dual tropism for cells of hematopoietic origin, including macrophages and B cells, as well as epithelial enterocytes.

Cellular factors that determine the viral tropism are also slowly being discovered. Specifically, secreted and cell-associated carbohydrates, particularly histo-blood group antigens (HBGAs), are critical determinants for HuNoV infection in the human host and also affect HuNoV replication in cell culture (Jones et al., 2014; Ettayebi et al., 2016). However, expression of functional fucosyltransferase 2 (FUT2), the enzyme that transfers fucose to HBGA precursors, is not sufficient to render otherwise permissive cells susceptible, suggesting that the key barrier to HuNoV infection is entry. This suggests that HBGAs alone do not open the gate to HuNoV infection and that a bona fide entry receptor for HuNoV is still missing. Another co-factor is bile acid, which is required for strain-dependent HuNoV replication in the enteroid model (Ettayebi et al., 2016).

Following glycan binding, caliciviruses are thought to engage a protein receptor for viral entry. The first proteinaceous entry receptor was identified for feline calicivirus (FCV), a member of the vesivirus genus. The feline junctional adhesion molecules 1 (fJam1), an immunoglobulin-like protein that localizes to lung epithelial and endothelial cells, determines the cellular tropism of FCV. Binding of FCV to fJam1 induces a conformational change in the viral capsid, inducing flexibility of the protruding domain leading to loss of icosahedral symmetry. Its human counterpart hJam1 was later shown to confer susceptibility to yet another vesivirus, the human-pathogenic Hom-1 strain of the San Miguel sea lion virus (SMSLV) (Sosnovtsev et al., 2017). This indicates a broad role of Jam1 in the ability of vesiviruses to cross the species barrier.

Recently, another immunoglobulin-like protein, murine CD300lf, and its paralog CD300ld were identified as functional receptors for MNV entry by means of a CRISPR-Cas9 technology (Orchard et al., 2016; Haga et al., 2016). CD300lf and CD300ld are both type I transmembrane proteins that share their luminal

N-terminal portion of the protein, which directly interacts with the viral capsid. Expression of CD300lf is both sufficient and necessary, as deleting CD300lf renders cells resistant to infection *in vitro* and *in vivo*, while expression in non-susceptible cell types facilitates infection.

CD300lf is expressed on immune cells and thereby determines this cellular tropism. Interestingly, MNV strains causing persistent infection exhibit a different cellular phenotype. While MNV-1 constitutes the prototype strain causing acute infection in wild-type mice that is cleared within a week, other strains including MNV-CR6 cause persistent infection, associated with fecal shedding for at least a month. Transplanting bone marrow between wild-type and CD300lf-deficient mice revealed that specialized epithelial cells called Tuft cells, rather than bone-marrow-derived immune cells, facilitate persistent infection of MNV-CR6 (Wilen et al., 2018). However, Tuft cells express surface proteins typical for immune cells, including CD300lf, and thereby serve as a major reservoir for persistently shed virus. The ability of MNV-CR6, but not MNV-1, to infect Tuft cells is hereby linked to a single amino acid exchange in the nonstructural protein NS1/2, making the virus resistant to the cellular interferon-lambda-driven antiviral response.

Murine CD300lf is key to MNV species specificity and limits infection to immune and Tuft cells expressing the receptor. Interestingly, CD300lf not only binds to the viral capsid, but also exosomes containing multiple norovirus particles when interacting with phosphatidyl serine present in the ER-derived lipid vesicles (Santiana et al., 2018). Thus, CD300lf plays a dual role as a bona fide receptor for the uptake of individual viral particles as well as a receptor for vesicle-cloaked clusters containing multiple copies of norovirus particles.

Structural analysis revealed that the CD300lf-binding site in the MNV capsid is located at the top side of the protruding domain mediated by hydrophilic and hydrophobic interactions (Kilic et al., 2018). Although a direct interaction of MNV with CD300lf was observed, the contact area of MNV protruding (P) domain of the viral capsid with the receptor was small and the monomeric affinity was unusually low (K_D of ~25 μM, in solution), suggesting that co-factors might contribute to the interaction (Nelson et al., 2018). Indeed, bile acids, cations, and phospholipids were identified as such. Bile acids, particularly glycochenodeoxycholic acid (GCDCA), promoted multimeric binding to the receptor and increased cell binding and infection, presumably by increasing avidity due to multivalent receptor engagement. Bile acids directly bound the MNV P domain in two deeply buried binding pockets, distant from the CD300lf binding site and without significant structural changes in the MNV P domain or N-terminal ectodomain of CD300lf. Long-range allosteric effects are likely involved in providing improved receptor interactions. It will be interesting to see whether structural changes become noticeable when complete VP1 is used instead of the truncated P domain as well as naturally glycosylated CD300lf compared to the non-glycosylated bacterially expressed ectodomain. In addition to bile acid, bile juice contains high concentrations of metal ions, which are also necessary for interaction of P domain and CD300lf (Kilic et al., 2018; Nelson et al., 2018).

In addition to CD300lf, Orchard and colleagues observed that two additional genes (*Sptlc1* and *Sptlc2*) were important for MNV replication (Orchard et al., 2018). These proteins belong to the serine palmitoyltransferase (SPT) complex and catalyze *de novo* ceramide and sphingolipid biosynthesis. The SPT complex is required for infection of the murine glial cell line BV-2 using both the acute strain MNV-1 and the persistent strain MNV-CR6. BV-2 cells that lack Sptlc2 showed significant reduction in viral infection 12 to 24 hr post infection and almost complete block of binding at time point 0, showing that Sptlc2 is an essential cofactor to infection. A mutation in the active center of Sptlc2 (R507A) and treatment of BV-2 cells with a functional inhibitor of the SPT complex further confirmed that Sptlc2 must be enzymatically active to facilitate infection. Surprisingly, Sptlc2 was not needed for surface localization of CD300lf; instead, it rendered the receptor inaccessible to the viral capsid and a conformational antibody. Orchard et al. (2018) conclude that Sptlc2 may induce a conformational change affecting the virus and antibody binding to CD300lf. The conformational change is hereby inferred by the observation that the protein is still detectable on the cell surface via a C-terminal flag tag but no longer by the conformational antibody. Previous observations revealed that MNV infection is cholesterol and lipid raft dependent. An alternative interpretation may be that the depletion of ceramide by blocking the SPT complex interferes with lipid raft formation, leading to an altered membrane environment that could impact CD300lf accessibility or receptor clustering. This could also explain why extracellular ceramide, which would restore lipid raft formation, is able to rescue virus and antibody binding. In addition, receptor clustering would increase avidity of virus-receptor interactions, which was hypothesized to occur in nature based on a low K_D of receptor-P domain interactions.

In conclusion, expression of the proteinaceous receptor CD300lf determines the cellular and tropism for immune cells and specialized intestinal epithelial cells (Tuft cells) (Wilen et al., 2018). Efficient binding to CD300lf depends on multiple co-factors, including metal irons and bile acid (Nelson et al., 2018), as well as sphingolipid biosynthesis (Orchard et al., 2018). This raises the question of whether HuNoVs follow a similar paradigm. A proteinaceous receptor for HuNoVs has not been determined but seems ever more likely, as carbohydrates do not account for major aspects of HuNoV tropism.

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Hijacking Host Angiogenesis to Drive Mycobacterial Growth

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In this issue of *Cell Host & Microbe*, Walton et al. (2018) uncover the mycobacterial factors that activate VEGF signaling and promote aberrant angiogenesis in the tuberculous granuloma. Preventing abnormal angiogenesis in the granuloma represents a potential therapeutic approach for tuberculosis.

Mycobacterium marinum, a close relative of the tuberculosis-causing *Mycobacterium tuberculosis*, causes an infection in zebrafish (*Danio rerio*) that resembles tuberculosis in humans, including the formation of aggregates of immune cells and bacteria, i.e., granulomas, which are characteristic features of tuberculosis (Tobin and Ramakrishnan, 2008; Parikka et al., 2012). This makes the zebrafish a suitable model for studying the host-pathogen relationship in tuberculosis (Myllymäki et al., 2016).

A low-dose *M. marinum* infection in adult zebrafish leads to a latent (or chronic) infection that can be reactivated upon immunosuppression and thus replicates the different outcomes and phases of an *M. tuberculosis* infection in human (Parikka et al., 2012; Myllymäki et al., 2018). While the adult zebrafish has been applied to study the innate and adaptive immune responses, the larval zebrafish can be used for studying the innate immunity in the absence of the adaptive arm. Taking advantage of the transparency of the developing zebrafish larvae, the interaction of immune cells with fluorescently labeled bacteria can be observed in real time, allowing investigation of the dynamic interplay between host and mycobacteria during the development of early granulomas (Davis et al., 2002). Since the granuloma-resident mycobacteria evade immune clearance, the factors that promote the survival of the pathogens inside granulomas and the factors that drive the reactivation of latent tuberculosis are of therapeutic interest.

Infection-triggered induction of vascular endothelial growth factor (VEGF) in host macrophages results in the development of novel and aberrant vasculature around the granuloma, which is beneficial for the proliferation of the pathogen inside the granulomas (Polena et al., 2016). Many host genes involved in the process have been identified, and the effect of angiogenesis preventing drugs on limiting bacterial growth has been demonstrated (Polena et al., 2016; Datta et al., 2015; Oehlers et al., 2017). However, the bacterial factors affecting the process remain poorly understood. In this issue of *Cell Host & Microbe*, Walton et al. (2018) have examined the driving factors behind granuloma-associated abnormal angiogenesis.

Using the larval zebrafish model, these authors have previously shown that granuloma formation in the trunk region leads to aberrant VEGF-driven angiogenesis, which is beneficial for bacterial expansion (Oehlers et al., 2015). In the current study, they use zebrafish larvae with a fluorescently labeled vasculature to investigate the *in vivo* behavior of mycobacteria. Using *M. marinum* mutants in this model, they show that bacteria lacking the gene coding for *proximal cyclopropane synthase of alpha mycolates* (*PcaA*) are not able to trigger abnormal angiogenesis around granulomas. PcaA acts by *cis*-propanating mycolic acids, especially trehalose-6,6-dimycolate (TDM), which is a known immunogenic component of the mycobacterial