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Review

NLRP6 inflammasome

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ABSTRACT

NOD-like receptor family pyrin domain containing 6 (NLRP6) is a novel NLR family member, that shows high expression in the intestine and liver (in contrast to NLRP3 in myeloid cells), to regulate inflammation and host defense against microbes. NLRP6 is reported to involved in inflammasome activation, regulation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling, antiviral interferon (IFN) signaling, mucus secretion, and antimicrobial peptide (AMP) production. Here, we discuss the recent findings as well as debates regarding: how NLRP6 is induced ("signal I") and activated ("signal II"); its roles in intestinal cells and immune cells; how NLRP6 and NLRP9 coordinate to regulate the anti-viral immune response in the intestine; potential targeting of NLRP6 in human diseases.

1. NLRP6: a novel NLR family member that forms the inflammasome

NLRP6 (originally termed PYPAF5) is a novel Nod-like receptor (NLR) family member that forms the inflammasome. The latter initiates maturation of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18, as well as the induction of pyroptosis (a proinflammatory form of cell death) (Levy et al., 2017). Similar to other NLRP family members (e.g., NLRP3), the structure of NLRP6 comprises a N-terminal pyrin domain, a central NACHT domain (shared by all NLRs) and a C-terminal leucine-rich repeat (LRR) domain. NLRP6 and NLRP3 share similarities in amino-acid sequences of 32% for humans and 33% for mice. The main variation arises from the C-terminal LRR domain, which is indicative of different ligand recognition with NLRP3.

Initial studies focused on co-expression of NLRP6 and the inflammasome component apoptosis-associated speck-like protein containing a CARD (ASC) in human cell lines. The *in vitro* data suggested that NLRP6 could activate caspase-1 (an indicator of inflammasome activation) and nuclear factor-kappa B (NF-κB) signaling (Grenier et al., 2002). Later, *in vivo* evidence from Nlrp6-deficient mice also suggested that NLRP6 might form the inflammasome. Reduced levels of IL-18 (but not IL-1β) were observed in Nlrp6-deficient mice under steady-state conditions or after dextran sulfate sodium (DSS)-induced colitis in comparison with those in wild-type (WT) littermates (Elinav et al., 2011a; Levy et al., 2015a), which might have been due to tissue-specific expression of NLRP6, IL-18, and IL-1β (Wang et al., 2015a; Zhu et al., 2017). Moreover, the research teams of Eran Elinav and Richard Flavell showed that NLRP6 co-localizes with ASC in goblet cells (a type of intestinal epithelial cell) under steady-state conditions to form the inflammasome. Those data are consistent with co-immunoprecipitation (co-IP) results that showed NLRP6 can pull-down ASC in intestinal tissue using a Flag-tag knock-in mouse into a NLRP6 locus (unpublished data from our research team). Those results suggest that NLRP6 assembles an inflammasome *in vitro* and *in vivo*, although the molecular details of assembly of the NLRP6 inflammasome are not available.

Most recently, a study from the team of Wu Hao shed light on the molecular mechanisms of the inflammasome. They provided cryogenic electron microscopy (cryo-EM) and crystal structures of the pyrin domain (PYD) of NLRP6. Those methods showed that NLRP6 PYD alone could self-assemble into filamentous structures accompanied by large conformational changes, and could recruit the ASC adaptor using PYD–PYD interactions (Shen et al., 2019). Revelation of the structure of the full length of NLRP6 (as well as the complex with its ligands or adaptor protein ASC) will deepen our understanding of assembly of the

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NLRP6 inflammasome. Notably, studies have shown that NLRP6 can also function independent of inflammation activation (e.g., negative regulators of NF-κB signaling during *Listeria* infection (Anand et al., 2012), positive regulation of interferon (IFN) and interferon-stimulated genes (ISGs) in response to encephalomyocarditis virus (EMCV) infection in the intestine (Wang et al., 2015b)), which will be discussed in sections 5 and 6.

2. Expression and regulation of NLRP6 - typeI IFNs and PPARγ activators might be the Signal I of the NLRP6 inflammasome activation

According to online databases and the literature illustrating its expression pattern, NLRP6 is expressed predominantly in mucosal tissues, which are full of microbial components. This information is indicative of the potential function of NLRP6 in regulating immune responses against microbes. The widely used gene-expression portal BioGPS shows that murine NLRP6 is expressed mainly in the small intestine, large intestine, stomach, liver, and kidney (http://biogps.org/#goto=genereport&id=101613). The research team of Richard Flavell showed that among 24 tissues, NLRP6 showed high expression in the kidneys, liver, lungs, and gastrointestinal tract by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) from WT mice (Elinav et al., 2011a). Subsequently, they generated a Flag-tag knock-in mouse in the NLRP6 locus, and western blotting showed NLRP6 to be expressed very specifically in the intestine and liver, but not in lymphoid tissue (Wang et al., 2015a). Those data explained why most studies on NLRP6 have focused on its role in the gastrointestinal tract.

With regard to transcriptional regulation of NLRP6, the research team of Gordon Smith showed that the promoter region of NLRP6 harbors binding sites for peroxisome proliferator-activated receptor (PPAR)γ, retinoid X receptor α, and chicken ovalbumin upstream promoter transcription factor 1. They used rosiglitazone (PPARγ agonist) to treat human colonic epithelial (Caco-2) cells and found that rosiglitazone increased expression of NLRP6 mRNA ≥2-fold (Kempster et al., 2011). *In vivo* data showed water-avoidance stress-induced enteritis is associated with inhibition of expression of NLRP6 (but not NLRP3) which could be prevented by rosiglitazone, which induced epithelial expression of NLRP6 (Sun et al., 2013a).

NLRP6 is expressed mainly in the intestine, which is the home of many microbes, so scholars are interested in whether a microbial signal can induce NLRP6 expression. The first study came from the team of Richard Flavell. They showed that expression of NLRP6 mRNA could be induced by EMCV, polyinosinic:polycytidylic acid [poly(I:C)] or IFNα in mouse embryonic fibroblasts, thereby suggesting that NLRP6 is an ISG (Wang et al., 2015a). The team of Gabriel Nunez showed that the typeI IFN pathway is required for the induction of NLRP6 expression in bone marrow-derived macrophages.

An increasing number of studies has shown that NLRP6 expression is regulated in multiple "layers". Interestingly, Mao and colleagues found that a *Candida albicans* strain inhibited NLRP6 expression in Caco-2 cells (although the underlying mechanism needs further investigation) (Mao et al., 2020). Another study showed that obese rats had reduced intestinal NLRP6 expression that could be restored by Roux-en-Y gastric bypass (RYGB) surgery but not by calorie restriction (Wang et al., 2020a). Studies have shown the epigenetic regulation of expression of NLRP6 mRNA. For example, micro (mi)R-650 targets the 3′-untranslated region of NLRP6 to inhibit its translation (Xu et al., 2019), and long noncoding RNA OIP5-AS1 can silence NLRP6 expression epigenetically by binding to epigenetic modifier EZH2 (Bai and Li, 2020).

Collectively, those data suggest that microbial signals (e.g. typeI IFNs) and metabolic signals (e.g. PPARγ activators) might serve as signal I for activation of the NLRP6 inflammasome (Fig. 1).

3. The ligands of NLRP6 - microbial RNA, metabolites, LTA and LPS might be the Signal II for NLRP6 inflammasome activation

The canonical inflammasome complex usually consists of a NLR sensor (or another PYD-containing proteins such as absent in melanoma 2 (AIM2) and pyrin), an effector protein (caspase-1, which has a caspase activation and recruitment domain (CARD)) and an adaptor ASC (which consists of a PYD and CARD) to bridge the sensor and effector (de Zoete et al., 2014; Strowig et al., 2012). Activation models for the NLRC4 inflammasome and NLRP3 inflammasome have been proposed, which could serve as references for activation of the NLRP6 inflammasome.

The team of Jijie Chai illustrated the crystal structure of NLRC4 and suggested an "autoinhibition" model (Hu et al., 2013). They postulated that the C-terminal LRR domain inhibits the nucleotide-binding domain (NBD) spatially and, consequently, sequesters NLRC4 in a monomeric form. Meanwhile, disruption of adenosine diphosphate (ADP)-mediated interactions between the central NBD and winged-helix domain result in constitutive activation of NLRC4 (Hu et al., 2013). The team of Wu reported a cryo-EM structure of inactive human NLRP3 in complex with the mitotic kinase NIMA related kinase 7 (NEK7), which licenses the assembly and activation of the NLRP3 inflammasome in interphase. Their work suggests that NEK7 bridges adjacent NLRP3 subunits with bipartite interactions to mediate activation of the NLRP3 inflammasome (Sharif et al., 2019).

Upon activation of NLRs, the interaction of PYD–PYD and CARD–CARD homeodomains, as well as the prion-like properties of PYD and CARD filaments, further facilitate inflammasome assembly; this notion is supported by the cryo-EM structure and structure-based mutagenesis (Cai et al., 2014; Lu et al., 2014). As a result of two nucleation-induced polymerization steps, released activated caspase-1 mediates the maturation of pro-IL-1β and pro-IL-18, as well as gasdermin D-induced pyroptosis (Kayagaki et al., 2015; Shi et al., 2015). Recently, the Hao Wu team reported the detailed filamentous and crystal structure of the PYD of NLRP6 (Shen et al., 2019). They identified (through molecular-dynamics simulations) the surface (W53 and R42) that is critical for the pyrin filaments of NLRP6 to recruit the PYD of ASC. Also, purified NLRP6 or NLRP6 PYD promote polymerization of the ASC PYD (Shen et al., 2019). NLRP6 has been shown to recognize bacterial metabolites (Levy et al., 2015a), viral RNA (Wang et al., 2015a), as well as bacterial lipoteichoic acid (LTA) (Hara et al., 2018). Hence, further biochemical studies on the ligand/NLRP6/adaptor complex are necessary to understand activation and assembly of the NLRP6 inflammasome.

In light of recent elegant studies that elucidated the ligands of NLRP6, the latter recognizes mainly microbial components, probably due to its high expression in the intestine. In 2015, the Eran Elinav team and Eran Segal team showed that microbiota-modulated metabolites regulate the NLRP6 inflammasome. They found that taurine promotes (whereas histamine and spermine inhibit) NLRP6 inflammasome-mediated IL-18 secretion (Levy et al., 2015a). Those data suggested that taurine might be the ligand to activate the NLRP6 inflammasome (although further biochemical analysis is required to prove direct binding). Notably, a recent study demonstrated that RYGB surgery restores NLRP6 activation in obese mice, probably due to the increased level of taurine and decreased level of histamine (Wang et al., 2020b). In the same year, the Richard Flavell team showed that NLRP6 binds viral RNA by associating an RNA helicase (DEAH-box helicase 15 (DHX15)) to promote production of anti-viral IFNs and ISGs (Wang et al., 2015b). Whether NLRP6 can bind directly to RNA was not shown because they used co-IP to show binding with NLRP6-overexpressed cell lysates (Wang et al., 2015b). Subsequently, the Richard Flavell team showed that NLRP6 can bind directly to high-molecular-weight poly (I:C) by glutathione S-transferase (GST) pull-down assays with GST-NLRP6 purified from *Escherichia coli* (Zhu et al., 2017), which suggested that long double-stranded RNA (dsRNA) from the virus might be a direct ligand for NLRP6. Whether NLRP6 can induce the inflammasome

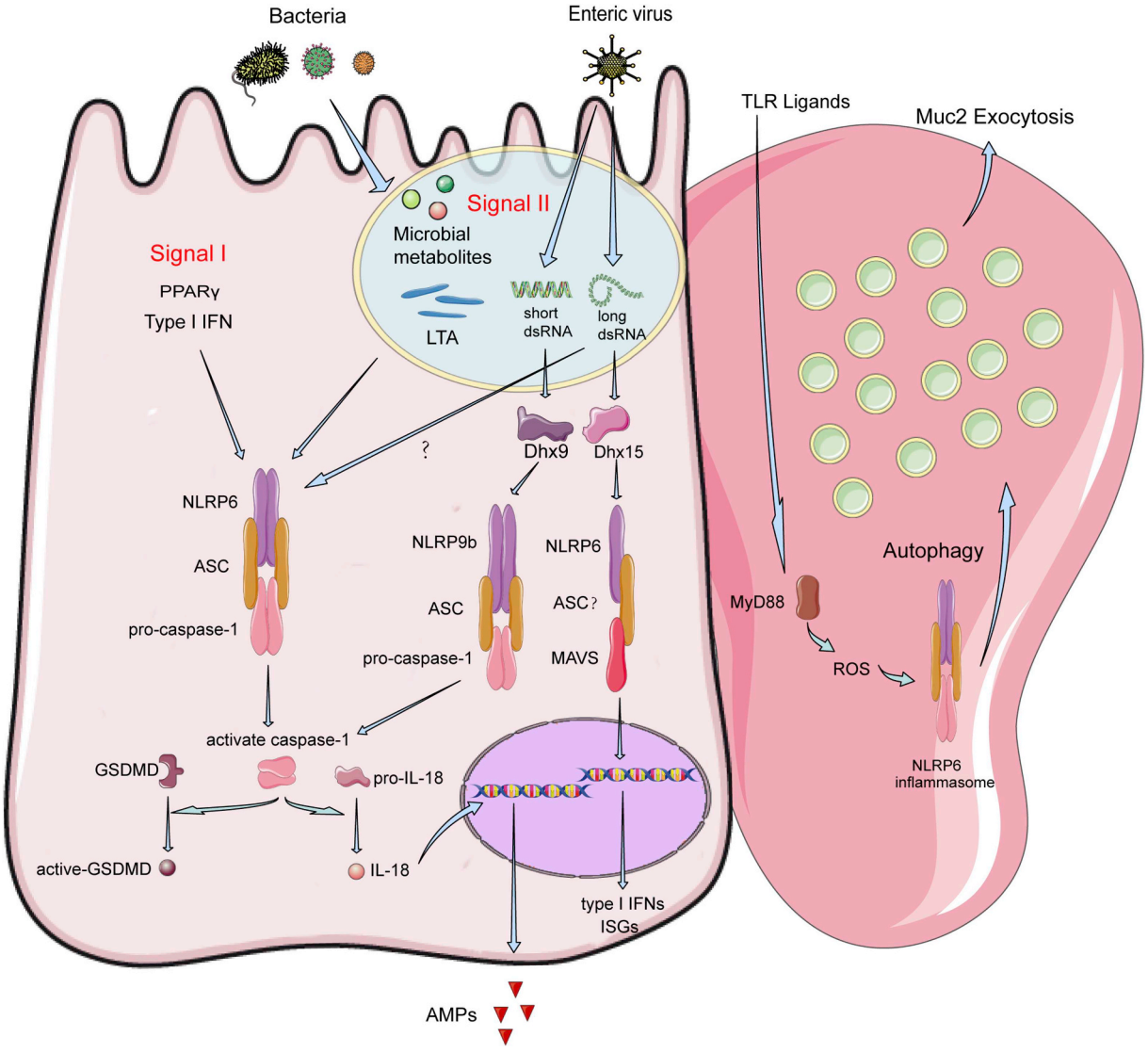

Fig. 1. Signaling pathways mediated by NLRP6
In intestinal epithelial cells, NLRP6 expression is regulated by microbial signals (e.g., typeI IFNs) and metabolic signals (e.g., PPARγ activators) ("signal I"). Next, microbial components (metabolites, RNA, LTA, LPS) might bind directly to NLRP6, and serve as "signal II" to induce inflammasome assembly or induce IFN expression for defense against bacteria and viruses. IL-18 released by activation of the NLRP6 inflammasome promotes AMP secretion to "shape" the host–microbiome interface. Conversely, NLRP6 and NLRP9b might cooperate in defense against different enteric viruses with different tropisms. They have complementary: expression patterns (duodenum *vs*. ileum); ligand-binding patterns; downstream signaling. In goblet cells, TLR ligands activate the MyD88-ROS pathway to activate the NLRP6 inflammasome, to facilitate exocytosis of mucin granules to form a colonic mucus layer above the epithelium.
IFN: interferon; PPAR, peroxisome proliferator activated receptor; LTA: lipoteichoic acid; ASC, apoptosis-associated speck-like protein containing a CARD; GSDMD: gasdermin-d; IL-18: interleukin-18; DHX15: DEAH-box helicase 15; MAVS: mitochondrial antiviral signaling protein; AMPs: antimicrobial peptides; MyD88: myeloid differentiation primary response-88; ROS: reactive oxygen species.

instead of typeI IFNs and ISGs in response to different types of viral RNAs must be investigated. In 2018, the Gabriel Nunez team showed that, in addition to microbial metabolites and RNA, LTA (a molecule produced by Gram-positive bacteria) binds and activates the NLRP6 inflammasome and leads to processing of caspase-11 and caspase-1, which regulates Gram-positive bacterial infection (Hara et al., 2018). Another work in 2020 provided structural insights into NLRP6 activation in response to the Gram-negative bacteria component lipopolysaccharide (LPS). The latter was shown to bind directly to NLRP6 and induce conformational changes and dimerization. Further stimulation with adenosine triphosphate (ATP) induced NLRP6 assembly into a linear molecular platform, and ASC was recruited to form higher-molecular-weight structures, which suggested a step-by-step activation mechanism (Leng et al., 2020).

Increasing evidence suggests that microbial components (metabolites, RNA, LTA, LPS) might bind directly to NLRP6, and serve as signal II to induce inflammasome assembly or induce IFN expression for defense against microbes (Fig. 1).

4. NLRP6 regulates IL-18 production, goblet-cell function, and microbial homeostasis in the intestine

Due to the expression pattern of NLRP6, most functional studies on NLRP6 have focused on the intestine. Early studies showed that an absence of NLRP6 accelerated DSS-induced colitis or colitis-associated tumor growth (Chen et al., 2011; Normand et al., 2011; Elinav et al., 2011b) because of the unknown function of NLRP6 in hematopoietic cells (Chen et al., 2011), deregulated regeneration and proliferation of intestinal epithelial cells (Normand et al., 2011), or an imbalance in the microbiota (dysbiosis) in NLRP6-deficient mice (Elinav et al., 2011b).

Water-avoidance stress-induced small-bowel inflammation (enteritis) has been shown to be associated with inhibition of NLRP6 expression mediated by corticotropin-releasing hormone (Sun et al., 2013b), which confirmed the role of NLRP6 in intestinal inflammation. Studies from the teams of Eran Elinav and Richard Flavell in mice revealed that NLRP6 deficiency leads to defective autophagy in goblet cells, which interfered with mucus secretion in the intestinal lumen. This situation rendered NLRP6 inflammasome-deficient mice unable to clear enteric pathogens such as *Citrobacter rodentium* from the mucosal surface, making them highly susceptible to persistent infection and inflammation (Wlodarska et al., 2014). Later, the Gunnar Hansson team identified the NLRP6 inflammasome could be activated by toll-like receptor (TLR)- and myeloid differentiation primary response 88 (MyD88)-dependent synthesis of NADPH-oxidases and dual oxidases and this, in turn, triggered calcium ion-dependent exocytosis of mucin-2 from goblet cells (Birchenough et al., 2016). The Eran Elinav team and Eran Segal team found, in response to microbiota-associated metabolites such as taurine, histamine, and spermine, that the NLRP6 inflammasome was regulated and, in turn, it controlled epithelial IL-18 secretion and downstream anti-microbial peptide (AMP) profiles (Levy et al., 2015b). That study also shed light into how microbiota in NLRP6-deficient mice are altered, which had been shown in an earlier study (Elinav et al., 2011b).

Very interestingly, Richard Flavell and colleagues identified unusual accumulation of the family Prevotellaceae and TM7 using 16s sequencing (Elinav et al., 2011b), leading to the hypothesis the accumulation of inflammatory commensals cause inflammation. Also, the transfer of the microbiota from NLRP6-deficient mice to WT mice also transferred the intestinal inflammation, and the WT mice harboring the microbiota from NLRP6-deficient mice developed severe colitis and non-alcoholic fatty liver disease (NAFLD) and obesity (Elinav et al., 2011b; Henao-Mejia et al., 2012). Supporting this observation, studies from the Grace Chen group showed that *Akkermansia muciniphila* might act as a pathobiont in IL-10-deficient mice to promote colitis, and that NLRP6 is a key regulator of its abundance to protect IL-10-deficient mice from colitis (Seregin et al., 2017a). However, two studies argued that NLRP6 did not impact the composition of gut microbiota (Lemire et al., 2017; Mamantopoulos et al., 2017). Also, Volk and colleagues detected a functional inner mucus layer (IML) barrier in littermate-controlled NLRP6-deficient mice, but they identified a defective IML in IL-18 deficient mice that they traced, ultimately, to a microbiota-driven, IL-18 independent effect. Those data suggested that the NLRP6 inflammasome is not required for colonic IML formation/function at baseline. They concluded that IML formation/function at baseline is independent of inflammasome activity and highlights the role of the microbiota in determining the function of the IML barrier (Volk et al., 2019). Also, in another work using allogeneic transplantation to evaluate the role of NLRP6 in allogeneic immune-mediated gastrointestinal graft-versus-host disease (GVHD), allogeneic [B6Ly5.2 to *Nlrp6*$^{-/-}$] mice had significantly improved survival compared with allogeneic [B6Ly5.2 to B6 WT] mice, suggesting that NLRP6 in intestinal stroma cells exacerbates GVHD. Although they did not ascertain the exact mechanism of action, they showed that the phenotype was independent of the microbiome (Toubai et al., 2019).

The teams of Till Strowig and Richard Flavell found evidence to explain the contradictory observations from different research teams. In conventionally housed Nlrp6-deficient mice, familial transmission has a significant effect on microbiota composition. However, upon rederivation of Nlrp6-deficient mice into standardized specific pathogen-free (SPF) conditions without pathobionts such as *Helicobacter* species and Prevotellaceae, microbiota composition as well as microbiota-dependent inflammation is indistinguishable between WT mice and Nlrp6-deficient mice (Galvez et al., 2017). However, upon reconstitution of pathobiont-containing microbiota, microbiota composition as well as transferrable colitis phenotypes reappear, suggesting that the impact of NLRP6 upon microbiota composition is dependent upon community structure and primarily influences pathobionts but not commensals (Galvez et al., 2017). Notably, NLRP3-mutant mice and NLRP12-deficient mice can remodel the intestinal microbiota (Yao et al., 2017; Lau and Dombrowski, 2018), but further investigations are needed to test whether this scenario is animal facility-dependent. Indeed, the "pitfalls" in future studies of host–microbiome must be noted, which has been discussed by Jayaraman and colleagues (Jayaraman, 2019).

Summarization of the functional studies on the NLRP6 inflammasome in the intestine reveals that NLRP6 has important roles in controlling production of IL-18 and AMPs from intestinal epithelial cells, as well as mucus secretion from goblet cells. Also, the effects of NLRP6 on the microbiota are dependent upon the community structure (e.g., whether there are pathobionts in the community).

5. NLRP6 and NLRP9 cooperate to regulate intestinal defense

In addition to bacteria, viruses are important components of the intestinal microbiota; the number of viruses in the gut is considered to be $> 10^{14}$ (Ley et al., 2005). Intriguingly, NLRP6 has been found to have a critical role in anti-viral responses in the intestine through an inflammasome-independent pathway (Wang et al., 2015b). The Erol Fikrig team and Richard Flavell team found that NLRP6-deficient mice and control mice challenged systemically with EMCV (a +ssRNA virus) had similar mortality; however, the gastrointestinal tract of NLRP6-deficient mice exhibited increased viral loads. Moreover, NLRP6-deficient mice infected with EMCV had increased mortality and viremia compared with control mice, suggesting a tissue-specific anti-viral role of NLRP6 in the intestine (Wang et al., 2015b). They also postulated that NLRP6 might associate with an RNA helicase (DHX15) to “sense” long dsRNA from EMCV. They found the NLRP6 partner DHX15 by IP/MS and further confirmed by co-Immunoprecipitation (coIP). After ligation of RNA to DHX15/NLRP6, an IFN pathway (instead of an inflammasome pathway) seemed to be activated because they found defective IFN/ISGs in NLRP6-deficient mice whereas caspase-1-deficient mice did not phenocopy NLRP6-deficient mice in an EMCV-infection model (Wang et al., 2015b). That study suggested that NLRP6 might not only regulate bacterial composition, but also regulate intestinal anti-viral immune responses. Whether NLRP6 can regulate the intestinal virome must be investigated. Notably, one study using GST pull-down assays with purified NLRP6 from bacteria showed that NLRP6 may bind to RNA directly but to a lesser extent compared with the binding affinity of RNA to DHX15 (Zhu et al., 2017). That finding suggests that NLRP6 might be an RNA sensor in the intestine. Testing if NLRP6 activates the inflammasome pathway in response to a specific type of RNA would be a very interesting study.

Interestingly, in the same GST pull-down assays, NLRP9b (a novel member of the NLRP family) did not show direct RNA-binding ability. Instead, it could bind viral RNA indirectly through another RNA helicase (DHX9) (Zhu et al., 2017), which is involved in RNA processing, cell survival, and anti-viral defense (Aktas et al., 2017; Lee et al., 2016; Zhang et al., 2011). EMCV infects the duodenum mainly, and locates in the proximal small intestine (Wang et al., 2015a), Flavell and colleagues used a rotavirus infection model, which was found to infect the ileum mainly and locate in the distal small intestine (Little and Shadduck, 1982). Notably, NLRP9 did not affect steady-state IL-18 secretion in the intestine, however, the rotavirus-induced IL-18 secretion and pyroptosis were reduced considerably in NLRP9b-deficient mice. They provided further evidence that NLRP9b can interact with the inflammasome adaptor ASC in response to rotaviruses, which suggests inducible assembly of the inflammasome. Moreover, mice with deletion of the components and downstream effectors of the NLRP9 inflammasome (e.g., NLRP9b, ASC, caspase-1, gasdermin-d) were susceptible to rotavirus infection. Those results suggested that the NLRP9 inflammasome, triggered by viral RNA ligation, induced pyroptosis to defend against rotavirus replication (Zhu et al., 2017). Notably, while NLRP6 tends to bind to long dsRNA, NLRP9b binds more strongly to short

dsRNA (Wang et al., 2015a; Zhu et al., 2017). Our recent data have shown high expression of NLRP6 in the duodenum (the proximal small intestine) whereas NLRP9b is expressed mainly in the ileum (the distal small intestine) (unpublished data), which matches exactly the infection patterns of each virus sensed by these two receptors.

Thus, NLRP6 and NLRP9b might cooperate in defense against different enteric viruses with different tropisms. The complementary expression pattern, ligand-binding pattern, and downstream signaling they activate make them the perfect pair of NLRs in the intestine (Fig. 1). Whether they cooperate in defense against other microbes in the gut or in other physiological functions of the intestine merits further investigation.

6. Role of NLRP6 in immune cells and other mucosal organs

High expression of NLRP6 in the liver has also been shown. NLRP6 expression is decreased in fibrotic and cirrhotic livers (Zhu et al., 2018). In a human hepatic stellate cell line (LX-2), NLRP6 exerts anti-fibrotic effects by suppressing cell proliferation, hydroxyproline accumulation, as well as expression of typeI and typeIII collagens, alpha-smooth muscle actin, and matrix metalloproteinase (MMP)2 and MMP9 (Zhu et al., 2018). One study showed that, during allogeneic hematopoietic stem cell transplantation, NLRP6-deficient mice had more severe liver damage compare to the WT control mice (Li et al., 2019). Those results indicate the protective roles of NLRP6 in the liver to prevent liver damage during transplantation and liver fibrosis.

Scholars have revealed the role of NLRP6 in immune cells, brain, kidneys, as well as gingival fibroblasts and synoviocytes (Anand et al., 2012; Toubai et al., 2019; Zhu et al., 2018; Li et al., 2019; Ydens et al., 2015; Lin and Luo, 2017; Seregin et al., 2017b; Wang et al., 2017; Ghimire et al., 2018; Liu et al., 2018; Lu et al., 2019; Meng et al., 2019; Radulovic et al., 2019; Valino-Rivas et al., 2019; Zhang et al., 2020). Although steady-state protein expression of NLRP6 in lymphoid tissues (or the other tissues mentioned above) has not been observed from Flag-tag knock-in mice according to western blotting (Wang et al., 2015a), NLRP6 expression might be induced in response to microbial signals (Wang et al., 2015a; Hara et al., 2018). Thirumala-Devi Kanneganti and colleagues showed Nlrp6-deficient mice to be highly resistant to infection by *Listeria monocytogenes*, *Salmonella typhimurium* and *Escherichia coli* (Anand et al., 2012). An *in vitro* study showed that *Nlrp6* deficiency in macrophages enhanced activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways after TLR ligation (Anand et al., 2012). NLRP6 in human periodontal ligament cells also suppress the inflammatory response by inhibiting expression of NF-κB and extracellular-signal-regulated kinase (ERK) signaling pathways (Lu et al., 2019), suggesting that NLRP6 might be a negative regulator of canonical NF-κB and MAPK pathways in these cells. Gabriel Nunez and colleagues showed that NLRP6- and caspase-1-deficient mice were less susceptible to *L. monocytogenes* infection compared with WT control mice, but provided another explanation of the phenotype, which was associated with reduced pathogen loads and impaired IL-18 production. Administration of IL-18 to NLRP6- and caspase-1-deficient mice restored the susceptibility of these mice to *L. monocytogenes* infection. That study suggested that sensing of cytosolic LTA by NLRP6 facilitated Gram-positive pathogen infection by promoting IL-18 secretion from macrophages (Hara et al., 2018). Also, Ghimire and coworkers showed that NLRP6 serves as a negative regulator of neutrophil-mediated host defense during Gram-positive *Staphylococcus aureus* infection in the lungs through regulation of the influx and function of neutrophils (Ghimire et al., 2018). Those works demonstrated a negative role of NLRP6 in regulating defense pathways against Gram-positive bacteria in immune cells and stroma cells with low NLRP6 expression. However, Seregin and colleagues showed defective expression of reactive oxygen species (ROS) and tumor necrosis factor (TNF)-α in $Ly6C^{hi}$ inflammatory monocytes from NLRP6-deficient mice (Seregin et al., 2017b), which is contrary to the aforementioned negative role of NLRP6 in myeloid cells.

One report has shown the role of NLRP6 in cluster of differentiation $CD4^+$ T cells. Increased caspase-1 activation and pyroptosis were found in the T cells of NLRP6-deficient mice following adoptive transfer into $Rag^{-/-}$ mice, indicating that the NLRP6 inflammasome might promote death of $CD4^+$ T cells (Radulovic et al., 2019). It will be interesting to calculate the T-cell populations in the intestines of NLRP6-deficient mice because intestinal T cells have important roles in defense and tolerance (Ma et al., 2019). NLRP6 can activate caspase-1 expression and induce pyroptosis in gingival fibroblasts, and may contribute to periodontitis (Liu et al., 2018). Two reports have revealed the role of NLRP6 in the neuronal system, but with conflicting results. One study showed that NLRP6 promotes recovery from peripheral-nerve injury by dampening inflammatory responses but independently of IL-1β and inflammasomes (Ydens et al., 2015). Another study showed the NLRP6 inflammasome to be mainly colocalized in glial fibrillary acidic protein-positive astrocytes, and that the NLRP6 inflammasome in perihematomal brain tissues attenuated intracerebral hemorrhage-induced brain injury (Wang et al., 2017). However, in a model of cerebral ischemia–reperfusion injury, NLRP6 had a proinflammatory role because it activated expression of caspase-1 and proinflammatory cytokines (Meng et al., 2019). A most recent study supports the notion that NLRP6 expressed in astrocytes promotes neuronal injury after oxygen-glucose deprivation/reperfusion by activating the inflammasome and pyroptosis (Zhang et al., 2020).

Taken together, the results mentioned above showed that NLRP6 can form an inflammasome in tissues with low NLRP6 expression outside the intestine. There are many controversial results in those studies, which may be due to: (i) low expression; (ii) different microbiota communities in different animal facilities because NLRP6-deficient mice tend to shape dysbiosis facilities with pathobionts.

7. NLRP6 in diseases

Most functional studies of NLRP6 have focused on mouse models, so insights of the role of NLRP6 in humans are limited. Encouragingly, NLRP6 in human chromosome 11 and its ortholog in mouse chromosome 7 are highly conserved in terms of sequence (68.6% identity by pairwise alignment). Moreover, the transcriptome and proteome analyses defined by RNA sequencing and antibody-based profiling in the human gastrointestinal tract have shown high expression of NLRP6, which is located specifically in intestinal epithelial cells according to immunohistochemistry (Gremel et al., 2015), thereby reinforcing the findings in mice (Elinav et al., 2011a; Wang et al., 2015a). However, NLRP6 expression in humans is found mainly in the small intestine whereas, in mice, NLRP6 is expressed in the small intestine and large intestine (Elinav et al., 2011a; Wang et al., 2015a), although we have found NLRP6 expression in mice to be higher in the proximal small intestine than in the distal small intestine and colon (unpublished data). Notably, NLRP6 transcripts are detectable in organoids cultured from human gut biopsies (Zhu et al., 2017), Another work also showed that NLRP6 is expressed in the human colon according to qPCR and western blotting, and that its expression in the colonic epithelium is decreased markedly in specimens from patients with Hirschsprung's disease compared with controls as measured by immunofluorescence (Tomuschat et al., 2019). However, differences in NLRP6 expression in samples of human colorectal cancer compared with those of healthy controls have not been documented (Liu et al., 2015). Further analyses using single-cell sequencing and mass cytometry could be used to measure NLRP6 expression in different segments and cell types in the human intestine. NLRP6 is expressed in the human kidney tubular epithelium, and expression is reduced during human-kidney injury. The regulatory mechanism of NLRP6 expression in humans needs further investigation. Intriguingly, a single nucleotide polymorphism (SNP) in NLRP6 has been linked to mean platelet volume in a large genome-wide association study (GWAS) (Gieger et al., 2011), which suggests the

potential involvement of NLRP6 in platelet function. More linkage analyses of GWAS data of various human diseases focusing on NLRP6 could provide more insights of the role of NLRP6 in human diseases.

Very interestingly, similar to NLRP3 (Ahn et al., 2018), natural microbes, dietary components or surgical procedures have been reported to regulate NLRP6 expression/activity. *Lactobacillus rhamnosus* GG (LGG) strain is a well-known probiotic. It has been shown to ameliorate the inflammation induced by *Salmonella* infection in the intestine of newly weaned pigs *via* inhibition of the canonical NF-kB pathway and expression of the NLRP6 inflammasome (Yu et al., 2017). Another regulatory commensal, *Clostridium butyricum*, exerts a beneficial action on visceral hypersensitivity in irritable bowel syndrome (IBS) by inhibiting NLRP6-mediated low grade inflammation in the colonic mucous (Zhao et al., 2018). *C. albicans* (an opportunistic fungal pathogen that colonizes human gastrointestinal mucosal tissues) has been shown to inhibit NLRP3 and NLRP6 pathways and dampen the activity of human intestinal mucosal barriers by reducing production of AMPs and tight-junction proteins (Mao et al., 2020). Flavones (a class of polyphenols found in many plant foods) exert their anti-inflammatory effects by regulating the gut microbiota *via* NLRP6; these anti-inflammatory effects are lost in the absence of NLRP6 (Radulovic et al., 2018), which is indicative of modulation of the Nlrp6 signaling pathway by dietary flavonoids to treat inflammatory bowel disease. Moreover, therapeutic treatment or surgery can also NLRP6 expression/activity. Mild moxibustion (a traditional Chinese formulation) can relieve low-grade gastrointestinal inflammation and alleviate visceral hypersensitivity in IBS by regulating intestinal microbes and controlling signaling of the NLRP6 inflammasome (Bao et al., 2019). Another aforementioned study showed that obese rats had decreased intestinal NLRP6 levels, which could be restored by RYGB, but not by calorie restriction. The authors also found that RYGB surgery restored abnormal gut permeability and reduced intestinal inflammation based on reactivation of NLRP6 expression (Wang et al., 2020b). Those works suggest that searching for NLRP6 agonists/antagonists to modulate NLRP6 expression may aid treatment of intestinal inflammation and the metabolic syndrome.

Encouragingly, scientists have screened small-molecule compounds as NLRP3 substitutes, and shown effects on treatment of neuronal inflammation, cryopyrin-associated autoinflammatory syndrome, peritonitis, gouty arthritis, NAFLD, typeII diabetes mellitus, diabetes mellitus-induced impaired fracture healing, and myocardial ischemia–reperfusion injury (Coll et al., 2015; Yang et al., 2016, 2019; Jiang et al., 2017; He et al., 2018; Marchetti et al., 2014; Huang et al., 2018). Thus, understanding the biology of NLRP6 and developing agonists and antagonist to modulate intestinal immune responses accordingly might benefit the inflammation/tumor conditions in the intestine.

NLRP6 may be a promising therapeutic target because of: (i) the conserved expression of NLRP6 in mice and humans; (ii) tight regulation of NLRP6 expression during many physiological and pathological processes; (iii) involvement of NLRP6 in infection, inflammation, cancer, and metabolic diseases; (iv) identification of natural functional agonists and antagonists of NLRP6.

8. An angiotensin-vasopressin receptor (AVR) encoded in the NLRP6 locus

NLRP6 has been suggested to function as an angiotensin II receptor and angiotensin-vasopressin receptor (AVR), which is different from its reported functions (Albrecht et al., 2003). The AVR is distinct from NLRP6 based on different mRNA and protein sizes, subcellular localization, and tissue-specific expression patterns. NLRP6 mRNA transcription starts from exon-1 whereas AVR mRNA transcription starts from exon 4 (Herrera et al., 2008). Glorioso and colleagues identified one NLRP6/AVR SNP associated with decreased susceptibility to hypertension in males (rs7948797G), revealed an association between NLRP6/AVR loci with male essential hypertension in males (Glorioso et al., 2013). Although the NLRP6-deficient mice used in the aforementioned studies had deletion of exons 1 and 2, *AVR* was not affected (Chen et al., 2011; Elinav et al., 2011b).

9. Conclusions

NLRP6 has a tissue-specific expression pattern that attracts attention because of its role in the intestine. It has a protective role in defense against Gram-negative bacteria as well as enteric RNA viruses because it mediates mucus secretion as well as production of AMPs and IFN. Its role in shaping the intestinal microbiota is controversial; likely highly dependent upon the microbiota community of each animal facility. However, NLRP6 is a negative regulator in host defense against Gram-positive and -negative bacteria in myeloid cells. The divergent role of NLRP6 in intestinal and immune cells might due to different: (i) expression of NLRP6 in intestinal epithelial cells and macrophages; (ii) binding patterns of NLRP6 in different cells; (iii) ligands from different environments that trigger activation of NLRP6 expression; (iv) subcellular localization of NLRP6 in different cell types (this requires further investigation). Most studies have mainly used NLRP6-deficient mice to study its function, so further studies using conditional knockout mice to delete NLRP6 in intestinal epithelial cells, myeloid cells, T cells, or other cell types are needed to address the tissue-specific function of NLRP6.

Studies have shed light on how NLRP6 senses ligands, initiates signals, or regulates signals to react to environmental cues. Further investigations (including more biochemical and structural studies) might be needed to address how the NLRP6 inflammasome is assembled, and how NLRP6 coordinates multiple signals (IL-18 production, IFN production, mucin secretion), and balances immune defense and homeostasis (Mishra and Kumar, 2018). NLRP6 and NLRP9 coordinate to exert anti-viral capabilities in different regions of the intestine, so maybe they recognize a specific type of RNA. Whether NLRP6 and NLRP9 shape the intestinal virome merits investigation.

We have discussed the potential implications of targeting NLRP6 in human diseases. Most of our understanding on the physiological and pathological roles of NLRP6 arise from studies on mice. Human NLRP6 has been found to be highly and specifically expressed in human intestinal epithelial cells, Broader investigations on the cellular functions and disease susceptibilities of human NLRP6, as well as the development of agonists/antagonists for NLRP6, will be the next “hot topics” for this unique and critical innate immune receptor.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.mam.2020.100859.

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