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The Goldilocks Conundrum: NLR Inflammasome Modulation of Gastrointestinal Inflammation during Inflammatory Bowel Disease

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Abstract

Recent advances have revealed significant insight into Inflammatory bowel disease (IBD) pathobiology. Ulcerative colitis and Crohn's disease, the chronic relapsing clinical manifestations of IBD, are complex disorders with genetic and environmental influences. These diseases are associated with the dysregulation of immune tolerance, excessive inflammation, and damage to the epithelial cell barrier. Increasing evidence indicates that pattern recognition receptors, including Toll-like receptors (TLRs) and nucleotide-binding domain and leucine-rich repeat-containing proteins (NLRs), function to maintain immune system homeostasis, modulate the gastrointestinal microbiome, and promote proper intestinal epithelial cell regeneration and repair. New insights have revealed that NLR family members are essential components in maintaining this immune system homeostasis. To date, the vast majority of studies associated with NLRs have focused on family members that form a multiprotein signaling platform called the Inflammasome. These signaling complexes are responsible for the cleavage and activation of the potent pleiotropic cytokines IL-1 β and IL-18, and they facilitate a unique form of cell death defined as pyroptosis. In this review, we summarize the current paradigms associated with NLR Inflammasome maintenance of immune system homeostasis in the gastrointestinal system. New concepts related to canonical and noncanonical Inflammasome signaling, as well as the implications of classical and alternative Inflammasomes in IBD pathogenesis, are also reviewed.

Keywords

microbiome; immune system; colon; pattern recognition receptor; Nod-like receptor; cancer

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I. Introduction

In the gastrointestinal (GI) tract, there is a delicate alliance between the immune system and microbiota. When optimally balanced, this alliance facilitates host immunity against pathogens, drives tolerance toward innocuous antigens, and protects the host against a diverse range of autoimmune and Inflammatory disorders.¹ Indeed, we now appreciate that any shift in this balance toward either a more aggressive or attenuated host immune response targeting elements of the microflora can have dramatic and often unpredictable consequences in disease pathogenesis. Inflammatory bowel disease (IBD) is one of the most common diseases associated with aberrant Inflammation in the GI tract. IBD is a complex disorder with genetic, immune system, microbiome, and environmental influences that can be subdivided into two distinct forms: ulcerative colitis (UC) and Crohn's disease (CD) (Fig. 1). Both of these disorders are associated with dysregulated Inflammation either restricted to the colon or throughout the gastrointestinal tract, respectively. The loss of immune system homeostasis in the gut is a hallmark of IBD and is commonly associated with dysbiosis.

Immune system homeostasis in the GI tract is maintained by pattern recognition receptors (PRRs). In the context of IBD, the prevailing literature has focused on the role of Toll-like receptors (TLRs), nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs), RIG-I-like helicase receptors (RLRs), and C-type lectin receptors (CLRs), all PRR family members.^{2,3} These PRR families sense highly conserved pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) and modulate the ensuing innate immune response following microbial exposure, damage, and cellular stress. The biological signaling cascades associated with each of these PRRs can have significant effects on IBD pathogenesis beyond the direct modulation of Inflammation, including the regulation of cell proliferation and death, tissue repair and remodeling, and the production of potent signaling molecules, such as reactive oxygen species (ROS), in the gut.⁴ To maintain immune system homeostasis in the GI tract, PRR activation is required to be robust and sufficient to facilitate the host immune response against pathogens and damage, while also being sufficiently constrained and permissive to promote a healthy gut microflora and avoid prolonged activation. Persistent stimulation and chronic dysregulation of PRR signaling in the GI tract is associated with significant collateral damage and a shift in the microbiome toward dysbiosis, which can contribute to chronic Inflammation, autoimmunity, and cancer.² Thus, we have previously described this phenomenon as a “Goldilocks conundrum,” where PRR expression, activation, and repression must be constantly maintained in balance to promote immune system homeostasis.^{2,5}

Over the last few years, we have witnessed a significant expansion in the number of reports associated with the contribution of NLR family members in IBD pathobiology.² These studies have shed new insight on the complex mechanisms modulated by NLRs in the GI tract. In large part, the interest in exploring the role of NLRs in IBD stems from human genetic association studies that have linked single-nucleotide polymorphisms (SNPs) in several NLR family members with either UC or CD.⁶ The most well-known of these mutations are in NOD2 and are associated with CD pathogenesis in patient subpopulations.⁷ However, beyond NOD2, genome-wide association studies (GWASs) have identified a number of other risk alleles in NLR genes associated with IBD, including mutations in

NOD1 and NLRP3, and dysregulated expression of a variety of NLR genes have recently been found in IBD patient populations.² Beyond these mutation and gene expression findings, mechanistic and functional insight in human subjects is currently lacking. The overwhelming majority of data pertaining to NLR function in IBD has been derived from mouse models.

There are more than 24 distinct NLR and NLR-like proteins in humans, and only about half of these have been well characterized.^{8–10} These initial characterization studies have revealed that the NLR family members can be divided into 3 primary subgroups: Inflammasome forming NLRs, regulatory NLRs, and reproductive NLRs.¹¹ Regarding these subgroups, the vast majority of studies have focused on identifying and characterizing the Inflammasome forming NLRs. NLR Inflammasomes are macromolecular platforms that sense cytosolic PAMPs and DAMPs, resulting in the maturation of pro-IL-1 β and pro-IL-18 into their bioactive cytokines (Fig. 2).¹² In addition to IL-1 β and IL-18 maturation, Inflammasome activation can also result in a unique form of Inflammatory cell death, termed pyroptosis or pyronecrosis.^{13–18} The core unit of the Inflammasome is composed of a specific NLR(s); the apoptosis-associated speck-like protein containing a caspase activating and recruitment domain (CARD) (ASC; encoded by the gene *PYCARD*); and a specific caspase (typically Caspase-1). NLRP1, NLRP3, NLRP6, NLRC4, and the functionally related protein absent in melanoma 2 (AIM2) have been clearly shown to form inflammasome complexes. In contrast, NLRC5, NLRP7, NLRP12, and NAIP cofactors are less defined, but they also appear to contribute or directly function through Inflammasome formation under cell type-, species-, stimuli-, and/or temporal-specific mechanisms.^{19–26} These NLR and NLR-like proteins sense specific PAMPs and/or DAMPs and form a specific NLR inflammasome following recognition and activation.⁹ In general, the mechanisms and signals associated with NLR activation are currently unclear. However, two mechanisms have been proposed: direct ligand binding or indirect milieu sensing.² Evidence supporting both mechanisms is prominent in the literature. For example, AIM2 directly binds dsDNA and NAIP5 directly binds to flagellin fragments.^{20,27–29} Conversely, NLRP3 does not appear to directly bind any specific PAMP- or DAMP-associated ligand/s, acting instead through indirect milieu sensing. NLRP3 has been suggested to sense changes in the intracellular environment and/or disruptions in the cytosol associated with pathogen infection, cellular damage, and stress.¹⁹ These changes include K⁺ efflux, generation of mitochondrial ROS, lysosomal damage, and/or cathepsin B release.¹⁹

Despite extensive study, a significant number of unsolved mysteries remain in NLR biology.³⁰ In the context of IBD, the clinical relevance of NLR Inflammasome function is still unclear, cell-type-specific effects have yet to be extensively characterized, and the role of NLRs outside of direct host-microbe sensing remains largely unexplored. Likewise, the seemingly conflicting data from an exponentially increasing number of mouse studies has generated a highly complex and often misinterpreted mechanistic overview of NLR biology in the gut. In this review, we summarize our current understanding of the emerging concepts associated with NLR inflammasome formation and maintenance of immune system homeostasis in the GI tract. We discuss new and emerging concepts in NLR Inflammasome biology, provide an overview of the current mouse model literature, discuss insights

pertaining to human IBD pathobiology, and attempt to reconcile divergent mouse model findings.

II. NLR Inflammasomes

A. The Canonical Inflammasome

The previous paradigm in innate immunology and the PRR field was that inflammasome organization and signaling proceeds through a relatively simple, linear process. In the “classical” Inflammasome formation model, the cell requires “Signal 1” for transcriptional priming (Fig. 2). In essence, the stimulation of TLRs results in increased transcription and translation of pro-IL-1 β . Similarly, this process also results in increased pro-IL-18; however, pro-IL-18 is constitutively expressed in a variety of cell types and increases following TLR stimulation.³¹ These pro-form cytokines are maintained in the cytosol in their immature states until Inflammasome formation occurs to drive post-translational processing and cleavage into the mature cytokines. In the classical model, the adaptor protein ASC (encoded by the *PYCARD* gene) links the NLR sensor to caspase-1 to form the basic Inflammasome subunit. ASC includes a pyrin domain and a CARD.³² The ASC protein interacts with NLR family members through pyrin–pyrin interactions, which trigger the formation of a large multimer of dimers termed the ASC speck.³² Following speck formation, monomers of pro-caspase-1 are recruited and form CARD–CARD interactions with the ASC. This interaction induces the self-cleavage of pro-caspase-1 and results in the formation of a heterotetrameric, active caspase-1. Both pro-IL-1 β and pro-IL-18 are proteolytic targets of caspase-1 and are thus cleaved when caspase-1 becomes activated. Several Inflammasome-forming NLR proteins contain CARD domains, such as NLRC4, and can thus activate caspase-1 without an ASC.³³ However, IL-1 β and IL-18 generation is significantly enhanced with ASC speck formation, and various cofactors are typically involved in pattern recognition to assist NLRC4 with signaling.³³ Following cleavage and activation, IL-1 β and IL-18 require a “Signal 2,” which typically includes exogenous ATP, to facilitate release from the cell (Fig. 2).

This classical overview of NLR Inflammasome formation and function has been predominately based on studies conducted utilizing knockout mouse models and human cell lines. However, it has long been realized that significant differences exist between human and mouse processing and secretion of IL-1 β /IL-18 and pyroptosis. For example, human cells typically do not require the exogenous ATP associated with signal 2 for IL-1 β and IL-18 release following PAMP stimulation; however, this is essential for mouse cells. Adding a level of complexity to this observation, ATP is only needed in the context of PAMP stimulation, as most pathogens are capable of initiating complex signaling cascades and generate sufficient levels of signal 2 to promote cytokine release without exogenous stimulation. Likewise, whole-pathogen exposure also negates the need for Signal 1, as the PAMPs and DAMPs associated with the infection also serve as the transcriptional priming step. Recently, the mechanism associated with the differences between mouse and human cells has come to light. It is now recognized that Inflammasome formation can occur through either the classical pathway or a newly described alternative pathway (Table 1).³⁴ Specifically, classical inflammasome formation, as defined in mouse monocytes/

macrophages, requires signal 1 (transcriptional priming) and signal 2 (ATP); whereas, Inflammasome signaling in human cells can proceed without the dual signals and can also occur independently of exogenous ATP, pyroptosome formation, and pyroptosis (Table 1). This “alternative” Inflammasome activation was defined in the context of the NLRP3 Inflammasome following LPS exposure.³⁴ Mechanistically, the alternative Inflammasome signals through a TLR4-TRIF-RIPK1-FADD-caspase 8 cascade, all upstream of NLRP3 activation in humans.³⁴ While more mechanistic insight is certainly needed to better define this mechanism, it is now clear that Inflammasome formation and function are much more complex than initially thought and a multitude of questions pertaining to this alternative Inflammasome remain unanswered. For example, it is unclear whether there is cell type specificity for classical and alternative Inflammasome formation. The vast majority of studies have characterized and defined Inflammasome function in monocyte/macrophage populations, but significantly less is known beyond these leukocyte populations. It is also unclear whether this is a stimuli-specific or an NLR-specific effect. The alternative Inflammasome was defined following LPS exposure and in the context of NLRP3 activation. It is unclear whether this applies to other PAMPs/stimuli or additional NLR Inflammasomes. Finally, it is also unclear whether these differences are species specific. NLR family members are highly conserved, and it is unclear whether the alternative Inflammasome functions in other mammals or even in other cell types in humans and mice. This question was partially addressed in the study that defined the alternative Inflammasome, wherein this mechanism was observed in both human and pig monocytes.³⁴

In addition to direct Inflammasome formation, some NLRs also require cofactors for inflammasome formation. These cofactors function to assist with signal recognition or to stabilize the activated NLR. The best-characterized cofactors are the NLR family, apoptosis inhibitory protein (NAIP) proteins. These cofactors have been characterized in mouse models associated with NLRC4 inflammasome formation and signaling. Because NLRC4 has a CARD protein, rather than a pyrin domain, the exact mechanism underlying Inflammasome formation remains somewhat unclear. However, recent findings have revealed that NLRC4 forms complexes with NAIP proteins to facilitate signaling. In this model, the NAIP proteins directly recognize the respective PAMPs through receptor–ligand interactions and subsequently complex NLRC4 to facilitate inflammasome formation. In mice, NAIP1 detects the type III secretion system needle, and NAIP2 senses the type III secretion system rod protein, while NAIP5 and NAIP6 detect flagellin prior to interactions with NLRC4.^{21,35,36} However, only a single NAIP orthologue has been identified in the human genome, and it functions similarly to the murine NAIP1 protein to sense the type III secretion system needle.³⁶ Thus, the mechanism used by NLRC4 in human cells to sense flagellin remains unclear, but it does not appear to utilize the same NAIP5/NAIP6 mechanism described in mice.

Pyroptosis, a unique form of inflammatory cell death that is also associated with Inflammasome formation, is similar to apoptosis in that both rely on the activation of specific repertoires of caspases to trigger programmed cell death. Apoptosis relies on a set of initiator caspases (caspase-2, -8, -9, -10) that respond to apoptotic signals and cleave effector caspases (caspase-3, -6, -7). These effector caspases proceed to cleave target proteins to drive apoptotic cell death. Conversely, pyroptosis is defined by the activation of a subset of

Inflammatory caspases (caspase-1, -4, -5, -11), which encode both the initiator and effector functions to orchestrate cleavage of target proteins and modulate cell-death pathways. Pyroptosis is defined by the following unique characteristics: (1) the activation of an Inflammatory caspase; (2) pore formation in the plasma membrane; (3) low levels of DNA damage and an intact nucleus; and (4) poly(ADP-ribose) polymerase (PARP) activity is not required.³⁷ Pyroptosis and Inflammation are intimately linked in host–pathogen responses. Indeed, pyroptosis is an efficient mechanism to restrict pathogens by destroying their protective, intracellular niche within infected cells.³⁷

The mechanistic link between caspase signaling and pyroptosis has recently been found to involve gasdermin D (GSDMD).^{38–40} The critical role of GSDMD in Inflammasome signaling hinges on caspase cleavage and activation of GSDMD from the “pro” full-length protein of 53 kDa to the active 30-kDa N-terminal fragment.⁴⁰ Interestingly, GSDMD proteolytic cleavage can be mediated via caspase-1,⁴⁰ as well as by caspase-11 in mouse cells or caspase-4 in human cells.³⁹ This potentially indicates some diversity regarding the role of GSDMD in canonical or noncanonical Inflammasome function. Functionally, cleaved GSDMD was found to selectively bind to plasma membranes containing lipids, such as the mitochondrial and bacterial lipid cardiolipin, and phosphatidylinositol phosphates that are located on the inner leaflet of the mammalian cell membrane.³⁸ The result of this binding is the formation of oligomeric pores that kill mammalian cells, which is restricted to the activated cell and does not kill bystander cells.³⁸ GSDMD was also shown to directly kill bacteria, but because active GSDMD binds selectively to phospholipids that are restricted to the inner leaflet of mammalian cell membranes, it remains unclear whether the antibacterial function of GSDMD is limited to killing bacteria that escaped the phagosome.³⁸

B. ASC and Caspase-1 in IBD Pathogenesis

ASC is considered an essential adaptor protein for Inflammasome function due to its critical role as a “bridge” between NLRs containing PYD domains and pro-caspase-1 and significantly enhances the activity of NLRs containing CARD domains.^{19,32} Indeed, ASC is a major player in multiple Inflammatory diseases and is implicated in several autoimmune diseases associated with Inflammasome dysfunction.⁴¹ Because of the central role of ASC in Inflammasome formation, *Asc*^{−/−} mice are commonly used in murine models to investigate Inflammasome function, as deletion of ASC results in the ablation of all inflammasome function. In murine models of chemically induced colitis with dextran sulfate sodium (DSS), the absence of ASC has been consistently shown to have a more severe phenotype than other individual NLR knockout genotypes,^{42–47} implying that loss of all Inflammasomes is more detrimental to IBD pathogenesis than the loss of any individual sensor. In general, these studies all demonstrate that *Asc*^{−/−} mice present with more severe clinical features associated with colitis progression, including increased mortality, weight loss, loose stool, dehydration, and decreased colon length. Histopathologically, mice lacking ASC have increased colon Inflammation and significant defects in the epithelial cell barrier. These barrier defects have been shown to be associated with increased levels of serum endotoxin, indicating enhanced permeability and bacteria translocation in the *Asc*^{−/−} mice.⁴⁴ Colitis sensitivity in the *Asc*^{−/−} mice is correlated with ablated levels of IL-1 β and IL-18, due to the loss of

Inflammasome function.^{42–47} Together, these data reveal that NLR Inflammasomes function to attenuate gastrointestinal Inflammation during experimental colitis.

Patients with IBD have a significantly increased risk of developing a distinct form of cancer, defined as colitis-associated cancer.⁴⁸ One of the distinct advantages of DSS-based experimental colitis models is the ability to robustly study not only gastrointestinal Inflammation but also colitis-associated cancer. In these models, mice are exposed to the mutagen azoxymethane (AOM) prior to DSS driven inflammation.⁴⁹ AOM induces mutations in intestinal epithelial cells, which leads to a well-defined pathological progression to colorectal cancer, typically adenocarcinoma, in genetically susceptible mouse genotypes. This process is significantly enhanced when DSS is used to drive Inflammation and a wound-healing response in epithelial cells. Increasing evidence has linked ASC and cancer progression. Indeed, prior to the discovery of the Inflammasome and ASC speck formation, ASC was originally defined as a target of methylation-inducing silencing 1 (TMS1) and was shown to be silenced through methylation in human breast and gastric cancers.^{50,51} Prior to what we now define as pyroptosis, these early studies suggested that ASC silencing was associated with reduced apoptosis and increased cancer severity.^{50,51} Subsequently, ASC expression has also been shown to be reduced in several cancers including melanoma, colorectal cancer, lung cancer, and oral squamous cell carcinoma.^{52–55} Thus, the majority of studies evaluating the *Asc*^{-/-} mice in the DSS models also included assessments of Inflammation driven tumorigenesis using the AOM+DSS model. Consistent with the experimental colitis findings, mice lacking ASC also demonstrate significantly increased tumor formation, with some studies identifying large macroscopic polyps and significantly enhanced morbidity and mortality in the *Asc*^{-/-} mice as early as 30 days after AOM treatment.⁴² These findings indicate that tumor formation occurs approximately twice as rapidly and much more aggressively in the *Asc*^{-/-} mice than in wild-type animals.⁴² Taken together, it seems that the *Asc*^{-/-} mouse model data are consistent with the *ASC* expression and silencing findings in human patient populations, which correlate decreased expression with increased cancer pathogenesis.

The findings associated with ASC were originally unexpected because the *Asc*^{-/-} mice were postulated to have reduced Inflammation and tumorigenesis due to the loss of pro-Inflammatory IL-1 β and IL-18 signaling. Thus, parallel studies were also conducted evaluating caspase-1. Caspase-1, formally known as IL-1 β cleaving enzyme (ICE), is considered the archetypal member of the Inflammatory caspases. In mammals, this family of Inflammatory caspases includes caspase-1, caspase-4, caspase-5, caspase-11, and caspase-12.⁵⁶ Some differences in these caspases have been observed between mice and humans. For example, in most humans, caspase-12 is mutated and encodes for a nonfunctional protein.⁸ In addition, caspase-11 is found in mice, while caspases-4 and 5 are found in humans and are considered to be the human homologs of caspase-11.⁸ Interestingly, all of the Inflammatory caspases, including caspase-1, contain an N-terminal CARD, which is responsible for binding ASC in the formation of the canonical Inflammasome.^{12,56} Like ASC, caspase-1 was originally considered essential for the function of all Inflammasomes and critical for the cleavage of pro-IL-1 β and pro-IL-18 into their mature forms.^{12,57–59} Due to its overwhelming role in many inflammatory processes, including Inflammasome formation, caspase-1 is the most fully characterized of the Inflammatory caspases.⁸ As

mentioned previously, caspase-1 activation is triggered by the oligomerization of NLRs via their NACHT domains and the subsequent interaction with ASC.⁸ When these molecules are in close proximity, the autocatalytic activity of caspase-1 is initiated, leading to its activation.⁶⁰ Compared to other caspases, such as caspase-3 and caspase-7, caspase-1 has a restricted substrate repertoire.⁶¹ However, caspase-1 has been shown to be involved in a variety of “Inflammation-independent” functions, such as cell death, glucose homeostasis, and triglyceride metabolism.^{62–64} Regardless, most of these other functions of caspase-1 are speculated to be dependent on caspase-1 activation via inflammasome formation.⁶²

As with ASC, due to the importance of caspase-1 to IL-1 β and IL-18 processing and Inflammasome formation, several mouse studies have evaluated the role of caspase-1 in experimental colitis models.^{42,47,65} One of the earliest of these by Siegmund et al. (2001) utilized *Casp1*^{-/-} mice in acute and chronic models of DSS colitis. Contrary to the findings associated with the *Asc*^{-/-} mice, this study found decreased weight loss, diarrhea score, and bleeding score in mice lacking caspase-1 put through both acute and chronic DSS models.⁶⁵ However, more recent studies have consistently shown that loss of *Casp1*^{-/-} results in worsened disease progression in similar mouse models.^{42,47,66,67} In each of these current studies, mice lacking caspase-1 displayed significantly increased morbidity and mortality, weight loss, rectal bleeding, and other pathological features of disease progression. Likewise, each of these studies correlated increased disease progression with ablation of IL-1 β and/or IL-18.^{42,47,66,67} Together, the consensus data are consistent with the findings for ASC and further support a protective role for the NLR Inflammasome in IBD pathobiology. Due to its protective role in IBD pathogenesis, caspase-1 has also gained attention in the context of colitis-associated cancer. Recent studies using the AOM-DSS model have shown that mice lacking *Casp1* display higher tumor load and tumor frequency than wild-type mice.⁶⁸ Interestingly, in the same study, there was no difference in inflammation between wild-type and *Casp1*^{-/-} mice.⁶⁸ This finding contrasts with the studies mentioned earlier, in which *Casp1*^{-/-} mice showed increased levels of Inflammation and tumorigenesis compared to wild-type mice.^{42,47,66,67}

While the DSS model is an excellent choice to evaluate Inflammation-driven tumorigenesis, one common critique is that DSS fails to accurately model many clinical and physiological features associated with human IBD. Thus, many investigators utilize spontaneous colitis models to complement DSS findings. For example, the most common approach used to study spontaneous colitis utilizes mice on an IL-10 defective background. Surprisingly, studies utilizing *Il-10*^{-/-} mice are relatively rare in the inflammasome field compared to DSS. In one of the few studies to evaluate caspase-1 in the *Il-10*^{-/-} colitis model, inhibition of either IL-1 receptor signaling or caspase-1 using chemical antagonists suppressed IL-1 β and IL-17 production from inflamed colon explants significantly ameliorated the development of spontaneous colitis.⁶⁹ These data are consistent with another recent spontaneous colitis and colitis-associated cancer model utilizing IEC *Clgalt*^{-/-} mice.⁷⁰ Core 1- and core 3-derived mucin-type O-linked oligosaccharides (O-glycans) are major components of the mucus layer in the gut.⁷⁰ When this mechanism was disrupted in the gut in mice lacking core 1-derived O-glycans (IEC *Clgalt*^{-/-}), the animals developed spontaneous colitis, followed by the appearance of invasive carcinomas as the mice age.⁷⁰ This spontaneous Inflammation-driven tumorigenesis is likely highly dependent on the host microbiota, which are able to have

increased interactions with the intestinal epithelial cells and translocate more readily due to the decreased integrity of the mucosal barrier.⁷⁰ Similar to the observations in the *Il-10*^{-/-} model, when these IEC *Clgalt*^{-/-} mice were crossed with mice deficient in *Casp-1*, the resultant animals showed significantly reduced development of colitis-associated tumorigenesis compared to IEC *Clgalt*^{-/-} mice.⁷⁰

In the DSS and spontaneous colitis studies, it is clear that caspase-1 deletion dramatically alters the phenotype. However, it is currently unclear whether caspase-1 protects against or augments IBD and colitis-associated cancer progression. Likewise, it is unclear whether this is associated with model differences, stimuli, temporal, or cell-type specific differences. Adding a level of complexity to these studies, the majority of commercially available *Casp1*^{-/-} mice used in prior experimental colitis and IL-1 β /IL-18 studies are actually *Casp1*^{-/-} and *Casp11*^{-/-} double-knockout animals, due to the nested nature of the *Casp1* and *Casp11* genes.⁷¹ Thus, it is difficult to reconcile the individual contributions of caspase-1 to disease pathogenesis without also considering the possible effects of caspase-11 deletion. Because all three of the described models have significant microbiome contributions, it is also highly likely that differences in the gut flora could significantly impact the phenotypes observed in each of these models.

C. The Noncanonical Inflammasome

NLR Inflammasome activation an essential pillar of innate immunity and maintains immune system homeostasis in the gut. However, in addition to the “canonical” Inflammasome, which includes classical and alternative activation and relies on caspase-1, a “noncanonical” Inflammasome has also been characterized and is associated with caspase-4/-5 in humans and caspase-11 in mice (Fig. 1; Table 1).⁷¹ These caspases are broadly expressed in both hematopoietic and nonhematopoietic derived cells.^{16,45,71} Caspase-11 has been found to significantly modulate the host immune response following exposure to *Escherichia coli*, *Citrobacter rodentium*, and *Vibrio cholera*.^{39,71} It appears that caspase-11 and the human paralogs caspase-4 and caspase-5 function through directly binding cytosolic LPS and lipid A from gram-negative bacteria.⁷² This binding has been suggested to be highly specific and to occur through the CARD domains.⁷² Once binding occurs, the caspases oligomerize and become activated.⁷² Subsequent findings have revealed that caspase-11 functions as an essential initiator of other caspases during acute pathological conditions,⁷³ such as sepsis, endotoxic shock, and acute bacterial infection. Indeed, it appears that the noncanonical Inflammasome also plays a dominant role during acute Inflammation, whereby activation of caspase-11 results in IL-1 β and IL-18 cleavage through a canonical Inflammasome-dependent mechanism.⁷¹ Beyond IL-1 β and IL-18 processing, the noncanonical Inflammasome also plays a potent role in pyroptosis. However, noncanonical Inflammasome-associated pyroptosis appears to proceed through a canonical Inflammasome-independent mechanism,⁷¹ which has yet to be well characterized. As mentioned above in the caspase-1 discussion, the majority of commercially available *Casp1*^{-/-} mice are actually *Casp1*^{-/-} and *Casp11*^{-/-} double-knockout animals.⁷¹ Subsequent studies using the *Casp11*^{-/-} targeted mice have revealed that caspase-11 functions in a variety of biological mechanisms once thought to be solely dependent on caspase-1 and has also revealed several caspase-1-independent functions.^{71,74,75}

The overwhelming majority of studies characterizing caspase-11, -4, -5 have focused on acute bacteria exposure models and sepsis.^{73,76,77} Thus, it is unclear whether other PAMPs beyond LPS and Lipid A signals associated with gram-negative bacteria can initiate noncanonical Inflammasome formation. Interestingly, recent work by our team and others, has revealed that noncanonical Inflammasome signaling may extend well beyond the currently identified mechanisms associated with cytosolic LPS recognition. Specifically, our research has recently revealed that caspase-11 modulates Inflammation during *Toxoplasma gondii* infection.⁷⁸ Using *Casp11*^{-/-} mice, our results revealed that noncanonical Inflammasome signaling significantly impacts neuro-Inflammation and cyst burden during the chronic phases of disease.⁷⁸ While the mechanism underlying these findings remains unclear, these data suggest a role of caspase-11 in the sensing and response to a currently unidentified molecular pattern associated with this eukaryotic parasite or imply that noncanonical Inflammasome signaling is functioning in response to intracellular changes driven by the parasite.⁷⁸ Thus, it is highly likely that future studies will continue to expand the role of noncanonical Inflammasome function and signaling.

D. Non-Canonical Inflammasome Associated Caspases in IBD

The majority of previous research regarding caspases and IBD has focused on caspase-1. However, additional studies have investigating caspase-11 and the noncanonical Inflammasome in the pathogenesis of IBD due to the recent revelation that the *Casp1*^{-/-} mice used previously in many IBD studies are truly *Casp1*^{-/-}/*Casp11*^{-/-} double-knockout mice. Similar to the prior caspase-1 studies, caspase-11 has also been shown to attenuate gastrointestinal Inflammation during experimental colitis in mice.⁴⁵ Specifically, *Casp11*^{-/-} mice in an acute DSS study had increased morbidity, mortality, and clinical parameters associated with disease severity.⁴⁵ Furthermore, *Casp11*^{-/-} mice displayed more Inflammation and epithelial cell damage in the colon than wild-type animals.⁴⁵ However, unlike *Asc*^{-/-} and *Casp1*^{-/-}/*Casp11*^{-/-} mice, the *Casp11*^{-/-} animals showed attenuated IL-1 β and IL-18 levels in colon culture supernatants, rather than full ablation, and that reconstitution of these cytokines in these mice attenuated experimental colitis pathogenesis.⁴⁵ Unlike the *Asc*^{-/-} and *Casp1*^{-/-}/*Casp11*^{-/-} mice, the protective influence of caspase-11 appears to be restricted to acute experimental colitis, as deletion of caspase-11 only had a minor impact in chronic DSS and AOM+DSS models.⁴⁵ These data are consistent with hypothesis that caspase-11 functions prevalently during severe damage and inflammation, rather than during chronic or minor insults. Consistent with these findings, a second study also found that *Casp11*^{-/-} mice were more susceptible to DSS induced experimental colitis.⁷⁹ Interestingly, *Casp11*^{-/-} mice were highly susceptible to acute DSS, independent of microbiome shifts.⁷⁹ The microbiome of *Casp11*^{-/-} mice contained a reduced abundance of *Prevotellaceae* compared to wild-type animals. However, cohousing *Casp11*^{-/-} and wild-type mice equilibrated *Prevotellaceae* and *Bacteroides* contents, but the mice lacking caspase-11 still displayed the same increased severity phenotype.⁷⁹

In humans, the caspase-11 orthologues caspase-4 and caspase-5 have recently been shown to be significantly upregulated in both CD and UC patient samples.⁸⁰ This study revealed that *caspase-4* expression is higher in noninflamed ileum and colon tissues from IBD patients, suggesting that caspase-4 may be a candidate for a biomarker of disease.⁸⁰ In contrast, both

caspase-1 and -5 expression was significantly higher in inflamed colonic, not ileal, tissue, suggesting their involvement with colitis, rather than ileitis.⁸⁰ Taken together, these data indicate that caspases-4 and -5 are involved in intestinal Inflammation observed during IBD. This study also evaluated colorectal cancer tissue and found the same trend of increased caspase-4 and -5 expression with some correlation to cancer stage.⁸⁰ Indeed, it has been hypothesized that caspase-5 mutations may be linked with gastrointestinal cancers that have a microsatellite mutator phenotype.⁸¹ However, despite some trends associated with progression of disease, there are currently no significant functional or mechanistic studies in humans directly linking these caspases and noncanonical Inflammasome signaling with mechanisms underlying IBD or colorectal cancer pathogenesis.

III. Inflammasome Forming NLRs in IBD- and Colitis-Associated Cancer

A. NLRP3

NLRP3 (also known as cryopyrin and NALP3) is the most well-characterized NLR and is known for sensing a diverse array of signals.^{2,19} In humans it is encoded by the *NLRP3* gene, sometime referred to as the cold induced auto-Inflammatory syndrome 1 (CIAS1) gene and is best characterized in leukocytes.⁸² While the exact mechanism of NLRP3 activation remains a mystery, currently at least three prevailing models attempt to address this cryptic process. In the first model, NLRP3 Inflammasome formation involves the ligation of extracellular ATP via pore formation associated with the ATP channel P2X7. Once this channel is formed, K⁺ can then exit the cell and subsequently recruit pannexin 1, allowing PAMPs to enter the cytosol (Fig. 3).² In the second model, NLRP3 activation involves the phagocytosis of particulates, such as asbestos and monosodium urate crystals, leading to a phenomenon known as “frustrated phagocytosis.”² Thus, lysosomal instability occurs and leads to the release of cathepsin B into the cytosol.^{2,83,84} In the third model, NLRP3 inflammasome formation occurs following the generation and subsequent recognition of ROS.² In each of these models, NLRP3 functions through an indirect sensing mechanism to drive Inflammasome formation. Thus, regardless of which mechanism is more accurate or widespread, this indirect strategy allows NLRP3 to sense broad changes in the intracellular environment and respond to a diverse range of pathogens and/or damage to drive the host innate immune response.

Recent studies have shown that NLRP3 attenuates disease progression during experimental colitis similar to the recent findings regarding ASC and Caspase-1.^{42,47,85} Specifically, mice deficient in *Nlrp3* show increased morbidity, mortality, and have higher levels of Inflammation in their gastrointestinal tract in acute DSS models.^{42,47,85} Interestingly, *Nlrp3*^{-/-} mice show a more subtle disease during the recurring or chronic model of experimental colitis, compared to mice deficient in other Inflammasome components (*Casp1*^{-/-} and *Asc*^{-/-}), although mice lacking *Nlrp3* were still shown to have worse disease than wild type mice.⁴² As with ASC and caspase-1, NLRP3 functions to attenuate colitis progression through the modulation of IL-1 β and IL-18.^{66,86} Specifically, lower levels of IL-1 β can significantly impact T-cell differentiation, leading to increased intestinal inflammation.⁸⁶ Likewise, decreased levels of IL-18 result in defective epithelial cell regeneration and repair, which contribute to increased barrier dysfunction in the *Nlrp3*^{-/-}

mice.⁴⁷ Together, the altered T-cell differentiation and reduced barrier function results in increased bacterial translocation and Inflammation in the gut.

Similar to its role in IBD pathogenesis, NLRP3 has been shown to play a protective role in mouse models of colitis-associated tumorigenesis, whereby loss of *Nlrp3* leads to significantly worsened disease progression in AOM+DSS models.^{42,47} Specifically, *Nlrp3*^{-/-} mice showed higher mortality, morbidity and significantly truncated colons during this model of Inflammation driven tumorigenesis.⁴² Importantly, *Nlrp3*^{-/-} mice show significantly higher tumor burdens compared to wild-type mice receiving the same treatment, indicating that NLRP3 indeed plays an important role in attenuating colitis-associated cancer.^{42,47} Mechanistically, the enhanced tumorigenesis seen in the *Nlrp3*^{-/-} mice appears to be associated with dysregulated IL-18 production.⁴⁷ While both IL-1 β and IL-18 levels are significantly attenuated in *Nlrp3*^{-/-} mice, IL-18 appears to play a more vital role in maintaining gastrointestinal homeostasis. Specifically, the levels of IL-18 were found to be much lower in colon homogenates from *Nlrp3*^{-/-} mice compared to wild-type mice treated with AOM+DSS, while the levels of other important Inflammatory cytokines (IL-6, IL-12 and TNF) were unchanged.^{42,66} As mentioned earlier, IL-18 has been shown to play an important role in promoting epithelial cell regeneration and repair during experimental colitis using DSS.^{47,67} However, it also inhibits hyperplasia and proliferation during the AOM+DSS model.⁶⁶ Thus, IL-18 plays multiple roles through the epithelial cell compartment that can significantly impact both IBD and cancer.

The role of NLRP3 in IBD and colitis-associated cancer has also been evaluated in human disease, mostly through the investigation of meta-analysis of polymorphisms and disease risk. In humans, 60 SNPs have been identified in the NLRP3 gene.⁸² One of the most prevalent of these is *NLRP3* rs35829419, which is a gain-of-function mutation that is heavily associated with human Inflammatory diseases, such as rheumatoid arthritis, celiac disease, and CD.⁸² In a recent meta-analysis, this SNP was closely associated with protection against UC and colorectal cancer (CRC).⁸² In colorectal cancer, this protection is hypothesized to come from the increased interaction with caspase-1 leading to Inflammasome activation and subsequent cell death.⁸² In the case of IBD, it was suggested that the rs35829419 polymorphism interacts with the rs2043211 polymorphism in the *CARD8* gene that encodes a human Inflammasome adaptor protein (CARD8; TUCAN).⁸² Specifically, this *CARD8* polymorphism creates a truncated CARD8 protein, which reduced inflammasome activation and thus attenuated IBD pathology.⁸² The finding that a gain-in-function mutation in *Nlrp3* is protective during IBD and colorectal cancer is in agreement with the animal studies showing that loss of function or deletion enhances disease progression.

B. NLRP6

The NLRP6 Inflammasome was first identified in 2002, where initial *in vitro* studies reported the colocalization of NLRP6 with ASC, and that NLRP6 and ASC coexpression resulted in production of IL-1 β .⁸⁷ NLRP6 has since been found to be widely expressed in immune cells such as neutrophils, dendritic cells, macrophages, and T-cells, but it is also highly expressed in epithelial cells, particularly those in the duodenum, ileum, and by

myofibroblasts within the stem-cell niche of the colon.⁸⁸ Therefore, NLRP6 and its role in regulating Inflammation in the gut has been a topic of considerable interest. Similar to NLRP3, *Nlrp6*^{-/-} mice are significantly more susceptible to intestinal Inflammation and tumorigenesis than wild-type animals when subjected to DSS-induced experimental colitis.^{89,90} *Nlrp6*^{-/-} mice exhibit significantly increased Inflammation and fail to properly repair damaged epithelium as efficiently as their wild-type counterparts.^{89,90} The combination of increased Inflammation and inefficient repair results in increased epithelial cell proliferation, ultimately promoting both spontaneous intestinal hyperplasia and exacerbation of colon tumorigenesis in the DSS model.^{89,90} As with the other Inflammasome deficient animals, *Nlrp6*^{-/-} mice were also found to have reduced IL-1 β and IL-18 levels. Subsequent studies, utilizing *Il-1 β* ^{-/-} and *Il-18*^{-/-} mice, adoptive transfer, and cohousing studies revealed that the sensitivity of the *Nlrp6*^{-/-} mice was associated with epithelial cell derived IL-18.^{46,88–90} However, monocyte-derived NLRP6 also appears to play a role in reducing susceptibility to experimental colitis.⁹¹ Adoptive transfer of wild-type Ly6Chⁱ monocytes into *Nlrp6*^{-/-} mice was sufficient to protect the mice during acute colitis through a mechanism associated with IL-18 dependent TNF production.⁹¹

Mechanistically, the protective nature of NLRP6 during experimental colitis is highly correlated with the gut flora, as the microbiome of *Nlrp6*^{-/-} animals is significantly different compared to that of wild-type mice (Fig. 4).⁹⁰ Specifically, the *Nlrp6*^{-/-} mice were characterized by an expansion of the Bacteroidetes, Prevotellaceae, and TM7 phyla.⁹⁰ This altered microbiome, and subsequent disease susceptibility, of *Nlrp6*^{-/-} mice was transmissible to wild-type animals when cohoused, with no preference or dependence on age as both animals cohoused early in postnatal life, as well as, during adulthood demonstrated the exacerbated DSS colitis phenotype.⁹⁰ The absence of NLRP6 results in decreased mucus production due to impaired mucin granule exocytosis and Muc2 secretion and subsequent impaired mucus layer formation throughout the GI tract.⁴⁶ This defective mucus layer results in mice that are more susceptible to enteric infections (Fig. 4).⁴⁶ Similar to *Nlrp6*^{-/-} animals, this phenotype was also seen in *Asc*^{-/-} and *Casp-1*^{-/-}/*Casp-11*^{-/-} mice.⁴⁶ In fact, a so-called “sentinel” goblet cell (senGC) at the base of the colonic crypt was recently discovered to be responsible for nonspecific endocytosis of any bacteria that penetrates the inner mucus layer.⁹² Evidence suggests that senGCs are able to activate the NLRP6 Inflammasome following endocytosis of TLR ligands, in turn leading to MyD88 Nox/Duos reactive oxygen species synthesis. This process initiates calcium ion signaling to drive secretion of Muc2 mucin from the senGC generating intercellular gap junction signaling, inducing Muc2 secretion from responsive and adjacent goblet cells.⁹² This action, in turn, expels bacteria from the opening of the crypt that penetrated the inner mucus layer, protecting both the lower crypt and intestinal stem cells.⁹² The absence of NLRP6 and the subsequent impaired mucus layer creates a permissive niche for *Prevotelloceae* to exploit; as a result, these bacteria colonize areas of the crypt where they would normally be excluded. This increased colonization results in chronic Inflammation in the colon, driven by other PRRs that likely compensate for the loss of NLRP6. Likewise, this shift in the host microbiome associated with the loss of NLRP6 results in dramatic changes in microbiota-modulated metabolites. For example, the microbiota-associated metabolites taurine, histamine, and spermine comodulate NLRP6 Inflammasome signaling and can significantly impact IL-18 production

and anti-microbial peptide (AMP) profiles.⁹³ The AMP distortion appears to be another critical component of the mechanism associated with NLRP6 deficiency as restoration of the AMP axis with Ang4 is able to restore microbial diversity in the absence of IL-18 production.⁹³

Beyond experimental colitis and colitis-associated tumorigenesis, *Nlrp6*^{-/-} mice are also more susceptible to infection with enteric pathogens than their wild-type counterparts. For example, *Nlrp6*^{-/-} mice that were subjected to infection with *Citrobacter rodentium*, which is often used as a model of altering the microbial community, were unable to clear the bacteria as effectively as wild-type mice exposed to the same dosage.⁴⁶ Taken together, it is clear that the relationship between NLRP6 and the control of microbial composition via mucus and AMP production has strong implications for the pathophysiology of a variety of diseases and pathogens associated with Inflammation of the gut. Additional studies are warranted to better define stimuli, cell-type, and time-specific mechanisms associated with NLRP6 Inflammasome activity and function in the gut and beyond.

C. NLRP1

NLRP1 was the first inflammasome to be identified.¹² The NLRP1 Inflammasome is activated following exposure to either anthrax Lethal Toxin (LeTx) or *Toxoplasma gondii* in rodents and muramyl dipeptide (MDP) in humans.^{94–99} Consistent with host-specific differences, the protein structure of NLRP1 is species specific. Human NLRP1 contains both an N-terminal PYRIN domain and a CARD domain; whereas, mouse NLRP1 only contains a CARD domain.² At the genomic level, rodents encode three different paralogs of *Nlrp1*, which are all poorly characterized.⁹⁵ Currently, it appears that NLRP1A plays a role in regulating hematopoiesis, NLRP1B is involved with sensing *Bacillus anthracis* LeTx, and NLRP1C is predicted to be a pseudogene.^{95,96,100} Because of the CARD domain, NLRP1 can bypass the requirement for ASC during Inflammasome formation similar to NLRC4 (Fig. 5). However, as with NLRC4, ASC significantly enhances Inflammasome formation and modulates the formation of this signaling platform optimizing caspase-1 activation.⁹⁴

Until recently, the multiple paralogs of *Nlrp1* in the mouse had confounded efforts to generate a NLRP1-deficient animal. However, two independent groups have now generated targeted *Nlrp1abc*^{-/-} and *Nlrp1b*^{-/-} mice.^{96,100} Although the extent of the literature on the involvement of NLRP1 in gastrointestinal Inflammation in both human and mouse studies is currently limited, the availability of these novel mouse lines now allows for robust assessment of murine NLRP1 function. To date, only a single study has evaluated NLRP1 in the experimental colitis model. Similar to the other Inflammasome forming NLRs, the *Nlrp1b*^{-/-} mice were found to have significantly more weight loss than their wild-type counterparts in both the acute DSS model and the chronic DSS model of relapsing remitting disease.⁴⁴ As with the other NLRs, NLRP1-deficient mice also demonstrated increased Inflammation and attenuated IL-1 β and IL-18 production.⁴⁴ Interestingly, reconstitution of the *Nlrp1b*^{-/-} mice with either recombinant IL-1 β or IL-18 partially restored protection in the acute DSS model, and inhibition with either anti-IL-1 β or anti-IL-18 in wild-type mice resulted in increased sensitivity.⁴⁴ Thus, it appears that NLRP1 attenuation of disease pathogenesis is, at least in part, associated with the production of both IL-1 β and IL-18.

When evaluated in the AOM+DSS model of colitis associated tumorigenesis, the absence of NLRP1 resulted in greater tumor burden and size than in wild-type mice.⁴⁴ Consistent with its dominate epithelial cell expression pattern, the protection associated with NLRP1 did not appear to be dependent on the hematopoietic compartment, as *Nlrp1*^{-/-} wild-type chimeras did not show improvement in the DSS-induced colitis model.⁴⁴ Similar to the findings for NLRP6, the microbiome in the *Nlrp1*^{-/-} mice appears to strongly modulate the progression of experimental colitis because antibiotic treatment and cohousing studies significantly impacted colitis pathogenesis.⁴⁴ The aspects of the host microbiome influencing colitis and cancer progression in the *Nlrp1b*^{-/-} mice remain unclear. However, as with NLRP6, future studies will further define this phenotype and will provide additional mechanistic insight.

In humans, *NLRP1* mutations have been linked to a range of autoimmune and autoinflammatory disorders, including vitiligo, celiac disease, rheumatoid arthritis, systemic lupus erythematosus, and type I diabetes.^{101–106} GWAS identified CD susceptibility mutations in NLRP1 associated with co-occurring extraintestinal manifestations in skin, although the data sample size was small.⁶ Additionally, polymorphisms in NLRP1 were associated with increased responsiveness to IBD steroid therapeutics in a study of pediatric IBD patients.¹⁰⁷ Beyond GWAS and SNP evaluations, NLRP1 expression levels have also been shown to be significantly altered during IBD. Retrospective pooled data from three distinct studies demonstrated that *NLRP1* expression was significantly increased in colon biopsies from patients with active ulcerative colitis compared to specimens from healthy patients.⁴⁴ Conversely, when investigating the expression of *NLRP1* from eight separate studies of patients that evaluated colon biopsies from tumors versus adjacent tissue or biopsies/tissue from colon cancer patients compared with healthy controls, *NLRP1* was significantly down regulated.⁴⁴ These cancer findings are consistent with a second retrospective study analyzing the expression patterns of several different NLRs in a cohort of colorectal cancer patients that also found NLRP1 significantly downregulated relative to healthy controls.¹⁰⁸ Thus, it appears that NLRP1 expression in humans is significantly altered in the colon in a disease-specific manner.

Interestingly, polymorphisms in the regulatory NLR NOD2 are the best-characterized mutations associated with IBD in select human subpopulations. Likewise, both NOD2 and NLRP1 have been shown to recognize MDP in humans and NOD2 has been shown to be necessary for NLRP1 inflammasome function.^{94,109,110} While the exact mechanism remains unclear, it is likely that NOD2, which has 2 CARD domains, functions to stabilize NLRP1 or acts as a cofactor to augment NLRP1 Inflammasome formation. The latter scenario is reminiscent of the mechanism described for the NAIP cofactors in the NLRC4 Inflammasome. It is interesting to speculate that mutations in either NOD2 or NLRP1 could significantly impact the function of one or both of these proteins and potentially impact IBD progression in human patients.

D. NLRC4

NLRC4, also known as IPAF, is unique compared to the other Inflammasome-forming NLRs mentioned, in that it is capable of acting in concert with NAIP proteins, which provides ligand specificity.^{21,36} The NLRC4 Inflammasome senses flagella, as well as, gram-negative

bacteria that use type III or IV secretion systems (Fig. 6).²⁴ The ligand-bound NAIP protein releases the autoinhibited NLRC4 to allow the NLRC4-NAIP-ligand multimeric complex, ASC, and caspase-1 to form the subsequent inflammasome.^{2,24} As mentioned previously, there are multiple differences between the single human NAIP and the multiple murine NAIP proteins.²⁴ In addition to utilizing the NAIP cofactors, the CARD domain of NLRC4 is likely also able to directly associate with the CARD domain of caspase-1, complicating the understanding of the role of ASC in NLRC4 Inflammasome formation.² In response to *Shigella flexneri*, ASC appears to be required for optimal caspase-1 activation and IL-1 β production, while ASC is not required for NLRC4- Inflammasome formation in response to *Pseudomonas aeruginosa* or caspase-1-dependent cell death in response to *Shigella*.^{111–113} Additionally, NLRC4 is unique compared to other NLRs in that Inflammasome formation is not dependent on potassium efflux, which could be due to direct interactions of bacterial PAMPs and NAIP proteins.¹¹⁴

Beyond the complexities associated with the NLRC4 Inflammasome, the role of NLRC4 in IBD is also quite convoluted. Originally, when NLRC4 was evaluated in DSS models using *Nlrc4*^{-/-} mice, no significant differences were observed between the *Nlrc4*^{-/-} and wild-type animals in clinical, histopathological, or immunological features of experimental colitis or colitis-associated tumorigenesis progression.⁴² However, subsequent studies found that NLRC4 did not appear to alter the progression of experimental colitis induced by blocking IL-10 using anti-IL-10R monoclonal antibody treatments, but it did appear to have a role in DSS-induced disease.¹¹⁵ Here, loss of NLRC4 was associated with increased disease severity due to increased epithelial cell barrier damage.¹¹⁵ In a third study, *Nlrc4*^{-/-} mice developed increased epithelial cell damage and Inflammation in the DSS and AOM+DSS models, similar to the other Inflammasome-forming NLRs discussed above.⁶⁸ While these data may appear discrepant, we can speculate that they can be reconciled by considering potential differences in the microbiome populations present in these different animals. It is highly likely that the loss of NLRC4 created permissive niches in these animals that were exploited by different populations of microflora. Unfortunately, these studies were conducted prior to the routine assessments of the microbiome in the NLR field. Thus, future studies targeting the microbiome may better define the mechanisms underlying these different phenotypes.

One must also consider the NAIP proteins when discussing the NLRC4 Inflammasome. In mice, there are 6 NAIP paralogs (*Naip1-6*), 4 of which are functional and 2 of which are noncoding.¹¹⁶ To thoroughly explore the contribution of the NAIPs in IBD, mice were generated with a targeted deletion of all 6 paralogs (*Naip1-6*^{-/-}). These mice were subjected to AOM+DSS models of colitis associated tumorigenesis and were found to significantly protect against cancer progression. The *Naip1-6*^{-/-} mice demonstrated a significant increase in colorectal tumors in an epithelially intrinsic manner.¹¹⁶ Interestingly, the increased tumorigenesis was not associated with exacerbated Inflammation, rather NAIP1-6 appear to protect the animals through the modulation of apoptosis and maintenance of proliferation.¹¹⁶ These findings were confirmed using AOM-only treatments, which is an Inflammation-independent model.¹¹⁶ The *Naip1-6*^{-/-} mice displayed STAT3 hyperactivation and p53 defects that were not observed in the *Nlrc4*^{-/-} animals.¹¹⁶ Together, these data suggest that the NAIPs attenuate tumor initiation and progression through facilitating the death and

removal of damaged epithelial cells. This appears to occur through an NLRC4 Inflammasome-independent mechanism. More work is certainly needed to translate the findings from these mouse models to humans, where only NAIP1 is encoded in the genome. Likewise, while much more difficult to address, additional studies are warranted to better resolve which NAIP paralogs may be responsible for the observed phenotypes.

E. AIM2 (Pyhin Family Member)

In addition to the NLRs, other proteins have been shown to form Inflammasomes. The best characterized of these proteins is AIM2 (absent in melanoma 2). AIM2 is a unique member of the HIN200 protein family, and it is capable of interacting with ASC through its PYRIN domain to form an “NLR-like” Inflammasome.^{27,28} AIM2 is a sensor of double-stranded DNA and is best characterized for its ability to instigate Inflammasome formation following the recognition of pathogen associated DNA.^{27,28} Some of the earliest studies evaluating the AIM2 inflammasome characterized its role in host–pathogen interactions following infection with *Francisella tularensis* and *Listeria monocytogenes*.^{28,117,118} Notably, AIM2 is also able to sense host-derived DNA in the cytoplasm, which has strong implications for a variety of autoimmune diseases and cancer.^{119,120} Of particular relevance to the current review, one of the earliest findings associated with AIM2 relates to its role in CRC.¹²⁰ AIM2 is often found inactivated in melanoma, breast cancer, mammary tumors, gastric cancer, and CRC. Restoration of AIM2 in a colon cancer cell line was found to suppress proliferation and drive G2/M cell cycle arrest.¹²⁰ Beyond CRC, the presence of antinuclear and double-stranded DNA antibodies has been linked to decreased efficacy of anti-TNF therapies and adverse outcomes in patients with both CD and UC.^{121,122} Indeed, one of these more common adverse outcomes in IBD patients is an increased frequency of a lupus-like syndrome that has been suggested to be associated with these elevated autoantibodies.^{121,122} Due to its role in lupus and sensing host DNA,¹²³ it is certainly possible that AIM2 may also play a role in this IBD-associated lupus-like syndrome.

In addition to indirect effects on IBD progression, recent findings have revealed a more direct role for AIM2 in modulating intestinal homeostasis and Inflammation (Fig. 7). Consistent with the data from human cell lines, AIM2-deficient mice have been shown to be highly sensitive to colonic tumor development.^{124,125} The loss of AIM2 resulted in increased intestinal stem cell proliferation that was associated with aberrant Wnt signaling.¹²⁴ Unlike the other NLRs, colon histopathology from *Aim2*^{-/-} mice revealed increased crypt dysplasia and hyperplasia compared to wild-type animals, despite any differences with regard to Inflammatory immune infiltrates in the colon.¹²⁵ Likewise, the increased tumorigenesis observed in the AIM2-deficient mice was independent of IL-1 β and IL-18.^{124,125} However, similar to the NLRs, the cancer progression was significantly enhanced by the dysbiotic microbiome.¹²⁴ The mechanism linking AIM2 and tumorigenesis appears to be, in part, associated with direct regulation of both Wnt signaling and the Akt pathway.^{124,125} While these prior two studies indicated that AIM2 function in tumorigenesis is independent of Inflammation, the dysbiosis noted in the AIM2 deficient mice resulted in higher experimental colitis susceptibility in subsequent studies.¹²⁶ Specifically, *Aim2*^{-/-} mice were found to have higher colonic levels of *Escherichia coli*.¹²⁶ In this study, *Aim2*^{-/-} mice were found to be sensitive to acute DSS in this single study, and reconstitution with IL-18

was found to reduce experimental colitis progression.¹²⁶ Similar to these findings, a more recent study using a *Salmonella typhimurium* colitis/acute infection model also found that AIM2 functions to protect against experimental colitis progression.¹²⁷ The mechanism identified suggests that AIM2 functions to promote tight junction formation through AKT activation, which would be consistent with the IL-18 and AKT results previously reported.^{125–127}

IV. The Effects of the Inflammasome on the Host Microbiome

The relationship between aberrant Inflammasome signaling and dysbiosis in the gut associated with IBD is a common theme that has recently emerged. Gut commensal bacteria function to protect the host from pathogenic bacteria by occupying and competing for biological niches. For the bacteria to exist and flourish in the gut, the immune system must create a permissive environment. The increasingly expanding interest in the contribution of the microbiome in health and disease is accompanied by an existing void as to mechanistic insights as to how microbiome components could be exerting such effects. Any breakdown in intestinal epithelial barrier function can lead to the translocation of microbial flora, and subsequent recognition by NLRs. A compelling hypothesis implicating NLRs in the role of maintaining homeostasis between the resident commensal microbial community and the host is that NLRs keep the unique microbial components in check. With a loss or depletion of mucosal barrier protection, as is so often observed in IBD and CAC patients,^{128,129} bacteria from the microbiome are able to come into contact with the epithelium and occupy niches within the gut where they would normally be excluded. In healthy populations, the translocation of the microbiome to the epithelium is sensed by Inflammasome-forming NLRs. However, with a decreased NLR repertoire, as seen in both IBD and CAC populations, the host immune response is suboptimal and creates microenvironments where bacteria are likely to hyperproliferate. Ultimately, this results in a loss of flora diversity and potentially leads to an increase in harmful commensal or pathogenic bacteria in the gut. This shift in the microbiome has far-reaching effects, including significant changes in the metabolomic profile of the host, which can have dramatic effects on IBD and beyond.

V. The Goldilocks Conundrum

Immune system homeostasis in the gut is a delicate balancing act. The immune system must maintain a significant level of vigilance to protect the host from harmful pathogens and environmental insults, while simultaneously suppressing overzealous inflammation in response to the probiotic and commensal flora present in the microbiome. PRRs are essential components of this process and are maintained at a critical threshold to enable a rapid response if needed, but they are insufficient to drive widespread gastrointestinal inflammation.¹³⁰ NLR family members are integral PRRs in the gut and play vital roles, both directly and indirectly, in maintaining immune system homeostasis. Under normal conditions, Inflammasome-forming NLRs in cells associated with the mucosal barrier and leukocytes function to maintain proper intestinal epithelial cell regeneration and repair, cell death, and a well-balanced inflammatory microenvironment. However, the loss of NLR signaling, as observed in knockout mouse models, results in increased inflammation and enhanced IBD pathogenesis, and it augments the progression of colitis-associated cancer

(Fig. 8). In general, the consensus data suggest that this is true for all of the NLR family members, cofactors, adaptor proteins, and caspases. In fact, we typically observe robust gastrointestinal Inflammation and colon tumorigenesis in mice lacking the common adaptor protein ASC or caspase-1/-11. However, while disease progression is still significantly increased over the wild-type animals, the loss of any individual NLR typically results in an attenuated phenotype compared to animals with completely defective Inflammasomes. This finding suggests that some NLR Inflammasomes function through redundant mechanisms in the gut to protect the host during IBD.

At first glance, the increased IBD progression in Inflammasome-deficient mice appear to be counterintuitive. One would predict, as most research groups originally did, that loss of NLR Inflammasome function would result in reduced IL-1 β and IL-18 levels and attenuated disease pathogenesis. However, both of these robust pro-Inflammatory cytokines have significant roles beyond simply driving Inflammation. For example, IL-18 is also critically important in epithelial cell regeneration and repair.¹³¹ Thus, in the context of IBD, the loss of IL-18, the subsequent increase in damage to the epithelial cell layer, and increase in microbial translocation across the barrier, actually promotes more robust Inflammation driven by other compensatory PRRs outside of the NLR family (i.e., TLRs). Likewise, the loss of specific NLRs creates permissive niches for the colonization of commensal and pathogenic components of the host microbiome^{46,90,93} (Table 2). In essence, without specific NLR recognition, some microbes can take advantage of the suboptimal immune response and colonize areas of the gastrointestinal tract where they are typically excluded. Ultimately, this can result in significant shifts in the local microbial microenvironment and changes in the metabolomic profile associated with the change in flora. In sum, this, too, results in increased Inflammation, IBD pathogenesis, and tumorigenesis. Conversely, if NLR Inflammasome signaling is enhanced and not properly resolved, as observed following pathogen infection or chronic damage to the epithelial cell barrier, then overzealous Inflammation can also occur (Fig. 8). This heightened level of Inflammation is directly associated with increased cytokine signaling and leukocyte activation. The robust pro-Inflammatory microenvironment promotes increased epithelial cell proliferation and Inflammation-driven colon tumorigenesis. Likewise, enhanced IL-1 β signaling can significantly impact Th cell differentiation through enhancing the effects of lineage-specifying cytokines.¹³² In the presence of specific cytokine milieu, IL-1 β can significantly impact the differentiation of Th cells into specific Th1, Th2, or Th17 subpopulations.¹³² Together, the functional studies associated with NLR Inflammasome maintenance of immune system homeostasis has revealed a highly complex network of both protective and detrimental roles in gastrointestinal health and disease. It is now clear that skewing NLR activation either toward reduced activity (i.e., to cold) or enhanced activity (i.e., to hot) have equally detrimental effects on IBD progression and associated tumorigenesis in the gut (Fig. 8).

VI. Conclusions

Significant progress has been made over the last decade in defining the contribution of NLR family members in IBD pathobiology. Early studies focused on Inflammasome-forming NLRs as potential therapeutic targets for CD and UC. These early studies postulated that if a

specific NLR could be linked to IBD pathogenesis and successfully targeted, inflammation could be strategically attenuated with minimal detrimental effects. However, it became quickly evident that deletion of any NLR Inflammasome components resulted in unpredictable gastrointestinal phenotypes in IBD models. These unexpected findings have fueled numerous and exciting new avenues of research associated with NLR function in gastrointestinal health and disease. Indeed, we now realize that NLR Inflammasome function in the gut is significantly more complex than originally thought. A key challenge in future studies will be translating these highly complex findings from mouse models to human patients.

As with any rapidly moving area of research, many published studies in the NLR field appear to offer seemingly contradictory findings. This is especially true in the context of the IBD models described in this review. In our opinion, we believe that these apparent discrepancies can be best reconciled by considering the impact and interaction between the various parameters that drive IBD pathogenesis. Many of the earlier studies associated with NLR Inflammasomes exclusively focused on genetics and immune system dysfunction. However, as we stated in the introduction, IBD is a disease that is also characterized by microbiome and environmental factors that are not well understood. It is now clear that NLRs can dramatically modulate the host microbiome and the absence of an individual Inflammasome results in dramatic shifts in the gastrointestinal flora that can significantly alter disease progression. The niches that are created in the absence of an NLR Inflammasome are open to exploitation by both commensal microbes already present in the host and microbes present in the specific environment unique to each individual vivarium. It is certainly clear that future studies must account not only for genetics and immune system function but also for the microbiome and specific environmental conditions present at the site of testing. Beyond microbiome compositional analysis, it will also be critical to gain greater insight into the functional differences associated with specific microbiome and metabolomics profiles that are directly associated with NLR Inflammasome function.

Further understanding of the mechanisms associated with NLR Inflammasome function, regulation, and effects on the host microbiome will provide significant insight into IBD pathobiology and pathogenesis. It is our hope that a better understanding of these highly interesting PRRs will lead to novel therapeutic strategies that will ultimately aid in the diagnosis, management, and treatment of IBD.

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Abbreviations

GI	gastrointestinal
IBD	Inflammatory bowel disease
UC	ulcerative colitis
CD	Crohn's disease
PRR	pattern recognition receptor
TLR	Toll-like receptor
NLR	nucleotide-binding domain and leucine-rich repeat-containing proteins
RLR	RIG-I-like helicase receptor
CLR	C-type lectin receptor
PAMP	pathogen-associated molecular pattern
DAMP	damage-associated molecular pattern
ROS	reactive oxygen species
SNP	single-nucleotide polymorphism
GWAS	genome-wide association studies
CARD	caspase activating and recruitment domain
ASC	apoptosis-associated speck-like protein containing a CARD
AIM2	absent in melanoma 2
NAIP	NLR family, apoptosis inhibitory protein
LPS	lipopolysaccharide
PARP	poly(ADP-ribose) polymerase
GSDMD	gasdermin D
DSS	dextran sulfate sodium

AOM	azoxymethane
TMS1	target of methylation-inducing silencing 1
ICE	IL-1 β cleaving enzyme
CIAS1	cold-induced auto-Inflammatory syndrome 1
CRC	colorectal cancer
senGC	sentinel goblet cell
AMP	antimicrobial peptide
LeTx	lethal toxin
MDP	muramyl dipeptide
LRR	leucine-rich repeat
GBP	guanylate-binding proteins
NBD	nucleotide binding domain
FIIND	function to find domain

Fig. 1.

Inflammatory Bowel Disease Pathogenesis. Crohn's disease (CD) and ulcerative colitis (UC) are complex disorders, which are driven by poorly understood genetic and environmental influences. These disorders are strongly associated with dysfunctional and overzealous immune system signaling in the gastrointestinal tract. It is also clear that dysbiosis and perhaps even mild-to-moderate changes in specific host microbiome populations can dramatically influence IBD progression and prognosis. Each of these mechanisms function in synergy to drive IBD pathobiology.

Fig. 2.

The Canonical and Noncanonical Inflammasome. The activation of the canonical NLR Inflammasome requires priming, typically via activation of a Toll-like receptor (TLR). Following ligation of the TLR, MyD88 is recruited and subsequently leads to the activation of NF-κB signaling. Activated NF-κB promotes the transcription of pro-IL-1β and pro-IL-18. Many mechanisms have been proposed for the activation of the NLR Inflammasome once priming has occurred: direct PAMP sensing, K⁺ efflux, pore forming channels and toxins, Ca²⁺ influx, reactive oxygen species from the mitochondria, mitochondrial DNA, cardiolipin translocation, FADD, caspase-8 and rupture of phagosomes. Upon activation from one of these mechanisms, the NLR, ASC and caspase-1 come together to form the core unit of the Inflammasome. This assembly leads to the cleavage of pro-IL-1β and pro-IL-18 into their mature forms and the induction of the pro-Inflammatory form of cell death defined as pyroptosis. In noncanonical Inflammasome activation, type I interferon is stimulated via the TLR-TRIF-mediated pathway, which drives STAT1 activation and leads to the induction of caspase-11 expression. Gram-negative bacteria in the cytosol that escape vacuoles release their LPS inside the cell. This mechanism requires rupture of the vacuoles via interferon-inducible guanylate-binding proteins (GBPs). The binding of cytosolic LPS to caspase-11 induces its activation leading to pyroptosis and potentially feedback into the canonical Inflammasome pathway. Gasdermin D functions as a substrate for caspase-11 and the

cleavage of gasdermin D is part of the driving force for the noncanonical Inflammasome mediated activation of pyroptosis, IL-1 β maturation, and also functions to regulate epithelial cell proliferation.

Fig. 3.

NLR Inflammasomes in Inflammatory Bowel Disease and Tumorigenesis. (A) NLRP3 and NLRP6 in epithelial cells maintain the secretion of IL-1 β and IL-18 to maintain immune system homeostasis in the gut. One major role of IL-18 is to maintain intestinal epithelial cell homeostasis through controlling proper proliferation and tissue repair. In addition, IL-18 is also responsible for the secretion of peptides for anti-microbial defense. Goblet cells also express NLRP6, which is responsible for the secretion of mucus into the lumen. This results in increased protection against harmful commensal microbes and pathogens. (B) Damage to the epithelial cell barrier drives acute Inflammation. When either NLRP3 or NLRP6 are absent, IL-18 generation is attenuated. This process leads to dysbiosis and the expansion of bacteria into niches where they are typically excluded. In mice lacking NLRP6, CCL5 is upregulated, resulting in an increase in leukocyte recruitment and infiltration. Without IL-18, there is a lack of epithelial cell repair, crypt proliferation, and secretion of anti-microbial peptides. In addition, the lack of NLRP6 leads to a decreased mucus production, which allows microbes from the lumen to gain better access to the epithelial cell barrier, increased bacterial translocation, and increased Inflammation. (C) Chronic Inflammation of the colon is commonly associated with tumorigenesis and is often referred to colitis-associated cancer in human patients. It has been shown that mice lacking NLRP3 and NLRP6 have decreased

production of IL-18 when put through a model for colitis associated cancer. Inflammasome formation has been shown to significantly impact Wnt, Notch, and AKT signaling, which are all highly associated with tumorigenesis and likely impact this aspect of IBD.

Fig. 4.

NLRP6 in Inflammatory Bowel Disease. When NLRP6 is present and active, it is heavily involved in the maintenance and regulation of the homeostasis of the gut via its production of mature IL-18. This includes the maintenance of intestinal epithelial cell proliferation, repair of injury, and secretion of the protective mucus layer. All of these functions play a role in maintaining the balance of pathogenic and commensal flora. However, in the absence of NLRP6, IL-1 β and IL-18 production is decreased, leading to dysfunctional mucus secretion and suboptimal host responses to specific components of the microbiome. This ultimately results in a disruption in the balance of pathogenic and commensal bacteria, leading to bacterial translocation and Inflammation.

Fig. 5.

Domain Structure and Activation of NLRP1. Murine NLRP1 contains a nucleotide binding domain (NBD), leucine-rich repeat (LRR) domain, function to find domain (FIIND), and caspase activating and recruitment domain (CARD). **(A)** In mice, NLRP1 has been shown to sense Anthrax lethal toxin (LeTx). Initially NLRP1 is unprocessed and considered to be in its immature form. However, the FIIND domain is able to cleave itself to create the mature form of NLRP1, which is then considered to be responsive to LeTx. NLRP1 can then undergo one of two pathways associated with activation. **(B)** The first is the Direct Activation Pathway. This pathway involves the cleavage of the N-terminal of NLRP1 via LeTx. This cleavage event activates NLRP1 and allows it to dimerize with caspase-1, leading to cleavage of pro-IL-1 β and pro-IL-18 into their mature forms. **(C)** The second pathway for LeTx-mediated NLRP1 activation is the Indirect Activation Pathway. This pathway involves the cleavage of a negative regulator of NLRP1 by LeTx. Once the negative regulator has been removed, NLRP1 becomes activated leading to caspase-1 recruitment/activation and maturation of IL-1 β and IL-18. Because murine NLRP1 contains a CARD domain, it is possible the NLRP1 utilizes cofactors and other CARD containing proteins to augment signaling. However, additional mechanistic insight is needed to better define NLRP1 activation and signaling triggers beyond LeTx.

Fig. 6.

Activation of NLRC4. Some bacteria utilize the type III secretion system, such as *Salmonella typhimurium*, to insert its pathogenic components into the cell. Once inside the cell, these components are detected by NAIPs, which serve as cofactors and provide specificity for the activation of NLRC4. Once NLRC4 is activated, ASC is recruited along with caspase-8 and caspase-1, which together form the NLRC4 Inflammasome. The formation of this Inflammasome activates caspase-1, which then cleaves pro-IL-1 β and pro-IL-18 into their mature forms. The formation of the NLRC4 Inflammasome is also responsible for pyroptosis via a caspase-1 dependent, but ASC independent mechanism. It has also been speculated that PK δ kinase is also involved in the activation of NLRC4, although it may not be a necessary component.

Fig. 7.

The AIM2 Inflammasome. Upon sensing of dsDNA, AIM2 forms an inflammasome resulting in caspase-1 activation and the subsequent cleavage of IL-1 β and IL-18. AIM2 has also been shown to modulate signaling through the Wnt and AKT signaling cascades, which have significant implications associated with gastrointestinal inflammation and tumorigenesis. Dysbiosis is a significant contributing factor associated with AIM2 inflammasome function.

Fig. 8.

The Goldilocks Conundrum. In the gut, NLRs function to maintain immune system homeostasis by modulating Inflammatory signaling pathways, either directly or indirectly. These pathways are balanced at a critical threshold throughout the gastrointestinal system. If NLR signaling is disrupted, then Inflammation and disease pathogenesis can be increased due to reduced barrier function, the formation and expansion of permissive niches for pathogenic microbes, and reduced tumor surveillance. Conversely, if NLR signaling increases and is not properly resolved, then the overzealous Inflammation can result in significant collateral damage to the epithelial cell barrier, promote epithelial cell proliferation, augment leukocyte activation, and drive IBD pathogenesis.

Table 1
Inflammasome terminology

Terminology	Defining feature	Species
Canonical Inflammasome	Caspase-1 dependent	Human/Mouse
Noncanonical Inflammasome	Capase-4/-5/-11 dependent	Human (Casp-4/-5); mouse (Casp-11)
Classical Inflammasome	Signal 1; Signal 2; Requires ATP	Mouse/Human (cell type/stimuli specific?)
Alternative Inflammasome	Utilized TRIF, RIPK1, FADD, and Casp8; ATP not required for IL-1 β release; only defined in macrophages	Human (cell type/stimuli specific?)

Table 2
Microbiome effects on NLR modulation of IBD and CAC

NLR	Bacteria	References
NLRP3	<i>Helicobacter pylori</i>	133, 134
	<i>Clostridium</i>	85, 135
	<i>Mycobacterium</i>	136–138
	<i>Collinsella</i>	85
	<i>Subdoligranulum</i>	85
	<i>Enterobacteriaceae</i>	139
	<i>Ralstonia</i>	85
NLRP6	<i>Prevotellaceae</i>	90
	<i>Helicobacter hepaticus</i>	90
NLRP1	Unknown Microbiome Component	44
NLRC4	<i>Salmonella</i>	140, 141
	<i>Citrobacter rodentium</i>	142
	<i>Shigella flexneri</i>	111–113