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Rip2: A Key Molecule that Regulates both Innate and Acquired Immunity

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Abstract: The Receptor interacting protein-2 (Rip2, also called RICK, CARDIAK) is an intracellular serine-threonine kinase that contains a carboxy-terminal caspase activation and recruitment domain (CARD). The initial biochemical analysis emphasized a role for Rip2 in the activation of nuclear factor-kappaB (NF- B) and apoptosis when overexpressed. The subsequent generation of mice with a targeted deletion of the gene for Rip2 and the description of a possible target for Rip2 kinase activity has clarified the role of Rip2. Following infectious challenges, the activation of a protective immune response relies on the coordinated interplay of contextual stimulation and inflammatory processes. All mammals must balance the need to combat dangerous pathogens from the destructive potential for mistaking autologous cells or proteins as appropriate targets for response. Rip2 has carved out an evolutionary niche serving as a regulator of inflammatory responses. Rip2 helps to direct or propagate signals towards cell-mediated immune responses and resolution of infection by modifying signals from pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (Nod) family members of innate immunity, the T cell receptor (TCR) complex of acquired immunity, and cytokine signaling of the interleukin (IL)-1 receptor family and IL-12 signaling pathways. Here we wish to outline the progress made in describing the biological significance of Rip2 and the mode of regulation of this kinase. Further studies considering Rip2 as a target of intervention have the potential to be of great clinical value.

INTRODUCTION

The immune system in mammals is comprised of two evolutionarily distinguished but interdependent branches, the innate and adaptive immune systems. Innate systems of immunity are phylogenetically more ancient and are found throughout the plant and animal kingdoms. They respond to conserved motifs in the life cycle of a microorganism termed pattern-associated molecular patterns (PAMPs) [1]. As such the targets of recognition are immutable without compromising the survival of the microorganism. Innate recognition mediates an immediate early response comprised of natural killer (NK) cell, complement, and macrophage activation, as well as chemokine and cytokine production. These players set in motion the multiple pathways of defense used to control an infectious event [2]. Indeed, it is arguable that in the absence of all innate activations, functional activation of the acquired immune systems is impossible.

Immunologically speaking, the evolutionary leap mammals have made is the ability to produce the antigen specific effector response described by adaptive immunity. Using a system of random gene fragment rearrangement, lymphocytes with antigen specific receptors can be selected and expanded to generate a specific response with almost limitless specificity. The evolutionary challenge is to design a system with the tools capable of distinguishing what antigen is an appropriate target for response and what type of response will be most effective. In large part, acquired immunity leaves this moral decision making to innate activation. This is apparent in disease models where lesions in particular innate activation pathways leave the host open to infection with defined classes of microorganisms. For example, patients with deficiencies in classical pathways of complement suffer repeated bacterial infections [3]. Mice with a targeted deletion of the pentraxin 3 gene (PTX3) are susceptible to fungal infection with *Aspergilis fumigatus* [4]. Suppression of interferon production as during an acute viral infection can lead to aggressive, fulminate, and often fatal infection [5]. Clearly, communication between the two defense mechanisms is critical in mounting an effective and protective response.

Being separately evolved, the innate and acquired immune systems need translators to bridge the communication gulf between two phylogenetically disparate systems. Receptors in the innate system come in various guises that reflect their different origins and the life cycle of the pathogens that produce the ligands. Soluble recognition systems that are carried in plasma are exampled by the C-reactive protein and complement proteins. Receptors expressed on the surface of cells are demonstrated by the TLRs that can respond to a broad range of PAMPs ranging from pathogen-specific motifs such as lipopolysaccharide (LPS) to polyunmethylated CpG motifs unique to prokaryotes. Finally intracellular receptors such as the protein kinase receptor (PKR) recognize double stranded RNA and describe a class of innate receptors that can respond specifically to intracellular infections. These receptors all activate immediate early systems that induce biological responses to control early infection. For instance, macrophage production of the proinflammatory cytokine tumor necrosis factor (TNF) in response to the TLR4 ligand Lipid A can activate the macrophage and induce the

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oxidative mechanisms required to combat infections. Interferon (IFN) produced by virally infected cells activates a broad range of antiviral responses. The acquired immune systems have commandeered these and other signals as ambassadors of infection. Subsequent to innate recognition, B and T lymphocytes respond to the catalogue of cytokines, antigen presentation events, and inflammatory stimuli generated by the innate activation events. These translation signals emphasize the flexibility of this system, as any particular infectious event will be broadcast to lymphocytes with an overlapping but potentially unique profile of innate amplifiers. The outcome is a spectrum of contextual interpretations that can generate acquired responses ranging from neutralizing antibody production by B lymphocytes to cytotoxic CD8+ T lymphocyte (CTL) responses.

Rip2 has emerged as a key modulator in activation events in both innate and acquired immune responses. As will be discussed, Rip2 may directly interact with and regulate signal transduction events in the TLR, Nod, and IL-1R family receptors, and regulate IL-12 signaling. Rip2 has also been shown to directly interact and signal in the TCR complex. Thus, its influence converges on the development of a cell-mediated response. The accumulated effect of Rip2 biology is best demonstrated in a murine model for Listeriosis. Mice with a targeted deletion of the gene for Rip2 universally succumb to a dose of the intracellular pathogen *Listeria monocytogenes* that wild type mice can almost always contain and eliminate. Examination of this infectious model illustrates a variety of innate and acquired interactions that make Rip2 an attractive target for therapeutic intervention.

THE RIP GENE FAMILY

The receptor interacting protein family now comprises four members. A homologous amino-terminal serine-threonine kinase domain combined with a variable carboxy-terminal protein interaction domain characterizes this family. Using the Fas receptor CD95 as bait, a yeast two-hybrid genetic screen identified the first member Rip1, as a 74 kDa protein comprised of an amino-terminal kinase domain linked by an intermediate domain (ID) to the carboxy-terminal protein interaction Death Domain (DD) [6]. Further two-hybrid screens using a TNF-Receptor-associated DD containing protein (TRADD), as bait described Rip1 as a member of the TNFR1 complex as well. Rip1 transient overexpression was shown to activate NF- B and induce apoptosis [7]. With this example of a novel kinase, public sequence databases revealed the second member of the family, Rip2. Rip2 showed significant homology in the kinase domain but expressed a carboxy-terminal CARD, a homophilic interaction domain initially described in the caspase family of proteins [8, 9]. EST libraries also elucidated the Rip4 protein with eleven ankyrin repeats coupled to the kinase domain [10]. Finally, cloned using Rip1 as bait in a yeast two-hybrid screen, Rip3 contains a RIP homotypic interaction motif (RHIM) in the ID that is also found in both RIP1 and the TLR adaptor protein TRIF, without a canonical carboxy-terminal protein interaction domain [11]. The RHIM allows assembly of a regulatory complex of these three proteins during TLR3 ligation. Biochemical analysis revealed the shared trend: The Rip family proteins activated NF- B and induced apoptosis when overexpressed. Notably, other than Rip4, no other members required functional kinase activity for activation of NF- B when overexpressed. However, these results must be taken in context, as kinase activity may be required at endogenous levels. As a family, it is also interesting that they have developed with kinase homologies ranging from 31 to 45% amino acid identity, but have totally unrelated carboxy-terminals. Teleologically, perhaps a useful domain has been adopted by a number of different receptor systems (Fig. **1**).

The presence of a DD and then a CARD in the first two described members of this family focused much initial attention on the role of Rip family proteins in apoptosis. Rip1 for example, was shown to be a member of both the Fas and TNFR1 signal transduction pathway, with a DD sufficient to propagate apoptosis. Furthermore, caspase-8 is able to cleave Rip1 at a glutamic acid near the kinase-ID junction thereby releasing a DD that can nucleate assembly of an apoptosis-inducing signaling complex [12]. Alternatively, Rip4 protein is susceptible to CD95-activated caspase-dependent processing causing the release of a dominant negative ankyrin domain that can inhibit NF- B activation [10]. In contrast, development of Rip1 knockout mice revealed that Rip1 was essential for TNF -induced activation of NF- B. It remains to be seen how the biological roles for Rip3 and Rip4 manifest in knockout mice.

FUNCTIONAL PROPERTIES OF RIP2

Human Rip2 is a 531 amino acid 61-kDa protein, whose locus is encoded on the long arm of chromosome 8. The murine homologue contains 86% nucleotide identity and 84% amino acid identity. The CARD, a homophilic interaction domain that directs many of the protein-protein interactions of Rip2, consists of a six helix structure [13]. Numerous studies have described specificity of Rip2 interactions with other CARD-containing proteins such as caspase-1, cellular inhibitor of apoptosis protein (cIAP)-1, Nod1 and 2, but not RAIDD, initiators of cell death caspase-2, and caspase-9, the pro-inflammatory caspase-4, or cIAP-2 [9, 14-16].

Initial biochemical characterizations of Rip2 can best be divided into two major functional categories: Activation of inflammatory pathways and induction of apoptosis. Regarding inflammation, when overexpressed in cells, recombinant Rip2 activates several transcription factor pathways that mediate immune and inflammation responses. These pathways include NF- B, Jun N-terminal kinase (JNK), extracellular signal-regulated kinases 1/2 (ERK), and p38 mitogen activated protein kinase (MAPK). Rip2 is also capable of autophosphorylation when overexpressed [8, 17], although numerous reports suggested that kinase activity is dispensable for NF- B activation [17, 18]. Furthermore, the ability of Rip2 to interact with multiple protein families predicted potential for broad functional diversity. Association with members of the TNF receptor-associated factor (TRAF) family such as TRAF1, 5, 6 [17], and TRAF 2 [9] potentially linked Rip2 to TRAF-dependent pathways of NF- B, JNK, and p38. Another study showed that Rip2 recruitment to the catalytic subunits of the I B kinase (IKK) complex, IKK and IKK , through interaction with the

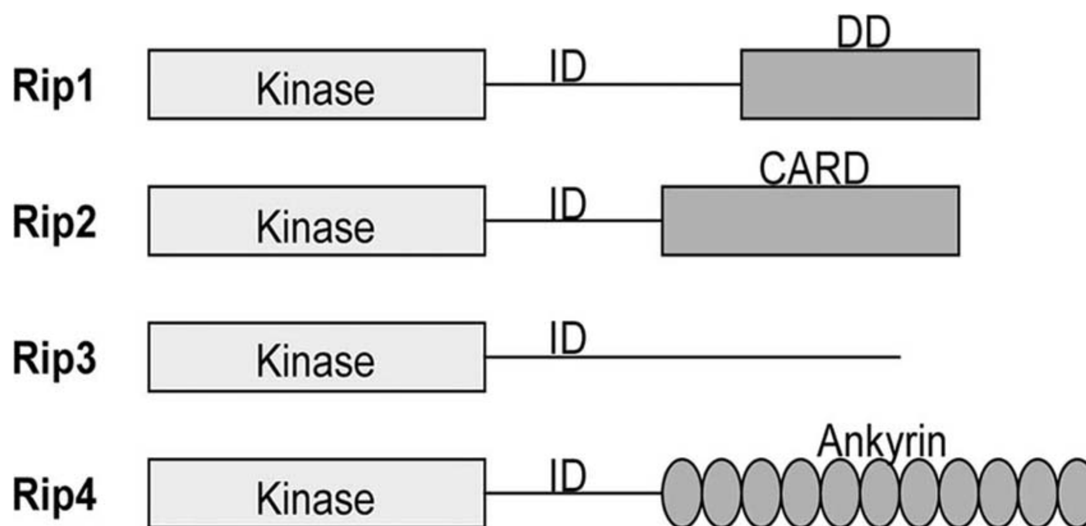

Fig. (1). The Rip Family. A protein family sharing homology in the amino-terminal serine-threonine kinase domain. An intermediate domain (ID) separates the kinase from the divergent carboxy-terminal protein interaction domains. The carboxy-terminal domains define the signaling complex to which each Rip family member is localized.

regulatory subunit of IKK activated NF- B independent of its CARD. Members of the NK- B family of transcription factors are bound and held inactive in the cytoplasm by I B proteins. Transcriptional activation of the B site-containing target genes requires the IKK complex for phosphorylation of I B in a signal-dependent manner. This results in degradation of I B and the subsequent nuclear translocation of NK- B. Expanding its role, Rip2 was shown to interact with and be subject to phosphorylation by Raf1, a MAPK kinase kinase, following TNF stimulation in a COS cell transient system. Activation of Rip2 by Raf1 led to phosphorylation of ERK1/2 that required Rip2 kinase activity [19]. While these overexpression studies directly implicated Rip2 kinase activity in ERK activation, a role of Rip2 in TNFR signal transduction has yet to be confirmed at endogenous levels of expression.

The Rip2 CARD was observed to interact with the amino-terminal CARD of pro-caspase-1. This association promoted the processing of pro-caspase-1 into its active form suggesting a role for Rip2 in regulating caspase-1 activity. Caspase-1, originally known as IL-1 converting enzyme (ICE), was characterized as the enzyme that cleaved pro-IL-1 and pro-IL-18, releasing the proinflammatory cytokines IL-1 and IL-18. Recent studies demonstrated that overexpression of caspase-1 can activate NF- B, with its activity inhibited by a kinase inactive mutant of Rip2 [9]. Caspase-1-deficient animals are resistant to lipopoly-saccharide (LPS)-induced toxic shock presumably due to reduced IL-1 production [20]. Interestingly, Rip2$^{-/-}$ mice are also LPS resistant, implicating Rip2 function in the inflammasome, a term coined to describe the regulated scaffold that assembles to direct the processing and production of mature IL-1 . However, production of IL-1 from bone marrow-derived macrophages lacking Rip2 protein showed no consistent differences compared to wild type (Unpublished data). More recently, Rip2 has been shown to be dispensable for *in vivo* regulation of the inflammasome [21].

Recent studies have expanded the understanding of caspase-1 function in other cell types such as neurons, suggesting that caspase-1 is an apical mediator of ischemic brain injury and neuronal cell death in a mouse model of stroke. Overexpression of Rip2 in primary mouse cerebrocortical neurons induced caspase-1-dependent apoptosis [22]. Further *in vitro* characterizations of Rip2 indicated additional roles for this kinase in apoptosis. Overexpression of Rip2-induced apoptosis in cell lines such as human embryonic kidney cells and MCF7 breast cancer cells. One mechanism suggested that Rip2 interaction with CLARP, a protein containing two death effector domains (DED) capable of binding to Fas-associated death domain (FADD) and caspase-8, mediated CD95-induced apoptosis. This activity required an intact CARD and kinase domain of Rip2 for its full effect [8]. Interaction with the CARD-containing cIAP-1 could also implicate Rip2 in modulation of apoptosis [9, 17]. cIAP-1 binds to and potently inhibits caspase activity. In this context, it is not clear if Rip2 interaction with cIAP-1 would augment or inhibit cIAP-1 activity. To date however, examination of Rip2-deficient mice has not revealed these mechanisms in an *in vivo* setting. Specifically, in an examination of a role for Rip2 in apoptosis, equivalent apoptosis in knockout and wild type cells was observed in embryonic fibroblasts and thymocytes to a variety of agents including TNF , Fas, etoposide, and cisplatinin [23, 24] (Unpublished data). Thus, despite overexpression evidence for a role of Rip2 in apoptosis, the lack of developmental defects in Rip2 knockout mice or apoptotic defects in Rip2$^{-/-}$ cells argues against a significant role in apoptosis such as those observed in mice lacking the CARD-containing proteins caspase-8 and Apaf-1.

Rip2 tissue expression is broad, with high levels of mRNA found in the spleen, peripheral blood, prostate, testis, heart, placenta, kidney, pancreas [17], and brain [8]. Inducibility of Rip2 mRNA upon activation of macrophages by LPS stimulation, in CD4+ T cells by CD3 stimulation [23, 24], upon infection of fibroblasts with human

cytomegalovirus (HCMV) [25], and during hypoxia in neuronal cells [22], suggested a method of regulating Rip2-dependent responses and emphasized Rip2 as a gene that is upregulated in response to a very broad spectrum of stress activators. Subsequent to the initial biochemical characterizations, three groups further investigated the physiological function of Rip2 through examination of knockout mice [23, 24, 26]. These studies explored new and unexpected twists of Rip2 biology and provided yet another testimony to the advantages of knockout models in clarifying physiological roles of proteins.

Pertinent negative findings revealed from the examination of Rip2-deficient mice narrowed the potential functions of Rip2. Rip2 knockout mice were viable, fertile, with genotypes of over 400 offspring approaching a Mendelian ratio from heterozygous breedings (Unpublished data). Splenocytes derived from Rip2 mice showed no differences in expression of typical surface markers on T lymphocytes, B lymphocytes, and myeloid lineage cells. Bone marrow and thymocytes were also equivalent in wild type and knockout mice. *In vitro* development of bone marrow-derived macrophages and dendritic cells revealed comparable expression of CD11b and CD11c and activation of major histocompatability (MHC) antigens upon treatment with IFN . In the B cell compartment, CD40 ligand or anti-IgM induced proliferation was equivalent in wild type and knockout mice. These data were interesting as the observed *in vitro* association between TRAF adaptors and Rip2 did not translate into a CD40 defect *in vivo* (Unpublished data). As indicated in the introduction, insight into Rip2 function came from challenging these animals with *L. monocytogenes.*

RIP2 IN INNATE IMMUNITY

The two main functions of innate immunity are first, to control and limit pathogen load early in infection. Secondly, to determine the scale and specificity of acquired immune responses via the antigens selected by innate immune systems and the context in which they are presented to acquired immunity. The impact of Rip2 on these processes is clearly demonstrated in Rip2-deficient animals infected with *L. monocytogenes*. Even challenges with limiting doses of *L. monocytogenes* that are one tenth of a 50% lethal dose are overwhelming and lethal in a Rip2-deficient mouse [23]. Clearly, in the absence of Rip2, innate systems are not proficient in clearing an acute infection. This is instructive as even though there are a number of deficiencies that cause increased sensitivity to *L. monocytogenes*, SCID mice lacking all lymphocytes but retaining a functional innate immune system can still control an acute infection although they remain chronically infected [27].

IFN production has been shown to be critical in early containment of *Listeria* infection. Disruption of granuloma and dissemination of infection can be achieved in SCID mice by neutralization of IFN or depletion of neutrophils. In addition, mice lacking either IFN or NK cells are extremely susceptible to *Listeria* infection [28]. Activation of macrophages by IFN results in priming of an oxidative burst and regulation of antigen presenting cell (APC) functions crucial in the defense against intracellular pathogens [29]. The primary source of immediate early IFN in mice and humans are in NK cells, with release stimulated by the cytokines IL-12 or IL-18 [30]. In the absence of Rip2, splenic NK cells produce no IFN in response to IL-12 or IL-18 [23, 24]. Subsequently, decreased or absent IFN expression effects acquired immune activation due to the altered activation of APCs and to an impaired bias of T helper cell differentiation to the Th1 subtype. This IFN response is specific to NK cells as treatment of splenocytes with IL-12 or IL-18 and analysis of intracellular expression indicated that IFN is restricted to NK cells (Unpublished data).

L. monocytogenes is a Gram-positive rod that in mice is phagocytosed by macrophages, escapes the phagosome by expression of the pore-forming toxin Listeriolysin O, and infects and replicates in the cytosol. The cell wall of bacteria such as *Listeria* is a rich source of ligands for certain TLR family members. For instance, peptidoglycan (PGN) or lipoteichoic acid (LTA) can stimulate TLR2 or TLR4 respectively [31] and this leads, in wild type mice, to increased expression of Rip2. This upregulation would suggest a regulatory role for Rip2 during infection. Indeed, TLR4 activation of NF- B was shown to be inhibited by a dominant negative Rip2 construct. Consistent with this, *L. monocytogenes* activation of NF- B was diminished in the absence of Rip2 [23]. In fact, stimulation of Rip2$^{-/-}$ macrophages with LPS, LTA, or PGN showed impaired production of inflammatory mediators such as IL-6, TNF , and IP10. Furthermore, overexpressed Rip2 could transiently associate with the TLR2 signaling complex upon stimulation with PGN [24]. Taken together, it seems possible that diminished TLR signaling due to the absence of Rip2 could ease innate recognition of *L. monocytogenes* and allow unrestricted expansion of the infection. These data are consistent with the observation that recognition of the TLR4 ligand LPS is diminished sufficiently in Rip2$^{-/-}$ mice to allow increased survival to an otherwise toxic dose. While this is an attractive model, it is confounded by several observations. First, the response to *L. monocytogenes* in TLR2 knockout mice is equivalent to wild type animals [32]. Clearly, complete absence of the TLR2 receptor had no effect on the ability to recover from infection with this infectious pathogen. The fact that mice lacking the shared TLR intracellular adaptor protein MyD88 are extremely sensitive to *L. monocytogenes* infection indicated a role for other TLRs or TLR heterodimers. Secondly, there has been speculation for contamination of commercial preparations of LPS and PGN with degraded components of these PAMPs activating non-TLRs. The recent description of a novel family of intracellular receptors, the Nod family, and their respective ligands has shed more light on this observation and places the role of Rip2 in TLR signaling in controversy.

The majority of Nod family of proteins have ligand binding domains composed of leucine rich repeats (LRR) homologous to those found in TLRs [33]. Functional homologues of Nod-LRR structures can be found throughout the plant and animal kingdoms and are associated with host responses. In the context of Rip2-dependent signal transduction, Nod1 and Nod2 are interesting in that they contain one or two CARDs, respectively [14-16]. Nod1 responds to g-D-glutamyl-meso-diaminopimelic (iE-DAP)

peptides while Nod2 responds to the muramyl dipeptide *N*-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP). Both ligands can be derived from PGN, and both receptors are expressed in the cytoplasm where they have access to intracellular pathogens such as *Listeria* [34-37]. The CARD domain of either Nod1 or Nod2 has been shown to interact with that of Rip2 [14, 18] and in the absence of Rip2, neither Nod1 nor Nod2 can stimulate activation of a NF– B reporter [23, 24]. More recently we have show MDP-stimulated activation of NF- B in bone marrow-derived macrophages was dependent on Rip2 expression (Unpublished data). This dependency is a significantly stronger effect than the diminished NF- B or cytokine response seen in LPS-stimulated Rip2$^{-/-}$ macrophages, an observation that lends more credence to a unique role for Nod proteins. During initial experiments with Nod, ligation was achieved by transfection or microinjection with LPS [38]. This treatment might model the delivery of Nod ligands to the cytoplasm as by infection with intracellular bacteria. In support of this model, *in vitro* analysis of the response to the invasive bacteria *Shigella flexneri* demonstrated that NF- B and JNK were activated in a Nod1-dependent TLR4-independent manner [39]. This signaling complex included Rip2. However, presentation and availability of Nod ligands on the surface of bacteria has not yet been described. PGN contains all the minimal Nod ligands within its structure, raising the possibility that endocytosed TLR ligands could be processed and subsequently recognized by intracellular Nod receptors. However, no evidence of cellular processing of PAMPs such as LPS and PGN has been identified. It is possible that MDP is accessible to Nod recognition in the context of intact PGN on bacterial surfaces.

The joint attention of an extracellular receptor for LPS or PGN acting in concert with an intracellular receptor for bacterially derived peptides would have the vocabulary to inform the host that an infection was intracellular. Both Nod and TLR together might contribute to a response appropriate and sufficient to contain a challenge against *L. monocytogenes*. There is a long experimental history of the adjuvant properties of the Nod2 ligand MDP [40]. Indeed, MDP was originally described as the minimal immunostimulatory activity in Freund's complete adjuvant (CFA) [41]. Treatment of macrophages with either PGN and MDP or LPS and MDP produced TNF in synergistic amounts [42]. Therefore it is possible that co-stimulation of cells with TLR and Nod ligands turns on a gene program unique to the synergistic recognition of an intracellular infection. As mentioned earlier, the shared sensitivity of either MyD88 or Rip2 deficient animals to *L. monocytogenes* infection hints at just such an arrangement. In a telling parallel, Nod2 knockout mice are partially protected from toxic shock following LPS treatment [43], replicating the effect seen in Rip2$^{-/-}$ mice. In this model, perhaps toxic shock to LPS treatment is contributed to both by a MyD88-dependent TLR response to LPS as well as a Rip2-dependent Nod response to its ligand.

There remains a broader role for the Nod2-Rip2 signaling complex that was discovered with the identification of Nod mutants associated with Crohn's disease. Crohn's disease is a complex chronic inflammatory condition regulated by multiple genetic and environmental factors. Human linkage analysis has clearly identified the Nod2 gene product as the IBD1 locus [44, 45]. It has also been linked to the Blau Syndrome, a chronic granulatomous disease [46]. Nod2 genes associated with Crohn's bear a variety of mutations most of which are clustered around the LRR domains. Genetically, these mutants appear to behave as loss of function mutations [34, 47]. This suggests Nod2 mutants that no longer recognize MDP can cause a chronic inflammatory condition due to an inability to respond properly to intracellular bacteria. One might therefore expect genetic linkage between Rip2 and Crohn's if individuals harbored a null mutation in this necessary Nod2 signaling adaptor. In fact there is no IBD locus located on chromosome 8 where the gene for Rip2 is found. Alternatively, mutations in Nod2 might induce gain of function mutations that when prompted by a mild inflammatory condition are exacerbated into a chronic condition. In this case, the condition would be alleviated by inhibition of Rip2 function. Rip2 might then provide an interesting target for therapeutic intervention in this debilitating disease.

Akin to induced Rip2 expression during intracellular bacterial infections, infection of human fibroblasts with HCMV upregulated Rip2 expression. Enforced expression of Rip2 during infection significantly inhibited replication of HCMV. This finding was correlated with activation of NF- B and its downstream inflammatory effectors. Additionally, Rip2 synergized with HCMV to greatly increase expression of the antiviral cytokine IFN [25]. While it is not yet clear which intracellular receptor system is responsible for HCMV recognition, viral pathogens, as obligate intracellular parasites, may share response requirements with intracellular bacteria. Thus, Rip2 may have a broader regulatory role during intracellular infectious events.

RIP2 IN ACQUIRED IMMUNITY

One of the primary means of translating innate activation into acquired immune responses is by the targeted production and release of inflammatory cytokines. The pro-inflammatory cytokine IL-12 bridges innate and adaptive immunity, in part, by inducing the production of IFN , a critical mediator of Th1, CTL, and NK cell responses [30]. These IFN -dependent responses are required for resistance to intracellular pathogens such as *L. monocytogenes*, *Toxoplasma gondii* [48], *Mycobacteria tuberculosis* [49], and *Leishmania major* [50]. IL-12 binding to the IL-12R heterodimer causes association with the Janus kinase family of tyrosine kinases, Tyk2 and Jak2. Activation of Jak kinases by IL-12 leads to tyrosine phosphorylation of IL-12R, and subsequent recruitment and activation of signal transducer and activator of transcription (Stat)4. Production of IFN subsequent to IL-12 receptor ligation requires phosphorylation and nuclear translocation of Stat4 for interaction with IFN promoter elements and IFN transcription.

The pro-inflammatory cytokine IL-18 can synergize with IL-12 to enhance Th1 development and induce IFN production by NK and Th1 cells [51]. However the biochemical route to IFN production is significantly different. IL-18 binds to the heterodimeric IL-18 receptor whose intracellular domain is homologous to those of IL-1R

and TLRs. Binding of IL-18 to its receptor recruits a signaling complex utilizing the adaptor proteins MyD88, TRAF6, and the serine-threonine kinase IL-1R-associated kinase (IRAK). This complex recruits the IKK complex to activate the NF- B pathway, and JNK to activate the AP-1 transcription factor pathway. Importantly, IL-18 has been shown to be necessary for resistance to intracellular pathogens such as *L. monocytogenes* and *Propionibacterium acnes* infections [51, 52].

Activated T helper cells differentiate into Th1 and Th2 effector subtypes. Simplistically, these subtypes mediate inflammatory or humoral immune responses respectively. Th1 cells secrete cytokines including IFN and LT (TNF), with Th1 lineage commitment driven by the presence of IL-12 [53]. Sterile immunity to *L. monocytogenes* infections requires an adaptive inflammatory response [27]. Consistent with susceptibility to *L. monocytogenes*, Rip2 is necessary for optimal development of activated T cells and the generation of Th1-mediated immunity. Impaired T cell activation in Rip2 deficiency was evidenced by decreased TCR-induced CD4+ T cell proliferation and NF- B activation [23, 24]. Furthermore, IFN production from draining lymph nodes cells of $Rip2^{-/-}$ mice immunized with chicken egg ovalbumin was diminished [23]. These findings were supported by *in vitro* experiments revealing a key role for Rip2 in the differentiation of naïve T cells to a Th1 phenotype and ultimately in mediating IL-12- and IL-18-induced IFN production in Th1 cells [23]. *In vivo* support of impaired cell-mediated immunity was observed in delayed allograft rejection in recipients of a fetal heart graft model in Rip2-deficient mice [26].

Several mechanisms by which Rip2 alters T cell activation and differentiation have been proposed. Rip2 may influence IFN production by altering IL-12-induced tyrosine phosphorylation of Stat4 [23]. Indeed, mice deficient in multiple aspects of the IL-12 signaling pathway have similar phenotypes as $Rip2^{-/-}$ mice and impaired resistance to challenge with intracellular pathogens such as *Listeria*. Stat4 deficient mice have demonstrated that Stat4 is critical for IFN production by Th1 cells [54, 55]. Interestingly, the B10.Q/J subline of B10.Q mice was initially described as susceptible to infection with the intracellular pathogen *T. gondii*. This strain of mice was also resistant to a collagen-induced arthritis model and had impaired IFN production in response to IL-12 treatment [56]. These mice have recently been shown to harbor a point substitution in the Tyk2 pseudokinase domain [57]. This similarity in phenotype implies that the decreased Stat4 phosphorylation observed in $Rip2^{-/-}$ T cells could contribute to increased *Listeria* sensitivity. However, whether Rip2 interacts in the IL-12R signaling complex is still unfounded. Additionally, the diminished response of $Rip2^{-/-}$ Th1 cells to IL-18 stimulation may share structural homology to the role of Rip2 in the related TLRs, although this has not been demonstrated.

By altering the Th1 response to IL-12 and IL-18, Rip2 may indirectly effect T cell activation. Another possibility is a direct role in TCR-mediated activation as decreased TCR-induced proliferation and NF- B activation was observed in $Rip2^{-/-}$ T cells. Naïve (Th0) $CD4^{+}$ T helper cells undergo clonal expansion and produce IL-2 upon recognition of specific MHC-peptide complexes on APCs by the TCR complex in conjunction with appropriate costimulatory signals; signals that are primarily derived from innate activation events. The NF- B family of transcription factors plays a central role in these biological processes including production of IL-2 [58]. Although significant advances in understanding the signaling pathways upstream of the IKK complex in TNFR and TLR signaling have been made, the mechanisms of TCR-induced NF- B activation remain less clear. TCR activation leads to clustering of the receptor complex forming the supramolecular activation complex (SMAC) at the interface of the TCR and APC. It is proposed that stimulation of the TCR activates the IKK complex through the recruitment and activation of PKC , in conjunction with the CARD-containing proteins CARD11 and Bcl10 [59]. CARD11, containing a membrane-associated guanylate kinase-like (MAGUK) domain, may function to recruit PKC , BCl10, and IKK together to the lipid rafts of the SMAC [60]. T cells lacking these intermediate signaling mediators are defective in TCR-mediated NF- B activation [61-64]. Recent evidence demonstrated Rip2 interaction with Bcl10 in a T cell line. Furthermore, T cells lacking Rip2 are defective in TCR-mediated Bcl10 phosphorylation correlating with decreased NF- B activation [26]. Bcl10-mediated NF- B activation could be partially rescued by expression of a kinase active Rip2 in fibroblasts. This experiment was the first describe an *in vivo* target for the kinase domain. The ability to rescue this phenotype with ectopic Rip2 expression also diminished the possibility that Rip2-deficient mice simply expressed a developmental block in the T cell population. These studies place Rip2 among a growing list of CARD-containing proteins, including Bcl10 and CARD11, implicated in TCR-mediated activation of NF- B, but raises additional questions regarding the formation of this CARD-containing protein complex. Whether Rip2 directly associates with CARD11 or forms a complex with Bcl10 and CARD11 remains to be determined.

THE FUTURE OF RIP2

The Rip family of proteins has emerged over the last few years as a fascinating group of kinases that are potent inducers of NF- B in a regulated manner. Through the varied use of protein-protein interaction domains and proteolytic processing, the Rip proteins present pathway specific mechanisms to regulate NF- B activation. In addition, Rip2 mediates signal processes ranging from activation of macrophages and NK cells to differentiation of Th1 cells (Fig. **2**). These diverse roles are a reflection of the multiple sites of integration involving Rip2 signaling. Between signaling through the PRRs, as well as a potential viral receptor, Rip2 is a key signaling member for intracellular infections. Furthermore, Rip2 modulation of IFN production by NK cells as well as Th1 cells highlights an IL-12 response by the acquired immune system. Thus, all the major biological effects of Rip2 are focused on modulating cell-mediated inflammatory responses. Whether sensitivity of Rip2-deficiency to pathogens other than *L. monocytogenes* occurs will be of great interest in the future. Furthermore, the relative importance of Rip2 contributions in

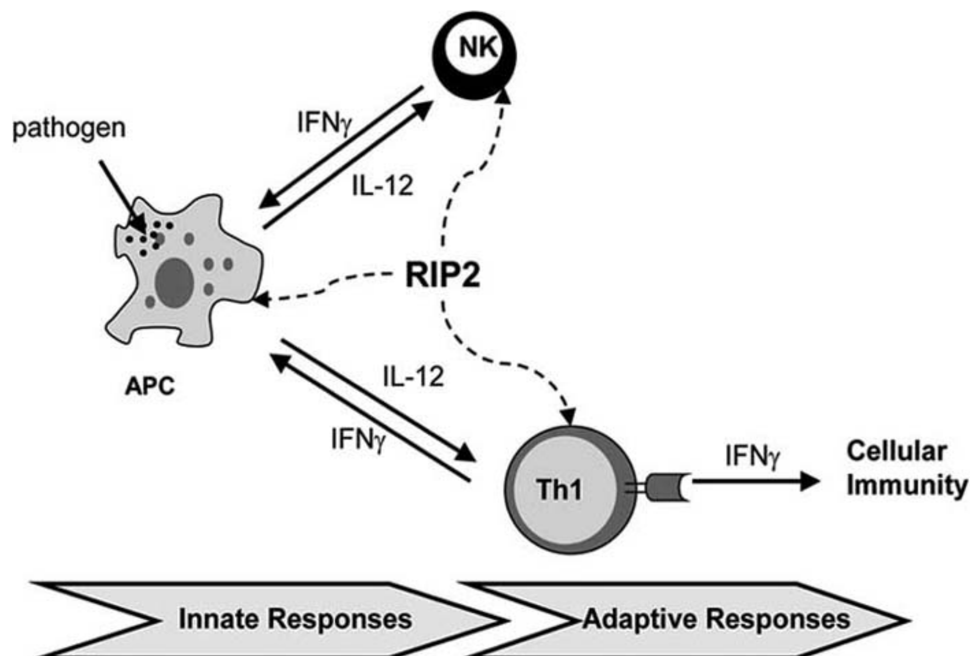

Fig. (2). Rip2 in Innate and Adaptive Immunity. Rip2 regulates multiple different pathways in innate and adaptive immune responses. The coordinated impact of this regulation is enhanced effector function of the cell-mediated immune response. A potential therapeutic target, Rip2 antagonism would intervene at multiple different levels.

innate versus adaptive immune responses to *L. monocytogenes* infection is unclear and will need to be elucidated. These cellular responses involving Rip2 can present as a continuum of activation events ranging from the excessive to ineffectual, or from chronic inflammation and autoimmunity to infection susceptibility (Fig. **3**). It is just this spectrum of inflammation that contrasts the possible roles ascribed to Rip2 in diseases ranging from graft rejection, Crohn's disease, collagen-induced arthritis, Type I diabetes, to susceptibility to intracellular infections. Rip2 presents an attractive target for the development of small molecule regulators both because of the plurality and the specificity of function. In developing models for inflammation and immune responses, Rip2 should be included in established paradigms

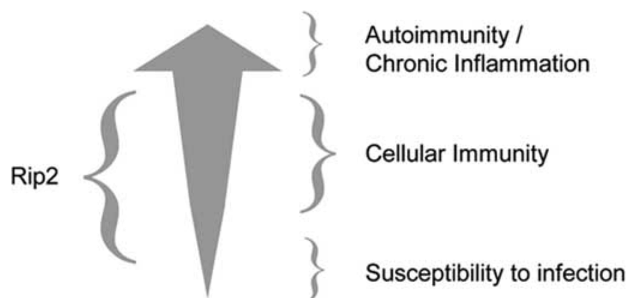

Fig. (3). Rip2 as Modulator of Inflammation. Rip2 serves to modify, amplify, or direct immune responses along this spectrum of inflammatory activation. Altered Rip2 protein may manifest as susceptibility to infection as in *Listeria monocytogenes* infection, biasing development of Th1 lymphocyte directed responses, or altering chronic inflammatory conditions such as Crohn's disease.

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