Physiological and Pathological Functions of CARD9 Signaling in the Innate Immune System

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Contents

1	Introduction.....	178
2	CARD9 Structure, Expression and Binding Partners.....	179
3	CARD9 Activation and Innate CBM Complex Assembly.....	179
4	Effector Functions of CARD9 Signaling.....	181
5	Negative Regulation of CARD9 Signaling.....	185
6	Physiological Functions of CARD9 Signaling in Host Protection.....	186
6.1	CARD9 Signaling in Antifungal Immunity.....	186
6.2	CARD9 in Antibacterial Defense.....	188
6.3	CARD9 in Immunity to Parasites.....	189
6.4	CARD9 Signaling upon Viral Recognition.....	189
7	CARD9 Signaling in Noninfectious Inflammation and Cancer Models.....	190
8	Genetic Alterations of <i>CARD9</i> in Human Disease.....	192
9	Conclusions and Therapeutic Perspective.....	194
	References.....	195

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Abstract Caspase recruitment domain protein 9 (CARD9) forms essential signaling complexes in the innate immune system that integrate cues from C-type lectin receptors and specific intracellular pattern recognition receptors. These CARD9-mediated signals are pivotal for host defense against fungi, and they mediate immunity against certain bacteria, viruses and parasites. Furthermore, CARD9-regulated pathways are involved in sterile inflammatory responses critical for immune homeostasis and can control pro- and antitumor immunity in cancer microenvironments. Consequently, multiple genetic alterations of human *CARD9* are connected to primary immunodeficiencies or prevalent inflammatory disorders in patients. This review will summarize our current understanding of CARD9 signaling in the innate immune system, its physiological and pathological functions and their implications for human immune-mediated diseases.

1 Introduction

The cells of the innate immune system make up our first line of defense against microbial pathogens, and they mediate homeostasis of the organism upon tissue damage. To mediate these effects, innate immune cells utilize germline-encoded pattern recognition receptors (PRRs) such as C-type lectin receptors (CLRs) to sense pathogen-associated molecular patterns (PAMPs) or endogenous danger-associated molecular patterns (DAMPs) (Gong et al. 2020; Rivera et al. 2016). The controlled activation of PRR-associated intracellular signaling pathways is critical for immune physiology and for host protection, and pathological dysfunctions of the same pathways are frequent causes of human immunodeficiencies and inflammatory disorders (Cao 2016; Huber-Lang et al. 2018; Zhang and Cao 2019).

Caspase recruitment domain protein 9 (CARD9) is a central signaling adapter molecule in the innate immune system. This protein integrates signals from the C-type lectin receptor and plays a very prominent role downstream of the antifungal CLRs Dectin-1, Dectin-2 and Mincle (Gross et al. 2006; Robinson et al. 2009; Shenderov et al. 2013). In addition, CARD9 can be activated by several intracellular nucleic acid-sensing PRRs and NOD-like receptors (Hsu et al. 2007; Poeck et al. 2010; Roth et al. 2014), and it controls innate immune responses to fungi, certain bacteria, viruses and parasites and is also involved in sterile danger pathways upon cellular damage (Brown et al. 2018). These CARD9-controlled pathways are highly relevant to human immune pathology, as genetic alterations of *CARD9* are causal for primary immunodeficiencies (Drummond and Lionakis 2016) or risk factors for prevalent human inflammatory disorders (Drummond et al. 2018). CARD9 has also been reported to play critical roles in tumor immune microenvironments (Bergmann et al. 2017; Wang et al. 2018; Zhong et al. 2019). In the next chapters, we discuss our current understanding of the molecular regulation of CARD9 signaling with a focus on CLR pathways and outline the known physiological and pathological functions of CARD9 signaling in human disease.

2 CARD9 Structure, Expression and Binding Partners

The CARD9 protein has a bipartite structure consisting of an amino-terminal caspase recruitment domain (CARD) (Bertin et al. 2000), which represents a homotypic protein–protein interaction module that can associate with other CARDS, and a carboxy-terminal coiled-coil region that serves as an oligomerization site (Bertin et al. 2000) (Fig. 1a). CARD9 is highly conserved between mice and humans, sharing approximately 87% similarity and 83% identity. CARD9 is specifically expressed in myeloid cells of the innate immune system, such as dendritic cells, macrophages and neutrophils, and is found in various tissues, including the spleen, liver, brain, placenta, peripheral blood and bone marrow (Bertin et al. 2000; Hsu et al. 2007; Roth and Ruland 2013). Initial studies have demonstrated that CARD9 can utilize its CARD to directly interact with the ubiquitously expressed CARD-containing adapter protein B-cell lymphoma 10 (BCL10) (Bertin et al. 2000). BCL10 in turn is constitutively associated with the multifunctional protein mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1), which is composed of a death domain, three immunoglobulin (Ig)-like domains and a catalytic caspase-like domain (Fig. 1a), which has proteolytic function. MALT1 is therefore also referred to as a paracaspase and is unique to the mammalian proteome (Uren et al. 2000). Activation of CARD9 by innate immune signals also leads to rapid activation of the BCL10-MALT1 module and the formation of a CARD9-BCL10-MALT1 complex (also called the CBM complex), which then mediates context-dependent inflammatory responses (Fig. 1b, c). The BCL10-MALT1 module can also be activated by a series of additional immune and inflammatory receptors in other cell types, including antigen receptors in T and B lymphocytes, via CARD11 (Ruland and Hartjes 2019).

3 CARD9 Activation and Innate CBM Complex Assembly

The activation of CARD9 is a highly regulated process that can be triggered by multiple and potentially all SYK-coupled CLRs, including Dectin-1 (Gross et al. 2006), Dectin-2 (Robinson et al. 2009) and Mincle (also known as CLEC4E) (Shenderov et al. 2013) (Fig. 1b). CARD9 is also activated by other ITAM-containing receptors on myeloid cells, including the Fc γ receptor Fc γ RIII, myeloid-associated immunoglobulin-like receptor II (MAIR-II) (also known as CD300D), osteoclast-associated immunoglobulin-like receptor (OSCAR) and triggering receptor expressed on myeloid cells 1 (TREM1) (Hara et al. 2007) (Fig. 1b). Furthermore, the cytosolic nucleic acid sensors retinoic acid-inducible gene 1 (RIG-I), melanoma differentiation-associated protein 5 (MDA5, also known as IFIH1) (Poeck et al. 2010) and RAD50 (Roth et al. 2014) as well as nucleotide-binding oligomerization domain-containing protein 2 (NOD2) (Hsu et al. 2007) can utilize CARD9 for signal transduction (Fig. 1c).

Fig. 1 CARD9-BCL10-MALT1 complex and signaling. **a** In myeloid cells, the CBM signalosome consists of CARD9-BCL10-MALT1. CARD9 has a bipartite structure with a CARD domain at the amino-terminal end and a coiled-coil domain at the carboxy-terminal end. TRIM62 binds CARD9 at its C-terminal end and is required for K125 ubiquitylation. Activating phosphorylation at T231 is mediated by PKC δ , whereas inhibitory phosphorylation at T531/T533 is mediated by CK2. BCL10 consists of amino-terminal CARD and a serine-/threonine-rich carboxy-terminal region. MALT1 comprises an amino-terminal death domain (DD), three immunoglobulin-like (Ig) domains and a caspase-like catalytic domain. **b** Upon extracellular DAMPs/PAMPs (e.g., fungi), CARD9 relays signals from SYK-coupled immunoreceptor tyrosine-based activation motif (ITAM)-containing CLR such as Dectin-1 and ITAM-associated CLR such as Dectin-2, Dectin-3 and Mincle, which lack a cytoplasmic ITAM domain and thus require ITAM-containing adaptor proteins such as DAP12 or Fc γ R for signal transduction. Further, ITAM-associated receptors engaging CARD9 are the Fc γ receptor Fc γ RIII, myeloid-associated immunoglobulin-like receptor II (MAIR-II, CD300D), osteoclast-associated immunoglobulin-like receptor (OSCAR) and triggering receptor expressed on myeloid cells 1 (TREM1). In contrast, the immunoreceptor tyrosine-based inhibition motif (ITIM)-bearing myeloid inhibitory C-type lectin-like receptors (MILs) generally suppress inflammatory pathways by recruiting tyrosine phosphatases such as SHP1 and SHP2. Alternatively, unopsonized fungi can be sensed via complement receptor 3 (CR3), which leads to granule-dependent fungal killing via Kindlin3/SYK/CARD9. **c** CARD9 is also activated by intracellular pathogens of bacterial or viral origin. The cytosolic nucleic acid sensors RIG-I and MDA5 and the DNA double-strand break repair protein RAD50 promote NF- κ B signaling via CARD9, which leads to inflammatory cytokine expression (e.g., IL-1 β), cellular activation and selective adaptive immunity. Furthermore, CARD9 mediates p38/ERK activation upon NOD2 engagement of intracellular bacteria

To date, the activation of CARD9 in the CLR pathways in the context of fungal recognition is best understood (Fig. 2). The early activation of CLRs, such as Dectin-1, Dectin-2 or Mincle, leads to Src family tyrosine kinase-mediated phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs),

ITAM-like regions within Dectin-1 or Dectin-2, or Mincle at associated transmembrane ITAM adapters, such as the Fc receptor γ -chain or DAP10 (Geijtenbeek and Gringhuis 2009; Rogers et al. 2005; Sato et al. 2006; Yamasaki et al. 2008). These receptor proximal events result in the recruitment of SYK to receptors and their activation, leading to the activation of PKC δ (Strasser et al. 2012) in cooperation with VAV proteins (Roth et al. 2016). Active PKC δ then triggers direct phosphorylation of CARD9 within its coiled-coil domain at position T231, and this modification is critical to induce assembly of the CARD9-BCL10-MALT1 complex (Strasser et al. 2012). Structural analysis revealed that CBM assembly upon CARD9 activation requires disruption of its autoinhibitory state, which leads to a conformational change that exposes CARD9-CARD for BCL10 binding (Holliday et al. 2019). CARD9-BCL10 interactions are then mediated via CARDs, whereby CARD9-CARD forms a helical assembly that serves as a template for unidirectional filamentous BCL10 polymerization, presumably dependent on Zn²⁺ (Holliday et al. 2018).

The assembled CBM complex in innate immune cells serves subsequently as a scaffold structure that likely places ubiquitin modifiers and protein kinases such as TAK1 in proximity for downstream signaling (Strasser et al. 2012). Although the precise collection of interaction partners in the CARD9-controlled innate immune pathways remains to be identified, the BCL10-MALT1 module can in principle recruit the E2 ubiquitin-conjugating enzymes UBC13 and UEV1a (Deng et al. 2000; Sun et al. 2004), the E3 ubiquitin ligases tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2) and TRAF6 (Oeckinghaus et al. 2007; Sun et al. 2004), cellular inhibitor of apoptosis protein 1 (cIAP1) and cIAP2 (Yang et al. 2016a), and the linear ubiquitin chain assembly complex (LUBAC) (Yang et al. 2016b). These factors have been reported to regulate BCL10-MALT1-dependent lymphocyte signaling (Ruland and Hartjes 2019). It is therefore conceivable that similar mechanisms would also control CARD9-BCL10-MALT1 signaling for innate immunity. Ubiquitylation reactions at CARD9-BCL10-MALT1 signalosomes are expected to recruit ubiquitin-binding proteins such as TAK1-binding protein 2 (TAB2) and TAB3 and for recruitment of TGF β -associated kinase 1 (TAK1) (Sun et al. 2004; Wang et al. 2001) to activate downstream pathways. Nevertheless, the exact regulation of individual CARD9 effectors upon CARD9 activation by distinct PRRs is still insufficiently resolved.

4 Effector Functions of CARD9 Signaling

One key output of activated CARD9-BCL10-MALT1 signalosomes is activation of the canonical NF- κ B signaling pathway (Roth and Ruland 2013) involving TAK1 and activation of the I κ B kinase- β (IKK β) (Fig. 2). This mechanism is particularly important for the control of the transcriptionally active NF- κ B subunits RelA/p65 and c-Rel (Hayden and Ghosh 2012; Liu et al. 2017), which are essential for orchestrating the expression of a large series of inflammatory cytokines, such as

◀**Fig. 2** CARD9 effector functions. After fungal engagement of CLRs (such as Dectin-1), the Src family kinase member Src phosphorylates the intracellular ITAM domain, allowing the recruitment of SYK. SYK subsequently activates the protein kinase C delta (PKC δ), which phosphorylates CARD9 at T231 in the coiled-coil domain in cooperation with VAV proteins, allowing CBM complex assembly. Subsequent K27-linked polyubiquitination of CARD9 at K125 by TRIM62 is required for NF- κ B activation. Additionally, TRAF6 is recruited to the CBM signalosome upon assembly to mediate its ubiquitination. Then, the IKK complex is recruited to the CBM complex and activated by TAK1 together with TAB2 and TAB3. Activated IKK phosphorylates I κ B α to induce proteasomal degradation, releasing the NF- κ B subunits p65 and c-Rel for nuclear translocation and subsequent inflammatory cytokine expression. Additionally, TAK1 activates JNK. The NF- κ B subunit RELB is counter-regulated by MALT1-mediated cleavage. Independent of BCL10/MALT1, Dectin-1 engagement initiates SYK-dependent Ras-GRF1 phosphorylation, which complexes with CARD9 and subsequently activates the H-Ras/ERK pathway. Moreover, CARD9 inhibits LyGDI-mediated suppression of RAC1, allowing NADPH oxidase activity, which is needed for microbial killing in the phagolysosome. For details, see text

TNF, IL-1 β , IL-6, IL-10, IL-12, IL-23 and GM-CSF, and chemokines, such as CCL3, CXCL1, CXCL2 and CXCL8, which can be induced upon CARD9 engagement (Drummond et al. 2015; Gavino et al. 2016; Gross et al. 2006; Jhingran et al. 2015; LeibundGut-Landmann et al. 2007; Németh et al. 2016; Poeck et al. 2010; Roth et al. 2014). To drive full NF- κ B activation, CARD9 recruits the ubiquitin ligase TRIM62, which binds to the C-terminal tail of CARD9 and mediates K27-linked ubiquitination of CARD9 at K125 (Cao et al. 2015). This ubiquitin-conjugating modification sterically disrupts the autoinhibitory state of the CARD9 CARD-coiled-coil interface, enabling BCL10 polymerization and subsequent NF- κ B activation (Holliday et al. 2019). CARD9 signaling is also implicated in CLR-induced ERK (Jia et al. 2014) and in NOD2-induced p38 and JNK signaling (Hara et al. 2007), which further contribute to fully inducing the inflammatory cascade.

Triggering of the CARD9-BCL10-MALT1 complex induces activation of MALT1 protease function (Yu et al. 2015). Although the specific role of MALT1-mediated proteolysis in CARD9-dependent innate immunity is still ill-defined, nine MALT1 substrates have been identified thus far, largely in activated T cells (Ruland and Hartjes 2019). The currently known MALT1 substrates include MALT1 itself and BCL10, the cleavage of which is thought to serve as a feedback loop to support optimal gene transcription (Baens et al. 2014). Furthermore, MALT1 can cleave and thereby inactivate the negative NF- κ B and MAPK regulators A20 (Coornaert et al. 2008) and CYLD (Staal et al. 2018) as well as HOIL1 (Douanne et al. 2016; Elton et al. 2016; Klein et al. 2015) to amplify the NF- κ B and JNK pathways. Similarly, upon lymphocyte activation, MALT1 can cleave the ribonuclease Regnase 1 (also known as ZC3H12A) (Uehata et al. 2013) or the mRNA decay regulators Roquin 1 (also known as RC3H1) and Roquin 2 (also known as RC3H2) (Jeltsch et al. 2014), which are important for controlling the stability of inflammatory mRNAs by binding to common stem-loop elements in their 3' untranslated region (3'-UTR) (Mino et al. 2015). Although A20 (Coornaert

et al. 2009), CYLD (Zhang et al. 2011), HOIL1 (MacDuff et al. 2017), Regnase 1 (Mao et al. 2017) or Roquin 1/2 (Athanasopoulos et al. 2016) are also involved in the regulation of innate immune responses, it is currently unknown whether and under what conditions MALT1 may inactivate these factors during CARD9-controlled innate immunity. Nevertheless, the fact that MALT1 cleaves overlapping substrates in innate immune cells and in activated lymphocytes is demonstrated in the case of the NF- κ B subunit RELB that is inactivated in stimulated T cells (Hailfinger et al. 2011) as well as in myeloid lineage cells upon CARD9-mediated Dectin-1 signaling (Xu et al. 2018). The NF- κ B RELB subunit acts primarily in the noncanonical NF- κ B pathway and controls upon Dectin-1 stimulation the expression of the Th-2 cytokine IL-5, which is an important example of how CARD9-triggered MALT1 proteolysis tunes the inflammatory response.

In addition to the activation of CARD9-BCL10-MALT1 complexes, CARD9 has also been reported to engage other immune effector pathways. In response to Dectin-1-initiated SYK-mediated Ras-GRF1 phosphorylation, CARD9 can associate with Ras-GRF1 to activate the H-Ras/ERK pathway for the full induction of inflammatory cytokines such as IL-6, TNF and IL-1 β (Jia et al. 2014). Moreover, CARD9 can control the production of ROS under certain conditions. Upon phagocytosis of *Listeria monocytogenes*, CARD9 is recruited to bacteria-containing phagolysosomes and suppresses the LyGDI-mediated inhibition of Rac1, enabling guanine nucleotide exchange factors (GEFs) to fuel Rac1 with GTP, which in turn drives NADPH complex-dependent ROS production required for antimicrobial killing (Wu et al. 2009). Furthermore, CARD9 can mediate granule-dependent killing of unopsonized fungal species that are recognized by complement receptor 3 (CR3) (Gazendam et al. 2014, 2016) through mechanisms that remain to be further defined.

In response to cytosolic PRR engagement, CARD9 plays additional selective roles. After the activation of NOD2, CARD9 selectively activates the MAP kinases p38 and JNK without affecting the NF- κ B pathway (Hara et al. 2007). Furthermore, in response to cytosolic RNA or DNA detection by the nucleic acid receptors RIG-I, MDA5 (Poeck et al. 2010) or RAD50 (Roth et al. 2014), CARD9 engages BCL10 presumably without MALT1 to drive canonical NF- κ B signaling for the production of inflammatory cytokines, particularly IL-1 β .

To induce the production of IL-1 β during innate immunity, CARD9 frequently collaborates with the activation of inflammasomes (Gross et al. 2009; Poeck et al. 2010), which are multiprotein complexes that induce caspase-1 activation to cleave pro-IL-1 β and related cytokines into their bioactive form (Guo et al. 2015). While CARD9 is not directly involved in the assembly of inflammasome complexes, it mediates pro-IL-1 β expression as well as NF- κ B-dependent inflammasome priming in response to fungal cell wall components (e.g., β -glucan) (Gross et al. 2009), mycobacterial glycolipids (e.g., TDM) (Schweneker et al. 2013; Shenderov et al. 2013) and viral nucleic acids (e.g., 3pRNA) (Poeck et al. 2010). Recent work has also reported that CARD9 can associate with necroptosis-inducing kinases RIPK1 and RIPK3 to regulate myeloid cell necroptosis upon fungal infection (Cao et al.

2019). While these noncanonical functions of CARD9 are currently emerging, putative BCL10-MALT1-independent mechanisms of CARD9 during cytosolic PRR signaling, ROS or necroptosis control are still insufficiently defined.

5 Negative Regulation of CARD9 Signaling

To dampen or downregulate CARD9 signaling to counter-regulate inflammation, the innate immune system has developed several mechanisms that operate at different levels of the signaling cascade (Fig. 3). In addition to ITAM-containing activating CLRs that activate CARD9, myeloid cells also express inhibitory CLRs that contain immunoreceptor tyrosine-based inhibition motives (ITIMs), such as the myeloid inhibitory C-type lectin-like receptor MICL (Redelinghuys and Brown 2011). These ITIM CLRs can recruit protein tyrosine phosphatases such as SHP1 and SHP2 to negatively regulate SYK-induced cellular activation (Marshall et al. 2004) and, consequently, CARD9 engagement. An additional mechanism that counteracts activating CLR signaling involves the E3 ubiquitin ligase CBLB (Wirnsberger et al. 2016; Xiao et al. 2016), which is active during antifungal immunity. CBLB mediates ubiquitin-dependent degradation of Dectin-1 and Dectin-2 as well as SYK, resulting in the negative regulation of CARD9 signaling (Wirnsberger et al. 2016; Xiao et al. 2016).

Direct negative regulation of the CARD9-BCL10-MALT1 complex itself is mediated by the regulator of autophagosome maturation and endocytosis called Rubicon (Yang et al. 2012). In response to fungal PAMPs or RNA viruses, Rubicon switches its interaction from the phosphoserine-/phosphothreonine-binding protein 14-3-3 β to CARD9 and thereby induces the disassembly of CARD9-BCL10-MALT1 complexes as an inhibitory feedback for CBM signaling in response to CLR and RIG-I ligands (Yang et al. 2012). Here, the S₂₄₈ phosphorylation status of Rubicon in its ST-N region is critical for selective 14-3-3 β or CARD9 binding.

CARD9 is also negatively regulated by phosphorylation-dependent events. In this context, casein kinase 2 (CK2) can directly phosphorylate the c-terminus of CARD9 at T531/T533 and mediate subsequent negative regulation of the NF- κ B pathway via the von Hippel–Lindau tumor suppressor protein (pVHL) (Yang et al. 2007). However, whether pVHL can directly bind CARD9 or requires an accessory protein remains unclear. Downstream of kinase 3 (DOK3) can induce protein phosphatase (PP)1-dependent dephosphorylation of CARD9 at threonine residues, which subsequently attenuates CARD9-dependent NF- κ B and JNK activation, and the subsequent reduction in cytokine production results in negatively regulated neutrophil antifungal immunity (Loh et al. 2019). Moreover, it is conceivable that additional inhibitory phosphorylation or the inhibition of activating phosphorylation and physical interference with activating binding partners or selective degradation of CARD9 complex components could further negatively regulate this pathway, although these hypotheses have been less studied.

Fig. 3 Negative regulation of CARD9 signaling. ITIM-containing CLRs such as MICL recruit SHP1/SHP2 to generally inhibit SYK-mediated activating receptor signaling. Serving as inhibitory feedback, Rubicon switches its interaction from 14-3-3 β to CARD9 upon stimulation and thereby induces disassembly of the CBM complex. Via pVHL, CK2 can directly phosphorylate the c-terminus of CARD9 at T531/T533 and mediate subsequent negative regulation of NF- κ B. Dephosphorylation of CARD9 by DOK3-induced PP1 activity can further attenuate NF- κ B signaling. CARD9 signaling during antifungal immunity is further counter-regulated by CBLB-mediated degradation of Dectin-1 and Dectin-2 and their associated SYK kinase

6 Physiological Functions of CARD9 Signaling in Host Protection

6.1 CARD9 Signaling in Antifungal Immunity

The nonredundant functions of CARD9 in innate immunity and antifungal defense were originally discovered in studies of CARD9-deficient mice using *Candida albicans* infections as a model (Gross et al. 2006). Subsequent investigations demonstrated that CARD9 signaling also mediates host protection against

Aspergillus fumigatus (Jhingran et al. 2012), *Coccidioides posadasii* (Hung et al. 2016), *Microsporum canis* (Mao et al. 2014), *Phialophora verrucosa* (Wu et al. 2016) and *Cryptococcus neoformans* (Yamamoto et al. 2014), which together establish CARD9 as one of the most important regulators of the mammalian antifungal immune response. These functions can be explained by the nonredundant role of CARD9 downstream of the SYK-coupled CLRs Dectin-1, Dectin-2, Dectin-3 and Mincle and the role of these receptors in sensing the fungal PAMPs β -glucan (Dectin-1) (Taylor et al. 2007), α -mannans (Dectin-2 and Dectin-3) (Saijo et al. 2010; Zhu et al. 2013) and α -mannose (Mincle) (Bugarcic et al. 2008).

To mediate antifungal defense, CARD9 triggers at least two important immune effector functions (Fig. 4). On the one hand, CARD9 signaling in monocytes and microglia induces the production of GM-CSF and CXCL1, respectively, which initiates the recruitment of neutrophils to the site of fungal infections (Drummond et al. 2015, 2019; Gavino et al. 2016). Neutrophils are required for fungal phagocytosis and killing and for the subsequent clearance of pathogens. The failure of CARD9-deficient mice to produce GM-CSF results in impaired neutrophil recruitment and allows invasive fungal growth (Drummond et al. 2015; Gavino et al. 2016; Jhingran et al. 2015). In addition to these immediate innate immune effects, CARD9 signaling in antigen-presenting cells also couples innate immune sensing to the activation of T cell-mediated adaptive immunity and controls, in particular, the differentiation of Th1 and Th17 cells (LeibundGut-Landmann et al. 2007; Robinson et al. 2009; Saijo et al. 2010; Sokol et al. 2013). CARD9 drives the

Fig. 4 Physiological functions of CARD9 in host defense and homeostasis. CARD9 signaling upon sensing DAMPs or PAMPs by myeloid cells leads to the expression of inflammatory cytokines and chemokines and presumably other effector molecules that orchestrate homeostatic and host defense function including T cell priming, control of the microbiota, stimulation of regeneration of epithelial cells, recruitment of neutrophils and the induction of neutrophilic myeloid-derived suppressor cells (nMDSCs). For details, see text

production of a specific set of cytokines, including IL-1 β , IL-6 and IL-23, which provide a milieu that induces Th17 differentiation of antigen-stimulated naïve T cells (LeibundGut-Landmann et al. 2007; Saijo et al. 2010). Th17 cells are crucial for host defense against extracellular pathogens, including fungi such as *Candida albicans* (LeibundGut-Landmann et al. 2007) and *Cryptococcus neoformans* (Yamamoto et al. 2014). The failure of CARD9-deficient mice to mount adequate Th17 immunity is an additional cause of defective antifungal defense. During later phases of fungal infection, CARD9 regulates the development of neutrophilic myeloid-derived suppressor cells (nMDSCs) in a manner that depends on ROS, caspase-8 and IL-1 β (Rieber et al. 2015). In general, nMDSCs can suppress T lymphocytes via ROS, arginase and NO to prevent pathological T cell and NK cell responses (Gabrilovich and Nagaraj 2009), and CARD9-mediated nMDSC generation after fungal recognition prevents tissue damage at later stages of infection.

6.2 CARD9 in Antibacterial Defense

Since CARD9-coupled CLRs sense not only fungi but also bacteria, it is not surprising that CARD9-deficient animals also exhibit pathological deficiencies in certain bacterial infection models. The mycobacterial cord factor trehalose-6,6-dimycolate (TMD) from *M. tuberculosis* is a potent proinflammatory PAMP detected by Mincle, and it induces potent CARD9-BCL10-MALT1 signaling (Shenderov et al. 2013; Werninghaus et al. 2009). This mechanism is particularly important for promoting the production of IL-10 during *M. tuberculosis* infection, which prevents neutrophil-mediated systemic hyperinflammation (Dorhoi et al. 2010). Furthermore, Mincle can mediate host protection against group A *streptococcus* (GAS) after recognition of the GAS-produced anchor of lipoteichoic acid monoglucosyldiacylglycerol (MDGD) by inducing the production of inducible nitric oxidase synthase (iNOS), ROS and inflammatory cytokines such as TNF, MIP-2 and IL-6, the latter of which is directly regulated by CARD9 (Imai et al. 2018). In the context of *Vibrio cholerae* infections, FcRg-CARD9 signaling induces inflammatory responses upon stimulation with the nontoxic cholera toxin subunit CTB (Phongsisay et al. 2015). Independent of CLRs, the ITAM-like motif bearing the CEACAM3 receptor engages the CARD9 pathway upon infection with *Moraxella catarrhalis* to induce the production of chemokines such as CXCL8 and CCL3 (Heinrich et al. 2016). The function of CARD9 in response to NOD2 stimulation has been implicated in the antibacterial defense against intracellular pathogens such as *Listeria monocytogenes* (Hsu et al. 2007). Moreover, CARD9-deficient animals are also hypersusceptible to bacterial infections with *Citrobacter rodentium*, which is commonly used to study experimental colitis (Lamas et al. 2018; Sokol et al. 2013). Linking innate to adaptive immunity, SYK-CARD9 signaling in APCs is also important for the optimal activation of T cells specific for bacterial proteins such as flagellin (Atif et al. 2015).

6.3 *CARD9 in Immunity to Parasites*

Parasites such as *Schistosoma mansoni*, which are causative for human schistosomiasis (hepatic and intestinal granulomatous inflammation), are also detected by the SYK-coupled CLRs Dectin-2, Mincle and SIGNR5, which synergistically activate CARD9 for IL-1 β and IL-23 production and subsequent Th17-mediated immunity (Kalantari et al. 2018; Ritter et al. 2010). The function of CARD9 is also required for host defense against the intracellular protozoan parasite *Toxoplasma gondii* by mediating IL-1 β production in primary human peripheral blood monocytes (Pandori et al. 2019), presumably upon activation of Dectin-1 and Mincle (He et al. 2016; Yan et al. 2014). Furthermore, CARD9-mediated IL-1 β production contributes to malaria-associated acute respiratory distress syndrome (ARDS) in response to *Plasmodium falciparum*, the most virulent species of malaria parasites (Maknitikul et al. 2017, 2018).

6.4 *CARD9 Signaling upon Viral Recognition*

Several SYK-coupled CLRs, such as DC-SIGN, langerin, MMR, DCIR, MDL-1 and LSECtin, have evolved to sense viruses such as HIV-1, HBV, influenza virus, measles virus, dengue virus and SARS corona virus (Geijtenbeek and Gringhuis 2009; Monteiro and Lepenies 2017), and it is therefore conceivable that these could signal via CARD9 during viral recognition. Furthermore, in vivo experiments with influenza virus (IFV) have demonstrated that CARD9-mediated innate activation plays a critical role in severe influenza pneumonia (Uematsu et al. 2015), presumably via DC-SIGN receptor engagement (Wang et al. 2008). In CARD9-deficient mice, pneumonia was drastically attenuated concomitant with reduced inflammatory cytokines and chemokines in the influenza-infected lungs. However, the type I interferon response and virus-specific adaptive lymphocyte immunity were not altered (Uematsu et al. 2015). Interestingly, CARD9 deficiency selectively altered the cytokine response to IFV in DCs in vitro but not in macrophages. Similarly, the Mincle-CARD9 signaling axis in DCs also controls the early antiviral response phase against the mosquito-transmitted La Crosse virus (LACV), whereas viral clearance was unaffected (Monteiro et al. 2019). Moreover, as indicated above, RNA and DNA viruses, which are detected by cytosolic nucleic acid sensors RIG-I, MDA5 (Poeck et al. 2010) and RAD50 (Roth et al. 2014), engage CARD9 for proinflammatory cytokine production. Consistently, RNA viruses such as vesicular stomatitis viruses (VSV) and DNA viruses such as vaccinia viruses (VV) activate CARD9 signaling in vitro, and deficiency of CARD9 results in impaired IL-1 β , TNF and IL-6 responses to these pathogens in vivo (Poeck et al. 2010). While these CARD9-dependent signals are important in

shaping the T cell response to viruses at later stages of infection, they are not involved in the immediate early type I interferon responses that mediate innate antiviral protection.

7 CARD9 Signaling in Noninfectious Inflammation and Cancer Models

More recent experiments with CARD9-deficient mice have demonstrated critical functions for CARD9 signaling in the regulation of noninfectious immune homeostasis (Fig. 4). Using the classical inflammatory bowel disease (IBD) model of chemically induced colitis with dextran sodium sulfate (DSS) (Chassaing et al. 2014), CARD9-deficient animals were found to be hypersusceptible compared with wild-type animals (Bergmann et al. 2017). Mechanistic studies have demonstrated that CARD9 signaling in innate immune cells regulates the production of cytokines such as IL-6, TNF and IFN γ and the Th17- and innate lymphoid cell (ILC)-related cytokines IL-17A, IL-17F and IL-22 during intestinal inflammation. These CARD9-induced cytokines are necessary to stimulate optimal IL-22 production by ILCs, which subsequently activate the IL-22 receptor on intestinal epithelial cells (IECs) to promote an epithelial regenerative response via STAT3 activation (Bergmann et al. 2017). Furthermore, CARD9 signaling shapes the composition of the gut microbiome (Lamas et al. 2016, 2018). Consequently, CARD9-deficient mice at baseline already exhibit an underrepresentation of bacterial species from the *Adlercreutzia* genus and *Lactobacillus reuteri*, which metabolize tryptophan into aryl hydrocarbon receptor (AHR) ligands, which are also crucial for the expression of IL-22 by lymphoid cells (Lamas et al. 2016). The impaired AHR ligand production is another reason for impaired IL-22 production and delayed epithelial regeneration in CARD9-deficient mice (Lamas et al. 2016). In line with the function of CARD9 in the antifungal response, the intestinal flora of CARD9-deficient mice also exhibits an aberrant fungal microbiome, which includes *Candida tropicalis* species that can increase the susceptibility to intestinal inflammation (Wang et al. 2018).

The effects of CARD9 signaling during intestinal inflammation have further consequences in models of colitis-associated cancer (CAC) (Bergmann et al. 2017; Hartjes and Ruland 2019; Malik et al. 2018; Wang et al. 2018). On the one hand, the CARD9-triggered IL-22-mediated proliferative IEC response can stimulate the growth of transformed colon epithelial cells and thereby enhance colon cancer growth (Bergmann et al. 2017). On the other hand, presumably dependent on the microbiota, CARD9 signaling can enhance granulocytic MDSC accumulation and activation in the lamina propria, which inhibits T cell-dependent antitumor immunity in the colon (Wang et al. 2018). Moreover, CARD9 signaling in the gut controls IL-18 production. This effect supports antitumor immunity during CAC development by promoting epithelial barrier integrity and by stimulating intestinal CD8 + T cells (Malik et al. 2018).

While these recent studies have demonstrated that CARD9 signaling can mediate pro- and antitumor immune effects during CAC (Hartjes and Ruland 2019), CARD9 signaling not only affects colon cancer growth at the primary site but also contributes to liver metastasis of colon carcinoma cells by promoting metastasis-associated macrophage polarization in response to VEGF CARD9 engagement and by inducing the production of tumor-promoting cytokines such as IL-10 and IL-1 α (Yang et al. 2014). In addition to colon cancer, CARD9 has been implicated in the pathogenesis of renal cell carcinoma (RCC), whereby loss of the RCC tumor suppressor molecule pVHL leads to CARD9-BCL10-TRAF6-mediated signaling, which subsequently leads to oncogenic hyperactive JNK activation (An et al. 2013; Yang et al. 2007). In Lewis lung carcinoma, CARD9 has been implicated in regulating the accumulation of MDSCs in tumor tissues and subsequent IDO production, which can negatively regulate tumor growth (Qu et al. 2019). CARD9 has thus emerged as an important regulator of tumor immune microenvironments, although the cancer-derived ligands that can engage CARD9 signaling remain largely unclear.

Endogenous DAMPs that could in principle activate CARD9 include cholesterol crystals, SAP130 (a subunit of the histone deacetylase complex) and β -glucosylceramide (a ubiquitous intracellular metabolite that indicates massive tissue damage if elevated), which can all trigger Mincle (cholesterol crystals, β -glucosylceramide, SAP130) (Kiyotake et al. 2015; Nagata et al. 2017; Seifert et al. 2016) and vimentin (an intermediate filament protein), which activates Dectin-1 (vimentin) (Thiagarajan et al. 2013). While the *in vivo* scenarios under which these specific DAMPs activate CARD9 signaling are not well explored, there is emerging evidence that CARD9 signaling contributes to noninfectious inflammatory responses under conditions beyond the cancer microenvironment. In acute pancreatitis, the severity of pancreatic injury is correlated with overexpression of CARD9 in mononuclear cells, which leads to enhanced NF- κ B and p38 MAPK signaling-mediated cytokine expression that promotes inflammatory pathology (Yang et al. 2016c, 2017). In the context of ischemic heart disease, CARD9 signaling promotes neutrophil infiltration following the acute ischemia/reperfusion (I/R) phase by inducing inflammatory cytokines such as TNF, IL-6, CXCL1 and MCP-1, thereby damaging the myocardium and promoting I/R injury (Qin et al. 2018). Similarly, after cerebral ischemic injury, which leads to an upregulation of triggering receptor expressed on myeloid cells (TREM)-1, the activation of CARD9 together with inflammasomes leads to pyroptosis and the release of inflammatory factors, which results in neuroinflammatory injury (Xu et al. 2019). Likewise, in a rat model of brain injury after subarachnoid hemorrhage (SAH), the sensing of necrotic cells by Mincle was linked to the activation of CARD9 signaling for inflammatory cytokine production, promoting neuroinflammation-induced brain damage following cerebral ischemia and reperfusion injury in the central nervous system (He et al. 2015). In contrast, in a murine model of atherosclerosis induced by a Western-type diet using *Ldlr*^{-/-} mice, CARD9 deficiency increased the atherosclerotic lesion size and severity, indicating that CARD9 signaling may be protective for the development of atherosclerosis (Thiem et al. 2019). Two

additional studies demonstrated further critical functions of CARD9 during neutrophil-mediated sterile inflammatory pathology. These reports demonstrated that CARD9 signaling can drive autoantibody-induced rheumatoid arthritis and dermatitis by controlling inflammatory chemokine/cytokine release in neutrophils (Németh et al. 2016) and that CARD9 engages in cross talk with SHP-1 to regulate IL-1-driven neutrophilic footpad inflammation in an animal model of human neutrophilic dermatosis (Tartey et al. 2018). Thus, these studies demonstrate that CARD9 signaling can mediate critical pathophysiological responses during sterile inflammatory stress in several organ systems.

8 Genetic Alterations of *CARD9* in Human Disease

Following the discovery of CARD9 as a nonredundant regulator of innate immunity and inflammation (Gross et al. 2006; Hara et al. 2007; Hsu et al. 2007) pathological *CARD9* loss-of-function (LOF) mutations and *CARD9* risk polymorphisms have been identified in human patients (Fig. 5). In line with the high susceptibility of CARD9-deficient mice to fungal infections (Gross et al. 2006), familial homozygous *CARD9* LOF mutations in patients also result in a high susceptibility to fungal infections (Glocker et al. 2009). Since the report of autosomal recessive *CARD9* LOF variants due to the Q295X mutation (Glocker et al. 2009), multiple *CARD9* LOF mutations have been identified in patients (Drummond and Lionakis 2016), including the homozygous *CARD9* missense mutations R18W (Lanternier et al. 2015a), R35Q (Lanternier et al. 2015b), R57H (Drummond et al. 2015), R70W (Lanternier et al. 2015b), Y91H (Gavino et al. 2014), R101C (Lanternier et al. 2013) and R101L (Grumach et al. 2015). All these alterations lead to impaired CARD9 signaling functions and a high susceptibility to fungal pathogens, including *Candida albicans*, *Aspergillus fumigatus*, *Phialophora verrucosa*, *Corynespora cassiicola*, several dermatophytes and some *Trichophyton* and *Exophiala* species, some of which cause lethal infections (Drummond and Lionakis 2016; Drummond et al. 2018). *Candida* infections are predominantly localized to the oral mucosa, subcutaneous tissues or the central nervous system (CNS), while *Aspergillus*, *Exophiala* and *Phialophora* species are recurrently detected in skin, CNS, abdominal organs or bone tissues (Drummond et al. 2018). Similar to CARD9-deficient mice, CARD9 signaling defects in patients lead to impaired Th17 cell responses (Glocker et al. 2009; LeibundGut-Landmann et al. 2007; Wang et al. 2014) and defective monocytic GM-CSF and C-X-C chemokine (e.g., CXCL1) production with impaired neutrophil recruitment to the site of infections (Drummond et al. 2015; Gavino et al. 2016).

In addition to *CARD9* LOF mutations, multiple human *CARD9* single-nucleotide polymorphisms (SNPs) have been associated with prevalent inflammatory disorders. These include IBDs, such as ulcerative colitis and Crohn's disease (Jostins et al. 2012; Rivas et al. 2011; Zhernakova et al. 2008) (McGovern et al. 2010) (Franke et al. 2010); ankylosing spondylitis, a severe form of arthritis affecting the

Fig. 5 Genetic alterations of CARD9 in human disease. While several CARD9 loss-of-function mutations lead to antifungal immunodeficiencies (red), the CARD9^{S12N} SNP leads to increased inflammatory disease risk (blue). Conversely, the CARD9^{Δ11} variant, which results from a skipping of exon 11, has protective functions in IBD (green)

spine (Evans et al. 2011; Pointon et al. 2010); primary sclerosing cholangitis, a complex inflammatory disorder gradually resulting in liver cirrhosis (Janse et al. 2011); and IgA nephropathy, in which IgA deposits trigger glomerulonephritis leading to renal failure (Kiryuk et al. 2014). While most of the *CARD9* disease SNPs do not affect the coding regions of *CARD9*, they presumably influence the level of *CARD9* expression and thereby modulate *CARD9* signaling strength. One prevalent *CARD9* SNP, which is correlated with an increased risk for IBD, encodes a missense variant at position 12 where the asparagine (N) is replaced by a serine (S) residue (Franke et al. 2010; McGovern et al. 2010). Experiments in knock-in mice that carry the respective CARD9^{S12N} allele demonstrated that this specific *CARD9* variant can deviate *CARD9* signaling to induce activation of the RELB NF-κB subunit for IL-5 production in response to CLR stimulation, resulting in a deviated T cell response toward Th2 differentiation (Xu et al. 2018). Consistently, human individuals with the CARD9^{S12N} variant exhibit an increased risk of Th2-mediated allergic bronchopulmonary fungal infections (Xu et al. 2018). Therefore, it is conceivable that related pathways might contribute to the inflammatory pathology in IBD patients with CARD9^{S12N} alteration.

Another human *CARD9* IBD risk variant that has been functionally studied is the protective *CARD9* isoform that decreases the risk of inflammatory bowel diseases and results from the exclusion of exon 11 within *CARD9*, creating a CARD9^{Δ11} protein (Rivas et al. 2011). Mechanistically, CARD9^{Δ11} can assemble trimeric signaling complexes with BCL10 and MALT1, but these CARD9^{Δ11}-BCL10-MALT1 complexes are defective in their ability to recruit TRIM62 for NF-κB activation. Therefore, it has been suggested that a reduction in TRIM62-mediated inflammation underlies the protective function of CARD9^{Δ11} in the intestinal immune system during IBD pathogenesis. While these investigations

into the function of aberrant CARD9 signaling during intestinal inflammation provide the first insights into the role of these pathways in human inflammatory disorders, mechanistic studies on the function of CARD9 in ankylosing spondylitis, primary sclerosing cholangitis or IgA nephropathy have not yet been carried out.

9 Conclusions and Therapeutic Perspective

Over the last decade, the myeloid cell-specific adapter molecule CARD9 has emerged as a key regulator of innate immunity and inflammatory responses. Multiple human patients with CARD9 deficiency have been identified (Drummond and Lionakis 2016), and their primary immunodeficiency is a phenocopy of that observed in CARD9-deficient mice (Gross et al. 2006), confirming that CARD9 function is highly conserved between humans and mice. The recently developed mechanistic understanding of the CARD9 signaling pathways has paved the way for rational strategies for the treatment of CARD9-dependent innate immune disorders. On the one hand, patients with inherited CARD9 deficiencies and defective immune responses have been successfully reconstituted with allogeneic stem cell transplantation to reconstitute leukocyte-mediated CARD9 immunity, resulting in complete clinical remissions (Queiroz-Telles et al. 2019). Other patients with CARD9 mutations have been successfully treated with GM-CSF, because this CARD9-controlled cytokine is critical for neutrophil recruitment and control of CNS candidiasis (Gavino et al. 2014). One additional strategy to enforce CLR-CARD9 signaling for the potential treatment of disseminated fungal infections could involve inhibition of the negative regulator CBLB, which has been experimentally tested in preclinical models (Wirnsberger et al. 2016; Xiao et al. 2016). Further strategies have been explored to boost CARD9-mediated immunity in vaccination regimens against mycobacterium tuberculosis (Ostrop et al. 2015), where selective triggering of the CLR-CARD9 signaling pathways with defined adjuvants can generate profound protective Th1 cell and Th17 cell responses (Werninghaus et al. 2009).

Conversely, in scenarios where aberrant CARD9 signaling might drive inflammatory pathologies, strategies are being developed to dampen the CARD9 pathway. Based on the knowledge that the IBD-protective CARD9^{Δ11} protein exhibits impairment of TRIM62 recruitment and subsequent NF-κB activation, small molecule inhibitors that disrupt the proinflammatory CARD9-TRIM62 interaction have been developed as candidate IBD therapeutics (Leshchiner et al. 2017). Other strategies to attenuate pathological CARD9 activity could potentially utilize MALT1 paracaspase inhibitors, which were initially developed for the treatment of malignant lymphomas that are driven by pathological BCL10-MALT1 signaling in transformed B lymphocytes (Ferch et al. 2009; Fontan et al. 2012; Hailfinger et al. 2011; Nagel et al. 2012). Although the functional identity and biological effects of MALT1 substrates in innate immune responses remain largely unclear, the activation of CARD9-BCL10-MALT1 signalosomes induces proteolytic activity of

MALT1 (Gringhuis et al. 2011), which likely controls important inflammatory pathways. Therefore, it is necessary to further study the innate immune function of the MALT1 protease in vivo and to explore whether its pharmacological inhibition could also be utilized to target CARD9-mediated immune pathology. Therefore, further mechanistic knowledge on the regulation of CARD9-BCL10-MALT1 signaling complexes in innate immunity and inflammation is required; as such, knowledge will open new avenues for therapeutic strategies that target inflammatory pathways during human pathology.

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Declaration of Interests

The authors declare no competing interests.

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