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Review

A Critical Role for CARD9 in Intestinal Microbiota Modulation and Colorectal Malignancies

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Abstract

The adaptor protein Caspase Recruitment Domain Family Member 9 (CARD9) plays an indispensable role in innate immunity. Recent studies indicate that dysregulated CARD9 is a critical risk factor in the progression of colorectal cancer (CRC). This review provides novel insights into the functions of CARD9 in CRC, particularly in delineating its role in disrupting the host microbe balance, fueling gut microbiota metabolism and inducing systemic immunoglobulin G (IgG) antifungal antibodies. These pathways provide important information that can potentially be used for therapeutic innovation in developing potential vaccines for CRC.

Keywords: CARD9; colorectal cancer; gut microbe; microbiota metabolism; systemic antifungal antibodies

1. Introduction

The human intestinal microbiota is composed of 10^{13} to 10^{14} microorganisms, encompasses appropriately 10 times more bacterial cells than human cells and forms a highly complex microbial ecosystem. Intestinal microbiota is now recognized as an "organ", which exerts a significant impact on human health and disease by maintaining intestinal homeostasis, modulating metabolites, enhancing immune functions [1,2], shaping the intestinal epithelium [3], facilitating energy harvest [4], protecting against pathogens [5] and altering host physiological functions.

Over the past few years, there has been a concerted effort to identify the role of the intestinal microbiota in colorectal carcinogenesis [6]. The tumor microenvironment in colorectal carcinoma (CRC) may derive from a deranged interplay between the host and microbiota [7,8]. The differences in the intestinal microbiota compositions between healthy individuals and CRC patients have been extensively explored, confirming that intestinal microbes in CRC patients are significantly depleted or enriched compared to healthy controls [6,9]. Interestingly, intestinal microbiome alterations typically occur in the early stages of CRC, serving as potential biomarkers for early cancer detection. In addition, studies have shown that the intestinal microbiome may represent a novel target for therapeutics and/or prevention of CRC [6,9]. Although the enormous diversity of microbiota has been demonstrated, it remains largely unknown in CRC.

Caspase Recruitment Domain Family Member 9 (CARD9), a myeloid cell-specific signal protein, is predominantly expressed in myeloid cells, particularly in macrophages and dendritic cells. CARD9 is an inflammation-related protein and is involved in the transduction of signals from different innate pattern recognition receptors (PPARs, e.g., C-type Lectin receptors, Toll-like receptors). This results in activation of downstream pathways (e.g., NF-κB, MAPK) which leads to the production of a cascade of inflammatory cytokines, elicits the host immune response and initiates the host defense against pathogen invasion. There is emerging evidence that CARD9 is involved in tumor progression in CRC [10,11]. A deeper understanding of the correlation between CARD9 function and gut microbiota in CRC carcinogenesis is crucial. It can provide fundamental insights into the precise functional effects of CARD9 in CRC and has potential critical translational implications for new treatment strategies for this type of cancer.

In this review, we summarize the novel functions of CARD9 in colorectal cancer, with a particular interest in its impact on (1) the disruption of the host microbe balance, (2) fueling gut microbiota metabolism and (3) inducing systemic immunoglobulin G (IgG) antifungal antibodies, providing valuable data for the development of vaccines for CRC. Some functions of CARD9 including signal transductions, innate and adaptive immunity, will be omitted in this review.

2. CARD9 in Colorectal Carcinoma Development

Although many studies have functionally investigated the role of CARD9 in intestinal carcinoma, a full characterization of the protein remains unclear. For exam-

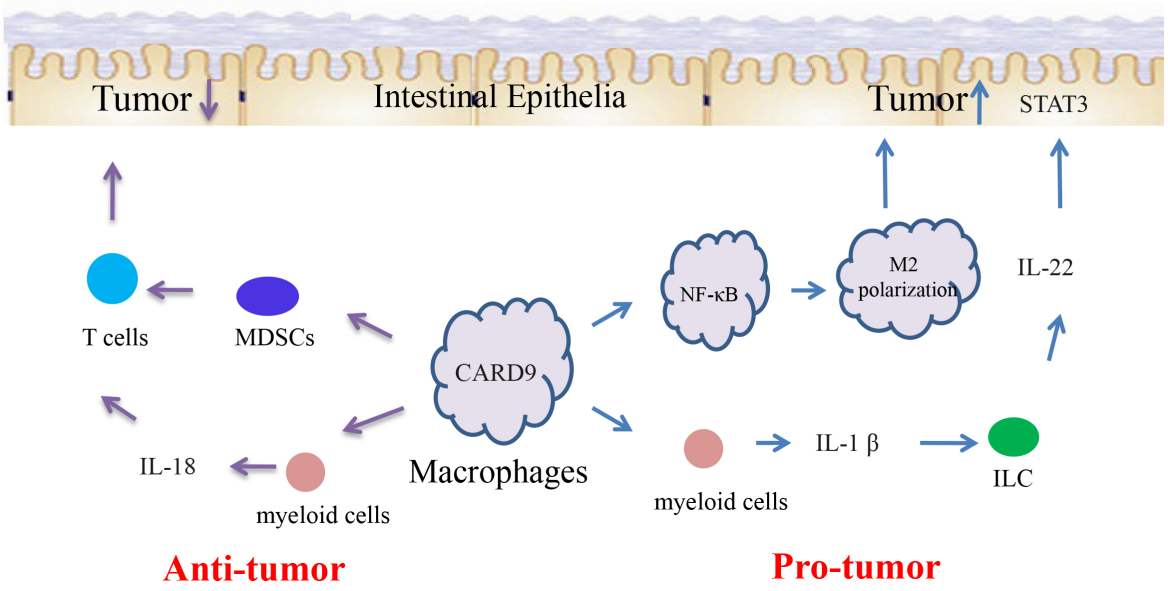

Fig. 1. The role of CARD9 in CRC. CARD9 promotes the tumor growth and metastasis of CRC via NF-κB activation in tumor-infiltrating macrophages and STAT3 secretion in intestinal epithelial cells. CARD9 inhibits the tumor growth and metastasis of CRC via T cells.

ple, it is uncertain whether CARD9 signal contributes to a tumor-promoting or tumor-suppressing immune environment in CRC, as highly expressed CARD9 was correlated with poor clinical outcomes in some reports, while not in others [12,13]. The mechanisms behind these differing effects of CARD9 are summarized below.

Early studies demonstrated the tumor-promoting role of CARD9 in preclinical models. For instance, CARD9 was found to be highly expressed in CRC tumor tissue when compared to adjacent normal tissue in an analysis of 48 patient samples [14]. CARD9 is highly expressed in tumor-infiltrating macrophages rather than cancer cells and is significantly associated with CRC tumor metastasis as well as advanced histopathologic stage [14]. Further exploration of the molecular mechanisms by which CARD9 acts as a promoter for CRC tumorigenesis demonstrate that CARD9 effectively triggers the activation of NF-κB, thus resulting in the induction of metastasis-associated macrophage polarization. It also facilitates spleen tyrosine kinase (SYK) activation in macrophages [14].

The tumor-promoting role of CARD9 was also investigated in murine CRC models which were developed through the oral administration of dextran sodium sulfate (DSS) and azoxymethane (AOM) causing AOM-DSS-induced colitis-associated CRC (CAC) [15]. Compared to littermate control mice, CARD9-deficient mice displayed an anti-tumour immune response with a lower rate of tumor cell proliferation and fewer gut polyps [15]. In addition, CARD9-driven CAC had dramatically enhanced interleukin-1β (IL-1β) production in myeloid cells with subsequently high IL-22 generation from group 3 innate lymphoid cells which eventually stimulated the tumor cell-intrinsic activation of STAT3 [15]. STAT3 is known as a key transcription factor that drives the survival and proliferation of malignant cells during CAC pathogenesis [16] which can be activated by IL-1β/IL-22 cytokines [17–20]. These reports addressed the functions of CARD9 in promoting the tumor growth and metastasis of CRC by mediating the activation of key signal including NF-κB and STAT3 (Fig. 1).

In contrast, investigations have uncovered opposing activities of CARD9 in CRC progression. Wang *et al*. [10] found that CARD9-deficient mice developed larger and increased numbers of tumors when compared to wild-type mice, displaying an anti-tumor immune response [10,21]. Pathways associated with CARD9 deletion dampened T cell immunity through intestinal accumulation of myeloid-derived suppressor cells (MDSCs) [10]. MDSCs are heterogeneous immature myeloid cells derived from the bone marrow. They can suppress T cell proliferation and T helper 17 (Th17) cell differentiation and negatively interfere with T cell-mediated antitumor immunity, thereby inducing the escape of tumor cells from immune surveillance leading to tumorigenesis [22]. Furthermore, CARD9 effectively enhances the production of the inflammatory IL-18 cytokine via a canonical SYK-dependent manner in myeloid cells [21,23]. IL-18 can stimulate interferon gamma (IFNγ) secretion by T cells and maintain intestinal epithelial integrity, triggering an antitumor immune response during CAC progression. In conclusion, these results have highlighted the functions of CARD9 in regulating the initiation of MDSCs and IL-18 secretion, therefore stimulating antitumor $CD8^+$ T cells activation in CAC development (Fig. 1).

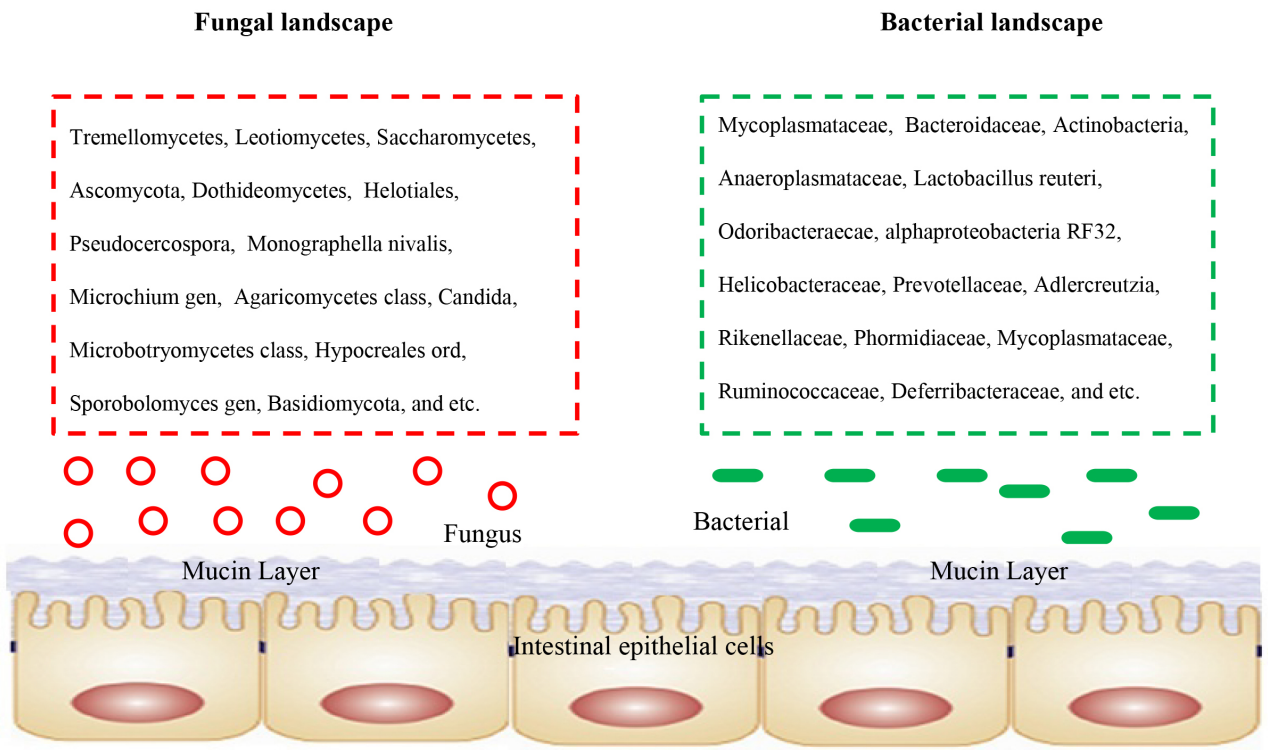

Fig. 2. CARD9 controls the microbiome composition in the gut, consisting of commensal bacteria and fungi.

3. CARD9 Regulates the Microbial Landscape in the Gut

3.1 Fungal Landscape

CARD9 is involved in the microbiota balance, playing a critical role in the fungal landscape in the gut (Fig. 2). Malik *et al.* [21] co-housed CARD9-deficient mice and littermate controls during the AOM/DSS treatment protocol. Subsequently, 18S internal transcribed spacer and 16S rRNA sequencing was used to assay the fungal diversity between the wild-type (WT) and CARD9-deficient mice [21]. Under these conditions, WT mice displayed a mycobiota predominantly consisting of Tremellomycetes (Basidiomycota) and Leotiomycetes (Ascomycota). CARD9-deficient mice revealed a distinct mycobial landscape, including an increased number of Saccharomycetes, particularly Diutinacatenulata and Cladosporium of Dothideomycetes, along with the decreased presence of several members of Ascomycota, including Pseudocercospora cordiana of Dothideomycetes and Agaricomycetes and Claussenomyces of Leotiomycetes [21]. As compared to the control group, the anti-fungal treatment group had a similar shift in the mycobial landscape leading to a significant decrease in several members of Ascomycota, particularly Leotiomycetes, Helotiales, and Pseudocercospora (Dothideomycetes) [21]. In addition, Lamas *et al.* [24] explored the composition of the fungal microbiota in CARD9$^{-/-}$ and WT mice. CARD9$^{-/-}$ mice developed a higher fungal load at the colonic tissues, and reached a peak at day 7. The study further analyzed the genus composition of fecal fungal microbiota via high-throughput internal transcribed spacer 2 sequencing revealing a major difference in Monographella nivalis, Microchium gen, Agaricomycetes class, Microbotryomycetes class, Ascomycota phy, and Hypocrealesord, and Sporobolomyces gen. Of note, diversity measurements in CARD9$^{-/-}$ mice exhibited a fungal microbiota dominated by members of the phyla Zygomycota, Basidiomycota, and Ascomycota. In a recent study, Wang *et al.* [10] reported the same results (i.e., that the total fungal burden in feces from tumor-bearing CARD9$^{-/-}$ mice were significantly higher than that from tumor-bearing WT mice) [24]. Importantly, a significant difference regarding fungal biodiversity was observed between tumor-bearing WT and CARD9$^{-/-}$ mice, showing the fungal microbiota in feces dominated by Candida (C. glabrata, C. tropicalis, and C. albicans) in tumor-bearing CARD9$^{-/-}$ mice [24]. In accordance with previous work, CARD9 null mice were found have a higher susceptibility with an increased *C. rodentium* fecal load compared with WT mice [25]. Although it was difficult to distinguish the normal and diseased fungi due to the diversity of the gut fungi [26], there was increasing evidence that *C. tropicalis* acted as an inducer of CRC [10]. Thus, CARD9 may correlate with CRC by controlling the gut fungal landscape.

3.2 Bacterial Landscape

In parallel, additional work also demonstrated that CARD9 was associated with the composition of the gut bacteria (Fig. 2) [10,21,24]. Ankit *et al.* [21] revealed

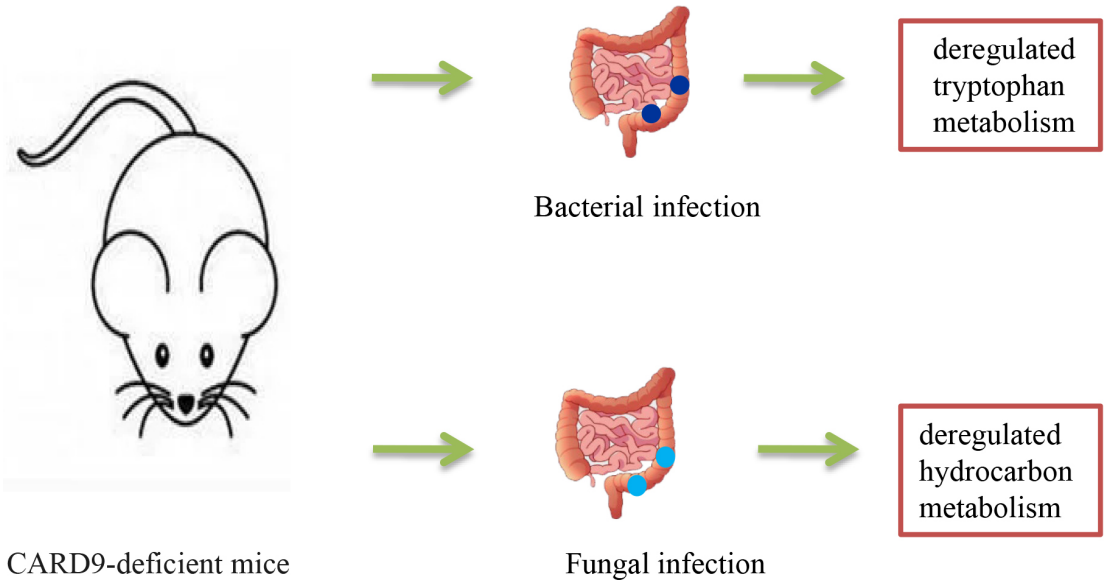

Fig. 3. CARD9 controls the microbiota metabolism in the gut. CARD9 deficiency is prone to decrease the number of *Allobaculumsp* and *L. reuteri* in the gut, and thus has no ability to metabolize tryptophan to metabolites which activates AHR as AHR ligand. CARD9$^{-/-}$ mice are more susceptible to *C. rodentium* infection. CARD9 is required for shaping an intestinal microbiota able to compete with *C. rodentium* for nutrients. The microbiota of CARD9$^{-/-}$ mice failed to outcompete the monosaccharide-consuming *C. rodentium*, worsening the infection severity.

a distinct bacterial landscape in WT and CARD9 null mice, showing a relative increase in members of Mycoplasmataceae, Bacteroidaceae, Anaeroplasmataceae, Rikenellaceae, and Odoribacteraecae, and a relative decrease in Alphaproteobacteria RF32, Helicobacteraceae, Prevotellaceae, Ruminococcaceae, Paraprevotellaceae, Deferribacteraceae, Acetobacteraceae, and Phormidiaceae in the CARD9 null mice. Under the condition of cohousing mice, the abundance of Mycoplasmataceae was higher while Phormidiaceae and Alphaproteobacteria RF32 was lower in the CARD9 null mice housed with the WT mice [21]. Similarly, Lamas *et al*. [24] identified the composition of the bacterial microbiota in feces by sequencing the 16S rDNA and revealed similar biodiversity in WT and CARD9$^{-/-}$ mice, but a decreased microbiota including Lactobacillus reuteri, Adlercreutzia (genus), and Actinobacteria (phylum) in the CARD9$^{-/-}$ mice. Recently, a study demonstrated that the baseline faecal bacterial composition was different between CARD9$^{-/-}\rightarrow$ germ-free (GF) mice and WT$\rightarrow$ GF mice, especially in an increase in *Ruminococcus* genera in the CARD9$^{-/-}\rightarrow$ GF mice [25]. GF mice are devoid of all microorganisms including bacteria, fungi and viruses. CARD9$^{-/-}$ GF mice refer to the CARD9$^{-/-}$ mice without microorganisms. CARD9$^{-/-}$ mice refer to the CARD9$^{-/-}$ mice with microorganisms. Different from the above reports, this study demonstrated no difference in the bacterial burden or composition between tumor-bearing WT and CARD9$^{-/-}$ mice [10].

To determine the contribution of the microbiome in the gut, mice were depleted of anaerobic bacteria with oral administration of metronidazole before AOM-DSS administration [21,27]. Fecal samples of these mice demonstrated a significant increase in Aerococcaceae, Enterobacteriaceae, Porphyromonadaceae, Lactobacillaceae, and Mycoplasmataceae, and a decrease in Rikenellaceae and S24_7 of Bacteroidales, Alphaproteobacteria, Deferribacteraceae. Of note, metronidazole treatment could depletecommensal anaerobes, led to distinct changes in the bacterial landscape, and eventually promoted CRC development [21]. Thus, CARD9 may correlate with CRC progression by controlling the bacterial landscape in the gut; however, further confirmation is needed.

4. Microbiota Metabolism in the Gut

CRC is associated with the load and composition of gut microbes [28]. The specific gut microbe-derived metabolites might be crucial to maintaining intestinal homeostasis, counteracting and/or enhancing tumorigenesis [29]. First, CARD9$^{-/-}\rightarrow$ GF and WT$\rightarrow$ GF mice were used to examine the metabolites derived from the gut fungal microbiome. A decreased resilience to *C. rodentium* infection was demonstrated between CARD9$^{-/-}\rightarrow$ GF and WT$\rightarrow$ GF mice, along with several functional differences. The most notable was a change in hydrocarbon metabolism such as fructose and mannose, galactose, pyruvate and butanoate metabolism (Fig. 3). As previously reported, hydrocarbon metabolism plays a key role in regulating microbiota energy homeostasis and intestinal mucosal immunity. Thus, CARD9$^{-/-}$ mice were prone to develop an infection with *C. rodentium*, exhibiting deregulated hydrocar-

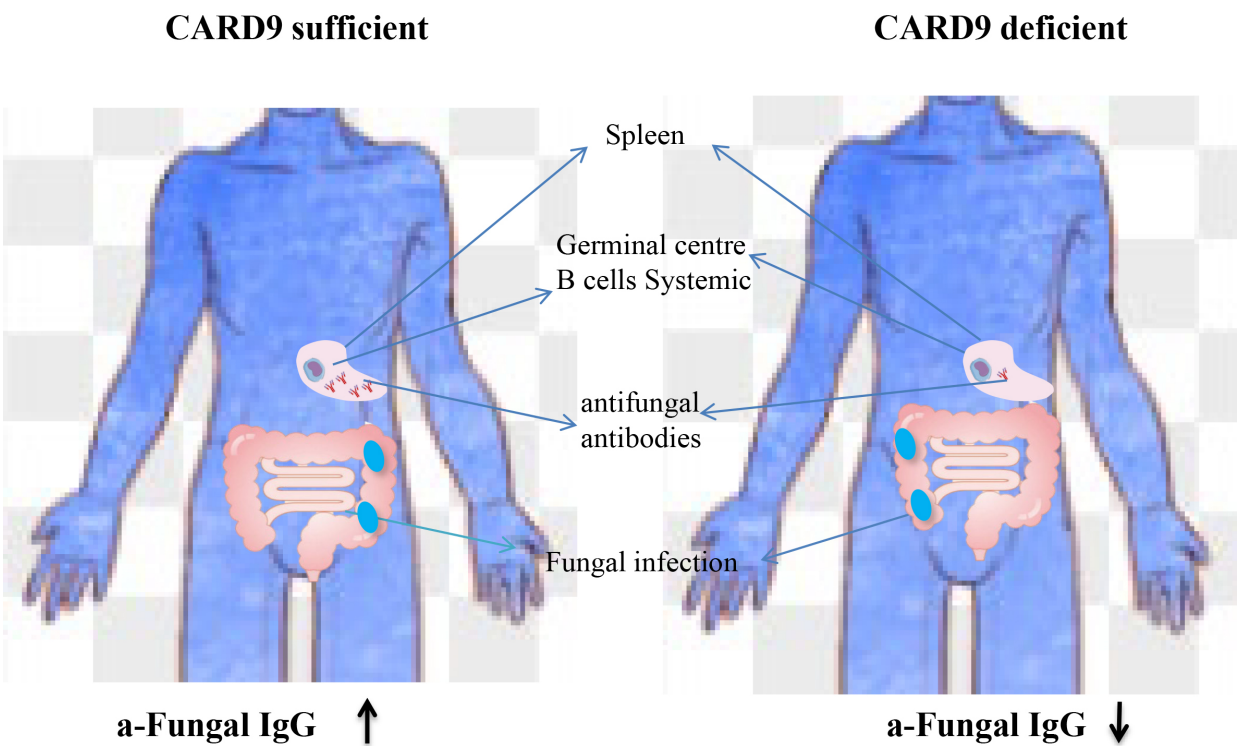

Fig. 4. Commensal *C. albicans* modulates antifungal immunity by systemic antifungal IgG antibodies produced germinal centre B cells dependent on CARD9 expressing macrophages.

bon metabolism of intestinal microbiota [25,30]. Second, CARD9$^{-/-}$ mice also were used to explore the metabolites derived from the gut bacterial microbiota. *Allobaculumsp* and *L. reuteri* bacteria were found in decreased abundance in the CARD9$^{-/-}$ mice. Aryl hydrocarbon receptor (AHR) activation enhances tumor malignancy and suppresses anti-tumor immunity, while these two bacteria are strongly responsible for activating the AHR. As expected, CARD9$^{-/-}$ mice failed to metabolize tryptophan, led to the inability to activate AHR (Fig. 3). AHR activation in CARD9$^{-/-}\rightarrow$ GF mice attenuated the symptom of colitis. Thus, CARD9$^{-/-}$ mice had impairment in microbiota required for hydrocarbon and tryptophan metabolism in the intestine. Additionally, AHR activity and tryptophan metabolites were reduced following analysis of fecal samples in patients [24].

5. Microbiota-Induced Immune Responses in the Gut

5.1 Fungi-Induced Immune Responses

C-type lectin receptors (CLRs), known as membrane-associated receptors, are critical for the detection of pathogenic and commensal fungal signals [31]. CARD9 functions as an adaptor molecule downstream of the CLRs. First, CLRs induce the activation of NF-κB in a CARD9 dependent manner [32]. After CLR activation, Protein Kinase C δ (PKCδ) is recruited to the immunological synapse. PKCδ enables CARD9 phosphorylation at T231 in the coiled-coil domain, forming the assembly of a Bcl10 and Malt1 proteins, termed the CARD9-Bcl10-Malt1 (CBM) complex. The CBM complex serves as a critical regulator to control the canonical NF-κB pathway, which regulates the expression of a variety of immune-regulatory factors, including TNF-α, IL-6, IL-1β, etc. Notably, chronic inflammation is linked to tumor carcinogenesis, which leads to inflammation-associated tumors [33]. NF-κB modulates inflammatory signal in the tumor microenvironment indicating potential crosstalk between inflammation and tumor [34]. Second, CARD9 signal stimulates inflammasome-mediated cytokine IL-18 secretion and regulated T cell immunity in the fungal-sensing pathways [21,35]. CLRs are activated by commensal gut fungi, which contributed to inflammasome production via the CARD9 signal. The inflammasome forms a platform to trigger the proteolytic processing of IL-18 [36]. IL-18 has been demonstrated to be critical to maintaining intestinal epithelial integrity and stimulating interferon gamma (IFN-γ) expression from intestinal CD8$^+$ T cells [37,38]. As expected, CARD9-deficient mice demonstrate greatly reduced inflammasome activation and IL-18 maturation and increased susceptibility to CRC [21]. Conversely, exogenous supplementation of IL-18 in CARD9-deficient mice has been shown to restore antitumor CD8$^+$ T cell function, increase IFN-γ production, ameliorate inflammation and reduce intestinal tumor development [21]. Third, in a study of the intestinal mycobiota, CARD9 deficiency increased the accumulation of myeloid-derived suppressor cells (MDSCs) in response

to fungal dysbiosis [10]. CARD9$^{-/-}$ macrophages exhibited impaired fungicidal functions, which led to an obvious increase in the gut fungal burden, particularly in *C. tropicalis*. Specific fungi could decrease the number of CD8^{+} and CD4^{+} T cells and promote the accumulation of T regulatory (Treg) cells and MDSCs in the colonic lamina propria, particularly with a notable increase in MDSCs [10]. It is well known that MDSCs are a group of immature myeloid cells that suppress the functions of effector T cells, negatively interfere with T cell-mediated antitumor immunity and are positively associated with the development of CRC [39,40]. CARD9 is a central adaptor protein in antifungal immune systems via CLRs signal, which is involved in CRC carcinogenesis and progression.

CARD9 is associated with cellular immunity in patients with systemic fungal infections. Little is known about its antifungal antibody response. Recently, a study revealed that commensal *C. albicans* boosted the production of systemic antifungal IgG antibodies in a process dependent on CARD9 macrophages (Fig. 4) [41,42]. *C. albicans* was identified as a strong inducer to elicit germinal centre B cell expansion in the spleen, which shapes the human antibody repertoire and induces the generation of high affinity antibodies. CARD9 deficiency is an inherited immune disorder that has been specifically linked to systemic fungal infections. Despite the normal function of germinal centre B cells, there is a failure to generate systemic antifungal IgG antibodies in the presence of a high systemic *Candida* burden in CARD9 deficiency. In contrast, increased expression of CARD9 was able to induce germinal centre B cells to produce systemic IgG antibodies against *C. albicans* [41,42]. Thus, these results suggest that CARD9 signal in macrophages elicits germinal centre B cell expansion for the production of antifungal antibodies.

5.2 Bacteria and Virus-Induced Immune Responses in the Gut

The receptor nucleotide-binding oligomerization domain containing protein 2 (NOD2) is an intracellular biosensor that can recognize a myriad of types of infectious bacteria. In this pathway, CARD9 signal plays a critical role in NOD2-mediated recognition of the microbiota [43]. Muramyl dipeptide (MDP), a bacterial cell wall component peptidoglycan produced Gram-positive bacteria, and whole *Listeria monocytogenes*, specifically activates NOD2, promotes the interaction of CARD9 with NOD2, leads to the activation of the JNK and MAP kinases p38 [43].

Mincle, a pattern recognition receptor, contributes to the detection of commensal bacteria and inflammation resolution in macrophages. Mincle has been shown to sense the Surface (S)-layer of the probiotic bacteria *Lactobacillus brevis* in a CARD9-dependent manner [44]. *Lactobacillus* is regarded as one of the major bacteria in the intestinal tract of mammals [45]. The macrophage inducible Mincle interacts with the S-layer of commensal bacteria that contributed to forming the Mincle/Syk/CARD9 axis. Importantly, this is accompanied by an altered release of both pro- and anti-inflammatory cytokines and impaired immune priming capacity of CD4^{+} T cells [44].

Toll-like receptors (TLRs) such as TLR3 and TLR7 on the cell surface detect viral infection [46]. In a study CARD9$^{-/-}$ macrophages failed to produce IL-6 and TNF-α after treatment with poly (I:C) (a TLR3 ligand), loxoribine (a TLR7 ligand), but not PGN (a TLR2 ligand), LPS (a TLR4 ligand). This suggested that CARD9 was involved in the antivirus immune via TLR3 and TLR7 [43]. In addition, CARD9 was required for the recognition of viral nucleic acids via the nucleic acid receptors including RIG-I [47] and RAD50 [48,49], which triggered the NF-κB pathway.

6. Conclusions

CARD9 has been confirmed to be critical for CRC development; however, to date the function of CARD9 in CRC is not well defined. Somestudies report a pro-tumor role of CARD9 in CRC, butothers have an anti-tumor role. These dual CARD9 mediated pro- and antitumor immune responses are required for further investigation.

CARD9 is central to the regulation of host immune responses in the presence of various microbial infections. Interestingly, in addition to fungal infections in the gut, CARD9 activity also correlates with certain bacteria and viruses. These CARD9 mediated interactions are critical in providing further insights into the complex interplay among the microbiota, the immune system and cancer development. Of note, a new study demonstrates that CARD9 signal in macrophages boosts systemic antifungal IgG antibodies production by germinalcentre B cells, leading to humoral antifungal immunity. As a result, mycobiota-induced IgG antibodies have a protective capability in gut fungal infections, suggesting that, in the future, potential vaccines and antifungal therapies could be developed to prevent colorectal cancer.

Author Contributions

PL wrote the original draft. ZM draw the figures. ZM and ZY edited the review. All authors have read and agreed to the published version of the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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