Fungi Enter the Stage of Colon Carcinogenesis

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The significant contribution of intestinal bacteria for the pathogenesis of colorectal cancer is widely accepted by now. In this issue of *Immunity*, two articles by Malik et al. (2018) and Wang et al. (2018) highlight the role of commensal fungi, which so far have been underestimated.

The importance of the intestinal microbiome for tissue homeostasis, as well as the pathogenesis and treatment of a wide range of inflammatory, immune, and malignant diseases, has become increasingly evident. So far, however, most studies have focused on the role of bacteria. In this issue of *Immunity*, two papers now provide compelling evidence that another class of commensals, namely the mycobiota, might also contribute substantially to the development of colitis-associated colorectal cancer (CAC).

The adaptor protein CARD9 is a member of the caspase recruitment domain family, which is essential for anti-fungal response and has been documented in both mice and humans (Gross et al., 2006; Drummond et al., 2018). *Card9*-deficient mice are susceptible to diverse fungal infections, such as those of *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*. In humans, CARD9 deficiency causes mostly *Candida* infections, although one recent study reported *Ascomycete* infection (Corvilain et al., 2018). The fungal β-glucans and α-mannans are recognized by the C-type lectin receptors Dectin-1, Dectin-2, Dectin-3, and Mincle (Zhu et al., 2013). Upon ligand binding and recruitment of the kinase Syk to the receptor, the assembly of the CARD9-MALT-BCL10 complex is triggered. This complex then leads to activation of the NFκB and MAPK signaling pathways, thereby controlling cytokine expression (Drummond et al., 2018).

The two new studies in this issue examine the function of CARD9 in the AOM-DSS (azoxymethane and dextran sodium sulfate) model of CAC. Whereas in both cases *Card9* deficiency leads to increased intestinal tumor load, the proposed underlying mechanisms and the contribution of fungi are seemingly different. After observing that CARD9 expression was increased in human CRC biopsies, Malik et al. (2018) went on to evaluate CARD9 function in the AOM-DSS model. Increased tumor load in *Card9*$^{-/-}$ mice was caused by an enhanced inflammatory response during acute colitis. Using various elegant genetic approaches, the authors unraveled a signaling cascade in myeloid cells wherein Syk-dependent activation of Card9 controlled caspase 1 activation and subsequent interleukin-18 (IL-18) maturation. Genetic deletion of *Card9* or myeloid-restricted deletion of *Syk* led to reduced amounts of cytoprotective IL-18, whereas administration of recombinant IL-18 mitigated colitis and reduced tumor incidence in these mice. Interestingly, the authors then noted that the colitogenic and associated tumor-promoting phenotype of *Card9*-deficient mice was transferrable to cohoused wild-type (WT) mice. Detailed analysis of the intestinal microbiome revealed striking differences in the bacterial and mycobial colonization of *Card9*$^{-/-}$ and WT mice that assimilated upon cohousing. Considering the relevance of CARD9 in host defense against fungal pathogens, the authors then demonstrated that depletion of the intestinal mycobiome in WT mice could mimic the phenotype of *Card9*$^{-/-}$mice in terms of colitis and tumorigenesis, which could be prevented by IL-18 administration.

Wang et al. (2018) also observed increased tumor load in *Card9*$^{-/-}$ mice in the AOM-DSS model. Curiously, they also reported a high frequency of invasive adenocarcinoma in both *Card9*$^{-/-}$ and WT mice with a tendency toward a more aggressive phenotype in *Card9*$^{-/-}$ mice. This represents a rather unexpected finding given that WT mice usually do not develop invasive tumors in the AOM-DSS model. Wang et al. then connected the pro-tumorigenic phenotype of *Card9*$^{-/-}$ to a distinct change in the fecal fungal composition after tumor induction and identified *C. tropicalis* as the dominating fungus in the *Card9*$^{-/-}$ intestine. *Card9*-deficient macrophages had an impaired ability to limit intracellular replication of *C. tropicalis*, and monocolonization of germ-free WT mice with *C. tropicalis*, but not *S. fibuligera*, promoted tumor growth. The authors propose that *C. tropicalis* can induce differentiation and activation of myeloid-derived suppressor cells (MDSCs)—especially the granulocytic subtype of MDSCs (G-MDSCs)—and also MDSCs from *Card9*$^{-/-}$ mice comprise a higher immunosuppressive potential. Accordingly, treatments with antibodies targeting Ly6G, which causes reduction of G-MDSCs, and the anti-fungal fluconazole reduced tumor incidence in *Card9*$^{-/-}$ mice. Interestingly, Wang et al. also illustrated that fungal burden, particularly of *C. tropicals* but not *C. albicans*, is higher in human CRC patients than in unaffected individuals and that CARD9 expression is inversely correlated with fungal burden.

Although these two studies, which used the same gene-targeted mice, seem to describe somewhat conflicting data at first sight, they also have several points in common. First, both studies clearly define a central role of CARD9 in both sensing fungal signals and controlling the mycobial composition, and both provide strong evidence for a significant contribution of fungal-induced signaling to colon carcinogenesis. Second, although Malik et al. (2018) focused on the early immune response during acute colitis and Wang et al. (2018) concentrated their analysis on established tumors, both studies demonstrated an increase in myeloid cell numbers as well as an attenuation of T cell function.

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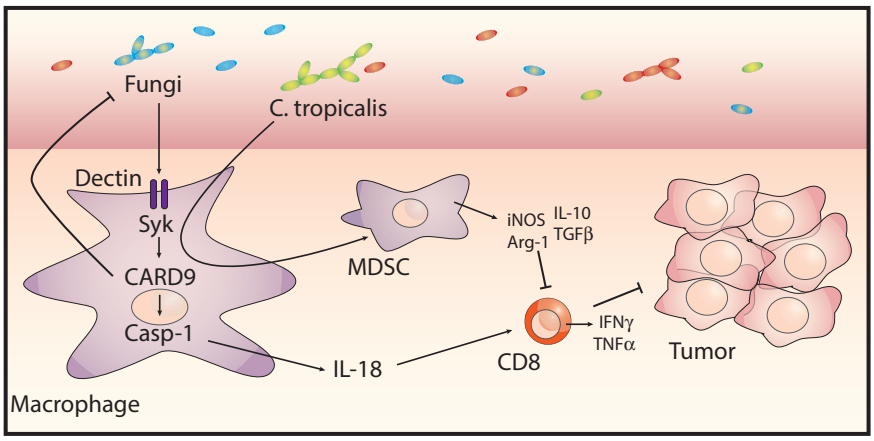

Figure 1. Fungal Sensing by CARD9 Orchestrates Immune Response to Limit Tumor Growth
Using *Card9*-deficient mice, two different studies by Malik et al. (2018) and Wang et al. (2018) propose various modes of how CARD9 activation in myeloid cells can affect colitis-associated tumorigenesis. Malik et al. suggest that fungi-induced Card9 activation suppresses tumor growth by controlling caspase 1 and subsequent IL-18 release, which suppresses inflammation and induces T cell activation, arguing that commensal fungi might be responsible for tumor-suppressive signaling events. In contrast, Wang et al. (2018) show that Card9 is involved in the control of *C. tropicalis* replication in macrophages. *C. tropicals* can induce differentiation of immunosuppressive MDSCs, which in turn suppress T cell activation, and monocolonization of germ-free mice with *C. tropicalis* enhances tumor load, arguing for tumor-promoting properties of this fungal species. Thus, on the one hand these studies provide clear support for an important fungal contribution to colitis-associated carcinogenesis. On the other hand, however, they also illustrate the complexity of the apparent pro-and anti-tumorigenic properties of different species.

Malik et al. did not further specify whether the elevated neutrophil population is actually composed of Ly6G$^+$ or Ly6C$^+$ cells; however, Wang et al. (2018) identified these myeloid cells as T-cell-suppressive G-MDSCs. Thus, both studies might actually describe the same phenomenon despite the different time points examined, and it is conceivable that both the Syk-CARD9-caspase1-dependent control of IL-18 maturation and the enhanced G-MDSC differentiation, which is most likely involved in controlling the expression of various immunosuppressive cytokines (i.e., IL-10, TGFβ, Cox2, and Arg1), ultimately impinge on CD4$^+$ and CD8$^+$ T cell activation (Figure 1). Yet, the greatest difference between these two studies seems to be the contribution of the intestinal mycobiome. To modify and deplete mycobiota, Malik et al. (2018) used the anti-fungals amphotericin B and itraconazole, both of which similarly increased susceptibility to colitis-associated colon cancer in WT mice. In stark contrast, Wang et al. (2018) nicely illustrated that fluconazole administration suppressed tumor growth in *Card9* $^{-/-}$ mice but did not affect tumorigenesis in WT mice. Moreover, *C. tropicalis* monocolonization increased tumor burden in germ-free mice, indicating a direct tumor-promoting role. Malik et al. (2018) did not test the effect of anti-fungal treatment in *Card9*$^{-/-}$ mice. Considering that both amphotericin B and itraconazole led to a significantly different bacterial composition in the intestine, an at least partial contribution of bacteria to CAC development in this context cannot be fully excluded. Although antibiotic treatment with metronidazole, which induced an overall increase in fungal burden, protected WT mice from colitis-associated colon cancer that could be prevented by anti-fungal treatment, the most likely reason for the opposing findings, however, is a difference in the composition of the fungal and bacterial microbiota in the intestine of WT mice used in the two studies. In line with this notion, a previous report suggested that *Card9* deficiency protects from CAC development (Bergmann et al., 2017). In that study, the authors cohoused mice for several weeks to minimize the environmental effects, including of the microbiota, in order to focus on the direct genetically driven effects of CARD9 in the host. Given that Malik et al. (2018) and Wang et al. (2018) did not detect a difference in tumor load between cohoused WT and *Card9*$^{-/-}$ mice, this indicates that not only the gene deletion but also the different vivaria could have had a profound effect on the composition of the respective commensals. Indeed, Malik et al. (2018) describe a decrease in Ascomycota in *Card9*$^{-/-}$ mice, whereas Wang et al. (2018) report a similar level of Ascomycota in unchallenged mice and a higher level of this phylum in *Card9*-deficient animals than in WT tumor-bearing mice. Furthermore, Wang et al. (2018) did not find significant differences in the bacterial composition between WT and *Card9*$^{-/-}$ tumor-bearing mice, whereas this was the case in the other study.

Despite their discrepancies, these two articles provide strong support for a decisive role of mycobiota in colitis-associated colon carcinogenesis—a role that has been underestimated so far. Indeed, most microbiota studies are focused on bacteria, and the effect of bacterial dysbiosis is assumed to be limited to bacteria. Therefore, careful analysis of both fungal and bacterial microbiomes will be necessary in the future. Considering the striking differences in the microbiome within the same genotype between those two studies, it is clear that the exact effect of the mycobiota is context dependent. One specific species, phylum, or even higher order can have a different outcome depending on the overall microbial composition. For laboratory animals, it means that observed results related to microbiota research are dependent on diet, which has been show to affect the microbiota, the exposition to microbiota from genetically modified mice, and pathogens present in the vivarium. This raises the concern that the data generated are highly vivarium specific. Therefore, the identification of "good" and "bad" species—bacteria or fungi—should always be considered within its microbial environment. By unveiling the role of fungi in CAC, these two studies, through their convergent but also divergent findings, further demonstrate the importance and the complexity of microbiota-dependent effects on the host. Therefore, a substantial amount of hard work, challenges, amazing findings, and exiting times still lie ahead for us to fully comprehend the complexity of the microbiome effect on diseases such as cancer.

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Gone with the Antibody

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Bacterial metabolites can reach distant organs, and in this issue of *Immunity*, Uchimura et al. (2018) show a fast systemic distribution of microbiota metabolites. This distribution is controlled by antibodies that accelerate bacterial transit through the small intestine, resulting in reduced local and systemic metabolite penetration and attenuation of immune responses.

The gut microbiota harbors trillions of bacteria that have co-evolved with the host and are essential for maintaining its health. The host has developed a series of physical barriers that allow the containment of the gut microbiota in the intestinal tract, therefore preventing bacterial spread to systemic circulation and distant organs. Altered composition of the gut microbiota has been associated with several pathologies in the gastrointestinal tract, such as inflammatory bowel diseases and colorectal cancer (Tilg et al., 2018). It is becoming obvious that besides the intestine, the gut microbiota modulates homeostasis and inflammation in virtually all host organs through a series of metabolic and immune regulatory functions (Martinez et al., 2017). Given the tight containment of the gut microbiota within the intestine, how can a host-wide reach be explained? The answer lies in the ability of the intestinal bacteria to produce a large spectrum of metabolites that act throughout the host and complement the host metabolism at the same time. In this issue of *Immunity*, Uchimura et al. (2018) propose that absorption of bacterial metabolites in the small intestine results in a fast systemic distribution that can cause inflammatory responses. The production of immunoglobulin A (IgA) can reduce this phenomenon by dragging bacteria in the colon, where the penetration of bacterial metabolites is minimal.

The idea that microbial metabolites can penetrate distant host organs emerges from several studies that have attempted to provide a deeper understanding of the dynamic interactions between host and gut microbiota. For instance, during conventionalization of germ-free mice, the establishment of a microbial flora has been shown to correlate with profound alterations in the transcriptomic and metabolomic profile of intestinal tissues, and significant changes have also been found in the urine metabolome, therefore suggesting a systemic impact of bacterial colonization (El Aidy et al., 2013). However, the above-mentioned study did not discriminate between microbial- and host-derived metabolites in response to conventionalization. In addition, it was not known how and whether the host immune responses could modulate the effect of bacterial metabolites.

In this issue of *Immunity*, by using a unique set of experimental tools, Uchimura et al. provide deeper insights into this matter.

In order to allow an unequivocal discrimination of the metabolite source, the authors colonized germ-free mice with ^{13}C-labeled HA107 *E. coli*. Therefore, ^{13}C-labeled metabolites of bacterial origin and ^{12}C-labeled host metabolites could be distinguished and efficiently traced by high-resolution mass spectrometry. Further, given that the HA107 strain of *E. coli* is engineered with cell-wall auxotrophy and was previously shown to be unable to replicate *in vivo*, the ^{13}C-labeled bacterial metabolites did not become diluted with ^{12}C host nutrient sources released during *in vivo* bacterial growth (Hapfelmeier et al., 2010).