Priority Brief

Cancer Immunology Research

CARD9 Promotes Sex-Biased Colon Tumors in the APC^{min} Mouse Model

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Abstract

Caspase recuitment domain–containing protein 9 (CARD9) functions in different inflammation pathways to elicit responses to microbial signals and is known to affect intestinal inflammation. Examining the APC^{min} mouse model of intestinal tumorigenesis and using stringently controlled, sex- and age-matched pairs of CARD9-competent and CARD9-deficient mice, we have found that CARD9 has a restricted but strong effect on tumorigenesis in the large intestine. We have found that CARD9 reduces viability specifically in males and promotes tumorigenesis specifically in the large intestines of these male mice. To our knowledge, this is the first gene ablation in APC^{min} mice that solely affects colon tumors in male subjects and, as such, may have significant clinical implications. Additional data suggest correlative disruption of plasma cytokine expression and immune infiltration of the tumors. We speculate that known sex-specific differences in human colorectal cancer may involve inflammation, particularly CARD9-dependent inflammation. *Cancer Immunol Res; 3(7); 721–6. ©2015 AACR.*

Introduction

The microbe-rich milieu in which colorectal cancer develops provides strong inflammatory signals (reviewed in ref. 1). Innate and adaptive immune responses induced by cancer cells, as well as by the associated intestinal-barrier damage, lead to cytokine release, and enhanced proliferation.

The caspase recuitment domain–containing protein 9 (CARD9) plays a role in chronic intestinal inflammation (2, 3) and may be an important factor in colorectal cancer progression (4). Its presence in mice is essential for full myeloid activation as well as antifungal response through the immunoreceptor tyrosine–based activation motif (ITAM) domain–containing Dectin transmembrane receptors (5).

CARD9 forms a complex with B-cell lymphoma 10 (BCL10) protein and mucosa-associated lymphoid tissue lymphoma translocation gene 1 (MALT1) in myeloid cells. At least three receptor signaling pathways are believed to signal through this complex, namely the nucleotide-binding oligomerization domain protein NOD2, which binds to intracellular peptidoglycans, the Dectin receptors, which recognize extracellular fungal-derived β-glucan products, and Mincle, which recognizes mycobacteria (5). Thus, CARD9 deletion can be used simultaneously to examine the role of a great number of non–Toll-like receptor (non-TLR) immune signals.

The APC^{min} mouse model mimics the genetic lesion associated with human familial adenomatous polyposis (FAP; ref. 6). Unlike human FAP, the tumor burden of the APC^{min} mouse is concentrated in the small intestine. Interestingly, the inflammation profiles of the colonic and small-intestinal tumors from APC^{min} mice are different, with the colonic tumors demonstrating more infiltrating immune cells and cytokine expression (7). Genetic evidence also suggests qualitative differences between APC^{min} small-intestinal and colonic tumors (8, 9). The APC^{min} mouse has sex-specific phenotypes, whereby male APC^{min} mice demonstrate higher multiplicity of colon tumors (10). Furthermore, several gene-ablation studies have demonstrated a female-specific effect on adenoma formation (8, 11, 12).

In this study, we have used *Card9*-deficient animals to probe its involvement in APC^{min} tumor incidence and progression. We found that CARD9 reduces viability and increases colonic tumor multiplicity in male mice. This effect correlates with decreased plasma IL6, G-CSF, and RANTES in male CARD9-deficient mice. Finally, male CARD9-deficient colonic tumors showed less macrophage and T-cell infiltration than wild-type tumors.

Materials and Methods

Animals

C57BL/6 backcrossed *APC*$^{+/min}$ (The Jackson Laboratory stock #002020) and *Card9*-deficient mice (13) were interbred to produce *APC*$^{+/min}$::*Card9*$^{+/+}$ and *APC*$^{+/min}$::*Card9*$^{-/-}$ pairs of mice. All pairs were cocaged from the time of weaning with 5% irradiated chow and water provided *ad libitum*. Humane endpoints provided for termination of the pair under conditions in which one mouse displayed rapid or extreme weight loss, evidence of

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suffering, unresponsive behavior, or rectal prolapse as approved under Singhealth IACUC protocol 2012/SHS/726.

Tissue extractions

Mice were sacrificed by CO_2 asphyxiation and blood was harvested by heart puncture. Blood was maintained in heparin-coated tubes on ice until plasma separation (4,000 × *g*, 5 minutes, 4°C). Plasma was isolated, snap-frozen in liquid nitrogen, and stored at −30°C. Intestines were removed, cleaned and rinsed internally and externally with PBS and then immediately fixed with 4% formaldehyde in PBS. Twenty-four hours post-mortem, fixed intestines were transferred to ethanol until they were counted. Tumor counting was performed, blinded of genotype for each animal, with the large intestine analyzed separately from the small intestine (14).

Plasma analysis

Plasma samples were thawed and analyzed, blinded of genotype, using the Bioplex Mouse Cytokine 23-plex Kit (Bio-Rad cat #M60-009RDPD) as per the manufacturer's specifications.

Immunohistochemistry

Fixed mouse tumors were excised from the intestine and embedded in paraffin with CARD9-competent and CARD9-deficient adenomas placed on the same slide for controlled staining. Sections were then stained for T cells using anti-CD3 (Dako, cat# A0452), macrophages using anti-F4/80 (Ab Serotec cat# MCA497GA), granulocytes using anti-Ly6G (Affimetrix, cat# 13-5931-82), and Ki67 (Thermo Fisher Scientific, clone# Sp6). Quantification of tumor infiltration was performed on scans of slides at ×40 magnification. Multiple images were captured for each slide and two random squares of neoplastic tissue for each image were used to count positive cells.

Statistical analyses

Data were tested for normality using the Shapiro–Wilk test. Where normality of data was not observed, statistical analyses were performed using nonparametric tests. The specific tests used are noted in the Results section. A *P* value of less than 0.05 on a two-tailed test was taken to be statistically significant. Analyses were performed using STATA version 12.1.

Results

To assess the role of CARD9 in intestinal cancer, we bred the *Card9* heterozygous mice into the APC^{min} background to get sex-matched pairs of $Apc^{min/+}$::$Card9^{+/+}$, hereafter termed "+/+," and $Apc^{min/+}$::$Card9^{-/-}$, hereafter termed "−/−" mice. For each pair, mice were born no more than 3 days apart. Cocaging of these mice from the time of weaning ensured that mice were exposed to the same physical and microbial environment. The phenotype was examined when one animal of each pair reached a humane endpoint as this provides maximum allowable phenotypic presentation (14).

CARD9 deficiency promotes APC^{min} survival

As expected, the −/− mice are viable, arising at similar frequencies consistent with the wild-type APC^{min} progeny. Our analysis of 33 pairs of mice included 16 pairs of female and 17 pairs of male mice. For each pair, we monitored health status daily, blinded to genotype. After harvest, we recorded the genotype of the animal that reached the humane endpoint or died first. Evaluation of survival was performed using the binomial test assessing the null hypothesis that mice with CARD9 wild-type and CARD9 deficiency had equal probability of survival against the alternative hypothesis that there is a survival difference. We found that 21 of the +/+ mice reached the endpoint before their −/− counterparts whereas 12 −/− mice reached the endpoint before their +/+ counterparts. Although not demonstrative of a statistically significant effect of genotype (Table 1), this indicated a trend toward increased survival of *Card9*-deficient APC^{min} mice.

Table 1. Male CARD9-deficient APC^{min} mice have improved viability

Genotype	Females	Males	Total
+/+	6/16 (37.5%)	15/17 (88.2%)	21/33 (63.6%)
−/−	10/16 (62.5%)	2/17 (11.8%)	12/33 (36.4%)
P	0.5	**0.002**	0.2

NOTE: Mice of indicated sex and Card9 status were counted upon reaching the humane endpoint or mortality. *P* value was determined by binomial test of the null hypothesis that genotype did not affect mouse viability.

CARD9 effect on APC^{min} survival is male specific

When we further stratified the pairs by sex, we found a striking sex-specific effect of the *Card9* deficiency. Female pairs of mice reached morbidity or mortality at statistically similar frequencies between the two *Card9* genotypes (Table 1). In contrast, the +/+ male mice reached the endpoint at greater frequency (15/17) than their cocaged −/− counterparts (Table 1). These data indicate that CARD9 increases APC^{min} lethality specifically in the male mice.

No apparent differences in small-intestinal tumor multiplicity or growth

To better characterize the nature of this male-specific effect for CARD9 in APC^{min} tumorigenesis, we excised and fixed the intestines for adenoma counting at the point when one animal of each pair reached the experimental endpoint. Seven pairs of animals presented in Table 1, for which one animal was found dead, were excluded from this analysis to avoid post-mortem artefact. Age at analysis differed between pairs from between 11 and 24 weeks of age (average, 17 weeks). Comparisons of tumor incidence and expression between paired CARD9-deficient and CARD9–competent mice were performed using the paired *t* test.

The effect of CARD9 on tumor multiplicity and burden between male and female mice were investigated using the two-sample *t* test or the Welch *t* test when variances of the two groups were not equal. Homogeneity of variances were assessed using the F test. Where a significant effect was observed between the sexes, comparison of tumor multiplicity and burden (separately) between paired CARD9-deficient and -competent mice was performed using the paired *t* test for male and female mice separately. No statistically significant differences could be found between the genotypes for small-intestinal tumor multiplicity (Supplementary Fig. S1A), either overall or with sex segregation between females and males. Similarly, when we examined total small-intestinal tumor burden (Supplementary Fig. S1B) or average tumor size in the small intestine (Supplementary Fig. S1C), we found no effect of *Card9* status overall or with sex stratification. We therefore propose that neither small-intestinal tumor incidence nor burden is sufficient to explain the increased viability of the CARD9-deficient APC^{min} animals.

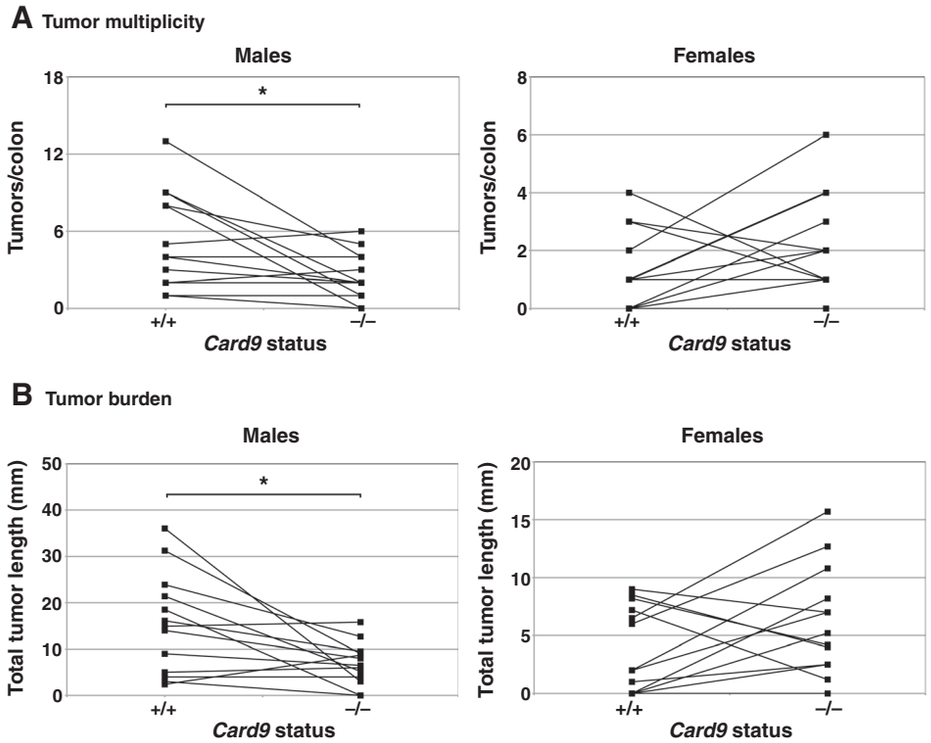

Figure 1.
CARD9 promotes colon tumor multiplicity specifically in male APC^{min} mice. Colons from cocaged, sex- and age-matched CARD9-competent (+/+) and CARD9-deficient (−/−) mice were assessed for colon tumor multiplicity (A) and total colonic tumor burden (B). Values from paired samples are connected by a line; *, $P < 0.05$ by Wilcoxon Signed Rank analysis inclusive of Bonferroni adjustment for location.

Decreased colonic tumor multiplicity in male CARD9-deficient mice

As seen with small-intestinal tumorigenesis, we noted no reduction in colon tumor multiplicity associated with Card9 deficiency [paired t test: t(25) = 1.48, $P = 0.2$]. When we examined the effect of sex on tumor multiplicity, however, an effect was evident ($P = 0.007$). Tumor multiplicity for male mouse pairs showed a statistically significant effect of genotype (Fig. 1A). Average tumor size was unchanged overall ($P = 0.177$) for females ($P = 0.117$) and for males ($P = 0.435$). Consistent with decreased colon tumor multiplicity without changes in average colon tumor size, the total tumor burden in CARD9-deficient mice was decreased in a sex-specific manner (Fig. 1B). By virtue of the correlation between improved mouse viability with decreased colon-tumor incidence in the CARD9-deficient mice, we propose that CARD9 promotes colon tumorigenesis in the APC^{min} mice to negatively impact viability, specifically in male mice.

Plasma IL6 correlates with APC^{min} colon tumorigenesis

CARD9 functions in several proinflammatory pathways. To better clarify which pathway(s) might associate with the male-specific CARD9-dependent increase in colonic tumor incidence, we collected and stored plasma from mice at the time of harvest. With representation of the two sexes (females, $n = 7$; males, $n = 6$), we analyzed plasma cytokine expression. Extremely high plasma cytokine levels might be indicative of an active systemic infection, which could affect viability. Statistical analyses of plasma cytokine levels were performed using the Wilcoxon signed-rank test. As shown in Fig. 2A, we found a male-specific decrease of plasma IL6, G-CSF, and RANTES concentrations in the −/− mice (T = 0, $n = 6$, $P < 0.05$). However, these individual findings did not withstand Bonferroni adjustment. No effect of genotype was evident for cytokine levels in female mice (Fig. 2A and Supplementary Fig. S1A). Only one female −/− mouse had levels that were extremely high for multiple plasma cytokines, suggestive of an active systemic infection. It should be noted that statistical analyses, by the Mann–Whitney U test, indicated no effect of sex on plasma cytokine expression. For individual male mice, no correlation was evident for plasma cytokine levels with either total colonic (Fig. 2B) or small-intestinal (Supplementary Fig. S2B) tumor burden, suggesting that plasma cytokines are not proxy markers of tumor-induced breakdown of the intestinal barrier. These data instead support a model whereby CARD9 functions, directly or indirectly, to increase plasma IL6, G-CSF, and RANTES in male APC^{min} mice.

Decreased T-cell infiltration in Card9-deficient colon tumors

The role of CARD9 in colonic proinflammatory Th17 response is documented (15, 16). Furthermore, IL6 and G-CSF are involved in granulopoeisis induction (17). We therefore examined whether there were differential immune infiltrates in colonic tumors from the male mice. Size- and location-paired tumors from paired male +/+ and −/− mice were isolated and stained for tumor-infiltrating immune cells, including T cells, neutrophils, and macrophages. Statistical analyses of plasma cytokine levels were performed using the Wilcoxon signed-rank test. As shown in Fig. 3, Ki67$^+$ proliferating cancer cells were present at similar levels in +/+ and −/− tumors. Whereas 4 of 5 tumors had decreased Ly6G$^+$ cell infiltration, the levels of this granulocyte marker did not reach statistical significance. In contrast, CD3$^+$ and F4/80$^+$ cells were present at lower frequency

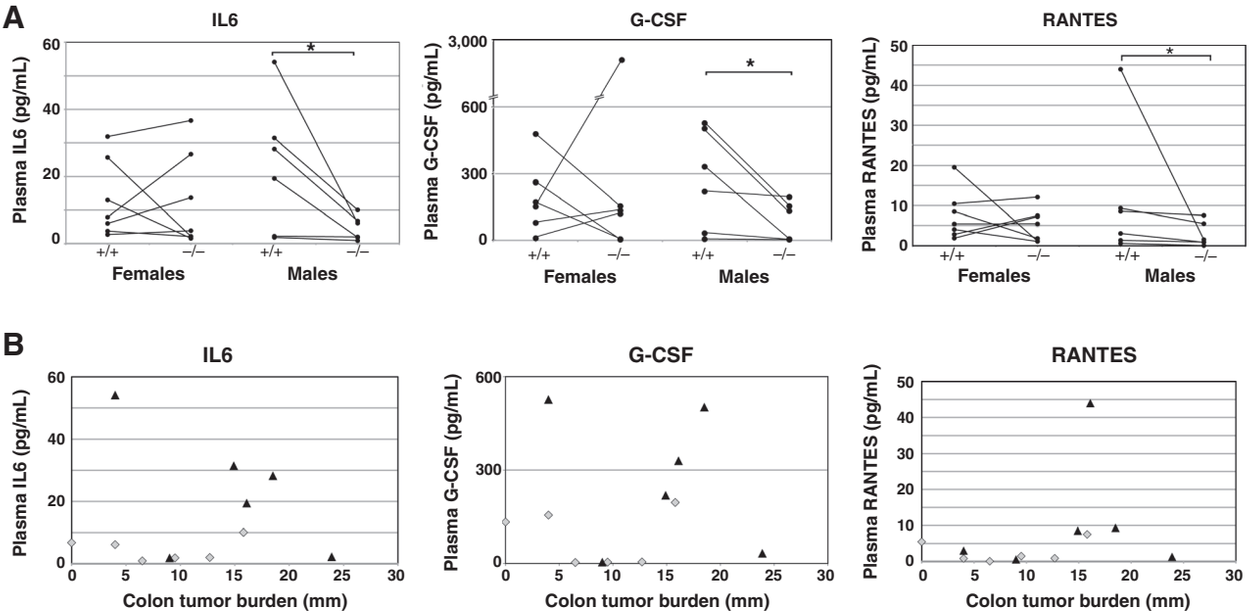

Figure 2.
Sex-specific disruption of plasma cytokine levels. A, plasma levels of IL6, G-CSF, and RANTES were examined for male and female CARD9-competent (+/+) and CARD9-deficient (−/−) APC^{min} mice at endpoint. Results from paired samples are connected by a line. B, plasma cytokine concentrations from male CARD9-competent (▲) and CARD9-deficient mice (◈) were plotted against colonic tumor burden; *, $P < 0.05$ by the Wilcoxon signed-rank test.

in each of the CARD9-deficient tumors as compared with their CARD9-competent controls. In the two pairs of size- and location-paired colon tumors from female mice, we did not see an apparent difference in leukocyte infiltration (Supplementary Table S1). These data suggest that CARD9 is promoting T-cell and macrophage infiltration into the tumor, which may advance early adenoma growth, and thereby tumor initiation in APC^{min} mice.

Discussion

Gender effects in colorectal cancer have been long recognized, but commonly overlooked aspects of the disease. Nevertheless, it is evident from the clinical studies that unknown environmental cues, likely involving inflammation, can induce a male bias in colorectal cancer incidence and severity of prognosis (18–20). This study presents the first gene ablation, to our knowledge, that caused a male-specific effect on adenomas in mice. CARD9 deletion resulted in increased viability and fewer colonic tumors in the APC^{min} background. In these mice, no effect was evident in females or in male small-intestinal tumors. Also, tumor size did not seem to be affected, suggesting a role in the early stage of adenoma formation. Our data indicate CARD9-dependent promotion of specific plasma cytokines, namely IL6, G-CSF, and RANTES, in APC^{min} male mice. Furthermore, decreased infiltration of immune cells was evident in male CARD9-deficient tumors compared with their paired CARD9-competent tumors of similar sizes and locations.

Immune infiltration of APC^{min} colonic tumors differs significantly between small-intestinal and colonic tumors (7). In this context, our finding of a colon-specific effect of an inflammation gene is not surprising. Indeed, it is somewhat surprising that other inflammation pathways do not manifest a similar colon-specific effect (10, 11, 14, 21).

Other inflammation-related genes that have been examined in the APC^{min} model either show no apparent sex specificity or show a female-specific effect (8, 11, 12). The male-specific effect in our study is somewhat surprising, but may have important clinical implications. Although we have found no reported gender bias in human FAP, demographics indicate that there are gender-based differences in human colorectal cancer (18, 19). Countries with high rates of colorectal cancer have a greater incidence among males (18). Furthermore, many studies have shown that men have poorer survival with colorectal cancer (18), as well as a greater associated risk from chronic inflammation (20). Taken together, the evidence suggests that males have a genetic predisposition to colorectal cancer under specific environmental conditions. It will be interesting to definitively answer whether a gender bias exists for FAP patients and whether risk correlates with differences in immune infiltration and/or known CARD9 polymorphisms.

We propose that this CARD9 deficiency may allow laboratory investigation of this important clinical phenomenon without confounding effects of gender-biased diet and lifestyle. Intriguingly, CARD9 has an established role in *Candida* response (15, 22), and simple sugars, prevalent in Western diets, can increase the presence of intestinal *Candida* (23, 24). Although this is just one of many microbes that may enhance CARD9-dependent inflammation, it is an attractive hypothesis that *Candida* species promote male-specific colorectal cancer associated with Western diets. Further elucidation of gut microbiota influence on this male-specific effect is warranted.

The plasma IL6 association with male colon adenoma formation in APC^{min} mice could be extremely relevant to

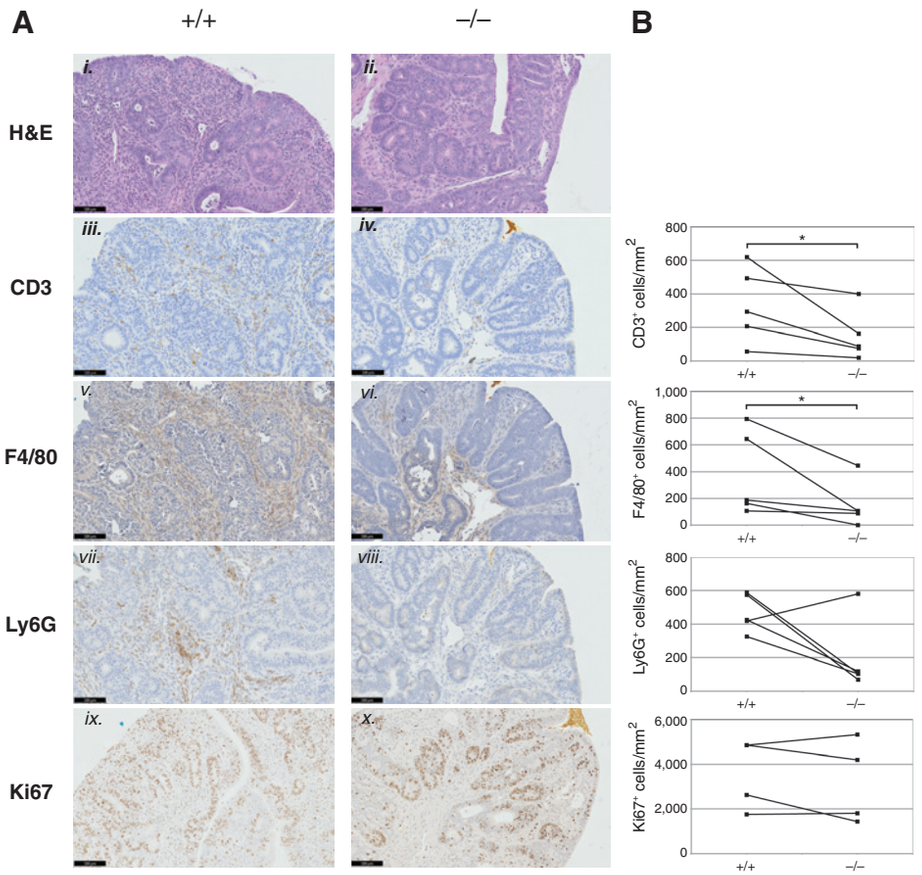

Figure 3.
CARD9-dependent changes in colon tumor immune infiltration. A, representative histologic images of colonic tumors from CARD9-competent (i, iii, v, vii, and ix) and CARD9-deficient (ii, iv, vi, viii, and x) stained with hematoxylin and eosin (i and ii), anti-CD3 (iii and iv), anti-F4/80 (v and vi), anti-Ly6G (vii and viii), or anti-Ki67 (ix and x). Scale bar, 100 microns. B, quantification of tumor infiltration from male CARD9-competent (+/+) and CARD9-deficient (–/–) APC^{min} colon tumors. Results from paired samples are connected by lines; *, $P < 0.05$ by the Wilcoxon signed-rank test.

understanding this phenomenon. In other studies, increased plasma IL6 is coincident with cachexia in male APC^{min} mice but not female APC^{min} mice (25). It may be that CARD9 is functioning upstream of plasma IL6 in male APC^{min} mice to enhance cachexia and reduce viability, potentially through Th17-cell induction. Unfortunately, our study was not designed to investigate cachexia. Further studies will be required to test this hypothesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: P.T. Reilly
Development of methodology: P.T. Reilly
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V.I. Leo, H. Bergmann, P.Y. Cheah, M.H. Chew, J. Ruland, P.T. Reilly
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V.I. Leo, S.H. Tan, H. Bergmann, P.Y. Cheah, M.H. Chew, K.H. Lim, P.T. Reilly
Writing, review, and/or revision of the manuscript: S.H. Tan, H. Bergmann, P.Y. Cheah, M.H. Chew, P.T. Reilly
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V.I. Leo, K.H. Lim, P.T. Reilly
Study supervision: P.T. Reilly

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