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1 **CARD9 in Neutrophils Protects from Colitis**  
2 **and Controls Mitochondrial Metabolism and Cell Survival**

3

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34

35

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38

39

#### 40 **ABSTRACT**

41 **Objectives** : Inflammatory bowel disease (IBD) results from a combination of genetic predisposition,  
42 dysbiosis of the gut microbiota and environmental factors, leading to alterations in the gastrointestinal  
43 immune response and chronic inflammation. Caspase recruitment domain 9 (*Card9*), one of the IBD  
44 susceptibility genes, has been shown to protect against intestinal inflammation and fungal infection.  
45 However, the cell types and mechanisms involved in the CARD9 protective role against inflammation  
46 remain unknown.

47 **Design** : We used dextran sulfate sodium (DSS)-induced and adoptive transfer colitis models in total  
48 and conditional CARD9 knock-out mice to uncover which cell types play a role in the CARD9  
49 protective phenotype. The impact of *Card9* deletion on neutrophil function was assessed by an *in vivo*  
50 model of fungal infection and various functional assays, including endpoint dilution assay, apoptosis  
51 assay by flow cytometry, proteomics and real time bioenergetic profile analysis (Seahorse).

52 **Results** : Lymphocytes are not intrinsically involved in the CARD9 protective role against colitis.  
53 CARD9 expression in neutrophils, but not in epithelial or CD11c+ cells, protects against DSS-induced

54 colitis. In the absence of CARD9, mitochondrial dysfunction in neutrophils leads to their premature  
55 death through apoptosis, especially in oxidative environment. The decrease of functional neutrophils in  
56 tissues could explain the impaired containment of fungi and increased susceptibility to intestinal  
57 inflammation.

58 **Conclusion** : These results provide new insight into the role of CARD9 in neutrophil mitochondrial  
59 function and its involvement in intestinal inflammation, paving the way for new therapeutic strategies  
60 targeting neutrophils.

61

62

### 63 **Summary box**

#### 64 **1. What is already known about this subject?**

- 65 • Inflammatory bowel disease (IBD) results from genetic predisposition, microbiota dysbiosis  
66 and environmental factors, but the alterations of the immune response leading to chronic  
67 intestinal inflammation are still not fully understood.
- 68 • Caspase recruitment domain 9 (*Card9*), one of the IBD susceptibility genes, has been shown  
69 to protect against intestinal inflammation and fungal infection.
- 70 • However, the cell types and cellular mechanisms involved in the CARD9 protective role  
71 against inflammation remain unknown.

#### 72 **2. What are the new findings?**

- 73 • CARD9 expression in neutrophils, but not in lymphocytes, epithelial cells or CD11c+ cells,  
74 protects against DSS-induced colitis.
- 75 • In the absence of CARD9, mitochondrial dysfunction in neutrophils leads to their premature  
76 death through apoptosis, especially in oxidative environment.
- 77 • The decrease of functional neutrophils in tissues could explain the impaired containment of  
78 fungi and increased susceptibility to intestinal inflammation.

#### 79 **3. How might it impact on clinical practice in the foreseeable future?**

- 80 • These results provide new insight into the role of CARD9 in neutrophil mitochondrial function  
81 and its involvement in intestinal inflammation.
- 82 • Understanding the role of neutrophils in chronic inflammation could lead to innovative  
83 therapeutic strategies targeting these key immune cells for various complex diseases.

84

## 85 INTRODUCTION

86 Inflammatory bowel disease (IBD) results from a combination of genetic predisposition,  
87 dysbiosis of the gut microbiota and environmental factors, leading to alterations in the gastrointestinal  
88 immune response and chronic inflammation<sup>1,2</sup>. Especially, the innate compartment of the immune  
89 system has been involved in IBD development, with a role for dendritic cells, macrophages and  
90 neutrophils<sup>3-6</sup>. Neutrophils, one of the most abundant and important mediators of innate immunity, are  
91 professional phagocytes that mount the acute inflammatory response and act as the first line of  
92 defense against invading pathogens<sup>7,8</sup>. An impaired neutrophil function may result in limited pathogen  
93 clearance and fuel a chronic inflammatory response with excessive lymphocyte activation. Patients  
94 with congenital disorders in neutrophil function such as chronic granulomatous disease (CGD) often  
95 develop IBD-like phenotypes<sup>9-12</sup>. Moreover, functional defects have been observed in neutrophils from  
96 IBD patients, including impaired chemotaxis, migration, phagocytosis or ROS production<sup>4,5</sup>.

97 CARD9, one of the numerous IBD susceptibility genes, encodes an adaptor protein that  
98 integrates signals downstream of pattern recognition receptors<sup>13-18</sup>. Especially, CARD9 is involved in  
99 the host defense against fungi via C-type lectin sensing<sup>19,20</sup>. CARD9 polymorphisms in humans are  
100 associated with multiple susceptibilities including IBD<sup>21</sup>, whereas loss-of-function mutations are  
101 associated with invasive fungal infections caused by species such as *Candida albicans*<sup>21-24</sup>. CARD9  
102 was shown to mediate its protective functions, at least in part, through the induction of adaptive Th17  
103 cell responses<sup>22,23,25</sup>. *Card9*<sup>-/-</sup> mice are more susceptible to colitis due to impaired IL-22 production  
104 and have an increased load of gut-resident fungi<sup>25</sup>. Indeed, CARD9 affects the composition and  
105 function of the gut microbiota, altering the production of anti-inflammatory microbial metabolites<sup>26,27</sup>.  
106 However, the cell types involved in the CARD9 protective role against intestinal inflammation remain  
107 unknown.

108 In this work, we show that CARD9 expression in neutrophils, but not in epithelial or CD11c+  
109 cells such as dendritic cells, protects against dextran sulfate sodium (DSS)-induced colitis in mice.  
110 The absence of CARD9 impacts neutrophil capacity to contain fungal dissemination, notably by  
111 impairing neutrophil mitochondrial function and survival. Indeed, *Card9* deletion induces a basal  
112 overactivation of mitochondria, increasing mitochondrial dysfunction and apoptosis in neutrophils.

113 These results provide new insight into the role of CARD9 in neutrophil mitochondrial function and its  
114 consequences in intestinal inflammation.

115

## 116 RESULTS

### 117 Lymphocytes have no intrinsic role in the *Card9*<sup>-/-</sup> susceptibility to colitis

118 CARD9 was previously reported to be mainly expressed in myeloid cells, especially dendritic  
119 cells, macrophages and neutrophils<sup>14,28</sup>. Using qRT-PCR analyses in various C57BL/6 mouse organs,  
120 we confirmed that *Card9* is mainly expressed in immune organs such as bone marrow, spleen and  
121 distal small intestine (ileum), but is low at baseline in proximal and mid small intestine, caecum, colon,  
122 stomach and liver, and not detectable in *Card9*<sup>-/-</sup> tissues (Fig. S1A). Consistently, western-blot analysis  
123 showed the expression of CARD9 protein in bone marrow, spleen and distal small intestine of WT  
124 mice (Fig. S1B). To dissect *Card9* expression at the cellular level, we sorted immune cell populations  
125 from spleen and bone marrow of WT mice. *Card9* is highly expressed in neutrophils (Ly6G<sup>+</sup>CD11b<sup>+</sup>  
126 cells), macrophages (CD11b<sup>+</sup>F4/80<sup>+</sup> cells), CD11c<sup>+</sup> cells, including dendritic cells, and monocytes  
127 (CD11b<sup>hi</sup>F4/80<sup>+</sup> cells); but the expression is lower in innate or adaptive lymphocytes (CD3<sup>+</sup>TCRγδ<sup>+</sup>  
128 lymphoid cells, CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells, CD3<sup>-</sup>CD19<sup>+</sup> B cells) (Fig. S1C). Thus, CARD9 likely  
129 plays a major role within the myeloid immune compartment.

130 Previous studies from our group and others started to investigate the role of *Card9* in murine  
131 models of experimental colitis, showing that *Card9* deletion increases colitis susceptibility<sup>25,26,29</sup>. In  
132 order to decipher the respective roles of lymphocytes and myeloid cells in the *Card9* susceptibility to  
133 intestinal injury and inflammation, we first induced colitis with DSS in *Rag2*<sup>-/-</sup> and *Rag2*<sup>-/-</sup>*xCard9*<sup>-/-</sup> mice  
134 that are deficient in functional T and B cells. Mice were euthanized after receiving 3% DSS in drinking  
135 water for 7d, as the severity limit was reached for the *Rag2*<sup>-/-</sup>*xCard9*<sup>-/-</sup> group. Indeed, disease severity  
136 (defined by weight loss, DAI (Disease Activity Index) score, colon length, and histologic score) was  
137 strongly increased in *Rag2*<sup>-/-</sup>*xCard9*<sup>-/-</sup> mice compared to *Rag2*<sup>-/-</sup> mice (Fig. 1A-E). In an adoptive  
138 transfer model of colitis, in which *Rag2*<sup>-/-</sup> mice lacking functional lymphocytes received T cells either  
139 from WT or *Card9*<sup>-/-</sup> mice, no difference was observed on colitis severity (Fig. 1F), meaning that *Card9*  
140 expression in T cells does not impact colitis susceptibility. However, the transfer of WT T cells into  
141 *Rag2*<sup>-/-</sup>*xCard9*<sup>-/-</sup> recipient mice did aggravate colitis compared to *Rag2*<sup>-/-</sup> simple KO recipients, with a

142 significantly stronger weight loss (Fig. 1F). These results demonstrate that *Card9* mediates its  
143 protective role against colitis through the innate immunity compartment, although its role in intestinal  
144 epithelial cells cannot be ruled out.

145

#### 146 ***Card9* expression in neutrophils, but not in epithelial or CD11c<sup>+</sup> cells, protects against colitis**

147 Based on these findings, we generated conditional KO mice using the cre-lox technology, and  
148 obtained mouse strains defective for *Card9* either in epithelial cells only (Villin<sup>cre</sup>*Card9*<sup>lox</sup> line),  
149 CD11c-expressing cells only, including dendritic cells, macrophages and monocytes  
150 (CD11c<sup>cre</sup>*Card9*<sup>lox</sup> line), or neutrophils only (Mrp8<sup>cre</sup>*Card9*<sup>lox</sup> line). To validate their phenotypes,  
151 we isolated epithelial cells from the colonic lamina propria of *Card9*<sup>Villin<sup>cre</sup> and *Card9*<sup>Villin<sup>wt</sup> mice, or used  
152 MACS separation columns to isolate either CD11c<sup>+</sup> or Ly6G<sup>+</sup> cell fractions from spleen or bone marrow  
153 of *Card9*<sup>CD11c<sup>wt</sup> and *Card9*<sup>CD11c<sup>cre</sup> mice or *Card9*<sup>Mrp8<sup>wt</sup> and *Card9*<sup>Mrp8<sup>cre</sup> mice, respectively. We  
154 performed qRT-PCR on these cell fractions (Fig. S2A), and western-blot analyses on the Ly6G<sup>+</sup> and  
155 Ly6G<sup>low/-</sup> fractions of *Card9*<sup>WT</sup>, *Card9*<sup>-/-</sup>, *Card9*<sup>Mrp8<sup>wt</sup> and *Card9*<sup>Mrp8<sup>cre</sup> mice (Fig. S2B). Results  
156 confirmed that *Card9* deletion is restricted to the expected cell types (Fig. S2A-B). Purity of  
157 Ly6G<sup>+</sup>CD11b<sup>+</sup> neutrophils isolated from the bone marrow of C57Bl/6 mice reached 95% by flow  
158 cytometry (Fig. S2C).</sup></sup></sup></sup></sup></sup></sup></sup>

159 We then assessed the susceptibility of these newly generated mice strains in a model of  
160 intestinal inflammation. DSS was administered for 7 days, followed by additional 5 days in which DSS  
161 was discontinued. The deletion of *Card9* in epithelial or CD11c<sup>+</sup> cells did not affect mouse  
162 susceptibility to colitis (Fig. 2A-B and S2D). However, the deletion of *Card9* in neutrophils aggravates  
163 colitis compared to WT littermate controls, with significantly increased weight loss from day 8 (Fig.  
164 2C), DAI score from day 5 (Fig. 2C), and histological score (Fig. 2E-F), as well as decreased colon  
165 length (Fig. 2D). Thus, the expression of *Card9* in neutrophils plays a crucial role in the protection  
166 against intestinal inflammation. The expression of myeloperoxidase (MPO), an anti-microbial enzyme  
167 abundantly expressed in neutrophils, was increased in *Card9*<sup>Mrp8<sup>cre</sup> compared to *Card9*<sup>Mrp8<sup>wt</sup> colon  
168 tissue at day 12, indicating a more important presence or activation of neutrophils in the absence of  
169 *Card9* (Fig. 2G). Similarly, the expression of the inflammatory marker lipocalin (*Lcn2*) was increased in  
170 *Card9*<sup>Mrp8<sup>cre</sup> colon tissue at day 12 (Fig. 2H).</sup></sup></sup>



171           These results suggest that *Card9*-deficient neutrophils are efficiently recruited to the inflamed  
172 tissue but likely exhibit functional defects preventing them from adequately controlling microbial  
173 invaders and thus maintaining inflammation within the intestinal mucosa. We previously showed that  
174 the gut microbiota of total *Card9*<sup>-/-</sup> KO lineage mice exhibit an altered production of AhR agonists  
175 compared to WT<sup>26</sup>. However, no difference was observed between *Card9*<sup>Mrp8wt</sup> and *Card9*<sup>Mrp8cre</sup> mice  
176 (Fig. S2E), suggesting that this aspect of the phenotype is not intrinsically related to the role of CARD9  
177 in neutrophils.

178

### 179 ***Card9* deletion affects the number of activated neutrophils in the inflamed colon**

180           To investigate neutrophil function in WT mice during DSS-induced inflammation, we analyzed  
181 RNA expression of the neutrophil-specific genes *Lcn2*, *Cxcr2* and *S100A8* in colon tissue at days 0, 4,  
182 7, 9, 12 and 16 of DSS-induced colitis (Fig. 3A and S2F). The neutrophil recruitment was maximal at  
183 day 9, corresponding to the peak of clinical inflammation, and remained high up to day 16 (Fig. 3A).  
184 Histological sections of the distal colon confirmed these findings (Fig. 3B). The fact that inflammation  
185 was higher in *Card9*<sup>Mrp8cre</sup> than in *Card9*<sup>Mrp8wt</sup> mice (Fig. S2E), and that *Lcn2*, *Cxcr2* and *S100a8*  
186 expression at day 9 in colon tissue were similar in both genotypes (Fig. 3C) excludes the hypothesis of  
187 a defect of neutrophil recruitment in *Card9*<sup>Mrp8cre</sup> mice. We then examined the immune cell populations  
188 recruited to the colon lamina propria (LP) of *Card9*<sup>Mrp8cre</sup> versus *Card9*<sup>Mrp8wt</sup> mice at day 9 of colitis.  
189 Surprisingly, although the total number of neutrophils was similar in the two genotypes, the percentage  
190 and count of mature and activated Ly6G<sup>+</sup>CD11b<sup>+</sup> neutrophils was decreased in the colon LP of  
191 *Card9*<sup>Mrp8cre</sup> (Fig. 3D-E). Moreover, the expression levels of both Ly6G and CD11b surface proteins,  
192 two major neutrophil maturation and activation markers, were significantly reduced in the overall  
193 neutrophil population, as shown by decreased MFIs (for Mean Fluorescence Intensity) (Fig. 3F-G).  
194 These findings suggest a structural or functional defect in neutrophils deleted for *Card9*.

195

### 196 ***C. albicans* killing capacities are impacted by *Card9* deletion in neutrophils**

197           To investigate the defect caused by *Card9* deletion in *Card9*<sup>Mrp8cre</sup> compared to *Card9*<sup>Mrp8wt</sup>  
198 neutrophils, we developed several *in vitro* assays with Ly6G<sup>+</sup> neutrophils purified from mouse bone

199 marrow using MACS separation columns. Immunofluorescence, scanning electron microscopy (SEM)  
200 and transmission electron microscopy (TEM) analyses did not reveal noticeable structural differences  
201 between both genotypes (Fig. S3). On the functional side, we tested the neutrophils ability to kill  
202 microorganisms, especially fungi, as CARD9 plays a crucial role in host defense against fungal  
203 infection in both humans and mice<sup>25,20</sup>. Indeed, *C. albicans* killing capacities are strongly affected by  
204 *Card9* deletion in neutrophils (Fig. 4). An endpoint-dilution survival assay in 96-well plates revealed  
205 that twice as many *C. albicans* cfu (colony forming unit) do survive after 24h co-incubation with *Card9*  
206 <sup>-/-</sup> or *Card9*<sup>Mrp8cre</sup> neutrophils compared to *Card9*WT or *Card9*<sup>Mrp8wt</sup> controls, respectively (Fig. 4A-B). A  
207 killing assay using the cfu counting method on agar plates confirmed that *Card9*<sup>Mrp8cre</sup> neutrophils have  
208 impaired abilities to kill *C. albicans* compared to *Card9*<sup>Mrp8wt</sup> (Fig. 4C). *Card9* deletion in neutrophils did  
209 not impact phagocytosis *per se*, as shown by flow cytometry experiments using fluorescein-  
210 isothiocyanate (FITC)-conjugated zymosan (from *Saccharomyces cerevisiae* cell wall), or cultures of  
211 live *C. albicans*-GFP or *E. coli*-GFP (Fig. S4A-B). Moreover, we did not observe a difference in the  
212 levels of Reactive Oxygen Species (ROS) production over time between neutrophils of the two  
213 genotypes in response to phorbol myristate acetate (PMA), a PKC-dependent neutrophil activator, or  
214 zymosan, a fungal stimulus (Fig. S4C-E). Even though *Card9* is involved in autophagy<sup>30</sup>, this cellular  
215 process did not seem to be affected by *Card9* deletion in neutrophils *in vitro*, as shown by the  
216 normality of p62 and LC3BII/I ratio on western-blot (Fig. S4F).

217 We thus followed the track of fungal killing to investigate whether the absence of *Card9*  
218 expression in neutrophils drives a general impairment of the immune system to control infection.  
219 During a DSS-induced colitis model, we detected a slight but non-significant increase in total  
220 fungi/bacteria DNA ratio at days 7 and 12 in the feces of *Card9*<sup>Mrp8cre</sup> compared to *Card9*<sup>Mrp8wt</sup> mice  
221 (Fig. S4G). These data suggest a reduced ability of *Card9*<sup>Mrp8cre</sup> mice to contain fungi expansion in the  
222 inflamed intestine. However, this effect might be difficult to point out due to the very low fungal  
223 abundance in SPF mouse microbiota. Thus, to go further and reveal a potential phenotype, we  
224 induced colitis in mice treated with a broad-spectrum antibiotic and antifungal cocktail and gavaged  
225 with *C. albicans* to expand the intestinal fungal load (Fig. 4D). The increased colitis severity in  
226 *Card9*<sup>Mrp8cre</sup> mice was maintained in this setting (Fig. 4E-G). *C. albicans* load was slightly increased  
227 (although not statistically significant) in the caecal content (Fig. 4H-I), colon and caecal tissues (Fig.  
228 4J) of *Card9*<sup>Mrp8cre</sup> mice, and the number of cfu recovered from liver, spleen and kidney were

229 significantly higher compared to *Card9*<sup>Mrp8wt</sup> mice (Fig. 4K). These results show that *Card9* expression  
230 in neutrophils is crucial to control the fungal load in the inflamed gut, the direct translocation of *C.*  
231 *albicans* from the gut to the liver, and to avoid its systemic dissemination.

232

### 233 **The absence of *Card9* impacts neutrophils survival by increasing apoptosis**

234 To explore the mechanisms underlying the impaired capacity of *Card9*<sup>Mrp8cre</sup> neutrophils to kill  
235 *C. albicans* despite intact phagocytosis, autophagy, and ROS production, we investigated neutrophils  
236 survival rates by flow cytometry using an AnnexinV-FITC assay coupled to Live/Dead staining (Fig. 5).  
237 AnnexinV reveals ongoing apoptosis, whereas Live/Dead only stains permeable dead cells.  
238 Interestingly, after 1h incubation at 37°C, *Card9*<sup>Mrp8cre</sup> neutrophils showed a significant increase in  
239 AnnexinV-FITC MFI compared to *Card9*<sup>Mrp8wt</sup> neutrophils (Fig. 5A-B). Consistently, an increase in  
240 percentages of apoptotic (Q1, AnnexinV<sup>+</sup>LD<sup>-</sup>) and dead (late apoptotic/necrotic) neutrophils (Q2,  
241 AnnexinV<sup>+</sup>LD<sup>+</sup>), and a decrease in viable neutrophils (Q4, AnnexinV<sup>-</sup>LD<sup>-</sup>), were observed for the  
242 *Card9*<sup>Mrp8cre</sup> genotype (Fig. 5C). Interestingly, the surface expression of the CD62L marker (a surface  
243 protein that is lost upon cell activation) was reduced in *Card9*<sup>Mrp8cre</sup>, with a lower percentage of CD62L<sup>+</sup>  
244 cells and a higher percentage of CD62L<sup>-</sup> cells (Fig. 5D). These results suggest an excessive basal  
245 activation of *Card9*<sup>Mrp8cre</sup> compared to *Card9*<sup>Mrp8wt</sup> neutrophils. Similar results were obtained with  
246 *Card9*<sup>-/-</sup> versus *Card9*WT neutrophils (Fig. S5A-D), confirming the impact of *Card9* on neutrophil  
247 survival.

248 To go further, we performed a proteomic analysis on *Card9*<sup>Mrp8cre</sup> versus *Card9*<sup>Mrp8wt</sup>  
249 neutrophils after 1h incubation at 37°C. This approach revealed that a large number of proteins related  
250 to cellular metabolism pathways were differentially regulated between both genotypes. Indeed, a Gene  
251 Ontology (GO) functional analysis showed the enrichment in numerous cellular metabolic processes,  
252 both in unstimulated and stimulated conditions, among proteins statistically down- or up-regulated in  
253 *Card9*<sup>Mrp8cre</sup> compared to *Card9*<sup>Mrp8wt</sup> neutrophils (Fig. 5E and S5E). Especially, we observed a high  
254 prevalence of mitochondrial proteins among the differentially regulated candidates between the two  
255 genotypes (Fig. 5F), suggesting that neutrophil mitochondrial functions are impacted by the absence  
256 of *Card9*.

257

## 258 **Card9 controls neutrophil survival by affecting mitochondrial function**

259 Subsequently, we analyzed neutrophil mitochondrial function using MitoTracker Green (reflecting  
260 mitochondrial mass) and MitoTracker Red (reflecting mitochondrial membrane potential) markers in a  
261 flow cytometry assay (Fig. 6A). No difference was observed in terms of MFIs, but we observed a  
262 significant increase in the percentage of MitoGreen<sup>+</sup>MitoRed<sup>-</sup> neutrophils (with dysfunctional or  
263 metabolically inactive mitochondria) in the *Card9*<sup>Mrp8cre</sup> compared to the *Card9*<sup>Mrp8wt</sup> genotype (Fig. 6A).  
264 Tetramethylrhodamine methyl ester (TMRM) assay evaluating the mitochondrial membrane potential  
265 confirmed the increase in apoptotic, metabolically stressed cells (TMRM<sup>-</sup>) among *Card9*<sup>Mrp8cre</sup>  
266 neutrophils (Fig. 6B). These results suggest that the survival defect of *Card9*<sup>Mrp8cre</sup> neutrophils may be  
267 due to an altered energetic metabolism. Real-time bioenergetic profile analysis using Seahorse  
268 technology showed that *Card9*<sup>Mrp8cre</sup> neutrophils have a higher basal Oxygen Consumption Rate  
269 (OCR) during a cell mito stress assay (Fig. 6C), indicating a higher oxidative phosphorylation activity  
270 compared to *Card9*<sup>Mrp8wt</sup> neutrophils. Basal respiration and ATP production rate were both highly  
271 increased in *Card9*<sup>Mrp8cre</sup> neutrophils (Fig. 6D). Moreover, a Seahorse real-time ATP rate assay  
272 demonstrated that *Card9*<sup>Mrp8cre</sup> neutrophils have an increased mitochondrial ATP (mitoATP) production  
273 rate, whereas glycolytic ATP (glycoATP) production rate is only mildly decreased, as shown by the  
274 ATP rate index and the energetic map (Fig. 6E). A Seahorse glycolytic rate assay confirmed the  
275 moderate decrease of the basal Extracellular Acidification Rate (ECAR) of *Card9*<sup>Mrp8cre</sup> neutrophils  
276 (Fig. S6A). However, metabolomics analysis on neutrophil supernatants incubated for 24h revealed a  
277 decreased in lactate production, leading to a reduced lactate/glucose ratio in *Card9*<sup>Mrp8cre</sup> neutrophils  
278 (Fig. S6B). Similar results were obtained when we compared *Card9*<sup>WT</sup> and *Card9*<sup>-/-</sup> neutrophils in the  
279 assays described above (Fig. S6C-E). Thus, *Card9* deletion in neutrophils induces an overactivation of  
280 mitochondria and tends to reduce glycolytic activity, which is the major energy source of normal  
281 neutrophils. Blocking glycolysis with 2DG (2-Deoxy-D-glucose) increased the apoptotic rate of  
282 *Card9*<sup>Mrp8wt</sup> but not of *Card9*<sup>Mrp8cre</sup> neutrophils; showing that glycolysis is more essential to *Card9*<sup>Mrp8wt</sup>  
283 than *Card9*<sup>Mrp8cre</sup> neutrophils (Fig.6F). Conversely, blocking the mitochondrial respiratory chain with  
284 oligomycinA increased the apoptotic rate of *Card9*<sup>Mrp8cre</sup> but not of *Card9*<sup>Mrp8wt</sup> neutrophils,  
285 demonstrating the crucial role of mitochondria as an energy source in *Card9*<sup>Mrp8cre</sup> neutrophils (Fig.  
286 6G). Thus, contrary to *Card9*<sup>Mrp8wt</sup> neutrophils that mainly rely on glycolysis as a source of energy,  
287 *Card9*<sup>Mrp8cre</sup> neutrophils present an altered metabolism with an overactivation of mitochondria

288 associated with a dysfunctional state, leading to apoptosis. Intestinal inflammation is associated with a  
289 high degree of oxidative stress. To evaluate the effect of oxidative stress on the phenotype of  
290 *Card9*<sup>Mrp8cre</sup> neutrophils, they were treated for 1h with H<sub>2</sub>O<sub>2</sub>. The increased apoptosis and necrosis  
291 rates observed in *Card9*<sup>Mrp8cre</sup> neutrophils were stronger in oxidative stress than in basal condition  
292 (Fig. 6H). These results show that the role of CARD9 in the survival capacity of neutrophils, especially  
293 in oxidative conditions, is mediated by effects on mitochondrial functions.

294

## 295 **DISCUSSION**

296 Altogether, this study reveals that, in the absence of CARD9, mitochondrial dysfunction in  
297 neutrophils leads to their premature death through apoptosis, especially in an oxidative environment.  
298 The decrease of functional neutrophils in the gut affects fungal containment and increases  
299 susceptibility to intestinal inflammation. CARD9 polymorphisms are associated with IBD. Mouse  
300 studies showed the contribution of CARD9 to host defense and intestinal barrier, notably through the  
301 production of IL-22 and the modulation of the gut microbiota metabolic activity<sup>25,26</sup>. However, the role  
302 of CARD9 in disease pathogenesis has not been elucidated at the cellular level. Our study reveals that  
303 *Card9* deletion in neutrophils, contrarily to epithelial or CD11c<sup>+</sup> cells, increases the susceptibility to  
304 DSS-induced colitis. We thus focused on studying the role of CARD9 expression in neutrophil  
305 functionality. Indeed, neutrophils have been studied in different models of IBD and fungal infection, but  
306 their direct contribution to pathogenesis and the role of CARD9 in these mechanisms remain poorly  
307 understood<sup>31,4,5,32</sup>.

308 Human CARD9 deficiency results in impaired neutrophil fungal killing, leading to a selective  
309 defect to contain invasive fungal infection<sup>33</sup>. In both humans and mice, CARD9 is required in microglia  
310 for neutrophil recruitment and control of fungal infection in the central nervous system<sup>34,35</sup>. CARD9  
311 signaling was also involved in neutrophil phagocytosis and NETosis (Neutrophil extracellular traps)  
312 functions, enhancing mouse survival to a lethal dose of *C. albicans*<sup>36</sup>. Moreover, CARD9 expression in  
313 neutrophils promotes autoantibody-induced arthritis and dermatitis in mice<sup>37</sup>, and inflammation levels  
314 in a mouse model of neutrophilic dermatosis<sup>38</sup>. In line with these studies, we found that *Card9* deletion  
315 affects the capacity of neutrophils to kill fungi *in vitro* and *in vivo*, with no impact on neutrophil  
316 structure, ROS production, autophagy or phagocytosis. We have not examined the role of CARD9 in

317 chemotaxis because, in the context of fungal infection in patients with CARD9 deficiency, neutrophil-  
318 intrinsic chemotaxis was not affected<sup>24</sup>. In DSS-induced colitis, *Card9* deficiency in neutrophils does  
319 not impact their recruitment to the colon, but reduces the number of mature neutrophils. Indeed, we  
320 discovered that the absence of CARD9 increases apoptosis rates in neutrophils, and that this  
321 premature death was caused by mitochondria overactivation. Indeed, the intrinsic pathway of  
322 apoptosis is initiated by the permeabilization of mitochondria, which releases proapoptotic factors into  
323 the cytosol<sup>39,40</sup>. Other CARD proteins mediate apoptotic signaling through CARD-CARD domain  
324 interactions<sup>13</sup>. In addition to mediating inflammation, CARD9, a member of the CARD proteins family,  
325 was recently shown to inhibit mitochondria-dependent apoptosis of cardiomyocytes under oxidative  
326 stress<sup>41</sup>. Here, we show that CARD9 also mediates mitochondrial function and apoptosis in  
327 neutrophils, especially in an oxidative environment. Further investigation is required to fully elucidate  
328 the impact of mitochondria overactivation on neutrophil function, especially *in vivo*. The impact of  
329 *Card9* on neutrophil mitochondrial function might not fully explain the increased susceptibility of  
330 *Card9*<sup>Mrp8cre</sup> mice to DSS colitis. Moreover, other cell types are likely involved in the *Card9*<sup>-/-</sup> mice  
331 phenotype, explaining the impact of *Card9* deletion on microbiota metabolic activity, as we show that  
332 this is not dependent on neutrophils<sup>25,26</sup>.

333 Neutrophils contain very few mitochondria compared to other leukocytes, and depend mainly  
334 on glycolysis to produce ATP, which is essential to perform their designated tasks. This allows energy  
335 generation in a low-oxygen environment and keeps oxygen available for neutrophil effector  
336 functions<sup>42</sup>. Thus, the dependence of neutrophils on glycolysis could be an adaptation to allow oxygen  
337 to be used in the anti-microbial response rather than entering oxidative phosphorylation<sup>42</sup>. Here, we  
338 show that in the absence of CARD9, mitochondria are overactivated in neutrophils, leading to their  
339 premature death and the loss of their anti-microbial functions. Alternatively, excessive oxidative  
340 phosphorylation could also lead to an overactivation of neutrophils by increasing ATP production,  
341 especially in inflammatory environments where their activity is typically tightly controlled by the low  
342 oxygen pressure. Neutrophil overactivation could damage the surrounding tissues and exacerbate  
343 inflammation, explaining the increased susceptibility to intestinal colitis in the absence of *Card9*. In  
344 humans, the « glycogen storage disease type Ib » induces a functional defect of neutrophils due to  
345 glycolysis dysfunction and impaired energy homeostasis<sup>43</sup>. This disease is associated with IBD-like  
346 phenotypes, highlighting the importance of neutrophil metabolism in intestinal health<sup>44,43</sup>.

347 Immunometabolism is a central concept as immunity and metabolism impact each other in both ways :  
348 (i) energetic metabolism impacts immune function, which is well-documented for lymphocytes and  
349 macrophages<sup>45</sup>, but not for neutrophils; and (ii) we show that a protein known for its roles in innate  
350 immunity, CARD9, can also impact cell metabolism. Further investigation is required to understand  
351 how CARD9 does interact with mitochondrial function in a direct or indirect manner.

352 Neutrophils are involved in various diseases, including infection, cardiovascular diseases,  
353 inflammatory disorders and cancer, which makes them exciting targets for therapeutic intervention<sup>46</sup>.  
354 Despite the complex implication of neutrophils in disease, various therapeutic approaches aim to  
355 enhance, inhibit or restore neutrophil function, depending on the pathology. In inflammatory diseases  
356 with excessive neutrophil activity, their attenuation could be desired, even though killing functions  
357 against microorganisms might still need to be preserved. Our work shows that increased apoptosis in  
358 neutrophils does not alleviate intestinal inflammation, even though these cells are known to contribute  
359 to disease development. Recent studies indicate substantial phenotypic and functional heterogeneity  
360 of neutrophils<sup>46</sup>. Thus, targeting a specific subpopulation may allow the attenuation of harmful aspects  
361 of neutrophils without compromising host defence. Some neutrophil-targeted therapeutic strategies  
362 have reached the clinic, notably in the context of IBD with positive effects of the growth factor  
363 granulocyte colony-stimulating factor G-CSF<sup>47</sup>. It opens promising options for numerous complex  
364 pathologies.

365

## 366 **Author Contributions**

367 C.D and H.S. conceived and designed the study, performed data analysis, and wrote the manuscript;  
368 C.D. designed and conducted all experiments, unless otherwise indicated; C.M., J.P., A.M., A.A., M.-  
369 L.M., B. L., G.D.-C., M.S., C. G., M.P., Y.W., A.L. provided technical help for the *in vitro* and/or *in vivo*  
370 experiments; J.S. and B.M. performed the immunofluorescence microscopy experiments; C.Pi. and  
371 S.C. conducted the proteomics analyses; F.B., E.C. and A.R. performed the metabolomics analysis;  
372 T.L. provided mice and C.O. performed genotyping; C.Pe., S.L. and J.E.-B. helped with ROS  
373 production assays; R.J.A provided Scenith kits for tests with neutrophils; C.D., C.M., J.P., M.L.-R.,  
374 P.L., N.R., J.E.-B., B. M. and H.S. discussed experiments and results.

375

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385

## 386 **Declaration of Interests**

387 The authors declare no competing interests.

388

## 389 **Material and Methods**

390

391 **Mice.** *Card9<sup>-/-</sup>*, *Rag2<sup>-/-</sup>xCard9<sup>-/-</sup>*, *Rag2<sup>-/-</sup>*, *Card9loxMrp8cre*, *Card9loxVillincre*, and  
392 *Card9loxCD11ccre* mice on C57BL/6J background were obtained from the Saint-Antoine Research  
393 Center and housed at the IERP (INRAE, Jouy-en-Josas) under specific pathogen-free conditions.  
394 Animal experiments were performed according to the institutional guidelines approved by the local  
395 ethics committee of the French authorities, the 'Comité d'Ethique en Experimentation Animale'  
396 (COMETHEA, CEEA45).

397

398 **Induction of DSS colitis and colonization with *C. albicans*.** Mice were administered drinking water  
399 supplemented with 2-3% (wt/vol) DSS (MP Biomedicals) for 5-7 days (depending on colitis severity of  
400 each experiment), and then water only for 5d. Animals were monitored daily for weight loss. For *C.*  
401 *albicans* colonization, mice were treated with 0.4mg/ml streptomycin, 300U/ml penicillin G and  
402 0.125mg/ml fluconazole as indicated on Fig. 4.



403

404 **Histology.** Colon samples were fixed, embedded in paraffin and stained with hematoxylin and eosin.

405 Slides were scanned and analyzed to determine the histological score (Table S1)<sup>25</sup>.

406

407 **Table S1. Histological grading of colitis**

Feature graded	Grade	Description
Inflammation severity	0	None
	1	Slight
	2	Moderate
	3	Severe
Inflammation extent	0	None
	1	Mucosa
	2	Mucosa and submucosa
	3	Transmural
Crypt damage	0	None
	1	Basal 1/3 damaged
	2	Basal 2/3 damaged
	3	Only surface epithelium lost
	4	Entire crypt and epithelium lost
Percent involvement	1	1-25%
	2	26-50%
	3	51-75%
	4	76-100%

For each feature, the product of the grade and the percentage involvement was established. The histological score was obtained by adding the subscores of each feature.

408

409 **Fecal DNA extraction and total bacteria and fungi quantification.** Fecal DNA was extracted as  
410 previously described<sup>26</sup>. Luna Universal qPCR Master Mix (New England Biolabs) was used for  
411 quantification of fungal ITS2 sequences and TaqMan Gene Expression Assays (Life Technologies) for  
412 quantification of bacterial 16S rDNA sequences.

413

414 **Cell preparation and stimulation.** Epithelial cells were isolated from colonic tissue using a  
415 DTT/EDTA buffer. Neutrophils were purified from mouse bone marrow using anti-Ly6G MicroBeads  
416 UltraPure and MACS separation columns (Miltenyi Biotec). CD11c<sup>+</sup> cells were purified from mouse  
417 spleen using anti-CD11c MicroBeads UltraPure and MACS separation columns (Miltenyi Biotec).  
418 Purity checks and cell counts were performed using a BD Accuri C6 flow cytometer (BD Biosciences).  
419 After purification, neutrophils were seeded in 96-well suspension plate (Sarstedt), rested for 30min in  
420 RPMI 1640 Medium (Gibco, ThermoFisher Scientific) before addition of 2% heat-inactivated Fetal  
421 Bovine Serum (FBS), 2-DG 10mM, oligomycine 1.5µM or H2O2 0.01M and incubated at 37°C as  
422 indicated. Isolation of lamina propria immune cells was performed as previously described<sup>48</sup>.

423

424 **Gene expression analysis using quantitative RT-PCR.** Total RNA was isolated from colon samples  
 425 or cell suspensions using RNeasy Mini Kit (Qiagen), and quantitative RT-PCR performed using  
 426 QuantiTect Reverse Transcription Kit (Qiagen) and Luna® Universal RT-PCR Kit (New England  
 427 Biolabs) in a StepOnePlus apparatus (Applied Biosystems) with specific mouse oligonucleotides  
 428 (Table S2). We used the  $2^{-\Delta\Delta^{Ct}}$  quantification method with mouse *Gapdh* as an endogenous control  
 429 and the WT group as a calibrator.

430

431 **Table S2 List of oligonucleotides and antibodies**

Oligonucleotides	SEQUENCE/SOURCE	IDENTIFIER
<i>Gapdh</i> (sense)	AAC TTT GGC ATT GTG GAA GG	
<i>Gapdh</i> (antisense)	ACA CAT TGG GGG TAG GAA CA	
<i>Card9</i> exon1F (sense)	CAG TGA CCC CAA CCT GGT CAT	
<i>Card9</i> exon3R (antisense)	TCT GCA GCT TCA TGA CCT CTG TC	
All fungi (sense) (ITS1, ITS2)	CTT GGT CAT TTA GAG GAA GTA A	
All fungi (antisense) (ITS1, ITS2)	GCT GCG TTC TTC ATC GAT GC	
All bacteria (sense) (16s)	CGG TGA ATA CGT TCC CGG	
All bacteria (antisense) (16s)	TAC GGC TAC CTT GTT ACG ACT T	
All bacteria (Probe) (16s)	6FAM-CTT GTA CAC ACC GCC CGT C-MGB	
<i>Il-23</i> mouse (sense)	AGC GGG ACA TAT GAA TCT ACT AAG AGA	
<i>Il-23</i> mouse (antisense)	GTC CTA GTA GGG AGG TGT GAA GTT G	
<i>Lcn2</i> mouse	Quantitect	Mn_Lcn2_1_SG QT00113407
<i>Cxcr2</i> mouse (sense)	CTC ACA AAC AGC GTC GTA GAA C	
<i>Cxcr2</i> mouse (antisense)	AGG GCA TGC CAG AGC TAT AAT	
<i>S100a8</i> mouse (sense)	TCA AGA CAT CGT TTG AAA GGA AAT C	
<i>S100a8</i> mouse (antisense)	GGT AGA CAT CAA TGA GGT TGC TC	
<b>Antibodies</b>		
CARD9 (A-8)	Santa Cruz Biotechnology	Cat# sc-374569
β-ACTIN (D6A8)	Cell Signaling Technology	Cat# 8457S
APC-labeled anti-mouse TCRγδ (GL3)	eBioscience	Cat# 17-5711-82
anti-CD16/32 (93)	eBioscience	Cat# 14-0161-85
PerCP5.5-labeled anti-mouse CD45 (30-F11)	eBioscience	Cat# 45-0451-82
FITC-labeled anti-mouse CD3ε (145-2C11)	eBioscience	Cat# 11-0031-85

BV605-labeled anti-mouse CD8 $\alpha$ (53-6.7)	BioLegend	Cat# 100744
PE-labeled anti-mouse CD4 (RM4-5)	eBioscience	Cat# 12-0042-83
AF700-labelled CD19 (6D5)	BioLegend	Cat# 115528
PE-labelled CD11c (N418)	BioLegend	Cat# 117308
APC-labelled F4/80 (BM8)	BioLegend	Cat# 123116
AF700-labelled CD11b (M1/70)	BioLegend	Cat# 101222
APCFire750-labelled CD45 (30-F11)	BioLegend	Cat# 103154
PerCP5.5-labelled Ly6G (1A8)	BioLegend	Cat# 127616
PE-labelled CD11b (M1/70)	eBioscience	Cat# 12-0112-82
BV421-labelled CD62L (MEL-14)	BioLegend	Cat# 104424

432

433 **Immunoblot Analysis.** Mouse tissue or cell suspensions were lysed using Laemmli buffer, loaded on  
434 a SDS-PAGE and analyzed with antibodies against CARD9 (A-8:sc-374569, Santa Cruz  
435 Biotechnology), or  $\beta$ -ACTIN (D6A8, CST).

436

437 **Flow cytometry, cell sorting and functional assays.** Flow cytometry was carried out by using LSR  
438 Fortessa X-20 (BD) and cell sorting FACS Aria machines (BD). For apoptosis assay,  $4 \times 10^5$  neutrophils  
439 were stained with AnnexinV-FITC in Binding Buffer (Miltenyi Biotec). For mitochondria analyses,  
440 MitoTracker™ Green and MitoTracker™ Red FM were added to neutrophils in MACS buffer for 15min  
441 at RT (ThermoFisher Scientific). Alternatively, neutrophils were incubated with TMRM in RPMI for  
442 20min at 37°C (Abcam). For phagocytosis assay,  $10^5$  neutrophils stimulated with zymosan-FITC  
443 (50 $\mu$ g/ml, Fluorescein zymosanA BioParticles conjugates, FisherScientific), *C. albicans*-GFP (MOI1:1)  
444 or *Escherichia coli*-GFP (MOI1:10) for 45min. Cells were stained with surface antibodies in MACS  
445 buffer (Table S2).

446

447 **Endpoint dilution survival assay.** Isolated mouse neutrophils were seeded at  $10^5$  cells/well in 96-  
448 well plates in RPMI+2% FBS and infected with *C. albicans* (serial fourfold dilutions of an OD=1  
449 solution were added by rows (row1, MOI20:1; row2, MOI5:1...). After 24h at 37°C, colonies were  
450 visualized with a Nikon TMS inverted microscope and counted at the lowest dilutions (row7-8).

451

452 **Killing assay.** Purified neutrophils were seeded in 96-well plate at  $10^6$  cells/well and stimulated with  
453 PBS, *C. albicans* (MOI1) or *E. coli* (MOI10) for 90min at 37°C. Cells were washed and lysed with  
454 200 $\mu$ l TritonX100 0.025%. Serial dilutions were plated on YEPD or LB plates.

455

456 **Oxydative burst.** Experiments were performed on a TriStar LB942 Reader using  $10^5$  neutrophils in  
457 200 $\mu$ L HBSS (ThermoFisher Scientific) and luminol 80 $\mu$ M (Sigma) and stimulated with phorbol 12-  
458 myristate 13-acetate (PMA; 0.1 $\mu$ g/mL; Sigma) or opsonized zymosan (20mg/mL zymosan A from  
459 *Saccharomyces cerevisiae*; Sigma). The indexed maximal relative luminescence (in relative light units  
460 [RLU]) was calculated as follow: indexed RLU max=(hemochromatosis patient maximal RLU)/[healthy  
461 donor maximal RLU] $\times$ 100. Alternatively, superoxyde dismutase (5Units/mL) and catalase A  
462 (10Units/mL) were added to differentiate total and intracellular ROS production. Absorbance was  
463 measured at 550nm for 30min.

464

465 **Real time bioenergetic profile analysis.** Mito Stress Test, Glycolytic Rate Assay and Real-time ATP  
466 assay were performed on a XF96 Extracellular Flux Analyzer (Seahorse Biosciences). Mouse  
467 neutrophils were seeded at  $2 \times 10^5$  cells/well in RPMI+2%FBS in 0.01% poly-L-lysine pre-coated plate.  
468 After 2h rest, cells were washed in Seahorse RPMI medium and incubated for 1h at 37°C without  
469 CO<sub>2</sub>. In the analyzer, oligomycin 1.5 $\mu$ M, FCCP 1 $\mu$ M, Rotenone+AntimycinA 0.5 $\mu$ M and 2DG 50 $\mu$ M  
470 were injected at the indicated times. Protein standardization was performed after each experiment,  
471 with no noticeable differences in protein concentration and cell phenotype.

472

473 **Electron microscopy.** Purified neutrophils were stimulated with *C. albicans* (MOI 2) or *E. coli* (MOI  
474 10) for 1h at 37°C, washed in PBS and fixed. Samples preparation and SEM/TEM analyses were  
475 performed at the Microscopy and Imaging Platform MIMA2 (Université Paris-Saclay, INRAE,  
476 AgroParisTech, Jouy-en-Josas, France, <https://doi.org/10.15454/1.5572348210007727E12>).

477

478 **Proteomics.** 5 $\mu$ g protein extracts were submitted to in-gel digestion. Desalting was performed as  
479 described before<sup>49</sup>. Peptides were analyzed on a nanoElute-timsTOF ProLC-MS/MS system  
480 (Bruker)<sup>50</sup>. Raw files were analyzed using MaxQuant v1.6.10.43: database UP000000589\_10090  
481 (21994 entries, 17-Jun-2020). Data filtering, imputation and statistical analysis were performed with  
482 ProStar Zero 1.20.0<sup>50</sup>. Proteins with FDR<5% (Pounds method) were significant with a fold change  
483 >1.2.

484

485 **Nuclear Magnetic Resonance.** 200µl culture media were analyzed by 1D 1H-NMR. All NMR spectra  
486 were recorded on a Bruker AvanceIII 800MHz spectrometer equipped with a QPCI 5mm cryogenic  
487 probe head. Spectra were acquired and processed using the Bruker Topspin 4.0 software.  
488 Quantification of glucose and lactate was performed using addition of 25% TSPd4 in D2O as internal  
489 standard.

490

491 **Statistical analysis.** Statistical analysis was performed using GraphPad Prism 7 software (see figure  
492 legends).

493

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- 609



610 **Main figure titles and legends**

611 **Figure 1. Lymphocytes have no intrinsic role in the *Card9*<sup>-/-</sup> phenotype in colitis models.** (A)  
612 Weight and (B) Disease Activity Index (DAI) score of DSS-exposed *Rag2*<sup>-/-</sup> or *Rag2*<sup>-/-</sup> x *Card9*<sup>-/-</sup> mice.  
613 (C) Colon length and (D) histological score of colon sections at day 7. Data points represent individual  
614 mice. One representative experiment out of three. (E) Representative H&E-stained images of mid  
615 colon cross-sections from DSS-exposed *Rag2*<sup>-/-</sup> (left) and *Rag2*<sup>-/-</sup> x *Card9*<sup>-/-</sup> (right) mice at day 7. Scale  
616 bars, 500 μm. (F) Weight of mice receiving naive T cells for adoptive transfer of colitis experiment.  
617 *Rag2*<sup>-/-</sup> received either WT or *Card9*<sup>-/-</sup> lymphocytes. *Rag2*<sup>-/-</sup> x *Card9*<sup>-/-</sup> mice received WT lymphocytes.  
618 Data are mean ± SEM of two independent experiments. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001  
619 as determined by two-way analysis of variance (ANOVA) with Sidak's post-test (A, B, F) and Mann-  
620 Whitney test (C, D). LT, lymphocytes T.

621 **Figure 2. *Card9* expression in neutrophils, but not in epithelial or CD11c<sup>+</sup> cells, protects against**  
622 **colitis.** (A) Weight and DAI score of DSS-exposed *Card9*<sup>Villin<sup>wt</sup></sup> or *Card9*<sup>Villin<sup>cre</sup></sup> mice. (B) Weight and  
623 DAI score of DSS-exposed *Card9*<sup>cd11c<sup>wt</sup></sup> or *Card9*<sup>cd11c<sup>cre</sup></sup> mice. (C) Weight and DAI score of DSS-  
624 exposed *Card9*<sup>Mrp8<sup>wt</sup></sup> or *Card9*<sup>Mrp8<sup>cre</sup></sup> mice. (D) Colon length, (E) histological score of colon sections and  
625 (F) representative H&E-stained images of mid colon cross-sections from DSS-exposed *Card9*<sup>Mrp8<sup>wt</sup></sup>  
626 (left) and *Card9*<sup>Mrp8<sup>cre</sup></sup> (right) mice at day 12. Scale bars, 500 μm. (G) Myeloperoxidase (MPO)  
627 concentration in total colon tissue at day 12. (H) Lipocalin (*Lcn2*) expression by qRT-PCR in total  
628 colon tissue at day 12, normalized to *Gapdh*. Data points represent individual mice. Data are mean ±  
629 SEM of three independent experiments. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001 as determined  
630 by two-way ANOVA with Sidak's post-test (A-C) and Mann-Whitney test (D-H).

631 **Figure 3. *Card9* deletion affects the number of neutrophils in the inflamed colon.** (A) Relative  
632 expression of *Lcn2*, *Cxcr2* and *S1008a* in distal colon tissue of C57BL/6 WT mice during a DSS colitis  
633 model relative to *Gapdh*. (B) Representative H&E-stained images of mid colon cross-sections from  
634 DSS-exposed WT mice at day 0, 4, 7, 9, 12 and 16 after DSS exposure. Scale bars, 50 μm. (C) *Lcn2*,  
635 *Cxcr2* and *S100A8* expression in total colon tissue from *Card9*<sup>Mrp8<sup>wt</sup></sup> and *Card9*<sup>Mrp8<sup>cre</sup></sup> mice at day 9 of  
636 DSS colitis by qRT-PCR analyses, normalized to *Gapdh*. (D) Representative flow cytometry plots of  
637 Ly6G<sup>+</sup>CD11b<sup>+</sup> cells (neutrophils) in the colon lamina propria (LP) of DSS-exposed *Card9*<sup>Mrp8<sup>wt</sup></sup> and  
638 *Card9*<sup>Mrp8<sup>cre</sup></sup> mice. (E) Percentage and count of Ly6G<sup>+</sup>CD11b<sup>+</sup> cells in the LP of DSS-exposed

639 *Card9*<sup>Mrp8wt</sup> and *Card9*<sup>Mrp8cre</sup> mice. (F) Ly6G and (G) CD11b expression (MFI for Mean Fluorescence  
640 Intensity) of Ly6G<sup>+</sup>CD11b<sup>+</sup> neutrophils from *Card9*<sup>Mrp8wt</sup> and *Card9*<sup>Mrp8cre</sup> mice. Data points represent  
641 individual mice. Data are mean ± SEM of two independent experiments. \*P<0.05, \*\*P<0.01,  
642 \*\*\*P<0.001 as determined by Mann-Whitney tests.

643 **Figure 4. *C. albicans* killing capacity is impacted by *Card9* deletion in neutrophils.** (A)  
644 Representative images of *C. albicans* cfu number at the endpoint dilution after infection of *Card9*<sup>WT</sup>,  
645 *Card9*<sup>-/-</sup>, *Card9*<sup>Mrp8wt</sup> or *Card9*<sup>Mrp8cre</sup> neutrophils for 24h, using a microscope (top) or directly showing *C.*  
646 *albicans* cfu in the 96-well plate, with a photography from above (middle picture) or a scan from below  
647 the plate (bottom picture). (B) *C. albicans* cfu after infection of neutrophils for 24h. CfU counting was  
648 performed in 96 well plates using a microscope. Data points represent individual mice. Data are mean  
649 ± SEM of three independent experiments. (C) *C. albicans* cfu number after infection of *Card9*<sup>Mrp8wt</sup> or  
650 *Card9*<sup>Mrp8cre</sup> neutrophils for 3 or 24h. CfU counting was performed after plating on YEPD agar plates.  
651 Data are mean ± SEM of two independent experiments. (D) Experimental design of antibiotic  
652 treatments and *C. albicans* inoculation in mice treated with 3% DSS. (E) Weight, (F) DAI and (G) colon  
653 length of DSS-exposed *Card9*<sup>Mrp8wt</sup> and *Card9*<sup>Mrp8cre</sup> mice after colonization with *C. albicans*. (H-K)  
654 Fungal burden in the feces (H), caecal content (I), caecal and colon tissue (J) and liver, spleen and  
655 kidney (K) of DSS-exposed *Card9*<sup>Mrp8wt</sup> and *Card9*<sup>Mrp8cre</sup> mice after colonization with *C. albicans*. Data  
656 are mean ± SEM of two independent experiments. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001 as  
657 determined by one-way ANOVA with Tukey's post-test (A), two-way ANOVA with Sidak's post-test (E,  
658 F) and Mann-Whitney test (C, G, I-K). Genta, gentamycin.

659 **Figure 5. The absence of *Card9* impacts neutrophils survival by increasing apoptosis.** (A)  
660 Representative flow cytometry plots of Ly6G<sup>+</sup>CD11b<sup>+</sup> neutrophils purified from bone marrow of  
661 *Card9*<sup>Mrp8wt</sup> (left) and *Card9*<sup>Mrp8cre</sup> (right) mice, co-stained with AnnexinV and a Live/Dead marker. (B)  
662 AnnexinV MFI of Ly6G<sup>+</sup>CD11b<sup>+</sup> neutrophils from *Card9*<sup>Mrp8wt</sup> and *Card9*<sup>Mrp8cre</sup> mice, incubated for 1h at  
663 37°C. (C) Percentage of apoptotic neutrophils (Q1: AnnexinV<sup>+</sup>LD<sup>-</sup> cells), dead (late apoptotic/necrotic)  
664 neutrophils (Q2: AnnexinV<sup>+</sup>LD<sup>+</sup> cells) and viable neutrophils (Q4: AnnexinV<sup>-</sup>LD<sup>-</sup> cells) amongst the  
665 Ly6G<sup>+</sup>CD11b<sup>+</sup> population. (D) Percentage of CD62L<sup>+</sup> and CD62L<sup>-</sup> neutrophils amongst the  
666 Ly6G<sup>+</sup>CD11b<sup>+</sup> population. Data represent one out of two independent experiments. (E) Histogram  
667 representing Gene Ontology biological processes significantly enriched amongst proteins down- (blue)

668 or up-regulated (red) in *Card9*<sup>Mrp8cre</sup> compared to *Card9*<sup>Mrp8wt</sup> neutrophils in the unstimulated condition.  
669 (F) Morpheus heat-map representing mitochondria-related proteins significantly down- or up-regulated  
670 in *Card9*<sup>Mrp8cre</sup> compared to *Card9*<sup>Mrp8wt</sup> neutrophils in the unstimulated condition. E and F obtained  
671 from proteomics data analysis. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001 as determined by Mann-  
672 Whitney test (B) and two-way ANOVA with Sidak's post-test (C-D).

673 **Figure 6. *Card9* controls neutrophil energetic metabolism by affecting mitochondrial function.**

674 (A) Representative flow cytometry plots and percentages of MitoGreen<sup>+</sup>MitoRed<sup>-</sup> cells (corresponding  
675 to cells with dysfunctional mitochondria) amongst Ly6G<sup>+</sup>CD11b<sup>+</sup> neutrophils. Neutrophils were purified  
676 from the bone marrow of *Card9*<sup>Mrp8wt</sup> or *Card9*<sup>Mrp8cre</sup> mice, and incubated for 1h at 37°C. (B)  
677 Representative flow cytometry plots and percentages of TMRM<sup>-</sup> cells (corresponding to cells with  
678 dysfunctional mitochondria/apoptotic or metabolically inactive cells) amongst Ly6G<sup>+</sup>CD11b<sup>+</sup>  
679 neutrophils. (C) Oxygen Consumption Rate (OCR) of *Card9*<sup>Mrp8wt</sup> and *Card9*<sup>Mrp8cre</sup> neutrophils  
680 measured during a Seahorse Cell Mito Stress assay. (D) Basal respiration (late rate measurement  
681 before oligomycin injection (t3) - non-mitochondrial respiration rate (t12)) and ATP production rate  
682 (late rate measurement before oligomycin (ATP synthase blocker) injection (t3) – minimum rate  
683 measurement after oligomycin injection (t6) obtained from the Seahorse Cell Mito Stress assay. (E)  
684 ATP rate index and energetic map of *Card9*<sup>Mrp8wt</sup> and *Card9*<sup>Mrp8cre</sup> neutrophils obtained from the  
685 Seahorse Real-time ATP rate assay. (F) Percentage of apoptotic cells amongst the Ly6G<sup>+</sup>CD11b<sup>+</sup>  
686 neutrophil population of *Card9*<sup>Mrp8wt</sup> and *Card9*<sup>Mrp8cre</sup> genotypes after treatment with 2-DG 10 mM or  
687 (G) oligomycin 1.5 μM for 1h. (H) Percentage of apoptotic and necrotic cells amongst the  
688 Ly6G<sup>+</sup>CD11b<sup>+</sup> neutrophil population of *Card9*<sup>Mrp8wt</sup> and *Card9*<sup>Mrp8cre</sup> genotypes after addition of H2O2  
689 0.01 mM for 1h to increase oxydative stress. Data are mean ± SEM of at least two independent  
690 experiments. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001 as determined by Mann-Whitney test (A,  
691 B, D, E, F) or two-way ANOVA with Sidak's post-test (G, H). Norm. Unit, normalized unit; FCCP,  
692 Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; Rot/AA, Rotenone/AntimycinA; 2DG, 2 deoxy-  
693 glucose; OligoA, oligomycinA.

694

695 **Supplemental Figure and Table**

696 **Figure S1. *Card9* is mainly expressed by myeloid cells.** (A) *Card9* expression in different organs of  
697 *Card9*<sup>WT</sup> and *Card9*<sup>-/-</sup> mice by quantitative RT-PCR analyses, normalized to *Gapdh*. (B) CARD9 and  
698 β-ACTIN expression in different organs of *Card9*<sup>WT</sup> and *Card9*<sup>-/-</sup> mice by Western Blot analyses. (C)  
699 *Card9* expression in different sorted cell populations from the spleen and bone marrow of *Card9*<sup>WT</sup>  
700 and *Card9*<sup>-/-</sup> mice by quantitative RT-PCR analyses, normalized to *Gapdh*.

701 **Figure S2. Validation of conditional knockout mice strains.** (A) *Card9* expression in colonic  
702 epithelial cells and tissue from *Card9*<sup>Villin<sup>wt</sup></sup> and *Card9*<sup>Villin<sup>cre</sup></sup> mice (up right panel), CD11c<sup>+</sup> purified cells  
703 and CD11c<sup>-</sup> fraction from the spleen of *Card9*<sup>Cd11c<sup>wt</sup></sup> and *Card9*<sup>Cd11c<sup>cre</sup></sup> mice (up left panel) and Ly6G<sup>+</sup>  
704 purified neutrophils and Ly6G<sup>-</sup> fraction from the bone marrow of *Card9*<sup>WT</sup>, *Card9*<sup>-/-</sup>, *Card9*<sup>Mrp8<sup>wt</sup></sup> and  
705 *Card9*<sup>Mrp8<sup>cre</sup></sup> mice (lower panels) by qRT-PCR analyses, normalized to *Gapdh*. (B) CARD9 and β-  
706 ACTIN expression in Ly6G<sup>+</sup> purified neutrophils and Ly6G<sup>-</sup> fraction from the bone marrow of *Card9*<sup>WT</sup>,  
707 *Card9*<sup>-/-</sup>, *Card9*<sup>Mrp8<sup>wt</sup></sup> and *Card9*<sup>Mrp8<sup>cre</sup></sup> mice by Western blot. (C) Flow cytometry plots representing total  
708 cell populations of the bone marrow of a C57BL/6 mouse (left panel), and Ly6G<sup>+</sup>CD11b<sup>+</sup> neutrophils  
709 purified from the bone marrow using Ly6G ultrapure magnetic beads and MACS column (right panel).  
710 (D) Colon length of DSS-exposed *Card9*<sup>Villin<sup>wt</sup></sup>, *Card9*<sup>Villin<sup>cre</sup></sup>, *Card9*<sup>Cd11c<sup>wt</sup></sup> and *Card9*<sup>Cd11c<sup>cre</sup></sup> mice. (E)  
711 AhR activity of feces from *Card9*<sup>Mrp8<sup>wt</sup></sup> and *Card9*<sup>Mrp8<sup>cre</sup></sup> mice at steady-state, using HepG2 reporting  
712 cells. (F) Weight and DAI score of DSS-exposed *Card9*<sup>Mrp8<sup>wt</sup></sup> or *Card9*<sup>Mrp8<sup>cre</sup></sup> mice for 9 days.

713 **Figure S3. Imaging of *Card9*-deleted neutrophils.** (A) Immunofluorescence staining of *Card9*<sup>WT</sup>,  
714 *Card9*<sup>-/-</sup>, *Card9*<sup>Mrp8<sup>wt</sup></sup> and *Card9*<sup>Mrp8<sup>cre</sup></sup> neutrophils unstimulated or stimulated with zymosan for 1 or 3h.  
715 Blue for DNA, red for β-ACTIN. Scale bars, 5 μm. (B) Scanning electron microscopy (SEM, left) or  
716 Transmission electron microscopy (TEM, right) of *Card9*<sup>Mrp8<sup>wt</sup></sup> and *Card9*<sup>Mrp8<sup>cre</sup></sup> neutrophils  
717 unstimulated or stimulated with *C. albicans* or *E. coli* for 1h. White arrows point *C. albicans* or *E. coli*  
718 phagocytosed by neutrophils. Scale bars, 1 or 1.5 μm.

719 **Figure S4. *C. albicans* killing capacity is impacted by *Card9* deletion in neutrophils, but not  
720 phagocytosis capacity, ROS production or autophagy.** (A) Representative flow cytometry plots  
721 showing percentages of Ly6G<sup>+</sup>FITC<sup>+</sup> and Ly6G<sup>+</sup>GFP<sup>+</sup> *Card9*<sup>WT</sup> or *Card9*<sup>-/-</sup> neutrophils stimulated by  
722 zymosan-FITC (50μg/ml, Fluorescein zymosan A BioParticles conjugates), *C. albicans*-GFP (MOI 1:1)  
723 or *E. coli*-GFP (MOI 1:10) for 45 min. (B) Graph representing phagocytosis rates obtained by flow

724 cytometry analysis. (C-D) Oxydative burst kinetics of *Card9*<sup>WT</sup>, *Card9*<sup>-/-</sup>, *Card9*<sup>Mrp8<sup>wt</sup></sup> and *Card9*<sup>Mrp8<sup>cre</sup></sup>  
725 neutrophils as measured by luminol-amplified chemiluminescence of *Card9*<sup>WT</sup> and *Card9*<sup>-/-</sup> (left  
726 panel) and *Card9*<sup>Mrp8<sup>wt</sup></sup> and *Card9*<sup>Mrp8<sup>cre</sup></sup> (right panel) neutrophils after stimulation with (C) PMA  
727 (phorbol 12-myristate 13-acetate; 0.1 µg/mL; Sigma) or (D) opsonized zymosan (20 mg/mL, zymosan  
728 A from *Saccharomyces cerevisiae* opsonized with 10% SVF). (E) Total and Intracellular Reactive  
729 Oxygen Species (ROS) produced by *Card9*<sup>WT</sup> and *Card9*<sup>-/-</sup> neutrophils (left), or *Card9*<sup>Mrp8<sup>wt</sup></sup> and  
730 *Card9*<sup>Mrp8<sup>cre</sup></sup> neutrophils (right) in unstimulated condition or after 90 min stimulation with non-opsonized  
731 zymosan (0.5 mg/mL). RLU, relative light unit. (F) Western blot showing p62 (62 KDa) and  
732 LC3BI/LC3BII (16 and 14 KDa) autophagy proteins in *Card9*<sup>WT</sup>, *Card9*<sup>-/-</sup>, *Card9*<sup>Mrp8<sup>wt</sup></sup> and *Card9*<sup>Mrp8<sup>cre</sup></sup>  
733 neutrophils purified from the bone marrow and incubated 1h in RPMI +2% FBS at 37°C. (G)  
734 Fungi/bacteria loads in feces from *Card9*<sup>Mrp8<sup>wt</sup></sup> or *Card9*<sup>Mrp8<sup>cre</sup></sup> mice during DSS colitis measured by  
735 qRT-PCR (ratio of 2<sup>-C<sub>t</sub></sup>).

736 **Figure S5. The absence of *Card9* impacts neutrophils survival by increasing apoptosis.** (A)  
737 Representative flow cytometry plots of Ly6G<sup>+</sup>CD11b<sup>+</sup> neutrophils purified from bone marrow of  
738 *Card9*<sup>WT</sup> (left) and *Card9*<sup>-/-</sup> (right) mice, co-stained with AnnexinV and a Live/Dead marker. (B)  
739 AnnexinV MFI of Ly6G<sup>+</sup>CD11b<sup>+</sup> neutrophils from *Card9*<sup>WT</sup> and *Card9*<sup>-/-</sup> mice, incubated for 1h at  
740 37°C. (C) Percentage of apoptotic neutrophils (Q1: AnnexinV<sup>+</sup>LD<sup>-</sup> cells), dead (late apoptotic/necrotic)  
741 neutrophils (Q2: AnnexinV<sup>+</sup>LD<sup>+</sup> cells) and viable neutrophils (Q4: AnnexinV<sup>-</sup>LD<sup>-</sup> cells) amongst the  
742 Ly6G<sup>+</sup>CD11b<sup>+</sup> population. (D) Percentage of CD62L<sup>+</sup> and CD62L<sup>-</sup> neutrophils amongst the  
743 Ly6G<sup>+</sup>CD11b<sup>+</sup> population. Data represent one out of two independent experiments. \*P<0.05, \*\*P<0.01,  
744 \*\*\*P<0.001, \*\*\*\*P<0.0001 as determined by Mann-Whitney test (B) or two-way ANOVA with Sidak's  
745 post-test (C-D).

746 **Figure S6. *Card9* controls neutrophil energetic metabolism by affecting mitochondrial function.**  
747 (A) Extracellular Acidification Rate (ECAR) of *Card9*<sup>Mrp8<sup>wt</sup></sup> and *Card9*<sup>Mrp8<sup>cre</sup></sup> neutrophils measured  
748 during a Seahorse Glycolytic rate assay in unstimulated condition (RPMI + 2% FBS). Graph  
749 represents basal glycolysis (t2). (B) Lactate/glucose ratio in 24h culture supernatant of *Card9*<sup>Mrp8<sup>wt</sup></sup> and  
750 *Card9*<sup>Mrp8<sup>cre</sup></sup> neutrophils measured by metabolomics data analyses. (C) Representative flow cytometry  
751 plots and percentages of MitoGreen<sup>+</sup>MitoRed<sup>-</sup> cells (corresponding to cells with dysfunctional  
752 mitochondria) amongst the Ly6G<sup>+</sup>CD11b<sup>+</sup> neutrophil population from *Card9*<sup>WT</sup> or *Card9*<sup>-/-</sup> mice

753 incubated for 1h at 37°C. (D) Representative flow cytometry plots and percentages of TMRM<sup>-</sup> cells  
754 (corresponding to cells with dysfunctional mitochondria/apoptotic or metabolically inactive cells)  
755 amongst the Ly6G<sup>+</sup>CD11b<sup>+</sup> neutrophil population incubated for 3h at 37°C. (E) Oxygen Consumption  
756 Rate (OCR) and Extracellular Acidification Rate (ECAR) of *Card9*<sup>WT</sup> or *Card9*<sup>-/-</sup> neutrophils measured  
757 during a Seahorse Cell Mito Stress assay or a Seahorse Glycolytic rate assay, respectively. Data are  
758 mean ± SEM of at least two independent experiments. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001  
759 as determined by Mann-Whitney tests. FCCP, Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone;  
760 Rot/AA, Rotenone/AntimycinA; 2-DG, 2-deoxy-D-glucose.

761 **Table S1. Histological grading of colitis**

762 **Table S2. List of oligonucleotides and antibodies**

763

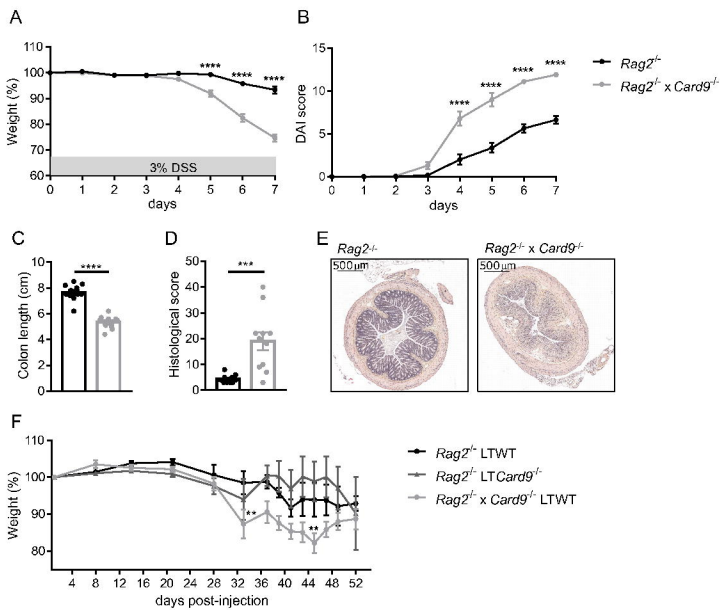


Figure 1

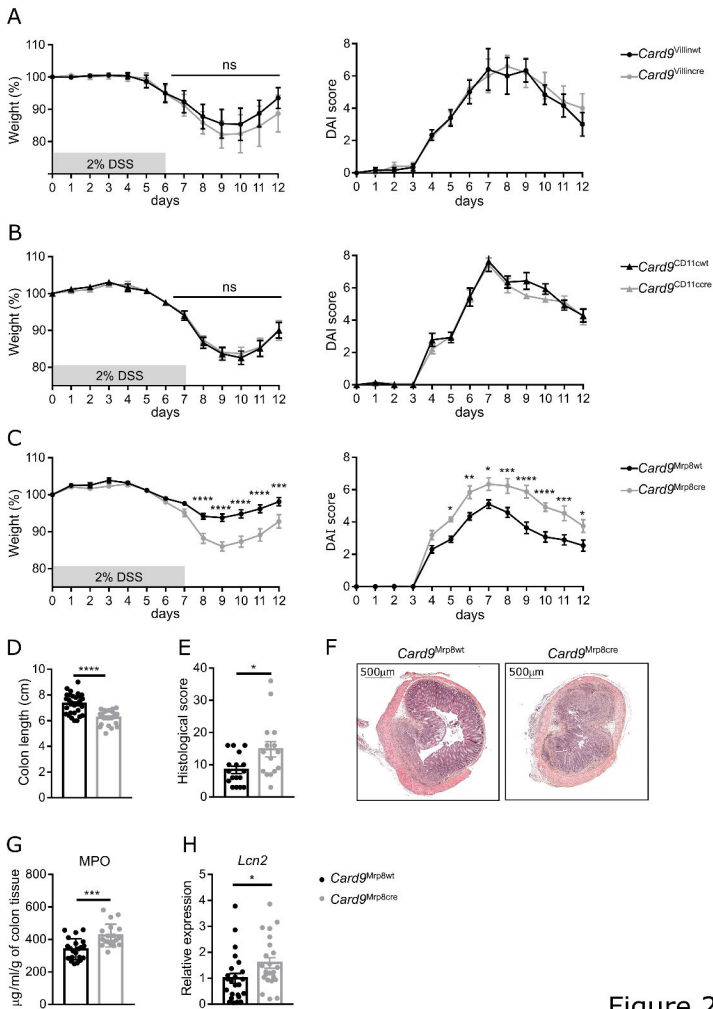


Figure 2



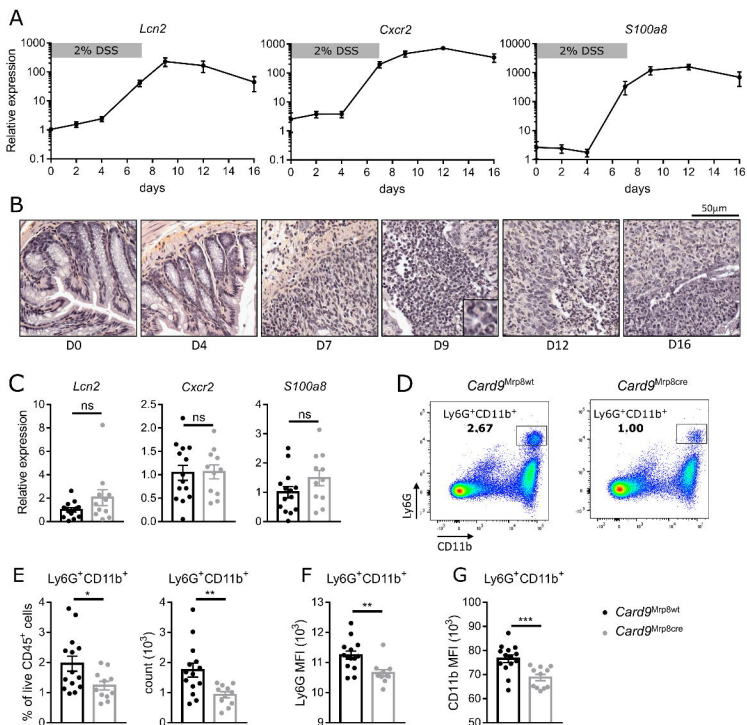


Figure 3

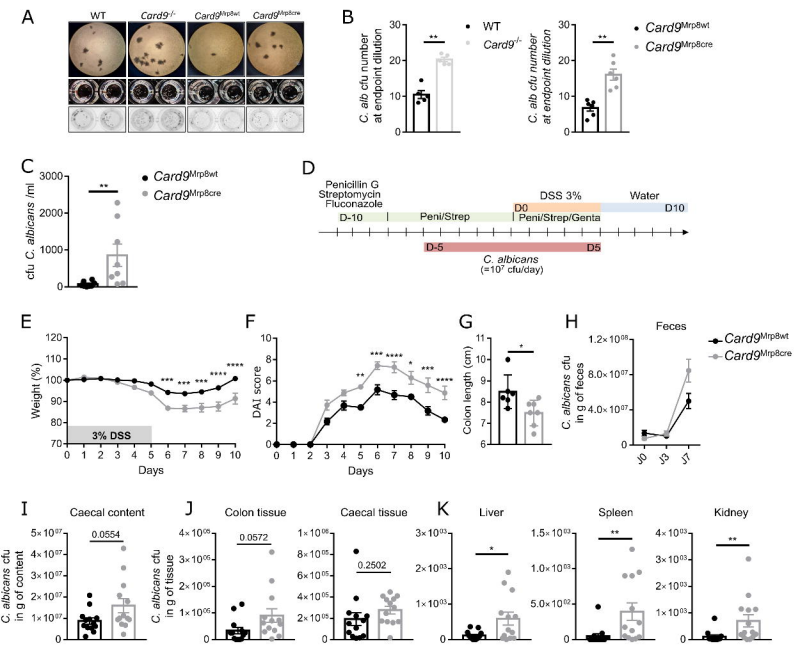


Figure 4

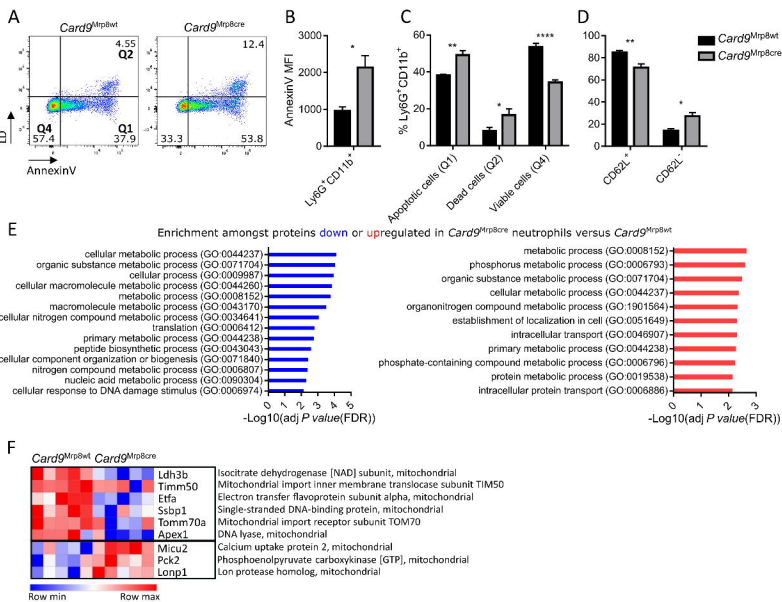


Figure 5

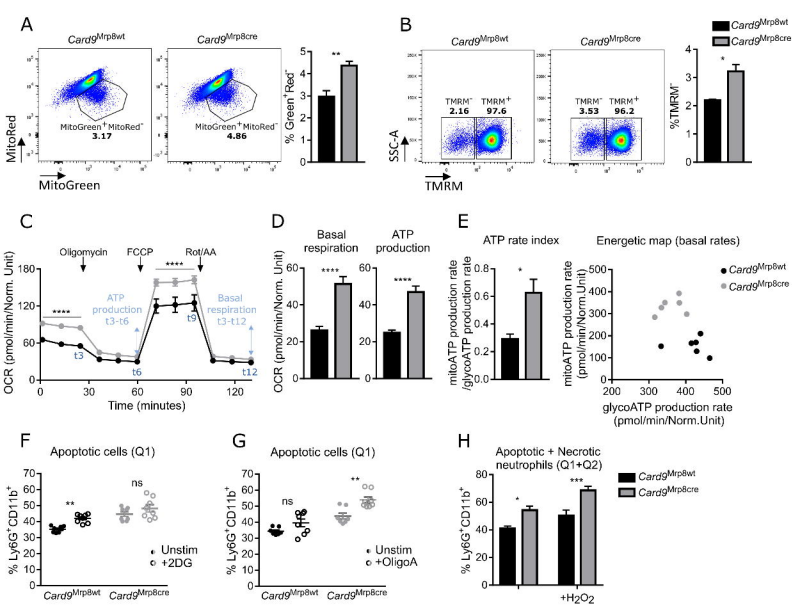


Figure 6