

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

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1	CARD9 in Neutrophils Protects from Colitis
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39

40 ABSTRACT

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

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

Results : Lymphocytes are not intrinsically involved in the CARD9 protective role against colitis.
 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 fonctional neutrophils in			
56	tissues could explain the impaired containment of fungi and increased susceptibility to intestinal			
57	inflammation.			
58	Conclu	ision : 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	targetir	ig neutrophils.		
61				
62				
63	Summa	ary 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 fonctional 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 immune response and chronic inflammation^{1.2}. Especially, the innate compartment of the immune 88 89 system has been involved in IBD development, with a role for dendritic cells, macrophages and 90 neutrophils³⁻⁶. 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^{7,8}. 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 develop IBD-like phenotypes⁹⁻¹². Moreover, functional defects have been observed in neutrophils from 95 96 IBD patients, including impaired chemotaxis, migration, phagocytosis or ROS production^{4,5}.

97 CARD9, one of the numerous IBD susceptibility genes, encodes an adaptor protein that integrates signals downstream of pattern recognition receptors ¹³⁻¹⁸. Especially, CARD9 is involved in 98 the host defense against fungi via C-type lectin sensing^{19,20}. CARD9 polymorphisms in humans are 99 100 associated with multiple susceptibilities including IBD²¹, whereas loss-of-function mutations are 101 associated with invasive fungal infections caused by species such as Candida albicans²¹⁻²⁴. CARD9 102 was shown to mediate its protective functions, at least in part, through the induction of adaptive Th17 cell responses^{22,23,25}. Card9^{-/-} mice are more susceptible to colitis due to impaired IL-22 production 103 and have an increased load of gut-resident fungi²⁵. Indeed, CARD9 affects the composition and 104 function of the gut microbiota, altering the production of anti-inflammatory microbial metabolites^{26,27}. 105 106 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^{-/-}* susceptibility to colitis

118 CARD9 was previously reported to be mainly expressed in myeloid cells, especially dendritic cells, macrophages and neutrophils^{14,28}. Using qRT-PCR analyses in various C57BL/6 mouse organs, 119 120 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 Card9⁷⁻ 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⁺CD11b⁺ 126 cells), macrophages (CD11b⁺F4/80⁺ cells), CD11c⁺ cells, including dendritic cells, and monocytes 127 $(CD11b^{h}F4/80^{+} cells)$; but the expression is lower in innate or adaptive lymphocytes $(CD3^{+}TCR\gamma\delta^{+})$ 128 lymphoid cells, CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells, CD3⁻CD19⁺ 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 models of experimental colitis, showing that Card9 deletion increases colitis susceptibility^{25,26,29}. In 131 132 order to decipher the respective roles of lymphocytes and myeloid cells in the Card9 susceptibility to intestinal injury and inflammation, we first induced colitis with DSS in Rag2^{-/-} and Rag2^{-/-} xCard9^{/-} mice 133 134 that are deficient in functional T and B cells. Mice were euthanized after receiving 3% DSS in drinking water for 7d, as the severity limit was reached for the Raa2^{-/-}xCard9^{-/-} group. Indeed, desease severity 135 136 (defined by weight loss, DAI (Disease Activity Index) score, colon length, and histologic score) was strongly increased in Rag2^{-/-}xCard9^{/-} mice compared to Rag2^{-/-} mice (Fig. 1A-E). In an adoptive 137 transfer model of colitis, in which Rag2^{-/-} mice lacking functional lymphocytes received T cells either 138 139 from WT or Card9^{-/-} 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^{-/-}xCard9^{-/-} recipient mice did aggravate colitis compared to Rag2^{-/-} simple KO recipients, with a significantly stronger weight loss (Fig. 1F). These results demonstrate that *Card9* mediates its protective role against colitis through the innate immunity compartment, although its role in intestinal epithelial cells cannot be ruled out.

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146 *Card9* expression in neutrophils, but not in epithelial or CD11c⁺ 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 (VillincrexCard9lox line), 149 CD11c-expressing cells only, including dendritic cells, macrophages and monocytes 150 (CD11ccrex Card9lox line), or neutrophils only (Mrp8crex Card9lox line). To validate their phenotypes, 151 we isolated epithelial cells from the colonic lamina propria of Card9^{Villincre} and Card9^{Villinwt} mice, or used 152 MACS separation columns to isolate either CD11c⁺ or Ly6G⁺ cell fractions from spleen or bone marrow of Card9^{CD11cwt} and Card9^{CD11ccre} mice or Card9^{Mrp8wt} and Card9^{Mrp8cre} mice, respectively. We 153 154 performed qRT-PCR on these cell fractions (Fig. S2A), and western-blot analyses on the Ly6G⁺ and Ly6G^{low/-} fractions of Card9WT, Card9^{/-}, Card9^{Mrp8wt} and Card9^{Mrp8cre} mice (Fig. S2B). Results 155 156 confirmed that Card9 deletion is restricted to the expected cell types (Fig. S2A-B). Purity of 157 Ly6G⁺CD11b⁺ neutrophils isolated from the bone marrow of C57BI/6 mice reached 95% by flow 158 cytometry (Fig. S2C).

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⁺ 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 abundantly expressed in neutrophils, was increased in Card9^{Mrp8cre} compared to Card9^{Mrp8wt} colon 167 168 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 Card9^{Mrp8cre} colon tissue at day 12 (Fig. 2H). 170

These results suggest that *Card9*-deficient neutrophils are efficiently recruited to the inflamed tissue but likely exhibit functional defects preventing them from adequately controlling microbial invaders and thus maintaining inflammation within the intestinal mucosa. We previously showed that the gut microbiota of total *Card9^{-/-}* KO lineage mice exhibit an altered production of AhR agonists compared to WT²⁶. However, no difference was observed between *Card9^{Mrp8wt}* and *Card9^{Mrp8cre}* mice (Fig. S2E), suggesting that this aspect of the phenotype is not intrinsically related to the role of CARD9 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 was higher in Card9^{Mrp8cre} than in Card9^{Mrp8wt} mice (Fig. S2E), and that Lcn2, Cxcr2 and S100a8 185 186 expression at day 9 in colon tissue were similar in both genotypes (Fig. 3C) excludes the hypothesis of a defect of neutrophil recruitment in Card9^{Mrp8cre} mice. We then examined the immune cell populations 187 188 recruited to the colon lamina propria (LP) of Card9^{Mrp8cre} versus Card9^{Mrp8wt} 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⁺CD11b⁺ neutrophils was decreased in the colon LP of Card9^{Mrp8cre} (Fig. 3D-E). Moreover, the expression levels of both Ly6G and CD11b surface proteins, 191 192 two major neutrophil maturationa 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*^{Mrp8cre} compared to *Card9*^{Mrp8wt} 198 neutrophils, we developed several *in vitro* assays with $Ly6G^+$ 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 infection in both humans and mice^{25,20}. Indeed, C. albicans killing capacities are strongly affected by 203 204 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 ^{/-} or Card9^{Mrp8cre} neutrophils compared to Card9WT or Card9^{Mrp8wt} controls, respectively (Fig. 4A-B). A 206 killing assay using the cfu counting method on agar plates confirmed that Card9^{Mrp8cre} neutrophils have 207 impaired abilities to kill C. albicans compared to Card9^{Mrp8wt} (Fig. 4C). Card9 deletion in neutrophils did 208 209 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 zymosan, a fungal stimulus (Fig. S4C-E). Even though Card9 is involved in autophagy³⁰, this cellular 214 215 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-blots (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 fungi/bacteria DNA ratio at days 7 and 12 in the feces of Card9^{Mrp8cre} compared to Card9^{Mrp8wt} mice 220 221 (Fig. S4G). These data suggest a reduced ability of Card9^{Mrp8cre} 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 antifongic cocktail and gavaged 225 with C. albicans to expand the intestinal fungal load (Fig. 4D). The increased colitis severity in 226 Card9^{Mrp8cre} 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. 4J) of Card9^{Mrp8cre} mice, and the number of cfu recovered from liver, spleen and kidney were 228

significantly higher compared to Card9^{Mrp8wt} 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.

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^{Mrp8cre} 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^{Mrp8cre} neutrophils showed a significant increase in AnnexinV-FITC MFI compared to Card9^{Mrp8wt} neutrophils (Fig. 5A-B). Consistently, an increase in 239 240 percentages of apoptotic (Q1, AnnexinV⁺LD⁻) and dead (late apoptotic/necrotic) neutrophils (Q2, 241 AnnexinV⁺LD⁺), and a decrease in viable neutrophils (Q4, AnnexinV⁻LD⁻), were observed for the 242 Card9^{Mrp8cre} genotype (Fig. 5C). Interestingly, the surface expression of the CD62L marker (a surface protein that is lost upon cell activation) was reduced in Card9^{Mrp8cre}, with a lower percentage of CD62L⁺ 243 244 cells and a higher percentage of CD62L cells (Fig. 5D). These results suggest an excessive basal activation of Card9^{Mrp8cre} compared to Card9^{Mrp8wt} neutrophils. Similar results were obtained with 245 246 Card9^{/-} versus Card9WT neutrophils (Fig. S5A-D), confirming the impact of Card9 on neutrophil 247 survival.

248 To go further, we performed a proteomic analysis on Card9^{Mrp8cre} versus Card9^{Mrp8wt} 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 Card9^{Mrp8cre} compared to Card9^{Mrp8wt} neutrophils (Fig. 5E and S5E). Especially, we observed a high 253 254 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⁺MitoRed⁻ neutrophils (with dysfunctional or metabolically inactive mitochondria) in the Card9^{Mrp8cre} compared to the Card9^{Mrp8wt} genotype (Fig. 6A). 263 264 Tetramethylrhodamine methyl ester (TMRM) assay evaluating the mitochondrial membrane potential confirmed the increase in apoptotic, metabolically stressed cells (TMRM) among Card9^{Mrp8cre} 265 neutrophils (Fig. 6B). These results suggest that the survival defect of Card9^{Mrp8cre} neutrophils may be 266 267 due to an altered energetic metabolism. Real-time bioenergetic profile analysis using Seahorse technology showed that Card9^{Mrp8cre} neutrophils have a higher basal Oxygen Consumption Rate 268 269 (OCR) during a cell mito stress assay (Fig. 6C), indicating a higher oxidative phosphorylation activity compared to Card9^{Mrp8wt} neutrophils. Basal respiration and ATP production rate were both highly 270 increased in Card9^{Mrp8cre} neutrophils (Fig. 6D). Moreover, a Seahorse real-time ATP rate assay 271 demonstrated that Card9^{Mrp8cre} neutrophils have an increased mitochondrial ATP (mitoATP) production 272 273 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 moderate decrease of the basal Extracellular Acidification Rate (ECAR) of Card9^{Mrp8cre} neutrophils 275 276 (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^{Mrp8cre} neutrophils 278 (Fig. S6B). Similar results were obtained when we compared Card9WT and Card9^{/-} 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 Card9^{Mrp8wt} but not of Card9^{Mrp8cre} neutrophils; showing that glycolysis is more essential to Card9^{Mrp8wt} 282 than Card9^{Mrp8cre} neutrophils (Fig.6F). Conversely, blocking the mitochondrial respiratory chain with 283 oligomycineA increased the apoptotic rate of Card9^{Mrp8cre} but not of Card9^{Mrp8wt} neutrophils. 284 285 demonstrating the crucial role of mitochondria as an energy source in Card9^{Mrp8cre} neutrophils (Fig. 6G). Thus, contrary to Card9^{Mrp8wt} neutrophils that mainly rely on glycolysis as a source of energy, 286 287 Card9^{Mrp8cre} neutrophils present an altered metabolism with an overactivation of mitochondria associated with a dysfunctional state, leading to apoptosis. Intestinal inflammation is associated with a high degree of oxidative stress. To evaluate the effect of oxidative stress on the phenotype of $Cardg^{Mrp8cre}$ neutrophils, they were treated for 1h with H₂O₂. The increased apoptosis and necrosis rates observed in *Cardg^{Mrp8cre}* neutrophils were stronger in oxidative stress than in basal condition (Fig. 6H). These results show that the role of CARD9 in the survival capacity of neutrophils, especially 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^{25,26}. 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⁺ 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^{31,4,5,32}.

308 Human CARD9 deficiency results in impaired neutrophil fungal killing, leading to a selective defect to contain invasive fungal infection³³. In both humans and mice, CARD9 is required in microglia 309 for neutrophil recruitment and control of fungal infection in the central nervous system^{34,35}. CARD9 310 311 signaling was also involved in neutrophil phagocytosis and NETosis (Neutrophil extracellular traps) 312 functions, enhancing mouse survival to a lethal dose of C. albicans³⁶. Moreover, CARD9 expression in 313 neutrophils promotes autoantibody-induced arthritis and dermatitis in mice³⁷, and inflammation levels in a mouse model of neutrophilic dermatosis³⁸. In line with these studies, we found that Card9 deletion 314 315 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, neutrophilintrinsic chemotaxis was not affected²⁴. In DSS-induced colitis, *Card9* deficiency in neutrophils does 318 319 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 the cytosol^{39,40}. Other CARD proteins mediate apoptotic signaling through CARD-CARD domain 323 324 interactions¹³. 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 stress⁴¹. 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^{Mrp8cre} mice to DSS colitis. Moreover, other cell types are likely involved in the Card9^{/-} mice 331 phenotype, explaining the impact of Card9 deletion on microbiota metabolic activity, as we show that this is not dependent on neutrophils^{25,26}. 332

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 functions⁴². Thus, the dependence of neutrophils on glycolysis could be an adaptation to allow oxygen 336 337 to be used in the anti-microbial response rather than entering oxidative phosphorylation⁴². 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 glycolysis dysfunction and impaired energy homeostasis⁴³. This disease is associated with IBD-like 345 phenotypes, highlighting the importance of neutrophil metabolism in intestinal health^{44,43}. 346

Immunometabolism is a central concept as immunity and metabolism impact each other in both ways :
(i) energetic metabolism impacts immune function, which is well-documented for lymphocytes and
macrophages⁴⁵, but not for neutrophils; and (ii) we show that a protein known for its roles in innate
immunity, CARD9, can also impact cell metabolism. Further investigation is required to understand
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 intervention⁴⁶. 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 neutrophils⁴⁶. Thus, targeting a specific subpopulation may allow the attenuation of harmful aspects 361 of neutrophils without compromising host defence. Some neutrophil-targeted therapeutic strategies have reached the clinic, notably in the context of IBD with positive effects of the growth factor 362 granulocyte colony-stimulating factor G-CSF⁴⁷. It opens promising options for numerous complex 363 364 pathologies.

365

366 Author Contributions

367 C.D and H.S. conceived and designed the study, performed data analysis, and wrote the manuscript; C.D. designed and conducted all experiments, unless otherwise indicated; C.M., J.P., A.M., A.A., M.-368 369 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 metobolomics 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

Card9^{-/-}, Rag2^{-/-}, 391 Mice. Rag2^{-/-}xCard9^{-/-}, *Card9*loxxMrp8cre, Card9loxxVillincre, and 392 Card9loxxCD11ccre 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

Induction of DSS colitis and colonization with *C. albicans*. Mice were administered drinking water supplemented with 2-3% (wt/vol) DSS (MP Biomedicals) for 5-7 days (depending on colitis severity of each experiment), and then water only for 5d. Animals were monitored daily for weight loss. For *C. albicans* colonization, mice were treated with 0.4mg/ml streptomycin, 300U/ml penicillin G and 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)²⁵.
- 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

Fecal DNA extraction and total bacteria and fungi quantification. Fecal DNA was extracted as
 previously described²⁶. Luna Universal qPCR Master Mix (New England Biolabs) was used for
 quantification of fungal ITS2 sequences and TaqMan Gene Expression Assays (Life Technologies) for
 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⁺ 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 described⁴⁸.

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
Gapdh (sense)	AAC TTT GGC ATT GTG	
	GAA GG	
Gapdh (antisense)	ACA CAT TGG GGG	
	TAG GAA CA	
Card9exon1F (sense)	CAG TGA CCC CAA CCT	
	GGT CAT	
Card9exon3R (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	
	GALGC	
All bacteria (sense) (16s)	CGG IGA ATA CGT ICC	
All bacteria (antisense) (16s)		
All bacteria (Probe) (168)	6FAM-CIT GTA CAC	
	ACC GCC CGT C-MGB	
<i>II-23</i> mouse (sense)		
// 22 mayon (anticanca)		
II-23 IIIOuse (alluselise)		
Lon2 mouso	AGG IGI GAA GII G	Mn Lon2 1 SC
	Quantitect	QT00113407
Cxcr2 mouse (sense)	CTC ACA AAC AGC GTC	
	GTA GAA C	
Cxcr2 mouse (antisense)	AGG GCA TGC CAG	
	AGC TAT AAT	
S100a8 mouse (sense)	TCA AGA CAT CGT TTG	
	AAA GGA AAT C	
S100a8 mouse (antisense)	GGT AGA CAT CAA TGA	
	GGT TGC TC	
Antibodies		
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 CD3c (145-2C11)	eBioscience	Cat# 11-0031-85

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BV605-labeled anti-mouse CD8α (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)	eBioscence	Cat# 12-0112-82
BV421-labelled CD62L (MEL-14)	BioLegend	Cat# 104424

432

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

436

Flow cytometry, cell sorting and functional assays. Flow cytometry was carried out by using LSR 437 438 Fortessa X-20 (BD) and cell sorting FACS Aria machines (BD). For apoptosis assay, 4x10⁵ neutrophils 439 were stained with AnnexinV-FITC in Binding Buffer (Miltenvi 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, 10⁵ neutrophils stimulated with zymosan-FITC 443 (50μg/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

Endpoint dilution survival assay. Isolated mouse neutrophils were seeded at 10⁵ cells/well in 96well plates in RPMI+2% FBS and infected with *C. albicans* (serial fourfold dilutions of an OD=1 solution were added by raws (row1, MOI20:1; row2, MOI5:1...). After 24h at 37°C, colonies were visualized with a Nikon TMS inverted microscope and counted at the lowest dilutions (row7-8).

451

Killing assay. Purified neutrophils were seeded in 96-well plate at 10⁶ cells/well and stimulated with
PBS, *C. albicans* (MOI1) or *E. coli* (MOI10) for 90min at 37°C. Cells were washed and lysed with
200µl TritonX100 0.025%. Serial dilutions were plated on YEPD or LB plates.

455

Oxydative burst. Experiments were performed on a TriStar LB942 Reader using 10⁵ neutrophils in 456 457 200µL HBSS (ThermoFisher Scientific) and luminol 80µM (Sigma) and stimulated with phorbol 12-458 myristate 13-acetate (PMA; 0.1µg/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])x100. 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

Real time bioenergetic profile analysis. Mito Stress Test, Glycolytic Rate Assay and Real-time ATP assay were performed on a XF96 Extracellular Flux Analyzer (Seahorse Biosciences). Mouse neutrophils were seeded at 2x10⁵ cells/well in RPMI+2%FBS in 0.01% poly-L-lysine pre-coated plate. After 2h rest, cells were washed in Seahorse RPMI medium and incubated for 1h at 37°C without CO2. In the analyzer, oligomycin 1.5µM, FCCP 1µM, Rotenone+AntimycinA 0.5µM and 2DG 50µM were injected at the indicated times. Protein standardization was performed after each experiment, with no noticeable differences in protein concentration and cell phenotype.

472

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

477

Proteomics. 5µg protein extracts were submitted to in-gel digestion. Desalting was performed as described before⁴⁹. Peptides were analyzed on a nanoElute-timsTOF ProLC-MS/MS system (Bruker)⁵⁰. Raw files were analyzed using MaxQuant v1.6.10.43: database UP000000589_10090 (21994 entries, 17-Jun-2020). Data filtering, imputation and statistical analysis were performed with ProStar Zero 1.20.0⁵⁰. Proteins with FDR<5% (Pounds method) were significant with a fold change >1.2.

484

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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). 493 494 References 495 1. Plichta DR, Graham DB, Subramanian S, et al. Therapeutic Opportunities in Inflammatory Bowel 496 Disease: Mechanistic Dissection of Host-Microbiome Relationships. Cell 2019;178:1041–1056. 497 2. Graham DB, Xavier RJ. Pathway paradigms revealed from the genetics of inflammatory bowel 498 disease. Nature 2020;578:527-539. 499 3. Dave M, Papadakis KA, Faubion WA. Immunology of Inflammatory Bowel Disease and 500 Molecular Targets for Biologics. Gastroenterology Clinics of North America 2014;43:405–424. 501 4. Wéra O, Lancellotti P, Oury C. The Dual Role of Neutrophils in Inflammatory Bowel Diseases. 502 JCM 2016;5:118. 503 Zhou GX, Liu ZJ. Potential roles of neutrophils in regulating intestinal mucosal inflammation of 5. 504 inflammatory bowel disease: Role of neutrophils in IBD. Journal of Digestive Diseases 505 2017;18:495-503. 506 Caër C, Wick MJ. Human Intestinal Mononuclear Phagocytes in Health and Inflammatory Bowel 6. 507 Disease. Front Immunol 2020;11:410. 508 7. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat 509 Rev Immunol 2013;13:159-175. 510 8. Segal AW. HOW NEUTROPHILS KILL MICROBES. Annu Rev Immunol 2005;23:197–223. 9. 511 Mulholland MW, Delaney JP, Foker JE, et al. Gastrointestinal Complications of Congenital 512 Immunodeficiency States: Annals of Surgery 1983;198:673-680. 513 10. Segal AW. The NADPH oxidase and chronic granulomatous disease. Molecular Medicine Today 514 1996;2:129-135. 515 11. Annabi B, Mansfield BC, Hiraiwa H, et al. The Gene for Glycogen-Storage Disease Type 1b Maps 516 to Chromosome 11q23. The American Journal of Human Genetics 1998;62:400-405. 517 12. Uzel G, Tng E, Rosenzweig SD, et al. Reversion mutations in patients with leukocyte adhesion 518 deficiency type-1 (LAD-1). Blood 2008;111:209-218.

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610 Main figure titles and legends

Figure 1. Lymphocytes have no intrinsic role in the Card9^{-/-} phenotype in colitis models. (A) 611 Weight and (B) Disease Activity Index (DAI) score of DSS-exposed Rag2^{-/-} or Rag2^{-/-} x Card9^{/-} mice. 612 (C) Colon length and (D) histological score of colon sections at day 7. Data points represent individual 613 614 mice. One representative experiment out of three. (E) Representative H&E-stained images of mid 615 colon cross-sections from DSS-exposed Rag2^{-/-} (left) and Rag2^{-/-} x Card9^{/-} (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^{-/-} received either WT or Card9^{-/-} lymphocytes. Rag2^{-/-} x Card9^{-/-} 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.

Figure 2. Card9 expression in neutrophils, but not in epithelial or CD11c⁺ cells, protects against 621 colitis. (A) Weight and DAI score of DSS-exposed Card9^{Villinvt} or Card9^{Villincre} mice. (B) Weight and 622 623 DAI score of DSS-exposed Card9^{cd11cwt} or Card9^{cd11ccre} mice. (C) Weight and DAI score of DSSexposed Card9^{Mrp8wt} or Card9^{Mrp8cre} mice. (D) Colon length, (E) histological score of colon sections and 624 (F) representative H&E-stained images of mid colon cross-sections from DSS-exposed Card9^{Mrp8wt} 625 626 (left) and Card9^{Mrp8cre} (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, Cxcr2 and S100A8 expression in total colon tissue from Card9^{Mrp8wt} and Card9^{Mrp8cre} mice at day 9 of 635 636 DSS colitis by gRT-PCR analyses, normalized to Gapdh. (D) Representative flow cytometry plots of Ly6G⁺CD11b⁺ cells (neutrophils) in the colon lamina propria (LP) of DSS-exposed Card9^{Mrp8wt} and 637 Card9^{Mrp8cre} mice. (E) Percentage and count of Ly6G⁺CD11b⁺ cells in the LP of DSS-exposed 638

639 $Card9^{Mrp8wt}$ and $Card9^{Mrp8cre}$ mice. (F) Ly6G and (G) CD11b expression (MFI for Mean Fluorescence 640 Intensity) of Ly6G⁺CD11b⁺ neutrophils from $Card9^{Mrp8wt}$ and $Card9^{Mrp8cre}$ 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 Card9WT, Card9^{/-}, Card9^{Mrp8wt} or Card9^{Mrp8cre} neutrophils for 24h, using a microscope (top) or directly showing C. 645 646 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 ± SEM of three independent experiments. (C) C. albicans cfu number after infection of Card9^{Mrp8wt} or 649 Card9^{Mrp8cre} neutrophils for 3 or 24h. Cfu counting was performed after plating on YEPD agar plates. 650 651 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 length of DSS-exposed Card9^{Mrp8wt} and Card9^{Mrp8wt} mice after colonization with C. albicans. (H-K) 653 654 Fungal burden in the feces (H), caecal content (I), caecal and colon tissue (J) and liver, spleen and kidney (K) of DSS-exposed Card9^{Mrp8wt} and Card9^{Mrp8cre} mice after colonization with C. albicans. Data 655 656 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) Representative flow cytometry plots of Ly6G⁺CD11b⁺ neutrophils purified from bone marrow of 660 Card9^{Mrp8wt} (left) and Card9^{Mrp8cre} (right) mice, co-stained with AnnexinV and a Live/Dead marker. (B) 661 AnnexinV MFI of Ly6G⁺CD11b⁺ neutrophils from Card9^{Mrp8wt} and Card9^{Mrp8cre} mice, incubated for 1h at 662 663 37°C, (C) Percentage of apoptotic neutrophils (Q1: AnnexinV⁺LD⁻ cells), dead (late apoptotic/necrotic) 664 neutrophils (Q2: AnnexinV⁺LD⁺ cells) and viable neutrophils (Q4: AnnexinV⁻LD⁻ cells) amongst the Ly6G⁺CD11b⁺ population. (D) Percentage of CD62L⁺ and CD62L⁻ neutrophils amongst the 665 666 Ly6G⁺CD11b⁺ population. Data represent one out of two independent experiments. (E) Histogram 667 representing Gene Ontology biological processes significantly enriched amongst proteins down- (blue)

or up-regulated (red) in *Card9^{Mrp8cre}* compared to *Card9^{Mrp8wt}* neutrophils in the unstimulated condition.
(F) Morpheus heat-map representing mitochondria-related proteins significantly down- or up-regulated
in *Card9^{Mrp8cre}* compared to *Card9^{Mrp8wt}* neutrophils in the unstimulated condition. E and F obtained
from proteomics data analysis. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 as determined by Mann-
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 precentages of MitoGreen⁺MitoRed⁻ cells (corresponding 675 to cells with dysfunctional mitochondria) amongst Ly6G⁺CD11b⁺ neutrophils. Neutrophils were purified 676 from the bone marrow of Card9^{Mrp8wt} or Card9^{Mrp8cre} mice, and incubated for 1h at 37°C. (B) 677 Representative flow cytometry plots and percentages of TMRM⁻ cells (corresponding to cells with 678 dysfunctional mitochondria/apoptotic or metabolically inactive cells) amongst Ly6G⁺CD11b⁺ neutrophils. (C) Oxygen Consumption Rate (OCR) of Card9^{Mrp8wt} and Card9^{Mrp8cre} neutrophils 679 680 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) ATP rate index and energetic map of Card9^{Mrp8wt} and Card9^{Mrp8cre} neutrophils obtained from the 684 685 Seahorse Real-time ATP rate assay. (F) Percentage of apoptotic cells amongst the Ly6G⁺CD11b⁺ neutrophil population of Card9^{Mrp8wt} and Card9^{Mrp8cre} genotypes after treatment with 2-DG 10 mM or 686 687 (G) oligomycin 1.5 µM for 1h. (H) Percentage of apoptotic and necrotic cells amongst the Ly6G⁺CD11b⁺ neutrophil population of *Card9^{Mrp8vrt}* and *Card9^{Mrp8cre}* genotypes after addition of H2O2 688 689 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<0.001 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.

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695 Supplemental Figure and Table

696Figure S1. Card9 is mainly expressed by myeloid cells. (A) Card9 expression in different organs of697Card9WT and Card9^{-/-} mice by quantitative RT-PCR analyses, normalized to Gapdh. (B) CARD9 and698β-ACTIN expression in different organs of Card9WT and Card9^{-/-} mice by Western Blot analyses. (C)699Card9 expression in different sorted cell populations from the spleen and bone marrow of Card9WT700and Card9^{-/-} mice by quantitative RT-PCR analyses, normalized to Gapdh.

701 Figure S2. Validation of conditional knockout mice strains. (A) Card9 expression in colonic epithelial cells and tissue from Card9^{Villinwt} and Card9^{Villincre} mice (up right panel), CD11c⁺ purified cells 702 and CD11c⁻ fraction from the spleen of Card9^{Cd11cwt} and Card9^{Cd11ccre} mice (up left panel) and Ly6G⁺ 703 704 purified neutrophils and Ly6G⁻ fraction from the bone marrow of Card9WT, Card9^{/-}, Card9^{Mrp8wt} and Card9^{Mrp8cre} mice (lower panels) by gRT-PCR analyses, normalized to Gapdh. (B) CARD9 and β -705 706 ACTIN expression in Lv6G⁺ purified neutrophils and Lv6G⁻ fraction from the bone marrow of Card9WT. Card9^{/-}, Card9^{Mrp8wt} and Card9^{Mrp8wt} mice by Western blot. (C) Flow cytometry plots representing total 707 708 cell populations of the bone marrow of a C57BL/6 mouse (left panel), and Ly6G⁺CD11b⁺ neutrophils 709 purified from the bone marrow using Ly6G ultrapure magnetic beads and MACS column (right panel). (D) Colon length of DSS-exposed Card9^{Villinwt}, Card9^{Villincre}, Card9^{Cd11cwt} and Card9^{Cd11ccre} mice. (E) 710 AhR activity of feces from Card9^{Mrp8wt} and Card9^{Mrp8cre} mice at steady-state, using HepG2 reporting 711 712 cells. (F) Weight and DAI score of DSS-exposed Card9^{Mrp8wt} or Card9^{Mrp8cre} mice for 9 days.

Figure S3. Imaging of *Card9*-deleted neutrophils. (A) Immunofluorescence staining of *Card9*WT, *Card9*^{-/-}, *Card9*^{Mrp8wt} and *Card9*^{Mrp8cre} neutrophils unstimulated or stimulated with zymosan for 1 or 3h. Blue for DNA, red for β-ACTIN. Scale bars, 5 µm. (B) Scanning electron microscopy (SEM, left) or Transmission electron microscopy (TEM, right) of *Card9*^{Mrp8wt} and *Card9*^{Mrp8cre} neutrophils unstimulated or stimulated with *C. albicans* or *E. coli* for 1h. White arrows point *C. albicans* or *E. coli* phagocytosed by neutrophils. Scale bars, 1 or 1.5 µm.

Figure S4. C. albicans killing capacity is impacted by Card9 deletion in neutrophils, but not phagocytosis capacity, ROS production or autophagy. (A) Representative flow cytometry plots showing percentages of Ly6G⁺FITC⁺ and Ly6G⁺GFP⁺ Card9WT or Card9^{/-} neutrophils stimulated by zymosan-FITC (50µg/ml, Fluorescein zymosan A BioParticles conjugates), C. albicans-GFP (MOI 1:1) 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 Card9WT, Card9^{-/-}, Card9^{Mrp8wt} and Card9^{Mrp8cre} 725 neutrophils as measured by luminol-amplified chemiluminescence of Card9WT and Card9^{-/-} (left panel) and Card9^{Mrp8wt} and Card9^{Mrp8cre} (right panel) neutrophils after stimulation with (C) PMA 726 727 (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 Card9WT and Card9^{-/-} neutrophils (left), or Card9^{Mrp8wt} and 730 Card9^{Mrp8cre} 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 LC3BI/LC3BII (16 and 14 KDa) autophagy proteins in Card9WT, Card9^{//-}, Card9^{Mrp8wt} and Card9^{Mrp8cre} 732 733 neutrophils purified from the bone marrow and incubated 1h in RPMI +2% FBS at 37°C. (G) Fungi/bacteria loads in feces from Card9^{Mrp8wt} or Card9^{Mrp8cre} mice during DSS colitis measured by 734 qRT-PCR (ratio of 2^{-Ct}). 735

736 Figure S5. The absence of Card9 impacts neutrophils survival by increasing apoptosis. (A) 737 Representative flow cytometry plots of Ly6G⁺CD11b⁺ neutrophils purified from bone marrow of 738 Card9WT (left) and Card9^{/-} (right) mice, co-stained with AnnexinV and a Live/Dead marker. (B) AnnexinV MFI of Ly6G⁺CD11b⁺ neutrophils from Card9WT and Card9^{-/-} mice, incubated for 1h at 739 740 37°C. (C) Percentage of apoptotic neutrophils (Q1: AnnexinV⁺LD⁻ cells), dead (late apoptotic/necrotic) 741 neutrophils (Q2: AnnexinV⁺LD⁺ cells) and viable neutrophils (Q4: AnnexinV⁻LD⁻ cells) amongst the Ly6G⁺CD11b⁺ population. (D) Percentage of CD62L⁺ and CD62L⁻ neutrophils amongst the 742 Ly6G⁺CD11b⁺ population. Data represent one out of two independent experiments. *P<0.05, **P<0.01, 743 744 ***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.

(A) Extracellular Acidification Rate (ECAR) of *Card9^{Mrp8wt}* and *Card9^{Mrp8cre}* neutrophils measured during a Seahorse Glycolytic rate assay in unstimulated condition (RPMI + 2% FBS). Graph represents basal glycolysis (t2). (B) Lactate/glucose ratio in 24h culture supernatant of *Card9^{Mrp8wt}* and *Card9^{Mrp8cre}* neutrophils measured by metabolomics data analyses. (C) Representative flow cytometry plots and percentages of MitoGreen⁺MitoRed⁻ cells (corresponding to cells with dysfunctional mitochondria) amongst the Ly6G⁺CD11b⁺ neutrophil population from *Card9*WT or *Card9^{-/-}* mice

incubated for 1h at 37°C. (D) Representative flow cytometry plots and percentages of TMRM cells (corresponding to cells with dysfunctional mitochondria/apoptotic or metabolically inactive cells) amongst the Ly6G⁺CD11b⁺ neutrophil population incubated for 3h at 37°C. (E) Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) of *Card9*WT or *Card9^{-/-}* neutrophils measured during a Seahorse Cell Mito Stress assay or a Seahorse Glycolytic rate assay, respectively. Data are mean \pm SEM of at least two independent experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 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





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Enrichment amongst proteins down or upregulated in Card9^{MrpScre} neutrophils versus Card9^{MrpSwt}



Isocitrate dehydrogenase (NAD) subunit, mitochondrial

Single-stranded DNA-binding protein, mitochondrial

Mitochondrial import receptor subunit TOM70 DNA lyase, mitochondrial

Calcium uptake protein 2, mitochondrial

Electron transfer flavoprotein subunit alpha, mitochondrial

Phosphoenolpyruvate carboxykinase [GTP], mitochondrial Lon protease homolog, mitochondrial

Mitochondrial import inner membrane translocase subunit TIM50

Card9Mrp8wt Card9Mrp8cre



