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## **An anti-inflammatory transcriptional cascade conserved from flies to humans**

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1 **Title** Gcm: a novel anti-inflammatory transcriptional cascade conserved from flies to  
2 humans  
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14  
15

## 16 Abstract

17 Innate immunity is an ancestral process that can induce pro- and anti-inflammatory states. A  
18 major challenge is to characterise the transcriptional cascades that modulate the response to  
19 chronic and acute inflammatory challenges. The *Drosophila melanogaster* Gcm transcription  
20 factor represents an interesting candidate for its potential anti-inflammatory role. Here we  
21 explore its evolutionary conservation and its mode of action. We found that the murine ortholog  
22 *Gcm2* (*mGcm2*) is expressed upon aging, which is considered as a state of chronic  
23 inflammation. *mGcm2* is found in a subpopulation of microglia, the innate immune cells of the  
24 central nervous system (CNS). Its expression is also induced by a lyso-phosphatidylcholine  
25 (LPC)-induced CNS demyelination (acute inflammation) and *mGcm2* conditional knock out  
26 mice show an increased inflammatory phenotype upon aging or LPC injection. In agreement  
27 with the role of this transcriptional cascade in inflammation, the human ortholog *hGCM2* is  
28 expressed in active demyelinating lesions of Multiple Sclerosis (MS) patients. Finally,  
29 *Drosophila gcm* expression is induced upon aging as well as during an acute inflammatory  
30 response and its overexpression decreases the inflammatory phenotype. Altogether, our data  
31 show that the inducible Gcm pathway is highly conserved from flies up to humans and  
32 represents a potential therapeutic anti-inflammatory target in the control of the inflammatory  
33 response.

34  
35 **Keywords:** immunity, neuro-inflammation, aging, transcription factors

## 36 Introduction

37

38 The immune response is one of the oldest processes of living organisms. It goes from simple  
39 enzymatic reactions in bacteria to cellular and humoral pathways in more complex animals (1,  
40 2). The main requirement for a successful immune response is the recognition of non-self  
41 through specific receptors that activate different immune pathways. The majority of both the  
42 receptors and their downstream pathways are highly conserved throughout evolution. The  
43 immune response is controlled by either pro- or anti-inflammatory cues. The pro-inflammatory  
44 JAK/STAT, Toll and NF- $\kappa$ B pathways are found in both insects and mammals (3-7). The TGF-  
45  $\beta$  signalling pathway is associated with promotion of anti-inflammatory properties in  
46 vertebrates upon resolution of inflammation, with similar molecules being present in flies (8,  
47 9). One of the most challenging issues is to discover transcription factors that coordinately  
48 block the inflammatory response.

49

50 Glial cells missing/Glial cell deficiency (*Gcm*/Glide, *Gcm* throughout the text) is expressed in  
51 the haemocytes of *Drosophila melanogaster*, functional orthologs of the vertebrate  
52 macrophages (10). *gcm* silencing in the fly macrophages does not on its own produce an overt  
53 phenotype, but it enhances the inflammatory phenotype triggered by the constitutive activation  
54 of the JAK/STAT pathway (11-13). While *gcm* is only expressed early and transiently in the  
55 haemocytes, its silencing also enhances the response to an acute inflammatory challenge  
56 performed well after *gcm* expression has ceased. Thus, *Gcm* in flies modulates the acute and  
57 chronic inflammatory responses, through mechanisms that are not fully understood.

58

59 *Gcm* is an atypical zinc finger transcription factor that is structurally conserved throughout  
60 evolution. The two mammalian orthologs are named *hGCM1* and *hGCM2* in humans, *mGcm1*  
61 and *mGcm2* in mice. *Gcm1* is required for the differentiation of trophoblasts in the developing  
62 placenta and its mutation is associated with pre-eclampsia (14). *Gcm2* is expressed and required  
63 mainly in the parathyroid glands, where it is necessary for the survival and the differentiation  
64 of the precursor cells but no role was described in the immune cells (15-17). Since the fly *gcm*  
65 gene, but not its orthologs, is also necessary in glia (18, 19), and since the fly glial cells  
66 constitute the immune cells of the nervous system, we speculated that this transcriptional  
67 pathway may have an anti-inflammatory role in microglia, the resident macrophages of the  
68 vertebrate nervous system. Microglia are not only responsible for the development and the

69 homeostasis of the central nervous system (CNS) but are also involved in neuroinflammation  
70 (20-24). Moreover, recent studies suggest that the increased inflammation in the aged brain is  
71 attributed, in part, to the resident population of microglia (25). Interestingly, the heightened  
72 inflammatory profile of microglia in aging is associated with a ‘sensitised’ or ‘primed’  
73 phenotype, which might be triggered by transcriptional pathways controlling the inflammatory  
74 response. This phenotype includes differences in morphology and gene expression of aged  
75 versus young microglia (26-30).

76

77 Here we show that the murine ortholog *mGcm2* starts being expressed in a subset of microglia  
78 upon aging. Loss of *mGcm2* enhances the aging phenotype in terms of microglia morphology  
79 and expression of pro-inflammatory markers, corroborating the hypothesis that this  
80 transcription factor has an anti-inflammatory role during chronic inflammation. Furthermore,  
81 *mGcm2* expression is induced in demyelinated lesions triggered by lysophosphatidylcholine  
82 (LPC) injection in the spinal cord and in *mGcm2* inducible conditional knock-out animals, the  
83 same challenge triggers a much stronger inflammatory reaction. Therefore, *mGcm2* is involved  
84 in chronic and acute responses. Most importantly, the human ortholog *hGCM2* is expressed in  
85 active demyelinating lesions of Multiple Sclerosis (MS) patients. Finally, chronic and acute  
86 challenges also induce the expression of *gcm* in flies. *De novo* expression of *gcm* counteracts  
87 the inflammatory phenotype, explaining its mode of action and highlighting its anti-  
88 inflammatory potential. Altogether, our data demonstrate that Gcm constitutes a highly  
89 conserved immune transcriptional cascade from flies up to humans and represents a novel  
90 potential therapeutic target in the control of the inflammatory response.

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## 99 Material and Methods

100

### 101 Mouse lines

102 *Cx3cr1-Cre* mice were obtained from TAAM Orléans and bred to maintain them on the  
103 C57Bl/6J background. The *mGcm2<sup>lox/lox</sup>* line was created at the Institute Clinique de la Souris  
104 (ICS, Strasbourg) (**Figure 1A**). Conditional knock-out (cKO) mice with their littermate  
105 controls derived from *Cx3cr1-Cre/+;mGcm2<sup>lox/+</sup>* males were crossed with *mGcm2<sup>lox/lox</sup>*  
106 females. The open field behavioural test was conducted at the ICS.

107 To generate tamoxifen-inducible conditional knock-out *mGcm2* mice specifically in microglia,  
108 *Cx3cr1<sup>CreER/+</sup>* knock-in mice (Parkhurst et al., 2013; JAX stock #021160) were crossed with  
109 *mGcm2<sup>lox/lox</sup>* mice. *Cx3cr1<sup>CreER/+</sup>;mGcm2<sup>lox/+</sup>* mice were crossed with *mGcm2<sup>lox/lox</sup>* animals to  
110 generate *Cx3cr1<sup>CreER/+</sup>;mGcm2<sup>lox/lox</sup>* mice (called icKO thereafter) and *Cx3cr1<sup>CreER/+</sup>;*  
111 *mGcm2<sup>lox/+</sup>* (control). WT and *mGcm2<sup>lox/lox</sup>* were also used as controls for demyelinating  
112 lesion experiments. All mice were maintained on a normal diet in a 12-hour light/dark cycle. 5  
113 to 10 mice were used per group (per gender, age and genotype).

114

### 115 Genotyping

116 DNA were extracted according to the Jacks Lab protocol. For primers, we used: *CX3Cr1<sup>cre</sup>*  
117 GTTCGCAAGAACCTGATGGACA and CTAGAGCCTGTTTTGCACGTTT, *mGcm2<sup>lox</sup>*  
118 CAATAGGGAAGTGATCCCTAGAGTC and GGGAAACTTGTCTGTTCTTTTCACACAG  
119 and *mGcm2<sup>WT</sup>* CAATAGGGAAGTGATCCCTAGAGTC and  
120 GGGAAACTTGTCTGTTCTTTTCACACAG, forward and reverse, respectively. Primer  
121 sequences for *Cx3cr1<sup>CreER</sup>* genotyping were: CX3 F- CTTCTTGCATTCTTGCAGG; CX3 R  
122 -CACTACCTCATCCATGA; *CX3CreER1*- CACGGGGGAGGCAGAGGGTTT;  
123 *CX3CreER2*-GCGGAGCACGGGCCACATTTT.

124

### 125 Tamoxifen treatment

126 To induce Cre recombination, adult *Cx3cr1<sup>CreER/+</sup>;mGcm2<sup>lox/lox</sup>* and *Cx3cr1<sup>CreER/+</sup>;* *mGcm2<sup>lox/+</sup>*  
127 mice (10 to 16week-old) were treated with tamoxifen (100mg/kg; Sigma) by intraperitoneal  
128 injections, during 5 consecutive days prior LPC-induced demyelination.

129

130

131

## 132 **LPC-induced demyelination of the mouse spinal cord**

133 C57Bl6/J 12week-old females from Janvier were used for focal spinal cord demyelinated  
134 lesions. To evaluate microglial response and oligodendrocyte differentiation, we used  
135 tamoxifen treated  $Cx3cr1^{CreER/+};mGcm2^{flox/flox}$ ,  $Cx3cr1^{CreER/+};mGcm2^{flox/+}$ ,  $Gcm2^{flox/flox}$  and WT  
136 mice. Twenty minutes before anaesthesia induction with 3% Isoflurane, animals were injected  
137 with Buprenorphine (0.1mg/kg). After induction, isoflurane concentration was increased to 2%  
138 for the surgical phase. Animals were placed on the stereotaxic frame, and small incision was  
139 made at the level of thoracic vertebrae (T8 and T9). Using a Hamilton syringe connected with  
140 a glass capillary, 1% lyso-phosphatidylcholine (LPC, sigma) was injected into the dorsal  
141 funiculus of the spinal cord. The injection site was marked with an active charcoal, and internal  
142 and external sutures were made. After surgery, animals were injected subcutaneously with  
143 Buprenorphine during 2 consecutive days and then treated *ad libitum* with a solution of  
144 Buprenorphine in the drinking water.

145

## 146 **P1 primary CNS cultures**

147 Postnatal day 1 (P1) cultures were produced as previously described (31). The cultures were  
148 kept in the incubator at 37°C and 5 % CO<sub>2</sub> for 14 days. The medium was changed at day 1 and  
149 day 3. *In vitro* cultures were fixed with 4 % PFA (Electron Microscopy Sciences) in PBS 0.1M  
150 and then proceed to the immunolabeling.

151

## 152 **Tissue dissections**

153 Mice were anesthetised by a solution of Ketamine (100mg/ml)/Xylazine (Rompun, 20mg/ml):  
154 130mg/kg of ketamine + 13mg/kg of xylazine, transcardially perfused with ice-cold PFA 4%  
155 in 0.1M PBS, and then the brains, the spinal cord, lungs and adipose tissue were dissected. The  
156 tissues were fixed overnight with 4 % PFA in 0.1M PBS and then the brains were cut into left  
157 and right hemisphere, the rest of the tissues were cut in half. Half of the samples were  
158 embedded with paraffin and the other half with cryomatrix. 8 µm and 50 µm thick sections  
159 were used for paraffin labelling and for cryo-section, respectively.

160 For LPC demyelinated lesions, mice were euthanised several days post LPC-injection (dpi),  
161 after lethal anaesthesia with Xylazine (10mg/kg) and pentobarbital sodium (150mg/kg), and  
162 then transcardially perfused 4% PFA in 0.1M PBS. Spinal cords were dissected and post-fixed  
163 2 h in 4% PFA. After post-fixation, spinal cords were cryoprotected in 20% sucrose solution  
164 O/N and then frozen in O.C.T. compound (Thermo Fisher) at -60°C in isopentane. Coronal

165 spinal cords were sections (12 $\mu$ m thickness) were performed at cryostat (Leica) and slides were  
166 kept on -80°C until use.

167

### 168 **Immunolabelling in mouse samples**

169 For immunolabeling, samples were permeabilized with PTX (0.1M PBS, 0.1 % Triton-X100)  
170 for 30 min and incubated with blocking buffer for 1 h at room temperature (RT). The samples  
171 were incubated with primary antibodies overnight at 4°C, then incubated with the appropriate  
172 secondary antibodies. Finally, they were incubated with DAPI, (Sigma-Aldrich)) to label the  
173 nuclei and the samples were mounted with Aqua Poly/Mount (Polysciences). Primaries and  
174 their appropriate secondary antibodies are on supplementary table 1.

175 For peroxidase immunolabelling of Iba1, sections were incubated with the primary antibody  
176 incubation overnight and then washed extensively in 0.1M PBS,0.1% Triton. Slides were  
177 incubated for 1 h in RT with biotinylated secondary antibody for 1h, washed extensively and  
178 then incubated with the avidin–biotin–peroxidase (ABC) complex (Vector Laboratories). After  
179 washes, slides were incubated with the chromogen 3,3' diaminobenzidine tetrahydrochloride  
180 (DAB; Sigma–Aldrich) until desired labelling intensity developed and counterstained with  
181 haematoxylin.

182

### 183 **Oil Red O staining**

184 For Oil Red O (ORO) that labels macrophages containing myelin debris, spinal cord sections  
185 were dried at RT, rinsed in 60% isopropanol, then stained with freshly prepared and filtered  
186 0.01% ORO solution. After 20 min, slides were rinsed in 60% isopropanol, counterstained with  
187 haematoxylin, rinsed in water and mount in aqueous mounting medium.

188

### 189 **MS tissue samples**

190 Snap frozen post-mortem brain and cerebellar samples from MS and control patients were  
191 obtained from the UK MS tissue bank (Imperial College, London, approved by the Wales  
192 Research Ethics Committee, ref. no. 18/WA/0238). For this study, we used 4 MS and 1 control  
193 samples (**Supplementary Table 2**). 12 $\mu$ m-thick sections were cut on a cryostat, and lesions  
194 were classified as active (N=2), chronic active (N=1) and chronic inactive (N=2), using Luxol  
195 fast blue/major histocompatibility complex class II (MHCII) staining, as previously described  
196 (32). For immunohistochemistry, sections were post-fixed 20 min in 2% PFA and  
197 immunofluorescence labelling was performed as mentioned above for mouse experiments.



## 198 **Quantitative RT-PCR analysis**

199 Animals were euthanised by CO<sub>2</sub> inhalation, and samples of spinal cords around of the  
200 injection site was dissected. Total RNA, from 4 dpi LPC- and saline-injected spinal cords, were  
201 purified using RNeasy Mini Kit (Qiagen 74104). RNA concentrations were measured using  
202 Nanodrop. Reverse transcription was performed by using High-capacity cDNA reverse  
203 transcription kit with RNase inhibitor (Applied Biosystem 4374967). qPCR was performed  
204 with the TaqMan Fast Advanced Master Mix (Thermo Fisher 4444556) with specific probes  
205 for *Hprt*, *mGcm2*, *iNOS*, *TLR2*, *ARG1*, *Il-4ra*, and *CD16* (**Supplementary Table 3**). qPCR  
206 reactions with the same concentration of cDNA were run in duplicates using LightCycler 96  
207 (Roche). *Hprt* was used as a housekeeping gene and used as endogenous control.  $\Delta$ Ct values  
208 were used to determine the relative gene expression change.

209

## 210 **RNAscope multiplex assay**

211 RNAscope *in situ* hybridisation (RNA ISH) was performed on fixed frozen sections of mouse  
212 and human post-mortem samples. Preparations, pre-treatment and RNA ISH steps were  
213 performed according to the manufacturer's protocols. All incubations were at 40°C and used a  
214 humidity control chamber (HybEZ oven, ACDbio). For mouse experiments, probe mixes used  
215 were as follows: *mCX3CR1* (Cat No.314221-C2), *mGcm2* (Cat No. 530481-C3), *mGcm1* (Cat  
216 No. 429661-C1), *Polr2a* (Cat No.312471, used a positive control) and *dapB* (No. EF191515;  
217 used a negative control). For RNA ISH on human tissues, probes used were: *hGCM2*  
218 (Cat.871081), *hCD68* (Cat.560591) and *Polr2a* (Cat No.310451, used as positive control); on  
219 *Drosophila*, we used the *gcm* probe (Cat No.1120751-C1)

220 Tyramide dye fluorophores (Cy3, Cy5: TSA plus; and Aykoya) or Opal fluorophores (Opal  
221 520, Opal 570, Opal 690, Aykoya) were used diluted appropriately in RNAscope Multiplex  
222 TSA dilution buffer. Slides were also counterstained with DAPI.

223

## 224 **Imaging**

225 Fluorescent and brightfield imaging were performed under a 20X objective using Axioscan  
226 (Zeiss) and Nanozoomer (Hammamatsu) for all quantitative analysis, and representative  
227 images in the figures were made using an Apotome (Zeiss).

228 Leica Spinning Disk microscope equipped with 20, 40 and 63X objectives was used to obtain  
229 confocal images with a step size of 0.2-1  $\mu$ m. For the quantifications, five or more fields per  
230 sample were used with more than 50 cells in total.

231



## 232 **Image Analysis**

233 Image analysis was performed with the Fiji image analysis program and with Imaris. Fiji was  
234 mainly used to produce images with sum of Z-projections. In all images, the signal was set to  
235 the same threshold in order to compare the different genotypes. Imaris (version 9.5.1) was used  
236 to analyse the morphology of microglial cells during aging using a semi-automatic protocol.  
237 The p-values were estimated after comparing control to cKO cells by two-way ANOVA test  
238 followed by bilateral student test. To analyse the activation state of microglia in LPC lesions  
239 at 7 and 14 dpi, we used the Visiopharm software. We selected 2 parameters: the number of  
240 branching points (ramifications) and roundness. Data were analysed by two-way ANOVA,  
241 followed by Tukey post hoc test.

242

## 243 **Transcriptome analysis of *gcmKD* haemocytes from *Drosophila* embryos and larvae**

244 Haemocytes were sorted by FACS from stage 16 embryos of the following genotypes:  
245 *srp(hemo)Gal4/+;UAS-RFP/+* for the control and *srp(hemo)Gal4/+;UAS-RFP/UAS-gcm-*  
246 *RNAi* (BDRC #31519) for the *gcmKD* (33). Haemocytes were also sorted from wandering third  
247 instar larvae of the following genotypes: *srp(hemo)Gal4/HmlΔRFP* (control) and  
248 *srp(hemo)Gal4/hmlΔRFP;UAS-gcm-RNAi/+* (BDRC #31519) for the *gcmKD*. 20000 to 50000  
249 cells were sorted for each replicate, three replicates were done for each condition. The RNA  
250 were extracted using Tri-reagent (SigmaAldrich) according to the manufacturer protocol.  
251 RNAseq libraries were prepared using the SMARTer (Takara) Low input RNA kit for Illumina  
252 sequencing. All samples were sequenced in 50-length Single-Read. At least  $40 \times 10^6$  reads were  
253 produced for each replicate. Data analysis was performed using the GalaxEast platform  
254 (<http://www.galaxeast.fr/>, RRID:SCR\_006281) as described in (33, 34). The differential  
255 expression analysis was carried out using HTseq-Count (RRID:SCR\_011867) and DESeq2  
256 (RRID:SCR\_015687) (35). The gene ontology analysis was done with ShinyGO 0.76 (36). The  
257 graphs were plotted using the packages pheatmap (RRID:SCR\_016418) and ggplot2  
258 (RRID:SCR\_014601) in R (version 3.4.0) (R Core Team, 2017). The RNAseq data were  
259 deposited in the ArrayExpress database at EMBL-EBI ([www.ebi.ac.uk/arrayexpress](http://www.ebi.ac.uk/arrayexpress)) under  
260 accession number E-MTAB-8702.

261

## 262 **Expression profile of the microglial markers in adult *Drosophila* haemocytes and glia**

263 The dataset GSE79488 for adult haemocytes and GSE142788 for adult glia were retrieved from  
264 GEO database, mapped using RNA-STAR and compared with DESeq2 as described above  
265 (37). The microglial genes conserved across evolution were determined by Geirsdottir (38).

266 The *Drosophila* orthologs were determined using DIOPT (39). The heatmap was drawn using  
267 pheatmap (RRID:SCR\_016418).

268

### 269 **Tracing *gcm* expression**

270 To assess the induction of *gcm* expression in *Drosophila* upon wasp infestation, a lineage  
271 tracing system was expressed under the control of *gcm*-specific promoters and a  
272 thermosensitive inhibitor. The detailed genotype is *UAS-FLP/+;act5c-FRT,y+,FRT-*  
273 *Gal4,UASmCD8GFP/gcmGal4,tubulinGal80<sup>TS</sup>;UAS-FLP,Ubi-*  
274 *p63E(FRT.STOP)Stinger/6KbgcmGal4 (gcm>g-trace)* for the experiment and *UAS-*  
275 *FLP/+;act5c-FRT,y+,FRT-Gal4,UASmCD8GFP/+;UAS-FLP,Ubi-*  
276 *p63E(FRT.STOP)Stinger/+* for the control (**Supplementary Figure 8A**). Embryos and larvae  
277 were raised at 18°C (tracing off) until the second instar larval stage, to avoid revealing the  
278 embryonic expression of *gcm*. Second instar larvae were infested with the parasitoid wasp  
279 *Leptopilina boulardi*. 20 female wasps were used to infest 100 *Drosophila* larvae for 2 h at RT.  
280 Infested larvae were then transferred at 29°C (tracing on) and let develop until wandering third  
281 instar larval stage for further analyses.

282

### 283 **Wasp infestation assays**

284 Wasp eggs were collected upon bleeding 50 infested larvae in PBS 1X added with some N-  
285 *Phenylthiourea* crystals (Sigma) to prevent haemocyte melanisation. Wasp eggs were fixed in  
286 4% PFA/PBS 1X for 30 min, washed with PTX (0.3% Triton X-100 in PBS 1X), incubated 1h  
287 in PTXN (NGS 5% in PTX, Vector Laboratories), incubated overnight at 4°C in primary  
288 antibodies, washed with PTX, incubated 1h in secondary antibodies, 1h with DAPI and TRITC-  
289 phalloidin (Sigma), then mounted with Vectashield mounting medium (Vector Laboratories).  
290 Haemocytes were labelled as previously published (12). Primaries and their appropriate  
291 secondary antibodies are on supplementary table 1.

292 For the gain and loss of function assays, we used control, gain of function (GOF) or loss of  
293 function (LOF) flies upon crossing *w;hmlΔGal4/CyoGFP;tubGal80<sup>TS</sup>* animals with *white-1118*  
294 (*control*), *UASTgcmF18A* (gain of function, GOF) or *UASgcmRNAi* (loss of function, LOF)  
295 animals, respectively (40,12). The infestation was conducted as described above and the  
296 tumour size was counted as previously described (12).

297

298

299

300 **Statistical analysis**

301 Variance analysis using bilateral student tests for unpaired samples was used to estimate the p-  
302 values for microglia ramifications, coverage area, iNOS, Arg1-positive microglia. In each case,  
303 at least five animals were counted. In all analyses, “ns” means not significant, “\*” for p-value  
304 < 0.05; “\*\*” for p-value < 0.01; “\*\*\*” for p-value < 0.001.

305 For cell quantification in LPC lesions, 3-5 spinal cord sections per animal were analysed, with  
306 at least N=3/group. Quantification of Olig2+ and CC1+ cells was performed using ZEN (Zeiss)  
307 and QuPath software. Statistical analyses were performed by using R and GraphPad (Prism)  
308 Software using Two-way ANOVA with Tukey post hoc comparison tests.

309

310 **Study approval.**

311 All animal experiments were conducted according to the European law for the welfare of  
312 animals. All animal procedures were reviewed and approved by the “Comités d’Ethiques en  
313 Expérimentation Animale” of IGBMC-ICS and of the Paris Brain Institute - ICM.

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## 334 Results

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### 336 **Expression of the murine *Gcm* genes *in vitro***

337 We assessed whether the mGcm1 and mGcm2 genes are expressed in microglia by  
338 characterising CNS primary cultures (41, 42). Microglia were labelled with the broad  
339 hematopoietic marker CD45 (43). Microglia adopt one of the three morphologies both *in vitro*  
340 and *in vivo*: ramified microglia with small cell body and long ramifications, round cells with a  
341 small cell body and no ramifications, amoeboid microglia with a big cell body and no  
342 ramifications (44-46). Round cells are considered as activated microglia, the other two as  
343 resting cells (47). mGcm2, but not mGcm1, is expressed in 30% of round shaped microglia  
344 (**Figure 1B and Supplementary figure 1A**). We ascertained the specificity of the mGcm2  
345 immunolabeling in a conditional knock out (cKO throughout the text) *mGcm2<sup>fllox/fllox</sup>* mouse line  
346 crossed it with the *Cx3cr1-Cre* line expressed in microglia. The *mGcm2<sup>fllox/fllox</sup>* line was  
347 produced by introducing two LoxP sites upstream and downstream of the *Gcm* exons 2,3 and  
348 4 (**Figure 1A**). mGcm2 labelling is absent in the cKO microglia, proving by the same token  
349 the specificity of the antibody and the efficiency of our cKO model (**Figure 1B**).

350

351 In sum, mGcm2 is expressed in active microglia of CNS cultures.

352

### 353 ***mGcm2* is expressed in the microglia of aged animals**

354 Based on the above results, we analysed neonatal microglia at postnatal day 14 (P14) but found  
355 no mGcm2 expression (**Figure 1C**). This could be explained by the fact that, *in vivo*, microglia  
356 display major differences from microglial cultures (48). The finding that mGcm2 expression is  
357 specific to activated microglia *in vitro*, prompted us to ask whether its expression is induced in  
358 an inflammatory condition such as aging. Brain and spinal cord were collected at different ages:  
359 P14, 2, 12 and 24 months. As cell-specific markers, we used the microglia-specific cocktail  
360 (CD11b, CD68, F4/80) that distinguishes microglia from the meningeal macrophages (49).  
361 mGcm2 is expressed in microglia at 12 and 24 months but not in 2month-old animals in  
362 different areas of the brain including the cortex (**Figure 1C**). Moreover, the number of mGcm2-  
363 positive microglia increases over time: 12 and 24month-old cortices show 25% and almost  
364 double mGcm2-positive microglia (48%), respectively (**Figure 1D**).

365

366 We also performed RNA ISH on brain sections and confirmed that *mGcm2* is expressed in aged  
367 brains (18month-old animals), while *mGcm1* is not expressed in either control or cKO brains

368 **(Supplementary figure 1B,C)**. This further validates the efficiency of our flox/flox line and  
369 shows that *mGcm1* does not compensate for the lack of *mGcm2* expression. Finally, to evaluate  
370 the expression in other resident macrophages, lung and adipose tissues were dissected from  
371 24month-old animals and cryo-sections were labelled for CD45 and mGcm2. No mGcm2  
372 labelling was observed in the resident macrophages associated with those tissues  
373 **(Supplementary figure 1D)**.

374

375 Thus, mGcm2 is expressed only in a subset of aged microglial cells.

376

### 377 **Microglia of *mGcm2* cKO mice have an activated morphology in homeostatic conditions**

378 To explore the role of mGcm2 in microglia *in vivo*, we characterised their morphology, which  
379 is tightly linked to the activation state of microglia: resting cells have long ramifications while  
380 activated cells have shorter ramifications (50). Sections of control and cKO brains from  
381 different age groups were labelled with the microglial marker Iba1 **(Figure 2A-C)** (51, 52).  
382 Our criteria include the number of ramifications and the coverage area of the cell, that is, the  
383 area where microglia extend their ramifications at, which thus represents the area they can  
384 survey. The former parameter is a direct measurement of their activation state while the latter  
385 is an indirect indicator, as activated microglia have shorter or less ramification, which results  
386 in a decreased coverage area.

387

388 We found that microglia morphology changes over time, but more in the cKO animals **(Figure**  
389 **2A-C)**. More specifically, the number of ramifications per cell decreases during aging in both  
390 genotypes **(Figure 1A)**. However, there is a significant decrease in the number of ramifications  
391 in *mGcm2* cKO compared to control microglia at 24 months **(Figure 2B)**. The same trend is  
392 also visible for the coverage area **(Figure 2C)** tendency to decreased coverage area in the  
393 *mGcm2* cKO).

394

395 Altogether, these results reveal for the first time that the lack of *mGcm2* has an impact on  
396 microglia morphology, indicative of a pro-inflammatory phenotype.

397

### 398 ***mGcm2* cKO animals display a pro-inflammatory profile**

399 We complemented the morphological data by labelling 2, 12 and 24month-old brains with pro-  
400 and anti-inflammatory markers. Microglia/macrophage activation states are classified as pro-  
401 inflammatory (or M1) or anti-inflammatory (or M2). We chose the markers inducible nitric

402 oxide synthase (iNOS) for the M1 and Arginase-1 (Arg1) for the M2 state, which were already  
403 used to characterise microglia *in vivo* (53). Labelling from different age groups shows that  
404 *iNOS* is present mostly in 12 and 24month-old animals and its expression is increased in both  
405 groups as they age (**Figure 3A,B**). Upon quantifying the iNOS-positive microglia, we found  
406 that *mGcm2* cKO animals specifically display an increased number of iNOS-positive microglia  
407 compared to control animals by 24 months (**Figure 2B**).

408  
409 Next we evaluated the number of Arg1-positive cells (**Figure 3C,D**). 2month-old control  
410 animals have a higher number of Arg1-positive microglia compared to 12 and 24month-old  
411 animals (**Figure 3D**). Likewise, *mGcm2* cKO animals show a significant decrease in the  
412 number of Arg1-positive microglia, from 2 (74%) to 12 months (47%). Importantly, only the  
413 percentage of Arg1-positive microglia in the cKO animals further decreases from 12 to 24  
414 months, resulting in a significant decline. Thus, the microglia lacking *mGcm2* display a  
415 stronger progression toward a pro-inflammatory phenotype compared to control microglia.

416  
417 The state of other CNS populations does not seem overtly affected in the mutant animals. The  
418 size of astrocytes increases in inflammatory conditions (54, 55), a phenomenon called  
419 astrogliosis that can be measured via GFAP labelling (56). We found no difference between  
420 the GFAP labelling of 24month-old control and mutant animals (**Supplementary figure**  
421 **2A,B**). Similarly, we found no difference in neuronal cell death by co-labelling the cell death  
422 marker caspase 3 with the pan neuronal marker NeuN (**Supplementary figure 2C-E**). No  
423 difference in oligodendrocyte number was found either (**Supplementary figure 2F,G**).  
424 Accordingly, mutant and control animals show similar behavioural habits in an open field test  
425 (**Supplementary figure 2H-J**), which assays general locomotor activity levels, anxiety, and  
426 willingness to explore.

427  
428 The morphological and the molecular data show that *mGcm2* has an anti-inflammatory role in  
429 murine microglia during chronic inflammatory conditions such as aging.

### 430 431 ***mGcm2* is expressed in acute LPC lesions and *hGcm2* in active MS lesions**

432 Since *mGcm2* expression is induced upon aging, we asked whether it is also expressed in  
433 immune cells following CNS injury, which represents a condition of acute inflammation. We  
434 induced acute demyelination by LPC injection in the mouse dorsal white matter spinal cord  
435 and analysed *mGcm2* expression profile. LPC lesions show limited amount of inflammation,



436 usually associated with myelin debris removal. The *mGcm2* protein was specifically detected  
437 in a subset of CD45<sup>+</sup> immune cells (**Figure 4A**). RNAscope ISH assays also showed *mGcm2*  
438 labelling in very few microglia specifically within the lesions from 2 to 21 dpi (**Figure 4B**).

439

440 To assess the relevance of these findings in humans, we analysed the expression of *hGCM2* in  
441 acute and chronic MS lesions, as well as in the normal appearing white matter from MS and  
442 non-neurological control cases (**Supplementary Table 1**). MS lesion subtypes were first  
443 characterised using Luxol Fast Blue and MHCII staining (**Figure 5A**) and classified as active,  
444 chronic active and chronic inactive (32). The *hGCM2* protein was specifically detected in few  
445 MHCII<sup>+</sup> immune cells located in active MS lesions, which have traditionally been defined as  
446 showing demyelination with inflammatory infiltrates, whereas chronic lesions show  
447 demyelination with little or no activity (**Figure 5B**). We next performed double RNA ISH for  
448 *hGCM2* and *hCD68* and found *hGCM2* expression in few *hCD68*-positive microglia only in  
449 active lesions and in the active rim of chronic active lesions (**Figure 5C**).

450

451 In sum, *hGCM2* and *mGcm2* gene expression is upregulated in demyelinating lesions, in a  
452 subset of cells belonging to the microglia/macrophage lineages.

453

#### 454 **Loss of *mGcm2* in microglia promotes a pro-inflammatory response after demyelination**

455 To decipher the functional role of *mGcm2* in microglia/macrophages in LPC lesions, we  
456 generated an inducible cKO mouse line. The *mGcm2* floxed strain (**Figure 1A**) was crossed  
457 with the *Cx3cr1<sup>CreER/+</sup>* mouse line (31). The deletion was induced in the F1 generation,  
458 specifically in microglia by tamoxifen injection (**Figure 6A**). We refer to this mouse strain as  
459 *Cx3cr1<sup>CreER/+</sup>;Gcm2<sup>flox/flox</sup>* (inducible cKO, icKO). *Cx3cr1<sup>CreER/+</sup>;Gcm2<sup>flox/+</sup>* heterozygous  
460 littermates (named control thereafter), *Gcm2<sup>flox/flox</sup>* and WT animals were used as controls for  
461 LPC lesions. 10-16 week-old mice were treated with tamoxifen during 5 consecutive days prior  
462 to LPC induced lesions. We then assessed the impact of *mGcm2* deletion in microglia on the  
463 different steps of the remyelination process, including OPC recruitment, differentiation and  
464 remyelination (**Figure 6B**). *mGcm2* deletion was first confirmed by qPCR analysis of RNA  
465 extracted from dissected spinal cord lesions at 4 dpi. *mGcm2* relative mRNA expression is  
466 indeed severely reduced in LPC demyelinated spinal cords of icKO mice with respect to  
467 controls (**Figure 6C**).

468



469 Once the tamoxifen-inducible Cre deletion of *mGcm2* in Cx3cr1-expressing cells had been  
470 validated, we monitored the microglia response to LPC lesions in *mGcm2* icKO mice.  
471 Immunohistochemistry for iNOS combined with the microglia cocktail markers (CD11b, F4/80  
472 and CD68) revealed a drastic increase of microglial cells in a pro-inflammatory state in  
473 demyelinated lesions of *mGcm2* icKO mice as compared to controls (**Figure 6D**). To further  
474 confirm that the *mGcm2* deletion promotes a microglial pro-inflammatory state after LPC-  
475 induced demyelination, we performed qPCR analysis of M1 and M2 marker relative  
476 expression. In agreement with the immunohistochemistry data as well as the aging data, the  
477 expression of the M1 gene *iNOS* (**Figure 6E**) and *TLR2* (**Figure 6F**) revealed a trend increase  
478 after spinal cord demyelination in the icKO mouse strain with respect to WT, *mGcm2*<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> and  
479 control mice. We also evaluated the relative gene expression of the M2 markers *Arg1* (**Figure**  
480 **6G**), *Il-4ra* (**Figure 6H**) and *CD163* (**Figure 6I**) in *mGcm2* icKO and control animals,  
481 however, our data did not reveal any significant difference.

482  
483 To further corroborate these findings, we examined microglial cell morphology in conditional  
484 *mGcm2* icKO and control mice in LPC demyelinated lesions, *in vivo*. Spinal cord sections  
485 throughout the demyelinated lesions were labelled with the microglial marker Iba1 and  
486 microglia morphology was evaluated with VisioPharm (57). In line with the above data, the  
487 number of ramifications point per microglial cell decreases significantly in *mGcm2* icKO  
488 compared to controls, specifically in demyelinated lesions at 7 and 14 dpi (**Figure 6J**). As an  
489 additional morphological parameter of activated microglia, we evaluated the roundness of the  
490 cells at the same time points. The number of microglial cells exhibiting a round morphology  
491 increases significantly in *mGcm2* icKO compared to controls (**Figure 6K**), further indicating  
492 that *mGcm2* loss-of-function in microglia favours a pro-inflammatory state.

493  
494 The above findings support a role of *mGcm2* as a new anti-inflammatory transcription factor  
495 in mouse microglia under pathological conditions.

496  
497 **Loss of *mGcm2* in microglia delays oligodendrocyte differentiation in demyelinated**  
498 **lesions**

499 Microglia activation state plays a critical role in the regulation of demyelination and  
500 remyelination (58). Therefore, we asked whether *mGcm2* loss-of-function in microglia  
501 hampers myelin debris clearance and oligodendrocyte differentiation in LPC induced  
502 demyelinated lesions. Oil-Red O staining was performed to evaluate the density of microglia

503 containing myelin debris (**Supplementary Figure 3A**). The Oil-RedO-positive areas as well  
504 as the percentage of Oil-RedO-positive areas in LPC lesions do not differ significantly between  
505 *mGcm2* icKO and control mice at similar time point post-demyelination (**Supplementary**  
506 **Figure 3B,C**), suggesting that *mGcm2* deletion in microglia does not affect the ability of these  
507 cells to phagocytose myelin debris.

508

509 We next examined whether oligodendrocyte differentiation could be hampered. Spinal cord  
510 sections of demyelinated lesions from icKO and control mice were immunolabeled for Olig2,  
511 a pan-oligodendrocyte marker, together with CC1, a specific marker of differentiated  
512 oligodendrocytes (**Supplementary Figure 4A**). Quantification of the percentage of  
513 Olig2+CC1+ mature oligodendrocytes and Olig2+CC1- OPCs revealed a significant increase  
514 of differentiated oligodendrocytes in both groups at 7 and 21 dpi (**Supplementary Figure 4B,**  
515 **D**). Nevertheless, oligodendrocyte differentiation is delayed in LPC lesions of the *mGcm2*  
516 icKO strain with respect to controls, between 7 and 14 dpi (**Supplementary Figure 4B,C**). It  
517 is worth noting that the overall number of Olig2+ oligodendroglial cells in demyelinated lesions  
518 increases in both groups but was significantly lower in the icKO mice compared to the controls  
519 at 21dpi (**Supplementary Figure 4D**), suggesting that the pro-inflammatory state of *mGcm2*  
520 icKO microglia hampered oligodendroglia cell survival or apoptosis.

521

### 522 ***gcm* expression is induced upon aging in *Drosophila melanogaster***

523 Since the Gcm pathway is induced in chronic (aging) and acute (LPC induced) inflammatory  
524 conditions in mice, we asked whether this is an ancestral process and evaluated it in aging  
525 *Drosophila* brains. In physiological conditions, Gcm is expressed in a subpopulation of  
526 haemocytes, in which it has an anti-inflammatory role (12). Gcm expression is confined to the  
527 haemocytes derived from the first hematopoietic wave that occurs in the procephalic mesoderm  
528 of the embryo (19). These haemocytes cease to express Gcm by the end of embryogenesis and  
529 survive to the adult where they coexist with those derived from the second wave occurring in  
530 the larval lymph gland, which is Gcm independent (59).

531 In the adult, haemocytes are mostly associated with peripheral tissues (60), we therefore first  
532 assessed the number of brain associated haemocytes over time by labelling dissected fly brains  
533 with macrophage markers (P1/NimC4 and Hemese) at different ages, from week 1 (young  
534 animals) to week 6 (old animals), (**Figure 7A,B**). The number of brains that display associated  
535 haemocytes increases over time and by 6 weeks all brains are associated with haemocytes  
536 (**Figure 7C**). The number of brain-associated haemocytes observed in each animal varies,

537 which likely also depends on the dissection protocol that may dissociate from the brain  
538 migratory cells as haemocytes. We next assessed the expression profile of Gcm in the adult  
539 brain upon aging, by performing *in situ* hybridization with a RNAscope probe. In young  
540 animals, Gcm is expressed only in two neuronal clusters located in the central brain  
541 (**Supplementary Figure 5A**) at lateral and dorsal positions (61). In 6week-old animals,  
542 however, Gcm labelling is also present at ectopic positions, indicating *de novo* expression  
543 (**Figure 7A,D**). As in the case of the haemocyte markers, *gcm* labelling is located at the surface  
544 of and not within the brain, indicative of cells that are associated with but do not belong to the  
545 tissue itself.

546

547 ***gcm* expression is induced upon acute challenge and counteracts the inflammatory state**  
548 *gcm* expression in the old haemocytes might be a transient process. Since the *in situ* assay only  
549 identifies cells that express *gcm* at the time of the dissection, this approach may miss cells that  
550 have expressed *gcm* earlier. For this reason, we performed lineage tracing using the g-trace tool  
551 and followed all the cells that express and/or have expressed *gcm* at some point (62). This may  
552 reveal more *gcm*-positive cells than the *in situ* assay. To make sure that we specifically look at  
553 *de novo* expression and not at remnant expression from the embryonic haemocytes, we  
554 activated the g-trace only after adult eclosion (**Figure 8A,B**). The results showed *gcm*-positive  
555 cells that are associated with the brain and do not express glial or neuronal markers. Of note,  
556 the number of *gcm*-positive cells that are associated with old brains is not higher than those  
557 revealed by *in situ*. Surprisingly, these cells do not express the P1/Hemese pan-haemocyte  
558 markers either (**Figure 7E and Supplementary Figure 5B**). This may indicate an  
559 uncharacterised population of haemocytes present at that location or these cells may have not  
560 yet acquired a proper haemocyte identity. The production of additional tools will be necessary  
561 to distinguish amongst these possibilities, but clearly non-neural *gcm* expressing cells become  
562 associated with the aged brain.

563

564 Based on the above results, we asked whether *gcm* is reactivated upon an acute inflammatory  
565 challenge. In *Drosophila*, the most studied acute inflammatory response is induced by wasp  
566 parasitisation. In brief, the parasitoid wasp *Leptopilina boulardi* is allowed to infest and lay  
567 eggs in *Drosophila* larvae. This leads to extensive haemocyte proliferation and activation,  
568 which consists in resting haemocytes transdifferentiating into lamellocytes (activated  
569 haemocytes) in the infested larvae (63). Lamellocytes are huge cells able to encapsulate the  
570 wasp egg, preventing it from hatching and hence allowing *Drosophila* to escape the infestation.

571 Upon activating the *g*-trace tool only during the larval life, no *gcm* expression can be detected  
572 in normal conditions in third instar larvae (**Supplementary Figure 5C**). Upon wasp  
573 infestation, however, *gcm* (*g*-trace)-positive cells surround the wasp eggs, revealing a *de novo*  
574 expression of *gcm* following the acute challenge (**Figure 8C**). We also found *gcm* expression  
575 in circulating lamellocytes (**Figure 8D**). Thus, *gcm* expression is induced by an acute  
576 inflammatory challenge in flies.

577  
578 Since *gcm* is expressed *de novo* upon wasp infestation, we asked how *gcm* gain or loss of  
579 function (GOF or LOF, respectively) would affect the response to wasp infestation (**Figure**  
580 **9A**). To this purpose, we specifically induced (GOF) or silenced (LOF) *gcm* expression in the  
581 larval haemocytes after wasp infestation (**Figure 8B**) and evaluated the so-called tumour  
582 phenotype as a readout of the inflammatory response. The infested animals of the three  
583 genotypes (control, LOF and GOF) carry tumours, but their number and/or size varies. Large  
584 tumours contain the wasp eggs encapsulated by the fly haemocytes, while small/medium size  
585 tumours are due to haemocyte aggregations. LOF animals have in average more tumours than  
586 control and GOF animals (**Figure 9B**). This is mostly due to a very large increase in the number  
587 of small tumours (**Figure 9C,D**). GOF animals, on the other hand, show a decreased number  
588 of large tumours compared to control animals.

589 In sum, silencing the *de novo* expression of *gcm* aggravates the inflammatory phenotype and  
590 inducing *gcm* expression *de novo* ameliorates it.

591

## 592 ***gcm* downregulation triggers a pro-inflammatory state in the *Drosophila* haemocytes**

593 Altogether, our data indicate that the conserved Gcm pathway is induced in response to a wide  
594 variety of challenges and counteracts acute as well as chronic inflammation. It seemingly has  
595 a priming role: the cKO mice are fully viable, fertile and do not display an overt inflammatory  
596 phenotype, much like the mutant flies (12). To investigate the molecular mechanisms  
597 underlying this priming process we proceeded to a high throughput analysis in flies, given the  
598 simplicity of this animal model. Since Gcm is also involved in gliogenesis and its mutation is  
599 embryonic lethal, we analysed the transcriptome of *srp(hemo)Gal4;UAS-gcm-RNAi (gcmKD)*  
600 animals in which Gcm is specifically affected in haemocytes. *gcmKD* haemocytes present  
601 overall increased levels of expression of immune related genes, such as anti-microbial peptides  
602 and components of major immune pathways (**Figure 10A**). In the embryo, the different

603 expression is restricted mostly to STAT92E and Toll, but in the larva more genes appear to  
604 have different expression levels between *gcmKD* and control haemocytes. The highest  
605 difference is found in Gene Ontology (GO) terms associated with immune regulation, such as  
606 regulation of immune response to bacteria and signalling pathway of recognition of  
607 peptidoglycans (**Figure 10B**).

608

609 Thus, silencing *gcm* triggers a pro-inflammatory state.

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## 634 Discussion

635 The present study identifies a novel and evolutionarily conserved anti-inflammatory  
636 transcriptional pathway. The *Gcm* transcription factor was known to regulate the development  
637 of fly immune cell populations (glia and haemocytes) and to modulate the inflammatory  
638 response in flies. Here we demonstrate that the expression of fly and murine *Gcm* genes is  
639 induced upon challenge, which helps counteracting the inflammatory state. Moreover, the  
640 human *Gcm2* ortholog is expressed in MS lesions. The identification of this conserved pathway  
641 opens exciting perspectives to study neuro and inflammatory diseases.

642

### 643 ***mGmc2* and its anti-inflammatory role in the microglia of aged mice.**

644 Aging results in gradual loss of normal function, due to changes at the cellular and molecular  
645 level (64). Microglia exhibit an exaggerated pro-inflammatory response during aging, a  
646 phenomenon referred to as microglia priming (26, 27). Morphologically, aged microglia have  
647 enlarged processes, cytoplasmic hypertrophy and a less ramified appearance (28, 65). They  
648 also express higher levels of activation markers than control microglia (66). All these changes  
649 lead to impaired remyelination and are associated with decreased number of M2 microglia,  
650 cells in an anti-inflammatory state (58).

651

652 Our data indicate that *mGcm2* expression helps keeping the inflammatory state under control  
653 during aging. Aged microglia of cKO mice have a pro-inflammatory morphology with less  
654 ramifications and coverage area. Furthermore, they show a statistically significant increase of  
655 the pro-inflammatory marker *iNOS* and a significant decrease of the anti-inflammatory marker  
656 *Arg1* between 12 and 24 months. The most parsimonious hypothesis is that *mGcm2* acts as a  
657 regulator of the activation state in microglia. Loss of its expression leads to an uncontrolled  
658 stimulation and thus a higher pro-inflammatory profile even under basal conditions. It is widely  
659 accepted that targeting anti-inflammatory (M2) regulators of microglia could lead to new  
660 therapeutic targets for aging, *mGcm2* may represent one of them.

661

### 662 **Vertebrate *Gcm2* is a new anti-inflammatory transcription factor in CNS demyelinating** 663 **lesions**

664 The *mGcm2* protein is present in few CD45-positive immune cells after acute demyelination  
665 induced by LPC injection, indicating that *mGcm2* expression is restricted to a subset of  
666 inflammatory cells. Moreover, using double RNA ISH for *mGcm2* and *cx3cr1*, we provided

667 compelling evidence supporting the expression of mGcm2 in a subset of  
668 microglia/macrophages, specifically in demyelinated lesions. Acute inflammation is one of the  
669 features of LPC-induced demyelination (67, 68). Interestingly, hGCM2 expression was also  
670 detected in a small fraction of MHCII/CD68-double positive microglia/macrophages located  
671 specifically in active lesions and in the active rim of chronic active lesions of MS, thus further  
672 supporting a conserved function of Gcm2 in microglial/macrophage lineage cells in humans.  
673 In order to evaluate the role of mGcm2 specifically during an acute inflammatory condition  
674 and not as a constitutive deletion, as we did during aging, we used the icKO model. Our data  
675 gained in the mGcm2 icKO, specifically in microglia/macrophages, clearly indicate that  
676 mGcm2 favours an anti-inflammatory M2 state, and could therefore promote indirectly myelin  
677 regeneration and repair by modulating OPC differentiation after acute demyelination (58). This  
678 emerging anti-inflammatory role of Gcm2 may open new therapeutic interventions, targeting  
679 this transcription factor in demyelinating diseases.

680

#### 681 **Gcm: glia, haemocytes and microglia**

682 Our data explain what was considered as a conundrum. The fly *gcm* gene was initially described  
683 for its developmental role in glia and haemocytes (10, 18). Surprisingly, while the genes  
684 necessary for neuronal differentiation are functionally conserved in evolution, the orthologs of  
685 Gcm, the fly glial promoting factor, are neither expressed nor required in the differentiation of  
686 vertebrate glial cells (69, 70). Moreover, none of the early transcription factors  
687 expressed/required in fly glial cells and target of Gcm such as the panglial Reverse Polarity  
688 (Repo) transcription factor is expressed in vertebrate glia and this gene is not even conserved  
689 in the vertebrate genome. The gliogenic pathway seems therefore not evolutionarily conserved,  
690 in line with the hypothesis that glia may have evolved several times, adapting to the needs of  
691 the organism (71). The glia of simple organisms play a neural role, as they control axon  
692 ensheathment, synapse activity and insulate the brain through the blood brain barrier (BBB).  
693 In addition, they act as the resident immune cells of the nervous system. By contrast, in  
694 complex organisms, the role of brain resident immune cells is taken by a new cell type of non-  
695 neural origin, the microglia, that infiltrate the nervous system before the BBB is formed. This  
696 division of labour guarantees a better and more targeted response to inflammatory challenges.  
697 We speculate that the haemocytes of simple, fast developing, animals migrate along the  
698 nervous system and contribute to remove dying cells, but as the BBB forms, they are excluded  
699 from the tissue, the immune function being taken up by the glial cells. According to this  
700 hypothesis, we should find orthologs of microglial markers expressed in fly haemocytes as well



701 as glia. A recent and elegant study characterised the microglia transcriptional programs across  
702 ten species spanning more than 450 million years of evolution (72). By using these data with  
703 the RNAseq data from adult fly haemocytes and glia, we created a heatmap of the microglia  
704 orthologs in *Drosophila* (37, 73). These orthologs are mostly shared between the two fly cell  
705 populations (**Figure 10C,D**). One microglial gene whose ortholog is shared by haemocytes and  
706 glia is Peli2, a member of E3 Ubiquitin ligases controlling the Toll signalling pathway (74, 75).  
707 The *Pellino* (*Pli*) fly gene antagonises Toll-mediated innate immune signalling by controlling  
708 MyD88 turnover in macrophages. Future studies will determine whether the pathway is also  
709 conserved in glia. In addition to the shared genes, some are specific to glia (*spn42Da*, *moody*,  
710 *CG42709*) or haemocytes (*CG7882*, *pgant9*, *eya* and *CG30345*). Interestingly, *moody* is one  
711 of the most known markers of the *Drosophila* BBB glia and its ortholog in mammals (*Gpr84*)  
712 is a well-known pro-inflammatory maker that is highly up regulated in microglia upon nerve  
713 injury (76). Revisiting the role of *moody* during neuroinflammation could shed light into the  
714 function of this gene in both species.

715  
716 As a corollary of the above and based on multiple pieces of evidence, we propose that *repo*  
717 constitutes the *bona fide* fly gliogenic gene. Accordingly, Repo misexpression in the mesoderm  
718 suppresses haematopoiesis and its lack triggers the expression of haemocyte markers in the  
719 nervous system (77). We speculate that the Gcm pathway has an ancestral, conserved, role is  
720 in immunity and may have been coopted in the differentiation of fly glia. One of the future  
721 challenges will be to characterize the Gcm-positive cells associated with the aged brain.

722

### 723 **The conserved role of the Gcm pathway in immune processes**

724 Together with the inhibitory role of Gcm on the JAK/STAT pathway and the increased  
725 response to wasp infestation, which relies on the Toll cascade, the present data strongly suggest  
726 that Gcm controls different inflammatory conditions, including aging (12). This is also in line  
727 with the transcriptomic data of the *gcmKD* animals, which confirm the induction of the  
728 JAK/STAT pathway observed *in vivo* and extend the inhibitory role of Gcm to other pathways  
729 such as IMD. Furthermore, *gcm* GOF can ameliorate the inflammatory phenotype in flies, thus  
730 paving the way for new therapeutic strategies against autoimmune diseases, such as MS, where  
731 the inflammatory response needs to be contained. We hypothesise that the induction of the  
732 Gcm cascade reduces the intensity of the inflammatory response and hence has a protective  
733 function. This hypothesis is corroborated by recent data obtained in other organisms. A peculiar  
734 macrophage population called pigment cells is present in the sea urchin (78). Such cells are

735 involved in the immune defence by the production of a pigment that has anti-microbial  
736 properties. Morpholino antisense oligonucleotides for *Spgcm* (*gcmMO*) injection showed that  
737 *gcmMO* animals are less resistant to challenging environmental conditions portrayed by  
738 decreased survival rate compared to the control (79-81). A recent study showed that a *gcm*  
739 ortholog is also expressed in glia-like cells of the freshwater crayfish (*Pacifastacus leniusculus*)  
740 upon an acute inflammatory response (82). Moreover, the expression of a planaria *gcm* ortholog  
741 was found to be induced upon regeneration in a subset of cells close to the wound; its silencing  
742 has no effect in homeostatic conditions but impairs neoblast repopulation upon wounding (83,  
743 84). Thus, more and more studies highlight an evolutionarily conserved mechanism.

744

745 In sum, we report here the discovery of an anti-inflammatory transcriptional cascade that is  
746 conserved from flies to humans. Given the strong potential of transcription factors in  
747 coordinating the expression of several genes and the scarce number of known transcription  
748 factors with a similar function, this work represents a major contribution to understand the  
749 molecular mechanisms controlling the inflammatory response. It also lays the ground for  
750 studying novel therapeutical targets for neuro-inflammatory diseases in humans.

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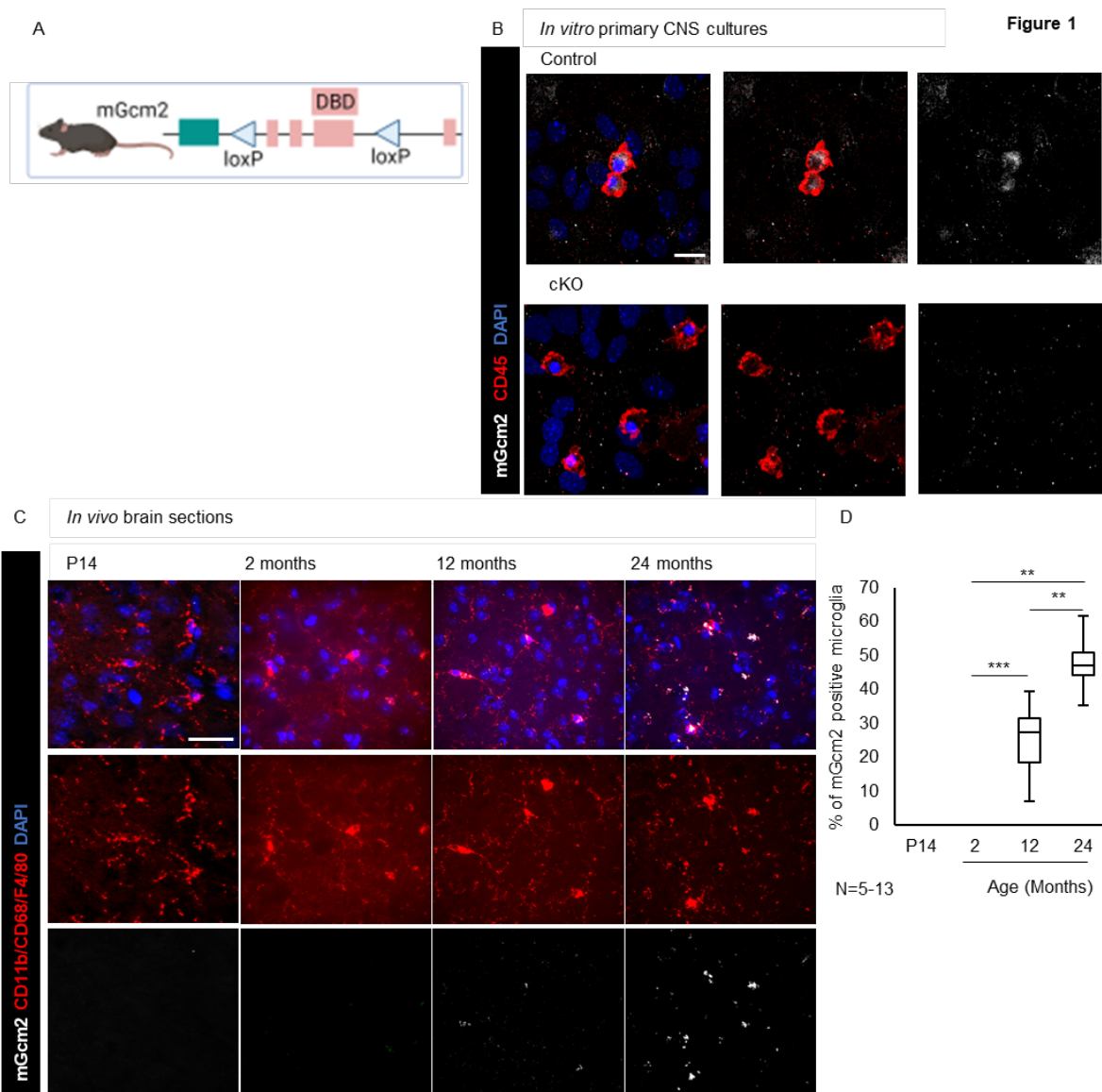
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767 Figure and Figure Legends

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769

770 **Figure 1. *mGcm2* locus and *mGcm2* expression.** (A) For the cKO production two loxP sites

771 were inserted between exons 2 and 4, DBD indicates the DNA Binding Domain. (B)

772 Immunolabelling of CNS primary cultures with CD45 (red), DAPI (blue) and mGcm2 (grey),

773 N=3; scale bar 10  $\mu$ m. (C) Immunolabelling of brain sections for mGcm2 expression *in vivo* at

774 P14 and in 2, 12 and 24-month-old animals. mGcm2 (grey), microglia (CD68, CD11b, F4/80,

775 red) and DAPI (blue). N=5-13/genotype; scale bar: 50  $\mu$ m. (D) Quantification of mGcm2-

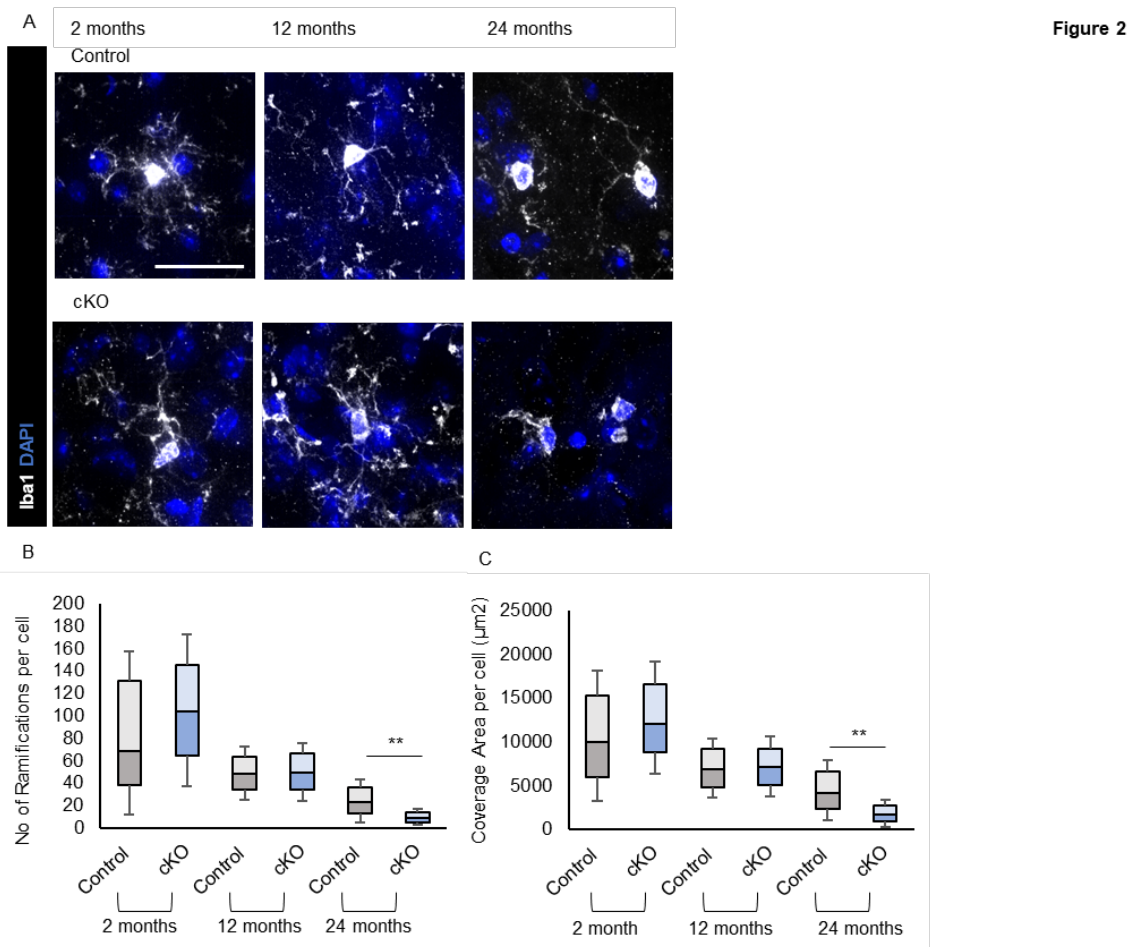
776 positive microglia in the cortex at different ages. “\*\*” for p-value < 0.05; “\*\*\*” for p-value <

777 0.01; “\*\*\*\*” for p-value < 0.001. Statistical significance was determined by one-way ANOVA

778 followed by 2-tailed, unpaired t-test. *Cx3cr1-Cre*<sup>+/-</sup>;*mGcm2*<sup>lox/+</sup> (control) and *Cx3cr1-Cre*<sup>+/-</sup>,

779 *mGcm2*<sup>lox/lox</sup> (cKO)

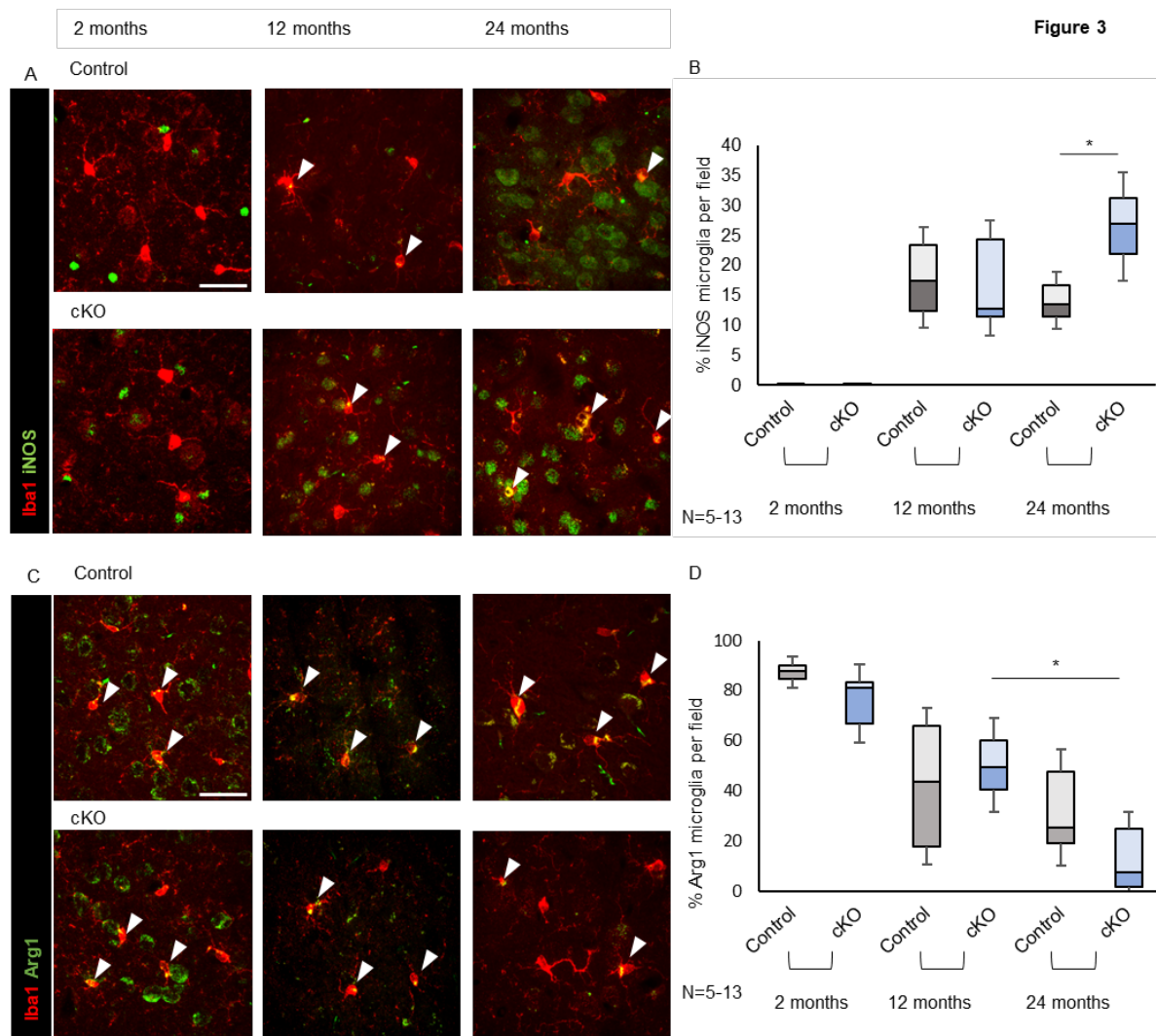
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781

782 **Figure 2. Impact of *mGcm2* deletion on microglia morphology *in vivo*.** (A) Microglia  
783 immunolabelling of *in vivo* brain sections with Iba1 (grey) and DAPI (blue) at 2, 12 and 24  
784 months. (B, C) Analysis of microglia morphology in 2, 12 and 24month-old animals of the  
785 different genotypes for number of ramifications (B) and coverage area ( $\mu\text{m}^2$ ) (C). N=5-13  
786 animals per genotype ; scale bar: 50  $\mu\text{m}$ ; p-value: \* $<0.05$ , \*\* $<0.01$  and \*\*\* $<0.001$ . Statistical  
787 significance was determined by 2-way ANOVA followed 2-tailed, unpaired t-test. *Cx3cr1-*  
788 *Cre*<sup>+/-</sup>; *mGcm2*<sup>fllox/</sup> (control) and *Cx3cr1-Cre*<sup>+/-</sup>; *mGcm2*<sup>fllox/fllox</sup> (cKO)

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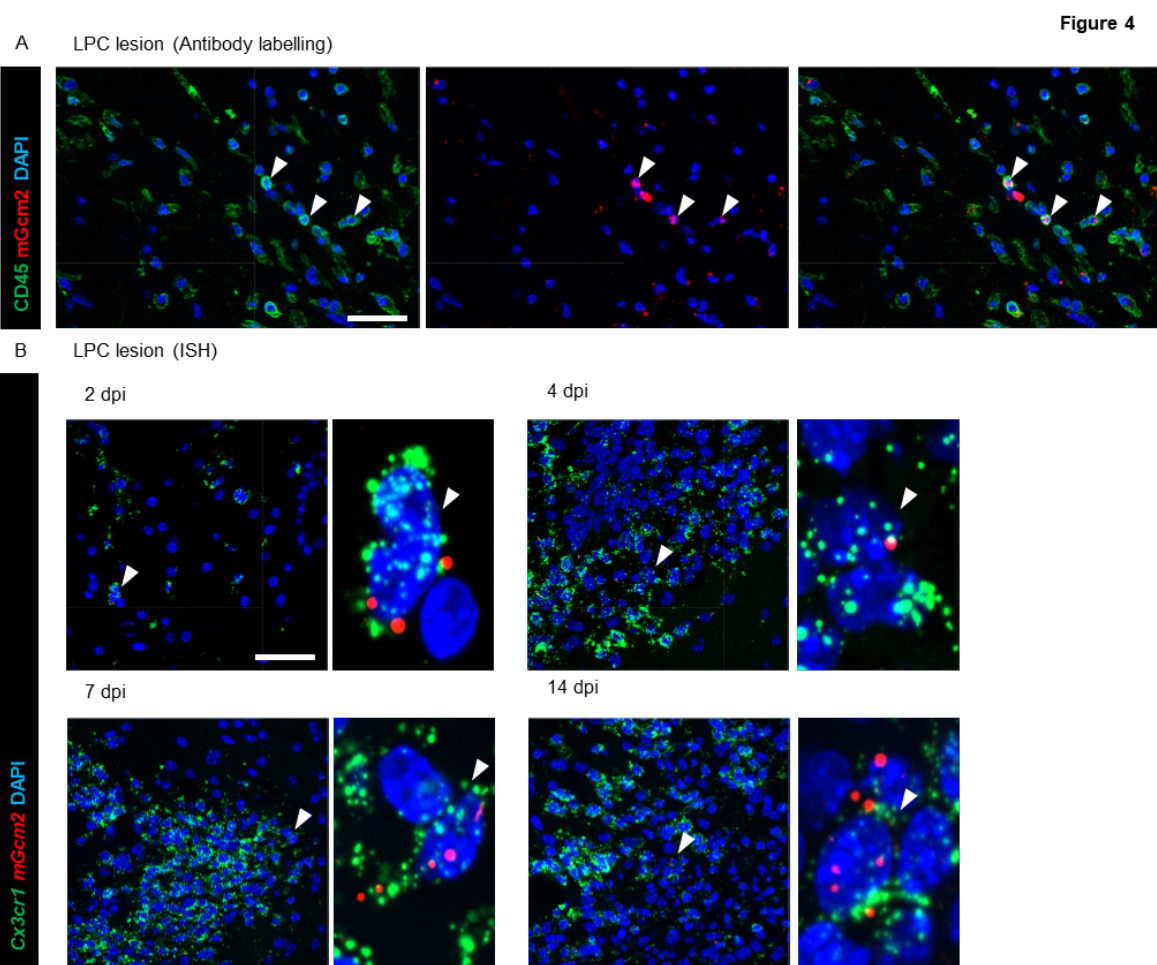


790

791 **Figure 3. Impact of *mGcm2* deletion on microglia expression of inflammatory markers *in***  
 792 ***vivo*.** (A) Immunolabelling of *in vivo* brain sections and (B) quantification of the pro-  
 793 inflammatory marker iNOS (green) with Iba1 (red), (C) Immunolabelling of *in vivo* brain  
 794 sections and (D) quantification of the anti-inflammatory marker Arg1 (green) with Iba1 (red),  
 795 Double positive cells with Iba1 and iNOS or Arg1 expression are indicated by arrows. N=5-  
 796 13; scale bar: 50  $\mu$ m. p-value: \* $<0.05$ , \*\* $<0.01$  and \*\*\* $<0.001$ . Statistical significance was  
 797 determined by 2-way ANOVA followed 2-tailed, unpaired t-test. *Cx3cr1-Cre*<sup>+/-</sup>;  
 798 *mGcm2*<sup>lox/+</sup>(control) and *Cx3cr1-Cre*<sup>+/-</sup>, *mGcm2*<sup>lox/lox</sup>(cKO).

799





**Figure 4. mGcm2 expression in LPC lesions of mouse spinal cord.** (A) Immunolabelling of brain sections for CD45 (green) and mGcm2 (red) in the spinal cord at 2 dpi. Double positive cells with nuclear mGcm2 expression are indicated by arrows. (B) RNA ISH of brain sections, *mGcm2* (red) are mainly detected in a subset of microglial cells expressing *Cx3cr1* (green) at 2, 4, 7 and 14 dpi. Nuclei are counterstained with DAPI (blue). Scale bars: (A), 20 $\mu$ m; (B), 50 $\mu$ m.

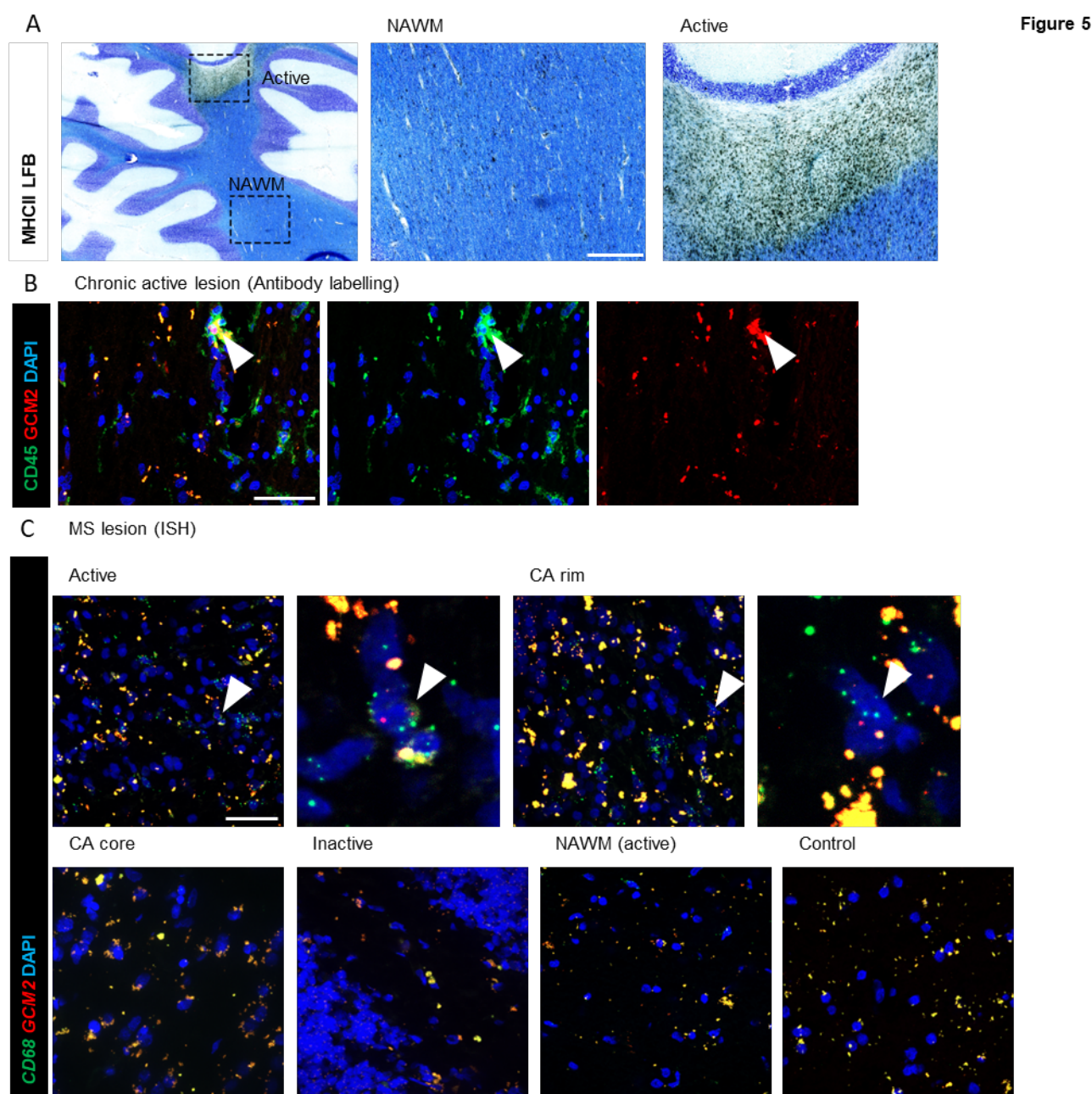


Figure 5

808

809 **Figure 5. hGCM2 is expressed in microglia in active lesions of MS patients.** (A) Luxol fast

810 blue (LFB)/ MHCII staining showing a typical active MS lesion in the cerebellar white matter.

811 The boxed areas illustrate an active lesion containing MHCII+ immune cells and the normal

812 appearing white matter (NAWM) (B) Immunolabelling for MHCII and hGCM2 in an active

813 lesion. Note that hGCM2 expression is only detected in few MHCII-positive immune cells

814 (arrow) of active MS lesions. (C) RNA ISH for *hGCM2* and *hCD68* microglial marker in active

815 plaques, in the active rim, chronic core of chronic active lesions, chronic inactive lesions,

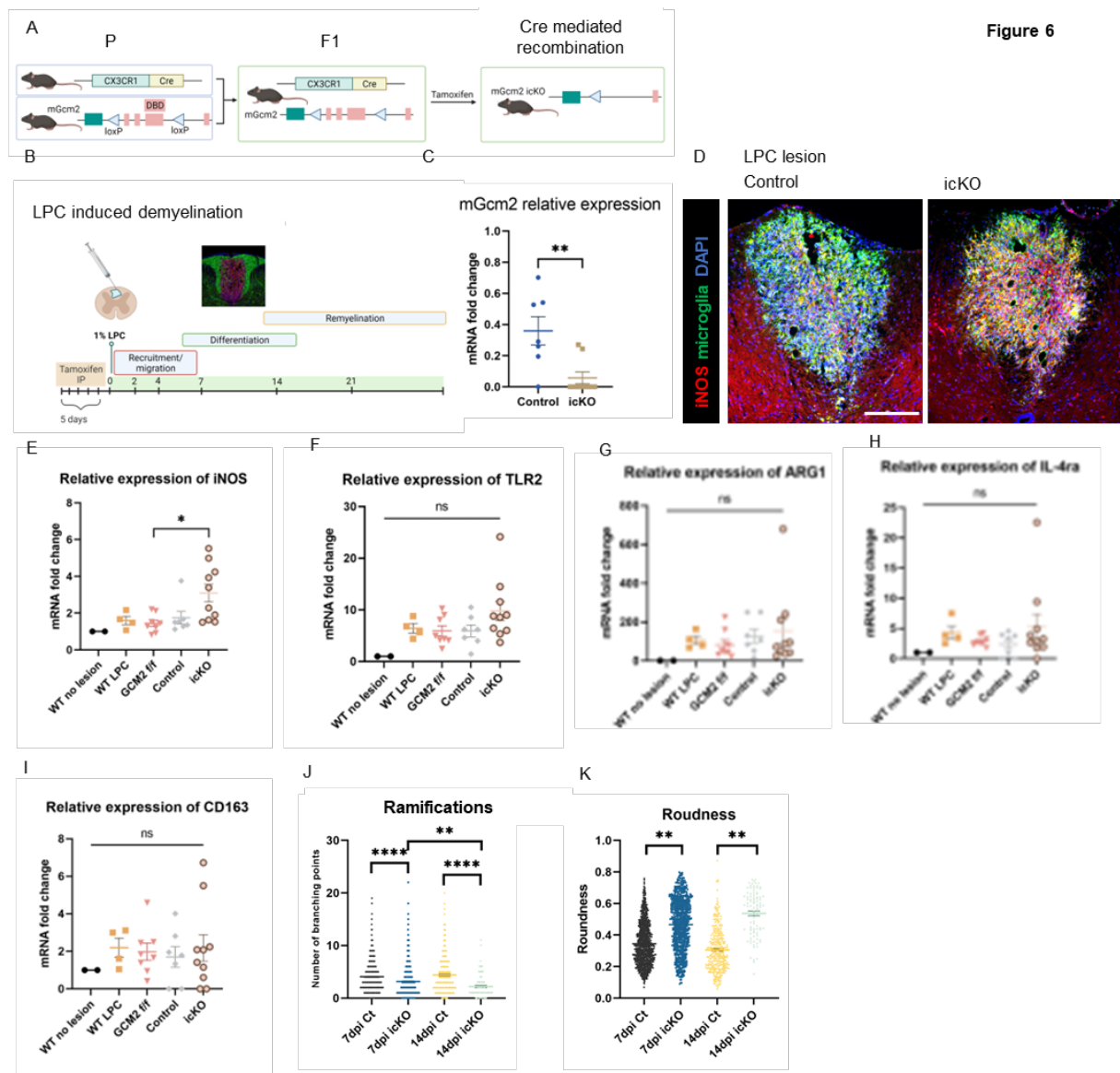
816 normal appearing white matter of MS and control cases. *hGCM2* transcripts are only detected

817 in few *hCD68*-expressing microglial cells located in active lesions and not in inactive lesions,

818 nor in normal appearing white matter of MS or control cases. Note that the yellow dots in

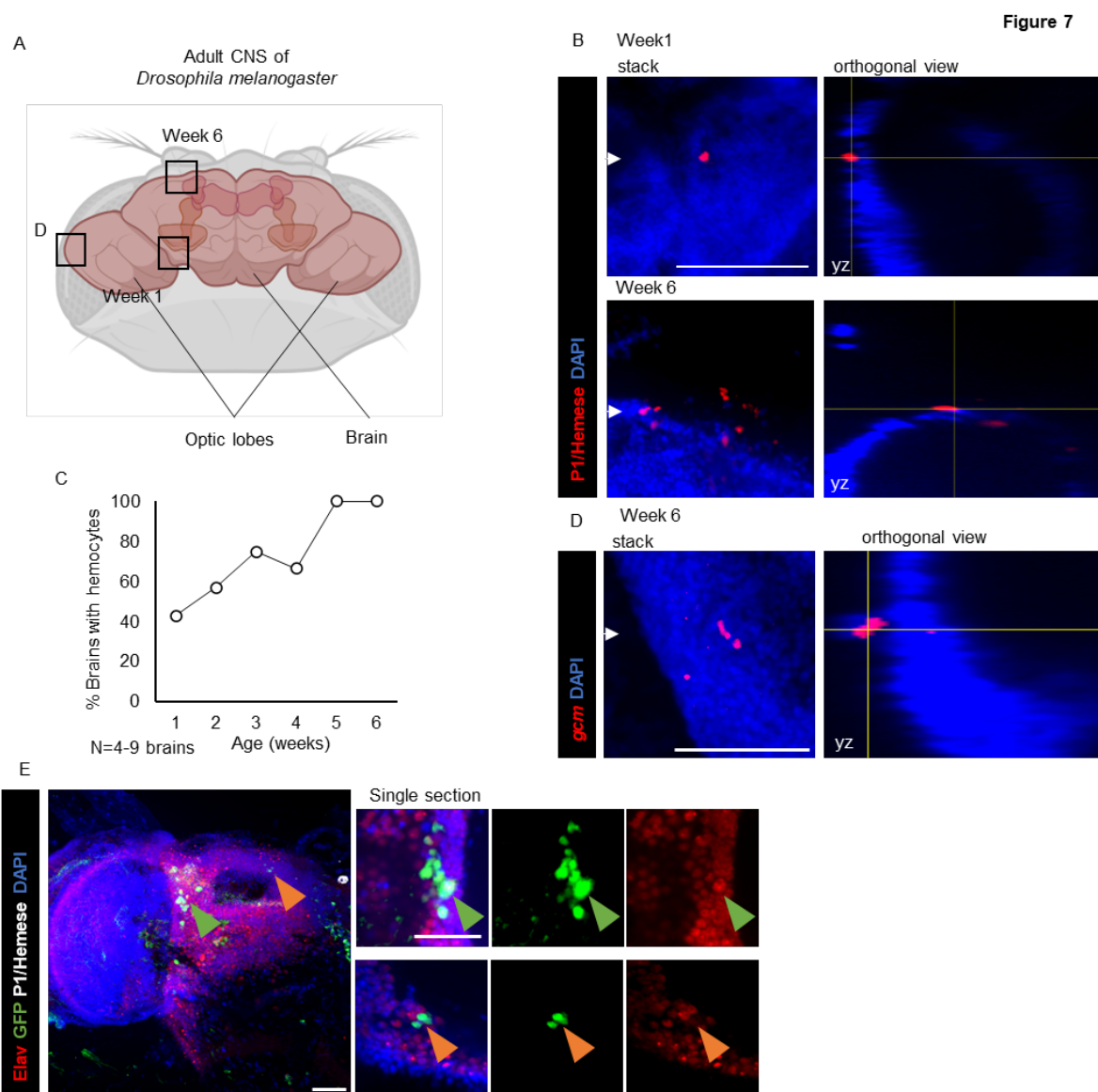


819 panels B and C are due to lipofuscin aggregates that are inherent to human brain tissue. Scale  
 820 bars: (A), 2mm and 500 $\mu$ m for magnification; (B and C), 50 $\mu$ m.  
 821



822  
 823 **Figure 6. Loss of *mGcm2* function in microglial cells favours a pro-inflammatory state**  
 824 **after demyelination.** (A) Schematic representation of the *Cx3Cr1-Cre<sup>ER</sup>* transgene and  
 825 *mGcm2* floxed allele and strategy used to generate tamoxifen-inducible *mGcm2* (icKO) in  
 826 microglia. (B) Schematic illustration of LPC-induced demyelination in the mouse spinal cord  
 827 dorsal funiculus and typical time frame of OPC migration/recruitment, differentiation and  
 828 remyelination (C) *mGcm2* gene expression in demyelinated spinal cords of *Cx3cr1-Cre<sup>ER+/-</sup>*  
 829 *;mGcm2<sup>lox/+</sup>* (icKO) and *Cx3cr1-Cre<sup>ER+/-</sup>;mGcm2<sup>lox/lox</sup>* (control) mice at 4 dpi. (C) *mGcm2*  
 830 mRNA fold changes were normalised relatively to demyelinated WT spinal cords at the same  
 831 time point. Data represent mean  $\pm$  SEM. Mann-Whitney U nonparametric test was used for the

832 statistical analysis (N=7-8/group). \*\* $p < 0.01$ . **(D)** Immunolabelling for M1 marker iNOS (red)  
833 and CD11b/ F4/80/ CD68 microglia (green) in the LPC lesion of control and *mGcm2* icKO, at  
834 14 dpi. The number of microglial cells expressing iNOS in LPC lesions increases in *mGcm2*  
835 icKO animals with respect to control. **(E-I)** qPCR analysis of the relative gene expression of  
836 M1 and M2 microglia markers in WT without LPC lesion, and LPC demyelinated spinal cords  
837 of WT, *Gcm2*<sup>flox/flox</sup>, control and *mGcm2* icKO mice. **(J-K)** Morphological analysis of Iba1-  
838 positive microglia with Visiopharm, in LPC lesions of control and *mGcm2* icKO animals, at 7  
839 and 14 dpi. Two microglia morphological parameters were evaluated: the number of  
840 ramifications **(J)** and the roundness **(K)**. Statistical analyses were performed with R and  
841 GraphPad Software using One-way ANOVA in **(E-I)** and two-way ANOVA **(J-K)**, followed  
842 with Tukey post-hoc test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ . Scale bar: **(D)**, 200  $\mu\text{m}$ .  
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846

847 **Figure 7. Haemocytes and *gcm* expressing cells associated with the aging *Drosophila* CNS.**

848 (A) Schematic of the adult *Drosophila* brain, which contains the medially located central  
849 complex and the lateral optic lobes. The squares indicate the position of the pictures (B and

850 D). (B) Haemocytes associated with the brain at different ages, from week 1 and 6 at 25°C. The  
851 rectangular panels show an orthogonal projection along the yz axes from the position indicated

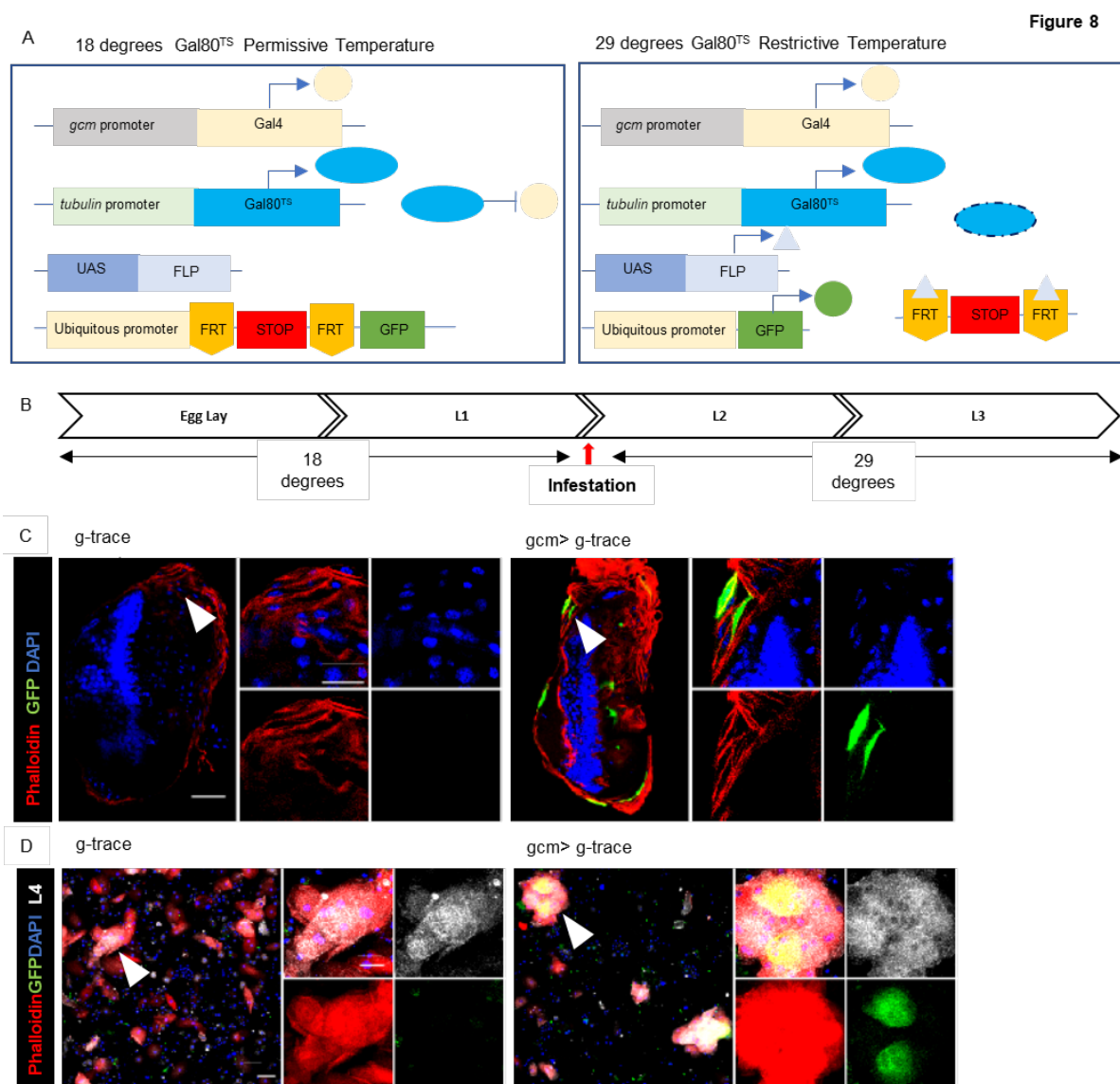
852 by the white arrowheads. (C) Percentage of brains with associated haemocytes at different ages.

853 (D) RNA ISH showing ectopic *gcm* expression in the aging brain, the orthogonal view shows  
854 that the signal is in cells closely associated with the brain but not within it. Scale bar: 50 µm.

855 (E) Immunolabelling of adult *Drosophila* brain with *gcm* tracing (*gcm*>*g-trace*) in green,  
856 P1/Hemese (grey), Elav (red) and DAPI (blue), Scale bar: 20 µm

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860 **Figure 8. Acute inflammation induces *gcm* expression in *Drosophila*.** (A) Inducible lineage

861 tracing using the UAS-Gal4 system. (i) The thermosensitive Gal80 protein (Gal80<sup>TS</sup>) blocks

862 Gal4 activity and hence Gal4-dependent gene transcription at 18°C. (ii) At 29°C, Gal80<sup>TS</sup> is

863 rapidly degraded and Gal4-dependent transcription can occur, FLP indicates the Flippase

864 recombinase, FRT the Flippase Recognition Target. (B) Timeline of the wasp infestation assay.

865 Larvae are kept at 18°C and upon infestation they are transferred at 29°C until the end of the

866 larval development. (C) Tracing of *gcm* expression in activated haemocytes (lamellocytes)

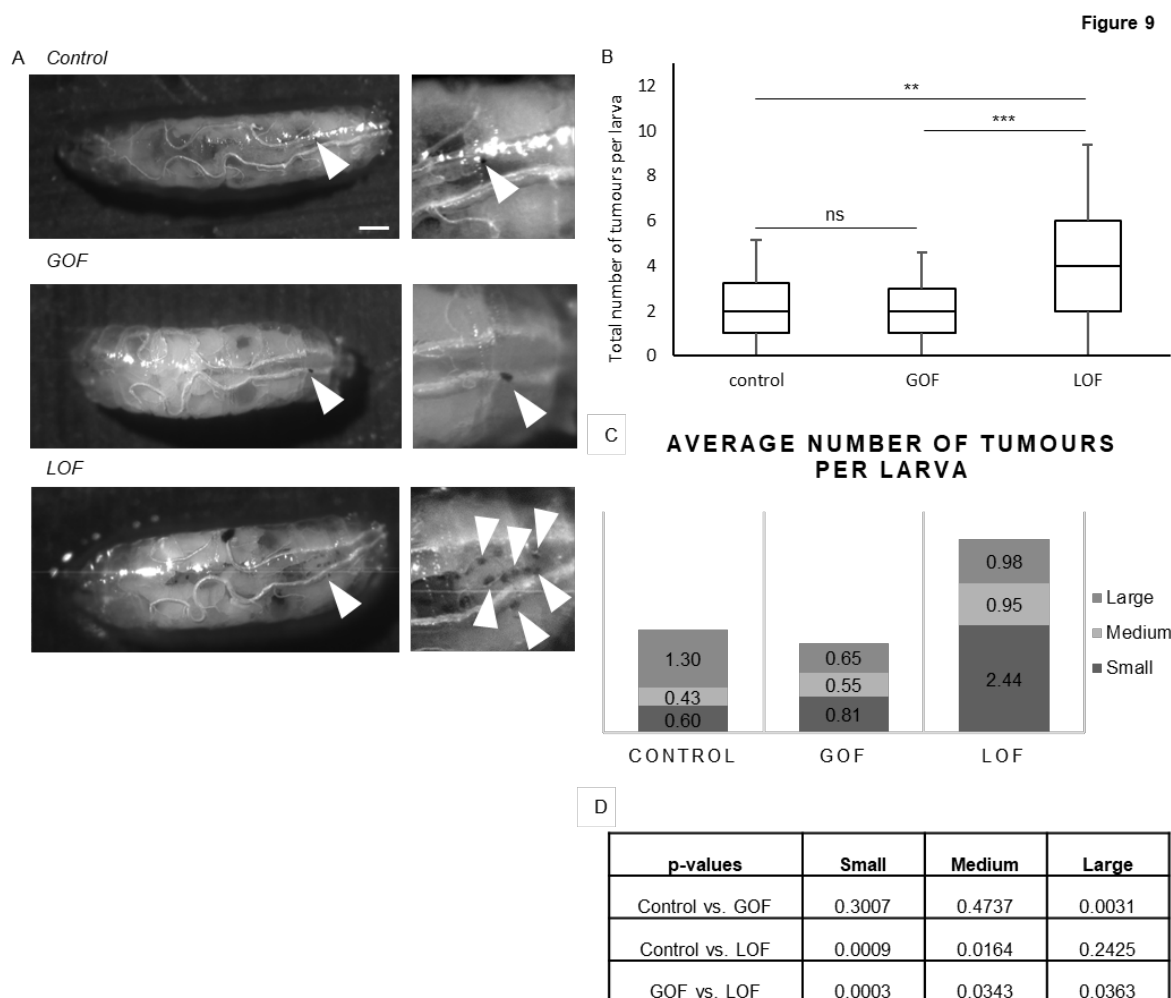
867 surrounding the wasp embryo (nuclei visible with DAPI (blue)) and (D) in haemocytes.

868 Phalloïdin (red), *gcm*>*g-trace* (green), lamellocyte marker L4 (grey) and DAPI (blue). N=3;

869 scale bar for enlarged picture: 50 µm, for magnification: 10 µm.

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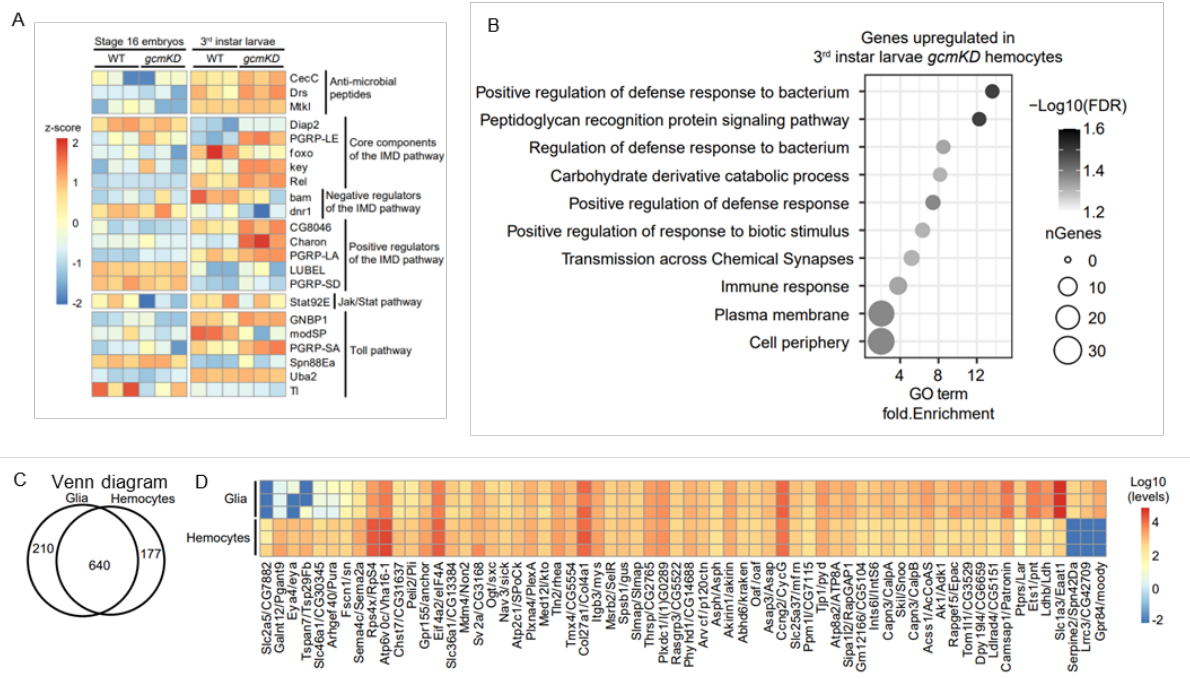


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873 **Figure 9** (A) Brightfield images of infested larvae for the control  
 874 (*w;hmlΔGal4/+;tubGal80<sup>TS</sup>/+*), GOF (*w;hmlΔGal4/+;tubGal80<sup>TS</sup>/UASTgcmF18A*) and LOF  
 875 (*w;hmlΔGal4/+;tubGal80<sup>TS</sup>/UASgcmRNAi*) genotypes.. The arrows indicate the small size  
 876 tumours; scale bar 100 μm. (B) Quantification of the total number of tumours per larva. (C)  
 877 Quantification of the average number of tumours per larva according to their size (D) and the  
 878 p-values. \**p* < 0.05, \*\**p* < 0.01, \*\*\*\**p* < 0.0001 and ns for not significant.

879

Figure 10



880

881 **Figure 10. RNaseq data from microglia and from fly haemocytes and glia** (A) Heatmap

882 from RNaseq data showing differentially expressed genes in hemocytes from late embryos

883 (E16) and wandering third instar larvae. (B) GO term analysis of the genes upregulated in

884 *gcmKD* haemocytes from wandering third instar larvae. (C) diagram representing the number

885 of microglial markers upregulated in glia, in haemocytes or expressed in both tissues at similar

886 levels. The genes upregulated in a specific tissue present an expression fold difference above

887 2 and a p-value < 0.05 (determined using DESeq2). (D) Heatmap representing the expression

888 levels (Log<sub>10</sub> scale) of the orthologs of microglial markers in *Drosophila* adult haemocytes

889 and glia. The markers presenting the strongest expressions (>500reads) were selected. The

890 expression levels were retrieved from the datasets GSE79488 (adult haemocytes) and

891 GSE142788 (adult glia) and analysed using DESeq2.

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## 901 Author contributions

902 AP, SM, PC, RP, BNO and AG designed the experiments and co-wrote the manuscript. AP,  
903 SM, CR, RP performed the experiments in mice, RP performed the experiments in humans,  
904 SM and AP performed the assays in *Drosophila melanogaster*, YY was responsible for the  
905 mGcm2<sup>flox/flox</sup> production and PC analysed the RNAseq data.

906

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