

TLR7/8 stress response drives histiocytosis in SLC29A3 disorders

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38 **Abstract**

39 SLC29A3, also known as ENT3, is a lysosomal transmembrane protein that transports
40 nucleosides from the lysosomes to the cytoplasm¹. Loss-of-function mutations in *SLC29A3*
41 cause lysosomal nucleoside storage and histiocytosis: phagocyte accumulation in multiple
42 organs^{2,3}. However, little is known about the mechanism through which lysosomal nucleoside
43 storage drives histiocytosis. Herein, histiocytosis in *Slc29a3*^{-/-} mice was demonstrated to
44 depend on TLR7, which senses a combination of nucleosides and oligoribonucleotides^{4,5}.
45 TLR7 responded to lysosomal nucleoside storage and enhanced proliferation of Ly6C^{hi}
46 CX3CR1^{low} immature monocytes and their maturation into Ly6C^{low} phagocytes in *Slc29a3*^{-/-}
47 mice. Because accumulated nucleosides primarily originated from cell corpse phagocytosis,
48 TLR7 in immature monocytes recognized nucleoside storage as lysosomal stress and
49 increased phagocyte numbers. This non-inflammatory compensatory response is referred to
50 as the TLR7 stress response where Syk, GSK3 β , β -catenin, and mTORC1 serve as
51 downstream signalling molecules. In SLC29A3 disorders, histiocytosis accompanies
52 inflammation^{6,7}. Nucleoside storage failed to induce pro-inflammatory cytokine production in
53 *Slc29a3*^{-/-} mice, but enhanced ssRNA-dependent pro-inflammatory cytokine production in
54 Ly6C^{hi} classical monocytes and peripheral macrophages, not proliferating immature
55 monocytes. Patient-derived monocytes harbouring G208R *SLC29A3* mutation showed higher
56 survival and proliferation in the presence of M-CSF and produced larger amounts of IL-6
57 upon ssRNA stimulation than did those derived from healthy subjects. A TLR8 antagonist
58 inhibited the survival/proliferation of patient-derived macrophages. These results
59 demonstrated that TLR7/8 responses to lysosomal nucleoside stress drive SLC29A3
60 disorders.

61 **Main**

62 TLR7 and 8 are known as lysosomal ssRNA sensors that initiate innate immune responses
63 during infections^{8,9}. However, their structures indicate that they respond to RNA degradation
64 products; TLR7 binds to guanosine (Guo) or deoxyguanosine (dGuo), as well as to uridine
65 (Urd)-containing oligoribonucleotides (ORNs), whereas TLR8 interacts with Urd and purine-
66 containing ORNs^{4,5,10}. RNA degradation in endosomes/lysosomes proceeds to nucleosides,
67 which are then transported to the cytoplasm for further degradation. SLC29A3, also known as
68 ENT3, is a lysosomal nucleoside transporter abundantly expressed in macrophages¹. Loss-of-
69 function mutations in *SLC29A3* cause monogenic diseases, including H syndrome, Faisalabad
70 histiocytosis, pigmented hypertrichosis with insulin-dependent diabetes mellitus syndrome,
71 and familial Rosai-Dorfman disease^{2,11,12}. These SLC29A3 disorders are characterised by
72 histiocytosis: mononuclear phagocyte accumulation in multiple organs^{2,11,12}. However, the
73 mechanisms by which lysosomal nucleoside storage increases phagocyte numbers have not
74 been elucidated. We hypothesised that the accumulated nucleosides act on TLR7 and TLR8
75 to drive histiocytosis in SLC29A3 disorders.

76 **TLR7-dependent histiocytosis in *Slc29a3*^{-/-} mice**

77 *Slc29a3*^{-/-} mice were obtained (Extended Data Fig. 1a-1c), and various organs of these mice
78 were examined by liquid chromatography-mass spectrometry (LC-MS) to evaluate
79 nucleoside accumulation. We found significant increases in cytidine (Cyd), Guo, 2'-
80 deoxycytidine (dCyd), dGuo, and thymidine (dThd) in the spleens of lysosomal nucleoside
81 transporter-deficient *Slc29a3*^{-/-} mice (Fig. 1a, Extended Data Fig. 2). Significant
82 accumulation of nucleosides was not observed in the other organs, such as the liver, kidney,
83 heart, and lung.

84 Because TLR7 responds to Guo and dGuo⁴, their accumulation in *Slc29a3*^{-/-} mice may
85 activate TLR7. Therefore, we generated *Slc29a3*^{-/-}*Tlr7*^{-/-} mice to evaluate the role of TLR7
86 in histiocytosis (Extended Data Fig. 1d-1f). Consistent with the previous report³, the spleens

87 of *Slc29a3*^{-/-} mice were larger and heavier than those of wild-type (WT) mice due to
88 increased cell number (Fig. 1b, Extended Data Fig. 3a). Concerning cell type-specific
89 changes in the spleen and peripheral blood, increase in numbers was restricted to monocytes
90 and macrophages, not T or B cells (Fig. 1c, Extended Data Fig. 3b-h). Peripheral blood
91 platelet counts decreased, probably, due to premature clearance by accumulated macrophages
92 (Extended Data Fig. 3i). All these changes were dependent on TLR7, as demonstrated in the
93 *Slc29a3*^{-/-} *Tlr7*^{-/-} mice (Fig. 1b-c, Extended Data Fig. 3).

94 **Cell corpse phagocytosis increases nucleoside storage**

95 Nucleoside storage was also observed in professional phagocytes, such as thioglycolate-
96 elicited peritoneal macrophages (pMphs) and bone marrow-derived macrophages (BM-Mphs)
97 (Extended Data Fig. 4a, 4b). In contrast, we observed much smaller nucleoside increases in
98 splenic B cells and low nucleoside accumulation in BM-pDCs (Extended Data Fig. 4c, 4d).
99 Given that B cells and BM-pDCs are less phagocytic than pMphs and BM-Mphs,
100 phagocytosis might increase nucleoside storage. To address this possibility, we exposed
101 dying thymocytes (cell corpse) to BM-Mphs (Fig. 1d, Extended Data Fig. 4e). We observed
102 increases in nucleosides such as dGuo, dCyd, and dThd, which peaked at 8-24 h after cell
103 corpse treatment. At 48 h, the levels of dGuo returned to normal in WT BM-Mphs but
104 remained high in *Slc29a3*^{-/-} BM-Mphs. A lower, but appreciable, increase in the levels of
105 Guo and Cyd was observed. In contrast to cell corpse phagocytosis, SRBC engulfment did
106 not increase the amounts of nucleosides (Fig. 1e, Extended Data Fig. 4f). As SRBCs do not
107 have nuclei, nuclear DNA and RNA from cell corpses are likely to be the major sources of
108 nucleosides accumulated in BM-Mphs. Nucleoside storage in pMphs and BM-Mphs
109 suggested their engulfment of cell corpses during elicitation by thioglycolate *in vivo* or by *in*
110 *vitro* culture with M-CSF, respectively (Extended Data Fig. 4a, 4b).

111 We, next, studied the localisation of TLR7 and SLC29A3 in the mouse macrophage cell
112 line J774.1, which engulfed the DAPI-labelled cell corpse. TLR7 and FLAG-tagged
113 SLC29A3 were recruited to the cell corpse-containing phagosomes (Fig. 1f). These results
114 suggest that SLC29A3 silences TLR7 by transporting phagosomal nucleosides to the
115 cytoplasm in WT macrophages.

116 **TLR7 drives proliferation to increase phagocytes**

117 Monocyte progenitors in the bone marrow give rise to Ly6C^{hi} monocytes/macrophages,
118 which mature into Ly6C^{low} monocytes/macrophages¹³. In the spleen and peripheral blood,
119 both Ly6C^{hi} and Ly6C^{low} monocytes increased in a TLR7-dependent manner in the *Slc29a3*^{-/-}
120 mice (Fig. 2a, 2b, Extended Data Fig. 5a). Both Ly6C^{hi} and Ly6C^{low} splenic monocytes
121 expressed TLR7 (Fig. 2c) and stored nucleosides, such as Guo and dGuo (Fig. 2d, Extended
122 Data Fig. 5b), suggesting that TLR7 is cell-autonomously activated in these subsets. To
123 characterise TLR7 responses in these monocyte subsets, we performed transcriptome
124 analyses comparing *Slc29a3*^{-/-} and *Slc29a3*^{-/-} *Tlr7*^{-/-} monocytes with WT monocytes. An
125 increase of 1.5-fold altered genes was observed in a TLR7-dependent manner in Ly6C^{hi}
126 monocytes (Extended Data Fig. 5c). Gene set enrichment analyses (GSEAs) of more than
127 1.5-fold altered genes revealed that proliferation-related gene sets such as “E2F targets”,
128 “G2M checkpoint”, and “mitotic spindle” were positively enriched in *Slc29a3*^{-/-} Ly6C^{hi}
129 monocytes (Fig. 2e). These changes were dependent on TLR7 because such changes were not
130 observed in *Slc29a3*^{-/-} *Tlr7*^{-/-} Ly6C^{hi} monocytes. To directly study the survival and
131 proliferation of monocytes, splenic Ly6C^{hi} and Ly6C^{low} monocytes were sorted and cultured
132 *in vitro* in the presence of M-CSF, which has been shown to promote histiocytosis in
133 *Slc29a3*^{-/-} mice³. Ly6C^{hi}, but not Ly6C^{low}, monocytes from *Slc29a3*^{-/-} mice showed higher
134 survival and proliferation in the presence of M-CSF at concentrations comparable to those *in*
135 *vivo* (Fig. 2f). As the cell surface expression of the M-CSF receptor CD115 was not

136 appreciably upregulated in *Slc29a3*^{-/-} splenic monocytes (Extended Data Fig. 5d), TLR7 did
137 not increase cell surface CD115 to augment M-CSF response. The two signals via TLR7 or
138 CD115 would synergistically drive the survival and proliferation of Ly6C^{hi} monocytes.

139 To study the *in vivo* proliferation of monocytes, mice were intravenously administered
140 with the thymidine analogue–EdU, and the percentages of EdU⁺ monocytes in bone marrow,
141 peripheral blood, and spleen were analysed 3-h post EdU administration. In WT mice, EdU⁺
142 proliferating monocytes were found only in bone marrow Ly6C^{hi} monocytes (Fig. 2g). The
143 percentage of EdU⁺ monocytes in the bone marrow TLR7-dependently increased in *Slc29a3*^{-/-}
144 mice. Even more drastic changes were observed in the spleen, where EdU⁺ Ly6C^{hi} monocytes
145 were found only in *Slc29a3*^{-/-} mice. We analysed EdU⁺ monocytes 3 days after EdU
146 administration and observed that the majority of EdU⁺ monocytes in the circulation and
147 spleen turned Ly6C^{low} (Fig. 2g), suggesting that proliferating Ly6C^{hi} monocytes mature into
148 Ly6C^{low} monocytes within 3 days in the *Slc29a3*^{-/-} mice. Ly6C^{low} monocytes in the *Slc29a3*^{-/-}
149 ^{-/-} mice stored deoxyribonucleosides more than Ly6C^{hi} monocytes (Fig. 2d, Extended Data
150 Fig. 5b). Because lysosomal deoxyribonucleosides were derived from cell corpses (Fig. 1d,
151 1e), Ly6C^{low} monocytes are likely to have engulfed cell corpses during or after maturation
152 from Ly6C^{hi} monocytes. Consistent with this, splenic Ly6C^{low} monocytes in the *Slc29a3*^{-/-}
153 mice engulfed intravenously administered dying thymocytes (Fig. 2h). TLR7, therefore,
154 increased the number of phagocytes in the *Slc29a3*^{-/-} mice. TLR7 is likely to recognise
155 lysosomal nucleoside storage as lysosomal stress and increases phagocyte number as a
156 compensatory mechanism.

157 **Mutually exclusive induction of proliferation and inflammation by TLR7**

158 To further narrow down the proliferating population of Ly6C^{hi} monocytes, we examined a
159 marker specifically expressed or not expressed in proliferating Ly6C^{hi} monocytes. The cell
160 surface expression of CX3CR1, a chemokine receptor for the membrane-tethered chemokine

161 CX3CL1¹⁴, increases with maturation from Ly6C^{hi} to Ly6C^{low} monocytes¹⁵. We found that
162 the CX3CR1^{low} population in Ly6C^{hi} monocytes increased in a TLR7-dependent manner in
163 the *Slc29a3*^{-/-} mice (Fig. 3a). When splenic monocytes were cultured *in vitro* for 1 h with
164 EdU, its uptake was detected in this immature monocyte subset but not in more mature
165 Ly6C^{hi} CX3CR1^{hi} classical monocytes (Fig. 3b). These results demonstrate that Ly6C^{hi}
166 CX3CR1^{low} immature monocytes proliferate in response to lysosomal nucleoside storage in
167 the *Slc29a3*^{-/-} mice.

168 Because SLC29A3 disorders are considered to be inflammatory diseases in which IL-6 has
169 pathogenic roles⁶, we examined TLR7-dependent inflammatory responses in the *Slc29a3*^{-/-}
170 mice. Unexpectedly, inflammation-associated gene sets such as “TNF α via NF- κ B” and
171 “inflammatory response” were negatively enriched in splenic Ly6C^{hi} monocytes (Fig. 2e).
172 Consistent with this, SLC29A3-deficiency in Ly6C^{hi} monocytes did not increase the
173 expression levels of mRNAs encoding proinflammatory cytokines, such as IFN- α , IFN- β ,
174 IFN- γ , IL-6, IL-17A, IL-23, and TNF- α (Extended Data Fig. 6a). Furthermore,
175 proinflammatory cytokines, such as TNF- α , IFN- β , IL-1 β , IFN- α , and IL-6, were not
176 identifiable in the serum using ELISA (Extended Data Fig. 6b). These results suggest that
177 nucleoside accumulation is not sufficient to induce TLR7-dependent inflammation. We
178 previously reported that TLR7 response to ssRNA is enhanced by Guo/dGuo⁴, which
179 accumulated in *Slc29a3*^{-/-} monocytes (Fig. 2d). Therefore, TLR7 response to ssRNA may be
180 enhanced in *Slc29a3*^{-/-} monocytes. As predicted, *Slc29a3*^{-/-} Ly6C^{hi} CX3CR1^{hi} classical
181 monocytes produced IL-6 upon poly U stimulation, whereas WT Ly6C^{hi} macrophages did not
182 respond to poly U (Fig. 3c). In contrast, *Slc29a3*^{-/-} Ly6C^{hi} CX3CR1^{low} proliferating immature
183 monocytes did not respond to poly U treatment (Fig. 3c). Consistent with this finding, when
184 splenic monocytes were treated with EdU and poly U, we only detected EdU⁺ or IL-6⁺
185 Ly6C^{hi} monocytes but not EdU⁺ IL-6⁺ double-positive monocytes (Fig. 3d). TLR7 is likely to

186 induce proliferation and inflammation in a mutually exclusive manner in immature and
187 classical monocytes, respectively.

188 *Slc29a3*^{-/-} Ly6C^{hi} classical monocytes responded to the TLR9 ligand CpG-B but produced
189 smaller amounts of IL-6 than WT Ly6C^{hi} classical monocytes (Fig. 3c). In addition, *Slc29a3*^{-/-}
190 Ly6C^{low} monocytes did not produce IL-6 in response to CpG-B, whereas WT Ly6C^{low}
191 monocytes did (Extended Data Fig. 7a). As TLR7-deficiency rescued impaired TLR9
192 response (Fig. 3c, Extended Data Fig. 7a), nucleoside-activated TLR7 is likely to inhibit
193 TLR9 responses.

194 An antibody array for cytokines showed that poly U-stimulated splenic monocytes
195 produced chemokines and cytokines, including CCL2, CCL3, CCL12, CXCL2, CXCL9, IL-
196 6, TNF- α , IL-12p40, and IL-10, in *Slc29a3*^{-/-} mice (Extended Data Fig. 7b, 7c). Furthermore,
197 *Slc29a3*^{-/-} professional macrophages, such as BM-Mphs and pMphs, exhibited stronger IL-
198 12p40 production in response to poly U than did WT macrophages, and their responses to
199 R848 and CpG-B were not impaired (Fig. 3e, Extended Data Fig. 7d). Inflammation in
200 SLC29A3 disorders might be driven by an enhanced TLR7 response to ssRNA in Ly6C^{hi}
201 splenic monocytes and peripheral macrophages. These results suggest that the TLR7 response
202 to nucleoside storage varies with monocyte maturation from proliferation to an excessive
203 inflammatory response to ssRNA (Fig. 3f).

204 **Signals involved in TLR7-dependent monocyte proliferation**

205 Next, we focused on growth-promoting TLR7 signalling. We cultured whole splenocytes *in*
206 *vitro* with 3 ng/ml M-CSF, where larger numbers of *Slc29a3*^{-/-} splenic monocytes survived
207 than WT monocytes (Fig. 2f). Inhibitors of MEK (PD0325901), Syk (PRT062607, R788), β -
208 catenin (PKF118-120), PI3K (wortmannin), AKT (Afuresertib), mTORC1 (rapamycin,
209 Torin1), and MyD88 (ST2825), but not JNK (JNK-IN-8), reduced the number of surviving

210 monocytes (Fig. 4a). We examined the *in vivo* TLR7-dependent activation of these signalling
211 molecules. Flow cytometry analyses of Ly6C^{hi} monocytes revealed TLR7-dependent
212 increases in the activated form of β -catenin and the phosphorylated forms of signalling
213 molecules including Syk, GSK3 β , and the ribosomal protein S6 (the mTORC1 downstream
214 effector) (Fig. 4b). In contrast, TLR7-dependent ERK phosphorylation was not significantly
215 observed. These results suggest that TLR7 activates signalling molecules such as Syk,
216 GSK3 β , β -catenin, and mTORC1 to promote proliferation/survival in *Slc29a3*^{-/-} Ly6C^{hi}
217 monocytes.

218 **TLR8-dependent histiocytosis in humans**

219 Finally, we investigated whether human monocytes from SLC29A3 disorders show enhanced
220 TLR7/8 responses. In human peripheral blood mononuclear cells (PBMCs) from a patient
221 harbouring the *SLC29A3* p.Gly208Arg (G208R) mutation²⁰, CD14^{low} CD16^{hi} monocytes,
222 which are equivalent to the mouse Ly6C^{low} monocytes^{13,21}, were increased by approximately
223 3-folds (Fig. 5a). The expression levels of TLR7 and TLR8 in both CD14^{hi} CD16^{low} and
224 CD14^{low} CD16^{hi} monocytes were not altered in the patient (Fig. 5b). To study monocyte
225 proliferation and survival, PBMCs were cultured *in vitro* with human M-CSF. A larger
226 number of HLA-DR⁺ CD11b⁺ monocytes from the patient survived *in vitro* culture than those
227 from three healthy subjects (Fig. 5c). PBMCs were allowed to differentiate into macrophages
228 by human M-CSF and IL-4 and were stimulated with the TLR7/8 ligands poly U and
229 RNA9.2S or TLR8 ligand ssRNA40⁴. Macrophages harbouring the G208R *SLC29A3*
230 mutation produced larger amounts of IL-6 than the control macrophages in response to
231 TLR7/8 and TLR8 ssRNA ligands, strongly suggesting that the *SLC29A3* mutation enhanced
232 TLR8 responses in macrophages (Fig. 5d). We also examined PBMCs from another patient
233 harbouring the *SLC29A3* p.Ser184Arg (S184R) mutation²². The percentage of monocytes did
234 not increase (Extended Data Fig. 8a); however, proliferation and survival *in vitro* in the

235 presence of M-CSF were significantly enhanced (Extended Data Fig. 8b). These results
236 demonstrate that the phenotypes in the *Slc29a3*^{-/-} mice were consistent with those of the
237 patients with SLC29A3 mutation.

238 To determine whether TLR7 or TLR8 drives histiocytosis in humans, PBMCs from a
239 patient harbouring the G208R or the S184R SLC29A3 mutations were cultured with human
240 M-CSF in the presence of TLR7 or TLR8 specific inhibitors. The TLR8 antagonist CU-
241 CPT9a²³, but not the TLR7 antagonist DSR-139970 (Cpd-7)²⁴, inhibited the *in vitro* survival
242 of human macrophages with SLC29A3 mutation as much as the Syk inhibitors R788 and
243 PD0325901 (Fig. 5e, Extended Data Fig. 8b). Additionally, we detected approximately 3-fold
244 higher amounts of Urd, the TLR8 ligand^{4,8,10}, in macrophages harbouring the G208R
245 *SLC29A3* mutation than in those from healthy subjects (Fig. 5f). These results suggest that
246 SLC29A3 negatively regulates TLR8 by transporting Urd from the lysosomes to the
247 cytoplasm, and that TLR8 drives histiocytosis in SLC29A3 disorders. The role of huTLR7 in
248 monocyte proliferation in SLC29A3 disorders remains unclear.

249 Here, we identified a previously unknown histiocytosis-driving program activated by
250 germline loss-of-function mutations in *SLC29A3*. This program recognises lysosomal
251 nucleoside storage as lysosomal stress and increases phagocytes by driving monocyte
252 proliferation and maturation. This program is reminiscent of another response activated by
253 haem following erythrocyte clearance. Although a haem sensor has not been identified, haem
254 stress in macrophages induces their differentiation into red pulp macrophages, which are
255 phagocytes specialised for red blood cell clearance²⁵. We refer to metabolite-dependent
256 macrophage proliferation and maturation as the lysosomal stress response. TLR7 and TLR8
257 serve as nucleoside sensors to activate lysosomal stress responses, which drive histiocytosis,

258 unless the stress is relieved. These results demonstrate that SLC29A3 disorders are lysosomal
259 stress diseases.

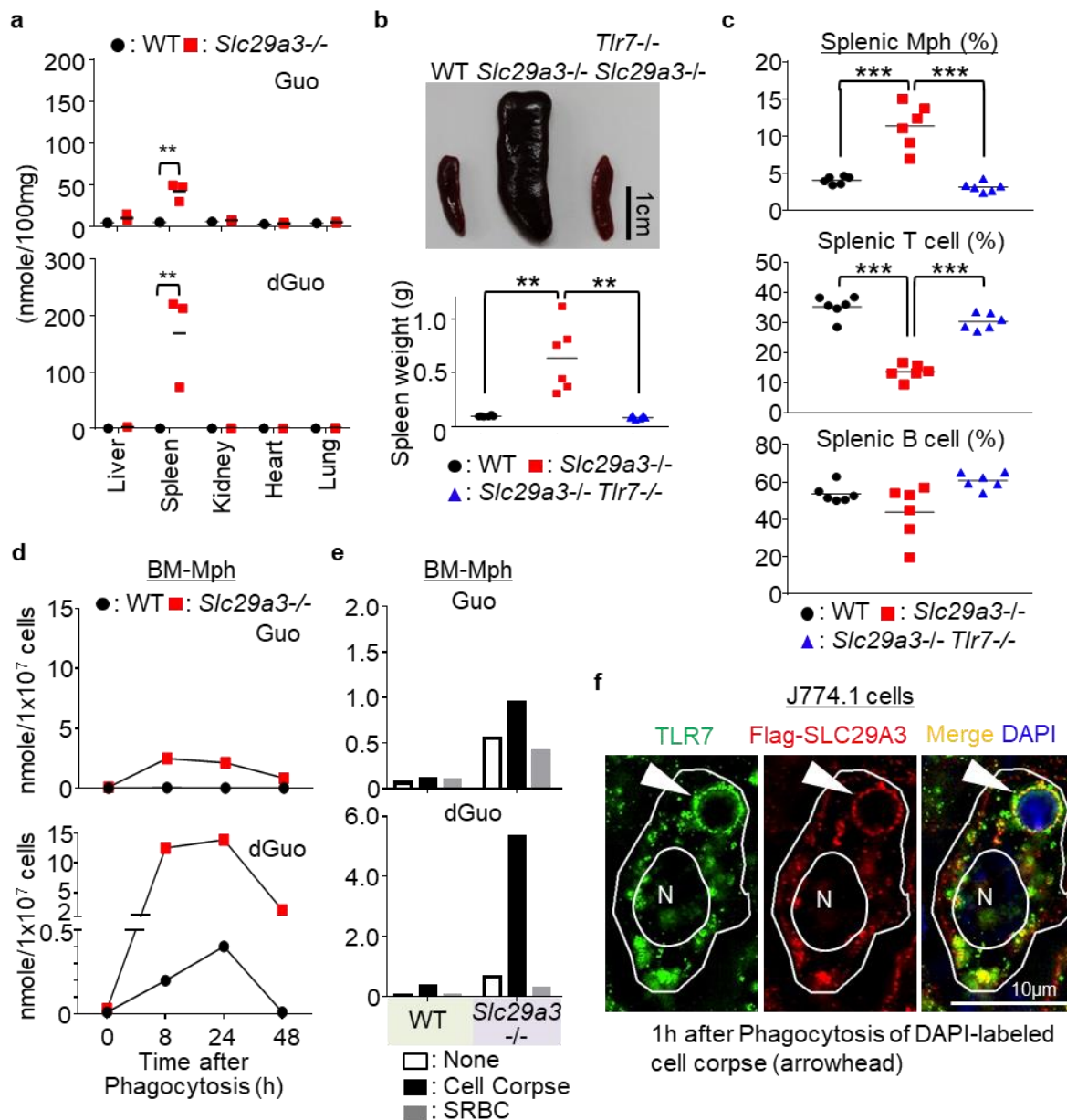
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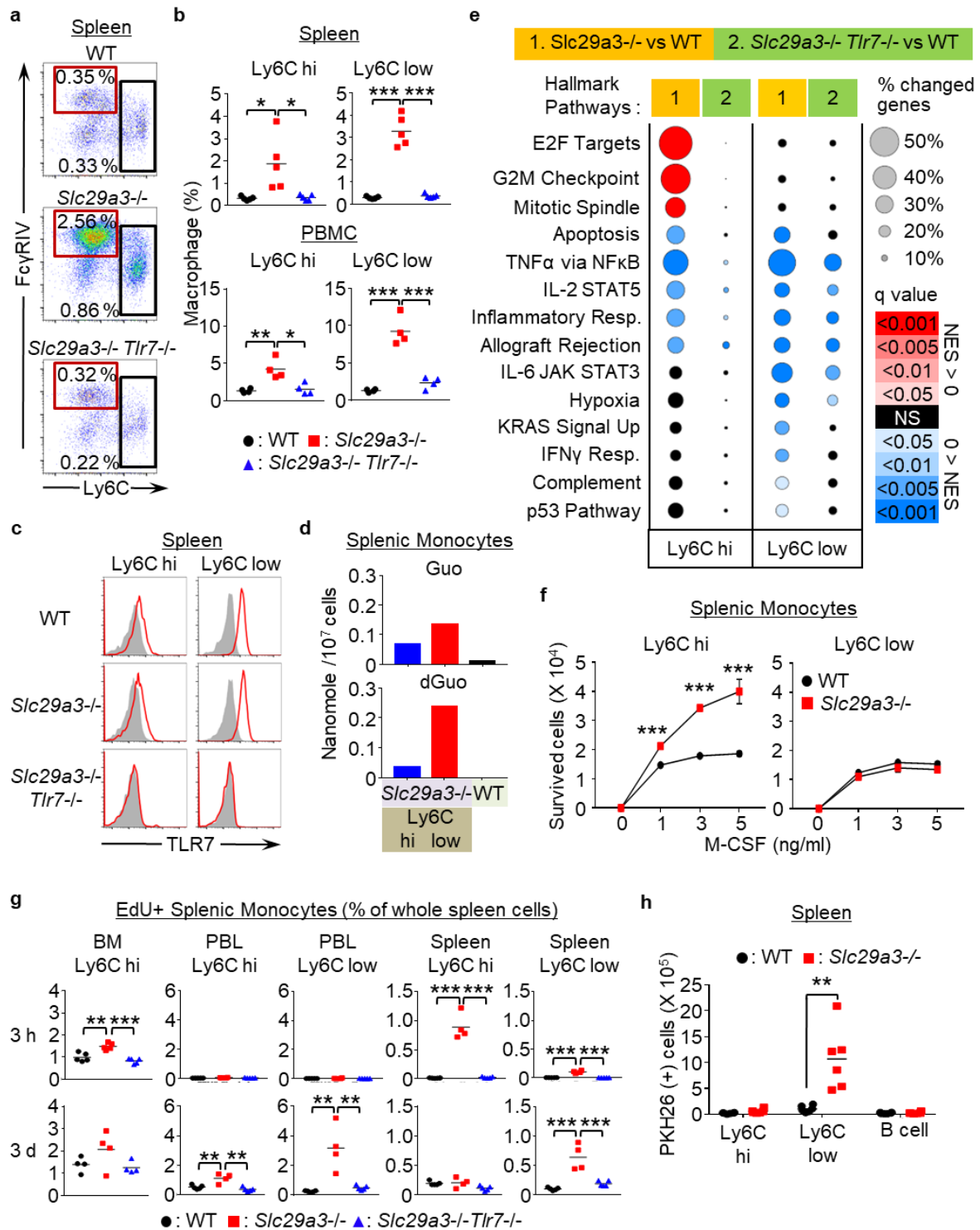


343

344 **Figure legends**

345 **Fig. 1. TLR7-dependent histiocytosis in *Slc29a3*^{-/-} mice**

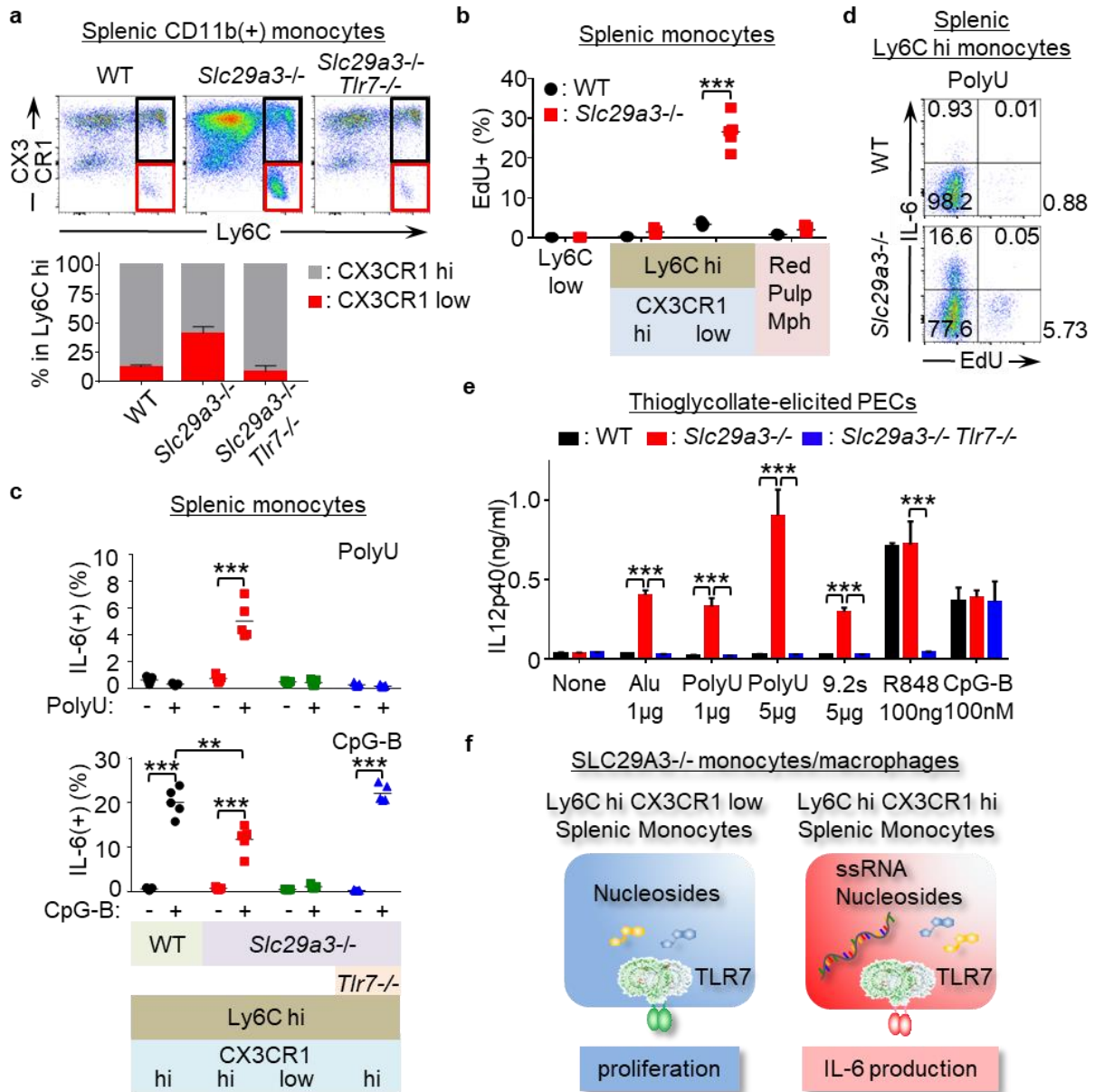
346 **a**, Amounts of guanosine (Guo) and 2'-deoxyguanosine (dGuo) in the indicated organs. Each
 347 dot represents a value (nanomole/100 mg tissue) from each mouse. **b**, Representative spleen
 348 images from 6-month-old mice (top). Scale bar, 1 cm. The bottom panel shows the spleen
 349 weight (n = 6). **c**, Percentages of NK1.1⁻ Ly6G⁻ CD11b⁺ monocytes, CD3e⁺ T cells, and
 350 CD19⁺ B cells in the spleen from the indicated mice (n = 6). **d**, **e**, The amount of accumulated
 351 nucleosides (nanomoles) in 10⁷ cells of WT and *Slc29a3*^{-/-} BM-Mphs after treatment with 10⁸
 352 dying thymocytes (cell corpse) or 10⁹ SRBCs for the indicated hours (**d**) or 24 h (**e**) were
 353 evaluated by LC-MS. **f**, Staining of TLR7 and Flag-SLC29A3 in the J774.1 macrophage cell
 354 line at 1 h after phagocytosis of the DAPI-labelled cell corpse. Arrowheads indicate a
 355 phagosome containing cell corpses. scale bar, 10 μm. ***p* < 0.01, ****p* < 0.001.



356

357 **Fig. 2. Ly6C^{hi} monocytes proliferate to increase Ly6C^{low} phagocytes**
 358 **a**, Representative FACS analyses of CD11b⁺ Ly6G⁻ NK1.1⁻ CD11c^{low} IA/IE^{low} splenic
 359 monocytes from WT, *Slc29a3*^{-/-}, and *Slc29a3*^{-/-} *Tlr7*^{-/-} mice. The red and black squares show
 360 the gates of Ly6C^{low} and Ly6C^{hi} monocytes, respectively. **b**, Dot plots show the percentages
 361 of Ly6C^{low} and Ly6C^{hi} monocytes in the peripheral blood and spleen from the indicated mice
 362 (n = 5). **c**, Red histograms show intracellular TLR7 expression levels in Ly6C^{hi} and Ly6C^{low}
 363 monocytes from the indicated mice. Grey histograms show staining with the isotype control
 364 antibodies. **d**, Amounts (nmol/10⁷ cells) of Guo and dGuo in WT CD11b⁺ splenic monocytes

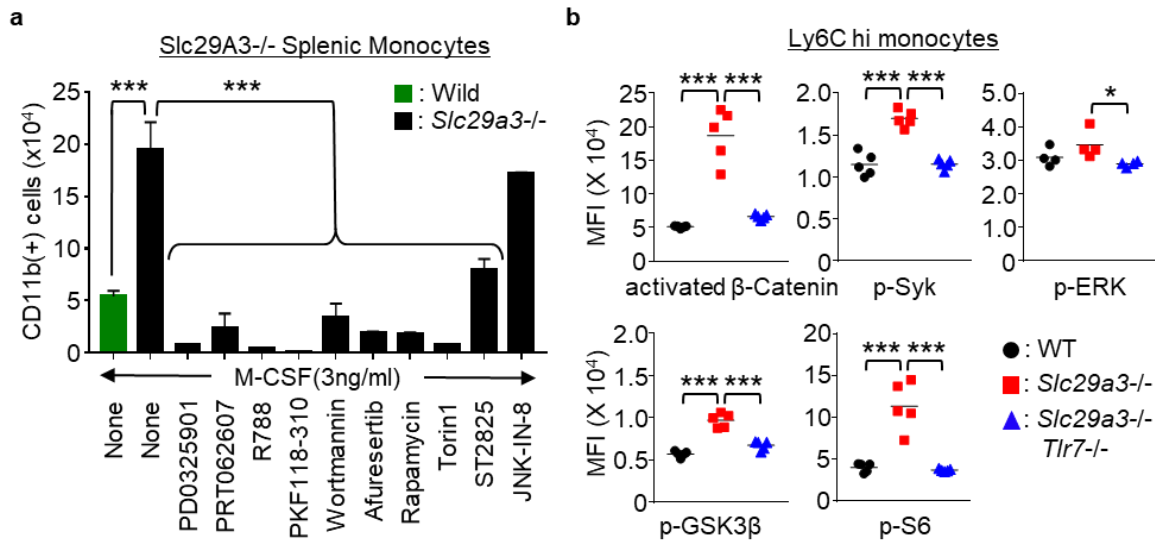
365 or in Ly6C^{hi} and Ly6C^{low} splenic monocytes from *Slc29a3*^{-/-} mice. **e**, Gene set enrichment
366 analysis (GSEA) of more than 1.5-fold changed genes in comparing Ly6C^{hi} and Ly6C^{low}
367 splenic monocytes from *Slc29a3*^{-/-} and *Slc29a3*^{-/-} *Tlr7*^{-/-} mice with those from WT mice.
368 Red and blue circles indicate positive and negative normalised enrichment scores (NES),
369 respectively. Their sizes indicate the percentage of genes with a >1.5-fold change in each
370 gene set. The colour gradation indicates the *q*-value of positive/negative enrichment. **f**,
371 Numbers of splenic Ly6C^{hi} and Ly6C^{low} monocytes from WT (black circle) and *Slc29a3*^{-/-}
372 (red square) mice that survived for 4 days in *in vitro* culture with M-CSF at the indicated
373 concentrations. Average values with s.d. from triplicate samples are shown. **g**, Uptake of the
374 thymidine analogue EdU *in vivo* by monocytes from WT, *Slc29a3*^{-/-}, and *Slc29a3*^{-/-} *Tlr7*^{-/-}
375 mice at 3 h (upper) and 3 d (below) after intravenous EdU administration. Percentages of
376 EdU⁺ cells in whole splenocytes are shown. n=5. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. **h**,
377 Number of PKH26(+) cells in the indicated cell populations from the mice that had received
378 PKH26-labelled dying thymocytes 1 h before analyses. Each dot represents the value for each
379 mouse (n = 6).



380

381 **Fig. 3. TLR7 differentially induces proliferation and inflammation**

382 **a**, Expression of CX3CR1 and Ly6C in splenic CD11b⁺ Ly6C⁺ NK1.1⁻ CD11c^{low} IA/IE^{low}
 383 monocytes from WT, *Slc29a3*^{-/-}, and *Slc29a3*^{-/-} *Tlr7*^{-/-} mice (top). Black and red gates show
 384 Ly6C^{hi} CX3CR1^{hi} and Ly6C^{hi} CX3CR1^{low} monocytes, respectively. The bottom panel shows
 385 the percentages of the two Ly6C^{hi} monocyte subsets in the indicated mice (n = 4). **b**, EdU
 386 uptake by each splenic monocyte subset during 1 h culture with 10 μM EdU. Each dot
 387 represents a value from a single mouse (n = 4). **c**, Percentage of IL-6⁺ cells in each monocyte
 388 subset after *in vitro* stimulation with poly U (10 μg/mL) or CpG-B (1 μM) for 4 h. Brefeldin
 389 A (10 μg/mL) was added during cell stimulation. Each dot shows the values for each mouse
 390 from the indicated mice (n = 5). **d**, Representative dot plot of EdU⁺ and IL-6⁺ cells in Ly6C^{hi}
 391 splenic monocytes treated with poly U + brefeldin A for 4 h. EdU was added during the last
 392 hour of stimulation. **e**, IL-12 p40 production by thioglycollate-elicited pMphs after stimulation
 393 with TLR7 and TLR9 ligands for 18 h. Alu: Alu retroelements. The results are represented as
 394 the average with s.d. from triplicate samples. **f**, Schematics showing the induction of two
 395 distinct TLR7 responses, proliferation and cytokine production, in *Slc29a3*^{-/-} mice. ****p* <
 396 0.01, ****p* < 0.001.



397

398

Fig. 4. FcR γ and DAP10 mediate TLR7 responses in *Slc29a3*^{-/-} mice

399

a, Number of splenic CD11b⁺ macrophages from WT (green) or *Slc29a3*^{-/-} (black) mice that

400

survived *in vitro* 4 day culture in the presence of 3 ng/mL M-CSF under serum-free

401

conditions. Cells were treated with inhibitors of MEK (PD0325901, 1 μ M), Syk

402

(PRT062007, 1 μ M; R788, 0.5 μ M), β -catenin (PKF118-120, 5 μ M), PI3K (wortmannin, 10

403

μ M), AKT (Afuresertib, 5 μ M), mTORC1 (rapamycin, 0.5 μ M), mTORC1 and 2 (Torin1,

404

250 nM), MyD88 (ST2825, 10 μ M), and JNK (JNK-IN8, 1 μ M). Bar graphs represent mean

405

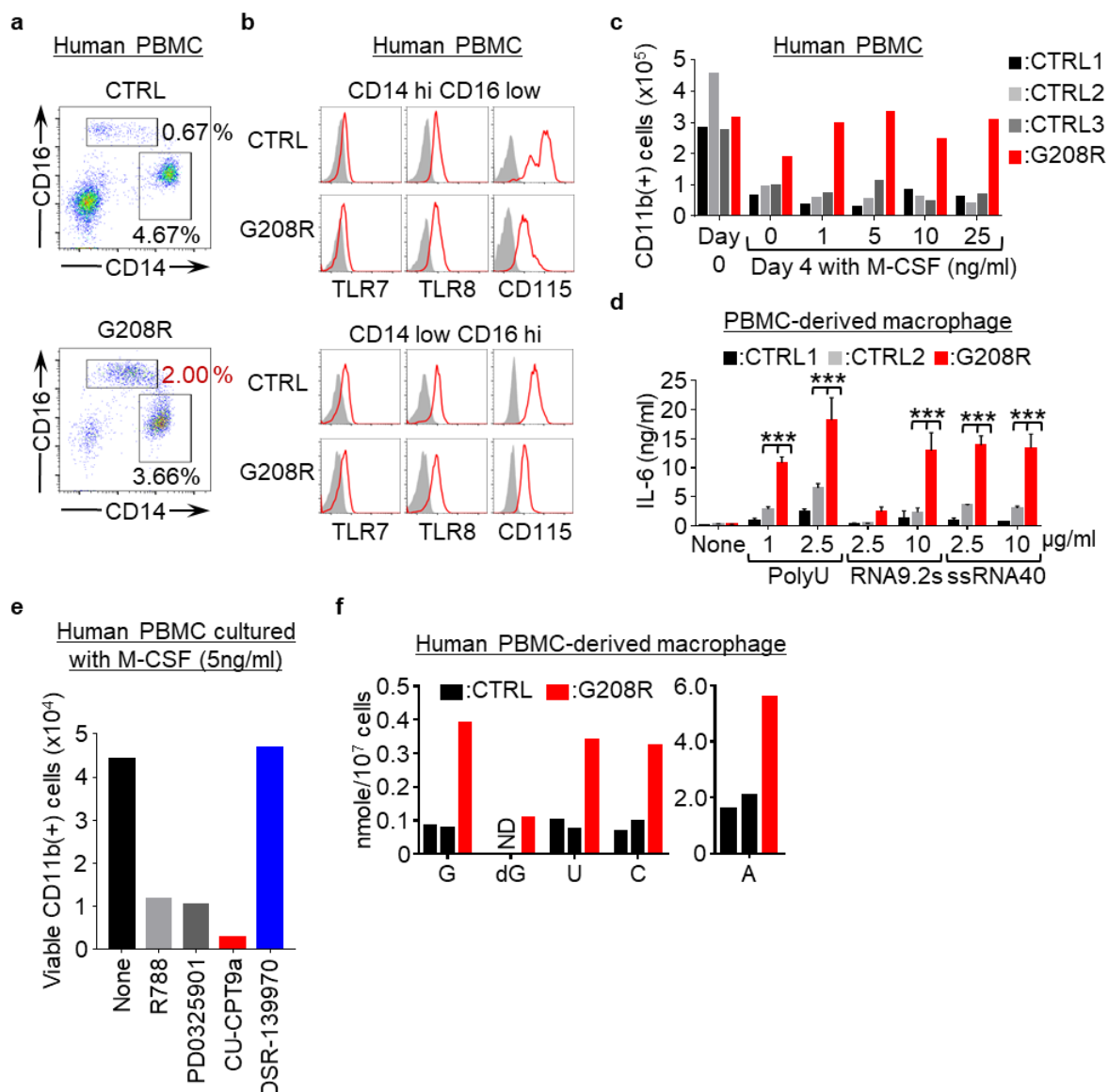
values \pm s.d. of triplicate samples. **b**, Mean fluorescence intensity (MFI) of staining with

406

activation- and phospho-specific Abs to signalling molecules in *Ly6C*^{hi} monocytes from the

407

indicated mice (n = 4-5). **p* < 0.05, ****p* < 0.001.



408

409 **Fig. 5. TLR8 drives histiocytosis in SLC29A3 disorders**

410 **a, b**, Expression of surface CD16/CD14/CD115 (**a, b**) and intracellular TLR7/TLR8 (**b**) in
 411 HLA-DR⁺ CD15⁻ CD56⁻ PBMCs from a patient with the G208R *SLC29A3* mutation and a
 412 healthy subject. Red and grey histograms show staining with the indicated and isotype-
 413 matched control antibodies, respectively. **c**, Number of surviving CD11b⁺ CD15⁻ CD56⁻
 414 monocytes in PBMCs from patients (red) or healthy subjects (black and grey) after 4 days of
 415 culture with M-CSF at the indicated concentrations. **d**, PBMC-derived macrophages from the
 416 patient (red) or two control subjects (black and grey) were stimulated with the indicated
 417 TLR7 or TLR8 ssRNA ligands at the indicated concentrations. IL-6 production was evaluated
 418 using ELISA. The bars represent the average values with s.d. from triplicate samples. **e**,
 419 CD11b⁺ cells that survived *in vitro* PBMC culture with 5 ng/mL M-CSF in the presence of
 420 the indicated inhibitors of Syk (1 µM R788), MEK1/2 (1 µM PD0325901), TLR8 (10 µM
 421 CPT9a), and TLR7 (10 µM DSR-139970). **f**, The amounts (nmol/10⁷ cells) of nucleosides
 422 accumulated in PBMC-derived macrophages. ND, not detectable. ***p* < 0.01, ****p* < 0.001.

423 **Methods**

424 **Generation of *Slc29a3*^{-/-} and *Tlr7*^{-/-} Mice**

425 C57BL/6 *Slc29a3*^{-/-} and *Tlr7*^{-/-} mice were generated using the CRISPR/CAS9 system. gRNA
426 target sites on *Slc29a3* and *Tlr7* were determined using CRISPRdirect software
427 (<http://crispr.dbcls.jp/>), and 5'-agcttcttgatggttactcg-3' and 5'-gaacagttggccaatctctc-3' sequences
428 were chosen for the construction of *Slc29a3*^{-/-} and *Tlr7*^{-/-} mice, respectively. These gRNA
429 target sequences were cloned into the BbsI site of the pKLV-U6 gRNA (BbsI)-
430 PGKpuro2ABFP vector (Addgene plasmid 50946). Using the constructed vectors as
431 templates, gRNAs were synthesised by *in vitro* transcription using the MEGA shortscript T7
432 Transcription Kit (Thermo Fisher Scientific). Additionally, hCAS9 mRNA was synthesised
433 from the hCAS9 sequence in pX458 (Addgene plasmid 48138) *in vitro* using the
434 mMESAGE mMACHIN T7 ULTRA Transcription Kit (Thermo Fisher Scientific, USA).
435 To generate *Slc29a3*^{-/-} *Tlr7*^{-/-} mice, synthesized gRNAs (50 ng/μL) targeting the *Slc29a3* and
436 *Tlr7* genes, hCAS9 mRNA (100 ng/μL), and the repair donor DNA (100 ng/μL) to introduce
437 a stop codon into the *Slc29a3* locus (5'-
438 tgcctctgaggacaatgtataccacagctccaatgctgtctacagagcccTGATAGCGTAAAGCACTGAGGAA
439 Gcgagtaaccatcaagaagctgaccaggaagccctgctggggaaactacta-3' ; Lower-case letters and capital
440 letters represent homology arm and insertion sequence, respectively) were injected into
441 zygotes from C57BL/6 mice at the pronuclei stage. The injected zygotes were then
442 transferred to the oviducts of pseudopregnant female C57BL/6 mice. Candidates for *Slc29a3*^{-/-}
443 *Tlr7*^{-/-} mice were typed by PCR using primer pairs for *Slc29a3* (Fw:5'-
444 CCAGCATGGACGAGAGATGTCTTC-3', Rv:5'-GCACCATTGAAGCGATCCTCTGG-3')
445 and *Tlr7* (Fw:5'- GAGGGTATGCCGCCAAATCTAAAGAATC-3', Rv:5'-
446 CTGATGTCTAGATAGCGCAATTGC-3'). The PCR product was analysed using the MCE-
447 202 MultiNA Microchip Electrophoresis System for DNA/RNA Analysis (SHIMAZU,

448 Japan), and *Slc29a3*^{-/-} *Tlr7*^{-/-} male mice were chosen for mating with WT C57BL/6 female
449 mice. *Slc29a3*^{+/-} *Tlr7*^{+/-} mice were mated to establish *Slc29a3*^{-/-} and *Slc29a3*^{-/-} *Tlr7*^{-/-} mice.
450 The sequences of the mutated allele in *Slc29a3*^{-/-} and *Tlr7*^{-/-} mice were confirmed by direct
451 sequencing performed by FASMAC (Japan).

452 **Mice**

453 WT C57BL/6 mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). All animals
454 were housed in SPF facilities at the Institute of Medical Science, University of Tokyo
455 (IMSUT). All animal experiments were approved by the Institutional Animal Care and Use
456 Committee of the IMSUT.

457 **Reagents**

458 PD0325901 (mirdametinib), PRT062607 (P505-15) HCl, R788 (fostamatinib), wortmannin
459 (KY 12420), afuresertib (GSK2110183), AY-22989 (Rapamycin), and JNK-IN-8 were
460 purchased from Selleck Chemical (USA). PKF118-310 was purchased from Sigma (USA).
461 Torin 1 and ST2825 were purchased from Calbiochem/Merck Millipore (USA) and
462 ChemScence (USA), respectively.

463 Stable isotope-labelled nucleosides, G (13C10, 98%;15N5, 96-98%), U (13C9, 98%;15N2,
464 96-98%), A (13C10, 98%;15N5, 96-98%), C (13C9, 98%;15N3, 96-98%), and dG (13C10,
465 98%;15N5, 96-98%) were purchased from Cambridge Isotope Laboratories (Massachusetts,
466 USA) for the quantification of nucleosides by LC-MS/MS. The EdU used in the *in vivo*
467 proliferation assay was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan).

468 DSR-139970 (Cpd7), a TLR7 inhibitor, was kindly provided by Sumitomo Pharma Co., Ltd.
469 (Japan). CU-CPT9a, a specific TLR8 inhibitor, and R848 were purchased from InvivoGen
470 (Hong Kong, China). Sheep red blood cells (SRBC) used in the phagocytic assay were
471 obtained from Cosmo Bio Co. (Japan).

472 RNA9.2s (20mer, UsGsUsCsCsUsUsCsAsAsUsGsUsCsCsUsUsCsAsA), ssRNA40
473 (GsCsCsCsGsUsCsUsGsUsUsGsUsGsUsGsAsCsUsC), PolyU (19mer,
474 UsUsUsUsUsUsUsUsUsUsUsUsUsUsUsUsUsUsUsU), and ODN1668 (20mer,
475 dTsdCsdCsdAsdTsdGsdAsdCsdGsdTsdTsdCsdCsTdsdGsdAsdTsdGsdCsdT), in which ‘s’
476 depicts a phosphothioate linkage, were synthesized by FASMAC (Japan).

477 **Establishment of anti-human TLR7/8 monoclonal antibodies**

478 To establish an anti-human TLR7/8 monoclonal antibody (mAb), WT Wistar rats and
479 BALB/c mice were immunised several times with purified huTLR7/8 ectodomain and Ba/F3
480 cells expressing huTLR7/8 mixed with TiterMax Gold. Four days after final immunisation,
481 splenocytes and SP2/O myeloma cells were fused with polyethylene glycol. After selection
482 by hypoxanthine/aminopterin/thymidine (HAT), antibodies to TLR7 and TLR8 were selected
483 by flow cytometry analyses using Ba/F3 cells expressing huTLR7/8. Anti-huTLR7 and
484 huTLR8 mAbs were designated rE3 (rat IgG2a/κ) and M7B (mouse IgG1/κ), respectively.
485 The purity of the mAbs was checked by SDS-PAGE and Coomassie brilliant blue staining,
486 and biotinylated mAbs were used for subsequent experiments.

487 **Flow cytometry**

488 For the preparation of samples for flow cytometry analysis, the spleens were minced using
489 glass slides, and the bone marrow cells were pipetted several times to disperse the cells in
490 RPMI1640 culture medium. The suspended samples were teased using nylon mesh to remove
491 tissue debris. All samples were treated with BD Pharm lysing buffer (BD Biosciences) to
492 remove red blood cells before being subjected to cell staining. Cell surface staining for flow
493 cytometry analysis was performed using fluorescence-activated cell sorting (FACS) staining
494 buffer (1×PBS with 2.5% FBS and 0.1% NaN₃). The prepared cell samples were incubated
495 for 10 min with an unconjugated anti-mouse CD16/32 blocking mAb (clone 95) to prevent

496 nonspecific staining in the staining buffer. The cell samples were then stained with
497 fluorescein-conjugated mAbs for 15 min on ice.

498 Peripheral blood mononuclear cells (PBMCs), bone marrow cells (BMs), and splenocytes
499 from mice were stained with fluorescent dye-conjugated monoclonal antibodies specific for
500 the following markers after blocking antibody treatment: CD11b (clone M1/70), FcγRIV
501 (clone 9E9), CD3ε (clone 145-2c11), CD19 (clone 6D5), CD11c (clone N418), CD71 (clone
502 R17217), Ter119/Erythroid Cells (clone Ter119), Ly6C (clone HK1.4), and Ly6G (clone
503 1A8).

504 Human PBMCs were assessed using fluorescent dye-conjugated antibodies specific for the
505 following markers: HLA-DR (clone G46-6), CD14 (clone M5E2), CD16 (clone 3G8), CD15
506 (clone HI98), CD56 (clone 5.1H11), CD3ε (clone SK7), and CD19 (clone HIB19). These
507 antibodies were purchased from eBioscience (USA), BioLegend (USA), BD Biosciences
508 (USA), and TONBO Biosciences (USA). Biotinylated anti-mouse TLR7 (A94B10) mAb was
509 previously established in our laboratory²⁹.

510 To detect endolysosomal TLR7 and TLR8, cells after cell surface staining were fixed and
511 permeabilised using a Fixation/Permeabilization Solution Kit (BD Biosciences, USA) and
512 stained again with biotinylated anti-mouse/human TLR7/8 mAb and PE streptavidin
513 (Biolegend, USA). To detect intracellular mouse IL-6 in splenocytes stimulated with various
514 ligands in the presence of Brefeldin A (10µg/ml), cells after cell surface staining were fixed
515 with Fixation Buffer (Biolegend, USA), permeabilised using 1X Click-iT saponin-based
516 permeabilisation and wash reagent (Invitrogen™ ThermoFisher Scientific, USA), and then
517 stained with PE-conjugated anti-mouse IL-6 mAb (clone MP5-20F3, Biolegend). Stained
518 cells were analysed using a BD LSR Fortessa cell analyser (BD Biosciences, USA) or an
519 ID7000 Spectral Cell Analyser (Sony Biotechnology, Japan). All flow data were analysed
520 using FlowJo software v10.7 (BD Biosciences, USA).

521 **Intracellular phospho-flow cytometry**

522 Phosphorylation of signalling molecules was detected by flow cytometry. First, $3-4 \times 10^6$
523 splenocytes after cell surface staining were fixed with Cyto-Fast Fix/Perm buffer
524 (BioLegend, USA) for 20 min at room temperature and washed twice with FACS staining
525 buffer. Fixed cells were further permeabilised by adding pre-chilled True-Phos Perm Buffer
526 (BioLegend, USA) and incubated at -20°C for 2–3 h. After washing twice with FACS
527 staining buffer, permeabilised cells were stained with PE-conjugated anti-p-Syk (clone
528 C87C1; 1:50 dilution; Cell Signaling Technology, Danvers, MA, USA), anti-p-S6 (clone
529 D57.2.2E; 1:100 dilution; Cell Signalling Technology), anti-p-GSK3 β (clone D85E12; 1:50
530 dilution; Cell Signalling Technology), anti-p44/42 MAPK (Erk1/2) (clone 137F5; 1:50
531 dilution; Cell Signalling Technology), or Fluor647-conjugated anti-active β -catenin (clone
532 8E7; 1:400 dilution; Merck Millipore) and subjected to flow cytometry analyses.

533 **Cell sorting of pDCs and splenic monocytes**

534 Cell staining for sorting was performed in a sorting buffer (1 \times PBS with 10% FBS, 10 mM
535 HEPES and 1 mM Sodium pyruvate). Flt3L induced bone marrow cells were stained with
536 anti-CD11c/B220 mAbs and CD11c+B220+ cells were sorted as bone marrow-derived pDCs.
537 For purification of Ly6C^{low} and Ly6C^{high} splenic monocytes, whole splenocytes from wild-
538 type and *Slc29a3*^{-/-} mice were sequentially incubated with biotinylated anti-mouse CD3
539 (clone 145-2C11)/CD19 (clone 6D5)/NK1.1 (clone PK136)/Ly6G (clone aA8)/TER-
540 119/erythroid cells (clone Ter-119) and Streptavidin MicroBeads (Miltenyi Biotec,
541 Germany). The magnetically labelled cells were removed using autoMACS (Miltenyi Biotec,
542 Germany), and the enriched cells were stained with anti-mouse
543 CD11b/Ly6C/Fcgr4/NK1.1/Ly6G/Siglec-F mAb. Ly6C^{low}Fcgr4^{high} and Ly6C^{high}Fcgr4^{low} in
544 CD11b+NK1.1-Ly6G cell populations were sorted as Ly6C^{low} and Ly6C^{high} monocytes,

545 respectively. Cell sorting was performed using a FACS ARIA III cell sorter (BD
546 Biosciences).

547 **RNA-seq analysis**

548 Ly6C^{low} and Ly6C^{high} splenic monocytes were obtained by FACS sorting from WT,
549 *Slc29a3*^{-/-}, and *Slc29a3*^{-/-} *Tlr7*^{-/-} mice. Total RNA was extracted using RNeasy Mini Kits
550 (Qiagen, Germany), and quality of RNA was evaluated using the Agilent Bioanalyzer device
551 (Agilent Technologies, Santa Clara, CA). The samples with RIN (RNA Integrity Number)
552 value more than 7.3 were subjected to library preparation. RNA-seq libraries were prepared
553 with 1 ng of total RNA using an Ion AmpliSeq Transcriptome Mouse Gene Expression kit
554 (Thermo Fisher Scientific) according to manufacturer's instructions. The libraries were
555 sequenced on Ion Proton using an Ion PI Hi-Q Sequencing 200 kit and Ion PI Chip v3
556 (Thermo Fisher Scientific). The FASTQ files were generated using AmpliSeqRNA plug-in
557 v5.2.0.3 in the Torrent Suite Software v5.2.2 (Thermo Fisher Scientific) and analysed by
558 ROSALIND (<https://rosalind.bio/>, OnRamp Bioinformatics, USA), which is a cloud-based
559 bioinformatics software. Raw reads were trimmed using Cutadapt, and quality scores were
560 assessed using FastQC2. Reads were aligned to the *Mus musculus* genome build mm10 using
561 the STAR aligner. Individual sample reads were quantified using HTseq and normalised via
562 relative log expression (RLE) using the DESeq2 R library. DESeq2 was used to determine the
563 fold changes and *p*-values. Genes showing more than a 1.5-fold change in expression (*p* <
564 0.05) were considered to be significantly altered. To interpret gene expression profiles, gene
565 set enrichment analysis (GSEA) was performed using MSigDB hallmark gene sets to explore
566 the pathways associated with SLC29A3 deficiency. Enriched pathways with FDR-adjusted *p*-
567 values lower than 0.05 are shown in Figure 2e.

568 **EdU proliferation assay**

569 *In vivo* and *in vitro* proliferation assays were performed using a Click-iT Plus EdU Alexa
570 Fluor 488 Flow Cytometry Assay Kit (Invitrogen) according to the manufacturer's
571 instructions. In brief, mice were injected intravenously with 1 mg 5-ethynyl-2'-deoxyuridine
572 (EdU) dissolved in 1xPBS. Spleen and blood samples were collected at 3 h or 3 days after
573 injection. Then, erythrocytes were completely lysed by BD Pharm Lyse lysing buffer (BD
574 Biosciences) to collect splenocytes and peripheral blood mononuclear cells (PBMCs). After
575 blocking splenocytes and PBMCs with anti-CD16/32 (clone:95) mAb, the samples were
576 stained with fluorescent dye-conjugated mAbs. The stained samples were then fixed with BD
577 Cytotfix (BD Biosciences) and permeabilised using 1× Click-iT saponin-based
578 permeabilisation and washing reagent. Finally, EdU incorporated into the genomic DNA was
579 stained using Click-iT EdU reaction cocktails. EdU-positive cells were detected using a BD
580 LSR Fortessa cell analyser (BD Biosciences, USA) or a spectral flow cytometer ID7000
581 (Sony Biotechnology).

582 **Proliferation assay *in vitro***

583 Proliferation assays were performed in serum-free AIM-V medium (Thermo Fisher
584 Scientific) supplemented with penicillin-streptomycin-glutamine (Thermo Fisher Scientific).
585 Whole mouse splenocytes and human PBMCs were plated at a density of 5×10^6 cells per well
586 in a Cepallete W-type 24 well microplate (DIC, Japan) and cultured for 4 days with or without
587 mouse/human M-CSF (Peprotech, USA). Surviving macrophages adhered to 24-well plates
588 were detached by lowering the temperature on ice. The collected cells were incubated with
589 both LIVE/DEAD fixable aqua fluorescent reactive dye (Invitrogen, USA) and SYTOX
590 Green dead cell stain (Invitrogen, USA) in 1×PBS for dead cell staining. Whole mouse
591 splenocytes and human PBMCs were stained with CD11b/Ly6G/NK1.1/Ly6C/Fcgr4 and
592 CD11b/HLA-DR/CD14/CD16 after blocking antibody treatment, respectively. The number

593 of live macrophages was estimated using flow-count fluorospheres (Beckman Coulter, USA)
594 and flow cytometry.

595 Sorted Ly6C^{low} and Ly6C^{high} monocytes were plated on 96-well plates (BD Falcon, USA) at
596 2×10^4 cells/well and cultured for 4 days with or without mouse M-CSF. The
597 surviving macrophages were detected by the CellTiter-Glo 2.0 Cell Viability Assay
598 (Promega, USA) following the manufacturer's protocol, and macrophage number was
599 estimated by comparing with FACS analyses to the control samples whose monocyte
600 numbers were counted.

601 **LC-MS analysis**

602 Quantitative nucleoside analysis was performed using an LC-MS system equipped with a
603 reversed-phase column (2.0 mm I.D. \times 100 mmL) packed with Develosil C30 UG (3 μ m
604 particle, Nomura Chemical) connected to a hybrid quadrupole-orbitrap mass spectrometer (Q
605 Exactive, Thermo Fisher Scientific) through an electrospray interface. For analyses of
606 nucleoside accumulation in mouse cells and human PBMC-derived macrophages, 1×10^7
607 cells were lysed using 400 μ L D solution (7M guanidine hydrochloride and 0.5M Tris-
608 HCl/10mM EDTANa₂, pH 8.5) containing stable isotope-labelled nucleosides (final standard
609 nucleoside concentration; 1 nmol/400 μ L of A/U/G/C/dG). For the analysis of nucleoside
610 accumulation in tissues, 100 mg of each tissue was lysed with 400 μ L D solution containing
611 stable isotope-labelled nucleosides (final standard nucleoside concentration: 1 nmol/400 μ L
612 of C/dG, 10 nmol/400 μ L of U/G, and 100 nmol/400 μ L of A). The extract was centrifuged at
613 $10,000 \times g$ for 30 min, and the supernatant was diluted 40- to 200-fold with 10 mM
614 ammonium acetate buffer (pH 6.0). Samples (ca. 1–100 pmol nucleosides/40 μ L) were loaded
615 into a reversed-phase column and eluted with a 30 min linear gradient from 2% to 12%
616 acetonitrile in 10 mM ammonium acetate buffer (pH 6.0) at a flow rate of 100 μ L/min. The
617 eluate from the first 6 min was automatically wasted by switching a 3-way electric valve to

618 remove guanidine hydrochloride from the system and was subsequently sprayed into a mass
619 spectrometer at 3.0 kV operating in positive-ion mode. Mass spectra were acquired at a
620 resolution of 35,000 from m/z 200 to 305. Each nucleoside in the sample cells or tissues was
621 quantified from the peak height relative to that of the corresponding isotope-labelled standard
622 nucleoside. All LC-MS data were processed and analysed using Xcalibur (version 3.0.63,
623 Thermo) and Excel 2013 (Microsoft).

624 **Platelet and cell counts**

625 Platelet numbers in PBMCs were analysed using an automatic haematology analyser (Celltac
626 α ; Nihon Kohden). Cell number was measured using an automated cell counter, CellDrop BF
627 (DeNovix).

628 **Preparation of splenic B cells**

629 Splenic B cells were purified by negative selection using CD43 MicroBeads (Miltenyi
630 Biotec). Splenocytes from WT and *Slc29a3*^{-/-} mice were labelled with CD43 magnetic
631 beads, and CD43-negative splenic B cells were enriched using autoMACS (Miltenyi Biotec,
632 Germany) and subjected to experiments.

633 **Preparation of bone marrow (BM)-derived macrophages and pDCs**

634 BM cells were collected from the tibiae, femora, and pelves of WT, *Slc29a3*^{-/-}, and *Slc29a3*^{-/-}
635 *Tlr7*^{-/-} mice, and red blood cells were removed using BD Pharm Lyse lysing buffer. For
636 preparation of BM-macrophages (BM-Mphs), BM cells were plated at a density of 7×10^6
637 cells per well on a non-tissue culture polystyrene 94 mm petri dish (Greiner Bio-
638 One, Germany) and cultured in 10 mL RPMI medium (Gibco™ ThermoFisher
639 Scientific, USA) supplemented with 10% FBS, penicillin-streptomycin-glutamine (Gibco™
640 ThermoFisher Scientific, USA), 50 μ M 2-ME, and 100 ng/mL recombinant murine
641 macrophage colony stimulating factor (M-CSF; PeproTech Inc., USA) for 6 days. Attached
642 cells on petri dishes were collected and used as BM-Mphs. For BM-plasmacytoid DCs (BM-

643 pDCs), BM cells were plated at a density of 2.5×10^7 cells per 10-cm cell culture dish
644 (Greiner Bio-One, Germany) and cultured in 10 mL RPMI 1640 medium (Gibco, Paisley,
645 UK) supplemented with 10% FBS, penicillin-streptomycin-glutamine, 50 μ M 2-ME, and 100
646 ng/mL recombinant murine FMS-like tyrosine kinase-3 ligand (Flt3L, PeproTech Inc., USA)
647 for 7 days. Flt3L-induced pDCs were stained with anti-CD11c/B220 mAbs, and
648 CD11c⁺B220⁺ cells were sorted as BM-pDCs using a FACSAria flow cytometer (BD
649 Biosciences, USA).

650 **Preparation of human PBMCs and macrophages**

651 All experiments using human samples were approved by the Institutional Ethics Review
652 Boards of the Institute of Medical Science at the University of Tokyo (IMSUT), Jichi
653 Medical University, and Hiroshima University.

654 To prepare human peripheral blood mononuclear cells (hPBMCs), 7 mL of EDTA-
655 anticoagulated whole blood was treated with 45 mL BD Pharm lysis buffer (BD Biosciences)
656 to completely lyse red blood cells. hPBMCs collected after centrifugation were subjected to
657 FACS analysis and survival assays or allowed to differentiate into macrophages. To induce
658 human macrophages, hPBMCs were plated in 94 \times 16 mm petri dishes (Greiner, Germany) at
659 a density of 1.0×10^7 cells per dish and cultured in 10 mL RPMI 1640 medium (Gibco, UK)
660 supplemented with 10% FBS, penicillin-streptomycin-glutamine (Gibco, UK), 50 μ M 2-ME,
661 100 ng/mL of recombinant human M-CSF (PeproTech Inc., USA), and 20 ng of recombinant
662 human IL-4 (PeproTech Inc., USA) for 7 days. After removing floating cells with 1xPBS, the
663 attached cells were collected as human macrophages and subjected to LC-MS and ELISA.

664 **Cytokine measurements by ELISA**

665 Mouse thioglycollate-elicited PECs and mouse BM-Mphs were cultured in flat-bottom 96-
666 well plates (BD Falcon, USA) at 1×10^5 cells/well. Human PBMC-derived
667 macrophages were cultured in flat-bottomed 96-well plates at 1×10^4 cells/well. All types of

668 immune cells were stimulated with the indicated ligands for 16–20 h, and cytokine
669 concentrations in the supernatant were measured using ELISA. Serum cytokine
670 concentrations in the mice were measured using ELISA. The concentrations of mouse IL-
671 12p40, mouse TNF- α , mouse IL-1 β , mouse IL-6, and human TNF- α in the supernatant were
672 measured using Ready-Set-Go! ELISA kits (eBioscience, USA). Mouse IFN- α and IFN- β
673 concentrations in the supernatant were measured using IFN- α/β ELISA kits (PBL Assay
674 Science, USA).

675 **Cytokine antibody array**

676 The production of 111 cytokines by splenic monocytes was quantified using the Proteome
677 Profiler Mouse XL Cytokine Array (R&D Systems). Cytokine antibody array was performed
678 according to the manufacturer's instructions. In brief, the antibody array membrane was
679 incubated with 200 μ L culture supernatant of splenic monocytes overnight at 4 °C. After
680 incubation with the samples, the membranes were sequentially treated with a detection
681 antibody cocktail and streptavidin-HRP. Finally, the membranes were treated with ECL
682 Select Western Blotting Detection Reagent (GE Healthcare), and the chemiluminescent
683 signal on the membranes was detected using an ImageQuant LAS 500 imager system (GE
684 Healthcare). The intensity of each spot was quantified using the Quick Spots image analysis
685 software (Western Vision Software).

686 After the cytokine antibody array, IL-6 production by the splenic monocyte population was
687 further determined by flow cytometry to confirm the results from the Proteome
688 Profiler Antibody Arrays.

689 **Cell death induction and phagocytosis assay *in vivo***

690 Cell death was induced by treatment of thymocytes at 47 °C for 20 min, and then, cells were
691 incubated at 37 °C for 3 h before subjecting the cell corpses to the phagocytosis assay. Before
692 the phagocytosis assay *in vivo*, cell corpses were stained with the PKH26 Red Fluorescent

693 Cell Linker Kit for General Cell Membrane Labelling (Sigma-Aldrich) according to the
694 manufacturer's instructions. Then, 5×10^7 PKH26 stained cell corpses were intravenously
695 administered to mice, and mouse spleens were collected 2 h after cell corpse administration.
696 Immune cells engulfing PKH26-positive cell corpses were detected using flow cytometry.

697 **Lentiviral transduction**

698 FLAG-tagged human SLC29A3 was expressed in the mouse macrophage cell line J774.1
699 cells using lentiviral transduction. The cDNA of FLAG-SLC29A3 was substituted with the
700 BFP2Apuro sequence in the lentiviral pKLV-U6gRNA(BbsI)-PGKpuro2ABFP vector
701 (Addgene plasmid 50946), excluding the U6gRNA(BbsI) site. The ViraPower Lentiviral
702 expression system (Thermo Fisher Scientific) was used to prepare the lentivirus for Flag-
703 SLC29A3 overexpression according to the manufacturer's instructions. Supernatants
704 containing lentivirus particles were collected 24 h post-transfection and used for transduction.

705 **Structured illumination microscopy**

706 Macrophages from the J774.1 cell line were allowed to adhere to collagen-coated coverslips
707 overnight and were stimulated by 1 mM Phorbol 12-myristate 13-acetate (PMA) for 2 h.
708 Cells attached on coverslips were treated with the heat treated dying thymocytes for 1 h.
709 After engulfment, the cells were fixed with 4% paraformaldehyde for 10 min and then
710 permeabilized with 1XPBS containing 0.2% saponin for 30 min.
711 After blocking with 2.5% BSA Blocking One (Nacalai Tesque, Japan) for 30 min, cells were
712 incubated with anti-TLR7 antibody and anti-HA antibody (Roche) at 37 °C for 90 min.
713 After washing the cells three times, the cells were incubated for 90 min at 37 °C with
714 AlexaFluor-488 conjugated goat anti-mouse and AlexaFluor-568 goat anti-rat antibodies and
715 DAPI (Invitrogen). Fluorescence microscopy was performed using a Nikon Structured
716 illumination microscope (N-SIM, Nikon) at excitation wavelengths of 405, 488, and 561 nm
717 with a CFI Apochromat TIRF 100 × objective lens (1.49 NA, NIKON). Data acquisition was

718 performed in 3D SIM mode before image reconstruction using NIS-Element software. Each
719 image represents more than three independent experiments.

720 **Statistical analysis**

721 Statistical significance between the two groups was determined using a two-tailed, unpaired
722 t-test with Holm–Sidak correction. To determine significant differences between more than
723 three groups, one-way ANOVA followed by Dunnett's multiple comparison test was
724 employed in this study. All data are represented as the mean \pm standard deviation (s.d.) and
725 graphs were made using PRISM. Statistical significance was set at $p < 0.05$. $*p < 0.05$, $**p <$
726 0.01 , $***p < 0.001$.

727

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790 Contributions

791 T.S. and K.M. conceived of and designed the experiments. T.S., M.O., and N.Y. constructed
792 knockout and transgenic mice. M.T., Y.Y. and T. Isobe conducted LC-MS analysis. R.S. and
793 S.S. performed high-resolution microscopy. T.S., E.F., M.K, M.O., E.L., and A.T. analysed
794 human blood samples from patients with H syndrome. K.Y. and F.Y. performed the
795 transcriptome analyses. T.S. performed the *in vivo* analyses with the help of S. G., Y. M., J.
796 K., K. I., Y. T., T. Ichinohe, R. H., R. F., T. K., and T. T.. R.S., K. Mukai, and T.T.
797 performed imaging and biochemical analyses. T.S. performed all other experiments in this
798 study and analysed the data for all figures. T.S. and K.M. wrote the paper with assistance
799 from U.O., and T. Shimizu. All authors have reviewed the manuscript.

800 Corresponding authors

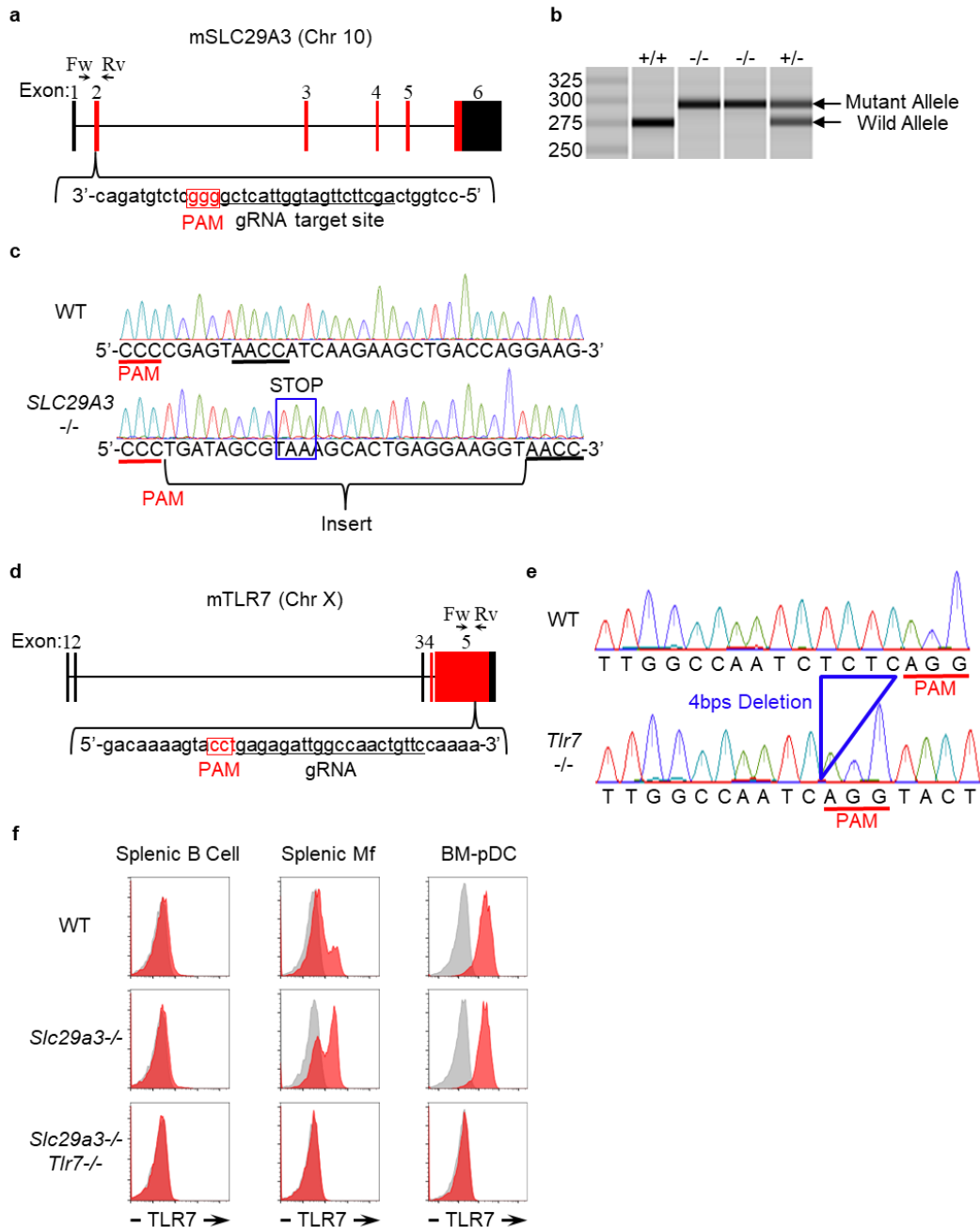
801 Correspondence to Kensuke Miyake

802 **Ethics declarations**

803 competing interests

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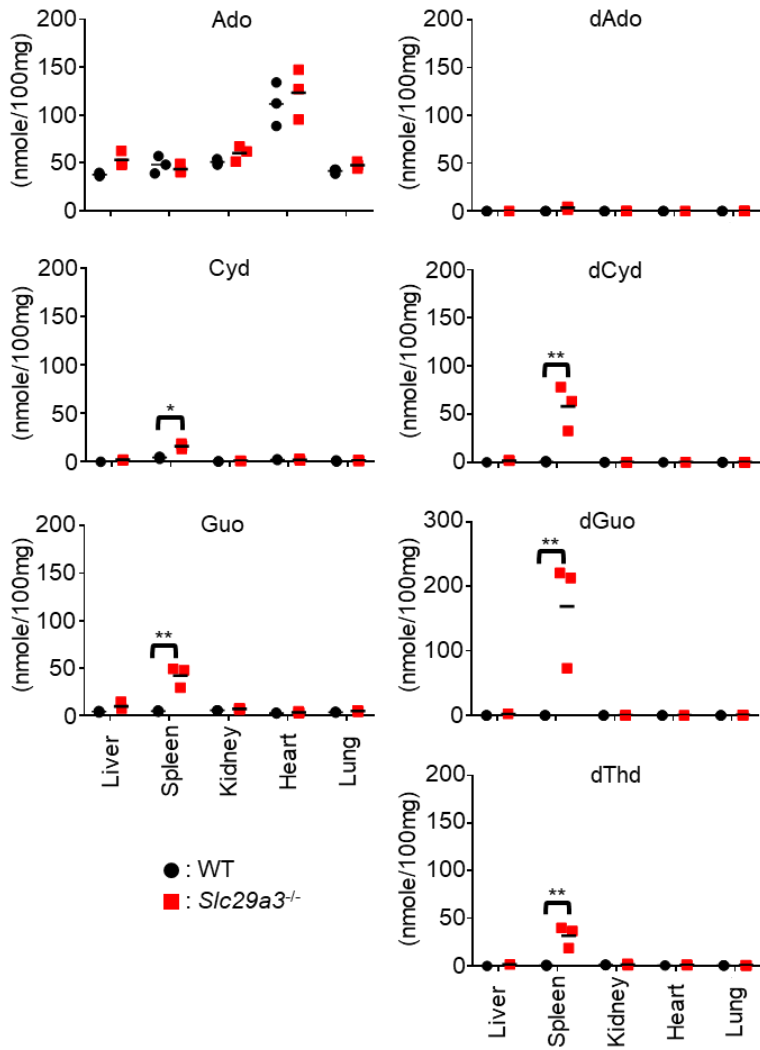
805 **Supplementary Materials:**



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807 **Extended Data Figure 1. Generation of *Slc29a3*^{-/-} and *Tlr7*^{-/-} mice**

808 **a, d**, Genomic configuration of *Slc29a3* and *Tlr7* genes showing 20mer gRNA target sites to
 809 introduce a mutation into exon 2 of *Slc29a3* and exon 5 of *Tlr7*. The PAM sequence is
 810 highlighted by the red box. **b**, Genomic PCR with the primer set (Fw and Rv) shown in (a) to
 811 reveal an insertional mutation in the targeted allele of *Slc29a3*. **c**, Direct sequencing of the
 812 gRNA target site of *Slc29a3*. The inserted sequence containing the stop codon is shown by
 813 the blue box. **e**, Sequence data of the gRNA target site on the *Tlr7* allele showing a 4-bp
 814 deletion in the 5th exon of *Tlr7* (blue). **f**, FACS analysis showing the lack of TLR7 protein in
 815 splenic B cells, splenic monocytes, and BM-derived plasmacytoid DCs from WT, *Slc29a3*^{-/-},
 816 and *Slc29a3*^{-/-} *Tlr7*^{-/-} mice. Red and grey histograms represent intracellular staining with and
 817 without anti-TLR7 mAb, respectively.

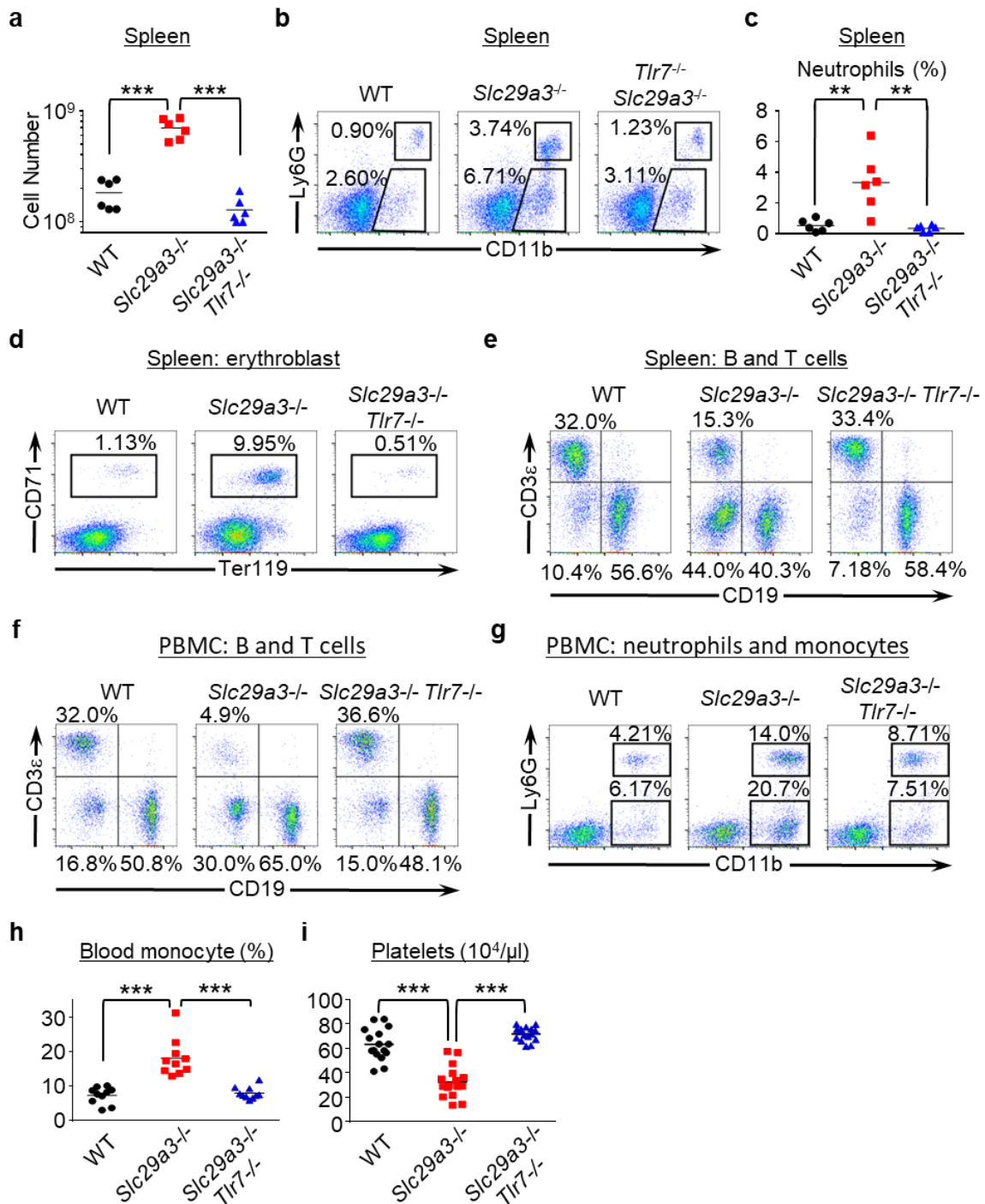


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819 **Extended Data Figure 2. Nucleoside storage in *Slc29a3*^{-/-} mice**

820 The amount of nucleosides (nmol/100 mg) in each organ from WT and *Slc29a3*^{-/-} mice was
821 determined by LC-MS analyses. Dot plots represent values from the indicated mice (n =
822 3). **p* < 0.05, ***p* < 0.01.

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Extended Data Figure 3. TLR7-dependent monocytosis in *Slc29a3*^{-/-} mice

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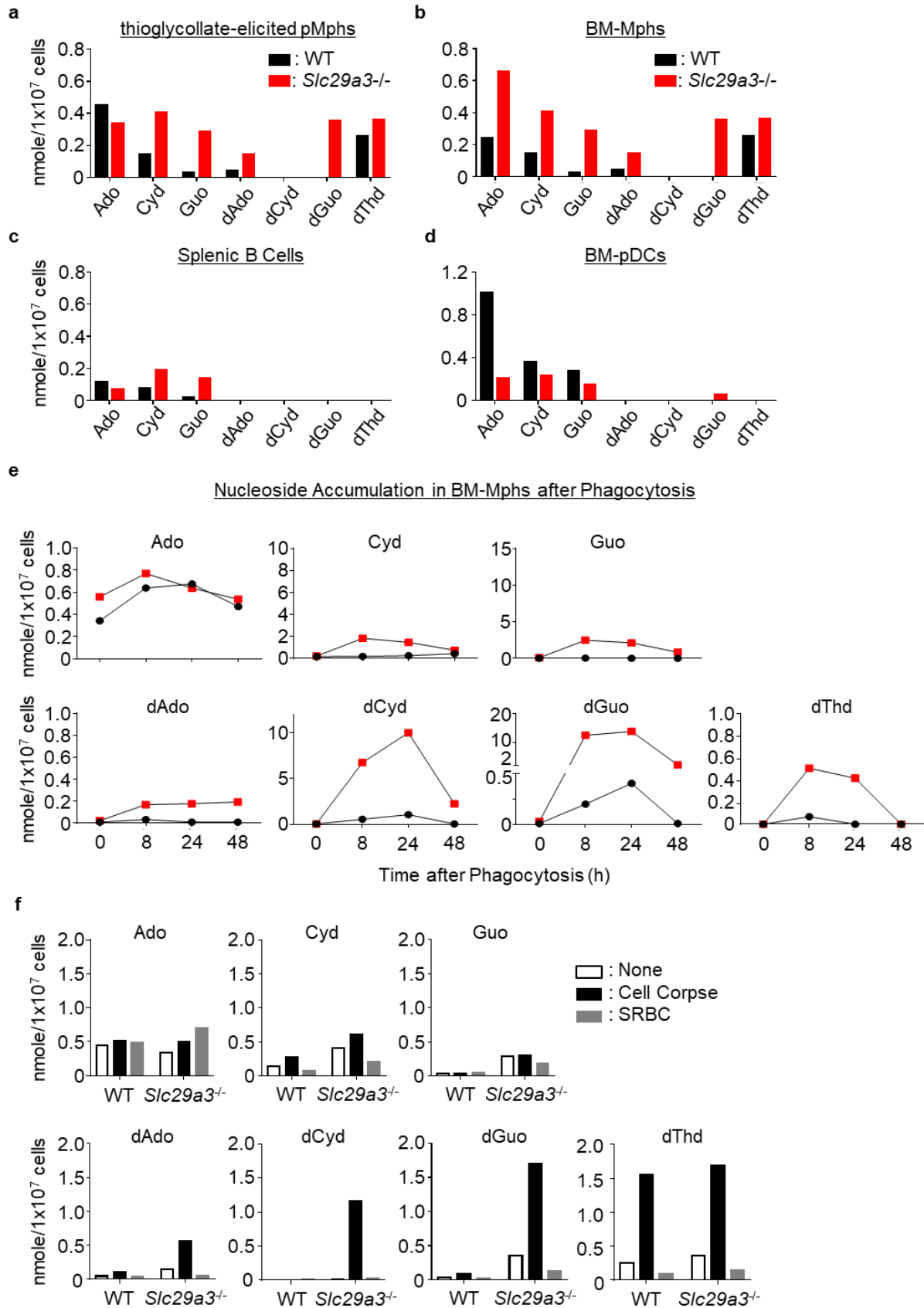
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a, Dot plots showing the number of splenocytes in the indicated mice (n = 6). **b**, Representative FACS analyses of Siglec F⁻ NK1.1⁻ splenocytes to show their expression of CD11b and Ly6G. **c**, Each dot shows the percentage of neutrophils in whole splenocytes from the indicated mice (n = 6). **d**, **e**, FACS analyses of CD71⁺ Ter119⁺ erythroblasts (**d**) or CD19⁺ B and CD3ε⁺ T cells (**e**) in the spleen. **f**, **g**, FACS analyses of B and T cells (**f**) or neutrophils and monocytes (**g**) in PBMCs. **h**, **i**, Percentages of CD11b⁺ Ly6G⁻ monocytes (n= 10) in PBMCs (**h**) and platelet counts (n = 16) in the peripheral blood (**i**) of 3-month-old mice.

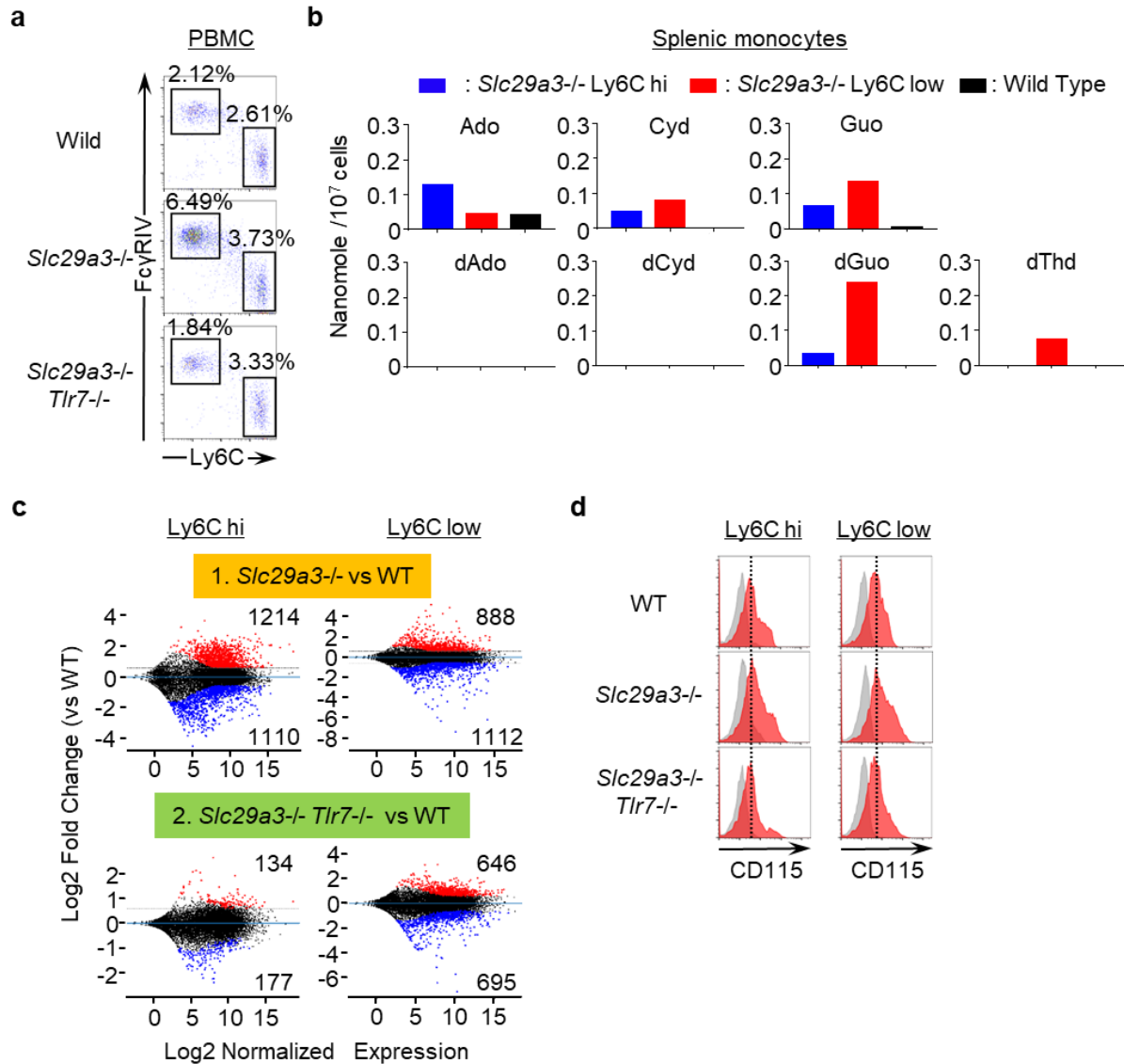


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Extended Data Figure 4. Nucleoside storage in *Slc29a3*^{-/-} phagocytes

836 **a-d**, The amount of nucleosides (nmol) in 10⁷ thioglycollate-elicited pMphs (**a**), bone marrow-
837 derived macrophages (BM-Mphs) (**b**), splenic B cells (**c**), and BM-plasmacytoid dendritic

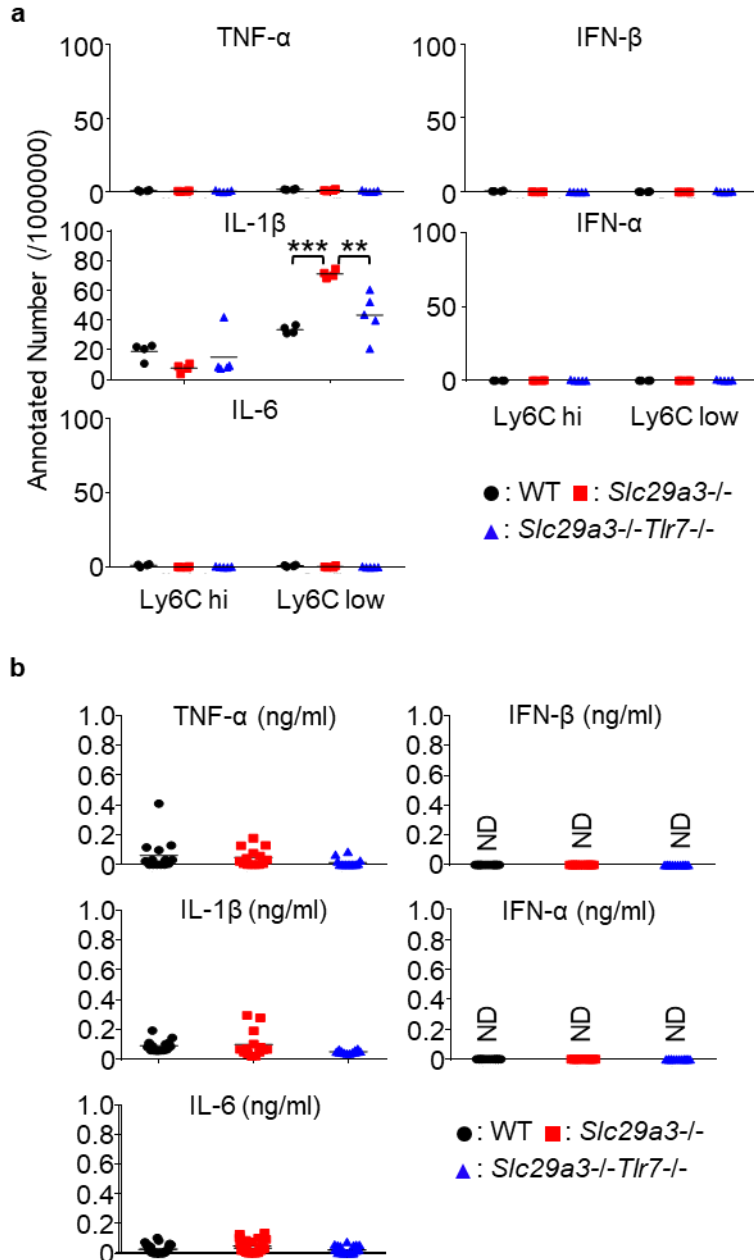
838 cells (BM-pDCs) (**d**) from WT (black) and *Slc29a3*^{-/-} (red) mice. **e**, The amount of
839 nucleosides (nanomoles) in 10⁷ BM-Mphs of WT and *Slc29a3*^{-/-} mice at the indicated time
840 points after treatment with 10⁸ dying thymocytes (cell corpse). **f**, The amount of nucleosides
841 (nanomoles) in 10⁷ BM-Mphs of WT and *Slc29a3*^{-/-} mice after 1 day treatment with 10⁸
842 dying thymocytes or 10⁹ SRBC.



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Extended Data Figure 5. Analyses of Ly6C^{hi} and Ly6C^{low} monocytes

a, Representative FACS analyses showing the expression of FcγRIV and Ly6C on CD11b⁺ Ly6C⁺ monocytes in the peripheral blood. The percentages of FcγRIV^{hi} Ly6C^{low} patrolling and FcγRIV^{low} Ly6C^{hi} classical monocytes among whole peripheral mononuclear cells are shown. **b**, The amount of nucleosides (nmole/10⁷ cells) in *Slc29a3*^{-/-} Ly6C^{hi} and Ly6C^{low} splenic monocytes or in WT CD11b⁺ splenic monocytes. **c**, Transcriptome analyses of Ly6C^{hi} and Ly6C^{low} monocytes from the spleen. MA-plots displaying Log₂ normalized expression (X-axes) and Log₂ fold change of expression (Y-axes) for the comparisons of *Slc29a3*^{-/-} (n = 4) vs WT (n = 4) monocytes and *Slc29a3*^{-/-} *Tlr7*^{-/-} (n = 4) vs WT (n = 4) monocytes. More than 1.5-fold upregulated and downregulated genes are shown in red and blue, respectively. **d**, Red histograms show cell surface expression of CD115 in Ly6C^{hi} and Ly6C^{low} monocytes in the spleens of WT, *Slc29a3*^{-/-}, and *Slc29a3*^{-/-} *Tlr7*^{-/-} mice. Grey histograms show staining with control mAb.



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Extended Data Figure 6. Low cytokine production in *Slc29a3*^{-/-} mice

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a, mRNA expression of cytokines in splenic Ly6C^{hi} and Ly6C^{low} monocytes from WT

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(black), *Slc29a3*^{-/-} (red), and *Slc29a3*^{-/-} *TLR7*^{-/-} (blue) mice. Each dot shows the normalised

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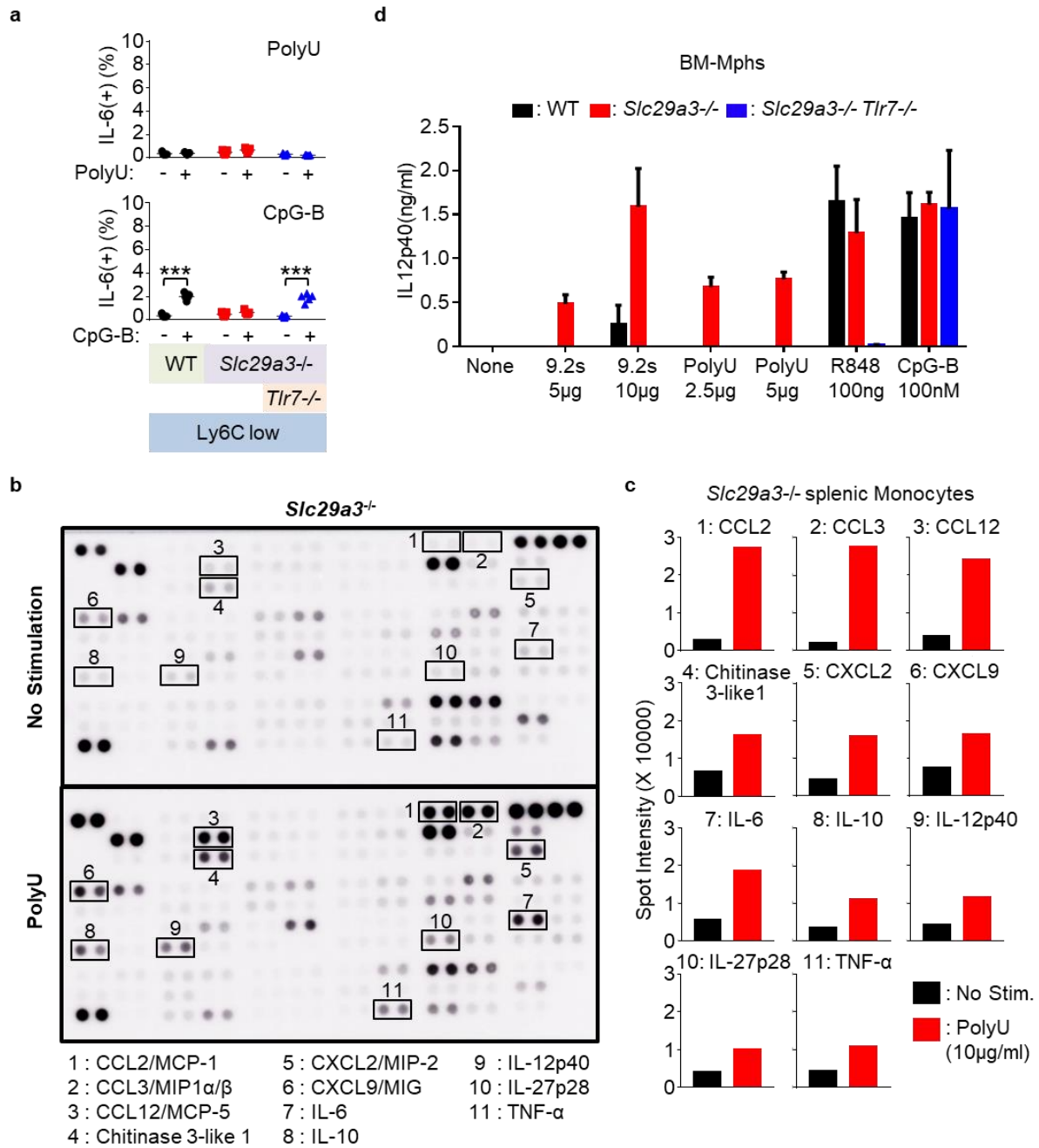
read count per 1 million reads from the RNA-seq analyses for each monocyte population (n =

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4). **b**, Serum cytokine levels were determined using ELISA. Sera were collected from 4–6

864

months old mice (n = 14). ***p* < 0.01, ****p* < 0.001. ND, not detected.



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Extended Data Figure 7. Enhanced TLR7 response to ssRNA in *Slc29a3*^{-/-} monocytes

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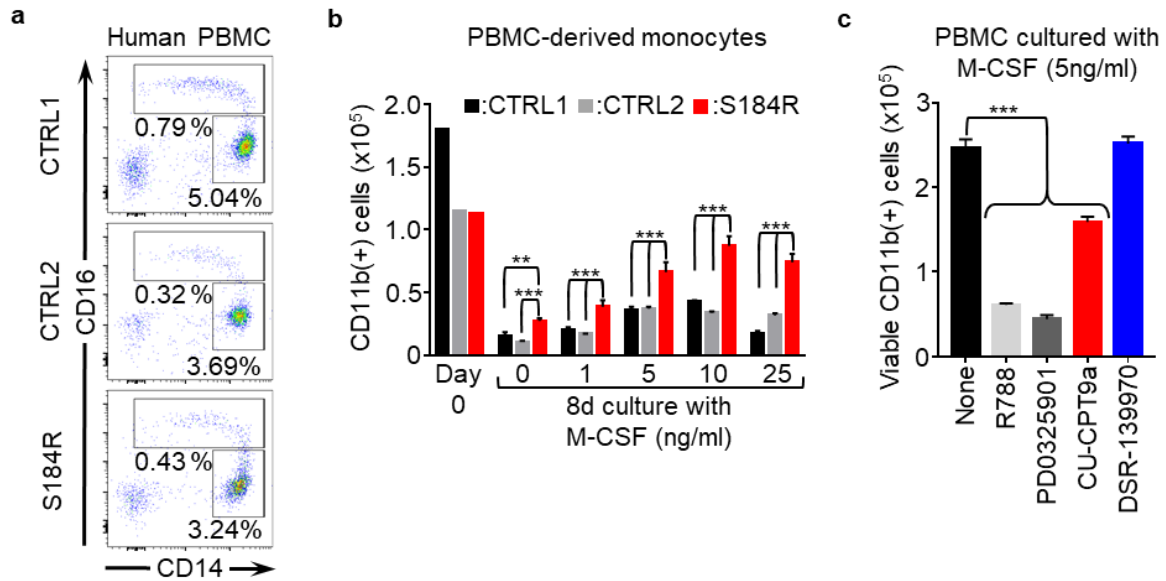
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a, The percentage of IL-6⁺ cells in Ly6C^{low} monocytes from indicated mice after *in vitro* stimulation with poly U (10 μg/mL) or CpG-B (1 μM) in the presence of brefeldin A (10 μg/mL) for 4 h. Each dot represents the value for each mouse (n = 5). **b**, **c**, *Slc29a3*^{-/-} splenic monocytes were stimulated with poly U (10 μg/mL) for 18 h. Cytokines in the supernatants were detected using a cytokine antibody array (**b**). The results are shown as the mean signal intensity value for each cytokine spot (**c**). **d**, IL-12 p40 production by BM-Mphs after stimulation with TLR7 and TLR9 ligands for 18 h. The results are represented as the mean ± s.d. from triplicate samples.



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876 **Extended Data Figure 8. Enhanced survival of PBMC derived monocytes from the**
 877 **patient harbouring the S184R *SLC29A3* mutation**

878 **a**, Expression of surface CD16/CD14 in HLA-DR⁺ CD15⁻ CD56⁻ PBMCs from the patient
 879 with the S184R *SLC29A3* mutation and from healthy subjects. **b**, Numbers of CD11b⁺ CD15⁻
 880 CD56⁻ monocytes in PBMCs from the patients (red) and healthy subjects (black and grey)
 881 that survived 4 days of culture with M-CSF at the indicated concentrations. **c**, Numbers of
 882 CD11b⁺ cells that survived 8 days PBMC culture with 5 ng/mL M-CSF in the absence or
 883 presence of the indicated inhibitors of Syk (1 μ M R788), MEK1/2 (1 μ M PD0325901), TLR8
 884 (10 μ M CPT9a), and TLR7 (10 μ M DSR-139970).