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1 **Systemic inflammation recruits fast-acting anti-inflammatory innate myeloid progenitors**
2 **from BM into lymphatics**

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29 **ABSTRACT**

30

31 Innate immune cellular effectors are actively consumed during systemic inflammation but the
32 systemic traffic and the mechanisms that support their replenishment remain unknown. Here we
33 demonstrate that acute systemic inflammation induces the emergent activation of a previously
34 unrecognized system of rapid migration of granulocyte-macrophage progenitors and committed
35 macrophage-dendritic progenitors, but not other progenitors or stem cells, from bone marrow
36 (BM) to lymphatic capillaries. The progenitor traffic to the systemic lymphatic circulation is
37 mediated by Ccl19/Ccr7 and is NFκB independent, Traf6/IκB-kinase/SNAP23 activation which
38 is responsible for the secretion of pre-stored Ccl19 by a subpopulation of CD205^{+}/CD172a^{+}
39 conventional dendritic cells type 2 (cDC2) and upregulation of BM myeloid progenitor Ccr7
40 signaling. The consequence of this progenitor traffic is anti-inflammatory with promotion of early
41 survival and initiation of replenishment of lymph node cDC.

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44 **INTRODUCTION**

45

46 Bacterial infections represent one of the major threats for the human immune system
47 and can lead to sepsis or death (Martin et al., 2003). A functional immune response is a key
48 factor to control the outcome of bacterial infections. Therefore, the human immune system has
49 evolved several effector mechanisms to fight bacterial infections which involve the innate and
50 the adaptive arms of the immune system. Antigen presentation is an essential mechanism of
51 activation that requires crosstalk between the innate and adaptive immune system to fight
52 bacterial infections. Dendritic cells are short-lived professional antigen presenting cells (APC)
53 and their life span is further reduced during the inflammatory response to pathogens (Kamath et
54 al., 2002). Upon inflammation, primed APC thus need to be replaced.

55 During inflammation, systemic signals alert and activate bone marrow (BM) myeloid
56 differentiation (Baldridge et al., 2010; Essers et al., 2009; Nagai et al., 2006). Inflamed
57 secondary lymphoid organs such as lymph nodes (LN) recruit antigen-presenting dendritic cells
58 (DC) (Legler et al., 1998; Luther et al., 2000; Saeki et al., 1999), while pathogen-associated
59 molecular pattern signals (PAMPs) trigger migration of tissue-resident DC to the LN (Kaisho and
60 Akira, 2001; Sallusto and Lanzavecchia, 2000). Circulation of hematopoietic stem cells and
61 progenitors (HSC/P) that enter the lymphatic vessels from the peripheral blood (PB) with ability
62 to amplify APCs has been described (Massberg et al., 2007). However, the circuits used by
63 these HSC/P populations, their characterization and the cellular and molecular mechanisms that
64 regulate this traffic in inflammatory conditions have not been addressed in detail.

65 Lymphatics form part of an open circulatory system that drains cells and interstitial fluid
66 from tissues. Recently, bone lymphatic endothelial cells have been shown to arise rapidly from
67 pre-existing regional lymphatics in inducible bone-expressing *Vegfc* transgenic mice through
68 Vegfr3, osteoclast activation and bone loss (Hominick et al., 2018; Monroy et al., 2020). Acute
69 endotoxemia is associated with osteoclast activation and bone loss (Hardy and Cooper, 2009;
70 Nason et al., 2009). We postulated the pre-existence of an anatomical and functional patent

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71 circuit that communicates BM and lymphatic tissues that can be induced upon severe
72 inflammatory conditions like endotoxemia.

73 Our work identifies an emergent traffic of DC-biased myeloid progenitors through direct
74 transit from BM to bone lymphatic capillaries. This traffic is highly activated in endotoxic
75 inflammation. In human reactive lymphadenitis or just after a single immune endotoxic
76 challenge, such as following lipopolysaccharide (LPS) stimulation in mice, a massive
77 mobilization of myeloid progenitors from the BM to lymph and retention in the LN takes place.
78 The mobilization is rapid, prior to their appearance in PB. LPS simultaneously induces cell-
79 autonomous Ccr7 expression on granulo-macrophage progenitors (GMP) and macrophage-
80 dendritic progenitors (MDP), and a non-cell autonomous myeloid cell-dependent secretion of
81 Ccl19 in the LN. In vivo blockade of LPS signaling in mature myeloid cells, deletion of
82 hematopoietic Ccl19 or neutralization of Ccr7 completely abrogated the GMP/MDP migration
83 from the BM to the LN and increased acute inflammation associated mortality. Moreover,
84 genetic and pharmacological approaches revealed that Traf6-Irak1/4-Ubc13-IκB kinase (IKK)
85 signaling mediates NF-κB-independent-SNAP23 phosphorylation and secretion of pre-formed
86 Ccl19 from a specific population of conventional dendritic cells (cDC). These findings indicate
87 that inflammation results in mobilization of cDC-forming cells directly from the BM to the lymph
88 and LN. As such, emergent myeloid lineage mobilization from the BM to lymph may be
89 important in inflammation by acutely replenishing antigen-presenting cells in lymph tissues and
90 impairing the inflammatory signaling responsible for mortality in endotoxemia.

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93 **RESULTS**

94 **Inflammation associates with emergent migration of myeloid progenitors, but not HSC,**
95 **from BM to lymphatics.**

96 To determine whether there is a circulation of HSC/P to human LN, we prospectively
97 analyzed the presence of side population (SP) cells in LN biopsies (**Figure S1A**) obtained from
98 lymphadenitis and lymphoma patients at diagnosis. Human and murine SP cells, with ability to
99 extrude the dye Hoechst 33342 through upregulated activity of multidrug resistance protein
100 complexes (Zhou et al., 2001) in BM and other tissues (Brusnahan et al., 2010; Challen and
101 Little, 2006; Goodell et al., 1996) are enriched in long-term reconstituting HSC and other more
102 committed populations of progenitors (Matsuzaki et al., 2004; Weksberg et al., 2008). We found
103 a SP population at a frequency higher than 0.01% in 36 out of 64 LN biopsies (53.12%).
104 However, the content of SP cells in the LN did correlate with the LN histological diagnosis. The
105 elevated frequency of SP cells in LN did correlate with the LN histological diagnosis (**Figure 1A**)
106 but not to the anatomical location of the lymphadenopathy (**Figure S1B; Table S1**). The
107 accumulation of SP cells was significantly higher in LN from lymphadenitis patients than in
108 lymphoma patients. Further dissection based on histological classifications by independent
109 pathology analysis resulted in the lymphadenitis specimens being sorted into distinct histological
110 categories which corresponded to follicular lymphadenitis with paracortical predominance (FL),
111 granulomatous lymphadenitis (GL), and lymphadenopathies with histological or molecular
112 evidence of viral etiology (viral lymphadenitis, VL). Interestingly, FL and GL LN contained a
113 median of 0.2% SP cells with a range from <0.01% to ~40%, which was significantly higher than
114 the content of SP cells in VL, Hodgkin's lymphoma, and non-Hodgkin's lymphoma LN
115 (**Figure1A; Figure S1A, Table S1**). The existence of myeloid-committed hematopoietic
116 progenitors was confirmed in myeloid colony-forming cell unit (CFU) assays (**Figure S1C**)
117 performed on samples from patients with FL. These data show that non-viral inflammatory
118 lymphadenitis results in a significantly increased frequency of primitive hematopoietic cells in
119 LN, while it does not reveal the type of progenitor cells. To confirm whether LN SP cells indeed

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120 contained HSC/P, we first sorted LN SP cells from patients with reactive lymphadenitis and
121 plated them in semisolid cultures containing rhIL-3, rhIL-6 and rhSCF cytokines CFU analysis
122 demonstrated that SP cells were indeed capable of producing myeloid colonies (**Figures S1D**),
123 while non-SP cells were devoid of measurable CFU-forming ability (data not shown).
124 Immunophenotypic analysis of SP-derived progenitors was also consistent with enrichment of a
125 heterocellular population of CD34 and CD133 expressing granulocyte-, granulocyte-
126 macrophage-, and cDC-biased progenitors (Bornhauser et al., 2005; Gorgens et al., 2013)
127 (**Figure S1D**). The vast majority of CD45^{+}/CD34^{+} cells co-expressed CD133^{+}, and the
128 CD45^{+}/CD34^{-} population was split ~50:50 into CD133^{+} and CD133^{-} cells (**FigureS1D**). In
129 combination, these data show the accumulation of a myeloid-committed HSC/P population in
130 human lymphadenitis. Adult inflammatory LN tissues therefore contain an increased number of
131 myeloid-committed HSC/P. This increase can result from either the recruitment of these cells to
132 LN via the bloodstream or the expansion of otherwise rare and already resident myeloid-
133 committed HSC/P in these LN.

134 The release of HSC/P from BM into the bloodstream follows circadian cycles (Mendez-
135 Ferrer et al., 2008) controlled by the activity and fate of inflammatory cells (Casanova-Acebes et
136 al., 2013; Chang et al., 2014). We postulated that if inflammation is responsible for the
137 recruitment of HSC/P to the LN and possibly other organs, we should be able to recapitulate the
138 process of BM egression, migration, and organ retention, in an inflammatory murine model
139 wherein the HSC/P migration process is highly conserved. Since the largest content of HSC/P in
140 human LN was found in biopsies from patients with lymphadenitis, we generated a mouse
141 model of Gram-negative sepsis by injection of *E. coli* LPS into C57Bl/6 mice at the early
142 timepoint of the circadian HSC/P mobilization cycle (*zeitgeber* time, ZT) (Bellet et al., 2013)
143 (**Figure1B**). *E. coli* LPS is able to activate a large number of Toll-like receptors (TLR), which
144 result in high-level activation of the inflammatory signaling cascade (Beutler and Rietschel,
145 2003). LPS is also a well-known inducer of HSC/P mobilization to PB(Cline and Golde, 1977;
146 Velders et al., 2004; Vos et al., 1972; Vos and Wilschut, 1979). In our experiments, the

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147 circadian mobilization pattern of HSC/P in the PB was severely modified by the administration of
148 LPS, with the increase in HSC/P appearing later and peaking at ZT10, 6 hours post-
149 administration (**Figure1C**), coincident with an increased neutrophil count in the PB (**Figure**
150 **S1E**). We observed similar kinetics of an increased numbers of HSC/P in the highly
151 vascularized kidney and liver tissues after LPS administration (**Figure 1D-E**), suggesting that
152 the presence of HSC/P in these tissues closely paralleled their presence in the PB. Interestingly,
153 LPS did not elicit a significant change in the level of splenic HSC/P within the first 12 hours after
154 inflammation (Esplin et al., 2011; Wright et al., 2001) (**Figure 1F**).

155 Notably, when the HSC/P content was reduced in the BM, the kinetics of their
156 subsequent mobilization to the PB was discordant. The BM HSC/P content decreased, which
157 supports the migratory nature of the increased HSC/P in the PB (**Figure 1G**); yet the nadir of
158 the BM HSC/P content occurred as early as 3 hours after LPS administration (at ZT7), returning
159 to normal values by 6 hours (ZT10, **Figure 1G**). The time lapse between the loss of retention of
160 HSC/P in the BM and their presence in the PB circulation suggested that the migration of
161 HSC/P from the BM to the PB required an intermediate step of circulation through other tissues.
162 Based on an earlier description of a lymphatic circulation of HSC/P (Massberg et al., 2007), we
163 hypothesized that this delay in the appearance of HSC/P in the PB was due to an intermediate
164 transit of HSC/P through the lymphatic circulation. Indeed, the lymphatic circulation in LPS-
165 treated animals did show a significant increase in the levels of circulating HSC/P in the LN and
166 the thoracic duct compared to controls that closely mirrored the decline of HSC/P in the BM
167 **(Figures 1H-I**).

168 We next characterized the type of primitive cell populations migrating into the LN via the
169 lymphatic circulation. We first analyzed whether the content of immunophenotypically
170 identifiable BM HSC populations changed concomitantly with the progenitor population changes
171 previously described. LPS induced expansion of BM Lin^-/$c\text{-}kit^+$/$Sca\text{-}1^+$ (LSK) and
172 immunophenotypically identified long-term (LT)-HSC, short-term (ST)-HSC and multipotential
173 progenitors (MPP) populations at later time points (Z10-ZT16) (**Figures S1F and S1H-K**) with

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174 no changes in the BM HSC content by ZT7, suggesting a differential effect of LPS signaling on
175 the HSC population. Interestingly, the reduction in the BM content of progenitors was not
176 homogenous throughout the hematopoietic progenitor populations. Confirming the egress of BM
177 CFU-GM described above, the GMP population was significantly decreased by ZT7 (**Figures**
178 **S1G, L**), while the content of immunophenotypically defined common myeloid progenitors
179 (CMP) only declined by ZT10 (**Figures S1G, M**), and the megakaryocyte-erythroid progenitor
180 (MEP) content was increased (**Figures S1G, N**), resulting in no significant net changes in the
181 content of BM Lin$^-$/c-kit$^+$/Sca1$^-$ (LK) cells (**Figures S1G, O**).

182 Functional in vivo assays of LN cell suspensions obtained at ZT7 demonstrated that the
183 accumulation of progenitors in LN did not contain any significant numbers of long-term or
184 medium-term repopulating HSC. The analysis of competitive-repopulating units (CRU) in the LN
185 (**Figure S2A**) demonstrated that inflamed LN did not contain increased levels of repopulating
186 cells by ZT7 (**Figure S2B**). LN contained a transient, ST-myelopoietic progenitor population
187 without medium- or long-term multilineage repopulation ability (**Figure S2C**). Lineage analysis
188 of donor-derived circulating cells demonstrated no significant change in T-cell transfer (**Figure**
189 **S2D**), and a diminished transfer of B-cells into the lethally irradiated recipients (**Figure S2E**)
190 indicating the presence of adoptively transferred lymphoid cells and the absence of mobilization
191 of competent lymphoid progenitors to the inflamed LN. Furthermore, LN SP cells from LPS-
192 treated mice are enriched in LK cells and depleted in LSK cells (**Figure S2F**). LN SP cells
193 contain exclusively ST-repopulating progenitors with the ability to differentiate into myeloid cells
194 (data not shown) and are depleted from any significant 8-16 weeks engrafting HSC, as
195 assessed using CRU assays (**Figures S2F-G**), unlike their BM SP counterparts which are
196 enriched in LSK cells and LT-repopulating activity (**Figures S2F, H**). These results confirmed
197 that, similar to human inflammatory LN, the LN SP cells from mice treated with *E. Coli* LPS
198 accumulate in inflamed LN, contain progenitors, and are depleted of stem cell activity.
199 Altogether, these data indicate that LPS induces a selective lymphatic circulation of myeloid
200 committed progenitors, but not other types of HSC/P populations.

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201 To explore the nature of the circuit of the myeloid progenitor migration to LN, we first
202 analyzed the ability of HSC/P to seed LN in non-myeloablated mice. For this experiment, we
203 labeled C57Bl/6-BM-derived lineage negative (Lin$^-$) cells containing the HSC/P fraction with the
204 lipophilic dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), and
205 adoptively transferred them into un-manipulated lymphatic vessel reporter Lyve1-eGFP mice.
206 Lyve1-GFP knock-in mice display enhanced green fluorescent protein (eGFP) fluorescence
207 driven by the promoter/enhancer of the lymphatic vessel hyaluronan receptor 1 Lyve-1
208 identifying lymphatic endothelial cells (Pham et al., 2010). We analyzed the 17-hour homing of
209 Lin$^-$ cells to the BM and LN **(Figure 2A).** The homing of cells to the BM in mice treated with LPS
210 was reduced by ~65% compared with their vehicle-treated counterparts (**Figure 2B**). To
211 determine whether Lin$^-$/DiI$^+$ homed cells leave BM in response to LPS, we first determined the
212 existence of transcortical vessels (**Figure 2C**)(Gruneboom et al., 2019) and the presence of
213 lymphatic vasculature inside the bone by two-photon microscopy in mice either treated with PBS
214 or LPS **(Figures 2D-E and Movies S1-2).** Lyve1-GFP knock-in mice revealed that the bone of
215 LPS-treated animals contains a Lyve1+ network, which was only rarely identified in PBS-treated
216 mice (**Figure 2D**), suggesting that LPS induced inflammation may upregulate the expression of
217 the hyaluronan receptor Lyve1 and render patent a pre-existing network of Lyve1+ bone cells.
218 Interestingly, Lin$^-$/DiI$^+$ homed cells were located closer to the endosteum in response to LPS at
219 as early as 1.5 hours after administration of LPS **(Figure 2E)**. Quantification of the distance of
220 Lin$^-$/DiI$^+$ homed cells to endosteum area showed significant differences between PBS and LPS
221 treatment, indicating increased proximity to the endosteum area after LPS **(Figure 2F).** We
222 found, albeit at a very low frequency, tiny lymphatics scattered and projected inside the bone
223 (**Figure 2G and Movie S3**). On the other hand, the seeding of BM-derived Lin$^-$/DiI$^+$ cells into LN
224 increased ~3-fold which mirrored the decline in BM homing (**Figure 2H-J**). Histological analysis
225 of BM-derived Lin$^-$/DiI$^+$ cells within the LN by confocal microscopy showed that the migrated
226 HSP/C are spatially positioned in the cortex area surrounding primary follicles (**Figure 2I**),
227 consistent with localization in T-cell zone for antigen presentation. These findings strongly

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228 suggest that the rapid egress of hematopoietic progenitors from BM during inflammation may
229 indeed occur through bone lymphatics draining into LN.

230

231 **Systemic inflammation recruits dendritic cell-committed phenotypic progenitors to LN**

232 To determine the potential of the myeloid progenitors mobilized to the LN, we further
233 determined their in vitro and in vivo differentiation profile. To this end we analyzed the
234 differentiation capabilities of LN myeloid progenitors in specific-cytokine driven clonal assays in
235 methylcellulose assays. The majority of the differentially accumulated myeloid progenitors in LN
236 by 3 hours post-administration of LPS were granulocyte-macrophage progenitors (CFU-GM)
237 and in a much lesser degree unipotent granulocyte progenitors (GPs, CFU-G) with no
238 differential accumulation of unipotent macrophage progenitors (MPs, CFU-M) (**Figure 3A**).
239 Next, we investigated whether GMP were able to home and migrate to LN after in vivo
240 administration of LPS. For this purpose, we adoptively transferred sorted β-actin/eGFP
241 transgenic GMPs into congenic mice. Transgenic GMPs were allowed to home to the BM and
242 after 17 hours recipient mice were treated with a single dose of LPS or vehicle control. On day 7
243 after PBS or LPS administration, murine BM and LN were analyzed for donor-derived
244 granulocytes ($Gr1^{++}/CD11b^{+}/CD11c^{neg}$), macrophages ($Gr1^{dim}/CD11b^{+}/CD11c^{neg}$) and cDC
245 ($Gr1^{neg}/CD11b^{+}/CD11c^{+}$) by flow cytometry (**Figure 3B**). We found that LPS induced differential
246 donor-derived specific GMP differentiation towards the formation and retention of cDC in LN
247 (**Figure 3C**), but not in the BM (**Figure 3D**). The content of macrophages and granulocytes did
248 not significantly change with LPS in either LN or BM (**Figures 3C-D**) confirming the specific
249 nature of the cDC differentiation of mobilized GMP in LPS-treated mice, similar to our
250 observations in human lymphadenitis.

251 To elucidate whether the LN cDC content was dependent on migration of committed
252 cDC precursors opposed to local specification of migrated macrophages or macrophage
253 progenitors, we analyzed the migration of macrophage-dendritic precursors (MDP). BM MDP is
254 a progenitor population that can differentiate into monocytes/macrophages or directly into cDCs

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255 without intermediate macrophage specification (Fogg et al., 2006; Geissmann et al., 2003;
256 Waskow et al., 2008). MDP are characterized by high expression of the chemokine receptor
257 Cx3cr1, c-fms (CD115) and Flt3, and intermediate expression of c-Kit. Serial gating of Lineage^{-}
258 /Cx3cr1^{++}/c-Kit^{int} cells (**Figure 3E**, P2) showed an ~3-fold accumulation of CD115^{++}/FLT3^{++} cells
259 in LN and a concomitant 65% depletion in the BM, as early as 3 hours after LPS administration
260 (**Figures 3F-G and Figures S3A-B**). In the absence of significant changes in the LN content of
261 macrophages, these data demonstrate that LPS-induced systemic inflammation results in robust
262 and specific recruitment of phenotypic GMP that are BM cDC committed progenitors to the LN.

263

264 **Myeloid progenitor migration to LN is Traf6-dependent and NF-κB-independent.**

265 Immune cells and HSC/P express TLR (Beutler and Rietschel, 2003; Nagai et al., 2006;
266 Takeuchi and Akira, 2010) which act as microorganism sensors. LPS stimulation of TLR recruits
267 MyD88 and TRIF through the canonical and endosomal pathways respectively. Both adaptors
268 subsequently recruit TRAF6, which acts as the molecular hub of both signaling branches (Akira
269 et al., 2001; Kawai and Akira, 2006). To determine whether Traf6 deficiency might affect the
270 migration of HSC/P in response to LPS, we exploited an animal model in which *Traf6* is deleted
271 only in hematopoietic cells (Kobayashi et al., 2003) (**Figure 4A, Figure S3C**). LN from
272 *Mx1Cre;WT* chimeric mice after LPS administration (at ZT7, corresponding with the peak of
273 progenitor content in LN, **Figure 1H**) revealed a 3-fold increase in the frequency of CFU-GM.
274 This increase was completely abrogated by the deficiency of Traf6 in hematopoietic cells
275 (*Mx1Cre;Traf6*$^{\Delta/\Delta}$ animals, **Figure 4B**) indicating that the signals which result in CFU-GM
276 mobilization to LN are mediated by hematopoietic Traf6.

277 Although HSC/P respond directly to PAMPs such as LPS (Nagai et al., 2006; Zhao et al.,
278 2014), direct Gram-negative infection-derived LPS sensing by HSC/P does not play an essential
279 role in emergency granulopoiesis, but rather requires TLR4-dependent signals within the
280 microenvironment (Boettcher et al., 2012; Kwak et al., 2015). To further test whether the
281 hematopoietic Traf6-dependent response to LPS resulting in mobilization of GMP from the BM

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282 to the LN by ZT7 is indeed cell autonomous and not determined by the microenvironment, we
283 used the conditional Traf6-deficiency model and analyzed the *in vitro* migration of myeloid
284 progenitors towards chemoattractant gradients generated by LPS-stimulated BM or LN cells in
285 assays designed to identify the hematopoietic cell population affected by LPS (**Figures 4C, E,**
286 **G**). We found that the cell-autonomous deficiency of Traf6 resulted in a relative decrease in
287 migration of ~40% (**Figure 4D**) of BM myeloid progenitors in presence of LPS, indicating that
288 Traf6 is required for LPS-dependent cell-autonomous BM myeloid progenitor migration.
289 Interestingly, analysis of non-cell autonomous migration of BM myeloid progenitors
290 demonstrated that LN-derived cells generated more potent chemoattractant signals resulting in
291 a much larger migration of WT BM myeloid progenitors (5-fold higher, ~30%) during the same
292 period (**Figures 4E-F**), which was drastically diminished (~50% reduction) by Traf6-deficiency in
293 LN cells, but not when using control BM cells as chemoattractant source (**Figures 4E-F**). These
294 data indicate that although LPS-mobilized myeloid progenitors depend on both cell-autonomous
295 and non-cell autonomous Traf6-dependent signals, the chemoattractant gradient generated by
296 LPS on LN cells is the predominant effect responsible for Traf6-dependent myeloid progenitor
297 migration.

298 To delineate the resident LN population to cause the migration of GMPs into the LN, we
299 isolated T-cells (CD3e$^+$), B-cells (B220$^+$) and myeloid cells (CD11b$^+$) from LN of WT or Traf6$^{\Delta/\Delta}$
300 mice and layered input cell equivalents on the bottom of the chamber with LPS, as in the
301 previous experiments (**Figure 4G**). Although only ~1% of LN cells are myeloid, we observed
302 that LN CD11b$^+$ cells, but not B or T cells, from Traf6$^{\Delta/\Delta}$ mice can recapitulate the same
303 reduction of progenitor migration achieved by complete LN tissue (**Figure 4H**). To confirm that a
304 Traf6-dependent signaling in LN CD11b+ cells is responsible for myeloid progenitor mobilization
305 and eliminate the possible inflammatory effect of previous treatment with polyI:C in Mx1-Cre Tg
306 mice, we crossed *Traf6$^{flox/flox}$* mice with *LysM-Cre* transgenic mice(Clausen et al., 1999; Cross et
307 al., 1988) and analyzed the migration to LN after LPS administration in mature myeloid lineage-
308 specific Traf6 deficient *(LysM-Cre;Traf6$^{flox/flox}$*) mice. Mature myeloid lineage-specific deletion of

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309 Traf6 abrogated the migration of myeloid progenitors to LN in response to LPS (**Figure 4I**). A
310 major consequence of the deficiency of Traf6 in mature myeloid lineage-specific cells was an
311 increase in the endotoxemia dependent mortality (**Figure 4J**) indicating that Traf6 expression in
312 mature myeloid cells is required for both migration of myeloid progenitors to LN and protection
313 of the inflammatory cytokine storm responsible for LPS-induced death. Altogether, these data
314 indicate that LPS/Traf6 signaling is required for migration of myeloid progenitors through
315 predominantly long-range acting, mature myeloid lineage-dependent chemoattractant signals
316 and that LPS/Traf6 signaling in $LysM^+$ cells is protective against endotoxin-induced
317 inflammation.

318 Activation of TLRs conserves inflammatory pathways which culminate in the activation of
319 the NF-κB transcription factors(Karin and Greten, 2005). The LPS binds TLR4/MD2 complexes
320 on the cell surface, and through a series of adaptors and kinases recruits Traf6. By an E3
321 ligase-dependent mechanism, Traf6 activates the IκB kinase (IKK) complex, which initiates IκBα
322 degradation. Subsequent nuclear translocation of NF-κB transcription factors results in the
323 expression of cytokine and chemokine genes. To determine whether emergent NF-κB signaling
324 is responsible downstream of LPS/Traf6 for the LPS-induced LN migratory effect of myeloid
325 progenitors, we overexpressed a degradation-resistant mutant of IκBα (IκBα super-repressor
326 [$I\kappa B\alpha_{SR}$]), in primary murine progenitors, which were then differentiated into macrophages/cDC
327 by macrophage colony-stimulating factor (M-CSF)(O'Keeffe et al., 2010) (**Figure S3D**).
328 Analysis of LPS-driven migration *in vitro* (**Figure S3E**) demonstrated that the expression of
329 $I\kappa B\alpha_{SR}$ does not reduce the effect of LPS on the migration of myeloid progenitors towards LPS-
330 stimulated macrophages/cDC (**Figures S3F-G**), indicating that NF-κB transcription factors are
331 dispensable for myeloid progenitor migration. In contrast, inhibition of intracellular protein traffic
332 using monensin dramatically decreased myeloid precursor migration (**Figures S3F-G**),
333 suggesting that intracellular protein trafficking is necessary for the migration phenotype.
334 Collectively, these data indicate that LPS-induced myeloid progenitor migration occurs through
335 an NF-κB-independent, intracellular protein traffic-dependent pathway, and suggests that the

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336 progenitor mobilizing effect of LPS may not require transcriptional activation, depending rather
337 on the intracellular traffic of secreted proteins.

338

339 **Myeloid progenitors home into LN in a CCL19/CCR7 dependent fashion but**
340 **independently of L-selectin**

341 The secretome of myeloid cells includes multiple cytokines/chemokines with short- and
342 long-range activities on activation, proliferation, survival, differentiation, and migration of target
343 cells. Specifically, secreted chemokines stimulate migration of target cells following chemokines
344 to the areas of highest concentration. It has been described that hematopoietic progenitor
345 migration is dependent on Cxcl12 gradients (Greenbaum et al., 2013; Mendez-Ferrer et al.,
346 2010). However, by ZT7, LPS induced upregulation of Cxcl12 expression in BM, but not in LN,
347 indicating that Cxcl12 tissue concentrations per se could not explain the mechanism of
348 migration to LN (**Figure S4A**). An array of tests on secreted chemokines and cytokines and
349 demonstrated distinct secretome signatures between BM and LN tissues after LPS
350 administration (**Figures S4B-N**). Several myeloid cell cytokines and chemokines with ability to
351 recruit and differentiate macrophages and cDC were found to be upregulated in the extracellular
352 fluid of LN rather than BM as early as one hour after LPS challenge (**Figure S4C-J**). However,
353 none of these candidate cytokines/chemokines were found to consistently generate a differential
354 tissue concentration *in vivo* between LN and BM at both ZT5 and ZT7 (**Figure S4B**). Similar to
355 Cxcl12, some cytokines/chemokines with potential chemoattractant ability were also found to be
356 upregulated in BM rather than in LN or in both tissues similarly (**Figure S4K-N**). The lack of in
357 vivo tissue differential levels strongly suggested that these BM-derived cytokines or chemokines
358 were unlikely to be responsible for the attraction of BM myeloid progenitors to the LN.

359 The C-C chemokine receptor type 7 (Ccr7) ligand macrophage-inflammatory protein
360 (MIP)-3b/Ccl19 has been reported as a chemoattractant for BM and cord blood CD34$^+$ cells *in*
361 *vitro*, mainly CFU-GM (Kim et al., 1998). Analysis of Ccl19 in the extracellular fluid of the
362 femoral cavity, LN, and plasma, demonstrated that *in vivo* administration of LPS promotes a

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363 secretion of Ccl19 in LN when compared with BM and PB (**Figure 5A**). This differential
364 secretion is specific to Ccl19 since Ccl21, a highly-related chemokine, did not show the
365 formation of similar differential tissue concentrations in LN after LPS administration (**Figure**
366 **S5A**). Ccl19 is secreted by LN myeloid cells after LPS stimulation and depends on Traf6
367 expression (**Figure S5B**). Ccr7-mediated signals control the migration of immune cells to
368 secondary lymphoid organs such as LN, facilitating efficient surveillance and targeted cellular
369 response (Forster et al., 2008). Also, LPS upregulates membrane Ccr7 expression on cDC and
370 their committed progenitors (Schmid et al., 2011). We therefore hypothesized that LN-trafficking
371 of phenotypic GMP/MDP is regulated by Ccr7, and that therefore the Ccl19/Ccr7 axis might
372 explain the coexistence of cell-autonomous and non-cell autonomous mechanisms required for
373 GMP migration from the BM to LN in response to LPS. To test our hypothesis, we first analyzed
374 whether the specific deficiency of either Ccl19 or Ccr7 modified the level of progenitor migration
375 to LN. To prevent the interference of long-term deficiencies of Ccl19 and Ccr7 expression
376 described in deficient murine models (Forster et al., 1999; Mori et al., 2001), we performed
377 short-term *in vivo* neutralization of Ccl19 ligand or the Ccr7 receptor by using specific antibodies
378 or isotype controls (**Figure 5B**) and determine the content of CFU-GM in LN after LPS
379 challenge or PBS control. We administered an anti-Ccl19 and an anti-Ccr7 neutralizing antibody
380 (or their controls) twice within 15 hours before LPS administration. We found a dramatic
381 reduction (>90%) in the number of CFU-GM in the LN of LPS-, anti-Ccl19 treated animals by
382 ZT7 (**Figure 5C**). Also, we confirmed that Ccr7-expressing GMP in BM rapidly decreased in
383 response to LPS and increased in local LN (**Figures S5D-F**). The abrogation of accumulation
384 of progenitors in LN was reproduced by the administration of anti-Ccr7 (**Figure 5D**). Noteworthy,
385 the administration of anti-Ccr7 phenocopied the effect of Traf6 deficiency in LysM^{+} cells by
386 decreasing the latency to death or increasing the mortality rate of mice treated with lethal
387 (**Figure 5E**) or sublethal (**Figure 5F**) doses of LPS in the first hours after LPS administration,
388 respectively. Second, we analyzed the membrane expression of Ccr7 on BM-derived GMP,
389 CMP and MEP from *Mx1Cre;WT or Mx1Cre;Traf6*$^{\Delta/\Delta}$ mice, with or without LPS stimulation.
390 Membrane Ccr7 levels were significantly upregulated as early as 1 hour after LPS

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391 administration on GMP in LPS-treated WT mice. Such upregulation was abrogated in LPS-
392 treated Traf6-deficient GMP (**Figures S5G**). Finally, we confirmed that hematopoietic chimeric
393 Ccl19$^{-/-}$ animals did not mount a migratory response of myeloid progenitors from BM to LN in
394 response to LPS **(Figures 5G-H).**

395 Traffic of myeloid progenitors to regional LNs was recapitulated in mice receiving
396 intrafemoral adoptive transfer of GMP (**Figures 5I-J**). In these mice, in vivo L-selectin blockade
397 did not abrogate GMP migration to regional LN while sinusoidal-dependent B-lymphocyte
398 mobilization into regional LN was significantly impaired **(Figures 5J-K),** indicating that the
399 migration of BM myeloid progenitors, unlike B cells, into the regional lymphatic circulation is L-
400 selectin independent and therefore unlikely to be mediated by LN high endothelial venules
401 (HEV) (Rosen, 2004). Altogether, these data strongly indicated that Ccl19/Ccr7 chemokine
402 signaling is required for the rapid migration of myeloid progenitors to LN upon LPS
403 administration and that Ccr7 signaling is required to prevent death within the first hours after
404 LPS administration. Given the strong time association of these events, these data support a role
405 for the Ccr7-dependent early traffic of myeloid progenitors in the amelioration or delay of the
406 endotoxic shock induced by LPS.

407

408 **Ccl19 is expressed and pre-stored in cDC2 and released upon activation of IKK/Snap23.**

409 Chemokine secretion requires endosomal fusion with the membrane which can be
410 detected by exposure of the phosphatidylserine (PS)-rich inner leaflet of the endosomes to the
411 external surface of the cell membrane, providing a venue to determine what cell types were
412 responsible for the secretion of Ccl19. We found an increase in the levels of PS residues on the
413 outer membrane leaflet of WT LN cDC2 (defined as CD11b^{+}/CD11c^{+}), but not in the CD11b^{-}
414 /CD11c^{+} population which comprises cDC1 and plasmacytoid DC (**Figure 6A**). Interestingly, the
415 exposure of PS residues was abrogated in Traf6$^{\Delta/\Delta}$ LN cDC (**Figure 6A**) indicating that Traf6
416 also mediates the process of vesicle secretion upon LPS stimulation. Having demonstrated that
417 LPS/Traf6 signaling is required for chemokine traffic/secretion in LN mature myeloid-lineage

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418 cells, and NF-κB transcriptional activation is dispensable for LPS-dependent myeloid progenitor
419 migration, we hypothesized that Traf6 acts through non-NF-kB dependent IKK activity. To
420 determine whether canonical LPS/TLR downstream effectors were involved in the process of
421 myeloid progenitor migration, we analyzed the chemotaxis of BM myeloid progenitors towards a
422 gradient generated by LN cells in the presence of LPS and specific inhibitors for interleukin
423 receptor associated kinase 1/4 (Irak1/4), ubiquitin-conjugating enzyme 13 (Ubc13), and IKKβ
424 (**Figure 6B**). Increased myeloid progenitor migration was reversed by all three specific inhibitors
425 (**Figure 6B**) indicating that the integrity of canonical signaling pathway upstream of NF-κB might
426 be required to attract myeloid progenitors from the BM to the LN. The Traf6/IKK dependent
427 rapid response to LPS strongly suggests that LPS induces secretion of Ccl19 through a
428 mechanism of rapid release from pre-stored pools. The release of pre-formed cytokines in pre-
429 pooled, stored late endosomes depends on IKK activity through the phosphorylation of
430 mediators of cell membrane fusion. SNAP23 is an essential component of the high affinity
431 receptor which is part of the general membrane fusion machinery and an important regulator of
432 transport vesicle docking and fusion (Karim et al., 2013; Suzuki and Verma, 2008). Phospho-
433 SNAP23(Ser95) is significantly upregulated by LPS in LN cDC (**Figure 6C**). Each of the
434 inhibitors for Irak1/4, Ubc13 and IKK abrogated the activation of SNAP23 (**Figures 6C and**
435 **S5H**). Altogether, this set of data indicates that the activation through Traf6/Irak1/4/Ubc13
436 induced by LPS activates vesicular fusion and vesicular cargo release of pre-formed Ccl19
437 accumulated in late endosomes of LN myeloid cells. Analysis of steady-state LN myeloid cell
438 populations identified a subpopulation of cDC but not pDC or macrophages, containing most of
439 the cytoplasmic expression of Ccl19. Further analysis of the subpopulations of LN cDC2
440 demonstrated that B220$^-$/CD8$^-$ cDC that expressed the endocytic receptor DEC-205 (CD205$^+$)
441 and the mannose receptor signal regulatory protein α (SIRPα, CD172a$^+$) distinctly stored high
442 levels of cytosolic Ccl19 (**Figures 6D and S6A)** unlike other DC populations which expressed
443 low levels of Ccl19 **(Figure S6B).** These data indicate that a subpopulation of cDC2 stores
444 intracellular Ccl19 and is potentially able to self-regulate the migration of its own progenitors in
445 inflammation.

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446 **DISCUSSION**

447

448 This study describes a previously unrecognized , rapid, emergent traffic of myeloid
449 progenitor cells from the BM via lymphatic vessels directly to lymphatic tissues that by-pass the
450 peripheral blood stream. Careful analysis of serial femoral sections has not unveiled the
451 existence of a communication between lymphatic and blood vessels in BM further suggesting
452 the lack of communication between both circuits within the BM cavity and thus likely functional
453 regional independence of each circuit. Our data thus also supports the recently described
454 existence of functional lymphatic vessels in the bone. High-resolution confocal and multiphoton
455 microscopy demonstrated the existence of Lyve1+ cells in which their transgenic reporter
456 illuminated upon exposure to high-dose LPS in vivo along with tiny projections of lymphatics
457 penetrating into the bone. Probably, bone processing and cleaning before fixation and
458 decalcification may have deprived us (and other investigators) from a better identification of
459 notable, anatomically identifiable lymphatic vessels within the network of transcortical capillaries
460 (Gruneboom et al., 2019).

461 Bone is a dynamic organ in constant remodeling. Upon inflammation for example,
462 cytokines and microbial LPS are capable to initiate bone absorption by activating osteoclasts
463 (Hardy and Cooper, 2009; Nason et al., 2009). Systemic inflammation has been associated with
464 osteoclast activation and osteoblast thinning (Hardy and Cooper, 2009; Nason et al., 2009) and
465 bone lymphatic endothelial cells have been shown to arise rapidly from pre-existing regional
466 lymphatics upon osteoclast activation (Hominick et al., 2018; Monroy et al., 2020). Osteoclast
467 activation and osteoblast thinning are likely to facilitate transcortical migration of cells and fluid
468 through existing transcortical vessels.

469 Our data showed that as early as 90 minutes after LPS administration, myeloid
470 progenitors to or are in closer proximity to the lymphatic endothelium in BM while 1.5 hours later
471 there is a ~70% reduction of myeloid progenitors within the BM and a marked increase of
472 myeloid progenitors in the LN tissue. This observation along with the need of a longer period of
473 time to detect an increase in the frequency of myeloid progenitors in peripheral blood suggests

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474 that two temporally distinct waves of progenitors take place, a fast one to the lymphatic
475 circulation followed by a slower one into the blood stream.

476 In our study, by using transgenic animals, we demonstrated that the administration of a
477 single dose of LPS suffices to induce migration of GMP/MDP while no other types of progenitors
478 or stem cells migrate to lymphatics in this first wave of egression before any significant
479 contribution from or to PB and replenish short-lived cDCs in murine acute model of inflammatory
480 signaling by LPS, and that therefore they may modulate the course of infectious diseases and
481 other inflammatory conditions. This traffic is also likely to happen in homeostatic conditions as
482 previously shown (Waskow et al., 2008) while our analysis provides compelling evidence on its
483 striking activation upon inflammation/LPS administration.

484 Our data support the migration of a distinctly immature progenitor population composed
485 of GMP/MDP with ability to generate cDC in LN upon traffic from BM to LN. This traffic of
486 myeloid progenitors from BM to LN can be recapitulated in mice receiving intrafemoral adoptive
487 transfer of GMPs. These GMP/MDP tend to localize in the T-cell areas of LN. Cheong et al.
488 reported that migratory monocyte-derived cDC2 can also localize in T cell areas of the LN and
489 acquire an inflammatory phenotype DC-SIGN/CD209a^{+} (Cheong et al., 2010). No significant
490 mobilization of M-CSF responding monocyte progenitors (CFU-M) can be found as early as 3
491 hours after LPS administration. Interestingly, the interference of this traffic by either blocking the
492 chemokine secretion by mature LysM-expressing myeloid cells, or by blocking the chemokine
493 receptor Ccr7 results in increased animal death, which strongly suggests that the traffic of
494 progenitors resulting from Ccl19/Ccr7 signaling is not only destined to the differentiation into
495 cDC but also to a more immediate anti-inflammatory role as suppressors of the endotoxic shock
496 effects. Ccr7^{+} GMP/MDP, but not other myeloid or lymphoid progenitors, egress BM. Such
497 egress follows differential tissue levels of Ccl19 resulting from activation of the secretion of the
498 pre-formed chemokine by LN LysM-expressing mature myeloid cells, specifically a
499 subpopulation of cDC expressing CD205 and CD172a. This process seems to be independent
500 of Cxcl12 levels since no changes in Cxcl12 levels in LN, PB or BM were observed and this

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501 effect seems to be exclusively dependent on activation of non-canonical Traft6/IKK activity
502 without need for transcriptional activation.

503 Schmid et al (Schmid et al., 2011) demonstrated that a population of common dendritic
504 progenitors (CDP), a non-GMP derived population of progenitors can also migrate from the BM
505 to lymphoid and non-lymphoid tissues in response to TLR agonists and generate both cDCs and
506 pDCs (pDCs). The type of migration though depended on combined downregulation of Cxcr4
507 and upregulation of Ccr7, which seem to imitate the mechanism of GMP migration. Interestingly,
508 Ccl21, which is expressed by lymphatic endothelial and stromal cells but not by myeloid cells
509 (Eberlein et al., 2010), does not induce any differential gradient of secretion between LN and
510 BM or blood suggesting that Ccl21 may not be a primary mediator of the myeloid progenitor
511 migration from BM to LN upon LPS challenge, while the hematopoietic deficiency of Ccl19
512 suffices to completely abrogate the mobilization of myeloid progenitors to LN induced by LPS.

513 Our data support the existence of a steady-state LN population of cDC which co-
514 expresses the maturation antigens CD205 and CD172a and store high levels of Ccl19 in their
515 cytoplasm. An interesting possibility is, as our data indicate, that upon bacterial antigen
516 challenge, differentiated myeloid cells of LN like cDCs, which respond to LPS by secreting
517 chemokine-containing pre-formed exosomes, accelerate a positive feedback activation loop to
518 recruit cDC progenitors to the lymphatic tissue. cDC in LNs might thus act as sensors for the
519 presence of bacterial products and release Ccl19 within minutes. Individual DCs have a short
520 half-life (1.5-2.9 days)(Kamath et al., 2002) and DC precursors have a short half-life in blood
521 circulation (Breton et al., 2015). We posit that the migration of DC progenitors through the lymph
522 tissues provides a direct afferent communication between the LN mature cDC population
523 responsible for the secretion of the chemokine Ccl19 and at the same time, allows the emergent
524 migration of functional cDC progenitors from the BM to replenish the repertoire of lymphatic
525 antigen presenting cells.

526 Finally, our data also support the key role of an alternative inflammatory signaling
527 pathway elicited by coordinated by Traf6/IKK responsible for SNAP23 phosphorylation and

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528 Ccl19 secretion, before resulting in transcriptional regulation by their downstream effector NF-
529 kB. Traf6 has been identified as a signaling molecule that can regulate splicing of downstream
530 targets without affecting NF-kB in hematopoietic stem cells and progenitors (Fang et al., 2017).
531 Our data further identifies non-canonical signaling pathways elicited by Traf6 in differentiated
532 myeloid cells to modulate the inflammatory response affecting the circulatory dynamics of
533 hematopoietic progenitors. Traf6 dependent, cytosolic mediated inflammatory response allows a
534 fast response before inflammatory transcriptional and post-transcriptional signatures are
535 mounted.

536 In summary, we describe, upon inflammation, a rapid trafficking of cDC biased myeloid
537 progenitors from the BM, via lymphatic vessels, directly to lymphatic tissues that by-passes the
538 blood stream. This GMP/MDP migration represents a mechanism for fast replenishment of
539 cDCs in lymphatic tissues. Rapid replenishment of cDC-biased progenitors in LN may represent
540 a major homeostatic function of this novel lymphatic circuit and may explain why the circulation
541 of myeloid progenitors is conserved during the postnatal life.
542
543

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544 **MATERIAL AND METHODS**

545

546 **Mice**

547 CD57Bl/6 (CD45.2$^+$) mice were used between 8-10 weeks of age and were purchased
548 from Jackson Laboratory, Bar Harbor, ME; Harlan Laboratories, Frederick, MD. *Mx1Cre$^+$;Traf6*-
549 floxed mice were generated by breeding Mx1-Cre transgenic mice(Mikkola et al., 2003) with
550 biallelic TRAF6 floxed mice (kindly provided by Dr. Yongwon Choi, University of
551 Pennsylvania)(Kobayashi et al., 2003). Full chimeric mice were generated by non-competitive
552 transplantation of *Mx1Cre$^{Tg/+}$;*WT or *Mx1Cre;Traf6$^{flox/flox}$* whole BM cells into lethally irradiated
553 B6.SJL$^{Ptprca\ Pepcb/BoyJ}$ (CD45.1$^+$) mice obtained from the CCHMC Animal Core. Traf6 was deleted
554 upon induced expression of Cre recombinase after 3-6 intraperitoneal injections (10 mg/Kg/b.w.
555 Poly(I:C); Amersham Pharmacia Biotech, Piscataway, NJ, USA) every other day at 6 weeks
556 after transplantation. LysM-Cre;Traf6 floxed mice were generated by non-competitive
557 transplantation of *LysMCre$^{Tg/+}$*;WT or *LysMCre$^{Tg/+}$; Traf6$^{flox/flox}$* whole BM cells into lethally
558 irradiated B6.SJL$^{Ptprca\ Pepcb/BoyJ}$ (CD45.1$^+$) mice obtained from the Division of Experimental
559 Hematology/Cancer Biology of Cincinnati Children's Hospital Research Foundation (CCHRF).

560 Lyve1-eGFP (Pham et al., 2010) and β-actin-eGFP (Okabe et al., 1997) and Cx3cr1-
561 GFP(Jung et al., 2000) transgenic mice were purchased from Jackson Laboratories. C57BL/6
562 mice for circadian cycle analysis of CFU-C were maintained on a 14-hour light / 10-hour
563 darkness lighting schedule. This study was performed in strict accordance with the
564 recommendations in the Guide for the Care and Use of Laboratory Animals of the National
565 Institutes of Health. All of the animals were handled according to approved institutional animal
566 care and use committee (IACUC) protocol #2019-0041 of Cincinnati Children's Hospital.

567

568 **Human specimens**

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569 Lymphadenopathies from patients were obtained through Institutional Review Board-
570 approved protocols of the Hospital Reina Sofia (Cordoba, Spain), donor informed consent and
571 legal tutor approval in the case of patients younger than 18 years old. Diagnostic
572 lymphadenopathy biopsies from sixty-four consecutive patients from 2009 until 2013 were
573 analyzed in this study. The median age of patients was 34 years old (range: 3-89). Diagnosis
574 and histological classification of the type of lymphadenopathy and tumors were based on
575 previously published criteria (Campo et al., 2011; Weiss and O'Malley, 2013). Anatomical
576 location of lymphadenopathies is described in Table 1. Specimens were blindly analyzed
577 through adjudication of unique identifiers.

578

579 **LPS injection and samples collection**

580 Mice received a single intraperitoneal injection of 30 mg per Kilo of E. coli LPS (Sigma-
581 Aldrich, St Louis, MO) or PBS as vehicle control and were executed always at ZT4 or 4 hours
582 after the initiation of light into the animal room. At different time points after PBS or LPS
583 administration, BM cells from femurs, tibias and pelvis were harvested by crushing in PBS
584 containing 2% of FBS and erythrocytes were lysed using a hypotonic buffer from BD
585 Biosciences. Blood was collected by retro-orbital bleeding or cardiac injection. Liver, kidney and
586 thoracic duct cells were harvested by enzymatic digestion solution with collagenase II (1 mg/mL,
587 ThermoFisher Gibco, Waltham, MA) and dispase (5 mg/mL, Gibco, Life Technologies) in a
588 shaking water bath at 37^{0}C for 1h. Spleen cells were isolated by scraping with slides in sterile
589 PBS following red blood cell (RBC) lysis (Pharm Lyse^{TM}; BD Bioscience, San Jose, CA).
590 Extracted LN were derived from the cervical and axillary chains exclusively.

591

592

593 **Myeloid progenitor counts assay *in vitro***

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594 Cells from BM, lymph nodes, thoracic duct, blood, spleen, liver and kidneys were
595 depleted of RBC by 2 minute-incubation in Pharm Lyse™ (BD Biosciences, San Jose, CA),
596 washed, counted and plated in semisolid methylcellulose media (Methocult 3434; StemCell
597 Technology, Vancouver, Canada) and cultured in an incubator (37ºC, 5% CO_2/>95% humidity)
598 and the number of CFU-C was scored on day 7 or 8 of culture using an inverted microscope. To
599 examine the type of myeloid progenitors migrating into LN, we used base methylcellulose
600 medium (Methocult 3134; StemCell Technology, Vancouver, Canada) supplemented with 30%
601 FCS, 1% protease-free, deionized BSA (Roche), 100 mM b-mercaptoethanol, 100 IU/mL
602 penicillin, 0.1 mg/mL streptomycin andany of the following: for CFU-GM, rm-GM-CSF
603 (100ng/mL, PeproTech Rocky hill NJ) for specific analysis of CFU-GM content, rm-M-CSF
604 (100ng/mL, PeproTech Rocky hill NJ) for specific analysis of CFU-M content or rh-G-CSF
605 (100ng/mL, Neupogen) for specific analysis of CFU-G content.

606

607 **Long-term competitive repopulation assay**

608 To analyze the long-term reconstitution capacity of HSC/Ps mobilized into LNs after LPS
609 administration at different time of periods, 4-5x10^6 of erythrocyte-depleted CD45.2$^+$ LN
610 suspension cells were prepared in sterile conditions and transplanted together with 2.5x10^5
611 CD45.1$^+$ BM competitor cells into lethally irradiated CD45.1$^+$ recipient mice. In some
612 experiments, 10^4 LN SP cells or 10^3 BM SP cells from CD45.2$^+$ mice were competitively
613 transplanted into CD45.1$^+$ recipient mice. Competitive repopulating units (CRU) analysis was
614 performed by flow cytometry analysis (BD Biosciences) at different time points post-
615 transplantation(Harrison, 1980).

616

617 **Flow cytometry analysis and cell sorting.**

618 For immunophenotype analysis of HSC/P populations by fluorescence-activated cell
619 sorter (FACS), erythrocyte-depleted BM cells were stained first for lineage markers with biotin-

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620 labeled mouse lineage panel (BD Biosciences, Pharmingen) containing anti-CD3e ($CD3_{\varepsilon}$ chain),
621 anti-TER-119/Erythroid cells (Ly-76), anti-Gr1 (Ly6G and Ly-6C), anti-CD45R (B220), anti-
622 CD11b (integrin α chain, Mac1α) followed of allophycocyanin and cyanine dye Cy7-(APC-Cy7)
623 conjugated streptavidin, allophycocyanin (APC)-conjugated anti-c-Kit (clon 2B8), R-
624 phycoerythrin and cyanine dye Cy7 (PECy7)-conjugated anti-Sca1 (clone D7), eFluor 450-
625 conjugated anti-CD34 (clone RAM34) (affymetrix eBioscience, San Diego CA), PerCP and
626 cyanine dye Cy5.5 (PerCP Cy5.5)-conjugated anti-Fcγ-RII/III (clone 2.4G2) (BD Biosciences).
627 FACS sequential discrimination on a Lineage negative gated population was used to identified
628 LK myeloid progenitor subpopulations: Lin^{-},$c\text{-}Kit^{+}Sca1^{-}CD34^{+}$ $Fc\gamma RII/III^{+}$ (granulocyte-
629 macrophage progenitors, GMP); Lin^{-},$c\text{-}Kit^{+}Sca1^{-}CD34^{+}Fc\gamma RII/III^{lo}$ (common myeloid progenitors,
630 CMP); Lin^{-} $cKit^{+}Sca1^{-}$ $CD34^{+}$ $Fc\gamma RII/III^{-}$. LSK (Lin-Sca1+c-Kit-) subpopulations were
631 distinguished as Lin^{-},$c\text{-}Kit^{-}Sca1^{+}$ $CD34^{-}Flt3^{-}$ for LT-HSC Lin^{-},$c\text{-}Kit^{-}Sca1^{+}CD34^{+}Flt3^{-}$ for ST-HSC
632 and Lin^{-},$c\text{-}Kit^{-}Sca1^{+}CD34^{+}Flt3^{+}$ for multipotent progenitors (MPP). For chimera analysis in
633 repopulated animals, 20 µL of red cell-depleted blood was stained with fluorescein
634 isothiocyanate (FITC)-conjugated anti-CD45.1 (clone A20), R-phycoerythrin and cyanine dye
635 Cy7 (PECy7)-conjugated anti-CD45.2 (clone 104), allophycocyanin (APC)-conjugated anti-
636 CD11b (clone M1/70), allophycocyanin and cyanine dye Cy7-(APC-Cy7)-conjugated anti-B220
637 (clone RA3-6B2), R-phycoerythrin (PE)-conjugated anti-CD3e (clone 145-2C11) and BD
638 Horizon™ V450-conjugated anti-Gr1 (clone RB6-8C5), PerCPefluor®710 anti-CD115 (clone
639 AFS98) and R-phycoerythrin conjugated anti-CD135 or anti-Flt3 (clone A2F0.1). All monoclonal
640 antibodies were purchased from BD, Pharmingen. Cell acquisition was performed by flow
641 cytometry (LSRFortessa I, BD Biosciences) equipped with FACSDIVA™ software (BD,
642 Biosciences) for multiparameter analysis of the data. FACS sorting strategies were: $CD45.1^{-}$
643 $CD45.2^{+}CD3_{\varepsilon}^{+}B220^{-}CD11b^{-}$ for LN-T cells, $CD45.1^{-}$ $CD45.2^{+}CD3_{\varepsilon}^{-}B220^{+}/CD11b^{-}$ for LN-B cells
644 and $CD45.1^{-}$ $CD45.2^{+}CD3_{\varepsilon}^{-}/B220^{-}/CD11b^{+}$ for LN-myeloid cells in a FACSAria II cell sorter (BD
645 Biosciences). For BM and LN -side population (SP) cells analysis and sorting, $2x10^{6}$ cells/mL
646 were stained with Hoescht 3342 (5 µg/mL) as described previously[15]. For intracellular analysis
647 of the phosphorylated state of SNAP23 protein, surface antigen-labeled cells were fixed with

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648 Cytofix buffer (BD Biosciences) for 20 min and then permeabilized using Cytofix/Cytoperm
649 buffer (BD Bioscience) for 20 minutes. After washing, cells were stained intracellularly using a
650 rabbit non-conjugated monoclonal anti-phospho-SNAP23(Ser^{95})(Karim et al., 2013) for 40
651 minutes in Perm/Wash Buffer 1x (BD Bioscience) with 0.5% of rabbit serum. Cells were then
652 incubated with a secondary Alexa Fluor® 488-conjugated (ThermoFisher-Invitrogen) goat anti-
653 rabbit antibody for 40 minutes in Perm/Wash Buffer 1x with 0.5% of goat serum. All incubations
654 after cell stimulation were done on ice and in darkness. Single cell analysis was performed by
655 flow cytometry and the histogram-overlay graphed (LSRFortessa I; FlowJo xV0.7 software; BD
656 Biosciences). The mean fluorescence intensity (MFI) ratio was calculated as the ratio of the
657 fluorescence intensities of LPS-stimulated to PBS-stimulated (control).

658

659 **Annexin-V binding**

660 LN suspension cells from *Mx1Cre;WT and Mx1Cre;Traf6*$^{\Delta/\Delta}$ mice were obtained to
661 performed LPS stimulation. 10^6 cells were plated into 24-well-plates and treated with PBS or
662 LPS for 1 hour. After 15 minutes labelling with surface antibodies against CD45.2 (Clone 104),
663 CD11c (clone HL3), CD11b (Clone M1/70) and B220 (clone RA3-6B2) the samples were
664 washed twice and then stained for annexin-V for 15 minutes and in darkness. All antibodies we
665 purchased from BD Biosciences - Pharmingen. Single cell analysis was performed using flow
666 cytometry and the histogram-overlay graphed (LSRFortessa I; FlowJo xV0.7 software; BD
667 Biosciences). The MFI ratio between LPS MFI and PBS MFI was calculated.

668

669

670 **Homing and seeding assays**

671 For homing assays, $2x10^6$ of Lin^- cells, previously depleted by immunomagnetic selection
672 (Lineage Cell Depletion kit, Miltenyi Biotec, Auburn CA), were stained by 1,1'-dioctadecyl-
673 3,3,3',3', tetramethylindocarbocyanine perchlorate;CILC18(3) (5 μM/mL DiI, ThermoFisher-

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674 Invitrogen) and adoptively transferred intravenously into non-myeloablated Lyve1-GFP+ mice.
675 Seventeen hours later, one single LPS dose (3mg/mL) or vehicle control (PBS) was
676 administered intraperitoneally. 3 and 6 hours later (ZT7 or ZT10) mice were euthanized with
677 pentobarbital (60-80 mg/Kg) and the whole body was fixed using a freshly made solution of PBS
678 plus 2% of paraformaldehyde (PFA) and 0.05% of glutaraldehyde infused by perfusion pump
679 through left ventricle of the animal. 15-20 minutes later the BM cells and LN organs were
680 harvested and the percentage of labeled Lin^- cells which had homed into BM was determined by
681 FACS analysis. The homing calculation was done as previously reported(Boggs, 1984).

682

683 **Microscopy**

684 Fixed LN organs were permeabilized for 15 minutes with PBS containing 0.2% of Triton X-100.
685 To detect GFP on the lymphatic endothelium, LN were incubated overnight with a primary
686 antibody anti-GFP+ (ThermoFisher-Invitrogen). LN were scanned by confocal microscopy
687 (Nikon A1R GaAsP) through multidimensional acquisition to construct 3D representations of the
688 whole organ at 10x magnification. The merged images of GFP/DiI or DAPI/DiI are presented
689 and the total cell number of labeled Lin^- cells was counted manually. Finally, harvested femurs
690 were decalcified for 14 days with 10% of EDTA (Sigma-Aldrich St Louis, MO) in PBS and
691 embedded in paraffin. Longitudinal section of bone were cut 4-μm thickness and then were des-
692 paraffinized and broke the protein cross-link before stain by antigen retrieval treatment with
693 citrate buffer pH 6 (Cancelas et al., 2005). Then bone sections were permeabilized with 0.2% of
694 Triton X-100 for 15 min and blocked with 5% of BSA for 1h. Slides were stained with primary
695 antibodies anti-GFP (chicken polyclonal, Abcam Inc. Cambridge MA) and rat anti-mouse
696 panendothelial cell antigen (clone, MECA-32, BD Pharmingen) at 4°C overnight. Then we
697 stained with secondary antibodies; goat anti-rat Alexa Fluor-488 and goat anti-chicken Alexa
698 Fluor-568, all from Invitrogen at 1:1000v/v concentration for 1h at room temperature. Blood and

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699 lymphatic vessels were scanned by confocal microscopy (Nikon A1R GaAsP) through
700 multidimensional acquisition to construct 3D representation.

701 To further characterized lymphatic system in bone tissue and BM cavity and to image the close
702 proximity of homed Lin$^-$/DiI$^+$ to lymphatic vessels into Lyve1-eGFP$^+$ mice, we utilized multi-
703 photon intravital microscopy (IVM) as previously described (Gonzalez-Nieto et al., 2012; Kohler
704 et al., 2009) . After LPS/PBS injections long bones were harvested and muscle were carefully
705 cleaned. Further, bone tissues were cautiously trimmed with an electric drill (Dremel) to get
706 better excess of the BM cavity for imaging by leaving a very thin (~30-40 μm) layer of bone
707 tissue. Bones were mounted in 2% low melting agarose to minimize movements during imaging
708 and covered with PBS. Multi-photon microscopy on the long bones (femur and tibia) was
709 subsequently performed using a Nikon A1R Multiphoton Upright Confocal Microscope equipped
710 with Coherent Chameleon II TiSapphire IR laser, tunable from 700 to 1000 nm and signal was
711 detected by low-noise Hamamatsu photomultiplier (PMT) tubes. Bone tissue was identified as
712 second-harmonic (SHG) signal (PMT). Bones were images in PBS using a 25X Apo 1.1 NA
713 LWD water Immersion objective and NIS image software. For Initial standardization, bones were
714 scanned at wavelength of 800, 850, 900 -nm detecting GFP (530 nm) and Dii red (580 nm). For
715 imaging, a 500 × 500-μm area was scanned in ~35 steps of 4 μm down to 120-150 μm depth
716 using an illumination wavelength of 800 nm detecting SHG signal (480 nm), green (530 nm) and
717 red (580 nm) fluorescence. Control C57BL/6 mice were used as a negative control for Lyve-1
718 GFP mice to detect specific signal for GFP-lymphatic system in bone tissue and bone marrow
719 cavity. Lymphatic vessels were well detected in the bone tissue using Lyve-1 GFP mice with DiI
720 labeled Lin$^-$ cells in the BM cavity. For quantification of proximity of Lin$^-$/DiI$^+$ with Lymphatic
721 system, Imaris software was used to measure distance between DiI labeled cells and GFP
722 positive lymphatic vessels using 3D images.

723

724 **Analysis of L-selectin dependence of femoral GMP/MDP migration to regional LN**

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725 C57Bl6 mice received single intraperitoneal injections of MEL14 (CD62L) antibody (Biox-cell)
726 200ug. Control mice received same amount of Rat IgG2a. Post 3 days of MEL14 antibody
727 treatment, LDBM cells from ß-actin eGFP mice were injected intrafemorally. LPS (30mg/kg,
728 BW) was injected post 1hr. of interfemoral injections. Mice were sacrificed at 3 hours post-
729 administration and ipsilateral and contralateral regional and distant LNs (inguinal, popliteal,
730 axillary and cervical) were isolated for analysis. LN cells were stained GMP markers and anti-
731 CD19-PECy7 (Cat#552854, BD Biosciences) and analyzed by flow cytometry and quantified the
732 GFP^+ GMP and B lymphocyte populations migrating to LN.

733

734 **Femoral GFP^+ progenitor migration in WT and $Ccl19^{-/-}$ hematopoietic chimeras:**

735 Hematopoietic chimeras of WT and $Ccl19^{-/-}$ (Link et al., 2007) BM cells were generated by
736 transplantation into CD45.1+ mice, similarly to Mx1Cre;$Traf6^{f/f}$ hematopoietic chimeras. Mice
737 were followed for 8 weeks and found to have >95% chimera of CD45.2+ cells in peripheral
738 blood. After 8 weeks, femoral LDBM cells from donor congenic -actin transgenic, CD45.2+ mice
739 were injected ($5x10^5$ per mouse) intrafemorally to both Wt and Ccl19 hematopoietic chimeric
740 mice. PBS or LPS (30mg/Kg. b.w.) were injected at 1 h post-intrafemoral injections and
741 sacrificed at 3 h post-administration of LPS. At that time, ipsilateral LN from inguinal and
742 popliteal regions were isolated. Suspension of LN cells were counted and stained with specific
743 antibodies for GMP and MDP characterization, and the frequency of different GFP^+ GMP and
744 MDP populations was analyzed by flow cytometry as mentioned above.

745

746 **Chemotaxis/Migration assays**

747 For non-cell autonomous effect analysis, $5x10^5$ of BM or LN nucleated cells from
748 *$Mx1Cre^+$;WT or $Mx1Cre^+$;$TRAF6^{\Delta/\Delta}$* $CD45.2^+$ were layered on bottom wells of 24-well transwell
749 plate (Corning Inc., Lowell, MA) together with 100 ng/mL of LPS, and $1x10^5$ WT $CD45.1^+$ LDBM
750 cells were layered on upper chamber at 37°C, 5% CO_2. For cell autonomous effect analysis,

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751 5x10^5 of BM or LN nucleated cells from WT CD45.1$^+$ mice were layered in the lower chamber
752 with 100 ng/mL of LPS, and 1x10^5 *Mx1Cre$^{Tg/+}$*;WT or *Mx1Cre$^{Tg/+}$; Traf6$^{\Delta/\Delta}$* CD45.2$^+$ LDBM cells
753 were layered in the upper chamber at 37°C, 5% CO_2. After 4 hours, cells were resuspended and
754 those adhered to the bottom layer were collected using an enzyme free cell dissociation buffer
755 (Cell Dissociation Buffer, enzyme free, PBS, ThermoFisher-Gibco). Progenitor responses
756 toward migratory gradient were analyzed by flow cytometry analysis of LK cells. The percentage
757 of migration were calculated by dividing the number of LK in the outputs by the number of LK in
758 the inputs and multiplied by 100. PBS was included as negative control. All assays were
759 performed in triplicate.

760

761 **NFκB activity repression in myeloid cells**

762 To analyze the NFκB-dependent or independent mechanism of myeloid progenitor
763 migration, BM Lin$^-$ were transduced with pMSCVpuro-eGFP bicistronic retroviral vector
764 encoding the full length of IκBα mutant (super-repressor) in the presence of the recombinant
765 fragment of fibronectin, CH296 (Takara Bio Inc, Madison, WI) for 16 hours at 37°C. 24 hours
766 later GFP$^+$ cells were sorted and macrophages were generated(Chang et al., 2014). To
767 characterize the expanded population, R-phycoerythrin (PE)-conjugated anti-CD169 (clone
768 3D6.112), PerCP-efluor® 710-conjugated anti CD115 (clone AF598) (affymetrix eBioscience),
769 allophycocyanin and cyanine dye Cy7-(APC-Cy7) conjugated anti-CD11b, efluor® 450-
770 conjugated anti-F4/80 (clone BM8) (affymetrix eBioscience), and Alexa Fluor® 647-conjugated
771 anti-CD68 (clone FA-11)(BD Biosciences) were used for FACS analysis. 50x10^3 differentiated
772 and transduced macrophages with empty or IκBα super-repressor were layered on bottom wells
773 of 24-well transwell plate in presence of 100 ng/mL of LPS and 1x10^5 WT LDBM cells were
774 layered on upper chamber at 37°C, 5% CO_2. Four hours later migrated LK cells were
775 determined by flow cytometry as described above. All assays were performed in triplicate.

776

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777 **Secretome and individual cytokine/chemokine analyses**

778 BM, plasma and LN cells were isolated in PBS containing a protease inhibitor cocktail
779 (Roche Diagnostics, Chicago IL) and Ccl19/Ccl21 levels were determined by indirect sandwich
780 of enzyme-linked immunosorbent assay (ELISA) following manufacturer's instructions (**R&D**
781 **Systems, Minneapolis, MN).** Multi-analytic profiling beads using Milliplex® Multiplex mouse
782 cytokine/chemokine panel I kit (EMD Millipore, Billerica, MA) according to manufacturer's
783 instructions were used to analyze chemokines and cytokines profile into BM and LN tissues at
784 different time periods after LPS or PBS administration into WT mice.

785

786 **In vivo administration of anti-Ccr7 and anti-Ccl19**

787 Monoclonal rat IgG2a antibody specific for Ccr7 (Clone 4B12) or polyclonal goat IgG
788 antibody for Ccl19 (AF880) and and control rat IgG2a or control goat purified IgG, were obtained
789 from R&D Systems. Fifty µg of antibodies were injected twice into C56BL/6 mice within 15 hours
790 (first dose i.v. and the second dose i.p.)

791

792 **Small molecule inhibitors**

793 The Irak1/4 Inhibitor I, ubiquitin-conjugating enzyme E2 N (UBE2N) inhibitor, Ubc13
794 inhibitor (Rhyasen et al., 2013) and IκB Kinase inhibitor (PS-1145 dihydrochloride) were
795 purchased from Sigma-Aldrich. LN cells from C57BL6 mice were treated with 10µM of IRAK-Inh,
796 0.2 µM of Ubc13-Inh and 10µM of IKK-Inh for 45 minutes and compared with the vehicle
797 dimethylsulphoxide (DMSO) at 0.1% in PBS. Monensin (eBioscience) was used at 2 µM.

798

799 **Statistical analysis**

800 Quantitative data is given as mean ± standard error of the mean (SEM) or standard deviation
801 (SD). Statistical comparisons were determined using an unpaired Student t-test, non-parametric

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802 Mann-Whitney test, one-way or two-way Anova with Bonferroni corrections. A value of $p < 0.05$
803 was considered to be statistically significant.
804

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1028 **FIGURE LEGENDS**

1029 **Figure 1. Inflammation induces early mobilization of HSC/Ps to lymph organs in humans**
1030 **and mice.**

1031 (A) Content of SP cells in human LN by flow cytometry. LN biopsies had been blindly
1032 identified histologically as lymphadenitis, subcategorized in follicular (FL, black circles n=21),
1033 granulomatous (GL, green circles N=7) and viral (VL, pink circles n=5) and lymphomas,
1034 subcategorized in Hodgkin's lymphoma (HL, blue circles n=12) or non-Hodgkin's lymphoma
1035 (NHL, orange circles n=19). (B) Strategy for LPS administration and collection of tissues (blood,
1036 kidneys, liver, spleen, BM, LN and TD) at specific times. LPS or vehicle control PBS was
1037 administered at the early rest phase into C57Bl/6 ($CD45.2^+$) mice and tissue specimens were
1038 collected before (ZT4), 1h (ZT5), 3 h (ZT7), 6 h (ZT10) or 12h (ZT16) later. (C-I). Myeloid
1039 colony-forming-cell unit (CFU-C) content in the collected tissues at different circadian cycle
1040 times. CFU-C contained in blood (C), kidneys (D) liver (E), spleen (F), BM (G), LN (H) and TD
1041 (I) in response to PBS (black circles) or LPS (blue squares) at different circadian cycle times
1042 (n=3-4 mice per time point and treatment). Results are shown as mean ± SEM. *P< 0.05,
1043 **P<0.01.

1044

1045 **Figure 2. Draining of BM-derived lineage negative cells into lymphaticstem.**

1046 (A) Schema for adoptive transfer of BM-derived Lineage negative cells (Lin^-) labeled with
1047 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) dye into lymphatic
1048 endothelium reporter Lyve1-$eGFP^+$ mice. By 16 hours after cell transplantation, LPS or PBS
1049 were administered into the Lyve1-$eGFP^+$ mice at ZT4 (0h). BM and LN cells were analyzed at
1050 ZT5.5 (1.5h), ZT7 (3h) and ZT10 (6h) for labeled Lin^- cells (Lin^-/DiI^+). (B) Frequency of Lin^-/DiI^+
1051 homed to BM (solid bars) (mosaic bars) after PBS (black solid bar) or LPS (blue solid bar)
1052 administration at ZT7 (3h) and ZT10 (6h). N=3 mice per time point and group in two
1053 independent experiments. Graph represent mean ± SEM. *P<0.05, **P<0.01.

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1054 (C) Representative 3D reconstruction images of the whole bone by two-photon microscopy
1055 showing lymphatic vessels from Lyve1 surface marker (red), nuclei with DAPI (Blue) and cortical
1056 bone with second harmonic signal (SHG, light blue). The scale bars, 100 μm.
1057 (D, i-iv) Intravital two-photon microscopy imaging (IVM) of long bones from Lyve1-eGFP mice
1058 showing lymphatic vessels (displayed in green) near to the surface of the bone (blue, detected
1059 by SHG signal) and homed Lin^-/DiI^+ cells (displayed in red). (i,iii) IVM of PBS specimen. (ii,iv)
1060 IVM of LPS specimen. (E,F) Analysis and quantification of the distance of homed Lin^-/DiI^+ (red)
1061 cells to Lyve1-EGFP+ (green) cells after PBS/LPS administration at ZT5.5 (1.5h) analyzed by
1062 Imaris 7.7.2 software. (G) Two-photon microscopy examples of images of longitudinal femoral
1063 sections stained with anti-Lyve1 antibody and DAPI, and analyzed for specific fluorescence
1064 signal and SHG for cortical bone. (H-I) Representative of 3D reconstitution images of PBS- and
1065 LPS-treated LN tissues (H) and cross-sections of LPS-treated LN (I) analyzed by confocal
1066 microscopy showing the location of mobilized Lin^-/DiI^+ cells (red; nucleus stained by DAPI in
1067 blue) in relation with Lyve-1+ cells (green; nucleus stained by DAPI in blue). The Z-stack
1068 dimensions of upper panels were: X=1266.95μm, Y=1266.95μm and Z=344μm. Calibrate:
1069 XY=2.47μm and Z=4μm. Resolution: 512 x 512 x 86. The Z-stack dimensions of lower panels
1070 were: X=1259.36μm, Y=1259.36μm and Z=132μm. Calibrate: XY=2.46μm and Z=4μm.
1071 Resolution of images were: 512 x 512 x 86. (J) Absolute count of mobilized Lin^-/DiI^+ cells
1072 counted within LN at ZT7 (3h, solid bars) and ZT10 (6h, mosaic bars) after PBS/LPS
1073 administration. N=4-14 lymph nodes analyzed per time point and graph represent mean ± SEM.
1074 *P< 0.05, **P<0.01.

1075

1076 **Figure 3. LN-mobilized GMP preferentially differentiate into dendritic cells.**

1077 (A) Comparative quantification of the content of bipotent and unipotent myeloid progenitors in
1078 LN at ZT7 (3h) after PBS/LPS administration at ZT4 (0h). (B) Schema of isolation and transfer
1079 of BM-derived GMP from β-Actin-GFP reporter mice ($CD45.2^+$) into C57Bl/6 ($CD45.2^+$) mice
1080 ($2x10^5$ GFP^+-GMP cells/mouse, n=3 mice per group). After BM homing (17h), mice were treated

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1081 with single and low dose of LPS (5mg/Kg) at ZT4 (day1) and 7 days later BM and LN tissues
1082 were analyzed for GFP expression in myeloid populations by flow cytometry. (C-D) Graphs
1083 represent the percentage of GFP^{+}-GMP differentiated to granulocytes (solid bars,
1084 $Gr1^{++}CD11b^{+}CD11c^{-}$), macrophages (left mosaic bars, $Gr1^{dim}CD11b^{+}CD11c^{neg}$) and cDC (right
1085 mosaic bars, $Gr1^{-}CD11b^{+}$ $CD11c^{+}$) 7 days post-transferring after PBS/LPS administration by
1086 flow cytometry into LN (C) and BM (D). Values represent as mean ± SEM. **P<0.01. (E) FACS
1087 strategy for MDP content in BM and LN tissues from $Cx3cr1^{gfp+}$ reporter mice (4-5 mice per
1088 group). Phenotypically, MDP are defined as lineage-negative with high expression of the
1089 chemokine receptor Cx3cr1, c-fms (CD115) and Flt3 (P2), and intermediate expression of c-Kit.
1090 (F-G) Graphs show absolute numbers of MDP present in BM (F) and LN (G) 3 hour later (ZT7
1091 [3h]) after PBS (black circles) or LPS (blue squares) administration. Values represent mean ±
1092 SEM *P<0.05, **P<0.01.

1093

1094 **Figure 4. Traf6 is key regulator for migration of BM-derived myeloid progenitors to lymph**
1095 **nodes in a non-cell autonomous manner.**

1096 (A) Schema of full chimeric mice made by non-competitive transplantation of $CD45.2^{+}$
1097 *Mx1Cre;WT* and *Mx1Cre;Traf6*$^{flox/flox}$ BM cells into lethally irradiated $CD45.1^{+}$ $B6.SJL^{Ptprca}$
1098 $^{Pep3b/BoyJ}$. 6 weeks later Traf6 gene were deleted by intraperitoneal injection of poly(I:C). 1 week
1099 later we performed PBS/LPS injection early in the rest phase (ZT4 [0h]) and LN-contained
1100 myeloid progenitors at ZT7 (3h) was scored by CFU assay. (B) Absolute number of CFU-GM
1101 presents in LN from *Mx1Cre;WT (solid bars)* and *Mx1Cre;Traf6*$^{\Delta/\Delta}$ (orange bars) full chimeric
1102 mice (n= 6-7 mice per group) after PBS (black and orange solid bars) or LPS (blue and mosaic
1103 bars) administration. Values are shown as mean ± SEM of two independent experiments with a
1104 minimum of 3 mice per group *P< 0.05, **P<0.01. (C-H) *In vitro* transwell migration assay for
1105 BM-derived LK cells. (C) Experimental design for migration of WT or $Traf6^{\Delta/\Delta}$ low-density
1106 (LD)BM cells ($CD45.2^{+}$) toward a WT microenvironment generated by BM ($CD45.1^{+}$) in the
1107 presence of LPS for 4 hours. (D) Graph represents the percentage migrated LK from WT (blue

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1108 solid bar) or Traf6$^{\Delta/\Delta}$ (orange mosaic bar) low-density BM (LDBM) cells to the bottom as
1109 depicted in C. (E). Experimental design for migration of WT LDBM cells (CD45.1^{+}) toward a
1110 gradient generated by WT/Traf6$^{\Delta/\Delta}$ (CD45.2^{+}) BM or LN cells in the presence of LPS for 4 hours.
1111 (F) Graph represents the percentage of LDBM LK migrated to the BM bottom (solid bars) or LN
1112 bottom (mosaic bars) as schemed in E. (G) Experimental design for WT LDBM cells (CD45.1^{+})
1113 migration toward gradient generated by WT/Traf6$^{\Delta/\Delta}$ LN-derived T cells (CD45.2^{+}/CD3e^{+}/CD11b^{-}
1114 /B220^{-}) or B cells (CD45.2^{+}/CD3e^{-}CD11b^{-}/B220^{+}) or myeloid cells (CD45.2^{+}/CD3e^{-}
1115 /CD11b^{+}/B220^{-}) in the presence of LPS for 4 hour. (H) Graph represents the percentage of
1116 migrated LK LDBM to the WT LN bottom (blue mosaic bars) or Traf6$^{\Delta/\Delta}$ LN bottom (orange
1117 mosaic bars) as schemed G. In all cases, LK cell migration was determined by CD45 allotype
1118 analysis using flow cytometry in triplicate. Values are presented as mean ± SD. (I) Absolute
1119 number of CFU-GM presents in LN from *LysM-Cre;WT* (solid bars) and *LysM-Cre;Traf6*$^{\Delta/\Delta}$
1120 (mosaic bars) full chimeric mice after PBS/LPS administration at ZT7 (3h). A minimum of 4 mice
1121 per group were analyzed. Values are presented as mean ± SD, *P<0.05. **P<0.01. (J) Graph
1122 represent cumulative survival of *LysM-Cre;WT* (blue line) and *LysM-Cre;Traf6*$^{\Delta/\Delta}$ (orange line)
1123 after 10 mg/Kg of LPS. **P<0.01. (J) Survival curve after 30 mg/Kg of b.w.injection in *LysM-*
1124 *Cre;WT* (blue line) *or LysM-Cre;Traf6*$^{\Delta/\Delta}$ (orange line). **P<0.01.
1125

1126 **Figure 5. GMP cells drain into local Lymphatics and not blood circulation in early**
1127 **inflammation *via* Ccl19/Ccr7**

1128 (A) Graph represents soluble Ccl19 chemokine in femoral or LN extracellular fluid and PB
1129 plasma after PBS (black lines) or LPS (blue lines) administration at different circadian cycle
1130 times (ZT4.5 [0.5h], ZT5 [1h] and ZT7 [3h]). Values represent mean ± SEM of two independent
1131 experiments in duplicate. (B) Strategy for *in vivo* neutralization of Ccr7 receptor or Ccl19 ligand
1132 by in2jections of anti-Ccr7 antibody or anti-Ccl19 antibody (50 µg/dose, two doses) into C57Bl/6
1133 mice. One day after the last dose of antibodies, PBS or LPS was administered at ZT4 (0h) and
1134 the myeloid progenitors-circulating cells from the LN and PB were measured by CFU assay at

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1135 ZT7 (3h). (C-D) Absolute number of progenitors present into LN from neutralized mice with anti-
1136 Ccl19/IgG (left graph) or anti-Ccr7/IgG2a (right graph) after PBS (black) or LPS (blue)
1137 administration as depicted in B (n=7-8 mice per group). Values represent mean ± SEM. *P<0.05
1138 **P<0.01. (E-F) Survival curves after 30 mg/Kg LPS of b.w. (F) and 10 mg/Kg LPS of b.w. or
1139 PBS as control (E, dashed lines) into WT C57BL/6 mice pre-treated with anti-Ccr7 (red line) or
1140 IgG2a (blue line). *P<0.05. (G) Generation of hematopoietic chimeric Ccl19 expressing (WT) or
1141 not (Ccl19$^{-/-}$) mice and isolation of LNs after administration of PBS or LPS. (H) Colony forming
1142 unit content of LN from either WT or Ccl19$^{-/-}$ hematopoietic chimeric animals treated with PBS or
1143 LPS (***p≤0.001). (I) Experimental design to analyze L-selectin dependence of femoral GMP
1144 migration to regional (or distant) LN after LPS administration. (J-K) Percentages of GFP^{+} cells
1145 in LN after administration of an isotype control or anti-L-selectin antibodies. (J) Frequency of
1146 GFP^{+} GMP cells in regional LN after administration of LPS was not modified by L-selectin
1147 blockade in vivo. (J) Inhibition of the migration of GFP^{+} B-lymphocytes to regional LNs in mice
1148 pre-treated with anti-L-selectin antibody (**p≤0.01). In B and C, LN were collected at ZT7 or 3h
1149 after LPS.
1150

1151 **Figure 6. In vivo analysis of Ccl19/Ccr7 axis during inflammation. Pharmacological**
1152 **regulation of LPS/TLR signaling pathway.**

1153 (A) Annexin-V binding to membrane PS on LN myeloid populations from WT (left mosaic bars)
1154 and Traf6$^{\Delta/\Delta}$ (right mosaic bars) (n=4 mice per group) after PBS or LPS administration. LN
1155 suspension cells were stained for myeloid surface markers including annexin-V and analyzed by
1156 flow cytometry. Values represent mean ± SEM. **P<0.01. (B) Transwell migration of LDBM-
1157 derived LK cells toward gradient generated by pre-treated LN cells with DMSO (solid bars) as
1158 vehicle control and inhibitors (mosaic bars) against Irak1/4 (right lined), Ubc13 (left lined), and
1159 IKK (white squares), and following TLR signaling pathway activation by PBS (black) or LPS
1160 (blue). (C) Analysis of SNAP23 phosphorylation (Ser95) in LN myeloid cells previously treated
1161 with DMSO (solid bar) as vehicle control, Irak1/4 (right lined mosaic bar), Ubc13 (left lined

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1162 mosaic bar) or IKK (white squares mosaic bar) inhibitors. Values represent two independent
1163 experiments as mean ± SEM of two independent experiments performed in triplicate. *P<0.05,
1164 **P<0.01. (D) Graph shows intracellular Ccl19 in LN-derived myeloid cells from non-
1165 manipulated mice by flow cytometry. Values represent mean ± SEM of three independent
1166 experiments, n=8-9. ****P<0.0001.
1167

1168 **Supplementary Figure Legends**

1169 **Figure S1. Clonogenic and long-term multilineage potential of human and murine HSC/P**
1170 **in LN.** (A) Representative flow cytometry dot plots of SP cells from human LN biopsies
1171 diagnosed with lymphadenitis (left flow panel) and non-Hodgkin Lymphoma (NHL, right flow
1172 panel). SP cells form a tail cluster on the left side. (B) Graph represents the percentage of SP
1173 cells in human LN biopsies categorized according to their anatomical location. Supra-
1174 diafragmatic location (solid circles) included neck and axillary LN and infra-diafragmatic location
1175 (open circles) included mesenteric and inguinal LN. (C) Representative colony-forming units
1176 (CFU-C) micrographs in human LN diagnosed with follicular lymphadenitis. Scale bar: 100 μm.
1177 (D) Clonogenic potential of lymphadenitis-derived SP cells (upper left; with magnification of a
1178 CFU-GM in outlined inset) and FACS analysis of LN SP-derived hematopoietic progenitors
1179 (upper right and bottom panels) maintained for 1 week in culture as previously described(*19*).
1180 All SP-derived progenitors were positive for pan-leukocyte surface marker CD45 but had
1181 heterogenic expression for CD34 and CD133 surface markers. (E) Absolute neutrophil count in
1182 PB from C57Bl/6 mice pre-treated with PBS (black circles) or LPS (blue circles) at different
1183 circadian cycle times. (F-G). Representative FACS profiles of murine BM HSC/P in response to
1184 *in vivo* administration of PBS (left panels) or LPS (right panels) at specified ZT. (H-O) Time
1185 response in the BM content of LSK (H), LT-HSC (I), ST-HSC (J), MPP (K), GMPs (L), CMP (\M),
1186 MEP (N) and LK cells (O) in response to PBS (black lines) or LPS (blue lines). Values represent
1187 mean ± SD of a minimum of 4 mice per group. *P<0.05, **P<0.01, ***P<0.001.
1188

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1189 **Figure S2. Myeloid progenitor migration to LN in response to LPS is independent of**
1190 **NFκB activation.** (A) Schema for the competitive long-term reconstitution potential of LN cells
1191 (donor cells) from C57Bl/6 ($CD45.2^+$) mice treated with PBS/LPS and harvested at ZT7(3h), into
1192 lethally irradiated $CD45.1^+$ $B6.SJL^{Ptprca\ Pep3b/BoyJ}$ recipient mice (n=3 mice per group). (B-E)
1193 Competitive repopulating unit (CRU) assay of LN cells as assessed by flow cytometry of
1194 allotype CD45.2 expressing cells in transplanted mice followed for up to 16 weeks. (B) Overall
1195 CRU as gated on $CD45.2^+$ cells in response to donor cells from mice pre-treated with PBS
1196 (black line) or LPS (blue line).(C-E) Myeloid cell (C), T-cell (D) and B-cell (E) contributing CRU
1197 in response to donor cells from mice pre-treated with PBS (solid bar) or LPS (blue mosaic bar).
1198 (F) Representative example of FACS analysis for SP cells (upper dot plots) and SP-LSK
1199 potential (lower dot plots) from PBS- and LPS-treated LN or BM tissues at ZT7 (3h). Hoechst:
1200 Hoechst 33342 staining for blue (blue) and red (red) fluorescence emissions. (G-H) Donor-
1201 derived chimera of sorted LN-or BM-SP cells obtained from LPS-treated mice.

1202

1203 **Figure S3. Myeloid progenitor migration to LN in response to LPS is independent of**
1204 **NFκB activation.**

1205 (A) Flow cytometry analysis of GFP MDPs in bone marrow showing decreased MDPs upon LPS
1206 treatment. (B) Flow cytometry analysis of migrated GFP MDPs in lymph node (per regional
1207 group) showing increased GFP^+MDPs in Inguinal and Popliteal lymph nodes. (Black bar $EGFP^+$
1208 and Blue Bar $EGFP^-$. (C) Representative PCR amplifications show Traf6 deleted in circulating
1209 cells in four *Mx1cre;Traf6*$^{flox/flox}$ mice after poly(I:C) injections (10mg/Kg/2 days x 5 doses). (D)
1210 Schema for BM-derived macrophages and exogenous expression of IκBα mutant which is
1211 resistant to proteasome degradation. BM Lin^- cells were transduced with Pmscv-puro-eGFP
1212 bicistronic retroviral vector encoding full length of IκBα mutant and GFP^+ protein. $EGFP^+$ Lin^-
1213 cells were sorted and differentiated in culture by M-CSF cytokine to macrophages. Green
1214 fluorescent CD11b+-macrophages were layered on bottom chamber and stimulated them with

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1215 LPS to generate myeloid chemotaxis gradient. (E) FACS strategy for migrated LK (Lin^-/c-
1216 Kit^+/$Sca1^-$) cells contained in LDBM from upper chamber to the transduced macrophage bottom
1217 stimulated with LPS and LPS+monensin (LPS+Mon) for 4 hours as depicted in J. (F)
1218 Representative FACS dot plots demonstrating gating strategy to identify migrating GMP
1219 populations from the transwell migration assays. (G) Graph represents LK cell migration
1220 towards transduced macrophages in presence of LPS (solid bars) or LPS+Mon (mosaic bars).
1221 Values represent mean±SD of three mice and per triplicate. NS: not significant. *P<0.05
1222 ***P<0.001.

1223

1224 **Figure S4. Inflammation induces temporal changes in chemokine and cytokine**
1225 **signatures in BM and LN.** (A) Cxcl12 in femoral or LN extracellular fluid and plasma after
1226 PBS/LPS *in vivo* administration at ZT7 (3h). (B) Heat map showing cytokine profiling release
1227 into the extracellular fluid of femora and LN in response to PBS/LPS at ZT5 (1h) and ZT7 (3h).
1228 (C-O). Graphs represent levels of relevant cytokines and chemokines associated with
1229 migration/inflammatory response and released into LN extracellular fluid (black and green bars)
1230 or into femoral extracellular fluid (black and orange bars) after PBS/LPS administration into
1231 C57Bl/6 mice at ZT5 (1h) and ZT7 (3h). (C-J) Extracellular LN levels of Gm-csf (C), G-csf (D),
1232 M-Csf (E), Mcp-1 (F), Ccl5 (G), Eotaxin/Ccl11 (H), IL-13 (I), IL-5 (J). (K-N) Extracellular BM
1233 levels of Ccl3 (K), Ccl4 (L), Tnfα (M) and IL-1α (N). Values are mean ± SE of two mice per
1234 treatment and experiment, pooled from two independent experiments *P<0.05, **P<0.01,
1235 ***P<0.001, ****P<0.0001.

1236

1237 **Figure S5. Myeloid expression of Ccl19 ligand in LN and short-term differentiation of pre-**
1238 **treated GMPs.** (A) Ccl21 in femoral or LN extracellular fluid and PB plasma after PBS (left
1239 lines) or LPS (right lines) administration at different circadian cycle times. (B) Ccl19 released
1240 into the supernatant from sorted WT and Traf6-deficient LN T^+ cells, B^+ cells and $CD11b^+$
1241 myeloid cells after LPS stimulation *in vitro*. Values represent mean ± SEM of two independent

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1242 experiments. **P<0.01, ***P<0.001. (C-D). Membrane Ccr7 expression on GMP at ZT5 (1h)
1243 after PBS/LPS administration into WT and Traf6 deficient mice shows significant differences
1244 between groups. (C) Representative overlap histograms of Ccr7 expression on GMP-WT cells
1245 (upper) and GMP-Traf6$^{\Delta/\Delta}$ (lower) cells after PBS (red line) or LPS (blue line). (D) Schema of
1246 transfer of Ccr7+ EGFP+ BM cells from b-actin-EGFP+ transgenic animals to femurs of WT
1247 recipient mice and administered PBS or LPS. (E-F) Femoral content of Ccr7-expressing GMP
1248 cells in BM (E) and regional (inguinal and popliteal) LN (F). (G) MFI of Ccr7 on GMPs from WT
1249 and Traf6 deficient mice in presence of PBS (black and orange solid bars) or LPS (blue solid
1250 bar and orange mosaic bar). Values represent mean mean ± SD of three mice. (H)
1251 Representative overlap histograms of phospho-SNAP23 (pSNAP23) into LN myeloid cells
1252 treated with DMSO (upper left), Irak1/4-inh (upper right), Ubc13-inh (lower left) and IKK-inh
1253 (lower right) and stimulated with PBS (red lines) or LPS (blue lines).
1254

1255 **Figure S6. Basal Ccl19 detection into LN populations.**

1256 (A) Gating strategy to determine Ccl19 pre-stored into LN-residing myeloid cells basally. (B-C)
1257 Mean fluorescence intensity (MFI) quantification of pre-stored Ccl19 into LN-residing
1258 CD11b^{low}/CD11c^{+} cDC (B) and pDC (C) populations in basal conditions.
1259
1260

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1261 **Supplementary Movies**

1262

1263 **Movie S1 caption**

1264 Representative example of multi-photon microscopy processed with Imaris software of a femur
1265 from one Lyve1-EGFP mouse treated with PBS at 1 hour after administration (ZT5). Bone tissue
1266 is identified as second-harmonic (SHG) signal (blue). Imaris software was used to measure
1267 distance between DiI labeled cells and GFP positive lymphatic vessels using 3D images.

1268

1269 **Movie S2 caption**

1270 Representative example of multi-photon microscopy processed with Imaris software of a femur
1271 from one Lyve1-EGFP mouse treated with LPS at 1 hour after administration (ZT5). Bone tissue
1272 is identified as second-harmonic (SHG) signal (blue). Imaris software was used to measure
1273 distance between DiI labeled cells and GFP positive lymphatic vessels using 3D images.

1274

1275 **Movie S3 caption**

1276 Representative example of transcortical lymphatics discovered by staining with anti-Lyve1 (red)
1277 in wild-type C57Bl/6 mice. Bone tissue is identified as second-harmonic (SHG) signal (blue).
1278 Green signal is autofluorescence.

1279

1280

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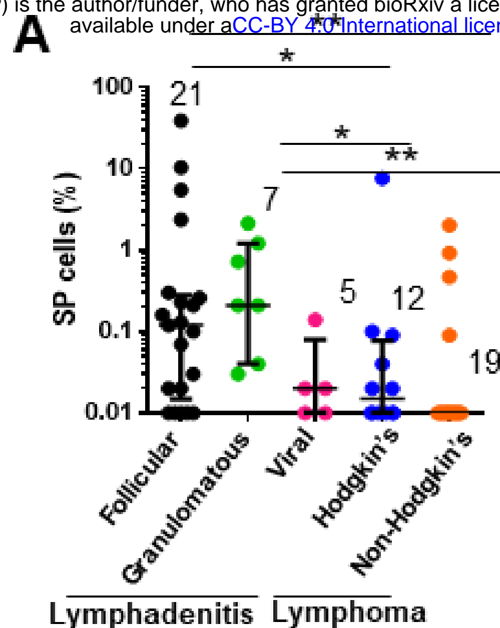
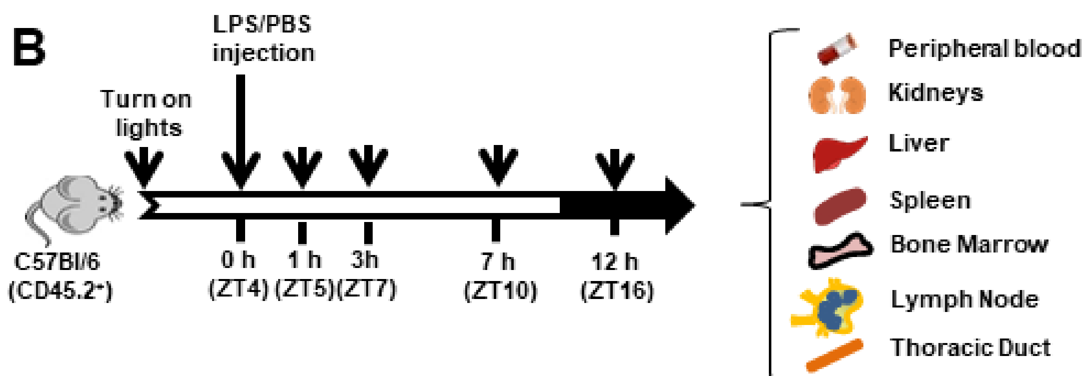
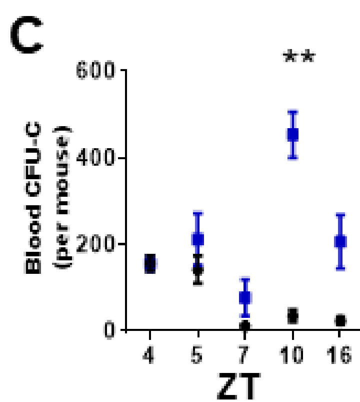
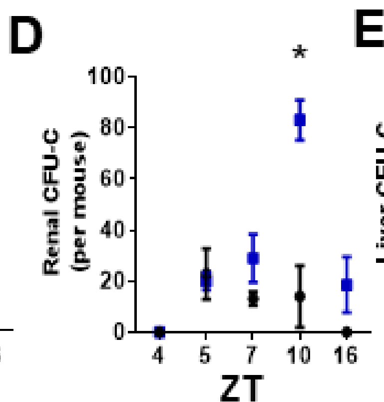
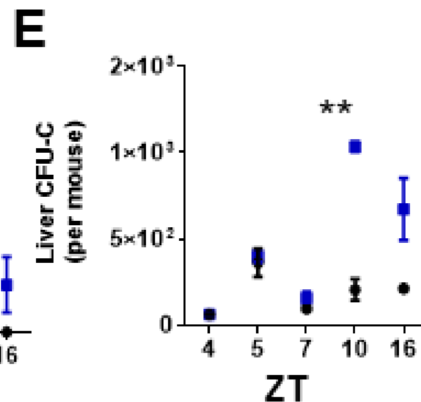
Figure 1

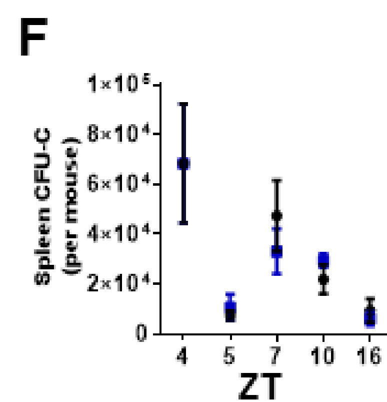


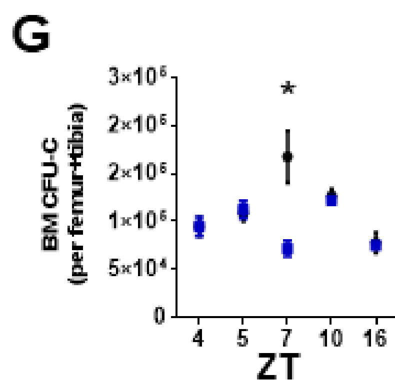


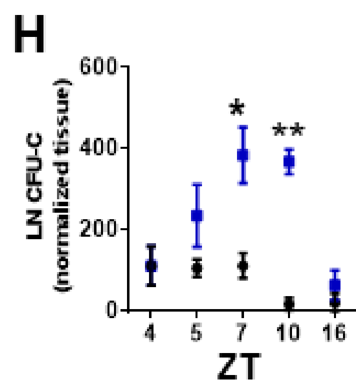


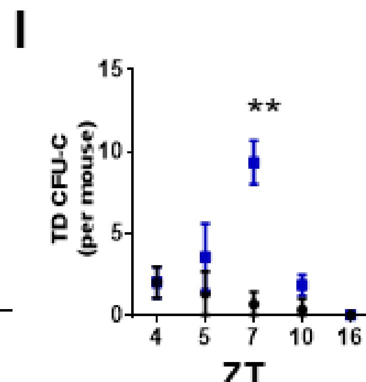












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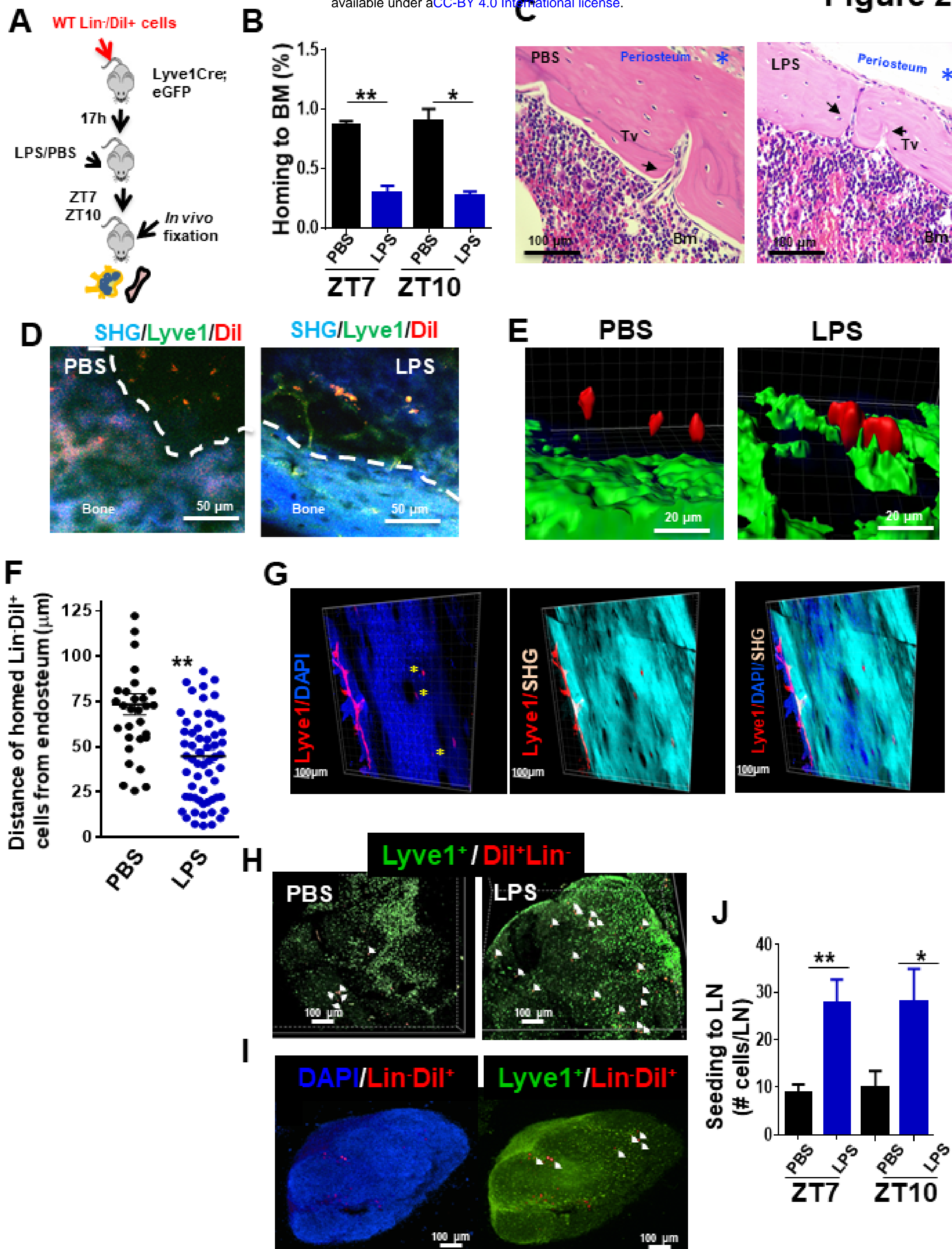

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Figure 3

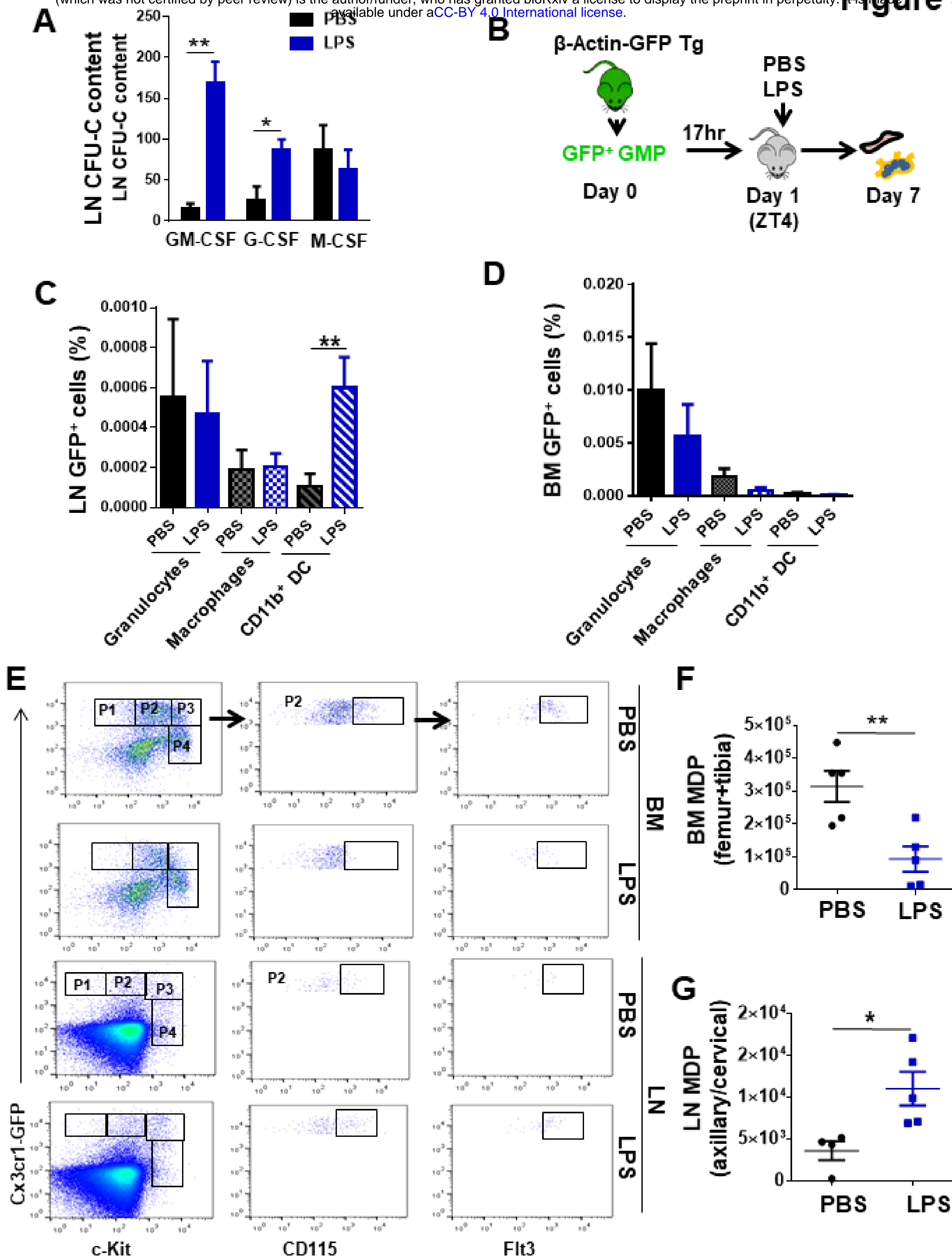

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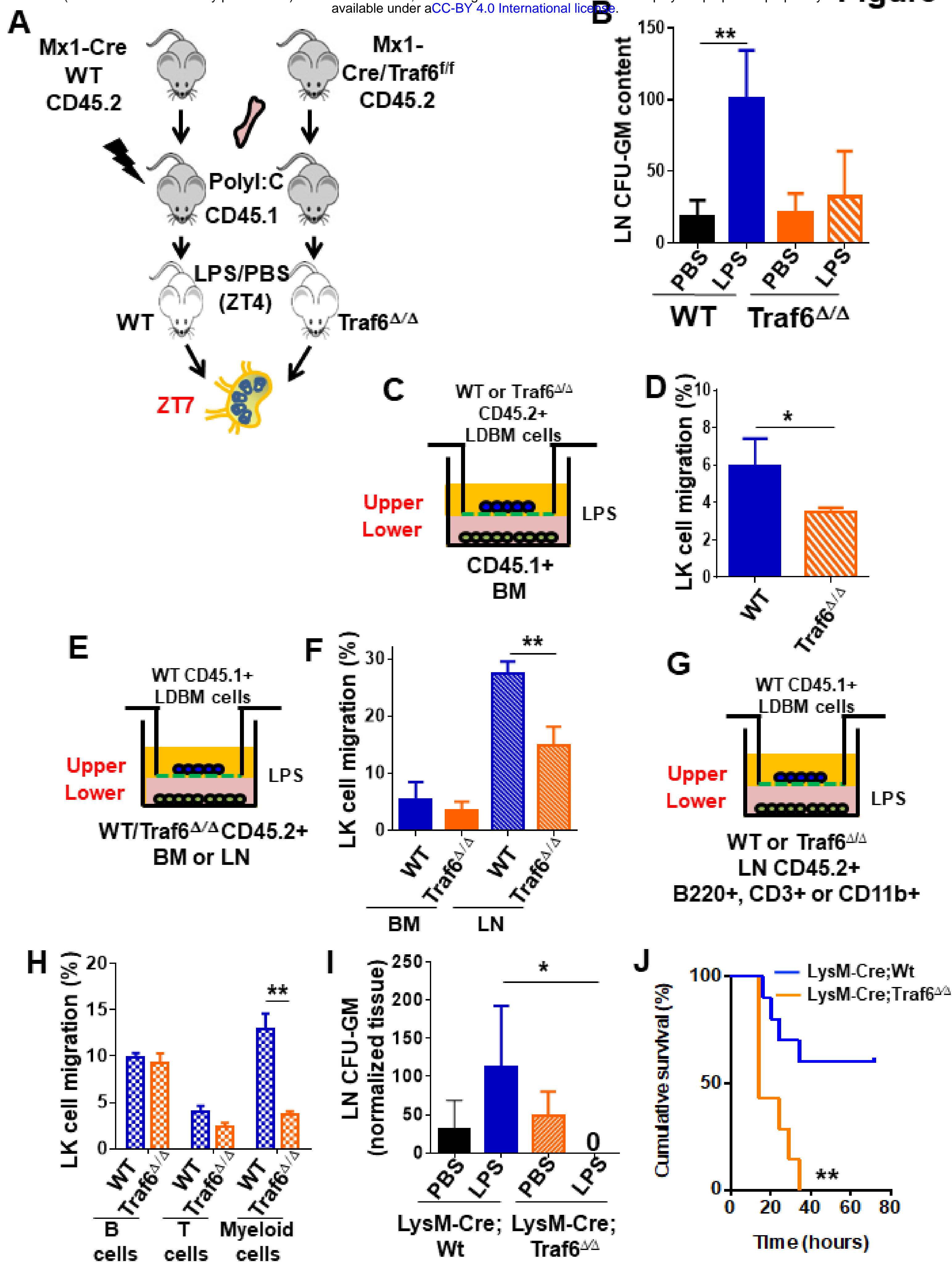
Figure 4



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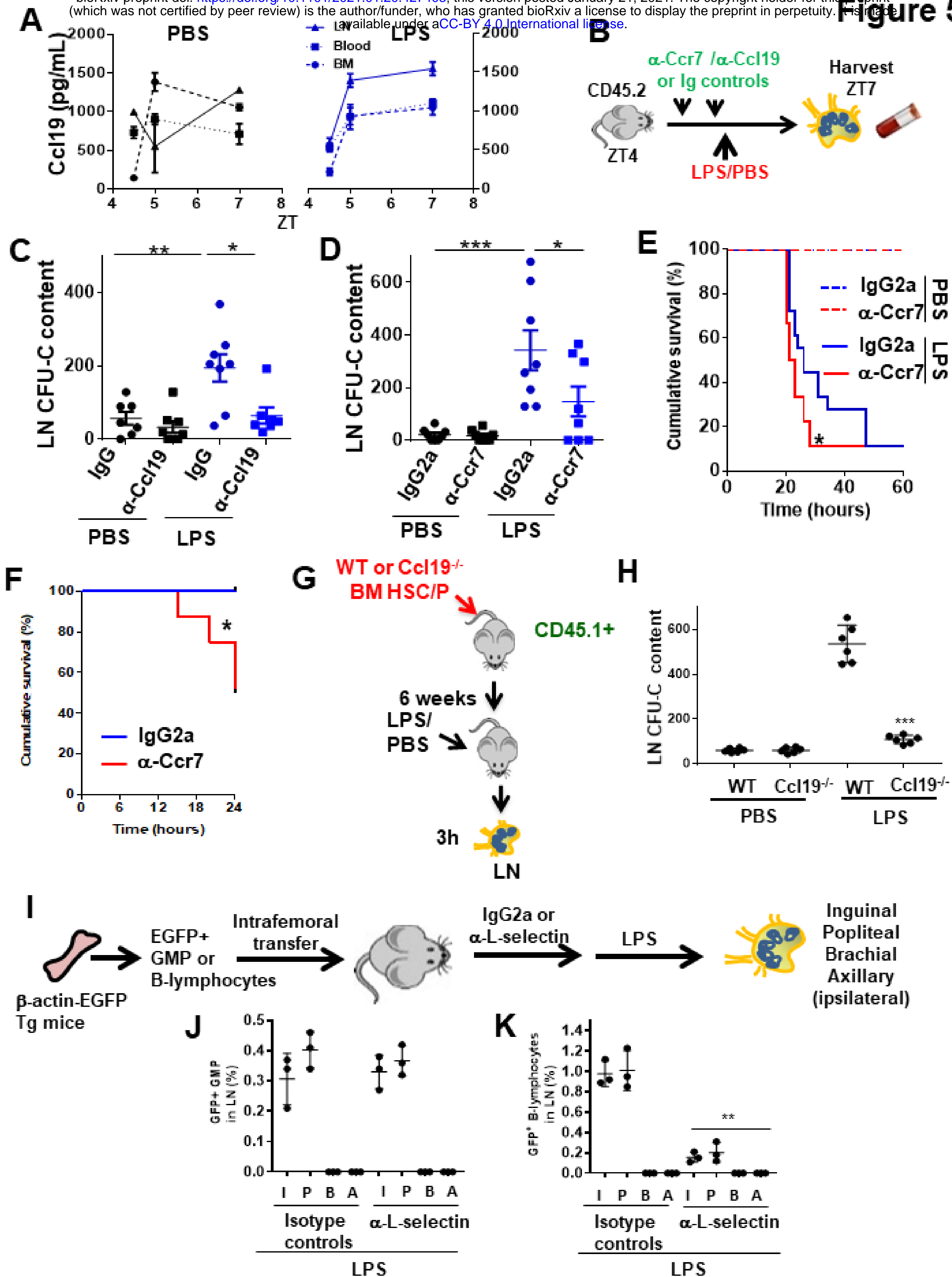

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Figure 6

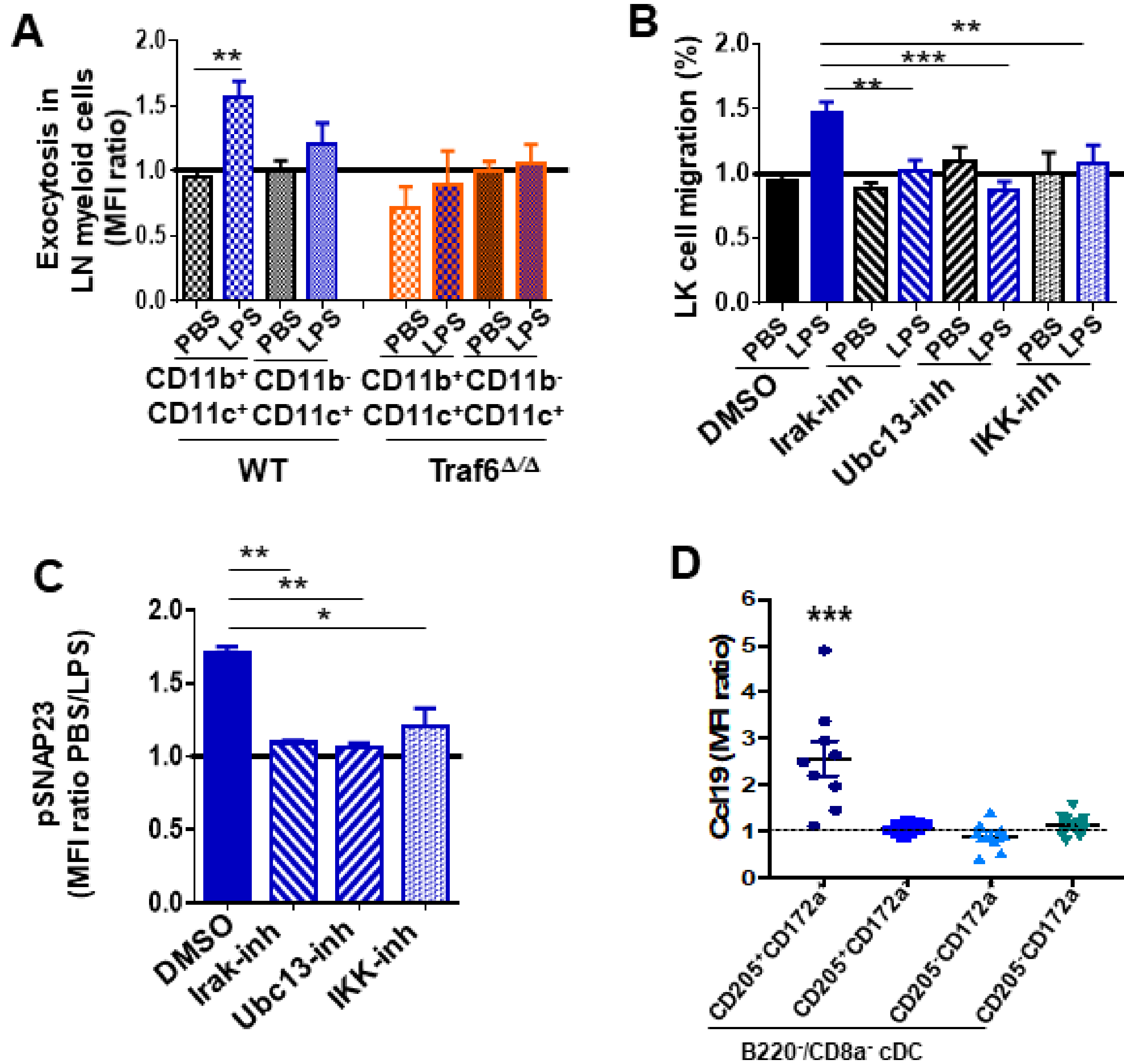

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Figure S1

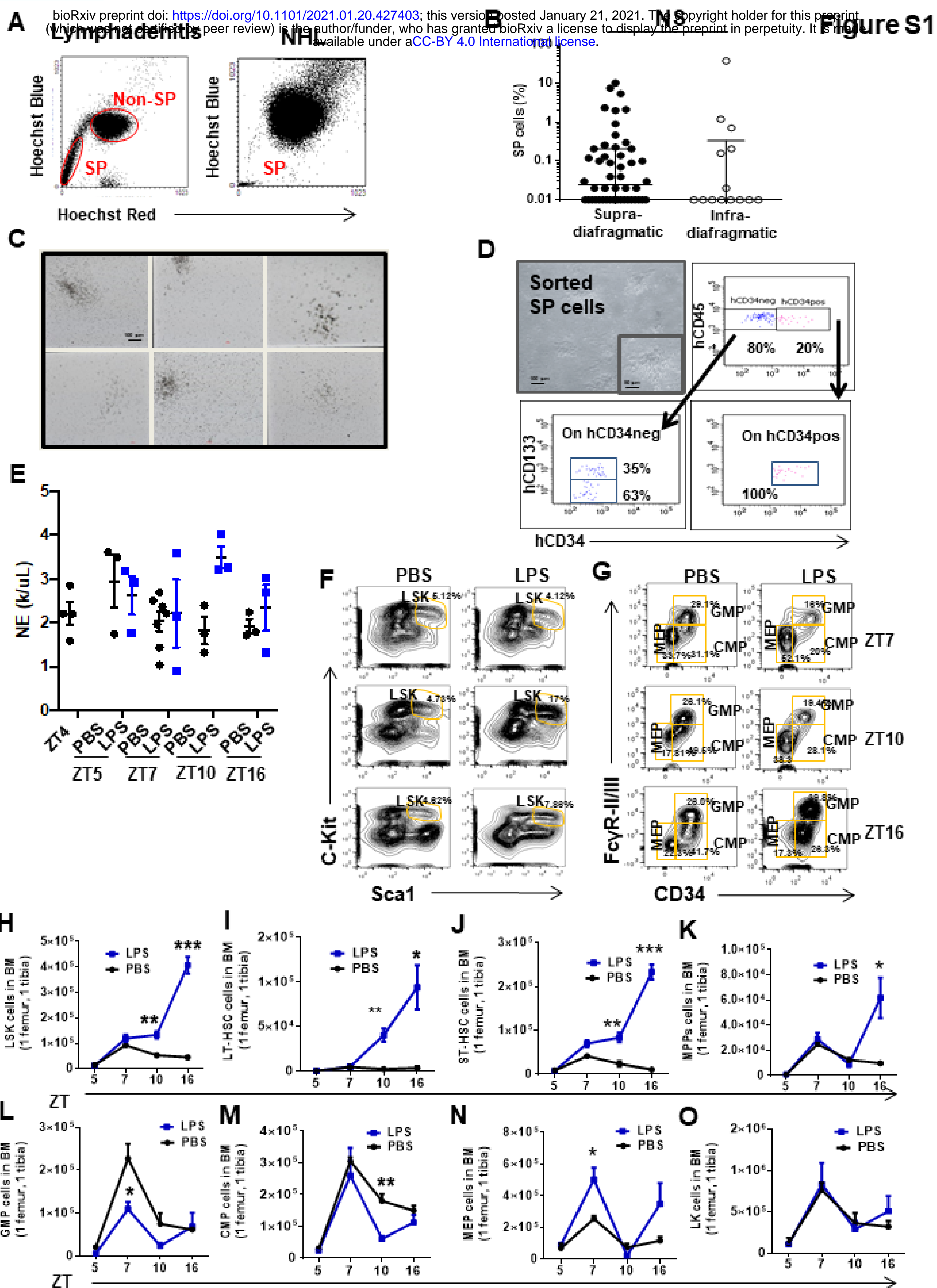

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Figure S2

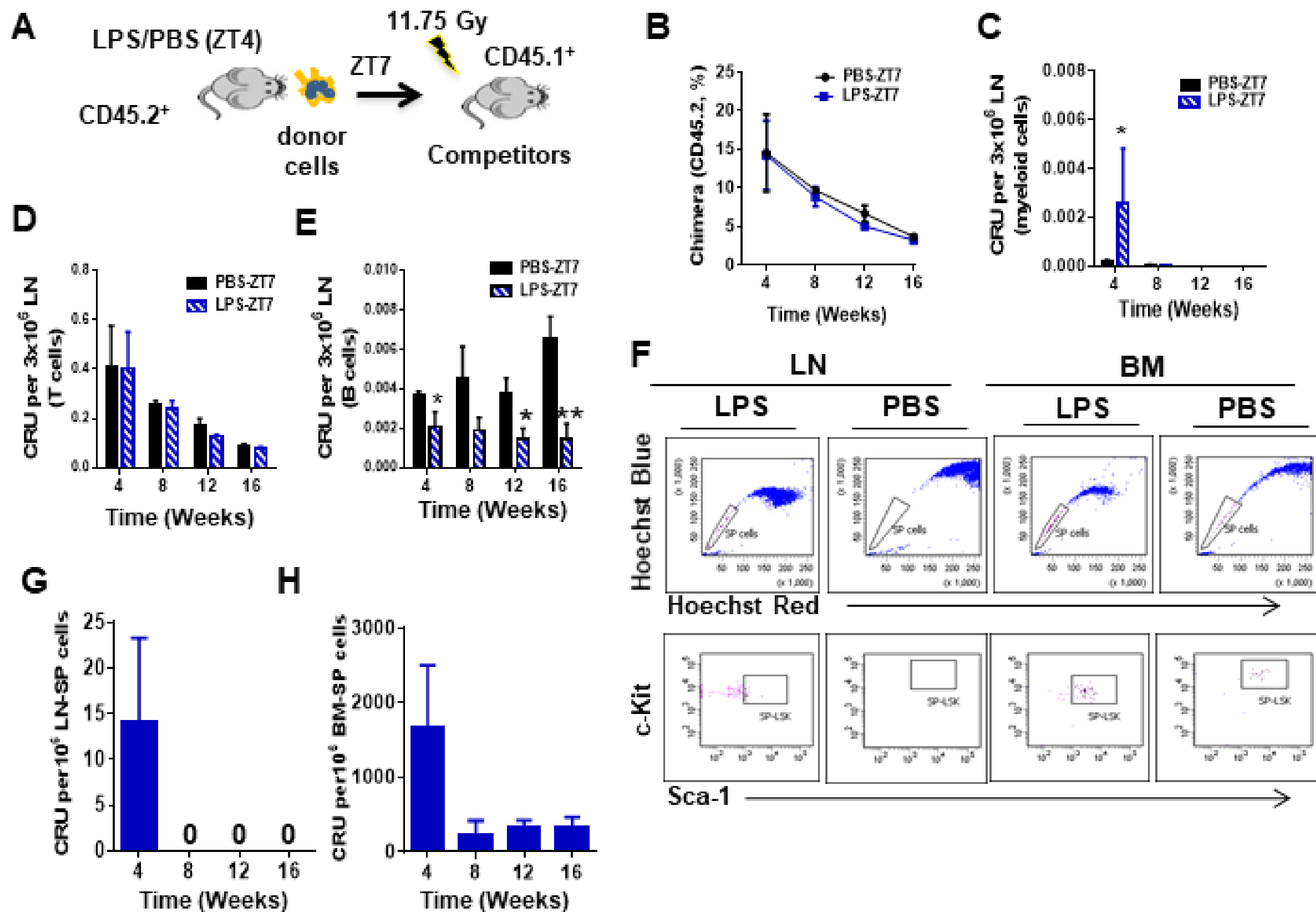

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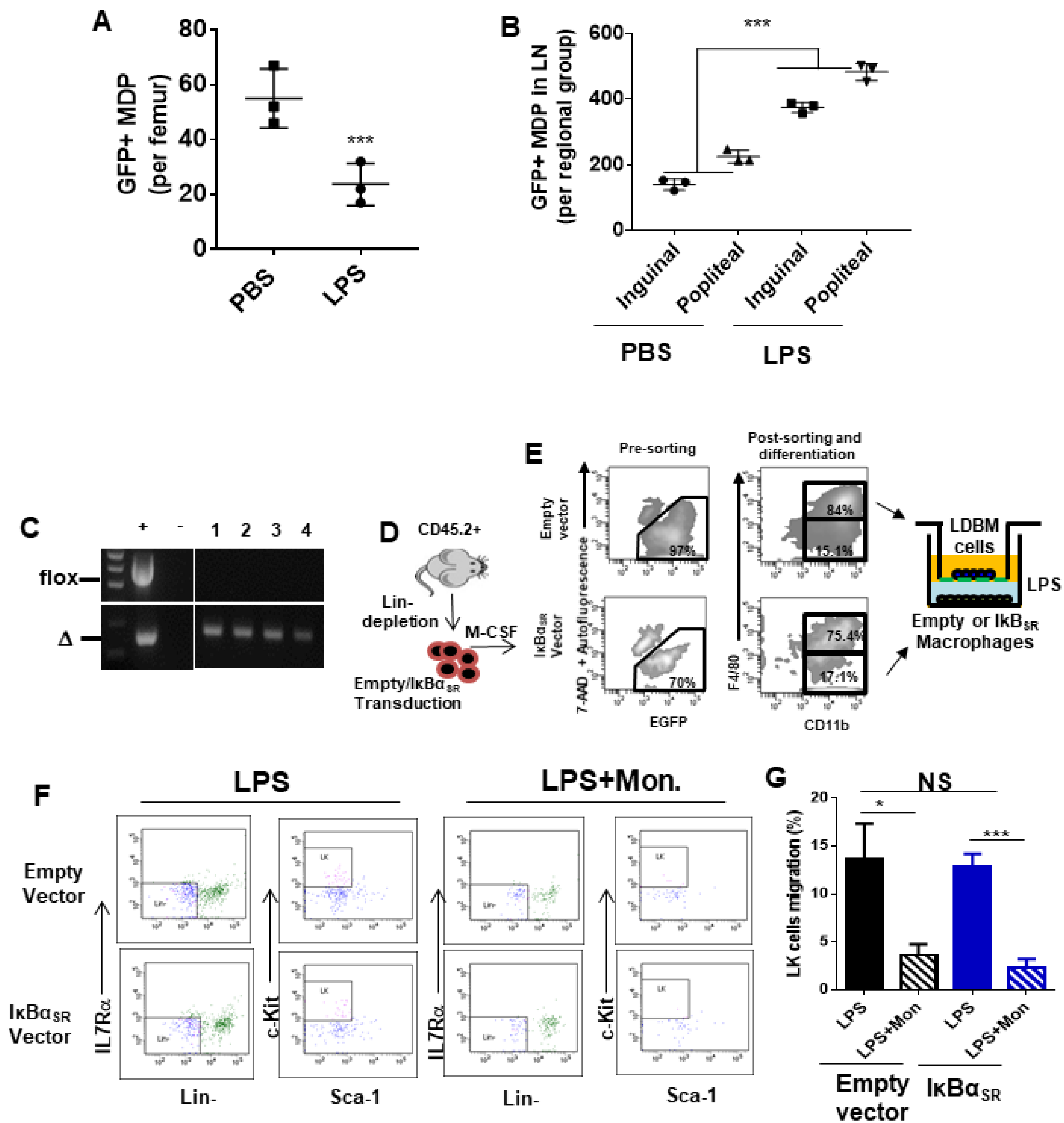
Figure S3



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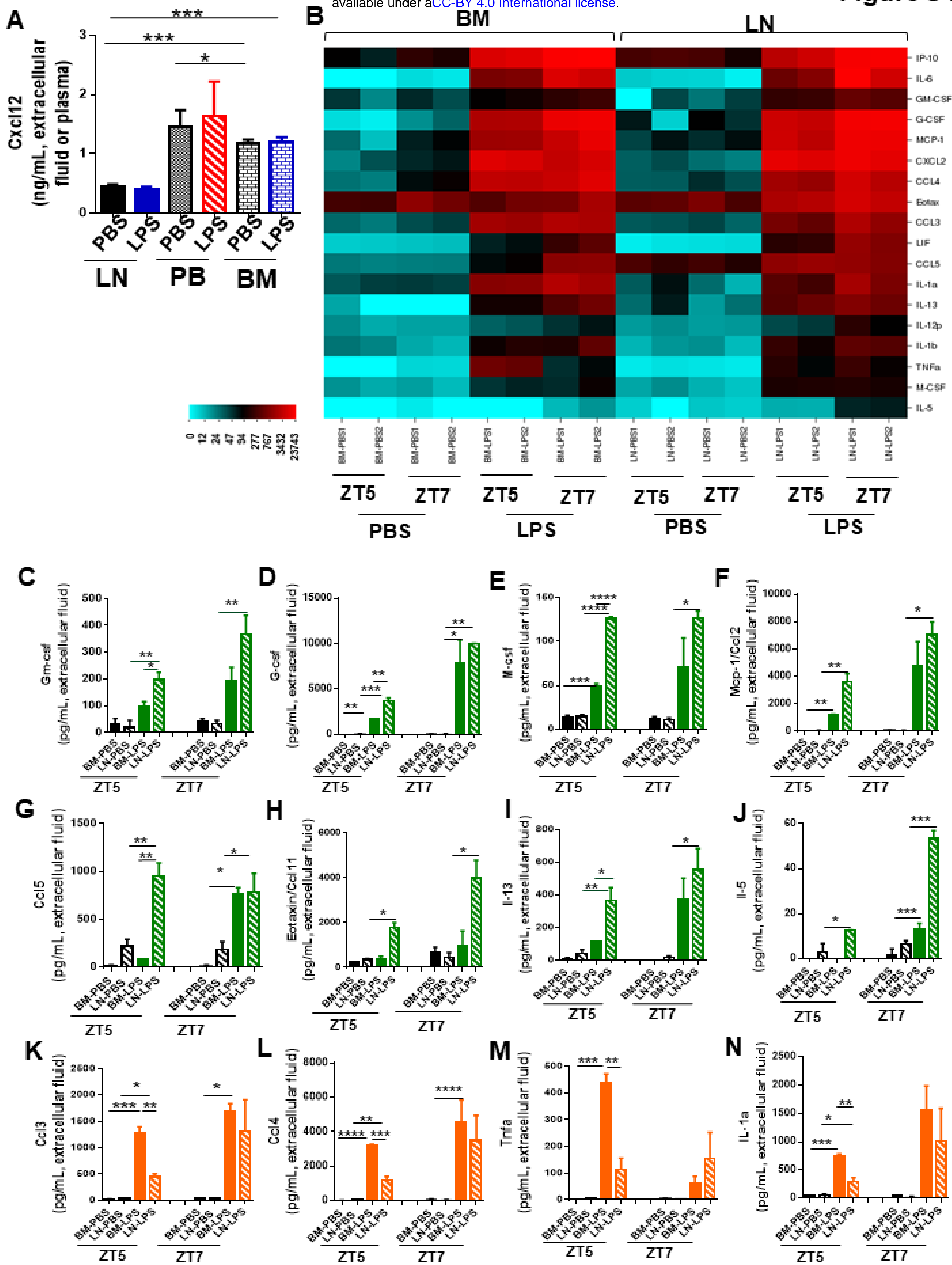

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Figure S5

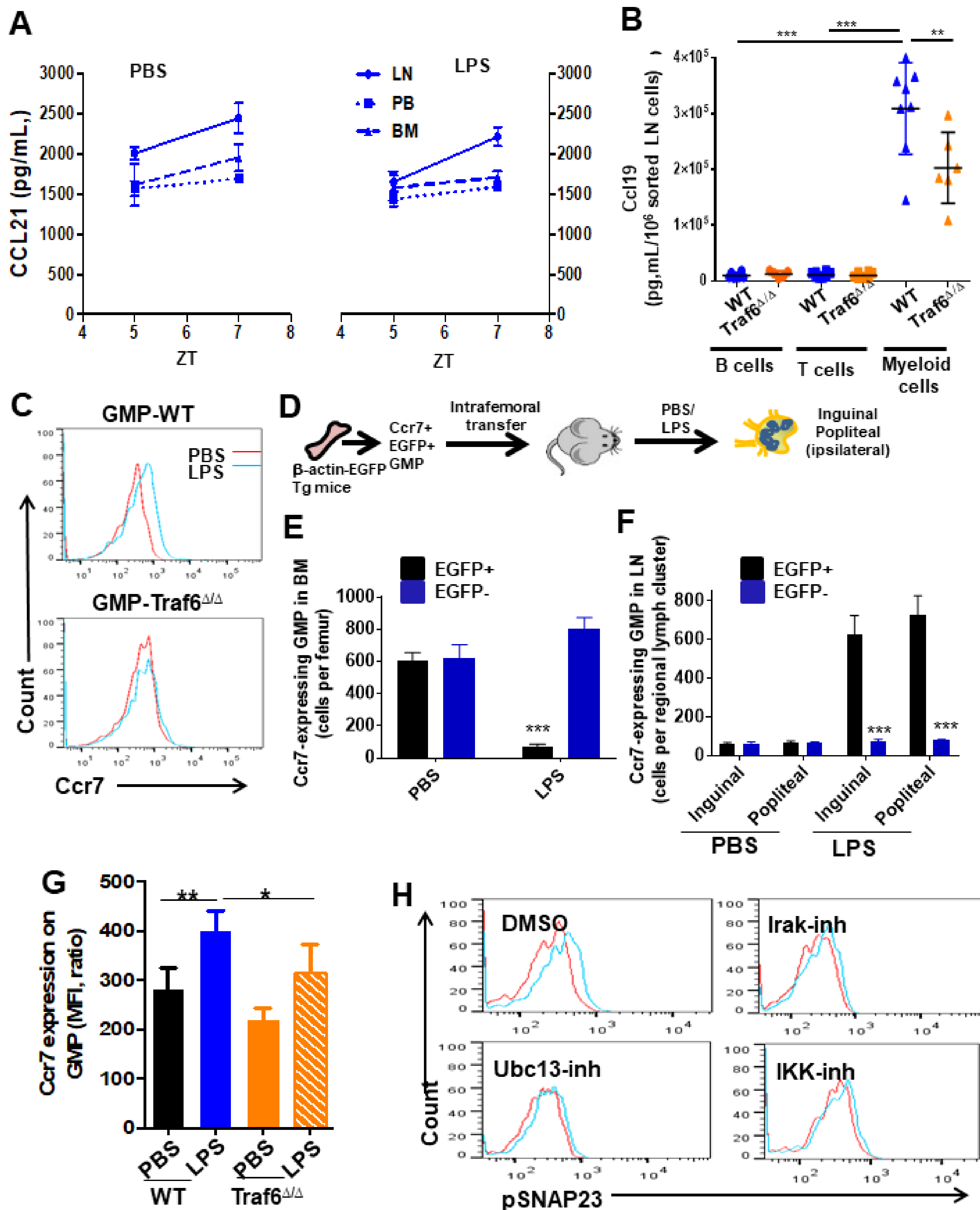

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Figure S6

A

Specimen_001-BFA negative mouse 2

B220 APC-Cy7-A

CD8 BV421-A

Specimen_001-BFA negative mouse 2

CD11c BV711-A

CD11b APC-A

Specimen_001-BFA negative mouse 2

CD205 PerCP-Cy5-5-A

CD172a PE-Cy7-A

B220-/CD8-/CD11b^{low}/CD11c+
CD205+/CD172a+

Specimen_001-BFA negative mouse 2

Count

Streptavidine DsRed-A

Specimen_001-BFA Ccl19 mouse 2

Count

Streptavidine DsRed-A

Specimen_001-BFA negative mouse 3

Count

Streptavidine DsRed-A

Specimen_001-BFA Ccl19 mouse 3

Count

Streptavidine DsRed-A

B