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Nuclear envelope disruption triggers hallmarks of aging in lung alveolar macrophages

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29

30

31 **Summary**

32 Aging is characterized by gradual immune dysfunction and increased risk for many diseases,
33 including respiratory infections. Genomic instability is thought to play a central role in the aging
34 process but the mechanisms that damage nuclear DNA in aging are insufficiently defined. Cells
35 that migrate or reside within confined environments experience forces applied to their nucleus,
36 leading to transient nuclear envelope (NE) ruptures. NE ruptures are associated with DNA damage,
37 and Lamin A/C is required to limit these events. Here, we show that Lamin A/C protects lung
38 alveolar macrophages from NE rupture and hallmarks of aging. Lamin A/C ablation in immune
39 cells results in a selective depletion of lung alveolar macrophages (AM) and a heightened
40 susceptibility to influenza infection. Lamin A/C-deficient AM that persist display constitutive
41 nuclear envelope rupture marks, DNA damage and p53-dependent senescence. In wild-type mice,
42 we found that AM migrate within constricted spaces *in vivo*, at heights that induce NE rupture and
43 DNA damage. AM from aged wild-type mice and from Lamin A/C-deficient mice share an
44 upregulated lysosomal signature with CD63 expression, and we find that CD63 is required to clear
45 damaged DNA in macrophages. We propose that induction of genomic instability by NE disruption
46 represents a mechanism of aging in alveolar macrophages.

47

48 **Introduction**

49 Age is a major risk factor for a large number of diseases including respiratory viral
50 infections such as influenza virus and SARS-CoV-2 (Schneider et al., 2021). Chronological aging
51 is characterized by gradual immune dysfunction, which limits protective responses (Nikolich-
52 Žugich, 2018). Studies in genetic models have shown that induction of immune dysfunction can
53 contribute to organismal aging, in part through inflammation (Desdín-Micó et al., 2020;
54 Yousefzadeh et al., 2021). At the cellular level, cells accumulate hallmarks of aging which include
55 genome instability, cellular senescence and altered proteolytic systems (López-Otín et al., 2013).
56 Genomic instability has been proposed to play a central role in driving the aging process
57 (Schumacher et al., 2021). However, the intracellular mechanisms that compromise genome
58 stability during aging in immune cells are ill defined.

59 Immune cells are highly migratory (Luster et al., 2005) and constantly go through narrow
60 spaces that lead to cellular deformation (Pflücke and Sixt, 2009; Raab et al., 2016). There is an
61 emerging recognition that genome instability can be driven by mechanical forces imparted on the
62 nucleus. Constricted or confined microenvironments *in vivo* exert pressure on the cells that migrate
63 through or reside within these spaces (Garcia-Arcos et al., 2019). These forces can deform the
64 nucleus – the largest and stiffest organelle in the cell (Nader et al., 2021a) – leading to transient
65 nuclear envelope ruptures (Denais et al., 2016; Irianto et al., 2017; Raab et al., 2016). These
66 ruptures cause DNA damage as a result of compromised nuclear compartmentalization (Cho et al.,
67 2019; Denais et al., 2016; Earle et al., 2020; Irianto et al., 2017; Raab et al., 2016; Shah et al., 2021;
68 Xia et al., 2018). The consequences of nuclear envelope rupture-induced DNA damage are largely
69 unknown and demonstrate cell-type specificity. It induces, for instance, senescence in a non-
70 transformed epithelial cell line, while it promotes tumor cell invasion in a breast cancer model
71 (Nader et al., 2021b). Nuclear envelope rupture also exposes genomic DNA to the cytosol, which

72 recruits the interferon-inducing cytosolic DNA sensor cGAS (Denais et al., 2016; Raab et al.,
73 2016). Several mechanisms keep cGAS activation in check following nuclear envelope rupture
74 (Gentili et al., 2019; Guey et al., 2020; Lan et al., 2014), thereby limiting interferon responses that
75 can themselves alter genomic stability (Banerjee et al., 2021; Moiseeva et al., 2006; Morales et al.,
76 2017). Among immune cells, nuclear envelope rupture has been observed in neutrophils and
77 dendritic cells *in vitro* (Raab et al., 2016; Thiam et al., 2020). However, the *in vivo* occurrence and
78 consequences of nuclear envelope rupture in the immune system are not known.

79 The Lamin meshwork, composed of Lamin A/C, B1 and B2, provides essential mechanical
80 protection of the nucleus (De Vos et al., 2011; Vargas et al., 2012). During migration of cell lines
81 in confined spaces *in vitro*, Lamin A/C is required to limit nuclear envelope rupture, DNA damage
82 and cell death (Denais et al., 2016; Raab et al., 2016). In tissues, Lamin A/C was shown to protect
83 against nuclear envelope rupture in the context of the beating heart and skeletal muscles, that are
84 mechanically strained tissues (Cho et al., 2019; Earle et al., 2020). In humans, mutations in the
85 Lamin A/C gene cause diverse and severe disease manifestations, including accelerated aging
86 phenotypes, and some of these mutations have been associated with decreased genome stability
87 and increased nuclear envelope rupture (De Vos et al., 2011; Earle et al., 2020). In immune cells,
88 Lamin A/C expression is regulated and impacts immune functions in disease contexts (Saez et al.,
89 2020). Whether Lamin A/C provides protection against aging in the immune system is not known.

90 **Results**

91 ***Lamin A/C is required to maintain alveolar macrophages (AM) in the lung***

92 To investigate the physiological consequences of nuclear envelope rupture in immune cells,
93 we sought to identify genetic models that would exacerbate it. The Lamin A/C protein is required
94 to limit nuclear envelope ruptures in constricted spaces (Cho et al., 2019; Earle et al., 2020; Nader
95 et al., 2020). We thus hypothesized that if immune cells undergo NE rupture *in vivo*, Lamin A/C
96 depletion should increase its occurrence. To avoid confounding cell-extrinsic effects of Lamin A/C
97 deficiency in non-immune cells, we analyzed the spleen, lymph nodes, bone marrow and lungs of
98 mice with immune-specific ablation of Lamin A/C (*Lmna*^{fl/fl} Vav1-Cre^{+/-}, referred to as Lamin A/C
99 CKO hereafter (de Boer et al., 2003; Kim and Zheng, 2013)). We did not detect a loss of a range
100 of major immune populations including dendritic cell, macrophage, T, B, natural killer cells and
101 hematopoietic stem cell populations in the spleen, lymph nodes and bone marrow (**Figure S1A-**
102 **C**). However, in the lung we found that alveolar macrophages (AMs) are specifically depleted in
103 Lamin A/C CKO mice (**Figure 1A**), whereas the frequency of lung dendritic cells populations,
104 eosinophils and interstitial macrophages was unperturbed (**Figure S2A, S2B**). This depletion of
105 AM was also confirmed upon myeloid-specific ablation of Lamin A/C (**Figure S2C**).

106 To determine if AM were reduced in Lamin A/C CKO as a result of cell loss or phenotypic
107 changes, we performed single cell RNA sequencing (scRNAseq) on CD45.2⁺ cells sorted from the
108 lungs of WT and Lamin A/C CKO mice. Clustering analysis identified immune populations known
109 to populate murine lung (**Figure 1B, top, Figure S3A, S3B, S3C, Table S1**). AM were found to
110 have the highest level of Lamin A/C expression within the immune compartment of the lung,
111 suggesting a particular dependency of this cell type on high Lamin A/C expression (**Figure 1B,**
112 **bottom**). Indeed, we found that Lamin A/C CKO cells were depleted within the AM cluster in
113 comparison to WT, whereas WT and Lamin A/C CKO cells were equally distributed among the

114 other immune clusters (**Figure 1C**). Lamin A/C CKO mice analyzed at 3-4 weeks of age did not
115 have a strong depletion of AM, in contrast to 8-13 week old mice, suggesting a progressive loss
116 (**Figure 1D**). High levels of Lamin A/C were also observed in AMs of human, pig and rat (**Figure**
117 **1E, Table S2**) (Raredon et al., 2019). Our data therefore demonstrate that Lamin A/C is required
118 for the presence of AM, but not other immune cells, within the lung immune compartment.

119

120 *A p53-dependent response to DNA damage drives AM depletion*

121 To determine how Lamin A/C deficiency leads to depletion of AM, we examined enriched
122 signatures in differentially expressed genes (DEGs) (**Table S3**). We found an enrichment for p53
123 targets and for genes involved in apoptotic signaling in response to DNA damage, in genes
124 upregulated in Lamin A/C CKO AMs (**Figure 2A, S4A**). We also found that Lamin A/C CKO
125 AMs have a higher level of the DNA damage marker γ H2AX at steady-state than that observed in
126 WT littermates (**Figure 2B**). In addition, these cells were more sensitive to etoposide, an inducer
127 of DNA damage, applied *ex vivo* (**Figure 2C**).

128 We thus asked if AM depletion was due to senescence associated with DNA damage. DNA
129 damage can induce cellular senescence (Gorgoulis et al., 2019). Expression of senescence-
130 associated cell cycle inhibitor p21 was increased in Lamin A/C CKO AM (**Figure S4B, Table S3**).
131 The dsDNA sensor cGAS can promote senescence by detecting damaged self DNA accumulating
132 in the cytosol (Gluck et al., 2017; Yang et al., 2017). We generated mice with double knockout of
133 Lamin A/C and cGAS (*Lmna*^{fl/fl} *Mab21d1*^{KO/KO} *Vav1-Cre*^{+/-}, referred to as Lamin A/C cGAS DKO).
134 Removal of cGAS did not rescue AM depletion in Lamin A/C CKO mice and elevated DNA
135 damage persisted (**Figure 2D**). In agreement with this result, we did not detect expression of cGAS-
136 responsive inflammatory cytokines in Lamin A/C CKO AM such as IL6, IL1 β and IL8 (**Table S3**)

137 (Gluck et al., 2017; Yang et al., 2017). Since p53 induces cellular senescence in response to DNA
138 damage (Reinhardt and Schumacher, 2012), we generated double knockout of Lamin A/C and p53
139 in immune cells (referred to as Lamin A/C p53 DKO). Conditional knockout of p53 was utilized
140 to delay the onset of tumor growth and create a time window to analyze AM in tumor-free
141 conditions (DeMicco et al., 2013). The depletion of AM was rescued in Lamin A/C p53 DKO mice,
142 despite γ H2AX levels remaining elevated in the surviving cells (**Figure 2D**, **Figure S4C**). These
143 findings demonstrate that AM depletion in Lamin A/C CKO mice is a consequence of the response
144 to DNA damage through p53 and is independent of cGAS.

145

146 *Alveolar macrophages (AM) migrate in constricted spaces in the lung*

147 Lamin A/C CKO AMs are progressively lost, consistent with an accelerated senescence
148 process triggered after birth (**Figure 1D**). At steady state, most AM are thought to be sessile and
149 stick to the alveolar epithelium. However slow displacement events within the same alveola or
150 between alveoli through the pores of Kohn have been observed (Neupane et al., 2020).
151 Interestingly, pores of Kohn are infrequent in newborns and increase in number during the first few
152 weeks of life (Amy et al., 1977). Furthermore, we noticed that AM located inside pores of Kohn
153 appeared to have deformed nuclei (Neupane et al., 2020). To determine if AM migrate in the lung
154 at heights that induce nuclear envelope rupture, we followed their movements by live imaging in
155 WT mice. We identified several examples of cell squeezing consistent with entry into pores of
156 Kohn (**Figure 3A and Movie 1**). At the most acute point of squeezing, we measured the confined
157 width of cells to be approximately 2–3 μ m (**Figure 3B**, **Movie 2**, **Figure S5A**). These observations
158 demonstrate constricted migration of AM *in vivo* within the lung, suggesting a potential

159 relationship between this migration and the onset of the senescence process in Lamin A/C CKO
160 AM.

161

162 *Lamin A/C protects against accumulation of nuclear envelope rupture marks in AM*

163 A wide range of cell types display nuclear envelope rupture when passing through
164 microchannels with 2 μm constrictions or following confinement at heights of 2 μm *in vitro* (Denais
165 et al., 2016; Raab et al., 2016). To determine if this height also induces rupture in AM, we isolated
166 bronchoalveolar lavage (BAL) cells from WT mice and confined these cells at 2 μm and 10 μm , a
167 control height that does not lead to nuclear deformation. SiglecF staining confirmed the identity of
168 AM in cells from the BAL (**Figure 3C**). Upon nuclear envelope rupture, the DNA-binding protein
169 Barrier-to-Autointegration Factor (BAF) forms characteristic foci on DNA herniations protruding
170 from the nucleus (Denais et al., 2016; Halfmann et al., 2019). We identified a fraction of cells at
171 2 μm that displayed nuclear DNA blebbing which was associated with foci of BAF (**Figure 3D**). In
172 contrast, nuclear blebbing and BAF foci were largely absent in AM confined at 10 μm .

173 Next, we examined nuclear envelope rupture in AM from Lamin A/C CKO mice following
174 BAL cell confinement at 2 μm and 10 μm (**Figure S5B**). We confirmed Lamin A/C ablation in
175 AM obtained from BALs at the protein level (**Figure S5C**). Consistent with a role of Lamin A/C
176 in nuclear shape, Lamin A/C CKO AM confined at 2 μm were characterized by nuclei with reduced
177 circularity and roundness and increased aspect ratio compared to WT (**Figure S5D**). Strikingly, a
178 discontinuous ring of BAF foci was present at the nuclear rim of Lamin A/C CKO AM (**Figure**
179 **3E**). This discontinuous ring of BAF foci was present also at 10 μm (**Figure 3E**), suggesting that
180 it was not induced by 2 μm confinement but was present constitutively. In HeLa cells, Lamin A/C
181 depletion is not sufficient to induce such a ring and instead favors BAF diffusion (Haraguchi et al.,

182 2008). Therefore, the discontinuous ring of BAF foci that we observed in Lamin A/C CKO is rather
183 in agreement with the cells undergoing repetitive nuclear envelope rupture and accumulating
184 multiple individual BAF foci. Lamin A/C therefore protects AM from accumulation of nuclear
185 envelope rupture marks.

186

187 *DNA damage in AM upregulates CD63 and a lysosomal signature*

188 We next examined the functional consequences of Lamin A/C deficiency and DNA damage
189 in AM. Differential gene expression analysis, together with a bootstrap validation step, was used
190 to identify the most robustly affected DEGs (**Figure 4A**). We identified a downregulation of
191 ribosomal genes that was common among clusters. We also detected upregulation of a set of genes
192 specifically in the remaining AM of Lamin A/C CKO mice. The second most induced gene was
193 *Cd63*, a tetraspanin that is associated with endosomal and lysosomal membranes. We validated
194 increased protein expression of CD63 in Lamin A/C-knockout AM via intracellular flow cytometry
195 (**Figure 4B**). We were also able to validate at the protein level the reduced expression of CD88,
196 also known as complement component 5a receptor 1 or C5AR1 (**Table S3, Figure S6A**). We also
197 identified upregulated expression of a number of proteases including the macrophage elastase
198 (*Mmp12*) and cathepsins (*Ctsk, Ctsd, Ctsl, Ctsz, Ctsb*) (**Figure 4A, Table S3**). We confirmed
199 increased Cathepsin L activity in the BAL of Lamin A/C CKO mice (**Figure S6B**). The BAL of
200 Lamin A/C CKO mice also contained elevated levels of neutrophils and T cells (**Figure S6C**)
201 indicative of altered alveolar homeostasis.

202 To test if the transcriptional signature of Lamin A/C CKO was the result of an initial DNA
203 damaging event, we analyzed a single-cell RNA seq dataset of whole lung, five months post-
204 irradiation, which is associated with lung fibrosis. We identified upregulated DEGs in the AM
205 cluster of irradiated mice (**Table S4**) and computed the overlap with the upregulated DEGs of

206 Lamin A/C CKO AM. Among the genes that overlapped between these two scRNAseq datasets,
207 were *Cd63*, lysosomal prosaposin (*Psap*), *Wfdc21* and cathepsins (**Figure 4C, Figure S6D**). We
208 next tested if DNA damage itself or the signaling response to DNA damage was inducing CD63,
209 using Lamin A/C p53 DKO AM. In contrast to Lamin A/C CKO, elevation of CD63 expression
210 was blunted in Lamin A/C p53 DKO AM (**Figure 4D**). We therefore conclude that AM upregulate
211 CD63 in Lamin A/C CKO AM in a p53-dependent manner, and in response to irradiation in WT
212 AM. Together with our previous results, this suggests that Lamin A/C deficiency causes DNA
213 damage in AM, leading to p53 activation, which induces CD63 and senescence.

214

215 *CD63 is required for damaged DNA clearance in macrophages*

216 CD63 is a tetraspanin that has been proposed to participate to a wide range of intracellular
217 trafficking processes (Pols and Klumperman, 2009). However, the function of CD63 in
218 macrophages is not known. CD63 and lysosomes have been associated with genomic DNA
219 accumulating in the cytosol following the induction of DNA damage in tumor cell lines (Shen et
220 al., 2015) and in senescent regions of human fibrotic lung (Borghesan et al., 2019). CD63 has also
221 been implicated in loading nuclear content into exosomes following micronuclei formation in
222 cancer cell lines (Yokoi et al., 2019). This raised the possibility that CD63 is implicated in the
223 response to damaged DNA in macrophages. We performed dsDNA staining on bone-marrow
224 derived macrophages and observed an accumulation of cytoplasmic dsDNA in CD63 KO cells
225 compared to WT cells (**Figure 5A**). To investigate whether CD63 may play a role within the
226 context of damaged DNA in AM, we assessed the level of DNA damage accumulating in CD63
227 KO AM upon *ex vivo* application of etoposide, a DNA damage inducer. We detected a higher
228 percentage of γ H2AX⁺ AM in CD63 KO compared to WT in response to etoposide (**Figure 5B,**

229 **Figure S6E**). Taken together, these data reveal that CD63 is required to limit the accumulation of
230 DNA damage and clear cytoplasmic DNA in macrophages.

231

232 *Lamin A/C protects against influenza virus infection.*

233 Next, we sought to determine whether the AM alterations that characterize the lungs of
234 Lamin A/C CKO mice have physiopathological consequences. AM play a particularly critical
235 protective role in protection against influenza virus infection (Purnama et al., 2014; Schneider et
236 al., 2014). We therefore infected WT and Lamin A/C CKO mice with the mouse-adapted Influenza
237 A strain PR8. Lamin A/C CKO mice had a lower probability of survival than their WT littermates
238 (**Figure 6A**). Lamin A/C CKO mice also had more dramatic weight loss at critical disease
239 timepoints (**Figure 6B**). Significant weight loss in comparison to the day of virus inoculation was
240 initiated a day earlier than WT littermates (**Figure S7A**). We observed similar results when testing
241 a higher dose of Pr8 (**Figure S7B, 7C, 7D**). We conclude that Lamin A/C is required in immune
242 cells to protect against influenza virus infection.

243

244 *Lamin A/C protects against acquisition of hallmarks of aging in AM*

245 Poor disease outcomes following infection by respiratory viruses, such as influenza virus,
246 in both mice and humans, is a well-characterized consequence of an aged immune system
247 (Schneider et al., 2021). This raised the possibility that Lamin A/C CKO leads to accelerated aging
248 of AM. We showed that WT AM can undergo nuclear envelope rupture at confinement heights that
249 mimic the constricted spaces these cells migrate through *in vivo*. AM are long-lived, self-renewing
250 cells of embryonic origin (Kopf et al., 2015; Sieweke and Allen, 2013). We therefore hypothesized
251 that if repeated rupture events accumulate over time *in vivo*, WT AM should gradually acquire a
252 signature related to Lamin A/C CKO signature. In mice aged for 63 weeks, we detected a reduction

253 of AM (**Figure S8A**), in agreement with a drop in AM levels previously reported in mice aged for
254 more than 80 weeks (Krishnarajah et al., 2021; Wong et al., 2017; Xin et al., 2015). Next, we
255 analyzed gene expression in AM from mice of increasing age using the Tabula Muris Senis (TMS)
256 scRNAseq atlas, a resource characterizing aging in mouse tissues (The Tabula Muris Consortium
257 et al., 2020). We performed a differentially expression analysis between “young” (1, 3 months) and
258 “old” (18, 21, 30 months) age groups to define a signature of aging in AM (**Figure 7A, Table S5**).
259 Strikingly, *Cd63* was the top upregulated hit in each of the aged timepoints compared to each of
260 the young timepoints. *Cd63* expression was induced between 3 and 18 months of age (**Figure 7B**).
261 We analyzed mice at 14 weeks (young) and 63 weeks (old) of age, that had an identical genetic
262 background and were housed in the same facility. We confirmed at the protein level that aged AM
263 have a higher level of CD63 than their young counterparts (**Figure 7C**). We also conducted a gene
264 set enrichment analysis comparing the TMS signature of aging in AM and the signature of Lamin
265 A/C ablation in AM to appreciate if there was overlap (**Figure 7D, Figure S8B**). The Lamin A/C
266 CKO AM signature was enriched in the AM transcriptome of aged mice and the TMS signature of
267 aging in AM was also reciprocally enriched in the transcriptome of Lamin A/C CKO AM. By
268 intersecting the DEG identified in the 24 months vs 3 months TMS signature of aging in AM with
269 those identified in the Lamin A/C CKO AM signature we observed that along with *Cd63*, *Psap*,
270 *Wfdc17* and cathepsins were also upregulated in both datasets (**Figure 7E, left**). To confirm this
271 data, we extracted the signature of aging in AM from an independent dataset, the Lung Aging Atlas
272 (LAA) that compared the age points 3 months and 30 months (Angelidis et al., 2019). Using this
273 LAA signature, we also observed that *Cd63*, *Psap*, *Wfdc17* and cathepsins were again shared
274 between the Lamin A/C AM CKO signature and the LAA aged AM signature (**Figure 7E, middle**).
275 Finally, to appreciate how divergent different signatures of AM aging can be, we compared the
276 TMS signature of aging with the LAA signature of aging in AM (**Figure 7E, right**). Interestingly,

277 the two signatures were not identical, which could be explained by different facilities and genetic
278 backgrounds. However, within the overlap, upregulated expression of *Cd63*, *Psap*, *Wfdc17* and
279 cathepsins persisted, suggesting that these changes withstand possible background and
280 environmental variables. We did not detect elevated DNA damage at steady state in aged AM at
281 63 weeks based on γ H2AX staining, but susceptibility to DNA damage, that characterizes Lamin
282 A/C CKO AM, was slightly increased in old macrophages (**Figure S8C**). We therefore found that
283 *Cd63*, cathepsin expression and enhanced susceptibility to DNA damage are common features of
284 aging and Lamin A/C ablation in AM.

285

286

287 **Discussion**

288 Our results show that AM require Lamin A/C for protection against accumulation of nuclear
289 envelope rupture marks, genome instability and accelerated aging.

290 Within the lung immune compartment, AM expressed the highest level of Lamin A/C. AM
291 are long-lived cells that self-renew in tissues (Sieweke and Allen, 2013). The longevity of this
292 population *in situ* may necessitate a higher level of nuclear protection to limit the accumulation of
293 DNA damage over time. Lamin A/C levels are thought to scale with tissue stiffness (Swift et al.,
294 2013). Therefore, high Lamin A/C expression in AM may be an adaptation to counter repetitive
295 nuclear deformation and subsequent envelope ruptures caused by constricted migration, such as
296 through pores of Kohn. Such migration may be further stimulated by lung infection (Neupane et
297 al., 2020). Additional mechanical effects, such as constant inflation and deflation that occurs during
298 breathing, could also be a source of biomechanical stress leading to increased nuclear envelope
299 rupture.

300 Multiple mechanisms likely contribute to the induction of DNA damage during nuclear
301 envelope rupture in AM. In addition to limiting nuclear envelope rupture, Lamin A/C also
302 participates in DNA repair (Gruenbaum and Foisner, 2015). The transient loss of nuclear lamina
303 integrity during rupture in WT cells may thus transiently compromise the repair process. Cytosolic
304 leakage of DNA repair proteins after nuclear envelope rupture may also contribute to DNA
305 damage (Irianto et al., 2017; Xia et al., 2018). Finally, in epithelial and cancer cell lines, the ER-
306 associated exonuclease TREX1 is a driver of this damage after translocation into the nucleus post-
307 rupture (Maciejowski et al., 2015; Nader et al., 2021b). DNA damage caused by nuclear envelope
308 rupture may therefore be the result of a combination of these and other, yet to be determined,
309 molecular events.

310 Our results provide *in vivo* evidence for a CD63/lysosomal pathway that clears damaged
311 DNA (Shen et al., 2015; Yokoi et al., 2019) and implicate it in the process of aging. CD63 KO AM
312 were more susceptible to DNA damage than WT counterparts, and CD63 KO BMDM
313 constitutively accumulated DNA damage. This indicates that CD63 promotes clearance of
314 damaged DNA in macrophages. Our results also show that CD63 marks macrophages that age over
315 time or through acceleration with increased DNA damage. CD63 induction in Lamin A/C CKO
316 required p53, suggesting that it is a response to genome instability. Accordingly, we found that the
317 CD63/lysosome signature is also induced by exposing lung to ionizing radiation, which is known
318 to induce senescence and accelerate aging, further suggesting that the upstream cause is the
319 presence of DNA damage. While we could show that aged AM are more sensitive to etoposide
320 than young AM, we did not detect increased DNA damage at baseline in aged vs young AM using
321 γ H2AX quantification. This suggests that very low yet chronic DNA damage, below the threshold
322 of γ H2AX induction or detection, accumulates over-time to induce a CD63/lysosomal response. In
323 support of this idea, whole-lung analyses have detected an increase in DNA damage with age
324 (Braidly et al., 2011; Lee et al., 2021) and an accumulation of DNA damage with age is emerging
325 as a generalized feature of aging (Schumacher et al., 2021).

326 Our results indicate that disruption of Lamin A/C results in endogenous DNA damage that
327 is sufficient to induce the CD63/lysosomal signature of aged AM. Cell-extrinsic mechanisms likely
328 play an additional role in AM aging. The lung microenvironment has been implicated in age-related
329 changes occurring in AM (McQuattie-Pimentel et al., 2021) and lung tissue mechanics evolve
330 during aging (Sicard et al., 2018). Phagocytosis of DNA or other debris accumulating in the lung
331 spaces could also contribute to lysosome congestion, reducing the ability of AM to clear their own
332 damaged DNA. Exogenous DNA damaging events can also contribute to inducing the AM aging

333 signature, as we found with lethal irradiation. We speculate that additional exogenous events, such
334 as inhaled agents or infection, could also serve as source of exogenous DNA damage. Finally, we
335 also detected increased Cathepsin L activity and immune cell infiltrate detected in the airspaces of
336 mice with immune-specific ablation of Lamin A/C, which could be triggered initially by an AM-
337 intrinsic defect, but subsequently exacerbated by cell-extrinsic responses to a changing
338 microenvironment.

339 While the progressive acquisition of senescent AM during aging is detrimental, as shown
340 by higher susceptibility to influenza infection in Lamin A/C CKO mice, senescence induction in
341 AM may be beneficial early in life, in agreement with the antagonistic pleiotropy hypothesis
342 (Blagosklonny et al., 2009). For example, it might be a safety mechanism to prevent expansion of
343 damaged cells that self-renew.

344 Advanced age is a major risk factor for the development of a range of lung diseases. These
345 include conditions such as chronic obstructive pulmonary disease, lung cancer and respiratory
346 infections such as influenza and most pressing, SARS-CoV-2 (Schneider et al., 2021). AMs play
347 central roles in these diseases (Casanova-Acebes et al., 2021; Purnama et al., 2014; Schneider et
348 al., 2014). We propose that Senescence INduction by Nuclear Envelope Rupture (Nader et al.,
349 2021b) (SINNER) is a mechanism of aging in lung alveolar macrophages. Targeting nuclear
350 envelope rupture may thus constitute a novel opportunity for therapeutic intervention in disease
351 and aging. Beyond AM, we propose that SINNER may be a mechanism of aging in other cell types,
352 that remain to be uncovered.

353

354

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371

372 **Declaration of interests**

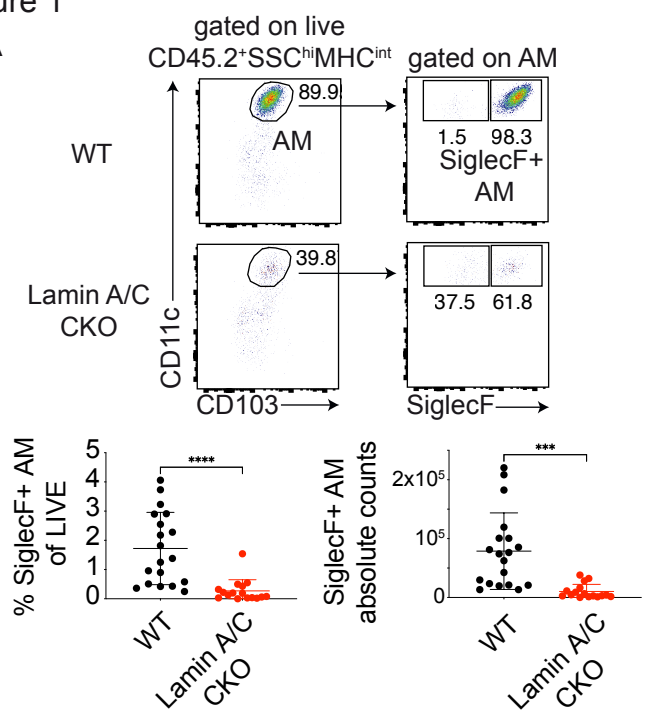
373 The authors do not have any competing interest to declare.

374 Movies S1 – S2

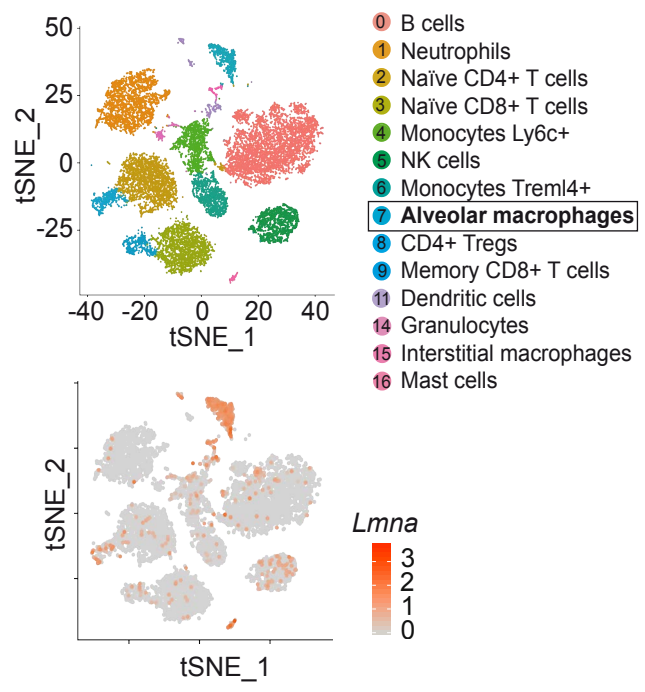
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Figure 1

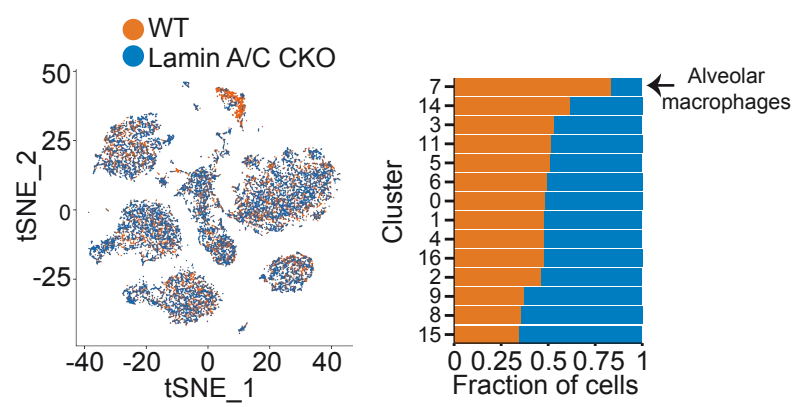
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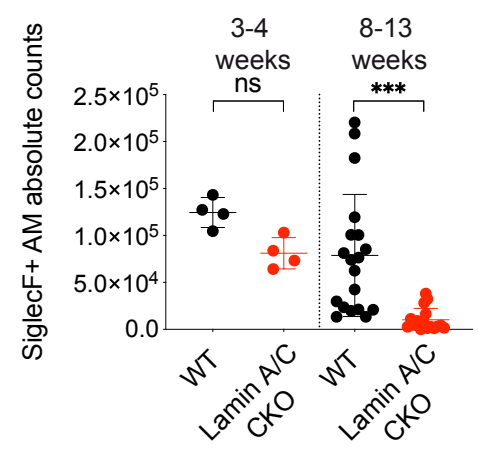
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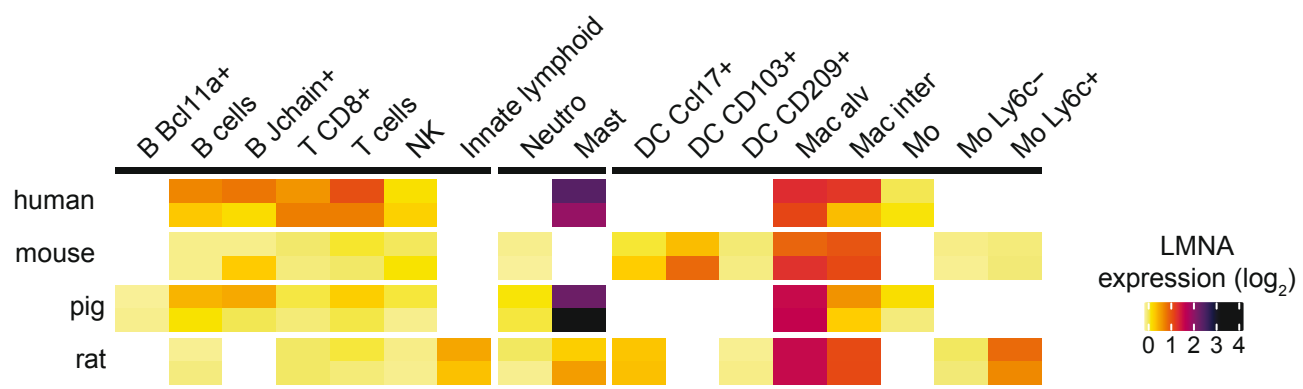
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D



E



376 **Figure 1 Lamin A/C protects alveolar macrophages from depletion.**

377 **(A)** Flow cytometric analysis of AM in WT and Lamin A/C CKO lungs. Top, representative
378 samples. Bottom, percentage of live and absolute counts of AM (n = 16-19 mice combined from
379 10 independent experiments, bar indicates mean \pm SD unpaired t-test).

380 **(B)** tSNE representation of cells identified via single cell RNA sequencing (scRNAseq) of
381 CD45.2+ lung immune cells sorted from WT and Lamin A/C CKO mice (n = 2 female mice per
382 genotype). Top, cluster labelling. Bottom, normalized *Lmna* expression.

383 **(C)** Annotation of WT and Lamin A/C CKO cells in each cluster. Left, tSNE representation. Right,
384 fraction of WT and Lamin A/C CKO per cluster.

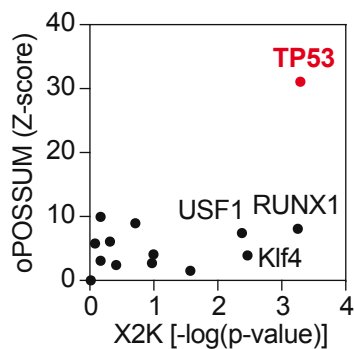
385 **(D)** Absolute counts of AM (CD45.2⁺SSC^{hi}MHC^{int}SiglecF⁺) in lung of WT vs Lamin A/C CKO
386 lung based on flow cytometric analysis at 3-4 and 8-13 weeks of age (n = 4-19 mice, combined
387 from 12 independent experiments, one-way ANOVA with Šidák).

388 **(E)** Expression of the Lamin A/C-coding gene in clusters of lung cells from human, rat, mouse and
389 pig.

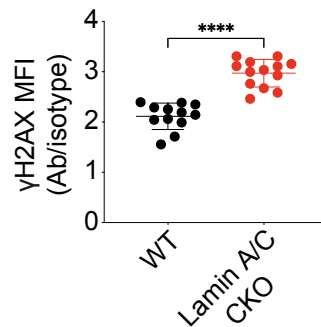
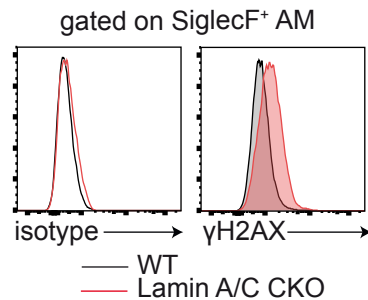
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Figure 2

A



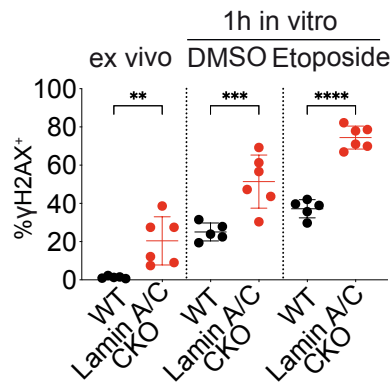
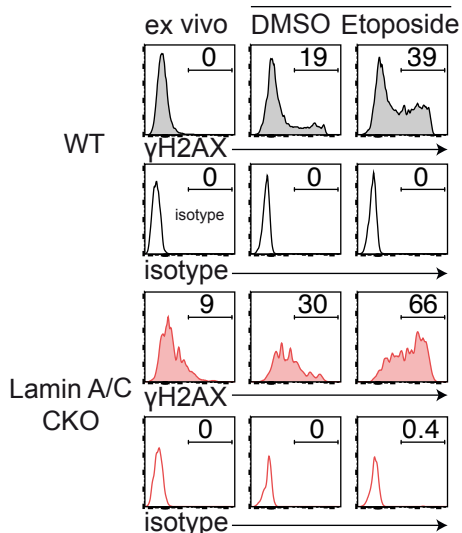
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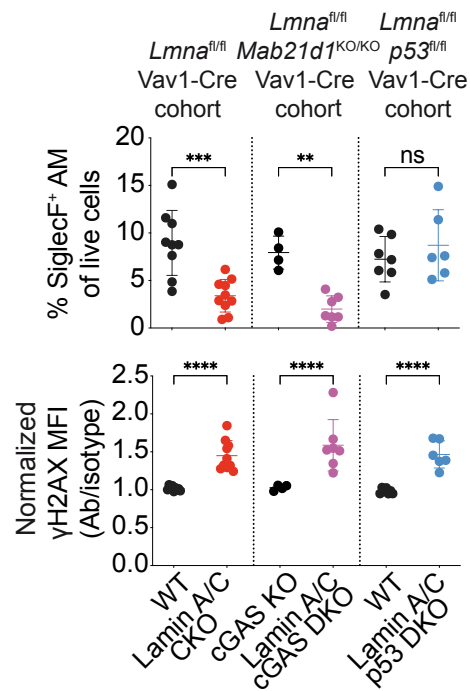
C

gated on SiglecF⁺ AM

1h in vitro



D



391 **Figure 2 Loss of alveolar macrophages is caused by a p53-dependent response to DNA**
392 **damage.**

393 **(A)** Transcription factor binding site enrichment in promoters of differentially expressed genes
394 (DEGs) upregulated in Lamin A/C CKO and specific to the AM cluster, using oPOSSUM and X2K
395 methods.

396 **(B)** Intracellular levels of γ H2AX within WT and Lamin A/C CKO AM
397 ($CD45.2^{+}SSC^{hi}MHC^{int}SiglecF^{+}$). Left, representative samples. Right, mean fluorescence intensity
398 (MFI) of γ H2AX signal normalized to isotype control signal (n = 12-13 mice combined from 11
399 independent experiments, bar indicates mean \pm SD, unpaired t test).

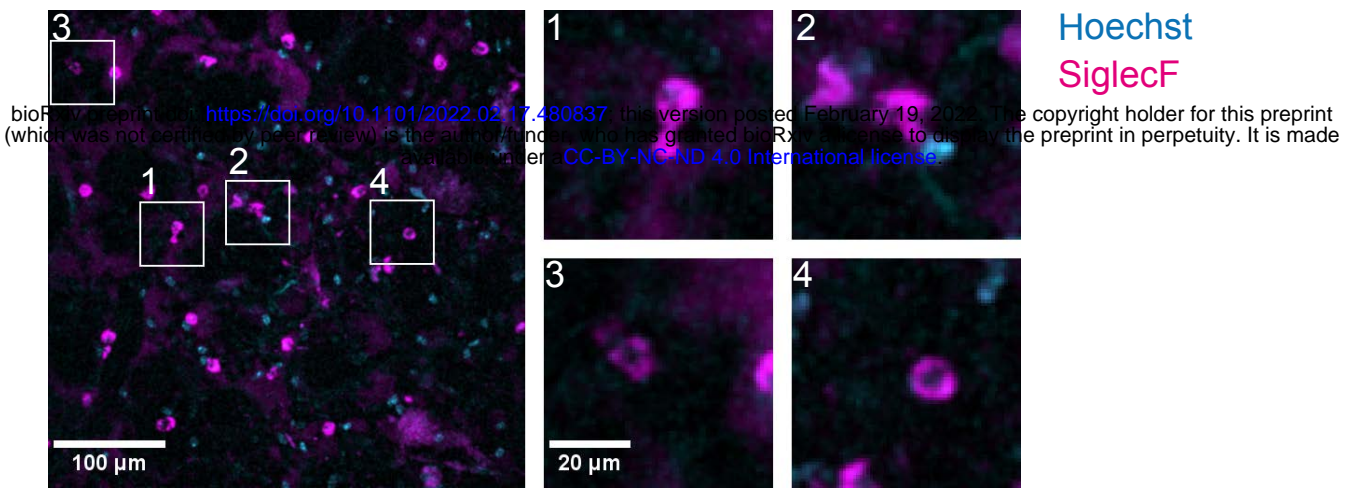
400 **(C)** γ H2AX response to DNA damage in AM ($CD45.2^{+}SSC^{hi}MHC^{int}SiglecF^{+}$). WT vs Lamin A/C
401 CKO lung single cell suspensions were analyzed directly or incubated with DMSO or 50 μ M
402 etoposide for 1 hour. Left, representative samples. Right, quantification of γ H2AX⁺ cells (n = 5 or
403 6 mice, combined from 3 independent experiments, bar indicates mean \pm SD, one-way ANOVA
404 with Šidák test).

405 **(D)** Impact of cGAS and p53 deficiencies on AM percentages and γ H2AX levels in Lamin A/C
406 CKO mice. 3 breeding cohorts are shown: WT ($Lmna^{fl/fl}Vav1-Cre^{-/-}$) vs Lamin A/C CKO ($Lmna^{fl/fl}$
407 $Vav1-Cre^{+/-}$), cGAS KO ($Lmna^{fl/fl}Mab21d1^{KO/KO}Vav1-Cre^{-/-}$) vs Lamin A/C cGAS DKO ($Lmna^{fl/fl}$
408 $Mab21d1^{KO/KO}Vav1-Cre^{+/-}$) and WT ($Lmna^{fl/fl}p53^{fl/fl}Vav1-Cre^{-/-}$) vs Lamin A/C p53 DKO
409 ($Lmna^{fl/fl}p53^{fl/fl}Vav1-Cre^{+/-}$). In each experiment, cGAS KO and Lamin A/C cGAS DKO or WT
410 and Lamin A/C p53 DKO were analyzed together with age-matched WT and Lamin A/C CKO
411 mice. Top, fraction of AM. Bottom, γ H2AX MFI is first normalized to isotype control level in each
412 sample, and next to WT analyzed in the same experiment (n = 4-10 mice combined from 8
413 independent experiments, bar indicates mean \pm SD, one-way ANOVA with Šidák test).

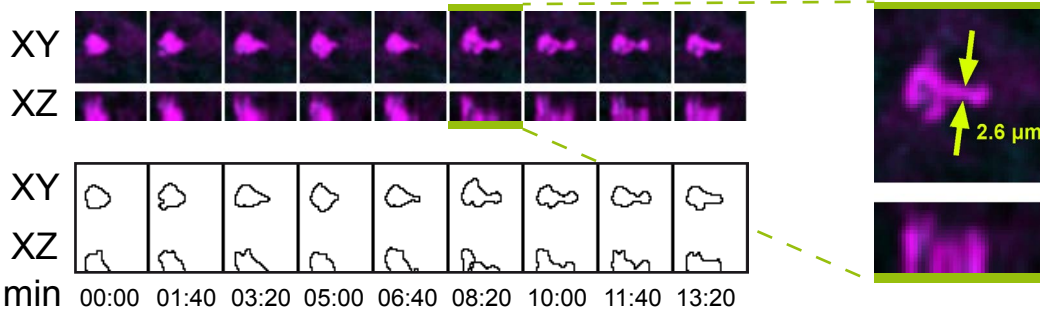
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Figure 3

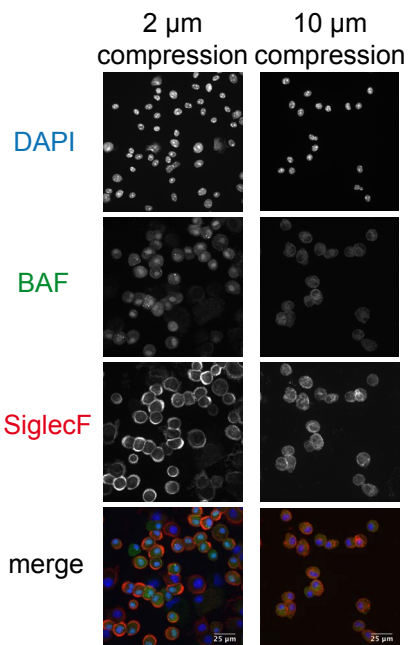
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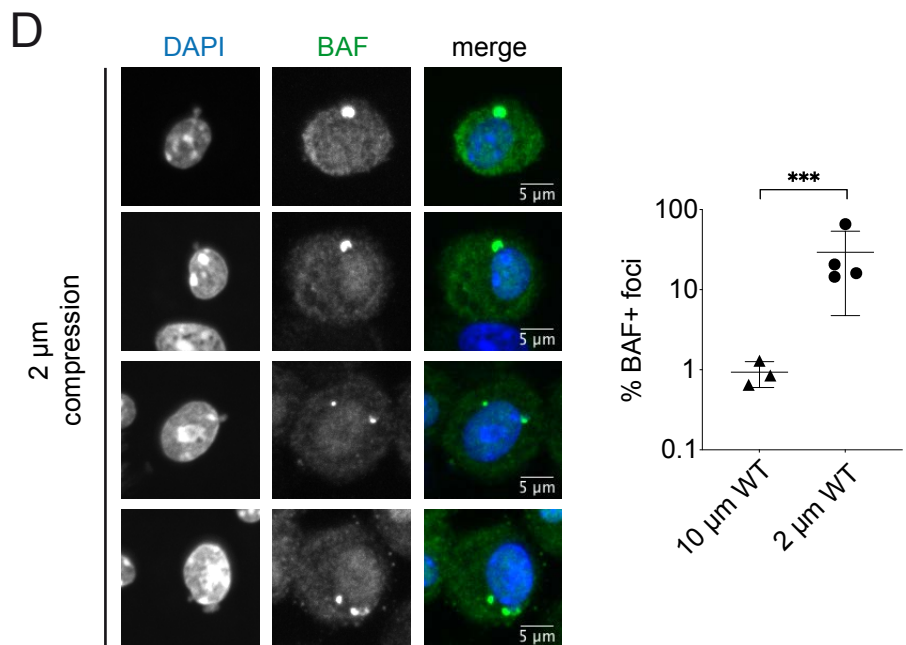
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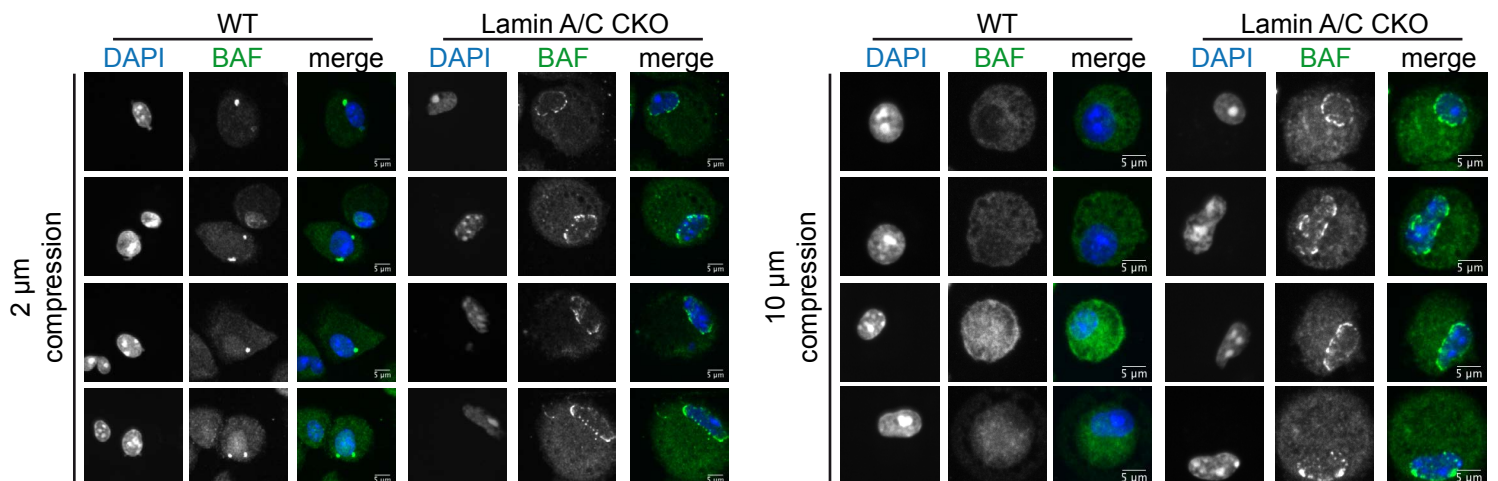
C



D



E



415 **Figure 3 Constricted migration and nuclear envelope rupture in alveolar macrophages.**

416 **(A)** Live imaging of WT mouse lung (female, 12 weeks old) after administration of Hoechst and
417 an anti-SiglecF antibody. Left, broad field of a lung region. Right, individual SiglecF⁺ AM
418 demonstrating constricted migration (see also Movie 1).

419 **(B)** Time-lapse of an AM undergoing constricted migration. Top left, images of XY and XZ planes
420 overt time. Bottom left, contour representation. Right, measurement of the cell width at the most
421 acute point of squeezing.

422 **(C)** Cells from the bronchialveolar lavage (BAL) of WT mice confined at a height of 2 μ M and 10
423 μ m for 1.5 hours and subsequently stained for DAPI, BAF and SiglecF (representative of n = 4
424 independent experiments, each time BAL was pooled from 2 mice aged 8-26 weeks).

425 **(D)** BAF foci in AM (SiglecF⁺) from WT BAL following confinement for 1.5 hours. Left,
426 representative images at 2 μ M height. Right, quantification of the percentage of BAF foci⁺ AM
427 after confinement at 2 μ m or 10 μ m (n = 3-4 independent experiments, each time BAL was pooled
428 from 2 mice aged 8-26 weeks, bar indicates mean \pm SD, unpaired t test on log-transformed data).

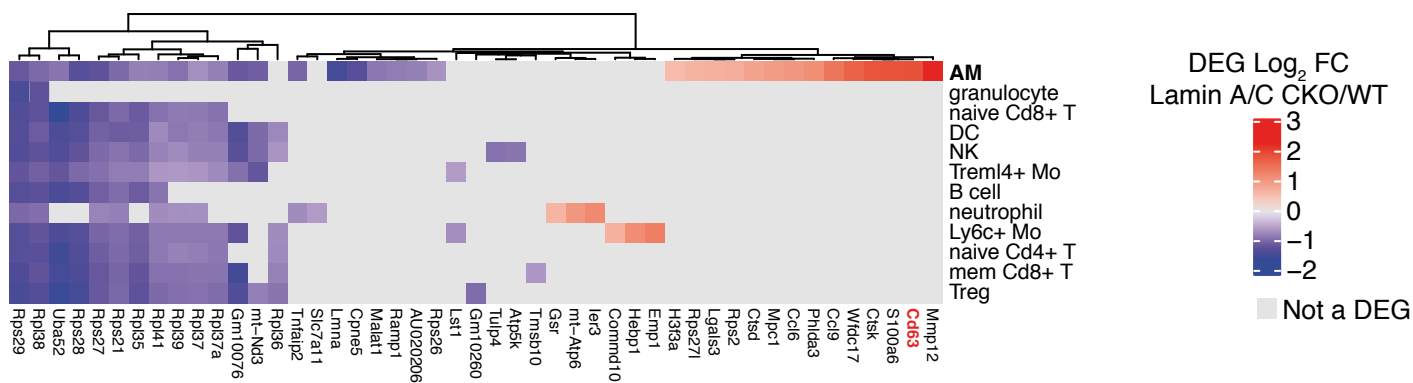
429 **(E)** BAF staining in AM (SiglecF⁺) from WT and Lamin A/C CKO BAL, following confinement
430 at 2 μ m and 10 μ m for 1.5 hours (representative of n = 3-4 experiments, each time BAL was pooled
431 from 2 mice aged 8-26 weeks). Different scale bars were used between 2 μ m and 10 μ m to
432 accommodate for cell spreading at 2 μ m.

433

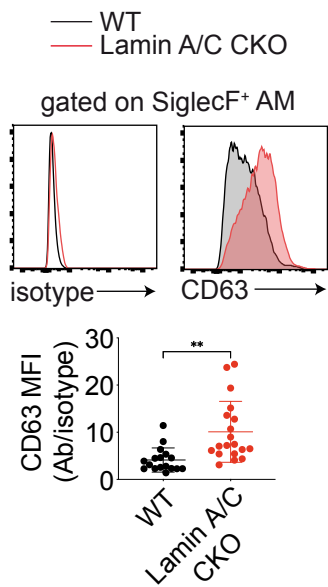
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Figure 4

A



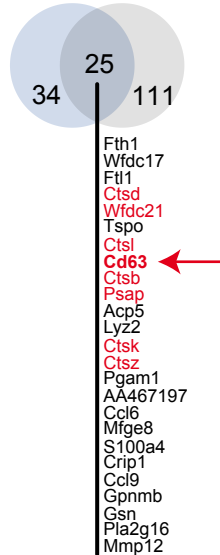
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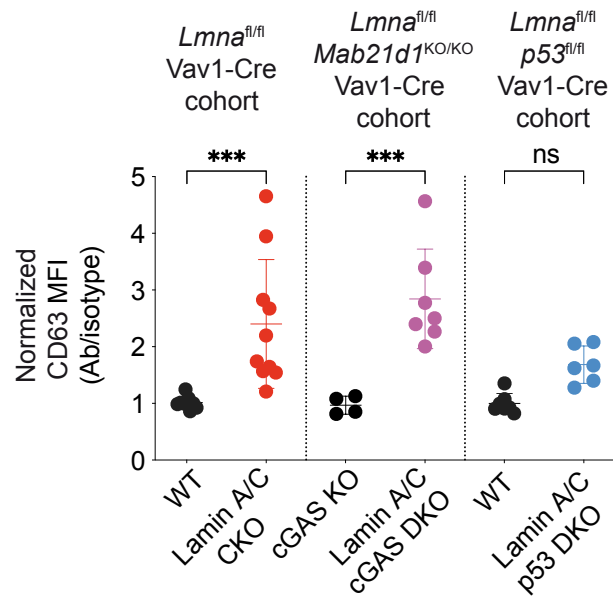
C

Lamin A/C CKO vs WT

Irradiated vs control



D



435 **Figure 4 A lysosomal/CD63 response is induced by DNA damage.**

436 **(A)** Heatmap showing the significant DEGs in Lamin A/C CKO vs WT cells within cell clusters.

437 Cell clusters are ordered by decreasing fraction of WT cells. Genes were clustered with a complete

438 linkage method computed on the Manhattan distances between genes, shown as dendrogram.

439 **(B)** Intracellular levels of CD63 in lung AM (CD45.2⁺SSC^{hi}MHC^{int}SiglecF⁺). Top, representative

440 samples. Bottom, CD63 MFI normalized to isotype control (n = 17-19 mice combined from 15

441 independent experiments, bar indicates average \pm SD, unpaired t test).

442 **(C)** Intersection of the DEGs identified in Lamin A/C CKO vs WT AM and the DEGs identified

443 in AM 5 months post-17 Gy irradiation vs no irradiation (control). Cd63, Psap and cathepsin genes

444 are highlighted in red.

445 **(D)** Intracellular levels of CD63 in lung AM (CD45.2⁺SSC^{hi}MHC^{int}SiglecF⁺) from 3 breeding

446 mouse cohorts: WT (*Lmna*^{fl/fl} Vav1-Cre^{-/-}) vs Lamin A/C CKO (*Lmna*^{fl/fl} Vav1-Cre^{+/-}), cGAS KO

447 (*Lmna*^{fl/fl} *Mab21d1*^{KO/KO} Vav1-Cre^{-/-}) vs Lamin A/C cGAS DKO (*Lmna*^{fl/fl} *Mab21d1*^{KO/KO} Vav1-

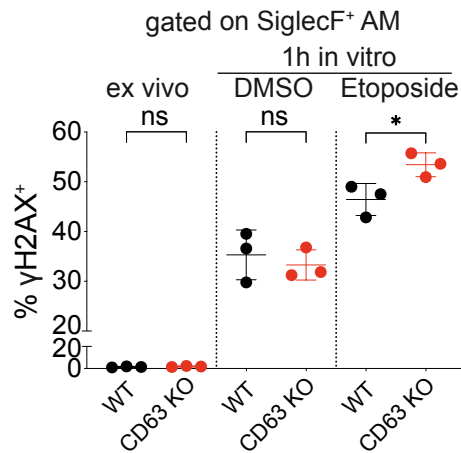
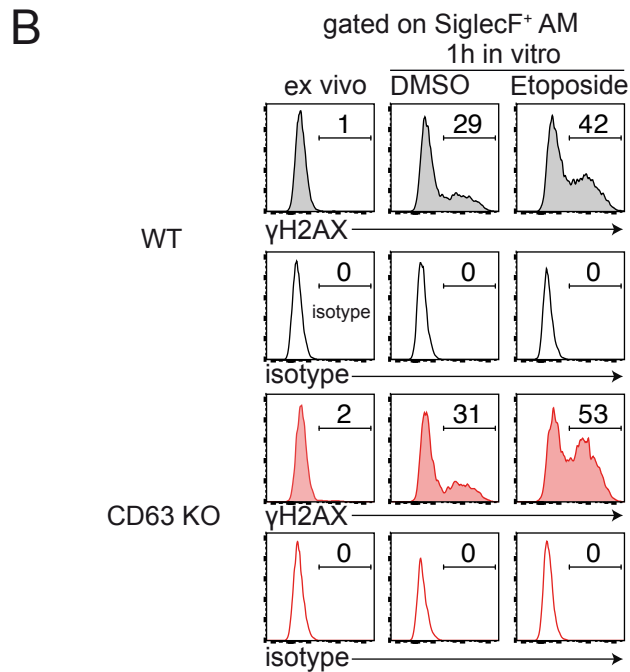
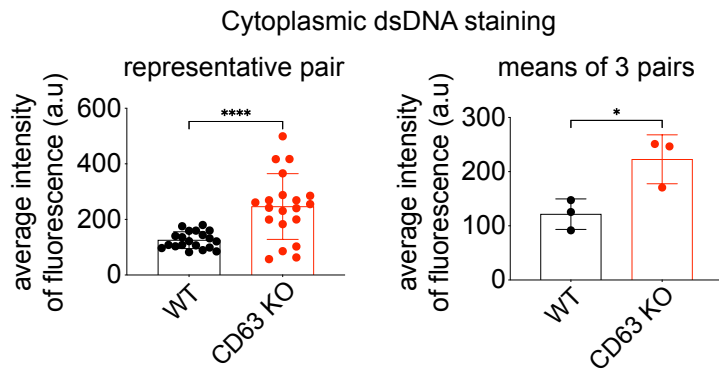
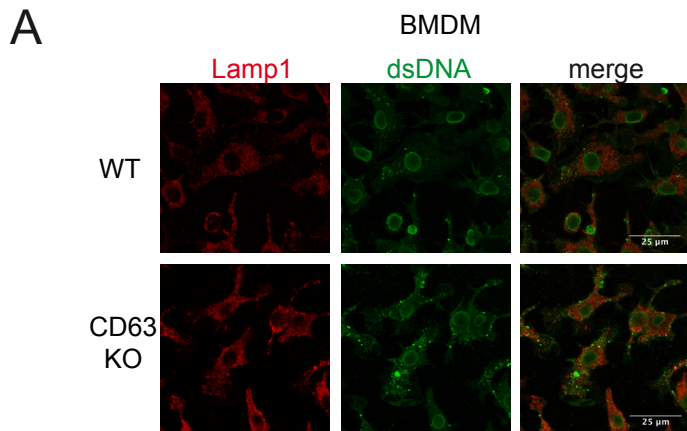
448 Cre^{+/-}) and WT (*Lmna*^{fl/fl} *p53*^{fl/fl} Vav1-Cre^{-/-}) vs Lamin A/C p53 DKO (*Lmna*^{fl/fl} *p53*^{fl/fl} Vav1-Cre^{+/-})

449 (n = 4-10 mice combined from 8 independent experiments, bar indicates mean \pm SD, one-way

450 ANOVA with Šidák test).

451

Figure 5



452 **Figure 5 CD63 is required for clearance of damaged DNA in macrophages.**

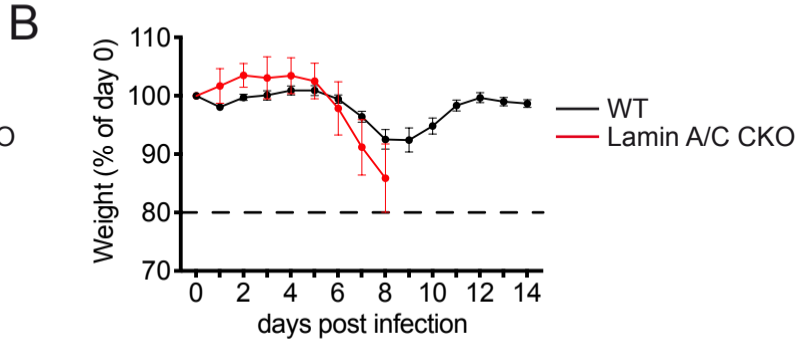
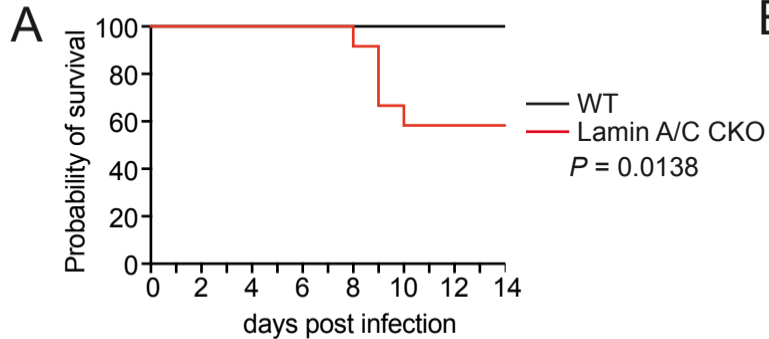
453 **(A)** Lamp1 and dsDNA staining in bone marrow-derived macrophages (BMDM) from WT and
454 CD63 KO mice. Left, representative images. Middle, average fluorescence intensity of the dsDNA
455 signal per cell quantified outside the nucleus in a representative pair of WT vs CD63 KO (each
456 symbol represents a single cell, Kolmogorov-Smirnov test). Right, average dsDNA signal per
457 mouse ($n = 3$ mice per genotype in one experiment, 49 weeks old, bar indicates mean \pm SD,
458 unpaired t-test).

459 **(B)** γ H2AX levels within AM ($CD45.2^+SSC^{hi}MHC^{int}SiglecF^+$) of WT vs CD63 KO lung single cell
460 suspension, directly ex vivo or after incubation with DMSO or 50 μ M etoposide for 1 hour. Left,
461 representative samples. Right, fraction of γ H2AX positive cells ($n = 3$ mice per genotype in one
462 experiment, 19 weeks old, bar indicates mean \pm SD, one-way ANOVA with Šidák test).

463

464

Figure 6



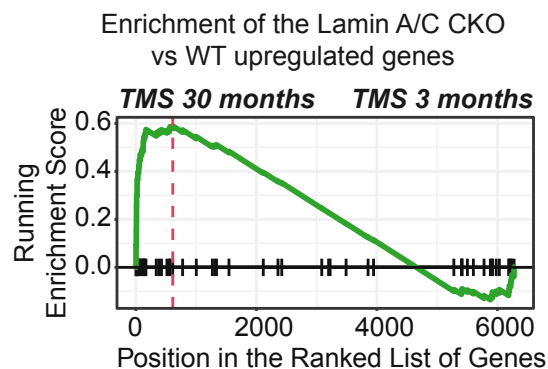
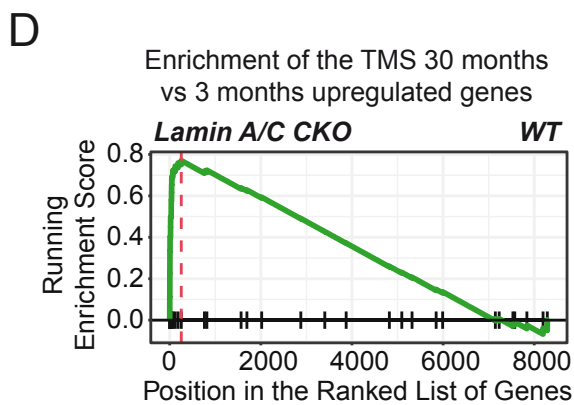
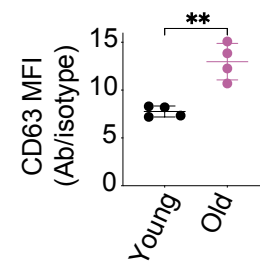
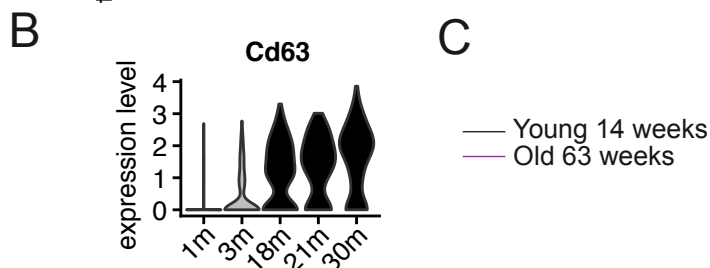
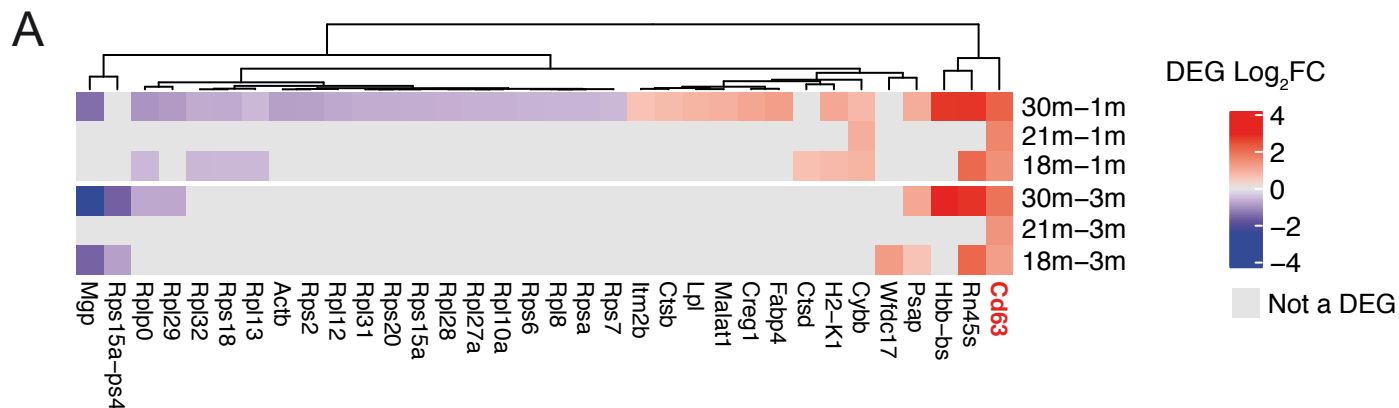
465 **Figure 6 Lamin A/C protects against influenza virus infection.**

466 **(A)** Survival of WT vs Lamin A/C CKO mice following infection with 50 pfu of Influenza A The
467 virus PR8 was delivered via the intranasal route (n = 12 female mice per genotype, combined from
468 2 independent experiments, Log-rank Mantel-Cox test).

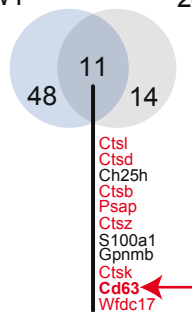
469 **(B)** Percentage of day 0 weight observed each day post infection described in (E). Curves of
470 average \pm SEM weights are shown and continued until a first death occurs in the group.

471

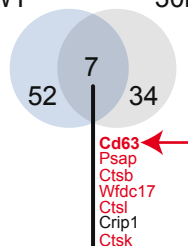
Figure 7



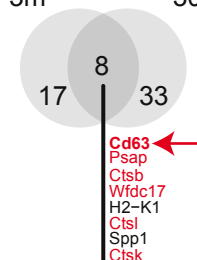
E Lamin A/C CKO vs WT Tabula muris senis 24m-3m



Lamin A/C CKO vs WT Lung Aging Atlas 30m-3m



Tabula muris senis 24m-3m Lung Aging Atlas 30m-3m



472 **Figure 7 Lamin A/C protects against hallmarks of aging in alveolar macrophages.**

473 **(A)** Heatmap showing the significant DEGs in old (18, 21, 30 months) vs young (1 month, 3
474 months) comparisons in AM in the TMS dataset. Genes were clustered with a complete linkage
475 method computed on the Manhattan distances between genes, shown as dendrogram.

476 **(B)** Violin plot showing the normalized expression of *Cd63* in the AM cluster at different ages.

477 **(C)** Intracellular CD63 levels in AM (CD45.2⁺SSC^{hi}MHC^{int}SiglecF⁺) of young (14 weeks) vs old
478 (63 weeks) mice. Left, representative samples. Right, CD63 MFI normalized to isotype control (n
479 = 4 mice combined from 2 independent experiments, bar indicates mean \pm SD, unpaired t test).

480 **(D)** Reciprocal gene set enrichment analyses, as indicated on figure.

481 **(E)** Intersection of the upregulated DEGs in Lamin A/C CKO vs WT, TMS 24 months vs 3 months
482 and LAA 30 months vs 3 months. Common genes are shown in red and *Cd63* is highlighted.

483

484

485 **STAR Methods**

486 Mice

487 All animals were used according to protocols approved by Animal Committee of Curie Institute
488 CEEA-IC #118 and maintained in pathogen-free conditions in a barrier facility. Experimental
489 procedures were approved by the Ministère de l'enseignement supérieur, de la recherche et de
490 l'innovation (APAFIS#24911-2020033119476092-v1) in compliance with international
491 guidelines. C57BL/6JMb21d1tm1d(EUCOMM)Hmgu (Mb21d1^{KO/KO}) mice were obtained from
492 The Jackson Laboratory. *Lmna*^{fl/fl} Vav1-Cre^{+/-} (Lamin A/C CKO mice, (de Boer et al., 2003; Kim
493 and Zheng, 2013)) and *Lmna*^{fl/fl} LysM-Cre^{+/-} (Clausen et al., 1999) were obtained from José María
494 González Granado. p53^{fllox/fllox} mice (Jonkers et al., 2001) were obtained from Renata Basto.
495 CD63^{KO/KO} were obtained from Paul Saftig (Schröder et al., 2009). The sex of mice used in the
496 experiments shown are listed in **Table S6**. Littermate controls were used in each experiment. The
497 age range was 6-13 weeks unless otherwise stated. Female C57BL/6J elderly mice and young
498 controls were purchased from Charles River Laboratories. Unless otherwise stated, WT controls
499 are *Lmna*^{fl/fl} Vav1-Cre^{-/-}. For analysis of DKO animals (Lamin A/C cGAS DKO, Lamin A/C p53
500 DKO), Lamin A/C CKO of the same age were analyzed with DKO animals in each experiment,
501 together with controls from each cohort.

502

503 Lung preparation for FACS analysis

504 Dissected lungs were mechanically disrupted using scissors and digested for 30 minutes with
505 0.4mg/mL collagenase (Sigma C2139) + 20µg/mL DNase I (Sigma 10104159001) in RPMI at 37
506 degrees in 12 well tissue culture plates. This was followed by homogenization of the tissue mix
507 over a 100µm filter using 1% BSA/1mM EDTA/PBS and a 5mL syringe plunger. Red blood cells

508 were lysed for five minutes at 4 degrees using red blood cell lysis buffer (Ozyme 42031) and the
509 cells were washed and filtered again over a 100 μ m filter. The resulting single cell lung suspension
510 was then used in FACS analysis (see **Table S7** for a list of antibodies used in the study) and cell
511 sorting.

512

513 Spleen and lymph node preparation for FACS analysis

514 Dissected spleen was injected with 0.125mg/mL Liberase (Sigma 5401020001) + 20 μ g/mL DNase
515 I (Sigma 10104159001) and inguinal and brachial lymph nodes were disrupted using scissors and
516 incubated with the same Liberase/DNaseI mix. The organs were digested for 20 minutes with
517 Liberase/DNaseI at 37 degrees in 12 well tissue culture plates. This was followed by
518 homogenization of the tissue mixes over a 40 μ m filter using 1% BSA/1mM EDTA/PBS and a 5mL
519 syringe plunger. Red blood cell lysis was performed as described above and the cells were washed
520 and filtered again over a 40 μ m filter. The resulting single cell suspensions were used in FACS
521 analysis.

522

523 Bone marrow extraction

524 Tibias and femurs were cleaned and cut at the ends. Bone marrow was flushed via centrifugation
525 at 2000 rcf for 20 seconds at 4 degrees. Extracted bone marrow was then used directly to generate
526 BMDMs or red blood cell lysis was performed as described above and the cells were washed and
527 filtered over a 40 μ m filter. The resulting single cell suspension was used in FACS analysis.

528

529 Flow cytometry (FACs) analysis

530 BAL cells, lung, spleen, lymph node or bone marrow single cell suspension was first stained
531 using a Fixable Viability Dye (ebioscience 65-0865-14) for 20 minutes at 4 degrees in PBS. The
532 cells were then washed with 1% BSA/1mM EDTA/PBS (FACs buffer) and stained with antibodies
533 specific for surface antigens for 30 minutes at 4 degrees in FACs buffer. If intracellular staining
534 was also performed, the Foxp3 Transcription Factor Staining Buffer Set (eBioscience 00-5523-00)
535 was used following the manufacturer's instructions. For surface staining of CD88, cells were
536 incubated with Fc block in FACs buffer for 15 minutes at 4 degrees after live/dead staining prior
537 to staining for surface antigens. See **Table S7** for details on antibodies used for cell surface and
538 intracellular staining. Cells were filtered over 40µm filter-cap FACs tubes in FACs buffer just prior
539 to analysis on an LSRII flow cytometer.

540

541 Cell sorting and scRNAsequencing

542 Lung single cell suspension was stained for FACs analysis as described above (live/dead and
543 surface staining) and CD45.2+ lung immune cells were sorted using a BD FACs Aria III from 2
544 $Lmna^{fl/fl}Vav1-Cre^{+/-}$ and 2 $Lmna^{fl/fl}Vav1-Cre^{-/-}$ littermate controls (all female and 10 weeks old).
545 A 100µm nozzle and 20 psi pressure was used during sorting. Cells were sorted into 20% FBS in
546 RPMI with penicillin-streptomycin, 50µM 2-Mercaptoethanol, 1X non-essential amino acids,
547 10mM HEPES and 1mM sodium pyruvate added and kept at 4 degrees. After cell counting using
548 a LUNA automated cell counter (Logos biosystems), 9600 CD45.2+ lung immune cells per sample
549 were processed using the Chromium Single Cell 3' Reagents Kits v3 following the manufacturer's
550 instructions and sequenced using 25,000 reads per cell on a Illumina NovaSeq 6000.

551

552 Ex vivo etoposide treatment

553 Lung single cell suspension was divided into three parts. One part was kept at 4 degrees
554 (unstimulated), and the remaining 2 parts were stimulated with DMSO or 50 μ M etoposide (Sigma
555 E1383) for 1 hr at 37 degrees 5% CO₂ in 6 well tissue culture plates. After 1 hour the DMSO and
556 etoposide-treated cells were re-harvested via flushing and these samples together with the
557 unstimulated sample, were stained for FACs analysis as described above (live/dead, surface and
558 intracellular staining).

559

560 Bronchialveolar lavage (BAL)

561 Mice were euthanized via gentle cervical dislocation under isoflurane-mediated anesthesia. An
562 incision was made at the voice box and a 1mL syringe with a 18G blunt safety transfer needle
563 (Dutscher 303129) attachment was inserted into the trachea. Five, 1mL washes of the lung
564 airspaces were performed using warm 0.5% BSA/2mM EDTA/PBS. For flow cytometry analysis
565 or cell confinement, red blood cells were lysed as described above and the BAL cells were washed
566 and filtered over a 40 μ m filter. For the measurement of cathepsin L activity in BAL, the first 1mL
567 BAL wash was stored separately and the supernatant was used immediately or aliquoted and frozen
568 for future analysis.

569

570 Multi-photon imaging

571 Two-photon intravital lung imaging was performed according to Dr Simon Cleary
572 recommendations (Cleary et al., 2020). Following anesthesia with ketamine/xylazine (80/15 mg/kg
573 i.p.), slow intratracheal administration of 50 μ l of a PBS solution containing (1mg/ml) Hoechst and
574 1 μ g of anti-siglecF-PE antibody was performed. Subsequently mice were ventilated with room air
575 plus 1.5% isoflurane at 125 breaths/min at 10 μ L/g body weight breath volume (Minivent, Harvard

576 Apparatus), with 2–3 cmH₂O positive end-expiratory pressure. A custom-made thoracic window
577 was then inserted into an intercostal incision and the left lung was immobilized against the window
578 with 10-30 mmHg negative pressure. Local temperature was monitored and maintained at 33°C
579 using an incubation chamber. The two-photon laser-scanning microscopy (TPL SM) set-up used
580 was a 7MP (Carl Zeiss) coupled to a Ti: Sapphire Crystal multiphoton laser (ChameleonU,
581 Coherent), which provides 140-fs pulses of near-infrared light, selectively tunable between 680
582 and 1050 nm and an optical parametric oscillator (OPO-MPX, Coherent) selectively tunable
583 between 1,050 and 1,600 nm. The NLO and the OPO beams were spatially aligned and temporally
584 synchronized using a delay line (Coherent). The excitation wavelength was 820 nm for the NLO
585 beam and 1070 nm for the OPO beam. The system included a set of external nondescanned
586 detectors in reflection with a combination of a LP-600-nm dichroic mirror (DM) followed by LP-
587 462-nm DM with 417-/60-nm emission filter (EF), LP-500-nm DM with 480-/40-nm EF, LP-
588 550nm DM with 525-/50-nm and 575/50 nm EFs. Real time movies were performed by imaging
589 every 10s by 5 consecutive 3µm z spacing image stack (total 12µm thickness). For all images the
590 objective was a water immersion, plan apochromat ×20 (numerical aperture = 1).

591

592 Cell confinement

593 Confinement of BAL cells was performed as described (Nader et al., 2021b). Briefly, a 6-well plate
594 cell confiner was used to confine BAL cells at 2µm and 10µm using coverslips containing a
595 microfabricated layer of PDMS micropillars of defined heights, with the height of the pillars
596 determining the height of spatial confinement of cells placed between the coverslip and 6-well plate
597 surface. The coverslips were applied to the cells using large PDMS pillars attached to a modified
598 6-well plate cover-lid. These large PDMS pillars served to push the confining coverslips onto the

599 cells to confine them at desired heights on the 6 well glass/plastic bottom plates. Imaging of the
600 cells was performed using the same plates (see below).

601

602 Immunofluorescence of confined BAL

603 For nuclear shape analysis, BAL cells were stained with Hoescht 33342 (Invitrogen R37605) prior
604 to cell confinement. In 1 of the 3 experiments, the cells were also stained for surface expression of
605 CD11c and SiglecF prior to confinement. The cells were imaged immediately while still confined
606 using an epifluorescence DMI-8 Leica inverted microscope equipped with a Hamamatsu OrcaFlash
607 4.0 Camera. For immunofluorescence staining, BAL cells were confined for 1.5 hours at at 37
608 degrees 5% CO₂. Staining was performed in the 6 well plate used during confinement after removal
609 of the pillars/coverlips. The cells were fixed with 4% paraformaldehyde for 20 minutes at room
610 temperature and quenched with 0.1M glycine (Life Technologies) for 10 minutes at room
611 temperature. Permeabilization was performed using 0.2% BSA/0.05% saponin in PBS (IF buffer)
612 for 30 minutes at room temperature. The samples were incubated with primary antibodies in IF
613 buffer + 10% goat serum (Sigma G9023) overnight at 4 degrees in a humidity chamber. After 4
614 washes with IF buffer the secondary antibodies were incubated for 1 hour at room temperature
615 protected from light. The samples were then washed 5 times with IF buffer and a further 2 times
616 each with PBS and then mounting in Fluoromount-G Mounting Medium with DAPI (Fisher
617 scientific 15596276). Samples were kept at 4 degrees, shielded for light prior to imaging. Imaging
618 was performed using spinning-disc confocal microscope with a Yokogawa CSU-X1 spinning-disc
619 head on a DMI-8 Leica inverted microscope equipped with a Hamamatsu OrcaFlash 4.0 Camera,
620 a Nano-ScanZ piezo focusing stage (Prior Scientific) and a motorized scanning stage (Marzhauser).
621 Both microscopes were controlled by MetaMorph software (Molecular Devices).

622 A macro was developed to measure nuclear shape of confined BAL cells using ImageJ in which
623 individual nuclear regions were identified after thresholding using the DAPI channel and the
624 Analyze Particles function was run. Three repeats of this experiment were performed, and the third
625 included manual selection of CD11c+SiglecF+ cells. Another macro was developed to quantify the
626 number of γ H2AX foci and their intensity in confined BAL cells. Here, individual nuclear regions
627 were identified after thresholding using a Z projection of the DAPI channel and SiglecF- cells were
628 excluded. The number of individual γ H2AX foci within nuclei was quantified using the Find
629 Maxima function on a Z projection of the γ H2AX channel, and the mean intensity of these spots
630 per nuclei was measured.

631

632 Measurement of Cathepsin L activity

633 Fresh or thawed BAL supernatant was analyzed using a Cathepsin L Activity Assay Kit (Abcam
634 ab65306) kit following the manufacturer's instructions and the sample fluorescence was measured
635 using a CLARIOstar microplate reader (BMG LABTECH). Readings were normalized by the
636 volume of BAL retrieved.

637

638 BMDM differentiation and immunofluorescence

639 Extracted bone marrow cells (see methods above) were plated at 1million/mL on untreated plates
640 in RPMI with 10% FBS, penicillin-streptomycin, 50 μ M 2-Mercaptoethanol, 1X non-essential
641 amino acids, 10mM HEPES and 1mM sodium pyruvate + 10ng/mL human M-CSF (Miltenyi
642 Biotec 130-096-492). Cells were left undisturbed for 6 days and then harvested following
643 detachment using trypsin. BMDM were first fixed in in 2% PFA for 15 minutes in cell culture
644 medium and then 2% PFA in PBS 1X. The cells were washed 3 times in PBS and quenched with

645 50mM Glycine in PBS for 20 minutes at room temperature, followed by 3 PBS washes. After
646 quenching, the cells were permeabilized in 0.2% BSA, 0.1% saponin in PBS for 15 minutes at
647 room temperature and blocked in 1% BSA, 0.2% saponin in PBS was 10 minutes at room
648 temperature. Primary and secondary antibodies were incubated in 1% BSA in PBS for 30 minutes
649 shielded from light. Cells was mounted using Immuno-mount (Thermo Scientific). BMDM were
650 imaged on SP5 Confocal microscope (Leica). Quantification of average intensity for cytoplasmic
651 dsDNA staining was performed using ImageJ software by selecting region of interest excluding
652 nucleus from each of the cells imaged for quantification.

653

654 Whole mouse irradiation

655 Ten to fourteen-weeks old females C57BL/6J mice were purchased from Charles River
656 Laboratories and exposed to whole thorax irradiation at a dose of 17 Gy under 2.5% isoflurane
657 anaesthesia. The irradiation procedure was approved by the ethics committee of the Institut Curie
658 CEEA-IC #118 (Authorization number APAFIS#5479-201605271 0291841 given by National
659 Authority) in compliance with the international guidelines. Mice, kept for five months after
660 irradiation, were weekly monitored and sacrificed if predefined ethical endpoints (i.e. loss of 20%
661 body weight, severe dyspnea) were reached.

662

663 Influenza infection

664 Influenza A virus PR8 (strain A/Puerto Rico/8/1934 H1N1) was a gift from Olivier Lantz.
665 Intranasal infection with 50 or 100 pfu was performed under Ketamine/Xylazine-mediated
666 anesthesia inside a PSMII hood. Mice were weighed daily and all husbandry was performed
667 underneath a PSMII hood with strict containment measures followed. An ethical endpoint of 20%
668 d0 weight loss was observed in all experiments. Animal care and use for these infection

669 experiments was performed in accordance with the recommendations of the European Community
670 (2010/63 / UE) for the care and use of laboratory animals. Experimental procedures were
671 specifically approved by the ethics committee of the Institut Curie CEEA-IC # 118 (Authorization
672 APAFIS # 32125-2021062516083243 v1 given by National Authority) in compliance with the
673 international guidelines.

674

675 Data analysis

676 Analysis of FACs data was performed using FlowJo v10.6.2. Statistical analyses were performed
677 using PRISM v9.1.2, the type of test performed is detailed in the relevant figure legends. ImageJ
678 Java 1.8.0_66 was used for analysis of immunofluorescence and intravital data. Figures were
679 compiled using Adobe illustrator v15.1.0

680

681 Bioinformatics analysis

682 **Quality check, read alignment, computation of the UMI counts, and cell calling.** FASTQ files
683 were obtained from BCF files using cellranger mkfastq command from CellRanger v3.0.2 (Zheng
684 et al., 2017). Sequencing quality was assessed using FastQC v11.8
685 (<https://www.bioinformatics.babraham.ac.uk>). cellranger count command (--expect cells 6000)
686 was used to map the reads to the annotated mm10 genome (accession: GCA_000001635.6, gene
687 build: 2016-01), compute UMI counts, and call cellular barcodes.

688 DoubletDetection (<https://doubletdetection.readthedocs.io>) was run on the gene-cell expression
689 matrix 50 times and a barcode was labelled as *doublet* (i.e., a droplet containing more than one
690 cell) if it was detected as such (p-value $\leq 10^7$) in at least 40 iterations.

691

692 **Processing of the gene-cell expression matrix.** The gene-cell expression matrix for each sample
693 was imported in R using Seurat v3.0.0 (Satija et al., 2015) and normalized as follows: the UMI
694 count of each gene i in cell j was augmented by 1, divided by the total UMI count for cell j ,
695 multiplied by 10000, and log-transformed. Doublets and cells with < 200 detected genes (UMI
696 count ≥ 1) were filtered out. Only the genes detected in ≥ 2 cells in at least one sample were
697 retained. The normalized matrices for the four samples were concatenated to obtain a joint
698 normalized matrix M and gene expression is standardized (Z-score) to obtain a scaled matrix M' .

699
700 **Generation of the clustering solution.** Feature selection methods and clustering parameters were
701 let vary in order to optimize the definition of the cell populations.

702 Feature selection was performed on M (defined above) using either mean.var.plot or vst method
703 from Seurat. They both model the expression dispersion of each gene in order to detect the
704 candidates that showed the highest variability across cells, called highly variable genes. For
705 mean.var.plot, the average expression was set in $[0.1, 0.5]$ and the scaled dispersion was required
706 to be $\geq d$, with $d = 0.5, 1, \text{ or } 1.5$. For vst, the number of top variable genes retained was set to 500,
707 1000, or 2000, respectively.

708 A principal component analysis (PCA) was possibly performed on M' (defined above) after feature
709 selection. The first 50 principal components (PCs) were calculated using an SVD approximation.
710 The number of top PCs to be retained was automatically defined using the following approach.
711 First, a p-value p_h was computed for each component PC_h using the JackStraw method (Seurat,
712 default parameters). To identify the contribution to the standard deviation that was likely due to
713 noise, we computed the standard deviation σ_{noise} explained on average by PC_{40}, \dots, PC_{50} . The
714 optimal number of top PCs was set to the maximal h such that the two conditions $\sigma_h \geq 1.25\sigma_{noise}$
715 and $p_h \leq 10^{-5}$ simultaneously hold.

716 Clustering was done using the Waltman and van Eck algorithm, either on the selected features or
717 on the top h PCs, by varying the number of neighbors k in $\{30, 40, 50\}$ and the resolution parameter
718 r from 0.1 to 1 in steps of 0.1. Overall, 360 clustering solutions were generated.

719
720 **Selection of the optimal clustering solution and identification of cell populations.** Given a
721 clustering solution C , a silhouette width $silh(j)$ was computed for each cell j using the $n \times n$ matrix
722 D , where n is the number of cells and D_{jk} is the euclidean distance between cell j and cell k ,
723 computed on the same space where C is calculated (features or PCs). The average silhouette width
724 across a cluster c will be referred to as $silh(c)$.

725 For each solution, the cluster with highest average expression ranking across the alveolar
726 macrophage (AM) markers (Marco, Mrc1, Siglec f, Lmna, Itgax) was defined as the putative AM
727 cluster and labelled as c_{AM} . For each feature selection method, with or without PCA, we selected
728 the solution such that $silh(c_{AM}) + silh(C)$ is maximal (12 solutions in total). Among them, we selected
729 the solution C such that $silh(C)$ is maxima, which contained 18 clusters ($k = 30, r = 0.7$) and was
730 computed on the top 20 PCs obtained from 399 features (mean.var.plot, $d = 1.5$; see “Generation
731 of the clustering solution”). A tSNE transformation was computed, with perplexity = 10, on the
732 same input used to compute C .

733
734 **Cluster labelling.** Clusters identify was assigned by manual curation using the ImmGen
735 MyGeneSet tool (<http://rstats.immgen.org/MyGeneSet/>). Clusters identified as thymic T cells,
736 endothelial cells and epithelial cells were removed from downstream analyses, resulting in 14
737 differentiated immune cell clusters. 7395 cells were retained for WT and 7493 for Lamin A/C
738 CKO.

739

740

741 **Differential expression analysis.** Differential expression analysis was performed between each
742 cluster and its complementary, and between the WT and *Lmna*^{fl/fl}Vav1-Cre cell subsets, c_{WT} and
743 c_{KO} , for each cluster c on solution C , using MAST method (Finak et al., 2015). Genes detected in
744 $\geq 10\%$ cells in either one of the two conditions with $|\log_2FC| > 0.5$ and adjusted p-value < 0.05
745 (Bonferroni correction) were defined as differentially expressed (DEGs).

746 For bootstrap validation, 10 cells subsets containing 50% of the cells were randomly generated for
747 both c_{WT} and c_{KO} for each immune cell cluster c containing at least 50 cells in both c_{WT} and c_{KO} (12
748 out of the 14 immune cell clusters identified).. A DEG ($|\log_2(FC)| > 0.5$, adjusted p-value < 0.05 ,
749 detected in $\geq 10\%$ of the cells in either a subset or its complementary) found in at least one
750 comparison between a subset and its complementary (within either c_{WT} or c_{KO}) was included in a
751 *null set*. 100 cross-comparisons were performed between each pair of subsets generated from c_{WT}
752 and c_{KO} and genes identified as DEGs are extracted ($|\log_2(FC)| > 0.5$, adjusted p-value < 0.05 ,
753 detected in $\geq 50\%$ of the cells in either one subset). A DEG detected in the full c_{WT} versus c_{KO}
754 comparison was labelled as *validated* if it did not belong to the null set, was detected in $\geq 50\%$ of
755 the cells in either c_{WT} or c_{KO} , and was found among the top 25% significant DEGs in $\geq 90\%$ of the
756 cross-comparisons.

757

758 **Single-cell RNA-Seq of irradiated murine AM.**

759 Lungs were resected and single cell suspensions were prepared by enzymatic dissociation before
760 loading into the Chromium controller (10x Genomics). Single-cell RNA-Seq of irradiated murine
761 AM were prepared using single cell 3'RNA-Seq V2 reagent kit. cDNA quality control was assessed
762 by capillary electrophoresis (Bioanalyzer, Agilent) before libraries preparation and sequencing on
763 HiSeq 2500 (Illumina). Initial data processing was performed using the Cell Ranger pipeline (v2.1

764 10x Genomics). Count matrices were processed with Seurat package v2.5. Briefly, gene counts
765 matrices were filtered ($n_{\text{Gene}} < 2500$; percentage of mitochondrial genes < 0.07), normalized and
766 aligned using the canonical correlation analysis method in Seurat. After dimensionality reduction,
767 clusters were annotated based on known cell type markers and DEG analysis performed on selected
768 AM cluster. DEGs were identified in AM 5 months post-17Gy irradiation vs non-irradiated controls
769 as genes that satisfy $|\log_2(\text{FC})| > 0.5$ with an adjusted p -value < 0.05 and are detected in at least
770 10% of the cells in either one of the two conditions. We obtained 141 up- and 216 down-regulated
771 genes for 5 months irradiation compared to control.

772

773

774 **Public single-cell RNA-Seq datasets of murine AM.**

775 ***Tabula Muris Senis (TMS)***. The 10x AM data were obtained from the of the Tabula Muris Senis
776 dataset (The Tabula Muris Consortium et al., 2020) (“alveolar macrophage” from
777 cell_ontology_class_reannotated ([https://s3.console.aws.amazon.com/s3/buckets/czb-tabula-](https://s3.console.aws.amazon.com/s3/buckets/czb-tabula-muris-senis/)
778 muris-senis/)) for each age (1, 3, 18, 21, 30 months). We clustered the AM ($k = 20$, $r = 0.1$) on the
779 top 10 PC computed on all genes and across all cells in the gene-cell matrix and computed the
780 average expression of AM markers (see above).

781 ***Aging atlas***. The cell-type-resolved differential gene expression testing between age groups was
782 obtained (Angelidis et al., 2019) and the DEGs between AM in aged (24 months) versus young
783 mice (3 months) were extracted (see paragraph “Differential expression analysis”).

784 ***Lung connectome***. The gene expression profile of human, mouse, rat, and pig (two samples per
785 species) across lung cells was obtained (Raredon et al., 2019) (accession: GSE133747). Based on
786 the available annotation, immune cells were extracted and the average LMNA log-normalized

787 expression (see paragraph “Processing of the gene-cell expression matrix”) across samples was
788 computed.

789 DEGs were converted to the corresponding official gene symbols (using limma v3.38.3 (Smyth,
790 2005)) before being possibly compared across datasets.

791
792 **Definition of the TMS aging signature in mouse AM.** Differential expression analysis was
793 computed on each age pair and the DEGs were extracted as in paragraph “Differential expression
794 analysis”. The DEGs for each old (18, 21, 30 months) versus young (1, 3 months) comparison are
795 extracted. Up-(down-)regulated DEGs in one old-young comparison that are also found either
796 down-(up-)regulated in another old-young comparison, or up-(down-)regulated in the 3 months
797 versus 1month comparison, are filtered out. The remainder DEGs were validated using a similar
798 bootstrap procedure as the one described in paragraph “Comparison of WT versus *Lmna*^{fl/fl}Vav1-
799 Cre in each cell population”, allowing to define a list of validated up- and down-regulated genes
800 for each of the six old-young comparison. The TMS aging signature was defined as the union of
801 the six validated DEG lists.

802
803 **Functional annotation.** For a given pair of conditions, functional annotation was performed
804 separately on the up-regulated and down-regulated DEGs (see paragraph “Differential expression
805 analysis”). Each DEG was first converted to the corresponding official gene symbol (using limma)
806 and then to the Entrez ID. GO-BP enrichment analysis is computed using all mouse genes having
807 a GO annotation as background (with clusterProfiler v3.10.0 (Yu et al., 2012) and org.Mm.eg.db
808 v3.7.0). Only GO terms containing ≥ 10 and ≤ 500 genes are considered. A cut-off of 0.1 on the
809 adjusted p-value (Benjamini-Hochbergh) is set to define the significantly enriched GO terms.

810 Redundant terms (Wang similarity measure > 0.7) are removed by keeping the most significant
811 representative.

812

813 **Gene set enrichment analysis.** Consistency between a defined gene set (A) and a phenotype (B)
814 was quantified using GSEA (Subramanian et al., 2005). A custom gene signature was defined as
815 the set of up-regulated DEG between case and control in A. The phenotype was defined as the
816 average \log_2 FC between case and control in B and used to rank the genes. Only the genes detected
817 in at least 10% of the cells in either case or control in the phenotype were considered for GSEA.

818

819 **Supplementary Tables**

820 **Table S1 Differentially expressed genes for cluster assignment.**

821 **Table S2 Expression of Lmna in the Lung Connectome dataset.**

822 **Table S3 Differentially expressed genes identified in clusters.**

823 **Table S4 Differentially expressed genes in the alveolar macrophage cluster of irradiated mice.**

824 **Table S5 Differentially expressed genes in the alveolar macrophage cluster of Tabula Muris**

825 **Senis.**

826

827 **Table S6 Sex of mice used in the study.**

<u>Figure 1</u>	
A	5 M WT 6 M Lamin A/C CKO 10 F WT 7 F Lamin A/C CKO *sex not noted for 4 WT and 3 CKO data points
B & C	2 F WT 2 F Lamin A/C CKO
D	Data shown in Figure 1A + 4 F WT 4 M Lamin A/C CKO
E	Sex not noted
<u>Figure 2</u>	
A	2 F WT 2 F Lamin A/C CKO
B	7 M WT 8 WT Lamin A/C CKO 5 F WT 5 F Lamin A/C CKO
C	3 M WT 4 M Lamin A/C CKO 2 F WT 2 F Lamin A/C CKO
D	7 M WT 8 M Lamin A/C CKO 2 F WT

	2 F Lamin A/C CKO 2 M cGAS KO 4 M Lamin A/C cGAS DKO 2 F cGAS KO 3 F Lamin A/C cGAS DKO 5 M WT 5 M Lamin A/C p53 DKO 2 F WT 1 F Lamin A/C p53 DKO
<u>Figure 3</u>	
A & B	1 F
C & D	representative images and data from 4 experiments in which BAL from 2 WT mice was pooled exp 1 - 2 M exp 2 - 2 M exp 3 - 1 M, 1F exp 4 - 2M
E	images representative of 4 experiments in which BAL from 2 mice of each genotype was pooled exp 1 - 2 M WT + 2 M Lamin A/C CKO exp 2 - 2 M WT + 2 M Lamin A/C CKO exp 3 - 1 M WT + 1 F WT + 2 F Lamin A/C CKO exp 4 - 2 M WT + 2 M Lamin A/C CKO
<u>Figure 4</u>	
A	2 F WT 2 F Lamin A/C CKO
B	10 M WT 13 M Lamin A/C CKO 7 F WT 6 F Lamin A/C CKO
C	2 F WT 2 F Lamin A/C CKO 2 F WT Control 2 F WT Irradiated
D	7 M WT 8 M Lamin A/C CKO 2 F WT 2 F Lamin A/C CKO 2 M cGAS KO 4 M Lamin A/C cGAS DKO 2 F cGAS KO 3 F Lamin A/C cGAS DKO 5 M WT 5 M Lamin A/C p53 DKO

	2 F WT 1 F Lamin A/C p53 DKO
<u>Figure 5</u>	
A	not noted
B	3 F WT 2 M Lamin A/C CKO 1 F Lamin A/C CKO
<u>Figure 6</u>	
A & B	12 F WT 12 F Lamin A/C CKO
<u>Figure 7</u>	
A & B	data from Tabula muris senis
C	8F
D & E	2 F WT 2 F Lamin A/C CKO and data from Tabula muris senis and Lung Aging Atlas
<u>Figure S1</u>	
	Sex not noted
<u>Figure S2</u>	
A & B	5 M WT 6 M CKO 8 F WT 5 F CKO *sex not noted for 4 WT and 3 CKO data points
C	sex not noted
<u>Figure S3</u>	
<u>A-C</u>	2 F WT 2 F Lamin A/C CKO
<u>Figure S4</u>	
A & B	2 F WT 2 F Lamin A/C CKO
C	representative example of data shown in Figure 2D and 4D
<u>Figure S5</u>	
A	1F
B	representative example of data shown in Figure 3C, D and E
C	representative images and data from 1 experiment in which BAL was pooled from 2 mice of each genotype 2 M WT

	2 M Lamin A/C CKO
D	representative images and data from 3 experiments in which BAL from 2 mice of each genotype was pooled exp 1 - 1 M WT + 1 F WT + 2 M Lamin A/C CKO exp 2 - 1 M WT + 1 F WT + 2 M Lamin A/C CKO exp 3 - 1 M WT + 1 F WT + 1 M Lamin A/C CKO + 1 F Lamin A/C CKO
<u>Figure S6</u>	
A	1 M WT 3 M Lamin A/C CKO 4 F WT 5 F Lamin A/C CKO
B	6 M WT 6 M Lamin A/C CKO 4 F WT 5 F Lamin A/C CKO
C	3 M WT 5 M Lamin A/C CKO 5 F WT 2 F Lamin A/C CKO
D	2 F WT Control 2 F WT Irradiated
E	3 F WT 2 M Lamin A/C CKO 1 F Lamin A/C CKO
<u>Figure S7</u>	
A	12 F WT 12 F Lamin A/C CKO
B-D	3 F WT 3 M WT 3 F Lamin A/C CKO 3 M Lamin A/C CKO
<u>Figure S8</u>	
A & C	8 F
B	2 F WT 2 F Lamin A/C CKO and data from <i>Tabula muris senis</i>

828

829

830 **Table S7 Antibodies used in the study.**

Surface FACs antibodies	Source	Identifier
V500 Mouse anti-Mouse CD45.2 Clone 104	BD Biosciences	562129
PE Mouse Anti-Mouse I-A[b] Clone AF6-120.1	BD Biosciences	553552
FITC Mouse Anti-Mouse I-A[b] Clone AF6-120.1	BD Biosciences	553551
Anti-Mouse CD11c PE-Cyanine7 Clone N418	eBioscience	25-0114-82
Anti-Mouse CD11b PerCP-Cyanine5.5 Clone M1/70	eBioscience	45-0112-82
Alexa Fluor® 700 Rat Anti-Mouse CD24 Clone M1/69	BD Biosciences	564237
APC anti-mouse CD64 (FcγRI) Clone X54-5/7.1	Ozyme	139306
Anti-Mouse CD103 (Integrin alpha E) eFluor® 450 Clone 2E7	eBioscience	48-1031-82
PE-CF594 Rat Anti-Mouse Siglec-F Clone E50-2440	BD Biosciences	562757
PerCP-Cy™5.5 Rat Anti-Mouse Siglec-F Clone E50- 2440	BD Biosciences	565526
PE anti-mouse TCR β chain Antibody Clone H57-597	Ozyme	BLE109208
Anti-Human/Mouse CD45R (B220) eFluor® 450 Clone RA3-6B2	eBioscience	48-0452-80
APC Rat Anti-Mouse Ly-6G and Ly-6C Clone RB6- 8C5	BD Biosciences	553129
PE anti-mouse CD88 (C5aR) Clone 20/70	Ozyme	BLE135805
PE Rat IgG2b, kappa Isotype control Clone RTK4530	Ozyme	BLE400607
Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block™) Clone 2.4G2	BD Biosciences	553142
V500 Rat anti-Mouse CD4 Clone RM4-5	BD Biosciences	560782
Pacific Blue™ Rat Anti-Mouse CD8a Clone 53-6.7	BD Biosciences	558106
APC Rat Anti-Mouse CD8a Clone 53-6.7	BD Biosciences	561093
PerCP-Cy™5.5 Mouse Anti-Mouse NK-1.1 Clone PK136	BD Biosciences	561111
PE-Cy™7 Rat Anti-Mouse CD62L Clone MEL-14	BD Biosciences	560516
Alexa Fluor® 700 Rat Anti-Mouse CD44 Clone IM7	BD Biosciences	560567

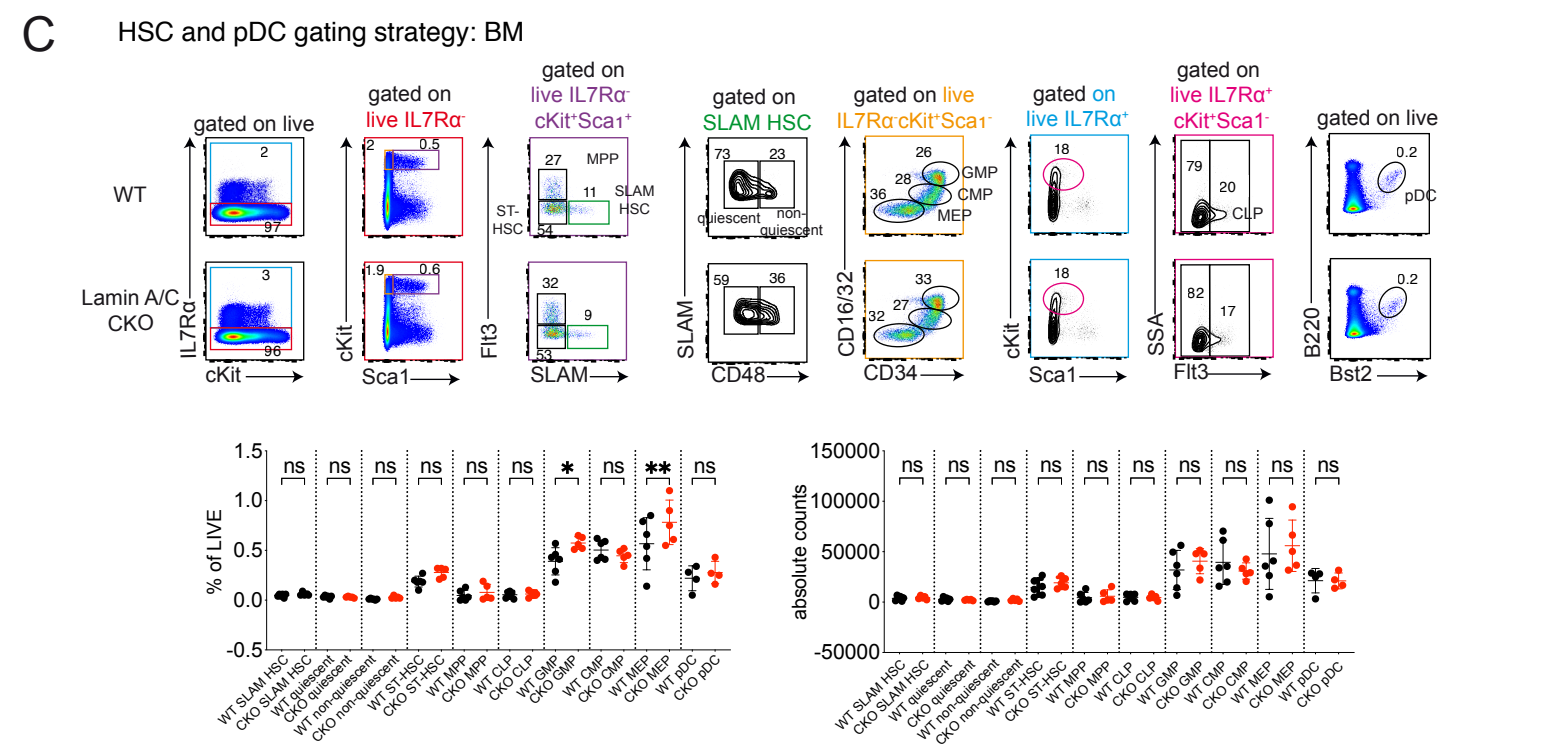
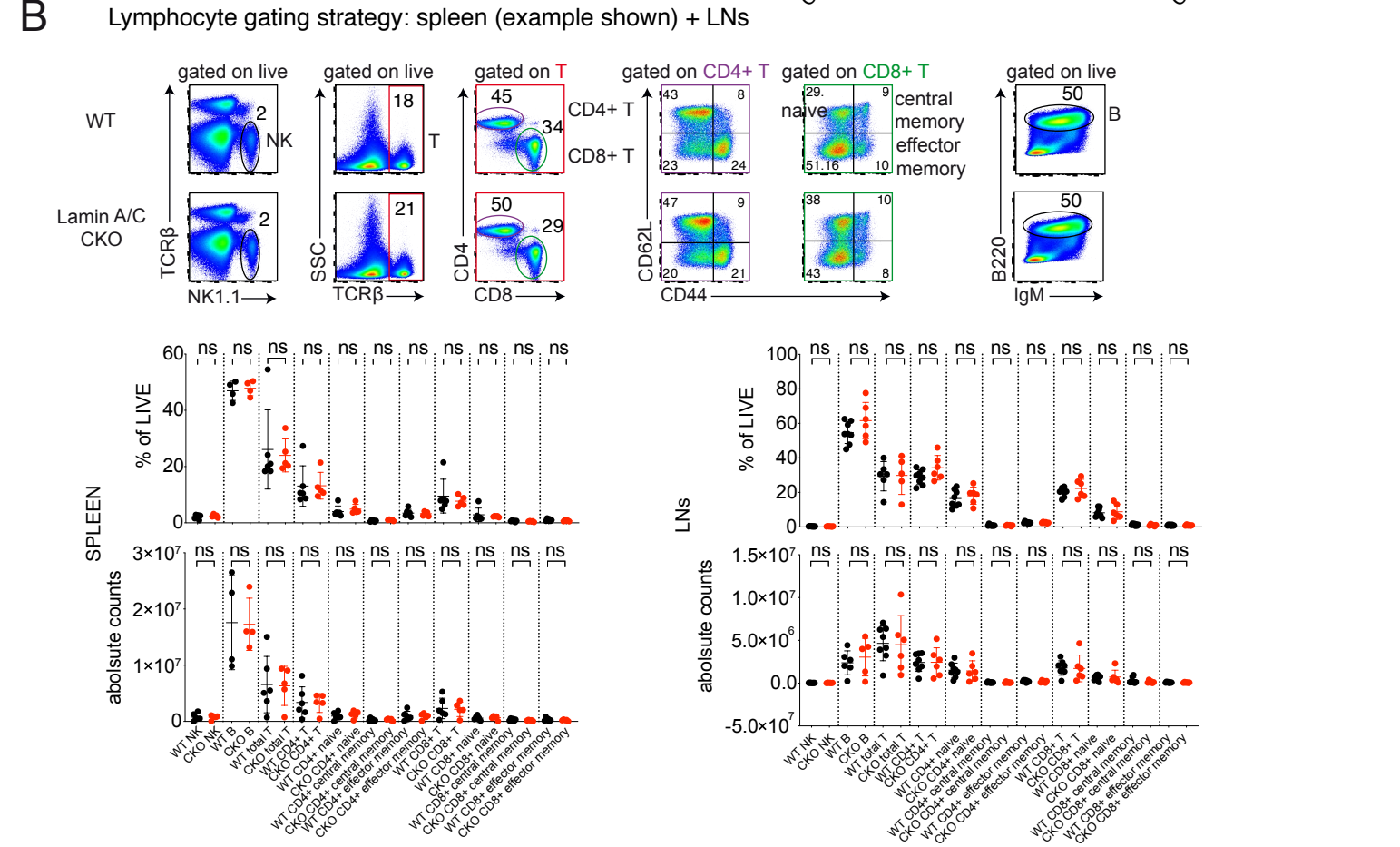
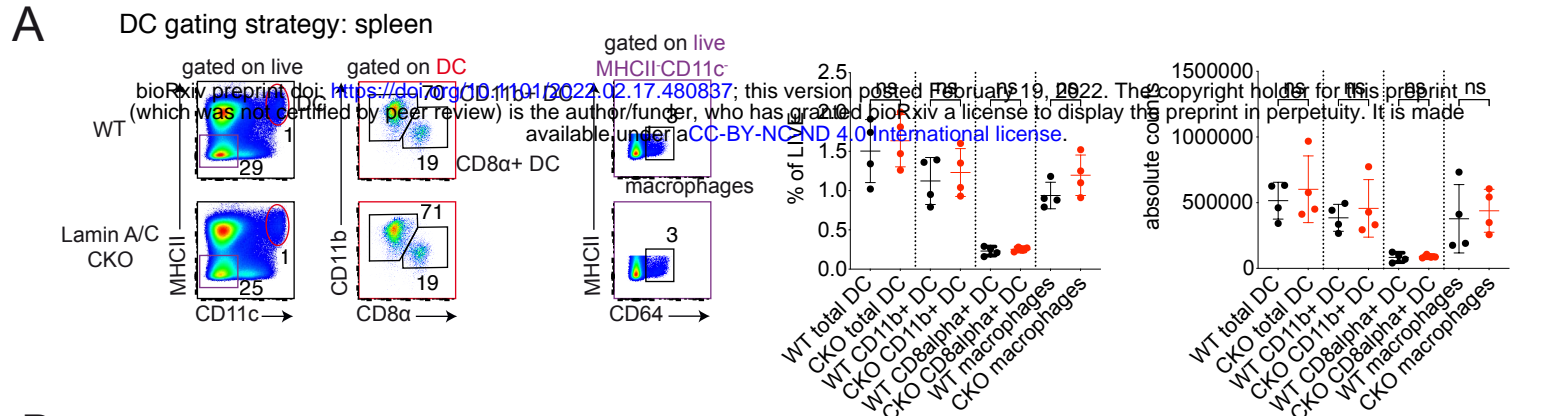
CD317 (PDCA-1) Antibody, anti-mouse Clone JF05-1C2.4.1	Miltenyi Biotec	130-102-828
PE-Cy TM 7 Rat Anti-Mouse IgM Clone: R6-60.2	BD Biosciences	552867
PerCP-Cy TM 5.5 Rat Anti-Mouse CD16/CD32 clone 2.4G2	BD Biosciences	560540
Anti-Mouse CD34 Alexa Fluor® 700 clone RAM34	eBioscience	56-0341-82
APC anti-mouse CD117 (c-Kit) Antibody clone 2B8	Biolegend	105812
Anti-Mouse CD135 (Flt3) PE clone A2F10	eBioscience	12-1351-82
Pacific Blue TM anti-mouse Ly-6A/E (Sca-1) Antibody clone D7	Biolegend	108120
PE/Cy7 anti-mouse CD150 (SLAM) Antibody clone TC15-12F12.2	Biolegend	115914
Brilliant Violet 510 TM anti-mouse CD48 Antibody Clone HM48-1	Biolegend	103443
PE/Dazzle TM 594 anti-mouse CD127 (IL-7R α) Antibody Clone A7R34	Biolegend	135031
Intracellular FACs antibodies	Source	Identifier
PE Rat Anti-Mouse CD63 Clone NVG-2	BD Biosciences	564222
PE Rat IgG2a, κ Isotype Control Clone R35-95	BD Biosciences	553930
Alexa Fluor® 647 Mouse anti-H2AX (pS139) Clone N1-431	BD Biosciences	560447
Alexa Fluor® 647 Mouse IgG1 κ Isotype control Clone MOPC-21	BD Biosciences	557783
PE Mouse Anti-H2AX (pS139) Clone N1-431	BD Biosciences	562377
PE Mouse IgG1, κ Isotype Control Clone MOPC-21	BD Biosciences	554680
Immunofluorescence antibodies (primary)	Source	Identifier
Purified Rat Anti-Mouse Siglec-F Clone E50-2440	BD Biosciences	552125
mouse Monoclonal antibody to BAF Clone A-11	Clinisciences	sc-166324
Monoclonal Anti-Lamin A/C antibody produced in mouse Clone 4C11	Sigma	SAB4200236
Anti-phospho-Histone H2A.X (Ser139) Antibody, Clone JBW301	Sigma	05-636
CD11c Monoclonal Antibody, Clone N418	eBioscience	14-0114-82
LAMP1 Polyclonal Antibody	ThermoFischer	PA1-654A
Anti-dsDNA Antibody, Clone HYB331-01	Santa Cruz Biotechnology	sc-58749
Immunofluorescence antibodies (secondary)	Source	Identifier

Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Invitrogen	A-21247
Goat anti-Hamster IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Invitrogen	A-21110
Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555	Invitrogen	A21434
Goat anti-Mouse IgG2a Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Invitrogen	A21131
Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Invitrogen	A21240
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Invitrogen	A-21245
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Invitrogen	A-11029

831

832

Figure S1



833 **Supplementary figure 1 Analysis of immune cells in Lamin A/C CKO mice.**

834 **(A)** Flow cytometric analysis of WT vs Lamin A/C CKO macrophage and dendritic cell (DC)
835 populations in spleen, with gating strategies for each population represented by colored gating.
836 Left, representative example of the gating strategy. Right, percentage of each population of live
837 spleen cells and absolute counts (n = 4 mice, combined from 2 independent experiments, bar
838 indicates mean \pm SD, one-way ANOVA with Šidák test).

839 **(B)** Flow cytometric analysis of WT vs Lamin A/C CKO Nature killer, T and B cell populations in
840 spleen and lymph nodes, with gating strategies for each population represented by colored gating.
841 Top, representative example of the gating strategy. Bottom, percentage of each population of live
842 spleen or lymph node cells and absolute counts (n = 5-8 mice, combined from 4 independent
843 experiments, bar indicates mean \pm SD, one-way ANOVA with Šidák test).

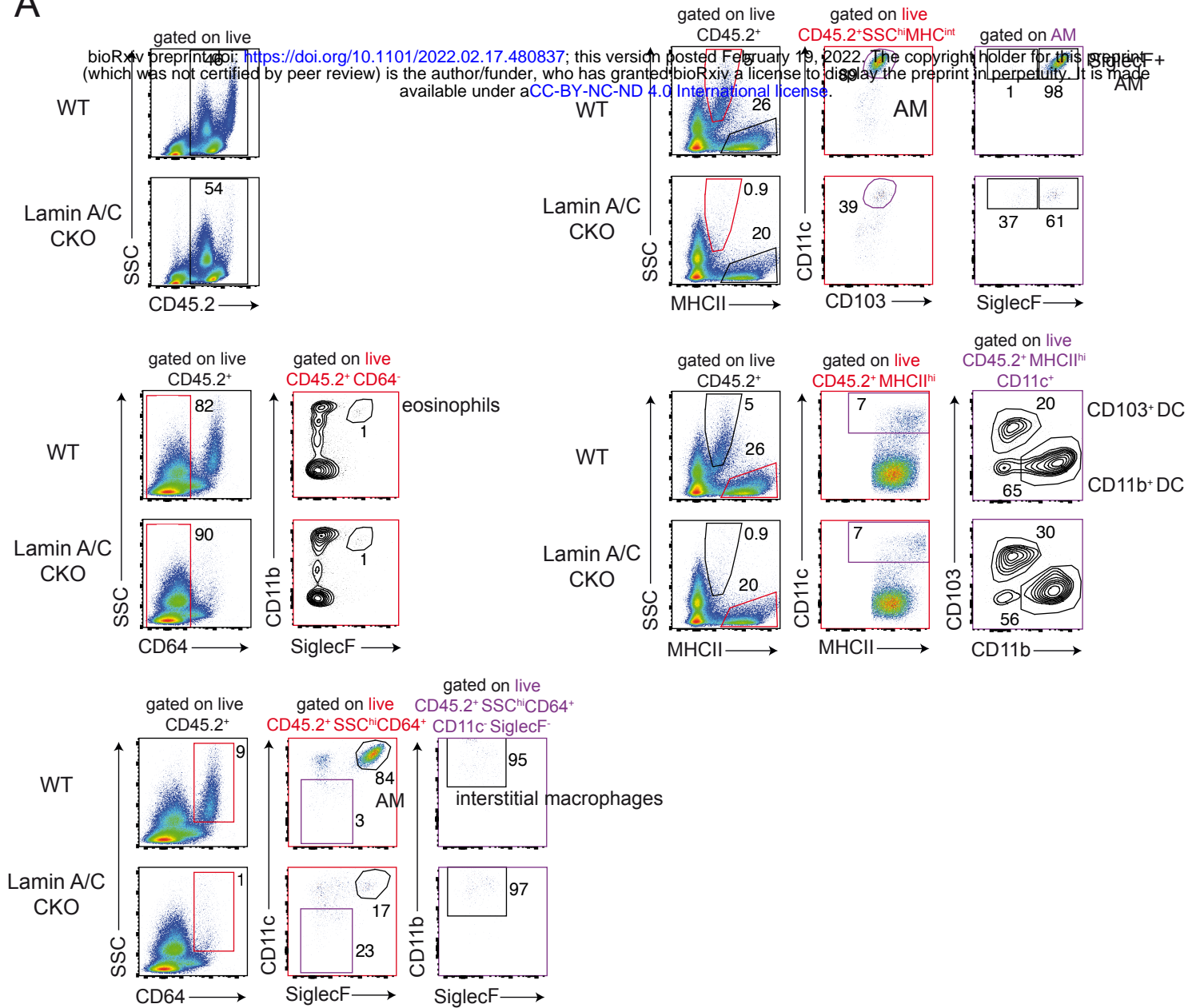
844 **(C)** Flow cytometric analysis of WT vs Lamin A/C CKO to analyze hematopoietic stem cell (HSC)
845 and plasmacytoid dendritic cell populations in bone marrow, with gating strategies for each
846 population represented by colored gating. Top, representative example of the gating strategy.
847 Bottom, percentage of each population of live bone marrow cells cells and the absolute counts (n
848 = 4-6 mice, combined from 3 independent experiments, bar indicates mean \pm SD, one-way
849 ANOVA with Šidák test). Acronyms used: Short term HSC (ST-HSC), Multipotent hematopoietic
850 progenitor (MPP), common myeloid progenitor (CMP), common lymphoid progenitor (CLP),
851 granulocyte macrophage progenitor (GMP), megakaryocyte-erythroid progenitor (MEP),
852 plasmacytoid dendritic cell (pDC).

853

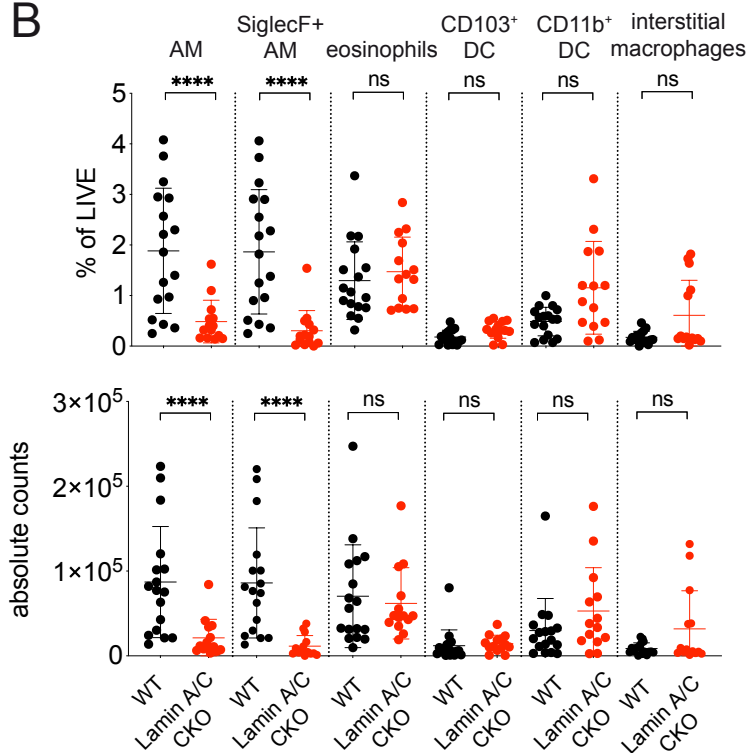
854

Figure S2

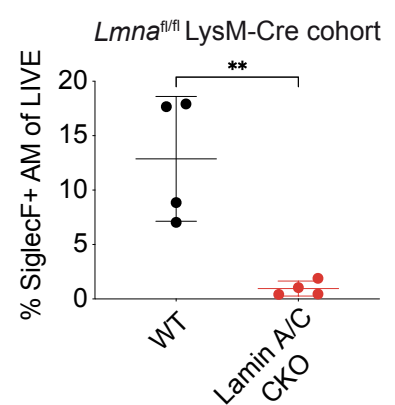
A



B



C



855 **Supplementary figure 2 Analysis of immune cells in lungs of Lamin A/C CKO mice.**

856 **(A)** Representative flow cytometric analysis of WT vs Lamin A/C CKO lung immune populations
857 including AM, eosinophils, interstitial macrophages, CD103⁺ DC and CD11b⁺ DC with gating
858 strategies for each population represented by colored gating.

859 **(B)** Percentages and absolute counts of AM, eosinophils, interstitial macrophages, CD103⁺ DC
860 and CD11b⁺ DC of live WT vs Lamin A/C CKO lung cells (n = 14-17 mice, combined from 9
861 independent experiments, bar indicates mean \pm SD, one-way ANOVA with Šidák test).

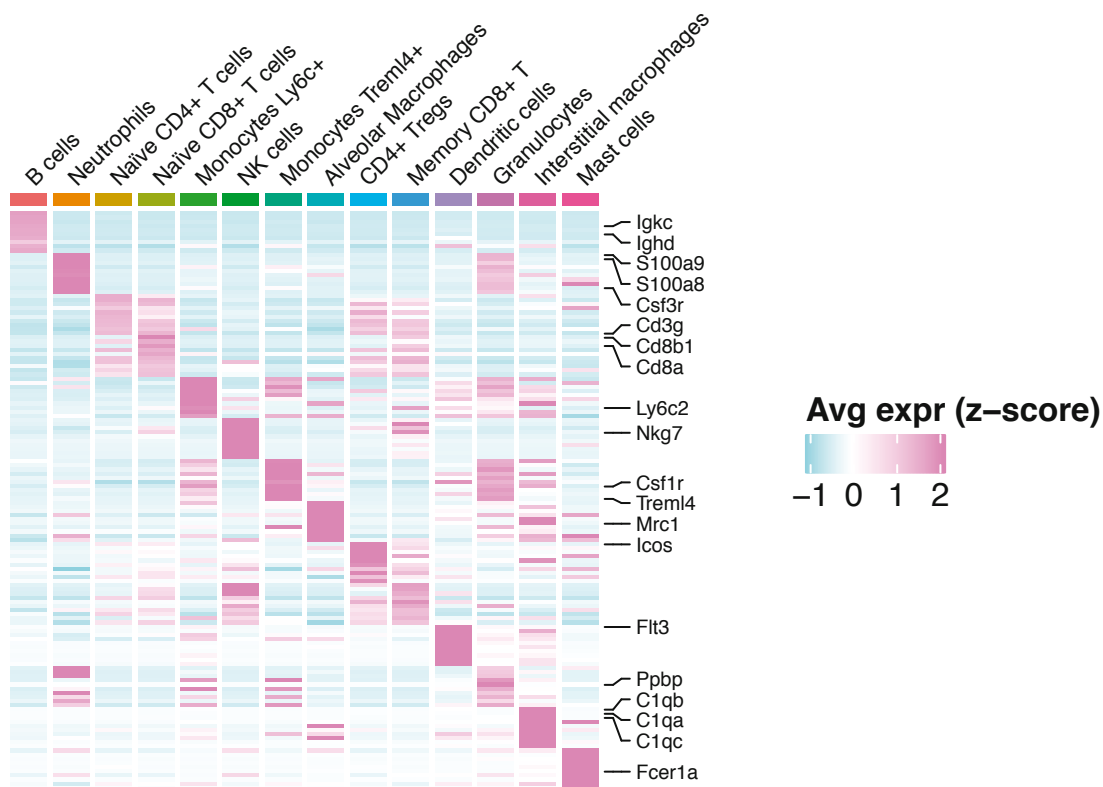
862 **(C)** Percentage of AM (CD45.2⁺SSC^{hi}MHC^{int}SiglecF⁺) following flow cytometric analysis of WT
863 (*Lmna*^{fl/fl} LysM-Cre^{-/-}) vs *Lmna*^{fl/fl} LysM-Cre^{+/-} lung (n = 4 mice combined from 2 independent
864 experiments, bar indicates mean \pm SD, unpaired t test).

865

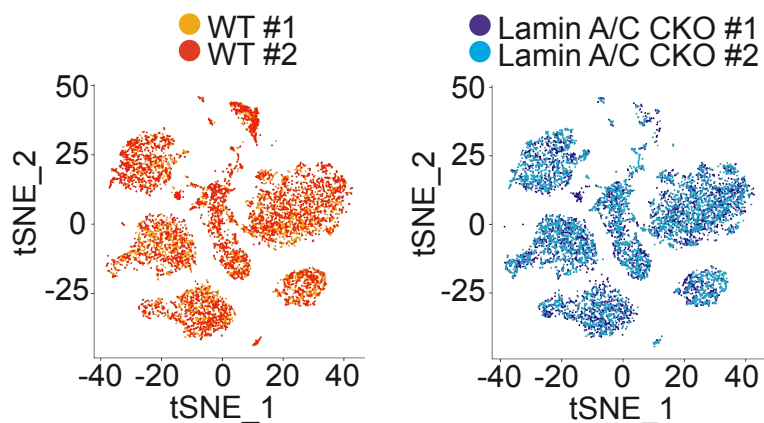
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Figure S3

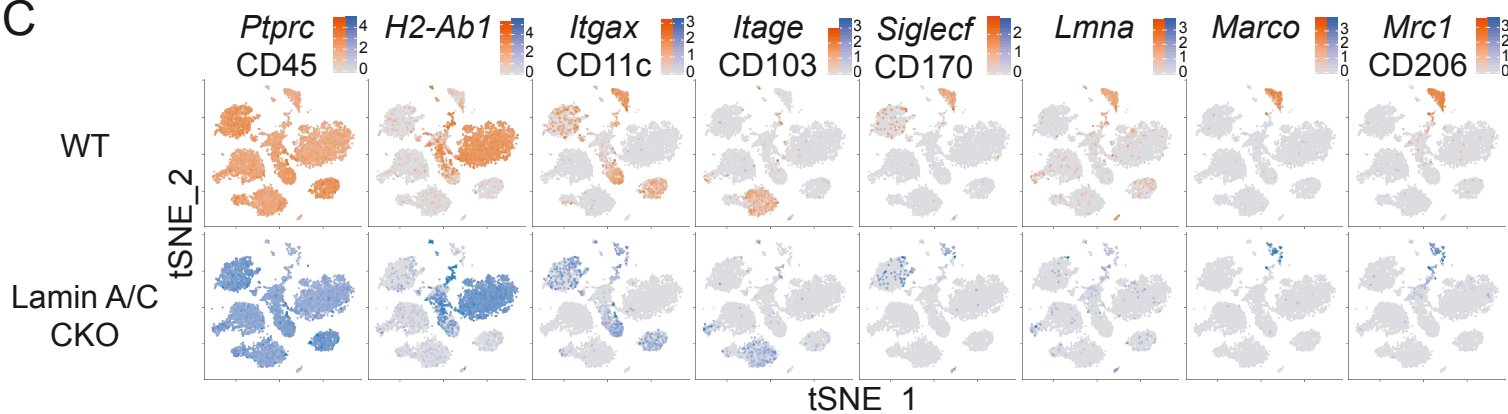
A



B



C



867 **Supplementary figure 3 scRNAseq analysis of lung immune cells in WT and Lamin A/C CKO**
868 **mice.**

869 **(A)** Heatmap showing the expression of cluster-specific gene markers. Differential expression
870 analysis is done for each cluster against all the other cells (including the contaminants). Among the
871 top 50 significant DEGs, the 10 DEGs with the top fold change are shown. Common DEGs between
872 clusters are reported only once. Entries are the z-scores of the normalized expression level, clipped
873 to fit the interval.

874 **(B)** tSNE representation showing WT (left) and Lamin A/C CKO (right) cells only, and colored by
875 replicate. In WT, replicate 1 contains 4075 cells and replicate 2 contains 3980. In Lamin A/C CKO,
876 replicate 1 contains 4000 cells and replicate 2 contains 3677.

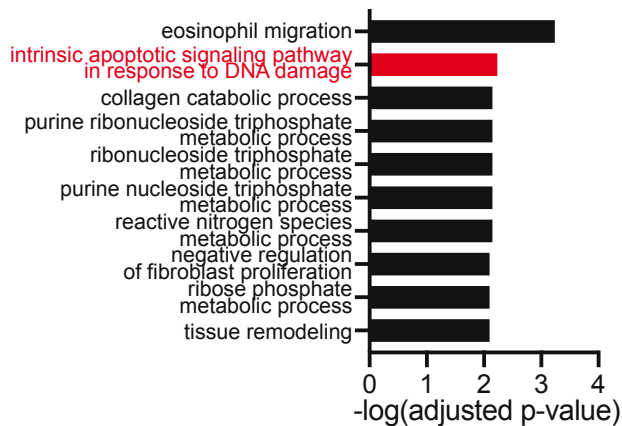
877 **(C)** tSNE representation showing WT cells (top) and Lamin A/C CKO cells (bottom). Cells are
878 colored with a gradient representing the normalized expression level of immune and alveolar
879 macrophage markers.

880

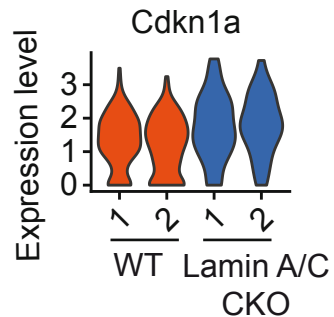
881

Figure S4

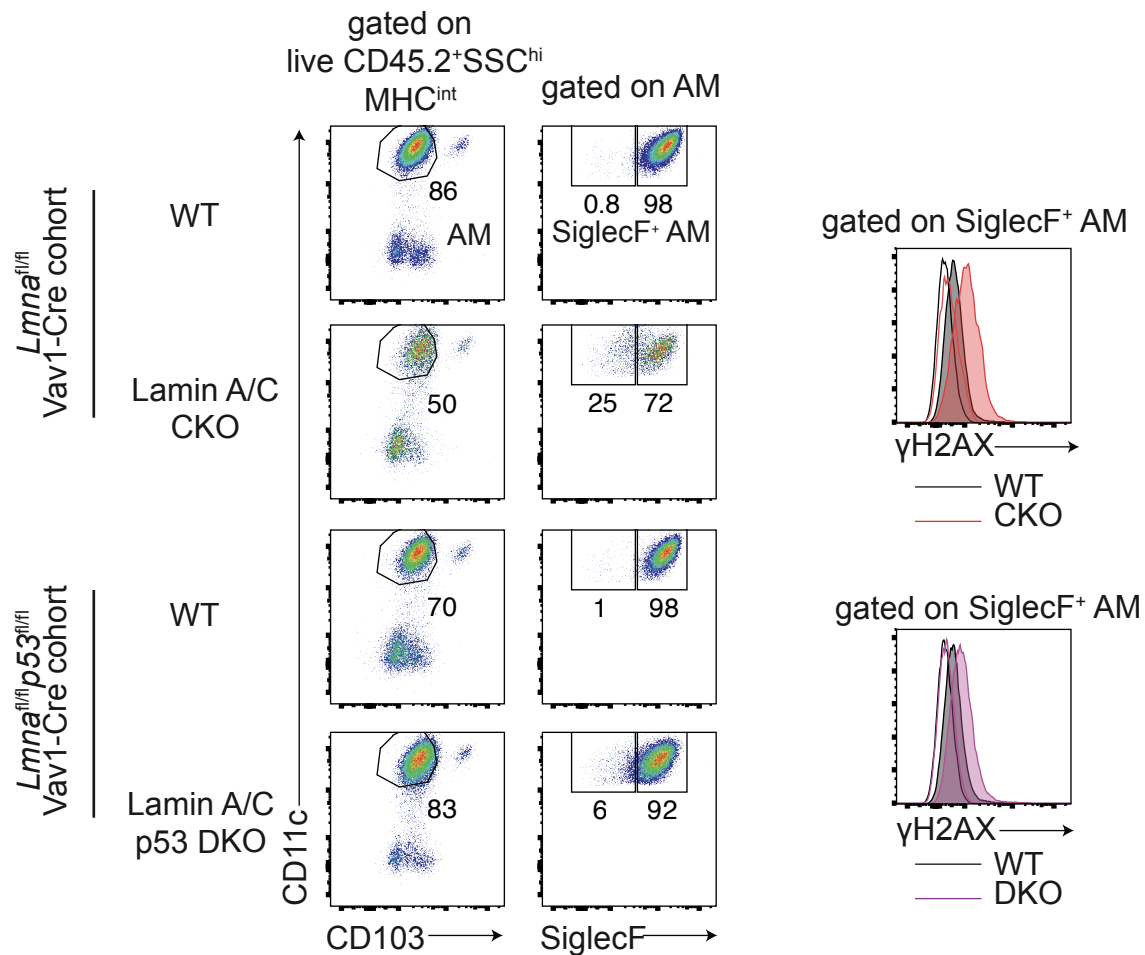
A



B



C



882 **Supplementary figure 4 DNA damage and related markers in alveolar macrophages of Lamin**
883 **A/C CKO mice.**

884 **(A)** Gene ontology enrichment analysis of DEGs upregulated in Lamin A/C CKO, specific to the
885 AM cluster. Significant terms (adjusted p-value < 0.01) from the biological process (BP) ontology
886 are shown.

887 **(B)** Violin plot showing the normalized expression of *Cdkn1a* in the AM cluster, with values for
888 individual WT and Lamin A/C CKO replicates shown.

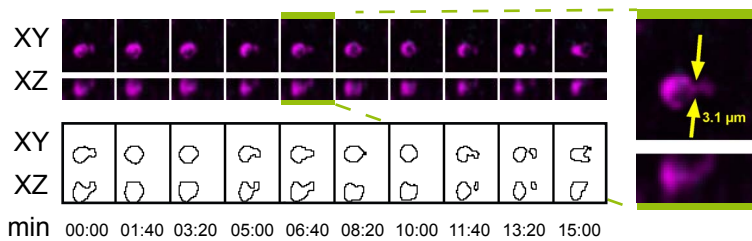
889 **(C)** Representative intracellular flow cytometric analysis of mice from WT (*Lmna*^{fl/fl} Vav1-Cre^{-/-})
890 vs Lamin A/C CKO (*Lmna*^{fl/fl} Vav1-Cre^{+/-}) and WT (*Lmna*^{fl/fl} *p53*^{fl/fl} Vav1-Cre^{-/-}) vs Lamin A/C
891 *p53* DKO (*Lmna*^{fl/fl} *p53*^{fl/fl} Vav1-Cre^{+/-}) to identify AM (CD45.2⁺SSC^{hi}MHC^{int}SiglecF⁺) and assess
892 γ H2AX levels.

893

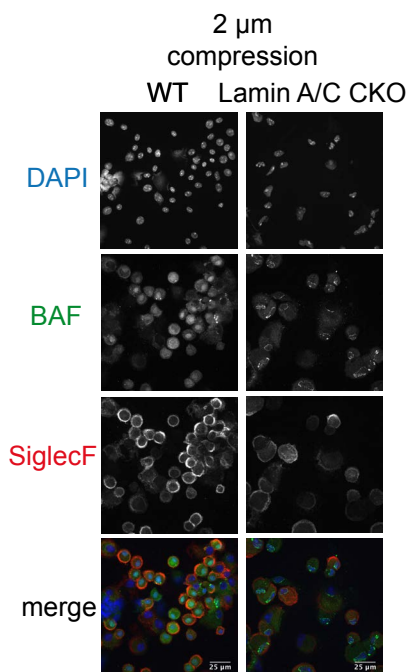
894

Figure S5

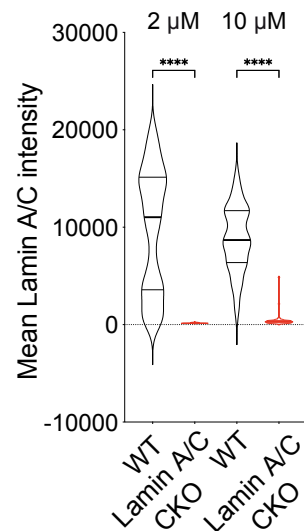
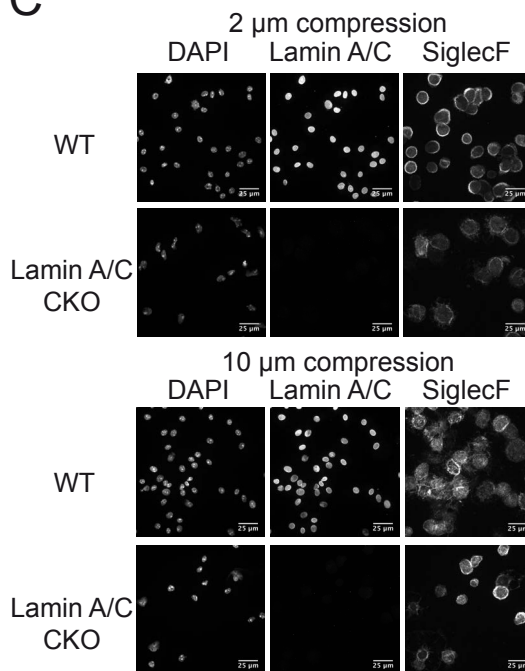
A



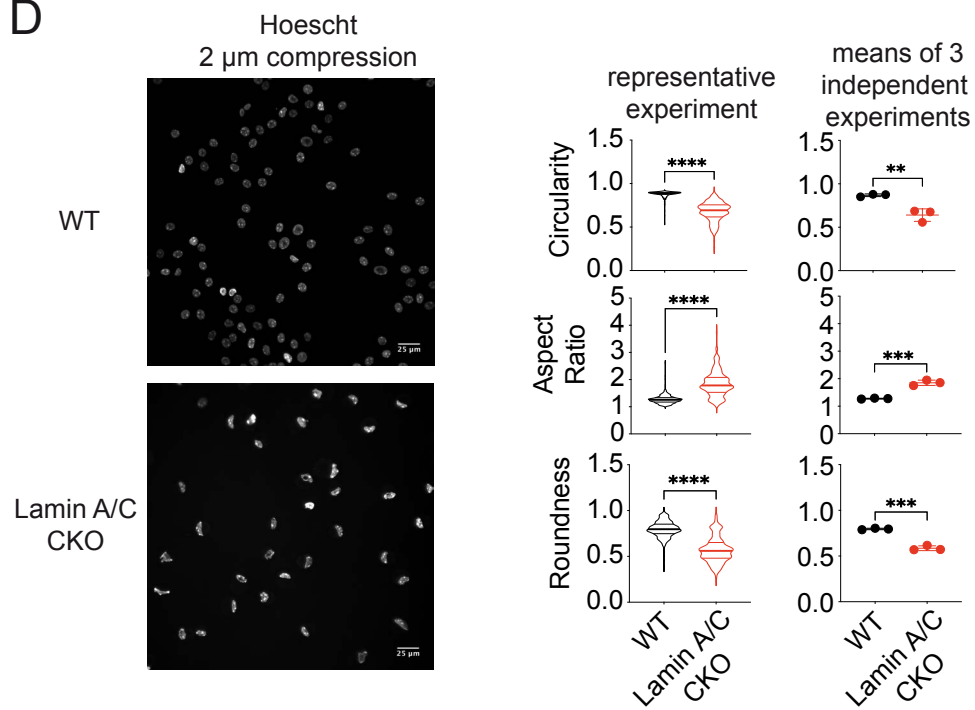
B



C



D



895 **Supplementary figure 5 Live imaging of alveolar macrophages in lungs.**

896 **(A)** AM from WT mouse lung (female) undergoing constricted migration *in vivo*. Top left, XY and
897 XZ planes. Bottom left, contour representation. Right, measurement of the cell width at the most
898 acute point of squeezing.

899 **(B)** BAL from Lamin A/C CKO mice confined at a height of 2 μm for 1.5 hours and subsequently
900 stained for DAPI, BAF and SiglecF (representative of $n = 4$ independent experiments, each time
901 BAL was pooled from 2 mice aged 8-26 weeks).

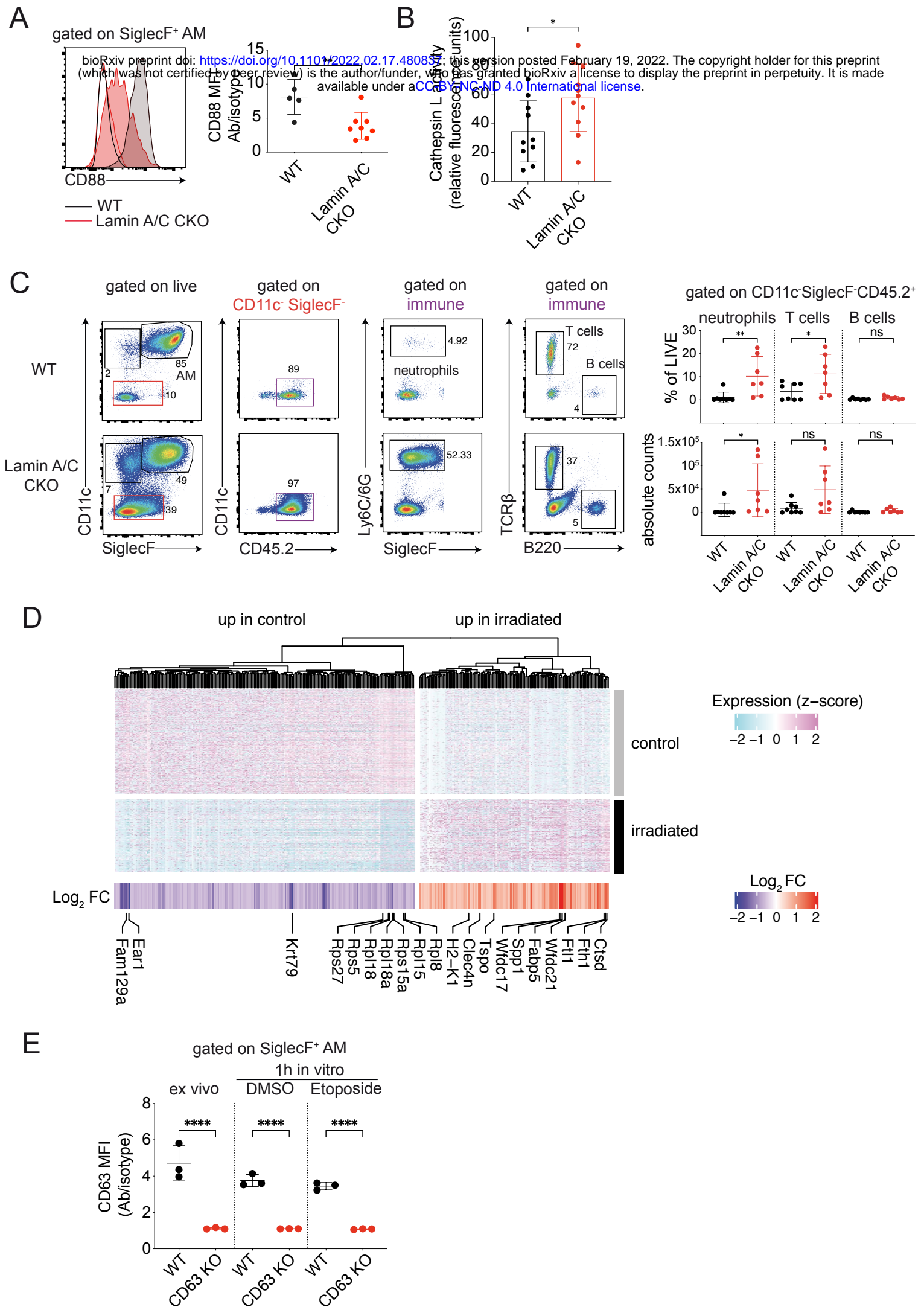
902 **(C)** BAL from WT and Lamin A/C CKO mice confined at 2 μm and 10 μm for 1.5 hours and
903 subsequently stained for DAPI, Lamin A/C and SiglecF. Left, representative field. Right,
904 quantification of mean intensity of the Lamin A/C intensity (violin plot with lines indicating the
905 median and quartile values, representative of 2 independent experiments, one-way ANOVA with
906 Tukey test, each time BAL was pooled from 2 mice of each genotype).

907 **(D)** Quantification of circularity, aspect ratio and roundness of nuclei from WT and Lamin A/C
908 CKO BAL. BAL was stained with Hoescht, confined at 2 μm and imaged immediately. Left,
909 representative field. Middle, values of individual cells in one experiment (violin plot with lines
910 indicating the median and quartile values, Kolmogorov-Smirnov test). Right, means of 3
911 independent experiments (unpaired t-test). For each experiment BAL was pooled from 2 mice of
912 each genotype aged 10-27 weeks. In the experiment shown, the cells were also stained for CD11c
913 and SiglecF prior to confinement to quantify nuclear shape specifically in AM. Bars indicate mean
914 \pm SD.

915

916

Figure S6



917 **Supplementary figure 6 Analysis of markers in Lamin A/C CKO mice, scRNAseq of**
918 **irradiated mice and CD63 expression in knock-out mice.**

919 **(A)** Left, intracellular flow cytometric analysis of WT vs Lamin A/C CKO lung for CD88
920 levels within AM (CD45.2⁺SSC^{hi}MHC^{int}SiglecF⁺). Right, mean fluorescence intensity (MFI) of
921 CD88 signal normalized to isotype control (n = 5-8 mice, combined from 3 independent
922 experiments, mice aged 28-39 weeks, bar indicates mean ± SD, unpaired t test).

923 **(B)** Cathepsin L activity in BAL extracted from WT vs Lamin A/C CKO mice (n = 10-11 mice
924 from 8 independent experiments, bar indicates mean ± SD, aged 13-34 weeks, unpaired t-test).

925 **(C)** Flow cytometric analysis of BAL obtained from WT vs Lamin A/C CKO mice to analyze
926 neutrophils, T and B cells with gating strategies for each population represented by colored gating.
927 Left, representative example. Right, percentage of each population of live BAL cells and absolute
928 counts of each population in extracted BAL (n = 7-8 mice, combined from 4 independent
929 experiments, mice aged 24-34 weeks, bars indicate mean ± SD, one-way ANOVA with Šidák test).

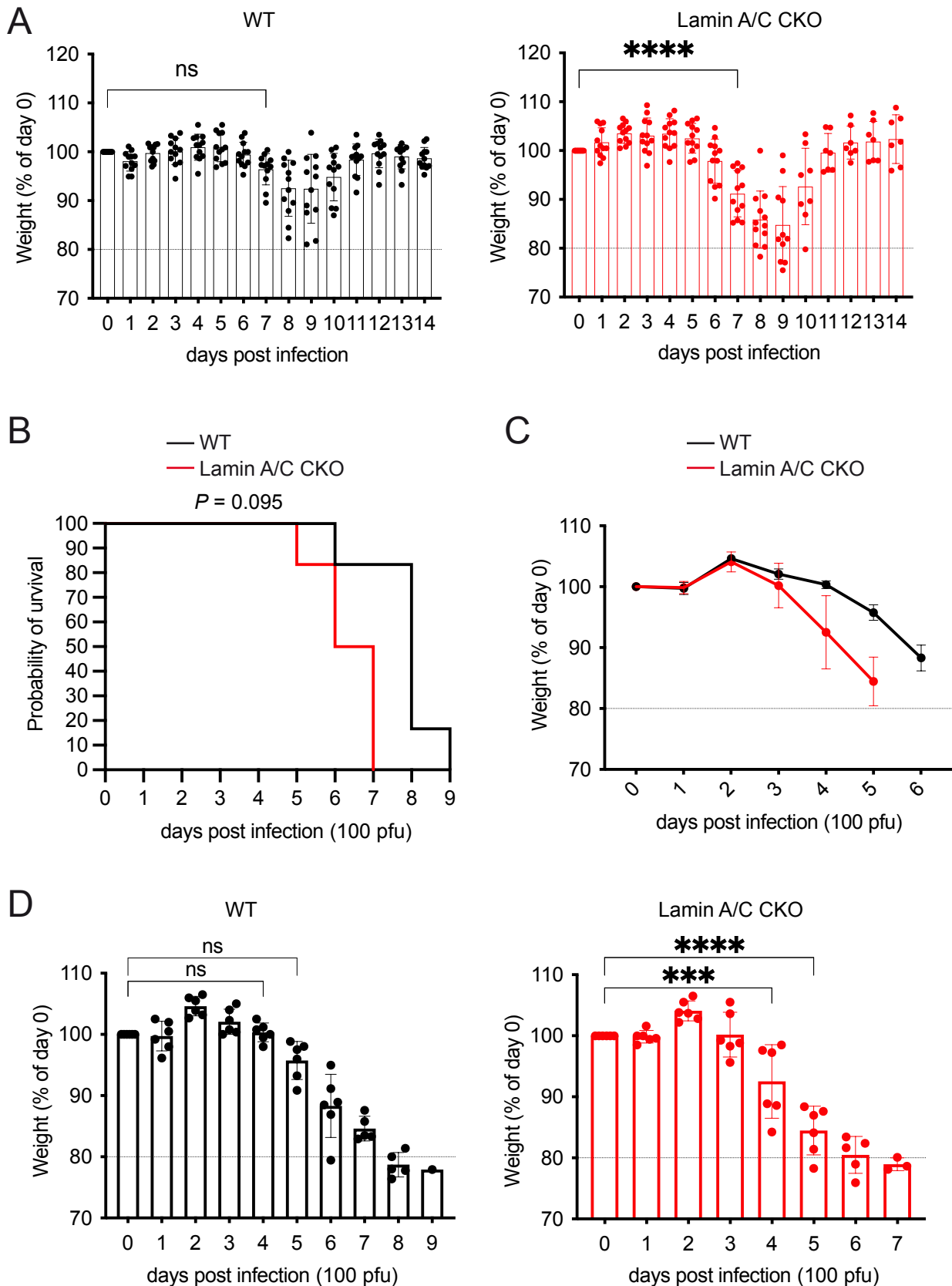
930 **(D)** Heatmap showing single cell gene expression (z-score) of DEGs in AM 5 months post-17Gy
931 irradiation and non-irradiated controls. Cells are grouped by condition. DEGs are divided among
932 up- and down-regulated in case vs control, clustered with a ward.D2 method computed on
933 euclidean distances and shown as dendrogram. DEGs are annotated with log₂ fold change (FC)
934 values.

935 **(E)** Intracellular flow cytometric analysis for CD63 levels within AM
936 (CD45.2⁺SSC^{hi}MHC^{int}SiglecF⁺) of WT vs CD63 KO lung single cell suspension, treated as
937 indicated. MFI of CD63 normalized to isotype control (n = 3 mice in one experiment, mice were
938 19 weeks of age, bars indicate mean ± SD, one-way ANOVA with Šidák test).

939

940

Figure S7



941 **Supplementary figure 7 Influenza infections in Lamin A/C CKO mice.**

942 **(A)** Percentage of day 0 weight observed each day post infection with 50 pfu influenza A virus via
943 the intranasal route in WT and Lamin A/C CKO mice (n = female 12 mice per genotype, combined
944 from 2 independent experiments, two-way ANOVA with Tukey test, bars indicate mean \pm SD).

945 **(B)** Survival of WT vs Lamin A/C CKO mice following infected with 100 pfu of influenza A virus
946 PR8 via the intranasal route (n = 6 mice per genotype in one experiment, Log-rank Mantel-Cox
947 test).

948 **(C)** Percentage of day 0 weight observed each day post infection described in (B). Curves of
949 average \pm SEM weights are shown and continued until a first death occurs in the group.

950 **(D)** Percentage of day 0 weight observed each day post infection described in (B) (two-way
951 ANOVA with Tukey test, bars indicate mean \pm SD).

952

953

954 **Supplementary figure 8 Hallmarks of aging in alveolar macrophages.**

955 **(A)** Percentage AM (CD45.2⁺SSC^{hi}MHC^{int}SiglecF⁺) of live lung cells in Young (14 weeks) vs Old
956 (63 weeks) mice (n = 4 mice combined from 2 independent experiments, bars indicate mean ± SD,
957 unpaired t test).

958 **(B)** Normalized enrichment scores (NES) and adjusted p-values following gene set enrichment
959 analysis of the indicated upregulated or downregulated genes. Left, TMS gene sets enrichment in
960 Lamin A/C CKO vs WT transcriptome. Geneset obtained after individual pairwise time-point
961 comparisons and overall aging signature were tested. Right, Lamin A/C CKO gene sets enrichment
962 in TMS transcriptome, ranked by the indicated time-point comparisons. n genes in sets indicates
963 the number of genes in given gene set detected in the tested transcriptome.

964 **(C)** Young (14 weeks) vs Old (63 weeks) lung single cell suspension was directly analyzed or
965 incubated with DMSO or 50 μM etoposide for 1 hour. Intracellular flow cytometric analysis for
966 γH2AX levels within AM (CD45.2⁺SSC^{hi}MHC^{int}SiglecF⁺) was then performed. The geoMFI of
967 γH2AX signal is shown normalized to isotype control (n = 4 mice from 2 independent experiments,
968 bars indicate mean ± SD, one-way ANOVA with Šidák test).

969

970 **Movie 1 Live imaging of a wild-type mouse lung.**

971 The lung was imaged after administration of Hoechst and an anti-SiglecF antibody. Left is a broad
972 field of a lung region, right features individual AM (SiglecF⁺) demonstrating constricted migration
973 (white boxes).

974

975 **Movie 2 A single AM undergoing constricted migration *in vivo*.**

976 Left, contour representation. Right, XY and XZ planes.

977

978

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