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1 **Severe combined immunodeficiency in Sting V154M/WT mice**

2

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40

41 **ABSTRACT**

42 **BACKGROUND:** Autosomal dominant gain-of-function (GOF) mutations in human STING
43 (Stimulator of Interferon Genes) lead to a severe autoinflammatory disease called SAVI
44 (STING Associated Vasculopathy with onset in Infancy), associated with enhanced expression
45 of interferon (IFN) stimulated gene (ISG) transcripts.

46 **OBJECTIVE:** The goal of this study was to analyze the phenotype of a new mouse model of
47 Sting hyperactivation, and the role of type I IFN in this system.

48 **METHODS:** We generated a knock-in model carrying an amino acid substitution (V154M) in
49 mouse Sting, corresponding to a recurrent mutation seen in human patients with SAVI.
50 Hematopoietic development and tissue histology were analyzed. Lymphocyte activation and
51 proliferation were assessed *in vitro*. Sting V154M/WT mice were crossed to IFNAR (IFN α / β
52 Receptor) knock-out mice in order to evaluate the type I IFN-dependence of the mutant Sting
53 phenotype recorded.

54 **RESULTS:** In Sting V154M/WT mice we detected variable expression of inflammatory
55 infiltrates in the lungs and kidneys. These mice showed a marked decrease in survival and
56 developed a severe combined immunodeficiency disease (SCID) affecting B, T and NK cells,
57 with an almost complete lack of antibodies and a significant expansion of monocytes and
58 granulocytes. The blockade in B and T cell development was present from early immature
59 stages in bone marrow and thymus. In addition, *in vitro* experiments revealed an intrinsic
60 proliferative defect of mature T cells. Whilst the V154M/WT mutant demonstrated increased
61 expression of ISGs, the SCID phenotype was not reversed in Sting V154M/WT IFNAR knock-
62 out mice. However, the anti-proliferative defect in T cells was partially rescued by IFNAR
63 deficiency.

64 **CONCLUSIONS:** Sting GOF mice developed an IFN-independent SCID phenotype with a T,
65 B and NK cell developmental defect and hypogammaglobulinemia, associated with signs of
66 inflammation in lungs and kidneys. Only the intrinsic proliferative defect of T cells was,
67 partially, IFN-dependent.

68 **KEY MESSAGES**

- 69 • A Sting V154M/WT murine model demonstrated a severe combined immunodeficiency
70 (SCID) affecting B, T and NK cells, with a significant expansion of monocytes and
71 granulocytes. This SCID phenotype was independent of the type I IFN pathway, despite
72 the observation of a significant upregulation of IFN induced gene transcripts.
- 73 • An anti-proliferative effect was noticed in T cells, which was partially IFN-dependent.
- 74 • Variable inflammation of lung and kidney was observed.

75

76 **CAPSULE SUMMARY**

77 Sting V154M/WT mice develop an IFN-independent severe combined immunodeficiency with
78 hypogammaglobulinemia, a partially IFN-dependent T cell proliferation defect, and variable
79 lung and kidney inflammation, providing new clues in the understanding of STING gain-of-
80 function pathophysiology.

81

82 **KEY WORDS**

83 Severe combined immunodeficiency, STING, V154M, type I interferon

84

85 **ABBREVIATIONS**

86	BM	Bone marrow
87	CD	Cluster of Differentiation
88	cGAMP	cyclic GMP-AMP
89	CLP	Common lymphoid progenitor
90	CMP	Common myeloid progenitor
91	Cxcl10/IP10	Interferon- γ -inducible protein 10
92	DD	Dimerization domain
93	DN	Double negative
94	DP	Double positive
95	ER	Endoplasmic reticulum
96	ETP	Early T cell progenitor
97	GMP	Granulocyte/monocyte progenitor
98	GOF	Gain-of-function
99	Ifit1	Interferon-induced protein with tetratricopeptide repeats 1

100	IFN	Interferon
101	IFNAR	Interferon α/β receptor
102	Ig	Immunoglobulin
103	IL	Interleukin
104	ILCs	Innate Lymphoid cells
105	IRF3	Interferon regulatory factor 3
106	Isg15	Interferon stimulated gene 15
107	ISGs	Interferon stimulated genes
108	KO	Knock-out
109	LT-HSC	Long-Term hematopoietic stem cells
110	LN	Lymph nodes
111	LT α	Lymphotoxin α
112	LTi	Lymphoid Tissue-inducing cells
113	MCP-1	Monocyte chemotactic protein-1
114	MEP	Megakaryocyte/erythroid progenitor
115	MPP	Multipotent progenitor
116	PAMPs	Pathogen associated molecular patterns
117	SAVI	Sting associated vasculopathy with onset in infancy
118	SCID	Severe combined immune deficiency
119	SLE	Systemic lupus erythematosus
120	STAT6	Signal transducer and activator of transcription 6
121	ST-HSC	Short-term hematopoietic stem cells
122	STING	Stimulator of interferon genes
123	TBK1	Tank-binding kinase 1
124	TNF α	Tumor necrosis factor α
125	Tregs	Regulatory T cells
126	WT	Wild-type

127 INTRODUCTION

128 Since its identification as an adaptor molecule in the cytosolic DNA-sensing pathway,
129 Stimulator of Interferon Genes (STING) has emerged as a central player in antiviral and
130 antibacterial immunity, autoinflammation and cancer¹⁻⁵. STING is an endoplasmic reticulum
131 (ER)-associated membrane protein, which is expressed in a wide variety of tissues including
132 endothelial, epithelial and hematopoietic cell types^{6,7}. Signaling through STING can be induced
133 by cyclic di-GMP, cyclic di-AMP and cyclic GMP–AMP (cGAMP). Such pathogen associated
134 molecular patterns (PAMPs) are recognized by STING, causing its activation and subsequent
135 migration from the ER to perinuclear vesicles where it recruits and activates the TANK-binding
136 kinase 1 (TBK1). STING-TBK1 then induces the phosphorylation, dimerization and nuclear
137 translocation of interferon regulatory factor 3 (IRF3), which drives a type I IFN response⁸ and
138 the expression of a set of interferon (IFN)-stimulated genes (ISGs). The STING–TBK1 axis is
139 also known to phosphorylate I κ B, resulting in NF- κ B release, nuclear translocation and
140 increased NF- κ B-dependent gene expression, a pathway implicated in cellular stress, tumor
141 progression, inflammation and immunity⁹. Finally, this axis can also lead to the phosphorylation
142 of Signal transducer and activator of transcription 6 (STAT6), which mediates immune
143 signaling in response to cytokines and to virus infection¹⁰.

144 STING is therefore an adaptor of DNA sensing, which appears to be critical for the detection
145 of diverse cytosolic cyclic dinucleotides and DNA¹¹. Usually confined to the nucleus and
146 mitochondria, in some circumstances abnormal host DNA can be found in the cytoplasm,
147 leading to the development of autoimmune features in mice and humans^{12,13}. For this reason, in
148 the homeostatic context, STING-mediated signaling has to be finely regulated, since chronic or
149 constitutive activation, for example due to genetic mutation, might otherwise lead to
150 autoinflammation¹⁴. Indeed, a number of gain-of-function (GOF) mutations in *TMEM173*,
151 encoding STING, have been described in patients with an autoinflammatory phenotype
152 designated Sting Associated Vasculopathy with onset in Infancy (SAVI)^{15,16}. SAVI is
153 considered part of a group of diseases referred to as type I interferonopathies, where type I IFN-
154 induced signaling is considered to play a central role in disease pathology^{17,18}. SAVI patients
155 variably demonstrate systemic inflammation, and inflammatory lesions of the skin and lungs.
156 T and NK lymphopenia is a consistent feature, although patients do not present with classical
157 symptoms of immunodeficiency such as severe susceptibility to pathogens. Autoimmunity is
158 also very rare^{7,15,16}. Today, the exact molecular mechanisms leading to these various features
159 remain uncertain.

160 Twenty two patients from 15 families have been reported with heterozygous GOF mutations in
161 *TMEM173* involving amino acids at positions 147, 154, 155 and 166, localized near to (V147L)
162 or within the dimerization domain (DD) (N154S, V155M, G166E) of STING protein^{15,16,19-24}.
163 These changes have been suggested to lead to the stabilization of the DD, and thus constitutive
164 activation of STING^{15,16}. Three other SAVI patients have been described, carrying distinct
165 mutations (namely C206Y in exon 6; R281Q and R284G in exon 7), located in the cGAMP
166 binding domain (CBD) of STING, also leading to constitutive activation of the protein, thereby
167 implicating a second region of STING important in type I IFN signaling⁷.
168 Here, we generated a new knock-in mouse model with the heterozygous V154M mutation,
169 corresponding to the V155M mutation in humans (the most frequent mutation described so far),
170 in order to better understand the impact of this mutation on the immune system and the role of
171 the type I IFN-pathway in SAVI. These mice developed a SCID phenotype, with a T, B and
172 NK lymphopenia, a strong hypogammaglobulinemia, and an expansion in the myeloid
173 population, which were IFN-dependent. They also developed variable lung and kidney
174 inflammation.

175

176 **MATERIALS AND METHODS**

177

178 **Generation of Sting V154M^{+/-} knock-in mice**

179 The V154M mutation was introduced in the *Tmem173* locus using a standard CRISPR-
180 mediated genome editing procedure (Taconic). In short, after administration of hormones,
181 superovulated C57BL/6NJ females were mated with C57BL/6NJ males. One cell stage
182 fertilized embryos were placed in a drop of M2 medium under mineral oil. A microinjection
183 pipette with an approximate internal diameter of 0.5 micrometers (at the tip) was used to inject
184 the mixed nucleotide preparation containing the Cas9-protein and the specific gRNA (Fig 1, A)
185 into the pronucleus of each embryo. After injection, 35 injected one-cell stage embryos were
186 transferred to one of the oviducts of pseudopregnant NMRI females. Founder animals were
187 identified by PCR of the targeted locus following *NcoI* digestion. PCR samples per founder
188 were subcloned and multiple clones were analyzed by DNA-sequencing to confirm the presence
189 of the V145M mutation and exclude the presence of additional mutations near the target
190 sequence. One founder animal was then selected for an *in vitro* fertilization procedure to
191 generate G1 heterozygous pups. Predicted potential off-target sites were analyzed in G1
192 heterozygous animals by targeted sequencing.

193

194 **Mice**

195 Mice were bred and backcrossed on a C57BL/6N background for 8 generations in specific
196 pathogen free (SPF) conditions at the animal facility of the Molecular and Cellular Biology
197 Institute (IBMC, Strasbourg). Littermate WT mice were always used as controls. As indicated
198 in the Results section for some experiments, Sting V154M/WT and WT littermate control mice
199 were treated the whole time with antibiotic in drinking water (Baytril® 10% oral solution,
200 Enrofloxacin 150µL per 100mL of filtrated and autoclaved water; drinking water changed every
201 week). Sting V154M/WT mice were crossed with IFNAR knock-out mice (provided by CDTA,
202 CNRS, Orléans, France)²⁵.

203 Genotyping of Sting V154M^{+/-} mice was assessed by PCR amplification of genomic DNA
204 extracted from tail samples, using the following primers: one forward primer in exon 4 (5'-
205 ATAGCAGTGCTGAGAGCAAGC -3') and one reverse primer in exon 6 (5'-
206 GGGATCTAATGCTCTCTTCTGG -3'). PCR products are then treated for one hour at 37°C
207 with NcoI restriction enzyme (New England Biolabs) in order to distinguish the WT allele (1
208 band) from the mutated one (3 bands). PCR was performed with initial denaturation (95°C, 5
209 min) followed by 35 cycles of denaturation (95°C, 30 sec), annealing (61.5°C, 30 sec) and
210 extension (72°C, 1 min). Genotyping of IFNAR KO mice was assessed by PCR amplification
211 with the following combination of primers: Forward 5'-
212 AAGATGTGCTGTTCCCTTCCTCTGCTCTGA -3', Reverse 5'-
213 ATTATTAAGAAAAGACGAGGCGAAGTGG -3' and a third one
214 5'CCTGCGTGCAATCCATCTTG-3'. PCR was performed as follow: 95°C, 3 min and 35
215 cycles 94°C 15 sec, 62°C 15 seconds and 72°C 30 sec.

216 All animal experiments were performed with the approval of the "Direction départementale
217 des services vétérinaires" (Strasbourg, France) and protocols were approved by the ethics
218 committee ("Comité d'éthique en matière d'Experimentation Animale de Strasbourg",
219 CREMEAS) under relevant institutional authorization ("Ministère de l'Education Nationale, de
220 l'Enseignement Supérieur et de la Recherche"), authorization number: 2015072907553237
221 (APAFIS#2387). All control mice used in experiments were littermate controls.

222

223 **Western Blotting**

224 Cell lysates were prepared from splenic-sorted CD43 negative naive B cells. Proteins were size-
225 fractionated by 4%-20% gradient SDS-PAGE, electrotransferred to a PVDF membrane for 1.5
226 h at 130V, and immunodetected with rabbit polyclonal anti-mouse Sting antibody
227 (ProteinTech). Sting protein (band of ~40kDa) was detected with a secondary horseradish

228 peroxidase-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch). Anti- β Actin
229 Ab (Santa Cruz) was used as protein loading control.

230

231 **Flow cytometry analysis**

232 Analyses of cell phenotype and proliferation rates were performed on splenic, thymic and bone
233 marrow lymphoid populations using a four-color fluorescence cytometer (FACSCaliburTM)
234 according to standard protocols. The following antibodies were used: FITC, PE, PerCP or APC
235 anti-mouse IgM, CD19, B220, CD3, CD4, CD8, CD21, CD23, CD44, CD62L, Mac1 (CD11b),
236 Gr1 (Ly6C/G), CD49b, NK1.1 (CD161), CD86, CD25, CD138, FoxP3 (all from BD
237 Biosciences). Propidium iodide (2.5 μ g/mL, Sigma) or FvD (Fixable viability dye) were used
238 for discrimination of live and dead cells. The following antibodies were used for bone marrow
239 and thymic progenitor analysis : PerCP Cy5.5, APC, Alexa 488, APC-e-Fluor780, BV 421, BV
240 605, PE, Alexa 700, PE-Cy7, FITC, Cy5, APC-Cy7, PE-CF594, PE-eFluor610 or e-Fluor450
241 anti-mouse CD11b, CD11c, CD317, Gr1, Lineage, ckit, Sca1, CD48 (from e-Biosciences),
242 Ter119, CD71, , CD127, CD34, CD16/32 (from BD Biosciences) and CD150 (from Biolegend).
243 Lineage staining correspond to the following antibodies: anti-mouse CD3, CD4, CD8, B220,
244 CD11b, CD11c, Gr1, CD49b and Ter119 (Ly76). These analyses were performed using a ten-
245 color fluorescence cytometer (FACS FORTESSATM), according to standard protocols. The
246 following antibodies were used for spleen, bone marrow or thymic proliferation and apoptosis
247 analysis, respectively: FITC anti-BrdU (see **5-bromo-2'-deoxyuridine (BrdU)**
248 **incorporation**) and FITC annexin V (from BD Biosciences). Data were analyzed with FlowJo
249 software (Treestar®).

250

251 **5-bromo-2'-deoxyuridine (BrdU) incorporation**

252 BrdU was firstly administered by intraperitoneal injection of 100 μ L BrdU (1mg/mL; Sigma)
253 72 hours prior to analysis, and also delivered to mice in their drinking water at a concentration
254 of 0.8 mg/ml. Bone marrow and thymus were collected 72 hours after the BrdU injection. Cells
255 were fixed with Cytotfix/Cytoperm buffer (BD Biosciences) and permeabilized with the
256 permeabilization buffer (BD Biosciences). DNA was then denatured with a DNase solution
257 (100 μ g/mL, BD Biosciences) in order to improve the accessibility of the incorporated BrdU to
258 the detection antibody. A FITC mouse anti-BrdU antibody was used to detect the incorporated
259 BrdU. Cells were then analyzed by flow cytometry.

260

261 **Complete Blood Count**

262 Blood (150µL) was collected in EDTA 2% anticoagulant solution (30µL per 100µL of blood).
263 Complete blood counts (WBC, RBC, Hemoglobin dosage) were performed in the Mouse Clinic
264 Institute (ICS, Illkirch, France).

265

266 **Antibody detection by ELISA**

267 Total IgM, IgG and IgA levels were measured in serum from 3 to 6-month-old mice or in culture
268 supernatant after stimulation. Sera were obtained by blood centrifugation at 6000 rpm for 6 min.
269 Levels of total immunoglobulins were measured as previously described²⁶. Absorbance was
270 then read at 492 nm with the MultiskanTM FC photometer (ThermoFisher) and analysed with
271 SkanItTM microplate reader software. The concentration of total serum Ig was evaluated by
272 comparison with a standard curve using purified mouse IgM, IgG or IgA standards (Sigma, ref.
273 M-5170; Jackson ImmunoResearch, ref. 015000003; BD, ref. 553476).

274

275 **Cell preparation and culture**

276 *In vitro* B- and T-cell stimulations were performed in complete RPMI-1640 medium containing
277 L-glutamine (Lonza) supplemented with 10% FCS (Dutscher), 50 mM β-Mercaptoethanol
278 (Gibco) 1% Penicillin/Streptomycin (Gibco), 10 mM HEPES (Lonza), and 1 mM Sodium
279 Pyruvate (Lonza). Splenic naïve (CD43 negative) B cells were purified by negative magnetic
280 selection (mouse CD43 (Ly-48) MicroBeads; MACS Miltenyi Biotech) and splenic T cells with
281 Dynabeads Untouched Mouse T cells kit (Invitrogen), according to the supplier's protocols. B
282 cells were stimulated with either LPS from *Salmonella typhosa* (10 µg/ml, Sigma-Aldrich) or
283 goat anti-IgM alone (10µg/mL, Jackson ImmunoResearch), a combination of anti-CD40
284 antibody and recombinant murine IL-4 (1µg/mL, 5ng/mL; 5 ng/mL, Sigma), or a combination
285 of anti-IgM and anti-CD40 for 3 or 4 days, depending on the experiment. For T cell stimulation,
286 plates were coated with anti-CD3 (BD Pharmingen, 10µg/mL) 4 hours at 37°C or O/N at 4°C
287 and cells were then stimulated with anti-CD28 (BD Pharmingen, 10µg/mL), for 3 days at 37°C.
288 For proliferation analysis, splenocytes or sorted splenic mature B cells were pre-treated with
289 CFSE dye (carboxyfluorescein diacetate succinimidyl ester) (Sigma), according to standard
290 protocols, before being cultured for 4 days at 37°C. Plasmablast differentiation
291 (B220^{low}CD138+ cells) was determined by flow cytometry.

292

293 **Quantitative real-time RT-PCR analysis**

294 RNA was extracted with RNeasy Kit (Qiagen) and cDNA was obtained with High Capacity
295 Reverse Transcription Kit (Applied Biosystems) using a T100™ Thermal cycler (Biorad).
296 Quantitative real-time PCR was performed on 10 ng of cDNA using Taqman Universal
297 Mastermix (Applied Biosystems) and Assays-on-Demand probes (Applied Biosystems)
298 (*Hprt1*: Mm01318743_m1, *Gapdh*: Mm03302249_g1, *Sting*: Mm01158117_m1, *Isg15*:
299 Mm01705338_s1, *Ifit1*: Mm00515153_m1, *Cxcl10*: Mm00445235_m1, *Ifna4*:
300 Mm00833969_s1, *Ifnβ1*:Mm00439552_s1). Each sample was amplified in triplicate in a
301 StepOnePlus® real-time PCR system (Applied Biosystems). mRNA levels were calculated
302 with the StepOne v2.1 software (Applied Biosystems), using the comparative cycle threshold
303 method, and normalized to the mean expression of *Hprt1* and *Gapdh* housekeeping genes.
304

305 **Histological analysis**

306 Histological processing and evaluation was performed in the Mouse Clinic Institute (iCS) on 5
307 μm thick sections obtained from formalin-fixed paraffin embedded tissues (bones have been
308 decalcified in rapid decalcifier for 2 hours [DC3, VWR Chemicals]). All tissues were stained
309 with hematoxylin and eosin (H&E). Lungs and kidneys were also stained using Masson's
310 trichrome method to demonstrate possible fibrosis.

311 **Lymph nodes mapping**

312 Mice were anesthetized with 2.5% isoflurane and injected subcutaneously with 5% Evans blue
313 (Sigma) dye into the front legs, the hindfoot and the ears. After 30 minutes of continuous
314 anesthesia, mice were euthanized and dissected to locate the lymph nodes²⁷.

315

316 **Statistical analysis**

317 Statistical significance was calculated with a two-tailed Mann & Whitney test using Prism
318 software (GraphPad) except for the survival curves which were evaluated using Kaplan-Meier
319 analysis (Log-rank test). All data were presented as mean ± Standard Error of the Mean (SEM).
320 P-values < 0.05 were considered statistically significant (*, p<0.05, **, p<0.01, ***, p<0.001,
321 ****, p<0.0001).

322

323 RESULTS

324

325 **Heterozygous Sting V154M mice demonstrate marked weight loss and reduced survival**

326 Heterozygous mutations in STING, including V155M mutation (V154M in mice), are
327 responsible for the development of SAVI^{15,16}. We generated heterozygous V154M mice using
328 CRISPR/Cas9 technology, by inserting an oligonucleotide containing two mutations (GTT into
329 ATG, resulting in the replacement of a valine 154 with a methionine) into exon 5 of *Tmem173*
330 (Fig 1, A, green and red). In order to facilitate genotyping, the oligonucleotide also contained a
331 silent mutation encoding for a NcoI restriction site (Fig 1, A, yellow). In this way, Sting
332 V154M/WT mice could be distinguished from wild-type (WT) animals by PCR (Fig 1, B). We
333 confirmed the presence of the mutation by Sanger sequencing of genomic DNA and
334 complementary DNA (Fig 1, C). We also analyzed Sting expression in this model. It has been
335 shown that STING expression is decreased in HEK293FT cells transfected with the V155M
336 STING mutation in comparison with cells expressing WT STING^{7,16}. In agreement with this,
337 we noticed a reduction of nearly 50% of *Sting* mRNA and Sting protein in Sting V154M/WT
338 mice (Fig S1, A and B).

339 Interestingly, V154M/WT mice showed a marked reduction in survival compared to WT
340 littermates, with none surviving beyond 150 days (Fig 1, D). Such a high mortality rate
341 precluded analysis in our animal facility conditions. Therefore, because the mice were not
342 raised in a germ-free environment, we treated them with enrofloxacin, a broad-spectrum
343 antibiotic. Survival was then significantly improved (Fig, 1D), but Sting V154M/WT mice still
344 exhibited weight loss compared to WT littermates (Fig S1, C). Sting V154M/WT females were
345 sterile, hence we were unable to breed mice homozygous for the Sting V154M allele.

346

347 **Sting V154M/WT mice variably develop lung and renal inflammation.**

348 SAVI is characterized by an inflammatory state^{15,16}. In particular, lung interstitial disease has
349 been observed in the majority of patients, as well as skin lesions, and systemic inflammation.
350 These features are not present in all patients, with rare familial cases of the disease confirming
351 sometimes marked variability in disease expression¹⁶. We noticed that 45% of Sting
352 V154M/WT mice developed slight (++) to severe (+++++) alveolar or perivascular inflammation,
353 whereas none of the WT littermate group developed these features (Fig S2). Of note, we did
354 not detect any lung fibrosis (Fig S2), nor did our mice demonstrate skin lesions, arthritis or
355 inflammation in the distal limbs (Fig S2). Almost thirty percent of the mice developed a
356 minimal renal inflammation (interstitial hypercellularity in the medulla), with some patches of

357 interstitial fibrosis, whereas WT littermate mice didn't develop any. However, similar lesions
358 have not been reported in human SAVI patients. Proteinuria was not recorded in the Sting
359 V154M/WT mice. We did not observe any correlation between organ inflammation in Sting
360 V154M/WT mice according to whether they were treated or not with antibiotic (Fig S2). In
361 addition, there was a significant increase in serum interleukin 6 (IL-6) and tumor necrosis factor
362 α (TNF- α) levels in mutant animals compared to WT littermates (1.2 ± 0.003 pg/mL *versus*
363 20.8 ± 8.1 pg/mL for IL6, 3.8 ± 0.003 pg/mL *versus* 5.7 ± 1.2 pg/mL for TNF α , in WT and
364 Sting V154M/WT mice, respectively, mean \pm SEM; $p < 0.05$, Mann and Whitney test). Thus we
365 conclude that Sting V154M/WT mice developed inflammatory manifestations with variable
366 expression.

367

368 **Sting V154M/WT mice develop severe combined immunodeficiency (SCID).**

369 Considering the T cell lymphopenia observed in SAVI patients^{15,16}, we analyzed the proportion
370 of immune cell subsets in peripheral lymphoid compartments. Because survival was improved
371 after treatment with enrofloxacin, all of the animals included in this analysis had received
372 antibiotic. Firstly, we noticed a complete absence of lymph nodes (LNs) in Sting V154M/WT
373 mice, even after coloration with Evans blue (Fig S3). In addition, we saw a significant decrease
374 in white blood cell counts in Sting V154M/WT mice compared to WT littermates (Fig 2, A).
375 Flow cytometry analysis showed that the distribution of blood leucocyte subpopulations was
376 different in Sting V154M/WT compared to WT animals, with a decrease of B, CD4+ and CD8+
377 T cells, and NK cells, associated with an increase of granulocytes and monocytes (Fig 2, A).
378 Furthermore, Sting V154M/WT mice developed anemia (Fig 2, B), consistent with the
379 description in SAVI patients^{15,16}, possibly reflecting systemic inflammation. In accordance with
380 the blood analysis, the spleens of Sting V154M/WT mice demonstrated a severe reduction in B
381 cells, CD4+ and CD8+ T cells, and NK cells, both in terms of percentage and absolute numbers,
382 particularly affecting T and NK cells. All B cell subsets (follicular, transitional T1 and T2, and
383 marginal zone B cells) were also affected (Fig 2, C). The same observation was made for T cell
384 subsets, where we recorded a decrease in effector memory, central (markedly in CD8+ T cells)
385 and naïve T cells, the latter being the most impacted T cell subpopulation (Fig 2, C). The
386 regulatory T cell compartment (Tregs) was also impacted (Fig. S4). However, even if the
387 percentage and absolute number of Tregs was strongly diminished (Fig. S4, A), the ratio of
388 Tregs on total CD4+ T cells remained unchanged between Sting V154M/WT and WT littermate
389 mice. Furthermore, the expression of functional marker CD25 on Tregs was comparable

390 between WT and Sting V154M/WT mice (Fig S4, B). In addition to the dramatic decrease in
391 all lymphoid populations, we noticed a significant increase of granulocyte and monocyte
392 populations in the Sting V154M/WT mice (percentages and absolute numbers) (Fig 2, C). In
393 agreement with these latter results, we detected an excess of serum MCP-1 (monocyte
394 chemotactic protein-1 or CCL2) production in the serum of Sting V154M/WT mice (3.9 ± 0.7
395 pg/mL *versus* $47.6 \pm 13.2 \text{ pg/mL}$ in WT and Sting V154M/WT mice, respectively, mean \pm
396 SEM ; $p < 0.05$, Mann and Whitney test). Finally, consistent with the reduction in B cells, total
397 immunoglobulin (Ig) M and IgG levels were profoundly decreased in serum Sting V154M/WT
398 mice in comparison to WT littermates, and IgA was undetectable (Fig 2, D). The introduction
399 of the V154M heterozygous mutation in mice was thus responsible for the development of a
400 SCID phenotype, affecting all lymphocyte populations, associated with a severe
401 hypogammaglobulinemia, and a developmental defect of LNs.

402

403 **The B- and T-cell developmental blockade in Sting V154M/WT mice originates at the**
404 **early immature stages in bone marrow and thymus.**

405 In order to understand the mechanisms of lymphocyte deficiency in the periphery, we analyzed
406 early developmental stages in the bone marrow (BM) and thymus. The Hematopoietic stem cell
407 (HSC) compartment in the BM, containing long-term HSC (LT-HSC), short-term HSC (ST-
408 HSC), and multipotent progenitors (MPP), was not affected by the Sting V154M mutation (Fig
409 S5, A). Similarly, the percentages and absolute numbers of common lymphoid progenitors
410 (CLP) were comparable in the two groups of mice (Fig S5, B). However, T and B cell lineages
411 were markedly affected after the CLP stage. Indeed, Sting V154M/WT mice displayed a strong
412 reduction in ProB and PreB subsets, and an even more profound decrease in immature and
413 mature stages (Fig 3, A). These results indicated that a developmental arrest between CLP and
414 further stages of B cell subsets is responsible for the peripheral B cell lymphopenia. In addition,
415 thymic T cell development seemed to be very impacted since Sting V154M/WT mice presented
416 a residual thymus consistent with markedly reduced cellularity (Fig 3, B). Percentages and
417 absolute numbers of early thymic progenitors (ETP) were comparable between Sting
418 V154M/WT and control animals (Fig 3, C). However, thymocyte development was profoundly
419 impaired at the double negative (DN) 1 and DN2 stages, and to a lesser extent at subsequent
420 stages (Fig 3, C). Finally, the absolute numbers of double positive (DP) and CD4 and CD8
421 single positive thymocytes were decreased in Sting V154M/WT mice (Fig 3, D), thus
422 explaining the peripheral T-cell lymphopenia. In order to analyze in more detail the
423 mechanisms of this lymphopenia, we also assessed the proliferation and death status in

424 progenitors both from BM and thymus, using annexin V and BrdU staining (Fig 3, E and F).
425 We observed that progenitors from B and T cell lineages are more prone to die by apoptosis
426 (Fig 3, E). We also observed an increased incorporation of BrdU indicating an increased
427 proliferation (Fig 3, F). We thus think that the increased apoptosis of lymphoid progenitors is
428 possibly accounting for the observed peripheral lymphopenia in Sting V154M/WT mice.
429 Considering the myeloid population and consistent with their expansion in periphery, we also
430 observed an increased percentage of monocytes in the BM (Fig S5, C). However, we did not
431 detect any increase in common myeloid progenitors (CMP) and granulocyte-monocyte
432 progenitors (GMP) (Fig S5, B), suggesting a homeostatic peripheral expansion of the myeloid
433 compartment as a consequence of impaired lymphocyte development. Finally, a slight reduction
434 of erythrocytes was noticed in the BM of mutant mice (Fig S5, D). Percentages and absolute
435 numbers of megakaryocyte and erythroid progenitors (MEP) and pro-erythroblasts were not
436 statistically different between the 2 groups of mice (Fig S5, B and D). This anemia could
437 therefore be a consequence of inflammation, or of a slight blockade in erythrocyte development
438 between the pro-erythroblast and erythrocyte stages, or both. In conclusion, the profound T and
439 B cell lymphopenia observed in Sting V154M/WT mice is at least partly a consequence of an
440 early developmental defect characterized by an increased apoptosis of progenitors.

441

442 **Sting V154M mutation results in intrinsic defects in mature T and B cells.**

443 Because of the T and B cell lymphopenia and reduced antibody levels in Sting V154M/WT
444 mice (Fig. 2), we further investigated the expression of activation markers and the proliferative
445 response of these cells after *in vitro* stimulation. We detected a 6-fold increase of the CD86
446 activation marker on unstimulated Sting V154M/WT B cells compared to WT counterparts.
447 Overexpression of the CD86 marker was also observed after stimulation with anti-CD40 and
448 IL-4, or with anti-IgM and anti-CD40 (Fig 4, A). Considering the profound
449 hypogammaglobulinemia in Sting V154M/WT mice (Fig 2, D), we quantified the secreted Ig
450 in supernatant after stimulation of total splenocytes *in vitro*. Immunoglobulin secretion was
451 strongly decreased in supernatants from Sting V154M/WT splenocytes, after stimulation with
452 LPS, LPS and IL-4 or anti-CD40 and IL-4 (Fig 4, B). This was not the consequence of an
453 intrinsic defect of proliferation or plasma cell differentiation (Fig 4, C and D). However, Sting
454 V154M/WT B cells were significantly more sensitive to apoptosis than WT B cells, as shown
455 by annexin V staining after 2 days of stimulation, with several protocols of stimulation,
456 including those mimicking T cell help (Fig 4, E). Similar to B cells, splenic CD4⁺ and CD8⁺ T

457 cells displayed a hyperactivated profile at steady-state, illustrated by an overexpression of the
458 CD25 activation marker (Fig 5, A). Sting V154M/WT T cells also displayed a profound
459 activation defect following stimulation with anti-CD3 and anti-CD28 (Fig 5, A) along with
460 decreased proliferation and increased cell necrosis and apoptosis (Fig 5, B and C). These results
461 showed that mature T cells from Sting V154M/WT mice have an intrinsic proliferative defect
462 that cannot compensate the increased mortality. Thus, the very low production of
463 immunoglobulins *in vivo* could be the combined consequence of an increased B and T cell
464 apoptosis leading to a low number of B and T cells, and a lack of T cell help required for B cell
465 activation and differentiation into plasma cells.

466

467 **The SCID observed in Sting V154M/WT mice is type I IFN-independent.**

468 Given that aspects of the SAVI phenotype have been proposed to reflect enhanced type I IFN
469 signaling, we analyzed the expression of ISGs in Sting V154M/WT mice. The expression of
470 *Isg15*, *Ifit1*, and *Cxcl10/IP10* was significantly increased in total splenocytes (Fig 6, A) and
471 confirmed in splenic sorted B cells from Sting mutant mice (Fig 6, B). The expression of IFN α
472 and IFN β was also higher in these cells (Fig 6, B), as expected with a Sting GOF mutation. In
473 order to evaluate the impact of excessive type-I IFN production on the phenotype of Sting
474 V154M/WT mice, we bred them with IFNAR knock-out (KO) animals. Expectedly, the
475 expression of ISGs returned to basal levels in Sting V154M/WT IFNAR KO animals (Fig 6,
476 A). However, the weight loss (Fig 6, C), the hypogammaglobulinemia (Fig 6, D), the SCID
477 phenotype (decrease of B, T and NK cells) and the expansion of monocytes and granulocytes,
478 were not reversed (Fig 6, E). This indicated that the blockade in T, B and NK cell development
479 observed in Sting V154M/WT mice is not type I IFN-dependent. However, the anti-
480 proliferative and pro-death effect of the Sting V154M mutation in T cells was partially reversed
481 in double-mutant animals (Fig 6, F and G), thus indicating both IFN dependent and independent
482 effects of Sting GOF.

483 CONCLUSIONS

484 The development of a lymphopenia affecting T and NK cells is a common feature of patients
485 carrying a STING V155M mutation, and other mutations such as N154S or V147L^{15,16}. Total
486 B cell counts in these patients are in the normal range, although memory and switched memory
487 B cells are decreased^{15,16}. IgG and IgA hypergammaglobulinemia is seen in several patients
488 with SAVI. Our data demonstrate that the introduction of a Sting V154M mutation in mice
489 (corresponding to the V155M mutation in humans) leads to the development of a SCID
490 phenotype (T, B and NK lymphopenia), more profound than that so far described in any SAVI
491 patient, as well as an important growth delay and a dramatic reduction in survival. These mice
492 also demonstrated a marked IgM, IgG and IgA hypogammaglobulinemia. We conclude that
493 both the T and B cell lymphopenia are at least partly the consequence of a blockade in early T
494 and B cell progenitor development, therefore highlighting an unforeseen role of STING in the
495 lymphoid development. Proliferation and apoptosis analyses performed on V154M/WT
496 lymphocyte progenitors showed both proliferative expansion and increased apoptosis.
497 Therefore, the increased apoptosis of lymphoid progenitors is possibly accounting for the
498 observed peripheral lymphopenia in Sting V154M/WT mice. Similar observations have been
499 previously made in different systems like *Drosophila* or Mouse Embryonic Fibroblasts²⁸⁻³⁰
500 where authors concluded on a causal link between apoptosis and proliferation. Indeed, a
501 compensatory proliferation after cell death can be triggered by caspases and be responsible for
502 the induction of adjacent surviving cells proliferation in order to replace dying cells^{28,29,31}.
503 Moreover, Cabatingan *et al.* have proposed a mechanism in which homeostatic B cell
504 proliferation is a regulatory process related to the intrinsic B cell defect³². Further experiments,
505 such as transcriptome analysis in successive stages of B and T development, are needed to better
506 understand the developmental arrest of lymphoid progenitors in Sting V154M/WT mice.
507 Several hypotheses can be proposed to explain the lymphocyte development blockade observed
508 in Sting V154M/WT mice but not in SAVI patients. Firstly, the level of STING expression
509 could be different in early T and B cell progenitors between humans and mice. Secondly, one
510 can speculate that partners of STING and/or target genes implicated in lymphocyte
511 development are distinct between species. These potential differences may lead to a toxic effect
512 on lymphoid progenitors that is more pronounced in mice, or not reversed by compensatory
513 mechanisms existing in humans.
514 In addition, we observed an absence of LNs in Sting V154M/WT mice. This could be, at least
515 in part, the consequence of an absence of Lymphoid Tissue-inducing cells (LTi), members of
516 an emerging family of innate lymphoid cells (ILCs) which play a key role in the development

517 of LNs and Peyer's patches. ILCs develop from the CILP (common innate lymphoid
518 progenitor) stage coming from CLP progenitor in bone marrow³³. Therefore, considering the
519 blockade of lymphoid cells development in Sting V154M/WT mice after the CLP stage, one
520 could speculate that the ontogeny of ILCs is also impacted. In addition, lymphotoxin alpha
521 (LT α) deficient mice are also lacking LN, demonstrating an important role for LT α -derived
522 LT α in secondary lymphoid organ development during embryogenesis³⁴⁻³⁶. Preliminary results
523 confirmed that the expression of LT α is decreased in T cells from Sting V154M/WT mice.
524 Finally, mature B and T cells displayed intrinsic defects, characterized by a hyperactivation
525 status at steady state, and by a defect of activation and proliferation in mature T cells. We
526 showed that the hypogammaglobulinemia was not the consequence of a B-cell intrinsic
527 proliferation or differentiation defect. However, we detected an increase of apoptosis in B and
528 T cells. It is therefore plausible that the low Ig titers accompanying B cell lymphopenia relate
529 to a lack of T cell help.

530 Another common molecular feature of the SAVI phenotype, for all the mutations described so
531 far, is an increased expression of ISGs. In Sting V154M/WT mice, we detected a significant
532 IFN signature in splenocytes and in sorted B cells. The magnitude of the IFN signature in our
533 mice (almost 5 fold) is close to that described in murine models of the type I interferonopathy
534 Aicardi-Goutières syndrome, carrying the homozygous RNaseH2 G37S point mutation^{37, 38},
535 and animals deficient for *Samhd1*³⁹. To our surprise, the KO of IFNAR in Sting V154M/WT
536 mice rescued neither the defect in B, T, and NK cell development, nor the profound
537 hypogammaglobulinemia, and did not reverse the expansion of myeloid cells, suggesting that
538 type I IFN does not play a causal role in the SCID phenotype of Sting V154M/WT mice.
539 However, the *in vitro* proliferative defect and the pro-death phenotype of mature T cells were
540 partially reversed by IFNAR deficiency. These data suggest potential multiple effects of Sting
541 GOF, comprising a type I IFN-independent blockade of lymphocyte development at early
542 stages on one hand, and an anti-proliferative effect in T cells which is partially type I IFN-
543 dependent on the other hand. A defect of T cell proliferation in SAVI patients has been
544 described recently, but was not reversed by type I IFN or TNF neutralization *in vitro*⁴⁰. One
545 difference between the two experimental models is the fact that T cells in our mutant mice
546 developed in an *in vivo* IFNAR KO environment, as opposed to an *ex vivo* / *in vitro* setting. In
547 addition, one cannot exclude the existence of species-specific mechanisms in the control of T
548 cell proliferation through the STING/type I IFN pathway. Finally, our data showed that the

549 partial rescue of the anti-proliferative effect of Sting in IFNAR KO T cells could not reverse
550 the low Ig production recorded in Sting V154M/WT mice.

551 We observed some inflammatory manifestations in Sting V154M/WT mice, although with
552 variable expression. These included signs of pulmonary and renal inflammation in 45% and
553 27% of the animals, respectively, which was associated to an increased production of
554 inflammatory cytokines. We did not record any lung fibrosis, or skin lesions, which is distinct
555 from the overall clinical picture of SAVI. The variable penetrance of pulmonary and renal
556 inflammation is in accordance with the human disease state which is also characterized by
557 variable expression, spanning early-onset systemic inflammation with mutilating skin lesions
558 and lethal pulmonary inflammation^{15,16}, through to “idiopathic” lung fibrosis^{21,23} and isolated
559 chilblain lupus inherited stably across several generations²⁴. In addition, an intrafamilial disease
560 expression variability is also observed in humans¹⁶. The inconstant expression of organ
561 inflammation in Sting V154M/WT model suggests that environmental factors (animal facility
562 environment and potential interaction with microbiota) are needed. Moreover, these results
563 confirmed that renal damages observed are not a consequence of the long-term antibiotic
564 treatment, as only some mutant mice (27%), but none of the treated controls, presented with
565 kidney inflammation.

566 Warner *et al.* recently described another model of Sting GOF, the N153S model, corresponding
567 to the N154S mutation in SAVI patients⁴¹. These mice developed an inflammatory disease with
568 pulmonary inflammation (~~lung involvement in 20% of animals~~) in the absence of fibrosis, and
569 skin inflammation, associated with an immune deficiency, including T cell lymphopenia. They
570 concluded that some features of N153S mice were different from those seen in STING N154S
571 SAVI patients. Altogether, the data obtained from Sting GOF mutations in mice (N153S and
572 V154M) are in support of partial differential effects of STING pathway in humans and mice.
573 The two animal models have common characteristics, such as T and NK cell lymphopenia, and
574 the existence of inflammatory features. The anti-proliferative effect of Sting N153S GOF was
575 not assessed by Warner *et al.* but an *in vitro* proliferative defect is seen both in the STING
576 V155M¹⁶ and in STING N154S human lymphocytes (F. Rieux-Laucat, unpublished results).
577 However, some differences between the Sting N153S and V154M models are also observed.
578 Indeed, the Sting V154M mice described here are characterized by a B cell lymphopenia and a
579 marked hypogammaglobulinemia, whereas Sting N153S mice demonstrated normal B cell
580 counts and hyper IgM. This hyperproduction, in the context of a T cell lymphopenia in Sting
581 N153S mice, is not explained for the moment, but it may possibly relate to a defective T-B
582 cooperation in germinal centers. The lack of hyper IgM in the V154M model suggests that B

583 cell precursors are more affected than those of the N153S model. Whether it relates to
584 differential expression levels of the mutants or to different impacts on STING partners remains
585 to be established. Finally, the expression of organic lesions was also different between the two
586 models. It has been shown that human N154S and V155M mutations result in constitutive ER
587 STING exit, and thus activate STING independently of cGAMP binding¹⁴. Moreover, both
588 mutations are localized in the DD domain of STING and form constitutive and stable dimers
589 ^{15,16}. However, some mutation-dependent effects might be envisaged to explain the phenotypic
590 differences between these two models. Further experiments (such as genome-wide
591 transcriptome profiling) comparing N153S and V154M murine cells will be necessary to
592 elucidate such differences. One may also question a possible difference in the stability of the
593 two mutant proteins. In addition, considering the important role of STING in viral and bacterial
594 sensing, we cannot exclude differences in environmental factors (animal facility environment
595 and health status) between the two models. The survival of the V154M/WT mice was
596 statistically increased after treatment with enrofloxacin, a broad-spectrum antibiotic, perhaps
597 arguing in favor of a microbial-dependent effect. However, an extensive bacteriological
598 analysis did not detect any infection related to pathogens targeted by this antibiotic in our mice.
599 An alternative possibility is that enrofloxacin treatment had an effect on the intestinal
600 microbiota composition, and that Sting interaction with the microbiota can impact on the mice
601 phenotype, notably because STING is activated by cyclic dinucleotides and that such CDNs
602 can be produced by gut microbiota^{42, 43}. Indeed, STING seems to be highly implicated in the
603 gut homeostasis, as shown in Sting deficient mice⁴². Thus, we can suppose that a fine regulation
604 of Sting is necessary to maintain this homeostasis and would explain variations between mice,
605 depending on interactions with the environment. Moreover, authors showed that IgA seem to
606 be essential for gut homeostasis, since they decrease the commensal bacteria penetration in the
607 intestinal epithelium⁴². Therefore, IgA deficiency in our GOF model could also have an impact
608 on intestinal microbiota and gut homeostasis.

609 Considering these findings, this new mouse model constitutes an important model in which to
610 explore the role of Sting in lymphocyte development, and to further improve our understanding
611 of pathophysiological mechanisms of lymphopenia in SAVI patients on the one hand, and of
612 SCID (including severe hypogammaglobulinemia) on the other. Finally, the type-I IFN
613 independent mechanism of SCID development may have important implications in the future
614 treatment strategy of SAVI patients.

615

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629

630 **AUTHOR CONTRIBUTIONS**

631 D.B., T.M., A.S.K., F.R.L. and P.S.S. designed the research.

632 D.B., F.A., D.L., V.D., C.E., H.J., and A.M.K. performed the research.

633 D.B., P.K., S.J., H.J., N.J., Y.J.C., I.A.S., A.S.K., F.R.L. and P.S.S. analyzed the data.

634 D.B., S.J., A.B., Y.J.C., I.A.S., A.S.K., F.R.L. and P.S.S. wrote the paper.

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750 **FIGURE LEGENDS**

751 **Figure 1. Murine *Sting* V154M heterozygous mutation is associated with a reduced**
752 **survival. (A)** Introduction of the murine mutation (V154M), corresponding to V155M *Sting*
753 GOF in humans, using CRISPR/Cas9 technology. **(B)** Agarose gel electrophoresis of PCR
754 amplified products using specific primers in exons 4 and 6 of *Tmem173*. Lane 1: DNA size
755 marker, lane 2: negative control of the PCR reaction, lanes 3-5: WT littermate mice, lanes 6-8:
756 *Sting* V154M/WT mice. **(C)** Visualisation of the heterozygous V154M mutation by Sanger
757 sequencing of genomic DNA (gDNA, upper panel) and complementary DNA (cDNA, lower
758 panel) from WT littermate (left) and transgenic mice (right). **(D)** Survival rates of *Sting*
759 V154M/WT mice, untreated and treated with antibiotic (see Materials and Methods), as well as
760 control WT littermate mice (n=15 per group) were evaluated by Kaplan-Meier analysis. The
761 result of the Log-rank test is indicated (***, p<0.0001).

762

763 **Figure 2. Murine *Sting* V154M heterozygous mutation is associated with a profound**
764 **combined immunodeficiency phenotype. (A)** Left: total WBC (white blood cells) count ($\times 10^3$
765 cells/ μ L) (n=5 in each group). Right: percentages of immune cell subpopulations in whole
766 blood cells assessed by flow cytometry (n \geq 9 in each group). **(B)** Left: RBC (red blood cells)
767 count ($\times 10^6$ cells/ μ L). Right: hemoglobin levels (g/dL) (n=5 in each group). **(C)** Flow cytometry
768 phenotypic characterization of splenic cells (indicated both as percentages and absolute
769 numbers) (n \geq 13 in each group). Upper panel: main splenic populations in the spleen of WT
770 littermate mice (open bars) and *Sting* V154M/WT mice (black bars). Middle panel, left: B cell
771 subpopulations gated on B220+IgM+ cells, with representative dot plots from WT littermate
772 mice and *Sting* V154M/WT mice. Gates show Transitional 1 (T1: CD21/CD35^{low} CD23^{low}),
773 Transitional 2 (T2: CD21/CD35^{high} CD23^{high}), Follicular (Fo: CD21/CD35^{int} CD23^{high}), and
774 Marginal Zone (Mz: CD21/CD35^{high} CD23^{low}) populations. Middle panel, right: representative
775 dot plots of granulocytes (Gr1^{high}Mac1⁺), monocytes (Gr1^{int}Mac1⁺) and NK cells
776 (CD49b⁺NK1.1⁺). Lower panel: T cell subpopulations gated on CD4⁺ T cells and CD8⁺ T cells
777 respectively, with representative dot plots from WT littermate and *Sting* V154M/WT mice.
778 Gates show effector memory (Eff. mem.: CD44⁺ CD62L⁻), central memory (C. m.: CD44^{high}
779 CD62L⁺) and naive (CD44^{low} CD62L⁺) T cell populations. **(D)** Total IgM, IgG and IgA
780 (μ g/mL) were measured in serum of WT littermate mice (open squares) and *Sting* V154M/WT
781 mice (black circles). Each dot represents the result for one animal. ND: non detectable. Mice
782 were 2 to 5 months old. Bars represent the mean \pm SEM. *, p<0.05, **, p<0.01, ***, p<0.001,
783 Mann-Whitney test.

784

785 **Figure 3. The defect observed in B and T cell populations occurs early in their**
786 **development in the bone marrow (BM) and the thymus. (A)** Immunophenotyping of B cells
787 in the BM. Left: representative dot plots from WT littermate and Sting V154M/WT mice. Gates
788 show ProB and PreB (IgM-B220^{low}), Immature (Imm.: IgM+B220^{low}) and Mature
789 (IgM+B220^{high}) B cells. Right: mean +/- SEM of percentages and absolute numbers in WT
790 (open bars) and Sting V154M/WT mice (black bars) (n≥6 in each group). **(B)** Left: total number
791 of thymocytes in WT littermate (open bars) and Sting V154M/WT mice (black bars) (n=17 in
792 each group). Right: pictures illustrating the abnormal thymus observed in Sting V154M/WT
793 mice compared to WT littermates (7 weeks old mice). **(C)** Immunophenotyping of ETP and DN
794 cells in the thymus. Left: representative dot plots from WT and Sting V154M/WT mice. Gates
795 show early T cell progenitors (ETP: Lin⁻CD44⁺CD25⁻ckit⁺), Double Negative fractions DN1
796 (Lin⁻CD44⁺CD25⁻), DN2 (Lin⁻CD44⁺CD25⁺), DN3 (Lin⁻CD44⁻CD25⁺) and DN4 (Lin⁻CD44⁻
797 CD25⁻). Right: mean +/- SEM of percentages and absolute numbers in WT littermate (open
798 bars) and Sting V154M/WT mice (black bars) (n≥6 in each group). **(D)** Immunophenotyping
799 of DP and SP cells in the thymus. Left: representative dot plots from WT littermate and Sting
800 V154M/WT mice. Gates show Double Positive fraction (DP: Lin⁻CD8⁺CD4⁺), and Simple
801 Positive fractions (CD4: Lin⁻CD4⁺CD8⁻ / CD8: Lin⁻CD4⁻CD8⁺). Percentages and absolute
802 numbers in WT littermate (open bars) and Sting V154M/WT mice (black bars) (n≥6 in each
803 group). **(E)** Cell death analysis by flow cytometry using annexin V staining. The first graph
804 represents the percentages of annexin V⁺ cells in CLP, ProB and PreB, Immature and Mature
805 stages in bone marrow. The second graph represents each stage of T cell lineage in thymus. WT
806 littermate mice: open bars; Sting V154M/WT mice: black bars (n=5 in each group) **(F)**
807 Proliferation analysis in progenitors by flow cytometry using BrdU staining. The first graph
808 represents the percentages of BrdU⁺ cells in bone marrow progenitors and the second one
809 represents thymus progenitors. WT littermate mice: open bars; Sting V154M/WT mice: black
810 bars (n=5 in each group). Mice were 2 to 5 months old. Bars represent the mean +/- SEM *,
811 p<0.05, **, p<0.01, ***, p<0.001, Mann-Whitney test.

812

813 **Figure 4. Sting V154M/WT B cells display intrinsic functional defects. (A)** Left: ratio of
814 CD86 MFI gated on B220⁺ cells after 3 days of culture of sorted splenic B cells, in unstimulated
815 cells (NS) or after stimulation with LPS (10μg/mL), anti-IgM (10μg/mL), anti-CD40 / IL4
816 (1μg/mL – 5ng/mL) and anti-IgM / antiCD40 (10μg/mL – 1μg/mL) (n≥5 in each group). Right:
817 histograms show representative overlays for WT littermate (white) and Sting V154M/WT mice

818 (grey). **(B)** Total IgM and IgG (ng/mL) were measured in supernatant after 3 days of culture of
819 total splenocytes from Sting V154M/WT (black bars) or WT littermate mice (open bars) (n>5
820 in each group), with LPS (10µg/mL), LPS / IL4 (10µg/mL – 5ng/mL) or anti-CD40 / IL4
821 (1µg/mL – 5ng/mL). ND: non detectable. **(C)** Percentage of proliferating B cells after 4 days
822 of stimulation with a combination of anti-IgM (10µg/mL) and anti-CD40 (1µg/mL) or with
823 LPS (10µg/mL). Histogram shows representative overlays (n=5 in each group). **(D)** Left:
824 percentage of plasmablasts (B220^{low/-}CD138⁺) after 3 days of stimulation of B cells with LPS
825 (10µg/mL). Open bars: WT littermate; black bars: Sting V154M/WT (n=6 in each group).
826 Right: representative dot plots. **(E)** Percentage of B cells apoptosis (Annexin V⁺ cells) after 2
827 days of stimulation of splenocytes with LPS (10µg/mL), LPS / IL4 (10µg/mL – 5ng/mL), anti-
828 IgM (10µg/mL), anti-CD40 / IL4 (1µg/mL – 5ng/mL) and anti-IgM / antiCD40 (10µg/mL –
829 1µg/mL) (n>10 in each group). Mice were 2 to 5 months old. Bars represent the mean +/- SEM.
830 *,p<0.05, **, p<0.01, ***, p<0.001, ****, p<0.0001, Mann-Whitney test.

831
832 **Figure 5. Sting V154M/WT T cells display intrinsic functional defects.** **(A)** Left: ratio of
833 CD25 MFI gated on CD4⁺ or CD8⁺ cells after 3 days of culture of splenocytes, in unstimulated
834 cells (NS) or after stimulation with anti-CD3/anti-CD28 (10µg/mL each) for WT littermate
835 (white) and Sting V154M/WT mice (grey) (n=6 in each group). **(B)** Left: percentage of
836 proliferating T cells after 4 days of stimulation of splenocytes from WT littermate (white) and
837 Sting V154M/WT mice (black) with anti-CD3/anti-CD28 (10µg/mL each) (n≥6 in each group).
838 Right: histograms show representative overlays. **(C)** Left panel: Percentage of T cell death (PI⁺
839 cells) at steady state and after stimulation of splenocytes with anti-CD3/anti-CD28 (10µg/mL
840 each) for 3 days (n≥6 in each group). Right panel: Percentage of T cell death (Annexin V⁺ cells)
841 at steady state and after stimulation of splenocytes with anti-CD3/anti-CD28 (10µg/mL each)
842 for 2 days (n>10 in each group). Mice were 2 to 5 months old. Bars represent the mean +/-
843 SEM. **, p<0.01, ***, p<0.001, ****, p<0.0001, Mann-Whitney test.

844
845 **Figure 6. The SCID phenotype in Sting V154M/WT mice is type I IFN-independent, but**
846 **the anti-proliferative effect in T cells is partly IFN-dependent.** **(A, B)** RT-qPCR analysis of
847 ISG in total splenocytes **(A)** and in sorted mature splenic B cells **(B)** of WT littermate (open
848 bars) Sting V154M/WT (black bars), Sting WT/IFNAR KO (light grey) and Sting
849 V154M/WT/IFNAR KO (dark grey). mRNA levels of 3 ISGs (n≥5 in each group) **(A, B)** and
850 of IFNα and IFNβ (n≥3 in each group) **(B)** were measured by qRT-PCR. **(C)** Weight curves of
851 Sting WT/IFNAR KO mice (light grey squares) and Sting V154M/WT/IFNAR KO mice (dark

852 grey squares). Each dot represents at least 8 mice. **(D)** Flow cytometry phenotypic
853 characterization of splenic cells from WT/IFNAR KO (light grey) and Sting
854 V154M/WT/IFNAR KO (dark grey) mice (n=10 in each group). Left: bar graphs show the
855 mean +/- SEM of percentages (n=10 in each group). Right: representative dot plots of each
856 population: B cells (IgM⁺ B220⁺), CD4⁺ T cells (CD4⁺CD8⁻), CD8⁺ T cells (CD4⁻CD8⁺),
857 Granulocytes (Gr.: Gr1^{high}Mac1⁺), Monocytes (Mo.: Gr1^{int}Mac1⁺) and NK cells
858 (CD49b⁺NK1.1⁺). **(E)** Total IgM and IgG (μg/mL) were measured in serum of WT/IFNAR KO
859 (open triangles) and Sting V154M/WT/IFNAR KO mice (grey diamonds). Each dot represents
860 one animal. **(F)** Left: percentage of proliferating T cells after 4 days of stimulation of
861 splenocytes from WT/IFNAR KO mice (light grey) and Sting V154M/WT/IFNAR KO mice
862 (dark grey) with anti-CD3/anti-CD28 (10μg/mL each) (n=10 in each group). Right: histogram
863 shows representative overlays. **(G)** Percentage of T cell death (PI⁺ cells) at steady state and
864 after stimulation of splenocytes with anti-CD3/anti-CD28 (10μg/mL each) for 3 days (n=10 in
865 each group). Mice were 2 to 5 months old. Bars represent the mean +/- SEM. *, p<0.05, **,
866 p<0.01, ***, p<0.001, ****, p<0.0001. Mann-Whitney test.

867

868 **Supplemental Figure 1. Weight loss and Sting expression in Sting V154M/WT mice.** **(A)**
869 Detection of Sting by immunoblot analysis on sorted splenic B cells (left: representative gel;
870 right: quantification of Sting expression, n=6 mice in each group). **(B)** Expression of *Sting*
871 mRNA assessed by RT-qPCR after RNA extraction of sorted splenic B cells from WT
872 littermate (open bars) and Sting V154M/WT mice (black bars) (n≥4 in each group). **(C)** Left:
873 weight curves of WT littermate mice (open squares) and Sting V154M/WT mice (black circles).
874 Each dot represents at least 17 mice. Right: representative picture of Sting V154M/WT *versus*
875 WT littermate mouse, both females (age at the analysis: 3 months). Bars represent the mean +/-
876 SEM. *, p<0.05, **, p<0.01, ****, p<0.0001, Mann-Whitney test.

877

878 **Supplemental Figure 2. Histological analysis showing that Sting V154M/WT mice present**
879 **incomplete penetrance of a lung and renal inflammatory phenotype.** **(A)** Summary of
880 histological analysis performed on 11 Sting V154M/WT mice *versus* 9 WT littermate mice.
881 Each individual experiment is presented with a different colour in the “mouse ID” column.
882 Some mice were treated with the antibiotic (enrofloxacin, see Materials and Methods), as
883 indicated in the second column. **(B)** Representative pictures of Sting V154M/WT mice with
884 organ damages (left, A, C, and E) and with absence of organ damages (right, B, D, and F). (A-
885 B) Hind paws; (C-D) lungs; and (E-F) kidneys (cortex). (A-D and G-H) H&E staining and (E-

886 F) Masson's trichrome (fibrosis is demonstrated in blue). Ellipses circle subcutaneous vascular
887 network (SVN) that are occasionally obstructed with cellular material. Dotted arrows indicate
888 inflammatory foci in lung and kidney medulla. Arrows point to fibrosis in renal cortex. A:
889 alveolar parenchyma; B: bone; Br: bronchia; BV: blood vessel; E: epidermis; G: glomeruli HF:
890 hair follicle; T: tubules. Scale bars correspond to 100 μm on (A-B); 500 μm on (C-D) and 250
891 μm on (E-H).

892

893 **Supplemental Figure 3. Absence of lymph node in Sting V154M/WT mice compared to**
894 **WT littermates.** Pictures from a WT littermate (left column) and a Sting V154M/WT mouse
895 (right column) (7-week-old mice). (A) auricular LNs. (B) axillary LNs. (C) inguinal LNs. (D)
896 popliteal LNs. LNs (arrow) were labelled with 5% Evans blue dye after subcutaneous injection
897 in front legs, hindfoot and ears. Pictures are representative of three distinct experiments. The
898 phenotype was identical at different ages (7-, 12- and 20-week-old mice).

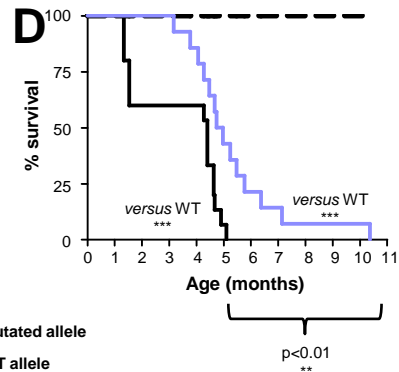
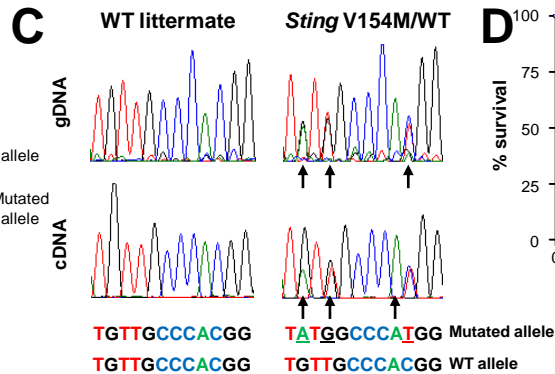
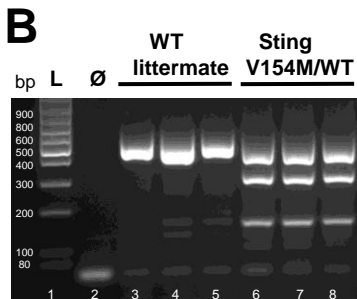
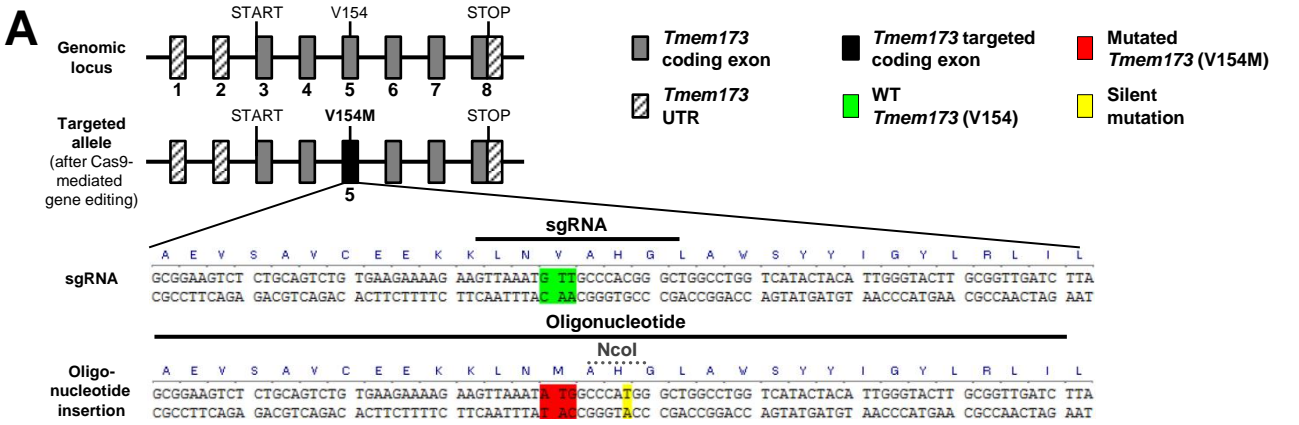
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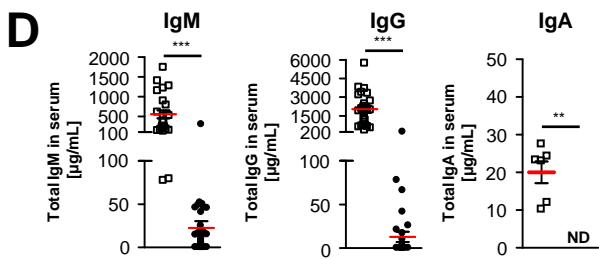
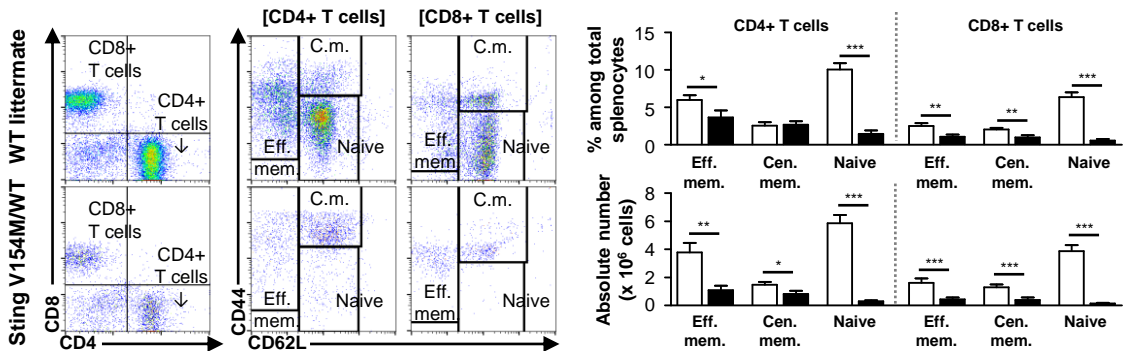
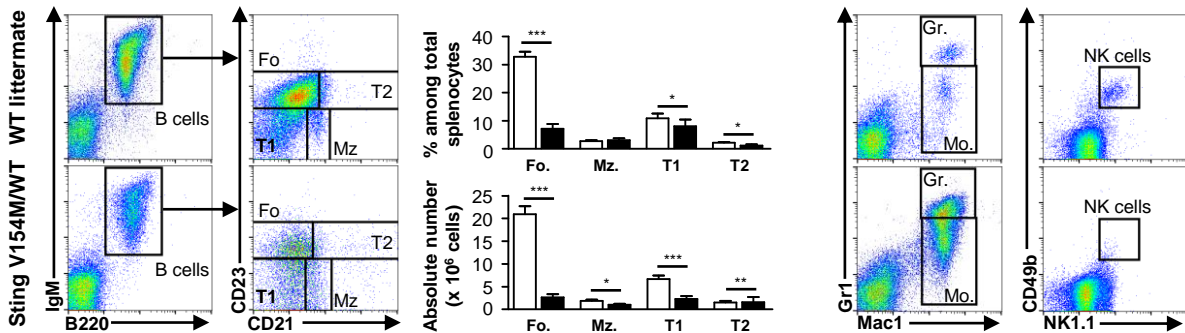
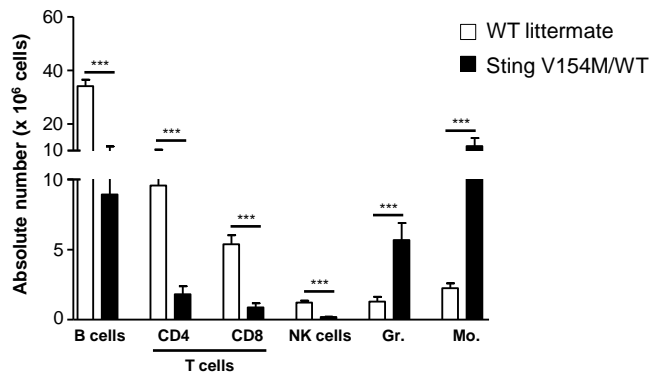
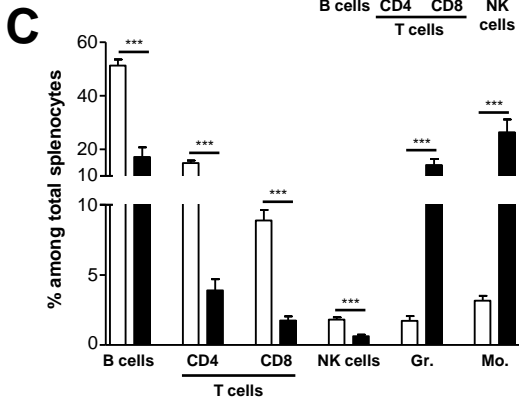
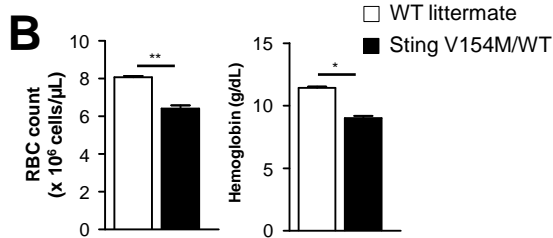
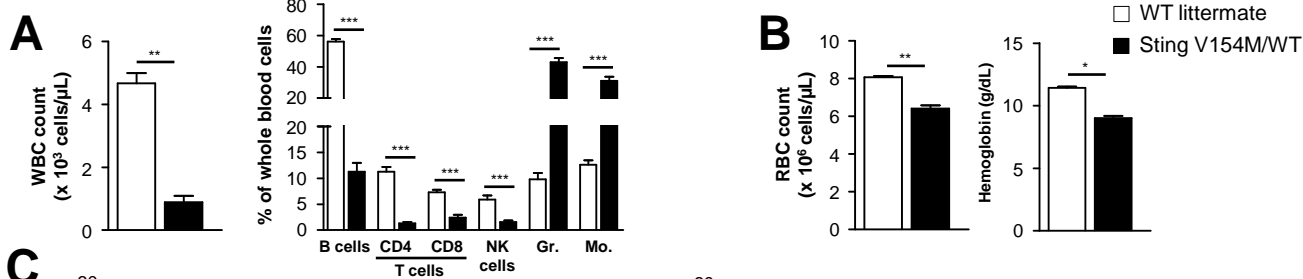
900 **Supplemental Figure 4. Impact of Sting GOF on Tregs and their activation status. (A)**
901 Panel on the left shows representative dot plots of the relevant population, both for WT
902 littermate and Sting V154M/WT mice ($n \geq 10$ in each group). Right: histograms showing
903 percentages and absolute numbers of Tregs ($\text{CD4}^+\text{Foxp3}^+$) and the ratio Tregs/total CD4^+ T
904 cells. WT littermate mice are represented with open bars and Sting V154M/WT mice with black
905 bars. **(B)** Expression of CD25 on Tregs. Histogram showing CD25 MFI ($n \geq 4$ in each group)
906 and a representative overlay. Mice were 2 to 5 months old. Bars represent the mean \pm SEM.
907 ***, $p < 0.001$, Mann-Whitney test.

908

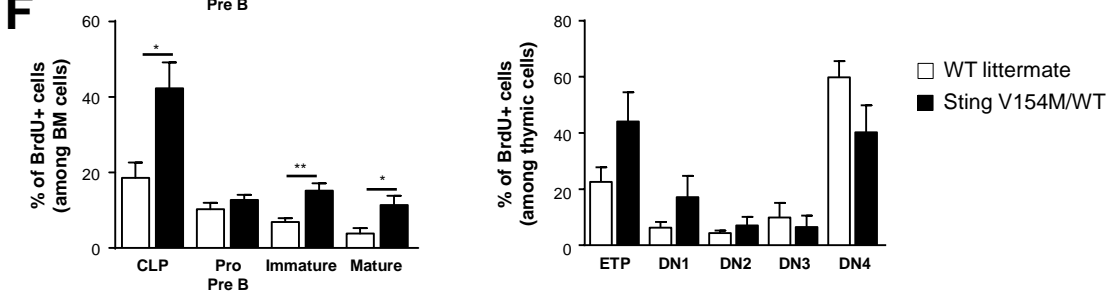
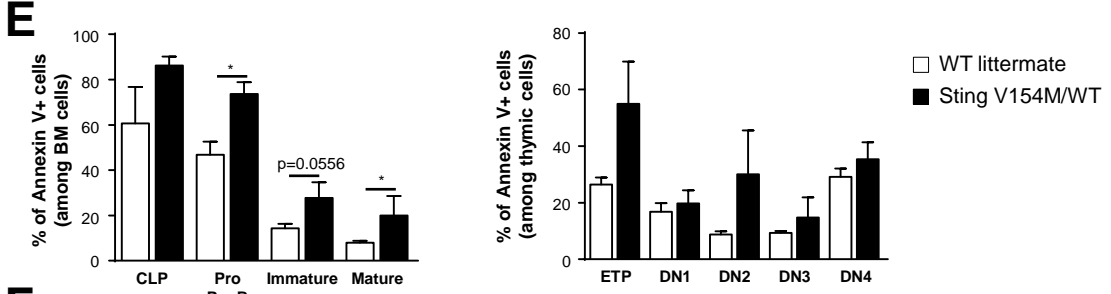
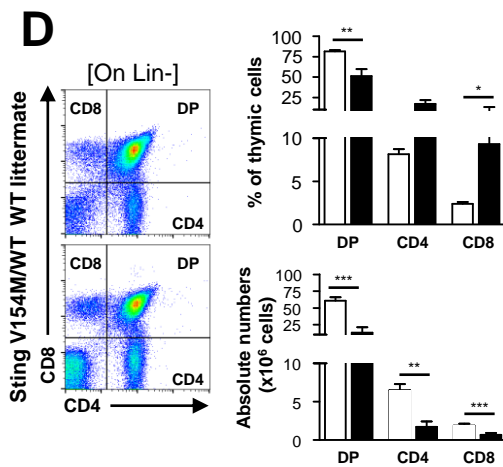
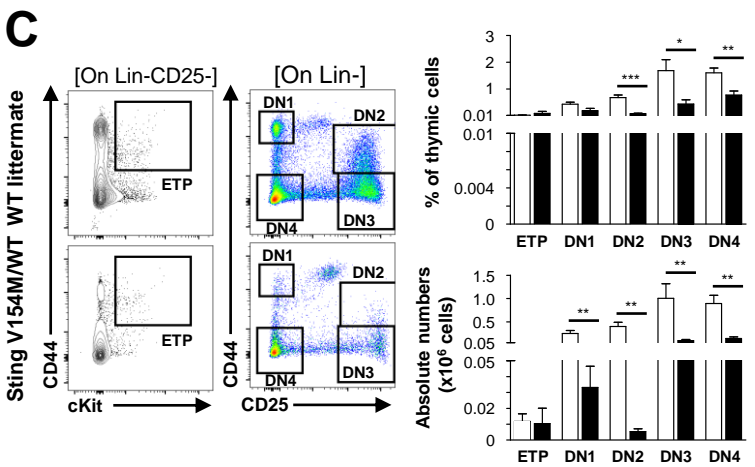
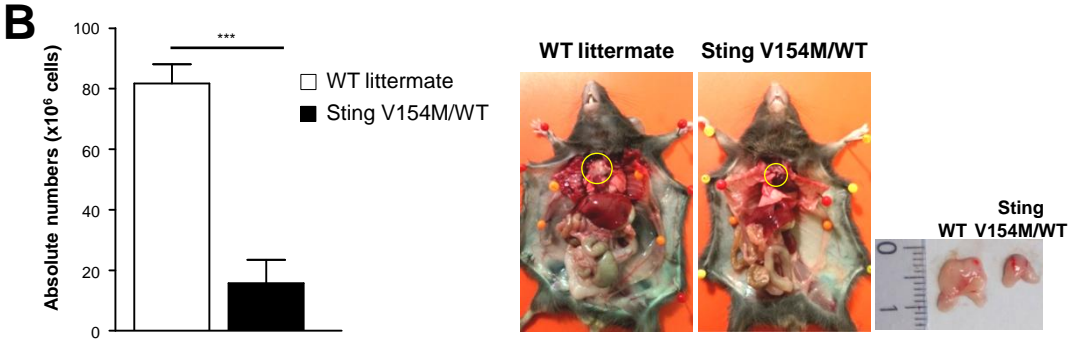
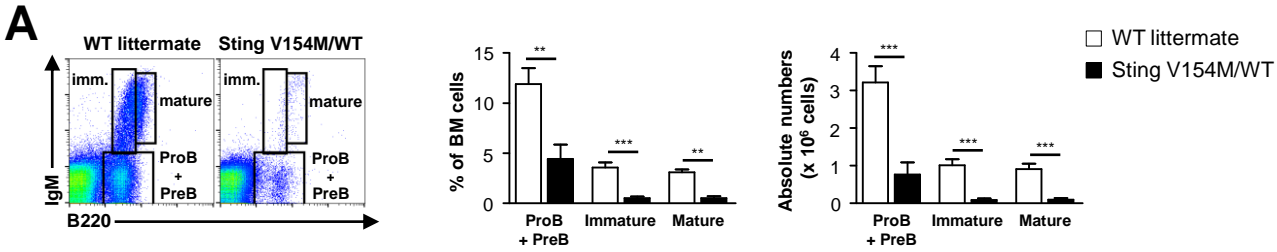
909 **Supplemental Figure 5. Despite a defect in hematopoietic cell development after**
910 **engagement in the B and T cell lineage, early bone marrow progenitors are not impacted**
911 **in Sting V154M/WT mice.** Each panel on the left shows representative dot plots of the relevant
912 population, both for WT littermate and Sting V154M/WT mice ($n \geq 3$ in each group). Each
913 population is shown in chronological order of appearance in the development in the BM.
914 Percentages (middle) and absolute numbers (right) represented for WT littermate (open bars)
915 and Sting V154M/WT (black bars) mice. **(A)** Left: gates show Long-Term (LT-HSC: Lin^-
916 $\text{Sca1}^+\text{ckit}^+\text{CD48}^-\text{CD150}^+$) and Short-Term (ST-HSC: Lin^- $\text{Sca1}^+\text{ckit}^+\text{CD48}^-\text{CD150}^-$)
917 Hematopoietic-Stem-Cells, and the Multi-Potent-Progenitor (MPP: Lin^-
918 $\text{Sca1}^+\text{ckit}^+\text{CD48}^+\text{CD150}^-$) populations. **(B)** Left: gates show the Common Lymphoid (CLP:
919 Lin^- $\text{CD127}^{\text{high}}\text{ckit}^{\text{low}}\text{Sca1}^{\text{low}}$), the Common myeloid (CMP: Lin^- $\text{ckit}^+\text{Sca1}^-\text{CD16/32}^{\text{int}}\text{CD34}^{\text{int}}$),

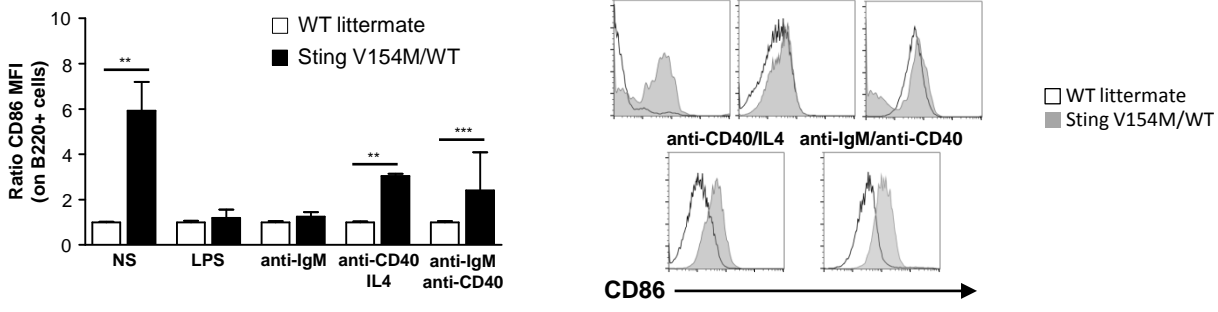
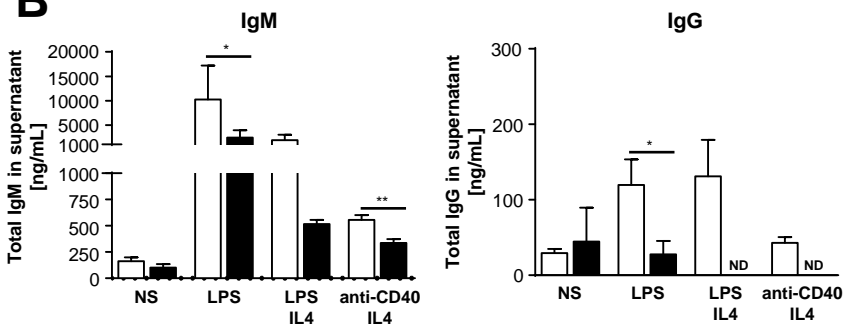
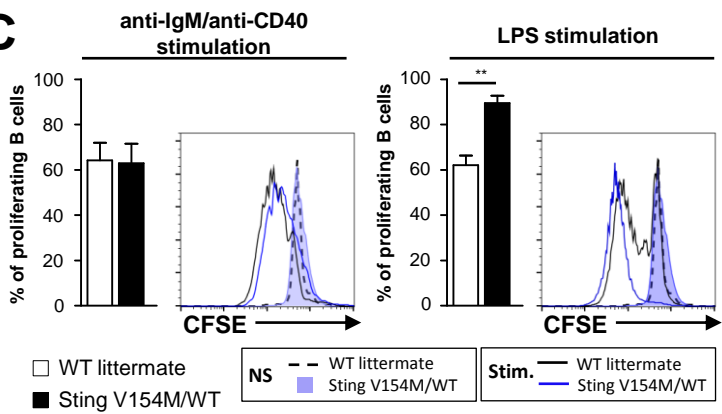
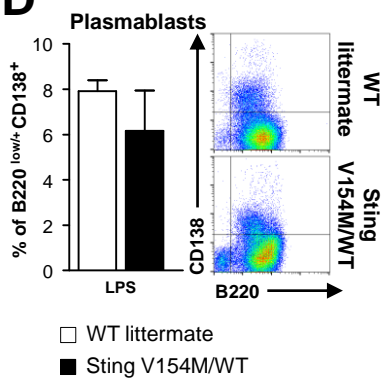
920 the Granulocyte/Macrophage (GMP: Lin⁻ckit⁺Sca1⁻CD16/32^{high}CD34^{high}) and the
921 Megakaryocyte/erythrocyte (MEP: Lin⁻ckit⁺ Sca1⁻CD16/32⁻CD34⁻) progenitor. (C) Left: gates
922 show conventional Dendritic cells (DCs: CD317⁺CD11c⁺), plasmacytoid Dendritic Cells
923 (pDCs: CD317⁺CD11c⁺), Monocytes (Mo.: Gr1^{int}Mac1⁺) and Granulocytes (Gr1^{high}Mac1⁺)
924 populations. (D) Left: gates represent the megakaryocyte/erythrocyte lineage with Pro-
925 erythrocytes (Pro Er.: CD71⁺Ter119^{low}) and Erythrocytes (Er.: CD71⁺Ter119⁺).
926 Bars represent the mean +/- SEM. **, p<0.01, Mann-Whitney test.





□ WT littermate ● Sting V154M/WT



A**B****C****D****E**