**Genetic differences between 129S substrains affect antiretroviral immune responses**

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Running title: Antiretroviral immune responses in 129S mice

**Abstract**

Inbred mouse lines vary in their ability to mount protective antiretroviral immune responses, and even closely related strains can exhibit opposing phenotypes upon retroviral infection. Here, we found that 129S mice inherit a previously unknown mechanism for the production of anti-murine leukemia virus (MLV) antibodies and control of infection. The resistant phenotype is controlled by two dominant loci that are independent from known MLV-resistance genes. We also show that production of anti-MLV antibodies in 129S7, but not 129S1 mice is independent of interferon gamma (IFNγ) signaling. Thus, our data indicate that 129S mice inherit an unknown mechanism for control of MLV infection and demonstrate that there is genetic variability in 129S substrains that affects their ability to mount antiviral immune responses.

**Importance**

Understanding the genetic basis for production of protective antiviral immune responses is crucial for the development of novel vaccines and adjuvants. Additionally, characterizing the genetic and phenotypic variability in inbred mice has implications for the selection of strains for targeted mutagenesis, choice of controls, and for broader understanding of the requirements for protective immunity.

18 **Introduction**

19 Resistance and sensitivity to viral infections depends greatly upon the genetic make-up of the
20 host. Model organisms such as inbred mice have proved to be invaluable for the investigation of
21 the pathways underlying protective immune responses due to the inability to perform genetic
22 manipulations in humans. In this regard, mouse models of murine retroviruses, such as murine
23 leukemia virus (MLV) and mouse mammary tumor virus (MMTV) have provided essential
24 insights into the molecular mechanisms underlying anti-viral immune responses. MLV is
25 transmitted as an exogenous virus through the blood and milk or as an endogenous stably
26 integrated provirus and primarily infects cells of lymphoid origin (1). Pathogenic MLVs are often
27 a mixture of ecotropic and polytropic [mink cell focus forming (MCF)] viruses, and some contain
28 an additional replication-defective spleen focus forming virus (SFFV) component that promotes
29 pathogenesis (1). These viruses cause a range of diseases in susceptible mice, including
30 lymphomas, leukemias, rhabdomyosarcoma, neurological disorders, and immunosuppression
31 (2-6). Mice from retrovirus-susceptible strains, such as BALB/c mice, sense retroviral
32 pathogens, as indicated by the fact that they initiate an antiretroviral response. However, this
33 response is not long-lasting and is unsuccessful in controlling virus replication (7, 8), which is
34 most likely due to the numerous mechanisms of immune evasion employed by retroviruses (9-
35 13). In contrast, pathogen detection in resistant mice translates into a robust, long-lasting, and
36 virus-neutralizing immune response comprised of both T and B cells responses (7, 9, 14, 15).

37 Classical genetics approaches in mice have identified several genes that play a role in
38 directly restricting MLV replication, or in the generation of protective antiviral immune responses
39 [Table 1 and recently reviewed in (16)]. *Fv1* is a capsid-specific restriction factor encoded by an
40 endogenous retroviral *Gag* gene that governs tropism of MLV. Alleles of *Fv1* are defined by their
41 ability to block specific subclasses of MLV, with incompatibility resulting in a post-entry block to
42 infection, (17-20) and in some cases, stimulation of cross-protective neutralizing adaptive
43 immune responses (21). *Fv2* is a dominant Friend MLV (FV) susceptibility gene that facilitates
44 splenomegaly and erythroleukemia induction by SFFV by promoting erythropoietin receptor

45 signaling (22, 23). *Rfv3* is a dominant FV resistance gene that is encoded by *Apobec3*. The
46 resistant allele of *Apobec3* inherited by C57BL/6 and C57BL/10 mice restricts FV replication and
47 promotes neutralizing antibody (Ab) responses (24). *Vic1* is a recessive MLV and MMTV
48 resistance gene that is encoded by *H2-Ob*, the beta subunit (Oβ in mice and DOβ in humans) of
49 the nonclassical major histocompatibility complex class II (MHC-II)-like molecule H2-O (HLA-DO
50 or DO in humans) (25). The resistant, functionally null allele of *vic1* inherited by I/LnJ mice
51 results in sustained production of virus-neutralizing Abs. Transfer of the I/LnJ *vic1* locus to
52 multiple retrovirus susceptible backgrounds, which contain a functional allele of *Ob*, such as
53 BALB/cJ (BALB/c$^{vic1I/LnJ}$ congenic mice) confers the ability to produce neutralizing Abs against
54 both MMTV and MLV (25, 26). Finally, adaptive immune responses against retroviruses in mice
55 (and humans) are also affected by classical MHC class I and II genes, with specific haplotypes
56 directing virus-specific cytotoxic T cell and Ab responses (16, 22).
57 Ab responses to soluble protein and carbohydrate antigens in mice are generally
58 restricted to the IgG1 and IgG3 isotypes (27, 28), while infection of mice with a variety of viral
59 and parasitic pathogens results in a unique bias in class switching to IgG2a (or IgG2c) Abs (29-
60 31), including antiretroviral Ab responses in I/LnJ mice (7). In mice, the Th1-type cytokine
61 interferon gamma (IFNγ) has been well established as the canonical signal for class switching to
62 the IgG2a/c isotype both in vitro and in the context of infection (32). Specifically, in B6 mice,
63 IFNγ is required for Ab responses to MLV infection and for long-term viral control (33), and in
64 I/LnJ mice, IFNγ is essential to produce antibodies of any isotype against retroviral infections (7,
65 26). However, we recently reported that in BALB/cJ$^{vic1i/i}$ mice, IFNγ is dispensable for the
66 production of anti-MLV IgG2a Abs (34), indicating that BALB/cJ mice inherit an alternative
67 mechanism for stimulating antiviral IgG2a Ab production upon viral infection. The ability to
68 generate IFNγ-independent Ab responses in BALB/cJ mice is controlled by a single recessive
69 locus on Chromosome (Chr) 9, which we have named *i*nterferon-*g*amma *i*ndependent *I*gG2a
70 (*igii*) (34).

71 Reverse genetics approaches in mice are a powerful tool to understand the function of
72 specific genes and have provided essential research models for numerous diseases. Prior to
73 the development of CRISPR technology, which allowed for targeted mutagenesis on multiple
74 genetic backgrounds, most ES cell lines used for targeted mutagenesis came from substrains of
75 129 mice. The 129 lineage has a complex history, with multiple outcrossing and bottleneck
76 events, resulting in three major lineages (Parental, Steel, and *Ter*), with substantial substrain
77 variability ((35, 36) and Figure S1). This complex history has important implications for
78 investigations using 129 mice, and on analysis of targeted mutations that have been crossed to
79 other genetic backgrounds. Here, we investigated anti-MLV immune responses in 129S
80 substrains and determined that 129S1 mice inherit two loci that control antiviral immunity. We
81 also demonstrate that genetic differences between 129S substrains affect IFNγ-independent
82 antiviral immune responses.
83
84 **Results**
85 **A dominant genetic mechanism controls anti-MLV immune responses in 129S1 mice.**
86 Previous reports demonstrated that 129P2 mice (representative of the Parental 129 substrains)
87 inherit the *Fv1*$^{nr}$ allele, which restricts B-tropic and some N-tropic, but not NB-tropic strains of
88 MLV (21, 37, 38). 129P2 infected with a B-tropic FV produce robust cross-protective neutralizing
89 Ab responses due to this *Fv1*-incompatibility. However, although they produce FV-specific Abs
90 upon infection with an NB-tropic FV, since they inherit susceptible alleles of *Fv2* and *Rfv3*, they
91 do not control the infection (21, 39). These reports additionally suggest that the entire 129
92 lineage is *Fv1*$^{nr/nr}$ and *Fv2*$^{s/s}$ (21, 23, 37, 38). Furthermore, genotyping of two Steel substrains
93 (see Materials and Methods) at the *Rfv3* locus indicates that the 129 lineage is *Rfv3*$^{s/s}$. To
94 investigate antiviral responses in 129 Steel substrains, we infected 129S1/SvImJ (129S1;
95 developed as a control for steel-derived ES cells (40)) mice with Rauscher-like-MLV (RL-MLV)
96 and monitored them for antiviral Ab production, development of splenomegaly, and viral titers in
97 the spleen. RL-MLV is a mixture of a NB-tropic ecotropic virus and a polytropic MCF virus that

98 causes erythroid leukemias; the mixture does not contain an SFFV component (3), therefore the
99 *Fv2* allele does not affect disease outcome. While susceptible control BALB/cByJ (ByJ) mice do
100 not produce a robust Ab response and were unable to control infection, 129S1 mice produced a
101 robust IgG2a Ab response against RL-MLV and the majority (83%) cleared infection and did not
102 develop splenomegaly (Figures 1 and S2). To determine whether the ability to produce
103 protective anti-MLV Ab responses is inherited in a dominant or recessive fashion, we crossed
104 129S1 mice to BALB/cByJ mice (Figure 2A). $F_1$ mice generated from these crosses were
105 infected with RL-MLV and screened for antiviral Abs. $F_1$ mice from crosses of both directions
106 [(129S1xByJ) and (ByJx129S1) produced antiviral Abs, although IgG2a productions was slightly
107 lower in (ByJx129S1) $F_1$ animals (Figure 1A). Additionally, the majority of $F_1$ mice eliminated the
108 virus and did not develop splenomegaly (Figure 1B-C), indicating that resistance to RL-MLV is
109 inherited in a dominant fashion.
110
111 **Two loci control anti-MLV Ab responses in 129S1 mice.** We next investigated how many loci
112 control anti-MLV Ab production by crossing resistant (129S1xByJ) $F_1$ mice to susceptible ByJ
113 mice to generate $N_2$ mice (Figure 2A). We found that 9 out of 40 (23%) $N_2$ mice produced
114 antiviral IgG2a Abs (Figure 2B). We then determined the viral titers in the spleen and spleen
115 weights of Ab negative and Ab positive $N_2$ mice and found that the Ab positive (resistant), but
116 not the Ab negative (susceptible) $N_2$ mice cleared the infection and had normal spleen weights
117 (Figure 2B). This three:one ratio of susceptible to resistant $N_2$ animals indicates that two loci
118 control the production of protective anti-RL-MLV Ab responses in 129S1 mice. Since the major
119 histocompatibility complex (MHC) locus is a major factor in controlling antiviral immunity,
120 including anti-MLV responses (16, 22), and 129S1 mice inherit the protective $H2^b$ haplotype
121 while ByJ mice inherit the susceptible $H2^d$ haplotype (Table 1), we next investigated whether the
122 MHC locus is one of the genetic determinants for anti-MLV Ab responses in 129S1 mice. MHC
123 haplotypes of $N_2$ mice were determined by staining peripheral blood lymphocytes with
124 haplotype-specific Abs for MHC Class I ($H2\text{-}D^b$, $H2\text{-}D^d$) and Class II ($I\text{-}A^b$, $I\text{-}A^d$). We did not

125 observe significant differences in Ab production, viral titers, and spleen weights in RL-MLV
126 infected $H2^{b/d}$ and $H2^{d/d}$ $N_2$ mice (Figure 2C-D and Table S1), indicating that control of infection
127 in $N_2$ mice is not determined by inheritance of the resistant $H2^b$ haplotype and that non-MHC
128 loci control antiviral responses in 129S1 mice.
129
130 **IFNγ-signaling is dispensable for anti-MLV IgG2a-specific Ab production in 129S7 mice.**
131 Following our previous finding that BALB/cJ mice congenic for the resistant allele of *vic1*
132 produce antiviral IgG2a Abs in the absence of IFNγ (34), we wondered whether this pathway
133 was unique to this genetic background. Earlier reports indicated that 129S mice also inherit a
134 pathway for IFNγ-independent production, as IFNγ-receptor 1 (IFNγR)-deficient 129S7 (G129)
135 mice produce IgG2a Abs against lymphocytic choriomeningitis virus (LCMV) (41) and (lactate
136 dehydrogenase-elevating virus) LV (42). We therefore investigated whether $IFN\gamma R^{-/-}$ 129S7
137 mice produce IFNγ-independent IgG2a Abs against RL-MLV. Control $IFN\gamma R^{-/-}$ ByJ (which do not
138 inherit the resistant *vic1* allele, Table 1) mice did not produce Abs of any isotype and did not
139 control infection, while $IFN\gamma R^{-/-}$ 129S7 mice produced IgG2a Abs against RL-MLV (Figure 3A
140 and Figure S2). Although Ab production was lower than in IFNγR-sufficient 129S1 mice, the
141 majority of $IFN\gamma R^{-/-}$ 129S7 mice (60%) cleared infection and did not develop splenomegaly
142 (Figure 3 and Table 1). Therefore, like BALB/cJ mice, 129S7 mice inherit a mechanism for
143 noncanonical, IFNγ-independent antiretroviral Ab production.
144
145 **IFNγ-independent anti-MLV IgG2a production in 129S7 mice is a complex trait.** We next
146 sought to investigate whether IFNγ-independent antiretroviral Ab production in 129S7 mice and
147 BALB/cJ mice is controlled by the same genetic mechanism. Since the resistant allele of *igii*-
148 inherited by BALB/cJ mice is recessive, we reasoned that $IFN\gamma R^{-/-}$ $F_1$ animals inheriting the
149 129S7 and BALB/c allele would only produce antiviral IgG2a Abs if the 129S7 allele of *igii* is
150 resistant. IFNγR-deficient mice are available on the BALB/cByJ background, and we crossed
151 these mice to $IFN\gamma R^{-/-}$ 129S7 mice. $F_1$ mice generated from these crosses were infected with

152 RL-MLV and screened for antiviral Abs. These $F_1$ progeny did not display a clear phenotype,
153 with ~30% of mice producing Abs and controlling infection while the majority did not produce
154 Abs, developed splenomegaly, and had infectious virus in their spleens (Figure 4 and Table S1).
155 Therefore, the phenotype is not fully penetrant, and the genetic basis for IFNγ-independent
156 antiretroviral Ab production in 129S7 mice cannot be determined from these crosses.
157 Furthermore, although these experiments suggest that 129S7 mice do not inherit a resistant
158 allele of *igii*, BALB/cByJ and BALB/cJ mice have been separated since 1935, and *vic1*
159 congenics are not available on the ByJ background, therefore we cannot exclude the possibility
160 that ByJ and BALB/cJ mice inherit different alleles of *igii*.
161
162 **Anti-MLV Ab production in 129S1 mice is IFNγ-dependent.** Since we were unable to
163 determine the genetic basis for IFNγ-independent antiretroviral Ab production in 129S7 mice by
164 crossing to IFNγR$^{-/-}$ ByJ mice, and these strains are deficient for the IFNγ receptor rather than
165 the cytokine, we decided to generate IFNγ-deficient 129S mice for the investigation of IFNγ-
166 independent Ab responses. Considering that wild-type mice of the precise genetic background
167 of 129S7-*Ifngr*$^{-/-}$ no longer exist, we selected the 129S1 background for the knock-out of IFNγ,
168 as they are the control strain recommended by the Jackson Laboratory for lines derived from
169 multiple 129S substrains. We used a previously validated CRISPR/Cas9 approach that targeted
170 introns 1 and 3 of the *Ifng* gene, thereby deleting exons 2 and 3, removing four of six alpha
171 helices ((43) and Figure S3A). We infected heterozygous control and IFNγ$^{-/-}$ mice from two
172 founder lines with RL-MLV and monitored them for antiviral Ab production. Although IFNγ
173 sufficient controls produced antiviral IgG2a Abs and cleared the infection, IFNγ$^{-/-}$ 129S1 mice
174 from either founder line failed to produce a robust antiviral Ab response of any isotype (Figure
175 5). Additionally, less than 20% of IFNγ$^{-/-}$ 129S1 mice cleared the infection, and the majority
176 developed splenomegaly (Figure 5B-C and Table S1), indicating that unlike 129S7 mice, 129S1
177 mice do not inherit a pathway for directing IFNγ-independent antiretroviral Ab responses.
178

179 **Genetic differences, not alternative receptor usage, underlie differential requirements for**
180 **IFNγ-signaling in 129S mice.** To determine if antiviral IgG2a Ab responses in 129S7 mice are
181 the result of IFNγ signaling through an alternative receptor or result from genetic differences
182 between 129S7 and 129S1 mice, we crossed IFNγR$^{-/-}$ 129S7 mice to IFNγ$^{-/-}$ 129S1 mice and
183 then intercrossed the resulting $F_1$ generation (Figure 6A). If IFNγ signaling through an
184 alternative receptor explained virus resistance in IFNγR$^{-/-}$ 129S7 mice, we would expect to
185 observe antiviral Ab production in IFNγR$^{-/-}$, but not IFNγ$^{-/-}$, or IFNγ/IFNγR$^{-/-}$ $F_2$ mice. On the other
186 hand, if 129S7 inherit an alternative pathway for IFNγ-independent antiviral Ab production, we
187 would expect a mixture of resistant and susceptible $F_2$ mice independently of inheritance of
188 IFNγR- or IFNγ-deficiency. As expected, $F_2$ mice sufficient for both cytokine and receptor
189 produced anti-MLV IgG2a-specific Abs and cleared the infection (Fig 6B-C). However, antiviral
190 Ab production was not observed in the majority of IFNγR$^{-/-}$ $F_2$ mice, and only 10% cleared the
191 infection (Figure 6B-C). Interestingly, more IFNγ$^{-/-}$ and IFNγ/IFNγR$^{-/-}$ $F_2$ mice produced anti-MLV
192 Abs and cleared infection than IFNγR$^{-/-}$ $F_2$ mice, and 64% of IFNγ$^{-/-}$ and 100% of IFNγ/IFNγR$^{-/-}$ $F_2$
193 mice cleared the infection (although only four double-deficient mice were infected) (Figure 6B-D
194 and Table S1). These data strongly suggest that IFNγ does not signal through an alternative
195 receptor to stimulate antiviral Abs in 129S7 mice and that an unknown genetic factor(s) present
196 in 129S7, but not 129S1 mice controls IFNγ-independent antiretroviral Ab responses.
197

198 **Discussion**

199 Here, we demonstrate that 129S1 mice inherit a previously unknown mechanism that controls
200 the production of protective antiviral Ab production upon MLV infection. This phenotype is
201 inherited in a dominant fashion and is controlled by two loci (Figures 1-2). Importantly, these loci
202 are distinct from previously characterized genes that restrict retroviral replication (*Fv1*, *Fv2*,
203 *Rfv3*) or promote antiviral immune responses (*vic1*, MHC locus, *Rfv3*), indicating that the
204 pathway in 129S1 mice potentially encodes for a novel mechanism for the stimulation of
205 protective immunity. Unlike I/LnJ and C57BL/6 mice, which fully clear RL-MLV infection (15, 26),

206 MLV resistance in 129S1 mice is not fully penetrant, as ~15% of infected mice fail to produce
207 antiviral Abs, develop splenomegaly, and retain infectious virus in their spleens (Figures 1 and
208 5, Table S1). Additionally, although this mechanism does not appear to be sex-linked, there was
209 a reduction in IgG2a Ab production in (ByJx129S1) $F_1$ mice, and fewer of them cleared the
210 infection than 129S1 or (129S1xByJ) $F_1$ mice (Figure 1 and Table S1), indicating that epigenetic
211 or environmental factors may play a role in controlling anti-MLV responses in 129S1 mice.
212 IFNγ is a well-established signal for stimulating CSR to the IgG2a isotype both *in vitro*
213 and in the context of various viral infections (21, 37, 38), including FV infection in B6 mice (22)
214 and RL-MLV and MMTV infection in I/LnJ mice (6, 15). We previously demonstrated that
215 BALB/cJ mice inherit a pathway for the generation of IFNγ-independent IgG2a antiviral Ab
216 responses that is controlled by a single recessive locus, *igii*, which is mapped to Chr. 9 (34).
217 Here, we demonstrate that 129S7 mice also inherit a pathway for IFNγ-independent anti-MLV
218 Ab production. However, the mechanism for IFNγR-independent anti-RL-MLV responses in
219 129S7 mice is a complex genetic trait, and we were unable to determine whether the pathway is
220 controlled by *igii* or another locus (or multiple loci). Nevertheless, the ability of both BALB/cJ and
221 129S7 mice to produce IFNγ-independent Abs against a variety of infections including
222 retroviruses, herpes simplex virus (HSV), LCMV, LV, and parasitic infections (34, 41, 42),
223 indicates that alternative pathways in addition to the canonical IFNγ-dependent pathway can
224 stimulate neutralizing Ab responses. Since production of IFNγ-independent Ab responses in
225 BALB/cJ mice is independent of *vic1* for HSV and VSV infections (but not MLV infections), this
226 indicates that the ability to produce anti-viral Ab responses and the ability to produce these
227 responses in the absence of IFNγ signaling are controlled by distinct genetic mechanisms.
228 Although we anticipate this is also the case for 129S7 mice, it cannot be definitively
229 demonstrated. These findings additionally highlight the importance of utilizing mice of multiple
230 genetic backgrounds to investigate the requirements for stimulating protective antiviral
231 immunity.

232 Following decades of widespread use of 129 ES cell lines, two reports from 1997
233 investigated the variation between 129 substrains and found substantial genetic variation
234 resulting from genetic drift, genetic contamination, and residual heterozygosity from
235 backcrossing programs (35, 36). This initiated efforts to clarify the nomenclature for the
236 improvement of gene targeting experiments and the selection and consideration of controls for
237 comparing phenotypes of genes targeted in ES cells of different 129 substrains (35, 36, 44).
238 These findings further suggested important features that should be considered for targeted
239 mutagenesis using 129 substrains 1) chimeras produced using 129 ES cells should be crossed
240 to a genetically matched substrain for maintenance on the 129 background and 2) selection of a
241 129 substrain for targeted mutagenesis should involve consideration of different genetic traits
242 between substrains (35). Like many mice generated prior to these recommendations, chimeric
243 IFNγR$^{-/-}$ 129S mice were not crossed to a genetically matched substrain, and the appropriate
244 wild-type control strain is unavailable. The 129S1 substrain is therefore recommended as the
245 control inbred strain for steel substrain-derived ES cell lines (40). However, we found that while
246 129S7 mice produce antiviral Ab responses in the absence of IFNγ, this mechanism is not
247 inherited by 129S1 mice (Figure 5). Some reports have suggested that IFNγ can signal through
248 an alternative receptor in the absence of *Ifngr1* (45-47), but this does not explain the differences
249 between 129S1 and 129S7 substrains reported here, as IFNγR$^{-/-}$ (129S1x129S7) $F_2$ mice did not
250 produce antiviral Abs (Figure 6). Therefore, for the purposes of investigating the requirements
251 for IFNγ in antiviral defenses, 129S1 mice are not an appropriate control for 129S7-*Ifngr*$^{-/-}$ mice.
252 The higher incidence of IFNγ-independent Ab production in IFNγ$^{-/-}$ and IFNγ/IFNγR$^{-/-}$ $F_2$ mice
253 than IFNγR$^{-/-}$ $F_2$ mice (Figure 6) was intriguing. However, while the number of $F_2$ animals
254 phenotyped here is sufficient to eliminate alternative receptor usage by IFNγ as the basis for
255 IFNγR-independent Ab production, retroviral resistance is not a fully penetrant phenotype in
256 either 129S1 or 129S7-*Ifngr*$^{-/-}$ mice, and it appears that IFNγ-independent antiviral Ab
257 production in 129S7 mice is a complex trait. Therefore, larger numbers of $F_2$ mice would be

258 required to draw any other conclusions regarding the genetic basis for these substrain
259 differences.

260 While 129 substrains have the same genotypes at known MLV-resistance loci [(21, 23,
261 37, 38) and this report], our findings clearly indicate that there are unappreciated differences in
262 their ability to mount protective antiviral immune responses. As such, it is unknown whether the
263 129P2/OlaHsd substrain utilized in many investigations on immune responses to FV infection
264 would exhibit a similar phenotype to 129S1 mice when infected with RL-MLV, or whether they
265 can produce IFNγ-independent antiviral immune responses. In fact, these substrains have been
266 separated since the re-establishment of the 129 line in 1948 following the fire at Jackson Labs
267 and have substantial genetic variation between them ((35, 36, 48) and Figure S1). The different
268 phenotypes observed here between Steel substrains are not entirely surprising since the 129S1
269 lineage has been separated from the 129/SvEv lineage, from which the IFNγR$^{-/-}$ 129S7 mice are
270 derived, since 1969. Furthermore, additional crosses occurred in the 129/SvEv lineage prior to
271 the introduction of the *Ifngr1* mutation in 129S7-derived AB1 ES cells [Figure S1 and (35, 48)],
272 thereby increasing the genetic divergence of these substrains. Ideally, we would conduct
273 experiments to confirm whether 129S7 and 129S1 mice inherit the same genetic mechanism
274 controlling the production of anti-MLV Abs and differ only their ability to produce these
275 responses in the absence of IFNγ. Unfortunately, such experiments cannot be conducted
276 because wild-type 129S7 mice no longer exist. Collectively, our findings further emphasize the
277 need for careful consideration of the genetic background of mutant and control mice and
278 indicate that genetic differences between 129S substrains can have consequences well beyond
279 affecting the efficiency of traditional gene targeting methods.
280

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**Author contributions**

Conception and design: M.K.; Acquisition of data: R.Z.Z., V.M., L.R., and M.K.; Analysis and interpretation of data: R.Z.Z. and M.K.; Drafting the article: M.K.; Revising the article: M.K., R.Z.Z., and V.M..

**Declaration of interests**

The authors declare no competing interests

299 **Materials and Methods**

300 **Mice**

301 129S1/SvImJ (129S1 stock #002448) and BALB/cByJ (stock #001026) were purchased from
302 the Jackson Laboratory. 129-*Ifngr1*$^{tm1Agt}$/J [(48) also known as G129] mice were purchased from
303 the Jackson Laboratory (via cryo recovery, stock #002702) and bred and maintained at the
304 University of Pittsburgh. IFNγR$^{-/-}$ 129 mice were generated using AB1 ES cells derived from
305 129S7/SvEvBrd-*Hprt*$^{b-m2}$ (129S7) mice, and chimeric founder males were crossed to an
306 unknown 129/SvEv substrain (although 129S8 mice are commonly referred to as 129/SvEv and
307 may have been used for these crosses) (48) for simplicity, we refer to these as 129S7-*Ifngr*$^{-/-}$
308 mice (Figure S1). C.129S7(B6)-*Ifngr1*$^{tm1Agt}$/J (IFNγR$^{-/-}$ ByJ) were purchased from the Jackson
309 Laboratory (via cryo recovery, stock #002286) and bred and maintained at the University of
310 Pittsburgh. Mice of both sexes were used in equal ratios for all experiments.

311

312 *Ifng*-deficient 129S1 mice were generated using CRISPR/Cas9 technology. The target
313 sequences (5′ guide sequence: 5′-GCTGTTTCCCTGCGTAGTTT-3′; 3′ guide sequence: 5′-
314 TAGAGGCTAACCAGAGCCGA-3′) were previously validated for the generation of conditional
315 knockout strains on the C57BL/6 background (43). Two male founders with complete deletions
316 of exons 2 and 3 (removing sequences coding for amino acids 38-120) were selected (Figure
317 S3A) and crossed to 129S1 females. Potential off-target sites with fewer than three mismatches
318 identified by the Cas-OFFinder algorithm (49) were sequenced in $N_1$ and $N_2$ mice, and mice with
319 no mutations at these sites were intercrossed to produce homozygous *Ifng*-deficient mice. Both
320 founder lines express a truncated IFNγ protein [lacking four of six alpha helices (50)] that is not
321 biologically active (Figure S3B-C).

322

323 Mice were genotyped from tail biopsies using real time PCR with specific probes designed for
324 each gene (Transnetyx, Cordova, TN) or by flow cytometry (see below). Mice of both sexes
325 were used in equal numbers for each experiment. All animal experiments were performed in the

326 American Association for the Accreditation of Laboratory Animal Care-accredited, specific-
327 pathogen-free facility Division of Laboratory Animal Resources, University of Pittsburgh School
328 of Medicine. Animal protocols were reviewed and approved by the Institutional Animal Care and
329 Use Committee at The University of Pittsburgh.
330
331 **Flow cytometry**
332 For MHC genotyping of [(129S1xByJ)xByJ]$N_2$ mice, blood was collected in sodium heparin
333 collection vials (SAI Infusion Technologies), and peripheral blood lymphocytes were isolated by
334 overlaying with Ficoll-Paque (Cytivia) and spinning at 11,000xg for 4min at 25ºC. Lymphocytes
335 were stained with antibodies against Class I [brilliant blue 700 (BB700)-conjugated anti-H-2Db
336 (BD Biosciences) and phycoerythrin (PE)-conjugated anti-H-2Dd (BD Biosciences)] or Class II
337 [peridinin chlorophyll (PerCP)-eFluor 710-conjugated anti-H2-Ab1 (I-Ab, Invitrogen) and PE-
338 conjugated anti-H2-Ad1 (I-Ad, BD Biosciences)] MHC and analyzed on an Attune NxT
339 cytometer (Life Technologies).
340
341 **MLV infection**
342 Rauscher-like MuLV (RL-MuLV), a mixture consisting of NB-tropic ecotropic and mink lung cell
343 focus-forming viruses, was described previously (69) and was provided by T. Golovkina,
344 University of Chicago. The virus was propagated in SC-1 embryonic mouse fibroblasts (ATCC).
345 Ecotropic (Eco) viral titers were determined by an infectious center assay (70). Experimental
346 mice were injected i.p. with $2 \times 10^4$ Eco PFUs at 5-8 weeks of age and screened for anti-virus
347 antibodies and plaque forming units 8-10 weeks later.
348
349 **ELISA**
350 To detect MuLV Abs in mouse sera, an enzyme-linked immunosorbent assay (ELISA) was
351 performed as previously described (6, 15). Virions isolated from RL-MLV infected SC-1 cells
352 were treated with 0.1% Triton X-100 and bound to plastic in borate-buffered saline overnight,

353 followed by incubation with mouse serum samples at 4°C for 90 minutes. All sera were used at
354 2 x $10^{-2}$ dilutions. Mouse IgG2a-specific, as well as total IgG-specific secondary antibodies
355 coupled to horseradish peroxidase (HRP) (Jackson ImmunoResearch) were used to detect anti-
356 virus antibodies. Ovalbumin (2%) was used as a blocking reagent. Backgrounds obtained from
357 incubation with secondary antibodies alone were subtracted from the values obtained from sera
358 of infected mice.
359
360 **Statistical analyses**
361 Statistical significance was determined using GraphPad software (one way ANOVA or unpaired
362 t test).
363
364
365

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367


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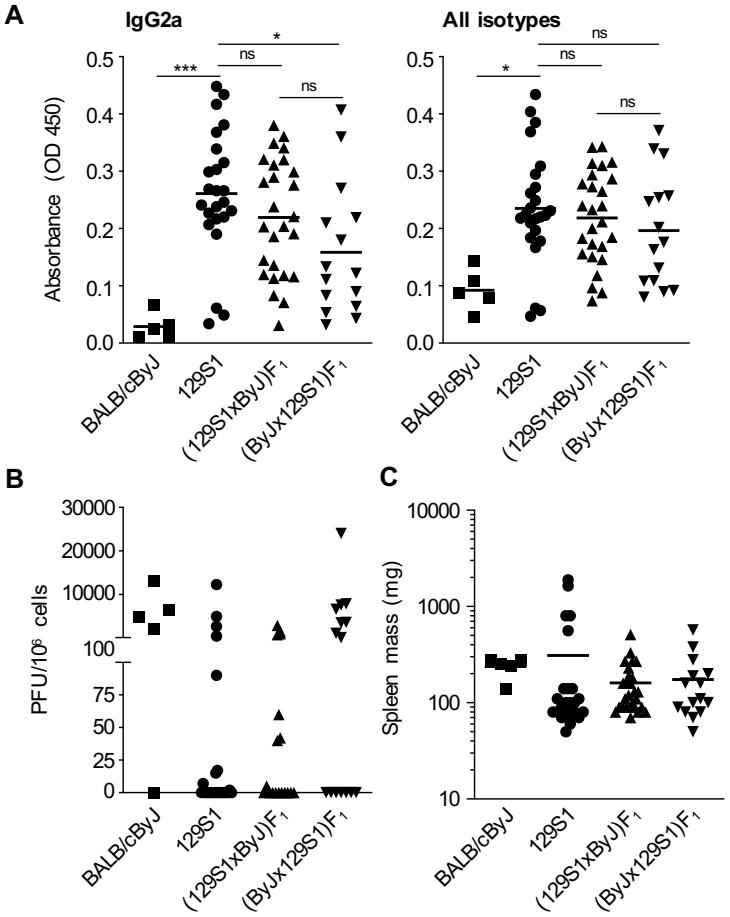



510

511 **Figure 1. Anti MLV-Ab production in 129S1 mice is controlled by a dominant genetic**

512 **mechanism.**

513 **A)** BALB/cByJ, 129S1, or $F_1$ mice were infected with RL-MLV and monitored for IgG2a-

514 specific antibodies (left) or total Igs (right) against RL-MLV virion proteins by ELISA 8-10

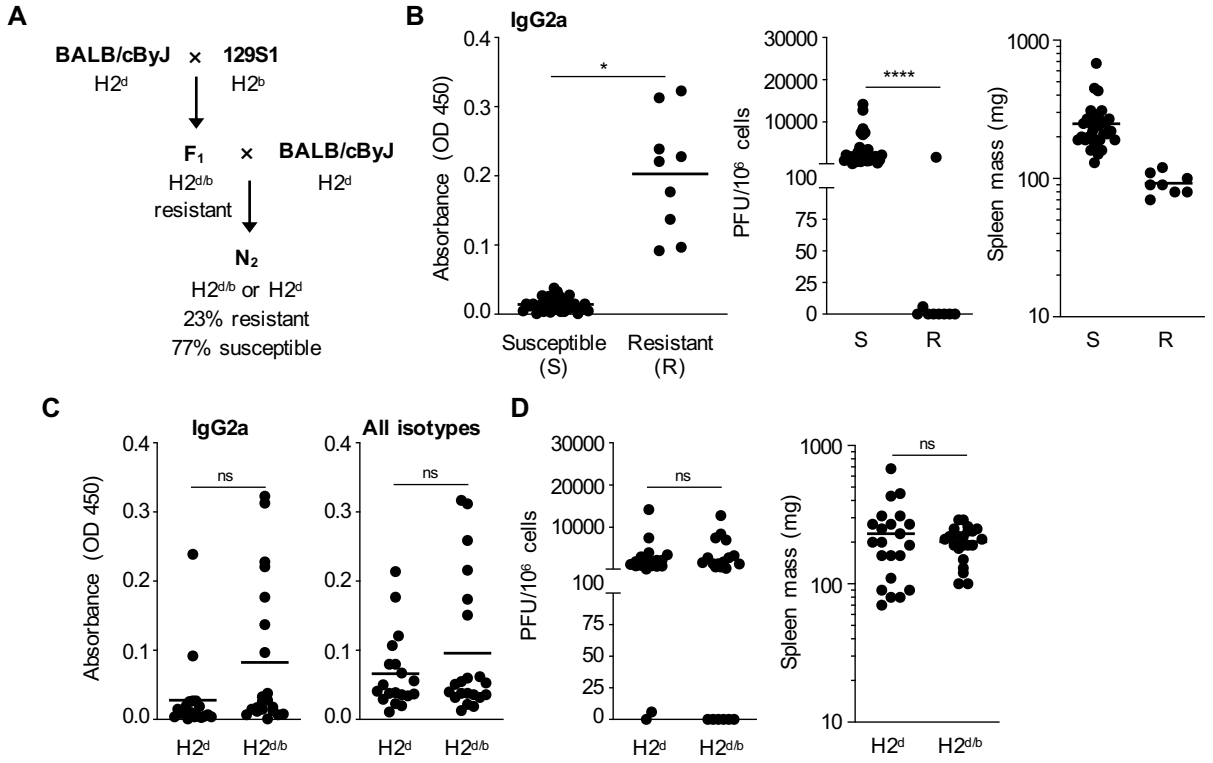
515 weeks post infection. ns, not significant; *, $p<0.05$; ***, $p<0.001$

516 **B)** Spleen cells from RL-MLV infected mice were subjected to an infectious center assay 8-

517 10 weeks post infection.

518 **C)** Spleen weights of RL-MLV infected mice at 8-10 weeks post infection.

519




520

521 **Figure 2. Anti MLV-Ab production in 129S1 mice is controlled by two non-MHC loci.**

522 **A)** Diagram of breeding scheme used to determine Mendelian inheritance of the

523 mechanism controlling IFNγ-independent IgG2a Ab production. $F_1$ and $N_2$ crosses were

524 conducted in both directions, with just one direction shown here for simplicity.

525 **B)** $N_2$ mice generated from crossing (129S1xByJ)$F_1$ and ByJ mice as shown in A) were

526 infected with RL-MLV. Eight weeks post infection, their sera were tested for IgG2a Abs

527 against MLV virion proteins by ELISA (left), mice were classified as "resistant" if their

528 sera reacted with MLV virion proteins with an absorbance greater than 0.1 at OD 450

529 with an IgG2a-specific secondary. Susceptible and resistant mice were then tested for

530 presence of infectious virions in their spleens by XC plaque assay (center), and spleen

531 weights were measured (right).

532 **C)** MHC haplotypes of $N_2$ mice from B) were determined by FACS analysis and then levels

533 of IgG2a-specific antibodies (left) or total Igs (right) reactive against RL-MLV virion

534 proteins by ELISA were compared.

535 **D)** Presence of infectious virions in spleen (left) and spleen weights (right) of $N_2$ mice

536 grouped by MHC haplotype.

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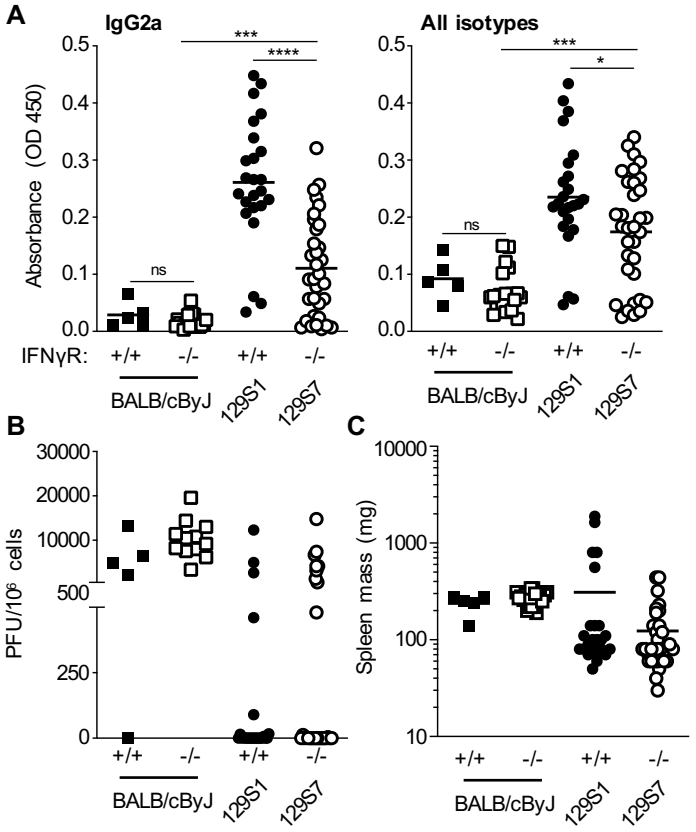


538

539 **Figure 3. IFNγR-independent anti-MLV IgG2a Ab production in 129S7 mice**

540 **A)** Mice of the indicated genotypes were infected with RL-MLV and monitored for IgG2a-
541 specific antibodies (left) or total Igs (right) against RL-MLV virion proteins by ELISA 8-10
542 weeks post infection. ns, not significant; *, $p<0.05$; ***, $p<0.001$; ****, $p<0.0001$

543 **B)** Spleen cells from RL-MLV infected mice were subjected to an infectious center assay 8-
544 10 weeks post infection.

545 **C)** Spleen weights of RL-MLV infected mice at 8-10 weeks post infection.

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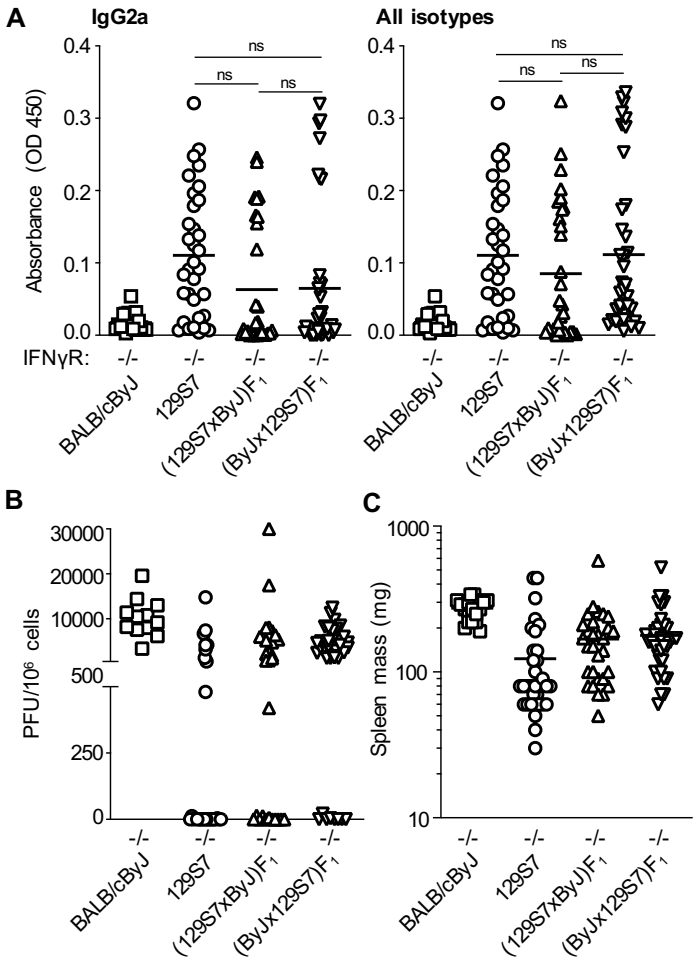


547

548 **Figure 4. IFNγR-independent anti-MLV Ab production in 129S7 mice is a complex trait**

549 **A)** IFNγR$^{-/-}$ BALB/cByJ, 129S7, and $F_1$ mice of the indicated genotypes were infected with

550 RL-MLV and monitored for IgG2a-specific antibodies (left) or total Igs (right) against RL-

551 MLV virion proteins by ELISA eight weeks post infection. ns, not significant

552 **B)** Spleen cells from RL-MLV infected mice were subjected to an infectious center assay

553 eight weeks post infection.

554 **C)** Spleen weights of RL-MLV infected mice at eight weeks post infection.

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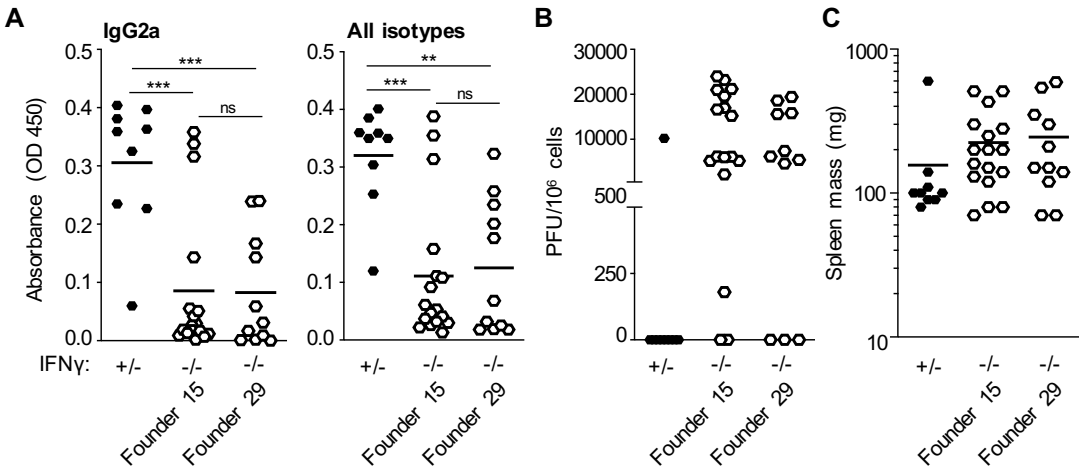


556

557 **Figure 5. Anti-MLV Ab responses in 129S1 mice are IFNγ-dependent**

558 **A)** IFNγ-deficient and heterozygous control mice from two 129S1 founder lines were

559 infected with RL-MLV and monitored for IgG2a-specific antibodies (left) or total Igs

560 (right) against RL-MLV virion proteins by ELISA eight weeks post infection. ns, not

561 significant; **, $p<0.01$; ***, $p<0.001$

562 **B)** Spleen cells from RL-MLV infected mice were subjected to an infectious center assay

563 eight weeks post infection.

564 **C)** Spleen weights of RL-MLV infected mice at eight weeks post infection.

565

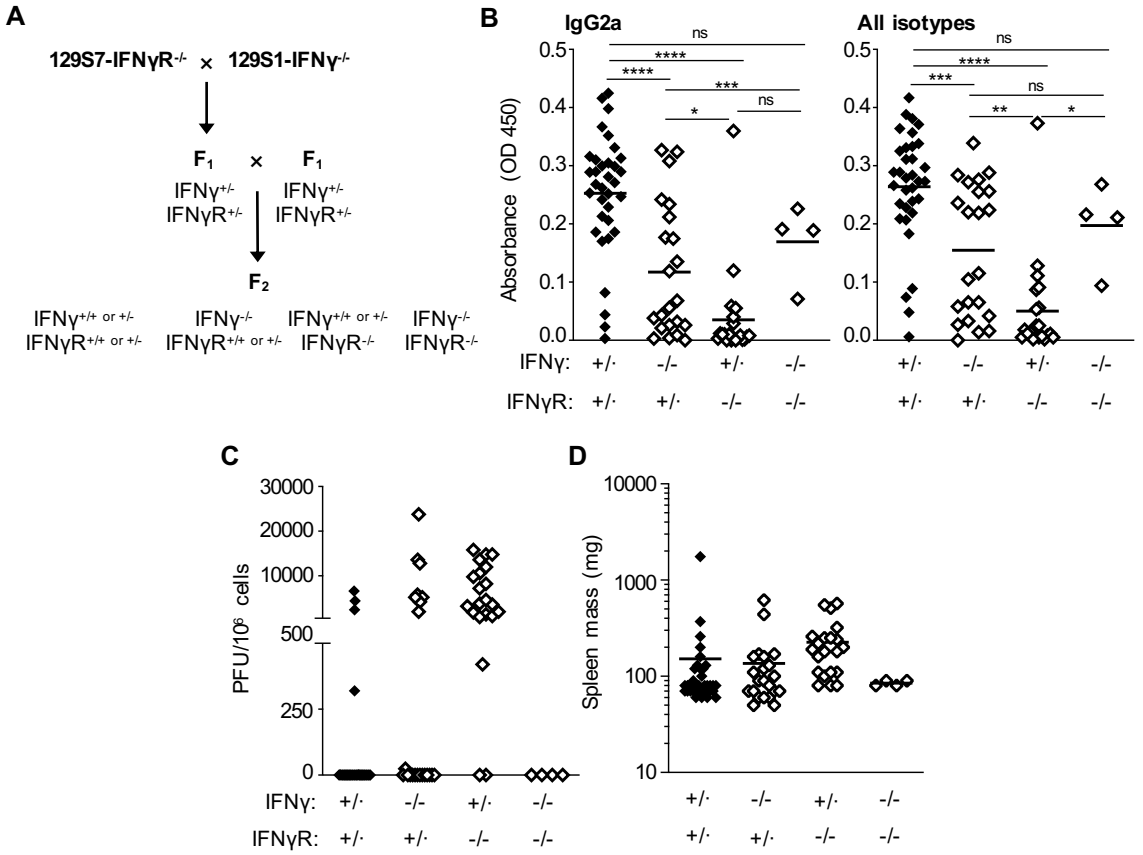


**Figure 6. Genetic differences between 129S substrains affect IFNγ-independent anti-MLV Ab responses**

**A)** Diagram of breeding scheme used to generate IFNγ, IFNγR, and IFNγ/IFNγR-deficient mice of mixed 129S7 and 129S1 genetic background.

**B)** $F_2$ mice of the indicated genotypes generated from intercrossing (129S7-*Ifngr1*$^{-/-}$ x 129S1-*Ifng*$^{-/-}$)$F_1$ mice as shown in A) were infected with RL-MLV. 8-10 weeks post infection, their sera were tested for IgG2a-specific antibodies (left) or total Igs (right) against RL-MLV virion proteins by ELISA. +/·, either heterozygous or homozygous wild type. ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

**C)** Spleen cells from RL-MLV infected $F_2$ mice were subjected to an infectious center assay eight weeks post infection.

**D)** Spleen weights of RL-MLV infected $F_2$ mice at eight weeks post infection.

**Table 1**. Genotypes of inbred strains at MLV-resistance loci

| | Genotype* | | | | | | |
|---|---|---|---|---|---|---|---|
| **Strain** | ***H2*** | ***Fv1*** | ***Fv2*** | ***Rfv3*** | ***vic1*** | ***igii*** | **References** |
| BALB/cJ | *d* | *b/b* | *s/s* | *s/s* | *s/s* | *r/r* | (24-26, 34, 39) |
| BALB/cByJ | *d* | *b/b* | *s/s* | *s/s* | *s/s* | ** | (24-26, 39) |
| BALB/cJ$^{vic1\ I/LnJ}$ | *j* | *b/b* | *s/s* | *s/s* | *s/s* | *r/r* | (24-26, 34, 39) |
| I/LnJ | *j* | | | *s/s* | *r/r* | *s/s* | (25, 26, 34) |
| 129S1/SvImJ | *b* | *nr/nr* | *s/s* | *s/s* | *s/s* | *s/s* | (21, 25) This study |
| 129S7/SvEvBrd-Hprt$^{+}$ | *b* | *nr/nr* | *s/s* | *s/s* | *s/s* | ** | (21, 25) This study |
| C57BL/ | *b* | *b/b* | *r/r* | *r/r* | *s/s* | *s/s* | (24, 39) |

s = Susceptible, r = Resistant, Blank cells = not tested or unknown

*- *H2* haplotype (MHC locus) affects T cell and NK cell responses; *Fv1* controls capsid-dependent tropism; *Fv2* is a dominant FV susceptibility gene that facilitates splenomegaly and erythroleukemia induction by SFFV; *Rfv3* is a dominant FV resistance gene that promotes neutralizing Ab responses; *vic1* is a recessive retroviral resistance gene that promotes neutralizing Ab responses; *igii* is a recessive locus linked to production of anti-viral IgG2a Abs in the absence of IFNγ.

**- Although BALB/cByJ mice likely inherit the resistant allele of *igii*, and the findings reported here suggest that 129S7 mice inherit a susceptible allele, these genotypes have not yet been definitively determined.

## Supplemental Materials and Methods

### Splenocyte isolation for stimulation

The spleens of mice were aseptically isolated, trimmed of all excess tissue and placed in sterile phosphate-buffered saline (PBS, Corning). Cell suspensions were erythrocyte-depleted by incubation in 450µl sterile distilled water (Gibco) for 10 seconds before the addition of 50µl of 10X PBS (Gibco) and 4mL of 1X PBS. Cell suspensions were pelleted at 300x*g* for 5 min at 25°C. Splenocytes were resuspended in RPMI 1640 Medium (Gibco), supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco) and 50µg/ml gentamicin (Gibco).

### Flow cytometry

Splenocytes ($2 \times 10^6$) were stimulated with 50ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and 750ng/ml ionomycin (Sigma-Aldrich) at 37°C with 5% $CO_2$ for six hours. For the last five hours, 1µg/ml GolgiPlug™ Protein Transport Inhibitor (BD Biosciences) was added to block cytokine secretion. Cells were surface stained with antibodies against AlexaFlour 532-conjugated CD45 (clone 30-F11, Invitrogen) in PBS supplemented with 0.5% BSA, 0.1% sodium azide, 3mM egtazic acid (EGTA), and 20ug/mL DNAse1. Cells were then fixed and permeabilized with eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (Invitrogen) and stained intracellularly with phycoerythrin (PE)-conjugated anti-IFNγ (clone XMG1.2 RUO, BD Bioscience) in permeabilization buffer. Cells were stained for viability with Zombie NIR™ Fixable Viability Kit (BioLegend) in PBS and Mouse Fc Block™ (BD Biosciences). Samples were analyzed on a Cytek® Aurora (Cytek Biosciences).

### IFNγ detection assay

IFNγ detection assay for biologically active mouse IFNγ was performed using B16-Blue™ IFNγ reporter cells (InvivoGen) engineered to produce secreted alkaline phosphatase (SEAP) in response to IFNγ stimulation. Cells were cultured following the manufacturer's protocol in Dulbecco's Modified Eagles Medium (DMEM, Gibco) supplemented with 10% FCS, 50µg/ml gentamicin, 100 µg/ml Normocin™ (InvivoGen), and 100 µg/ml Zeocin® (InvivoGen).

Splenocytes ($2 \times 10^6$) were stimulated with 50ng/ml PMA and 750ng/ml ionomycin in 200µl of cell culture medium at 37°C/5% $CO_2$ for 16 hours. After stimulation, 20µl of supernatant were added per well in a 96-well flat bottom plate. To generate a standard curve, 20µl recombinant murine IFNγ (MilliporeSigma™ 40732020UG) was added at a starting concentration of 100ng/ml and serially diluted (five-fold dilutions). After addition of samples, $7.2 \times 10^4$ B16-Blue™ IFNγ cells were added in 180µl of cell culture medium for a final working volume of 200µl. Samples were incubated at 37°C/5% $CO_2$ for 24 hours.

For QUANTI-Blue detection of SEAP, 20µl of supernatant was added to a 96-well flat bottom plate followed by addition of 180µl of QUANTI-Blue Solution™ (InvivoGen) and incubated at 37°C for 24 hours. SEAP levels were determined using a Synergy H1 microplate reader (BioTek) by reading the optical density (OD) at 650nm. Background was subtracted with a negative control of QUANTI-Blue Solution™ incubated with 20µl of complete cell culture media. Levels of IFNγ in splenocyte supernatants were determined based on a standard curve for recombinant murine IFNγ.

**Immunoblot Analysis**

RL-MLV virions were isolated from supernatants of infected SC-1 cells via centrifugation at 95,000x*g*. MLV virions were lysed in NuPage LDS sample buffer (Novex), separated by electrophoresis on NuPage 4–12% Bis-Tris gels (Invitrogen) and blotted onto polyvinylidene fluoride (PDVF, BioRad Laboratories). Membranes were incubated with sera at $5 \times 10^{-3}$ dilution. For the second step, membranes were incubated with goat anti-mouse-IgG2a-specific antibodies coupled to HRP (Jackson ImmunoResearch). Blots were developed with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) and imaged on the ChemiDoc Imaging System (Bio-Rad).

**Table S1.** Summary of mice utilized in this investigation and their phenotype upon MLV infection

| | Genotype | | MLV infections | | |
|---|---|---|---|---|---|
| **Mice** | **IFNγ** | **IFNγR** | **# Infected** | **# with ≤ 100PFU/ $10^6$cells** | **% Cleared** |
| BALB/cByJ | +/+ | +/+ | 5 | 1 | 20% |
| BALB/cByJ | +/+ | -/- | 12 | 0 | 0% |
| 129S1/SvImJ (129S1) | +/+ | +/+ | 24 | 20 | 83% |
| 129S1 | +/- | +/+ | 9 | 8 | 89% |
| 129S1 | -/- | +/+ | 28 | 5 | 18% |
| (129S1xByJ)$F_1$ | +/+ | +/+ | 18 | 14 | 78% |
| (ByJx129S1)$F_1$ | +/+ | +/+ | 15 | 7 | 47% |
| ([129S1xByJ)xByJ]$N_2$ resistant | +/+ | +/+ | 9 | 8 | 89% |
| ([129S1xByJ)xByJ]$N_2$ susceptible | +/+ | +/+ | 31 | 0 | 0% |
| ([129S1xByJ)xByJ]$N_2$ $H2^d$ | +/+ | +/+ | 2 | 19 | 11% |
| ([129S1xByJ)xByJ]$N_2$ $H2^{d/b}$ | +/+ | +/+ | 6 | 21 | 29% |
| 129S7 | +/+ | -/- | 20 | 12 | 60% |
| (129S7xByJ)$F_1$ | +/+ | -/- | 30 | 11 | 37% |
| (ByJx129S7)$F_1$ | +/+ | -/- | 38 | 8 | 21% |
| (129S7x129S1)$F_2$ | +/· | +/· | 34 | 30 | 88% |
| (129S7x129S1)$F_2$ | -/- | +/· | 22 | 14 | 64% |
| (129S7x129S1)$F_2$ | +/· | -/- | 21 | 2 | 10% |
| (129S7x129S1)$F_2$ | -/- | -/- | 4 | 4 | 100% |

+/· - either heterozygous wild-type/knock-out or homozygous wild-type

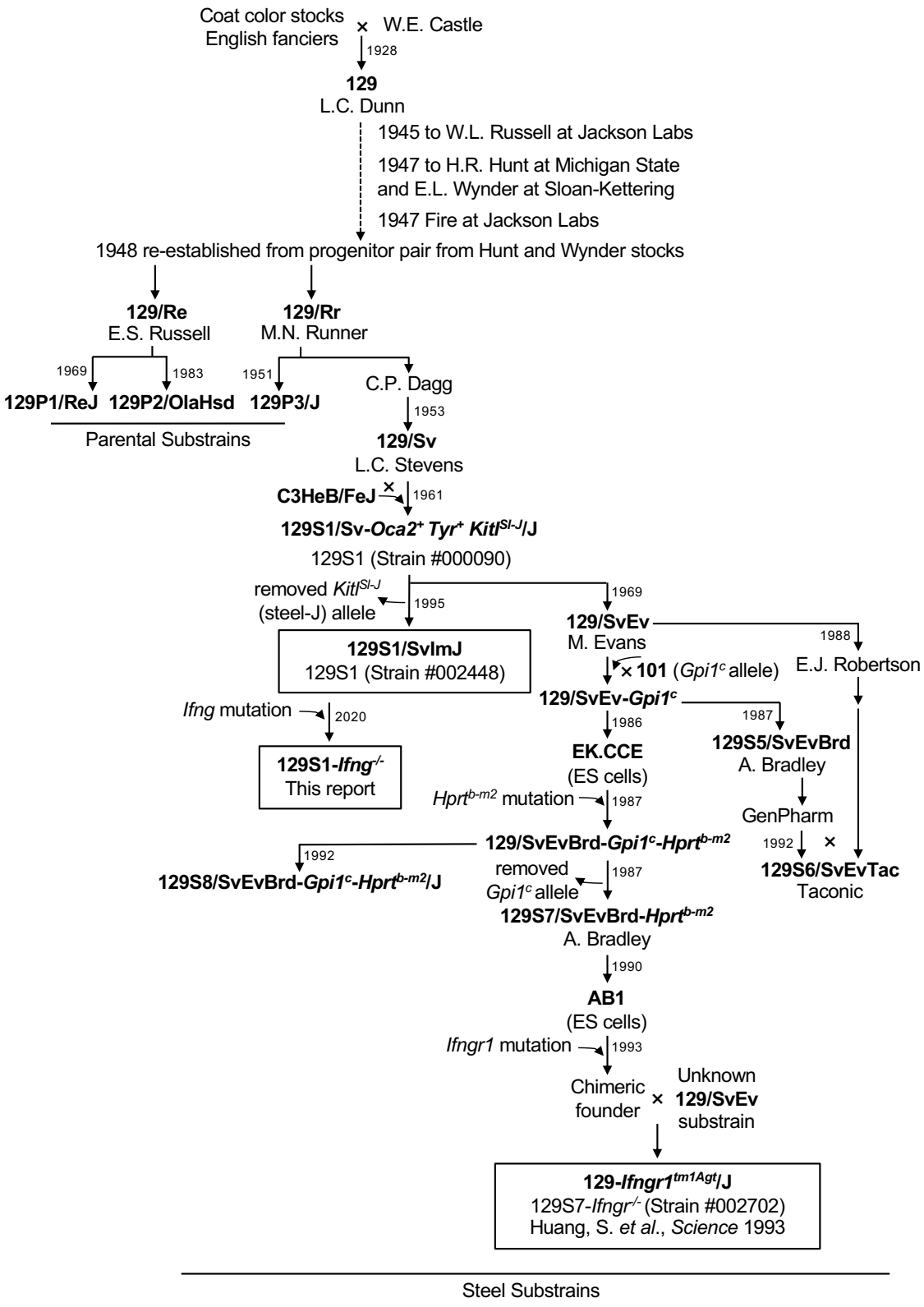


**Figure S1. History of selected 129 sublines.** Schematic of the genealogy of 129S1 and 129S7-*Ifngr*$^{-/-}$ mice. Adapted from (35).

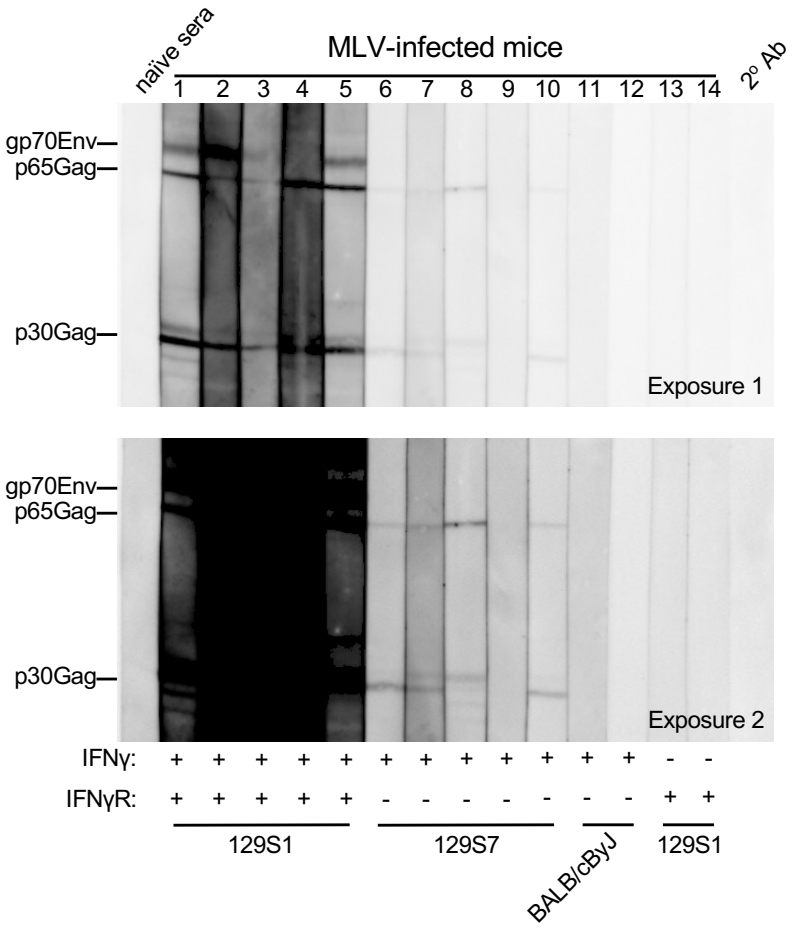


**Figure S2. Specificity of antiviral Abs.** Sera from 129S and BALB/cByJ mice of the indicated genotypes were tested for reactivity against MLV virion proteins by immunoblot 10 weeks post infection. Goat anti-mouse IgG2a-specific Abs coupled to HRP were used at the second step. Numbers correspond to individual mice. Two different exposures are shown.

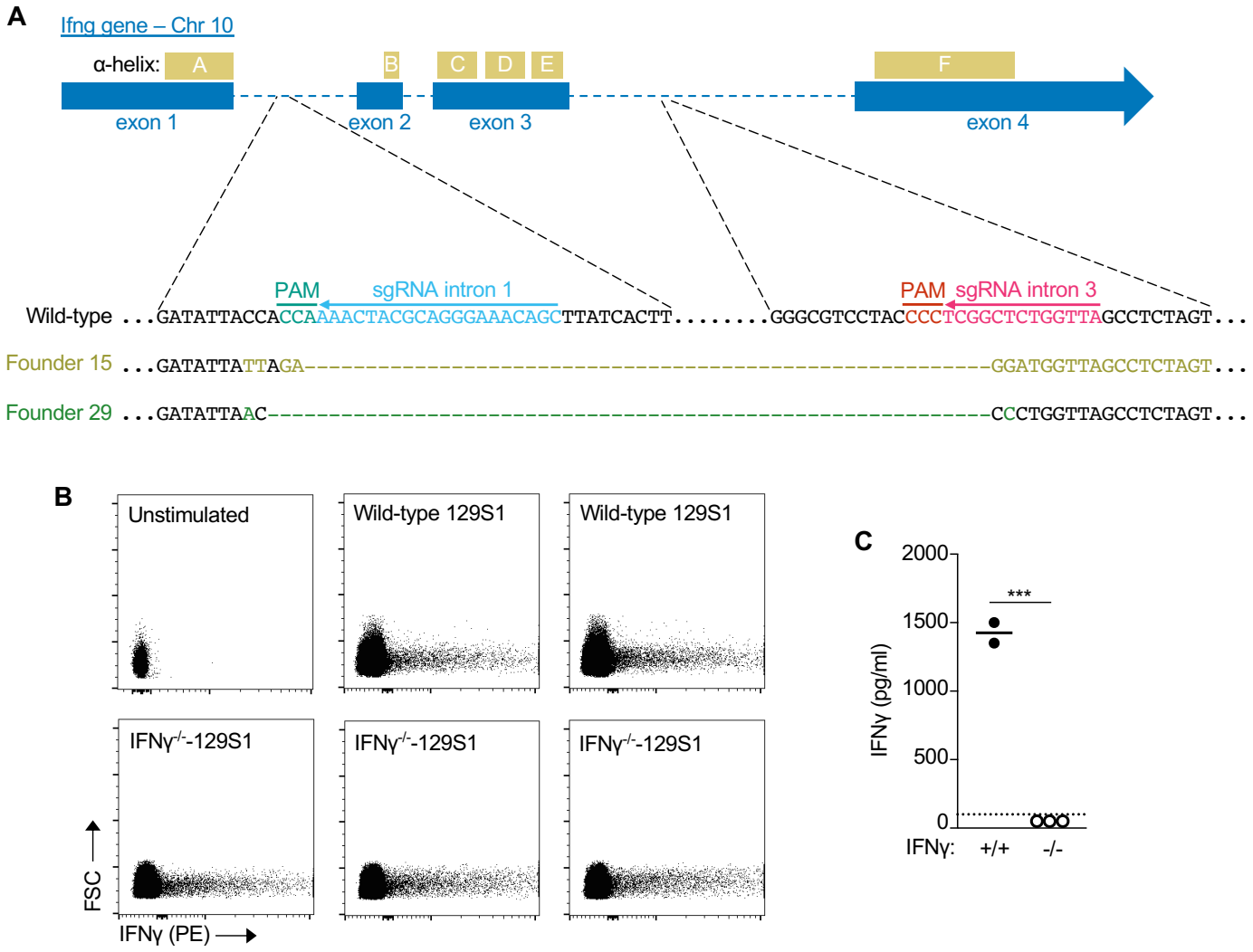


**Figure S3. Generation of IFNγ-deficient 129S1 mice. A)** Diagram of the *Ifng* locus, with sgRNA targeting sited in introns 1 and 3 indicated. The six α-helices based on the human IFNγ structure (REF) are indicated above the exons. Sequence of wild-type mice at targeted sites, with sgRNA and PAM sites indicated. Dots indicate sequences flanking those shown in detail. Sequences of the locus in selected founder mice with mismatches to wild-type sequence highlighted and deleted sequence indicated by dashes. Both founder lines lack exons 2 and 3, which encode for amino acids 38-120 (helices B through E). **B)** Truncated IFNγ is expressed in IFNγ$^{-/-}$-129S1 mice. Flow-cytometric plots showing intracellular IFNγ in unstimulated and PMA/ionomycin-stimulated CD45$^{+}$ splenocytes from wild-type 129S1 and IFNγ$^{-/-}$-129S1 (Founder 29) mice. **C)** The truncated IFNγ expressed in 129S1-*Ifng*$^{-/-}$ mice is not biologically active. Detection of IFNγ in supernatants from PMA/ionomycin-stimulated splenocytes from wild-type 129S1 and IFNγ$^{-/-}$-129S1 (Founder 29) mice by B16-Blue™ IFNγ cells. Dashed line indicates the limit of detection (100pg/ml).