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 a
27 mixture of ecotropic and polytropic [mink cell focus forming (MCF)] viruses, and many 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, detect 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 (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 [recently reviewed in (16)]. *Fv1* is a capsid-specific restriction factor encoded by an endogenous
40 retroviral *Gag* gene that governs tropism of MLV. Alleles of *Fv1* are defined by their ability to
41 block specific subclasses of MLV, with incompatibility resulting in a post-entry block to infection,
42 (17-20) and in some cases, stimulation of cross-protective neutralizing adaptive immune
43 responses (21). *Fv2* is a dominant Friend MLV (FV) susceptibility gene that facilitates
44 splenomegaly and erythroleukemia induction by SFFV (22, 23). *Rfv3* is a dominant FV

45 resistance gene that is encoded by *Apobec3*. The resistant allele of *Apobec3* inherited by
46 C57BL/6 and C57BL/10 mice restricts FV replication and promotes neutralizing antibody (Ab)
47 responses (24). *Vic1* is a recessive MLV and MMTV resistance gene that is encoded by *H2-Ob*,
48 the beta subunit (Oβ in mice and DOβ in humans) of the nonclassical major histocompatibility
49 complex class II (MHC-II)-like molecule H2-O (HLA-DO or DO in humans) (25). The resistant,
50 functionally null allele of *vic1* inherited by I/LnJ mice results in sustained production of virus-
51 neutralizing Abs. Transfer of the I/LnJ *vic1* locus to multiple retrovirus susceptible backgrounds,
52 which contain a functional allele of *Ob*, such as BALB/cJ (BALB/c$^{vic1I/LnJ}$ congenic mice) confers
53 the ability to produce neutralizing Abs against both MMTV and MLV (25, 26). Finally, adaptive
54 immune responses against retroviruses in mice (and humans) are also affected by classical
55 MHC class I and II genes, with specific haplotypes directing virus-specific cytotoxic T cell and
56 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 (Figure 1). To determine whether the ability to produce protective anti-
103 MLV Ab responses is inherited in a dominant or recessive fashion, we crossed 129S1 mice to
104 BALB/cByJ mice (Figure 2A). F_1 mice generated from these crosses were infected with RL-MLV
105 and screened for antiviral Abs. F_1 mice from crosses of both directions [(129S1xByJ) and
106 (ByJx129S1) produced antiviral Abs, although IgG2a productions was slightly lower in
107 (ByJx129S1) F_1 animals (Figure 1A). Additionally, the majority of F_1 mice eliminated the virus
108 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, we next investigated whether the MHC
122 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 and Table 1), indicating that control of infection in N_2
127 mice is independent of the MHC haplotype and that non-MHC loci control antiviral responses in
128 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 inherit a noncanonical IFNγ-independent
132 pathway for antiviral IgG2a Ab production (34), we wondered whether this pathway was unique
133 to this genetic background. Earlier reports indicated that 129S mice also inherit a pathway for
134 IFNγ-independent production, as IFNγ-receptor 1 (IFNγR)-deficient 129S7 (G129) mice produce
135 IgG2a Abs against lymphocytic choriomeningitis virus (LCMV) (41) and (lactate dehydrogenase-
136 elevating virus) LV (42). We therefore investigated whether $IFN\gamma R^{-/-}$ 129S7 mice produce IFNγ-
137 independent IgG2a Abs against RL-MLV. Control $IFN\gamma R^{-/-}$ ByJ mice did not produce Abs of any
138 isotype and did not control infection, while $IFN\gamma R^{-/-}$ 129S7 mice produced IgG2a Abs (Figure
139 3A). Although Ab production was lower than in IFNγR-sufficient 129S1 mice, the majority of
140 $IFN\gamma R^{-/-}$ 129S7 mice (60%) cleared infection and did not develop splenomegaly (Figure 3 and
141 Table 1). Therefore, like BALB/cJ mice, 129S7 mice inherit a mechanism for noncanonical,
142 IFNγ-independent antiretroviral Ab production.
143
144 **IFNγ-independent anti-MLV IgG2a production in 129S7 mice is a complex trait.** We next
145 sought to investigate whether IFNγ-independent antiretroviral Ab production in 129S7 mice and
146 BALB/cJ mice is controlled by the same genetic mechanism. Since the resistant allele of *igii*-
147 inherited by BALB/cJ mice is recessive, we reasoned that $IFN\gamma R^{-/-}$ F_1 animals inheriting the
148 129S7 and BALB/c allele would only produce antiviral IgG2a Abs if the 129S7 allele of *igii* is
149 resistant. IFNγR-deficient mice are available on the BALB/cByJ background, and we crossed
150 these mice to $IFN\gamma R^{-/-}$ 129S7 mice. F_1 mice generated from these crosses were infected with
151 RL-MLV and screened for antiviral Abs. These F_1 progeny did not display a clear phenotype,

152 with ~30% of mice producing Abs and controlling infection while the majority did not produce
153 Abs, developed splenomegaly, and had infectious virus in their spleens (Figure 4 and Table 1).
154 Therefore, the phenotype is not fully penetrant, and the genetic basis for IFNγ-independent
155 antiretroviral Ab production in 129S7 mice cannot be determined from these crosses.
156 Furthermore, although these experiments suggest that 129S7 mice do not inherit a resistant
157 allele of *igii*, BALB/cByJ and BALB/cJ mice have been separated since 1935, therefore we
158 cannot exclude the possibility that ByJ and BALB/cJ mice inherit different alleles of *igii*.
159
160 **Anti-MLV Ab production in 129S1 mice is IFNγ-dependent.** Since we were unable to
161 determine the genetic basis for IFNγ-independent antiretroviral Ab production in 129S7 mice by
162 crossing to IFNγR$^{-/-}$ ByJ mice, and these strains are deficient for the IFNγ receptor rather than
163 the cytokine, we decided to generate IFNγ-deficient 129S mice for the investigation of IFNγ-
164 independent Ab responses. Considering that wild-type mice of the precise genetic background
165 of 129S7-*Ifngr*$^{-/-}$ no longer exist, we selected the 129S1 background for the knock-out of IFNγ,
166 as they are the control strain recommended by the Jackson Laboratory for lines derived from
167 multiple 129S substrains. We used a previously validated CRISPR/Cas9 approach that targeted
168 introns 1 and 3 of the *Ifng* gene, thereby deleting exons 2 and 3, removing four of six alpha
169 helices ((43) and Figure S2). We infected heterozygous control and IFNγ$^{-/-}$ mice from two
170 founder lines with RL-MLV and monitored them for antiviral Ab production. Although IFNγ
171 sufficient controls produced antiviral IgG2a Abs and cleared the infection, IFNγ$^{-/-}$ 129S1 mice
172 from either founder line failed to produce a robust antiviral Ab response of any isotype (Figure
173 5). Additionally, less than 20% of IFNγ$^{-/-}$ 129S1 mice cleared the infection, and the majority
174 developed splenomegaly (Figure 5B-C and Table 1), indicating that unlike 129S7 mice, 129S1
175 mice do not inherit a pathway for directing IFNγ-independent antiretroviral Ab responses.
176
177 **Genetic differences, not alternative receptor usage, underlie differential requirements for**
178 **IFNγ-signaling in 129S mice.** To determine if antiviral IgG2a Ab responses in 129S7 mice are

179 the result of IFNγ signaling through an alternative receptor, or result from genetic differences
180 between 129S7 and 129S1 mice, we crossed IFNγR$^{-/-}$ 129S7 mice to IFNγ$^{-/-}$ 129S1 mice and
181 then intercrossed the resulting F_1 generation (Figure 6A). If IFNγ signaling through an
182 alternative receptor explained virus resistance in IFNγR$^{-/-}$ 129S7 mice, we would expect to
183 observe antiviral Ab production in IFNγR$^{-/-}$, but not IFNγ$^{-/-}$, or IFNγ/IFNγR$^{-/-}$ F_2 mice. On the other
184 hand, if 129S7 inherit an alternative pathway for IFNγ-independent antiviral Ab production, we
185 would expect a mixture of resistant and susceptible F_2 mice independently of inheritance of
186 IFNγR- or IFNγ-deficiency. As expected, F_2 mice sufficient for both cytokine and receptor
187 produced anti-MLV IgG2a-specific Abs and cleared the infection (Fig 6B-C). However, antiviral
188 Ab production was not observed in the majority of IFNγR$^{-/-}$ F_2 mice, and only 10% cleared the
189 infection (Figure 6B-C). Interestingly, more IFNγ$^{-/-}$ and IFNγ/IFNγR$^{-/-}$ F_2 mice produced anti-MLV
190 Abs and cleared infection than IFNγR$^{-/-}$ F_2 mice, and 64% of IFNγ$^{-/-}$ and 100% of IFNγ/IFNγR$^{-/-}$ F_2
191 mice cleared the infection (although only four double-deficient mice were infected) (Figure 6B-D
192 and Table 1). These data strongly suggest that IFNγ does not signal through an alternative
193 receptor to stimulate antiviral Abs in 129S7 mice and that an unknown genetic factor(s) present
194 in 129S7, but not 129S1 mice controls IFNγ-independent antiretroviral Ab responses.
195

196 **Discussion**

197 Here, we demonstrate that 129S1 mice inherit a previously unknown mechanism that controls
198 the production of protective antiviral Ab production upon MLV infection. This phenotype is
199 inherited in a dominant fashion and is controlled by two loci (Figures 1-2). Importantly, these loci
200 are distinct from previously characterized genes that restrict retroviral replication (*Fv1*, *Fv2*,
201 *Rfv3*) or promote antiviral immune responses (*vic1*, MHC locus, *Rfv3*), indicating that the
202 pathway in 129S1 mice potentially encodes for a novel mechanism for the stimulation of
203 protective immunity. Unlike I/LnJ and C57BL/6 mice, which fully clear RL-MLV infection (15, 26),
204 MLV resistance in 129S1 mice is not fully penetrant, as ~15% of infected mice fail to produce
205 antiviral Abs, develop splenomegaly, and retain infectious virus in their spleens (Figures 1 and

206 5, Table 1). Additionally, although this mechanism does not appear to be sex-linked, there was
207 a reduction in IgG2a Ab production in (ByJx129S1) F_1 mice, and fewer of them cleared the
208 infection than 129S1 or (129S1xByJ) F_1 mice (Figure 1 and Table 1), indicating that epigenetic
209 or environmental factors may play a role in controlling anti-MLV responses in 129S1 mice.
210 IFNγ is a well-established signal for stimulating CSR to the IgG2a isotype both *in vitro*
211 and in the context of various viral infections (21, 37, 38), including FV infection in B6 mice (22)
212 and RL-MLV and MMTV infection in I/LnJ mice (6, 15). We previously demonstrated that
213 BALB/cJ mice inherit a pathway for the generation of IFNγ-independent IgG2a antiviral Ab
214 responses that is controlled by a single recessive locus, *igii*, which is mapped to Chr. 9 (34).
215 Here, we demonstrate that 129S7 mice also inherit a pathway for IFNγ-independent anti-MLV
216 Ab production. However, the mechanism for IFNγR-independent anti-RL-MLV responses in
217 129S7 mice is a complex genetic trait, and we were unable to determine whether the pathway is
218 controlled by *igii* or another locus (or multiple loci). Nevertheless, the ability of both BALB/cJ and
219 129S7 mice to produce IFNγ-independent Abs against a variety of infections including
220 retroviruses, herpes simplex virus, LCMV, LV, and parasitic infections (34, 41, 42), indicates
221 that alternative pathways in addition to the canonical IFNγ-dependent pathway can stimulate
222 neutralizing Ab responses. These findings additionally highlight the importance of utilizing mice
223 of multiple genetic backgrounds to investigate the requirements for stimulating protective
224 antiviral immunity.
225 Following decades of widespread use of 129 ES cell lines, two reports from 1997
226 investigated the variation between 129 substrains and found substantial genetic variation
227 resulting from genetic drift, genetic contamination, and residual heterozygosity from
228 backcrossing programs (35, 36). This initiated efforts to clarify the nomenclature for the
229 improvement of gene targeting experiments and the selection and consideration of controls for
230 comparing phenotypes of genes targeted in ES cells of different 129 substrains (35, 36, 44).
231 These findings further suggested important features that should be considered for targeted
232 mutagenesis using 129 substrains 1) chimeras produced using 129 ES cells should be crossed

233 to a genetically matched substrain for maintenance on the 129 background and 2) selection of a
234 129 substrain for targeted mutagenesis should involve consideration of different genetic traits
235 between substrains (35). Like many mice generated prior to these recommendations, chimeric
236 IFNγR$^{-/-}$ 129S mice were not crossed to a genetically matched substrain, and the appropriate
237 wild-type control strain is unavailable. The 129S1 substrain is therefore recommended as the
238 control inbred strain for steel substrain-derived ES cell lines (40). However, we found that while
239 129S7 mice produce antiviral Ab responses in the absence of IFNγ, this mechanism is not
240 inherited by 129S1 mice (Figure 5). Some reports have suggested that IFNγ can signal through
241 an alternative receptor in the absence of *Ifngr1* (45-47), but this does not explain the differences
242 between 129S1 and 129S7 substrains reported here, as IFNγR$^{-/-}$ (129S1x129S7) F_2 mice did not
243 produce antiviral Abs (Figure 6). Therefore, for the purposes of investigating the requirements
244 for IFNγ in antiviral defenses, 129S1 mice are not an appropriate control for 129S7-*Ifngr*$^{-/-}$ mice.
245 The higher incidence of IFNγ-independent Ab production in IFNγ$^{-/-}$ and IFNγ/IFNγR$^{-/-}$ F_2 mice
246 than IFNγR$^{-/-}$ F_2 mice (Figure 6) was intriguing. However, while the number of F_2 animals
247 phenotyped here is sufficient to eliminate alternative receptor usage by IFNγ as the basis for
248 IFNγR-independent Ab production, retroviral resistance is not a fully penetrant phenotype in
249 either 129S1 or 129S7-*Ifngr*$^{-/-}$ mice, and it appears that IFNγ-independent antiviral Ab
250 production in 129S7 mice is a complex trait. Therefore, larger numbers of F_2 mice would be
251 required to draw any other conclusions regarding the genetic basis for these substrain
252 differences.

253 While 129 substrains have the same genotypes at known MLV-resistance loci [(21, 23,
254 37, 38) and this report], our findings clearly indicate that there are unappreciated differences in
255 their ability to mount protective antiviral immune responses. As such, it is unknown whether the
256 129P2/OlaHsd substrain utilized in many investigations on immune responses to FV infection
257 would exhibit a similar phenotype to 129S1 mice when infected with RL-MLV, or whether they
258 can produce IFNγ-independent antiviral immune responses. In fact, these substrains have been
259 separated since the re-establishment of the 129 line in 1948 following the fire at Jackson Labs

260 and have substantial genetic variation between them ((35, 36, 48) and Figure S1). The different
261 phenotypes observed here between Steel substrains are not entirely surprising since the 129S1
262 lineage has been separated from the 129/SvEv lineage, from which the IFNγR$^{-/-}$ 129S7 mice are
263 derived since 1969. Furthermore, additional crosses occurred in the 129/SvEv lineage prior to
264 the introduction of the *Ifngr1* mutation in 129S7-derived AB1 ES cells [Figure S1 and (35, 48)],
265 thereby increasing the genetic divergence of these substrains. Collectively, our findings further
266 emphasize the need for careful consideration of the genetic background of mutant and control
267 mice and indicate that genetic differences between 129S substrains can have consequences
268 well beyond affecting the efficiency of traditional gene targeting methods.
269

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276

277 **Author contributions**
278 Conception and design: M.K.; Acquisition of data: R.Z.Z., V.M., L.R., and M.K.; Analysis and
279 interpretation of data: R.Z.Z. and M.K.; Drafting the article: M.K.; Revising the article: M.K.,
280 R.Z.Z., and V.M..
281

282 **Declaration of interests**
283 The authors declare no competing interests
284
285
286

287 **Materials and Methods**

288 **Mice**

289 129S1/SvImJ (129S1 stock #002448) and BALB/cByJ (stock #001026) were purchased from
290 the Jackson Laboratory. 129-*Ifngr1*^{tm1Agt}/J [(48) also known as G129] mice were purchased from
291 the Jackson Laboratory (via cryo recovery, stock #002702) and bred and maintained at the
292 University of Pittsburgh. IFNγR$^{-/-}$ 129 mice were generated using AB1 ES cells derived from
293 129S7/SvEvBrd-*Hprt*$^{b-m2}$ (129S7) mice, and chimeric founder males were crossed to an
294 unknown 129/SvEv substrain (although 129S8 mice are commonly referred to as 129/SvEv and
295 may have been used for these crosses) (48) for simplicity, we refer to these as 129S7-*Ifngr*$^{-/-}$
296 mice (Figure S1). C.129S7(B6)-*Ifngr1*^{tm1Agt}/J (IFNγR$^{-/-}$ ByJ) were purchased from the Jackson
297 Laboratory (via cryo recovery, stock #002286) and bred and maintained at the University of
298 Pittsburgh.

299

300 *Ifng*-deficient 129S1 mice were generated using CRISPR/Cas9 technology. The target
301 sequences (5′ guide sequence: 5′-GCTGTTTCCCTGCGTAGTTT-3′; 3′ guide sequence: 5′-
302 TAGAGGCTAACCAGAGCCGA-3′) were previously validated for the generation of conditional
303 knockout strains on the C57BL/6 background (43). Two male founders with deletions of exons 2
304 and 3 were selected (Figure S2) and crossed to 129S1 females. Potential off-target sites with
305 fewer than three mismatches identified by the Cas-OFFinder algorithm (49) were sequenced in
306 N_1 and N_2 mice, and mice with no mutations at these sites were intercrossed to produce
307 homozygous *Ifng*-deficient mice.

308

309 Mice were genotyped from tail biopsies using real time PCR with specific probes designed for
310 each gene (Transnetyx, Cordova, TN) or by flow cytometry (see below). Mice of both sexes
311 were used in equal numbers for each experiment. All animal experiments were performed in the
312 American Association for the Accreditation of Laboratory Animal Care-accredited, specific-
313 pathogen-free facility Division of Laboratory Animal Resources, University of Pittsburgh School

314 of Medicine. Animal protocols were reviewed and approved by the Institutional Animal Care and
315 Use Committee at The University of Pittsburgh.
316
317 **Flow cytometry**
318 For MHC genotyping of [(129S1xByJ)xByJ]N_2 mice, blood was collected in sodium heparin
319 collection vials (SAI Infusion Technologies), and peripheral blood lymphocytes were isolated by
320 overlaying with Ficoll-Paque (Cytivia) and spinning at 11,000xg for 4min at 25°C. Lymphocytes
321 were stained with antibodies against Class I [brilliant blue 700 (BB700)-conjugated anti-H-2Db
322 (BD Biosciences) and phycoerythrin (PE)-conjugated anti-H-2Dd (BD Biosciences)] or Class II
323 [peridinin chlorophyll (PerCP)-eFluor 710-conjugated anti-H2-Ab1 (I-Ab, Invitrogen) and PE-
324 conjugated anti-H2-Ad1 (I-Ad, BD Biosciences)] MHC and analyzed on an Attune NxT
325 cytometer (Life Technologies).
326
327 **MLV infection**
328 Rauscher-like MuLV (RL-MuLV), a mixture consisting of NB-tropic ecotropic and mink lung cell
329 focus-forming viruses, was described previously (69) and was provided by T. Golovkina,
330 University of Chicago. The virus was propagated in SC-1 embryonic mouse fibroblasts (ATCC).
331 Ecotropic (Eco) viral titers were determined by an infectious center assay (70). Experimental
332 mice were injected i.p. with 2 $\times 10^4$ Eco PFUs at 5-8 weeks of age and screened for anti-virus
333 antibodies and plaque forming units 8-10 weeks later.
334
335 **ELISA**
336 To detect MuLV Abs in mouse sera, an enzyme-linked immunosorbent assay (ELISA) was
337 performed as previously described (6, 15). All sera were used at 2 x 10^{-2} dilutions. Mouse
338 IgG2a-specific, as well as total IgG-specific secondary antibodies coupled to horseradish
339 peroxidase (HRP) (Jackson ImmunoResearch) were used to detect anti-virus antibodies.

340 Backgrounds obtained from incubation with secondary antibodies alone were subtracted from
341 the values obtained from sera of infected mice.
342
343 **Statistical analyses**
344 Statistical significance was determined using GraphPad software (one way ANOVA or unpaired
345 t test).
346
347
348

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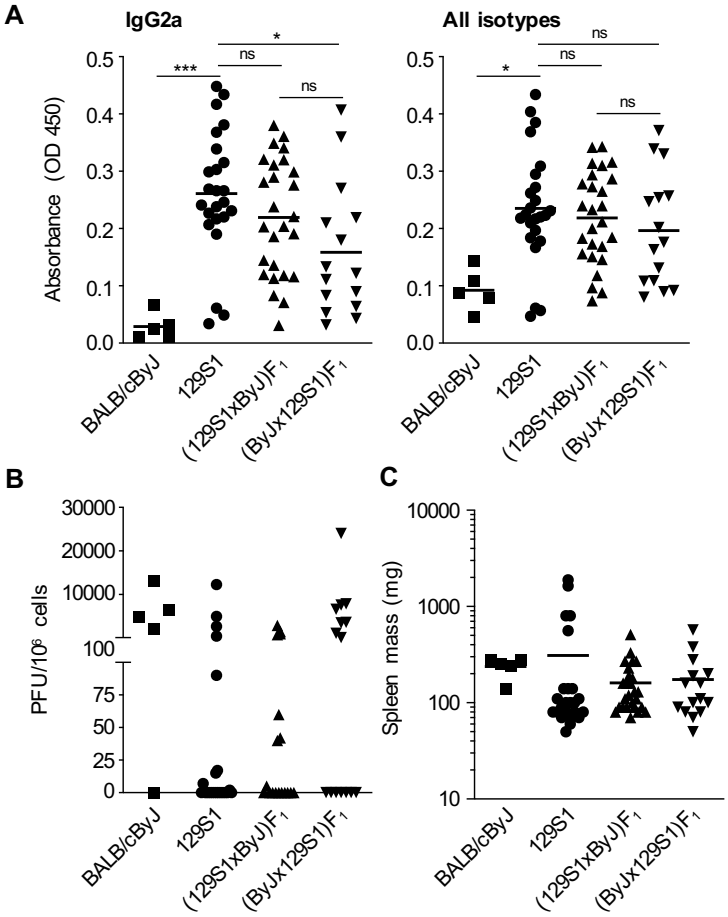


490

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

492 **mechanism.**

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

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

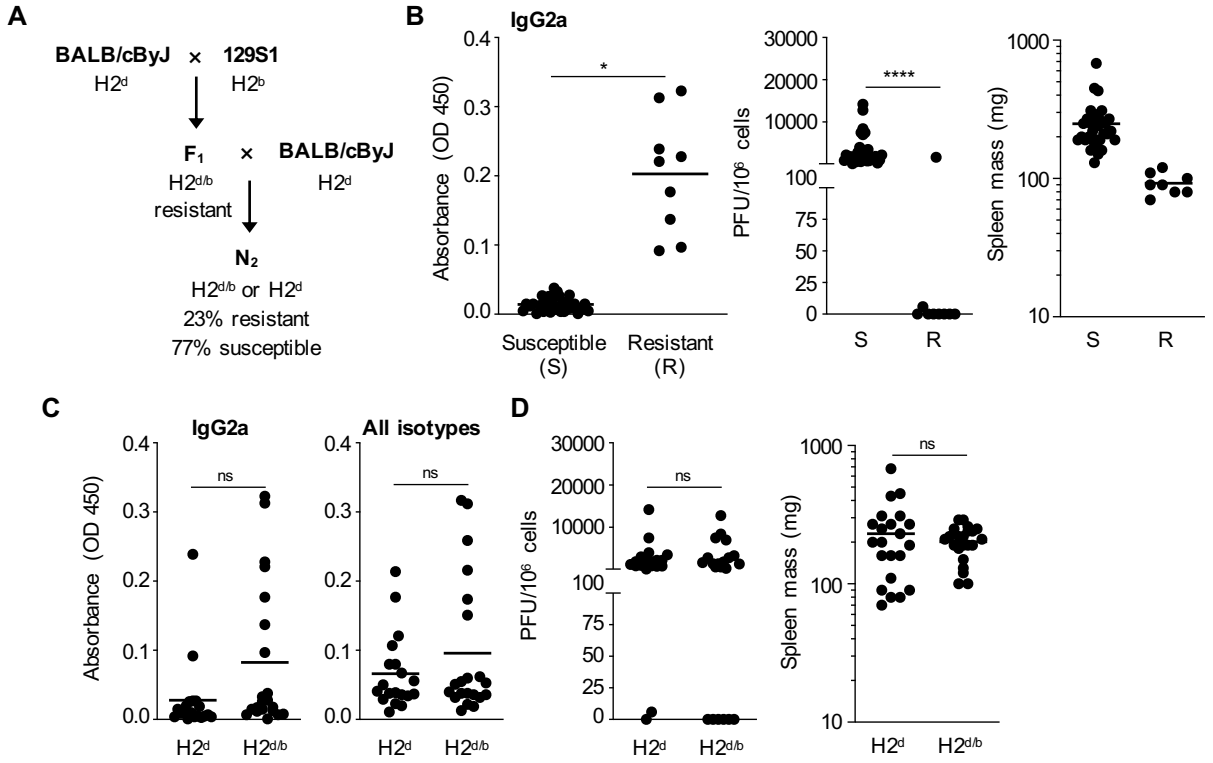
495 weeks post infection. ns, not significant; *, $p<0.05$; ***, $p<0.001$

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

497 10 weeks post infection.

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

499



500

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

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

503 mechanism controlling IFN□-independent IgG2a Ab production.

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

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

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

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

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

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

510 weights were measured (right).

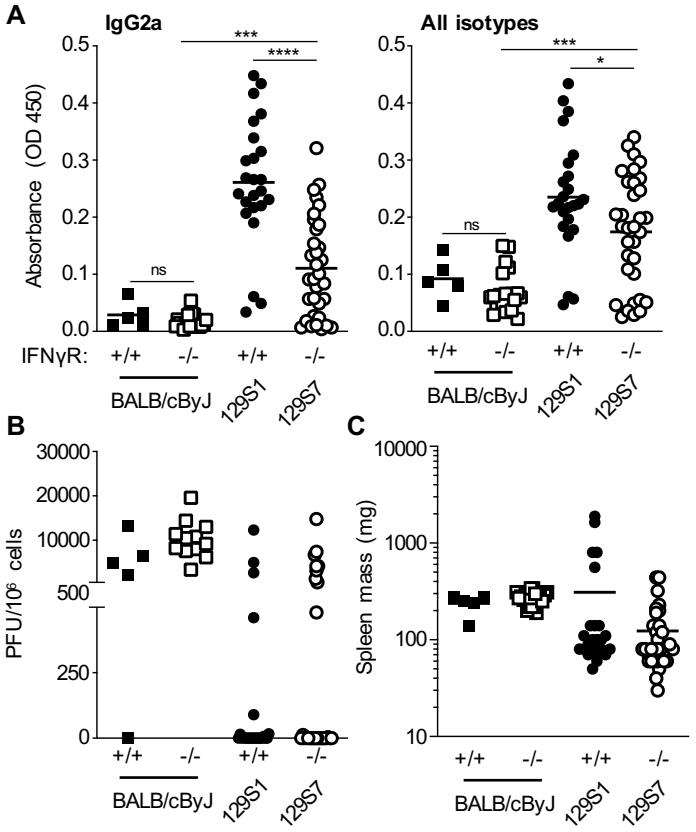
511 **C)** MHC haplotypes of N_2 mice from B) were determined by FACS analysis and then levels

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

513 proteins by ELISA were compared.

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

515 grouped by MHC haplotype.



516

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

518 **A)** Mice of the indicated genotypes were infected with RL-MLV and monitored for IgG2a-

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

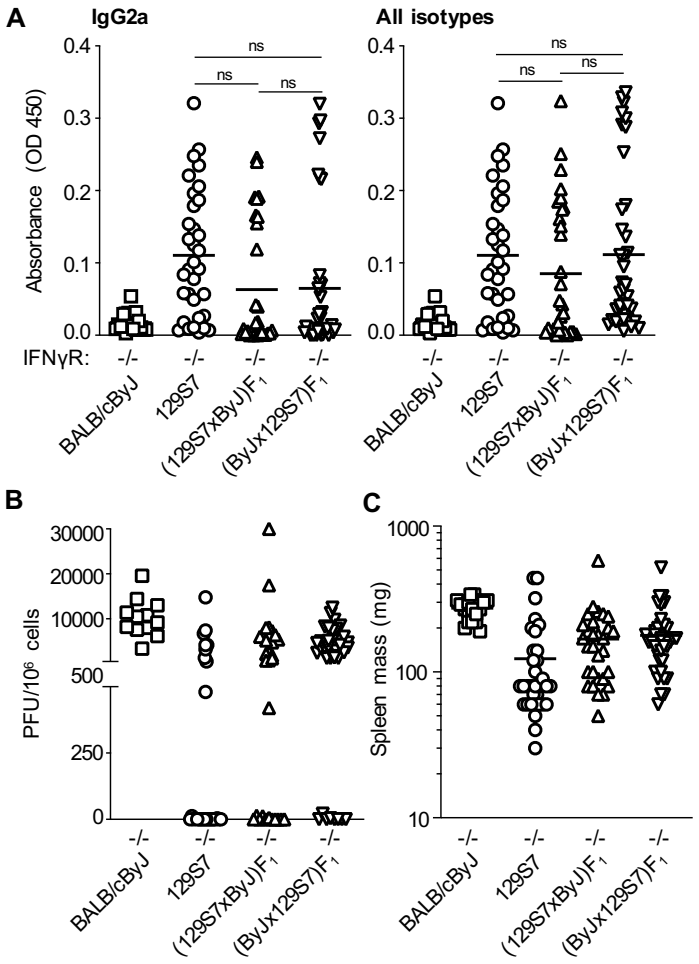
520 weeks post infection. ns, not significant; *, $p<0.05$; ***, $p<0.001$; ****, $p<0.0001$

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

522 10 weeks post infection.

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

524



525

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

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

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

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

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

531 eight weeks post infection.

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

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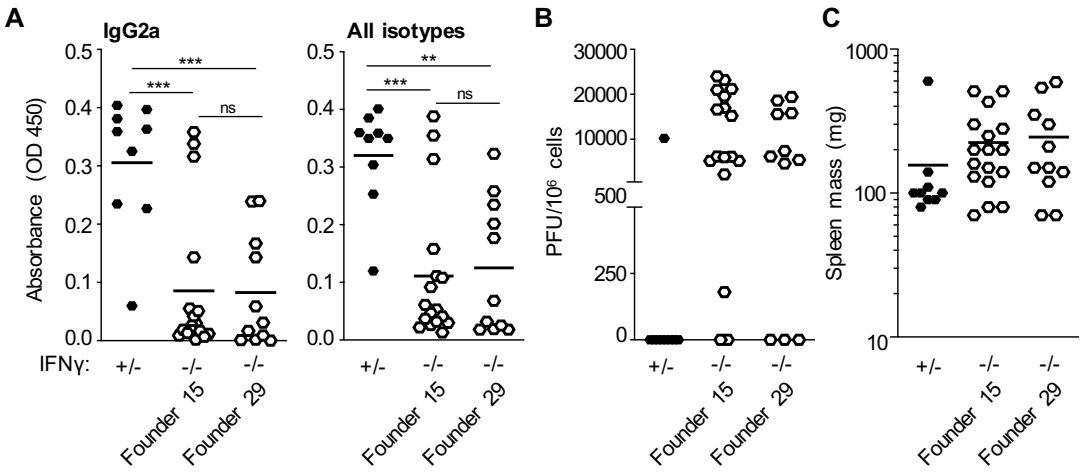

534

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

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

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

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

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

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

541 eight weeks post infection.

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

543

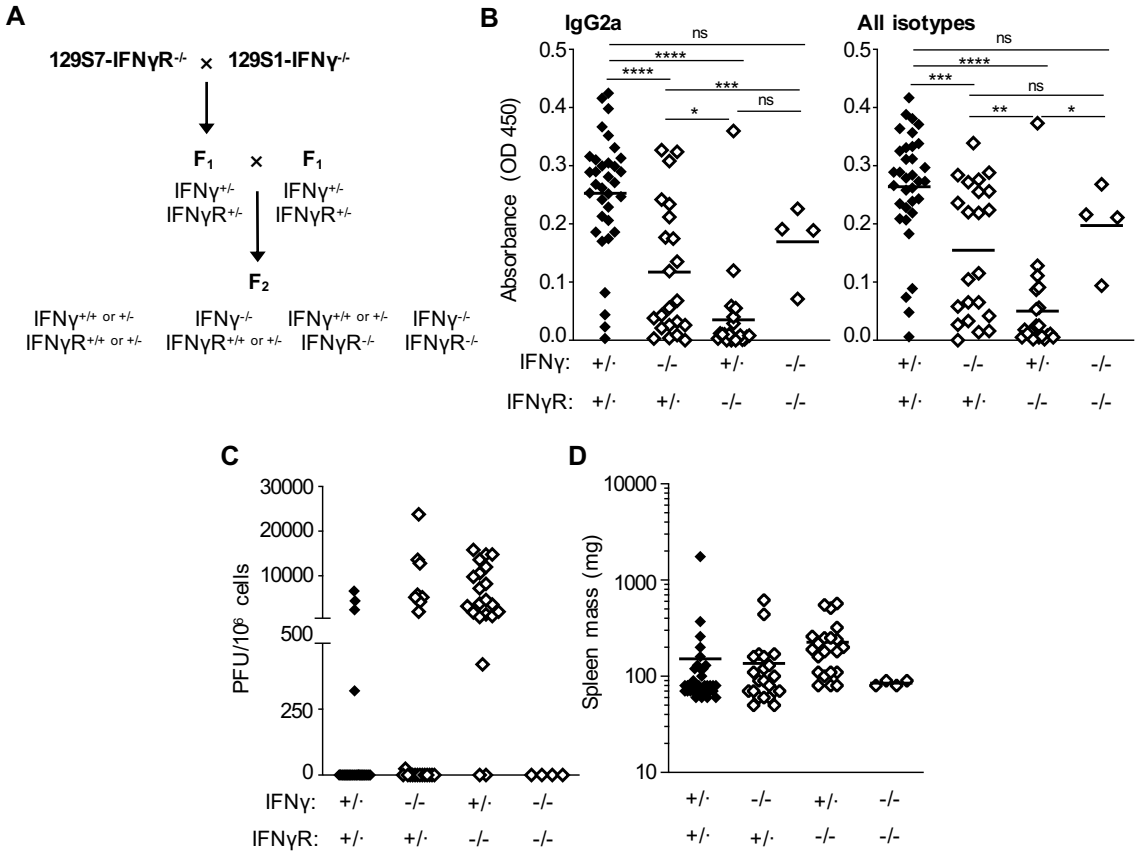

Figure 6. Genetic differences between 129S substrains affect IFNγ-independent anti-MLV Ab responses

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.

558 **Table 1.** Summary of mice utilized in this investigation and their phenotype upon MLV infection

	Genotype		MLV infections		
Mice	**IFNγ**	**IFNγR**	**# Infected**	**# with ≤ 100PFU/ 10^6cells**	**% 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%

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

560