Expression of a single inhibitory Ly49 receptor is sufficient to license NK cells for effector functions

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Abstract

Natural killer (NK) cells recognize target cells through germline-encoded activation and inhibitory receptors enabling effective immunity against viruses and cancer. The Ly49 receptor family in the mouse and killer immunoglobin-like receptor family in humans play a central role in NK cell immunity through recognition of MHC class I and related molecules. Functionally, these receptor families are involved in licensing and rejection of MHC-I-deficient cells through missing-self. The Ly49 family is highly polymorphic, making it challenging to detail the contributions of individual Ly49 receptors to NK cell function. Herein, we showed mice lacking expression of all Ly49s were unable to reject missing-self target cells *in vivo*, were defective in NK cell licensing, and displayed lower KLRG1 on the surface of NK cells. Expression of Ly49A alone on a H-2D^{d} background restored missing-self target cell rejection, NK cell licensing, and NK cell KLRG1 expression. Thus, a single inhibitory Ly49 receptor is sufficient to license NK cells and mediate missing-self *in vivo*.

Introduction

Natural killer (NK) cells are innate lymphoid cells (ILCs) that can mediate effective immunity against viruses and cancer through direct lysis and cytokine production (Huntington et al., 2020; Piersma and Brizić, 2021). NK cells recognize their target cells through integration of signals by germline-encoded activation and inhibitory receptors (Long et al., 2013). These inhibitory receptors include members of the Ly49 family in the mouse and killer immunoglobin-like receptor (KIR) family in humans and they prevent killing of healthy cells through recognition of MHC class I (MHC-I) (Colonna and Samaridis, 1995; Karlhofer et al., 1992). Host cells may lose surface MHC-I expression in response to virus infection or malignant transformation. As a result, these cells become invisible to $CD8^+$ T cells, but simultaneously become targets for NK cells through "missing-self" recognition (Kärre et al., 1986).

The inhibitory Ly49 molecules appear to be responsible for missing-self recognition in mice (Babić et al., 2010; Belanger et al., 2012; Gamache et al., 2019; Parikh et al., 2020; Zhang et al., 2019). However, it has been sometimes challenging to draw definitive conclusions because the Ly49 family is highly polymorphic and differs between mouse strains. Moreover, multiple inhibitory Ly49 receptors within a single host can recognize a given MHC-I molecule while others apparently have no ligands and instead recognize other MHC-I alleles (Schenkel et al., 2013). Yet, the Ly49s for non-host MHC-I alleles are still expressed. For example, in the C57BL/6 background, Ly49C and Ly49I can recognize H-2^b MHC-I molecules, while Ly49A and Ly49G cannot recognize H-2^b molecules and instead they recognize H-2^d alleles. Still these Ly49s are expressed in C57BL/6 mice, so their individual contributions to missing-self rejection are unclear. Ly49A has also been implicated in recognition of the non-classical MHC-I molecule H2-M3, which is upregulated in response to exposure to N-formylated peptides (Andrews et al., 2012; Chiu et al., 1999). Importantly, the specificities of several Ly49s have been clearly established while others remain to be confirmed. For example, the binding of Ly49A to H-$2D^d$ has been confirmed by crystallographic studies (Tormo et al., 1999), and validated by mutational analysis of both Ly49A and H-$2D^d$ (Matsumoto et al., 2001; Wang et al., 2001). By contrast, the MHC-I specificities of other Ly49s have been primarily studied with MHC tetramers containing human β_2-microglobulin (B2m) which is not recognized by Ly49A (Mitsuki et al., 2004) on cells overexpressing Ly49s (Hanke et al., 1999). Thus, the contributions of individual Ly49 receptors to NK cell effector function are confounded by expression of multiple receptors, some of which may be irrelevant to a given self-MHC haplotype, and multiple Ly49 alleles whose specificities are less well defined.

In addition to effector function in missing-self, when inhibitory Ly49 receptors recognize their cognate MHC-I ligands *in vivo*, they license or educate NK cells for potent effector functions including IFNγ production and degranulation in response to activation receptor stimulation (Elliott et al., 2010; Kim et al., 2005). Like missing-self recognition, inhibitory Ly49s require SHP-1 for NK cell licensing which interacts with the ITIM-motif encoded in the cytosolic tail of inhibitory Ly49s (Bern et al., 2017; Kim et al., 2005; Viant et al., 2014). Moreover, lower expression of SHP-1, particularly within the immunological synapse, is associated with licensed NK cells (Schmied et al., 2023; Wu et al., 2021). Thus, inhibitory Ly49s have a second function that licenses NK cells to self-MHC-I thereby generating appropriate self-tolerant NK cells, but it has not been possible to exclude contributions from other co-expressed Ly49s.

The complex nature of the Ly49 family confounds our fundamental understanding of these receptors, particularly regarding their function *in vivo*. To better understand Ly49 function

in vivo, several groups made mutant mouse lines with altered expression of the *Ly49* locus. This was pioneered by the Makrigiannis group, which targeted the *Ly49o* promoter in 129 ES cells and resulting mice were subsequently backcrossed to C57BL/6 background (Belanger et al., 2012). These mice were defective in rejection of MHC-I deficient target cells *in vivo* and exhibited reduced tumor control as well (Tu et al., 2014). However, there was limited surface expression of NKG2A as well as Ly49s, so these target defects were not solely dependent on absence of Ly49s. Moreover, these mice likely carried 129 alleles of other genes in the NK gene complex (NKC) that are expressed on NK cells, display allelic polymorphisms and are genetically linked to *Ly49*. Following the development of mouse CRISPR engineering, the Dong group deleted the entire 1.4 Mb *Ly49* locus in the C57BL/6 NKC and showed that Ly49-deficient mice were unable to reject MHC-I deficient cells *in vivo* at steady state (Zhang et al., 2019). Besides loss of Ly49 expression, surface expression of other receptors, including KLRG1, NKG2A, NKG2D and CD94 were also reduced in these mice. Around the same time, we generated a mouse that contains a 66 Kb and 149 Kb deletion in the C57BL/6 Ly49 locus, resulting in loss of 4 Ly49 molecules including Ly49A and Ly49G (Parikh et al., 2020). The resulting ΔLy49-1 mice were also deficient in $H2D^d$-restricted control of murine cytomegalovirus (MCMV), which was rescued by knock-in of *Ly49a* into the *Ncr1* locus. Thus, available genetic evidence suggest inhibitory Ly49 receptors are essential for licensing and missing-self recognition.

The current data, however, do not take into account that individual Ly49s are stochastically expressed on NK cells, and multiple receptors are simultaneously expressed on individual NK cells, resulting in a diverse Ly49 repertoire of potential specificities on overlapping subsets of NK cells (Dorfman and Raulet, 1998; Kubota et al., 1999; Smith et al., 2000). As a result, not all NK cells express a specific Ly49 receptor and most NK cells express multiple Ly49 molecules, making it difficult to study the biology of a specific Ly49 receptor without genetic approaches. However, *Ly49* genes are highly related and clustered together, resulting in a high concentration of repetitive elements (Makrigiannis et al., 2005), complicating the capacity to target individual *Ly49* genes for definitive analysis. Moreover, in ΔLy49-1 mice which had an intact *Ly49d* coding sequence, the percentage of NK cells expressing Ly49D was markedly reduced, even though Ly49D was otherwise expressed at normal levels, suggesting a regulatory locus control region within the deleted fragments (Parikh et al., 2020). Such data raise the possibility that the large genetic deletions of the Ly49 locus may affect other NK cell receptors in the NKC that contribute to NK cell function. Thus, expression of individual Ly49 receptors without confounding effects of other Ly49s and potentially other NKC genes is needed to validate the conclusions from study of mice lacking Ly49 expression.

Here we studied the role of an individual Ly49 receptor in NK cell function. To this end, we deleted all NK cell Ly49 genes using CRISPR/Cas9 and confirmed the role of the *Ly49* family in missing self and licensing. Subsequently, we expressed Ly49A in isolation under control of the *Ncr1* locus in Ly49-deficient mice on a $H\text{-}2D^d$ background to show that a single inhibitory Ly49 receptor expressed by all NK cells is sufficient for licensing and mediating missing-self *in vivo*.

147 **Results**
148
149 *NK cells from CRISPR-generated mice lacking all NK cell-related Ly49 molecules display*
150 *reduced KLRG1 expression.*
151 To investigate the role of individual Ly49 molecules, we generated a mouse that lacked all
152 expressed Ly49 receptors. We targeted the remaining *Ly49* region in our previously published
153 ΔLy49-1 mouse (Parikh et al., 2020) with guide RNAs targeting *Ly49i* and *Ly49q*. The resulting
154 mouse contained a fusion between *Ly49i* and *Ly49q* with a deletion of the start codon and
155 insertion of a fusion sequence that did contain a potential start site for a putative 5 amino acid
156 polypeptide (Figure 1A). We confirmed that the 3' deletion reported in the ΔLy49-1 mouse was
157 unaffected, resulting in a frameshift and a premature stop codon after 9 amino acids in the fused
158 *Ly49a/g* gene. Thus, genetic and sequencing analysis revealed all Ly49 genes were disrupted and
159 we termed this mouse line Ly49KO (Figure 1A).
160 Flow cytometry confirmed loss of cell surface expression of Ly49 molecules in
161 homozygous Ly49KO mice (Figure 1B). NKG2A, CD94, and NKG2D molecules that are
162 encoded by the NKG2 locus, located next to the Ly49 locus, were still expressed, albeit at
163 marginally lower frequencies (Figure 1C) (Yokoyama and Plougastel, 2003). Unrelated
164 molecules 2B4 and CD122 were unaffected. In heterozygous Ly49KO mice, the percentages of
165 NK cells expressing Ly49A, Ly49C, Ly49D, Ly49G2, Ly49H, and Ly49I were reduced by 33-
166 41%. The median fluorescent intensity (MFI) for Ly49I was reduced by 26% in Ly49I^{+} NK cells
167 in heterozygous Ly49KO mice, while the other Ly49s did not display significant differences in
168 MFI. Consistent with apparent dependence of normal KLRG1 expression on MHC-I expression
169 (Corral et al., 2000) and previous reports (Zhang et al., 2019), Ly49KO NK cells showed a 51%
170 reduction in KLRG1 expression (Figure 1C). NK cells in Ly49KO mice displayed similar
171 maturation to wildtype NK cells, based on expression of the surface markers CD27 and CD11b
172 (Figure 1D). Thus, Ly49KO mice specifically lack all Ly49 molecules and display moderate
173 alterations in select surface molecules while showing otherwise normal numbers of apparently
174 mature NK cells.
175
176 *Ly49-deficient NK cells are defective in licensing and rejection of MHC-I deficient target cells.*
177 Inhibitory Ly49-positive NK cells can be licensed through recognition of cognate MHC-I
178 molecules, resulting in a phenotype of increased IFNγ production following plate-bound anti-
179 NK1.1 stimulation (Kim et al., 2005). Stimulation of Ly49KO NK cells anti-NK1.1 resulted in a
180 73% reduction in IFNγ production as compared to wildtype NK cells, similar to unlicensed NK
181 cells from MHC-I-deficient H-2K^{b} x H-2D^{b} deficient (KODO) mice (Figure 2A). Both Ly49KO
182 and KODO NK cells produced high amounts of IFNγ in response to phorbol 12-myristate 13-
183 acetate (PMA) plus ionomycin (Figure 2B), indicating that their IFNγ production machinery is
184 intact. Thus, these results confirm that Ly49 molecules are required for the NK cell licensed
185 phenotype.
186 To investigate the capability of Ly49KO NK cells to reject MHC-I deficient target cells,
187 we challenged anti-NK1.1 NK cell-depleted, wildtype, and Ly49KO mice with a mixture of
188 wildtype, B2m deficient x KODO (MHC-I KO), H-2D^{b} KO, and H-2K^{b} KO splenocytes that
189 were differentially labeled with CellTrace Far Red (CTFR) and CellTrace Violet (CTV) (Figure
190 2C). While wildtype mice efficiently rejected MHC-I KO and H-2K^{b} KO target cells, only 13%
191 of H-2D^{b} KO target cells were rejected by wildtype mice, indicating that missing-self is
192 dominated by H-2K^{b} in the C57BL/6 background. None of the target cell populations were

193 rejected in the Ly49KO mice, comparable to wild type controls depleted of NK cells. Thus, Ly49
194 molecules mediate NK cell-dependent MHC-I deficient target cell killing *in vivo* under steady-
195 state conditions and rejection of MHC-I deficient target cells is predominantly controlled by H-
196 $2K^b$ in the $H\text{-}2^b$ background.
197
198 *Expression of Ly49A in Ly49-deficient H-2D^d transgenic mice rescues KLRG1 expression.*
199 To investigate the potential of a single inhibitory Ly49 receptor on mediating NK cell
200 licensing and missing-self rejection, the Ly49KO mice were backcrossed to $H\text{-}2D^d$ transgenic
201 KODO (D8-KODO) Ly49A KI mice that express *Klra1* cDNA encoding the inhibitory Ly49A
202 receptor in the *Ncr1* locus and its cognate ligand $H\text{-}2D^d$ but not any other classical MHC-I
203 molecules (Parikh et al., 2020). Ly49A expression in the resulting Ly49KO/Ly49A KI D8-
204 KODO mice closely follows NKp46 expression because $NKp46^-$ $NK1.1^+$ NK cells in the bone
205 marrow of these mice do not express Ly49A, while virtually all the $NKp46^+$ $NK1.1^+$ NK cells in
206 the bone marrow and spleen express Ly49A (Figure 3A). Ly49KO/Ly49A KI D8-KODO NK
207 cells expressed robust levels of Ly49A, albeit at lower MFI as compared to Ly49A expression on
208 D8-KODO NK cells, consistent with prior observations with wild type Ly49A and $H2D^d$ (Held
209 et al., 1996; Karlhofer et al., 1994). NK cells were able to fully mature in Ly49KO D8-KODO
210 and Ly49KO/Ly49A KI D8-KODO mice as we observed similar percentage of mature $CD27^-$
211 $CD11b^+$ NK cells in spleen, bone marrow, and liver (Figure 3B). While there was a modest
212 significant increase in immature $CD27^+$ $CD11b^-$ NK cells in the bone marrow of Ly49KO D8-
213 KODO and Ly49KO/Ly49A KI D8-KODO mice, no differences were observed in NK cell
214 maturation in spleen and liver. The decrease in the frequency of $KLRG1^+$ NK cells observed in
215 the Ly49KO NK cells on the $H\text{-}2^b$ background was recapitulated in the D8-KODO background
216 (Figure 3C). Intriguingly, expression of Ly49A in Ly49KO/Ly49A KI D8-KODO mice rescued
217 KLRG1 expression and resulted in similar levels of KLRG1 as D8-KODO NK cells. Taken
218 together, Ly49A engineered to be encoded within the NKp46 locus was efficiently expressed as
219 an isolated Ly49 receptor and supported KLRG1 expression.
220
221 *NK cells expressing Ly49A in isolation are fully licensed and capable of rejecting MHC-I*
222 *deficient target cells.*
223 Next, we investigated the potential of Ly49A expression alone to mediate NK cell licensing and
224 rejection of MHC-I deficient target cells. In D8-KODO mice, $Ly49A^+$ NK cells displayed
225 increased levels of IFNγ production and degranulation measured by CD107 in response to plate
226 bound anti-NK1.1 stimulation as compared to all NK cells including unlicensed cells (Figure
227 4A). Similar to Ly49KO NK cells on the $H\text{-}2^b$ background, Ly49KO NK cells on the D8-KODO
228 background showed a 72% decrease in IFNγ production, but also showed a 59% decrease in
229 degranulation as compared to $Ly49A^+$ NK cells in D8-KODO mice. This impaired IFNγ
230 production and degranulation was reversed in Ly49A KI NK cells on the Ly49KO D8-KODO
231 background, that showed similar IFNγ production and degranulation to $Ly49A^+$ NK cells on the
232 D8-KODO background, indicating that Ly49A KI NK cells are licensed. Importantly, there were
233 no differences among all NK cell populations in IFNγ production and degranulation in response
234 to PMA/Ionomycin (Figure 4B), indicating that the IFNγ production and degranulation
235 machinery are not affected in any of the mouse strains. Thus, the Ly49KO/Ly49A KI D8-KODO
236 NK cells displayed a fully licensed phenotype comparable to licensed $Ly49A^+$ NK cells in D8-
237 KODO mice.

238 Finally, we interrogated whether a single inhibitory Ly49 molecule would be sufficient to
239 mediate missing-self rejection of MHC-I deficient cells. To this end D8-KODO, Ly49KO D8-
240 KODO, and Ly49KO/Ly49A KI D8-KODO mice were challenged with a mixture of D8-KODO
241 and KODO (MHC-I deficient) splenocytes that were differentially labelled with CTV. D8-
242 KODO mice efficiently rejected KODO target splenocytes, while Ly49KO D8-KODO mice
243 were unable to reject these cells. However, this defect was completely restored in the
244 Ly49KO/Ly49A KI D8-KODO mice, demonstrating that a single inhibitory Ly49 receptor is
245 sufficient to mediate missing-self rejection of cells lacking its MHC class I ligand.
246

247 **Discussion**
248 Several mice with genetic modifications in the Ly49 complex have been developed to study the
249 role of Ly49 receptors but they have limitations (Belanger et al., 2012; Bern et al., 2017;
250 Gamache et al., 2019; Parikh et al., 2020; Zhang et al., 2019). A complicating factor in these
251 studies is that multiple Ly49s, often with incompletely understood specificities, may be involved
252 in target cell recognition. Here, we studied a mouse where a single Ly49 was under control of the
253 *Ncr1* locus which is expressed on all NK cells on the background of a complete Ly49 KO.
254 Consistent with previous reports (Belanger et al., 2012; Parikh et al., 2020; Zhang et al., 2019),
255 Ly49-deficient NK cells were deficient in licensing and missing-self rejection, both on a H-2^b
256 background and in the presence of a single classical MHC-I allele, H-$2D^d$. Moreover, expression
257 of the inhibitory Ly49A in isolation did not alter NK cell numbers or maturation, indicating that
258 the Ly49s do not affect these parameters of NK cells. Yet the NK cells were fully licensed in
259 terms of their functional phenotype, including capacity to be activated by an activation receptor
260 *in vitro* and efficient rejection of MHC-I deficient target cells *in vivo*. Thus, a single Ly49
261 receptor confers the licensed phenotype *in vitro* and *in vivo*.
262 We observed that rejection of H-$2K^b$-deficient targets was more potent than H-$2D^b$-
263 deficient target splenocytes, comparable to previous observations (Johansson et al., 2005). These
264 data indicate that H-$2K^b$ is more efficiently recognized by Ly49s as compared to H-$2D^b$. On the
265 other hand, early studies using Ly49 transfectants binding to Con A blasts found that Ly49C and
266 Ly49I can bind to H-$2D^b$-deficient but not H-$2K^b$-deficient cells (Hanke et al., 1999), but these
267 studies have the caveat of testing binding to cells overexpressing Ly49s. Our studies indicate that
268 the efficiency of Ly49-dependent missing-self rejection depends on characteristics of specific
269 MHC-I alleles recognized by cognate Ly49 receptors *in vivo*, raising a caution to solely
270 depending on Ly49 specificities based on *in vitro* studies alone.
271 KLRG1 is an inhibitory receptor that recognizes E-, N-, and R- cadherins to inhibit NK
272 cell cytotoxicity (Ito et al., 2006). KLRG1 is expressed on a subset of mature NK cells and can
273 be upregulated in response to proliferation in a host with lymphopenia (Huntington et al., 2007).
274 Consistent with previously published results (Zhang et al., 2019), we observed decreased
275 KLRG1 expression in Ly49-deficient NK cells. The *Ly49* gene family as well as *Klrg1* is located
276 within the NKC (Yokoyama and Plougastel, 2003), thus an effect of regulatory elements deleted
277 in Ly49KO mice cannot be excluded. This is further emphasized by studies of our ΔLy49-1
278 mouse which expresses Ly49D on fewer NK cells (Parikh et al., 2020). The *Ly49d* gene appears
279 intact and $Ly49D^+$ NK cells expressed Ly49D at normal levels, suggesting the absence of a
280 regulatory element in the deleted regions. Here, we showed expression of only Ly49A in
281 Ly49KO mice on a H-$2D^d$ background restored KLRG1 expression in NK cells from different
282 tissues, indicating that inhibitory Ly49 receptors rather than regulatory elements influence
283 KLRG1 expression. Moreover, NK cell KLRG1 expression is modulated by MHC-I molecules
284 (Corral et al., 2000). Therefore, KLRG1 expression may be modulated as a consequence of NK
285 cell licensing through inhibitory Ly49 receptors.
286 The Ly49 family is stochastically expressed on NK cells, resulting in a NK cell repertoire
287 with different combinations of Ly49 receptors on individual NK cells. Not all Ly49 alleles are
288 equally expressed which has been suggested to be dependent on allelic exclusion (Held et al.,
289 1995). Epigenetic control including DNA-methylation, histone modification, and regulatory
290 elements have been linked to Ly49 expression (Kissiov et al., 2022; McCullen et al., 2016;
291 Rouhi et al., 2006; Saleh et al., 2004). We observed that in Ly49KO heterozygous mice the
292 percentage of $Ly49^+$ NK cells was reduced for each Ly49 molecule, yet the expression level

293 measured by MFI was not affected except for Ly49I, indicating that loss of one allele does not
294 affect Ly49 surface levels. Nonetheless, alternate mechanisms may control Ly49 expression as
295 was observed for Ly49I. Expression of *Ly49a* within the *Ncr1* locus resulted in ubiquitous
296 Ly49A expression in NK cells, albeit at lower levels compared to Ly49A^{+} D8-KODO NK cells.
297 Despite the lower expression levels of Ly49A, Ly49A KI NK cells were fully licensed and
298 efficiently eliminated MHC-I-deficient target cells, suggesting that minor alterations in
299 expression levels of various Ly49s on individual NK cells may not affect NK cell functions. In
300 conclusion, these data show that expression of a single inhibitory Ly49 receptor is necessary and
301 sufficient to license NK cells for missing self-rejection under steady state conditions *in vivo*.
302

Materials and Methods

Animals

C57BL/6 (stock # 556) mice were purchased from Charles Rivers laboratories, B2m-deficient (stock # 2087) were purchased from Jackson laboratories, H-2K^b x H-2D^b double-deficient (stock # 4215; KODO) mice were purchased from Taconic Farms. D8 is a H-2D^d transgenic mouse that has been previously described (Bieberich et al., 1986) and was provided by D. Marguiles, National Institute of Allergy and Infectious Diseases, Bethesda, MD. D8-KODO mice have been previously generated by crossing D8 transgenic mice to a KODO background (Choi et al., 2011). ΔLy49-1 and Ly49A KI mice were previously generated in our laboratory (Parikh et al., 2020). All mice were maintained within the Washington University animal facility in accordance with institutional ethical guidelines under protocol number 21-0090. All experiments utilized sex- and age-matched mice.

Generation of Ly49KO mice

The remaining Ly49 receptors in ΔLy49-1 mice were targeted using CRISPR/Cas9 as previously described (Parikh et al., 2015a). Briefly, the Ly49 locus was targeted with gRNAs directed against Ly49q (5'-ACCCATGATGAGTGAGCAGG-3') and Ly49i (5'-TGAGACTTCATAAGTCTTCAAGG-3'), with the PAM sequence underlined. For the mRNA microinjections, 20ng of each guide and 100ng of Cas9 mRNA were used. Deletions in the Ly49 locus were screened using the primers 5'-GCCCATCTGGCTTCCTTTCT-3' (*Ly49q*-Rv), 5'-CAAGCCCCGATGAGATGGAT-3' (*Ly49i*-Rv), and GGATCAGTCCATGTCAGGGTT (*Ly49i*-Fw) yielding a 409 bp wildtype and a 552 bp mutant band and confirmed using Southern blot analysis (data not shown). To minimize off-target CRISPR/Cas9 effects, candidate founder mice were backcrossed to C57BL/6 mice for 2 generations then crossed to derive homozygous Ly49KO mice. Deletions were verified by Sanger sequencing (Azenta Life Sciences) in homozygous Ly49KO mice using the PCR primers *Ly49q*-Rv and *Ly49i*-Fw for the *Ly49q/i* fusion sequence and the primers AACCAAGCCCCAATGAGATC (*Ly49g*-Rv) and TGGGTCAGTCCATGTCAGTG (*Ly49a*-Fw) for the *Ly49a/g* fusion sequence resulting in 552bp and 409bp products, respectively.

Flow cytometry

Fluorescent-labeled antibodies Ly49D (clone 4D11), Ly49EF (CM4), Ly49F (HBF-719), Ly49G2 (eBio4D11), Ly49H (3D10), Ly49I (YLI-90), 2B4 (eBio244F4), CD122 (TM-b1), NKG2AB6 (16a11), NKG2ACE (20D5), CD94 (18d3), NKG2D (CX5), CD27 (LG.7F9), CD11b (M1/70), IFNγ (XMG1.2), CD107a (eBio1D4B), NKp46 (29A1.4), CD3(145-2C11), CD4 (RM4-5), CD8 (53-6.7), TCRB (H597), and CD19 (eBio1D3) were purchased from Thermo Fisher Scientific; Ly49A (YE1/48.10.6), KLRG1 (2F1), and NK1.1 (PK136), were purchased from Biolegend; Ly49C (4LO311) was purchased from Leinco Technologies. Cells were stained with fixable viability dye eF506 (Thermo Fisher Scientific), continued by staining of cell surface molecules in 2.4G2 hybridoma supernatant to block Fc receptors. For intracellular staining, cells were fixed and stained intracellularly using the BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Bioscience) according to manufacturer's instructions. Samples were acquired using FACSCanto (BD Biosciences) and analyzed using FlowJo software (BD Biosciences). NK cells were defined as singlet Viability-NK1.1+NKp46+CD3-CD19- or Viability-NK1.1+NKp46+CD4-CD8-TCRB-CD19-.

349
350 ***In vitro* stimulation assays**
351 Stimulation of splenic NK cells was performed as previously described (Parikh et al., 2020;
352 Piersma et al., 2019). Briefly, 1 - 4 µg/ml anti-NK1.1 (clone PK136, Leinco Technologies) in
353 PBS was coated in 24-well plates for 90 min at 37ºC. Plates were washed with PBS and 5 x 10^6
354 splenocytes were added per well. In parallel, splenocytes were stimulated with 200 ng/ml
355 Phorbol myristate acetate (PMA; Sigma-Aldrich) and 400 ng/ml Ionomycin (Sigma-Aldrich).
356 After 30 min incubation at 37ºC, Monensin (Thermo Fisher Scientific) and fluorescently labelled
357 anti-CD107a antibody were added, cultures were incubated for an additional 7 hours at 37ºC and
358 subsequently analyzed by flow cytometry.
359
360 ***In vivo* killing assays**
361 *In vivo* killing assays were performed as previously described (Parikh et al., 2015b). Briefly,
362 target splenocytes were isolated from C57BL/6, MHC-I deficient (TKO), H-$2K^b$-deficient, H-
363 $2D^b$-deficient, KODO and D8-KODO mice. Indicated target splenocytes were differentially
364 labelled with CFSE, CellTrace violet, and/or CellTrace far red (Thermo Fisher Scientific). Target
365 cells were mixed at equal ratios for each target and 2 x 10^6 splenocytes per target were injected
366 i.v. into naïve hosts. Where indicated NK cells were depleted with 100µg anti-NK1.1 (Leinco
367 technologies) 2 days before target cell injection. Two days after challenge splenocytes were
368 harvested and analyzed by flow cytometry. Target cell rejection was calculated using the formula
369 $[(1-(\text{Ratio(KO target/wildtype target)}_{\text{sample}}/\text{Ratio(KO target/wildtype target)}_{\text{control}}))\times 100]$.
370
371 **Statistics**
372 Statistical analysis was performed with Prism (GraphPad software) using unpaired *t*-tests and
373 two-way ANOVA with corrections for multiple testing. Error bars in figures represent the SEM.
374 Statistical significance was indicated as follows: ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$;
375 *, $p < 0.05$; ns, not significant.
376
377
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382

383 **Figure Legends**
384
385 **Figure 1** *Mice generated to lack all NK-related Ly49 molecules using CRISPR have NK cells*
386 *that display a less mature phenotype and impaired KLRG1 expression.* (**A**) Genetic map of the
387 Ly49 locus of wildtype C57BL/6 and Ly49KO mice and Sanger sequencing of the fusion
388 sequences in the Ly49KO mice. (**B**) Ly49 receptor expression on splenic NK cells of the
389 indicated mice. (**C**) Surface receptor expression on splenic NK cells from indicated mice. (**D**)
390 Expression of the maturation markers CD27 and CD11b on splenic NK cells from indicated
391 mice. MFI, median fluorescent intensity. Error bars indicate SEM; ns, not significant; *$p < 0.05$,
392 **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.
393
394 **Figure 2** *NK cell licensing and rejection of MHC-I deficient target cells is defective in Ly49KO*
395 *mice.* Splenocytes from the indicated mice were stimulated with plate bound anti-NK1.1 (**A**) or
396 PMA/ionomycin (**B**) and IFNγ production by NKG2A- NK cells and analyzed by flow
397 cytometry. (**C**) *In vivo* cytotoxicity assay against H-2K^b, H-2D^b and full MHC-I deficient targets.
398 Splenocytes from WT, H-2Kb, H-2Db and MHC-I deficient mice were differentially labeled
399 with CFSE, CTV, and CTFR as indicated. Mixture of labeled target cells were injected i.v. into
400 wildtype, Ly49KO, and anti-NK1.1-depleted mice. Target cells were analyzed in spleens by flow
401 cytometry 2 days after challenge. KODO, H-2K^b x H-2D^b knock out; MHC-I KO, KODO x B2m
402 knockout; MFI, median fluorescent intensity. Error bars indicate SEM; ns, not significant; *$p <$
403 0.05, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.
404
405 **Figure 3** *Ly49A is efficiently expressed in Ncr1-Ly49A knockin mice and rescues KLRG1*
406 *expression in NK cells.* Flow cytometric analysis of NK cells in D8-KODO, Ly49KO D8-
407 KODO, and Ly49KO/Ly49A KI D8-KODO mice (**A**) Ly49A expression in NKp46$^+$ and NKp46$^-$
408 NK1.1$^+$ NK cells in bone marrow and spleen of the indicated mice. (**B**) Expression of the
409 maturation markers CD27 and CD11b on NK cells in spleen, bone marrow, and liver of indicated
410 mice. (**C**) KLRG1 expression by NK cells in spleen, bone marrow, and liver of indicated mice.
411 MFI, median fluorescent intensity. Error bars indicate SEM; ns, not significant; *$p < 0.05$, **$p <$
412 0.01, ***$p < 0.001$, and ****$p < 0.0001$.
413
414 **Figure 4** *Expression of Ly49A in isolation is sufficient for NK cell licensing and missing-self*
415 *rejection.* Splenocytes from the indicated mice were stimulated with plate bound anti-NK1.1 (**A**)
416 or PMA/ionomycin (**B**). IFNγ production and degranulation (CD107a) by NKG2A- NK cells
417 were analyzed by flow cytometry. (**C**) Splenocytes from D8-KODO and KODO mice were
418 differentially labeled with CFSE and CTV as indicated. A mixture of labeled target cells was
419 injected i.v. into D8-KODO, Ly49KO D8-KODO, and Ly49KO/Ly49A KI D8-KODO mice.
420 Specific rejection of target cells was analyzed in spleens by flow cytometry 2 days after
421 challenge. Error bars indicate SEM; ns, not significant; ***$p < 0.001$, and ****$p < 0.0001$.
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Figure 1 - Piersma et al.

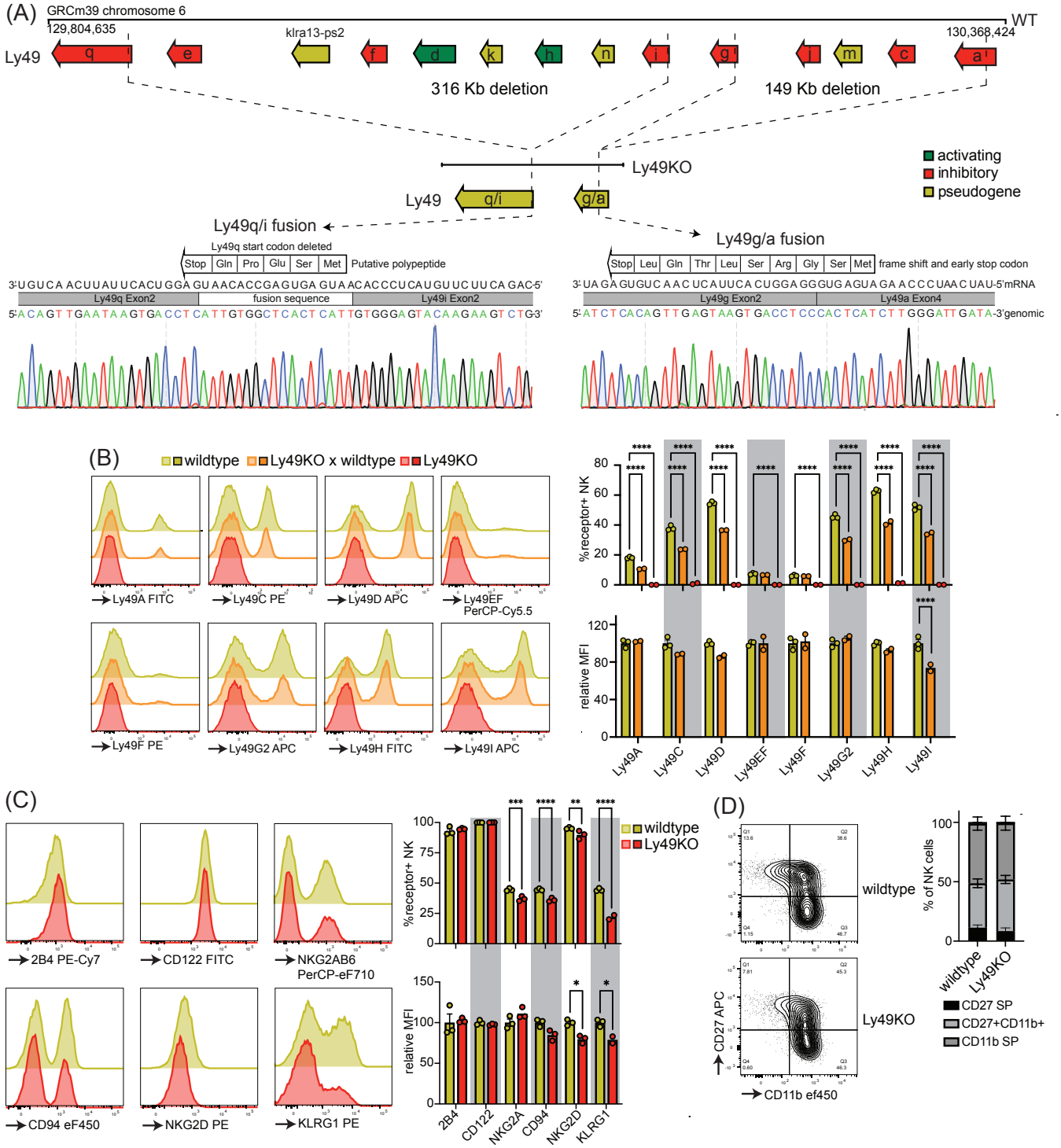

Figure 1 *Mice generated to lack all NK-related Ly49 molecules using CRISPR have NK cells that display a less mature phenotype and impaired KLRG1 expression.* (**A**) Genetic map of the Ly49 locus of wildtype C57BL/6 and Ly49KO mice and Sanger sequencing of the fusion sequences in the Ly49KO mice. (**B**) Ly49 receptor expression on splenic NK cells of the indicated mice. (**C**) Surface receptor expression on splenic NK cells from indicated mice. (**D**) Expression of the maturation markers CD27 and CD11b on splenic NK cells from indicated mice. MFI, median fluorescent intensity. Error bars indicate SEM; ns, not significant; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Figure 2 - Piersma et al.

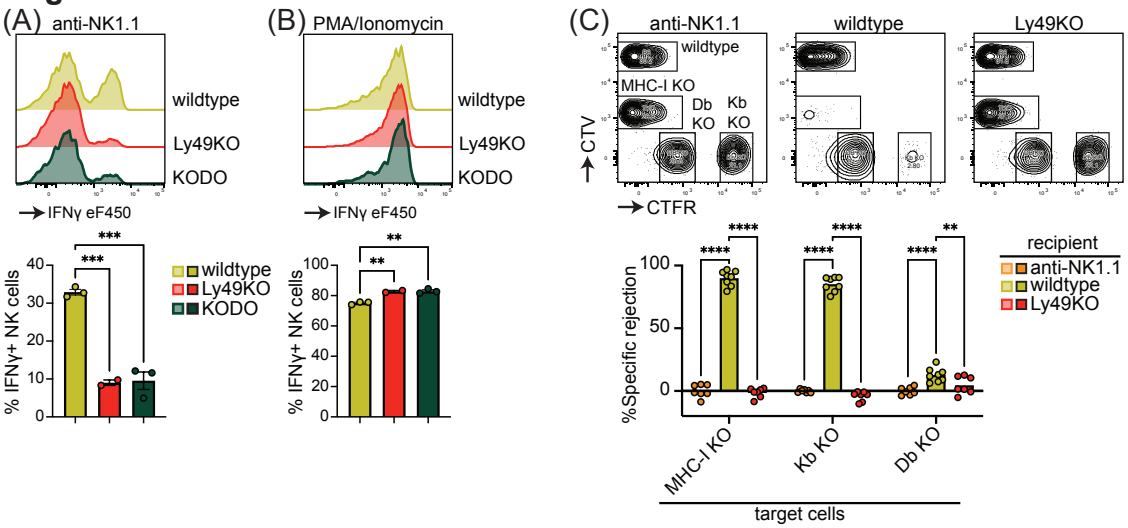

Figure 2 *NK cell licensing and rejection of MHC-I deficient target cells is defective in Ly49KO mice.* Splenocytes from the indicated mice were stimulated with plate bound anti-NK1.1 (**A**) or PMA/ionomycin (**B**) and IFN γ production by NKG2A- NK cells and analyzed by flow cytometry. (**C**) In vivo cytotoxicity assay against H-2Kb, H-2Db and full MHC-I deficient targets. Splenocytes from WT, H-2Kb, H-2Db and MHC-I deficient mice were differentially labeled with CFSE, CTV, and CTFR as indicated. Mixture of labeled target cells were injected i.v. into wildtype, Ly49KO, and anti-NK1.1-depleted mice. Target cells were analyzed in spleens by flow cytometry 2 days after challenge. KODO, H-2Kb x H-2Db knock out; MHC-I KO, KODO x B2m knock-out; MFI, median fluorescent intensity. Error bars indicate SEM; ns, not significant; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Figure 3 - Piersma et al.

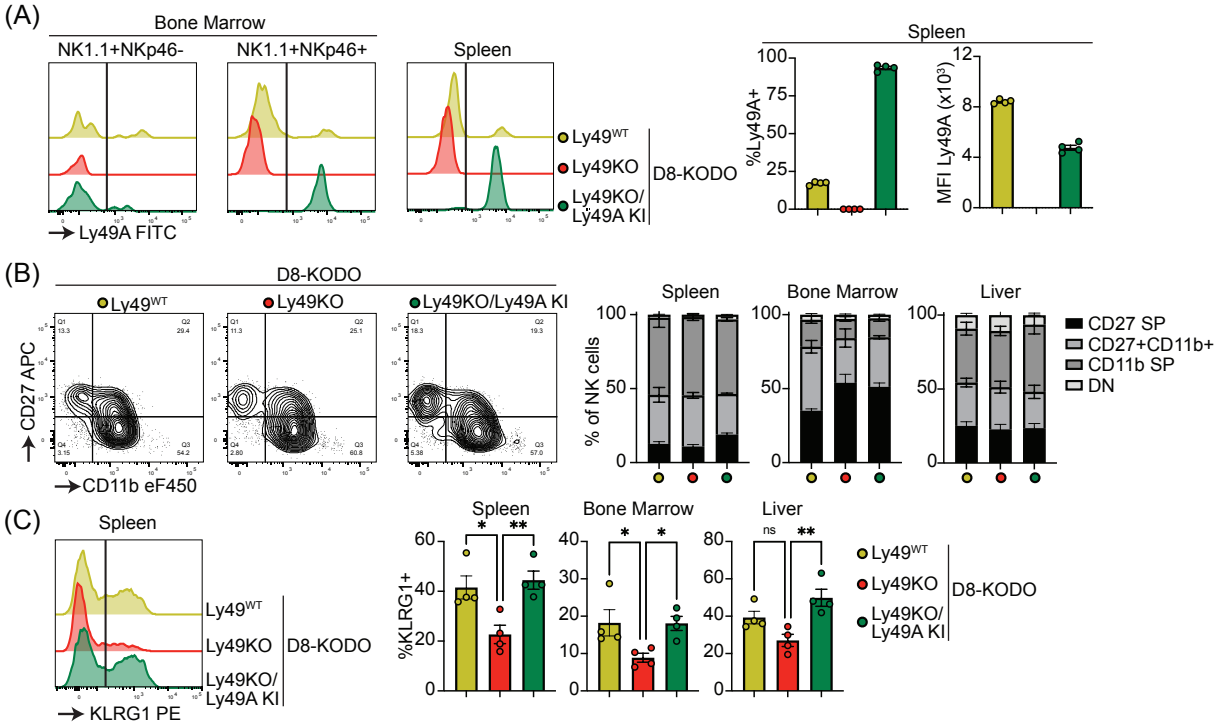

Figure 3 Ly49A is efficiently expressed in Ncr1-Ly49A knockin mice and rescues KLRG1 expression in NK cells. Flow cytometric analysis of NK cells in D8-KODO, Ly49KO D8-KODO, and Ly49KO/Ly49A KI D8-KODO mice (**A**) Ly49A expression in NKp46+ and NKp46- NK1.1+ NK cells in bone marrow and spleen of the indicated mice. (**B**) Expression of the maturation markers CD27 and CD11b on NK cells in spleen, bone marrow, and liver of indicated mice. (**C**) KLRG1 expression by NK cells in spleen, bone marrow, and liver of indicated mice. MFI, median fluorescent intensity. Error bars indicate SEM; ns, not significant; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.000**1.**

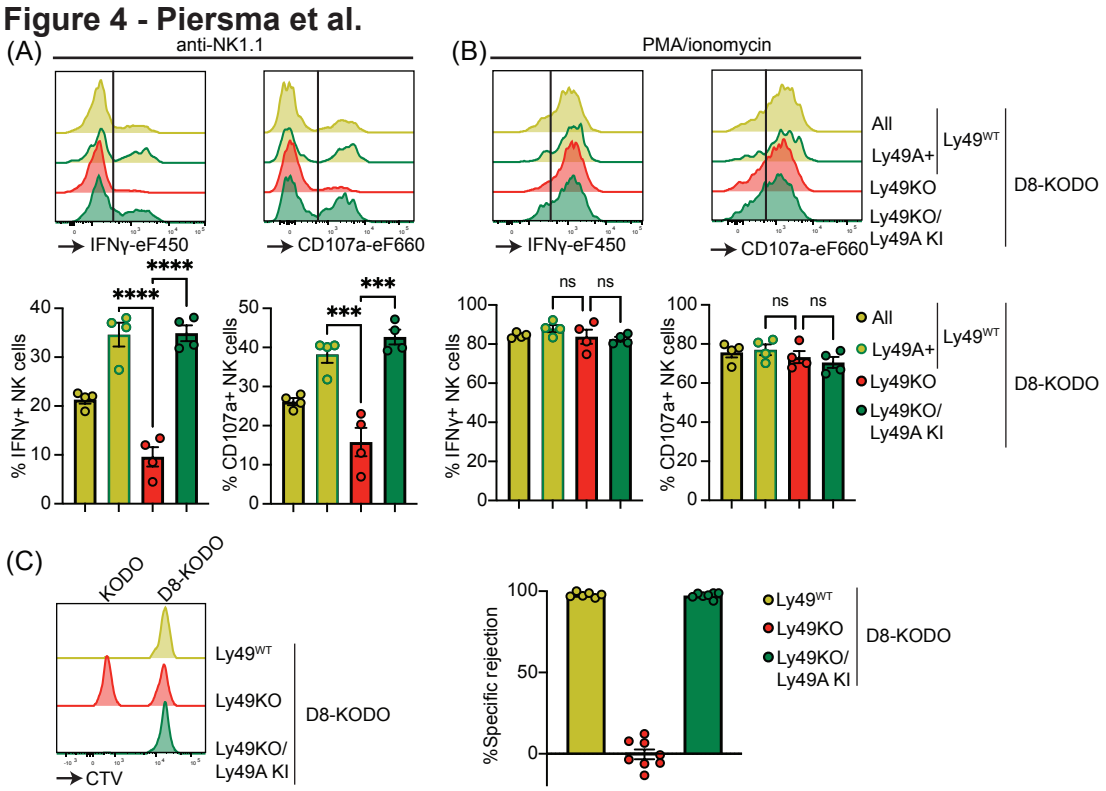

Figure 4 *Expression of Ly49A in isolation is sufficient for NK cell licensing and missing-self rejection.* Splenocytes from the indicated mice were stimulated with plate bound anti-NK1.1 (**A**) or PMA/ionomycin (**B**). IFNγ production and degranulation (CD107a) by NKG2A- NK cells were analyzed by flow cytometry. (**C**) Splenocytes from D8-KODO and KODO mice were differentially labeled with CFSE and CTV as indicated. A mixture of labeled target cells was injected i.v. into D8-KODO, Ly49KO D8-KODO, and Ly49KO/Ly49A KI D8-KODO mice. Specific rejection of target cells was analyzed in spleens by flow cytometry 2 days after challenge. Error bars indicate SEM; ns, not significant; ***$p < 0.001$, and ****$p < 0.0001$.