Published in final edited form as:
Nat Immunol. 2012 December ; 13(12): 1171–1177. doi:10.1038/ni.2468.

Recognition of H2-M3 by Ly49A regulates natural killer cell licensing and activation

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Abstract

Natural killer cell development and function is regulated by the interaction of inhibitory Ly49 receptors with distinct peptide-laden Major Histocompatibility Complex allotypes (pMHC-I), although whether Ly49 could bind to other MHC-I like molecules was unclear. We show the prototypic inhibitory receptor Ly49A binds the highly conserved non-classical class I molecule, pH2-M3 with similar affinity to that with H-2D^{d}. Ly49A specific recognition of H2-M3 regulates NK cell licensing and mediates missing-self recognition of H2-M3 deficient-bone marrow. Host-peptide H2-M3 was required for optimal NK cell activity against experimental metastases and

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Author Contributions

D.M.A conceived, designed and performed the experiments with assistance from L.C.S., N.B., C.J.C, R.B., C.L.C., H.H, J.L, S.V.W, J.P-L. and M.J.S. C-R.W, A.A.S, W.M.Y, J.R, A.G.B and M.J.S provided reagents and critical input to the analysis of results. D.M.A, L.C.S., J.R., A.G.B. and M.J.S wrote the manuscript.

carcinogenesis. Accordingly, non-classical MHC-I molecules can act as cognate ligands for Ly49 molecules and provide insight into the various mechanisms that lead to NK cell tolerance.

Natural Killer (NK) cells contribute to immune responses against cancer[1, 2] and viruses[3]. Unlike adaptive immune lymphocytes such as B and T cells, the receptor repertoire of NK cells is independent of somatic rearrangement. Rather, target cell specificity results from complex expression patterns of a large array of both activating and inhibitory receptors many of which interact with Major Histocompatibility Complex (MHC) Class I molecules. Binding of MHC class I molecules by inhibitory receptors of the Ly49 and killer cell immunoglobulin-like receptor families blocks NK cell activation[4, 5]. The relevance of these interactions was demonstrated in a setting where the absence of MHC class I rendered tumours more susceptible to control by NK cells[6]. This lead to the development of the "missing-self" hypothesis[7], which was subsequently supported by the observation that H-2D^d could protect tumour cells from cytotoxicity by NK cells[4]. A recognition system for the "missing self" hypothesis was elucidated when the inhibitory receptor, Ly49A, was specifically shown to directly recognise MHC class I[4, 8] As well as suggesting a mechanism for preferential targeting by NK cells of tumours with low expression of MHC class I, these data also provided a basis for the mechanism by which NK cells developed tolerance to self.

Principal among the early models of tolerance was the "at least one" hypothesis, which suggested that each NK cell expresses an inhibitory receptor specific for self-MHC[9, 10]. However, many mouse NK inhibitory receptors do not recognise syngeneic MHC class I[11] and populations of NK cells exist that lack inhibitory receptors that recognise self-encoded H-2D and H-2K class I molecules[12]. Recognition of MHC-I is critical for the acquisition of full effector function as NK cells from MHC class I-deficient mice exhibit reduced cytotoxicity and cytokine production[13]. In light of these discoveries, many mechanisms of NK cell tolerance have been proposed (reviewed in[14]), but much recent attention is focused on the role of "licensing". During this process, inhibitory receptors are proposed to recognise a self- MHC class I and "license" the cells to acquire cytotoxic potential and cytokine producing capacity[15].

MHC-I specific receptors from a number of different families regulate NK cell responses[16]. Inhibitory members of the Ly49 family have been shown to recognise specific allotypes of classical MHC class I molecules whereas the highly conserved CD94-NKG2 receptors recognise the non-classical molecules Qa-1^b in mice[17, 18] and HLA-E in humans[19, 20]. However little is known of the capacity of other non-classical class I molecules to regulate NK cell responses. H2-M3 is an MHC class Ib molecule from the same non-classical region as Qa-1^b[21]. While most cells retain H2-M3 in the endoplasmic reticulum it is constitutively expressed on B cells[22]. H2-M3 plays a specialised role in the presentation of N-formylated peptides of bacterial or mitochondrial origin[23, 24] of which the best characterised are the *Listeria monocytogenes* peptide LemA (f-MIGWII)[25] and the mitochondrial natural ligand peptide derived from the N-terminus of the NADH dehydrogenase subunit 1 (f-MFFINIL termed ND1)[26]. Peptide-H2-M3 complexes can be recognised by a specialised population of CD8 T cells that are important for immunity to certain bacterial infections[27]. However, recent studies have also demonstrated an impaired capacity of lymphocytes from H2-M3-deficient mice to kill NK cell sensitive target cells such as YAC-1 cells[28], suggesting that H2-M3 may regulate NK cell responses.

We demonstrate that peptide-H2-M3 is a ligand for the NK cell inhibitory receptor, Ly49A. Given that no definitive ligand of Ly49A has been recognised on the H-2^b background we investigated the role of H2-M3 to Ly49A$^+$ NK cell responses. The absence of H2-M3 results in NK cell hypo-responsiveness, missing-self rejection and increased tumour burden in a Ly49A-dependent fashion. These results demonstrate that the ligands for the Ly49 family of

molecules may exist outside of classical MHC class I and imply a re-examination of the "at least one" hypothesis.

Results

H2-M3 tetramers bind to Ly49A

Given the reduction in NK cell killing in H2-M3 deficient mice[28], we sought to determine the relationship of H2-M3 to other ligands of well characterised NK cell receptors. Sequence alignment of the alpha 1 and alpha 2 domains of H2-M3 (Accession NP_038847.1) showed homology with H-2D^d (Accession AAA39581) and H-2D^b (Accession NP_034510) (Fig. 1a). As H-2D^d and H-2D^b may be ligands for Ly49A[4, 8, 11, 29–33] H2-M3 tetramers loaded with f-MIGWII[25] or ND1[26] were assessed for the capacity to stain transfected cell-lines expressing Ly49A, Ly49D or Ly49G2 molecules[11, 34]. Ly49A expressing cells bound H2-M3 tetramers but not Ly49D or Ly49G2 expressing cells (Fig. 1b). As expected an H-2D^d tetramer loaded with the motif peptide AGPARAAAL-bound cells expressing Ly49A[8] but not those transfected with either Ly49D or Ly49G2. Neither was there binding of a HLA-B8 tetramer loaded with immuno-dominant epitope of EBV[35] to any of the Ly49-expressing cells further demonstrating the specificity of the interaction. Moreover H2-M3 tetramer binding to Ly49A expressing cells could be blocked by pre-treatment with the Ly49A-specific monoclonal antibody YE1/48 (Fig. 1c). These data demonstrate that Ly49A can recognise the non-classical class I molecule H2-M3.

H2-M3 binds to Ly49A with a similar affinity to H-2D^d

To provide further evidence that H2-M3 binds Ly49A we assessed the interactions between recombinant H2-M3 refolded with f-MIGWII (*LemA*) or f-MFFINIL (ND1) peptides, H-2K^b and H-2D^d with Ly49A using surface plasmon resonance (SPR). The data was fitted to a Langmuir 1:1 model, with the Chi^2 values (≤5) and residuals indicating a good data fit (Fig. 2). H2-M3 complexed with either f-MIGWII or f-MFFINIL (Fig. 2a and b) bound to Ly49A with similar affinity (2.75 ± 0.071 μM and 4.06 ± 0.33 μM respectively) to that of H-2D^d. The affinity of AGPARAAAL-loaded H-2D^d for Ly49A (2.05 ± 0.07 μM, Fig. 2c) was similar to previously reported values[36]. In contrast, we observed no interaction between Ly49A and SIINFEKL-loaded H-2K^b (Fig. 2d) as has been previously reported[11, 29], demonstrating specificity in these interactions.

Normal NK and MHC class I in H2-M3-deficient mice

Given the reduced capacity of lymphocytes from H2-M3^{tm1Crw} mice (which lack H2-M3) to kill YAC-1 cells[28] and the reported observation that certain NK cell receptors may regulate NK cell development[37], we next examined whether the absence of H2-M3 impacted on NK cell development. Using monoclonal antibodies specific for CD122 and NK1.1, CD27 and CD11b, and CD43 and CD11b to identify NK cells and their maturation phenotypes[38, 39], we determined that NK cell maturation and total number were unaffected in H2-M3-deficient mice (Fig. 3a,b). All known members of the Ly49 and the NKG2 family were expressed normally, however there was a small but significant (P=0.0177) increase in the frequency of DNAM-1$^+$ NK cells in H2-M3-deficient mice (Fig. 3b) (Table 1).

To confirm that the absence of H2-M3 did not alter the levels of classical MHC class I molecules, we assessed expression of H-2K^b and H-2D^b in the spleen and bone marrow. Expression of these classical H-2 molecules was unaffected by the absence of H2-M3 (Fig. 3c). In line with previous experiments[22], H2-M3 was constitutively expressed on B cells in the spleen and bone marrow (representing approximately 50% of all leukocytes in these organs) (Fig. 3c). Thus, while H2-M3 was required for the lymphocyte-mediated killing of YAC-1 cells, its absence resulted in little or no differences in NK cell development.

H2-M3 is required for NK cell suppression of tumours

Given that peptide-H2-M3 can be recognized by Ly49A, we next defined the role of H2-M3 during melanoma metastasis and fibrosarcoma development, both models where NK cell mediated control is critical. Following intravenous injection of a range of doses of B16F10 tumour cells, H2-M3-deficient mice had significantly increased (* P=0.0491, ** P= 0.0002 at 10^5 and 5×10^4 and P=0.0006 at 1×10^4) numbers of lung metastases when compared to wild type controls (Fig. 4a). Increasing the number of B16F10 cells inoculated decreased the difference in metastatic load between wild type and H2-M3-deficient mice (compare 2×10^5 with 1×10^4) (Fig. 4a). These results demonstrated a dose-dependent defect in the NK cell-mediated resistance to melanoma lung metastases in the absence of H2-M3.

In concert with the experimental B16F10 lung metastases results, we also observed a carcinogen dose-dependent effect of host H2-M3 on the penetrance of MCA-induced fibrosarcoma (Fig. 4b). A significant increase (* P=0.0365) in the proportion of H2-M3-deficient mice developing fibrosarcoma was observed at 25 μg MCA compared with wild type mice while, despite a similar trend, no statistical difference was observed in wild type or H2-M3-deficient mice challenged with higher doses (100 or 400 μg) of MCA (Fig. 4b). The difference in carcinogenesis between wild type and H2-M3-deficient mice was completely abrogated in the absence of NK cells, with depleting anti-asialoGM1 resulting in a comparable but higher tumour penetrance in both strains. In an independent experiment, heterozygous H2-$M3^{+/-}$ mice displayed a similar sensitivity to MCA as H2-$M3^{+/+}$ mice, both groups having a higher proportion of mice that remained tumour free relative to mice that completely lacked H2-M3 (Supplementary Fig. 1). Overall, in two models (one experimental and one *de novo* carcinogenesis), where host NK cell protection from tumour was critical, host H2-M3 was required for optimal NK cell activity.

H2-M3 licenses Ly49A$^+$ NK and regulates tumour control

Given that we had observed decreased NK cell activity in H2-M3-deficient mice, we assessed the possibility that H2-M3-dependent licensing may be required for NK cells to obtain full effector function. In order to confirm this hypothesis, we used a well-established licensing assay designed to detect differences in the acquisition of NK cell interferon-γ (IFN-γ) production[15, 40]. NK cells expressing known inhibitory receptors Ly49C/I, Ly49G2 and NKG2ACE (Ly49F/H were also excluded as a result of antibody cross reactivity) were excluded and the Ly49A positive population (containing Ly49D and NKG2D) was analysed for cytokine production. IFN-γ production in the Ly49A positive NK cell pool was significantly reduced (** P=0.0072 * P=0.0141) in H2-M3-deficient mice (Fig. 5a, 5b). The Ly49C/I$^+$ Ly49G2$^-$/Ly49A$^-$/NKG2ACE$^-$ and Ly49G$^+$ Ly49C/I$^-$/Ly49A$^-$/NKG2ACE$^-$ populations were licensed equivalently between WT and H2-M3-deficient mice, thereby demonstrating the specificity of the Ly49A$^+$ NK-H2-M3 axis (Fig. 5b).

In order to determine whether the reduction in licensing of Ly49A$^+$ NK cells in H2-M3-deficient mice had any pathophysiological consequences, we assessed the ability of wild type and H2-M3-deficient mice to control B16F10 tumours after depletion of Ly49A$^+$ NK cells. Mice were treated with YE1/32 antibody, which specifically depletes the Ly49A$^+$ subset[41], prior to injection with B16F10 tumour cells. Depletion of this subset in WT mice resulted in a significant increase (*** P=0.0004) in the number of lung metastases (Fig. 5c). Consistent with the results from Fig. 4, H2-M3-deficient mice showed a significant increase in the number of lung metastases (*** P=0.0001) that was not altered upon depletion of Ly49A$^+$ NK cells (Fig. 5c). Thus, H2-M3 can act to provide a “licensing” signal to Ly49A$^+$ NK cells, resulting in the generation of effector function.

H2-M3 is a "missing-self" determinant

The "missing self" hypothesis predicts that absence of MHC class I on naive hematopoietic cells should result in their rejection. Indeed, transfer of resting lymphocytes from $\beta 2m^{tm1Unc}$ mice (which lack β2m) results in their rejection in an NK cell-dependent manner[42]. We therefore sought to determine whether H2-M3 could act as a "missing self" determinant by transferring bone marrow cells (BMC) (which are H2-$M3^{+}$ Fig. 3) from wild type and H2-$M3^{-/-}$ mice into wild type recipients. Freshly isolated BMC from WT and H2-$M3^{-/-}$ mice were labelled with CFSE or Cell Trace Violet (CTV) respectively, mixed in a 1:1 ratio and injected into WT mice. BMC cells from H2-$M3^{-/-}$ mice showed significantly increased rejection when compared to wild type mice (* $P<0.0001$ spleen and liver) (Fig. 6). This rejection was Ly49A- and NK-cell dependent as treatment with depleting antibodies that targeted Ly49A or NK1.1 abrogated rejection and resulted in retention similar to that observed in the WT to WT transfer (Fig. 6 3rd and 4th panel). Given that the bone marrow constitutively expresses H2-M3, the observed increase in NK cell-dependent rejection of H2-M3-deficient cells is consistent with models of "missing self"[42].

Discussion

The relevance of Ly49A to NK cell responses has received much attention since its identification over 20 years ago[43–45]. Using cellular, biochemical and functional assays, we have demonstrated that the conserved non-classical class I molecules H2-M3 is a ligand for Ly49A. The existence of a classical MHC class I ligand for Ly49A on the H-2^{b} background remains controversial with some groups showing no interaction with H-$2K^{b}$ or -D^{b}[4, 8, 11, 29, 30] while other groups showed a weak interaction with H-$2D^{b}$[31–33]. Here we have shown that H2-M3, which is highly conserved across mouse strains and is expressed in mice with H-2^{b} haplotypes such as C57BL/6, interacts with Ly49A. Moreover, H2-M3 can both license NK cells and regulate rejection in a Ly49A-dependent manner, results consistent with the current theories of NK cell education[14] and models of "missing self"[42].

Although H2-M3 contributed to licensing of $Ly49A^{+}$ NK cells, these cells are weakly licensed in H-2^{b} mice compared to H-2^{d} mice[46]. Our data has shown that Ly49A has similar affinity for H2-M3 and H-$2D^{d}$, which suggests that other factors in addition to the affinity of the interaction between NK cell receptors and their ligands contribute to NK cell education. It is worth noting that when multiple ligands for a single Ly49 molecule are present the effects on NK cell education are greater than when a single ligand is available[31]. For example, the presence of H-$2D^{b}$ increases licensing of $Ly49C^{+}$ NK cells but this is further increased when both H-$2D^{b}$ and H-$2K^{b}$ are present[31]. Thus, in H-2^{d} mice where H2-M3 and H-$2D^{d}$ are both present engagement of both of these ligands by Ly49A could account for the increased licensing observed on the H-2^{d} background. In line with this hypothesis, transgenic mice expressing H-$2D^{d}$ in the absence of H-$2D^{b}$ or H-$2K^{b}$ (but presence of H2-M3) demonstrate increased levels of licensing of $Ly49A^{+}$ cells compared to H-2^{b} mice[46, 47]. However, it is also possible that the weak licensing of $Ly49A^{+}$ NK cells by H2-M3 results from the low level of constitutive surface expression of H2-M3 relative to classical MHC-I. Two separate studies have addressed the role of quantitative MHC expression on $Ly49A^{+}$ NK cell licensing. The generation of mice expressing H-$2D^{d}$ in a hemizygous ($D^{d+/-}$) and homozygous ($D^{d+/+}$) fashion allowed the analysis of $Ly49A^{+}$ NK cell education in the presence of differing levels of MHC[46, 47]. While the expression level of H-$2D^{d}$ had no effect in one study [46] a quantitative effect on licensing was observed in the other[47]. As H2-M3 expression in naive mice is largely restricted to B cells this would further reduce the capacity of $Ly49A^{+}$ cells to interact with H2-M3 in comparison with H-$2D^{d}$, which is constitutively expressed on almost all cells. Thus the low level of H2-M3-expressed in relatively restricted number of cells may account for the low level of licensing observed in H-2^{b} mice, even though H2-M3 and H-$2D^{d}$ have similar affinities.

The "at least one" model proposed that every mature NK cell expresses an inhibitory receptor specific for a self-MHC class I molecule[9, 10]. Subsequent experiments have demonstrated that subsets of NK cells exist which achieve self-tolerance without expressing self-MHC–I reactive receptors[12]. Furthermore, the "licensing" hypothesis has provided significant mechanistic data regarding NK cell tolerance[14, 48]. These observations have lead to the suggestion that the "at least one" model is not correct. However, similar to what is observed for CD8 T cell education, it is possible that NK cell self-tolerance occurs through multiple interactions. Given that Ly49A$^+$ NK cells are weakly licensed on the H-2^b background but clearly have a self ligand this suggests that "at least one" may be valid for this subset of cells. Our data show that the Ly49 molecules may be capable of recognising non-classical MHC class I, thereby providing a source of ligands for those inhibitory receptors which do not bind classical MHC class-I. Identifying and quantifying these interactions will provide significant benefit to the study of NK cell education.

Materials and Methods

Mice

C57BL/6 wild-type mice were purchased from WEHI. C57BL/6 H2-M3^{tm1Crw} mice[28] were bred at the Peter MacCallum Cancer Centre. All mice were used at the ages of 6–10 weeks. All experiments were performed in accordance with the animal ethics guidelines ascribed by the National Health and Medical Research Council of Australia. All experiments were approved by the Peter MacCallum Cancer Centre Animal Ethics Committee.

Flow cytometry

NK cells—Spleens were harvested from wild type and H2-M3-deficient mice. Two million cells were blocked in the presence of 2.4G2 monoclonal antibody (mAb) prior to staining with mAbs reactive with NK1.1 (PK136 eBioscience, CA USA), CD3 (17A2 Biolegend, CA USA), CD27 (LG.7F9 eBioscience) and Ly49A (YE1/48.10 Biolegend), Ly49C/I/F/H (14B11 eBioscience), Ly49D (4E5 Biolegend), Ly49E/F (CM4 eBioscience), Ly49G2 (eBio4D11 eBioscience), Ly49H (3D10 eBioscience), NKG2ACE (20d5 eBioscience), NKG2D (C7 Biolegend) and DNAM-1 (480.1 Biolegend). Fluorogold was used to exclude dead cells and 5000 NK cells (NK1.1$^+$/CD3$^-$) were electronically collected using an LSR-II (BD Bioscience, CA USA).

Ly49 expressing cells—Cells were plated onto Corning suspension dishes at 2×10^5/ml for 2 days and were removed from the dishes with 50 mM EDTA and washed twice in PBS before blocking in the presence of 2.4G2. Cells were then stained with the Ly49 antibodies (above) or tetramers. Cells were then fixed in 2% paraformaldehyde, washed and 20000 events collected on an LSR-II (BD Biosciences, CA USA).

Cloning and expression of recombinant proteins

RNA from C57BL/6 mice was reverse transcribed and used to amplify a cDNA fragment encoding the entire extracellular domain of Ly49A (Ly49A EC, residues 67-262) using the following primers (5′ to 3′) GGCCGGATCCAAAATTTTTCAGTATG and CCGCTAGCTCAATGAGGGAATTTATC. The construct was then cloned into the pHLsec vector for mammalian expression, that contained secretion tags as well as an N-terminal 6×His for purification and a BirA signal peptide for biotinylation. Ly49A EC was expressed from transiently transfected HEK 293T cells and the secreted protein was harvested from the culture media 3 days after transfection by dialysing against 10mM Tris pH 8.0, 500mM NaCl before purification on nickel affinity resin. The Ly49A EC was eluted from the nickel resin with 10mM Tris pH 8.0, 500mM NaCl, 500mM imidazole. The protein was purified by gel filtration chromatography using an S200 16/60 column (GE Healthcare).

Following reverse transcription of RNA prepared from C57BL/6 splenocytes, cDNA encoding residues 1-274 of H2-M3 was generated by PCR and cloned into a pET-30 based vector that allowed for an inframe fusion of a substrate peptide for the enzyme BirA. The H2-M3 heavy chain and murine β_2-microglobulin were expressed separately in *E. coli*, purified from inclusion bodies and refolded in the presence of either LemA (fMIG, f-MIGWII) or ND1 (f-MFFINIL) peptides and purified essentially as described previously[49]. The cloning and expression of H-2D^d, H-2D^b and H-2K^b has been described elsewhere[11].

Surface Plasmon Resonance

A ProteOn XPR36 protein-interaction array system (Bio-Rad) was used for surface plasmon resonance as described[50]. Streptavidin was coupled to a GLC Sensor Chip (Bio-Rad) by amine coupling (~500 response units, RU), and biotinylated Ly49A EC produced from 293T cells was captured on the surface (~80 RU). An empty flow cell containing streptavidin alone served as a control surface. Monomeric H2-M3 f-MIGWII, H2-M3 f-MFFINIL, H-2Dd AGPARAAAL or H-2Kb SIINFEKL were serially diluted (16-1 μM) and simultaneously injected over test and control surfaces at a rate of 30 μl/min for 240 sec. After subtraction of data from the control flow cell, kinetic interactions were analyzed with ProteOn Manager software version 3.0.1. All interactions were tested minimally in duplicate.

In vivo tumor assays

B16F10 model—Single cell suspensions of B16F10 were prepared in PBS and 10^4 to 2×10^5 cells were injected i.v. in the tail vein. Lungs were harvested on day 14 and fixed in Bouin's solution, and tumour nodules were counted with the aid of a dissection microscope. For blockade or depletion studies mice were treated with control Ig, or anti-asialoGM1, anti-CD8β (53.5.8), anti-NK1.1 (PK136) at 100 μg i.p. on day −1, 0 and 7 or anti-Ly49A (YE1/32) at 300 μg on days −2 and 5.

MCA model—Groups of 10–25 male WT, H2-M3$^{-/-}$ mice (or groups of 14–25 male WT, H2-M3$^{+/-}$ and H2-M3$^{-/-}$ in F1 studies) were inoculated s.c. in the hind flank with 25, 100, or 400 μg of 3-methylcholanthrene (MCA; Sigma–Aldrich) in 0.1 mL of corn oil. Some mice received control Ig or weekly depletion of NK cells (anti-asialoGM1) from the time of MCA inoculation to day 42. Development of fibrosarcomas was monitored weekly over the course of 300 days. Tumours >3 mm in diameter and demonstrating progressive growth were recorded as positive. Measurements were made with a caliper square as the product of two perpendicular diameters (cm^2).

Licensing Assays

Licensing assays were performed as described[40, 46].

"Missing Self" Rejection

"Missing Self" assays were performed as described[42] with minor modifications. For depletion studies mice were treated with control Ig or anti-NK1.1 (PK136) at 100 μg i.p. on day −1 and 0 or anti-Ly49A (YE1/32) at 300 μg on days −2 and 0.

Statistical analysis

Statistical significance between data sets not involving survival was performed using the non-parametric, two-tailed Mann–Whitney U-test. $P<0.05$ was considered significant. Survival data sets were analyzed using a Log-rank (Mantel–Cox) Test. $P<0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Health & Medical Research Council of Australia (NHMRC), Cancer Australia and the Cure Cancer Australia Foundation. DMA was supported by an NH&MRC Peter Doherty and Career Development Fellowship. LCS was supported by an NH&MRC Career Development Fellowship. RB was supported by an NH&MRC Peter Doherty Fellowship. CR-W was supported by a grant from the NIH (NIH AI040310). WYM is an investigator of the Howard Hughes Medical Institute. MJS and JR were supported by NH&MRC Australia Fellowships.

We thank Christophe Paget for critical analysis of the manuscript and Edwin Hawkins, Qerime Mundrea, Ben Venville, Nicole McLaughlin and Janelle Sharkey for their excellent technical assistance. Bill Murphy is gratefully acknowledged for providing the anti-Ly49A YE1/32 antibody.

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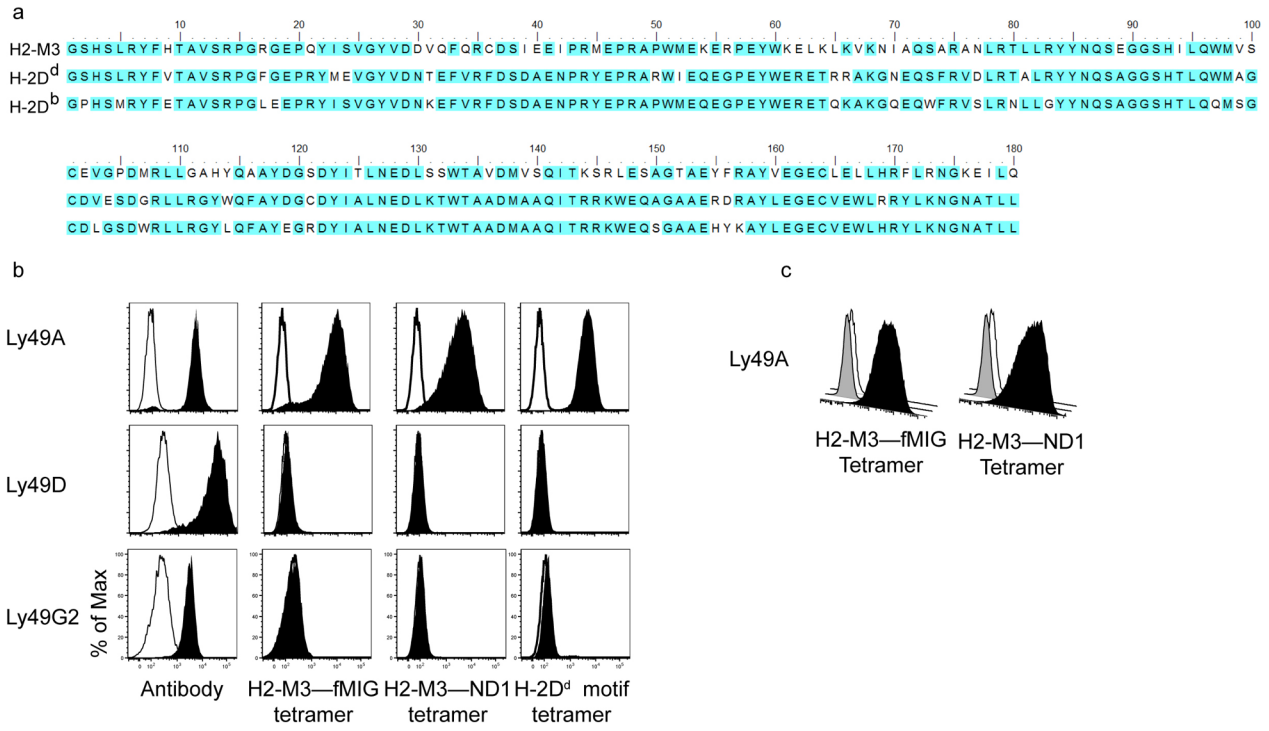

Figure 1. H2-M3 is recognised by C57BL/6 Ly49A

(**a**) Sequence alignment of H2-M3, H-2-D^{d} and H-2D^{b} demonstrates homology between the two ligands at the protein level. (**b**) Tetramer staining of cells expressing Ly49A, Ly49D and Ly49G2 molecules demonstrates that H2-M3 tetramers bind to Ly49A. Black solid histograms are staining with Ly49 specific antibody (left panel), fMIG loaded H2-M3 tetramer (middle panels) or ND1 loaded H2-M3 tetramer (right panel). Open histograms are isotype control antibody (left panel) or human HLA-B8 tetramer (middle and right panel). Results are representative of at least 4 independent experiments. (**c**). Antibody against Ly49A prevents binding of H2-M3 tetramers. CHO cells expressing Ly49A were pre-treated with isotype control antibodies or YE1/48 (Ly49A). Cells were then stained with H2-M3 tetramers loaded with fMIGWII or fMFFINIL. Black solid histograms are staining with H2-M3 tetramers on cells pre-treated with isotype control, grey histograms are cells pre-treated with Ly49 specific antibodies and open histograms are untreated cells stained with irrelevant human HLA-B8 tetramer. Histograms have been offset and angled by a factor of 5 to allow visualisation of all three populations. Results are representative of at least 3 independent experiments.

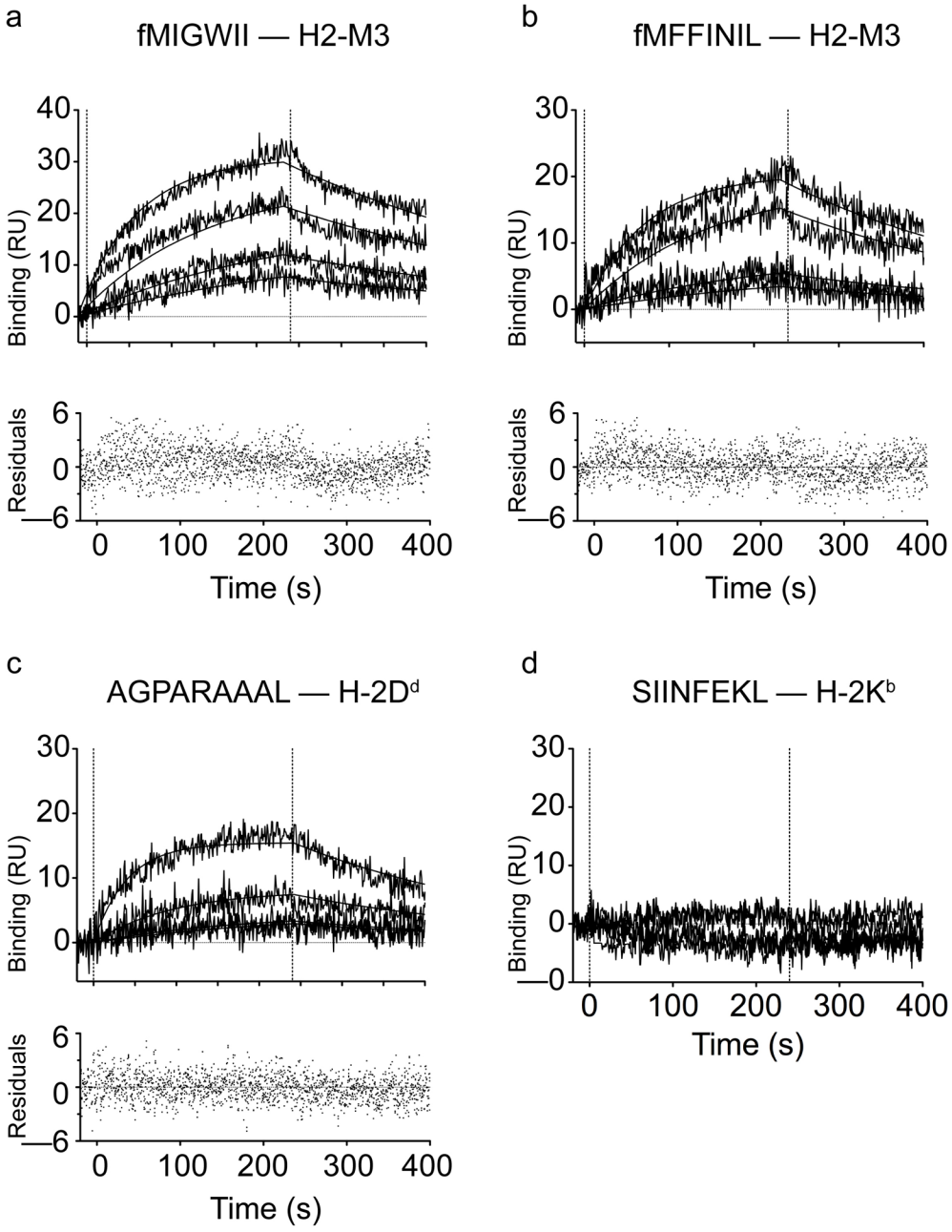

Figure 2. Ly49A binds H2-M3 with similar affinity to H-2D^d

H2-M3 refolded with f-MIGWII (**a**) or ND1 (**b**) peptides was serially diluted and injected over Ly49A immobilized by streptavidin on a BioRad ProteOn GLC chip. Serial dilutions of H-2D^d (**c**) and H-2K^b (**d**) served as positive and negative controls respectively. Sensorgrams show the binding (solid lines, response units, RU) of decreasing concentrations (16, 6.4, 2.6, 1 μM) of MHC following baseline subtraction. Binding affinities (K_D) were determined by kinetic analysis (lines show kinetic fits) using the ProteOn Manager 3.0.1 software. Dotted lines at 0 and 240 sec indicate injection start and injection stop respectively. Binding values are as follows: fMIGWII – H2-M3 $K_a = 9.5 \times 10^2\ M^{-1}\ s^{-1}$, $K_D = 2.75\ \mu M$, $k_d = 2.62 \times 10^{-3}\ s^{-1}$ and $Chi^2 = 3.1$; fMFFINIL – H2-M3 $K_a = 8.28 \times 10^2\ M^{-1}\ s^{-1}$, $K_D = 4.06\ \mu M$, $k_d = 3.36$

$\times 10^{-3}$ s^{-1} and Chi^2 = 2.83; AGPARAAAL – H-2D^d $K_a = 1.65 \times 10^3$ M^{-1} s^{-1}, K_D = 2.05 μM, $k_d = 3.38 \times 10^{-3}$ s^{-1} and Chi^2 = 2.14.

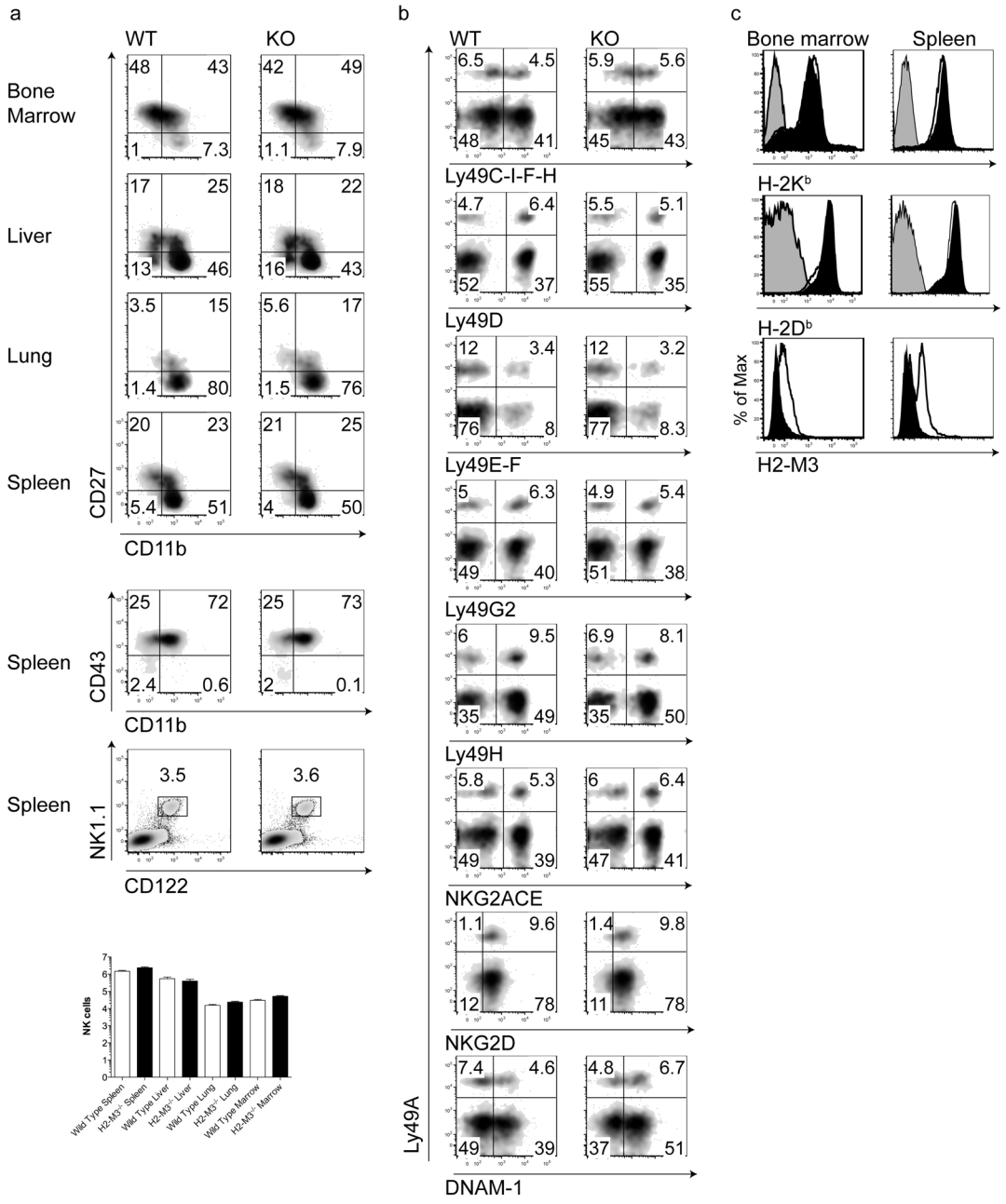

Figure 3. H2-M3 is not required for NK cell homeostasis

Analysis of NK cells from wild type and H2-M3-deficient mice demonstrated no difference in NK cell development and homeostasis. (**a**) Maturation of NK ($NK1.1^+CD3^-$) cells (assessed by CD27 and CD11b, CD43 and CD11b and $NK1.1^+$ and $CD122^+$ and $CD3^-$ cells) in the spleen, liver, lung and bone marrow of wild type and H2-M3-deficient mice is presented. The density plots are representative of two independent experiments using 4 mice per time point (N=8). Frequencies of the populations are presented in the quadrants. Absolute numbers of NK cells in each organ were pooled from the two independent experiments (N=8) and are presented as mean ± SEM (lower panel). (**b**) The expression of Ly49, NKG2 and DNAM family receptors on splenic NK cells are presented. The density

plots are representative of 3 independent experiments using 4 mice per time point (N=12). Frequencies of the populations are presented in the quadrants. (**c**) The expression of H-2K^b, H-2D^b and H2-M3 on bone marrow and spleen cells. Histograms are representative of 2 independent experiments using 4 mice per time point (N=8). The filled histogram is staining on H2-M3-deficient mice while the open histogram is wild type mice. Shaded histograms are isotype controls for H-2D^b and H-2K^b.

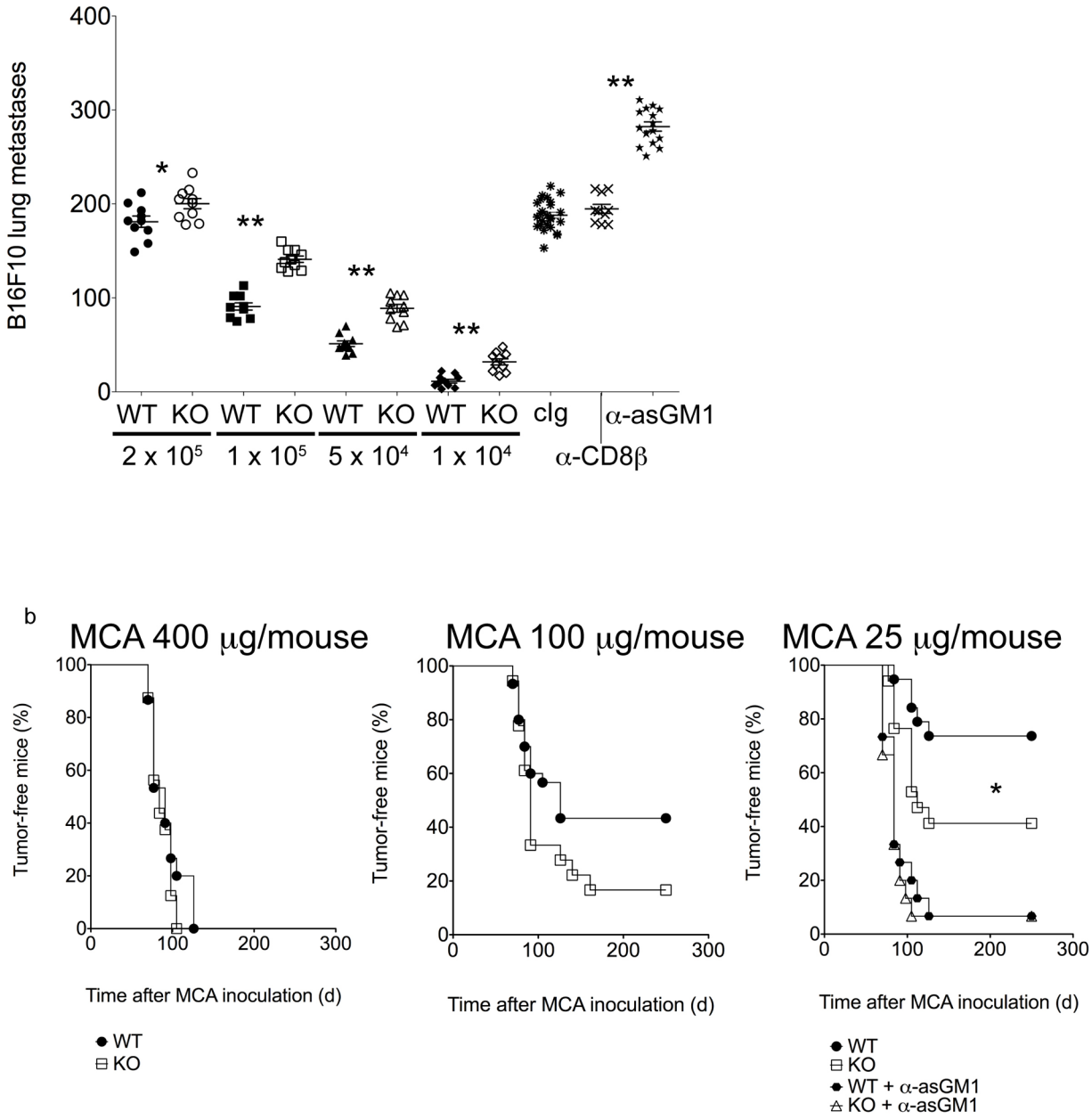

Figure 4. H2-M3 is required for NK cell control of B16F10 metastases and MCA-induced fibrosarcoma

Two independent models of NK cell-dependent control of tumours were used to demonstrate the role of H2-M3 in NK cell licensing. (**a**) WT and H2-M3-deficient mice were challenged intravenously with different doses of B16F10 as indicated and tumour metastases counted 14 days post injection. NK cells and CD8 T cells were depleted using anti-asialoGM1 or anti-CD8β, respectively. Results are pooled from two independent experiments using 5 mice per time point (N=10) and show the mean ± SEM. (* P=0.0491, ** P= 0.0002 at 10^5 and 5×10^4 and P=0.0006 at 1×10^4) (**b**). WT and H2-M3-deficient mice were challenged with MCA and the development of fibrosarcoma assessed for 300 days (left panel). The numbers above the panels define the amount of MCA administered in micrograms/mouse. The panels show the percentage of tumour free mice. Some groups of mice were depleted of NK cells

using anti-asialoGM1 on day −1,0 and weekly until day 42 after MCA inoculation. (* P=0.0365)

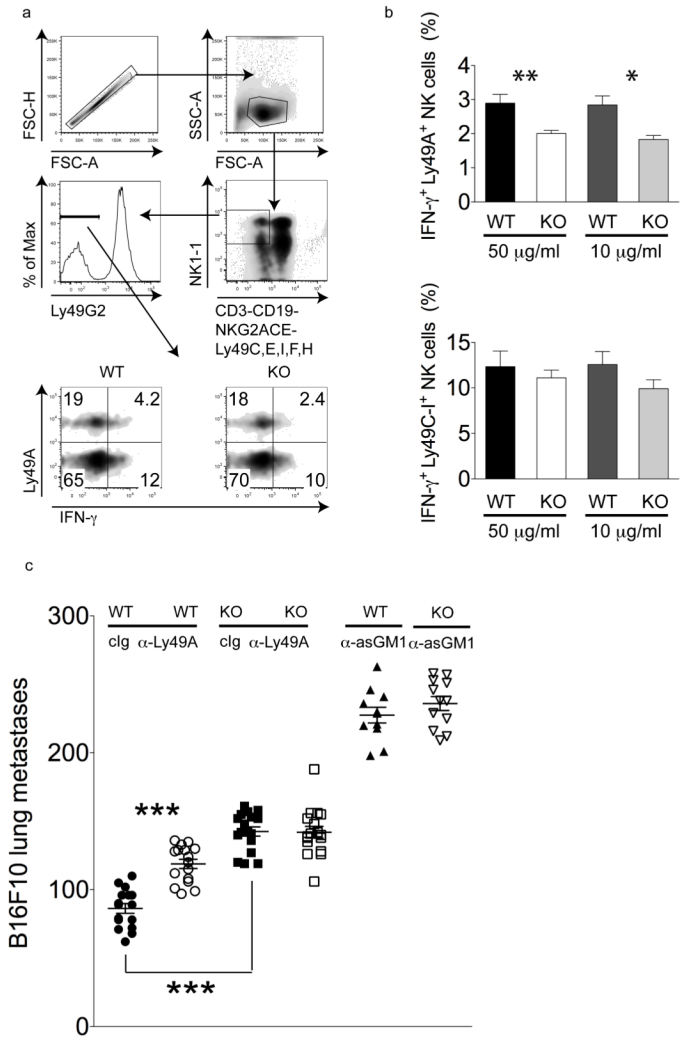

Figure 5. Ly49A$^+$ NK cells from H2-M3-deficient mice are not licensed

(**a**) Representative gating scheme for analysis of intracellular IFN-γ production by Ly49A$^+$ NK cells (Ly49A$^+$ NK1.1$^+$ CD3$^-$ CD19$^-$ NKG2ACE$^-$ Ly49C$^-$ Ly49F$^-$ Ly49I$^-$). In the top left panel singlet cells are gated and then lymphocytes are segregated on the basis of morphology (top right panel). NK1.1$^+$CD3-CD19-NKG2ACE-Ly49CEIFH negative cells are then gated (middle right panel) and Ly49G2 on these cells is determined (middle left panel). On these cells (which are inhibitory receptor negative except Ly49A) the production of IFN-γ is then determined in the Ly49A$^+$ and Ly49A$^-$ (includes Ly49D and NKG2D) as shown in the bottom panels. The numbers in the density plot represents the percentage of cells. The density plots are representative of 4 independent experiments using 3 mice per time point (N=12). (**b**) The frequencies of IFN-γ production by the Ly49A$^+$ and Ly49C/I$^+$ populations from the 4 experiments were pooled and show the mean ± SEM (N=12). (**c**) B16F10 cells were i.v. injected into C57BL6 mice (1×10^5 cells/mouse). Lungs were harvested at 14 days post injection. Results for YE1/32 and 2A3 are pooled from 3 independent experiments using 5–6 mice per experiment (N=16–17) while anti-asialoGM1 is from two experiments using 5–6 mice per experiment (N=11–12). Results are presented as mean ± SEM. *** P<0.0001 comparing WT control Ig to WT YE1/32 and *** P<0.0001 comparing WT control Ig to H2-M3$^{-/-}$ control Ig.

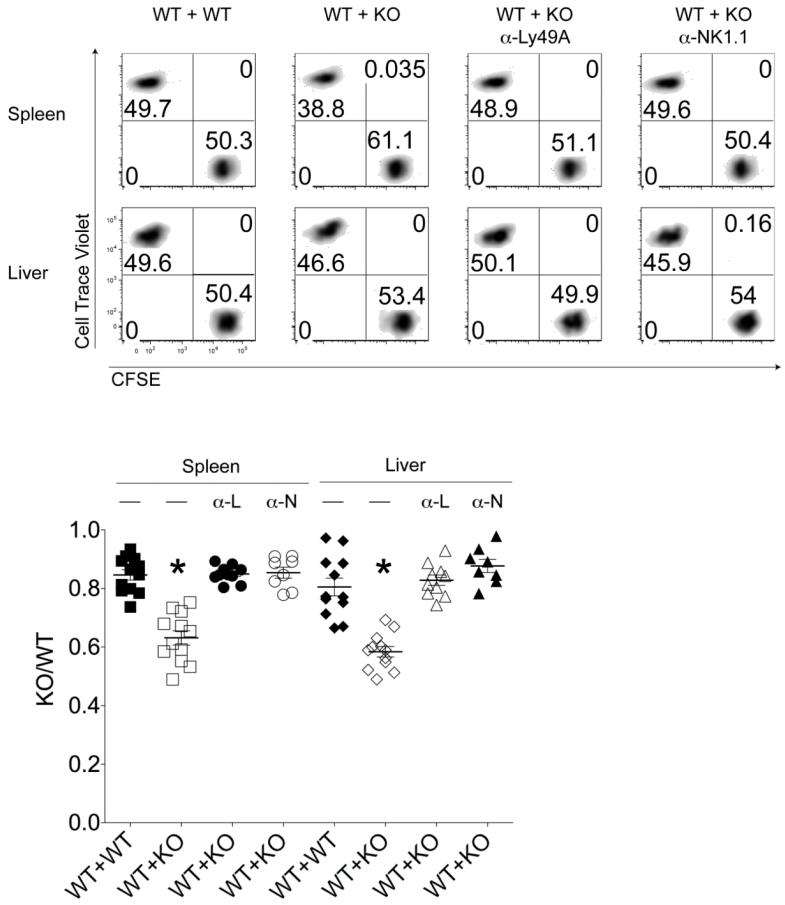

Figure 6. Rejection of H2-M3$^{-/-}$ bone marrow demonstrates "missing self" recognition

Bone marrow from wild type or H2-M3-deficient mice was fluorescently labelled and injected into wild type recipients for analysis of rejection by NK cells. Rejection of H2-M3-deficient bone marrow occurs in wild type mice in an NK cell-dependent manner. Bone marrow from wild type (CFSE labelled) and H2-M3-deficient (Cell Trace Violet) mice were labelled and mixed prior to injection into wild type mice. After 24 h leukocytes were harvested from spleen and liver and analysed by flow cytometry. The density plots are electronically gated to exclude host cells and are representative of 2–3 independent experiments using 3–5 mice per experiment (N=8 to 12). The numbers in the quadrants represent the frequency of cells within the region. The bottom panel is pooled data from the 2 independent experiments and shows the mean ± SEM (N=12). * P<0.0001 (spleen and liver). α-L is the Ly49A depleting antibody YE1/32 and α-N is the NK cell depleting antibody PK136.

Table 1

Frequency of major activating and inhibitory NK cell receptors in WT and H2-M3-deficient mice.

Receptor Frequency	Ly49A	Ly49C/I/F/H	Ly49D	Ly49E/F	Ly49G2	Ly49H	NKG2ACE	NKG2D	DNAM-1
WT	10.7±0.2	43.4±1.2	45.4±0.9	10.6±0.4	45.2±0.6	56.1±2.6	46.9±1.0	87.1±0.3	51±2.2
H2-M3^{tm1Crw}	11.8±0.3	41.8±1.4	43.5±1.3	12.8±0.5	46.6±0.5	57.9±10.5	48.5±0.7	86.8±0.1	58±0.6

Frequencies were calculated using results from 2 independent experiments using 4 mice per experiment (N=8). Frequencies are presented as Mean±SEM.