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Impaired NK cell education diminishes resistance to murine CMV infection

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Abstract

Ly49G2 (G2+) NK cells mediate murine (M)CMV resistance in MHC D^k-expressing mice. Bone marrow transplantation (BMT) studies revealed that G2+ NK cell-mediated MCMV resistance requires D^k in both hematopoietic and nonhematopoietic cells. As a Ly49G2 ligand, D^k in both cell lineages may contribute to lysis of virus-infected cells. Alternatively, cellular differences in self-MHC D^k may have affected NK-cell education, and consequently NK cell-mediated viral clearance. We investigated the D^k-licensing effect on BM-derived NK cells in BMT recipients by analyzing cytokines, cytotoxicity and MCMV resistance. In BMT recipients with lineage-restricted D^k, G2+ NK-cell reactivity and cytotoxicity was diminished in comparison to BMT recipients with self-MHC in all cells. Reduced G2+ NK-mediated MCMV resistance in BMT recipients with lineage-restricted self-MHC indicates that licensing of G2+ NK cells is related to NK cell reactivity and viral control. Titrating donor BM with self-MHC-bearing hematopoietic cells, as well as adoptive transfer of mature G2+ NK cells into BMT recipients with self-MHC in non-hematopoietic cells only, enhanced NK cell licensing and rescued MCMV resistance. This disparate self-MHC NK cell education model would suggest that inadequately licensed NK cells corresponded to inefficient viral sensing and clearance.

Keywords

allogeneic transplantation; Ly49G2; Ly49I/U; H-2^k; MHC; NK cell receptor; NKp46; immunogenetics

Introduction

NK cells mediate vital protection against viruses, especially herpesviruses, which involves direct lysis of viral targets and production of pro-inflammatory cytokines [1, 2]. NK cell function is regulated by many different cell surface NK receptors (NKRs) that are structurally related to either the immunoglobulin superfamily (e.g. KIR, ILT-2 and NKp46)

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or the C-type lectin superfamily (NKG2D, NKG2A and Ly49). Their stochastic expression results in diverse combinations of NKRs expressed in NK cells and considerable NK cell heterogeneity.

Inhibitory KIR and Ly49 NKRs bind self-MHC class I molecules, which hinders NK cell effector function and prevents lysis of autologous target cells. Paradoxically, NK cells that express inhibitory NKRs for self-MHC acquire increased functionality in an educational process referred to as NK cell licensing [3–5]. Licensed NK cells are generally more responsive to *ex vivo* stimulation through activation receptors than are unlicensed NK cells [3–7]. Hence, inhibitory NKRs apparently ratchet NK reactivity upwards against target cells without self-MHC class I expression, while at the same time helping to maintain self-tolerance [8, 9]. However, less is known about how NK cells become licensed or the cellular self-MHC class I requirements.

Prior studies have shown that inhibitory receptor G2+ NK cells licensed by self-MHC D^k mediate MCMV resistance [10, 11]. Interestingly, after MHC class I allogeneic BM transplantation (BMT), G2+ NK cell-mediated MCMV resistance was much less effective in BMT chimeric mice with expression of self-MHC D^k restricted to either the hematopoietic or the non-hematopoietic cell lineage [10]. However, the underlying mechanism has not been investigated.

We sought to elucidate how cell lineage-restricted expression of the self-ligand D^k affects NK cells, their ability to license normally, and to investigate a possible link between NK-cell reactivity and NK cell-mediated viral control. Analysis of intrinsic NK features, *ex vivo* responsiveness and capacity to mediate missing-self lysis of cellular targets *in vivo* established the critical importance of faithful self-ligand expression in hematopoietic and nonhematopoietic cells. In every case, we observed that NK education corresponded with NK mediated MCMV resistance. Discordant cellular expression of MHC I, especially among hematopoietic cells, resulted in impaired NK-cell reactivity and further corresponded to poor NK cell-mediated MCMV resistance. Nonetheless, adoptive transfer of mature NK cells into BMT recipients with NK cell deficiency was adequate to reverse the ability of NK cells to respond to *ex vivo* stimulation and also rescued their capacity to mediate MCMV resistance. The results of these experiments indicate that BM-derived reconstituting and adoptively transferred mature NK cells have distinctive licensing requirements dependent on the expression characteristics of MHC class I molecules, which relates to their capacity to mediate viral control. We infer that NK cells acquire the capacity to mediate viral control in a way that is sensitive to subtle quantitative and/or cellular variations in MHC class I expression.

Results

Lineage-restricted self-MHC D^k expression specifically affects G2+ NK cells

Previously it was shown that G2+ NK cell-mediated MCMV resistance requires self-MHC D^k expression in hematopoietic and non-hematopoietic cells [10]. Because D^k licenses G2+ NK cells [10, 12], these data suggested that both cell lineages might be required in normal NK cell licensing. We therefore examined the effect of lineage-restricted D^k on G2+ NK

cells and effector cell reactivity in BMT chimeric mice established using C57L (H-2^b) mice expressing a genomic D^k transgene (Tg-D^k) or not (non-Tg).

As expected, peripheral blood leukocytes in BMT recipients reconstituted with Tg-D^k BM (i.e. T-T and T-N) displayed cell surface D^k at levels comparable to Tg-D^k control mice (Fig. 1A), similar to previous results [10]. In contrast, D^k was not detected on peripheral blood cells from BMT recipients reconstituted with non-Tg BM (i.e. N-T and N-N) (Fig. 1A). Because inhibitory Ly49 NKR interaction with self-MHC results in reduced receptor median fluorescence intensity (MFI) display, an indication of NK cell licensing [13, 14], we first examined the effect of lineage-restricted D^k on Ly49+ NK cells.

In BMT recipients without hematopoietic D^k expression (N-T and N-N), larger percentages of G2+ NK cells with higher G2 MFI were detected by comparison to NK cells in BMT recipients with hematopoietic D^k expression (T-T and T-N) (Fig. 1B and C). This effect is not likely explained by a difference in total NK cells since NKp46+ cell numbers were equivalent in control and chimeric host mice (Fig. 1B and C). A larger fraction of G2+ NK cells with higher G2 MFI was also detected in the non-Tg BMT recipients of hematopoietic cell-restricted D^k (T-N) expression compared to control T-T mice. Thus, for NK cells reconstituting after BM transplantation, self-MHC D^k in both hematopoietic and nonhematopoietic cells affected both the percentages of G2+ NK cells and Ly49 receptor display.

To test whether the D^k effect on NK cells is G2-specific, we examined additional NK subsets marked by defined NKR expression. Whereas little, if any, effect on the percentages of NKG2A/C/E+ and Ly49I/U+ NK cells was observed, NKG2D+ NK cells were slightly more sensitive to the BMT setting (Fig. 1B and C). Nonetheless, compartmentalized D^k held little correspondence with NKG2D or with NK cell maturation as assessed by CD27 and CD11b expression (Fig. 1B and C). Thus, lineage-restricted D^k most specifically affected G2+ NK cells.

Self-MHC D^k in hematopoietic and nonhematopoietic cells licenses G2+ NK cells

We next assessed the effect of lineage-restricted D^k on reconstituted G2+ NK effector reactivity in blood and spleen. BMT NK cells were stimulated *ex vivo* with immobilized mAb against NKR-P1 and then analyzed by flow cytometry for intracellular IFN-γ or the degranulation marker CD107a among defined NK cell subsets. Receptor-stimulated G2+ NK reactivity was significantly diminished in BMT recipients with either lineage-restricted (T-N and N-T) or no (N-N) self-MHC D^k in comparison to T-T control mice (Fig. 2A and B). No other differences were observed. Similar results were obtained after *ex vivo* stimulation of splenic NK cells via NK1.1, NKG2D or CD16 cross-linking (Fig. 2C). We further examined the self-MHC D^k effect in BMT recipients established using congenic MA/My strains, M.$H2^b$ and M.$H2^b$-Tg1D^k. Licensing assays with NK cells from MA/My-derived chimeric recipients recapitulated what was seen on the C57L background (Supporting Information Fig. 1). A failure to adequately license G2+ NK cells in recipients with lineage-restricted D^k, therefore, was not due to background genetic effects. Thus, G2+ NK cells that developed posttransplantation were sensitive to differences in D^k expression among hematopoietic and nonhematopoietic cells.

To address whether the effect of D^k on G2+ NK cells is specific, we examined Ly49I+ NK cells using mAb 1F8 that binds $Ly49I^{129}$ [12, 15]. The high relatedness of NKC-Ly49 haplotypes in 129/J and C57L [16] suggested 1F8 mAb might also bind Ly49I+ NK cells in C57L. Indeed, we observed a distinct subset of I/U+ (14B11+) NK cells in Tg-D^k and non-Tg mice were stained with the 1F8 mAb (Supporting Information Fig. 2A). However, we observed similar percentages of 1F8+ NK cells and MFI in both strains (Supporting Information Fig. 2B). In addition, although 1F8+ NK cells produced more IFN-γ than IF8− NK cells (Supporting Information Fig. 2C), the effect was evident in both strains. Thus, Ly49I+ NK cells appear to license in the C57L background irrespective of D^k expression. Altogether the data demonstrate that self-MHC D^k expressed in both hematopoietic and nonhematopoietic cells is required to specifically license G2+ NK cells.

Self-MHC licensed G2+ NK cells mediate missing-self recognition of cellular targets and MCMV resistance

The missing-self hypothesis posits that the absence of MHC I on resting hematopoietic cells increases their susceptibility to NK cell-mediated killing [17]. Moreover, NK cell reactivity is tuned by different levels of expression of Ly49 inhibitory receptors [18–21]. Hence, we expected poorly licensed G2+ NK cells to display diminished cell-mediated killing in *in vivo* assays of G2+ NK cellular cytotoxicity. As predicted, control T-T BMT recipients specifically eliminated non-Tg cell targets, whereas BMT recipients with restricted or no self-MHC D^k had a significantly higher ratio of non-Tg / Tg-D^k residual targets (Fig. 3A and B). The data indicate that NK cells were unable to effectively eliminate cellular targets when self-MHC D^k was compartmentally restricted or altogether missing.

To verify their role in cytotoxicity, distinct NK populations were depleted before transferring labeled target cells into T-T BMT mice. Higher ratios of non-Tg/Tg-D^k targets confirmed that pretreatment with total (PK136) and G2-specific (4D11 and AT8) NK cell depleting mAbs abrogated the specific loss of non-Tg cellular targets in liver and spleen, and that missing-self recognition contributed to *in vivo* elimination of cellular targets mediated by G2+ NK cells. In contrast, mAb 1F8 had no effect on missing-self rejection of non-Tg targets in spleen (Fig 3C). A slightly increased rejection of non-Tg targets in 1F8-treated liver was significant for unknown reasons. Taken together, *in vivo* missing-self recognition and *ex vivo* licensing assays show that self-MHC expressed in hematopoietic and nonhematopoietic cells elicits functionally reactive NK cells.

The importance of self-MHC expression in both cell lineages for NK cell education and effector function further suggests that it may be required to mediate viral clearance. To explore the relationship in depth, we first compared the degree of MCMV resistance in BMT recipients differing in self-MHC D^k expression. As expected, MCMV levels were higher in BMT recipients with lineage-restricted self-MHC D^k compared to T-T control mice (Fig. 4A). However, these mice had lower viral loads than observed in control N-N mice, which suggests that T-N and N-T NK cells mediated partial MCMV resistance, particularly in the spleen (Fig. 4A). Moreover, MCMV levels and licensing ratios in spleen ($r=-0.5829$, $p=0.0003$) and liver ($r=-0.4790$, $p=0.0042$) negatively correlated (Fig. 4B). Thus, normal licensing of G2+ NK cells requires self-MHC D^k in hematopoietic and non-hematopoietic

cells, which corresponded to missing-self detection and viral clearance after MCMV infection.

Licensed NK cell reactivity tuned to self-ligand expression in hematopoietic cells needed to mediate viral control

Prior studies showed that the responsiveness of developing NK cells is determined by the MHC environment in which they mature. In particular, it was shown that developing NK cells are sensitive to variable MHC I expression among hematopoietic cells, which results in diminished NK cell reactivity and poor lysis of MHC I deficient target cells [22–24]. In line with this reasoning, we wanted to know how reduced NK reactivity due to variable MHC I expression among hematopoietic cells might affect NK-mediated lysis of viral targets. To address the question, we generated mixed-BM chimeras by reconstituting Tg-D^k and Non-Tg recipients with different (1:1 or 9:1) mixtures of BM cells from Tg-D^k and Non-Tg mice, respectively. We then analyzed NK-cell reactivity, receptor expression and MCMV resistance in the mixed BMT recipients. In agreement with previous results, we observed that reconstituting G2+ NK cells were less responsive in the mixed BM (M-T) environment (Fig. 5A), even though self-MHC D^k was expressed in hematopoietic cells and the percentage of G2+ NK cells and G2 MFI were equivalent by comparison to control T-T mice (Fig. 5B–D). The data suggest that G2+ NK cells were weakly licensed in the M-T BMT recipients, analogous to H-2^q-licensed Ly49A+ NK cells with marked *cis* engagement of Ly49A, but relatively lower *ex vivo* responsiveness [25]. Still, M-T recipients controlled MCMV almost as effectively as control T-T mice, whereas viral loads were much higher in N-T, N-N and M-N BMT recipients at d6 post infection (Fig. 5E). Similar results were obtained after 3.5 days of MCMV infection (Supporting Information Fig. 3). Further titration of the hematopoietic compartment in mixed BMT recipients which yielded higher percentages of cells with self-MHC D^k resulted in increased NK licensing and lower viral loads overall, regardless of D^k's presence or absence in nonhematopoietic cells (Fig. 5A and E). Collectively the results demonstrate that G2+ NK cells were most responsive to stimulation when the self-ligand was expressed equivalently in both cell compartments and its increased expression in hematopoietic cells led to a concomitant increase in NK reactivity and the capacity to mediate MCMV resistance.

Adoptive transfer of mature NK cells rescues G2+ NK cell impairment in N-T BMT mice

Although NK cells acquire functional reactivity with expression of inhibitory receptors for self-MHC ligands, recent studies have shown that mature NK cells display plasticity in licensing and can be re-educated after adoptive transfer into a different MHC I environment [22, 26]. As we observed that mature BM-derived G2+ NK cells in N-T BMT mice displayed impaired effector cell reactivity and viral clearance (Figures 2–4), it was therefore suggested that reconstituting BM-derived and transferred mature NK cells might have distinct licensing requirements which could be further related to their ability to mediate lysis of viral targets. To address the question, we adoptively transferred CTV-labeled splenocytes with mature NK cells from Tg-D^k or non-Tg into N-T BMT mice and then measured NK reactivity and MCMV resistance. After transfer, G2+ NK cells in N-T BMT recipients displayed significantly increased reactivity to *ex vivo* stimulation, irrespective of the donor source of splenocytes (Fig. 6A). Adoptive transfer recipients further displayed greater

MCMV resistance compared to control mice, again irrespective of the donor source of splenocytes (Fig. 6B). The results suggest that mature non-Tg G2+ NK cells without prior licensing by self-MHC D^k were 're-educated' by D^k-bearing non-hematopoietic cells in N-T mice, which coincided with more efficient missing-self lysis of MCMV-infected targets. However, the inflammatory state of the recipients after cell transfer could have resulted in non-specific NK reactivity. To test this, we further analyzed adoptively transferred NK cells in BMT chimeric mice without self-MHC expression in non-hematopoietic cells. In contrast to the above results, NK reactivity and MCMV clearance in adoptively transferred T-N and N-N BMT recipients was unaffected (Fig. 6C and D). We infer from the results that re-education of mature NK cells required self-MHC D^k only in non-hematopoietic cells, whereas reconstituting BM-derived NK cells also required MHC I in hematopoietic cells to realize their full capacity to mediate viral sensing and clearance.

Discussion

Our results delineate that self-MHC D^k expression in both hematopoietic and non-hematopoietic cells was required to adequately license G2+ NK cells, while lineage-restriction of the self-ligand resulted in impaired NK cell functionality and less effective viral clearance. The findings are in agreement with previous reports that linked G2+ NK cells with MCMV resistance [10] and selective expansion after MCMV exposure in mice with self-MHC D^k [27]. The current study firmly establishes that NK cells reconstituted after allogeneic BM transplantation had increased reactivity due to self-MHC education, which directly corresponded to more effective NK-mediated virus resistance. A long-standing question has been what role NK stimulatory receptors might have in D^k-dependent MCMV resistance. The current results demonstrate that NK recognition of MCMV targets in D^k mice is inadequate without optimally self-licensed G2+ NK cells, irrespective of stimulatory NK receptor expression. We propose that self-licensed G2+ NK cells efficiently recognize MCMV targets and then subsequently promote killing of infected cells via NK stimulatory receptor dependent mechanisms [28]. Although we were unable to discern a role for D^k on NK cells themselves or other hematopoietic cells, recent work suggests that self-ligand display in both NK and T cells contributes to the license effect [29, 30]. We infer that BM-derived NK cells required exposure to equivalent levels of self-MHC on hematopoietic and nonhematopoietic cells in order to be optimally educated to confer effective viral control.

In support of this conclusion, G2+ and G2− NK cells displayed equivalent responsiveness to stimulation in mixed BM chimeric mice. A lower G2 MFI on NK cells in the M-T BM chimeric mice implied that G2 had engaged D^k in *cis* on NK cells with the self-ligand expressed, and were therefore potentially weakly licensed. However, the effect was lost in M-N chimeric mice, which further substantiates the importance of self-MHC expression on non-hematopoietic cells in NK cell licensing. These effects were more dramatically observed in mixed BM chimeras with titrated expression of the self-ligand in the hematopoietic compartment, which again corresponded with NK-mediated MCMV resistance. The discrepancy between inhibitory NKR expression features and NK reactivity to stimulation further implies that more than one mechanism is involved in licensing, which may progress through stages depending on self-MHC expression patterns. Two models have

been proposed to explain the difference in responsiveness of NK cells that either express or lack self-MHC binding receptors. The licensing model predicts that NK cells must express inhibitory NKR to acquire reactivity [7], while the disarming model proposes that the reactivity of NK cells without self-ligand specific inhibitory NKR is negatively impacted due to chronic low-level stimulation [31]. Our results demonstrate that NK cell licensing was impaired when self-ligand expression in hematopoietic cells was limiting. However, hematopoietic cells without self-ligand expression could have 'disarmed' NK reactivity, or a combination of licensing and disarming could have resulted in diminished NK cell reactivity in the same environmental context [32]. Further investigation of NK cell licensing and its relationship to viral control is warranted.

This study further distinguishes reconstituting BM-derived from adoptively transferred mature NK cells with regards to their sensitivity to different self-ligand D^k expression requirements. Whereas BM-derived NK reactivity required self-ligand display in hematopoietic and non-hematopoietic cells, the transferred spleen NK cells re-licensed in BMT mice with the self-ligand restricted to non-hematopoietic cells. Thus, BM-derived NK cells are especially sensitive to hematopoietic cell self-MHC expression in the BMT environment. We have not investigated whether transferred mature NK cells diminish in reactivity if exposed for a longer period to hematopoietic cells without the self-ligand. This outcome seems unlikely, however, since hematopoietic-restricted D^k failed to re-educate transferred NK cells. Our results differ somewhat from those obtained in mice with induced MHC I expression, which concluded that NK cell licensing depended primarily on interactions between NK receptors and self-ligand+ hematopoietic cells [33]. However, whereas transgenic self-ligand induction in these mice only occurred well after BM transplantation and reconstitution, the results seem most analogous to re-licensing of mature NK cells after adoptive transfer. Recently, Sungur and colleagues showed that donor BM-derived NK cells in MHC allogeneic hematopoietic stem cell transplantation (HSCT) recipients acquired responsiveness to MCMV in a way consistent with licensing by donor MHC I on hematopoietic cells [34]. Licensed NK subset depletions prior to viral exposure effectively abrogated NK mediated MCMV resistance in the allo-HSCT setting [34]. An additional licensing effect of nonhematopoietic MHC I in the current study might be related to the simplified experimental design with transplantation across only a single MHC class I disparity. Differences in donor BM and/or a longer duration of donor cell reconstitution also may have contributed. Nonetheless, we infer that self-MHC expression in both compartments affected BM-derived reconstituting NK cells, in agreement with prior studies [22–24], which related to their resultant reactivity and capacity to mediate viral control.

Many prior studies have investigated the HSCT effect on NK cells since HLA and NK receptor polymorphisms might contribute critical graft versus leukemia effects [35–38]. A promising model system to investigate the effect of MHC and NK receptor polymorphisms on NK-cell education and reactivity is now established. Inasmuch as BMT affected NK mediated viral immunity, the current work further suggests that differences in NK cell education due to MHC and NKR polymorphisms might be related to vulnerability to infectious diseases and/or latent viral reactivation after HSCT in addition to putative anti-leukemic effects [39–41].

The proposed discontinuity theory of immunity [42] suggests that immune responsiveness is prompted by discontinuity in molecular motifs recognized by immune cell receptors, whereas continuous motif expression begets tolerance. Environmental context differences might therefore explain immune response discrepancies. On the other hand, mature NK cells taken from an environment with equivalent expression of a given self-ligand in both cellular compartments might be differently competent to reset reactivity in a way that prioritized expression of the self-ligand on non-hematopoietic cells. These data are in keeping with adoptive transfer [22, 26] and acute inducible MHC I expression studies [33] that have shown that mature NK cell responsiveness can be reprogrammed after exposure to different MHC class I environments. Distinct self-MHC requirements in hematopoietic and non-hematopoietic cells therefore affect NK cell licensing at different developmental stages.

In summary, our findings delineate key educational requirements for developing and mature NK cells that result in significant differences in effector function potential and their capacity to mediate viral control, which may provide useful information for clinical therapies by modulating NK cell functions to treat cancer or viral infections.

Materials and methods

Mice

C57L and MA/My breeders were purchased from Jackson Laboratory. C57L-derived transgenic C57L.Tg3-D^k (Tg-D^k) mice and MA/My-derived strains MA/My.L-$H2^b$ (M.$H2^b$) and MA/My.L-$H2^b$-Tg1D^k (M.Tg1) were generated at the University of Virginia and maintained in specific pathogen-free conditions and used in accordance with University of Virginia Institutional Animal Care and Use Committee oversight.

BM transplantation and adoptive transfer

Tg-D^k and control C57L mice (two doses of 5.75-Gy given 3 h apart) and M.$H2^b$ and M.Tg1 mice (one dose 9-Gy) were lethally irradiated and then i.v. injected with donor BM cells ($0.5–1.0 \times 10^7$). Following transplantation, BMT mice were given sulfate water for three weeks. For adoptive transfers, BMT mice (8–12 weeks after transplantation) were i.v. injected with Tg-D^k or non-Tg splenocytes (5×10^7) labeled with 5 μM CellTrace Violet (CTV, LifeTechnology).

In vivo cytotoxicity assay

In vivo cytotoxicity was measured essentially as described previously [43]. Briefly, a 1:1 mixture of CFSE- and CTV-labeled (5 μM dye label) non-Tg and Tg-D^k BM target cells (2×10^6/ 200 μl), respectively, was i.v. injected into recipient mice. After 20h, spleen and liver mononuclear cells were harvested and analyzed for residual dye-labeled target cells by flow cytometry on a FACSCanto II. A survival ratio comparing non-Tg and Tg-D^k cells was calculated as follows: (% residual non-Tg cells / % non-Tg cells injected) / (% residual Tg-D^k / % Tg-D^k cells injected). For immunodepletion of NK or other subsets of NK cells, mice were i.p. injected with mAbs against NK1.1 (PK136, 200 μg) on days −3 and −1 or with mAbs against G2 (4D11 or AT8) or Ly49I (1F8, kindly provided by Dr. Ortaldo, NIH/NCI) also at a dose of 200 μg on days −3 and −1 prior to injection of labeled BM targets.

Ex vivo stimulation of NK cells and intracellular cytokine staining

Peripheral blood mononuclear cells (PBMC) or splenocytes collected from BMT (8 wk post-transplant) or adoptive transfer (7d post-transfer) recipients were stimulated with immobilized anti-NK1.1 (PK136, 32 μg/mL), anti-NKG2D (C7, 50 μg/mL) or anti-CD16 (2.4G2, 50 μg/mL) mAb in the presence of Brefeldin A (GolgiPlug; BD) and 200 U/ml recombinant human IL-2 (Peprotech) for 6h. Afterward, cells were stained for NK cell surface receptors and CD107a. After fixation and permeabilization (Cytofix/Cytoperm; BD), cells were also stained for intracellular IFN-γ. The licensing ratio was calculated as described [3, 44] by dividing the percentage of IFN-γ+ or CD107a+ G2+ NK cells by the corresponding percentage of cytokine producing or degranulating G2− NK cells.

Antibodies and flow cytometry

Anti-mouse CD3e (145-2C11), Ly49G2 (4D11), CD107a (1D4B), NK1.1 (PK136), NKp46 (29A1.4), NKG2A/C/E (20d5) were purchased from BD Pharmingen. Anti-mouse Ly49I/U (14B11), CD19 (1D3), IFN-γ (XMG1.2) were purchased from BioLegend. Anti-mouse NKG2D (CX5), CD27 (LG.7F9), CD11b (M1/70), H-2D^k (15-5-5) and H-2D^b (28-14-8) were from eBioscience. Stained cells were analyzed by flow cytometry on a FACSCanto II (BD Biosciences). Data were collected using FACSDiva software (BD Biosciences) and analyzed using FlowJo (version 9.4; Tree Star).

MCMV infection and quantitation

Salivary gland stock virus (SGV) was prepared and titered on NIH3T3 monolayers as described previously [11]. Experimental mice were i.p. infected with 2×10^4 PFU of SGV MCMV. MCMV genomes in spleen and liver samples were measured using quantitative real-time PCR as described [11].

Statistical analysis

For comparisons between two independent treatment groups, an unpaired Student's t test was performed. For multi-group comparisons, one-way ANOVA (Tukey's post-test) was performed. Linear correlation was analyzed using the Pearson correlation coefficient. Data are reported with mean and standard error (mean ± SEM). P values <0.05 were considered significant. Statistical analyses were performed with Prism (GraphPad Version 5.0).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

BM bone marrow

BMT	bone marrow transplant
G2	Ly49G2
I/U	Ly49I/U
KIR	Killer Ig-like receptor
MCMV	murine CMV
MFI	median fluorescence intensity
M.H2^{b}	MA/My.L-H2^{b}
M.Tg1	MA/My.L-H2^{b}-Tg1D^{k}
NKR	NK cell receptor
PBMC	peripheral blood mononuclear cells
qPCR	quantitative real-time PCR
Tg-D^{k}	C57L.Tg3-D^{k}

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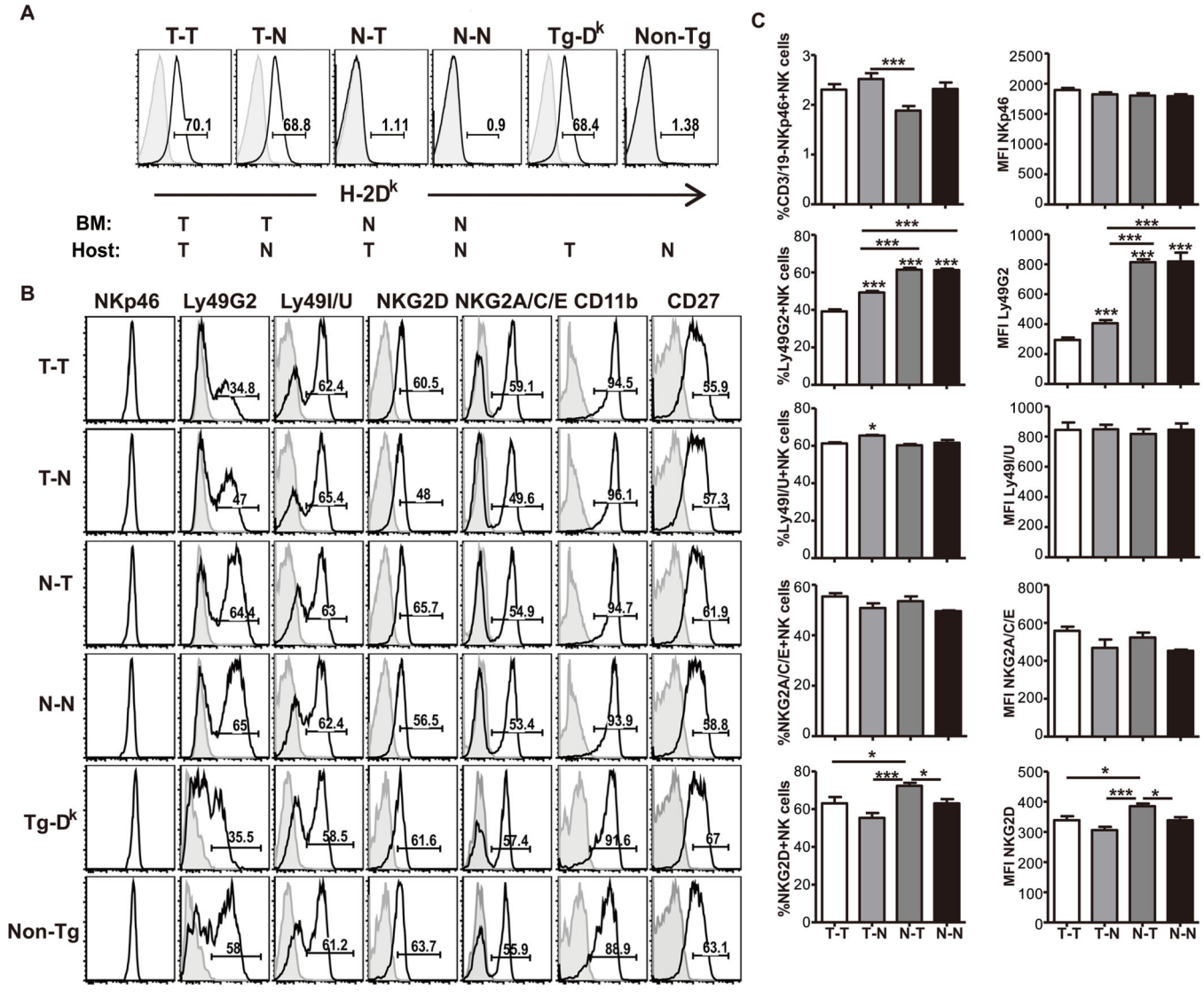

Figure 1.

Lineage-restricted D^k expression specifically affects G2+ NK cells. (A) Lethally irradiated Tg-D^k (T) and non-Tg (N) mice were reconstituted with Tg-D^k (T-T or T-N) or non-Tg BM (N-T or N-N), respectively. Representative histograms show isotype control (filled) and D^k (open) staining on peripheral leukocytes for the indicated BMT recipients 10–12 wk post-transplantation. (B) Representative histograms show expression of NK cell receptors and maturation markers (CD27 and CD11b) (open) and isotype control (filled) on live, gated NK cells (CD3− CD19− NKp46+) from indicated mice. (C) Bars represent the mean (±SEM) percentage and mean fluorescence intensity (MFI) for defined NK receptors for the indicated BMT recipients. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with T-T. Data are representative of three independent experiments. Each experiment contains 4–5 mice per group.

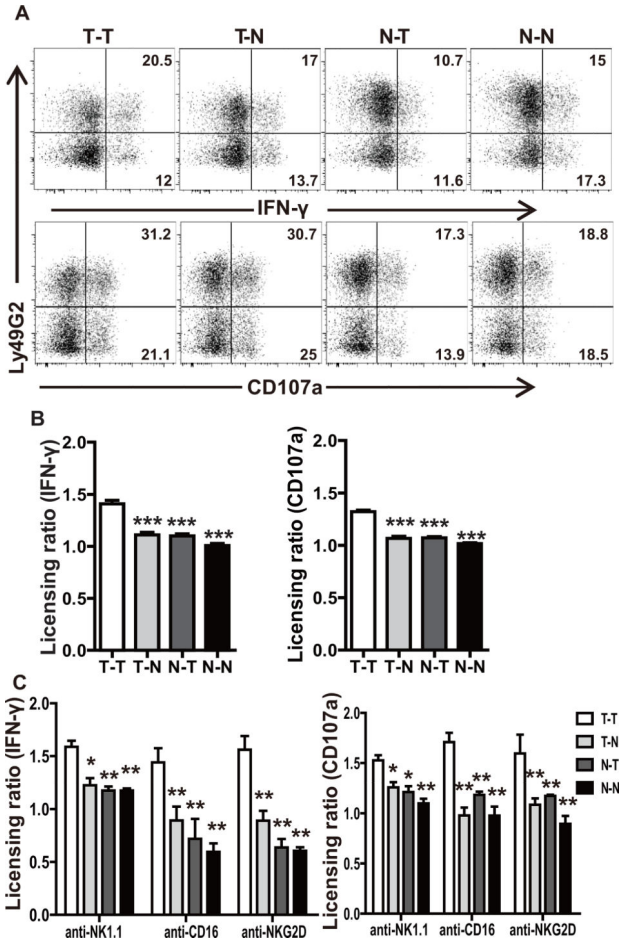

Figure 2.

MHC I D^k expression in hematopoietic and non-hematopoietic cells regulates G2+ NK cell licensing. Peripheral blood leukocytes or splenocytes were incubated with immobilized anti-NK1.1, anti-CD16, or anti-NKG2D and then permeabilized and stained for intracellular IFN-γ. (A) Representative dot plots show the percentage of IFN-γ and CD107a on peripheral blood NK cells after *ex vivo* stimulation with anti-NK1.1. (B and C) Bars represent the mean (±SEM) NK licensing ratio based on cytokine production or degranulation from peripheral blood (B) and spleen (C) for the indicated BMT recipients following *ex vivo* stimulation with anti-NK1.1 mAb (B, C), anti-CD16 or anti-NKG2D (C). Data are representative of eight independent experiments. Each experiment contains 4–5 mice per group.

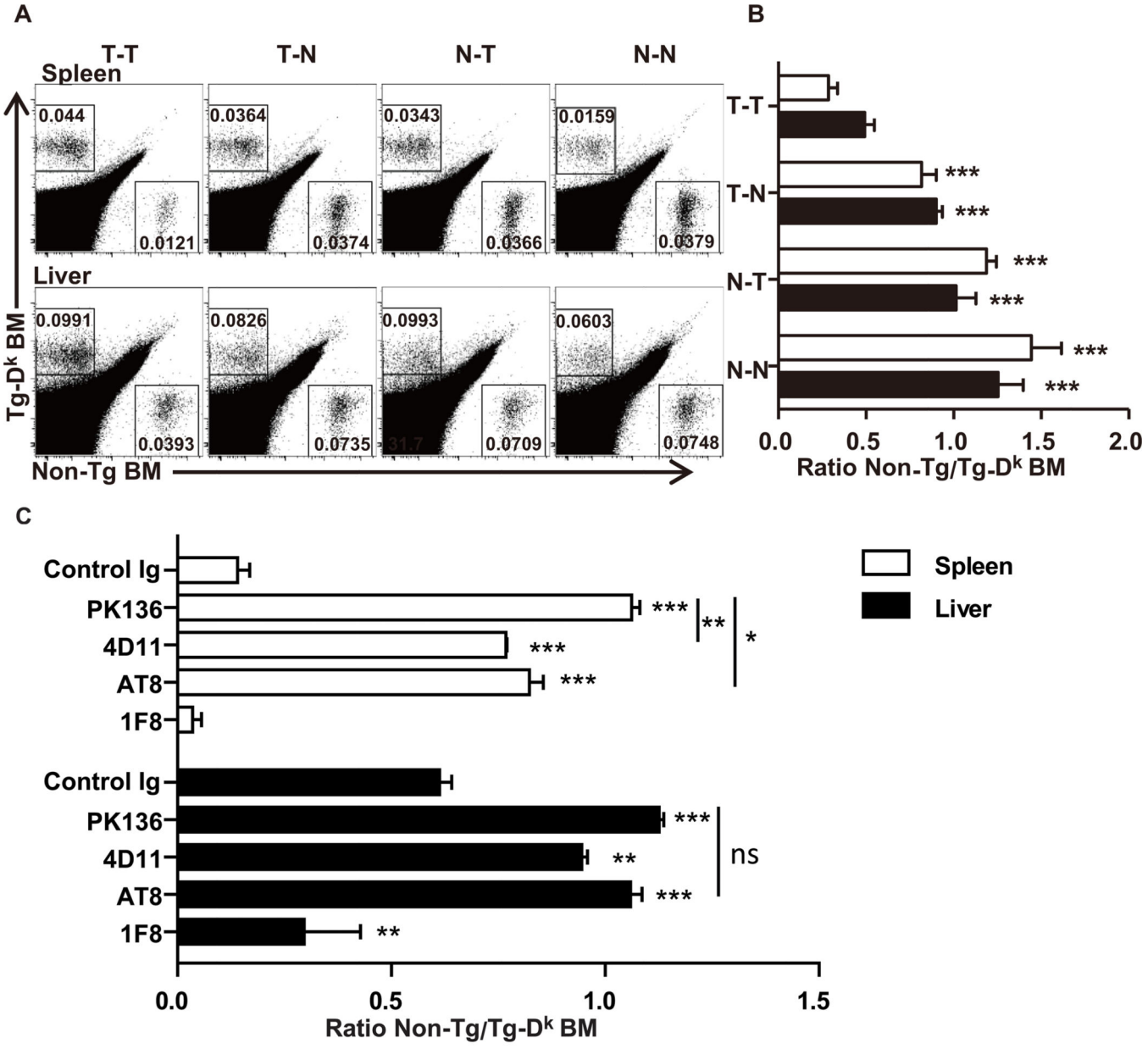

Figure 3.

G2+ NK cells mediate *in vivo* missing-self rejection of BM cellular targets without D^k in BMT chimeric mice. BMT recipients were i.v. injected with a 1:1 mixture of BM cells from Tg-D^k (CTV-labeled) and Non-Tg (CFSE-labeled) mice. After 20 h, spleen and liver leukocytes were collected and analyzed by flow cytometry. (A) Representative dot plots show residual labeled target cells in spleen and liver for the indicated BMT recipients. (B) The results from (A) are summarized in the graph with bars representing the mean (±SEM) ratio of the percentage of residual non-Tg / Tg-D^k BM target cells from the indicated BMT mice. (C) Rejection of Non-Tg BM cells is shown as a ratio of remaining Non-Tg to Tg-D^k BM cells in control Ig, total NK cells, G2+ NK cell (4D11 and AT8) depleted, and Ly49I+ NK cell (1F8) depleted BMT (T-T) mice. Data are shown as the mean ±SEM. $^{**}p < 0.01$;

***$p < 0.001$ compared with T-T. Data are representative of two independent experiments. Each individual experiment contains four mice.

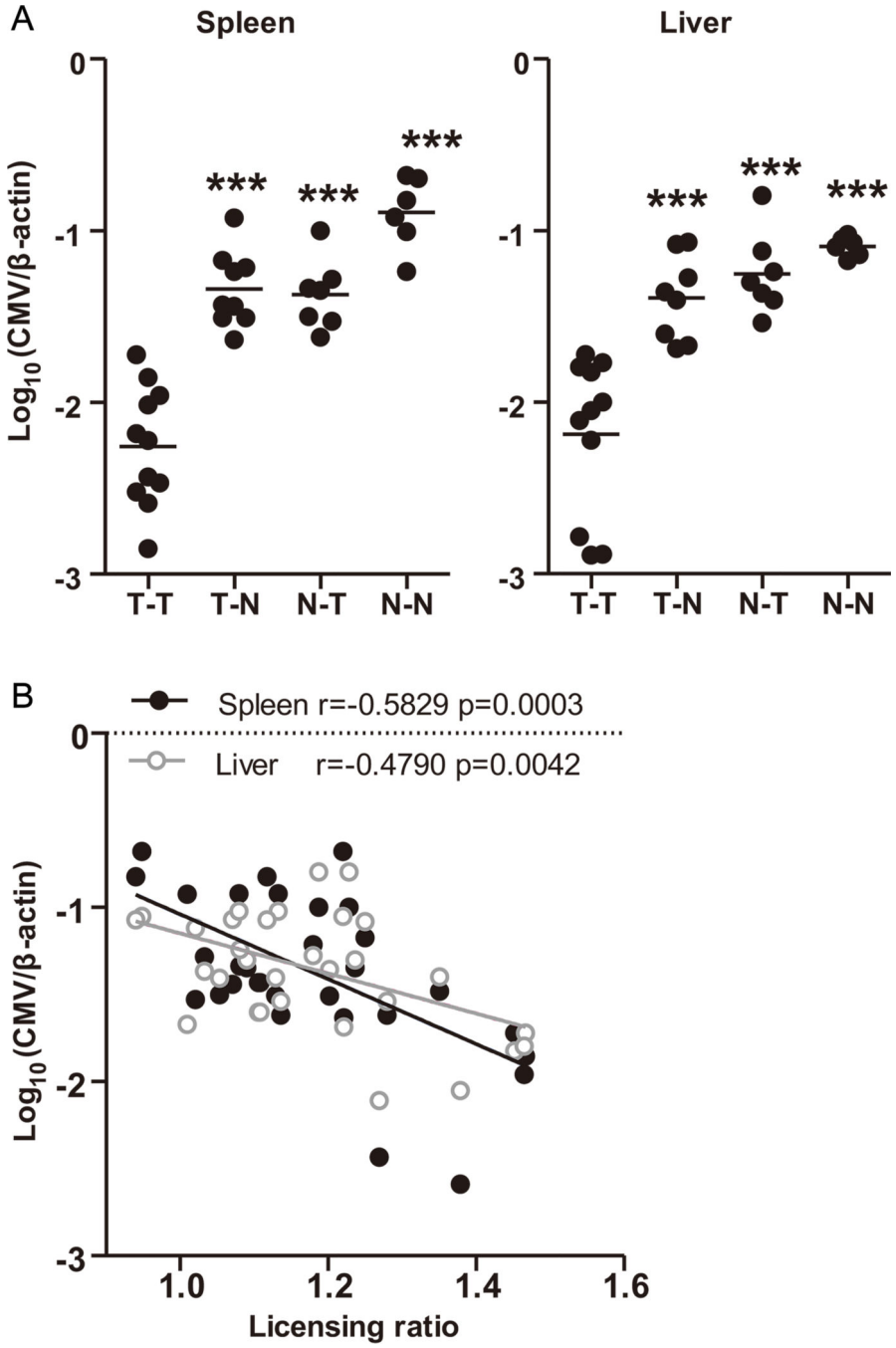

Figure 4.

MCMV resistance in BMT recipients corresponds with licensed G2+ NK cell reactivity. BMT recipients were infected with MCMV and then spleen and liver tissues were measured for MCMV genomes. (A) MCMV genome values (d 3.5) determined by qPCR for spleen and liver of the indicated BMT recipients are shown. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with T-T by ANOVA; # $p < 0.05$, ## $p < 0.01$ compared with N-N by ANOVA. (B) The scatter plot correlations relate the licensing ratio with viral load in spleen and liver

for individual BMT recipients. Solid line, linear growth trend; r, correlation coefficient. *p* values are shown.

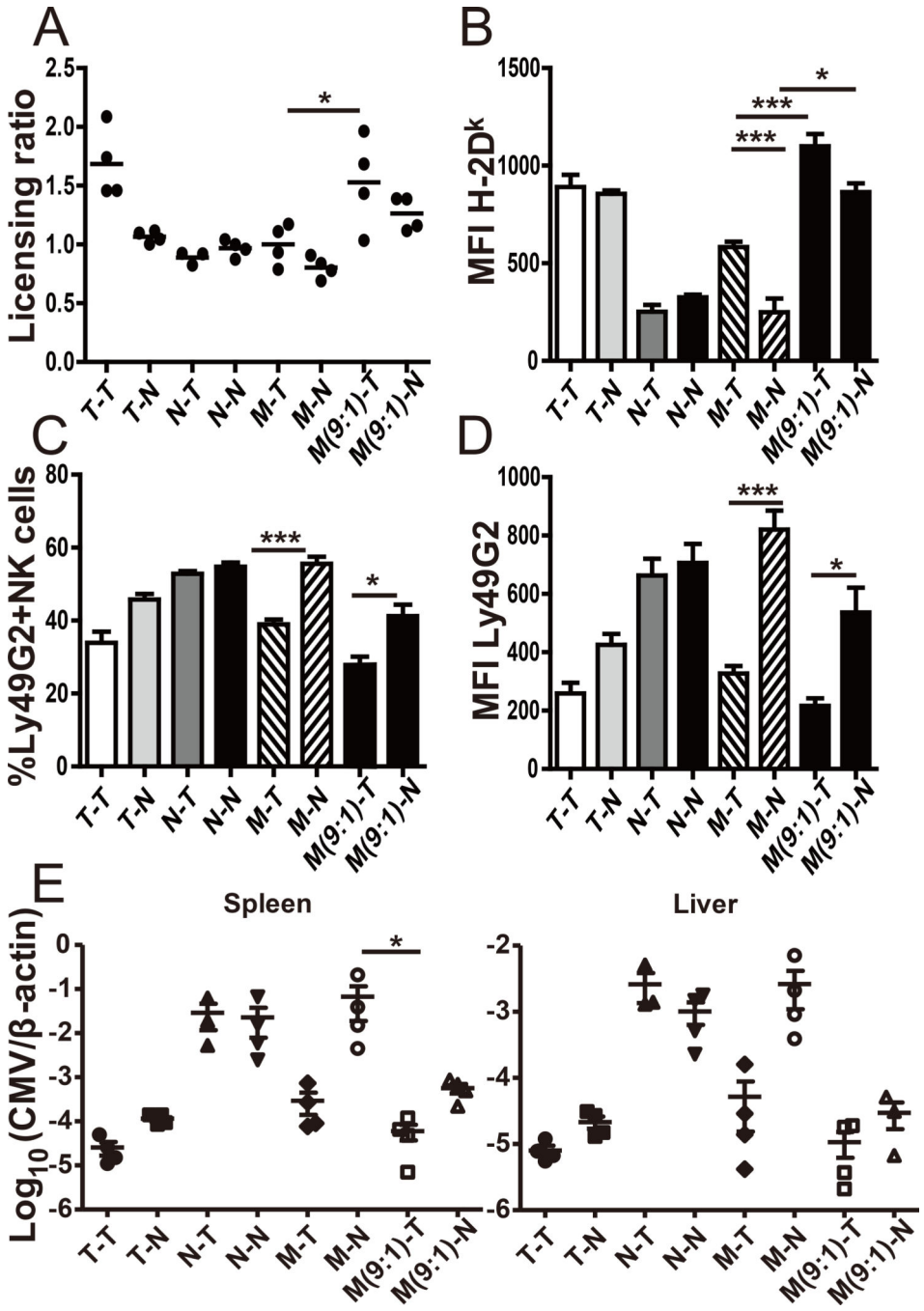

Figure 5.

NK cell reactivity tuned to self-ligand expression in hematopoietic cells dominantly affects NK-mediated MCMV resistance. Mixed BMT chimeric mice were established using 1:1 and 9:1 mixtures (M) of Tg-D^k and Non-Tg donor BM cells injected into either Tg-D^k or non-Tg recipients, respectively. Four different groups of mixed BM chimeras were analyzed, including (M-T) and (M-N) that had received 1:1 BM mixtures and (M(9:1)-T) and (M(9:1)-N) that had received 9:1 BM mixtures. Uninfected BMT and mixed BMT recipient PBMC were stained and treated as described for Figures 1 and 2. (A) NK licensing ratio based on

IFN-γ production was shown from the indicated BMT recipients following *ex vivo* stimulation with anti-NK1.1. (B) Bars represent the mean (±SEM) H-2 D^k MFI on peripheral blood leukocytes. (C, D) Bars represent the mean (±SEM) percentage of G2+ NK cells (C) and G2 MFI (D) for peripheral blood NK cells. Data are representative of three independent experiments, however mixed BMT chimeric mice established using a 9:1 ratio were only studied once. (E) BMT and mixed BMT recipients were i.p. infected with MCMV. Virus genome values (day 6) for spleen and liver are shown. Data are representative of two independent experiments. *$p < 0.05$; ***$p < 0.001$ by ANOVA.

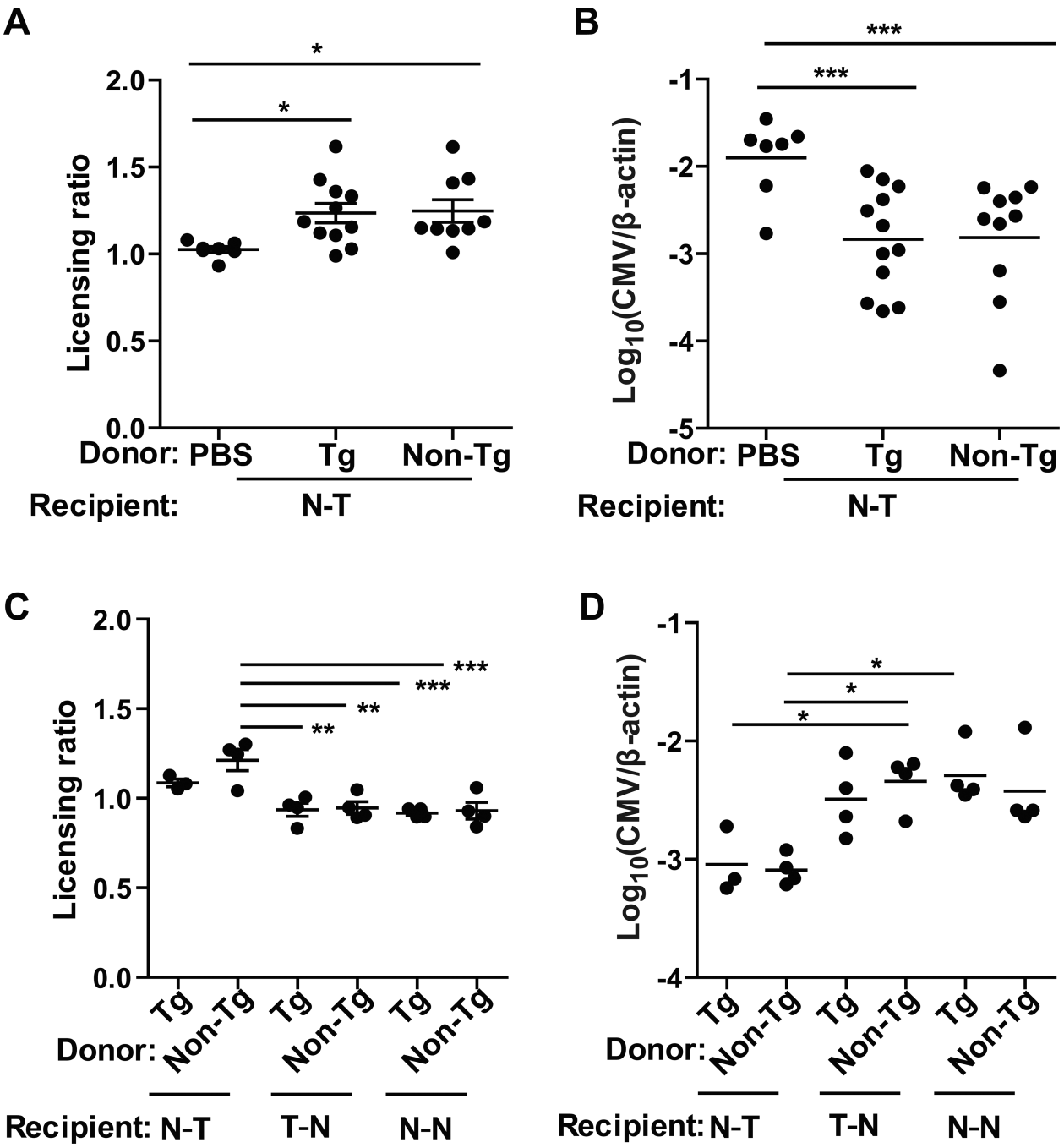

Figure 6.

Adoptive transfer of mature NK cells rescues G2+ NK cell impairment in N-T BMT mice. BMT recipients were i.v. injected with splenocytes from Tg-D^k or non-Tg mice. On d7 G2+ NK cell reactivity and viral control was assessed as described for Figures 2 and 4. (A, C) Peripheral blood leukocytes were incubated with immobilized anti-NK1.1 mAb and analyzed for intracellular IFN-γ. The graphs depict NK cell licensing ratios for individual BMT recipients after adoptive transfer with Tg-D^k or non-Tg splenocytes. (B, D) The graphs depict MCMV genome levels (d 3.5) for individual BMT recipients after adoptive transfer

with Tg-D^k or non-Tg splenocytes. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Each dot represents one mouse.