Title

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Differential expression of the Ly49G^{B6} and not Ly49G^{BALB} receptor isoform during NK cell reconstitution after hematopoietic stem cell transplantation

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Abstract

Inhibitory NK cell receptors specific for major histocompatibility complex class I (MHC-I) molecules include Ly49 receptors in mice and killer immunoglobulin-like receptors (KIR) in humans. The “licensing” or “arming” models imply that engagement of these receptors to self-MHC-I molecules during NK cell development educate NK cells to be more responsive to cancer and viral infection. We recently reported that hematopoietic stem cell transplantation (HSCT) induced rapid and preferential expansion of functionally competent Ly49G^{+}, but not other Ly49 family members, NK cells independently of NK-cell licensing via Ly49-MHC-I interactions. We now extend these studies to determine the expression of the two Ly49G receptor isoforms, Ly49G^{B6} and Ly49G^{BALB}, using mice with different MHC-I haplotypes that express one or both of the isoforms. NK cells from CB6F$_1$ (H-2^{bxd}) hybrid mice express two different alleles for Ly49G receptor (Ly49G^{B6} and Ly49G^{BALB}). Here, we found that CB6F$_1$ mice had more Ly49G^{B6+} than Ly49^{BALB+} NK cells, and that only Ly49G^{B6+} NK cells increased in relative numbers and in Ly49G MFIs after HSCT similar to the B6 parental strain. We further observed that Ly49G^{+} NK cells in BALB/c (H-2^{d}) and BALB.B (H-2^{b}), which have the same background genes, hosts slowly recover after HSCT, in contrast to Ly49G^{+} NK cells in B6 (H-2^{b}) recipients. The difference in expression of Ly49G^{B6} relative to Ly49G^{BALB} was linked to differences in the activity of the Pro1 promoter between the two alleles. Therefore, we conclude that the Ly49G^{B6}

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AUTHOR CONTRIBUTIONS

I.B. conducted experiments, analyzed data, and wrote the manuscript. S.K.A and P.W. conducted experiments, analyzed data, and assisted in writing of the manuscript. C.M.S. conducted experiments. D.R. had experimental oversight and discussed data. W.J.M had experimental oversight, discussed data and assisted in the writing of the manuscript.

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The authors have nothing to disclose.

receptor dominates Ly49G expression on NK cells after HSCT in strains where that allele is expressed. The data suggest that Ly49 allelic polymorphism within a particular Ly49 family member can differentially affect NK cell recovery after HSCT depending on the background genes of the recipient and not on the MHC-I haplotype.

INTRODUCTION

Natural Killer (NK) cells provide early immune protection against pathogens and cancer. NK cells express inhibitory receptors for major histocompatibility complex class I (MHC-I), Ly49 in mice and killer immunoglobulin-like receptors (KIR) in human, which inhibit NK cell function.

Several models have been proposed to explain the educational effects of MHC-I molecules on NK-cell development, function and self-tolerance. If self-MHC-I is down-regulated or absent, lack of inhibition triggers “missing self” killing [1]. NK cells developing in the absence of MHC-I or lacking inhibitory receptors for self-MHC-I are hypo-responsive [2]. The “licensing” or “arming” model implies that NK cells are initially hypo-responsive and become functional competent or “licensed” when their Ly49 receptors engage self-MHC-I during NK cell development [3, 4]. In addition, the rheostat model proposes that coexpression of numerous self-MHC-I-specific inhibitory receptors in NK cells results in increased capacity for MHC-I-dependent NK cell function [5].

Ly49 allelic polymorphism together with the arbitrary coexpression of MHC-I-specific receptors on NK cells generates diversity in the way individual NK cell interact with MHC-I molecules on targets [6, 7]. The acquisition of a self-MHC-I-specific receptor ensures NK cell tolerance to normal host cells and efficient killing of tumor and virus-infected cells. However, in humans and mice, the randomness of receptor distribution also generates NK cells that have unengaged or no inhibitory MHC-I receptors [3, 8], and it is now known that unengaged Ly49 receptors play a substantial role in reducing NK cell function [9]. The question of how MHC-I alleles influence NK cell development and responsiveness is important for the understanding of hematopoietic stem cell transplantation (HSCT) across KIR/human leukocyte antigen (HLA) donor-recipient mismatched barriers, in which donor NK cells elicit therapeutically beneficial rejection of recipient leukemic cells [10].

The “licensing” or arming” model has been brought into question recently with our finding that HSCT induced rapid and preferential expansion of $Ly49G^{+}$ NK cells independently of the host MHC haplotype [11]. This NK cell subset (unlicensed in H-2^{b} hosts) was responsible for mediating tumor killing and critical resistance to mouse cytomegalovirus (MCMV) infection [11, 12]. We sought to extend these studies to determine whether Ly49G allelic variation can differentially affect NK cell subset recovery after HSCT through the use of strains of mice expressing different MHC-I haplotypes but bearing the same background genes or mice expressing both Ly49G alleles. We observed that $CB6F_1$ (H-2^{bxd}) hybrid mice had more $Ly49G^{B6+}$ than $Ly49G^{BALB+}$ NK cells, and that only $Ly49G^{B6+}$ NK cells increased in relative numbers and in Ly49G MFIs after HSCT. We further observed that $Ly49G^{+}$ NK cells in both BALB/c (H-2^{d}) and BALB.B (H-2^{b}) hosts slowly recover after HSCT, in contrast to $Ly49G^{+}$ NK cells in B6 (H-2^{b}) recipients indicating this effect was independent of MHC. Analysis of Pro1 promoter elements controlling the BALB/c and B6 alleles revealed a more active promoter in the B6 allele, consistent with the increased subset of NK cells that express $Ly49G^{B6}$. We conclude that the $Ly49G^{B6}$, but not the $Ly49G^{BALB}$, allele dominates Ly49G receptor expression on NK cells post-HSCT. In aggregate, these data suggest that Ly49G allele receptor expression on NK cells is dependent on allele-specific differences in control elements and not on self- MHC-I molecules and that expression of a particular allele has an impact on reconstitution after HSCT.

METHODS

Mice

Female C57BL/6 (B6, H-2^b), Ly5.2 congenic B6 (B6, H-2^b), BALB/c (H-2^d), BALB.B (H-2^b), and $CB6F_1$ (B6xBALB/c, H-2^{bxd}) hybrid mice were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were 8–12 weeks of age and were housed in the Animal Facility at the University of Nevada, Reno, under specific pathogen-free conditions. All protocols involving animal work have been approved by the institutional laboratory animal care and use committee. Beginning one week before HSCT the mice received an antibiotic prophylaxis for four weeks with 100 mg of Ciprofloxacin (Ciprobay, Bayer, Germany) per liter drinking water.

Antibodies

The following fluorochrome-conjugated mAbs and streptavidin-conjugates were purchased from eBioscience (San Diego, CA): Pacific blue (PB)-anti-CD45 (30-F11) and -anti-CD45.1 (A20), R-Phycoerythrin (PE)- Cy7- or biotinylated-anti-CD3 (145-2C11), biotinylated-anti-CD122 (TM-β1), and allophycocyanin (APC)- or Alexa Fluor 647-(AF)-anti- NK1.1 (PK136), and fluorescein isothiocyanate (FITC-anti-$Ly49G^{B6}$ (Cwy3); from BD Biosciences (San Jose, CA): PE-anti-Ly49CI (5E6), FITC-anti-Ly49G2 (4D11) and -anti-$Ly49G^{BALB}$ (AT8), PE-anti-Ly49A (A1), and FITC-anti- Ly49D (4E5). Isotype-matched mouse and rat IgG mAbs were used as negative staining controls. Indirect staining was performed by using APC-conjugated streptavidin (Sav-APC or -APC-Cy7) purchased from eBioscience. In order to block FcγII/III receptor-mediated unspecific binding, anti-CD32/CD16 antibody (2.4G2) from eBioscience was used.

Hematopoietic Stem Cell Transplantation (HSCT)

Bone marrow (BM) cells were flushed out from femur and tibia of donors [Ly5.2 congenic B6, BALB/c, BALB.B, and $CB6F_1$] under aseptic conditions. Ly5.2 congenic B6 and $CB6F_1$ were treated with 200 μg of anti-NK1.1 mAb [PK136, in 0.2 ml phosphate-buffered saline (PBS) intraperitoneal (i.p) injection], and BALB/c and BALB.B with 20 μL anti-asialo-GM1 (1:20 dilution, Wako Chemicals), two days prior to HSCT to deplete donor NK cells prior to harvest. Single-cell suspensions were prepared, followed by T cell depletion using anti-Thy1.2 mAb (30H12) and rabbit-complement as previously described [13]. Donor NK cell and T cell depletion from the BM was confirmed by flow cytometric assessment using NK1.1 or CD122 with CD3 as discerning markers. The recipients were exposed to a lethal dose of gamma-irradiation from a ^{137}Cs source (950 cGy for B6, BALB/c and BALB.B; 1000 cGy for $CB6F_1$). Immediately after irradiation, the recipients were injected intravenously into the tail veins with congeneic or syngeneic or allogeneic HSCs cells (5×10^6 BMC in 0.5 ml of PBS) of sex- and age-matched mice. Each experiment was performed with 3–4 mice per group per harvest time point.

Cell preparation and flow cytometry

Recipient mice were sacrificed at 14 days after HSCT. For flow cytometry, splenocyte single-cell suspensions were first incubated with 2.4G2 mAb to block nonspecific antibody binding and then stained with combinations of the indicated fluorochrome-conjugated or biotinylated mAbs as previously described [13]. When biotinylated mAbs were used, cells were further incubated with APC-streptavidin. Stained cells were analyzed with a LSRII (BD, San Jose, CA) flow cytometer. Data analysis was performed by using FlowJo software (TreeStar). Percentages of donor-derived NK cells [$CD45.1^+NK1.1^+CD3^-$ (B6; congeneic) and $CD45^+NK1.1^+CD3^-$ ($CB6F_1$; syngeneic) or $CD45^+CD122^+CD3^-$ (BALB/c and BALB.B; syngeneic)] were determined. The mean fluorescence intensity (MFI) of each

receptor was defined for the subset of NK cells expressing the relevant receptor with MFI values ranging from 0–10,000.

Generation of Ly49G Pro1 core promoter luciferase reporter plasmids

Promoter fragments were generated by polymerase chain reaction (PCR) using primers corresponding to the sequence shown in Figure 4A and cloned into the TOPO-TA vector (Invitrogen, Grand Island, NY). Inserts were excised with either *SacI/XhoI* or *XhoI/HindIII* and cloned into pGL3 (Promega, Madison, WI) to generate constructs in both forward and reverse orientations. All constructs were verified by sequencing with specific primers.

Cell transfection and luciferase assays

LNK cells were transfected by electroporation with a BTX ECM 830 (Gentronics, Welshpool, Australia) set at 250mV, with 3 pulses of 7ms at an interval of 100ms. A total of 5×10^6 cells in 0.5 ml of serum free RPMI medium were transfected with 10μg of the specific reporter construct plus 0.1μg of the Renilla luciferase pRL-SV40 vector. Luciferase activity was assayed at 48hr using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The luciferase activity of the Ly49G Pro1 promoter constructs was normalized relative to the activity of the Renilla luciferase produced by the pRL-SV40 control vector and each construct was tested in at least three independent experiments.

Ly49G Pro1 transcript detection by PCR

RNA from spleens obtained at day 7 post-HSCT was extracted using the RNeasy® Mini Kit (Qiagen, Georgetown, MD) following the manufacturer's instructions. 2ug of RNA was used to generate cDNA using the SuperScript First Strand cDNA Synthesis Kit (Invitrogen). PCR to detect the Pro1 forward transcript of the $Ly49G^{B6}$ and $Ly49G^{BALB}$ genes was performed using 1 ul of cDNA and 35 cycles of (94°–20sec, 58°–30sec, 73°–20sec). Primers specific for the Pro1 transcript of each gene are listed below. The specificity of each primer set was confirmed by sequencing the PCR products. The sequence of the primers are as follows: $Ly49G^{BALB}$ exon-1a Forward: ACTACAGTCCAAGGATCTGACTAG and $Ly49G^{BALB}$ exon 3 Reverse: GCTTCCAGGGGACTGAATACTTTC (predicted product size: 292 bp); $Ly49G^{B6}$ exon-1a Forward: AGGATTTCTCATCACAAACTGAAG and $Ly49G^{B6}$ exon 3 Reverse: GAGCTTCCAGGGGACTGAATACTC (predicted product size: 268 bp). Quantitative PCR was performed with SYBR green to determine levels of Pro1 transcripts relative to an actin control.

Statistical analysis

Statistical significance was tested by using non-parametric tests. p values were considered statistical significant when $p<0.05$.

RESULTS

Monoallelic $Ly49G^{B6+}$ NK cells dominate in $CB6F_1$ hybrid mice

Previous studies using Ly49G mAb 4D11 have shown that Ly49G is a commonly expressed inhibitory receptor on NK cells, but the level of expression varies between different mouse strains [14]. We first confirmed the percentages of $Ly49G^+$ NK cells in different mouse strains using the 4D11 mAb which binds both allelic isoforms of Ly49G. $Ly49G^+$ NK cells comprised 45–50% of the NK cells in B6 ($H\text{-}2^b$) mice (Figure 1A). The frequencies of NK cells expressing the Ly49G receptor were lower in BALB/c ($H\text{-}2^d$) and BALB.B ($H\text{-}2^b$) (~ 20% and 35%, respectively; Figure 1B,C). $CB6F_1$ ($H\text{-}2^{bxd}$) hybrid mice had approximately 35% $Ly49G^+$ NK cells, with ~ 15% $Ly49G^{dim}$ and ~ 24% $Ly49G^{bright}$ (Figure 1D).

Two distinct Ly49G alleles have been identified in mice [15]. Using the Ly49G allele-specific mAbs, AT8 (specific for $Ly49G^{BALB}$) and Cwy-3 (binds $Ly49G^{B6}$), we first verified their ability to distinguish Ly49G alleles in $CB6F_1$ hybrid mice. As shown in Figure 1E and F, very few NK cells were biallelic (~1.7%), and more NK cells expressed the $Ly49G^{B6}$ than the $Ly49G^{BALB}$ allele (21.6% ± 1.2% *vs.* 9.4% ± 0.61%, respectively, $p<0.05$; $Ly49G^{B6}$: $Ly49G^{BALB}$ ratio = 2.23). Moreover, monoallelic $Ly49G^{B6}$ NK cells displayed higher MFI for Ly49G receptor than $Ly49G^{BALB+}$ monoallelic NK cells (Cwy-3 MFI of 1098 ± 128, and AT8 MFI of 357 ± 63, $p<0.05$, Figure 1G). Our data indicate that expression of the Ly49G receptor is differentially regulated in NK cells depending on the genetic background of the mouse strain.

Rapid and preferential recovery of $Ly49G^{B6+}$ NK cells early after HSCT in $CB6F_1$ hybrid mice

Murine HSCT models have provided important insights into NK cell development. We recently reported that NK cells were the first lymphoid cells to repopulate, as early as 7 days after transplant in mice, analogous to clinical HSCT [11]. This study showed a preferential expansion of cytotoxic donor-derived $Ly49G^+$ NK cells independently of MHC-I haplotype. We now extended these studies and determined whether this phenomenon occurs in other mouse strains. Lethally irradiated BALB/c ($H\text{-}2^d$), BALB.B ($H\text{-}2^b$) and $CB6F_1$ hybrid mice were transplanted with 5×10^6 syngeneic BMCs depleted of NK and T cells. Splenic $Ly49G^+$ NK cells were analyzed at day 14 after HSCT. As shown in Figure 2A, the frequency of Ly49G-expressing NK cells (4D11 positive) significantly increased in $CB6F_1$ recipients after HSCT [44.3 % ± 3.7 % *vs.* 35.6 % ± 1.1 % (control), 1.3 fold increase, $p<0.05$]. Interestingly, after HSCT, $Ly49G^{B6+}$ NK cells were present at higher relative numbers than $Ly49G^{BALB+}$ NK cells [$Ly49G^{B6+}$: 36.5 % ± 2% *vs.* 21.6% ± 1.2% (control) $p<0.05$; and $Ly49G^{BALB+}$: 5.23% ± 0.5% *vs.* 9.4% ± 0.6% (control) $p<0.05$; $Ly49G^{B6}$: $Ly49G^{BALB}$ ratio = 4.42, $p<0.05$] (Figure 2B,C,D) suggesting that the $Ly49G^{B6+}$ NK cell subset was selected for rapid recovery. In addition, only $Ly49G^{B6}$ NK cells exhibited increased Ly49G MFI (Cwy-3 MFI of 1778 ± 156 in transplanted *vs.* 1098 ± 128 in control, $p<0.05$, Figure 2E). Furthermore, the frequencies of $Ly49G^+$ NK cells in BALB/c and BALB.B were markedly reduced after HSCT in comparison to control adult resting mice [BALB/c: 13.3% ± 0.89% *vs.* 19% ± 2.6% (control), $p<0.05$; and BALB.B: 24.8% ± 1.2% *vs.* 35.5% ± 0.45%, (control), $p<0.05$; Figure 3A,B,D], and no alterations in the MFI values for Ly49G occurred [BALB/c: 264 ± 25 (control); and BALB.B: 620 ± 14 (control); Figure 3E]. This is in contrast to what we previously observed for the B6 black mouse strains [11], which was a marked increase in the frequency and MFI values of $Ly49G^+$ NK cells, as confirmed in Figure 3C,D,E. The percentage of Ly49G expressing cells in B6 mice at day 14 post-transplant was 51.6% ± 1.5% *vs.* 23.6% ± 0.56% in control mice ($p<0.05$), and the MFI values of Ly49G (detected by 4D11 mAb) were 6071 ± 235 in transplanted *vs.* 2843 ± 232 in control ($p<0.05$). NK cells expressing Ly49A and Ly49D (which also binds $H\text{-}2D^d$) are present at low frequencies in our HSCT models [11]. Our data show that the $Ly49G^{B6}$ allele dominates Ly49G receptor expression during NK cell reconstitution after HSCT even in mice expressing both alleles.

The $Ly49G^{B6}$ allele contains a Pro1 promoter element that is more active than the $Ly49G^{BALB}$ Pro1 promoter

Our previous study has shown that Ly49G Pro1 transcription dominates during NK cell reconstitution in B6 mice, providing a molecular explanation for the preferential expression of Ly49G after HSCT [11]. We therefore investigated whether there were allelic differences in the Ly49G Pro1 promoters between B6 and BALB/c mice that might account for their differential expression. Figure 4A shows the presence of two nucleotide substitutions in the BALB/c Ly49G Pro1 element as compared to B6. Although the altered nucleotides are not

contained within any of the transcription factor binding sites previously shown to play important roles in Pro1 function [16], they do create a potential TATA-binding element that might compete with the TATA elements required for forward or reverse transcription from Pro1. In order to determine if the nucleotide changes affect promoter activity, the BALB/c and B6 Ly49G Pro1 elements were cloned into the pGL3 luciferase reporter vector, and promoter activity was determined by transfection into the LNK cell line. Figure 4B demonstrates that the Ly49G Pro1 element from BALB/c mice possesses only 50% of the activity of the B6 allele, consistent with the decreased frequency of expression observed for BALB/c Ly49G. RT-PCR was performed with primers specific for either the BALB/c or B6 *Ly49g* Pro1 forward transcripts using RNA purified from spleen cells from either B6 or $CB6F_1$ recipients of $CB6F_1$ bone marrow collected 7 days post-transplant. (Figure 4C). As predicted by the 2-fold greater activity of the Ly49G Pro1 element, there was approximately twice as much forward transcript from the $Ly49G^{B6}$ allele. Taken together, these results indicate that allelic differences in the Pro1 promoters of $Ly49G^{B6}$ and $Ly49G^{BALB}$ are responsible for the preferential expression of $Ly49G^{B6}$ after HSCT.

DISCUSSION

MHC-I molecules play a critical role in NK cell function. According to the “licensing” or “arming” NK education model, during ontogeny, NK cells are initially hypo-responsive and become functionally competent or “licensed” when their Ly49 inhibitory receptors engage self-MHC-I [3, 4]; functional maturation does not occur in NK cells expressing exclusively unengaged or no inhibitory MHC-I receptors. However, this theory with regard to development and receptor acquisition has been brought into question with our recent finding that HSCT induced rapid and preferential expansion of $Ly49G^+$ NK cells independently of MHC-I haplotype [11]. However, it is quite possible that Ly49G may be different than the other Ly49 family members and is expressed as a marker for activation. The $Ly49G^+$ NK cell subset (unlicensed in $H\text{-}2^b$ hosts) was responsible for mediating tumor killing and critical resistance to mouse cytomegalovirus (MCMV) infection but these reflected general activity of the NK cell subpopulations [11, 12]. How NK cell responsiveness is impacted by different Ly49G repertoires during reconstitution following HSCT is still not known.

Ly49 allelic polymorphism together with the stochastic co-expression of MHC-I-specific receptors on NK cells generates diversity in the way an individual NK cell interacts with MHC-I molecules on targets [7, 6]. On the basis of our previous finding that $Ly49G^+$ NK cells are the primary NK population to recover post-HSCT, we examined whether Ly49G allelic variation could differentially affect NK cell recovery after HSCT using strains of mice with different MHC-I haplotypes. Two distinct Ly49G alleles have been identified in mice [15]. We observed that $Ly49^{B6+}$ NK cells (which comprise the majority of the $Ly49G^+$ NK cells in $CB6F_1$ mice) but not $Ly49^{BALB+}$ NK cells significantly increased their relative numbers at 14 days after HSCT in these recipients. Moreover, transplanted BALB/c and BALB.B mice had lower frequencies of splenic $Ly49G^+$ NK cells compared to control adult resting mice, and this NK cell subset dominated repopulation in B6 recipients after HSCT [11]. The data show that the two Ly49G alleles, $Ly49G^{B6}$ and $Ly49G^{BALB}$, are not expressed co-dominantly in $CB6F_1$ mice, and that the $Ly49G^{B6}$ allele dominates Ly49G expression during NK cell reconstitution after HSCT.

There is evidence for variability in MHC-I ligand recognition and binding strength of the different Ly49G alleles [17]. The $Ly49G^{BALB}$ receptor more efficiently binds to $H\text{-}2D^d$ and $H\text{-}2L^d$ than the $Ly49G^{B6}$ receptor as determined by target cell lysis and soluble MHC tetramer binding using different strains of hybrid mice [18, 19]. It is possible that in our HSCT models, $Ly49G^{BALB+}$ NK cells that develop in $H\text{-}2^d$ hosts have more affinity for $H\text{-}2D^d$ than $Ly49G^{BALB+}$ NK cells developing in an $H\text{-}2^b$ background, which may result in

strong inhibition after Ly49G engagement to ligand and reduced NK cell recovery. Makrigiannis et al., previously observed that $CB6F_1$ hybrid mice have more $Ly49^{B6+}$ NK cells than $Ly49^{BALB+}$ NK cells, and very few biallelic NK cells [15]. The presence of $H\text{-}2D^d$ ligand in these mice can be a determinant factor for NK cell development, as hybrid mice in $H\text{-}2^b$ and other MHC-backgrounds have more Ly49G biallelic NK cells [20]. However, in $CB6F_1$ mice, NK cells expressing the $Ly49G^{B6}$ allele exhibited higher proliferation in response to IL-2, and NK cells expressing the B6 allele were capable of activating the BALB/c allele, suggesting that $Ly49G^{B6}$ delivers a weaker inhibitory signal than $Ly49G^{BALB}$ [15]. We observed that adult resting BALB.B mice have elevated frequencies of $Ly49G^+$ NK cells compared to BALB/c mice, and the MFI for Ly49G is higher. This is probably a consequence of lack of self-MHC-I in BALB.B, and strong binding of Ly49G to $H\text{-}2D^d$ in BALB/c, possibly masking $Ly49G^{BALB}$ expression due to cis interaction with $H\text{-}2D^d$. $Ly49G^{B6+}$ but not $Ly49G^{BALB+}$ NK cells from $CB6F_1$ hosts showed an increase in the Ly49G MFI values. No alteration in the MFI values for Ly49G occurred in BALB/c and BALB.B recipients after transplantation. Of note, HSCT is associated with a significant rise in endogenous cytokine levels such as IL15 and IL7 [21]. IL15, in particular, is required for in vivo expansion and survival of NK cells [22]. This may be why the Ly49G subset dominates after HSCT and activation in general and the cytokine milieu after HSCT may have differential effects on the two Ly49G alleles as was seen with IL-2 stimulation.

We previously published that $Ly49G^+$ NK cells developing early after HSCT are cytotoxic against tumors and MCMV [11]. Our group also described that both $Ly49G^{B6+}$ and $Ly49G^{BALB+}$ NK cell subsets in $CB6F_1$ hybrid mice displayed lower cytotoxicity toward $H\text{-}2D^d$ targets. However, sorted $Ly49G^{B6+}$ NK cells displayed higher cytotoxicity against $H\text{-}2D^d$ target cells than $Ly49G^{BALB+}$ NK cells after IL-2 stimulation [15]. In the current study, due to the low frequencies of NK cell subsets developing early after HSCT, it was not possible to sort cells expressing one or both of the Ly49G alleles and compare NK cell functional capabilities in the HSCT models studied. Nevertheless, the data indicate that Ly49G allelic variation differentially affects NK cell recovery after HSCT.

Ly49A (inhibitory receptor) and Ly49D (activating receptor) also bind $H\text{-}2D^d$ ligands [23].It is unlikely that inhibition of NK responses due to co-expression of Ly49A with Ly49G account for the lack of expansion of $Ly49G^{BALB+}$ NK cells because low frequencies of $Ly49A^+$ NK cells were present after HSCT in all the B6 and BALB mouse strains used [11]. In addition, a previous study has shown that Ly49A is equally expressed on $Ly49G^{B6+}$ and $Ly49G^{BALB+}$ NK cells in adult resting $CB6F_1$ hybrid mice [15]. Activation signaling can override MHC-I-dependent cell inhibition and increase NK responsiveness [24]. $CB6F_1$ hybrid mice have more $Ly49G^{BALB+}$ NK cells expressing Ly49D than $Ly49G^{B6+}$ NK cells [15]. It is possible that NK cell activation induced by Ly49D in $H\text{-}2^d$ hosts is overridden by inhibitory signals derived from $Ly49G^{BALB}$ receptor engagement to $H\text{-}2^d$ (stronger binding to the ligand than the $Ly49G^{B6}$ receptor) and reduced NK cell recovery occurs. The lack of Ly49D receptor on NK cells from BALB/c and BALB.B hosts may be a contributing factor for the lesser NK cell activation and reduced NK cell recovery after HSCT.

Previous studies of the human KIR3DL1 gene have shown that allelic differences in the KIR bidirectional promoter can affect the relative levels of forward versus reverse promoter activity, and this correlates with differences in the frequency of NK cells expressing a given allele [25]. In the current study, we demonstrate that allelic differences in the Ly49G Pro1 bidirectional promoter are also associated with changes in subset expression of Ly49G. The relative promoter activity of the Ly49G Pro1 elements 129>B6>BALB/c correlates with the size of the $Ly49G^+$ subset found in each strain. It would therefore be of interest to determine

if the Ly49G^{129} allele behaves similar to the B6 allele in HSCT due to a more active Pro1 element, even though it has a higher affinity for H2D^{d} like the BALB/c allele.

NK cells have been under intensive study for their potential use in HSCT and cancer therapy after reports demonstrating that the use of the appropriate KIR or HLA mismatches in allogeneic HSCT improved clinical outcome of patients with leukemia [26, 27, 28]. Ly49 allelic polymorphism and haplotype size vary in mice, and this situation parallels KIR variability in humans [29]. Because Ly49 in mice (and KIR in human) receptor expression is stochastic, one advantage of allelic polymorphism and heterozygosity is that individual NK cells have more receptors with different MHC-I specificities to choose from during their development in the bone marrow. It remains to be determined if human KIR isoforms exist that exhibit these patterns of differential expression.

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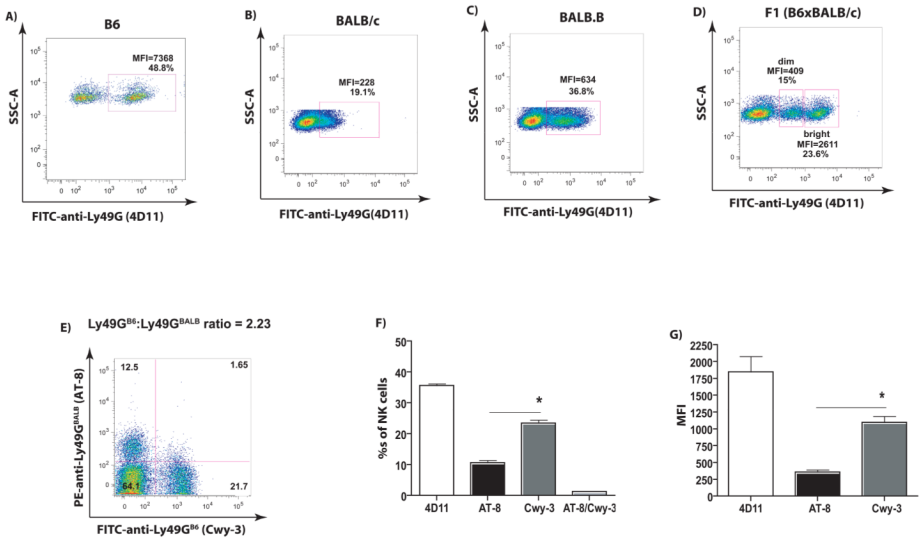

Figure 1. Expression of different Ly49G receptor alleles in NK cells

Splenic CD45.1$^+$NK1.1$^+$/CD122$^+$CD3$^-$ NK cells from (A) B6 (H-2^b), (B) BALB/c (H-2^d), (C) BALB.B (H-2^b) and (D) CB6F$_1$ (B6xBALB/c; H-2^{bxd}) hybrid mice were stained with FITC-anti-Ly49G (4D11). The percentage of cells staining positively and the MFI values are indicated. (E) Splenic NK cells from CB6F$_1$ mice were stained with FITC-Cwy-3 (Ly49G^{B6}) and PE-AT8 (Ly49G^{BALB}) mAbs. The percentage of cells expressing the different Ly49G receptor alleles is indicated. (F) Percentages of monoallelic and biallelic NK cells. (G) Intensity levels of Ly49G allele expression. Data are representative of 2–3 experiments using 3–4 mice per group in each experiment (mean ± SEM). *p<0.05

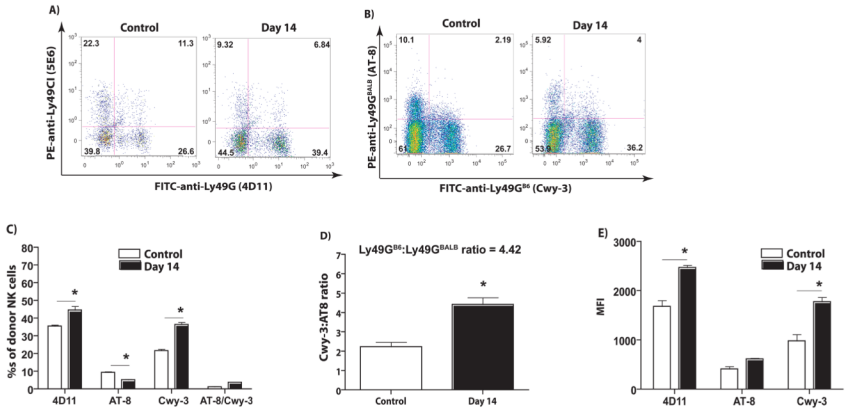

Figure 2. Ly49G^{B6+} NK cells dominate in CB6F$_1$ hybrid mice after HSCT

Lethally irradiated CB6F$_1$ (H-2^{bxd}) mice were transplanted with 5×10^6 of syngeneic NK and T cell depleted HSCs. Splenic CD45.1$^+$NK1.1$^+$CD3$^-$ NK cells from recipients were stained with FITC-anti-Ly49G (4D11) (A), FITC-Cwy-3 (Ly49G^{B6}) and PE-AT8 (Ly49G^{BALB}) mAbs (B) at day 14 after syngeneic HSCT and compared to adult resting mice (control). The percentage of cells staining positively is indicated in the flow plots. (C) Percentages of monoallelic and biallelic Ly49G$^+$ NK cells. (D) Ly49G^{B6+}: Ly49G^{BALB+} ratio. (E) Ly49G receptor MFIs. Data are representative of 2 experiments using 3 mice per group in each experiment (mean ± SEM). *$p<0.05$

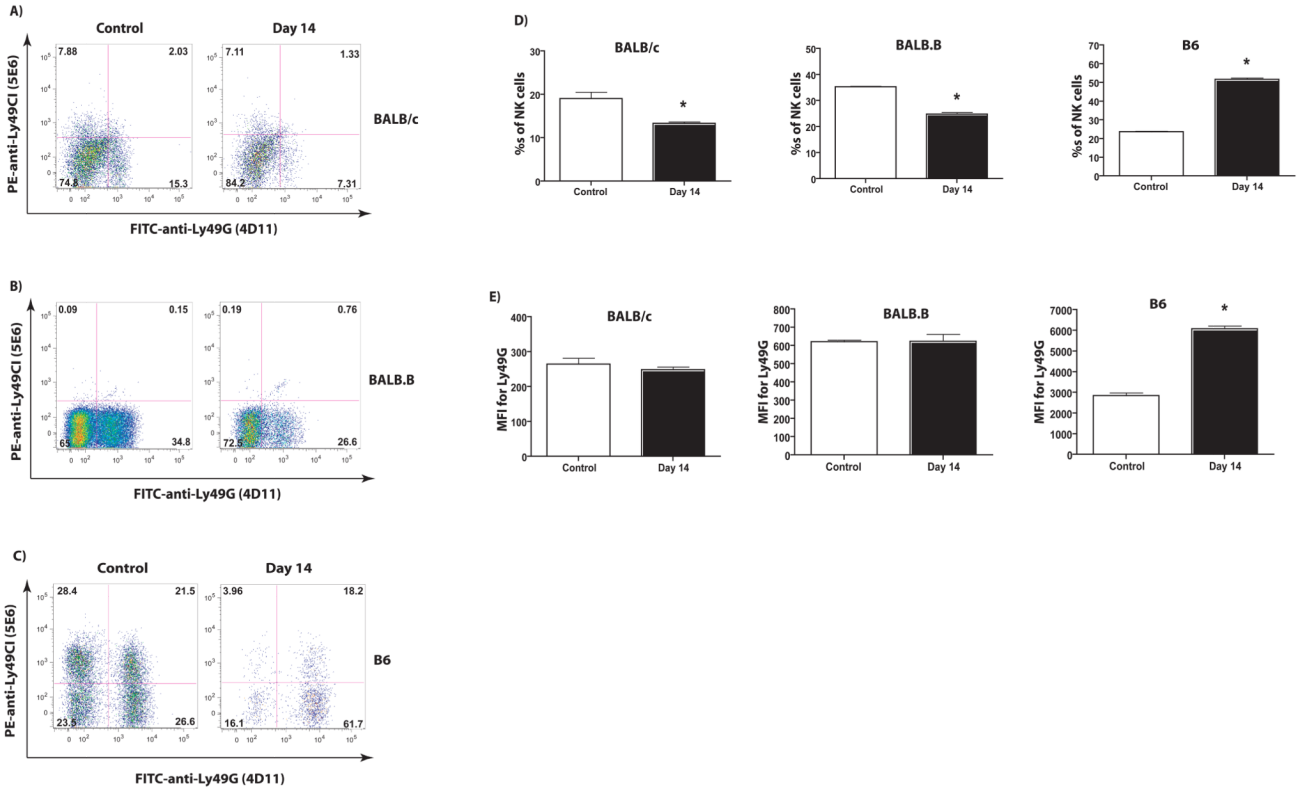

Figure 3. Low frequencies of Ly49G^{BALB+} NK cells after HSCT

Lethally irradiated C57BL/6 (B6, H-2^b, CD45.2) and BALB background mice (H-2^d) mice were transplanted with 5×10^6 of Ly5.2 congenic (H-2^b, CD45.1) and syngeneic HSCs depleted of NK and T cells, respectively. Splenic CD45.1$^+$NK1.1$^+$/CD122$^+$CD3$^-$ NK cells from (A) BALB/c (H-2^d), (B) BALB.B (H-2^b) and (C) B6 (H-2^b) mice were stained with FITC-anti-Ly49G (4D11) and PE-anti-Ly49C/I (5E6) mAbs. The percentage of cells staining positively is indicated. (D) Percentages of Ly49G$^+$ NK cells. (E) Intensity levels of Ly49G receptor alleles expression. Data are representative of 2–3 experiments using 3–4 mice per group in each experiment (mean ± SEM). *p<0.05

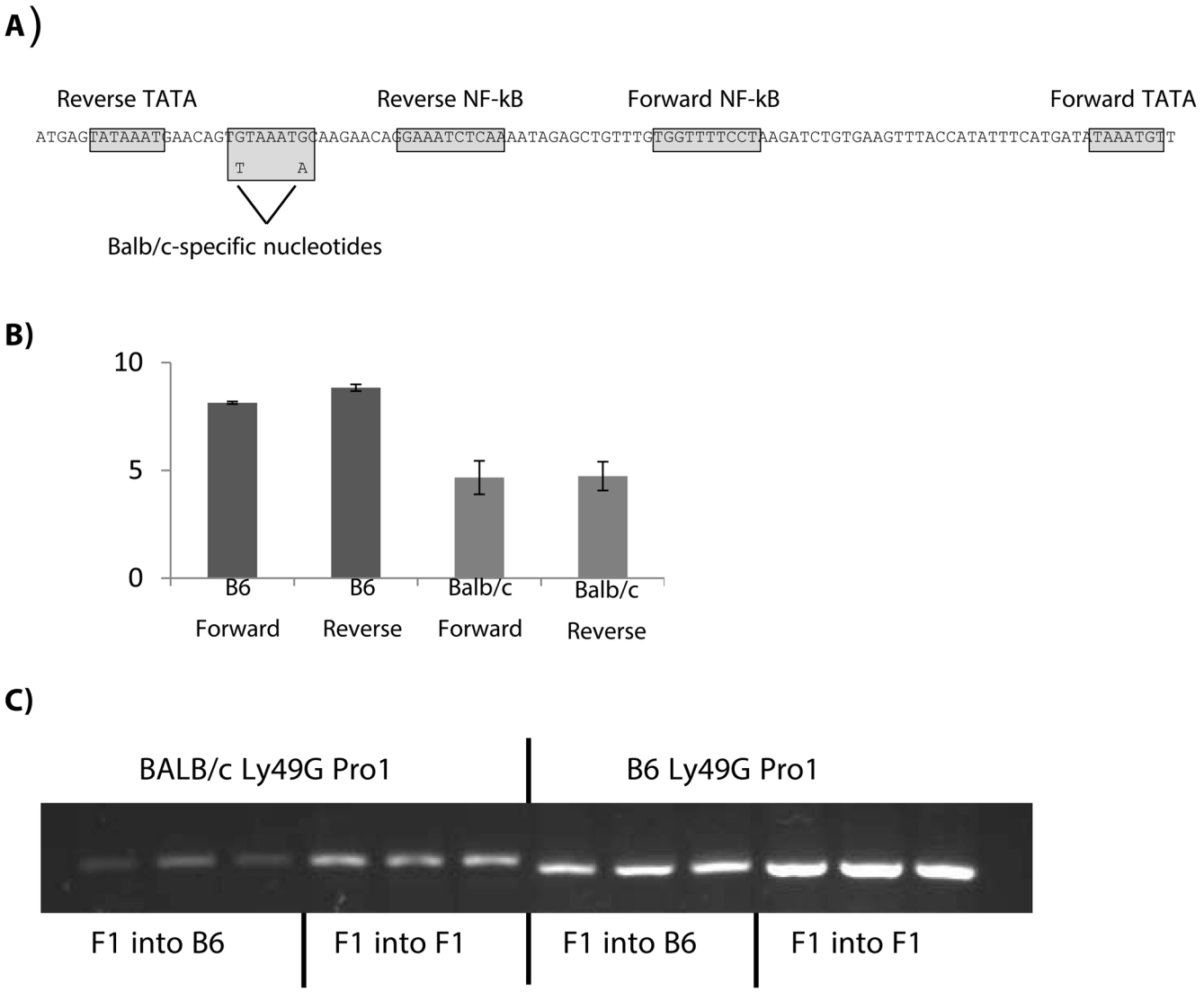

Figure 4. The Pro1 promoter of Ly49G^{B6} is more active than the BALB/c allele

Lethally irradiated CB6F$_1$ (H-2^{bxd}) and C57BL/6 (B6, H-2^{b}, CD45.2) mice were transplanted with 5×10^6 of CB6F$_1$ HSCs depleted of NK and T cells. Splenic RNA was extracted and cDNA generated and PCRs performed. (A) The sequence of the B6 Ly49G Pro1 promoter is shown with two nucleotide differences found in the BALB/c allele indicated below. Transcription factor binding sites shown to play a role in Pro1 activity, as well as a potential TATA element generated by the BALB/c-specific nucleotides are indicated by labeled rectangles. (B) Analysis of BALB/c and B6 Ly49G Pro1 promoter activity in LNK cells is shown. The promoter sequence shown in A was cloned in either the forward or reverse orientation into the pGL3 reporter vector. Values represent the average fold activity relative to an empty vector control and error bars indicate the standard deviation of at least 3 independent experiments. (C) RT-PCR analysis of BALB/c and B6 Ly49G Pro1 forward transcripts. Splenic RNA was isolated 7 days post-transplant from either B6 (F1 into B6) or CB6F$_1$ (F1 into F1) mice receiving a CB6F$_1$ HSCT. PCR with primers specific for either the BALB/c or B6 Ly49G Pro1 forward were used to amplify 1 μl of cDNA from each sample as described in Methods. Three mice were analyzed for each group.