

# Antigen-specific expansion and differentiation of natural killer cells by alloantigen stimulation

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**Natural killer (NK) cells provide important host defense against microbial pathogens and can generate a population of long-lived memory NK cells after infection or immunization. Here, we addressed whether NK cells can expand and differentiate after alloantigen stimulation, which may be important in hematopoietic stem cell and solid tissue transplantation. A subset of NK cell in C57BL/6 mice expresses the activating Ly49D receptor that is specific for H-2D<sup>d</sup>. These Ly49D<sup>+</sup> NK cells can preferentially expand and differentiate when challenged with allogeneic H-2D<sup>d</sup> cells in the context of an inflammatory environment. H-2D<sup>d</sup> is also recognized by the inhibitory Ly49A receptor, which, when coexpressed on Ly49D<sup>+</sup> NK cells, suppresses the expansion of Ly49D<sup>+</sup> NK cells. Specificity of the secondary response of alloantigen-primed NK cells was defined by the expression of activating Ly49 receptors and regulated by the inhibitory receptors for MHC class I. Thus, the summation of signals through a repertoire of Ly49 receptors controls the adaptive immune features of NK cells responding to allogeneic cells.**

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Abbreviations used: HCMV, human cytomegalovirus; ITAM, immunoreceptor tyrosine-based activating motif; MCMV, mouse cytomegalovirus; pi, postinfection.

NK cells recognize abnormal or allogeneic cells by using a repertoire of NK cell receptors that regulates their activation and effector functions (Lanier, 2005). Although NK cells were considered unable to differentiate into memory cells, accumulating evidence demonstrates that NK cells have adaptive immune features, which include antigen-specific expansion and differentiation into a long-lived memory subset (O'Leary et al., 2006; Cooper et al., 2009; Sun et al., 2009a, 2010; Paust et al., 2010; Min-Oo et al., 2013). In some mouse models, NK cells are activated after exposure to pathogens, antigens, and cytokines, and subsequently differentiate into long-lived memory or memory-like NK cells with augmented effector functions in response to a variety of secondary stimuli, as compared with naive NK cells (O'Leary et al., 2006; Cooper et al., 2009; Sun et al., 2009a). The existence of memory NK cells in humans is supported by the specific expansion and persistence for months of NKG2C<sup>high</sup> NK cells after human cytomegalovirus (HCMV) infection (Gumá et al., 2004; Lopez-Vergès et al., 2011; Foley et al., 2012a,b; Min-Oo et al., 2013). We have previously demonstrated that mouse NK cells

bearing the activating Ly49H receptor, which specifically recognizes the m157 mouse cytomegalovirus (MCMV) glycoprotein on the infected cells (Arase et al., 2002; Smith et al., 2002), undergo activation, expansion, contraction, differentiation into memory NK cells, and persistence for several months after MCMV infection (Sun et al., 2009a, 2010). These MCMV-specific memory NK cells are capable of mounting a recall response and provide more effective host protection against rechallenge with MCMV than naive NK cells (Sun et al., 2009a). The immunoreceptor tyrosine-based activating motif (ITAM)-containing DAP12 adapter protein, the proinflammatory cytokine IL-12, and the co-stimulatory DNAM-1 receptor are essential not only for optimal expansion of effector Ly49H<sup>+</sup> NK cells, but also for the generation of long-lived memory Ly49H<sup>+</sup> NK cells after MCMV infection (Sun et al., 2009a, 2012; Nabekura et al., 2014). However, specific receptors, other than Ly49H, that are able to drive

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the clonal expansion and differentiation of NK cells have not been identified. Furthermore, the specificity of the secondary responses of memory NK cells bearing multiple activating receptors also remains unknown, because an experimental system that allows NK cells to expand and differentiate into memory NK cells in a defined receptor-ligand specific manner has not been established, except for MCMV infection.

Cudkowicz and Stimpfling (1964) observed that in certain strains of mice parental bone marrow grafts are rejected by the F1 recipient, and this was subsequently demonstrated to be mediated by NK cells (Kiessling et al., 1977). The inhibitory Ly49 receptors that recognize polymorphic MHC class I ligands are expressed in a stochastic manner on subsets of NK cells in the host (Lanier, 1998; Anderson et al., 2001). As a consequence, in a F1 host, some of the NK cells will lack an inhibitory Ly49 receptor specific for the parental H-2 haplotype. Because they are not inhibited by the parental H-2 ligands, these NK cells are responsible for rejection of the parental graft. Although most Ly49 receptors function as inhibitory receptors for MHC class I, some members of the Ly49 family are activating receptors that transmit signals through the DAP12 and DAP10 signaling molecules (Orr et al., 2009). In C57BL/6 mice a subset of NK cells expresses the activating Ly49D receptor that recognizes H-2<sup>d</sup> alloantigens (George et al., 1999a,b). Some of the Ly49D<sup>+</sup> NK cells in C57BL/6 mice (H-2<sup>b</sup>) coexpress the inhibitory Ly49A receptor that recognizes H-2D<sup>d</sup>, which inhibits rejection of allogeneic cells bearing H-2D<sup>d</sup> (Karlhofer et al., 1992). Because of the structural and signaling similarities shared by Ly49H and Ly49D, we addressed whether an activating signal through Ly49D would result in the expansion and differentiation of Ly49D<sup>+</sup> NK cells in response to alloantigens, similar to the generation of memory Ly49H<sup>+</sup> NK cells during MCMV infection. Here, we established an experimental system for alloantigen-driven expansion and differentiation of Ly49D<sup>+</sup> NK cells. Using this system, we investigated the roles of activating and inhibitory Ly49 receptors in the generation and recall response of NK cells specific for alloantigens.

## RESULTS

### Ly49D<sup>+</sup> NK cells expand and differentiate in response to alloantigen stimulation

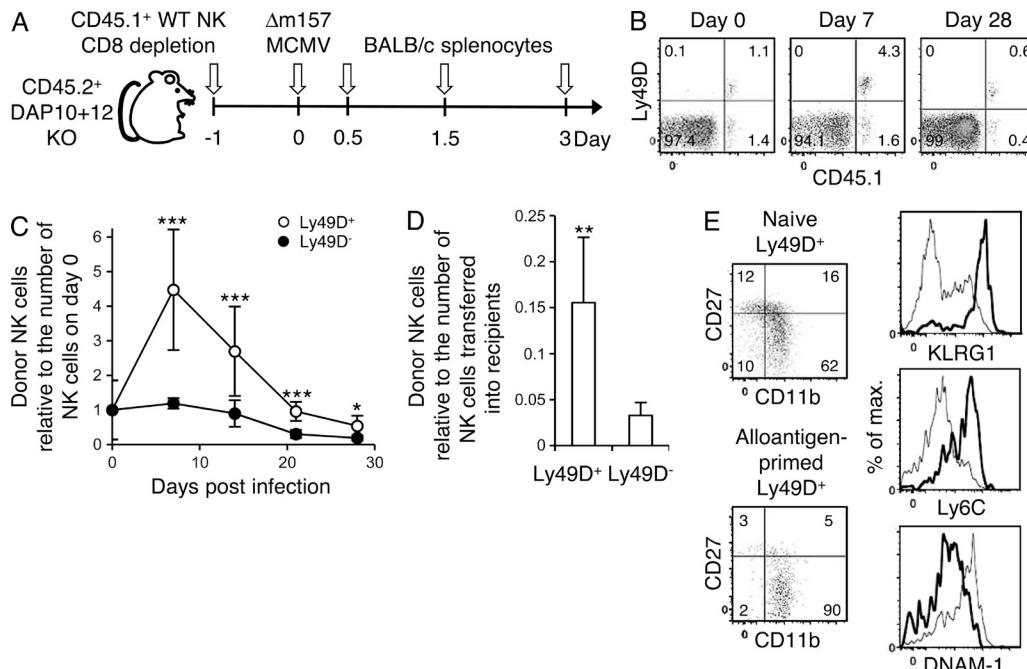
The Ly49D and Ly49H receptors associate with common adapter molecules for their activating signaling (Smith et al., 1998; Lanier, 2009); hence, we speculated that activation through Ly49D, which specifically recognizes H-2D<sup>d</sup>, might induce the expansion and differentiation of Ly49D<sup>+</sup> NK cells in response to alloantigen stimulation. To investigate the fate of Ly49D<sup>+</sup> NK cells that respond to challenge with allogeneic cells, we adoptively transferred WT C57BL/6 NK cells (~50% of NK cells are positive for Ly49D) into syngeneic DAP10 and DAP12 double-deficient (DAP10+12 KO) mice that lack expression of Ly49D and Ly49H, treated the mice with a depleting anti-CD8 mAb to ablate alloreactive CD8<sup>+</sup> T cells, and then injected allogeneic BALB/c splenocytes (depleted from NK cells and T cells to avoid graft-versus-host reactions). Although prior studies have shown that Ly49D<sup>+</sup>

NK cells in C57BL/6 mice reject H-2D<sup>d</sup> splenocytes *in vivo* (George et al., 1999a,b), we observed no expansion of the adoptively transferred Ly49D<sup>+</sup> NK cells nor did we observe alterations in the phenotype of the Ly49D<sup>+</sup> NK cells that would indicate their differentiation. Specifically, we did not detect the up-regulation of KLRG1 and Ly6C and down-regulation of CD27 and DNAM-1 that accompany the transition of Ly49H<sup>+</sup> NK cells from naive to memory cells after MCMV infection (unpublished data).

The expansion and generation of MCMV-specific memory Ly49H<sup>+</sup> NK cells requires IL-12, which is efficiently induced during viral infection (Sun et al., 2012). Therefore, we speculated that the expansion of alloantigen-specific Ly49D<sup>+</sup> NK cells might similarly require robust production of cytokines, such as IL-12, that are not induced by challenge with alloantigen alone. Therefore, as schematically outlined in Fig. 1 A, after adoptive transfer of WT NK cells into DAP10+12 KO recipient mice treated with a depleting anti-CD8 mAb on day −1, the mice were infected with a sublethal dose of a MCMV mutant strain lacking m157 (Δm157 MCMV) to elicit an inflammatory response. The m157-deleted MCMV strain was used to prevent expansion of MCMV-specific Ly49H<sup>+</sup> NK cells, which might complicate interpretation of the results given that a subset of the Ly49D<sup>+</sup> NK cells coexpresses Ly49H. To stimulate the adoptively transferred Ly49D<sup>+</sup> NK cells, BALB/c splenocytes (depleted from T and NK cells to prevent graft-versus-host reactions) were injected intravenously into recipient mice on days 0.5, 1.5, and 3 postinfection (pi; hereafter referred to as Ly49D alloantigen stimulation). In these conditions, donor Ly49D<sup>+</sup> NK cells in the blood preferentially proliferated after Ly49D alloantigen stimulation, whereas Ly49D<sup>−</sup> NK cells did not expand (Fig. 1, B and C). Subsequently, Ly49D<sup>+</sup> NK cells underwent contraction, but were detectable in the blood and the spleen up to a month after Ly49D alloantigen stimulation (Fig. 1, C and D). The same trend was observed in the differentiation of long-lived, alloantigen-primed NK cells on days 28–30 pi in the spleen and blood (unpublished data). The kinetics of the alloantigen-specific Ly49D<sup>+</sup> NK cell response strikingly resemble those of Ly49H<sup>+</sup> NK cells during MCMV infection (Sun et al., 2009a), although the magnitude of the Ly49D<sup>+</sup> NK cell response is less than the MCMV-induced response of Ly49H<sup>+</sup> NK cells. Long-lived, alloantigen-primed Ly49D<sup>+</sup> NK cells displayed the same phenotype (KLRG1<sup>high</sup>, CD27<sup>−</sup>, CD11b<sup>+</sup>, Ly6C<sup>+</sup>, and DNAM-1<sup>−</sup>) as MCMV-specific memory Ly49H<sup>+</sup> NK cells (Fig. 1 E; Sun et al., 2009a; Nabekura et al., 2014). These findings demonstrate that Ly49D is capable of providing the activating signal for expansion and differentiation of Ly49D<sup>+</sup> NK cells, if accompanied by inflammation or infection.

### Expansion and differentiation of Ly49D<sup>+</sup> NK cells are dependent on H-2D<sup>d</sup> and an optimal inflammatory condition

We addressed the factors necessary for the expansion and differentiation of Ly49D<sup>+</sup> NK cells after alloantigen stimulation. As compared with the response of donor Ly49D<sup>+</sup> NK cells after stimulation with BALB/c splenocytes and Δm157 MCMV



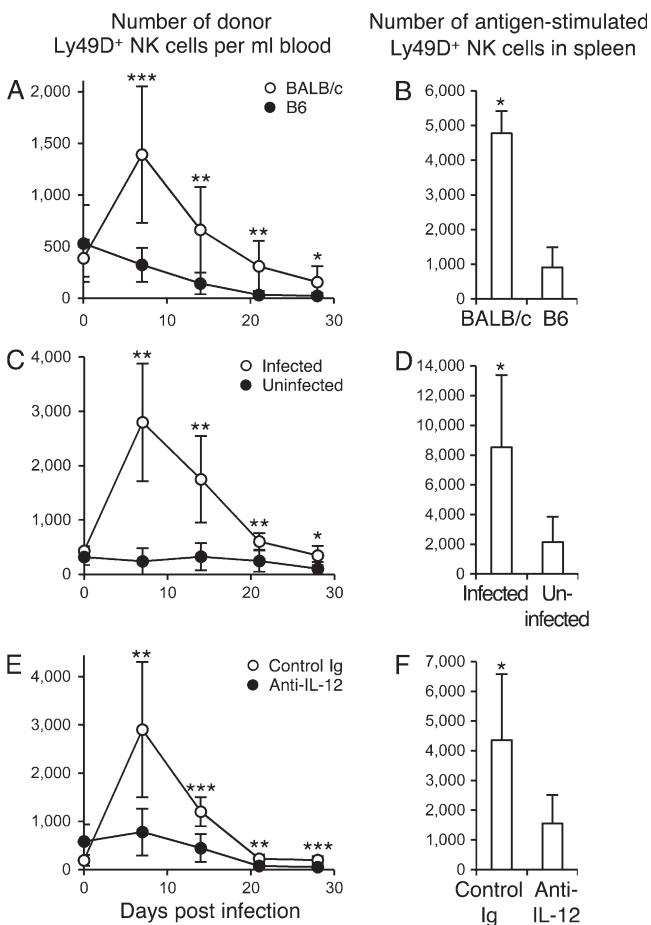
**Figure 1. Ly49D<sup>+</sup> NK cells expand and differentiate after alloantigen stimulation.** (A) 300,000 CD45.1<sup>+</sup> WT NK cells were transferred into CD45.2<sup>+</sup> DAP10+12 KO mice that had been depleted of CD8<sup>+</sup> T cells on the day before infection, and were infected with 5 × 10<sup>4</sup> pfu Δm157 MCMV on day 0. 4,000,000–5,000,000 T cell- and NK cell-depleted BALB/c splenocytes were injected on days 0.5, 1.5, and 3 pi. (B) Percentages of CD45.1<sup>+</sup> donor NK cells in the blood on day 0 (before infection), 7, and 28 pi. (C) The kinetics of the absolute number of Ly49D<sup>+</sup> and Ly49D<sup>-</sup> NK cells in the blood were represented as the ratio relative to the number of Ly49D<sup>+</sup> and Ly49D<sup>-</sup> NK cells in the blood on day 0 (before infection and alloantigen stimulation). (D) Long-lived alloantigen-stimulated Ly49D<sup>+</sup> NK cells in the spleen. The y axis represents the number of long-lived donor Ly49D<sup>+</sup> and Ly49D<sup>-</sup> NK cells detected in the spleen on day 28 pi compared with the number of naive Ly49D<sup>+</sup> and Ly49D<sup>-</sup> NK cells adoptively transferred into the recipient mice on the day before infection. Data are representative of 4 experiments ( $n = 6$ –8 mice per experiment). (E) Phenotype of alloantigen-primed Ly49D<sup>+</sup> NK cells on day 28 pi in the spleen. Expression of CD11b, CD27, KLRG1, Ly6C, and DNAM-1 on NK cells is shown. Bold and thin lines represent alloantigen-primed Ly49D<sup>+</sup> NK cells and naive Ly49D<sup>+</sup> NK cells. Data are representative of 3 experiments ( $n = 3$ –4 mice per experiment). \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.005 versus Ly49D<sup>-</sup>. Error bars show SEM.

infection, donor Ly49D<sup>+</sup> NK cells exhibited neither expansion nor differentiation when stimulated with syngeneic splenocytes accompanying Δm157 MCMV infection (Fig. 2, A and B). Similarly, injections of BALB/c splenocytes without Δm157 MCMV infection did not support expansion and differentiation of Ly49D<sup>+</sup> NK cells (Fig. 2, C and D). Neutralization of IL-12 on the day before Δm157 MCMV infection and day 3 pi impaired the expansion and differentiation of Ly49D<sup>+</sup> NK cells induced by alloantigen stimulation (Fig. 2, E and F), consistent with our prior studies documenting the role of IL-12 in the differentiation of memory Ly49H<sup>+</sup> NK cells during MCMV infection (Sun et al., 2009b, 2012). These results indicate that both the interaction of Ly49D with its ligand and an optimal inflammatory condition to provide IL-12 are required for the acquisition of adaptive immune features by Ly49D<sup>+</sup> NK cells.

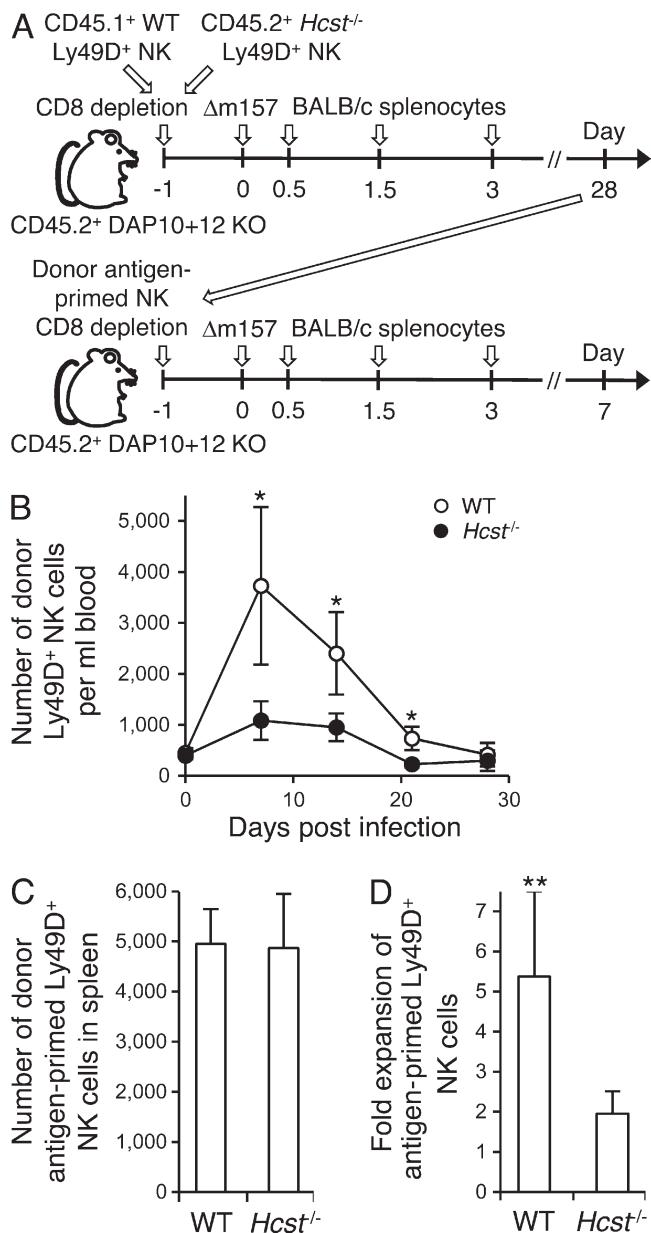
#### DAP10 is required for optimal expansion of alloantigen-specific Ly49D<sup>+</sup> NK cells

Ly49D and Ly49H associate with DAP10 in addition to DAP12 to form activating receptor complexes (Smith et al., 1998; Lanier, 2009). To examine the role of DAP10 in expansion

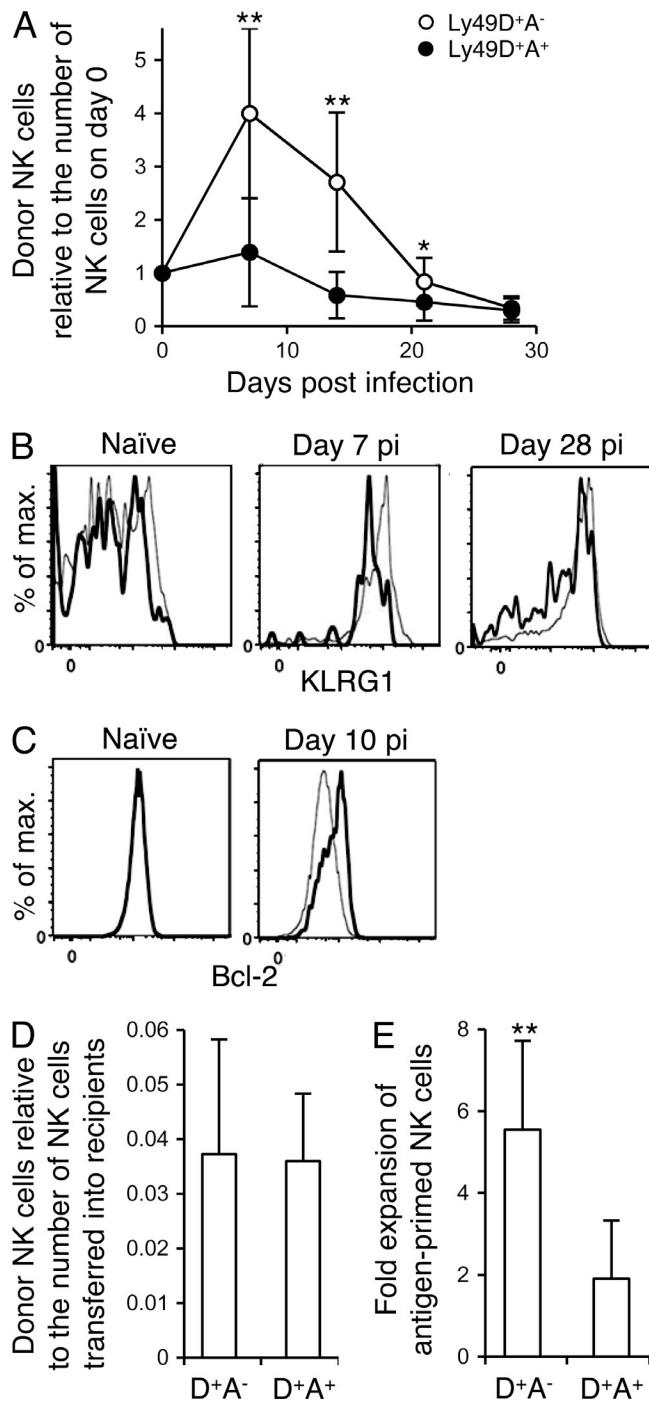
and differentiation of Ly49D<sup>+</sup> NK cells after alloantigen stimulation, CD45.1<sup>+</sup> WT Ly49D<sup>+</sup> NK cells and CD45.2<sup>+</sup> DAP10-deficient (*Hst*<sup>-/-</sup>) Ly49D<sup>+</sup> NK cells were admixed and adoptively transferred into DAP10+12 KO mice, and then stimulated with BALB/c splenocytes after Δm157 MCMV infection (Fig. 3 A). DAP10-deficient Ly49D<sup>+</sup> NK cells showed a significant impairment in the expansion of Ly49D<sup>+</sup> NK cells (Fig. 3 B), consistent with our prior study analyzing the response of Ly49H<sup>+</sup> NK cells to MCMV infection (Orr et al., 2009). However, long-lived Ly49D<sup>+</sup> NK cells were still detected a month after the primary alloantigen stimulation (Fig. 3, B and C), and these DAP10-deficient alloantigen-primed Ly49D<sup>+</sup> NK cells displayed the prototypic memory phenotype (KLRG1<sup>high</sup> CD11b<sup>+</sup> CD27<sup>-</sup> Ly6C<sup>+</sup> DNAM-1<sup>low</sup>) equivalently to WT alloantigen-primed Ly49D<sup>+</sup> NK cells (unpublished data). Naive, alloantigen-stimulated, and long-lived alloantigen-primed DAP10-deficient NK cells and WT NK cells equivalently expressed Ly49D (unpublished data). When DAP10-deficient alloantigen-primed Ly49D<sup>+</sup> NK cells isolated from the primary recipient mice 28 d after the original Ly49D alloantigen stimulation were transferred into naive DAP10+12 KO recipient mice (Fig. 3 A), they again proliferated



**Figure 2.** H-2D<sup>d</sup>, MCMV-induced inflammation, and IL-12 are required for alloantigen-induced expansion and differentiation of Ly49D<sup>+</sup> NK cells. 150,000 CD45.1<sup>+</sup>Ly49D<sup>+</sup> WT NK cells were transferred into CD45.2<sup>+</sup> DAP10+12 KO mice that had been depleted CD8<sup>+</sup> T cells on the day before infection. (A) Recipient mice with transferred Ly49D<sup>+</sup> NK cells were infected with  $5 \times 10^4$  pfu  $\Delta m157$  MCMV on day 0. 4,000,000–5,000,000 T cell- and NK cell-depleted DAP10+12 KO C57BL/6 (B6) splenocytes or BALB/c splenocytes were injected on days 0.5, 1.5, and 3 pi. The absolute number of Ly49D<sup>+</sup> NK cells in the blood is shown. (B) The absolute number of alloantigen-stimulated Ly49D<sup>+</sup> NK cells in the spleen on day 28–30 pi. Data were pooled from 3 experiments ( $n = 7$ –11 mice). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$  versus B6. (C) Recipient mice with transferred Ly49D<sup>+</sup> NK cells were infected on day 0 with  $5 \times 10^4$  pfu  $\Delta m157$  MCMV or left uninfected. T cell- and NK cell-depleted BALB/c splenocytes were injected on days 0.5, 1.5, and 3 pi. The absolute number of Ly49D<sup>+</sup> NK cells in the blood is shown. (D) The absolute number of alloantigen-stimulated Ly49D<sup>+</sup> NK cells in the spleen on day 28 pi. Data were pooled from 2 experiments ( $n = 5$ –8 mice). \*,  $P < 0.05$ ; \*\*,  $P < 0.005$  versus uninfected. (E) Recipient mice with transferred Ly49D<sup>+</sup> NK cells were infected with  $5 \times 10^4$  pfu  $\Delta m157$  MCMV, and T cell- and NK cell-depleted BALB/c splenocytes were injected on days 0.5, 1.5, and 3 pi. Mice were injected with 200  $\mu$ g control Ig or anti-IL-12 neutralizing mAb on the day before infection and day 3 pi. The absolute number of Ly49D<sup>+</sup> NK cells in the blood is shown. (F) The absolute number of alloantigen-stimulated Ly49D<sup>+</sup> NK cells in the spleen on day 29 pi. Data were pooled from 2 experiments ( $n = 6$  mice). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$  versus anti-IL-12. Error bars show SEM.



**Figure 3.** DAP10 is required for optimal expansion of alloantigen-specific NK cells. (A) 150,000 CD45.1<sup>+</sup> WT Ly49D<sup>+</sup> NK cells and CD45.2<sup>+</sup> DAP10-deficient Ly49D<sup>+</sup> NK cells were cotransferred into DAP10+12 KO mice that had been depleted of CD8<sup>+</sup> T cells followed by infection with  $5 \times 10^4$  pfu  $\Delta m157$  MCMV on day 0. 4,000,000–5,000,000 T cell- and NK cell-depleted BALB/c splenocytes were injected on days 0.5, 1.5, and 3 pi. Alloantigen-primed NK cells were isolated from spleens of recipient mice 28 d after primary alloantigen stimulation, transferred into naive DAP10+12 KO mice, and then challenged with Ly49D alloantigen stimulation. (B) The absolute number of Ly49D<sup>+</sup> NK cells in the blood after the primary Ly49D alloantigen stimulation is shown. (C) The absolute number of alloantigen-primed Ly49D<sup>+</sup> NK cells in the spleen on day 28 pi. Data are representative of 2 experiments ( $n = 4$  mice per experiment). (D) Secondary expansion of alloantigen-primed Ly49D<sup>+</sup> NK cells in the spleen on day 7 after alloantigen stimulation was represented as the fold-expansion relative to the number of alloantigen-primed NK cells detected in the spleen of mice adoptively transferred without rechallenge by alloantigen stimulation. Data are pooled from 3 experiments ( $n = 9$  mice). \*,  $P < 0.05$ ; \*\*,  $P < 0.005$  versus  $Hcst^{-/-}$ . Error bars show SEM.



**Figure 4. Ly49A suppresses expansion of naive and alloantigen-stimulated Ly49D<sup>+</sup> NK cells.** (A) 300,000 WT NK cells were transferred into DAP10<sup>+</sup>12 KO mice that had been depleted of CD8<sup>+</sup> T cells on the day before infection, infected with  $\Delta$ m157 MCMV on day 0, and then T cell- and NK cell-depleted BALB/c splenocytes were injected on days 0.5, 1.5, and 3 pi. The kinetics of the absolute number of donor NK cells in the blood were represented as the ratio relative to the number of NK cells in the blood on day 0 (before infection). Data are pooled from 3 experiments ( $n = 9$  mice). (B) Expression of KLRG1 on naïve and alloantigen-stimulated Ly49D<sup>+</sup> NK cells on days 7 and 28 pi in the blood is shown. Bold and thin lines represent Ly49D<sup>A+</sup> NK cells and Ly49D<sup>A-</sup> NK cells, respectively. (C) Expression of Bcl-2 on naïve and alloantigen-stimulated Ly49D<sup>+</sup> NK cells in the spleen on day 10 pi is shown. Bold and thin lines represent Ly49D<sup>A+</sup> NK cells and Ly49D<sup>A-</sup> NK cells. Data are representative of 2 experiments ( $n = 2$  mice per experiment). (D) Alloantigen-primed Ly49D<sup>+</sup> NK cells in the spleen. The y-axis represents the number of donor Ly49D<sup>+</sup> NK cells detected in the spleen on day 28–30 pi compared with the number of naïve Ly49D<sup>+</sup> NK cells adoptively transferred into the recipient mice on the day before infection. (E) Alloantigen-primed NK cells were isolated from spleens of recipient mice 28–30 d after primary alloantigen stimulation, transferred into naïve DAP10<sup>+</sup>12 KO mice, and rechallenged by alloantigen stimulation. Secondary expansion of alloantigen-primed NK cells in the spleen on day 7 after rechallenge by alloantigen stimulation is represented as the fold-expansion relative to the number of NK cells detected in the spleen of mice adoptively transferred with alloantigen-primed NK cells without alloantigen stimulation. Data are pooled from 3 experiments ( $n = 9$  mice). \*,  $P < 0.05$ ; \*\*,  $P < 0.005$  versus D<sup>A+</sup>. Error bars show SEM.

poorly in response to a secondary alloantigen stimulation (Fig. 3 D). These results demonstrate that DAP10 is required for the optimal expansion of naïve and alloantigen-primed Ly49D<sup>+</sup> NK cells, but dispensable for differentiation of alloantigen-stimulated Ly49D<sup>+</sup> NK cells.

#### Ly49A suppresses expansion of alloantigen-stimulated Ly49D<sup>+</sup> NK cells

Both the activating Ly49D receptor and the inhibitory Ly49A recognize H-2D<sup>d</sup> (Karlhofer et al., 1992; George et al., 1999a). We examined the impact of the inhibitory signal through Ly49A on the alloantigen-induced expansion and differentiation of Ly49D<sup>+</sup> NK cells expressing Ly49A (Ly49D<sup>A+</sup>) compared with Ly49D<sup>+</sup> NK cells lacking Ly49A (Ly49D<sup>A-</sup>). Ly49D<sup>A-</sup> NK cells were more proliferative than Ly49D<sup>A+</sup> NK cells at the peak of the initial expansion after alloantigen stimulation (Fig. 4 A). Although both Ly49D<sup>A+</sup> NK cells and Ly49D<sup>A-</sup> NK cells highly expressed KLRG1 after alloantigen stimulation as compared with naïve Ly49D<sup>+</sup> NK cells, alloantigen-stimulated Ly49D<sup>A+</sup> NK cells showed a less activated phenotype than alloantigen-stimulated Ly49D<sup>A-</sup> NK cells 7 and 28 d after the alloantigen stimulation, as indicated by lower expression of KLRG1 (Fig. 4 B). When the alloantigen-stimulated Ly49D<sup>+</sup> NK cells were examined a month after the alloantigen challenge, the skewing toward the Ly49D<sup>A-</sup> NK cell subset was no longer evident (Fig. 4, A and D). Interestingly, Ly49D<sup>A+</sup> NK cells expressed higher amounts of Bcl-2 compared with Ly49D<sup>A-</sup> NK cells in the contraction phase of the response (Fig. 4 C). These results demonstrate that expression of the inhibitory Ly49A receptor suppresses activation of Ly49D<sup>+</sup> NK cells and confers a selective survival advantage during the contraction phase after alloantigen stimulation. When these alloantigen-induced, long-lived Ly49D<sup>+</sup> NK cells were isolated from the primary recipient mice a month after initial stimulation and transferred into naïve recipient mice, followed by a secondary alloantigen challenge, Ly49D<sup>A-</sup> NK cells again showed better secondary

Data are representative of 3 experiments ( $n = 3$ –4 mice per experiment). (C) Expression of Bcl-2 on naïve and alloantigen-stimulated Ly49D<sup>+</sup> NK cells in the spleen on day 10 pi is shown. Bold and thin lines represent Ly49D<sup>A+</sup> NK cells and Ly49D<sup>A-</sup> NK cells. Data are representative of 2 experiments ( $n = 2$  mice per experiment). (D) Alloantigen-primed Ly49D<sup>+</sup> NK cells in the spleen. The y-axis represents the number of donor Ly49D<sup>+</sup> NK cells detected in the spleen on day 28–30 pi compared with the number of naïve Ly49D<sup>+</sup> NK cells adoptively transferred into the recipient mice on the day before infection. (E) Alloantigen-primed NK cells were isolated from spleens of recipient mice 28–30 d after primary alloantigen stimulation, transferred into naïve DAP10<sup>+</sup>12 KO mice, and rechallenged by alloantigen stimulation. Secondary expansion of alloantigen-primed NK cells in the spleen on day 7 after rechallenge by alloantigen stimulation is represented as the fold-expansion relative to the number of NK cells detected in the spleen of mice adoptively transferred with alloantigen-primed NK cells without alloantigen stimulation. Data are pooled from 3 experiments ( $n = 9$  mice). \*,  $P < 0.05$ ; \*\*,  $P < 0.005$  versus D<sup>A+</sup>. Error bars show SEM.

expansion than Ly49D<sup>+</sup>A<sup>+</sup> NK cells, similar to the primary expansion of naive Ly49D<sup>+</sup> NK cells (Fig. 4 E). Collectively, these findings demonstrate that Ly49A suppresses activation and expansion of both naive and long-lived, alloantigen-stimulated Ly49D<sup>+</sup> NK cells, but provides a survival advantage during contraction to the long-lived alloantigen-stimulated Ly49D<sup>+</sup> NK cell subset.

#### Activating and inhibitory Ly49 receptors regulate effector functions and antigen specificity of alloantigen-primed Ly49D<sup>+</sup> NK cells

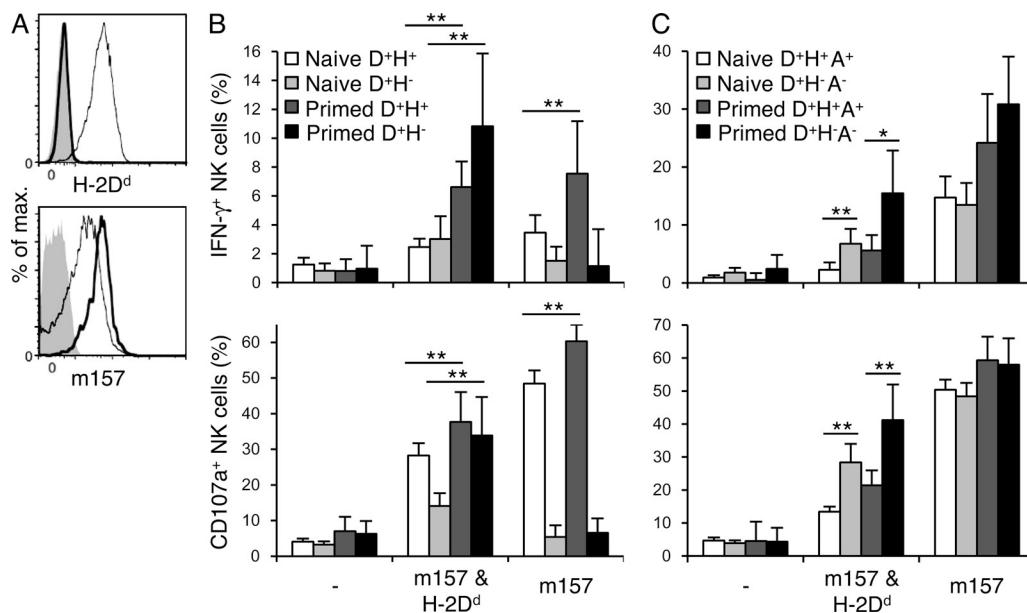
To determine whether Ly49D<sup>+</sup> NK cells generated by alloantigen stimulation exhibit enhanced effector functions as compared with naive Ly49D<sup>+</sup> NK cells, naive and long-lived, alloantigen-stimulated Ly49D<sup>+</sup> NK cells were co-cultured with RMA (H-2<sup>b</sup>) transfectants expressing both m157 and H-2D<sup>d</sup> or m157 alone (Fig. 5 A). Alloantigen-primed Ly49D<sup>+</sup> NK cells bearing Ly49H (Ly49D<sup>+</sup>H<sup>+</sup>) and not expressing Ly49H (Ly49D<sup>+</sup>H<sup>-</sup>) produced more IFN- $\gamma$  and degranulated more efficiently than naive Ly49D<sup>+</sup>H<sup>+</sup> and Ly49D<sup>+</sup>H<sup>-</sup> NK cells after co-culture with target cells expressing m157 and H-2D<sup>d</sup>, respectively (Fig. 5 B). Alloantigen-primed Ly49D<sup>+</sup>H<sup>+</sup> also showed stronger effector functions than naive Ly49D<sup>+</sup>H<sup>+</sup> NK cells when they were co-cultured with m157-expressing target cells in the absence of H-2D<sup>d</sup> (Fig. 5 B). Ly49A suppressed effector functions of naive and alloantigen-primed Ly49D<sup>+</sup>H<sup>+</sup> NK cells only when Ly49D<sup>+</sup>H<sup>+</sup> NK cells were co-cultured with target cells expressing H-2D<sup>d</sup> (Fig. 5 C).

These results show that the Ly49D alloantigen stimulation generates Ly49D<sup>+</sup> NK cells with enhanced effector functions and Ly49A modulates effector functions of alloantigen-primed Ly49D<sup>+</sup> NK cells against H-2D<sup>d</sup>-expressing target cells.

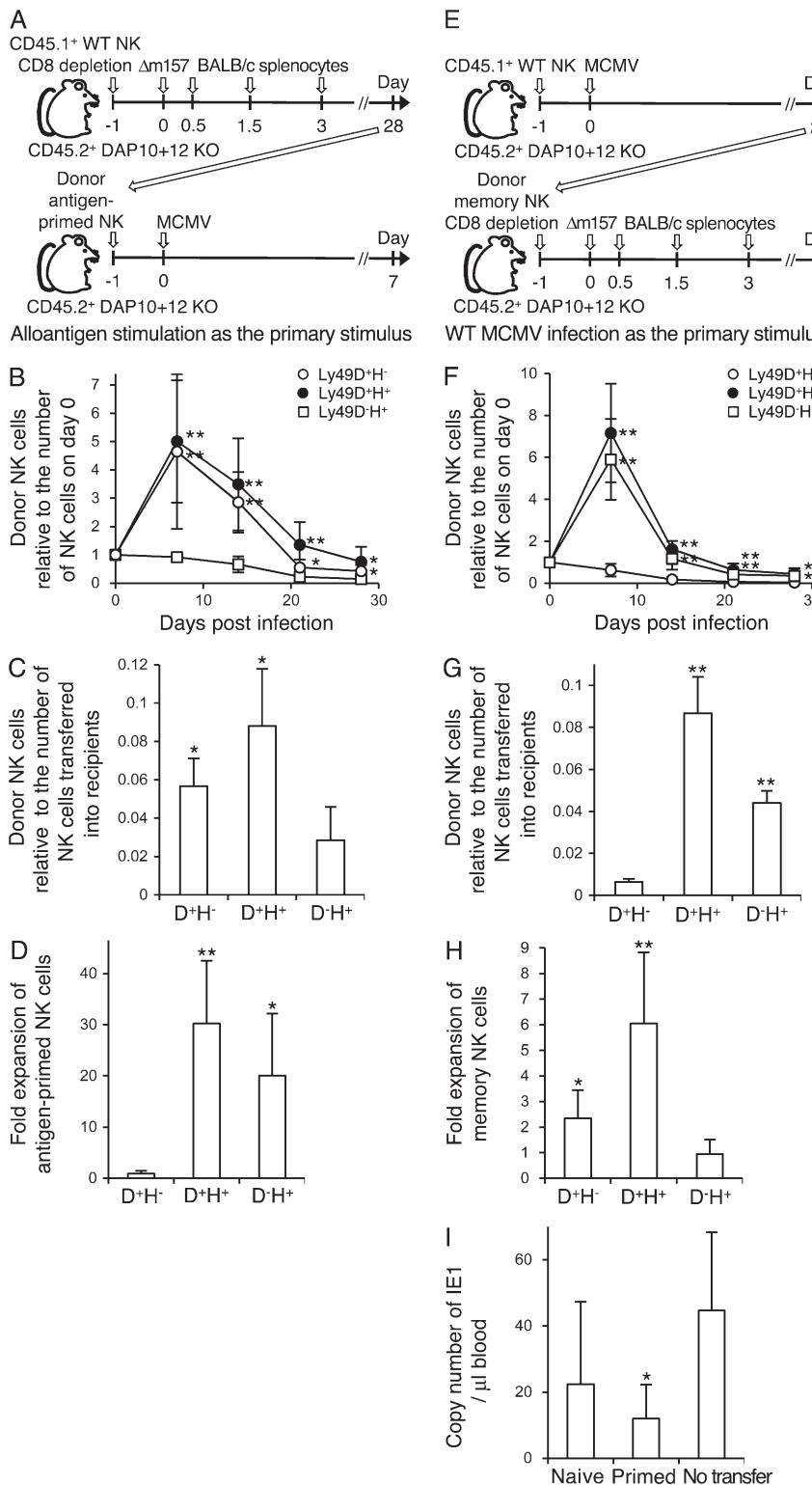
#### The antigen specificity of alloantigen-primed NK cells is defined by expression of activating Ly49 receptors

Because a subset of the alloantigen-stimulated Ly49D<sup>+</sup> NK cells coexpresses Ly49H, we assessed the antigen specificity of the secondary responses of the alloantigen-primed Ly49D<sup>+</sup> NK cells (Fig. 6 A). Ly49D<sup>+</sup>H<sup>-</sup> NK cells and Ly49D<sup>+</sup>H<sup>+</sup> NK cells, but not Ly49D<sup>-</sup>H<sup>+</sup> NK cells, underwent expansion, contraction, and differentiation after the primary Ly49D alloantigen stimulation (Fig. 6, B and C). Ly49D<sup>-</sup> NK cells and long-lived Ly49D<sup>+</sup> NK cells were isolated from the primary recipients 28 d after the primary alloantigen stimulation and were transferred into naive DAP10+12 KO mice, followed by infection with MCMV (Fig. 6 A). Alloantigen-primed Ly49D<sup>+</sup>H<sup>+</sup> NK cells showed a robust expansion after the MCMV infection (Fig. 6 D).

We investigated the specificity of recall responses of memory Ly49H<sup>+</sup> NK cells that had been generated after MCMV infection (Fig. 6 E). When WT NK cells were transferred into DAP10+12 KO mice followed by infection with MCMV, donor NK cell subsets bearing Ly49H efficiently expanded and differentiated into memory NK cells, consistent with our prior studies (Fig. 6, F and G). Donor Ly49H<sup>+</sup> and Ly49H<sup>-</sup> NK cells were isolated from these mice 28 d after infection



**Figure 5. Alloantigen-stimulated Ly49D<sup>+</sup> NK cells exert enhanced effector functions.** Alloantigen-primed Ly49D<sup>+</sup> NK cells were isolated from spleens of recipient mice 25–29 d after alloantigen stimulation. (A) Expression of H-2D<sup>d</sup> and m157 on RMA cell lines. Thin and bold lines represent RMA cells expressing m157 and H-2D<sup>d</sup> and RMA cells expressing m157. Shaded histograms represent RMA cells. (B) IFN- $\gamma$  production and degranulation of naive Ly49D<sup>+</sup>H<sup>+</sup>, naive Ly49D<sup>+</sup>H<sup>-</sup>, and alloantigen-primed Ly49D<sup>+</sup>H<sup>+</sup>, and alloantigen-primed Ly49D<sup>+</sup>H<sup>-</sup> NK cells after co-culture with RMA or RMA cells expressing m157 and H-2D<sup>d</sup> or m157 alone. (C) IFN- $\gamma$  production and degranulation of naive Ly49D<sup>+</sup>H<sup>+</sup>A<sup>+</sup>, naive Ly49D<sup>+</sup>H<sup>+</sup>A<sup>-</sup>, and alloantigen-primed Ly49D<sup>+</sup>H<sup>+</sup>A<sup>+</sup>, and alloantigen-primed Ly49D<sup>+</sup>H<sup>+</sup>A<sup>-</sup> NK cells after co-culture with RMA or RMA cells expressing both m157 and H-2D<sup>d</sup> or m157 alone. Data are pooled from 3 experiments ( $n = 9$ –12 in each group). \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ . Error bars show SEM.



MCMV-specific memory NK cells without alloantigen stimulation. \*, P < 0.05; \*\*, P < 0.01 versus D<sup>-</sup>H<sup>-</sup>. (I) Alloantigen-primed Ly49D<sup>+</sup>H<sup>+</sup> were purified from spleens of recipient mice 28 d after primary Ly49D alloantigen stimulation. Alloantigen-primed Ly49D<sup>+</sup>H<sup>+</sup> NK cells and naive Ly49D<sup>+</sup>H<sup>+</sup> NK cells were transferred into Ly49H-deficient or DAP12-deficient mice and infected with WT MCMV. The copy number of MCMV IE1 gene in blood on day 3 pi was analyzed by quantitative PCR. Data were pooled from 2 experiments (n = 6–10 mice per group). \*, P < 0.05 versus naive. Error bars show SEM.

**Figure 6. Antigen specificity of alloantigen- and MCMV-stimulated NK cells is defined by expression of Ly49D and Ly49H.** (A) 300,000 WT NK cells were transferred into DAP10+12 KO mice followed by alloantigen stimulation. Donor long-lived NK cells were isolated from spleens of recipient mice 28 d after primary alloantigen stimulation, transferred into naive DAP10+12 KO mice, and infected with WT MCMV. Ly49D<sup>+</sup> NK cells were stimulated by the alloantigen stimulation as the primary stimulus for the differentiation of alloantigen-primed Ly49D<sup>+</sup> NK cells (B–D and I). (B) The kinetics of the absolute number of donor NK cells in the blood were represented as the ratio relative to the number of NK cells in the blood on day 0 (before infection). (C) Long-lived alloantigen-primed Ly49D<sup>+</sup> NK cells in the spleen. The y-axis represents the number of long-lived, alloantigen-primed donor Ly49D<sup>+</sup> NK cells detected in the spleen on day 28 pi compared with the number of naive Ly49D<sup>+</sup> NK cells adoptively transferred into the recipient mice on the day before infection. \*, P < 0.05; \*\*, P < 0.005 versus D<sup>-</sup>H<sup>-</sup>. (D) Secondary expansion of long-lived alloantigen-primed NK cells in the spleen on day 7 after infection was represented as the fold-expansion relative to the number of NK cells detected in the spleen of mice adoptively transferred with long-lived, alloantigen-primed NK cells without infection. \*, P < 0.01; \*\*, P < 0.005 versus D<sup>-</sup>H<sup>-</sup>. Data are pooled from 2 experiments (n = 6–8 mice). (E) 600,000 WT NK cells were transferred into DAP10+12 KO mice and infected with WT MCMV. Donor long-lived NK cells were isolated from spleens of recipient mice 28 d after primary MCMV infection, and transferred into naive DAP10+12 KO mice, followed by alloantigen stimulation. Ly49H<sup>+</sup> NK cells were stimulated by WT MCMV infection as the primary stimulus for the differentiation of memory Ly49H<sup>+</sup> NK cells (F–H). (F) The kinetics of the absolute number of donor NK cells in the blood were represented as the ratio relative to the number of NK cells in the blood on day 0 (before infection). (G) Long-lived MCMV-specific memory Ly49H<sup>+</sup> NK cells in the spleen. The y-axis represents the number of long-lived donor Ly49H<sup>+</sup> NK cells detected in the spleen on day 28 pi compared with the number of naive Ly49H<sup>+</sup> NK cells adoptively transferred into the recipient mice on the day before infection. \*, P < 0.01; \*\*, P < 0.005 versus D<sup>-</sup>H<sup>-</sup>. (H) Secondary expansion of long-lived MCMV-specific memory NK cells in the spleen on day 7 after Ly49D alloantigen stimulation is represented as the fold expansion relative to the number of NK cells detected in the spleen of mice adoptively transferred with long-lived

and were transferred into recipient DAP10+12 KO mice followed by alloantigen stimulation (Fig. 6 E). MCMV-induced memory Ly49D<sup>+</sup>H<sup>+</sup> NK cells, but not MCMV-induced memory Ly49D<sup>-</sup>H<sup>+</sup> NK cells, proliferated in response to the Ly49D alloantigen stimulation (Fig. 6 H). Moreover, when alloantigen-primed Ly49D<sup>+</sup>H<sup>+</sup> NK cells generated by the primary alloantigen stimulation were adoptively transferred into naive Ly49H-deficient or DAP12-deficient recipient mice, which lack functionally competent Ly49H<sup>+</sup> NK cells and are unable to control early replication of MCMV (Sjölin et al., 2002; Sun et al., 2009a), they showed an improved protective effect against MCMV challenge on day 3 pi (Fig. 6 I). These results indicate that NK cells coexpressing both Ly49D and Ly49H can be primed to mount a more effective antiviral response when initially stimulated either by alloantigen or viral antigen.

## DISCUSSION

Prior studies have established that NK cells can mount an immune response with many of the features of adaptive immunity when challenged with viruses or chemical haptens (O’Leary et al., 2006; Sun et al., 2009a, 2010; Paust et al., 2010; Min-Oo et al., 2013). Here, we have examined whether alloantigens also elicit the expansion of alloantigen-specific NK cells with enhanced responses upon rechallenge. Because the Ly49D receptor specific for H-2D<sup>d</sup> and the Ly49H receptor specific for MCMV m157 are both expressed in C57BL/6 mice and use the same signaling subunits (e.g., DAP10 and DAP12; Karlhofer et al., 1992; Smith K.M. et al., 1998; Arase et al., 2002; Smith H.R. et al., 2002; Lanier, 2009), we compared the response of Ly49D<sup>+</sup> NK cells to alloantigen stimulation with the well characterized response of Ly49H<sup>+</sup> NK cells to MCMV infection. Although prior studies have demonstrated that Ly49D<sup>+</sup> NK cells in C57BL/6 mice can reject transplanted allogeneic H-2D<sup>d</sup> splenocytes (George et al., 1999a,b), we have determined that alloantigen alone is insufficient to induce the expansion and differentiation of these alloantigen-specific Ly49D<sup>+</sup> NK cells. However, Ly49D<sup>+</sup> NK cells can be induced to expand and differentiate, and mount a more potent recall response, as determined by ex vivo stimulation, when the alloantigen stimulation is accompanied by inflammation, in this case provided by viral infection at the time of alloantigen challenge. As with the generation of MCMV-specific Ly49H<sup>+</sup> memory NK cells (Sun et al., 2012), the alloantigen-specific expansion of Ly49D<sup>+</sup> NK cells was dependent on IL-12 produced in response to the accompanying viral infection. As with allogeneic challenge, when we have injected C57BL/6 mice with syngeneic tumor cells transduced to express m157 these m157-bearing tumors are eliminated by Ly49H<sup>+</sup> NK cells, but m157 alone did not induce expansion and generation of long-lived Ly49H<sup>+</sup> memory NK cells (unpublished data). Rather, the optimal expansion and differentiation of NK cells requires not only a specific ligand for the activating Ly49 receptor but also an appropriate inflammatory environment to provide the necessary growth and differentiation factors.

H-2D<sup>d</sup>-driven expansion of Ly49D<sup>+</sup> NK cells after the Ly49D alloantigen stimulation was less efficient than m157-driven expansion of Ly49H<sup>+</sup> NK cells during MCMV infection (Sun et al., 2010). This is likely explained by the differences in binding affinities of Ly49D and Ly49H to H-2D<sup>d</sup> and m157, respectively. We previously reported that Ly49H has a high affinity for m157 ( $K_d = 0.936 \mu\text{M}$ ; Adams et al., 2007). In contrast, Ly49D has a much lower binding affinity to H-2D<sup>d</sup> than Ly49A and Ly49G, which are inhibitory receptors recognizing H-2D<sup>d</sup> (Ly49A:H-2D<sup>d</sup>  $K_d = 4.4 \mu\text{M}$ ; and Ly49G:H-2D<sup>d</sup>  $K_d = 46.1 \mu\text{M}$ ; Deng et al., 2008; Ma et al., 2014), which was directly quantified by using H-2D<sup>d</sup>-tetramers (Hanke et al., 1999). Therefore, the less efficient expansion of Ly49D<sup>+</sup> NK cells is likely attributed to the lower binding affinity of Ly49D to H-2D<sup>d</sup> and the subsequently weaker activating signaling. Our results demonstrate that DAP10 is required for optimal expansion of alloantigen-specific Ly49D<sup>+</sup> NK cells. The cytoplasmic domain of DAP10 is only 19 aa, and the only motif that is evolutionarily conserved is the YINM sequence that we and others have shown binds to PI3 kinase and Vav1 (Lanier, 2009). Therefore, these signaling molecules would not be recruited to the Ly49D receptor in DAP10-deficient NK cells when it is activated. We also have previously shown that in the absence of DAP10 there is less downstream activation of ERK1/2 when Ly49H is engaged (Orr et al., 2009). Therefore, the diminished expansion of DAP10-deficient alloantigen-stimulated Ly49D<sup>+</sup> NK cells is almost certainly caused by the lack of recruitment of Vav1 and PI3 kinase to the receptor complex, which augments ERK1/2 signaling.

The antigen-specific expansion of both Ly49H<sup>+</sup> and Ly49D<sup>+</sup> NK cells is influenced by coexpression of inhibitory Ly49 receptors on the responding cells. Both the expansion and effector functions of the H-2D<sup>d</sup>-specific response of Ly49D<sup>+</sup> NK cells are restrained in Ly49D<sup>+</sup> NK cells that coexpress Ly49A, an inhibitory receptor for H-2D<sup>d</sup>. However, the long-lived alloantigen-stimulated Ly49D<sup>+</sup> NK cells that persisted for more than a month after the initial challenge were not skewed to a Ly49A-negative subset, compared with the impairment of expansion of Ly49D<sup>+</sup> NK coexpressing Ly49A during the peak of expansion. Similarly, we previously observed that the Ly49H<sup>+</sup> NK cells expanding during MCMV infection preferentially lacked the inhibitory Ly49 receptors recognizing self H-2<sup>b</sup> ligands at the peak of expansion (Orr et al., 2010), but at later times this skewing was no longer evident within the memory NK cell pool (unpublished data). The role of inhibitory receptors is to dampen the activating signals by recruitment of phosphatases, often SHP-1, and the dephosphorylation of key substrates such as Vav1 (Lanier, 2005). The recruitment of phosphatases would impair proliferation initiated through an activating receptor, but also likely suppress activation-induced cell death induced by strong signaling, accounting for the preferential survival of the Ly49A-bearing Ly49D<sup>+</sup> NK cells during the contraction phase of the response. We observed high levels of Bcl-2 in the alloantigen-stimulated Ly49D<sup>+</sup> NK cells coexpressing Ly49A during the contraction phase of the response, likely accounting for their

preferential survival. Thus, we speculate that the inhibitory Ly49 receptors are selected against during the acute phase of an NK cell response, when the inhibitory receptors engaging their self-ligands would restrain expansion and effector functions, but during the contraction phase of the response these inhibitory receptors may provide a survival advantage by dampening signaling to prevent apoptosis.

Because the Ly49 receptors are expressed on overlapping subsets of NK cells, this provided us with the opportunity to determine whether NK cells coexpressing both Ly49H and Ly49D primed by stimulation through one of the receptors would demonstrate an enhanced immune response when subsequently activated in an antigen-specific manner through the other receptor. This was clearly the case. Ly49D<sup>+</sup>H<sup>+</sup> NK cells that had been generated weeks earlier by alloantigen stimulation via Ly49D in vivo exerted enhanced effector functions on a per cell basis in response ex vivo to target cells expressing either m157 and H-2D<sup>d</sup> or m157 alone. More importantly, these alloantigen-primed Ly49D<sup>+</sup>H<sup>+</sup> NK cells provided more potent antiviral activity when challenged with MCMV infection, when compared with naive Ly49D<sup>+</sup>H<sup>+</sup> NK cells. Reciprocally, we previously observed that MCMV-specific Ly49H<sup>+</sup> memory NK cells showed enhanced responses when stimulated ex vivo through other activating receptors, for example NK1.1 (Sun et al., 2009a).

Our studies in the Ly49D model system have important implications for allogeneic transplantation in humans. As with Ly49D, some of the activating KIR in humans have specificity for polymorphic HLA class I ligands, for example KIR2DS1 specifically recognizes HLA-C alleles with a lysine at residue 80 (referred to as group 2 HLA-C; Colonna et al., 1993; Chewning et al., 2007). Our findings would predict that grafted tissues or cells from a donor expressing group 2 HLA-C proteins into a recipient lacking group 2 HLA-C alleles, but expressing KIR2DS1<sup>+</sup> NK cells, likely would be insufficient to cause the expansion and enhanced effector function of these alloantigen-specific NK cells—unless the transplant recipient experienced a viral infection at the time of the transplantation. Viral infection, in particular HCMV reactivation, is common in transplant patients, particularly in patients receiving hematopoietic stem cell transplants. Remarkably, subjects who undergo allogeneic hematopoietic stem cell transplantation for the treatment of acute myeloid leukemia (AML) have a reduced risk of relapse and an advantage of overall survival when the donor and/or recipient are HCMV-seropositive before the transplantation (Behrendt et al., 2009; Elmaagacli et al., 2011). Although the cellular and molecular mechanisms of this anti-leukemia effect in HCMV-seropositive patients are not understood, these observations raise the intriguing possibility that virus-induced memory NK cells bearing activating receptors capable of recognizing AML cells might contribute to the prevention of relapse. Previous studies have reported that AML patients who receive an allogeneic hematopoietic stem cell transplant from a donor with polymorphic inhibitory KIR that are mismatched with the recipients HLA haplotype have a significantly reduced frequency of relapse and

a survival advantage (Miller et al., 2005; Ruggeri et al., 2002, 2007). Moreover, AML patients that receive allogeneic hematopoietic stem cell grafts with a gene encoding the activating KIR2DS1 have a low rate of relapse, and patients receiving allogeneic hematopoietic stem cell grafts with a gene encoding the activating KIR3DS1 exhibit decreased mortality (Venstrom et al., 2012). Collectively, these results suggest that alloantigen-specific or virus-specific stimulation of NK cells may have a beneficial effect on the host response to transplantation, pathogens, and cancer.

## MATERIALS AND METHODS

**Mice and MCMV.** C57BL/6, congenic CD45.1<sup>+</sup> C57BL/6, and BALB/c mice were purchased from the National Cancer Institute. WT, DAP10, and DAP12 double-deficient (DAP10+12 KO; Inui et al., 2009; provided by T. Takai, Tohoku University, Sendai, Japan), DAP10-deficient (*Hcrf*<sup>-/-</sup>; Hykai-Nouspikel and Phillips, 2006), Ly49H-deficient (*Khr8*<sup>-/-</sup>; Fodil-Cornu et al., 2008; provided by S. Vidal, McGill University, Montreal, Quebec, Canada), and DAP12-deficient (*Tyrobp*<sup>-/-</sup>) mice (Bakker et al., 2000) on a C57BL/6 background were maintained at the University of California, San Francisco, in accordance with the guidelines of the Institutional Animal Care and Use Committee. Smith strain WT MCMV and  $\Delta$ m157 MCMV (Bubić et al., 2004; generously provided by U. Koszinowski, Max von Pettenkofer-Institut, Munich, Germany) were prepared by using C57BL/6 3T3 cells as described previously (Bubić et al., 2004). Mice were infected by intraperitoneal injection of  $5-10 \times 10^4$  plaque-forming units (pfu) WT MCMV or  $5 \times 10^4$  pfu  $\Delta$ m157 MCMV.

**NK cell enrichment and adoptive transfer.** NK cells were enriched by incubating splenocytes with purified rat mAbs against mouse CD4, CD5, CD8, CD19, Gr-1, and Ter119, followed by anti-rat IgG antibodies conjugated to magnetic beads (QIAGEN) as described previously (Nabekura et al., 2014). In some experiments, NK cells were stained with antibodies against CD45.1 or CD45.2 and purified by using a FACS Aria III (BD).

**Ly49D alloantigen stimulation and MCMV infection.** Splenocytes from BALB/c mice were depleted of T cells and NK cells by incubating with purified rat mAbs against mouse CD4, CD8, and NKP46, followed by anti-rat IgG antibodies conjugated to magnetic beads. For Ly49D alloantigen stimulation, DAP10+12 KO recipient mice were inoculated intraperitoneally with 200  $\mu$ g of a depletion mAb against mouse CD8 (clones 2.43 or YTS169.4) to prevent cytotoxic T lymphocyte-mediated rejection of BALB/c splenocytes, and  $3 \times 10^5$  WT NK cells or  $1.5 \times 10^5$  WT Ly49D<sup>+</sup> NK cells were injected intravenously into DAP10+12 KO mice on the day before  $\Delta$ m157 MCMV infection. 4,000,000–5,000,000 T cell- and NK cell-depleted BALB/c splenocytes were injected intravenously on days 0.5, 1.5, and 3 pi. In some experiments, mice were inoculated intraperitoneally with 200  $\mu$ g of a neutralizing mAb against mouse IL-12 p40 (clone C17.8) or an isotype-matched rat IgG2a on the day before  $\Delta$ m157 MCMV infection and on day 3 pi. For WT MCMV infection,  $6 \times 10^5$  WT NK cells or  $10^5$  WT Ly49H<sup>+</sup> NK cells were injected intravenously into Ly49H-deficient or DAP10+12 KO mice on the day before infection with MCMV.

**Flow cytometry.** Fc receptors (CD16 and CD32) were blocked with 2.4G2 mAb before surface or intracellular staining with the indicated fluorochrome-conjugated mAbs or isotype-matched control antibodies (BD, eBioscience, BioLegend, or TONBO Biosciences). Samples were acquired on an LSR II or a FACSCalibur (BD) and analyzed with FlowJo software (Tree Star).

**Measurement of MCMV load.** 10,000 naive or alloantigen-primed Ly49D<sup>+</sup> NK cells expressing Ly49H were transferred separately into Ly49H-deficient or DAP12-deficient mice and infected with WT MCMV. The

copy number of MCMV IE1 gene in DNA prepared from peripheral blood on day 3 pi was determined by quantitative PCR analysis using a SYBR green master mix reagent (Roche) as described previously (Nabekura et al., 2014).

**Ex vivo stimulation of NK cells.** 100,000–150,000 NK cells were cocultured with  $1.5 \times 10^5$  RMA or RMA transfectants expressing m157 alone or both m157 and H-2D<sup>d</sup> for 6 h at 37°C in the presence of PE-conjugated anti-CD107a mAb and GolgiStop (BD), followed by staining for surface molecules and intracellular IFN- $\gamma$ .

**Statistical methods.** The Student's *t* test was used to compare results. The Mann–Whitney *U* test was used to compare MCMV viral titers. *P* < 0.05 was considered statistically significant.

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