Natural killer (NK) cells are innate lymphocytes that exhibit adaptive features, such as clonal expansion and memory, during viral infection. Although activating receptor engagement and proinflammatory cytokines are required to drive NK cell clonal expansion, additional stimulatory signals controlling their proliferation remain to be discovered. Here, we describe one such signal that is provided by the adrenergic nervous system, and demonstrate that cell-intrinsic adrenergic signaling is required for optimal adaptive NK cell responses. Early during mouse cytomegalovirus (MCMV) infection, NK cells up-regulated Adrb2 (which encodes the β2-adrenergic receptor), a process dependent on IL-12 and STAT4 signaling. NK cell-specific deletion of Adrb2 resulted in impaired NK cell expansion and memory during MCMV challenge, in part due to a diminished proliferative capacity. As a result, NK cell-intrinsic adrenergic signaling was required for protection against MCMV. Taken together, we propose a novel role for the adrenergic nervous system in regulating circulating lymphocyte responses to viral infection.

Introduction

Natural killer (NK) cells are innate lymphocytes with the ability to kill virally infected, stressed, or transformed cells through the recognition of ligands normally absent in healthy cells, or detection of missing ligands normally present (Lanier, 2008; Yokoyama et al., 2004). Because they express germline-encoded receptors and do not undergo antigen receptor rearrangement, NK cells have traditionally been categorized as a component of the innate immune system. Nonetheless, recent evidence suggests that NK cells exhibit adaptive features during their response against pathogens (Geary and Sun, 2017; Sun and Lanier, 2011; Vivier et al., 2011). Following viral infection in humans, nonhuman primates, and mice, subsets of NK cells have been described to undergo a clonal-like expansion and form a pool of long-lived memory-like cells (Daniels et al., 2001; Dokun et al., 2001; Gumá et al., 2004; Lopez-Vergès et al., 2011; Reeves et al., 2015; Sun et al., 2009). During mouse cytomegalovirus (MCMV) infection, adaptive NK cell responses are triggered through the engagement of the activating receptor Ly49H, expressed by a subset of NK cells, with the virally encoded glycoprotein m157, expressed on infected cells (Arase et al., 2002; Sun et al., 2009). In addition to this receptor–ligand engagement, Ly49H+ NK cells require pro-inflammatory cytokine signals to drive clonal expansion and memory formation (Geary et al., 2018; Madera et al., 2016; Madera and Sun, 2015; Sun et al., 2012). However, the contribution of additional signals driving these adaptive features in NK cells during viral infection remains to be elucidated.

It is now becoming clear that catecholamines released by the adrenergic nervous system (ANS), such as epinephrine and norepinephrine, play a prominent role in regulating innate immune responses to pathogens such as bacteria and helminths (Godinho-Silva et al., 2019; Klose and Artis, 2019; Quatrini et al., 2018a). Stimulation of the β2 adrenergic receptor (β2AR) on tissue-resident immune cells (e.g., macrophages and innate lymphoid cells) by adrenergic neurons results in reduced barrier inflammatory responses during various infectious settings (Gabanyi et al., 2016; Moriyama et al., 2018). Whether β2AR signaling directly impacts circulating lymphocytes, however, has not been clearly addressed. Although global effects of epinephrine on NK cell circulation and function have been described for a variety of settings (Bigler et al., 2015; Breen et al., 2016; Liu et al., 2017; Tarr et al., 2012), the direct cross-talk between the ANS and NK cells during viral infection has not been carefully investigated. In this study, we sought to determine whether adrenergic signaling plays a role in modulating the NK cell response to viral infection, and to elucidate the mechanisms underlying such regulation.
**Results and discussion**

**NK cells localize near splenic adrenergic neurons during viral infection**

Secondary lymphoid organs are heavily innervated by the ANS. In the spleen, most sympathetic nerve fibers, characterized by tyrosine hydroxylase (TH⁺) expression, are located in the white pulp, particularly surrounding central arteries (Murray et al., 2017; Rosas-Ballina et al., 2008). Since interactions between TH⁺ fibers and lymphocytes in the spleen have been shown to be involved in coordinating immune responses to multiple infectious and noninfectious insults (Murray et al., 2017; Prass et al., 2003), we investigated the dynamics of NK cell trafficking in the spleen during viral infection and their relative proximity to splenic adrenergic neurons. Consistent with previous reports (Andrews et al., 2001; Bekiaris et al., 2008; Grégoire et al., 2008), we observed that most NK cells reside in the red pulp area of the spleen at steady-state, resulting in a spatial separation between adrenergic nerve fibers and NK cells (Fig. 1 A). However, during MCMV infection, NK cells trafficked into the white pulp (Fig. 1 B), a process thought to be regulated by pro-inflammatory chemokines (Grégoire et al., 2008). Consequently, NK cells were found in closer proximity to TH⁺ fibers at day 2 post-infection (PI), with the median NK cell–neuron distance decreasing by more than twofold (Fig. 1 C).

**NK cells up-regulate Adrb2 during viral infection in an IL-12- and STAT4-dependent manner**

We next determined whether NK cells possessed the cell-intrinsic ability to directly receive neuron-derived adrenergic signals. Among the nine known adrenoceptor subtypes in mice, resting NK cells predominantly express Adrb2 (encoding β2AR) at steady-state (Fig. 2 A). Interestingly, Adrb2 expression in activated NK cells increased at day 2 PI (Fig. 2 A and Fig. S1 A), coinciding with NK cell infiltration into the white pulp (Fig. 1 B). Early activation of NK cells during viral infection has been previously shown to be mediated through activating receptors such as Ly49H, along with pro-inflammatory cytokines secreted by dendritic cells, including type I IFNs and IL-12 (Madera et al., 2016; Sun et al., 2012). To determine which signals contributed toward the up-regulation of Adrb2 in NK cells during MCMV infection, we investigated the role of IL-12 and STAT4 in this context. We found that IL-12 and STAT4 were required for the up-regulation of Adrb2 in NK cells during MCMV infection. The role of these signals in mediating Adrb2 expression in NK cells during viral infection is further supported by the observation that NK cells from STAT4−/− or IL-12−/− mice were unable to up-regulate Adrb2 upon MCMV infection (Fig. 2 B and Fig. S1 B, right). These findings suggest that IL-12 and STAT4 play a critical role in the up-regulation of Adrb2 in NK cells during viral infection.
infection, we performed RNA-seq on purified NK cells stimulated ex vivo with either IFN-α or IL-12. Adrb2 expression in NK cells increased after activation with IL-12, but not IFN-α (Fig. 2 B and Fig. S1 B), suggesting that IL-12 signaling is sufficient to induce the up-regulation of Adrb2. Interestingly, Adrb2 expression increased further upon costimulation with IL-12 and IL-18, suggesting a synergistic effect of NF-κB and STAT4 signaling (Fig. S1 C). To confirm whether Adrb2 induction on NK cells was similarly regulated during MCMV infection, we generated mixed WT:KO bone marrow chimeric (mBMC) mice, and performed RNA sequencing (RNA-seq) on activated NK cells derived from WT NK cells at steady-state and day 2 PI, as assessed by RNA-seq. Differential expression of Adrb2 is shown for various KO compared with WT NK cells (n = 2 to 3 samples per condition). (C) Normalized counts of fragments binned at 200 bp across a 10-kb window centered on the transcriptional start site (TSS). Data are representative of two independent experiments. (D) Representative tracks of the chromatin accessibility around the Adrb2 locus on purified splenic Ly49H+ NK cells at steady-state and day 2 PI (top), as assessed by ATAC-seq. ATAC-seq peaks with a log2 fold-change > 1 between both conditions are shown in boxes and quantified in graphs (bottom). Data are representative of three independent experiments with three or four replicates per condition. Statistical differences (P < 0.05) are indicated with a single star (*) and were determined using multiple hypothesis-corrected P values as calculated by DESeq2. Norm, normalized, ns, not significant; Unstim, unstimulated. All bar plots show mean ± SEM.

Adrenergic signaling regulates optimal NK cell homeostasis, maturation, and effector function in a cell-intrinsic manner Our observation that NK cells migrate toward adrenergic nerve fibers while up-regulating Adrb2 during viral infection suggests that adrenergic signaling may be required for NK cell responses against MCMV. To address this possibility, we generated Nkp46Cre/+ Adrb2fl/fl mice, in which Adrb2 is specifically deleted in NK cells (referred to as NK-Adrb2−/− mice hereafter). At
steady-state, peripheral NK cell numbers, maturation, and receptor repertoire were comparable to those of littermate control mice (NKp46+/+ Adrb2fl/fl), suggesting that adrenergic signaling is dispensable for NK cell development and homeostasis (Fig. 3 A and Fig. S1, E and F). These findings are consistent with similar transgenic mice generated by Wieduwild et al. in this issue. However, in an mBMC setting, where NK-Adrb2−/− NK cells develop in the same environment as WT NK cells, we observed a reduction in the frequency of NK-Adrb2−/− NK cells across various organs (Fig. 3 B). To further confirm these findings, we...
generated Adrb2-deficient NK cells using two alternative mouse models, Vav1cre/+ Adrb2fl/fl mice and germline Adrb2−/− mice. In a mBMC setting, deletion of Adrb2 in NK cells using either genetic model resulted in a similar reduction in the frequency of circulating NK cells, while Adrb2fl/fl (littermate WT) NK cells were found at a similar frequency compared with CD45.1 WT NK cells in mBMCs (Fig. S1 G).

We also observed modest differences in the acquisition of several maturation markers in the mBMC setting (Fig. 3 C). Interestingly, the maturation differences became even more pronounced when mBMC were challenged with MCMV (Fig. 3 D), suggesting that β2AR may regulate the activation and terminal maturation of NK cells in a cell-intrinsic manner during viral infection.

We next investigated whether direct adrenergic signaling was required for NK cell effector function. During MCMV infection, NK cell activation results in the rapid production and release of IFN-γ and cytotoxic granules containing perforin and granzymes. NK-Adrb2−/− and littermate NK cells degranulated similarly during ex vivo stimulation with pro-inflammatory cytokines, or PMA and ionomycin (Fig. S1, H and I), consistent with findings from Wieduwilt et al. (2020). In the mBMC infected with MCMV, NK-Adrb2−/− NK cells exhibited a modest albeit reproducible defect in IFN-γ production, which was independent of their maturation stage (Fig. 3 E and (Fig. S1 J). To test whether such defect could have a functional relevance for NK cell-mediated killing, we transferred mi517-expressing target splenocytes and nontarget controls into either NK-Adrb2−/− or littermate NK cells, and assessed their ability to clear target cells in vivo. Compared with WT mice, NK-Adrb2−/− mice cleared target cells less efficiently (Fig. 3 F), suggesting that adrenergic signals present during a viral infection, but absent in an ex vivo setting, may be able to control certain effector functions of NK cells.

Adrenergic signaling drives optimal adaptive NK cell responses and protection during viral challenge

During MCMV infection, Ly49H+ NK cells can expand 100–1,000-fold to increase the pool of effector cells, an “adaptive” feature required for the proper control of the virus (Beaulieu et al., 2014; Sun et al., 2009). Given its modest role in modulating early NK cell effector functions, we investigated whether adrenergic signaling is required for adaptive NK cell responses. To directly test this, we adoptively transferred equal numbers of congenically distinct NK-Adrb2−/− and WT NK cells into Rlra8−/− (Ly49H-deficient) mice, infected these recipients with MCMV, and measured the expansion of transferred Ly49H+ NK cells in peripheral blood at various time points (Fig. 4 A). Whereas WT Ly49H+ NK cells underwent robust expansion, NK-Adrb2−/− Ly49H+ NK cells exhibited impaired expansion, representing less than half of all transferred effector cells at day 7 PI (Fig. S2 A and Fig. 4 B). NK cells from littermate controls expanded comparable to WT controls in this setting, while Adrb2-deficient NK cells generated using two alternative genetic models exhibited a similar expansion defect (Fig. S2, B and C). Diminished numbers of NK-Adrb2−/− NK cells were also observed at memory time points (Fig. S2 A), likely a consequence of defects observed in early expansion, as overall WT:KO ratios remained stable past day 7 PI, during the contraction and memory phases (Fig. S2 D).

Adrenergic signaling has been involved in the rapid redistribution of lymphocytes between lymphoid and nonlymphoid organs (Leiper, 1904; Pedersen et al., 2016), raising the possibility that the observed expansion defect of NK-Adrb2−/− NK cells in peripheral blood could be due to impaired circulation or trafficking. However, following adoptive transfer and MCMV infection, we observed a consistent reduction in NK-Adrb2−/− NK cell frequencies in both lymphoid and nonlymphoid organs (Fig. 4 C), suggesting that adrenergic signaling is required for general NK cell expansion and not restricted to any particular organ. We next investigated whether direct signaling by adrenergic fibers was required for robust NK cell expansion. We antagonized the activity of adrenergic fibers with oxidopamine (6-OHDA) and metyrosine (MTR), as described previously (Murray et al., 2017; Staedtke et al., 2018). WT NK cells transferred into vehicle-treated Ly49h−/− recipient mice expanded significantly more at day 7 PI than Adrb2-deficient NK cells, as reported in Fig. 4 C, and also significantly more than WT NK cells transferred into antagonist-treated Ly49h−/− mice, suggesting that adrenergic signaling is required for the optimal expansion of WT NK cells (Fig. 4 D). Furthermore, the expansion of Adrb2-deficient NK cells transferred into either vehicle- or antagonist-treated mice was comparable to that of WT NK cells in the antagonist-treated mice, suggesting that the impaired NK cell expansion observed in antagonist-treated mice occurs in an Adrb2-dependent manner (Fig. 4 D).

We next sought to determine how β2AR deficiency limited NK cell expansion during MCMV infection, and whether this would result in decreased host protection. We adoptively transferred NK cells labeled with the division-tracking dye CellTrace Violet (CTV), and found that NK-Adrb2−/− NK cells did not divide as efficiently as their WT counterparts (Fig. 5, A and B). NK-Adrb2−/− NK cells did not have elevated levels of apoptosis as measured by FLICA staining (Fig. S2 E), suggesting that a proliferation defect underlies the diminished clonal expansion observed.

To confirm that the diminished proliferative capacity of NK-Adrb2−/− NK cells is critical for protection against MCMV infection, we transferred purified NK cells from either NK-Adrb2−/− mice or littermate controls into Rag2−/− Il2rg−/− mice (which lack T, B, and NK cells), and measured their survival and viral load after MCMV challenge (Fig. 5, C and D). Mice receiving NK-Adrb2−/− NK cells were significantly more susceptible to MCMV than those receiving WT NK cells (Fig. 5 C). Furthermore, although mice receiving Adrb2-deficient NK cells had a comparable viral load to those receiving WT NK cells at earlier infection time points, they bore a much higher viral load at later infection time points, coinciding with the peak of NK cell expansion in this model (Fig. 5 D and Fig. S2 F). Consequently, transfer of WT NK cells, but not NK-Adrb2−/− NK cells, increased the median survival of infected Rag2−/− Il2rg−/− mice compared with mice that received no cells. Thus, our findings suggest that cell-intrinsic adrenergic signaling is required for optimal adaptive NK cell responses and host protection against viral infection (Fig. 5 E).

A growing body of evidence supports the notion that the adaptive features of NK cell responses, including prolific expansion and memory formation, are critical for host protection against viral infection (Lau and Sun, 2018; O’Sullivan et al., 2015;
Here, we reveal that costimulatory signals provided through β2ARs regulate these processes and are required for NK cell-mediated protection against MCMV. Global, transient stimulation of glucocorticoid and adrenergic pathways, as occurs during the fight-or-flight response or stroke episodes, have previously been described to modulate NK cell recruitment and function (Breen et al., 2016; Izumo et al., 2013; Prass et al., 2003; Quatrini et al., 2017, 2018b). However, whether NK cells can directly sense adrenergic signals had not been previously determined. Consistent with findings from Wieduwild et al. (2020), cell-intrinsic adrenergic signaling was largely dispensable for NK cell development, homeostasis, and early effector function against MCMV infection. In contrast to the early innate responses, in the present study we show that cell-intrinsic adrenergic signaling was necessary to elicit robust adaptive NK cell responses, revealed in adoptive transfer experiments where NK cells could undergo large proliferative bursts. Mainly, the proliferative capacity of NK cells was essential to control viral replication in immune-deficient hosts and improve survival against MCMV.

Interestingly, Wieduwild et al. (2020) show that indirect, cell-extrinsic adrenergic signaling acting through a non-neuronal, nonhematopoietic compartment negatively regulates IFN-γ production by liver NK cells, reducing host immune defense against viral challenge. In this case, the improved survival of Adrb2−/− mice was attributed to increased IFN-γ production, a hallmark of the early NK cell effector function. Thus, we believe that adrenergic signals control the function and proliferation of NK cells through distinct, nonexclusive mechanisms that differ in the timing and nature of the interactions between the immune and ANS. These complementary layers of regulation should be considered when designing therapies to target both NK cell activity and adrenergic pathways, or when given in combination with β-blockers commonly used in the clinic.

Recent reports have suggested that neural mediators (e.g., neuropeptides and catecholamines) can modulate group 2 innate lymphoid cell (ILC2) responses in the gut and lung (Cardoso et al., 2017; Klose et al., 2017; Moriyama et al., 2018; Wallrapp et al., 2017). Although they share a common lymphoid progenitor, conventional NK cells and ILC2s have distinct developmental requirements and functions. Whereas ILC2s are tissue-resident and exert their immune-modulatory properties through cytokine signals to mediate type II inflammation, NK cells are circulating and respond against MCMV infection via antigen receptor engagement (along with pro-inflammatory cytokines) to drive a potent type I immune response. Thus, we believe that signals traditionally associated with the ANS differentially regulate tissue-resident versus circulating lymphocytes during inflammatory or infectious states. Although NK cells are found in proximity to adrenergic neurons shortly after infection, and ablation of adrenergic neurons impairs NK cell proliferation in an Adrb2-dependent manner, the precise nature and duration of the interaction require further investigation. Future studies will also test whether the response of other circulating lymphocytes, such as virus-specific CD8+ T cells, may be controlled by adrenergic signaling in a similar fashion to Ly49H+ NK cells. In summary, our findings suggest that cell-intrinsic adrenergic signaling promotes adaptive NK cell responses during viral infection and provide new insights into how the nervous system may coordinate the immune response against pathogens.

Materials and methods

Mice

Mice used in this study were housed and bred at Memorial Sloan Kettering Cancer Center (MSKCC) under specific pathogen-free conditions. Experiments were conducted using age- and gender-matched mice (typically 6–10 wk old) in accordance with
approved institutional protocols from the Institutional Animal Care and Use Committee at MSKCC. The strains used in this study, all on the C57BL/6 background, are listed below: C57BL/6 (CD45.2), B6.SJL (CD45.1), Adrb2fl/fl (Hinoi et al., 2008), Klra8−/− (Ly49H-deficient; Fodil-Cornu et al., 2008), Nkp46iCre (referred to as NKp46Cre; Gazit et al., 2006), Nkp46iCre (referred to as NKp46Cre; Gazit et al., 2006), Nkp46iCre (referred to as NKp46Cre; Gazit et al., 2006), Nkp46iCre (referred to as NKp46Cre; Gazit et al., 2006), Vav1-iCre (referred to as Vav1cre; The Jackson Laboratory), Adrb2−/− (Chruscinski et al., 1999), Rag2−/− Il2rg−− (Taconic), Rosa26tdTom (The Jackson Laboratory), Stat1−/− (Meraz et al., 1996), and Stat4−/− (Kaplan et al., 1996). B6 CD45.1 × CD45.2, Klra8−/− CD45.1 × CD45.2, Nkp46Cre × Rosa26tdTom, and Nkp46Cre × Adrb2fl/fl mice were generated at MSKCC. Adrb2fl/fl mice were generously provided by Dr. Daniel Mucida (The Rockefeller University, New York, NY) with the permission of Dr. Gerard Karsenty (Columbia University, New York, NY). Adoptive transfer studies and the generation of mixed bone marrow chimeric mice were performed as previously described (Beaulieu et al., 2014; Sun et al., 2009).

Virus infection

MCMV (Smith strain) viral stocks were prepared by serial passage through BALB/c hosts (twice), and later dissociation of salivary glands 3 wk PI. For adoptive transfer studies, animals were infected i.p. with 7.5 × 10^2 PFU of MCMV. For mBMC infections, mice received 7.5 × 10^3 PFU of MCMV. For survival studies in Rag2−/− Il2rg−− recipients, mice were infected by i.p. injection of 10^4 PFU of MCMV.

Histology

For immunofluorescence staining, spleens from Nkp46-tdTomato or NK1.1-GFP reporter mice were incubated overnight at room temperature with fixative solution (1% paraformaldehyde, 1% PFA).

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80 mM L-lysine, and 10 μM sodium periodate in phosphate buffer), washed with PBS, and dehydrated in 30% sucrose (in PBS) overnight. Spleens were placed in TissueTek optimal cutting temperature compound (Sakura) and frozen in dry ice and ethanol. 20-μm spleen cryosections were rehydrated in PBS, blocked with 5% goat serum and 2% FCS in PBS, and stained with rabbit anti-TH (ABI52, Millipore) in antibody diluent (2% FCS in PBS) overnight at 4°C. Slides were washed twice with antibody diluent and stained with Alexa 470 goat anti-rabbit IgG (A21244, Invitrogen), and Alexa 488 rabbit anti-GFP (A21311, Invitrogen) when necessary. Slides were stained with DAPI nuclear staining and preserved with Pro-Long glass mountant (Thermo Fisher). Images were acquired using a Zeiss LSM 880 confocal microscope (Zeiss) and processed as TIFF files using ImageJ software (open source). For quantification of NK cell–neuron distances, single-channel images were converted into a binary format, and black pixels were quantified using the “analyze particle” function. The resulting regions of interest were measured with the “measure” function to obtain the xy-positions of the regions of interest. The cell contours and the xy-positions were exported, and the distances between neurons and NK cells were measured with the R software.

Flow cytometry and cell sorting

Cell surface staining of single-cell suspensions from various organs was performed using fluorophore-conjugated antibodies (BD Biosciences, eBioscience, BioLegend, Tonbo, R&D Systems). Intracellular staining was performed by fixing and permeabilizing with the BD Cytofix/Cytoperm Fixation/Permeabilization Solution kit for staining cytosolic proteins and meabilizing with the BD Cytofix/Cytoperm Fixation/Permeabilization Solution kit for staining cytosolic proteins and cytokines.

Flow cytometry and cell sorting were performed on the LSR II and Aria II cytometers (BD Biosciences), respectively. For RNA-seq experiments, quantitative real-time PCR (RT-qPCR), and for NK cell transfers into Rag2−/−IItg2−/−, cell populations were sorted to >95% purity. Data were analyzed with FlowJo9 software (Tree Star). Flow cytometry of purified lymphocytes was performed using the following fluorochrome-conjugated antibodies: CD3ε (17A2), TCRβ (H57-597), CD19 (ID3), F4/80 (BM8.1), Ly6G (IA8), NK1.1 (PK136), NKp46 (29A1.4), Ly49H (3D10), CD45.1 (A20), CD45.2 (104), CD11b (M1/70), CD27 (LG.3A10), KLRG1 (2F1), Ly49D (4ES), Ly49A (YE1/48.10.6), Ly49C/L (5E6), CX3CR1 (SAO1F11), CD25 (PC61), CD69 (H1.2F3), Granzyme B (G911), IFN-γ (XMG1.2), CD107α (ID4B), and CD49b (DX5). NK cell proliferation was analyzed by labeling cells with 5 μM CTV (Invitrogen) before transfer, and CTV labeling was performed according to manufacturer protocol. NK cell apoptosis was measured by staining transfected cells with FAM-DEVD-FMK FLICA reagent (FLICA, ImmunoChemistry Technologies) to measure active caspase-3/7 in live cells according to the manufacturer protocol.

Assessment of in vivo NK cell clearance of target cells

To study the killing of target cells by Ly49H+ NK cells in vivo, equal numbers of m157-expressing splenocytes (targets) and Ly49H-deficient splenocytes (controls) were transferred into either NK-Adrb2−/− or littermate mice. Blood was collected 4–6 h following transfer, and the percentage of target killing was calculated as the ratio of remaining targets to control cells, normalized to the initial ratio before transfer.

Ablation and antagonization of adrenergic neurons

Chemical ablation of adrenergic nerves was performed by treating Ly49h−/− recipient mice with two doses of 6-OHDA (250 mg/kg, Sigma) and MTR (80 mg/kg, Santa Cruz Biochemicals, sc-219470) at day -1 and day 3 PI. Equal numbers of Vav1cre−/−Adrb2fl/fl and congenically distinct WT Ly49H+ NK cells were transferred intravenously, and numbers and frequency of transferred cells analyzed at day 7 PI.

RNA-seq, H3K4Me3 chromatin immunoprecipitation (ChIP), DNA sequencing, and analysis

Isolation of RNA, library preparation, and data analysis of ex vivo NK cells PI and in vitro stimulated NK cells have been previously described (Geary et al., 2018; Lau et al., 2018; Rapp et al., 2017). Briefly, 1.5–10 × 10⁴ Ly49H+ NK cells (TCRβ−CD19−CD3εNK1.1+CD49b+Ly49H+) were sorted from C57BL/6 mice, or WT:KO (Stat4−/−IIfn1−/−, Statt−/−) mBMC mice at days 0 and 2 after MCMV infection. For in vitro stimulations, 5–8 × 10⁴ NK cells were sorted for purity and incubated overnight with 20 ng/ml of IL-12, or 100 U/ml of IFNα. RNA was extracted by Trizol and prepared for library construction and Illumina Next generation sequencing.

Isolation of DNA for H3k4me3 ChIP-seq and data analysis was performed and analyzed as described previously (Rapp et al., 2017). Briefly, 5–10 × 10⁶ NK cells (TCRβ−CD19−CD3εNK1.1+CD49b+) from WT or Stat4-deficient mice were sorted from pooled spleens and incubated overnight (~18 h) with IL-12 (20 ng/ml) and IL-18 (10 ng/ml). DNA and proteins were cross-linked for 8 min using 1% formaldehyde. ChIP was performed using 1 mg of rabbit polyclonal anti-trimethyl Histone H3 (Lys4) antibody (H3K4me3, Millipore, 07474), and prepared for Illumina next-generation sequencing.

RT-qPCR

5 × 10⁴ purified (>95% purity) NK cells were stimulated with IL-12 (20 ng/ml), IL-18 (10 ng/ml), or a combination of both, or were left untreated for 3 h, and RNA was extracted with the PicoPure RNAIsolation Kit (Arcturus). cDNA was synthesized using the QuantiTect Reverse transcription kit (Qiagen). RT-qPCR with cDNA as a template was performed in triplicate on an ABI QuantStudio 6 Flex RT-qPCR machine, using Taqman Probes for Agrp2 (Mm02524224_s1, Thermo Fisher) and Actb (Mm02668550_g1, Thermo Fisher). Relative Agrp2 gene expression was normalized to that of Actb using the 2−ΔΔCt method.

Quantification of viral titers from peripheral blood

At indicated time points, 200 μl of peripheral blood was drawn retro-orbitally, and total DNA was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN). Viral copy number in eluted DNA was determined in triplicate by quantitative PCR using iQ SYBR mastermix (Bio-Rad) using the MCMV-specific primers IE-1 (forward: 5′-TCGCACCATGTTCGAGA-3′, reverse: 5′-TCTGAAAATACCCCTCACTGAG-3′) and IE-2 (forward: 5′-ATGCGGTTTATTGCATGCTG-3′, reverse: 5′-CTCTTCACGAGTGATGAGTG-3′) and analyzed using a 7900HT real-time PCR machine (Applied Biosystems). The viral copy number was corrected to the input viral copy number of 1 × 10⁵.
GTAGGGCTCCACTGACCGA-3'). A standard curve of MCMV plasmid DNA was used to calculate the viral copy number in sample DNA by correlating their quantitation cycle (Cq) values.

Data availability
The sequencing data supporting these findings (RNA-seq, ATAC-seq, and ChIP-seq) have been deposited in the Gene Expression Omnibus public database under accession no. GSE106139. All other data presented in the current study are available from the corresponding author upon reasonable request.

Statistical analyses
Unless otherwise indicated, data represented as bar graphs are plotted as mean ± SEM. For experiments in which one variable was compared across two conditions, statistical differences were calculated using a Tukey’s multiple comparison test (one-way ANOVA). For experiments in which two variables were compared across multiple conditions, statistical differences were calculated using Sidak’s or Tukey’s multiple comparisons test (two-way ANOVA). Statistical differences in survival curves were determined by Mantel–Cox test analysis. A P value of <0.05 was used as the significance cutoff (*). Statistical analysis was performed using GraphPad Prism software.

Online supplemental material
Fig. S1 shows the expression of Adrb2 in NK cells under IL-12 stimulation and infection as assayed with RT-qPCR; the maturation state and ex vivo stimulation of NK-Adrb2−/− NK cells compared with littermate controls; and the mBMC reconstitution of Adrb2−/− NK cells (obtained with different genetic models) and IFN-γ production upon infection compared with WT controls. Fig. S2 shows the expansion of Adrb2−/− NK cells (obtained with different genetic models) and littermate controls compared with congenically distinct WT cells throughout infection; the percentage of KO and WT NK cells undergoing apoptosis during infection; and the change in viral titers during infection.

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Figure S1. **Adrb2 is dispensable for NK cell effector function in vitro and maturation in a noncompetitive setting.** Related to Figs. 2 and 3. (A) Real-time PCR analysis of Adrb2 mRNA in WT NK cells sorted at steady-state or day 2 PI (n = 3 samples per condition). (B and C) Real-time PCR analysis of Adrb2 mRNA in WT NK cells stimulated with IL-12 or left unstimulated (B), or stimulated with a combination of IL-12 and/or IL-18 (C) normalized to mRNA levels in unstimulated NK cells (n = 3 samples per condition). (D) Real-time PCR analysis of Adrb2 mRNA in Stat-deficient Ly49H+ NK cells sorted at steady-state or day 2 PI from WT: Stat1−/− and WT: Stat4−/− mBMCs, normalized to mRNA levels in WT Ly49H+ NK cells at each time point (n = 2 or 3 mice per group). (E) Representative flow plots showing the expression of maturation markers CD11b and CD27 NK cells from NK-Adrb2−/− or littermate Adrb2fl/fl mice at steady-state. Graph shows the quantification of the percentage of NK cells expressing these markers. Representative of three independent experiments with five mice per group. (F) Percentage of circulating NK cells expressing various activating (Ly49H, Ly49D) and inhibitory (Ly49A) receptors, and markers of terminal maturation (KLRG1, CX3CR1) at steady-state. Representative of two independent experiments with four to six mice per group. (G) Quantification of circulating NK cells (NK1.1+ CD49b+ Lin−) as percentage of total CD45+ cells in mixed WT(CD45.1): Adrb2-deficient (CD45.2) mBMC mice at steady-state, generated using the genetic mouse models indicated, or WT(CD45.1): Adrb2fl/fl littermate (CD45.2) mBMCs. Representative of two independent experiments with at least five mice per group. Statistical differences (P < 0.05) were calculated using Sidak’s multiple comparisons test (two-way ANOVA) and indicated with a single star (*). (H and I) Representative histogram of the percentage of splenic NK cells from NK-Adrb2−/− or littermate Adrb2fl/fl mice producing IFN-γ (H) or degranulating (I) after 4 h ex vivo stimulation with IL-12 and IL-18 or PMA and ionomycin, respectively. Representative of two independent experiments with two samples per condition. (J) Quantification of the production of IFN-γ (shown as MFI) by Adrb2−/− and WT NK cells at each maturation stage 2 d following MCMV infection in a mBMC setting. Representative of two independent experiments with four mice per group. Statistical differences (P < 0.05) were calculated using Sidak’s multiple comparisons test (two-way ANOVA) and indicated with a single star (*). Iono, ionomycin. All bar plots show mean ± SEM.
Figure S2. β2AR is required for NK cell expansion but not memory maintenance. Related to Figs. 4 and 5. (A) Representative flow plots showing the percentages of transferred WT and NK-Adrb2−/− Ly49H+ NK cells of total circulating NK cells at day 7 and 28 PI. (B) The percentage of transferred WT and littermate Adrb2fl/fl Ly49H+ NK cells was quantified in peripheral blood as indicated. Data are pooled from two independent experiments with four or five mice per group. (C) The ratio of transferred WT and Adrb2-deficient Ly49H+ NK cells from the indicated genetic mouse models, or littermate Adrb2fl/fl controls, was calculated in peripheral blood at day 7 PI. Representative of two independent experiments with five mice per group. (D) The ratio of transferred WT and NK-Adrb2−/− Ly49H+ NK cells was quantified in peripheral blood weekly as indicated. Representative of three independent experiments with five mice per group. (E) As in Fig. 4 C. The percentage of apoptotic WT and NK-Adrb2−/− Ly49H+ NK cells (as detected by FLICA stain, marking active caspases in live cells) was measured at day 4 PI. Data are representative of two independent experiments with four mice per group. (F) As in Fig. 5 D. The viral replication rate for each mouse is presented as fold increase in viral titers between each two consequent time points. Statistical differences (P < 0.05) were calculated using Sidak’s multiple comparisons test (two-way ANOVA) and indicated with a single star (*). All bar plots show mean ± SEM.