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$\alpha 2\beta 1$ integrin is required for optimal NK cell proliferation during viral infection but not for acquisition of effector functions or NK cell-mediated virus control

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Abstract

Natural killer (NK) cells play an important role in antiviral resistance. The integrin $\alpha 2$ ($\alpha 2$), which dimerizes with integrin $\beta 1$ ($\beta 1$), distinguishes NK cells from Innate Lymphoid Cells 1 (ILC1) and other leukocytes. Despite its use as an NK cell marker, little is known about the role of $\alpha 2\beta 1$ in NK cell biology. Here we show that in mice, $\alpha 2\beta 1$ deficiency does not alter the balance of NK cell/ILC1 generation, and slightly decrease the number of NK cells in the bone marrow and spleen without affecting NK cell maturation. NK cells deficient in $\alpha 2\beta 1$ had no impairment at entering or distributing within the draining lymph node (dLN) of ectromelia virus (ECTV) infected mice or at becoming effectors, but proliferated poorly in response to ECTV and did not increase in numbers following infection with mouse cytomegalovirus (MCMV). Still, $\alpha 2\beta 1$ deficient NK cells efficiently protected from lethal mousepox and controlled MCMV titers in the spleen. Thus, $\alpha 2\beta 1$ is required for optimal NK cell proliferation but is dispensable for protection against ECTV and MCMV, two well established models of viral infection where NK cells are known to be important.

Introduction

Natural killer (NK) cells are leukocytes of the innate immune system that belong to the Group 1 of innate lymphoid cells (ILCs). After developing in the bone marrow, NK cells circulate between blood and tissues patrolling the body for infections and tumors. Following viral infections, NK cells are rapidly recruited from the blood to secondary draining lymph nodes and other infected tissues where they produce interferon gamma (IFN- γ), kill infected cells and proliferate, playing a critical role as a first line of anti-viral defense (1, 2). For example, C57BL/6 (B6) mice, which are normally resistant to mouse cytomegalovirus (MCMV) and ectromelia virus (ECTV), become susceptible when NK cells are eliminated or dysfunctional (3-7). Also in humans, NK cell-deficient individuals become sick or succumb to normally non-life-threatening infections with human cytomegalovirus (HCMV) or with varicella zoster (8-10).

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The phenotypes and functions of NK cells overlap extensively with those of ILC1s, the only other member of the Group1 ILCs. For example, both ILC1s and NK cells express the typical NK cell activating receptors NKp46 and, in B6 mice, NK1.1. Both ILC1s and NK cells need the transcription factor T-bet for their development (11). Moreover, NK cells and ILC1s produce IFN- γ upon stimulation. Because of this, ILC1s can participate in virus control in tissues (12). Yet, ILC1s and NK cells also exhibit differences that make them unique. ILC1s are tissue resident and do not circulate in the blood, whereas NK cells circulate in the blood and are transient within tissues. NK cells but not ILC1s are cytolytic and express the transcription factor eomesodermin (Eomes) (13-16). At the cell surface, the major differences between ILC1s and NK cells is in the expression of integrins. ILC1s but not NK cells express the integrin β 3 and some, but not all ILC1s, express integrin α 1 (12). On the other hand, NK cells, but not ILC1s, express the integrin α M (CD11b, also expressed by other unrelated leukocytes) and the integrin α 2 (herein α 2, aka CD49b).

Integrins are cell surface heterodimeric receptors conformed by an α and a β chain (17). Integrins function in the adhesion of leukocytes to the endothelium and their extravasation into lymph nodes (LNs) and inflamed tissues. Integrins are also important for the displacement of leukocytes within tissues and can participate in their development and activation (18). α 2 dimerizes with the integrin β 1 (herein β 1, aka CD29) to form α 2 β 1 (aka VLA-2), a receptor for collagens Type I and III. Of note, β 1 is thought to be the only partner of α 2 while β 1 can heterodimerize with 12 different α chains (herein α x β 1) (19, 20) including α 1 (α 1 β 1) in ILC1s. Notably, α 2 β 1 is also expressed by human NK cells (21).

Despite α 2 being a reliable NK cell marker, the role of α 2 β 1 in the biology of NK cells is not well understood. When the Lanier group discovered α 2 on murine NK cells, they also showed that two different anti- α 2 mAbs, DX5 and HM α 2, do not block the binding of NK cells to collagen-coated plates, despite that HM α 2 blocks the α 2 β 1-dependent binding of platelets to collagen. In addition, Lanier's group demonstrated that immobilized anti- α 2 mAbs did not stimulate IFN- γ production in NK cells, nor did they block NK cell killing of YAC-1 cells (22, 23). Together, these data suggested that α 2 β 1 may not be essential for NK cell function. Later on, others showed that positive purification of NK cells using DX5 mAb negatively affected their ability to produce IFN- γ *in vitro*, kill target cells *in vitro* and *in vivo*, and impaired their motility in LNs (24). However, these data did not directly demonstrate a physiological role for α 2 β 1. More recently, it was shown that NK cells interacted with collagen fibers in the draining LNs (dLNs) of mice infected in the ear flaps with *Toxoplasma gondii* (*T gondii*), and that administration of HM α 2 in the ear flap decreased the presence of NK cells in *T gondii* infected foci in dLNs (25). This suggested that α 2 β 1 interactions with collagen might be involved in NK cells migration to infected foci. Similarly, a study with human NK cells *in vitro* also suggested that α 2 β 1 might participate in the interaction of NK cells with collagen but the data was correlative (21).

To further understand the role of α 2 β 1 in NK cell biology, we produced mice with specific genetic ablations of α 2 in NKp46⁺ cells. Using these mice, we show that α 2 β 1 affects but is not critical for NK development and maturation. Moreover, we also show that during ECTV infection, α 2 β 1 is not necessary for NK recruitment to or distributing within the dLN of ECTV infected mice or at becoming effectors after ECTV or MCMV infection. Notably,

$\alpha 2\beta 1$ is required for optimal NK proliferation in response to ECTV and increase in numbers in response to MCMV, but is dispensable for survival to ECTV and the control of MCMV titers in the spleen. These results indicate that $\alpha 2\beta 1$ is not essential for NK cell differentiation or effector function. Moreover, we demonstrate that NK cells with defective proliferation can still protect from ECTV lethality and can control MCMV replication.

Materials and Methods

Mice

All the procedures involving mice were carried out in strict accordance with the recommendations in the Eight Edition of the Guide for the Care and Use of Laboratory Animals of the National Research Council of the National Academies. All protocols were approved by Thomas Jefferson University's Institutional Animal Care and Use Committee. All mice used in experiments were 6-12 weeks old *Ncr1Cre⁺-Itgb1^{fl/fl}* or *Ncr1Cre⁺-Itga2^{fl/fl}* with appropriate littermate controls. No sex differences were observed. C57BL/6 (B6) and B6.CD45.1 mice were purchased from Charles river directly for experiments or as breeders. Aged B6 mice (18-20 months) were obtained from the National Institute of Aging (NIA) aged colony at Charles river. B6.Cg-*Itga2^{tm1.1Tkun/J}* ($\alpha 2^{fl/fl}$), and B6;129-*Itgb1^{tm1Efu/J}* ($\beta 1^{fl/fl}$) mice were purchased from Jackson Laboratories. Of note, the $\beta 1^{fl/fl}$ mice originally obtained from Jackson were in mixed 129/B6 background and carried the NK complex in chromosome 6 from the 129 strain. Therefore, they lacked NK1.1. To solve this problem, the original $\beta 1^{fl/fl}$ mice were backcrossed to C57BL/6 (B6) mice for two generations to generate $\beta 1^{fl/fl}$ mice expressing NK1.1 homozygously. C57BL/6-*Ncr1^{tm1.1(iCre)^{Viv}/Orl}* mice (*Ncr1-Cre*) were a gift of Dr. Eric Vivier (Marseille, France). Colonies were bred at Thomas Jefferson University under specific pathogen free conditions.

Viruses and Infection

ECTV-Moscow strain (ATCC VR-1374) and ECTV-mCherry were propagated in tissue culture as previously described (26). Mice were infected in the footpad with 3,000 or 100,000 plaque forming units (PFUs) ECTV as indicated. For the determination of survival, mice were monitored daily and, to avoid unnecessary suffering, mice were euthanized and counted as dead when imminent death was certain as determined by lack of activity and unresponsiveness to touch. Euthanasia was according to the 2013 edition of the AVMA Guideline for the Euthanasia of Animals. For virus titers, the entire spleen or portions of the liver were homogenized in 2.5% FBS RPMI (Corning) using a Tissue Lyser (QIAGEN). Virus titers were determined on BSC-1 cells as previously described (26).

For MCMV infections, indicated strains were infected with 2.5×10^5 pfu I.P. of MCMV K181 (K181) or 1.0×10^4 pfu I.P. of MCMV V70 (V70) for 120 hours. K181 stocks were produced by infecting 4×10^6 M210-B4 (ATCC CRL-1972TM) with a multiplicity of infection of 0.01 for 4-5 dpi, or until cytopathic effect in culture was observed. Cells and supernatant were collected, centrifuged at 2600 x g for 10 minutes, and resuspended in 10 mL complete RPMI 1640 (Corning) supplemented with 10% FBS, 10mM Hepes (Corning), 1mM Sodium Pyruvate (Corning), 100 units Penicillin (Corning), and 100 μ g/mL Streptomycin (Corning). Debris were then lysed with a Dounce homogenizer while on ice

and spun. Supernatant was collected, transferred to ultracentrifuge tubes (Beckman), and spun at 50,000 x g for 1 hour at 4° C. The supernatant was discarded and the pellet resuspend pellet in 500 µL of complete RPMI. V70 stocks were produced by infecting BALB/c mice (> 3 weeks) with 10 pfu of tissue-cultured V70 for 21 days. Whole salivary glands were harvested and placed in 1 mL sterile PBS (Corning), processed with a tissue grinder, and diluted to a 10% mixture of homogenate to media with a final concentration of 5% DMSO. Virus titers were determined by infecting M210-B4 monolayers for 4-5 days and counting the resulting plaques. This protocol was adapted from a published protocol (27).

Cell Depletions

NK cells were depleted by i.p. inoculation of 100 µg NK1.1 mAb (PK136, BioXcell) one day before injection and one dpi with ECTV. For MCMV infection, NK cells were depleted similarly one day before, one and three dpi with MCMV.

Immunofluorescent Microscopy

Preparation of ndLNs and dLNs was as previously described (28). 10-micron sections were stained with APC-NK1.1 (PK136; Biolegend) and mounted in Prolong Diamond plus DAPI (Thermo-Fisher). Images were collected with a Nikon A1R laser scanning microscope (LSCM). For better visualization, all the photographs were assembled in a single file and the contrast and brightness was increased in unison using Adobe Photoshop. In addition, APC-NK1.1 is shown in the green channel to distinguish NK cells more clearly from virally infected cells.

Cell isolation

Mice were euthanized by cervical dislocation. Single-cell suspensions were prepared from spleen and bone marrow and lysed for red blood cells using Ammonium-Chloride-Potassium (ACK) lysis buffer, and cells were washed with RPMI (Corning) supplemented with 5% FCS and later used for flow cytometric analysis. To obtain single-cell suspensions, LNs were first incubated in Liberase TM (1.67 Wünsch units/mL) (Sigma) in PBS with 25 mM HEPES for 30 minutes at 37°C before adding PBS with 25 mM HEPES + 10% FBS to halt the digestion process, followed by mechanical disruption of the tissue through a 70-µm filter.

Flow Cytometry

Flow cytometry to characterize NK cells in the bone marrow and spleen was performed as previously described (29). To determine NK cell responses in the LNs, intact LNs were incubated at 37°C for 1 h in media containing 10 µg/ml brefeldin A and then made into single cell suspensions. The cells were then stained for cell surface molecules, fixed, permeabilized, and stained for intracellular molecules using the Cytofix/Cytoperm kit (BD) or eBiosciences™ Foxp3/Transcription factor staining kit (Invitrogen) according to the manufacturer's instructions. The following Abs were used: FITC-CD3e (Clone 145-2C11; Biolegend), APC/Fire™750-CD11b/ BV605-CD11b (Clone M1/70; Biolegend), PerCP/Cy5.5-CD27 (Clone LG.3A10; Biolegend), PE/Cy7-CD29 (Clone HMβ1-1; Biolegend), PE-CD45.1 (Clone A20; biolegend), Percp-cy5.5-CD45.2 (104; Biolegend), BV421-CD49b

(Clone RB6-8C5; Biolegend), AF488-Eomes (Clone Dan11mag; eBiosciences), PE/Cy7-IFN γ (XMG1.2; Biolegend), APC-Ki67 (16A8; Biolegend), PE/ FITC-Ly49H (3D10; Biolegend), APC-NK1.1/BV605-NK1.1 (PK136; Biolegend), APC-NKp46 (Clone 29A1.4; Biolegend), BV786-TCR β (Clone H57-597; BD), and either Pacific Blue-GzmB (Clone GB11; Biolegend) or PE-labeled anti-human GzmB (Thermo Fisher) that cross-reacts with mouse GzmB. For analysis, samples were acquired using a BD Fortessa flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (TreeStar).

Adoptive Transfers of Lymphocytes

Splenocytes were obtained from the indicated strains of mice, mixed in a 1:1 ratio, then labeled with 4 μ M CFSE (Thermo Fisher), and a total of 2×10^7 cells (1×10^7 cells each) in 0.2 ml PBS was inoculated i.v. into the recipient mice.

RNA and DNA Preparation and RT-qPCR

Total RNA from LNs was obtained with the RNeasy Mini Kit (QIAGEN) as previously described (30, 31). First-strand cDNA was synthesized with High Capacity cDNA Reverse Transcription Kit (Life Technologies). For *EVM003* and *GAPDH* RT-qPCR was performed using iTaq Universal SYBR Green with the following primers: *Gapdh*: tgtccgctcgtggatctgac and cctgcttcaccacctcttg, *EVM003*: tctgtccttaacagcatagatgtaga and tgtaactcggagttgatgtaga. For viral loads, RNA (ECTV) from naïve mice were used as controls and no amplification was observed. Thus, for quantification purposes, their CT values were adjusted to 40.

Total DNA was extracted from the spleen using the Genra Puregene Tissue Kit (QIAGEN) and following the manufacturer's instructions for extraction of DNA from tissues. The spleen was homogenized in 10 mL of 2.5% FBS RPMI (Corning) and 1 mL was taken for DNA extraction. RNA and protein were removed by adding RNase A solution and Proteinase K. Extracted DNA was quantified by nanodrop and two microliters DNA were used as a template in each qPCR reaction using the TaqMan Fast Advanced Master Mix (Thermo-Fisher) with the following primer for MCMV-E1: tcgccatcgtttcgaga and tctcgtagtcactgacgga. Genome copy numbers were calculated based on a standard curve of a plasmid containing the MCMV-E1 gene.

Statistical analysis

Statistical analysis was performed with Prism software (Graphpad Software Inc., La Jolla, CA, USA) software. For survival studies, *P*-values were obtained with the log-rank (Mantel-Cox) test. *P*-values were determined using Mann-Whitney test, and when multiple groups had to be compared, we used one-way ANOVA and Fisher's LSD Test for multiple comparisons.

Results

Generation and characterization of mice with NK cells deficient in $\alpha 2\beta 1$

To investigate the role of $\alpha 2\beta 1$ in NK cell maturation and function, we crossed *Ncr1Cre⁺* mice (*Ncr1* encodes NKp46) (32) with either *Itgb1^{fl/fl}* or *Itga2^{fl/fl}* mice to respectively

generate mice with NK cells deficient in $\beta 1$ (*Ncr1Cre⁺-Itgb1^{fl/fl}*, herein *Cre⁺-Itgb1^{fl/fl}*) or $\alpha 2$ (*Ncr1Cre⁺-Itga2^{fl/fl}*, herein *Cre⁺-Itga2^{fl/fl}*). Flow cytometry analysis of bone marrow and spleen cells showed that most but not all CD3⁻ NK1.1⁺ cells in *Cre⁻-Itgb1^{fl/fl}* littermate control mice but only a few *Cre⁺-Itgb1^{fl/fl}* mice expressed $\alpha 2$ and $\beta 1$. This indicates that in NK1.1⁺ cells, $\alpha 2$ is only expressed at the cell surface if $\beta 1$ is also expressed. T-cells (CD3e⁺ NK1.1⁻) did not express $\alpha 2$ and had low levels of $\beta 1$ in either *Cre⁻* or *Cre⁺-Itgb1^{fl/fl}* mice (Figure 1A). Meanwhile, $\alpha 2$ in CD3⁻ NK1.1⁺ was expressed at high levels in *Cre⁻-Itga2^{fl/fl}* mice but was absent in *Cre⁺-Itga2^{fl/fl}* mice, which mostly maintained $\beta 1$. On the other hand, $\alpha 2$ was not expressed by T-cells in either *Cre⁻* or *Cre⁺-itga2^{fl/fl}* mice (Figure 1B). This demonstrates that $\alpha 2$ is efficiently deleted in NK1.1⁺ cells of *Cre⁺-Itga2^{fl/fl}* mice. Thus, *Cre⁺-Itga2^{fl/fl}* mice have $\alpha 2\beta 1$ deficiency at the surface of NK1.1⁺ cells. However, because $\beta 1$ was still expressed, $\beta 1$ likely pairs with other integrins at the surface of NK1.1⁺ cells. Thus, subsequent experiments are described with *Cre⁺* and *Cre⁻-Itga2^{fl/fl}* mice as in addition to $\alpha 2\beta 1$ loss, NK1.1⁺ cells in *Cre⁺-Itgb1^{fl/fl}* have deficiencies in additional $\beta 1$ pairs. Also, *Itgb1^{fl/fl}* mice are in a B6;129 mixed background, which could complicate the analysis. Of note, the deficiency of $\alpha 2$ in NK1.1⁺ cells did not redirect NK cells to the ILC1 lineage because the frequencies of NK cells (Eomes⁺) and ILC1s (Eomes⁻) within Group 1 ILCs (NK1.1⁺ CD3⁻) was similar in the bone marrow, spleen and peripheral LN (pLN) of *Cre⁻* and *Cre⁺-Itga2^{fl/fl}* (Figure 1C) where NK cells were the predominant population.

$\alpha 2\beta 1$ affects, but not critically, the development of NK cells.

We next investigated whether intrinsic $\alpha 2\beta 1$ deficiency affects NK cell development. Expression of CD27 and CD11b defines different maturational stages of NK cells where CD27⁺ CD11b⁻ (herein R1) are immature, CD27⁺ CD11b⁺ (herein R2) are transitional but with effector capacity, and CD27⁻ CD11b⁺ (herein R3) are the most mature and have the strongest cytolytic function (33) (Figure 2A). We found that in *Cre⁺-Itga2^{fl/fl}* mice, the frequency of NK1.1⁺ cells were decreased in the bone marrow (Figure 2B) and in the spleen (Figure 2C), but not in the blood (Figure 2D). However, the frequencies of the different maturation stages (R1, R2 and R3) were not altered in any of the organs (Figure 2 B, C, and D). Thus, the absence of $\alpha 2\beta 1$ seems to slightly decrease the frequency of NK cells in the bone marrow and spleen, but has no effect on NK cell maturation.

Response to viral infection

Deficiency in $\alpha 2\beta 1$ does not impair the constitutive or ECTV-induced accumulation of NK cells in LNs, their distribution within the LN or the acquisition of an effector phenotype—NK cells are present in LNs at low numbers at steady state and increase their numbers by migrating from the circulation during inflammation. We have previously shown that mature R3 NK cells rapidly enter the dLN after ECTV infection (7). Given the importance of integrins in lymphocyte adhesion and migration it was possible that $\alpha 2\beta 1$ was required in this process. *Cre⁺* and *Cre⁻-Itga2^{fl/fl}* mice were challenged with ECTV and the recruitment of NK cell into the dLNs was measured by comparing frequencies of NK cells in the non-draining LN (ndLN) (i.e. at steady state) and dLN at 48 hours post infection (hpi). We observed that *Cre⁺-Itga2^{fl/fl}* mice preferentially recruited NK cells to the dLN but slightly albeit significantly less than *Cre⁻-Itga2^{fl/fl}* mice, which could be an effect of the decreased number of NK cells in the bone

marrow and spleen of $Cre^+ - Itga2^{fl/fl}$. Nevertheless, similar to Cre^- controls, the accumulation of NK cells in the dLN of Cre^+ mice resulted in a relative increase in the frequency of mature R3 NK cells and concomitant decrease in immature R1 NK cells (Figure 3A-B).

It was possible that in $Cre^+ - Itga2^{fl/fl}$ mice NK cells deficient in $\alpha 2\beta 1$ migrated normally to the dLN because of a lack of competition. Thus, splenocytes from Cre^+ and $Cre^- - Itga2^{fl/fl}$ mice were mixed in a 1:1 ratio, labeled with CFSE, transferred into B6 mice, which were subsequently infected with ECTV in the footpad. At 48 hpi, the ratios of $Cre^+ / Cre^- - Itga2^{fl/fl}$ NK cells in the ndLN and the dLN were similar, confirming that deficiency in $\alpha 2\beta 1$ does not affect the constitutive or virus induce entry of NK cells into the dLN (Figure 3C).

Next, we looked at NK cell localization within LNs. As mentioned previously, antibody blockade suggested that $\alpha 2\beta 1$ directs NK cells to sites of bacterial infection within the dLN *via* interactions with collagen (24). Thus, we analyzed whether absence of $\alpha 2\beta 1$ results in NK cell mislocalization within the dLN during a viral infection. Cre^+ and $Cre^- - Itga2^{fl/fl}$ mice were infected with ECTV expressing mCherry and ndLNs and dLNs were visualized by confocal microscopy after staining with anti-NK1.1 mAb. The analysis confirmed recruitment because there was an increase in NK1.1⁺ cells (green) in the dLN vs the ndLN of both, Cre^+ and Cre^- mice. Furthermore, similar to Cre^- controls, NK1.1⁺ cells in Cre^+ mice distributed throughout the dLN including infected and uninfected areas (Figure 3D).

We also analyzed effector functions. At 60 hpi, the NK cells in the dLN of B6 mice are highly activated as determined by granzyme B (GzmB) and interferon gamma (IFN- γ) expression and these effector molecules are critical for the control of ECTV dissemination from the dLN to the liver and the spleen (7). Thus, we tested whether $\alpha 2\beta 1$ deficiency affects expression of GzmB and IFN- γ in NK cells. We found that Cre^- and $Cre^+ - Itga2^{fl/fl}$ had similarly increased frequencies of both, IFN- γ^+ and GzmB⁺ NK cells in the dLN at 60 hpi with ECTV when compared to the ndLN (Figure 3E).

$\alpha 2\beta 1$ is necessary for optimal NK cell proliferation in the spleen during ECTV and MCMV infections but is not required for the acquisition of effector functions

—In addition to curbing virus spread from LNs, NK cells play an important role in the control of ECTV by becoming activated in the spleen and liver until the T-cell response develops (3, 7). Thus, we also looked at NK cell responses in the spleen. At 120 hpi with ECTV, which is the peak of the NK response, the frequency and absolute numbers of NK cells in the spleens of Cre^+ mice were significantly lower than in $Cre^- - Itga2^{fl/fl}$ and naïve mice (Figure 4A and B). A similar effect was observed in the liver and blood (Figure S1). However, the frequency of GzmB⁺ NK cells was not significantly different between Cre^+ and Cre^- mice and was significantly higher than in naïve mice (Figure 4A and C), indicating that NK cells do not need $\alpha 2\beta 1$ to acquire effector functions in the spleen or liver during ECTV infection.

To determine whether reduced numbers of NK cells with efficient effector differentiation was a characteristic of the ECTV NK cell response or could be extended to other viral infections, we infected Cre^+ and $Cre^- - Itga2^{fl/fl}$ mice with MCMV. As with ECTV, Cre^+ -

Itga2^{fl/fl} mice had reduced frequencies and numbers of NK cells than *Cre⁻ -Itga2^{fl/fl}* mice at 120 hpi. Compared to naïve mice, MCMV-infected *Cre⁻ -Itga2^{fl/fl}* had increased total number (but not frequency) of NK cells (Figure 4D and E). As with ECTV, the upregulation of GzmB in *Cre⁺ -Itga2^{fl/fl}* mice remained intact (Figure 4D and F).

During MCMV infection of B6 mice, NK cells expand in two phases, the early phase occurs within the first two days of infection, is dependent on cytokines and most NK cells expand (34, 35). The late phase occurs ~120 hpi and happens mostly in Ly49H⁺ NK cells that recognized the viral protein m157 at the surface of infected cells (36-40). Analysis of Ly49H⁺ and Ly49H⁻ NK cells revealed that Ly49H⁺ NK cells from *Cre⁺ -Itga2^{fl/fl}* mice did not increase in number whereas the Ly49H⁺ NK cells from *Cre⁻* mice did (Figure 4G and H). Yet, similar frequencies of NK1.1⁺ Ly49H⁺ cells produced GzmB in *Cre⁻* and *Cre⁺ Itga2^{fl/fl}* mice (Figure 4G and I). Therefore, during MCMV infection, Ly49H⁺ NK cells deficient in $\alpha 2\beta 1$ failed to increase in numbers but MCMV-specific NK cells (i.e., Ly49H⁺) still acquired an effector phenotypes.

Given the reduced NK cell numbers, we next used the ECTV model to test whether $\alpha 2\beta 1$ is necessary for NK cell proliferation. At 120 hpi with ECTV, *Cre⁺ -Itga2^{fl/fl}* mice had significantly lower frequency of Ki67⁺ NK1.1⁺ cells in the spleen than *Cre⁻ -Itga2^{fl/fl}* mice indicating that in the absence of $\alpha 2\beta 1$, fewer NK cells entered into G1 (Figure 4J). To determine whether reduced Ki67 expression translated to impaired cell division, we adoptively transferred CFSE-labeled splenocytes from *Cre⁺* and *Cre⁻ Itga2^{fl/fl}* mice in a 1:1 ratio into a CD45.1 host, which were then infected with ECTV in the footpad. Proliferation of *Cre⁺* and *Cre⁻* NK cells (identified by $\alpha 2$ staining) was assessed by CFSE dilution at 144 hpi. The results indicated significantly decreased proliferation in *Cre⁺* compared to *Cre⁻ -Itga2^{fl/fl}* mice (Figure 4K). This indicates that $\alpha 2\beta 1$ is required for optimal proliferation of NK cells during ECTV infection.

$\alpha 2\beta 1$ -deficient NK cells protect from lethal mousepox and control MCMV titers in the spleen—B6 mice are naturally resistant to lethal mousepox but become highly susceptible when depleted of NK cells with anti-NK1.1 or Asialo-GM antibody or when NK cells are dysfunctional as demonstrated by increased virus titers in spleen and liver at 3 and 5 dpi, and by high lethality (29, 41). When challenged with ECTV, *Cre⁻* and *Cre⁺ -Itga2^{fl/fl}* had similar levels of viral gene expression in the dLNs at 72 hpi (Figure 5A) and similar titers of replicating virus in the spleen and liver at 120 hpi as determined by plaque assay (Figure 5B). Treatment with anti-NK1.1 mAb resulted in a significant increase in lethality in both *Cre⁻* and *Cre⁺ -Itga2^{fl/fl}* mice, while undepleted *Cre⁻* and *Cre⁺ -Itga2^{fl/fl}* mice were resistant indicating that $\alpha 2\beta 1$ is not required for NK cell-mediated resistance to lethal mousepox (Figure 5C). Moreover, *Cre⁺ -Itga2^{fl/fl}* mice were resistant when challenged with a significantly higher dose of ECTV, but control aged B6 mice, which have immature and dysfunctional NK cells, succumbed to ECTV infection (Figure 5D). Also, at 5 dpi with tissue cultured (K181) and salivary gland passaged (V70) MCMV, *Cre⁻* and *Cre⁺* mice had similar virus loads in the spleen, which increased similarly after NK cell depletion (Figure 5E and F). These results indicate that the $\alpha 2\beta 1$ in NK cells and optimal NK cell proliferation are dispensable for effective NK-cell mediated control of two mouse-specific viruses in the acute phase of infection.

Discussion

In this study, we show that $\alpha 2\beta 1$ expression by NK cells is not essential for their maturation or commitment to the NK cell lineage, entry to and distribution within lymph nodes, or the induction of an anti-ECTV effector response. However, $\alpha 2\beta 1$ is required for optimal NK cell proliferation during ECTV infection and for increase in NK cell during MCMV infection. Despite this, $\alpha 2\beta 1$ -deficient NK cells were still able to protect from ECTV and control MCMV titers in the spleen, demonstrating that mature and functional NK cells can mediate protection without extensive proliferation in two viral infection models where the anti-viral role of NK cells is very well established.

Previous research has shown that the VLA family of integrin heterodimers has roles in metastasis of cancer cells, immunomodulation, and leukocyte extravasation and retention in tissue (42-47). While $\alpha 2\beta 1$ is used as a marker to identify NK cells across mouse strains as well as to distinguish NK cells from ILC1s, its role in NK cell biology remains unclear. Bone marrow stromal cells synthesize types 1 and 3 collagen fibers (48) and it has been shown their reduction in bone marrow stromal cells leads to decreased collagen synthesis (49, 50). Previously, we have shown that the NK cells in the bone marrow and other organs of aged mice are immature with increased R1 and decreased R3 populations (29, 51). We also showed that NK cells in the bone marrow of aged mice have lower expression of $\alpha 2\beta 1$ (51) and that NK-cell extrinsic factors in the bone marrow stroma are responsible for the defective maturation of NK cells in aged mice, leading us to hypothesize that $\alpha 2\beta 1$ and its interaction with collagen are necessary for NK cell maturation. Thus, we also hypothesized that expression of $\alpha 2\beta 1$ on developing NK cells and their interaction with collagen could be required for their proper lineage commitment and maturation in the bone marrow. However, we found that $\alpha 2\beta 1$ does not influence lineage commitment of the common innate lymphoid progenitor to NK cells vs. ILC1s. Also, while $\alpha 2\beta 1$ deficiency slightly decreases the frequency of NK cells in bone marrow and spleen, it does not affect NK cell maturation because the proportions of R1s and R3s were similar within the bone marrow, peripheral blood, and spleen of Cre^+ - and $Cre^- -Itga2^{fl/fl}$ mice. Thus, the data strongly suggests that the deficient NK cell maturation in aged mice (51) is not due to deficient $\alpha 2\beta 1$ expression.

It is known that CXCR3 directs homing of NK cells to the dLN during viral infection (52, 53), but the identity of the integrin(s) involved in entry into the dLN remains unknown. We show that $Cre^+ -Itga2^{fl/fl}$ NK cells enter ndLN and dLNs normally, whether or not they are in competition with $Cre^- -Itga2^{fl/fl}$ NK cells. This indicates that $\alpha 2\beta 1$ is not required for NK cell constitutive entry into the ndLN and ECTV-induced entry into the dLN. Also, using confocal microscopy, we found that $\alpha 2\beta 1$ -deficient NK cells distribute similarly within the ndLN and dLN including areas of virus infection, indicating that $\alpha 2\beta 1$ is not required for intra-nodal displacement. Thus, adhesion molecules other than $\alpha 2\beta 1$ may be responsible for the NK cell extravasation into and distribution within ndLNs and dLNs.

It has been reported that $\alpha 2$ in mouse NK cells and $\beta 1$ in human NK cells are needed to control *Toxoplasma gondii* and *cryptococcal neoformans* infections respectively, *via* direct or indirect activation of NK cells (25, 54). Here we show that during viral infections, $\alpha 2\beta 1$ is not required for NK cell activation because $Cre^+ -Itga2^{fl/fl}$ NK cells upregulated IFN- γ or

GzmB normally during ECTV and MCMV infections. Furthermore, *Cre*⁻ and *Cre*⁺ -*Itga2*^{fl/fl} mice controlled ECTV and MCMV similarly while depletion of $\alpha 2\beta 1$ NK cells resulted in high susceptibility to lethal mousepox and higher MCMV titers in spleen, indicating $\alpha 2\beta 1$ is dispensable at conferring NK cell-mediated control against ECTV and MCMV.

Interactions with $\beta 1$ and collagen have previously been shown to affect the expansion of pancreas β -cells as well as the morphogenesis of submandibular epithelial cells (55, 56). Thus, we believe $\alpha 2\beta 1$ induces proliferation of NK cells but does not affect their trafficking or localization during viral infections because of the global reduction in NK cells within the blood, liver, and spleen of infected *Cre*⁺ -*Itga2*^{fl/fl} mice. It remains possible, however, that NK cells are increased at other sites that we did not test. Importantly, we showed that $\alpha 2\beta 1$ is required for optimal NK cell proliferation. Future experiments should identify the ligands and signaling mechanisms of this $\alpha 2\beta 1$ -dependent proliferation and whether $\beta 1$ plays a role in the proliferation of other leukocytes.

It is well established that survival to ECTV and efficient control of MCMV requires NK cells, and that the NK cell responses to both viruses includes acquisition of effector functions and proliferation. Our results show that the NK cell-mediated resistance to ECTV and control of MCMV virus loads can still occur with significantly decreased proliferation. It is also known that innate immune mechanisms including NK cell effector functions in the dLN play a critical role in protection against lethal ECTV infection (29, 59). Notably, at 48-72 hpi the frequency of NK cells in the dLN of *Cre*⁺ -*Itga2*^{fl/fl} is only slightly decreased. Thus, the successful migration of mature and functional $\alpha 2\beta 1$ -deficient NK cells to the dLN at 48-72 hpi and their activation in the spleen allows for ECTV control even when NK numbers are altered. These findings further highlight the importance of controlling viral replication in the dLN during an acute virus infection.

During primary infection with MCMV, Ly49H⁺ NK cells interacting with the viral protein m157 expand and protect mice from MCMV (5, 39, 60, 61). These MCMV-specific NK cells then contract and give rise to long-lived memory NK cells that contribute to protection upon reinfection (57, 62). Our data suggests that in addition to Ly49H interacting with m157, $\alpha 2\beta 1$ acts as an additional signal for optimal Ly49H⁺ NK cell proliferation. Future experiments could look whether this affects the induction of memory NK cells.

In summary, $\alpha 2\beta 1$ is viewed as a phenotypic hallmark of NK cells, but its role in NK cell biology is not well understood. Our work shows that $\alpha 2\beta 1$ is not required for NK cell maturation, migration to LNs or acquisition of effector functions. However, $\alpha 2\beta 1$ is required for optimal virus-induced proliferation and accumulation of NK cells. Unexpectedly, despite impaired proliferation, $\alpha 2\beta 1$ -deficient NK cells still protected from two viral infections in their natural hosts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key Points

$\alpha 2\beta 1$ deficiency does not alter the balance of NK cell/ILC1 generation

$\alpha 2\beta 1$ deficiency does not alter NK cell maturation

$\alpha 2\beta 1$ deficient NK cells efficiently protect from lethal mousepox and control MCMV

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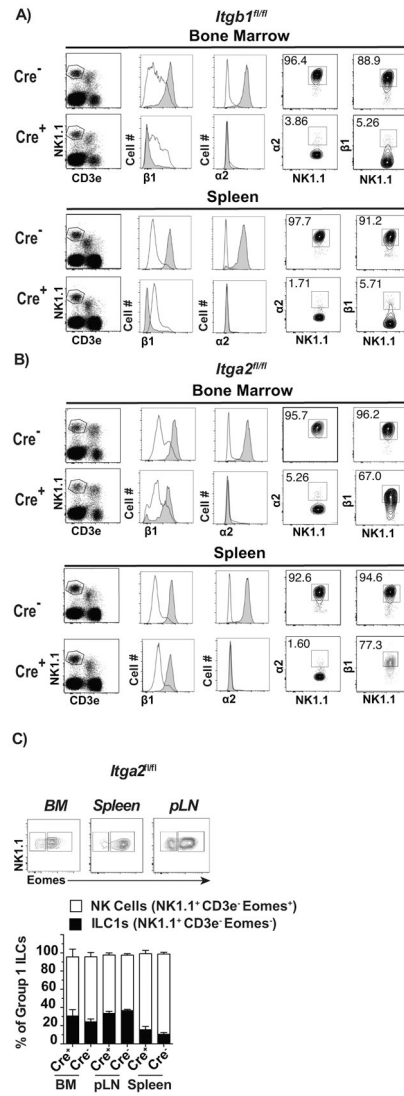


Figure 1: Generation and characterization of mice with NK cells deficient in $\alpha 2\beta 1$.

Ncr1Cre⁺ mice were crossed with *Itgb1^{fl/fl}* or *Itga2^{fl/fl}* mice to generate *Cre⁺-Itgb1^{fl/fl}*, *Cre⁺-Itga2^{fl/fl}* and *Cre⁻* littermate controls. **A-B)** Representative flow cytometry analysis in the indicated tissues and mouse strains: NK1.1 and T-cells were distinguished by CD3e and NK1.1 staining (left panels) on cells previously gated on FSC-A vs. FSC-H to eliminate doublets and on lymphocytes by FSC-H and SSC-H. From these plots, expression of $\alpha 2$ and $\beta 1$ was determined on gated NK cells (CD3e⁻ NK1.1⁺, gray histogram) or T-cells (CD3e⁺ NK1.1⁻, white histograms). These were used to establish the definitive gates for NK cells shown as contour plots on the right. **C)** Representative flow plots showing the gating strategy to identify ILC1s and NK cells in the indicated tissues based on NK1.1 and Eomes, and stacked columns depicting the frequencies of ILC1s and NK cells as mean \pm SEM in different tissues. Data correspond to 2 independent experiments combined with a total of 6-8 mice/group.

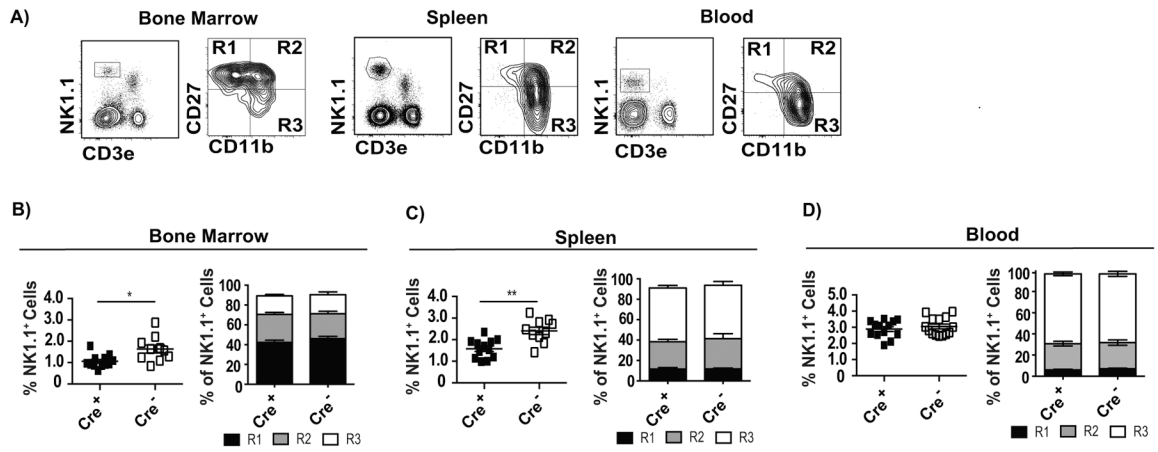


Figure 2: $\alpha 2\beta 1$ affects, but not critically, the development of NK cells.

The indicated tissues from *Cre*⁺ and *Cre*⁻ *Itga2^{fl/fl}* mice were analyzed for the frequency of NK1.1⁺ cells and their maturation stages (R1, R2 and R3) according to CD27 and CD11b expression. **A)** Representative flow cytometry plots indicating the gating strategy. **B-D)** Summary graphs indicating the frequency of NK1.1⁺ cells in individual mice with mean \pm SEM (left panels) and the mean \pm SEM frequencies of R1 (CD27⁺ CD11b⁻), R2 (CD27⁺ CD11b⁺), and R3 (CD27⁻ CD11b⁺) cells within the NK1.1⁺ CD3e⁻ gate in the indicated tissues and mice. Data correspond to three or four independent experiments combined with a total of 11-27 mice/group. *P*-values were calculated using the Mann-Whitney U statistical test or ANOVA as necessary. For all, **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.

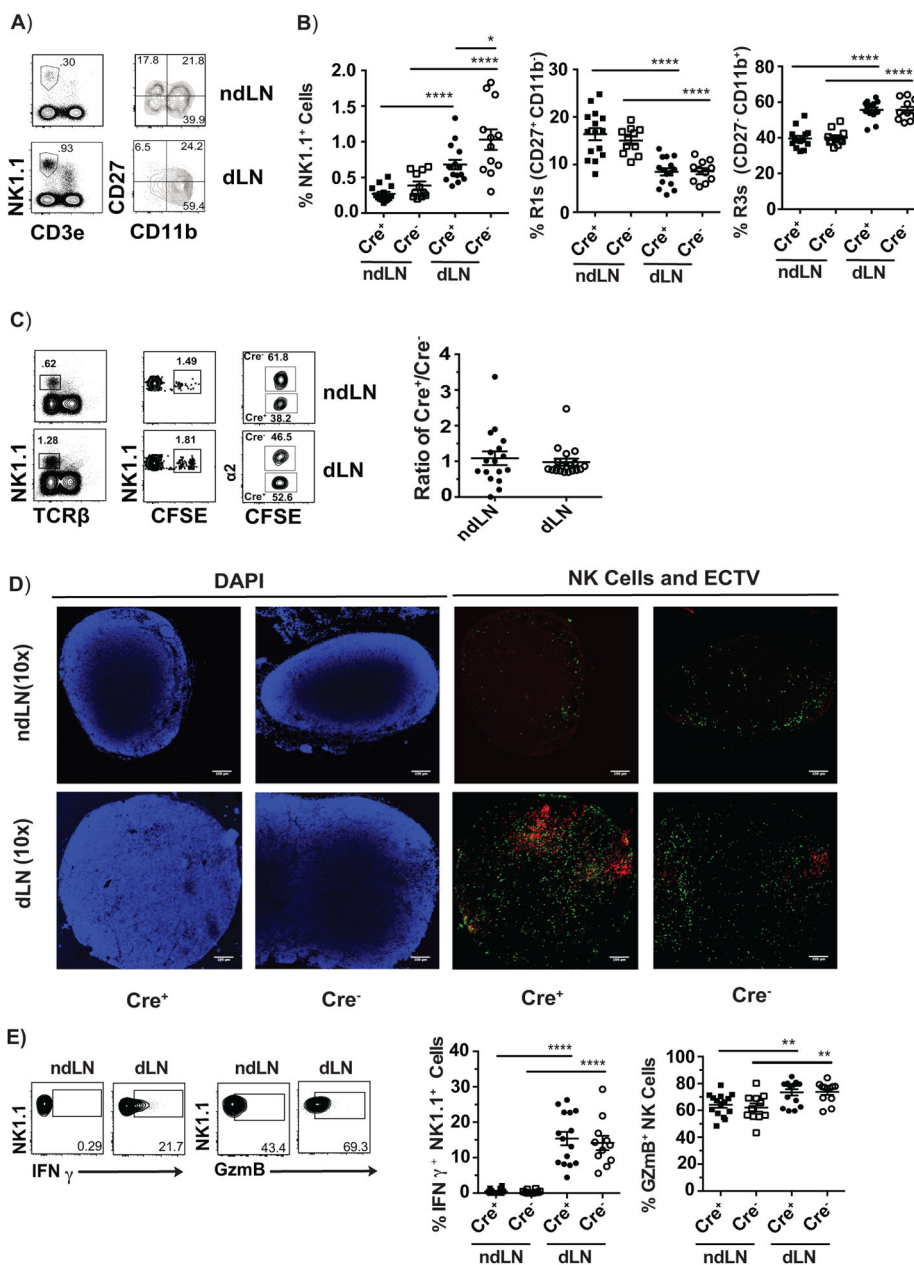


Figure 3: Deficiency in $\alpha 2\beta 1$ does not impair the constitutive or ECTV-induced accumulation of NK cells in LNs, their distribution within the LN or the acquisition of an effector phenotype. **A-E)** *Cre*⁺ and *Cre*⁻ *Itga2^{fl/fl}* mice were infected with 3,000 pfu WT ECTV or ECTV mCherry and their ndLN and dLNs were analyzed at 48-72 hpi. **A)** Representative flow cytometry plots showing the gating strategy. **B)** Summary graphs indicating the frequency of NK1.1⁺ cells in individual mice with mean \pm and frequencies of R1 and R3 cells in individual mice with mean \pm SEM in *Cre*⁻ and *Cre*⁺ *Itga2^{fl/fl}* mice. In A and B, data correspond to three individual experiments combined with a total n of 11-15 mice/group. **C)** Splenocytes from *Cre*⁺ *Itga2^{fl/fl}* and *Cre*⁻ *Itga2^{fl/fl}* were mixed at a 1:1 ratio labeled with 4 μ M CFSE and transferred into recipient B6 mice. The mice were infected with 3,000 pfu WT ECTV one day post transfer and their ndLN and dLNs analyzed at 48 hpi.

Representative flow plots show the gating strategy for identification of adoptively transferred cells and WT and $\alpha 2\beta 1$ -deficient NK cells. Dot plots show the ratio of $\alpha 2\beta 1$ /WT NK cells in the ndLNs and dLNs of individual mice with mean \pm SEM. All experiments were repeated 2-3 times. Data are displayed as a combination of all the repeats with a total n of 17 or 18 mice/group. **D)** *Cre⁺* and *Cre⁻ Itga2^{fl/fl}* mice were infected with ECTV-mCherry. At 72 hpi, their ndLN and dLN were visualized by confocal microscopy with 10X magnification. Blue: DAPI, Green: NK1.1 staining. Red: mCherry. Pictures are from the ndLN or dLN of ECTV infected mice. Experiments were repeated 2 times with a total n of 6 mice/group. Representative lymph nodes from one mouse in each group are shown. **E)** *Cre⁺* and *Cre⁻ Itga2^{fl/fl}* infected with 3,000 pfu WT ECTV and their ndLN and dLNs were analyzed at 48 hpi. Representative flow cytometry plots showing the gating strategy for expression of intracellular IFN- γ or GzmB. Dot plots depict the frequencies with mean \pm SEM of NK1.1⁺ cells that were IFN- γ ⁺ (left) or GzmB⁺ (right) in the ndLN and dLN of individual *Cre⁻* or *Cre⁺ Itga2^{fl/fl}* mice as indicated. Data corresponds to three individual experiments combined with a total n of 11-15 mice/group. *P*-values were calculated using the Mann-Whitney U statistical test or ANOVA when necessary. For all, **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.

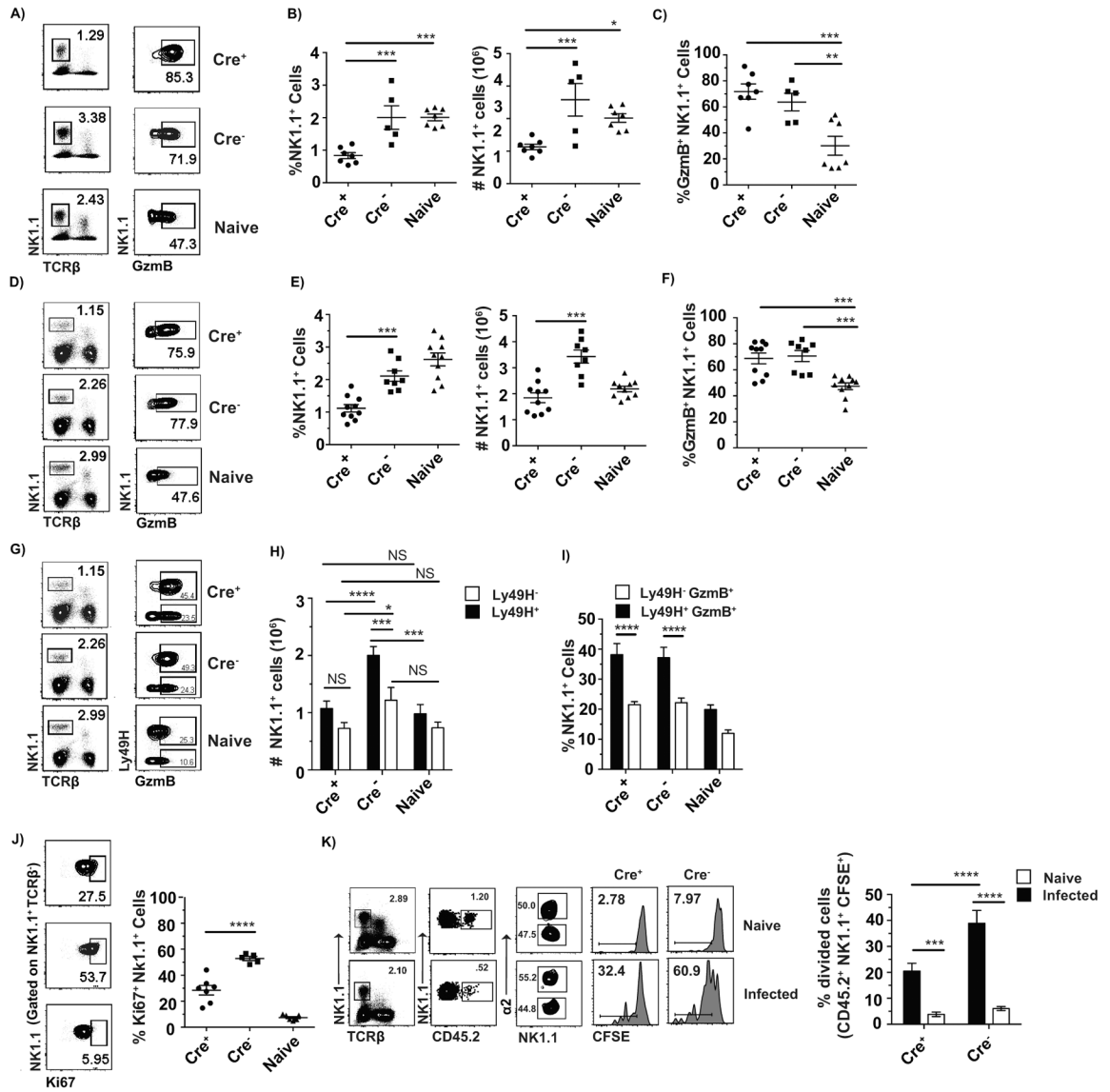


Figure 4: $\alpha 2\beta 1$ is necessary for optimal NK cell proliferation in the spleen during ECTV and MCMV infections but is not required for the acquisition of NK cell effector functions.

Cre⁺ and *Cre⁻ Itga2^{fl/fl}* mice were infected with either 3,000 pfu of WT ECTV or 250,000 pfu of MCMV K181 (MCMV), and their spleens were analyzed at 120 hpi. Naïve B6 mice were used as an additional control. **A)** Representative flow cytometry plots showing the gating strategy for identification of total NK cells in indicated mice infected with ECTV, as well as intracellular expression of GzmB in total NK1.1⁺ cells. **B)** Dot plots depict the frequencies and total numbers with mean \pm SEM of NK1.1⁺ cells that were in the spleen of ECTV infected individual *Cre⁻* or *Cre⁺ Itga2^{fl/fl}* mice as indicated. Data are displayed as a combination of two individual experiments with a total n of 5-7 mice/group **C)** Dot plots depict the frequency with mean \pm SEM of GzmB⁺ NK1.1⁺ cells that were in the spleen of ECTV infected individual *Cre⁻* or *Cre⁺ Itga2^{fl/fl}* mice as indicated. Data correspond to two individual experiments combined with a total n of 5-7 mice/group. **D)** Representative flow cytometry plots showing the gating strategy for identification of total NK cells in indicated

mice infected with MCMV, as well as intracellular expression of GzmB in total NK1.1⁺ cells. **E)** Dot plots depict the frequencies and total numbers with mean \pm SEM of NK1.1⁺ cells in the spleen of MCMV infected individual *Cre*⁻ or *Cre*⁺ *Itga2^{fl/fl}* mice as indicated. Data are displayed as a combination of three individual experiments with a total n of 8-10 mice/group. **F)** Dot plots depict the frequency with mean \pm SEM of GzmB⁺ NK1.1⁺ cells in the spleen of MCMV infected individual *Cre*⁻ or *Cre*⁺ *Itga2^{fl/fl}* mice as indicated. Data are displayed as a combination of three individual experiments with a total n of 8-10 mice/group. **G)** Representative flow cytometry plots showing the gating strategy for identification of total NK cells, as well as intracellular expression of GzmB in Ly49H⁺ or Ly49H⁻ cells within the NK1.1⁺ population in mice infected with MCMV. **H)** Bar graphs depict the number of Ly49H⁺ NK1.1⁺ or Ly49H⁻ NK1.1⁺ cells with mean \pm SEM of NK1.1⁺ in the spleen of MCMV infected individual *Cre*⁻ or *Cre*⁺ *Itga2^{fl/fl}* mice as indicated. **I)** Bar graphs depict the frequency of GzmB⁺ cells with mean \pm SEM in NK1.1⁺ Ly49H⁺ or Ly49H⁻ cells in the spleen of MCMV infected individual *Cre*⁻ or *Cre*⁺ *Itga2^{fl/fl}* mice as indicated. Data are displayed as a combination of three individual experiments with a total n of 8-10 mice/group. **J)** Dot plots depict the frequency with mean \pm SEM of Ki67⁺ NK1.1⁺ cells in the spleen of ECTV infected individual *Cre*⁻ or *Cre*⁺ *Itga2^{fl/fl}* mice as indicated. Data are displayed as a combination of two individual experiments with a total n of 5-7 mice/group. **K)** Splenocytes from *Cre*⁺ *Itga2^{fl/fl}* and *Cre*⁻ *Itga2^{fl/fl}* were mixed at a 1:1 ratio labeled with 4 μ M CFSE and transferred into recipient CD45.1 mice. Recipient mice were either naïve or infected with 3,000 pfu WT ECTV one day post transfer and their spleens analyzed at 120 hpi. Representative flow plots show the gating strategy for identification of adoptively transferred cells either WT and α 2 β 1-deficient NK cells and the dilution of CFSE by either WT or α 2 β 1-deficient NK cells. Bar graphs show the frequency of WT or α 2 β 1-deficient NK cells that diluted their CFSE in the spleen of individual mice with mean \pm SEM. Data corresponds to three individual experiments combined with a total n of 8-11 (naïve) and 10-12 (infected) mice/group. *P*-values were calculated using the Mann-Whitney U statistical test or ANOVA when necessary. For all, **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.

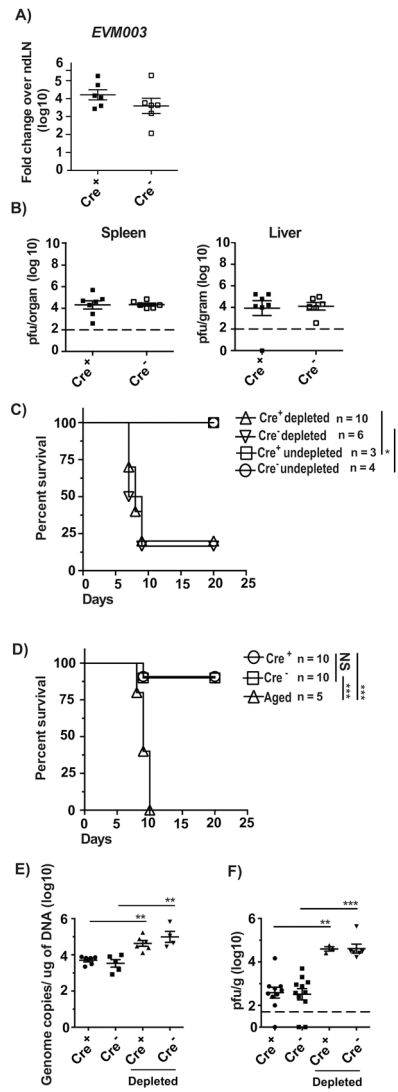


Figure 5. $\alpha 2\beta 1$ -deficient NK cells protect from lethal mousepox and from MCMV:
A) The indicated mice were infected with ECTV and at 72 hpi, the expression of the viral gene *EVM003* in individual mice with mean \pm SEM was determined in the dLN by RT-qPCR. Data correspond to two individual experiments combined with a total of 6-8 mice/group. **B)** The indicated mice were infected with ECTV and at 120 hpi, the virus loads in spleens or livers of individual mice with mean \pm SEM were determined by plaque assay. Data correspond to two individual experiments combined with a total of 6-9 mice/group. The limit of detection is indicated by the dotted line (2 log). **C)** The indicated mice were depleted or not of NK cells with anti-NK1.1 mAb PK136 one day before and one day after infection with 3,000 pfu ECTV in the footpad. Survival was monitored. Data are displayed as a combination of two independent experiments with a total of 3-10 mice/group. Statistical analysis was performed by Log-Rank test. For all, *p < 0.05; **p < 0.01. **D)** The indicated mice infected with 100,000 pfu ECTV in the footpad. Survival was monitored. Data are displayed as a combination of two independent experiments with a total of 5-10 mice/group. Statistical analysis was performed by Log-Rank test. For all, *p < 0.05; **p < 0.01; ***p <

0.001. **E-F**) The indicated mice were depleted or not of NK cells with anti-NK1.1 mAb PK136 one day before and one, three, and five days after infection with 250,000 pfu of tissue-cultured MCMV K181 (**E**) or 10,000 pfu of salivary gland passaged MCMV V70 (**F**) i.p. Spleens were collected at 120 hpi and genome copies of MCMV E1 gene (**E**) or pfu (**F**) were calculated. Data are displayed as a combination of two or three independent experiments with a total of 3-11 mice/group. The limit of detection for pfu is indicated by the dotted line (1.7 log). *P*-values were calculated using the Mann-Whitney U statistical test or ANOVA when necessary. For all, **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.