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The inhibitory NKR-P1B receptor regulates NK cell-mediated mammary tumor immunosurveillance in mice

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ABSTRACT

Natural killer (NK) cells are an important component of anti-cancer immunity, and their activity is regulated by an array of activating and inhibitory receptors. In mice, the inhibitory NKR-P1B receptor is expressed in NK cells and recognizes the C-type lectin-related protein-b (Clr-b) ligand. NKR-P1B:Clr-b interactions represent a 'missing-self' recognition system to monitor cellular levels of Clr-b on healthy and diseased cells. Here, we report an important role for NKR-P1B:Clr-b interactions in tumor immunosurveillance in MMTV-PyVT mice, which develop spontaneous mammary tumors. MMTV-PyVT mice on NKR-P1B-deficient genetic background developed mammary tumors earlier than on wild-type (WT) background. A greater proportion of tumor-infiltrating NK cells downregulate expression of the transcription factor Eomesodermin (EOMES) in NKR-P1B-deficient mice compared to WT mice. Tumor-infiltrating NK cells also downregulated CD49b expression but gain CD49a expression and exhibit effector functions, such as granzyme B upregulation and proliferation in mammary tumors. However, unlike the $EOMES^+$ NK cells, the $EOMES^-$ NK cell subset is unable to respond to further *in vitro* stimulation and exhibits phenotypic alterations associated with immune dysfunction. These alterations included increased expression of PD-1, LAG-3, and TIGIT and decreased expression of NKp46, Ly49C/I, CD11b, and KLRG-1. Furthermore, tumor-infiltrating NKR-P1B-deficient NK cells exhibited an elevated dysfunctional immune phenotype compared to WT NK cells. These findings demonstrate that the NKR-P1B receptor plays an important role in mammary tumor surveillance by regulating anti-cancer immune responses and functional homeostasis in NK cells.

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Introduction

NK cells are the third major lymphocyte population after B and T cells. They make up 5–15% of lymphocyte populations in blood and peripheral lymphoid tissues in humans and mice.[1] NK cells are classified as innate lymphoid cells due to their rapid response without prior antigenic exposure and their lack of antigen-specific receptors. However, NK cells have been shown to possess some degree of antigen specificity and adaptive memory-like functions.[2–4] The mechanisms of these adaptive functions in NK cells are not known. The target cells for NK cells include pathogen-infected cells, damaged or stressed cells, and transformed malignant cells. NK cells play an important role in anti-cancer immune responses by mediating direct killing of cancer cells and by modulating the activity of other immune cells via production of cytokines, including interferon γ (IFNγ) and tumor necrosis factor α (TNF-α).[1] Deficiencies in NK cell functions is associated with increased susceptibility to cancer in humans.[5–7] Similarly, depletion of NK cells and genetic manipulations that disrupt NK cell functions in mice increase tumorigenesis,[8,9] demonstrating that NK cells are an essential component of anti-cancer immunity in both humans and mice.

The function of NK cells is regulated by an array of activating and inhibitory cell surface receptors.[10] Integration of signals from these receptors interacting with their ligands expressed on target cells determines the outcome of NK cell activity. Increased interactions involving activating receptors and their ligands result in NK cell activation and target cell killing, while interactions involving inhibitory receptors and their ligands result in the suppression of NK cell activation.[11] Known ligands for activating receptors include viral proteins, such as m157 and m12 proteins of the mouse cytomegalovirus (MCMV)[12,13] and self-MHC-like proteins, such as MICA and MICB in humans[14] and Rae1 in mice,[15] which are upregulated in cells undergoing neoplastic transformation and genotoxic stress.[16] The ligands for inhibitory NK cell receptors include self proteins, such as class I major histocompatibility (MHC-I) molecules, which are expressed at high levels in normal healthy cells.[17] Inhibitory ligands are often downregulated in diseased cells, which can trigger NK cell activation in a process called the 'missing-self' response.[18]

Among the NK cell receptors, the NKR-P1 receptor family is unique in that these receptors are C-type lectin proteins conserved in multiple species and recognize non-MHC ligands

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belonging to the C-type lectin-related (Clr) family of proteins.[19,20] Genes encoding for NKR-P1 receptors and Clr ligands are genetically linked to the NK gene complex (NKC). In mice, five expressed members of the NKR-P1 receptors (NKR-P1A, B, C, F, and G), and seven members of the Clr proteins (Clr-a, b, c, d, f, g, and h) have been reported.[21–23] The known interactions between NKR-P1 and Clr proteins include NKR-P1B:Clr-b; NKR-P1F:Clr-c/d/g; and NKR-P1G:Clr-d/f/g.[24–27] In C57BL/6 mice, NKR-P1B is an inhibitory receptor expressed on approximately 60% of the splenic NK cells.[28,29] Its ligand, Clr-b, is broadly expressed in hematopoietic cells, but its expression is often downregulated in tumor cell lines and in cells undergoing genotoxic and cellular stress *in vitro*.[24,30] NKR-P1B:Clr-b interactions represent a 'missing-self' recognition system to monitor cellular levels of Clr-b. NKR-P1B:Clr-b interactions are also involved in cancer immunosurveillance by NK cells and in cancer immunoevasion. Genetic deficiency of NKR-P1B in mice was found to delay development of B cell lymphoma, which expresses Clr-b at high levels.[29]

Alterations in NK cell phenotype and functions have been noted in multiple cancers. T-box transcription factors, Eomesodermin (EOMES) and T-bet, play important roles in NK cell development, maturation, and function. Both transcription factors are expressed in NK cells.[31–33] EOMES regulates expression of CD122, the β-chain of IL-2 and IL-15 receptors, which is required for IL-15 signaling and NK cell development.[32] Genetic deficiency of EOMES in hematopoietic cells results in impaired NK cell development, while T-bet deficiency results in reduced numbers of NK cells in the peripheral organs, which exhibit an immature phenotype.[31,34] EOMES and T-bet appear to function in a stage-specific manner during NK cell development, with EOMES being essential during the early immature stages and T-bet in more mature NK cells.[35,36] EOMES and T-bet expression are upregulated when NK cells are stimulated with IL-12, IL-15, and IL-18, and correlates positively with IFNγ production.[31,34,37] Downregulation of EOMES and T-bet expression, accompanied by molecular signatures of immune exhaustion, such as downregulation of activating receptors, upregulation of inhibitory receptors, and expression of programmed cell death protein 1 (PD-1), T cell immunoglobulin, and ITIM domain protein (TIGIT), and lymphocyte-activation gene 3 (LAG-3) has been shown in various cancer models. As well, exhausted NK cells have a reduced capacity to produce IFNγ, perform cytotoxic activity, and fail to control tumor growth.[37–46] NK cells may also be induced to convert to type 1 innate lymphoid cells in the tumor microenvironment, which have reduced capacity for tumor immunosurveillance.[47]

Immune dysfunction is an established phenomenon that occurs in the tumor microenvironment, which has been extensively studied in T cells. Antigen-specific T cells progressively differentiate into distinct dysfunctional states in tumors. Early during tumorigenesis, T cells receive suboptimal activation and co-stimulatory signals in a non-inflammatory environment resulting in an anergy-like dysfunctional state characterized by cell surface expression of PD-1 and LAG-3.[48–50] As tumors progress, T cells receive continual antigenic stimulation, leading to an exhausted dysfunctional state characterized by the expression of inhibitory receptors CD38, CD39, CD101, and T-cell immunoglobulin domain and mucin domain 3 (TIM3), in addition to PD-1 and LAG-3.[51–53] While T cells in the early dysfunctional state can regain effector function when removed from the tumors, the late dysfunctional state appears to be irreversible in T cells. Progression to dysfunctional states is likely driven by multifaceted immunosuppressive mechanisms in the tumor microenvironment. Unlike T cells, there are no established phenotypic markers to distinguish various dysfunctional states of NK cells. Moreover, the mechanisms leading to NK cell dysfunction in the tumor microenvironment are not fully understood.

In the present study, we have used the MMTV-PyVT mouse model of breast cancer with a genetic ablation of the gene encoding for the inhibitory NKR-P1B receptors to study the role of NKR-P1B:Clr-b interactions in mammary tumor immunosurveillance. We demonstrate that NKR-P1B plays a role in mammary tumor immunosurveillance mediated by NK cells. Compared to WT mice, mammary tumors develop earlier in NKR-P1B-deficient mice, possibly due to the defective 'missing-self' response against Clr-b-deficient mammary tumor cells in these mice. Interestingly, inhibitory signals from the NKR-P1B receptor are important for maintaining effector functions in tumor-infiltrating NK cells. In the absence of the NKR-P1B receptor, NK cells exhibit a higher level of phenotypic and functional alterations associated with NK cell dysfunction. Together, the defective 'missing-self' response against Clr-b-deficient target cells and increased NK cell dysfunction in NKR-P1B-deficient mice renders them more susceptible to mammary tumor development.

Materials and Methods

Mice

NKR-P1B-deficient mice (*Nkrp1b*$^{-/-}$) have been reported earlier.[29] Wild-type (WT) C57BL/b (B6) and MMTV-PyVT transgenic mice on B6 background (strain: B6.FVB-Tg (MMTV-PyVT)634Mul/LellJ) were obtained from The Jackson Lab. Male MMTV-PyVT mice were bred with female *Nkrp1b*$^{-/-}$ mice to obtain the F1 progeny, which were *Nkrp1b*$^{+/-}$ and were either positive or negative for the MMTV-PyVT transgene. To obtain MMTV-PyVT^{+}*Nkrp1b*$^{-/-}$ and MMTV-PyVT^{+}*Nkrp1b*$^{+/+}$ mice, male MMTV-PyVT^{+}*Nkrp1b*$^{+/-}$ were bred with female *Nkrp1b*$^{+/-}$ mice. To increase the number of mice with appropriate genotype for experiments, we also established male MMTV-PyVT^{+}*Nkrp1b*$^{-/-}$ with female *Nkrp1b*$^{-/-}$ breeders, and male MMTV-PyVT^{+}*Nkrp1b*$^{+/+}$ with female WT (*Nkrp1b*$^{+/+}$) breeders. All mice were genotyped by polymerase chain reaction (PCR) using primers described before.[9,29] The female MMTV-PyVT^{+}*Nkrp1b*$^{-/-}$ (referred to as *Nkrp1b*$^{-/-}$) and MMTV-PyVT^{+}*Nkrp1b*$^{+/+}$ (referred to as WT) mice of similar age were co-housed throughout the duration of the experiments. All mice were housed in a specific pathogen-free (SPF) facility at the University of Windsor. All experiments were approved by the University of Windsor's Animal Care Committee in accordance with the Canadian Council for Animal Care (CCAC) guidelines.

Tumor measurements

Female mice were monitored weekly for the onset of the first palpable tumor. Subsequently, tumor growth was monitored biweekly, and tumor diameter measurements were recorded using a digital caliper. The size of tumors was recorded as an average of two diameter measurements at two different angles at each time point. MMTV-PyVT mice develop tumors in multiple mammary glands. Mice were euthanized when the tumor diameter was between 10 and 15 mm.

Preparation of tumor-infiltrating lymphocytes

Tumors from female MMTV-PyVT^{+} and MMTV-PyVT^{+}*Nkrp1b*$^{-/-}$ mice were harvested after euthanasia. Tumors were categorized by size; small (<5 mm), medium (5–10 mm), and large (>10 mm), representing early- (small) and late-stage (large) mammary tumors. Mammary glands #3 and 4 were harvested from normal WT and *Nkrp1b*$^{-/-}$ mice after removing the lymph nodes. Tumors and normal mammary glands were cut into fine pieces in 5 mL serum-free RPMI medium (Invitrogen) containing 200 µg/mL collagenase D (Wortnin) and 20 µg/mL DNase1 (Roche) and incubated at 37°C for 30 minutes with gentle shaking every few minutes. EDTA (Sigma) was added at a final concentration of 1 mM to stop digestion. The digested tumors were passed through a 70- µm cell strainer (Fisher Scientific) to obtain single-cell suspensions in phosphate buffered saline (PBS) solution containing 1 mM EDTA to prevent clumping of cells. Cells were spun down at 500xg for 5 min and treated with 5 mL Ammonium-Chloride-Potassium (ACK) lysis buffer for 5 min to lyse red blood cells. Subsequently, cells were washed and resuspended in PBS. Live cells were counted using trypan blue dye and a hemocytometer.

Flow cytometry analysis

The following antibodies were purchased from BioLegend: AF700-CD45 (30-F11), PE/Dazzel 594-NK1.1 (PK136), BV510-TCRβ (H57-597), PE-CY7-CD49b (Dx5), FITC-Ki67 (11 F6), PerCP-CY5.5-CD11b (M1/70), BV421-NKp46 (29A1.4), biotin-NKG2D (C7), FITC-PD-1 (29 F.1A12), PE-CY7-KLRG-1 (2F1/KLRG-1), BV421-TIGIT (1 G9), PerCP-CY5.5-DNAM-1 (10E5), biotin-LAG-3 (C9B7W), AF647-EOMES (W17001A), PE-EOMES (W17001A), BV421-Granzyme B (QA18A28), FITC-CD107a (1D4B), PE-IFNγ (XMG1), BV650-streptavidin, PerCP-Cy5.5-streptavidin, and PE-CY7-streptavidin. The following antibodies were purchased from BD Bioscience: PerCP-CY5.5-T-bet (O4-46), AF647-CD161b (2D9), BV421-CD161b (2D9), PE-CD49a (Ha31/8), APC-CD49a (Ha31/8), and biotin-Ly49C/I (5E6). Fixable viability dye (APC-eF780) was purchased from Invitrogen. Anti-Clr-b (4A6) antibody has been described previously.[24]

Approximately, 2×10^6 cells were stained with fluorescently labeled antibodies for flow cytometry analysis. Tumor cell suspensions were treated with TRUstain Fc block reagent (Biolegend) according to the manufacturer's instructions and then incubated with appropriate dilutions of the primary antibodies cocktail in FACS staining buffer (2.5% bovine serum albumin and 0.1% NaN_3 in PBS) at 4°C and protected from light for 20 min. Cells were washed once with FACS staining buffer, and if required, cells were incubated with fluorophore-conjugated streptavidin in FACS staining buffer at 4°C and protected from light for 20 min. For intracellular staining and staining of transcription factors, cells were washed with FACS buffer once after surface staining, fixed and permeabilized using transcription factor fixation and permeabilization buffers (Invitrogen), and incubated with appropriate dilutions of the antibodies in the permeabilization buffer (Invitrogen) following the manufacturer's instructions. Data were acquired using BD LSR Fortessa X20 flow cytometer and analyzed using FlowJo software.

In vitro stimulation

NK cell stimulation was performed by treating 1×10^6 cells prepared from tumors, as described above, with PMA (20 ng/mL) and ionomycin (1 µg/mL), or IL-12 (10 ng/mL) and IL-18 (10 ng/mL) in 200 µL RPMI-1640 (Sigma) medium containing 10% FBS (Gibco), 100 U/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco), 10 mM HEPES (Gibco), 1 mM sodium pyruvate (Gibco), 55 µM β-mercaptoethanol (Gibco), and 1% non-essential amino acids (HyClone). Cell stimulations were performed in a 96-well round-bottom plate. Unstimulated control cells were treated similarly but without the addition of stimuli. After 30–60 min of incubation at 37°C and 5% CO_2, brefeldin A (BioLegend), monensin (BioLegend), and FITC-CD107a antibody at appropriate dilution were added to the cells. Cells were incubated for another 4 h at 37°C and 5% CO_2. After incubation, the plate was spun at 500xg for 5 min, the supernatant was removed, and cells were stained using NK cell surface markers followed by intracellular staining for EOMES and IFNγ, as described above.

Statistical analysis

Statistical analysis was performed by log-rank test (tumor-free survival), two-tailed unpaired Student's *t*-test, and one-way or two-way ANOVA followed by Tukey or Sidak post-hoc test, respectively. Differences with *p* values of less than or equal to 0.05 were deemed statistically significant.

Results

Mammary tumors develop earlier in NKR-P1B-deficient MMTV-PyVT mice

MMTV-PyVT mice express the Polyoma virus middle T antigen in the mammary glands under the control of the mouse mammary tumor virus promoter and spontaneously develop mammary tumors.[54] To understand the role of the NKR-P1B:Clr-b recognition axis in mammary tumor immunosurveillance, we bred MMTV-PyVT mice on NKR-P1B-deficient (*Nkrp1b*$^{-/-}$) background. Female MMTV-PyVT mice on WT and NKR-P1B-deficient backgrounds were monitored for palpable tumor onset and progression. We observed that the WT mice remained tumor-free

significantly longer than the NKR-P1B-deficient mice (Figure 1a), suggesting a role for NKR-P1B in mammary tumor immunosurveillance. We did not observe a significant difference in tumor progression determined by an increase in tumor diameter over time (Figure 1b) or tumor growth rate (Figure 1c) between WT and NKR-P1B-deficient mice. Clr-b is highly expressed in hematopoietic cells, and its expression is reduced in tumor cell lines.[24] We analyzed Clr-b expression in mammary tumor cells from MMTV-PyVT mice by flow cytometry. Compared to the $CD45^+$ tumor-infiltrating lymphocytes which expressed Clr-b at high level, only a subset of mammary tumor cells ($CD45^-$) expressed Clr-b, which was highly variable between tumors (Figure 1d). Although a smaller proportion of $CD45^-$ tumor cells expressed Clr-b in large tumors from NKR-P1B-deficient mice compared to WT mice, this difference was not statistically significant (Figure 1e). We did not detect any statistically significant differences in Clr-b expression level quantified by median fluorescent intensity (MFI) of Clr-b staining in tumors from NKR-P1B-deficient and WT mice. We have previously shown that NKR-P1B-deficient mice exhibit defective NK cell-mediated missing-self response against target cells lacking Clr-b expression.[29] Since a majority of tumor cells (>70%) in MMTV-PyVT mammary tumors lack expression of Clr-b, the defective Clr-b-dependent missing-self response in the NKR-P1B-deficient mice may render them more susceptible to mammary tumors. Together, these data demonstrate the role of NKR-P1B receptors in mammary tumor immunosurveillance in MMTV-PyVT transgenic mice.

Tumor-infiltrating NK cells in NKR-P1B-deficient mice lose EOMES expression

NKR-P1B receptor plays an important role in Clr-b-dependent 'missing-self' responses and immunosurveillance mediated by NK cells in B6 mice.[29] Therefore, we analyzed the NK cell population in the mammary tumors from WT and NKR-P1B-deficient mice to reveal any differences that may impact mammary tumor immunosurveillance. Flow cytometry analysis of NK cells ($CD45^+NK1.1^+TCR\beta^-$) in the large (>10 mm in diameter), medium (5–10 mm in diameter), and small (<5 mm in diameter) mammary tumors from MMTV-PyVT mice revealed equal infiltration of NK cells into the tumors from both NKR-P1B-deficient and WT mice (**Suppl. Fig. 1**). NK cells express EOMES and T-bet transcription factors.[33] We detected loss of EOMES expression, but not T-bet expression,

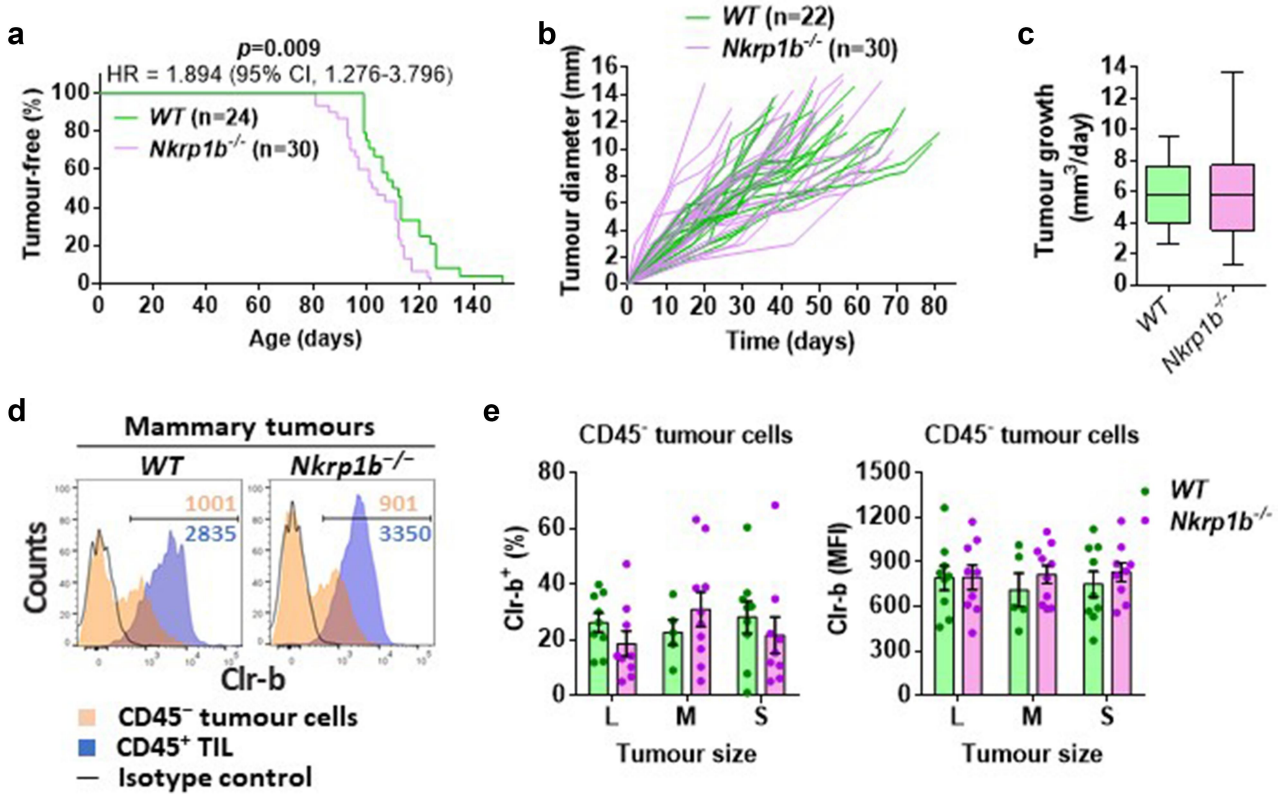

Figure 1. Mammary tumors develop earlier in NKR-P1B-deficient MMTV-PyVT mice. Mammary tumor development and growth in MMTV-PyVT mice on C57BL/6 (*WT*) and *Nkrp1b*$^{-/-}$ genetic backgrounds. **a**) Mammary tumor development in *WT* and *Nkrp1b*$^{-/-}$ MMTV-PyVT mice. Statistical analysis was performed by *Log-rank* test, and the *p*-value, hazard ratio (HR) and confidence interval (CI) are indicated. **b**) Weekly measurements of tumor diameter (mm) in *WT* and *Nkrp1b*$^{-/-}$ mice from the time tumors were detected. Each line represents a single tumor. **c**) Tumor growth rate calculated as increase in tumor volume (mm^3) over time (days). Top and bottom limits of the box represent the 25th and 75th percentiles, with whiskers indicating minimum and maximum values (n = 20–30). **d**) Clr-b expression on tumor-infiltrating lymphocytes ($CD45^+$) and tumor cells ($CD45^-$) from *WT* and *Nkrp1b*$^{-/-}$ MMTV-PyVT mice. Representative histogram overlay plots for anti-Clr-b (colored area) and isotype control (solid line) antibody staining of cells from mammary tumors are shown. Representative median fluorescence intensity (MFI) values of Clr-b staining for $CD45^-$ and $CD45^+$ cells are shown. e) Percentage of Clr-b^+ cells and Clr-b MFI in $CD45^-$ cells from large (L: >10 mm in diameter), medium (M: 5–10 mm in diameter), and small (S: <5 mm in diameter) mammary tumors are shown as mean ± SEM. Each symbol represents a single tumor from one mouse.

in tumor-infiltrating NK cells in the mammary tumors (Figure 2a). A greater frequency of NK cells lacking EOMES expression was detected in the tumors from NKR-P1B-deficient mice compared to the WT mice (Figure 2a). EOMES is a transcription factor essential for NK cell development and function.[31] Loss of EOMES expression associated with NK cell dysfunction has been previously shown in adaptively transferred NK cells in the mouse lymphoma model.[37] While we observed that the proportion of EOMES$^+$ and EOMES$^-$ NK cells remained stable in mammary tumors of different sizes in WT mice, EOMES$^-$ NK cell frequency increased progressively with tumor size in NKR-P1B-deficient mice (Figure 2a). There were significantly

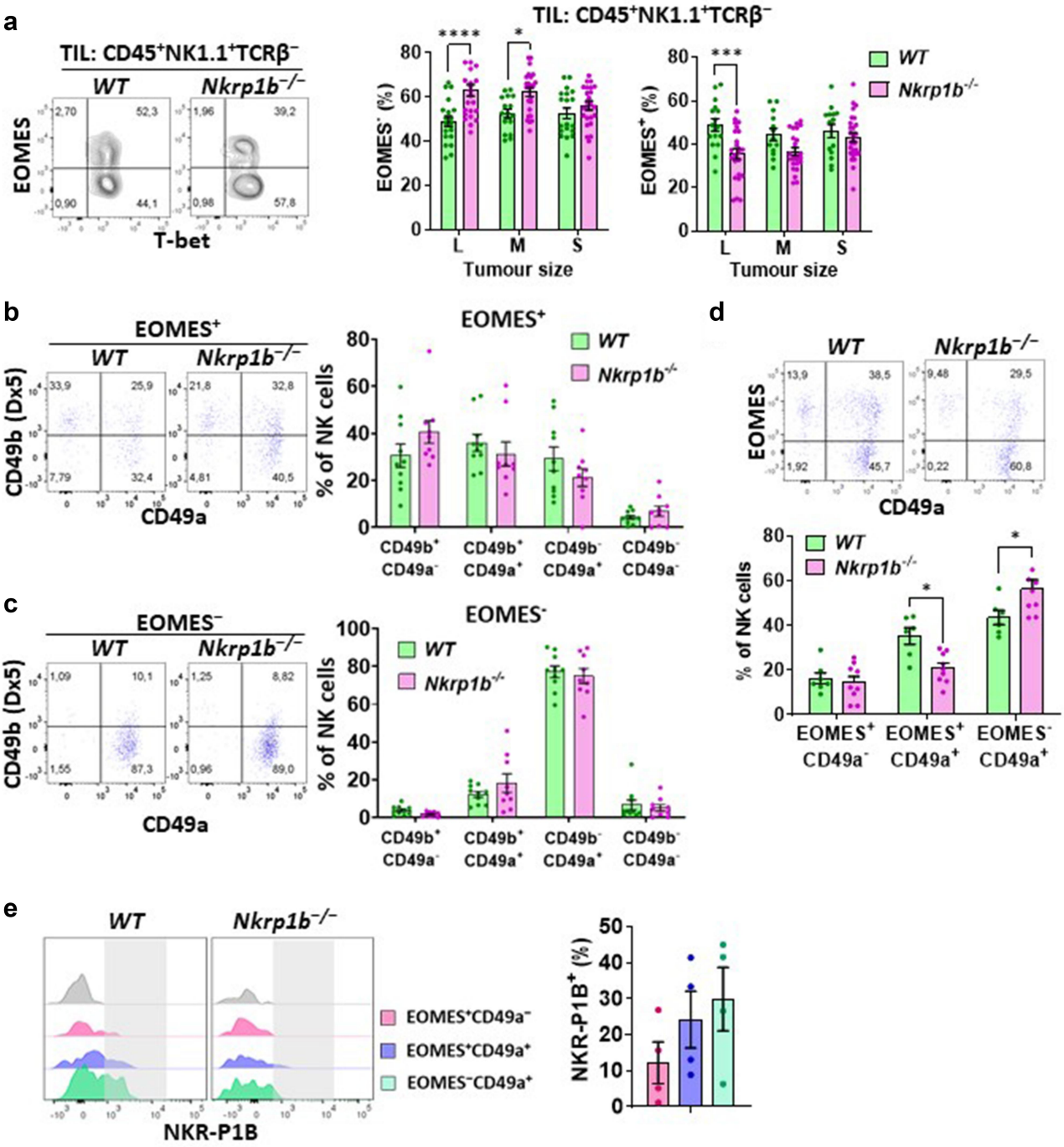

Figure 2. Altered phenotype of tumor-infiltrating NK cells in NKR-P1B-deficient mice. Flow cytometry analysis of NK cells from large (L: >10 mm in diameter), medium (M: 5–10 mm in diameter), and small (S: <5 mm in diameter) mammary tumors. **a**) A representative flow cytometry analysis plot for EOMES and T-bet expression in tumor-infiltrating NK cells (CD45$^+$NK1.1$^+$TCRβ$^-$) from *WT* and *Nkrp1b*$^{-/-}$ mice. Bar graphs show the percentages of EOMES$^+$ and EOMES$^-$ tumor-infiltrating NK cells in *WT* (n = 12–17) and *Nkrp1b*$^{-/-}$ (n = 24–28) mice. **b** and **c**) CD49a and CD49b expression in EOMES$^+$ (b) and EOMES$^-$ (c) subsets of tumor-infiltrating NK cells from *WT* and *Nkrp1b*$^{-/-}$ mice. **d**) Tumor-infiltrating NK cell subsets based on the expression of EOMES and CD49a in large tumors from *WT* (n = 7) and *Nkrp1b*$^{-/-}$ (n = 9) mice. **e**) NKR-P1B expression (shaded box) in EOMES$^+$CD49a$^-$, EOMES$^+$CD49a$^+$, and EOMES$^-$CD49a$^+$ subsets of tumor-infiltrating NK cells from WT and *Nkrp1b*$^{-/-}$ mice. Data are presented as mean ± SEM. Each symbol represents a single tumor from one mouse. Statistical significance was determined by two-way ANOVA followed by *Sidak* post-hoc tests.

greater proportions of EOMES$^-$ NK cells in the large- and medium-sized tumors from NKR-P1B-deficient mice compared to WT mice (Figure 2a). Analysis of integrins CD49a and CD49b in tumor-infiltrating NK cells revealed major differences in the expression of both integrins in the EOMES$^+$ and EOMES$^-$ NK cell subsets, but no statistically significant differences in the proportion of cells were observed in tumors from WT and NKR-P1B-deficient mice (Figure 2b and c). EOMES$^+$ NK cells were composed of roughly equal proportions of CD49b$^+$CD49a$^-$, CD49b$^+$CD49a$^+$, and CD49b$^-$CD49a$^+$ cells (Figure 2b); however, EOMES$^-$ NK cells were predominantly CD49b$^-$CD49a$^+$ (Figure 2c). CD49a is normally expressed in tissue resident NK cells but not in NK cells in circulation or in lymphoid tissues.[55] Based on the expression of EOMES and CD49a in tumor-infiltrating NK cells, three distinct subsets of NK cells could be distinguished: EOMES$^+$CD49a$^-$, EOMES$^+$CD49a$^+$, and EOMES$^-$CD49a$^+$ (Figure 2d), with a significantly lower frequency of EOMES$^+$CD49a$^+$ and larger frequency of EOMES$^-$CD49a$^+$ NK cells present in tumors from NKR-P1B-deficient mice compared to WT mice (Figure 2d). All three NK cell subsets expressed the inhibitory NKR-P1B receptor, but to varying degrees, with the highest frequencies of NKR-P1B$^+$ NK cells being present in the EOMES$^-$CD49a$^+$ subset followed by the EOMES$^+$CD49a$^+$ and EOMES$^+$CD49a$^-$ subsets (Figure 2e). The data together reveal that mammary tumor-infiltrating NK cells acquire a tissue-resident phenotype accompanied by a loss of EOMES expression, which occurs at a higher frequency when NKR-P1B receptor signals are disrupted via genetic deletion of the *Nkrp1b* gene.[56,57]

Tumor-infiltrating EOMES$^-$ NK cells exhibit an activated phenotype but fail to respond to further stimulation in vitro

To determine how CD49a expression and loss of EOMES affected effector functions of mammary tumor-infiltrating NK cells, we first analyzed the expression of granzyme B and Ki67 *ex vivo* in three subsets of tumor-infiltrating NK cells: EOMES$^+$CD49a$^-$, EOMES$^+$CD49a$^+$, and EOMES$^-$CD49a$^+$. Granzyme B is a cytotoxic effector molecule present in NK cell granules, and its expression is increased in activated NK cells. Activated NK cells in a proliferative state also upregulate expression of Ki67, a nuclear protein involved in cell proliferation. We observed a marked increase in both granzyme B and Ki67 expressiond in CD49a$^+$ NK cells (both EOMES$^+$ and EOMES$^-$ subsets) compared to the CD49a$^-$ NK cell subset, indicating that CD49a$^+$ NK cells constitute the effector subset of NK cells in mammary tumors from both WT mice (Figure 3a and b) and NKR-P1B-deficient mice (**Suppl. Fig. 2A**). We also observed that in large tumors, significantly fewer EOMES$^-$CD49a$^+$ NK cells expressed Ki67 compared to the EOMES$^+$CD49a$^+$ subset (Figure 3b), suggesting a reduced proliferative capacity in the EOMES$^-$ subset of tumor-infiltrating effector NK cells.

To further study tumor-infiltrating NK cell functionality in the MMTV-PyVT mice, NK cell degranulation (CD107a expression) and IFNγ production was evaluated following *in vitro* stimulation of tumor-infiltrating NK cells with PMA and ionomycin, or IL-12 and IL-18. PMA and ionomycin treatment induced IFNγ production and degranulation in a significantly larger proportion of EOMES$^+$CD49a$^-$ NK cells compared to EOMES$^-$CD49a$^+$ NK cells with EOMES$^+$CD49a$^+$ NK cells showing an intermediate response in both WT (Figure 3c) and NKR-P1B-deficient mice (**Suppl. Fig. 2B**). Similarly, IL-12 and IL-18 stimulation induced IFNγ production in a significantly higher proportion of EOMES$^+$CD49a$^-$ NK cells compared to EOMES$^+$CD49a$^+$ and EOMES$^-$CD49a$^+$ NK cells (Figure 3c). Together, the data demonstrate that CD49a$^+$ NK cells in mammary tumors constitute effector subset of tumor-infiltrating NK cells; however, they can lose expression of EOMES and enter into a dysfunctional state characterized by lower proliferative capacity and impaired response to stimulation *in vitro*.

EOMES$^-$ NK cells exhibit phenotypic alterations associated with a dysfunctional state

To determine whether EOMES$^-$ NK cells exhibit phenotypic characteristics of dysfunctional NK cells, we analyzed the expression of markers associated with differentiation and immune dysfunction *ex vivo* in three subsets of tumor-infiltrating NK cells from MMTV-PyVT mice: EOMES$^+$CD49a$^-$, EOMES$^+$CD49a$^+$, and EOMES$^-$CD49a$^+$.

Dysfunctional NK cells in cancers lose expression of activating receptors and acquire expression of markers associated with immune exhaustion. Therefore, we assessed expression of PD-1, LAG-3, and TIGIT, as well as the natural cytotoxicity receptor NKp46, and activating receptors NKG2D and DNAM-1. We observed a significant upregulation of PD-1 expression in EOMES$^-$CD49a$^+$ NK cells compared to EOMES$^+$CD49a$^-$ NK cells (Figure 4a). Similarly, EOMES$^-$CD49a$^+$ NK cell subset contained significantly higher proportion of cells expressing LAG-3 compared to the other two subsets (Figure 4b). On the other hand, TIGIT expression was detected in significantly larger proportion of EOMES$^+$CD49a$^+$ NK cell subset (Figure 4c). EOMES$^-$CD49a$^+$ NK cells also exhibited significant loss of the activating NKp46 receptor compared to other two subsets (Figure 4d); however, DNAM-1 expression was significantly increased in this subset of tumor-infiltrating NK cells (Figure 4e). NKG2D expression remained largely unchanged in three NK cell subsets of mammary tumors (figure 4f). Additionally, EOMES$^+$CD49a$^+$ and EOMES$^-$CD49a$^+$ NK cells exhibited significant loss of expression of Ly49C/I (Figure 4g), CD11b (Figure 4h), and killer cell lectin-like receptor G1 (KLRG1) (Figure 4i), which are associated with NK cell function, maturation, and homeostasis.[51,52] Normal mammary glands harbor a small population of NK1.1$^+$ cells, which include small subsets of EOMES$^-$ and CD49a$^+$ cells (**Suppl. Fig 3A** and **B**). However, NK cells in normal mammary glands do not express PD-1, TIGIT, or LAG-3 unlike tumor-infiltrating NK cells (**Suppl. Fig. 3D** and **E**). The data together indicate that EOMES$^-$ NK cells in mammary tumors exhibit phenotypic alterations characterized by a loss of expression of cell surface proteins associated with NK cell maturation and homeostasis and upregulation of markers associated with immune cell dysfunction in MMTV-PyVT mice.

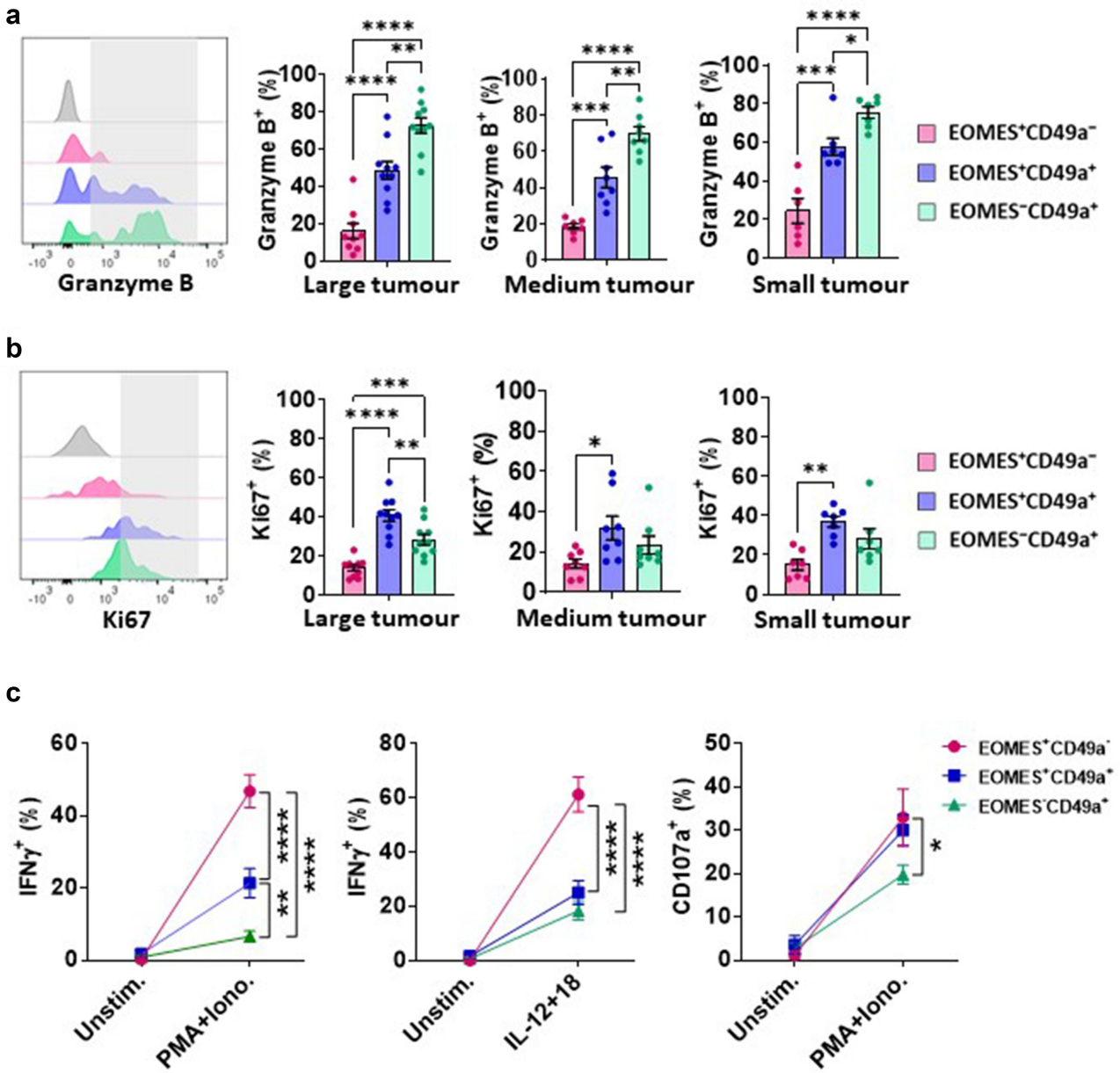

Figure 3. EOMES$^-$ NK cells exhibit effector phenotype in the mammary tumors but are less responsive toward external stimuli than EOMES$^+$ NK cells. A and B) Granzyme B (a) and Ki67 (b) expression in EOMES$^+$CD49a$^-$, EOMES$^+$CD49a$^+$, and EOMES$^-$CD49a$^+$ subsets of tumor-infiltrating NK cells in the large (n = 10), medium (n = 8), and small (n = 7) mammary tumors from *WT* mice. Each symbol represents a single tumor from one mouse. Representative histogram plot for cells stained with anti-granzyme B (**a**) and anti-Ki67 (**b**) antibodies, and isotype control antibodies (gray histogram) in large tumors is shown. **c**) Tumor-infiltrating lymphocytes were treated with PMA and ionomycin, or IL-12 and IL-18, followed by analysis of degranulation (CD107a expression) and IFNγ production in EOMES$^+$CD49a$^-$, EOMES$^+$CD49a$^+$, and EOMES$^-$CD49a$^+$ subsets of tumor-infiltrating NK cells. Data are presented as mean ± SEM of n = 5 independent tumors. Statistical significance was determined by one-way ANOVA (a and b), and two-way ANOVA (c) followed by *Tukey* post-hoc test.

Tumor-infiltrating NK cells experience higher immune dysfunction in NKR-P1B-deficient mice

The increased frequency of tumor-infiltrating EOMES$^-$ NK cells in NKR-P1B-deficient mice compared to the WT mice suggested accumulation of dysfunctional NK cells in the tumors from these mice (Figure 2a and d). To determine if tumor-infiltrating NK cells also exhibited higher levels of phenotypic changes associated with a dysfunctional state in the NKR-P1B-deficient mice, we compared expression of phenotypic markers, described above, in three subsets of tumor-infiltrating NK cells from the WT and NKR-P1B-deficient mice. We observed Ki67 expression in a significantly larger proportion of EOMES$^+$CD49a$^+$ NK cells from NKR-P1B-deficient mice compared to the WT mice, indicating higher proliferation in this subset of NK cells in the NKR-P1B-deficient mice (Figure 5a). Ki67 expression was reduced in EOMES$^-$CD49a$^+$ NK cells compared EOMES$^+$CD49a$^+$ NK cells to a comparable level in both WT and NKR-P1B-deficient mice (Figure 5a). Both the EOMES$^+$CD49a$^+$ and EOMES$^-$CD49a$^+$ subsets of tumor-infiltrating NK cells from NKR-P1B-deficient mice exhibited a significantly higher frequency of Ly49C/I expression and lower frequency of NKp46 expression compared to the corresponding NK cell subsets from the WT mice (Figure 5b and c). PD-1 expression was also significantly higher in the EOMES$^-$CD49a$^+$ NK cells from NKR-

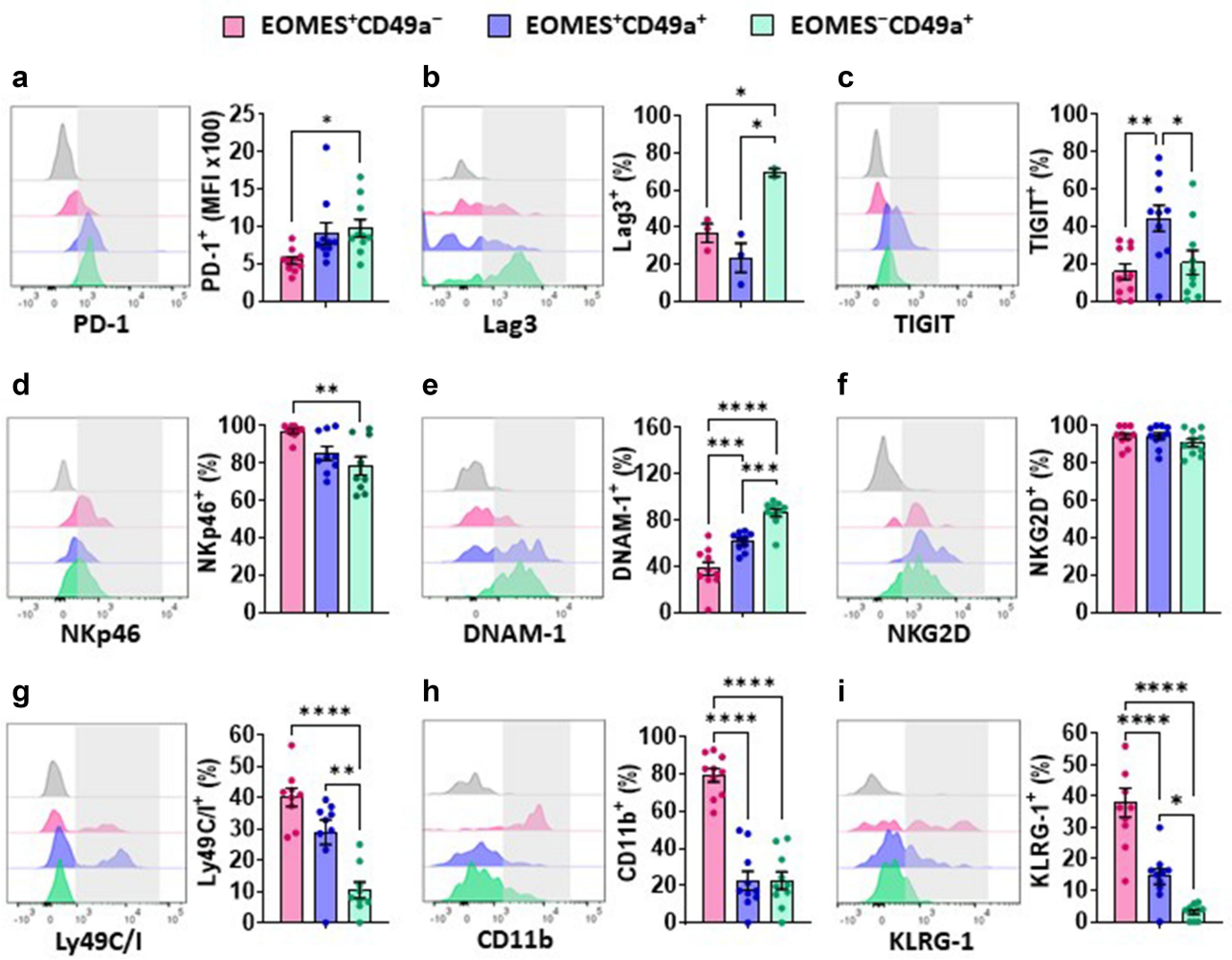

Figure 4. EOMES$^-$ NK cells exhibit an exhausted phenotype in the mammary tumors. Analysis of phenotypic markers, PD-1 (a), LAG-3 (b), TIGIT (c), NKp46 (d), DNAM-1 (e), NKG2D (f), Ly49C/I (g), CD11b (h), and KLRG-1 (i) in EOMES$^+$CD49a$^-$, EOMES$^+$CD49a$^+$, and EOMES$^-$CD49a$^+$ subsets of tumor-infiltrating NK cells in mammary tumors from *WT* mice (n = 10, except for Lag 3 (**b**; n = 3)). Data are presented as mean ± SEM of percentage of gated NK cell subsets positive for the analyzed markers (shaded box) or mean fluorescence intensity (MFI) for PD-1 expression (shaded box). Each symbol represents a single tumor from one mouse. Isotype control staining is represented by the gray histogram. Statistical significance was determined by one-way ANOVA followed by *Tukey* post-hoc test.

P1B-deficient mice compared to the WT mice (Figure 5d). In contrast to mammary tumors, no phenotypic differences were observed in NK cells in normal mammary glands from WT and NKR-P1B-deficient mice (**Suppl. Fig. 3**). Additionally, when stimulated *in vitro* (Figure 5e-g), tumor-infiltrating NKR-P1B-deficient NK cell subsets exhibited lower degranulation compared to WT-NK cells (Figure 5g), however, this difference did not reach statistical significance. Together, the data suggest that in addition to the increased frequency of EOMES$^-$ NK cells in tumors from NKR-P1B-deficient mice compared to WT mice, the NKR-P1B-deficient NK cells also exhibit increased phenotypic changes associated with NK cell dysfunction compared to WT-NK cells in mouse mammary tumors.

Discussion

NK cells are a major component of anti-cancer immune responses owing to the rapid priming of their effector functions without prior antigen exposure, and their natural ability to kill tumor cells, meaning that they hold great potential for use as effector cells in cancer immunotherapy. Several studies have shown that immune checkpoint receptor blockade (ICB) can improve NK cell functions and enhance their anti-cancer activity.[41,42,46] NK cells exhibit dysfunctional states similar to T cells, which can be resolved by ICB, but the nature of the dysfunctional states in NK cells and the mechanisms driving them are not fully understood. The functions of NK cells are regulated by an array of activating and inhibitory receptors, many of which have been shown to play important roles in cancer immunosurveillance.[8,9,29,58] A better understanding of the functions of these receptors in cancer immunosurveillance and their role in regulating functional homeostasis of NK cells during anti-cancer immune responses will be crucial to fully realize the potential of NK cells for cell-based cancer immunotherapy. Here, we report an important role of the inhibitory NKR-P1B receptor in the immunosurveillance of mammary tumors lacking expression of Clr-b in MMTV-PyVT transgenic mice and its role in regulating NK cell anti-cancer immune responses. We show that NKR-P1B-deficient mice develop mammary tumors earlier than WT mice. We also show that a greater frequency of NK cells exhibit phenotypic and

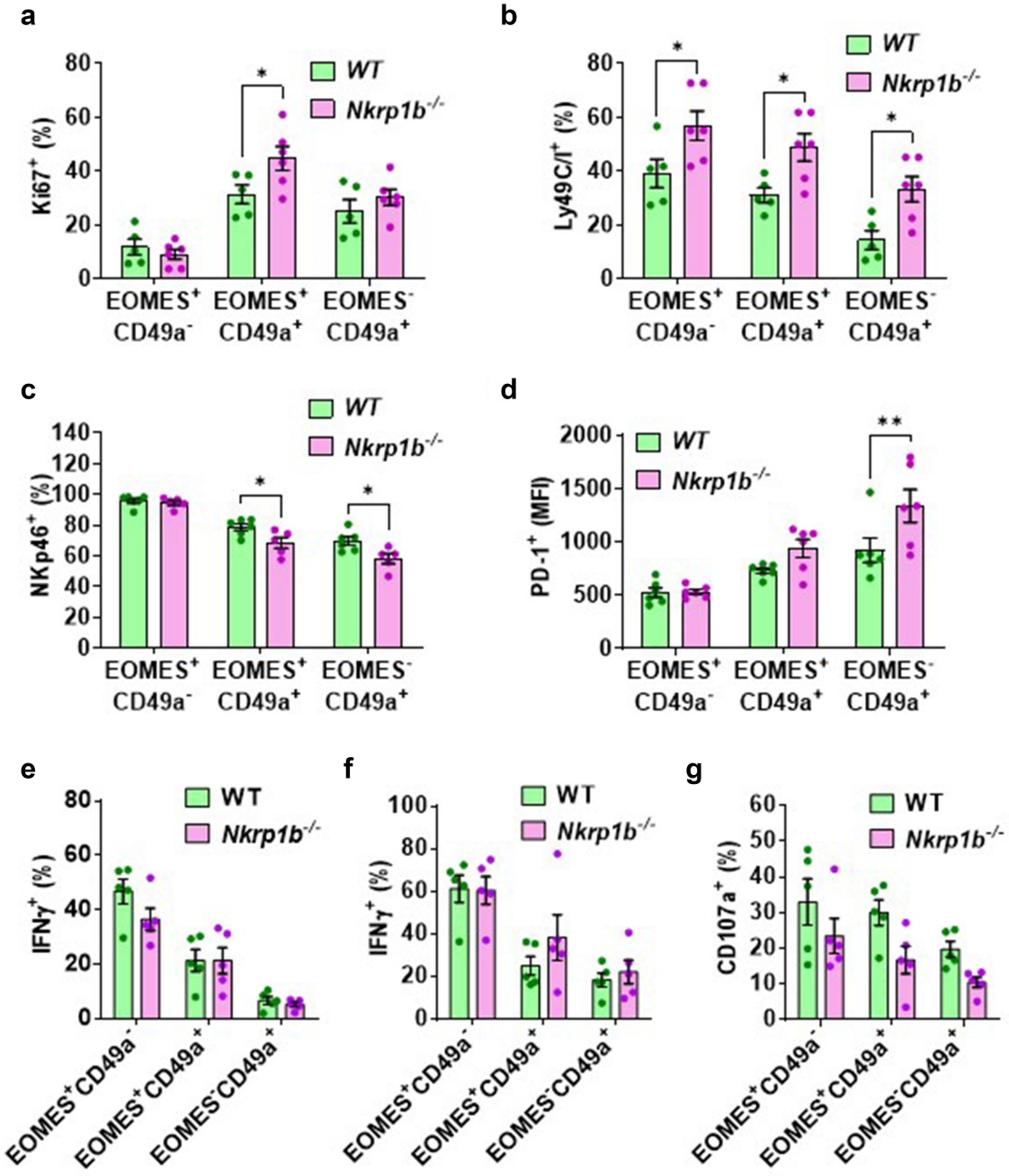

Figure 5. NKR-P1B-deficient tumor-infiltrating NK cells exhibit increased exhaustion phenotype than the *WT* NK cells. Comparison of the phenotype and function of NK cells in the large (>10 mm in diameter) mammary tumors from WT and *Nkrp1b*$^{-/-}$ mice. **a-c**) Percentage of cells expressing Ki67 (a), Ly49C/I (b), and NKp46 (c) amongst the three subsets (EOMES^{+}CD49a^{-}, EOMES^{+}CD49a^{+}, and EOMES^{-}CD49a^{+}) of tumor-infiltrating NK cells from WT (n = 6) and *Nkrp1b*$^{-/-}$ (n = 6) mice. **d**) Mean fluorescence intensity (MFI) values for PD-1 expression in the three subsets (EOMES^{+}CD49a^{-}, EOMES^{+}CD49a^{+}, and EOMES^{-}CD49a^{+}) of tumor-infiltrating NK cells from WT (n = 6) and *Nkrp1b*$^{-/-}$ (n = 6) mice. **e-g**) Production of IFNγ and degranulation (CD107a expression) in the three subsets (EOMES^{+}CD49a^{-}, EOMES^{+}CD49a^{+}, and EOMES^{-}CD49a^{+}) of tumor-infiltrating NK cells from WT (n = 5) and *Nkrp1b*$^{-/-}$ (n = 5) mice stimulated with PMA and ionomycin (e and g) or IL-12 and IL-18 (f) *in vitro*. Data are presented as mean ± SEM. Each symbol represents a single tumor from one mouse. Statistical significance was determined by two-way ANOVA followed by *Sidak* post-hoc test.

functional alterations associated with dysfunctional states in mammary tumors from NKR-P1B-deficient mice compared to WT mice, demonstrating that signals from the NKR-P1B receptor aid in maintaining effector functions in tumor-infiltrating NK cells.

Increased susceptibility to mammary tumors in NKR-P1B-deficient mice is not completely unexpected since mammary tumors in MMTV-PyVT are composed of a large proportion of cells devoid of Clr-b expression, and NKR-P1B-deficient NK cells display impaired 'missing-self' response against Clr-b-deficient target cells.[29] NKR-P1B^{+} NK cells could mediate a Clr-b-dependent 'missing-self' response against MMTV-PyVT mammary tumors, hence delaying the development of mammary tumors in WT mice. This is similar to the increased susceptibility of NKC^{KD} mice, which lack expression of inhibitory Ly49C/I receptors for self-class I major histocompatibility (MHC-I) molecules, to develop mammary tumors induced by the injection of MHC-I-deficient but not MHC-I-sufficient E0771 mammary

adenocarcinoma cells into the mammary glands.[9] The 'missing-self' response against MHC-I-deficient target cells is impaired in NKC^{KD} mice, and hence, they display an increased susceptibility to MHC-I-deficient tumor development.[58] Similar to Ly49 receptors, the function of the NKR-P1B receptor in tumor immunosurveillance also depends on the expression of its ligand in the tumor cells. While NKR-P1B-deficient mice are more susceptible to Clr-b-deficient mammary tumors compared to WT mice, they are more resistant to B cell lymphoma, which expresses Clr-b at high levels.[29] In the B cell lymphoma model, the tumor cells appear to exploit the inhibitory NKR-P1B:Clr-b interactions to inhibit NK cells in WT but not in NKR-P1B-deficient mice. It remains to be seen if Clr-b expression in mammary tumors (or other solid tumors) can also aid in evasion of NK cells by engaging the inhibitory NKR-P1B receptor.

In addition to mediating Clr-b-dependent 'missing-self' responses, NKR-P1B receptors also appear to play a role in the homeostasis of NK cell responses in mammary tumors. In the absence of inhibitory NKR-P1B signals, NK cells display higher levels of phenotypic alterations associated with dysfunctional NK cells in mammary tumors. Tumor-infiltrating NK cells assume a tissue-resident phenotype (expressing CD49a), which also represents the NK cell subset with effector functions, i.e. exhibiting high granzyme B expression and undergoing proliferation in tumors. A significant proportion of effector NK cells in mammary tumors loses expression of EOMES and exhibits phenotypic and functional features of dysfunctional immune cells. These include loss of NCR (NKp46) expression, increased expression of checkpoint receptors TIGIT, LAG-3, and PD-1, and reduced capacity to respond to external stimuli. Interestingly, NKR-P1B is highly expressed on the activated $EOMES^{+}CD49a^{+}$ and $EOMES^{-}CD49a^{+}$ NK cells, suggesting that this receptor may have a similar role as immune checkpoint receptors important for regulating NK cell activation. Genetic disruption of NKR-P1B function leads to a progressive loss of EOMES expression in tumor-infiltrating NK cells, resulting in the accumulation of higher frequencies of $EOMES^{-}$ NK cells mammary tumors from NKR-P1B-deficient mice compared to WT mice. Therefore, the inhibitory signals from the engagement of NKR-P1B receptors by its ligand in the tumor microenvironment appear to prevent the rapid loss of EOMES expression and reduce NK cell dysfunction. Interestingly, NKR-P1B-deficient tumor-infiltrating NK cells express higher levels of the inhibitory Ly49C/I receptor than those from WT mice, which could be a compensatory mechanism for the lack of inhibitor NKR-P1B signals.

While several dysfunctional states of T cells (ignorance, anergy, exhaustion, and senescence) have been described in cancers,[59] the dysfunctional states in NK cells are not well defined. Alterations in NK cell subset composition, activity, and phenotype have been reported in breast cancer patients, as seen by the downregulation of activating receptors NKp30, NKG2D, DNAM-1, and CD16 and upregulation of the inhibitory receptor NKG2A.[43,60] In mice, adaptively transferred NK cells were found to undergo exhaustion and lose their capacity to control tumors, accompanied by the downregulation of EOMES and activating receptors, and an upregulation of checkpoint receptors.[37] In tumors lacking MHC-I expression, mouse NK cells were shown to be induced into an anergic state that could be rescued by cytokine treatment.[61] Anergy was not induced in NK cells infiltrating into MHC-I-sufficient tumors, demonstrating a potential role for the inhibitory Ly49 receptor interactions with MHC-I in preventing NK cell dysfunction in tumors – similar to the role of NKR-P1B:Clr-b interactions reported here. It should be noted that MMTV-PyVT tumors express MHC-I as has been shown previously.[9] The $EOMES^{-}$ NK cells in MMVT-PyVT mammary tumors appear to be different from anergic NK cells in MHC-I-deficient tumors since these cells form in MHC-I-sufficient tumors and do not respond to external stimuli, including IL-12 and IL-18 treatment. Interestingly, in addition to NK cell dysfunction, TGF-β in methylcholanthrene (MCA)-induced fibrosarcoma microenvironment was shown to cause differentiation of NK cells into ILC1 cells, which were less efficient in controlling tumor growth in mice.[47] In this study, induced ILC1s in the tumor microenvironment downregulated CD49b expression and gained the expression of CD49a, similar to the $EOMES^{-}CD49a^{+}$ NK cells in mammary tumors. However, unlike the ILC1 cells in MCA-induced fibrosarcomas, which could be induced to produce IFNγ after treatment with PMA and ionomycin,[47] similar treatment failed to induce IFNγ production from $EOMES^{-}CD49a^{+}$ NK cells in the mammary tumors. Nonetheless, our data are unable to fully resolve whether the $EOMES^{-}$ NK cells in MMVT-PyVT mammary tumors represent true ILC1 or exhausted NK cells, nor their true origin.

While this study demonstrates an important role for NKR-P1B:Clr-b interaction in mammary tumor immunosurveillance mediated by NK cells, it does not reveal the cellular partners mediating these interactions in the tumor microenvironment. In addition to a variegated expression of Clr-b in MMTV-PyVT mammary tumors, $CD45^{+}$ tumor-infiltrating leukocytes also express Clr-b. Immune cells in the tumor microenvironment include NK and T cells, which mediate anti-cancer immune responses, along with regulatory T cells (Treg), myeloid-derived suppressor cells (MDSC), and tumor-associated macrophages (TAM), which possess immune suppressive functions and aid in tumor progression.[62] The role played by the NKR-P1B:Clr-b axis in cellular interactions within the mammary tumor microenvironment to regulate anti-tumor immune responses remains to be determined.

In conclusion, NKR-P1B:Clr-b interactions appear to impact cancer immunosurveillance in mice in at least two ways: (i) surveillance of self ligand (Clr-b) expression in tumor cells, and (ii) aid in maintaining functional homeostasis in tumor-infiltrating NK cells. Exploration of the mechanisms that regulate NK cell function in cancer can aid in fully realizing the potential for NK cells in cancer immunotherapy.

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Disclosure statement

The authors report there are no competing interests to declare.

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