NK cells are highly efficient at preventing cancer metastasis but are infrequently found in the core of primary tumors. Here, have we demonstrated that freshly isolated mouse and human NK cells express low levels of the endo-\(\beta\)-D-glucuronidase heparanase that increase upon NK cell activation. Heparanase deficiency did not affect development, differentiation, or tissue localization of NK cells under steady-state conditions. However, mice lacking heparanase specifically in NK cells (\(Hpse^{fl/fl}\) \(NKp46\)-iCre mice) were highly tumor prone when challenged with the carcinogen methylcholanthrene (MCA). \(Hpse^{fl/fl}\) \(NKp46\)-iCre mice were also more susceptible to tumor growth than were their littermate controls when challenged with the established mouse lymphoma cell line RMA-S-RAE-1\(\beta\), which overexpresses the NK cell group 2D (NKG2D) ligand RAE-1\(\beta\), or when inoculated with metastatic melanoma, prostate carcinoma, or mammary carcinoma cell lines. NK cell invasion of primary tumors and recruitment to the site of metastasis were strictly dependent on the presence of heparanase. Cytokine and immune checkpoint blockade immunotherapy for metastases was compromised when NK cells lacked heparanase. Our data suggest that heparanase plays a critical role in NK cell invasion into tumors and thereby tumor progression and metastases. This should be considered when systemically treating cancer patients with heparanase inhibitors, since the potential adverse effect on NK cell infiltration might limit the antitumor activity of the inhibitors.
NK cell heparanase controls tumor invasion and immune surveillance

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NK cells are highly efficient at preventing cancer metastasis but are infrequently found in the core of primary tumors. Here, we have demonstrated that freshly isolated mouse and human NK cells express low levels of the endo-β-D-glucuronidase heparanase that increase upon NK cell activation. Heparanase deficiency did not affect development, differentiation, or tissue localization of NK cells under steady-state conditions. However, mice lacking heparanase specifically in NK cells (Hpse<sup>−/−</sup> NKp46<i>−iCre</i> mice) were highly prone to tumor when challenged with the carcinoagen methylcholanthrene (MCA). Hpse<sup>−/−</sup> NKp46<i>−iCre</i> mice were also more susceptible to tumor growth than were their littermate controls when challenged with the established mouse lymphoma cell line RMA-S-RAE-1<i>β</i>, which overexpresses the NK cell group 2D (NKG2D) ligand RAE-1<i>β</i>, or when inoculated with metastatic melanoma, prostate carcinoma, or mammary carcinoma cell lines. NK cell invasion of primary tumors and recruitment to the site of metastasis were strictly dependent on the presence of heparanase. Cytokine and immune checkpoint blockade immunotherapy for metastases was compromised when NK cells lacked heparanase. Our data suggest that heparanase plays a critical role in NK cell invasion into tumors and thereby tumor progression and metastases. This should be considered when systemically treating cancer patients with heparanase inhibitors, since the potential adverse effect on NK cell infiltration might limit the antitumor activity of the inhibitors.

Introduction
Extracellular matrix (ECM) comprises more than 50 different proteins, with the main components being large insoluble proteins such as type IV collagen, laminin, and heparan sulphate proteoglycans (HSPGs), creating a barrier that is difficult for immune cells to cross. Many solid tumors are encapsulated by a dense layer of the ECM that makes it particularly difficult for immune cells to infiltrate. Several studies have shown that NK and other immune cells tend to accumulate in the stroma of the invasive margin rather than invade the tumor core itself (1, 2), ultimately limiting effective antitumor immunity (3).

HSPGs constitute a major part of the ECM (4). In mammals, the only enzyme known to degrade HSPGs is the endo-β-D-glucuronidase heparanase. There is only 1 enzymatically active form of heparanase in mammals that is expressed at very low levels in normal tissues, and heparanase deficiency in mice causes no obvious pathophysiology (5, 6). Heparanase is secreted as an enzymatically inactive pro-heparanase and requires reuptake into the cell and processing by cathepsin-L in lysosomes (7) or α granules (8) in order to give rise to the enzymatically active heparanase protein. In recent years, it has become evident that heparanase is a complex protein with both enzymatic and nonenzymatic activities, depending on its location and cleavage (9–13). The degradation of HSPGs and remodeling of the ECM require the enzymatically active heparanase protein to be secreted into the extracellular space. HSPGs bind a range of proteins including angiogenic factors, growth factors, and cytokines that are released into the ECM upon degradation by heparanase and induce tissue remodeling, angiogenesis, and chronic inflammation. Tumor cell–derived heparanase contributes to all these processes, thereby sustaining tumor cell proliferation, vascularization, and metastasis. It is therefore not surprising that the expression of heparanase is frequently upregulated in malignant cells and correlates with poor prognosis and metastatic potential (14–16). Heparanase is thus considered a highly promising target for anticancer therapy, and the first clinical trials using heparan sulfate (HS) mimetics have enrolled patients.

Besides tumor cells, a number of normal cell types, such as endothelial and immune cells including NK cells, express heparanase (17). Recently generated heparanase gene–targeted (Hpse<sup>−/−</sup>) mice were used to investigate the role of heparanase in immune cell subtypes in response to different stimuli. Whereas heparanase was proven important for the migration of DCs (6, 18) and eosinophils (19) in response to inflammation and allergic sensitization, heparanase appeared dispensable for the extravasation of mouse neutrophils and T cells under inflammatory conditions (19, 20). In contrast, heparanase reportedly enhances the tumor

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metastases of tumor cell lines (B16F10, LWT1, RM-1, and E0771) were exacerbated in Hpse fl/fl NKp46-iCre mice. Thus, this study is the first to our knowledge to define heparanase expression and activity of major importance in the tumor-invasive potential and antitumor activity of NK cells.

Results

Activated NK cells express enzymatically active heparanase. Whereas heparanase is highly abundant in platelets and tumor cells, its expression is rather limited in the majority of other tissues and immune cell types (8). Human NK cells freshly isolated from peripheral blood mononuclear cells (PBMCs) (f-NK cells) expressed low levels of HPSE mRNA (Figure 1A) and protein (Figure 1, B and C), comparable to what has been observed with immature human DCs (i-DCs) (22). Activation of NK cells with B-LCL and IL-2 in culture for 18 days (a-NK cells) significantly induced the transcription of the HPSE gene (Figure 1A) and enhanced heparanase protein levels by approximately 2-fold (Figure 1, B and C). Notably, the heparanase expression was analyzed by Western blotting. FACS-purified mouse TCRβ–NK1.1+NKp46+DX5+ NK cells were analyzed ex vivo or after stimulation for the indicated durations by cytokines (500 U/ml IL-2, 1 ng/ml IL-12, 10 ng/ml IL-15, and 10 ng/ml IL-18) or by NK cell receptor cross-linking (α-Ly49D or α-NK1.1). The enzymatic activity of heparanase was determined by a TR-FRET-based HS degradation assay. Splenic NK cells were isolated by negative depletion from WT mice that had been injected with 250 μg poly(I:C) 24 hours prior to the analysis or were left untreated (mean ± SD; n = 3). Statistically significant differences between the groups were determined by 1-way ANOVA with Tukey’s post test (A, C, and D) or unpaired Student’s t test (G). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Figure 1. Activated NK cells express enzymatically active heparanase. (A–E) NK cells isolated from human donors were assayed as f-NK or a-NK cells. i-DCs were included as a control. (A) mRNA expression of HPSE relative to UBC was assessed by quantitative PCR (qPCR) (mean ± SD; n = 3 individual donors; 1 representative experiment of 2 experiments). (B and C) Heparanase protein expression was determined by intracellular staining and flow cytometry (mean ± SEM; n = 5–13 donors per group). MFI, mean fluorescence intensity. (B) HPSE enzymatic activity was determined by incubating 2 × 10^5 f-NK or a-NK cells with ^3H-HS for 16 hours ± 1 U heparin. Human platelet heparanase (2.5 ng) was included as a control (mean ± SEM; n = 4–11 per group; data were pooled from 2 independent experiments). (E) a-NK cells (2 × 10^6) from 2 individual donors were cultured on ^35S-ECM plates ± 2 ng/ml PMA/0.1 μM ionomycin (IO) ± 200 μg/ml PI-88. ECM degradation was measured after 20 hours (mean ± SD; n = 3 technical replicates; data are representative of 5 individual donors). (F) Heparanase expression was analyzed by Western blotting. FACS-purified mouse TCRβ–NK1.1+NKp46+DX5+ NK cells were analyzed ex vivo or after stimulation for the indicated durations by cytokines (500 U/ml IL-2, 1 ng/ml IL-12, 10 ng/ml IL-15, and 10 ng/ml IL-18) or by NK cell receptor cross-linking (α-Ly49D or α-NK1.1). (G) The enzymatic activity of heparanase was determined by a TR-FRET-based HS degradation assay. Splenic NK cells were isolated by negative depletion from WT mice that had been injected with 250 μg poly(I:C) 24 hours prior to the analysis or were left untreated (mean ± SD; n = 3). Statistically significant differences between the groups were determined by 1-way ANOVA with Tukey’s post test (A, C, and D) or unpaired Student’s t test (G). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
Heparanase present in f-NK cells did not possess any measurable enzymatic activity. However, upon activation, NK cells clearly exhibited enzymatic activity (Figure 1D) and an improved ability to degrade artificial ECM (Figure 1E), which was abrogated by the natural heparanase antagonist heparin (Figure 1D) and the pharmaceutical heparanase inhibitor PI-88 (Figure 1E), respectively.

Likewise, mouse NK cells freshly isolated from splenocytes expressed low levels of heparanase (Figure 1F, lanes 1 and 8), whereas heparanase was strongly induced upon NK cell activation, irrespective of the nature of the in vitro stimulation (Figure 1F). The upregulation of heparanase was a rather slow process that showed the highest expression levels 4–5 days after stimulation by cytokines (IL-2, IL-12, IL-15, and IL-18) or NK cell receptor cross-linking (anti-NK1.1) (Figure 1F). To understand whether the heparanase produced by NK cells in vivo was enzymatically active, mice were challenged with immune-activating poly(I:C). The upregulation of heparanase was a rather slow process that showed the highest expression levels 4–5 days after stimulation by cytokines (IL-2, IL-12, IL-15, and IL-18) or NK cell receptor cross-linking (anti-NK1.1) (Figure 1F). To understand whether the heparanase produced by NK cells in vivo was enzymatically active, mice were challenged with immune-activating poly(I:C). Compared with NK cells from naive mice, we found that activated mice were challenged with immune-activating poly(I:C).

In order to investigate the molecular function of heparanase, we generated gene-modified mouse strains: (1) carrying the conditional deletion of heparanase in NKp46+ cells, (2) Hpsefl/fl NKp46-iCre knock-in mice, in which the heparanase gene is deleted in the individual tumors was previously shown to be highly dependent on NK cells (23). Fibrosarcomas arose in 39% of the control mice and Hpsefl/fl NKp46-iCre mice over the course of 200 days. In contrast, within the same observation period, 88% of Hpsefl/fl NKp46-iCre mice developed fibrosarcomas (Figure 2A). Although the prevalence was significantly higher in Hpsefl/fl NKp46-iCre mice, the growth kinetics of the individual tumors was similar in Hpsefl/fl NKp46-iCre and Hpsefl/fl NKp46-WT mice once the tumors were established (Figure 2B and C). This is consistent with the early role of NK cells in preventing tumor initiation but not tumor growth.

NK cells can be strongly activated in vivo by tumors overexpressing ligands that activate NK cell receptors (24, 25). To test the role of NK cell heparanase in such a setting, we injected Hpsefl/fl NKp46-iCre and Hpsefl/fl NKp46-WT control mice s.c. with the lymphoma cell line RMA-S-RAE-1β. This cell line is deficient in typically recirculate (Supplemental Figure 2). Additionally, the number and phenotype of liver-resident NKp46-expressing type 1 innate lymphoid cells (ILC1) cells were unaltered by heparanase deficiency (Supplemental Figure 3, A–D). It is noteworthy that we failed to detect heparanase protein in ILC1 or c-NKs freshly isolated from the livers of WT mice (Supplemental Figure 3E).

In summary, naive mouse and human NK cells express only low levels of enzymatically active heparanase, but upon activation, heparanase expression is increased. Heparanase deficiency does not appear critical for normal tissue residency and recirculation of NKp46+ immune cells, nor for the development and differentiation of the NK cell lineage.

**NK cell–intrinsic heparanase is indispensable for efficient tumor surveillance.** Although heparanase is dispensable for NK cell localization within healthy tissues, NK cells may require heparanase to migrate through tumor stroma. Therefore, we challenged Hpsefl/fl NKp46-iCre and Hpsefl/fl NKp46-WT mice with the de novo fibrosarcoma model, in which mice were administered a s.c. injection of 100 μg methylcholanthrene (MCA). Host resistance to tumor initiation was previously shown to be highly dependent on NK cells (23). Fibrosarcomas arose in 39% of the control mice and Hpsefl/fl NKp46-WT mice over the course of 200 days. In contrast, within the same observation period, 88% of Hpsefl/fl NKp46-iCre mice developed fibrosarcomas (Figure 2A). Although the prevalence was significantly higher in Hpsefl/fl NKp46-iCre mice, the growth kinetics of the individual tumors was similar in Hpsefl/fl NKp46-iCre and Hpsefl/fl NKp46-WT mice once the tumors were established (Figure 2B and C). This is consistent with the early role of NK cells in preventing tumor initiation but not tumor growth.

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MHC class I expression, but overexpresses the NK cell group 2D (NK2D) ligand RAE-1β and thus represents a highly immunogenic tumor that is typically rejected in an NK cell–dependent manner. RMA-S-Rae-1β tumors grew significantly larger in Hpsefl/fl mice when compared with tumor growth in Hpsefl/fl control mice, indicative of an ineffective or delayed NK cell response (Figure 2D). Although the tumor suppression was slightly delayed in Hpsefl/fl NKp46-iCre mice, all inoculated mice eventually rejected their tumors (Figure 2D), consistent with the very strong effect of RAE-1 ligands.

**NK cell heparanase is critical for the effective suppression of tumor metastases.** Host control of experimental metastases acts in both the periphery upon tumor seeding and within the lung niche issue, where the tumor colonies develop. Interestingly, Hpsefl/fl NKp46-iCre mice were significantly more susceptible to experimental tumor metastasis than were control mice when challenged with the prostate carcinoma cell line RM-1 (Figure 3A) or the melanoma cell lines LWT1 (Supplemental Figure 4A) or B16F10 (Figure 3B). Similar experiments using the global deletion of heparanase (Hpse−/−) revealed a minor increase in the experimental metastasis of RM-1 and B16F10 compared with that seen in WT controls, but Hpsefl/fl NKp46-iCre mice were significantly more susceptible to metastasis (Supplemental Figure 5). All of these experimental metastatic tumors were previously reported to be critically controlled by host NK cells (26, 27) (Supplemental Figure 4B). Further, the occurrence of spontaneous metastasis of orthotopically transplanted E0771 mammary carcinoma cells into the lungs was significantly higher in Hpsefl/fl NKp46-iCre mice than in Hpsefl/fl NKp46-WT mice (Figure 3C). In line with previous reports (26, 27), NK cell depletion in Hpsefl/fl NKp46-WT mice significantly enhanced the spontaneous formation of metastases (Figure 3C). In contrast, NK cell depletion in Hpsefl/fl NKp46-iCre mice did not significantly increase the spontaneous metastasis of E0771, suggesting that the antimitastatic properties of heparanase-deficient NK cells were negligible in this model. Given the impact of heparanase on innate NK cell antimitastatic activity, we next tested its importance in immunotherapy. Here, we inoculated B16F10 melanoma cell–bearing Hpsefl/fl NKp46-iCre and Hpsefl/fl NKp46-WT mice with high doses of recombinant IL-2. While this treatment significantly decreased the number of lung metastases in both Hpsefl/fl NKp46-iCre and Hpsefl/fl NKp46-WT mice, the antimitastatic effect of IL-2 was significantly stronger in Hpsefl/fl NKp46-WT mice (Figure 3D). Similarly, the contemporary therapy for advanced human melanoma, which involves the combination of anti-CTLA4 and anti–PD-1 treatment, efficiently reduced B16F10 melanoma cells and treated with either 500 μg control Ig (–) or 250 μg each of anti-CTLA4 and anti–PD-1 (+) on days 0 and 3 after injection, respectively. Lungs were harvested on day 14 and macrometastases counted (mean ± SEM; n = 6–8 mice per group). (**P < 0.01, ***P < 0.001, ****P < 0.0001).

Collectively, our data indicated that NK cell–intrinsic expression of heparanase plays a crucial role in the ability of NK cells to confer antitumor immunity in mouse models of carcinogen-induced tumor initiation, primary tumor growth, and tumor metastasis.

**NK cell proliferation and function are unchanged by loss of heparanase.** The increased tumor susceptibility of Hpsefl/fl NKp46-iCre mice (Figures 2 and 3) might potentially be linked to defects in NK cell survival, proliferation, or function in the absence of heparanase. To test these hypotheses, we isolated NK cells from Hpsefl/fl NKp46-WT, and Hpsefl/fl NKp46-iCre mice were injected i.v. with 1 × 105 RM-1 prostate carcinoma cells. Lungs were harvested on day 14 and macrometastases counted (mean ± SEM; n = 4–16 mice per group; data were pooled from 2 independent experiments). **(B)** Hpsefl/fl NKp46-WT, Hpsefl/fl NKp46-iCre, Hpsefl/fl WT and Hpsefl/fl iCre mice were injected i.v. with 2 × 106 B16F10 melanoma cells. Lungs were harvested on day 14 and macrometastases counted (mean ± SEM; n = 6–22 mice per group; data were pooled from 3 independent experiments). **(C)** Hpsefl/fl NKp46-WT and Hpsefl/fl NKp46-iCre mice were injected with 2 × 105 E0771 cells into the mammary fat pad and treated with either 50 μg control Ig (–) or anti-asialo-GM1 (+) (NK cell depletion) on days -1, 0, 7, 14, and 23 after tumor transplantation. Tumors were removed surgically on day 12. Lungs were harvested on day 35 and macrometastases counted (mean ± SEM; n = 6–8 mice per group). **(D)** Hpsefl/fl NKp46-WT and Hpsefl/fl NKp46-iCre mice were injected i.v. with 5 × 105 B16F10 melanoma cells and treated i.p. with either PBS or 100,000 IU IL-2 on days 0, 1, 2, 3, and 4. Lungs were harvested on day 14 and macrometastases counted (mean ± SEM; n = 10–11 mice per group; data were pooled from 2 independent experiments). **(E)** Hpsefl/fl NKp46-WT and Hpsefl/fl NKp46-iCre mice were injected i.v. with 5 × 105 B16F10 melanoma cells and treated with either 500 μg control Ig (–) or 250 μg each of anti-CTLA4 and anti–PD-1 (+) on days 0 and 3 after injection, respectively. Lungs were harvested on day 14 and macrometastases counted (mean ± SEM; n = 5–7 mice per group). (**A–E**) Statistically significant differences between the groups were determined by 1-way ANOVA with Tukey’s post test (⁎P < 0.05, ⁎⁎P < 0.01, ⁎⁎⁎P < 0.001, and ⁎⁎⁎⁎P < 0.0001).
**Figure 4.** NK cell proliferation and function are unchanged by loss of heparanase. (A and B) Purified BM NK cells from Hpse<sup>fl/fl</sup> Nkp46-WT or Hpse<sup>fl/fl</sup> Nkp46-iCre mice were labeled with CTV and cultured for 3 days in IL-15 as indicated (mean ± SD; n = 2 biological replicates). (A) Apoptosis was determined by annexin V and propidium iodide staining. (B) Proliferation was assessed by CTV dilution. (C) Purified splenic CFSE-labeled NK cells (2 × 10<sup>5</sup>) were injected i.v. into B6.Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> mice. After 3 days, the proliferation of CD45<sup>+</sup>TCR<sub>β</sub>−NK1.1+DX5<sup>+</sup> NK cells in the indicated organs was determined by flow cytometry. (D) The cytotoxicity of freshly isolated splenocytes or IL-2–activated NK cells (1,000 U/ml for 5 days) against YAC-1 and B16F10 target cells was tested at the indicated E/T ratios after 4 hours (mean ± SD; n = 3 biological replicates; 1 representative experiment of 2 experiments). (E) Splenocytes (5 × 10<sup>5</sup>) were stimulated for 4 hours with 1 ng/ml IL-12, 100 ng/ml IL-15, and 10 ng/ml IL-18, and the expression of CD107a was assessed on TCR<sub>β</sub>−NK1.1+DX5<sup>+</sup> NK cells (mean ± SD; n = 4 mice per group). (F) Lung cells were stimulated for 4 hours in 1 ng/ml IL-12, 100 ng/ml IL-15, and 10 ng/ml IL-18, and the production of IFN-γ was measured by intracellular staining (mean ± SEM; n = 10; data were pooled from 3 independent experiments). (G) Purified splenic NK cells were stimulated in 50 ng/ml IL-15, 100 ng/ml IL-21, 1 ng/ml IL-12, 10 ng/ml IL-18, or anti-NK1.1 precoated wells. The release of IFN-γ was measured after 24 hours by CBA (mean ± SD; n = 2 biological replicates; 1 representative experiment of 2 experiments).

**iCre** and Hpse<sup>fl/fl</sup> Nkp46-WT mice and cultivated them in vitro for 3 days in the presence of different concentrations of recombinant IL-15. We did not detect any differences in NK cell survival as assessed by annexin V and propidium iodide staining (Figure 4A). The in vitro proliferation of Hpse<sup>fl/fl</sup> Nkp46-iCre and Hpse<sup>fl/fl</sup> Nkp46-WT NK cells was similar as measured by CellTrace Violet (CTV) dilution (Figure 4B). In concert with these findings, we did not observe any differences in the in vivo proliferation of CFSE-labeled Hpse<sup>fl/fl</sup> Nkp46-iCre or Hpse<sup>fl/fl</sup> Nkp46-WT NK cells after transplantation into immunodeficient Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> mice (Figure 4C).

Since the impaired antitumor function of heparanase-deficient NK cells was not related to alterations in NK cell proliferation or survival, we next asked whether a lack of heparanase impacted NK cell effector functions. In vitro cytotoxicity assays performed with either freshly isolated splenocytes or IL-2–stimulated splenic NK cells from Hpse<sup>fl/fl</sup> Nkp46-iCre and Hpse<sup>fl/fl</sup> Nkp46-WT mice against YAC-1 and B16F10 target cells in various effector-to-target (E/T) ratios showed no differences between the strains (Figure 4D). Accordingly, Hpse<sup>fl/fl</sup> Nkp46-iCre and Hpse<sup>fl/fl</sup> Nkp46-WT NK cells possessed the same capacity to degranulate after stimulation in vitro, as measured by CD107a staining (Figure 4E). Besides direct killing of tumor cells, NK cells produce significant amounts of cytotoxic and immunomodulatory cytokines that are crucial for antitumor responses. However, loss of heparanase did not affect the production (Figure 4F) or release of IFN-γ (Figure 4G), TNF (Supplemental Figure 4C), or the chemokines CCL3, CCL4, and
CCL5 (data not shown) by cytokine-activated or receptor–cross-linked NK cells. Similarly, when challenged in vivo with LPS, Hpsefl/fl NKp46-iCre and Hpsefl/fl NKp46-WT NK cells produced comparable levels of IFN-γ and TNF (Supplemental Figure 4, D and E).

In summary, the increased tumor susceptibility of heparanase-deficient mice could not be explained by differences in NK cell survival, proliferation, cytotoxicity, or cytokine production. NK cell invasion is significantly impaired in the absence of heparanase. Considering the ample evidence of the involvement of heparanase in the transmigration of cells through the ECM and the basal membrane (28), we next examined whether heparanase deficiency affected NK cell migration and invasion. Loss of heparanase did not affect the surface expression of the migration marker CD62L or the chemokine receptors CXCR3, CXCR4, or CCR2 on NK cells when analyzed ex vivo in different organs or after stimulation in vitro (Figure 5A and Supplemental Figure 6). Importantly, the expression of heparanase was clearly dispensable for the simple chemokine-induced migration of NK cells as determined in a Transwell assay (Figure 5B). In contrast, heparanase deficiency significantly impaired the ability of NK cells to degrade HS chains in vitro (Figure 5C) and to invade the artificial ECM in vivo (Figure 5D). Matrigel plugs introduced s.c. contained fewer invading NK cells in Hpsefl/fl NKp46-iCre mice than in Hpsefl/fl NKp46-WT mice, whereas other immune cell types, including CD4+ and CD8+ T cells, infiltrated the plugs to the same degree (Figure 5D).

Considering the increased tumor susceptibility of Hpsefl/fl NKp46-iCre mice, we next assessed whether heparanase impacted the migration of NK cells into the tumor-bearing organs. We found that fewer Hpsefl/fl NKp46-iCre NK cells infiltrated s.c. injected RMA-S-RAE-1β tumors (Figure 5, F–H), while CD4+ and CD8+ T cell frequencies were unchanged (Supplemental Figure 7A). The tumors were harvested 5 days after injection, and at this early...
time point, the tumor weights were similar in both \( \text{Hpse}^{\text{WT}} \) NKp46-iCre and \( \text{Hpse}^{\text{WT}} \) NKp46-WT mice (Supplemental Figure 7B). As assessed by immunofluorescence staining, heparanase-deficient NK cells showed an impaired invasive capacity, which was quantified by the distance traveled into the tumor (Figure 5G). Ultimately, significantly fewer \( \text{Hpse}^{\text{WT}} \) NKp46-iCre NK cells were found in the tumor (Figure 5H), a finding that could not be related to any alterations in the proliferation of intratumoral NK cells (Supplemental Figure 7C). NK cells infiltrated the lungs of \( \text{Hpse}^{\text{WT}} \) NKp46-WT mice within 24 hours after i.v. injection of B16F10 cells. In contrast, we did not observe this increase in NK cell numbers in the tumor-bearing lungs of \( \text{Hpse}^{\text{WT}} \) NKp46-iCre mice (Figure 5E).

We found that T cell numbers and proportions were unchanged (Supplemental Figure 7D).

In summary, these data showed that heparanase plays an important role in NK cell invasion of Matrigel and tumors, probably by facilitating the breakdown of HS chains and the subsequent degradation of the ECM. The heparanase-mediated effect on NK cell invasion was independent of the expression of migration markers or chemokine receptors by NK cells.

Discussion

Despite the potency of NK cells against metastases and hematological cancers (29), their utility against solid tumors and our knowledge about their requirements for tumor infiltration are limited (1, 2, 30–32). Here, we report that NK cells express significant levels of heparanase that are strongly induced upon cell activation. Heparanase produced by NK cells exhibits enzymatic activity and is able to degrade ECM, which is reportedly a prerequisite for cell invasion and migration across a basement membrane (28). By using gene-modified mice with heparanase specifically deleted in NK cells, we showed that NK cell–intrinsic heparanase was indispensable for efficient tumor immunosurveillance. \( \text{Hpse}^{\text{WT}} \) NKp46-iCre mice were highly prone to de novo MCA-induced fibrosarcoma, transplanted lymphoma overexpressing NKG2D ligand, and experimental lung metastases of B16F10 melanoma, IWT1 melanoma, and RM-1 prostate carcinoma. \( \text{Hpse}^{\text{WT}} \) NKp46-iCre mice were also more susceptible to the spontaneous metastasis of E0771 mammary carcinoma. The increased tumor susceptibility correlated with significant impairments in NK cell, but not T cell, infiltration of Matrigel and tumor-bearing organs in the absence of NK cell heparanase. Furthermore, immunotherapies, such as high-dose IL-2 and immune checkpoint anti–PD-1/anti-CTLA4 mAbs in combination, were suboptimal in the absence of NK cell heparanase. Our data suggest that heparanase plays a critical role in NK cell invasion and tumor immunosurveillance and highlight the importance of inducing and maintaining heparanase activity to optimize NK cell functions against tumors.

The presence of tumor-infiltrating NK cells is associated with good prognosis (33–35), however, whether the primary function of tumor-infiltrating NK cells is to directly kill the malignant cells or to produce cytokines that will modulate the tumor, the stroma, or other immune cells in the microenvironment is still a matter of investigation. In the Matrigel and tumor model assays we examined, loss of heparanase in NK cells decreased NK cell localization in tumors, but was without an indirect effect on T cell subsets. In the MCA-induced fibrosarcoma model, NK cell–derived IFN-\( \gamma \) is a critical factor responsible for the formation of a fibrotic reaction enclosing the carcinogen and preventing tumor outgrowth (e.g., the foreign body reaction) (36). We hypothesize that the higher incidence of fibrosarcoma observed in the absence of NK cell heparanase is a result of fewer tumor-infiltrating NK cells, a reduced fibrotic reaction, and, consequently, a more frequent tumor initiation and outgrowth. Interestingly, some tumor tissues were shown to contain high levels of chemokines, which promoted the infiltration of T cells, but failed to do so for NK cells (1). It thus seems that, although the recruitment signals might be provided by the tumor microenvironment, NK cells are unable to infiltrate a solid tumor in sufficient numbers. NK cell accumulation in tumors was previously shown to be regulated by IFN-\( \gamma \) and CXCL10 (37). Our data demonstrated that the expression of CXCR3 — the receptor for CXCL10 — and other adhesion molecules, such as \( \lambda \)-selectin (also known as CD62L), was unaffected by the absence of NK cell heparanase. Therefore, we believe that the diminished invasive potential of heparanase-deficient NK cells is not attributable to defects in chemokine signaling but rather a result of impaired degradation of the ECM.

Even though we have shown that a function of heparanase in NK cells is to break down the ECM, the role of heparanase extends beyond tissue invasion in other immune cell subsets. For example, pro-heparanase can induce cell signaling via PI3-kinase–mediated phosphorylation of AKT (38, 39) and even act as a transcription factor regulating genes involved in cell differentiation, inflammation, and glucose metabolism (40–42). Other studies reported that heparanase enzymatic activity upregulates proinflammatory cytokines from human peripheral blood leukocytes (IL-1\( \beta \), IL-6, IL-8, IL-10, and TNF), as well as mouse splenocytes (IL-6, MCP-1, and TNF) (43). Furthermore, heparanase silencing by siRNA has been shown to reduce the capacity of Jurkat T cells to produce cytokines such as IFN-\( \gamma \) and IL-2 (41). Recently, Gutter-Kapon et al. reported a 2-fold slower growth of Lewis lung carcinoma (LLC) implanted s.c. into heparanase-deficient mice when compared with WT mice (44). They showed that heparanase-deficient macrophages had reduced motility, reduced infiltration into LLC tumors, and an altered phagocytic capacity that was partly independent of heparanase enzymatic activity. These data are interesting in light of the different levels of metastasis we observed between \( \text{Hpse}^{\text{WT}} \) (WT), \( \text{Hpse}^{\text{WT}} \), and \( \text{Hpse}^{\text{WT}} \) strains, since it is possible that deleting \( \text{Hpse} \) in NK cells promotes metastasis, while deleting \( \text{Hpse} \) in some myeloid cell populations may reduce metastasis. Consequently, the (global) \( \text{Hpse}^{\text{WT}} \) phenotype appears more similar to that of WT controls than does the conditional deficiency of \( \text{Hpse} \) in NK cells (\( \text{Hpse}^{\text{WT}} \)). Another study found that the antitumor effect of the HS mimetic PG545, an inhibitor of heparanase, in mice was dependent on DC-mediated IL-12 production that led to NK cell activation and accumulation in the tumor (45). In comparison, our findings show that NK cell–intrinsic loss of heparanase did not affect NK cell development, survival, cytotoxic function, or cytokine production, but significantly impaired tumor immunosurveillance leading to enhanced tumor incidence, growth of the primary tumor, and tumor metastases. These data highlight the diverse roles that heparanase plays in different cell types, tissues, and immune cell activation states.

Heparanase has an emerging role in major human diseases, such as cancer, inflammatory diseases, thrombosis, atherosclero-
also have adverse effects on the ability of NK cells to be recruited
patients may not only affect tumor growth and metastasis but may
or activity. Our data suggest that inhibiting heparanase in tumor
include various methods to reduce NK cell heparanase expression
adaptive resistance mechanisms used by tumors could potentially
for combination therapies, these findings suggest that innate or
potential consequences for combination therapies, these findings suggest that innate or
ly infiltrated solid tumors in vivo, ultimately leading to improved
T cells showed improved ECM degradation in vitro and efficient-
translation of heparanase function has been exploited in humans
would avoid the potentially adverse effect of reducing effector T
cells lacked heparanase. In addition to potential consequences
inflammation sites caused by TLR agonists or similar danger signals, in
tions such as Herpes virus infections or recruitment into inflam-
tion into Matrigel and tumors, but it might be interesting to assess
sis, and various rare diseases (10, 46–48). We have explored the
impact of heparanase loss in other NK cell–dependent condi-
tions such as Herpes virus infections or recruitment into inflam-
atory sites caused by TLR agonists or similar danger signals, in
NK cells are known to be critical in host defense (49, 50).
However, heparanase is best known for its involvement in tumor
growth and angiogenesis, metastasis, and chemoresistance (10,
cluding that heparanase is a promising therapeutic target
for cancer therapy. Preclinical studies in mice clearly showed that
inhibition of heparanase reduces the growth and metastasis of
solid tumors (51) and hematological malignancies (52, 53). The
first clinical trials targeting heparanase by HS mimetics (PI-88,
Pgs45, roneparstat, and neocuparanib) in patients look partially
promising (46). However, the increased tumor susceptibility of
Hpsefl/fl Nkp46-iCre mice indicates that the cell-intrinsic produc-
tion of heparanase in NK cells is important for their potential to
invade and suppress tumors. It is also notable that we found that
potent immunotherapies such as high-dose IL-2 and the anti-
PD-1/anti-CTLA4 combination were poorly effective when NK
cells lacked heparanase. In addition to potential consequences
for combination therapies, these findings suggest that innate or
adaptive resistance mechanisms used by tumors could potentially
include various methods to reduce NK cell heparanase expression
or activity. Our data suggest that inhibiting heparanase in tumor
patients may not only affect tumor growth and metastasis but may
also have adverse effects on the ability of NK cells to be recruited
at the appropriate position to exert their function. Our data
advocate a more selective targeting of heparanase in tumor cells that
would avoid the potentially adverse effect of reducing effector T
cell or NK cell infiltration into tumors. In line with this theory, the
translation of heparanase function has been exploited in humans
by engineering CAR T cells overexpressing heparanase (21). Such
T cells showed improved ECM degradation in vitro and efficiently
infiltrated solid tumors in vivo, ultimately leading to improved
antitumor activity. Our study strongly suggests that similarly
maintaining and/or enhancing heparanase expression in NK cells
will improve NK cell–based anticancer immunotherapy.

Methods

Enrichment of human cells from PBMCs
PBMCs were isolated from buffy coats and blood using Ficoll-Paque
Premium (GE Healthcare). For the generation of iDCs, PBMCs were
resuspended in HBSS (Invitrogen, Thermo Fisher Scientific) contain-
ing 5% heat-inactivated fetal calf serum (HIFCS) and incubated for 30
to 45 minutes. Adherent monocytes were cultured in mixed leukocyte
culture–conditioned (MLC-conditioned) media (consisting of DMEM
supplemented with 4 mg/ml D-glucose, 6 μg/ml folic acid, 3.6 μg/ml
L-asparagine, 116 μg/ml L-arginine, 3.7 mg/ml NaHCO3, 10% FCS,
2 mM L-glutamine, 1 mM HEPES, 1 mM sodium pyruvate, 0.1 mM
2-mercaptoethanol, 100 U/ml penicillin, 50 μg/ml streptomycin, and
100 μg/ml neomycin) supplemented with 50 ng/ml granulocyte macro-
phage–CSF (GM-CSF) and 20 ng/ml IL-4. On day 4, nonadherent
monocyte-derived i-DCs were purified by FACS sorting of CD3 CD14
CD56 CD19 CD209+ cells on a BD FACS Vantage SE Diva Option
Sorter (BD Biosciences). NK cells were isolated by negative depletion
using the RosetteSep Human NK cell Enrichment Cocktail (STEM-
CELL Technologies; catalog 15025) as described previously (54). The
purity of NK cells was assessed by flow cytometry after staining with
the BD Simulset CD3/CD16*CD56 Kit (BD Biosciences).

Human NK cell activation and culture
The B lymphoblastoid cell line (B-LCL) used to activate NK cells in
culture has been described elsewhere (55). B-LCL and NK cells were
resuspended in MLC media supplemented with 80 U/ml recombinant
human IL-2 and cocultured at a ratio of 105:104, respectively. After 18
days of cocultivation, NK cells were further stimulated for 20 hours with
2 ng/ml PMA and 0.1 μM ionomycin in order to generate α-NK cells.

Mice
C57BL/6j WT mice were purchased from the Walter and Eliza Hall
Institute for Medical Research or bred in-house at the QIMR Ber-
ghofer Medical Research Institute and the La Trobe Animal Research
and Teaching Facility. C57BL/6j mice were on a C57BL/6J WT
background and bred at the La Trobe Institute for Molecular Science
and the QIMR Berghofer Medical Research Institute and were used
between the ages of 6 and 16 weeks. Studies involving Hpsefl/fl Nkp46-
iCre mice were conducted in a blinded manner. Animals were ran-
domly assigned to groups.

Experimental tumor models
B16F10 (ATCC), RM-1 (provided by Pamela Russell, University of
Sydney, Sydney, Australia), and E0771 (provided by Robin Anderson,
Peter MacCallum Cancer Centre, Melbourne, Australia) cell lines
have all been previously described (26, 59). The cell lines were main-
tained in complete DMEM (cDMEM) containing 10% FBS, 100 U/ml
penicillin, 100 μg/ml streptomycin, and 2 mM glutamax (Gibco, Ther-
mo Fisher Scientific). LWT1 (60) and RMA-S-RAE-1β (25) cell lines
were cultured in complete RPMI 1640 (cRPMI) media containing 10% FBS,
100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamax,
55 μM 2-mercaptoethanol, HEPES, and sodium pyruvate. For primary
tumor growth, 5 × 104 RMA-S-RAE-1β cells in a volume of 100 μl
media were transplanted s.c. onto the right hind flank of male mice.
The tumor growth was measured every 2 to 3 days with a caliper square
as the product of 2 perpendicular diameters (mm2). For the MCA-
induced fibrosarcoma model, male mice were injected s.c. into the
right hind flank with 100 μg methylcholanthrene (Sigma- Aldrich) in
0.1 ml corn oil. Mice were monitored weekly for the development of
fibrosarcoma. Tumors greater than 3 mm in diameter with progress-
ive growth were counted as positive. For experimental metastasis, 1
× 105 RM-1 prostate cancer cells, 1 × 104 to 5 × 104 B16F10 melan-
oma cells, or 7.5 × 105 LWT1 (BRAFV600E-mutant) melanoma cells
were injected i.v. in a volume of 200 μlplain media. Some groups of mice
were injected i.p. with PBS or 100,000 IU IL-2 on days 0, 1, 2, 3, and
4 after tumor inoculation; some groups of mice received i.p. injec-
tions of clg (hamster Ig) or anti–PD-1 (RMP1-14, rat IgG2a) plus anti-
CTLA4 (UC10-4F10, hamster IgG, provided by Jeffrey Bluestone
[UCSF, San Francisco, California, USA]) on days 0 and 3 after tumor
inoculation. NK cells present in the lung were quantified by flow cytom-
ey 24 hours after the i.v. injection of 5 × 104 B16F10 cells. For sponta-
neous lung metastases, 2 × 104 E0771 mammary carcinoma cells were
injected orthotopically into the mammary gland in 50 μl plain media. Some mice were treated with 50 μg clg (rabbit Ig) or anti-α1-α3 integrin antibody under a dissecting microscope. The number of macrometastases under a dissecting microscope was counted. At least 5 × 10^6 enriched human NK cells were pelleted and lysed in TRIzol (Invitrogen, Thermo Fisher Scientific) or Ribo-Zol RNA Extraction Reagent (Ambrosia) and processed according to the manufacturer’s instructions. The dried RNA pellet was reconstituted in 50 μl RNA Storage Solution (Ambion, Applied Biosystems). cDNA was generated from total RNA using the iScript Select cDNA Synthesis Kit (Bio-Rad). For human HPSE amplification, reverse transcription PCR (RT-PCR) was performed using the Power SYBR Green System (Applied Biosystems). Primer sequences amplifying the human cDNA were as follows: forward: AAGTGCATCTCTCGCTCTGTTGGA; reverse: GGATATCGTGCAGGAGCTCA. HPSE was cloned into the pcDNA3 vector and UTBC into the pCR3.I vector. The housekeeping gene UBC was used as the baseline control for internal gene expression (62). Mouse HPSE cDNA was amplified using FastStart SYBR Green Master (Roche) with the Agilent Mx3000P qPCR System (Agilent Technologies) and the following primers: forward: CTCTCGCTCTGTTGGA; reverse: GGATATCGTGCAGGAGCTCA. HPSE and HPSE alleles were used to set up the standard curves to obtain absolute copy numbers. HPSE was stained by using a TR-FRET–based (TR-FRET–based) assay (Cisbio). Briefly, NK cells were enriched from splenocytes using an EasySep NK Enrichment Kit (STEMCELL Technologies) and lysed in 1% CHAPS/DMG. Equal concentrations of lysates were diluted and used to produce immunoblotting samples. Purified mouse NK cells were lysed in RIPA buffer (Sigma-Aldrich) supplemented with Complete Protease Inhibitor Cocktail (Roche) for 16 hours. A pony vial was filled with Ready Safe Scintillation Fluid (Packard) and counted in a Tri-Carb 1900CA or Tri-Carb 1500 scintillation system (Packard) for 1 to 5 minutes.

Heparanase enzymatic activity assay

Functional human HPSE activity was measured as described previously (63, 64) with the following modification: for inhibition studies, 1 IU heparin was diluted in water. Samples were incubated at 37°C for 16 hours. A pony vial was filled with Ready Safe Scintillation Fluid (Beckman Coulter), distilled H₂O, and 2 μl sample mixture (5 μmol ³H-heparan sulphate (provided by C. Freeman [Australian National University, Canberra, Australia]), 2 μg BSA, 10 mM Na-acetyl mannosamine, 80 mM sodium acetate buffer, pH 5.1, and 0.15 % Triton X-100. Samples were counted in a Tri-Carb 1900CA or Tri-Carb 1500 scintillation system (Packard) for 1 to 5 minutes. For mouse HPSE, heparanase enzymatic activity was determined using a time-resolved fluorescence energy transfer–based (TR-FRET–based) assay (Cisbio). Briefly, NK cells were enriched from splenocytes using an EasySep NK Enrichment Kit (STEMCELL Technologies) and lysed in 1% CHAPS/DMG. Equal concentrations of lysates were diluted 1:1 in buffer (20 mM Tris-HCl, 0.15 M NaCl, and 0.1% CHAPS, pH 5.5) before the addition of biotin-HS-Eu(K) (0.7 μg/ml biotin-HS-Eu(K)) and 50 μg/ml streptavidin at 37°C. The dried RNA pellet was reconstituted in 50 μl RNA Storage Solution (Ambion, Applied Biosystems). cDNA was generated from total RNA using the iScript Select cDNA Synthesis Kit (Bio-Rad). For human HPSE amplification, reverse transcription PCR (RT-PCR) was performed using the Power SYBR Green System (Applied Biosystems). Primer sequences amplifying the human cDNA were as follows: forward: AGTTTGAGAATCCCGGACAT; reverse: ACCGACCACTGTCGAACACTG. HPSE and HPSE alleles were used to set up the standard curves to obtain absolute copy numbers. HPSE was stained by using a TR-FRET–based (TR-FRET–based) assay (Cisbio). Briefly, NK cells were enriched from splenocytes using an EasySep NK Enrichment Kit (STEMCELL Technologies) and lysed in 1% CHAPS/DMG. Equal concentrations of lysates were diluted 1:1 in buffer (20 mM Tris-HCl, 0.15 M NaCl, and 0.1% CHAPS, pH 5.5) before the addition of biotin-HS-Eu(K) (0.7 μg/ml biotin-HS-Eu(K)) and 50 μg/ml streptavidin.
Ea(K) and 0.2 M NaCH₃CO₂, pH 5.5), and the reaction was incubated at 37°C. After 2 hours, 1 μg/ml streptavidin-XL665 (in 0.1 M sodium phosphate, pH 7.5, 1.2 M KF, 0.1% BSA, and 2 mg/ml heparin) was added, and the reaction was incubated in the dark for 16 hours at room temperature. After excitation at 315 nm, the emission was measured at both 620 nm and 668 nm. The percentage of HS degradation was calculated in relation to FRET-positive or -negative samples (i.e., the presence or absence of XL665-conjugated streptavidin, respectively, in the presence of heparanase). To determine the activity per microgram of protein, the final percentage of HS degradation was divided by the absolute mass of the lysates assayed.

**Enzymatic degradation of the ECM**

15S-labeled ECM (15S-ECM) plates were prepared as described by Vlodavsky (65) with the following modification: 6-well plates were precoated with 2 ml of 0.2% (w/v) gelatine/PBS for 16 hours at 4°C. PF-HR9 cells were seeded at 5 × 10⁷ cells per well in PF-HR9 ECM media (10% FCS/high-glucose DMEM supplemented with 50 μg/ml ascorbic acid, 100 μM l-menthol, 100 μg/ml streptomycin, 100 μg/ml neomycin and 4% [w/v] dextran T40) in a pretreated 6-well plate, together with 40 μCi 35S (Na2SO4; PerkinElmer). On day 2, cultures were fed with 0.5 ml PF-HR9 ECM media. On day 4, cultures were fed with 0.5 ml PF-HR9 media and 20–40 μCi 35S. On day 6, cells were lysed by discarding culture supernatant and incubating with warm NH4OH lysis solution (PBS, 20 mM NH4OH, and 0.5% Triton X-100) at 37°C for 5 to 10 minutes. Cells (2 × 10⁶) per 15S-ECM plate were seeded in culture media (0.074 g/l NaHCO3/MLC, 10% FCS, 0.1 M l-glutamine, 0.1 M sodium pyruvate, 0.1 M 2-mercaptoethanol, 100 μM penicillin, 50 μg/ml streptomycin, 100 μg/ml neomycin, and 20,000 μM recombinant human IL-2, pH 6.0) and incubated for 16 to 22 hours at 37°C. Culture supernatant was harvested and centrifuged at 320 RCF. Culture supernatant was passed through Amicon Centriprep Ultracel YM-10 or Ultra Ultracel 10k Filter Units (EMD Millipore) at 2,150 RCF. Supernatant was harvested and centrifuged at 320 RCF for 10 minutes before supernatant was passed through Amicon Centriprep Ultracel YM-10 or Ultra Ultracel 10k Filter Units (EMD Millipore) at 2,150 RCF. Supernatant (400 μl) was added to 3.6 ml Ready Safe Scintillation Fluid (Packman Beckman). Samples were counted on a Tri-Carb 1900CA or Tri-Carb 1500 scintillation machine (Packard) for 5 minutes.

**Flow cytometry**

Single-cell suspensions were prepared as described above and incubated in 2.4G2 (anti-CD16/32) to block nonspecific Fc receptor binding. Cells were washed with FACS buffer (1% FBS and 2 mM EDTA in PBS) and incubated for 20 minutes with diluted antibodies. The following reagents and anti-mouse antibodies (clones) were purchased from BioLegend: 7-AAD; anti-CD4 (clone RM4-5); anti-CD8 (clone 53-6.7); anti-CD11b (clone M1/70); anti-CD107a (clone BD4); anti-CD226 (clone 480.1); anti-IFN-γ (clone XMG1.2); anti-NKp46 (clone 29A1.4); anti-CD62L (clone Mil-E1); anti-CXCR4 (clone L276F12); anti-TCRβ (clone LG-7F9); anti-CD56 (clone 6H3); and anti-Ki-67 (clone B56); from Sigma-Aldrich: propidium iodide; from R&D Systems: anti-CCR2 (clone 475301); from eBioscience: anti-CD27 (clone L7.2F1); anti-CD49d (clone DX5); anti-CXCR3 (clone CXCR3-173); anti-KLRG1 (clone 2F1); and anti-NKG2A/C/E (clone 20d5); and from Miltenyi Biotec: anti-CD45.2 (clone 104-2); anti-CD49a (clone REA493); and anti-NKG2D (clone CX5). The following anti-human antibodies were purchased from R&D Systems: anti-CD209 (catalog FAB161P); BD Biosciences: anti-CD3 (catalog 553322); and anti-CD14 (catalog 347493); and anti-CD56 (catalog 340410); BioLegend: anti-CD19 (catalog 302260); Insight Biopharmaceuticals: anti-heparanase (catalog INS-26-1-0000-21); and Chemicon: sheep anti-mouse IgG-PE F(ab’2) (AQ326F). Apoptosis was determined by staining with annexin V and propidium iodide in Annexin V Binding Buffer (BD Biosciences). Degranulation was measured by CD107a staining for 4 hours in the presence of GolgiPlug and GolgiStop (BD Biosciences). Intracellular IFN-γ staining was performed using BD Fixation and Permeabilization Solution. Cytokine release into cell culture supernatants was determined with a CBA Flex Set Multiplex (BD Biosciences). To obtain absolute counts, equal amounts of Liquid Counting Beads (BD Biosciences) were added to the samples shortly before analysis. Single-cell suspensions were analyzed on a BD FACScan, a BD LSR1, or a BD FACS Fortessa Flow Cytometer (4 or 5 lasers), and the analysis was performed using FlowJo software, version 10 (Tree Star).

**Proliferation assays**

To assess in vitro proliferation, purified NK cells were labeled with 1 μM CTV (Thermo Fisher Scientific) and incubated at 37°C for 72 hours with different concentrations of IL-15 before flow cytometric analysis. To measure in vivo proliferation, FACS-sorted NK cells were labeled with 0.5 μM CFSE (BioLegend) and injected i.v. into recipient Rag2–/– II12rg–/– mice (2 × 10⁶ cells/200 μl/mouse). The indicated organs were processed for flow cytometric analysis 3 days after transplantation.

**Migration and invasion assays**

To measure in vitro migration, FACS-purified splenic NK cells were activated overnight in 100 μg/ml IL-15, 1 ng/ml IL-12, and 10 ng/ml IL-18 before being resuspended in plain RPMI containing 1% FBS and loaded onto Corning Transwell inserts (7.5 × 10⁴ cells per 24-well plate, 8-μm pores). NK cells were allowed to migrate toward chemotractant-containing media (20 ng/ml CXCL10 [R&D Systems] in cRPMI). The number of migrated cells was determined after 17 hours by manual cell counting using a Neubauer chamber. In vivo invasion of lymphocytes into Matrigel plugs was determined 72 hours after the s.c. injection of 100 μl ice-cold growth factor–reduced Matrigel diluted to a concentration of 5 mg/ml in PBS (Corning Matrigel Growth Factor Reduced Basement Membrane Matrix). The plugs were digested by 1 mg/ml collagenase type 4 (Worthington Biochem) and 20 μg/ml DNAse I (Roche) in plain DMEM, with slight agitation for 45 minutes at 37°C, followed by flow cytometric analysis.

**Cytotoxicity assays**

NK cell cytotoxicity against YAC-1 and B16F10 target cells was tested using freshly isolated splenocytes or splenic NK cells stimulated with IL-2 (1,000 U/ml) for 5 days. Target cells were labeled with 1 μM CTV to distinguish them from effector cells and coincubated with effector cells for 4 hours at different E/T ratios. Tumor cell lysis was determined by staining with annexin V/7-AAD in Annexin V Binding Buffer (BD Biosciences).
a Zeiss 780 laser-scanning confocal microscope (Oberkochen) using a ×20 objective (0.8 NA). NKP46+ cells were automatically detected using Imaris (Bitplane). The boundary of the tumor was identified by a minimum of 2 independent reviewers to calculate the tumor area. The distance of NKP46+ cells from the edge of the tumor as a percentage of the total distance from the edge to the center of the tumor was calculated using MATLAB (MathWorks). Frequency distribution statistics were performed on the percentage of distance from the edge to the center of the tumor using a bin width of 10% (GraphPad Software).

**Statistics**

Statistical analysis was performed using Graphpad Prism, version 7.01 (GraphPad Software). Data were considered statistically significant at a p value of 0.05 or less. Data were compared using a Mann-Whitney U test, Student’s t test, 1-way ANOVA with Tukey’s post test, 2-way ANOVA, or log-rank Mantel-Cox test.

**Study approval**

Human peripheral blood was obtained with consent from healthy donors by ACT Pathology at the Canberra Hospital (Garran, Australia). Fresh buffy coats were obtained from the ACT Red Cross Blood Transfusion Service (Canberra, Australia). Ethics approval for the collection and use of human blood was given by the ACT Health Research Ethics Committee and the Australian National University Human Ethics Committee (FHE09/R16). Anonymity of donors was achieved by labeling buffy coat donor samples with numbers corresponding to the serial found on the buffy coat, and individual donors were given a code only known within the laboratory. All mouse experiments were approved by the QIMR Berghofer Medical Research Institute Animal Ethics Committee and the La Trobe Animal Ethics Committee.

**Author contributions**

EMP, MDH, and MJS designed the research study; EMP, AJM, KK, DSB, KN, LT, KJG, DYY, IKHP, NB, and FSFG conducted experiments and acquired and analyzed the data; EMP, MDH, and MJS wrote the manuscript. All authors contributed to the writing of the manuscript.

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