NK cell heparanase controls tumor invasion and immune surveillance

Eva M. Putz, Alyce J. Mayfosh, Kevin Kos, Deborah S. Barkauskas, Kyohei Nakamura, Liam Town, Katharine J. Goodall, Dean Y. Yee, Ivan K.H. Poon, Nikola Baschuk, Fernando Souza-Fonseca-Guimaraes, Mark D. Hulett, and Mark J. Smyth

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 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 (Hpsefl/fl Nkp46-iCre mice) were highly tumor prone when challenged with the carcinogen methylcholanthrene (MCA). Hpsefl/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-1β, which overexpresses the NK cell group 2D (NKGD2) ligand RAE-1β, 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 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 (Hpsefl/fl Nkp46-iCre mice) were highly tumor prone when challenged with the carcinogen methylcholanthrene (MCA). Hpsefl/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-1β, which overexpresses the NK cell group 2D (NKGD2) ligand RAE-1β, 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.

Conflict of Interest: M.J. Smyth has research agreements with Bristol-Myers Squibb, Aduro Biotech, and Corvus Pharmaceuticals.

Submitted: January 20, 2017; Accepted: April 6, 2017.

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 and activity of major importance in the tumor-invasive potential and antitumor activity of NK cells.

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. (D) 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.

infiltration and effectiveness of adoptively transferred cytotoxic T cells (21). Interestingly, while short-term activation of T cells induced the expression of heparanase, long-term in vitro expansion of human T cells downregulated heparanase expression, which was responsible for the impaired invasion of stroma-rich tumors in vivo and Matrigel in vitro (21). These studies highlight the importance of heparanase, but also the differences between immune cell types and model systems.

Here, we investigated the expression and function of heparanase in human and mouse NK cells. Whereas freshly isolated NK cells express very low levels of heparanase, the expression is upregulated upon cytokine stimulation or NK cell receptor cross-linking. By generating conditional heparanase gene–targeted (floxed) mice specifically lacking the Hpse gene in NKp46+ cells (HpseΔ/Δ NKp46-iCre mice), we showed that heparanase expression in NK cells was indispensable for efficient invasion and subsequent tumor surveillance. The initiation of methylcholanthrene-induced fibrosarcoma, the growth of primary RMA-S-RAE-1β tumors, and the lung 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.
nase 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 where heparanase was strongly induced upon NK cell activation 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 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 Hpsel+/- NKp46-iCre and Hpsel+/- NKp46-WT mice in 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 Hpsel+/- NKp46-WT mice over the course of 200 days. In contrast, within the same observation period, 88% of Hpsel+/- NKp46-iCre mice developed fibrosarcomas (Figure 2A). Although the prevalence was significantly higher in Hpsel+/- NKp46-iCre mice, the growth kinetics of the individual tumors was similar in Hpsel+/- NKp46-iCre and Hpsel+/- NKp46-WT mice once the tumors were established (Figure 2, B 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.**

![Figure 2. NK cell–intrinsic heparanase is indispensable for efficient surveillance of MCA-induced fibrosarcoma and RAE-1-expressing lymphoma. (A–C) Hpsel+/- NKp46-WT and Hpsel+/- NKp46-iCre mice were inoculated s.c. in the hind flank with 100 μg MCA in 0.1 ml corn oil. Mice were then monitored over a 200-day period for fibrosarcoma development. Tumors were measured every week with a caliper (n = 24–28 per group; data were pooled from 2 independent experiments). (A) Tumor growth curves of individual (B) Hpsel+/- NKp46-WT and (C) Hpsel+/- NKp46-iCre mice. (D) Hpsel+/- NKp46-WT and Hpsel+/- NKp46-iCre mice were injected s.c. with 5 × 10^5 RMA-S-RAE-1β cells. Tumor growth was measured every 2 to 3 days with a caliper (mean ± SEM; n = 7 per group; 1 representative experiment of 2 experiments). Statistically significant differences were determined by log-rank Mantel-Cox test (A) and Mann-Whitney U test (B). *P < 0.05, **P < 0.01, and ****P < 0.0001.
Mice were injected with $2 \times 10^4$ E0771 cells into the mammary fat pad and treated with either 50 Hpsefl/fl RMA-S-RAE-1 $\beta$ tumor that is typically rejected in an NK cell–dependent manner and thus represents a highly immunogenic $\beta$ (NKG2D) ligand RAE-1 significantly higher in Hpsefl/fl NKp46-iCre transplanted E0771 mammary carcinoma cells into the lungs was ther, the occurrence of spontaneous metastasis of orthotopically 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 antitumor properties of heparanase-deficient NK cells were negligible in this model. Given the impact of heparanase on innate NK cell antitumor 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 antitumor 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 50,000 IU IL-2 on days 0, 1, 2, 3, and 4. 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 3. Heparanase-deficient NK cells display impaired control of lung metastases.** (A) Hpsefl/fl NKp46-WT, Hpsefl/fl NKp46-iCre, Hpsefl/fl NKp46-WT (B6.WT), and Hpsefl/fl NKp46-iCre mice were injected i.v. with $1 \times 10^4$ 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 NKp46-WT (B6.WT), and Hpsefl/fl NKp46-iCre mice were injected i.v. with $2 \times 10^6$ B16F10 melanoma cells. Lungs were harvested on day 14 and macrometastases counted (mean ± SEM; n = 6–12 mice per group; data were pooled from 3 independent experiments). (C) Hpsefl/fl NKp46-WT and Hpsefl/fl NKp46-iCre mice were injected with $2 \times 10^5$ 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 $5 \times 10^4$ 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 $5 \times 10^4$ 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 = 4–16 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 $^{****}P < 0.0001$).
NK cell effector functions. In vitro cytotoxicity assays performed with either freshly isolated splenocytes or IL-2–stimulated splen-ic NK cells from \( \text{Hps}^{\text{ef}1/\text{fl}} \text{NKp46-iCre} \) and \( \text{Hps}^{\text{ef}1/\text{fl}} \text{NKp46-WT} \) 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, \( \text{Hps}^{\text{ef}1/\text{fl}} \text{NKp46-iCre} \) and \( \text{Hps}^{\text{ef}1/\text{fl}} \text{NKp46-WT} \) 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-\( \gamma \) (Figure 4G), TNF (Supplemental Figure 4C), or the chemokines CCL3, CCL4, and CCL5.

**Figure 4.** NK cell proliferation and function are unchanged by loss of heparanase. (A and B) Purified BM NK cells from \( \text{Hps}^{\text{ef}1/\text{fl}} \text{NKp46-WT} \) or \( \text{Hps}^{\text{ef}1/\text{fl}} \text{NKp46-iCre} \) 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 \( \times \) 10\(^6\) per group) were injected i.v. into B6.\( \text{Rag2}^{\text{−/−}} \text{Il2rg}^{\text{−/−}} \) mice. After 3 days, the proliferation of CD45\(^{+}\)TCR\(^{+}\)NK1.1\(^{+}\)DX5\(^{+}\) NK cells in the indicated organs was determined by flow cytometry. Flow cytometric plot shows a representative proliferation profile. Data in the bar graph were pooled from 2 independent experiments (mean ± SEM; \( n = 8 \) per group). (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 \( \times \) 10\(^6\)) 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\(^{+}\)NK1.1\(^{+}\)DX5\(^{−}\) 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-\( \gamma \) 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-\( \gamma \) was measured after 24 hours by CBA (mean ± SD; \( n = 2 \) biological replicates; 1 representative experiment of 2 experiments).
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 5F–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 \( \textit{Hpse}^{+/+} \) \( \textit{NKp46-iCre} \) and \( \textit{Hpse}^{-/-} \) \( \textit{NKp46-WT} \) 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 \( \textit{Hpse}^{-/-} \) \( \textit{NKp46-iCre} \) 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 \( \textit{Hpse}^{+/+} \) \( \textit{NKp46-WT} \) 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 \( \textit{Hpse}^{-/-} \) \( \textit{NKp46-iCre} \) 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. \( \textit{Hpse}^{+/+} \) \( \textit{NKp46-iCre} \) mice were highly prone to de novo MCA-induced fibrosarcoma, transplanted lymphoma overexpressing NKG2D ligand, and experimental lung metastases of B16F10 melanoma, IW1 melanoma, and RM-1 prostate carcinoma. \( \textit{Hpse}^{-/-} \) \( \textit{NKp46-iCre} \) 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 (I). 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 \( \alpha \)-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 (4I). 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 \( \textit{Hpse}^{+/+} \) (WT), \( \textit{Hpse}^{-/-} \), and \( \textit{NKp46-iCre} \) strains, since it is possible that deleting \( \textit{Hpse} \) in NK cells promotes metastasis, while deleting \( \textit{Hpse} \) in some myeloid cell populations may reduce metastasis. Consequently, the (global) \( \textit{Hpse}^{-/-} \) phenotype appears more similar to that of WT controls than does the conditional deficiency of \( \textit{Hpse} \) in NK cells (\( \textit{NKp46-iCre} \)). 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-
sis, and various rare diseases (10, 46–48). We have explored the importance of heparanase production by NK cells in their infiltration into Matrigel and tumors, but it might be interesting to assess the impact of heparanase loss in other NK cell–dependent conditions such as Herpes virus infections or recruitment into inflammatory sites caused by TLR agonists or similar danger signals, in which 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, 46), indicating 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, PG545, roneparstat, and necuparanib) in patients lack promising trials (46). However, the increased tumor susceptibility of HpseΔ/Δ NKp46-iCre mice indicates that the cell-intrinsic production 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 to 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) containing 5% heat-inactivated fetal calf serum (HI-FCS) 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, 0.1% 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 macrophage–CSF (GM-CSF) and 20 ng/ml IL-4. On day 4, nonadherent monocyte-derived iDCs 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 (Br-LCL) used to activate NK cells in culture has been described elsewhere (55). Br-LCL and NK cells were resuspended in MLC media supplemented with 80 U/ml recombinant human IL-2 and cocultured at a ratio of 10:1, 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 a-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 Berghofer Medical Research Institute and the La Trobe Animal Research and Teaching Facility. HPse–/–, Nkp46-iCre mice were conducted in a blinded manner. Animals were randomly 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 maintained in complete DMEM (cDMEM) containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamax (Gibco, Thermo 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 plain 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 progressive growth were counted as positive. For experimental metastasis, 1 × 10⁶ BRAFV600E-mutant) melanoma cells were injected i.v. in a volume of 200 μl plain 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. injections 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 cytometry 24 hours after the i.v. injection of 5 × 10⁴ B16F10 cells. For spontaneous lung metastases, 2 × 10⁶ E0771 mammary carcinoma cells were
injected orthotopically into the mammary gland in 50 μl media. Some mice were treated with 50 μg clg (rabbit Ig) or anti–αsialo-GM1 on days -1, 0, 7, 14, and 23 after tumor transplantation. The tumors were removed surgically on day 12. Lung metastases were quantified on day 12 after the injection of RM-1, on day 14 after the injection of B16F10 and LWT1, or on day 35 after the injection of EO771 by counting the number of macrometastases under a dissecting microscope.

Organ preparation and purification of mouse conventional NK and ILC1 cells

Mouse livers and lungs were perfused with cold PBS postmortem before harvesting the organs. Livers were prepared as described elsewhere (61). Lungs were cut into fine pieces and incubated in 1 mg/ml DNAse I (Roche) in plain DMEM for 45 minutes at 37°C. Single-cell suspensions from all organs (blood, liver, lung, spleen, and bone marrow) were treated with ammonium chloride potassium (ACK) buffer (0.15 M NH4Cl, 1 mM KHCO3, and 0.1 mM Na2 EDTA) to deplete erythrocytes. For NK cell sorting, splenocytes or bone marrow single-cell suspensions from 2 to 6 mice were pooled and enriched for NK cells using the NK Cell Isolation Kit II (Miltenyi Biotec). Following magnetic separation on an autoMACS Separator (Miltenyi Biotec), conventional NK cells were gated as TCRβ+NK1.1+NKp46+DX5+ cells and sorted on a FACSaria II (4 lasers; BD Biosciences). ILC1 cells were FACS sorted from liver lymphocytes and gated as CD45+ TCRβ+DX5+DX5+CD49a+ cells.

Mouse NK cell activation

In vitro. Sorted mouse NK cells were plated at a density of 1 × 10^5 to 5 × 10^5 cells/96-well plate/200 μl in eRPMI. Cytokine concentrations included 300–600 U/ml recombinant human IL-2 (provided by Chiron Corporation), 3–100 ng/ml recombinant IL-15/IL-15R complex (eBioscience, referred to herein as IL-15), 1 ng/ml recombinant IL-12 (eBioscience), and 10 ng/ml recombinant IL-18 (R&D Systems). For NK receptor cross-linking, wells were coated with 2 μg anti-NK1.1 (PK136) or 8 μg anti-Ly49D per 96-well plate and incubated overnight at 4°C.

In vivo. Mice were injected i.p. with 250 μg polyclonal rabbit antiserum. The dried RNA pellet was reconstituted in TRIzol (Invitrogen, Thermo Fisher Scientific) or RiboZol RNA Extraction Reagent (Ambrosec) 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: HPSE, forward: GTTCCTGTCCGTACCATTGA; HPSE, reverse: CTTGAGAAGCCCGAGGAGT; ubiquitin C (UBC), forward: TGAAGAGATCCAGAGAACATA; and UBC, reverse: CAA-CAGGAACCTGTAACACTG. UBC and HPSE amplicons were used to set up the standard curves to obtain absolute copy numbers. HPSE was cloned into the pcDNA3 vector and UBC into the pCR3.1 vector. The housekeeping gene UBC was used as the baseline control for internal gene expression (62). For mouse Hpse, cDNA was amplified using FastStart SYBR Green Master (Roche) with the Agilent Mx3000P qPCR System (Agilent Technologies) and the following primers: Hpse, forward: CGTCTATCCCCACCGATAC; Hpse, reverse: CAGTTGGAGACAGATGAAGA; Gapdh, forward: TCTCGGTGCTTACACCCCA; and Gapdh, reverse: GCTTCACCCCTCCTGTAGTC.

FACS analysis. Human NK cells (2 × 10^4 to 4 × 10^4) were stained for cell-surface antigens before using the Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences) for the intracellular staining of human heparanase. Samples were analyzed on BD FACsCan or BD LSR I flow cytometers (BD Biosciences).

Western blot analysis. Purified mouse NK cells were lysed in RIPA buffer (Sigma-Aldrich) supplemented with complete Protease Inhibitor (Roche) for 15 minutes. Equal amounts of protein (1–5 μg) were run on SDS-page gels (4%–15% Precast Mini PROTEAN TGX; Bio-Rad). Proteins were transferred to PVDF membranes using the TurboBlot System (Bio-Rad). The membrane was blocked for 1 hour in 5% skim milk powder before the addition of one of the following primary antibodies: anti-HPSE (Abcam; catalog ab85543; diluted 1:1,000 or Insight Biotechnology; catalog TA332866; diluted 1:4,000) or anti–β-actin (Cell Signaling Technology; catalog 4967; diluted 1:1,000). After overnight incubation, the HRP-conjugated anti-rabbit antibody (Cell Signaling Technology; catalog 7074; diluted 1:5,000–1:20,000) was applied for 40 minutes. Proteins were visualized by x-ray film detection using ECL Prime Western Blotting Detecting Agent (GE Healthcare Life Sciences).

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 porcine royal with was filled with Ready Safe Scintillation Fluid (Beckman Coulter), distilled H2O, and 20 μl sample mixture (5 pmol H2-heparan sulphate (provided by C. Freeman [Australian National University, Canberra, Australia]), 2 μg BSA, 100 mM N-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 machine (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-
μC 35S. On day 6, cells were lysed by discarding media and 20–40 PF-HR9 ECM media. On day 4, cultures were fed with 0.5 ml PF-HR9 Vlodavsky (65) with the following modification: 6-well plates were pre-

Enzymatic degradation of the ECM

35S-labeled ECM (35S-ECM) plates were prepared as described by Vlodavsky (65) with the following modification: 6-well plates were pre-coated with 2 ml of 0.2% (w/v) gelatine/PBS for 16 hours at 4°C. PF-HR9 cells were seeded at 5 × 10^5 cells per well in PF-HR9 ECM media (10% ECS/high-glucose DMEM supplemented with 50 μg/ml ascorbic acid, 100 μM penicillin, 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 × 10^6) per 35S-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 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 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 (Beckman Coulter). 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 Bio-

Legend: 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 Mel-14); anti–CXCR4 (clone L276F12); anti–TCRβ (clone H57-597); and a Zombie Yellow Fixable Viability Kit; from BD Biosci-

ences: annexin V; anti-CD122 (clone TM-β1); anti-NK1.1 (clone PK136); and anti–Ki-67 (clone B56); from Sigma-Aldrich: propidium iodide; from R&D Systems: anti–CCR2 (clone 475301); and from eBioscience: anti–CD27 (clone LG.77F9); anti–CD49f (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 555332); anti–CD14 (catalog 347493); and anti–CD56 (catalog 340410); BioLegend: anti–CD19 (catalog 302206); Insight Biopharmaceuticals: anti–heparanase (catalog INS-26-1-0000-21); and Chemicon: sheep anti-mouse IgG-PE F(ab’2) (AQ326F). Aprotinin 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 FACSscan, 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−/−Il2rg−/−mice (2 × 10^5 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 ng/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 × 10^4 cells per 24-well plate, 8-μm pores). NK cells were allowed to migrate toward chemo-attractant-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 for annexin V/7-AAD in Annexin V Binding Buffer (BD Biosciences).

Immunofluorescence and image analysis

RMA-S-RAE-1β tumors were excised on day 5 and fresh frozen in PELCO Cryo-embedding Compound. Frozen sections were cut from the tumors and fixed in 4% paraformaldehyde. Sections were stained with anti-nKp46 (R&D Systems; catalog AF2225), detected by tyra-
mide signal amplification (Cy3; PerkinElmer), and counterstained with DAPI. Tiled images of the whole tumor section were captured on
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.

**Acknowledgments**

The authors are grateful to Kate Elder (QIMR Berghofer, Herston, Australia) for the breeding, genotyping, maintenance, and care of the mice used in this study. We thank Hilary Warren (Australian National University, Canberra, Australia) for help with the isolation and culturing of human NK cells. We are deeply grateful to Kestutis Barkauskas (Case Western Reserve University, Cleveland, Ohio, USA) for his help in developing the MATLAB algorithm for the quantification and localization of intratumoral NK cells. We thank Eric Vivier (Centre d’Immunologie de Marseille-Luminy, Marseille, France) for the provision of the NKp46-icre knock in mice. EMP was supported by an Erwin Schrodinger Fellowship of the Austrian Science Fund (J-3635). KN was supported by the Naito Foundation. FSFG was supported by a National Health and Medical Research Council (NHMRC) Peter Doherty Early Career Fellowship (1088703); a National Breast Cancer Foundation (NBFC) Fellowship (PF-15-008); and a Cancer Cure Australia Priority-Driven Young Investigator Project Grant (1082709). MJS was supported by a NHMRC Senior Principal Research Fellowship (1078671). MDH was supported by a NHMRC Project Grant (471424).

Address correspondence to: Mark J. Smyth, Immunology in Cancer and Infection Laboratory, QIMR Berghofer Medical Research Institute, 300 Herston Road, Herston, Queensland 4006, Australia. Phone: 61.7.3845.3957; Email: mark.smyth@qimrberghofer.edu.au. Or to: Mark D. Hulett, Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Science Drive, Bundoora, Victoria 3083, Australia. Phone: 61.3.9479.6567; Email: m.hulett@latrobe.edu.au.


41. He YQ, et al. The endoglycosidase heparanase enters the nucleus of T lymphocytes and modulates H3 methylation at actively transcribed genes via the interplay with key chromatin modifying enzymes. Transcription. 2012;3(3):130–145.


