The Natural Killer cell granule protein NKG7 regulates cytotoxic granule exocytosis and inflammation

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Immune-modulating therapies have revolutionised treatment of chronic diseases, particularly cancer. However, their success is restricted and there is need to identify new therapeutic targets. Here, we show that Natural Killer Cell Granule protein 7 (NKG7) is a regulator of lymphocyte granule exocytosis and downstream inflammation in a broad range of diseases. NKG7 expressed by $CD4^+$ and $CD8^+$ T cells played key roles in promoting inflammation during visceral leishmaniasis and malaria, two important parasitic diseases. Additionally, NKG7 expressed by NK cells was critical for controlling cancer initiation, growth and metastasis. NKG7 function in NK and $CD8^+$ T cells was linked with their ability to regulate translocation of CD107a to the cell surface and kill cellular targets, while NKG7 also had a major impact on $CD4^+$ T cell activation following infection. Thus, we report a novel therapeutic target expressed on a range of immune cells with functions in different immune responses.

Immunity and related inflammation are critical defence mechanisms against infection and tumors, as well as an integral part of tissue repair [1]. Lymphocytes are crucial for these responses and two important immune processes employed by them are the production and secretion of pro-inflammatory cytokines and cytotoxic granule exocytosis [1, 2]. If these cellular activities are not appropriately stimulated then infection or tumor growth can progress uncontrolled [3, 4, 5]. However, these responses also need to be tightly regulated to prevent tissue damage and associated disease [6].

Infectious diseases such as malaria and visceral leishmaniasis (VL) require the generation of IFN-γ-producing $CD4^+$ T (T_H1) cells to help phagocytes kill captured or resident parasites [7]. However, inflammation generated by these cells can also damage tissue, including destruction of reticuloendothelial networks that limits removal and killing of parasites, thereby contributing to persistence of infection [8]. In the case of malaria, inflammation can also activate vascular endothelium, thereby allowing parasite sequestration into various tissues with detrimental consequences for organ function [9]. However, the immunoregulatory pathways that emerge to control this inflammation can also have a negative impact on parasite control [10]. Thus, chronic infectious diseases such as malaria and VL can be characterised by an imbalance between pro- and anti-inflammatory immune responses.

Tumor microenvironments are often typified by the presence of unresponsive immune cell populations [11], whereby their anti-tumor activities are inhibited by local expression of immunoregulatory molecules [12]. Thus, the transient suppression of these molecules or activation of suppressed pro-inflammatory pathways is often a goal of cancer treatments. Indeed, metastatic melanoma patients with durable clinical responses following immune checkpoint blockade exhibit an immune signature characterised by increased expression of genes associated with IFN-γ-producing T_H1 cells and cytotoxic $CD8^+$ T cells [13, 14]. Success in treating cancer using host-directed therapies highlight the significant clinical potential of manipulating immune cells for patient benefit [4]. However, treatment success varies considerably between individuals, even for the same cancer types [14, 15], underlining the need to uncover new immunoregulatory molecules that can be targeted to improve disease outcomes.

Here we report that the Natural Killer Cell Granule protein 7 (NKG7) is a novel mediator of inflammation in several different inflammatory contexts. NKG7 was first identified in NK cells and T cells [16], but since then there have been few studies on this molecule, and as such, the function of NKG7 in health and disease remains poorly characterised. However, the recent increase in the reporting of whole transcriptome data sets has resulted in accounts of differential expression of *NKG7* in various experimental contexts [14, 17]. Herein we show that NKG7 functions to regulate cytotoxic granule exocytosis in effector lymphocytes, thus acting as a critical mediator of inflammation in a broad range of infectious and non-infectious diseases.

Results

The identification of NKG7 on CD4$^+$ T cells exposed to chronic inflammation

Experimental VL caused by infection of C57BL/6 mice with the human parasite *Leishmania donovani* is characterised by an organ-specific response to infection [18]. The liver is a site of acute, resolving infection, while infection persists in the spleen, accompanied by a breakdown in tissue architecture driven by unchecked inflammation and an accumulation of CD4$^+$ T cells that produce a range of pro-inflammatory molecules, but are unable to control parasite growth [10]. To better understand the inflammatory response in this organ, we isolated CD4$^+$ T cells from the spleen and liver of naive and *L. donovani*-infected C57BL/6 mice at day 56 post-infection (p.i.), to identify differentially expressed genes (DEGs) in effective anti-parasitic CD4$^+$ T cell responses (liver) and dysregulated CD4$^+$ T cell responses (spleen) (Supplementary Fig. 1). Concurrently, we also isolated CD4$^+$ T cells from VL patients on presentation to clinic and 30 days after drug treatment (when effective anti-parasitic immunity had developed [19]; Supplementary Table 1), and used these samples to identify DEGs associated with human VL using RNA-seq (Supplementary Fig. 1; Supplementary Table 2). We next compared mouse and human DEGs to identify a "core" signature shared between all CD4$^+$ T cell populations analysed (Supplementary Tables 2, 3 and 4). We also identified an "inflammatory" signature associated with persistent infection (i.e., DEGs shared between human VL patient and mouse spleen CD4$^+$ T cells; Supplementary Tables 2 and 4) and an "immune" signature associated with controlled infection (i.e., DEGs shared between human VL patient and mouse liver CD4$^+$ T cells; Supplementary Tables 2 and 3). We predict that molecules associated with the "inflammatory" signature contain DEGs strongly associated with inflammatory networks (Fig. 1a, Supplementary Tables 3 and 4), and the top DEG identified on this list encoded NKG7, a molecule predicted to be a multi-pass membrane protein comprising a helical bundle (Fig. 1b-c) expressed by multiple immune cells [14, 16, 17, 20]. We confirmed increased expression of *NKG7* by CD4$^+$ T cells from VL patients, relative to CD4$^+$ T cells from the same patients 30 days later and the same cell population from endemic controls (ECs) (Fig. 1d). We also measured increased expression of *Nkg7* by conventional (T_{conv}) and Foxp3$^+$ regulatory T (T_{reg}) CD4$^+$ T cells in the spleen and liver of *L. donovani*-infected mice at day 56 p.i. (Fig. 1d). Although *Nkg7* gene expression was highest in liver T_{conv} CD4$^+$ T cells both in the naive state and following infection, the increased

expression by splenic conventional $CD4^+$ T cells following infection was greatest and likely contributed to the ranking at the top of our "inflammatory" signature list (Fig. 1a). Thus, NKG7 is a highly expressed by $CD4^+$ T cells in infected and inflamed tissues.

Tissue-specific and temporal changes in Nkg7 expression during infection

Given a paucity of NKG7 detection reagents, we generated an *Nkg7* transcriptional reporter mouse to allow cellular analysis by flow cytometry and microscopy. Mice expressing the *Cre* gene under the control of the *Nkg7* promoter were crossed to a membrane reporter line [21] to generate mice in which cells with an active *Nkg7* promoter expressed green fluorescent protein (GFP) (Fig. 2a). At steady state, *Nkg7* expression was mainly detected in NK cells and a subset of $CD8^+$ T cells, with relatively minimal expression by $CD4^+$ T cells (Fig. 2b) and other immune cell subsets in the spleens of naive mice (Extended Data Fig. 1).

To establish patterns of *Nkg7* expression by $CD4^+$ T cells following activation, we first isolated these cells from the spleens of reporter mice and cultured them under neutral (T_H0), T_H1, T_r1 (type 1 regulatory; IL-10-producing T_H1 cells [10]), T_H2, T_H17 and inducible T_{reg} cell conditions [22] (Supplementary Table 5). We found little *Nkg7* expression under neutral, T_H2, T_H17 and inducible T_{reg} cell conditions (Fig. 2c). However, *Nkg7* expression increased under T_H1 cell conditions, as previously reported [20], and was further amplified following the addition of IL-27 to generate T_r1 cells [23] (Fig. 2c). Interestingly, IL-27-induced *Nkg7* expression was suppressed by the addition of TGF-β to cell cultures and this occurred in a dose-dependent manner (Fig. 2d). The decrease in *Nkg7* expression was not related to any increase in cell death under the latter cell culture conditions. Therefore, *Nkg7* expression appears to be linked to T_H1 cells, enhanced by IL-27-mediated polarization of T_r1 cells and inhibited by the presence of TGF-β.

Nkg7-expressing $CD4^+$ T cells were detected in naive C57BL/6 mice, but spleen and liver NK cells expressed the highest levels of *Nkg7*, followed by NKT cells in the liver, and $CD8^+$ T cells in both tissues (Fig. 2b and 3a, Extended Data Fig. 2a). This pattern of expression changed following *L. donovani* infection, with $CD4^+$ and $CD8^+$ T cells emerging as the main *Nkg7*-expressing cells after day 14 p.i. (Fig. 3a, Extended Data Fig. 2b). A closer examination of $CD4^+$ T cells at day 14 p.i. showed *Nkg7* expression was similarly upregulated by T_H1 and T_r1

cells *in vivo* (Extended Data Fig. 2c), both critical cellular determinants of *L. donovani* infection [10]. After resolution of hepatic infection and establishment of chronic infection in the spleen at day 56 p.i., *Nkg7* expression was highest amongst $CD8^+$ T cells, although $CD4^+$ T cells still comprised a substantial proportion of this population (Fig. 3a). Throughout the course of infection the frequency of *Nkg7*-expressing mononuclear cells was approximately 2-3 times higher in the liver than the spleen (Fig. 3A, Extended Data Fig. 2d). The control of hepatic infection is dependent on the accumulation of immune cells in foci surrounding infected Kupffer cells [24], and examination of liver tissue around the peak of this response (day 28 p.i. [24]) revealed an accumulation of *Nkg7*-expressing $CD4^+$ T cells in these inflammatory foci, relative to surrounding tissue (Fig. 3b). Thus, $CD4^+$ and $CD8^+$ T cells were the main immune cells expressing *Nkg7* throughout the course of *L. donovani* infection, and *Nkg7*-expressing $CD4^+$ T cells were located at the site of parasite control in inflammatory foci in the liver.

Nkg7 expression by $CD4^+$ T cells is required to control L. donovani infection

The results above suggested that *Nkg7* expression by T cells may be involved in protective immune responses in the liver, despite originally being identified as part of an "inflammatory" signature in the spleen (Fig. 1d, Fig. 3a). To investigate the role of NKG7 in disease outcome, we examined the response of *Nkg7*-deficient C57BL/6 mice [25] to *L. donovani* infection. Male and female mice were examined by the International Mouse Phenotyping Consortium (https://www.mousephenotype.org/data/genes/MGI:1931250), and no significant hematological changes were identified in *Nkg7*-deficient mice relative to WT mice [25]. Additionally, we found few changes in frequencies of leukocyte subsets in the spleen, thymus, bone marrow, lung or blood of *Nkg7*-deficient mice relative to WT mice (Supplementary Table 6). Following *L. donovani* infection, mice lacking NKG7 had a reduced capacity to control parasite growth in both liver and spleen, compared with WT controls (Fig. 4a). This was associated with minimal change in the development of hepatosplenomegaly (Extended Data Fig. 3a), a major feature in this model of VL [8, 18]. However, serum levels of the key pro-inflammatory cytokines IFN-γ, TNF and MCP-1 in *Nkg7*-deficient C57BL/6 mice were significantly reduced at day 14 p.i. (Fig. 4b). After this time point, serum IFN-γ remained consistently lower in *Nkg7*-deficent mice, compared to

WT controls. Importantly, infection failed to resolve in the livers of mice lacking NKG7 by the end of the experiment (day 56 p.i.) (Fig. 4a). Thus, NKG7 plays an important role in the control of parasite growth.

We next examined cellular responses at day 14 p.i. when differences in liver parasite burden and serum pro-inflammatory cytokine levels, were greatest between *Nkg7*-deficent mice and WT controls. We found reduced recruitment of mononuclear cells into the liver and limited expansion of these cells in the spleen at day 14 p.i. (Fig. 4c). Recruitment of $CD4^+$ T cells to the liver was also reduced (Extended Data Fig. 3b), although $CD4^+$ T cell expansion in the spleen was unaffected (Extended Data Fig. 3c) in *Nkg7*-deficient mice, compared to WT controls. To establish the importance of *Nkg7* expression by $CD4^+$ T cells in this infection, we adoptively transferred *Nkg7*-deficient or WT $CD4^+$ T cells into T and B cell-deficient *Rag1*$^{-/-}$ mice the day prior to *L. donovani* infection and measured parasite burdens 14 days later. We found an approximate 2-fold increase in liver parasite burdens of *Rag1*$^{-/-}$ mice that received *Nkg7*-deficient $CD4^+$ T cells, compared with those that received WT $CD4^+$ T cells (Fig. 4d). Thus, *Nkg7* expression by $CD4^+$ T cells was required for these cells to develop their full anti-parasitic potential during experimental VL caused by *L. donovani*.

In the liver, reduced control of parasite growth in *Nkg7*-deficient mice was associated with reduced frequency and number of T_H1 cells (Fig. 4e), as well as $CD4^+$ T cells recently exposed to antigen ($CD11a^+$ $CD49d^+$ [26]) (Fig. 4f), relative to WT mice. *Nkg7*-deficient $CD4^+$ T cells also expressed less IFN-γ mRNA but not TNF mRNA (Extended Data Fig. 3d). Expression of PD-1, CTLA-4 and ICOS on hepatic $CD4^+$ T cells were also reduced in *Nkg7*-deficient mice, compared to WT controls (Extended Data Fig. 3e), suggesting an overall reduction in $CD4^+$ T cell activation and differentiation in the absence of NKG7.

To further examine the role of NKG7 in $CD4^+$ T cells, we employed a *Leishmania*-specific MHCII tetramer presenting the PEPCK peptide [27] to measure antigen-specific $CD4^+$ T cell populations at day 14 p.i. (Extended Data Fig. 3f). *Nkg7*-deficient mice had a reduced number, but not frequency of PEPCK-positive $CD4^+$ T cells in the liver following *L. donovani* infection. However, both number and frequency of PEPCK-positive T_H1 cells in the liver was reduced at this time in *Nkg7*-deficient mice, compared to WT controls (Extended Data Fig. 3g). Additionally, we found that the activation (phosphorylation) of STAT4 by IL-12 (Fig. 4g), but not activation of STAT3 by IL-6 (Extended Data Fig. 3h), in hepatic polyclonal and PEPCK-

positive $CD4^+$ T cells was reduced in the absence of NKG7. Together, these results show that NKG7 plays an important role in the expansion and/or recruitment of $CD4^+$ T cells to the liver, as well as the production of IFN-γ in this critical anti-parasitic immune cell population.

NKG7 promotes inflammation in an experimental model of severe malaria

To examine the role of NKG7 in a parasitic disease where inflammation is detrimental, we infected *Nkg7*-deficient and WT mice with *Plasmodium berghei* ANKA (*Pb*A) and examined the development of experimental cerebral malaria (ECM). This pre-clinical model of severe malaria is characterised by systemic inflammation leading to the accumulation of parasitised red blood cells (pRBC) in the micro-vasculature of various tissues, including the brain [28]. Although WT mice developed severe neurological sequelae on day 6-8 p.i., as expected [29], *Nkg7*-deficient mice failed to develop these severe symptoms and instead survived up to day 13-14 p.i. (Fig. 5a-b). Although *Nkg7*-deficient mice had a small increase in blood parasitemia at days 7 and 8 p.i., relative to WT controls (Fig. 5c), when parasite biomass was measured using luciferase transgenic *Pb*A, parasite burden in the whole body (Fig. 5d), as well as in the brain (Fig. 5e), was significantly reduced. These latter measurements take into account pRBC that have accumulated in tissue microvasculature and indicate limited activation of vascular endothelium in *Nkg7*-deficient mice, compared to WT controls, resulting in less parasite biomass in the infected mice.

This disease model is characterised by the rapid recruitment of antigen-specific, cytotoxic $CD8^+$ T cells to the brain, which then cause damage to cerebral vascular endothelium [30]. In the absence of NKG7, there were limited changes in NK and $CD4^+$ T cell recruitment to the brain, but recruitment of $CD8^+$ T cells was reduced at the time when WT mice succumbed to disease (Extended Data Fig. 4a). The activation status of *Nkg7*-deficient $CD8^+$ T cells in the brain, as indicated by CD11a/CD49d and granzyme B expression, was also significantly reduced (Extended Data Fig. 4b). We also measured the recruitment of parasite-specific $CD8^+$ T cells to the brain using a *Plasmodium* peptide-MHCI tetramer [31] and found reduced recruitment of these cells to the brains of *Pb*A-infected *Nkg7*-deficient compared to WT mice (Fig. 5f). Therefore, in the absence of *Nkg7*-mediated inflammation there was reduced accumulation of *Pb*A pRBC in tissue, associated with diminished $CD8^+$ T cell recruitment and activation in the brain.

To test whether any of the above NKG7-mediated changes in $CD8^+$ T cell activation were cell intrinsic, we crossed the *Nkg7*$^{-/-}$ mice with PbT-I T cell receptor (TCR) transgenic mice that have $CD8^+$ T cells recognising a MHC-I-restricted parasite peptide found in all rodent *Plasmodium* species (PbT-I$^{\Delta Nkg7}$) [32]. Control mice were generated by crossing PbT-I TCR transgenic mice with congenic (CD45.1) C57BL/6 mice to produce mice expressing both CD45.1 and CD45.2 alleles (PbT-I^{WT}). $CD8^+$ T cells were isolated from the spleens of both lines and adoptively transferred in equal numbers into the same congenic (CD45.1) recipient C57BL/6 mice prior to *PbA* infection to allow a direct comparison between PbT-I^{WT} and PbT-I$^{\Delta Nkg7}$ cells in the same tissue environment. Recipient mice developed neurological symptoms on day 5 p.i., 24-48 hours earlier than normal, likely reflecting the increased kinetics of parasite-specific effector $CD8^+$ T cell expansion and recruitment to the brain. At the time when recipient mice developed ECM, we found reduced expansion of PbT-I$^{\Delta Nkg7}$ cells in the spleen (Extended Data Fig. 4c) and recruitment to the brain (Fig. 5g), compared to PbT-I^{WT} cells. Nevertheless, there was minimal difference in the frequencies of PbT-I$^{\Delta Nkg7}$ and PbT-I^{WT} cells producing granzyme B or perforin (Fig. 5h; Extended Data Fig. 4d), indicating a limited role for NKG7 in the generation of these effector molecules. However, PbT-I$^{\Delta Nkg7}$ cells had a clear reduction in the expression of CD107a, a molecule required for degranulation of cytotoxic molecules [33], on their cell surface in both the spleen (Extended Data Fig. 4e) and brain (Fig. 5i), compared to PbT-I^{WT} cells. Together, these results show that NKG7 plays an important, cell-intrinsic role in the expansion and recruitment of $CD8^+$ T cells to sites of infection and inflammation, as well as in the exocytosis of cytotoxic proteins by these cells, as indicated by reduced CD107a expression.

NKG7 co-localises with CD107a and plays a critical role in $CD8^+$ T cell-mediated killing of targets cells

To better understand how NKG7 might contribute to exocytosis of cytotoxic proteins, we employed a retroviral expression system to transduce $CD8^+$ T cells with NKG7-GFP to establish cellular localisation. We found strong co-localisation of NKG7-GFP with intracellular vesicles containing cytotoxic granules, as identified by LysoTracker (Fig. 6a). Furthermore, when transduced cells were stimulated with phorbol ester and calcium ionophore to induce exocytosis of cytotoxic molecules, we found co-localisation of GFP with

CD107a (Fig. 6b). Finally, to confirm a role for NKG7 in $CD8^+$ T cell-mediated killing, we compared the ability of PbT-I^{WT} and PbT-$I^{\Delta Nkg7}$ cells to kill peptide-pulsed target cells and found a deficit in PbT-$I^{\Delta Nkg7}$ cells, compared to PbT-I^{WT} cells (Fig. 6c). Together, these data identify an important role for NKG7 in the translocation of CD107a from intracellular vesicles to the cell surface by activated $CD8^+$ T cells, thereby promoting their efficient killing of cellular targets.

NKG7 is needed to control experimental tumor metastasis

To investigate whether the pro-inflammatory role of NKG7 extended beyond infectious diseases, we first interrogated the Cancer Genome Atlas: Skin Cutaneous Melanoma data set (TCGA:SKCM), because of the requirement for host immune cells and inflammation for control in this type of cancer [34]. To test whether there was any association between *NKG7* expression in tumors and disease outcome, melanoma patients were ordered by *NKG7* expression, following which, the highest and lowest quartiles were assessed for survival probability. A highly significant survival advantage was observed in patients with high *NKG7* expression, compared to those with low *NKG7* expression (Fig. 7a), suggesting that NKG7 may have a positive role in anti-tumor immunity. Furthermore, *NKG7* expression in these tumors was strongly associated with the expression of NK cell signature molecules [35] (Extended Data Fig. 5a).

Given NKG7 was abundantly expressed by NK cells (Fig. 2a-b, Fig. 3a), we next examined the role of NKG7 in NK cell-dependent pre-clinical models of experimental metastasis using B16F10 and LWT1 melanoma cell lines. When *Nkg7*-deficient mice were injected intravenously with either tumor cell line, there was a significant increase in the number of lung metastases compared to WT mice (Fig. 7b). Similar findings were also made in another experimental prostate carcinoma metastasis model, RM-1 (Extended Data Fig. 5b). Importantly, increased spontaneous metastasis to the lung post-resection of orthotopically injected mammary carcinoma (E0771) was also observed in *Nkg7*-deficient mice despite tumors being equivalent in size at surgery (Extended Data Fig. 5b). RMA-s is a classical MHC class I-deficient tumor target for NK cell perforin-mediated killing, previously characterized *in vivo* when injected into the peritoneum [36]. Survival of *Nkg7*-deficient mice was reduced compared with WT mice post-RMA-s injection and this difference was abrogated with

316 further reduced survival in both WT and *Nkg7*-deficient mice depleted of NK cells (Extended
317 Data Fig. 5b). NK cells are also critical in preventing the initiation of MCA carcinogenesis [37],
318 and herein the *Nkg7*-deficient mice were more sensitive to MCA-induced carcinogenesis
319 than WT mice (Extended Data Fig. 5b). Thus, in a number of mouse cancer models where NK
320 cells are known to be critical in host control, the *Nkg7*-deficient mice were defective
321 compared with WT mice. To examine the anti-tumor defect caused by *Nkg7*-deficiency, the
322 LWT1 and B16F10 metastasis models were further investigated. Increased LWT1 lung
323 metastasis was associated with limited changes in lung hematopoietic cells (Extended Data
324 Fig. 5c), but reduced recruitment of NK and T cells (Extended Data Fig. 5d). Furthermore,
325 the reduced recruitment of NK cells into the LWT1 burdened lung in *Nkg7*-deficient mice
326 was limited to $CD27^+$ $CD11b^+$ NK cells (Extended Data Fig. 5e), a mature inflammatory
327 population of NK cells previously associated with effector function [38]. Importantly, and
328 consistent with results from $CD8^+$ T cells above, *Nkg7*-deficiency resulted in a reduction in
329 the frequency and expression of CD107a on NK cells, but no change in the frequency and
330 expression of IFN-γ, compared to WT NK cells (Fig. 7c).

331 Lung metastasis of B16F10 is controlled by NK cells, IFN-γ and perforin-mediated
332 cytotoxicity [39]. We observed increased metastasis in mice transplanted with B16F10
333 following NK cell, but not $CD8^+$ T cell depletion, indicating that NKG7-mediated control of
334 metastasis was NK cell-dependent (Fig. 7d). While IFN-γ is important for host control of
335 B16F10 lung metastasis, consistent with a lack of impact of *Nkg7* loss on NK cell IFN-γ
336 production (Fig. 7c), the effects of *Nkg7* deficiency and IFN-γ neutralization on metastasis
337 were independent (Fig. 7d). To confirm that *Nkg7* expression by NK cells was required for
338 optimal control metastasis, we also adoptively transferred either *Nkg7*-deficient or WT NK
339 cells into *Rag2cγ*$^{-/-}$ mice 6 days prior to transplanting B16F10 cells. While reconstitution of
340 NK cells was equivalent at the time of tumor inoculation, there was a clear reduction in the
341 ability of *Nkg7*-deficient NK cells to control B16F10 lung metastases (Fig. 7e).

342 To gain further insight into how *Nkg7* expression influenced tumor metastasis, we identified
343 the 50 top up-and down-regulated genes in the high and low *NKG7* expressing individuals
344 from the TCGA:SKCM data set (Fig. 7f). We then identified the top 10 cytokine upstream
345 regulators (Fig. 7g). Many of these molecules have been shown to play important roles in
346 the maintenance or activation of NK cells, including the NK cell growth factor IL-2 [40]. When

Nkg7-deficient and WT mice were transplanted with a high number of B16F10 cells and then treated with therapeutic IL-2 or IL-15, another important NK cell growth factor, the anti-metastatic effect in WT mice was highly significant for both cytokines. However, these clinically-relevant cytokines had a much more limited effect in *Nkg7*-deficient mice (Fig. 7h). Hence, NKG7 plays an important role in promoting the anti-metastatic activity of NK cells, as well as the ability of these cells to respond to cytokine therapy.

NKG7 plays a critical role in NK cell-mediated killing of targets cells

The above results concerning defective CD107a expression, suggest that NKG7 might also be important for NK cell-mediated killing. To examine this possibility, we first tested the requirement of *Nkg7* for efficient control of the RMA-s-Rae1β lymphoma, a primary tumor transplant, and confirmed the requirement for NK cells, but not $CD8^+$ T cells in this model (Fig. 8a). Again, the effects of *Nkg7* deficiency and IFN-γ neutralization were independent (Fig. 8b). Next, we compared the ability of *Nkg7*-deficient and WT NK cells to kill YAC-1 and RMA-s-Rae1β lymphoma targets *in vitro* (Fig. 8c) and RMA-s-Rae1β lymphomas *in vivo* (Fig. 8d). In all settings, *Nkg7*-deficient NK cells had a defect in killing ability, relative to WT control NK cells. Hence, NKG7 was critical for efficient NK cell-mediated killing of target cells, and unlike WT NK cells, the killing activity of *Nkg7*-deficent NK cells could not be rescued to WT levels by prior activation with IL-2 (Fig. 8c). Interestingly, we found no difference in the expression of the activation markers DNAM-1, NKG2D, CD11a, granzyme B or perforin between *Nkg7*-deficient and WT NK cells (Extended Data Fig. 6a), even if they were IL-2-activated (Extended Data Fig. 6b). Furthermore, we observed no differences in the formation of cell conjugates between *Nkg7*-deficient or WT NK cells and their YAC-1 targets (Extended Data Fig. 6c). An evaluation of synapse formation between NK cells and YAC-1 targets also revealed no NKG7-dependent changes (Extended Data Fig. 6d). Together, these results show that NKG7 plays a critical role in NK cell-mediated killing of target cells, but has no measurable impact on the expression of effector molecules or the ability of NK cells to form contacts with cellular targets.

Discussion

Here we show that NKG7 is a novel mediator of inflammation. Several earlier studies reported that *NKG7* expression was associated with cell cytotoxicity [20, 41, 42]. This is consistent with our data showing less efficient killing of target cells by *Nkg7*-deficient NK and $CD8^+$ T cells. Importantly, the *Nkg7*-dependent defect in cytotoxic activity was not related to changes in expression of effector molecules such as granzyme B, perforin and IFN-γ. Furthermore, *Nkg7*-deficient NK cells had no reduction in their capacity to form conjugates or an immunological synapsis with target cells. Instead, the *Nkg7*-dependent killing defect was linked with reduced expression of CD107a on the surface of *Nkg7*-deficient NK cells in metastasis models and $CD8^+$ T cells in ECM. CD107a is critical for NK cells to deliver perforin and granzyme B to target cells [43]. Hence, our findings support a key role for NKG7 in the translocation of CD107a to the cell surface, associated with exocytosis of cytotoxic molecules.

T_H1The T_H1 cell canonical transcription factor T-bet binds to the *NKG7* promoter in T_H1 cells, and increased expression of *NKG7* and *PRF1* (encoding perforin), along with *IFN-G* (encoding IFN-γ) [20]. Our findings support a role for NKG7 in T_H1 cell IFN-γ production, with evidence that *Nkg7*-deficient $CD4^+$ T cells have a limited capacity to respond to IL-12 signalling, as indicated by reduced STAT4 activation. Interestingly, a recent study of blood samples from genetically susceptible mice infected with *Mycobacteria tuberculosis,* as well as from human tuberculosis (TB) patients and individuals with latent TB who progressed to active disease, revealed that *NKG7*, along with other genes associated with effector and cytotoxic NK and T cell responses, was downregulated [44]. These findings are consistent with our discoveries in VL, a disease also caused by infection of tissue-resident macrophages requiring an effective T_H1 cell response for control. However, we did not establish a cell-intrinsic role for NKG7 in $CD4^+$ T cell IFN-γ production. In fact, data from cell adoptive transfer studies of WT and *Nkg7*-deficient $CD4^+$ TCR transgenic cells indicated that that the effect of NKG7 on IFN-γ production is cell extrinsic and downstream of the NKG7-mediated impact on cytotoxic granule exocytosis (data not shown). Previous studies showed that perforin- and granzyme B-deficient mice have enhanced T_H1 cell responses compared to WT mice following *Pb*A infection[30]. Thus, one possibility is NKG7-mediated cytotoxic granule exocytosis by NK cells or $CD8^+$ T cells modulates antigen presenting cell numbers and/or function early during infection to influence $CD4^+$ T cell IFN-γ production.

Increased expression of *NKG7* in mouse IL-27-induced T_r1 cells [45] and human IL-10$^+$ T_H17 cells [17] has been reported. Although, we found limited *Nkg7* expression by mouse T_H17 cells, the association with human IL-10-producing T_H17 cells may indicate *NKG7* is expressed by highly differentiated CD4$^+$ T cell populations. This is supported by our findings that *Nkg7* expression was linked to T_H1 cells, enhanced by IL-27-mediated polarization to T_r1 cells, and the reduced levels of co-inhibitory receptor molecules associated with highly activated T cells on *Nkg7*-deficient CD4$^+$ T cells during infection. However, the use of our *Nkg7*-reporter mice in *in vivo* studies revealed that *Nkg7* was expressed by different T cell subsets, including conventional CD4$^+$ T and CD8$^+$ T cells, T_H1, T_r1, T_{reg} and NKT cells, as well as innate cell populations, most notably NK cells. Results from our studies in disease models also indicate functional roles for NKG7 in CD4$^+$ T cells (VL), CD8$^+$ T cells (malaria) and NK cells (cancer). Hence, *NKG7* is expressed by a range of immune cell populations, suggesting a diversity of immunological roles during disease that is context-dependent.

In the absence of CD4$^+$ T cell *NKG7* expression, the generation of an inflammatory response following *L. donovani* infection was limited, and this resulted in increased parasite growth, demonstrating a critical role for this molecule in host defence. However, inflammation can also cause disease, as in severe malaria syndromes [19]. Indeed, our results from a pre-clinical model of severe malaria showed that *Pb*A infection resulted in a NKG7-mediated inflammatory response that promoted the accumulation of parasites in host tissues such as the brain. In the absence of NKG7 there was reduced CD8$^+$ T cell recruitment to the brain, associated with less cell activation. We also found evidence for a cell-intrinsic reduction in parasite-specific CD8$^+$ T cell expansion in the spleen and recruitment to the brain in the absence of NKG7, as well as translocation of CD107a to the cell surface following activation, but a limited cell-intrinsic requirement for granzyme B or perforin production. Therefore, NKG7 likely acts at multiple steps in the activation, expansion and delivery of effector functions in CD8$^+$ T cells.

We also identified an important role for NKG7 in NK cell-mediated control of cancer metastasis, predicted by the strong, positive association between *NKG7* expression in skin cutaneous melanomas and patient survival probability. Recently, metastatic melanoma patients responding to combined PD-1 and CTLA-4 blockade were shown to have greater expansion of CD8$^+$ T cell clones that over-expressed genes associated with cytotoxicity,

including NKG7, than non-responding patients [14], supporting an important role for this molecule in tumor-associated immune responses. Together with our data, these findings reinforce of a role for NKG7 in anti-tumor immunity, and suggests that targeting this molecule for activation may represent a new approach for cancer treatment. Alternatively, maintaining NKG7 expression or stimulating overexpression may also have beneficial outcomes in this disease setting.

In summary, we have identified NKG7 as a critical mediator of inflammation in a range of diseases. NKG7 is expressed on different immune cells at different stages of disease and data from *Nkg7*-deficient mice indicates that targeting this molecule via blockade of function (antagonist) represents a novel approach to dampening inflammation in diseases such as severe malaria, while activating this molecule (agonist) may be employed to enhance immune responses during infectious diseases or cancer.

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METHODS

EXPERIMENTAL MODEL AND SUBJECT DETAILS

MICE

Mice greater than 6 weeks of age were used for all experiments unless specified otherwise. Mice were group-housed with a maximum of 6 mice per cage, in a passive air flow, environmentally ventilated cage system, and maintained under pathogen-free conditions at the QIMR Berghofer Medical Research Institute Animal Facility (Herston QLD, Australia). Mouse cages were housed in an environmentally controlled room that was maintained at 20–21°C with 60% relative humidity and a 12-hour light cycle (8.00 am to 8.00 pm), with no external or natural light sources. All exhaust air from the mouse racks were vented external to the building.

B6.Tg(*Nkg7*-cre)/J (B6.*Nkg7*-cre) mice were crossed to B6.Tg(*Nkg7*-cre)/J, B6.129(Cg)-*Gt(ROSA)26Sor*$^{tm4(ACTB–tdTomato,–EGFP)Luo}$/J (B6.mT/mG; RRID: IMSR_JAX:007676) [21] mice once to generate a transcriptional reporter of *Nkg7* (*Nkg7*-cre x mT/mG). *C57BL/6J* (RRID: IMSR_JAX:000664) mice were sourced from the Walter and Eliza Hall Insitute (WEHI) (Kew VIC, Australia) and B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (B6.*Cd45.1*; RRID: IMSR_JAX:002014) were sourced from the Animal Resources Centre (ARC; Canning Vale WA, Australia).

All other mice were bred in-house including *C57BL/6NJ* (C57BL/6N; RRID: IMSR_JAX:005304), C57BL/6-*Foxp3*^{tm1flv}/J (Foxp3-RFP; RRID: IMSR_JAX:008374, *Nkg7*$^{tm1.1(KOMP)Vlcg}$ (B6N.*Nkg7*$^{–/–}$; RRID: IMSR_KOMP:VG11445-1.1-Vlcg) [25], B6.129S7-*Rag1*^{tm1Mom}/J (*Rag1*$^{–/–}$; RRID: IMSR_JAX:002216) [46], and C;129S4-*Rag2*$^{tm1.1Flv}$ *Il2rg*$^{tm1.1Flv}$/J (*Rag2*$^{–/–}$γc$^{–/–}$; RRID: IMSR_JAX:014593) [47] mice. B6N.*Nkg7*$^{–/–}$ (*Nkg7*$^{tm1.1(KOMP)Vlcg}$) mice [25] were generated by the University of California Davis (UC Davis, Davis CA, USA) as part of the trans-NIH Knockout Mouse Project (KOMP) and obtained from the KOMP repository (http://www.komp.org/). Transgenic PbT-I mice [32] were crossed to B6.*Cd45.1* mice to generate PbT-I x B6.*Cd45.1* (PbT-I^{WT}; CD45.1^{+} CD45.2^{+}) mice, and crossed to B6J.*Nkg7*$^{–/–}$ mice to generate Nkg7-deficient PbT-I mice (PbT-I$^{\Delta Nkg7}$; CD45.1$^{–}$ CD45.2^{+}).

When the same mouse strain was used across multiple experimental groups, littermates of the same sex were distributed randomly into groups. Experimental use was in accordance

with the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" (Australian National Health and Medical Research Council (NHMRC)) and approved by the QIMR Berghofer Medical Research Institute Animal Ethics Committee (Herston QLD, Australia; approval numbers: A02-633M, A02-634M, A1707-615M, A19619M, A19620M).

HUMAN SUBJECTS

Blood samples were collected from 23 symptomatic VL patients at the Kala-azar Medical Research Center (Muzaffapur, Bihar, India). Patients were diagnosed either by microscopic detection of amastigotes in splenic aspirate smears or using a rk39 (*L. donovani* antigen) dipstick test. Clinical data from these patients are summarised in Table 1. Five ml of blood was collected from each patient on the day of admission (day 0), and 30 days following treatment with AmBisome (Gilead Sciences, Inc., Foster City CA, USA) (day 30). Five ml of blood was also collected from endemic controls (EC). Involvement of human subjects in this research was in line with recommendations from the Helsinki declaration. Human ethics approval was provided by the ethical review board of Banaras Hindu University (BHU), Varanasi, India (Dean/2011-12/289) and the QIMR Berghofer Medical Research Institute Human Ethics Committee (HREC; HREC reference number P1411). Written informed consent was obtained from all participants, and where participants were below 18 years of age, written informed consent was obtained from their legal guardian.

METHOD DETAILS

GENERATION OF *C57BL/6J-Nkg7$^{em1(cre)WEHI}$* MICE

C57BL/6J mice expressing the cre recombinase under the control of the *Nkg7* promoter (B6J.*Nkg7*-cre) were generated by the Melbourne Advanced Genome Editing Centre (MAGEC) at the Walter and Eliza Hall Institute (WEHI) using CRISPR/Cas9 mediated gene editing. Briefly, based on methods previously described [48], the single guide (sg)RNA (sequence: CATGGAGCCCTGCCGGTCCC) was used to induce double stranded breaks in the *Nkg7* locus to stimulate homologous recombination and a targeting vector containing ~2kb homology arms [49] was used to introduce the cre recombinase coding sequence.

Forward (ACGACCAAGTGACAGCAATG) and reverse (GCTAACCAGCGTTTTCGTTC) primers to detect the cre recombinase sequence were used to screen viable pups for integration of the

targeting vector by polymerase chain reaction (PCR). A 301bp amplicon was detected where the cre recombinase sequence was present. F0 mice expressing the cre sequence were selected for backcrossing that resulted in heterozygous F1 mice. The PCR described above was used to screen F1 mice for the cre sequence. Further validation by long-range PCR was performed to verify correct positional integration of the targeting vector.

LEISHMANIA INFECTION IN MICE

L. donovani (LV9; MHOM/ET/67/HU3) was originally isolated from a patient in Ethiopia in 1967 [50] and maintained by passage in B6.*Rag1*$^{-/-}$ mice. Passage mice were euthanised and the spleen was excised into 5 ml of sterile Roswell Park Memorial Institute Medium 1640 (RPMI 1640; Gibco™, Life Technologies, Carlsbad CA, USA) + 100 µg/ml penicillin–streptomycin (PS; Gibco™, Life Techonologies); RPMI/PS) medium. The excised spleen was homogenized using a glass tissue grinder and the cell suspension was centrifuged in an Eppendorf Centrifuge 5810 R (Fisher Scientific™, Thermo Fisher Scientific) at 115 *g* for 5 minutes at room temperature (RT), with brake off. The supernatant was transferred to a new tube, and the pellet discarded. The supernatant was centrifuged at 1960 *g* for 15 minutes at RT. The supernatant was discarded, and the pellet was incubated for 5 minutes in 1 ml of Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma-Aldrich®), following which, sterile RPMI/PS was added, and the parasites centrifuged at 1960 *g* for 15 minutes at RT. After discarding the supernatant, sterile RPMI/PS was added to the pellet and the centrifugation step was repeated at 1960 *g* for 15 minutes at RT. After discarding the supernatant, the parasite pellet was re-suspended in sterile RPMI/PS. The parasite suspension was taken up through a 26G x ½" needle on a 1 ml syringe (Terumo® Medical, Somerset NJ, USA) and dispensed, then the process repeated until a homogenous suspension was achieved. Two µl of the parasite suspension were loaded onto a Thoma cell counting chamber (Weber Scientific International, West Sussex, UK) and parasites were counted in the 4 x 4 grid in triplicate. An average count was used to determine the number of parasites/ml using the following equation:

$$\frac{average}{16} \times 2 \times 10^7 = parasites/ml$$

Parasites were diluted to a final concentration of 1 x 10^8 parasites/ml in sterile RPMI/PS. Each mouse received 2 x 10^7 parasites in 200 µl, injected intravenously (i.v.).

QUANTIFYING *LEISHMANIA* PARASITE BURDENS IN MURINE SPLEEN OR LIVER

Where indicated, parasite burden was quantified from spleen and liver impression smears stained in Giemsa (Sigma-Aldrich®). The number of amastigotes per 1000 host nuclei were counted under x1000 magnification using a light microscope (Olympus CX31; Olympus Life Science, Shinjuku, Tokyo, Japan) and multiplied by the organ weight (g) to derive Leishman-Donovan Units (LDU).

PLASMODIUM INFECTION IN MICE

Murine *Plasmodium* infections were established from parasites passaged in C57BL/6J mice once. Briefly, 200 µl of transgenic *P. berghei* ANKA (*Pb*A; 231c11) parasites (in house laboratory stock, frozen at -80°C) expressing luciferase and GFP under the control of the ef1-α promoter [51], were thawed at room temperature and injected i.p. into a passage mouse. A blood smear was prepared and stained with 20% (v/v) Giemsa (Sigma-Aldrich) from day 2 onwards. Parasitemia (% parasitised red blood cells (pRBC)) was quantified under a light microscope (Olympus CKX41; Olympus Life Science). The passage mouse was sacrificed at >1% pRBC. Blood was collected from the passage mouse by cardiac puncture, into RPMI/PS containing 1 IU/ml heparin and centrifuged at 290 *g* for 7 minutes at room temperature. RBCs were counted on a haemocytometer (Pacific Laboratory Products). A parasite inoculum containing 5 x 10^5 pRBC/ml was prepared and mice were injected with 200 µl of the inoculum i.v. (total of 1 x 10^5 pRBC per mouse).

Mice were tail bled to obtain a drop of blood, which was collected in 250 µl RPMI/PS with 1 IU/ml heparin. Fifty µl of blood preparation was stained with Hoechst 33342 (Sigma-Aldrich) and Syto™ 84 (Invitrogen™, Life Technologies) in RPMI/PS for 30 minutes at room temperature. Three hundred µl of RPMI/PS was then added and each sample acquired on a BD FACSCanto™ II or BD LSRFortessa through BD FACSDiva™V8.0 (BD Biosciences).

*Pb*A-infected mice were scored daily, from day 4 p.i., for symptoms of experimental cerebral malaria (ECM) including hunching in posture, piloerection, lethargy, and wobbly gait. At the peak of ECM, mice were anaesthetised with IsoThesia® NXT (HenrySchein, Melville NY, USA) and injected i.p. with 100 µl of 5 mg/ml D-Luciferin Firefly, Potassium Salt (Caliper Life Sciences, Waltham MA, USA). Whole body bioluminescence was visualised using the IVIS® Spectrum *in vivo* imaging system (Xenogen Corporation, Caliper Life Sciences). Mice were sacrificed by CO_2 asphyxiation and spleens were collected for cellular analysis. Mice were subsequently perfused through the heart with 10 ml of phosphate buffered saline (PBS).

Brains were excised and imaged as previously described for whole body bioluminescence. Bioluminescence was quantified using the Living Image 4.5 software (PerkinElmer, Waltham MA, USA) and expressed as average photons/second/cm^2/steer radiant (p/sec/cm^2/sr).

ISOLATION OF MOUSE PERIPHERAL BLOOD MONONUCLEAR CELLS (MNCs)

Mice were sacrificed by CO_2 asphyxiation and blood collected via cardiac puncture using an insulin syringe (BD Biosciences). The blood was then diluted with and equal volume of Dulbecco's PBS (DPBS) and overlaid onto 1.5 ml of Lympholyte®-Mammal (Cedarlane, Burlington, Canada) and processed as per manufacturer's instructions.

PREPARATION OF SPLEEN AND THYMUS SINGLE CELL SUSPENSIONS

A mid-sagittal incision was made on the abdominal cavity of sacrificed mice, from which the spleen was excised. Incisions were made on either side of the ribcage to expose the thoracic cavity and the thymus was collected. The spleen and thymus were weighed and placed into 1% (v/v) FCS in PBS (1% FCS.PBS). Spleens and thymi were mechanically passed through an EASYstrainer™ 100 µm cell strainer (Greiner Bio-One, Kremsmünster, Austria) using the back of a 5 ml syringe plunger (Terumo® Medical). Cells were resuspended in 1% FCS.PBS and centrifuged at 350 *g* in an Eppendorf Centrifuge 5810 R (Fisher Scientific™, Thermo Fisher Scientific) and lysed by incubating in Red Blood Cell Lysis Buffer Hybri-Max™ (Sigma-Aldrich®) for 7 minutes at RT. Cells diluted in DPBS (Gibco™) and Trypan Blue Stain (Invitrogen™) were counted using Countess™ Cell Counting Chamber Slides on the Countess II FL (both from Invitrogen™), as per manufacturer's protocol.

PREPARATION OF LIVER SINGLE CELL SUSPENSIONS

Mice were sacrificed by CO_2 asphyxiation. A mid-sagittal incision was made on the abdominal cavity. The liver was perfused via the hepatic portal vein with 1x phosphate-buffered saline (PBS). The excised liver was weighed and collected in 1% (v/v) FCS.PBS and mechanically passed through an EASYstrainer™ 100 µm cell strainer (Greiner Bio-One) using a 10 cc/ml syringe plunger (Terumo® Medical). The homogenized liver was washed twice in 1x PBS by centrifuging at 390 *g* in an Eppendorf Centrifuge 5810 R. Hepatocytes were separated from leukocytes using a 33% (v/v) Percoll™ Density Gradient Media (GE Healthcare, Little Chalfont, U.K.) and centrifugation at 575 *g* for 15 minutes at RT with the brake off. Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma-Aldrich®) was added to the leukocyte pellets and incubated for 7

minutes at RT. This was followed by a single wash in PBS as described above. Cells diluted in DPBS (Gibco™, Life Technologies™) and Trypan Blue Stain (Invitrogen™), then counted using Countess™ Cell Counting Chamber Slides on the Countess II FL (both from Invitrogen™), as per manufacturer's protocol.

ISOLATING BONE MARROW MONONUCLEAR CELLS (MNCs)

The femurs of sacrificed mice were collected and cleaned of surrounding muscle. Once clean, the ends of the femurs were removed using a pair of scissors and the shaft placed in an Eppendorf tube containing 1m of 1% (v/v) FCS.PBS. Tubes were centrifuged at 13523 *g* in a microcentrifuge for 3 minutes at 4°C. Upon removal of bones from the Eppendorf tubes, the remaining cell pellet was resuspended using a pipette. Cells were transferred into a 10 ml tube and centrifuged at 390 *g* for 6 minutes at room temperature. The supernatant was discarded by pouring and red blood cells were lysed by adding 1 ml of Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma-Aldrich®) to each sample and incubating for 5 minutes at room temperature. Cells were washed once in 1% (v/v) FCS.PBS by centrifuging as described above and pouring off the supernatant.

ISOLATING BRAIN MONONUCLEAR CELLS (MNCs)

Cardiac perfusion was performed with cold DPBS prior to removal of brains. Excised brains were dissected into smaller pieces and incubated in 2 mg/ml Collagenase from *Clostridium histolyticum* and 1 mg/ml Deoxyribonuclease (DNase) I from bovine pancreas (both from Sigma-Aldrich) resuspended in 2 ml HBSS (without phenol red, calcium chloride, and magnesium sulfate) per sample. Samples were incubated on an Incu-Shaker™ Mini (Benchmark Scientific, Sayreville NJ, USA) at 200 rpm for 20 minutes at 37°C. Samples were then homogenised through an EASYstrainer™ 70 µm cell strainer (Geiner Bio-One) using the back of a 10 ml syringe plunger (Terumo® Medical). Brain single cell suspensions were washed by resuspending in 1% FCS.PBS and centrifuging at 350 *g* in an Eppendorf Centrifuge 5810 R (Fisher Scientific™, Thermo Fisher Scientific). The supernatant was discarded by pouring and the pellet resuspended in a 33% (v/v) Percoll™ Density Gradient Media (GE Healthcare, Little Chalfont, U.K.) followed by centrifugation at 575 *g* for 15 minutes at RT with the brake off. The debris and supernatant were discarded by pouring, following which, MNCs were incubated in 500 µl Red Blood Cell Lysis Buffer Hybri-Max™ (Sigma-Aldrich®) for 4 minutes at RT. MNCs were washed once as described above, and incubated in 2x Monensin Solution

(BioLegend) diluted in complete media (as described above) for 3 h at 37°C in the presence of 5% CO_2. Flow cytometry staining panels for brain MNCs included the use of anti-mouse/human CD11b (Brilliant Violet 421™, M1/70; BioLegend) and anti-mouse F4/80 (Brilliant Violet 421™, BM8; BioLegend) to eliminate microglia.

PREPARATION OF LUNG SINGLE CELL SUSPENSIONS

Mice were sacrificed by CO_2 asphyxiation and lungs were perfused intraventricularly with cold PBS to remove blood. The perfused lungs were cut finely and digested in 3 ml RPMI 1640 media containing 1 mg/ml Collagenase IV (Worthington Biochemical Corporation, Australia) and 20 µg/ml DNase I (Roche Diagnostics Corporation, Indiana, USA) in a 6-well plate for 30 minutes at 37°C, set on a shaker at 150 rpm. Then digested lung tissue was homogenized through a 40 µm strainer and washed with PBS. Cells were centrifuged at 456 *g* for 4 minutes and resuspended in 1 ml FACS buffer with anti-CD16/32 (clone 2.4G2) to block Fc receptors and incubated for 20 minutes on ice. One fifth of the cell suspension was stained with fluorophore-conjugated antibody cocktails for 20 minutes on ice. Before samples were run on a flow cytometer, liquid-counting beads (BD Biosciences) were added directly to determine absolute cell counts in samples.

ISOLATION OF $CD4^+$ T CELLS FROM HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS (MNCs)

Approximately 5 ml of blood was collected from each patient on the day of admission (day 0) and 30 days after treatment with AmBisome (Gilead Sciences, Inc., Foster City CA, USA) (day 30) in a BD Vacutainer® Lithium Heparin^N (LH) 170 I.U. Plus Blood Collection Tubes (BD Biosciences). Blood was layered over Ficoll-Paque™ PLUS (GE Healthcare) to isolate PBMCs. PBMCs were counted using a haemocytometer (Pacific Laboratory Products, Blackburn VIC, Australia). $CD4^+$ T cells were enriched by magnetic activated cell sorting (MACS) using the anti-human CD4 MicroBeads (Miltenyi Biotec, Bergisch Gladback, Germany) according to manufacturer's instructions.

FLOW CYTOMETRY

All flow cytometry staining was performed in Falcon® 96-Well Clear Round Bottom Tissue Culture (TC)-Treated Cell Culture Microplates (Corning Inc., Corning NY, USA). Single cell suspensions were incubated with 50 µl of TruStain fcX™ (anti-mouse CD16/32; clone: 93) and

Zombie Aqua™ Fixable Viability Dye cocktail (both from BioLegend, San Diego CA, USA) for 15 minutes at RT. CD107a (LAMP-1) staining was performed during the 3-hour stimulation with PMA/ionomycin or incubation with Monensin (see below) by adding 5 µg/ml of anti-Mouse CD107a (clone: 1D4B from BioLegend or eBio1D4B from eBioscience, Life Technologies) to the stimulation cocktail. Cells were washed once with staining buffer (PBS, 0.02% (v/v) FCS, 5mM EDTA, 0.01% (w/v) NaN_3) by centrifuging in an Eppendorf Centrifuge 5810 R (Fisher Scientific™, Thermo Fisher Scientific) at 575 *g* for 1 minutes at 4°C. Samples were then incubated with 50 µl of a cocktail of fluorescence-conjugated antibodies reactive against surface molecules for 30 minutes. A list of antibodies and concentrations used can be found in Supplementary Table 7. Where samples were stained with a biotin-conjugated antibody, samples were subsequently incubated with 1 µg/ml Streptavidin PE-Cy7 Conjugate (Thermo Fisher Scientific).

After two washes with staining buffer, as described above, samples were incubated with 100 µl of fixation buffer from either the BD Cytofix™ Fixation Buffer Set (for cells that were subsequently stained with antibodies against cytokines) (BD Biosciences, San Diego CA, USA) or the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) (for cells that were subsequently stained with antibodies reactive against transcription factors) for 20 minutes. Cells were then washed twice with wash buffers from the respective kits, by centrifuging at 575 *g* for 1 minute at room temperature, following which, cells were incubated with 50 µl of cocktail containing fluorescence-conjugated antibodies against intracellular molecules for 35 minutes. All staining was performed at RT, and samples were incubated in the dark.

Samples were stored at 4°C before acquisition on a BD LSRFortessa™ (special order research product; BD Biosciences) through BD FACSDiva™ v8.0, or on the Cytek Aurora™ 5 laser through the SpectroFlo® software package v2.2 (Cytek Biosciences, Fremont CA, USA) and analysed on FlowJo v10 OSX (FlowJo, LLC, Ashland OR, USA). Graphing and statistical analyses were performed on GraphPad Prism 7 (Version 7.0c; GraphPad Software, La Jolla CA, USA). A p value ≤ 0.05 was considered statistically significant.

PMA/IONOMYCIN RESTIMULATION

Cells were incubated in complete media (10% (v/v) FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (penicillin–streptomycin), 1x GlutaMAX™, 1x non-essential amino acids, 110

mg/L (1 mM) Sodium Pyruvate, 5 mM HEPES (all from Gibco™), and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich®), in RPMI 1640 containing L-Glutamine (Gibco™)) containing 25 ng/ml of Phorbol 12-myristate 13-acetate (PMA) and 1 µg/ml (1.33 nM) Ionomycin calcium salt in the presence of 10 µg/ml Brefeldin A (all from Sigma-Aldrich) or 2x Monensin Solution (BioLegend). PMA/ionomycin re-stimulation was performed for 3 h at 37°C in the presence of 5% (v/v) CO_2.

TETRAMER STAINING

Detection and quantification of antigen-specific cells was performed using fluorophore-conjugated tetramers added alongside other fluorophore-conjugated antibodies detecting surface markers. 0.7 µg/well APC-conjugated I-A^bPEPCK$_{335-351}$ [27] and 0.3 µg/well PE-conjugated H2-K^b-NVF [31] tetramer was used.

DETECTION OF PHOSPHORYLATED STAT3 AND STAT4

Following staining for surface markers, cells were incubated in 400 ng/ml Recombinant Mouse IL-12 (p70; carrier-free) (BioLegend; for detection of phosphorylated STAT4) or Recombinant Mouse IL-6 (carrier-free) (BioLegend) for detection of phosphorylated STAT3) for 4 minutes at RT, in a 96-well U-bottom plate. Cells were then centrifuged at 575 *g* for 1 minute at RT and the supernatant was discarded by flicking. Cells were then incubated in pre-warmed 1x BD Phosflow™ Lyse/Fix Buffer (BD Biosciences) for 10 minutes at 37°C and centrifuged at 1860 *g* for 2 minutes. Supernatant was discarded by flicking. Cells were then washed twice with eBioscience™ Flow Cytometry Staining Buffer (Invitrogen) as described above. Following this, cells were incubated in -20°C pre-chilled BD Phosflow™ Perm Buffer III (BD Biosciences) for 30 minutes on ice. Cells were washed thrice in with eBioscience™ Flow Cytometry Staining Buffer (Invitrogen) as above. Finally, cells were stained with either PE-conjugated Mouse anti-Stat4 (pY693) (clone: 38/p-Stat4) or PE-conjugated Mouse anti-Stat3 (pY705) (clone: 4/P-STAT3) (both from BD Biosciences), followed by two washes in eBioscience™ Flow Cytometry Staining Buffer (Invitrogen) as described above. Samples were acquired on a BD LSRFortessa through BD FACSDiva™ V8.0 (BD Biosciences).

***EX VIVO* LUNG NK CELL FUNCTION ASSAY**

One fifth of a whole lung suspension was incubated at 200 µl/well in a 96-well U-bottom plate in complete RPMI 1640 media. Cells were incubated in the presence of the eBioscence ™ Cell

Stimulation Cocktail (plus protein transport inhibitors) (diluted 1000x; Invitrogen™) and 2.5 µg/ml Alex Fluor 647-conjugated anti-CD107a antibody (BioLegend). Four hours later, cells were stained for surface markers and intracellular cytokine production and analysed as described above.

EVALUATION OF THE IMMUNOLOGICAL SYNAPSE

Splenic NK cells were isolated using the NK Cell Isolation Kit II (Miltenyi Biotec) according to manufacturer's instructions and activated with 1,000 U/ml recombinant IL-2 for 4–5 days. Activated effector NK cells were labelled with 5 µM CellTrace™ Violet Cell Proliferation Kit, for flow cytometry (CTV; Thermo Fisher Scientific) and co-cultured with carboxyfluorescein succinimidyl ester (CFSE)-labelled YAC-1 target cells [52] for 15 minutes at an effector to target (E:T) ratio of 1:2 in polyproprylene tubes followed by fixing with 2% (w/v) paraformaldehyde in PBS.

Evaluation of the immunological synapse was performed using imaging flow cytometry as previously described [53]. Briefly, fixed cells were stained with PerCP-Cy5.5-conjugated anti-mouse 1 µg/ml CD11a/CD18 (LFA-1) (clone: H155-78; BioLegend) for 20 minutes at room temperature. Cells were washed with 2% FCS in PBS (2% FCS.PBS) and centrifuged at 300 *g* for 4 minutes, followed by aspiration of the supernatant using a pipette. Permeabilisation was performed with 0.1% (v/v) Triton-X in 2% FCS.PBS (Sigma, Cat. No T8787) containing 0.4 Units of Alexa Fluor 647-conjugated Phalloidin (Invitrogen Cat. No. A22287) by incubating at RT for 30 minutes. Cells were washed as described above and resuspended gently in 30 µl of 2% (w/v) formaldehyde in PBS. Samples were immediately acquired on the four laser, 12 channel Amnis® ImageStream®XMark II (Luminex Corporation, Austin TX, U.S.A) at a 60-fold magnification at low speed. Data analysis was performed using the Image Data Exploration and Analysis Software (IDEAS®), version 6.2 (Luminex Corporation). The gating strategy involved selection of cells in focus based on "gradient RMS". After sub-gating on double-positive CTV^+ and $CFSE^+$ events, focused and centered doublets were selected and at least 50 doublets per group were analysed. The interface mask was then applied with the NK-cells (CTV^+) defined as the target of interest. The mean fluorescence intensity of LFA-1 and Phalloidin within the Interface mask served as a surrogate marker for the strength and intensity of the immunological synapse. Statistical significance was determined using a non-parametric Mann-Whitney U test.

ISOLATION OF MOUSE SPLEEN AND LIVER CD4$^+$ T CELLS BY FLUORESCENCE ACTIVATED CELL SORTING (FACS)

CD4$^+$ T cells were isolated by MACS using the CD4$^+$ T cell isolation kit, mouse (Miltenyi Biotec) according to manufacturer's instructions. The flow through, containing enriched CD4$^+$ T cells were stained with propidium iodide (PI) (Sigma-Aldrich®), anti-mouse TCRβ (fluorescein isothiocyanate (FITC), H57-597) and anti-mouse CD4 (Allophycocyanin (APC), GK1.5) (both from BioLegend).

Isolation of CD4$^+$ T cells for real time quantitative polymerase chain reaction (RT-qPCR) was achieved by cell sorting after staining for anti-mouse CD90.2 (Thy-1.2; PerCP/Cyanine5.5 (PerCP/Cy5.5), 53-2.1) and anti-mouse CD4 (APC, GK1.5) (both from BioLegend).

Dead cells were excluded by positive staining for either PI or SYTOX™ Blue Dead Cell Stain, for flow cytometry (used according to manufacturer's instructions). CD4$^+$ T cells were identified as TCRβ$^+$ CD4$^+$ or CD90.2$^+$ CD4$^+$. Cells were sorted on the BD FACSARIA II (Becton Dickinson) and stored in buffer RLT (QIAGEN®, Hilden, Germany) at -80°C.

MICROARRAY

FACS™-sorted mouse spleen and liver CD4$^+$ T cells, stored in buffer RLT, were homogenised in QIAshredder columns prior to RNA extraction using the RNeasy Mini Kit (All from QIAGEN®) according to manufacturer's instructions. Each replicate within the naive liver CD4$^+$ T cell group consisted of 4 pooled samples. Samples were run using the Mouse Whole-Genome (WG)-6 v2.0 Expression BeadChip Kit (Illumina, San Diego CA, USA). Quality control was assessed using the Lumi package [54], run on R (https://www.r-project.org/). Differential gene expression was analysed using Limma [55].

RNA-SEQUENCING (RNA-SEQ)

CD4$^+$ T cells isolated from the PBMCs of 9 symptomatic VL patients (8 males, 1 females) at days 0 and 30 were homogenised in QIAshredder columns prior to RNA extraction using the RNeasy Mini Kit (both from QIAGEN®) according to manufacturer's instructions. Isolated RNA was treated with the RNase-free DNase Set (QIAGEN®). mRNA was isolated using the NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs Inc., Ipswich MA, USA). Libraries were prepared using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs Inc., Ipswich MA, USA). Libraries were quantified using the KAPA Library

Quantification Kit (Roche Sequencing, Pleasanton CA, USA) and RNA integrity number (RIN) obtained using the RNA 6000 Pico Kit (Agilent Technologies, Santa Clara CA, USA). Expression profiling was performed by 50 bp single-end mRNA-sequencing with a read-depth of ~10 M reads, on the Illumina HiSeq platform (performed by the Australian Genome Research Facility (AGRF), Parkville VIC, Australia).

REAL TIME QUANTITATIVE POLYMERASE CHAIN REACTION (RT-qPCR)

Cells sorted from naive and infected mice were stored in RLT buffer and homogenised in QIAshredder columns (both from QIAGEN®). RNA was extracted using the RNeasy Mini Kit (QIAGEN®) according to the manufacturer's instructions. The concentration of RNA (ng/µl) and sample purity (260/280 ratio) was measured using the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). Extracted RNA was reverse transcribed to complementary DNA (cDNA) using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems®) as per manufacturer's instructions.

QuantiTect® Primer Assays (specific for *M. musculus B2m*, *Hprt*, *Nkg7*; QIAGEN®) were used with the GoTaq® qPCR Master Mix (Promega Corporation), and Taqman® Gene Expression Assays (specific for *M. musculus Ifng, Tnf, Hprt, Pml*; Life Technologies) were performed with GoTaq® Probe qPCR Master Mix (Promega Corporation) based on the standard cycling conditions recommended by the manufacturer. Reactions were performed in a final volume of 10 µl containing 1 µl of template cDNA. RT-qPCR was performed in Hard-Shell® 384-Well Plates, thin wall, skirted, clear/clear (Bio-Rad, Hercules CA, USA), sealed with Microseal® 'B' PCR Plate Sealing Film, adhesive, optical (Bio-Rad). QuantiTect® Primer Assays were run on the QuantStudio 5 Real-Time PCR System (Applied Biosystems®) and Taqman® Gene Expression Assays were performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Relative quantification was performed using the comparative C_T method relative to the average of two internal control genes: *B2m* and *Hprt* (for QuantiTect® Primer Assays) and *Pml* and *Hprt* (for Taqman® Gene Expression Assays).

For human samples, $CD4^+$ T cells were enriched by MACS using the anti-human CD4 MicroBeads (Miltenyi Biotec) according to manufacturer's instructions. RNA was then extracted, and reverse transcribed to cDNA as previously described [10]. RT-qPCR for *NKG7* was performed on an ABI Prism® 7500 real-time PCR system (Applied Biosystems®) using the TaqMan® Gene Expression Assay (Assay ID: Hs01120688_g1; Applied Biosystems®). Relative

quantification was performed using the comparative C_T method [56] relative to 18S ribosomal RNA (rRNA) (Assay ID: Hs99999901_s1; Applied Biosystems®).

IN VITRO POLARIZATION OF NAIVE CD4$^+$ T CELLS

Splenic mononuclear cells (MNC) suspensions were stained with 30 µl of a master mix containing Zombie Aqua and TruStain fcX anti-mouse CD16/32 (both from BioLegend) for 15 minutes at room temperature. Cells were washed once in DPBS (Gibco) by centrifuging at 575 *g* for 1 minute at RT, and stained in 30 µl of master mix containing monoclonal anti-mouse CD90.2 (PerCP-Cy5.5, 53-2.1), CD4 (Brilliant Violet 605, GK1.5 or RM4-5), CD25 (PE-Cy7, PC61), CD44 (Alexa Fluor 700, IM7), and CD62L (PE, MEL-14) (all from BioLegend) for 30 minutes at RT. After two washes, cells were resuspended in staining buffer without sodium azide (1x PBS, 0.02% (v/v) FCS, 5 mM EDTA) and naive cells were purified by fluorescence activated cell sorting (FACS) on the BD FACSARIA III (Becton Dickinson). Naive CD4$^+$ T cells were identified as Zombie Aqua$^-$, CD90.2$^+$, CD4$^+$, CD25$^-$, CD44$^-$, CD62L$^+$.

One hundred µl of Ultra-LEAF™ Purified α-mouse CD3ε (clone:145-2C11; BioLegend; 4 µg/ml, diluted in DPBS (1x) (Gibco™)) monoclonal antibody (mAb) was added to each well of a 96-well U bottom plate and incubated either for 2 h in a 37°C incubator or overnight at 4°C to coat the wells. Following this, the purified anti-mouse CD3ε mAb was discarded and 100 µl of 2x polarization cocktails (Table 2, previously described in [22]) prepared in mouse T cell media (10% (v/v) FCS, 1x MEM Non-essential solution (NEAA), 100 U/ml penicillin and 100 µg/ml streptomycin (penicillin–streptomycin) (all from Gibco™), and 0.05 µM 2-mercaptoethanol (Sigma-Aldrich®), in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L D-Glucose, L-Glutamine, and 110 mg/L (1 mM) Sodium Pyruvate (Gibco™)) were added to the respective wells. Two hundred thousand naive CD4$^+$ T cells were seeded per well, in duplicate, and left to incubate at 37°C in 5% CO_2 for 72 h.

After 72 h, 50 µl of supernatant was collected and stored at -20°C for cytokine analysis by cytometric bead array. Cells in duplicate wells were pooled for flow cytometry staining.

CYTOTOXICITY ASSAYS

In vitro killing assays were performed as previously described [52]. Briefly, NK cells were isolated from the spleen using the NK Cell Isolation Kit II (Miltenyi Biotec). NK cells that were freshly isolated or stimulated with recombinant IL-2 (1,000 U/ml) for 4–5 days were used as effector

cells. YAC-1 target tumor cells (provided by Joseph Trapani, Peter MacCallum Cancer Centre, Melbourne, Australia) were labelled with 5 µM CTV (Thermo Fisher Scientific). 1 x 10^4 YAC-1 target cells were co-cultured with effector NK cells at the indicated effector-to-target (E:T) ratios for 4 hours. Tumor cell death in CTV^+ cells was determined using Annexin V/7-AAD in Annexin V Binding Buffer (BD Biosciences).

For $CD8^+$ T cell *in vitro* killing assays, 96-well U-bottom plates were coated with 5 µg/ml [57] Ultra-LEAF™ Purified α-mouse CD3ε (clone: 145-2C11; BioLegend; diluted in DPBS (1x) (Gibco™)) mAb in 100 µl per well by incubating at 37°C for 2 hours. Effector $CD8^+$ T cells were purified from PbT-I^{WT} and PbT-$I^{\Delta Nkg7}$ splenocytes by MACS using the $CD8a^+$ T Cell Isolation Kit, mouse (Miltenyi Biotec) according to manufacturer's instructions. Effector cells were cultured in the presence of plate-bound α-CD3ε mAb, 2 µg/ml LEAF™ Purified α-mouse CD28 (clone: 37.51; BioLegend) mAb and 20 ng/ml rmIL-2 (BioLegend) in T cell media (10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (penicillin–streptomycin), 1x Glutamax, 1x MEM Non-essential solution (NEAA), 1mM sodium pyruvate, 1x D-glucose (all from Gibco™), 5mM HEPES, and 0.05 µM 2-mercaptoethanol (Sigma-Aldrich®), in RPMI 1640 (Gibco™) for 48 hours. Splenocytes prepared from B6.*Cd45.1* mice were pulsed with 1 µM NVF peptide (sequence: NVFDFNNL) [31] by incubating in a water bath at 37°C for 1 hour. NVF-pulsed cells were labelled with 1 µM CTV (BD Horizon) by incubating in a water bath at 37°C for 15 minutes. CTV-labelled NVF-pulsed target cells were counted and combined in equal parts with control target cells (non-peptide-pulsed, no CTV-labelling). 2 x 10^5 activated effector $CD8^+$ T cells were co-cultured with target cells at the indicated E:T ratios for 8 hours. Cells were stained with TruStain FcX™ (anti-mouse CD16/32 mAb) (clone: 93; BioLegend) and Zombie NIR™ Fixable Viability Kit (BioLegend), prior to staining with BUV395 Mouse anti-Mouse CD45.2 (clone: 104; BD Horizon™) mAb to distinguish effector cells ($CD45.2^+$) from target cells ($CD45.2^-$). Cell viability was assessed by positive staining for Zombie NIR™. The frequency of target cell killing was determined using the following formula:

$$100 - \left(100 \times \frac{\left(\frac{CTV^+NVF^+}{CTV^-NVF^-}\right)_{with\ effector\ cells}}{\left(\frac{CTV^+NVF^+}{CTV^-NVF^-}\right)_{without\ effector\ cells}} \right)$$

To assess NK cell-mediated cytotoxicity *in vivo*, 5 x 10^6 RMA-s-Rae1β cells [52] were injected i.v. via the tail vein. Lungs were harvested 2 hours after tumor challenge, followed by digestion using collagenase IV (1 mg/ml, Worthington Biochemical). Numbers of RMA-s-Rae1β cells (Rae-1^+ H-$2Kb^-$ cells) in lung single-cell suspensions were determined by flow cytometry. Biotin H-$2K^b$ (AF6-88.5) was detected using 1 µg/ml APC-conjugated streptavidin (both BD Biosciences).

RETROVIRAL TRANSDUCTION OF PRIMARY MOUSE CD8$^+$ T CELLS

Codon-optimized NKG7 cDNA (NCBI Reference Sequence: NM_024253.4) was synthesized by Integrated DNA Technologies (Coralville, IA). eGFP gene was PCR amplified from MSCV.IRES.eGFP (Addgene plasmid # 20672; gift from Tannishtha Reya, University of California, San Diego, CA), with introduction of a linker sequence in the N-terminus. The final insert encodes an NKG7-eGFP fusion protein with NKG7 in N-terminus, fused to eGFP at the C-terminus via a (T)GGGGS linker, with the Threonine introduced by a restriction site during the cloning process. The insert was cloned into an MSCV backbone (Addgene plasmid #52114; gift from Dario Vignali, University of Pittsburgh, PA, USA) and verified by Sanger sequencing at Australian Genome Research Facility (Brisbane, Australia).

Replication incompetent retroviral particles were generated by transient transfection of HEK293T cells with the packaging plasmid (EcoPak) and the retroviral vector plasmid MSCV.NKG7-GFP. Retrovirus-containing supernatants were harvest at 48 and 72 hours and stored at -80°C until use.

Splenocytes (1 x 10^6) were activated with plate-bound CD3ε (clone 2C11) and CD28 (clone N3751) mAbs for 24 hours in Iscove's Modified Dulbecco's Medium (IMDM) with 10% heat-inactivated fetal bovine serum, 2 mM L-alanyl-L-glutamine dipeptide (GlutaMAX™, Gibco), 1% Non-Essential Amino Acids (NEAA), 1 mM Sodium Pyruvate, 50 mM 2-mercaptoethanol and 100 U/ml Penicillin-Streptomycin. The retroviral supernatant was centrifuged (1500 *g* for 60 mins at 4°C) on a RetroNectin (Takara, Kusatsu, Shiga, Japan) coated plate, and the supernatant removed. The activated splenocytes were incubated (37°C, 5% CO2) with plate-bound retroviral particles for 4 hours in IMDM complete medium with rhIL-2 (Aldesleukin, 100 U/ml) and hexadimethrine bromide 16 µg/ml (Polybrene, Sigma-Aldrich). The splenocytes were then harvested and expanded in the presence of rhIL-2 (100 U/ml) for 5–6 days before downstream application.

IMMUNOFLUORESCENCE (IF) MICROSCOPY

Immunofluorescence (IF) microscopy was performed on 7 µm frozen liver sections. Tissue was fixed in 4% (w/v) PFA for 2 h before overnight incubation in 30% (w/v) sucrose and embedding in Tissue-Tek® Optimal Cutting Temperature (OCT) Compound (VWR Chemicals, Radnor PA, USA). Following cutting, sections were stored frozen until use. Sections were air dried then rehydrated in tris-buffered saline with Tween 20 (TBS-T; 0.1 M TRIS-HCl, 0.15 M NaCl and 0.001% (v/v) Tween20 in MilliQ) and incubated in Background Sniper (Biocare Medical, Pacheco CA, USA) diluted 1/10 in TBS-T, for 30 min. Following this, sections were incubated in 5µg/ml Alexa Fluor 647-conjugated anti-mouse CD4 (RM4-5, BioLegend) mAb diluted in Van Gogh Yellow Diluent (Biocare Medical) for 1 hr at RT. Sections were washed in TBS-T after each incubation step. Sections were then incubated in DAPI (Sigma-Aldrich) diluted 1/20,000 in PBS for 10 min at RT. Finally, sections were mounted in ProLong™ Gold Antifade (Invitrogen™) under a glass cover slip.

IF microscopy was also performed on cell suspensions following retroviral transduction. C57BL/6N transduced splenocytes were incubated with LysoTracker™ Red DND-99 (Life Technologies) diluted to 75 nM in 5% FCS.PBS for 30 min at 37°C before imaging. PbT-I transduced cells were incubated with 5 µg/ml Alexa Fluor 647-conjugated anti-mouse CD107a/LAMP-1 (1D4B, BioLegend) mAb diluted in 200 µl re-stimulation media (containing 25 ng/ml phorbol 12-myristate 13-acetate, 2 µg/ml ionomycin calcium salt and 4 µM monensin in complete RPMI) for 2 hr at 37°C before imaging. Images were acquired on either a Zeiss 780-NLO laser-scanning confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) or a ScanScope FL slide scanner (Aperio, Leica Biosystems, Wetzlar, Germany).

Quantitative data was analysed automatically using QuPath, v0.1.2 (University of Edinburgh, Edinburgh, Scotland) with $CD4^+$ $NKG7^+$ cells counted and expressed per mm^2 of area. Regions of immune cell accumulation around infected Kupffer cells (inflammatory foci) in the livers of *L. donovani*-infected mice at day 28 post-infection were defined and the number of $NKG7^+$ $CD4^+$ T cells in 21 defined areas (average size = 0.017 mm^2) within inflammatory foci and 21 areas (average size = 0.024 mm^2) outside these zones for each infected mouse liver were measured using the above software. All cells were manually checked for accuracy before data was plotted and analysed in Prism (GraphPad).

CYTOMETRIC BEAD ARRAY (CBA)

Cytokine levels were assessed using the BD Cytometric Bead Array (CBA) Mouse Inflammation Kit or Mouse $T_H1/T_H2/T_H17$ Cytokine Kit (BD Biosciences) as per manufacturer's instructions. Serum or plasma samples from mouse blood was used neat while cell culture supernatants were diluted 1:5 in PBS for the detection of most cytokines. Supernatants were diluted 1:50 in PBS for the detection of IFN-γ. CBA data was analyzed using the FCAP Array Software v3.0 (BD Biosciences).

$CD4^+$ T CELL ADOPTIVE TRANSFER

$CD4^+$ T cells were purified by MACS from the spleens of either C57BL/6N or B6N.*Nkg7*$^{-/-}$ donor mice using the $CD4^+$ T Cell Isolation Kit, mouse (Miltenyi Biotec) according to manufacturer's instructions. 1 x 10^6 $CD4^+$ T cells were injected i.v. into each *Rag1*$^{-/-}$ host mouse the day prior to infection.

PbT-I CELL ADOPTIVE TRANSFER

$CD8^+$ T cells were isolated from the spleens of PbT-I^{WT} and PbT-$I^{\Delta Nkg7}$ mice using the CD8a+ T Cell Isolation Kit, mouse (Miltenyi Biotec) according to manufacturer's instructions. Single cell suspensions were prepared as described above. PbT-I^{WT} and PbT-$I^{\Delta Nkg7}$ cells were counted and combined in equal proportions at a final concentration of 5 x 10^6 cells/ml. 200 µl (1 x 10^6 cells) was subsequently injected i.v. into the tail vein of B6.*Cd45.1* recipient mice one day prior to infection with *Pb*A. The proportion of PbT-I^{WT} ($CD45.1^+$ $CD45.2^+$; refer to section on 'Mice') to PbT-$I^{\Delta Nkg7}$ ($CD45.1^-$ $CD45.2^+$) cells in the single cell suspension was verified by flow cytometry using anti-mouse CD45.1 (Alexa Fluor 700, A20; BioLegend) and anti-mouse CD45.2 (BUV395, 104; BD Biosciences).

TUMOR MODELS

Antibodies

Purified control antibodies (cIg) (hamster cIg or 1-1) or antibodies to deplete CD8 T cells (53.5.8) were purchased from BioXcell (West Lebanon, NH, USA). Anti-asialoGM1 (asGM1) was purchased from Wako Pure Chemicals, Japan. Antibody to neutralize IFN-γ (H22) was purchased from Leinco Technologies (St Louis, MO, USA).

Cell Lines

Mouse B16F10 melanoma, RM-1 prostate carcinoma, and E0771 mammary carcinoma cells [58] were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) FCS (Bovogen), 1% glutamine (Gibco), 1% HEPES (Gibco) and 1% penicillin/streptomycin (Gibco). LWT1 melanoma cells were cultured in RPMI 1640, supplemented with 10% FCS, 1% glutamine, and 1% penicillin-streptomycin. The YAC1, RMA-s and RMA-s-Rae-1β lymphoma cell lines [59] were cultured in complete RPMI 1640 media containing 10% FCS, 1% glutamine, and 1% penicillin-streptomycin, 2 mM glutamax, 55 μM 2-mercaptoethanol, HEPES, and sodium pyruvate. Cell lines were maintained at 37°C, 5% CO_2. All cell lines tested mycoplasma negative.

Experimental metastasis

Mice were injected i.v. with either 1 x 10^5 B16F10 melanoma cells (ATCC, 2007), 5 x 10^5 LWT1 melanoma cells [60] (derived in house), or 2 x 10^5 RM-1 prostate carcinoma cells, as previously described [60, 61, 62]. Some groups of mice were treated i.p. with either: cIg (100 μg); anti-CD8β (53.5.8, 100 μg), anti-asGM1 (50 μg) or anti-mIFN-γ (H22, 250 μg) on days -1, 0, and 7, relative to tumor inoculation. For NK cell-based cytokine immunotherapy, some mice were treated i.p. with PBS or IL-15 (0.5 μg)/IL-15Ra (3 μg) complexes (R&D Systems) on days 0 and 3 after tumor inoculation, or PBS or IL-2 (100,000 IU) on days 0, 1, 2, and 3 relative to tumor inoculation. Lungs were harvested from mice injected with B16F10 cells at day 14 post-injection. Lungs were harvested from mice injected with LWT1 cells at day 14 post-injection and perfused with India ink. Metastatic burden in all three models were quantified by counting colonies on the lung surface under a light microscope.

NK cell adoptive transfer

Groups of *Rag2*$^{-/-}$γc$^{-/-}$ mice were injected intravenously (i.v.) with 2 x 10^5 WT or *Nkg7*$^{-/-}$ NK cells isolated by fluorescence-activated cell sorting (FACS). Six days later, naive or adoptively-transferred *Rag2*$^{-/-}$γc$^{-/-}$mice received either 1 x 10^5 or 1 x 10^4 B16F10 tumor cells. Lungs were harvested from mice injected with B16F10 cells at day 14 post-injection. As above, metastatic burden was quantified by counting colonies on the lung surface under a light microscope.

Spontaneous metastasis

For spontaneous metastases, 1 x 10^5 E0771 mammary carcinoma cells (provided by Robin Anderson, Peter MacCallum Cancer Centre) were injected into the fourth mammary gland of

C57BL/6N (B6N; wild-type) or B6N.*Nkg7*-KO mice. Twelve days after tumor inoculation, the primary tumors were equivalent in size between WT and *Nkg7*-deficient mice (mean 15.8 ± 1.6 mm^2 versus 17.7 ± 1.6 mm^2, respectively), and the primary tumors were surgically resected. Mice were then sacrificed at day 35 and lungs harvested and the metastatic burden was quantified by counting colonies on the lung surface under a light microscope.

Intraperitoneal RMA-s lymphoma model

C57BL/6N (B6N; wild-type) or B6N.*Nkg7*-KO mice were injected i.p. with 1 x 10^5 RMA-s lymphoma cells (provided by John Ortaldo, NCI-Frederick Cancer Research Facility, Frederick MD, USA). Percent survival of each group was monitored for 150 days as previously described [36].

Subcutaneous RMA-s-Rae1β lymphoma model

C57BL/6N (B6N; wild-type) or B6N.*Nkg7*-KO mice were injected s.c. with 5 x 10^6 RMA-s-Rae1β lymphoma cells as previously described [52]. The tumor growth was measured every 2 to 3 days with a caliper square as the product of 2 perpendicular diameters (mm^2). Some groups of mice were treated with either: cIg (100 µg); anti-CD8β (100 µg), or anti-asGM1 (50 µg) on days -1, 0, 7, 14, and 20, relative to tumor inoculation.

MCA carcinogenesis

The MCA-induced fibrosarcoma model was performed as previously described [37]. Briefly, C57BL/6N (B6N; wild-type) or B6N.*Nkg7*-KO mice were injected subcutaneously with 100 µl of corn oil containing 25 µg MCA on the right-hand flank. Development of fibrosarcomas was monitored weekly over the course of 250 days. Percent tumor free mice were recorded.

QUANTIFICATION AND STATISTICAL ANALYSIS STATISTICAL ANALYSES

Statistical analyses were performed using Prism 7 (Version 7.0c; GraphPad Software). *p* values are shown as *, **, ***, and ****, which represented $p < 0.05$, 0.01, 0.001, and 0.0001 respectively. Where possible, the D'Agostino–Pearson omnibus normality test was used to test for normal distribution. Non-parametricity was assumed in cases where this was not possible. Statistical testing was performed using either the Kruskal–Wallis test and Dunn's multiple comparisons test (single variable data), or a regular two-way analysis of variance (ANOVA) with multiple comparisons (two-variable data), unless stated otherwise.

MODELLING THE NKG7 TERTIARY PROTEIN STRUCTURE

The NKG7 structure was modelled using I-TASSER protein structure and function prediction software [63]. The protein structure which was the highest ranked threading template used in the modelling of the human and mouse sequences was the mouse claudin-19 in complex with the C-terminal fragment of *Clostribium perfringens* enterotoxin (PDB ID code 3X29). The models with the highest C-scores were chosen for display, and corresponded to values of 0.15 and 0.51 for the human and mouse proteins respectively. The C-score represents the confidence in the model; values are typically in the range -5 to 2, and higher values correspond to models with higher confidence.

T-DISTRIBUTED STOCHASTIC NEIGHBOUR EMBEDDING (tSNE)

tSNE was performed as outlined in a protocol and R script written by T. Ashhurst (https://github.com/sydneycytometry/tSNEplots/releases). Briefly, cells were gated on lymphocytes, followed by exclusion of doublets and dead cells using FlowJo v10 OSX (FlowJo, LLC). Each sample was assigned a sample number and the live cell population down-sampled to 15,000 events on FlowJo v10 OSX, prior to concatenation into a single file. tSNE was performed using the lineage markers: CD11b, B220, NK1.1, TCRβ, CD4, I-A/I-E (MHCII), Ly-6C, CD11c, and CD8, with the following settings applied:

- Iteration: 2000
- Perplexity: 35
- Eta (learning rate): 200
- Theta: 0.5

A second tSNE was performed only on NK cells and T cells by excluding $NK1.1^{-}$ $TCR\beta^{-}$ cells from live cells. Biexponential scales were then applied for all fluorophores of interest. Scales were adjusted by increasing the width basis to reduce the spread of negative data. The gated population for each sample was assigned a sample number and down-sampled to 50,000 events on FlowJo v10 OSX. Down-sampled populations from all samples were then concatenated into a single file. NK1.1, TCRβ, CD4, and CD8 were selected as tSNE parameters and the same settings as above, were applied.

Each sample within the concatenated file was distinguished on the basis of the previously assigned sample number. Colourised tSNE plots were generated in R using exported channel values as an input to the script (v1.4) written by T Ashhurst (https://github.com/sydneycytometry/tSNEplots/releases).

RNA-SEQ ANALYSIS

Quality control was performed using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were mapped to the reference genome (GRCh38.p3), and assigned to transcripts (Ensembl v82) using Spliced Transcripts Alignment to a Reference (STAR) [64].

EdgeR run on R [65] was used to generate a list of differentially-expressed genes from the RNA-seq dataset consisting of n = 11 paired-samples. Briefly, the transcript with the highest read count for each gene was retained. Further filtering was performed by the elimination of genes with < 1 count-per-million (cpm) in more than 2/3rd of samples. The effective library size was determined using the default trimmed mean of M-value (TMM) normalization based on which, counts were normalized. Data exploration by hierarchical clustering revealed 2 failed biological samples that were removed with their corresponding pairs, resulting in n = 9 biological replicates. Testing for differential expression was performed using gene counts fitted to a negative binomial generalized log-linear model. Genes with a false discovery rate (FDR) < 0.05 were considered to be differentially-expressed, unless stated otherwise.

k-means clustering was performed using the eclust function within the factoextra package (https://rpkgs.datanovia.com/factoextra/) with the following parameters: FUNcluster = "kmeans", k.max = 10, nstart = 25, and nboot = 100.

Upstream regulators for genes of interest were identified using Ingenuity Pathway Analysis (IPA; QIAGEN®). In addition to drugs that were predicted on IPA, genes of interest were submitted to the Drug-Gene Interaction Database (DGIdb) [66] to identify currently available drugs. The International Mouse Strain Resource (IMSR) [67] was used in search of transgenic or knockout mouse strains that are available for each target gene. Localization of target genes were determined by functional annotation based on data from Gene Ontology: Cellular Component [68] using the Database for Annotation, Visualization and Integrated Discovery (DAVID; v6.8 Beta, National Institute of Allergy and Infectious Diseases (NIAID), National

Institutes of Health (NIH), Maryland, U.S.A) [69]. Graphs were plotted using Prism 7 (Version 7.0c; GraphPad Software).

ANALYSIS OF THE CANCER GENOME ATLAS (TCGA) DATA

Skin cutaneous melanoma (SKCM) RNA-seq data previously published by TCGA Research Network (https://cancergenome.nih.gov/) was downloaded and processed using the TCGAbiolinks package [70] on R [65]. Briefly, transcript counts were normalized by gene length and genes in the bottom quartile (<25%) of expression mean across all samples were filtered out. Samples were ranked according to expression values for the gene of interest and stratified into two groups where the uppermost quartile (>75%) were designated high expressers, and the lowermost quartile (<25%) were designated low expressers. Overall survival between these groups was analyzed and plotted as Kaplan–Meier curves with *p* values determined using a log-rank test. Correlation analyses were then performed between the 2 genes of interests and the relationship expressed as *r*.

NKG7 was excluded from a 20-gene signature for NK cells recently published [35]. TCGA:SKCM samples were ranked based on *NKG7* expression and correlated against a moving average of the remaining 19 genes–collectively termed the "NK cell signature".

INGENUITY PATHWAY ANALYSIS (IPA)

Gene symbols, $\log_2$ fold-change (logFC), and false discovery rate (FDR) from each data set was used as an input into IPA (version 43605602; QIAGEN®). Where there was a concatenation of genes that overlapped between two or more datasets, the logFC and FDR values for the human $CD4^+$ T cell dataset was used. Initial interrogation of each dataset was performed using default values and parameters set on IPA. In contrast, examination of the pathways involved in the core, chronic, and resolving gene signatures were restricted to molecules and/or relationships experimentally observed in *H. sapiens* or *M. musculus* and specifically in immune cells.

DATA AVAILABILITY

The materials, data and any associated protocols that support the findings of this study are available from the corresponding author upon request. The RNA-seq and microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) with the accession numbers GSE135965 (human RNA-

seq data) and GSE135857 (mouse microarray data). The list of differentially expressed genes in $CD4^+$ T cells isolated from PBMCs of visceral leishmaniasis patients is available within Supplementary Table 1. Supplementary Table 2 contains the list of differentially-expressed genes in liver $CD4^+$ T cells of *L. donovani*-infected mice, and supplementary Table 3 contains the list of differentially-expressed genes in splenic $CD4^+$ T cells of *L. donovani*-infected mice.

Acknowledgements

We thank the staff at the Kala-Azar Medical Research Centre (KAMRC), Muzaffarpur, Bihar, India for help in collecting blood samples, as well as patients and volunteers for allowing the use of blood samples. We thank staff in the QIMR Berghofer flow cytometry laboratory for assistance and staff in the QIMR Berghofer animal facility for animal husbandry. We acknowledge the facilities, and the scientific and technical assistance of the Melbourne Advanced Genome Editing Centre (MAGEC), Walter and Eliza Hall Medical Research Institute. The MAGEC is supported by the Australian Phenomics Network (APN) and the APN is supported by the Australian government through the National Collaborative Research Infrastructure Strategy program. We thank the NIH tetramer facility (Atlanta, GA) for production of the I-A^b-$PEPCK_{335-351}$ tetramer used to detect *L. donovani* PEPCK-specific $CD4^+$ T cells in these studies. This work was made possible through Queensland State Government funding and grants and fellowships from the National Health and Medical Research Council of Australia (NHMRC; grant numbers 1037304, 1058685, 1078671, 1132519, 1132975 and 1154265). Funding was also provided through a National Institute's of Health Tropical Medicine Research Centre (TMRC) grant (U19 AI074321), as well as Australian Post-graduate Awards through Griffith University, Institute of Glycomics and School of Natural Sciences to Patrick Bunn and Susanna Ng, respectively, a Dr Mildred Scheel Stiftung fuer Krebsforschung scholarship from Deutsche Krebshilfe to Matthias Braun, and an INSPIRE Faculty grant (LSBM-109/IF-14) provided by the Indian government Department of Science and Technology (DST) and Banaras Hindu University and University Grants Commission (M-14-70) to Rajiv Kumar. Bhavana Singh was supported by a Junior Research Fellowships from the Indian Council of Medical Research.

Author contributions

S.S.N. and C.R.E. conceived and designed the study with input from M.J.S., S.S., R.Kum., G.R.H., K.N., S-K.T., T.B., M.D-E., M.W.L.T., A.G.B., W.C.D. and A.H., and led and coordinated the study with M.J.S. and S.S.. S.S.N. and C.R.E. co-wrote the manuscript together with M.J.S. and G.R.H.. S.S.N. performed the bioinformatics analysis with D.C., S.W. and N.C.. S.B.C., B.S., S.S.S., O.P.S. and R.Kum. collected and processed VL patient samples under the coordination of S.S.. I.D., P.Z. and R.Kun. performed early experiments in inflammatory models, F.D.L.R., T.C.M.F., E.M., J.N., J.A.E., M.S.F.S., M.M.d.O., P.T.B., Y.W., F.H.A. and C.L.E.

performed all experimental malaria and VL experiments, while J.Y., J.H., X-Y.L., A.R.A., M.C., M.B., K.N. and M.J.S. performed all cancer model experiments. A.J.K. and M.J.H. generated the C57BL/6J-*Nkg7*$^{em1(cre)WEHI}$ mouse, A.L. and N.L.D. performed modelling of the NKG7 tertiary protein structure, B.A.M., S-K.T. and T.C.M.F. performed all retrovirus transductions and confocal microscopy, J.U. developed the PEPCK tetramer and provided advice on its' use, while N.G. and W.R.H. produced the *Plasmodium* peptide MHCI tetramer and helped design PbT-I cell killing assays. W.C.D., A.G.B. and M.D-E. provided important discussions for the project and critical feedback on the manuscript. All co-authors, read, reviewed and approved the manuscript.

Competing interests

The authors declare no competing interests.

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Figure Legends

Figure 1 *NKG7* is highly upregulated in the splenic CD4$^+$ T cells during *Leishmania donovani* infection.

(A) Up-regulated genes found in CD4$^+$ T cells isolated from mouse spleen at day 56 p.i., and human peripheral blood mononuclear cells (PBMCs) from visceral leishmaniasis (VL) patients at the time of admission to clinic for treatment.

(B) Cellular location of proteins encoded by the up-regulated genes in (A) are indicated on the cellular map using information obtained from the Gene Ontology: Cellular Component knowledgebase.

(C) The protein structure of NKG7 generated using Iterative Threading ASSEmbly refinement (I-TASSER). Predicted extracellular loops (indicated by the black arrows) of human and mouse NKG7 are highlighted in green and purple, respectively.

(D) Validation of *NKG7* up-regulation in VL patients before treatment (D0; *n* = 14) compared to the same patients after treatment (D30) and endemic controls (EC, *n* = 14) by real-time quantitative polymerase chain reaction (RT-qPCR). RT-qPCR validation was also performed in conventional T cells (T_{conv}) and regulatory T cells (T_{reg}) from the spleen and liver of naive and infected (day 56 p.i.) mice. Statistical significance was determined using a one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test (left) or two-way ANOVA with Sidak's multiple comparisons test (right). Centre line in data sets indicates the median, box limits indicate the upper and lower quartiles, and the whiskers indicate the maximum and minimum measures.

Figure 2 *Nkg7* expression is enriched in natural killer (NK) cells at steady state and inducible in CD4$^+$ T cells.

(A) A mouse expressing the *Cre* gene behind the promoter of *Nkg7* was generated (B6.*Nkg7*-cre) and crossed to a membrane reporter (B6.mT/mG) to generate *Nkg7* reporter mice (*Nkg7*-cre x mT/mG). Validation of green fluorescent protein (GFP) expression was performed on splenic NK cells in naive *Cre*$^+$ mice.

(B) t-Distributed Stochastic Neighbour Embedding (t-SNE) plot of splenocytes from a naive mouse, pre-gated to exclude doublets, dead cells, and NK1.1-APC/Cy7$^-$ TCRβ-BUV737$^-$ cells.

The remaining cells were clustered using NK1.1-APC/Cy7, TCRβ-BUV737, CD4-BUV395, and CD8α-PE/Cy7. Equal numbers of cells (50,000 cells) are shown for Cre^- and Cre^+ plots. *n* = 1 per genotype, performed once.

(C) The expression of *Nkg7* (GFP^+) under T_H0 (α-CD3 + α-CD28 + rIL-2), T_H1 (rIL-12 + α-IL-4 + T_H0 conditions), T_R1 (rIL-27 + T_H0 conditions), T_{reg} (rIL-27 + rTGF-β + T_H0 conditions), T_H2 (rIL-4 + α-IFN-γ + T_H0 conditions), and T_H17 (rIL-6 + rIL-1β + rIL-23 + α-IFN-γ + α-IL-4 + T_H0 conditions) cell polarising conditions. *n* = 1 per genotype, plots are representative of two independent experiments.

(D) The expression of *Nkg7* (GFP^+) when rIL-27 was titrated, or when rTGF-β was titrated in the presence of rIL-27. *n* = 1 per genotype, performed once.

T_H, T helper; r, recombinant; IL, interleukin; T_R1 cells, type 1 regulatory T cells; T_{reg} cells, regulatory T cells; TGF-β, transforming growth factor β; IFN-γ, interferon γ.

See also Extended Data Fig. 1.

Figure 3 *Nkg7* is expressed by mouse spleen and liver $CD4^+$ T cells during *Leishmania donovani* infection.

(A) *Nkg7* reporter mice were infected with *L. donovani* and the overall expression of GFP was assessed in the liver and spleen prior to infection (naive) and at days 14, 28, and 58 post-infection (p.i.). The size of the pie charts and the associated frequency represents the relative percentage of GFP^+ cells in the liver (above timeline) and spleen (below timeline). Within these pie charts, each slice represents the proportion of the indicated immune cell subset that made up the GFP^+ population. Measurements were taken from distinct samples at each timepoint. The data shown is representative of two independent experiments, each consisting of *n* = 3 mice per genotype, per timepoint. cDCs, conventional dendritic cells; pDCs, plasmacytoid dendritic cells; NKT, natural killer T.

(B) Confocal immunofluorescence (IF) microscopy was used to determine the tissue localisation of *Nkg7*-expressing (GFP^+) cells in the liver of mice at day 28 p.i.. The co-localisation of *Nkg7* and CD4 expression is shown in the merged image. Scale bar, 50 µm. The number of $CD4^+$ $NKG7^+$ cells within inflammatory foci (granulomas), relative to surrounding tissue are shown in the accompanying graph. Statistical significance was determined using the Mann–Whitney test.

See also Extended Data Fig. 2.

Figure 4 *Nkg7*-deficiency promotes elevated parasite burdens during *Leishmania donovani* infection.

(A) Wild-type (WT) and *Nkg7*$^{-/-}$ mice were infected with *L. donovani* and parasite burdens were measured in the liver and spleen at days 14, 28, and 56 post-infection (p.i.). Statistical testing performed using a two-way analysis of variance (ANOVA) with Sidak's multiple comparisons test. LDU, Leishman–Donovan Units.

(B) Quantification of serum pro-inflammatory cytokines in infected mice. Statistical testing performed using a two-way ANOVA with Sidak's multiple comparisons test.

(C) The total number of leukocytes in the liver and spleen at day 14 p.i. Statistical testing performed using the Mann–Whitney test.

(D) Day 14 p.i. liver parasite burdens in *Rag1*$^{-/-}$ mice that received CD4^{+} T cells isolated from either WT (n = 5) or *Nkg7*$^{-/-}$ (n = 5) mice. Statistical testing performed using the Mann–Whitney test.

(E) The graphs show the frequency and total number of T_H1 cells (gated on lymphocytes, singlets, live cells, NK1.1-APC/Cy7^{-} TCRβ-BUV737^{+}, CD4-BUV395^{+} CD8α-Alexa Fluor 700^{-}, Foxp3-Alexa Fluor 488^{-}, IFN-γ-APC^{+} IL-10-PE^{-} cells) in the liver of naive mice and infected mice at day 14 p.i. Statistical testing performed using the two way ANOVA with Sidak's multiple comparisons test. APC, Allophycocyanin; Cy, Cyanine.

(F) Representative flow cytometry plots were gated on lymphocytes, singlets, live cells, NK1.1-APC/Cy7^{-} TCRβ-BUV737^{+}, CD4-BUV395^{+} CD8α-Alexa Fluor 700^{-} cells. The graphs show the frequency and total number of CD11a^{+} CD49d^{+} CD4^{+} T cells in the liver at day 14 p.i. Statistical testing performed using the Mann–Whitney test. FITC, fluorescein isothiocyanate; PE, Phycoerythrin.

(G) The graphs show the frequency and median fluorescence index (MFI) of phosphorylated (p)-STAT4 within CD4^{+} TCRβ^{+} (polyclonal) and I-A^{b}PEPCK$_{335\text{-}351}$ (tetramer)-PE^{+} cells. Statistical significance was determined using the Mann–Whitney test. Representative plots show the expression of p-STAT4 upon treatment with rIL-12. n = 5 WT and 6 *Nkg7*$^{-/-}$ mice. Data is representative of two independent experiments.

p values are indicated where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Error bars represent mean ± standard error of mean (SEM). The data shown in A–C and E–F is representative of two independent experiments, each with $n = 3$ naive WT and *Nkg7*$^{-/-}$ mice, and $n = 5$ WT and 4 *Nkg7*$^{-/-}$ mice at days 14, 28 and 58 p.i.. Measurements in A and B were obtained from distinct samples at each timepoint.

See also Extended Data Fig. 3.

Figure 5 *Nkg7* promotes parasite accumulation in tissues and the onset of experimental cerebral malaria following *Plasmodium berghei* ANKA (*Pb*A) infection.

WT and *Nkg7*$^{-/-}$ mice were infected with *P. berghei* ANKA, which causes experimental cerebral malaria (ECM).

(A) ECM scores obtained as repeated measures at each timepoint.

(B) Survival analysis between WT and *Nkg7*$^{-/-}$ mice. Statistical testing performed using the log-rank (Mantel–Cox) test.

(C) Levels of parasitised red blood cells (pRBCs) in circulation obtained as repeated measures at each timepoint. Statistical significance was determined using a two-way ANOVA with Sidak's multiple comparisons test.

(D and E) Parasite biomass was quantified using luciferase-expressing *Pb*A (*Pb*A-luc) parasites, in the body (D) and brains (E) of infected mice. $n = 4$ WT and 5 *Nkg7*$^{-/-}$ mice. Statistical significance was determined using the Mann–Whitney test.

(F) The graph shows the frequency of H2-K^{b}-NVF (tetramer)-PE^{+} cells within the brain CD8^{+} T cell population at peak of ECM. Statistical significance was determined using the Mann–Whitney test in one experiment where $n = 3$ mice per group.

(G) The graph depicts the proportion of co-transferred transgenic PbT-I^{WT} and PbT-I$^{\Delta Nkg7}$ cells as a frequency of TCRβ^{+} CD8^{+} cells in the brain of naive and infected mice (at peak ECM).

(H) The graphs show the frequencies of Granzyme B^{+} or Perforin^{+} PbT-I^{WT} and PbT-I$^{\Delta Nkg7}$ cells after incubation with monensin. Statistical significance was determined using a two-way ANOVA with Sidak's multiple comparisons test.

(I) The representative histograms illustrate CD107a (LAMP1) expression by PbT-I^{WT} and PbT-I$^{\Delta Nkg7}$ cells after incubation with monensin or stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, in the presence of monensin. The graphs to the right of histograms present the frequency and MFI of CD107a expression by PbT-I^{WT} and PbT-I$^{\Delta Nkg7}$ cells.

Data in A–E is representative of three independent experiments. *n* = 5 mice per group in A–C. Experiment in G–I was performed once where *n* = 4 naive or 5 infected mice per strain. Error bars represent mean ± SEM. *p* values are indicated, with * and *** representing $p < 0.05$ and 0.001, respectively.

See also Extended Data Fig. 4.

Figure 6 NKG7 co-localises with cytotoxic vesicles expressing CD107a.

(A) The representative images show the co-localisation of NKG7 and LysoTracker in WT splenocytes transduced to expressed NKG7- GFP. Scale bars, 10 µm.

(B) Splenocytes from PbT-I^{WT} T cell transgenic mice were also transduced to express NKG7-GFP. The representative images depict the co-localisation of NKG7 and the cytotoxic granule-associated protein CD107a. Scale bar, 10 µm. AF647, Alexa Fluor 647.

(C) The graph shows a comparison of cytotoxic ability between PbT-I^{WT} and PbT-I$^{\Delta Nkg7}$ transgenic CD8^{+} T cells against NVF peptide-pulsed splenocytes *in vitro*. Data obtained from one experiment. Statistical significance was determined using a two-way ANOVA for effector:target (E:T) ratio and group as variables. Data is representative of two independent experiments.

Figure 7 *Nkg7* deficiency increases metastatic burden.

(A) Survival analysis between individuals in the top and bottom 25% of *NKG7* expressers in The Cancer Genome Atlas (TCGA): Skin Cutaneous Melanoma (SKCM) dataset was performed using a log-rank test.

(B) WT mice and *Nkg7*$^{-/-}$ mice were injected with either B16F10 (*n* = 8 WT and 6 *Nkg7*$^{-/-}$ mice) or LWT1 cells (*n* = 9 WT and 7 *Nkg7*$^{-/-}$ mice). Lung metastatic burdens were quantified at day 14 post-injection. The data shown is representative of two independent experiments. The Mann–Whitney test was used to determine statistical significance.

(C) The lungs of WT and *Nkg7*$^{-/-}$ mice (*n* = 5 mice per group), injected with LWT1 cells, were assessed for differences in the frequency of IFN-γ^{+} or CD107a^{+} NK cells and the MFI of IFN-γ or CD107a on these cells at 14 days post-injection is shown. The data is representative of two independent experiments. The Mann–Whitney test was used to determine statistical significance.

(D) WT and *Nkg7*$^{-/-}$ mice injected with B16F10 cells were treated with isotype control (cIg) (*n* = 27 WT and 26 *Nkg7*$^{-/-}$; pooled from 4 experiments), or antibodies against asialoGM1 (asGM1) (*n* = 10 WT and 11 *Nkg7*$^{-/-}$), CD8β (*n* = 9 WT and 5 *Nkg7*$^{-/-}$), or IFN-γ (*n* = 10 WT and 12 *Nkg7*$^{-/-}$; pooled from two experiments). Lung metastases were quantified at day 14 post-injection of B16F10 cells. A two-way ANOVA with Sidak's multiple comparisons test was used to determine statistical significance between groups.

(E) Differences in lung metastatic burden in *Rag2*$^{-/-}$γc$^{-/-}$ mice that received either WT (*n* = 9) or *Nkg7*$^{-/-}$ (*n* = 11) NK cells, prior to injection with B16F10 cells, are shown. Controls consisted of *Rag2*$^{-/-}$γc$^{-/-}$ mice that did not receive NK cells, but were injected with either 1 x 10^4 (*n* = 9) or 1 x 10^5 (*n* = 13) B16F10 cells. The data shown is representative of two independent experiments. The Mann–Whitney test was used to determine statistical significance.

(F) The waterfall plot shows the top 50 up- or down-regulated genes between high and low *NKG7* expressers from the TCGA:SKCM dataset. logFC, $\log_2$ fold-change.

(G) The top 10 upstream regulator cytokines between high and low *NKG7* expressers from the TCGA:SKCM dataset identified by Ingenuity Pathway Analysis (IPA) is shown.

(H) WT and *Nkg7*$^{-/-}$ mice injected with B16F10 cells were treated with recombinant IL-2 (*n* = 10 WT and 5 *Nkg7*$^{-/-}$) or IL-15/IL-15Rα (*n* = 11 WT and 5 *Nkg7*$^{-/-}$) and compared to phosphate buffered saline (PBS)-treated controls (*n* = 10 WT and 5 *Nkg7*$^{-/-}$). Mice injected with LWT1 cells were treated with recombinant IL-2 (*n* = 8 WT and 16 *Nkg7*$^{-/-}$; pooled from two experiments) and compared to PBS-treated controls (*n* = 7 WT and 14 *Nkg7*$^{-/-}$; pooled from two experiments). A one-way ANOVA with multiple comparisons was used to test for statistical significance.

p values are shown as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. For graphs in B-E and H, the centre line in data sets indicates the median, box limits indicate

the upper and lower quartiles, and the whiskers indicate the maximum and minimum measures.

See also Extended Data Fig. 5.

Figure 8 NKG7 plays a role in cytotoxicity.

(A) WT and *Nkg7*$^{-/-}$ mice were sub-cutaneously injected with RMA-s-Rae1β cells, followed by treatment with cIg, or antibodies against asGM1 or CD8β and the tumor size measured as repeated measures at each timepoint. A one-way ANOVA with Tukey's multiple comparisons test was used to determine statistical significance. The data shown is from one experiment performed where $n = 5$ mice per group.

(B) WT and *Nkg7*$^{-/-}$ mice were given a sub-cutaneous injection of RMA-s-Rae1β cells at day 0. Mean tumour size was derived from repeated measures at each timepoint. Mice were treated with either cIg ($n = 6$ WT and 6 *Nkg7*$^{-/-}$) or α-IFN-γ ($n = 6$ WT and 8 *Nkg7*$^{-/-}$) at days -1, 0, 7, 14, and 21. Statistical significance was determined using a two-way ANOVA with Tukey's multiple comparisons test. The statistical significance between cIg and α-IFN-γ-treated WT or *Nkg7*$^{-/-}$ mice at day 24 are shown, where **** indicates $p < 0.0001$. Statistical significance between WT cIg and α-IFN-γ groups was detected from day 14 (* $p < 0.05$), whereas statistical significance between *Nkg7*$^{-/-}$ cIg and α-IFN-γ groups was detected on day 18 (* $p < 0.05$). Data obtained from one experiment.

(C) WT and *Nkg7*$^{-/-}$ NK cell-mediated cytotoxicity against YAC-1 target cells or RMA-s-Rae1β *in vitro*, in the absence or presence of IL-2-induced activation, is shown. Statistical significance was assessed using a Mann–Whitney test at each E:T ratio. $n = 6$ per group, pooled from two independent experiments.

(D) The difference in WT and *Nkg7*$^{-/-}$ NK cell-mediated cytotoxicity against RMA-s-Rae1β *in vivo* is depicted in the representative plots and the number of target cells remaining in the lung of WT and *Nkg7*$^{-/-}$ mice were quantified. A Mann–Whitney test was used to determine statistical significance. $n = 11$ mice per group. Data pooled from two independent experiments.

See also Extended Data Fig. 6.

Extended data

Extended Data Fig. 1 *Nkg7* is only expressed by NK cells and a subset of CD8$^+$ TCRβ$^+$ cells in the spleen in naive mice.

t-SNE plot of splenocytes from a naive mouse, pre-gated to exclude doublets and dead cells. The remaining cells were clustered using TCRβ-BUV737, CD4-BUV395, CD8α-PE/Cy7, CD11b-PerCP/Cy5.5, CD11c-BV785, MHC-II-Pacific Blue, B220-BV650, NK1.1-APC/Cy7 and Ly-6C-BV605. Equal numbers of cells (15,000 cells) are shown for *Cre*$^-$ and *Cre*$^+$ plots. The black oval indicates the GFP$^+$ population. *n* = 1 per genotype, performed once. BV, brilliant violet.

Extended Data Fig. 2 Changes in the frequencies of *Nkg7*-expressing NK cells and CD8$^+$ T cells during *Leishmania donovani* infection.

(A) The gating strategy used to assess changes in the key immune cell subsets including NK cells, CD4$^+$ T cells, CD8$^+$ T cells, B cells, cDCs, pDCs, CD11b^{hi} Ly6C^{int} monocytes, inflammatory monocytes, macrophages, and NKT cells.

(B) The graphs show changes in GFP within each of the key immune cell subsets in the spleen and liver during *L. donovani* infection. Statistical significance was determined using the Kruskal–Wallis one-way analysis of variance (ANOVA) with Dunn's multiple comparisons test.

(C) The frequencies of GFP$^+$ T_H1 (gated on NK1.1$^-$ TCRβ$^+$ CD8$^-$ CD4$^+$ IFN-γ$^+$ IL-10$^-$) and T_R1 cells (gated on NK1.1$^-$ TCRβ$^+$ CD8$^-$ CD4$^+$ IFN-γ$^+$ IL-10$^+$) in the spleen and liver during the course of infection are shown. A two-way ANOVA with Sidak's multiple comparisons test was performed to test for statistical significance.

(D) Changes in the frequency and total number of GFP$^+$ cells in the spleen and liver over the course of infection are shown.

p value is indicated where * $p < 0.05$. Error bars represent mean ± SEM. The data shown is representative of two independent experiments, each with *n* = 3 mice per genotype, per timepoint.

Extended Data Fig. 3 *Nkg7* deficiency results in reduced CD4$^+$ T cell responses during *Leishmania donovani* infection.

(A) The liver and spleen weights of WT and *Nkg7*$^{-/-}$ mice during *L. donovani* infection. A two-way ANOVA with Sidak's multiple comparisons test was used to determine statistical significance. Data is representative of two experiments, where *n* = 3 naive WT and *Nkg7*$^{-/-}$ mice, and *n* = 5 WT and 4 *Nkg7*$^{-/-}$ mice at days 14, 28 and 58 p.i. groups.

(B and C) The frequency and total number of conventional (Foxp3^{-}) CD4^{+} T cells in the liver (B) and spleen (C) at day 14 p.i. are shown. Statistical significance was determined using a two-way ANOVA with Tukey's multiple comparisons test. The data shown is representative of two independent experiments, each with *n* = 3 naive WT and *Nkg7*$^{-/-}$ mice, and *n* = 5 WT and 4 *Nkg7*$^{-/-}$ mice at day 14 p.i..

(D) The expression of *Ifng* and *Tnf* mRNA by spleen or liver CD4^{+} T cells in naive or infected (day 14 p.i.) mice was determined by RT-qPCR. A two-way ANOVA with Sidak's multiple comparisons test was used to determine statistical significance. *n* = 4 naive and 5 infected mice in each group.

(E) The representative histograms show PD-1, CTLA-4, and ICOS staining on CD4^{+} T cells in the liver at day 14 p.i. The graphs indicate the frequencies of PD-1^{+}, CTLA-4^{+}, and ICOS^{+} CD4^{+} T cells. Statistical significance was determined using the Mann–Whitney test. Data is derived from one experiment, where *n* = 3 naive WT and *Nkg7*$^{-/-}$ mice, and *n* = 5 infected WT and 4 infected *Nkg7*$^{-/-}$ mice.

(F) The frequency and total number of I-A^{b}PEPCK$_{335\text{-}351}$ (tetramer)-PE^{+} cells in the liver of naive and infected (day 14 p.i.) mice are shown. A two-way ANOVA with multiple comparisons test was used to test for statistical significance. *n* = 4 WT or *Nkg7*$^{-/-}$ naïve, and 5 WT or 6 *Nkg7*$^{-/-}$ infected mice. Data is representative of two independent experiments.

(G) Representative plots depict the differences in T$_{H}$1 (IFN-γ^{+} T-bet^{+} cells) frequencies in WT or *Nkg7*$^{-/-}$ I-A^{b}PEPCK$_{335\text{-}351}$ (tetramer)-PE^{+} cells. The frequencies and numbers are shown in the accompanying graphs below. Statistical significance was determined using the Mann–Whitney test. *n* = 5 mice per group. Data is representative of two independent experiments.

(H) The frequency of WT or *Nkg7*$^{-/-}$ CD4^{+} TCRβ^{+} cells and I-A^{b}PEPCK$_{335\text{-}351}$ (tetramer)-PE^{+} cells expressing IL-6-stimulated phosphorylated (p)-STAT3 are shown. Statistical significance was determined using the Mann–Whitney test. *n* = 5 mice per group. Data is representative of two independent experiments.

p value is indicated where * $p < 0.05$. Error bars represent mean ± SEM.

Extended Data Fig. 4 The absence of NKG7 results in decreased CD8$^+$ T cell cytotoxicity during *Pb*A infection.

(A) The graphs show the frequency and total number of NK cells, CD4$^+$ T cells and CD8$^+$ T cells in the brain of naive and infected (peak of ECM) WT (*n* = 3 naive and 5 infected) and *Nkg7*$^{-/-}$ (*n* = 3 naive and 5 infected) mice. Cell subsets frequencies are expressed as a percentage of CD45$^+$ cells. Data is representative of two independent experiments.

(B) Representative flow cytometry plots were gated on lymphocytes, singlets, live cells, NK1.1-APC/Cy7$^-$ TCRβ-BUV737$^+$, CD8α-PerCP/Cy5.5$^+$ cells and the frequencies of CD11a$^+$ CD49d$^+$ cells and Granzyme B$^+$ cells are shown. *n* = 3 naïve and 4 infected mice per strain. Data is representative of two independent experiments.

(C) The graph shows the proportions of PbT-I^{WT} and PbT-I$^{\Delta Nkg7}$ cells in the spleen of naive (*n* = 4) or infected mice at peak of ECM (*n* = 5).

(D) The frequencies of splenic PbT-I^{WT} and PbT-I$^{\Delta Nkg7}$ transgenic CD8$^+$ T cells expressing Granzyme B or Perforin, in the presence of monensin, are shown. *n* = 4 naïve and 5 infected mice at peak of ECM.

(E) The representative histograms show differences in the expression of CD107a by splenic PbT-I^{WT} and PbT-I$^{\Delta Nkg7}$ cells incubated with monensin or stimulated with PMA and ionomycin in the presence of monensin. The frequency and MFI of CD107a expression is shown in the accompanying graphs. *n* = 4 naïve and 5 infected mice at peak of ECM.

Statistical significance in all graphs was determined using a two-way ANOVA with Tukey's (A) or Sidak's (B–E) multiple comparisons test.

Extended Data Fig. 5 *Nkg7*-deficiency results in increased cancer metastasis in experimental models.

(A) A correlation (*r*) between the moving average of a 19-gene natural killer (NK) cell signature genes and *NKG7* expression in *n* = 472 samples from TCGA:SKCM dataset.

(B) The graphs indicate differences in the number of lung metastases between WT and *Nkg7*$^{-/-}$ mice following injection of RM-1 prostate carcinoma cells (*n* = 6 WT and 7 *Nkg7*$^{-/-}$) and spontaneous metastasis of E0771 mammary carcinoma cells (*n* = 19 WT and 21 *Nkg7*$^{-/-}$,

from two pooled experiments). The survival of WT and *Nkg7*$^{-/-}$ mice treated with either cIg (*n* = 7 WT and 13 *Nkg7*$^{-/-}$) or α-asGM1 (*n* = 6 WT and 12 *Nkg7*$^{-/-}$) in an intraperitoneal RMA-s lymphoma model was also assessed. Statistical significance between groups was tested using the Log-rank (Mantel–Cox) test. Additionally, the difference in percentage of tumour-free mice between WT (*n* = 17) and *Nkg7*$^{-/-}$ (*n* = 14) mice following MCA-induced fibrosarcoma generation is shown. The log-rank (Mantel–Cox) test was used to determine statistical significance. ** and *** represents $p < 0.01$ and 0.001 respectively. ns, not significant.

(C) The lungs of WT and *Nkg7*$^{-/-}$ mice, injected with LWT1 cells, were assessed for differences in the frequency and total cell number of hematopoietic cells, at 14 days post-injection.

(D) The frequency and total cell number of NK cells and T cells were quantified in the lungs of mice injected with LWT1 cells, at 14 days post-injection.

(E) The differences in the frequency of NK cells at different stages of maturation, based on CD27 and CD11b expression at 14 days post-injection of LWT1 cells is shown.

The data shown in C-E is representative of two independent experiments where *n* = 5 mice per group. The Mann–Whitney test was used to determine statistical significance. *p* values are shown as follows: * $p < 0.05$ and ** $p < 0.01$.

Extended Data Fig. 6 *Nkg7*$^{-/-}$ NK cells do not have reduced abilities to conjugate with target cells or to form synapses.

(A and B) The representative histograms show expression of DNAM-1 (CD226), NKG2D (CD314), CD11a, Granzyme B, and Perforin by WT (*n* = 6) or *Nkg7*$^{-/-}$ (*n* = 5) NK cells in the naive state (A) or when activated with rIL-2 (B). The MFI for the expression of each marker in naive NK cells is also shown. Data is pooled from 2 independent experiments. Sv, Streptavidin.

(C) Representative plots depict the frequency of cell conjugates formed when CellTrace Violet (CTV)-labelled WT or *Nkg7*$^{-/-}$ NK cells were co-cultured with carboxyfluorescein succinimidyl ester (CFSE)-labelled YAC-1 target cells for 30 minutes at an E:T ratio of 1:2. The frequency of conjugated NK cells at 5, 15, 30, and 60 minutes is shown in the accompanying graph. *n* = 3 mice per group. Data is pooled from 2 independent experiments.

(D) Representative images of effector NK cell–YAC-1 target cell conjugates visualised using an Amnis® ImageStream®XMark II after *in vitro* co-culture of WT or *Nkg7*$^{-/-}$ cells with target cells for 15 minutes. The graphs show the MFI of Phalloidin or LFA-1 at the interface between effector and target cells. Data obtained from one experiment.