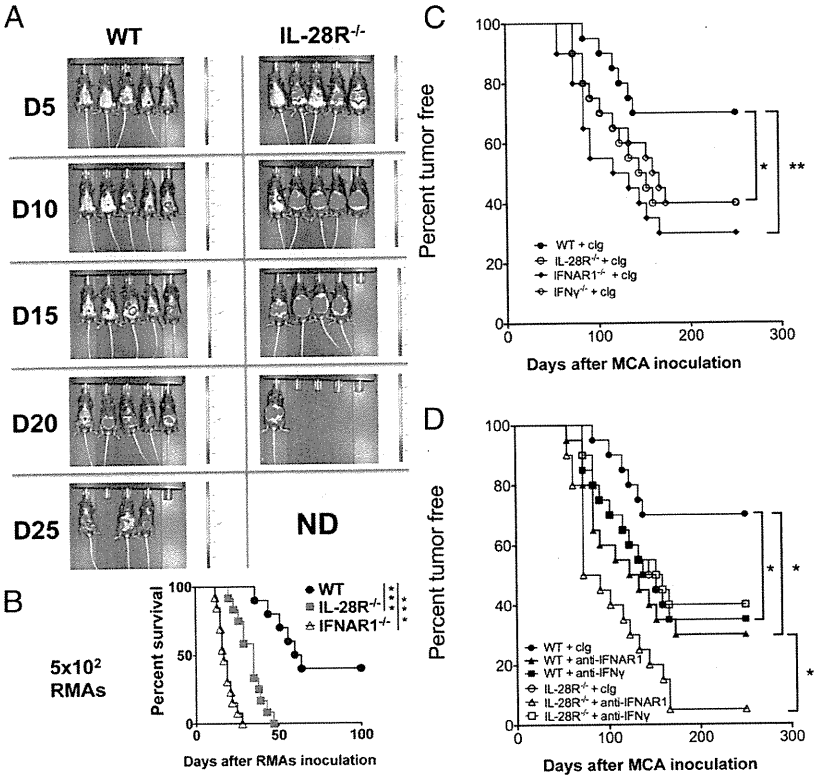

Fig. 6. IL-28R is necessary for effective control of NK cell-sensitive lymphoma and MCA-induced sarcoma in vivo. (*A*) Enhanced RMAs (5×10^4 Luc$^+$ RMAs cells) lymphoma growth in vivo in IL-28R$^{-/-}$ mice after i.p. inoculation is depicted by images taken every 5 d from one of these independent experiments in *SI Appendix*, Fig. S10*A*; ND, not determined. (*B*) IL-28R$^{-/-}$ mice display decreased survival after i.p. challenge with 5×10^2 NK cell-sensitive RMAs lymphoma cells. Statistical analysis was performed using a Mantel–Cox test; ***$P < 0.001$, $n = 10$–13 per group. (*C* and *D*). Groups of 20 male C57BL/6 WT or gene-targeted mice as indicated were inoculated s.c. with 5 μg of MCA in corn oil and subsequently monitored for tumor development over 250 d. WT, IL-28R$^{-/-}$, or IFNAR1$^{-/-}$ mice were treated with (*C* and *D*) control Ig (clg) or (*D*) neutralized for IFNAR1 or IFN-γ (250 μg i.p. at day −1 and 0 and then weekly until week 8). Results are shown as survival curves defined as the percentage of tumor-free mice at each time point. Statistical differences in tumor incidence were determined by Mantel-Cox Log-rank test (*$P < 0.05$; **$P < 0.01$).

cytokines in vivo. In particular, NK cells are critical endogenous IFN-αβ targets during the development of protective antitumor responses (11, 54), and NK cells may require a combination of IFNAR1 and IL-28R signaling (direct or indirect) to be completely antimetastatic. One alternate mechanism is inhibited angiogenesis in vivo because type I and type III IFNs up-regulate Mig and IP-10, both of which suppress neoangiogenesis within tumors. In addition, IFN-λ has been shown to augment the expression of MHC class I molecules, which subsequently increased the expression levels of putative tumor antigens (55). Alternatively, several experimental models showed that activated NK cells were primarily responsible for IFN-λ–mediated antitumor effects (17, 19). However, an antitumor role of type I and III IFN via additional mechanisms, such as regulating tumor cell proliferation, apoptosis, and autophagy, needs to be explored. These studies suggest that mechanisms of type III IFN-mediated antitumor effects are dependent on the tumor model used and that many factors influence the type III IFN-induced activities. Combinatorial therapy using IFN-αβ and IFN-λ may achieve antimetastatic activity by inducing complementary mechanisms and engaging both IFNAR1 and IL-28R.

Materials and Methods

Mice. C57BL/6J WT mice were purchased from the Walter and Eliza Hall Institute for Medical Research and housed at the QIMR Berghofer Medical Research Institute. C57BL/6 *IL-28R*$^{-/-}$ mice, described by Ank et al. (21), were kindly provided by Bristol-Myers Squibb. C57BL/6 *IFNAR1*$^{-/-}$, *Ifnγ*$^{-/-}$, and *RAG2*$^{-/-}\gamma c^{-/-}$ mice have been previously described (11, 28, 56) and were bred at the QIMR Berghofer Medical Research Institute. *IL-28R*$^{-/-}$ x *IFNAR1*$^{-/-}$ mice were generated at the QIMR Berghofer Medical Research Institute by crossing the strains as above. These mice were maintained on a C57BL6 background at the QIMR Berghofer Medical Research Institute. All mice were used between the ages of 6 and 14 wk. All experiments were approved by the QIMR Berghofer Medical Research Institute animal ethics committee.

Cell Culture. B16F10 melanoma and RM-1 prostate adenocarcinoma cell lines were cultured as previously described (42, 57) in Dulbecco's modified Eagle medium supplemented with 10% (vol/vol) heat-inactivated FCS (Thermo), glutamax (Gibco), and penicillin-streptomycin (Gibco). B16F10 were sourced from the American Type Culture Collection whereas RM-1 was obtained from Pamela Russell, Queensland University of Technology, Brisbane, Australia. YAC-1 (a Moloney murine leukemia virus-induced T-cell lymphoma of the A/Sn strain) and RMAs [a TAP2^{neg}/H-2b^{neg} variant of RMA cells (a Raucher virus-induced T-cell lymphoma RBL-5, H-2b$^+$)] cell lines were cultured as previously described (58) in RPMI medium 1640 supplemented with 10% heat-inactivated FCS (Thermo), glutamax (Gibco), and penicillin-streptomycin (Gibco). The generation of RMAs stably transduced with luciferase was performed in the same growth medium with 8 μg/mL polybrene at 75% confluency with 10 multiplicity of infection of lentivirus carrying the venus-luciferase (v2luc) expression plasmid. V2luc was generated by inserting the luciferase coding sequence into the LeGO-iV2 parent vector and was kindly provided by Michael Milsom, German Cancer Research Center, Heidelberg, Germany. After 4 h of incubation at 37 °C, virus- and polybrene-containing medium was replaced with fresh complete growth medium. Cells were kept for an additional 48 h in culture and were subsequently fluorescence-activated cell sorted on the basis of venus expression. All cell lines were tested for *Mycoplasma* detection by the QIMR Berghofer Medical Research Institute's scientific services.

In Vivo LPS Challenge. As previous described (28), LPS (from *E. coli* 0127:B8; Sigma-Aldrich) suspended in PBS was injected intraperitoneally into mice at the described doses (0.10, 0.75, 1.00, or 1.25 mg/30 g mouse). For survival experiments, mice were checked hourly for symptoms of endotoxicosis. Serum from these mice was taken for cytokine analysis by retroorbital or cardiac bleeding. Spleens were also taken from mice after 6 h post-LPS injection to analyze CD69 and intracellular IFN-γ expression by NK cells.

In Vivo CLP-Induced Septic Shock. CLP was performed as previously described (33). Briefly, mice were individually anesthetized by isoflurane, the abdomen was shaved and disinfected by betadine antiseptic spray, a midline incision was made, and 1 mL of saline was injected to prevent tissue dehydration. Cecum was externalized, and a 75% portion was ligated and punctured once using a 25-gauge needle to extrude a small amount of cecal content and induce a high-grade sepsis (100% mortality within 10 d). The cecum was returned to the abdomen, the peritoneum was closed via suture, and the skin was sealed using an auto clip wound clip applier (Becton Dickinson). Buprenorphin (Reckitt Benckiser Pharmaceutical) was applied at 0.05 mg per kg body weight at the incision site for postoperative analgesia.

NK Cell Activation in Vitro. Spleens from the indicated strains of mice were stained with anti-NK1.1, anti-NKp46, and anti-TCRβ mAbs, and NK cells were sorted by FACS (BD FACSAria II; BD Biosciences). Two hundred thousand freshly purified NK cells were plated in 96-well U bottom plates in NK cell media (RPMI supplemented with 10% FCS, non essential amino acids, Pyruvate, Hepes, glutamax, 2-mercaptoethanol, penicillin/streptomycin) in the presence of rIL-10 (Biolegend), rIL-12 (eBiosciences), rIL-15/IL-15Rα complex (eBiosciences), rIL-18 (R&D Systems), and PEG-IL-28A (kindly donated by Sean Doyle, Zymogenetics, Seattle) for 24 h. For NK cell-mediated cytotoxicity assays, sorted NK cells were either cultured for 5 d in NK cell media with supplementation of 10 ng/mL of rIL-15/IL-15Rα complex (in vitro priming) or sorted from mice post 24 h Poly I:C (100 μg per mice) i.p. injection (in vivo priming). Target B16F10 or YAC-1 cells, labeled with 100 μCi/1 × 10^6 cells of ^{51}Cr, were cocultured for 4 h with the indicated ratio of primed NK cells.

Flow Cytometry Analysis. Cells harvested from in vitro cultures or single cell suspensions from various organs were incubated for 15 min in Fc blocking buffer (2.4G2 antibody). Cells were then stained with the following antibodies: anti-mouse-CD3 (17A2), -CD11b (M1/70), -CD27 (LG.3A10), -CD43 (eBioR2/60), -CD69 (H1.2F3), -DNAM-1 (480.1), -IFN-γ (XMG1.2), -Ly49A (A1), -Ly49C/I (14B11), -NKG2D (CX5), -NKG2A/C/E (20d5), -NKp46 (29A1.4), -NK1.1 (PK136), and -TCRβ (H57-597). All of the mAbs were purchased from eBiosciences, BD Biosciences, or Biolegend. A Zombie Yellow or Zombie UV Fixable Viability Kit (Biolegend) was used to assess viability. Acquisition was performed using an LSR II Fortessa Flow Cytometer (BD Biosciences). Analysis was achieved using Flowjo (Treestar) software. For NK cell purification, spleen homogenates were first stained with Mouse NK Cell Isolation Kit II (Miltenyi Biotec) and enriched by a depleting program by an AutoMACS-Pro (Miltenyi Biotec). NK cell-enriched samples were then stained with NK1.1, NKp46, TCR-β, or viability stain and sorted with high purity (viable, NK1.1$^+$. NKp46$^+$, TCRβ^{neg}) using a FACS Aria II (BD Biosciences).

Cytokine Detection. All cytokines from in vivo assays were detected using Cytometric Bead Array (CBA) technology (BD Biosciences) according to the manufacturer's instructions. IFN-γ detection from purified NK cell supernatants from in vitro assays was measured by ELISA with the IFN-γ Duoset Kit (R&D Systems) according to the manufacturer's instructions. For intracellular cytokine detection, isolated splenocytes from LPS-injected mice or in vitro-activated NK cells were stained for the indicated surface markers, fixed, and permeabilized using BD cytofix/cytoperm (BD Biosciences) and then stained with an anti–IFN-γ antibody (XMG1.2).

In Vivo Tumor Imaging. Single cell suspensions of RMAs (5 × 10^2 to 5 × 10^4) or RMAs-Luciferase$^+$ cells (5 × 10^4) were injected i.p. into the indicated strains of mice at day 0 (D0). Tumor burden was measured by bioluminescence imaging and expressed as photon flux (photons per second) as previously described (44). Luminescence was assessed at 5-d intervals by injection of 0.5 mg/mL D-luciferin (Everest) per mouse for 5 min, and luminescence measure for 1 min in a Xenogen IVIS Caliper (Perkin-Elmer). Overall survival was calculated in parallel to the imaging kinetics.

Tumor Metastasis and MCA-Induced Fibrosarcoma. Single cell suspensions of RM-1 (5 × 10^3) or B16F10 melanoma cells (5 × 10^4 to 2 × 10^5) were injected i.v. into the tail vein of the indicated strains of mice. Lungs were harvested on day 14, and tumor nodules were counted under a dissection microscope. On day −1, 0, and 7 after tumor inoculation, some mice were treated with i.p. injections of control Ig (cIg) (2A3, 250 μg i.p), anti–mIFN-γ mAb (H22, 250 μg i.p), anti-mIFNAR1 mAb (MARI-5A3, 250 μg i.p), or asGM1 mAbs (100 μg i.p each). For MCA carcinogen-induced fibrosarcoma formation, groups of 8–20 male WT, IFNAR1$^{-/-}$, IFN-γ$^{-/-}$, or IL-28R$^{-/-}$ mice were injected s.c. on the right flank with 5 μg, 25 μg, or 300 μg of MCA and were monitored over 250 d for fibrosarcoma development. Data were recorded as the percentage of mice tumor-free. Some mice in these experiments were treated with control Ig (cIg) (2A3, 250 μg i.p), anti-mIFNAR1 mAb (MARI-5A3, 250 μg i.p), or asGM1 mAbs (100 μg i.p each) as indicated in the legends.

NK Cell Adoptive Transfer. NK cells (2 × 10^5) freshly purified (NK1.1$^+$, NKp46$^+$, TCRβ^{neg}) from WT or IL-28R$^{-/-}$ mice were injected via the tail vein into RAG2$^{-/-}$γc$^{-/-}$ mice. Five days later, mice were injected either i.p with LPS (0.1 mg/30 g mouse) or i.v with B16F10 melanoma cells (5 – 10^4). The NK cell reconstitution of each mouse was analyzed by flow cytometry in the peripheral blood before and after the completion of each experiment, and we observed no differences between IL-28R$^{-/-}$ and WT NK cell injected mice.

In Vivo Treatment with IFN-αβ and IFN-λ. Single tumor cell suspensions of 2 – 10^5 B16F10 were injected i.v. into the indicated strains of mice at day 0. Treatment groups consisted of rMOCK IFN-αβ, rIFN-αβ (50,000 U per mouse per day), PEG-IL-28A (25 μg per mouse per day), or both rIFN-αβ + PEG-IL-28A (same concentration per mouse per day). Treatments were applied i.p. daily from day 0 to day 5 post tumor inoculation, and overall survival was calculated. PEG-IL-28A was kindly provided by Sean Doyle, Zymogenetics, Seattle, and rMOCK IFN-αβ and rIFN-αβ were kindly provided by Antonella Sistigu, Institut Gustave Roussy, Paris.

Statistical Analysis. Statistical analysis was achieved using GraphPad Prism Software V6. Data were considered to be statistically significant where the *P* value was equal to or less than 0.05. Statistical tests used were the unpaired Student's *t* test, Mann–Whitney test, and the Mantel–Cox Log Rank test for survival.

Supplementary figures and legends are detailed in *SI Appendix*.

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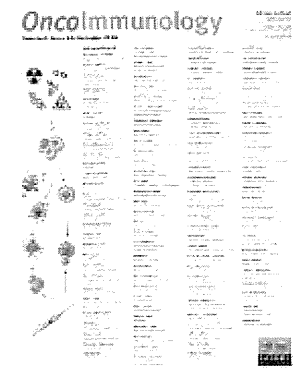

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Toll-Like Receptor 3 regulates NK cell responses to cytokines and controls experimental metastasis

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Toll-Like Receptor 3 regulates NK cell responses to cytokines and controls experimental metastasis

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Running Title: TLR3 in cancer immunosurveillance

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Conflict of interest

The authors have no conflict of interest to declare.

Abbreviations:

Abs: antibodies; **Batf3**: basic leucine zipper transcription factor, ATF-like 3; **DC**: Dendritic cell; **dsRNA**: double stranded ribonucleic acid; **IFN**: interferon; **IL**: interleukine; **IRF**: Interferon Regulatory Factor; **iv**: intravenous; **MCA**: methylcholanthrene; **MDA5**: melanoma

differentiation associated protein 5; **MHC**: Major Histocompatibility Complex **NF-κB**: Nuclear Factor kappa-light chain enhancer of activated B cells; **NK**: Natural Killer; **PMA**: phorbol 12-myristate 13-acetate; **Poly(I:C)**: polyriboinosinic-polyribocytoidylic acid; **sc**: subcutaneous; **TIR**: Toll-interleukin I receptor; **TLR**: Toll-like receptor; **TRIF**: TIR domain-containing adaptor inducing IFN-β; **WT**: wild-type

Abstract

The Toll-like receptor 3 (TLR3) agonist poly(I:C) is a promising adjuvant for cancer vaccines because it induces strong anti-tumor responses, mainly through the activation of dendritic cells (DCs) and natural killer (NK) cells. However, little is known about the role of TLR3 sensing of endogenous ligands in tumor immunosurveillance. Here, we investigated whether TLR3 could modulate immune responses and facilitate tumor control without administration of an agonist. We observed only limited impact of TLR3 deficiency on spontaneous carcinogenesis and primary growth of B16F10, E0771 or MC38 tumors when injected subcutaneously to mice; but importantly, TLR3 limited experimental B16F10 lung metastasis. Metastasis control was dependent on both IFN-γ secretion and NK cells. Interestingly, we observed that NK cells from $TLR3^{-/-}$ mice were hyporesponsive to cytokine stimulation. Indeed, compared with WT, $TLR3^{-/-}$ NK cells produced significantly less pro-inflammatory cytokines, including IFN-γ, when incubated in the presence of different combinations of IL-12, IL-18 and IL-15. Bone-marrow chimera experiments established that competent NK cell responses required TLR3 sensing on radio-sensitive immune cells. Intriguingly, although CD8α DCs express high levels of TLR3, we found that those cells were not necessary for efficient IFN-γ production by NK cells. Moreover, the defective NK cell phenotype of $TLR3^{-/-}$ mice appeared to be independent of the gut microbiota. Altogether, our data demonstrate a pivotal role of endogenous TLR3 stimulation for the acquisition of full NK cell functions and protection against experimental metastasis.

Introduction

Toll like receptor (TLRs) initiate immune responses upon recognition of pathogen- and damage-associated molecular patterns. Therefore, members of the TLR family play a crucial role in the protection against microbial infections and the maintenance of host homeostasis. [1] Among them, TLR3 is an endosomal receptor that senses viral dsRNA. [2] TLR3 is expressed by various immune or non-immune cells, including DCs, [3] macrophages, [4] NK cells, [5, 6] fibroblasts [7] and epithelial cells. [8] Sensing of viral dsRNA by TLR3 leads to the secretion of type I IFN and other pro-inflammatory cytokines. [9] TLR3 interacts with the adaptor protein TIR domain-containing adapter-inducing IFN-β (TRIF), also known as Toll-interleukin I receptor domain containing molecule 1 (TICAM), to induce the activation of IRF-3 and NF-κB transcription factors. [10] Interestingly, a TRIF independent pathway of TLR3 signalling has recently been described. [11] This pathway involves the non-receptor tyrosine kinase c-Src and regulates cell migration.

Well known for its role in viral infections, [12, 13] TLR3 can also protect against cancer. Indeed, the TLR3 agonist polyriboinosinic-polyribocytoidylic acid (poly I:C) efficiently suppresses tumor growth in mice [14, 15] and TLR3 agonists are in phase I/II trials as adjuvants for therapeutic vaccination against melanoma and breast cancer. [16] Anti-cancer properties of TLR3 agonists have been attributed to the direct apoptosis of TLR3-expressing tumor cells [17, 18] together with the induction of immune responses that overcome tumor tolerance. [19-21] Yet, the contribution of TLR3 to the anti-tumor properties of poly(I:C) remains unclear since this dsRNA analogue can also be recognised by the cytosolic sensor melanoma differentiation associated protein 5 (MDA5). [22, 23] In addition, little is known about TLR3 function in the absence of administration of its agonist. Interestingly, a study reported that transplanted mouse prostate adenocarcinoma grew faster in $TLR3^{-/-}$ mice compared with WT mice,

supporting a protective role for endogenous triggering of TLR3. [20] In humans, high levels of TLR3 expression have been associated either with good [24, 25] or poor [26] prognosis, depending on the malignancies. Thus, the exact role of TLR3 in tumor immunosurveillance remains to be characterized.

Among the different cellular mediators of the poly(I:C) induced-response, NK cells represent a major anti-tumor effector. [20, 21] NK cells are innate lymphocytes that recognize and directly kill transformed cells. [27] In addition, activated NK cells release of pro-inflammatory factors, including IFN-γ, TNF-α, GM-CSF, MIP1-α (CCL3), MIP1-β (CCL4) and RANTES (CCL5).[28] NK cell responses are controlled by the integration of signals from germline-encoded activating and inhibitory receptors that recognize molecules expressed on the surface of the target cells. Yet, the acquisition of full effector functions by NK cells requires additional signals provided by cytokines such as IL-2, IL-12, IL-15, IL-18 and type I IFN or by direct contact with accessory cells, often DCs. [29] Poly(I:C) has been shown to induce efficient NK cells responses, either by the direct activation of TLR3 on NK cells [5, 30] or via the activation of accessory cells. [21, 22, 23]

Here, we investigated the role of TLR3 in NK cell activation and cancer immunosurveillance in the absence of administration of exogenous dsRNA. We showed that TLR3 modulates NK cell responses by endowing them with the ability to release high amounts of IFN-γ in response to cytokine stimulation. In addition, we established that TLR3 signaling pathway controlled the growth of Rae-1β expressing tumors as well as experimental B16F10 tumor metastasis, all tumors known to be highly controlled on NK cell effector function. This study demonstrates that TLR3 expression on immune cells regulates IFN-γ secretion by NK cells independently of the gut microbiota and is essential to control metastatic spread of cancer.

Results

NK cells from TLR3$^{-/-}$ mice are hyporesponsive to cytokine stimulation

The ability of the TLR3 ligand poly (I:C) to activate NK cells is well established. [5, 22] However, nothing is known about the influence of TLR3 on NK cell priming in the absence of administration of its agonist. To determine whether TLR3 signaling modulates NK cell ability to respond to cytokine stimulation, we purified NK cells from WT or TLR3$^{-/-}$ mice (Sup. Fig. 1) and cultured them in the presence of different combinations of recombinant IL-12, IL-18 and IL-15. Interestingly, we observed that TLR3$^{-/-}$ NK cells produced significantly less IFN-γ than WT NK cells in response to cytokine stimulation (Fig. 1A). By contrast, when cultured with PMA/ionomycin, no difference between TLR3$^{-/-}$ and WT NK cells could be observed in terms of IFN-γ production (Fig. 1B). Thus, the inherent ability of TLR3$^{-/-}$ NK cells to produce IFN-γ was not compromised. In addition, despite low IFN-γ production, TLR3$^{-/-}$ NK cells were efficiently activated upon IL-12/IL-18 stimulation, as assessed by their up-regulation of CD69 (Fig. 1C). Flow cytometry data confirmed the decreased IFN-γ production of IL-12/IL-18 stimulated TLR3$^{-/-}$ NK cells when compared with WT NK cells: both the percentage of IFN-γ producing cells and the intensity of signal were reduced (Fig. 1D). Finally, we detected lower levels of MIP-1α, MIP-1β, RANTES, IL-6 and GM-CSF in the supernatant of TLR3$^{-/-}$ NK cells when cultured in the presence of IL-12/IL-18 or IL-12/IL-15 (Fig. 1E and F), indicating the signaling pathway controlling the production of all pro-inflammatory cytokines and chemokines by NK cells is defective in the absence of TLR3. Altogether, these data demonstrates that the presence of TLR3 regulates NK cell ability to produce of high levels of pro-inflammatory factors in response to cytokine stimulation.

NK cell development and killing activity are not affected by TLR3 deficiency

The decreased ability of NK cells from TLR3$^{-/-}$ mice to produce cytokines could have been caused by a defect in NK cell development. To explore this possibility, NK cell numbers and maturation status were analyzed by flow cytometry. TLR3$^{-/-}$ and WT mice showed similar percentages of NK cells in every organ analyzed, including bone marrow, liver, lung and spleen (Fig. 2A). In addition, the absence of TLR3 did not affect NK cell maturation status, as determined by the combined expression of CD11b and CD27 [31] (Fig. 2B). Moreover, WT and TLR3$^{-/-}$ NK cells expressed identical levels of NK cell activating (NKG2D, Ly49D, DNAM-1) or inhibitory (Ly49C/I, CD96, KLRG1) receptors (Fig. 2C). We found that NK1.1 crosslinking induced IFN-γ production and degranulation of TLR3$^{-/-}$ NK cells, even though the response was slightly decreased compared to WT NK cells (Fig. 2D). Very low degranulation was observed following anti-Ly49D stimulation, with no difference between WT and TLR3$^{-/-}$ NK cells. Finally, we analyzed the ability of NK cells from TLR3$^{-/-}$ mice to kill target cells *in vitro*. TLR3 deficiency did not affect NK cell cytotoxic activity against YAC-1 or B16F10 tumor cells (Fig. 2E). Taken together, these results demonstrate that NK cells develop, mature and acquire killing capacities independently of TLR3. However, TLR3 is probably required at later stages for conditioning mature NK cells to release high levels of pro-inflammatory cytokines upon stimulation and to be fully responsive to NK1.1 ligation.

Production of high levels of IFN-γ by NK cells requires TLR3 expression on hematopoietic cells

TLR3 is expressed on a wide range on immune cells but also on non-immune cells, including fibroblasts and epithelial cells. [2] To determine the contribution of TLR3 expression on non-hematopoietic cells to NK cell conditioning, we performed bone marrow chimera experiments in which lethally irradiated WT or TLR3$^{-/-}$ mice were reconstituted with bone

marrow cells from WT or TLR3$^{-/-}$ mice. Eight weeks later, NK cells were purified from these mice and stimulated with IL-12/IL-18 (Fig. 3A and B). As expected, NK cells obtained from TLR3$^{-/-}$ mice reconstituted with TLR3$^{-/-}$ bone marrow produced much less IFN-γ than NK cells from WT mice reconstituted with WT bone marrow. Interestingly, NK cells from TLR3$^{-/-}$ mice reconstituted with WT bone marrow were proficient in IFN-γ production whereas lower IFN-γ levels were produced by NK cells from WT mice reconstituted with TLR3$^{-/-}$ bone marrow. These data indicate that TLR3 expression on immune cells only is necessary and sufficient for the pre-conditioning of NK cells to cytokine stimulation.

CD8α DCs are not required for TLR3-dependent NK cell conditioning

To get further insight on which immune cells were responsible for TLR3-dependent NK cell conditioning, we performed intracellular TLR3 staining on mouse splenocytes. We found that TLR3 was expressed at much higher levels on CD8α DCs than on other immune cells (Fig. 3C). However, NK cells from Batf3$^{-/-}$ mice that lack CD8α DCs [32] produced IFN-γ to similar levels to those produced by WT NK cells, indicating that CD8α DCs are not necessary for NK cell conditioning (Fig 3C, D and E). Taken as a whole, our results indicate that the acquisition of full effector functions by NK cells requires TLR3 expression on immune cells other than CD8α DCs.

Role of the gut-microbiota in TLR3-dependent NK cell conditioning

Although TLR3 is well known for recognizing virus dsRNA, [9] we do not know what drives TLR3 signaling in the absence of infection. Given that NK cell responses are compromised in germ-free mice [33] and that bacterial RNA can potentially activate TLR3, [34] we hypothesized that a defective sensing of the microbiota would account for the defective NK cells conditioning in TLR3$^{-/-}$ mice. To explore this possibility, WT and TLR3$^{-/-}$ mice were co-housed for at least 6 six weeks before testing NK cell IFN-γ production (Sup. Fig. 2A). NK

cells from TLR3$^{-/-}$ mice were still poor IFN-γ producers compared with WT NK cells, even when the mice were co-housed. To confirm these data, we treated TLR3$^{-/-}$ and WT mice with broad-spectrum antibiotics known to strongly reduce the gut microbiota [35] for 3 to 4 weeks. IFN-γ production by TLR3$^{-/-}$ NK cells was still reduced compared with WT (Sup. Fig. 2B). These results strongly indicate that the acquisition of full effector functions by NK cells is independent of bacterial RNA sensing.

TRIF is not necessary for efficient NK cell response to cytokines

The adaptor protein TRIF interacts with the intracellular domain of TLR3 [36] and poly(IC)-induced responses are abolished in TRIF$^{-/-}$ mice. [10] However, a TRIF-independent pathway of TLR3 has recently been described. [11] Thus, we asked whether TRIF was involved in the TLR3-dependent sensitization of NK cells. For that, NK cells were purified from WT and TRIF$^{-/-}$ mice and cultured together with IL-12/IL-18 or IL-15/IL-12. Interestingly, NK cells from both mice secreted comparable levels of IFN-γ (Sup. Fig. 3A). Similar results were obtained regarding MIP-1α production (Sup. Fig. 3B). These data demonstrate that TLR3 signals through a TRIF-independent pathway to regulate NK cell responses to cytokine stimulation.

TLR3 is involved in NKG2D-mediated suppression of tumor progression

NK cells recognize various molecules up-regulated on the surface of transformed cells. [28] Among them, Rae-1 is a stress-induced molecule that triggers the NK cell activating receptor NKG2D. [37] We asked whether TLR3 would be involved in the recognition and/or killing of Rae-1 expressing tumor cells by NK cells. To this aim, WT and TLR3$^{-/-}$ mice were inoculated subcutaneously with RMAS tumor cells transfected with an empty vector (RMAS-MSCV) or with the Rae-1β molecule (RMAS- Rae-1β). Both mouse strains had equal outgrowth of the RMAS-MSCV cell line (Fig. 4A). By contrast, the growth of RMAS-Rae1β cells was

significantly reduced in WT compared with TLR3$^{-/-}$ mice (Fig. 4B). These results demonstrate that TLR3 is also involved in NKG2D-dependent NK cell-mediated tumor suppression.

TLR3 is dispensable for the control of primary tumors or carcinogen-induced fibrosarcoma

To further investigate the role of TLR3 in tumor control, we used the B16F10 melanoma and MC38 colon adenocarcinoma syngeneic mouse models. We observed that both cell lines had identical growth rates in WT and TLR3$^{-/-}$ mice (Fig. 5A and B). Next, we employed an orthotopic, syngeneic mouse tumor model by injecting E0771 mammary carcinoma cells into the mammary fat pad. Here again, no difference in tumor growth was observed between WT and TLR3$^{-/-}$ mice (Fig. 5C). We next wanted to explore whether TLR3 signaling could participate in the immunosurveillance of *de novo* models of tumorigenesis. For that, we took advantage a model of carcinogen-induced fibrosarcoma in which NK cell-mediated suppression of tumor initiation has been previously demonstrated. [38] WT and TRL3$^{-/-}$ mice were injected with MCA and monitored for tumor development over 300 days (Fig. 5D). Our results indicate a trend toward higher tumor incidence in TLR3$^{-/-}$ mice but this did not reach significance. Overall, our data suggest that TLR3 signaling is not critical for the control of both carcinogenesis and primary tumor growth.

TLR3 protects against experimental metastasis through NK cells and IFN-γ

Previous studies have established a fundamental role of NK cells in the control of B16F10 experimental metastasis. [39] This prompted us to investigate the role of TLR3 in this model. We found that TLR3$^{-/-}$ mice displayed significantly higher numbers of lung metastases than WT mice following intravenous inoculation of B16F10 cells (Fig. 6A). Thus, despite a negligible effect upon primary tumor growth, TLR3 controls metastasis of B16F10 cells. By

contrast, carcinogenesis, primary tumor growth and metastasis were unaffected in the TRIF$^{-/-}$ mice (Sup. Fig. 4). These experiments establish the importance of TRIF-independent signaling of TLR3 in the control of experimental B16F10 metastasis.

Finally, we aimed to investigate the mechanisms by which TLR3 controls metastasis. More particularly, we asked whether NK cells and the secretion of IFN-γ were involved in this process. Mice injected intravenously with B16F10 cells were treated with anti-asGM1 antibodies (Abs) or anti- IFN-γ Abs to deplete NK cells or neutralize IFN-γ, respectively. Both treatments significantly increased metastasis burden in WT and TLR3$^{-/-}$ mice (Fig. 6C and D). These data confirm the role of NK cells and IFN-γ in the protection against B16F10 metastasis [39, 40] and indicate that these two components are still protective in the absence of TLR3 signaling. Importantly, no significant difference was observed between WT and TLR3$^{-/-}$ mice when those mice were treated with anti-asGM1 or anti-IFN-γ Ab, suggesting that the TLR3-mediated control of B16F10 metastasis requires both NK cells and IFN-γ production. Altogether, these data demonstrate the importance of the TLR3 signaling pathway for the protection against experimental metastasis and establish that both NK cells and IFN-γ secretion are necessary for such protection.

Discussion

The present study highlights the critical role played by TLR3 signaling in the immunosurveillance of cancers and metastases. Importantly we showed that in the absence of administration of any agonist, TLR3 considerably increased NK cell production of pro-inflammatory cytokines and protected against experimental metastasis.

NK cells are innate effector cells that were initially thought to recognize and eliminate their targets with fast kinetics, without prior sensitization. However, NK cell activity is tightly regulated because only NK cells that can engage self-MHC become fully competent, a

processed called licensing. [41] In addition, similarly to T cells, NK cells need to be primed to acquire full effector functions. [42] Here, we extended those concepts by demonstrating that constant triggering of TLR3 keeps mature NK cells in a "ready to go" state. This process takes place at steady-state and thus differs from NK cell-priming that occurs in a highly inflammatory context and from NK cell-licensing that ensures self-tolerance during NK cell development. Hence, we propose that TLR3 allows the conditioning of NK cells, an essential step to ensure immediate and potent NK cell responses to cytokine stimulation.

Data from the literature indicate that, unlike human NK cells, [5, 6, 30] purified mouse NK cells fail to respond to poly(I:C) *in vitro*. [23, 44] Thus, even if our results did not formally exclude a direct role of TLR3 signaling on NK cells, it is probable that accessory cells are required for TLR3-dependent NK cell conditioning. Using BM-chimera mice, we demonstrated that the expression of TLR3 on hematopoietic cells is necessary and sufficient for the acquisition of full NK cell functions. These data are reminiscent of results showing that TLR3 signaling in BM-derived accessory cells, but not in stromal cells, drives NK cell activation. [22] Flow cytometry analyses established that TLR3 protein is preferentially expressed in CD8ı DCs, a finding consistent with the high levels of TLR3 mRNA in these cells. [23, 43] It has been described, CD8α DCs co-cultured with NK cells induce IFN-γ production in response to poly(I:C). [23] However, the use of BAFT3$^{-/-}$ mice (that lack CD8α DCs) excluded a predominant role for this DC subset in NK cell conditioning. It is noteworthy that such a negligible role of CD8α DCs has been previously reported following *in vivo* administration of poly(I:C). [22] Further work would be needed to identify the cell type(s) necessary for TLR3-dependent conditioning of NK cells. Macrophages constitute a likely candidate since they have been found to induce IFN-γ production by NK cells when stimulated with poly(I:C). [44]

IL-18 has been described to regulate NK cell ability to produce IFN-γ in response to cytokine stimulation. [45] An attractive hypothesis would be that TLR3 signaling controls IL-18

production by accessory cells. However, we measured similar levels of IL-18 in the sera of naïve WT and TLR3$^{-/-}$ mice (data not shown). In addition, we failed to detect any production of TNF-α or IL-12 p70 in the sera of naïve mice. These data do not exclude the possibility that cytokine levels are regulated locally in a TLR3-dependent manner.

Our result that NK cells are hyporesponsive in TLR3$^{-/-}$ mice suggests that repetitive stimulation by TLR3-ligand(s) controls NK cell activity. These ligands required for proper NK cell conditioning are still unknown. Although defective sensing of commensal bacterial mRNA could have explained NK cell hyporesponsiveness, [33] our data obtained in co-housed or antibiotic treated mice excluded a role of the gut microbiota. Interestingly, endogenous mRNA released from necrotic cells has the potential to activate TLR3. [46, 47] Therefore, NK cell responsiveness could be modulated by TLR3-sensing of host-derived mRNAs. Alternatively, TLR3 could recognize nucleic acids from endogenous retrovirus. [48] Eventually, TLR3 might interact with other signaling pathways in a ligand-independent manner. Future studies should aim to define the nature of the ligand(s) recognized by TLR3, if any.

The absence of phenotype in TRIF$^{-/-}$ mice despite defective NK cell responses and increased metastasis in TLR3$^{-/-}$ mice was somehow surprising. Indeed, TLR3 was initially believed to signal exclusively through the adaptor protein TRIF. [10] However, an interesting study highlighted a new branch of TLR3 signaling that is TRIF-independent but does require the non-receptor tyrosine kinase Src. [11] TRIF-independent response to TLR3 stimulation affected the migration, adhesion and proliferation of multiple cell types and did not require gene-induction. Thus, although TRIF is essential for TLR3-mediated response to virus or poly(I:C) leading to IRF3 and NF-ı B activation, another facet of TLR3-signaling may regulate a different kind of response in a TRIF-independent manner. It is tempting to speculate that different pathways of TLR3 signaling could be triggered in response to different signals (e.g. endogenous mRNA versus viral mRNA) or in different contexts (e.g. inflammation versus

homeostasis). Further investigation is warranted to determine whether the Src pathway or another yet unknown pathway is responsible for the TLR3-mediated control of NK cell reactivity.

Despite substantive data emphasizing the anti-cancer properties of poly (I:C), [16] it remained unclear whether TLR3 could promote immunosurveillance without administration of its agonist. Interestingly, one study reported a role for endogenous TLR3 in restricting the primary growth of transplantable tumors. [20] The present work broadens TLR3 function to the control of Rae1β-expressing tumor cells and metastasis. Conversely, we found no significant contribution of TLR3 to carcinogenesis in the MCA-induced fibrosarcoma model. Moreover, the presence of TLR3 did not influence primary growth of subcutaneous tumors that did not express NKG2D-ligand. Hence, the tumor microenvironment probably determines the importance of TLR3 signaling for the induction of efficient anti-tumor immune responses. The availability of TLR3 ligands and the presence TLR3-expressing cells at the tumor site are likely to be determinant factors for TLR3 function.

Intriguingly, the tumor models controlled by TLR3 (e.g. RMAS-Rae1β tumor growth and B16F10 metastasis) have been previously shown to be suppressed by NK cells. [37, 49, 50] Despite normal *in vitro* NK cell cytotoxic activity and expression levels of NKG2D in TLR3$^{-/-}$ mice, TLR3 deficiency might have affected NK cell ability to kill tumor cells *in vivo*. Alternatively, the defect in NK cells conditioning we observed might account for the higher sensitivity of TLR3$^{-/-}$ mice to tumor challenge. We can hypothesize that cytokines present in the tumor microenvironment stimulate NK cell IFN-γ production in WT mice, but hyporesponsive NK cells in TLR3$^{-/-}$ mice would fail to secrete sufficient IFN-γ. Our finding that protection against experimental B16F10 metastasis in WT mice requires both NK cells and IFN-γ support this hypothesis. Yet, the whole picture must be much more complicated and probably includes several feedback loops and interactions between the various actors of

the immune system. For instance, TLR3 signaling stimulates DCs or macrophages to prime NK cells. [22, 44] In turn, NK cells regulate DC functions as well as T cell responses. [29, 51] Thus, TLR3 may control the priming of innate responses (of NK cells more particularly), further necessary to turn on adaptive immunity against cancer.

Overall, we provide evidence that TLR3 is involved in the NK cell-mediated suppression of experimental tumor metastasis. Notably, this study demonstrates a role of TLR3 in cancer immunosurveillance in the absence of injection of its agonist. However, metastases development in TLR3 competent-host suggests that either TLR3 itself is not sufficient to prevent metastatic spread or that some mechanisms allow tumor to escape TLR3-dependent immune responses. Indeed, one attracting possibility would be that tumors would modulate the availability of TLR3 ligands and/or TLR3 expression to escape from the immune system. Our data provide clues in how the lack of TLR3 signaling down-regulates NK cell functions and leads to defective immune responses unable to control metastases.

Materials and Methods

Mice

C57BL/6 WT mice were purchased from the Walter and Eliza Hall Institute of Medical Research (Victoria, Australia). C57BL/6 TLR3$^{-/-}$, C57BL/6 TRIF$^{-/-}$ and C57BL/6 BATF3$^{-/-}$ mice were bred in house at the Peter MacCallum Cancer Center (Victoria, Australia) and then at the QIMR Berghofer Medical Research Institute (Queensland, Australia). Mice were used at 6-12 weeks of age and housed in specific pathogen free conditions.

Antibodies and reagents

The flow cytometry antibodies anti-CD69 (H1.2F3), CD107a (1D4B) and NKp46 (29A1.4) were purchased from Biolegend. Anti-IFN-γ (XMG1.2), NK1.1 (PK136), CD3ξ (145-2C11)