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Type I IFN Contributes to NK Cell Homeostasis, Activation, and Antitumor Function[1]

Jeremy B. Swann,* Yoshihiro Hayakawa,* Nadeen Zerafa,* Kathleen C. F. Sheehan,† Bernadette Scott,‡ Robert D. Schreiber,† Paul Hertzog,‡ and Mark J. Smyth[2]*

This study demonstrates that type I IFNs are an early and critical regulator of NK cell numbers, activation, and antitumor activity. Using both IFNAR1- and IFNAR2-deficient mice, as well as an IFNAR1-blocking Ab, we demonstrate that endogenous type I IFN is critical for controlling NK cell-mediated antitumor responses in many experimental tumor models, including protection from methylcholanthrene-induced sarcomas, resistance to the NK cell-sensitive RMA-S tumor and cytokine immunotherapy of lung metastases. Protection from RMA-S afforded by endogenous type I IFN is more potent than that of other effector molecules such as IFN-γ, IL-12, IL-18, and perforin. Furthermore, cytokine immunotherapy using IL-12, IL-18, or IL-21 was effective in the absence of endogenous type I IFN, however the antimetastatic activity of IL-2 was abrogated in IFNAR-deficient mice, primarily due to a defect in IL-2-induced cytotoxic activity. This study demonstrates that endogenous type I IFN is a central mediator of NK cell antitumor responses. ***The Journal of Immunology,*** **2007, 178: 7540–7549.**

Interferons have long been associated with regulating the immune response to infection inflammation and tumorigenesis. Studies in mice deficient for IFN signaling have demonstrated an altered susceptibility to infections, inflammation, and tumor development (1–4). In addition to their definitive antiviral activity, IFNs inhibit the proliferation of tumor cells and promote immune responses toward them, and have had limited success in the clinical treatment of certain tumors (5, 6). Although we are making rapid progress in understanding the molecular and cellular mechanisms of IFN actions in the TLR-mediated response to infection and inflammation, it is less clear in antitumor responses how IFN production is stimulated, which cells produce it, which cells are the major targets, and what is the relative importance of type I IFNs in the complex antitumor cytokine milieu.

The type I IFN family constitutes a number of related cytokines that all act through the type I IFN receptor, which is composed of two subunits, IFNAR1 (7) and IFNAR2 (8). IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, and IFN-τ, as well as limitin, all signal through the type I IFN receptor, however only the first two discovered, IFN-α and IFN-β, have been studied in depth (9). The IFNs were discovered and so named due to their ability to interfere with viral replication (10). Type I IFNs are pleiotropic cytokines that influence immune responses through the effects on myeloid cells (11, 12), T cells (13, 14), B cells (15), chemokinesis (16), and chemotaxis (17), as well as promote the acquisition of cytotoxic activity by both T and NK cells (18–20).

The prompt induction of type I IFNs during early infection makes this cytokine family important modulators of cells of the innate immune system such as macrophages, dendritic cells and NK cells (21). Indeed, type I IFNs are critical for early NK cell responses to viral infections, such as lymphocytic choriomeningitis virus and murine cytomegalovirus, and are thought to enhance NK cell cytotoxicity, migration, and cytokine production in such models (21). NK cells are also important contributors to tumor immunoediting and immunotherapy. Mice depleted of NK cells are more susceptible to methylcholanthrene (MCA)[3]-induced sarcomas (22), and in this model, NK cells appear to use the NKG2D pathway in part to protect the host from tumor development (23). NK cells have also been implicated in controlling the growth of B cell lymphomas, arising in mice deficient for both perforin and β_2-microglobulin (24), and are able to recognize and kill some tumors with absent or low class I MHC expression (25–27). In addition to their natural protective role in tumor models, NK cells are essential mediators of the antimetastatic effects of a number of recombinant cytokines such as IL-2, IL-12, IL-18, and IL-21 (28–30).

Although type I IFNs have long been known to have antitumor effects, it is only recently that a role for type I IFN in tumor immune surveillance has been described (4). IFNAR1-deficient 129/Sv mice were found to be more susceptible to MCA-induced sarcomas, and transplant experiments demonstrated that tumors arising in IFNAR1-deficient mice exhibit an "unedited" phenotype, as they were rejected in a T cell-dependent manner when transplanted into wild-type (WT) mice. A role for endogenous type I IFN in restricting the growth of transplantable tumors has also been documented by others (31–33), however the mechanisms of suppression are presently unclear.

*Cancer Immunology Program, Trescowthick Laboratories, Peter MacCallum Cancer Centre, St. Andrews Place, East Melbourne, Victoria, Australia; †Department of Pathology and Immunology, Center for Immunology, Washington University School of Medicine, St. Louis, MO 63110; and ‡Centre for Functional Genomics and Human Disease, Monash Institute of Medical Research, Monash University, Clayton, Victoria, Australia

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[2] Address correspondence and reprint requests to Dr. Mark Smyth, Cancer Immunology Program, Peter MacCallum Cancer Centre, Locked Bag 1, A'Beckett St., 8006, Victoria, Australia. E-mail address: mark.smyth@petermac.org

[3] Abbreviations used in this paper: MCA, methylcholanthrene; WT, wild type; 3LL, Lewis lung carcinoma; asGM1, asialo GM1; pDC, plasmacytoid dentritic cell.

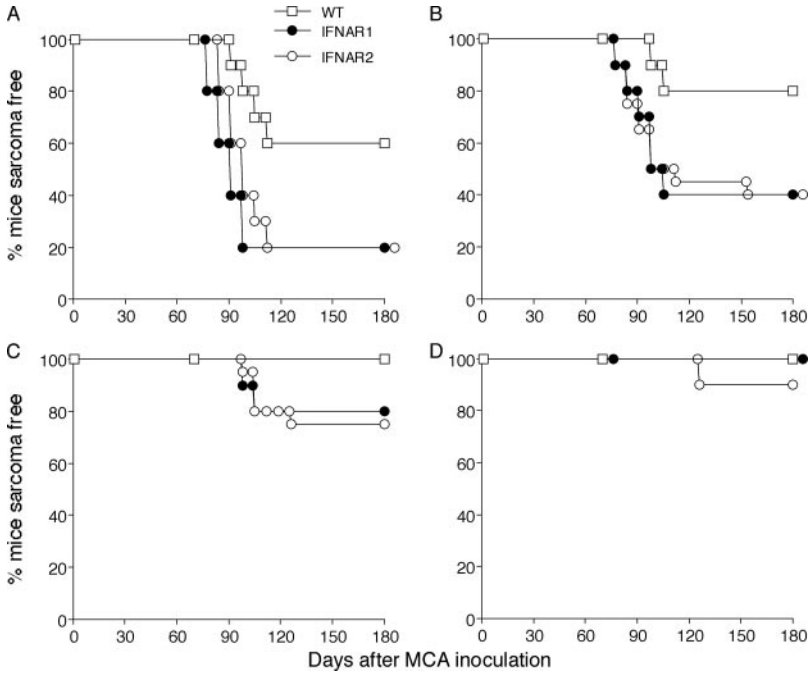

FIGURE 1. Endogenous type I IFN signaling is required for protection from carcinogen-induced sarcomas. WT, IFNAR1$^{-/-}$, and IFNAR2$^{-/-}$ mice were injected with 100 μg (*A*), 25 μg (*B*), 5 μg (*C*), or 1 μg MCA (*D*) as described in *Materials and Methods* and subsequently monitored for tumor development over 200 days (n = 10–20 mice/group).

In this study, we characterize the NK cell response in both IFNAR1- and, for the first time, in IFNAR2-deficient mice, because recent studies have shown that the IFNAR1 and IFNAR2 chains can have distinct functions in controlling immune responses (34). Our studies have demonstrated that endogenous type I IFN is critical for controlling NK cell-mediated antitumor responses in a number of experimental tumor models, including protection from MCA-induced sarcomas, resistance to the NK cell-sensitive, class I low RMA-S tumor, and cytokine immunotherapy of lung metastases. Ab neutralization and depletion experiments demonstrated that type I IFN signaling was essential early in the NK cell-mediated antitumor immune response. Cytokine immunotherapy using IL-12, IL-18, or IL-21 was effective in the absence of endogenous type I IFN, however the antimetastatic activity of IL-2 was abrogated in IFNAR-deficient mice, primarily due to a defect in IL-2-induced cytotoxic activity.

Materials and Methods

Mice and cell lines

C57BL/6 (B6) mice were purchased from the Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). IFNAR1$^{-/-}$ (2) and IFNAR2$^{-/-}$ (34) mice were backcrossed 10 times onto the B6 background before use in experiments. Perforin, IFN-γ, IL-12, IL-18, IL-12/IL-18, and TAP1 gene-targeted mice have been previously described, and are all on the B6 background. All gene-targeted mice were bred at the Peter MacCallum Cancer Centre (Melbourne, Australia). Cell lines RMA-S, RMA-S MSVC, RMA-S Rae1β, Lewis lung carcinoma (3LL), and B16F10 (all H-2^b) and Yac-1 (H-2^a) were maintained in RPMI 1640, supplemented with 10% FCS, penicillin/streptomycin, and L-glutamine.

Antibodies

Anti-asialo GM1 (asGM1) for depletion of NK cells was obtained from Wako Pure Chemical. IFNAR1-blocking Ab for in vivo experiments was purified from the hybridoma MAR1–5A3 (4, 35). In some experiments, the mAb GIR-208 (36) was used as a control for MAR1–5A3 treatment. Abs for flow cytometry anti-NK1.1-allophycocyanin, anti-TCR-β-FITC or PE, anti-CD122-FITC, anti-NKG2D-PE, anti-CD11b-FITC, anti-CD94-FITC, anti-Ly49 C plus I-FITC, anti-H-2D^b-biotin, anti-H-2K^b-PE, anti-IFN-γ-allophycocyanin, and relevant isotype control Abs were purchased from BD Pharmingen or eBioscience.

In vivo tumor assays

For induction of carcinogen-induced fibrosarcomas, male mice were injected in the hind flank with the indicated amount of 3-methycholanthrene (Sigma-Aldrich) in corn oil. Mice were monitored weekly for tumor growth over a period of 250 days. For RMA-S tumor challenges, single-cell suspensions were prepared in PBS and injected s.c. in the hind flank. The day of tumor onset was identified as the day at which a palpable tumor was first detected. In some experiments, NK cells were depleted by i.v. injection of anti-asGM1, starting on the indicated day, and subsequently repeated once per week thereafter. Blocking of the IFNAR1 receptor was achieved by i.p. injection of 0.5–1.0 mg purified MAR1–5A3 every 3 days. For lung and liver metastasis assays, single-cell suspensions of 3LL or B16F10 were prepared in PBS, and 5×10^5 cells were injected i.v. in the tail vein of recipient mice (for lung metastasis) or intrasplenically (for liver metastasis). Cytokine treatment was commenced on the day of tumor injection, and all cytokines were administered i.p. at the following dosing schedules: IL-2, 100,000 U/day for 5 days; IL-12, 50 ng/day for 5 days; IL-18, 2 μg/day for 5 days, and IL-21, 20 μg/day for 4 days. IL-2 was a gift from Chiron. IL-12 was provided by Genetics Institute, IL-18 was obtained from GlaxoSmithKline, and IL-21 was provided by ZymoGenetics. Lungs or livers were harvested on day 14 and fixed in Bouin's solution, and tumor nodules were counted with the aid of a dissection microscope.

Cell preparations, NK cell culture, and cytotoxicity assays

Single-cell suspensions were prepared from spleens and depleted of erythrocytes by ammonium chloride lysis. For purification of NK cells by FACS, splenocytes were depleted of T cells by incubation of anti-Thy1.1 and subsequent treatment with rabbit complement. The remaining cells were stained for NK1.1 and TCR, and NK1.1$^+$TCR-β^- cells were sorted on a FACSDiva (BD Biosciences). For purification of NK cells by MACS, splenocytes were prepared and depleted of T cells as above, then incubated with anti-DX5-coated MACS beads (Miltenyi Biotec), and cells were sorted by autoMACS (Miltenyi Biotec). NK cell medium consisted of RPMI 1640 containing 10% FCS, 2-ME, nonessential amino acids, sodium pyruvate, L-glutamine, and penicillin/streptomycin. NK cells were expanded in NK cell medium containing 500 U/ml IL-2 for 5 days, split 1/2 in medium with fresh cytokine, and cultured for an additional 5 days. For IFN-α stimulation, splenocytes were stimulated overnight in NK cell medium containing IL-2, IFN-α, or both at 100 U/ml. For IL-12 and IL-18 stimulation of IFN-γ production, total splenocytes were incubated for 6 h in NK cell medium containing 100 ng each of both cytokines. Golgistop was added for the final 2 h. Liver mononuclear cells were prepared by resuspending liver cell suspensions in 33% Percoll and centrifuging at 500 $\times$ g for 15 min,

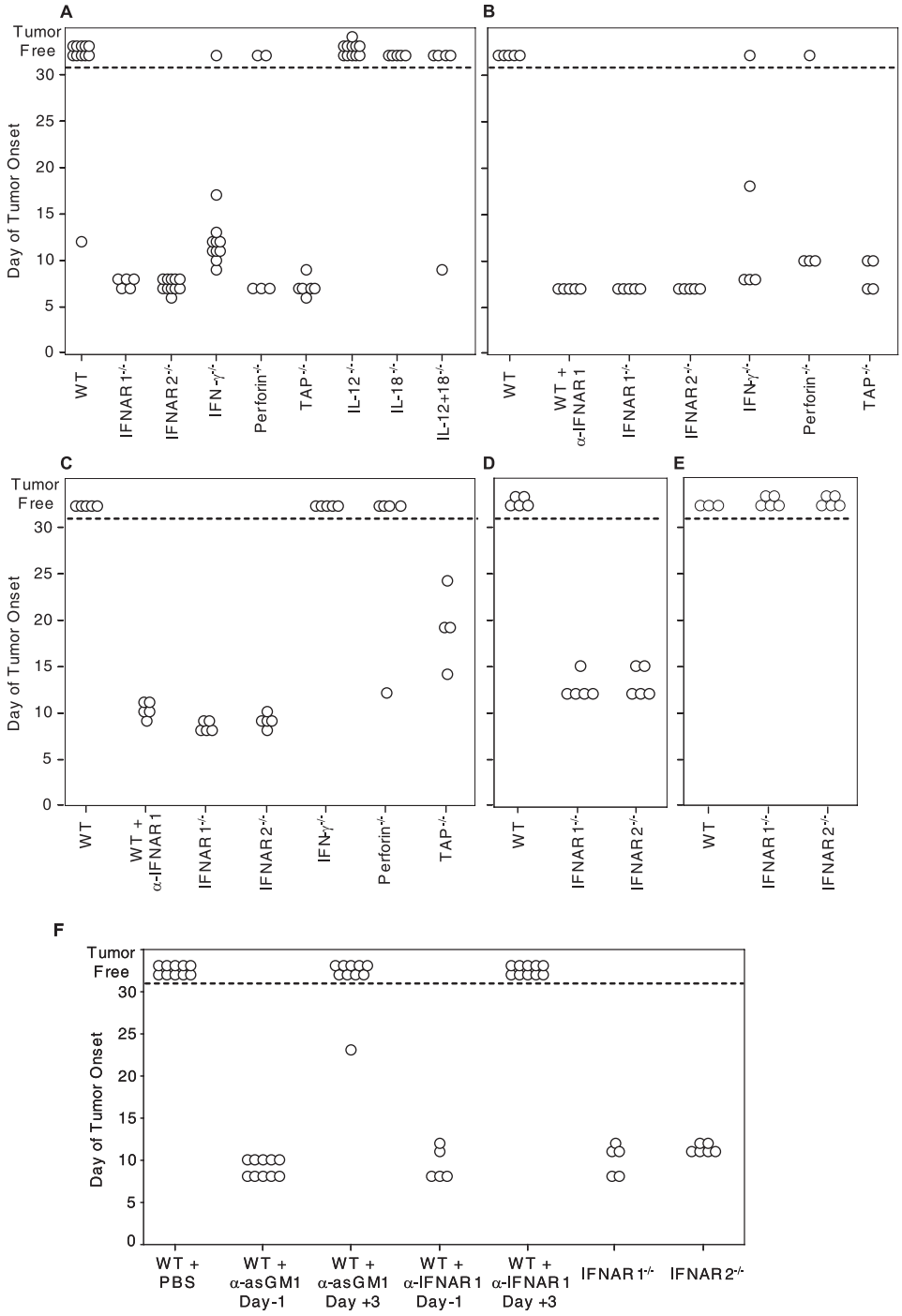

FIGURE 2. Requirements for the rejection of RMA-S lymphomas. Mice of the indicated genotype were challenged s.c. in the hind flank with 1×10^5 (*A*), 5×10^4 (*B*), 1×10^4 (*C*), 1×10^3 (*D*), and 1×10^2 (*E*) RMA-S tumor cells on day 0 and subsequently monitored for tumor onset. In *C* and *D*, the type I IFN pathway was blocked by treatment with 1 mg of MAR1–5A3 every 3 days, starting on day −1. Control mice received PBS. *F*, WT or IFNAR-deficient mice were challenged s.c. in the hind flank with 1×10^4 RMA-S tumor cells on day 0 and subsequently monitored for tumor onset. NK cells were depleted by treatment with anti-asGM1 starting on the indicated day, and for blocking of the type I IFN pathway, mice were injected with 1 mg of MAR1–5A3 every 3 days, starting on the indicated day. In all plots, each data point represents an individual mouse, and data points falling above the dashed line represent mice that remained tumor free after 60 days.

after which cell pellets were collected, washed, and resuspended in the appropriate medium for further analysis. Cytotoxicity was assessed by standard 4-h chromium release assays.

FACS analysis

Splenocytes were prepared as above and resuspended in FACS buffer consisting of 0.5% BSA in PBS with 0.04% sodium azide. For surface staining, 1×10^6 cells per sample were stained with the appropriate Abs in the presence of 2.4G2 (anti-CD16/32, to block Fc receptors) on ice, then washed and resuspended in FACS buffer containing Fluorogold for the determination of viable cells. Intracellular staining for the detection of IFN-γ was performed using the Cytofix/Cytoperm buffer system (BD Pharmingen), as per the manufacturer's instructions. Samples were acquired on a LSR II (BD Biosciences Pharmingen) and analyzed using FCS Express (De Novo Software).

Statistical analysis

Statistical significance was assessed through the use of the Mann-Whitney rank-sum test, Student's t test, or Fischer's exact test as appropriate.

Results

Type I IFN protects mice from carcinogen-induced sarcomas

Type I IFNs are potent regulators of the innate immune system, which plays a critical role in the control of MCA-induced sarcomas. To investigate the role of type I IFNs in the immunoediting of carcinogen-induced tumors, IFNAR1$^{-/-}$ and IFNAR2$^{-/-}$ and WT B6 male mice were injected s.c. with 100, 25, 5, or 1 μg of MCA, and then monitored for tumor onset

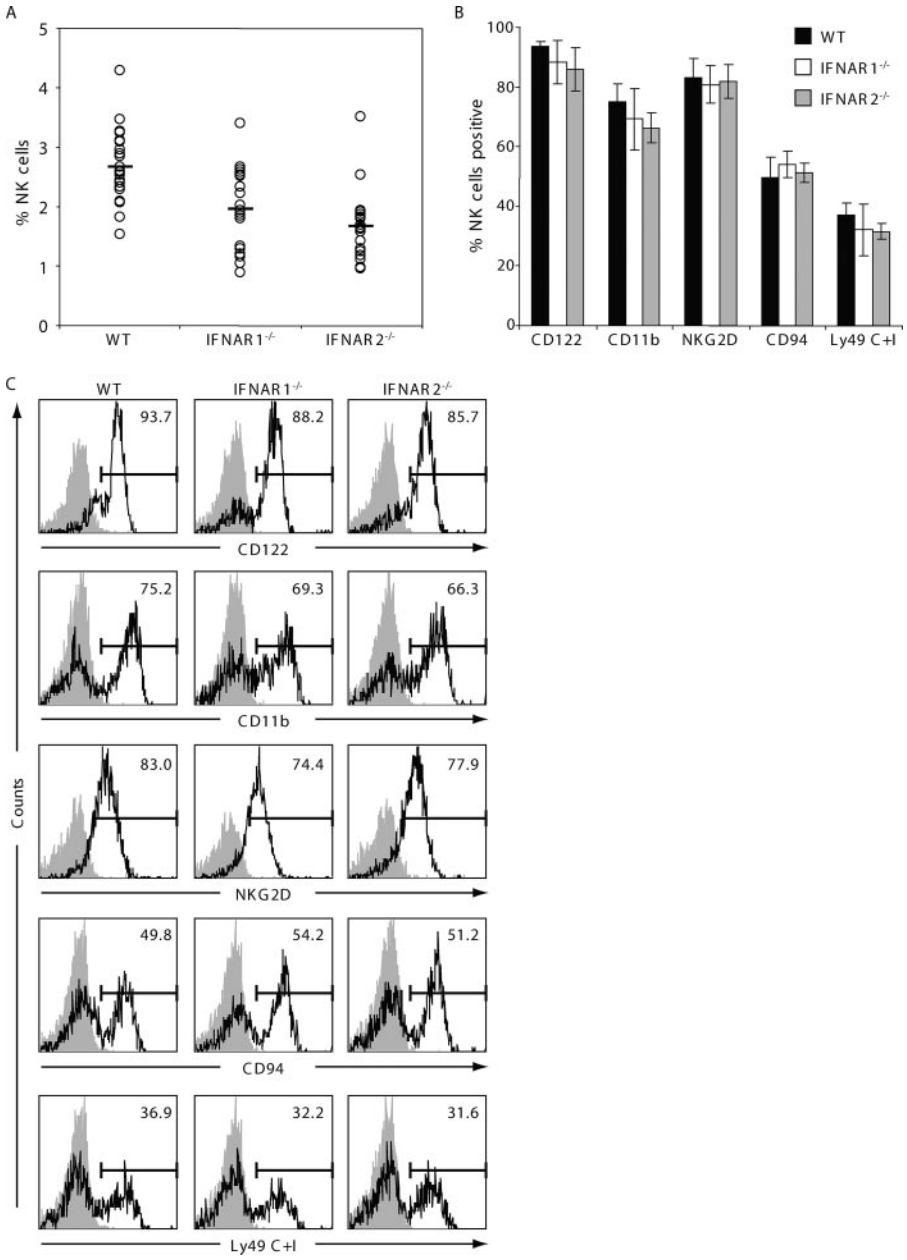

FIGURE 3. NK cell proportions and phenotype in IFNAR-deficient mice. *A*, Splenocytes were harvested from WT, IFNAR1$^{-/-}$, and IFNAR2$^{-/-}$ mice and NK cell proportions were determined by flow cytometry. NK cells were identified as TCR^{-}NK1.1^{+}. Each data point indicates an individual mouse. Horizontal bars, Mean for each group (n = 19–22/genotype). *B*, Gated NK cells from the spleens of WT and IFNAR-deficient mice were examined for the expression of the indicated surface markers. Data are presented as the percentage of NK cells positive for a given marker (±SD) (n = 7–10/genotype). Plots showing representative staining for the indicated NK cell markers (black tracings), compared with control staining (gray) are depicted in *C*. Plots are gated on TCR^{-}NK1.1^{+} events, and are displayed as the number of cells vs fluorescence intensity. Numbers in the top right-hand corner of each plot indicate the percentage of NK cells falling within the marker displayed.

and survival over a period of 250 days (Fig. 1). Both IFNAR1- and IFNAR2-deficient mice displayed increased susceptibility to sarcoma formation when treated with 100, 25, or 5 μg of MCA (Fig. 1, *A–C*). Differences in the number of tumor-free WT and IFNAR-deficient mice were significant at 25 μg (WT vs IFNAR1$^{-/-}$, p = 0.045 and WT vs IFNAR2$^{-/-}$, p = 0.023, by Fischer's exact test), and between WT and IFNAR2$^{-/-}$ mice at 5 μg (p = 0.047). Because NK cells play a prominent role in protecting mice from MCA-induced tumors (22), we next examined a simplified model that more directly investigated the role of type I IFNs in the NK cell antitumor response.

Type I IFN is required for resistance to RMA-S lymphomas

RMA-S is a variant of the RMA thymoma that is sensitive to NK cell-mediated rejection in vivo due to low class I MHC expression (25). To investigate the role of type I IFN in controlling RMA-S tumor growth, and to compare the importance of this cytokine family to other effector molecules known to be involved in NK cell effector function and maturation, we tested the ability of a sublethal dose of RMA-S (1×10^5/mouse) to grow s.c. in a panel of different gene-targeted mice (Fig. 2*A*). At this dose of cells, only 9% (1 of 11 of mice injected) of WT mice succumbed to tumor growth, whereas 100% of IFNAR1$^{-/-}$ (5 of 5), IFNAR2$^{-/-}$ (11 of 11), and TAP1$^{-/-}$ (5 of 5) mice developed tumors. IFN-$\gamma^{-/-}$ and perforin-deficient mice also demonstrated increased susceptibility to RMA-S growth, with 91% (10 of 11) and 60% (3 of 5) of gene-targeted mice developing tumors, respectively. IL-12 and IL-18, which are potent inducers of IFN-γ, were dispensable for protection from sublethal doses of RMA-S, as none of the mice deficient for these molecules developed tumors (Fig. 2*A*). Mice deficient for both IL-12 and IL-18 together were also resistant to RMA-S, with only 1/5 mice developing a tumor. To further investigate the relative contributions of the various effector pathways identified as important for control of RMA-S tumors in the original screen, gene-targeted mice were challenged with graded doses of RMA-S tumor cells (5×10^4, 10^4, 10^3, and 10^2; Fig. 2, *B–E*, respectively) and monitored for tumor onset. At the 10^4 cells/

mouse dose, IFNAR1- and IFNAR2-deficient mice were the most susceptible to RMA-S growth, because all IFNAR-deficient mice developed tumors, while the majority of IFN-$\gamma^{-/-}$ or perforin$^{-/-}$ mice were protected from RMA-S growth (Fig. 2*C*). In agreement with previous observations (37), TAP1$^{-/-}$ mice, which have defects in NK cell maturation, were also susceptible to tumor growth, with 100% (4 of 4) of mice injected with 10^4 RMA-S cells developing tumors. TAP1$^{-/-}$ mice were slightly more resistant to tumor growth than IFNAR-deficient mice however, because palpable tumors took longer to arise in TAP1$^{-/-}$ mice (19.0 $\pm$ 3.5 days) compared with both IFNAR1$^{-/-}$ (8.4 $\pm$ 0.5) and IFNAR2$^{-/-}$ mice (9.0 $\pm$ 0.6) (Fig. 2*C*). As few as 10^3 RMA-S cells were sufficient for tumor initiation in 100% (5 of 5) of the challenged IFNAR-deficient mice (Fig. 2*D*), but extremely low doses of RMA-S (10^2 cells) were rejected (Fig. 2*E*). Additionally, WT mice injected with a monoclonal IFNAR1-specific blocking Ab (MAR1–5A3; Ref. 35) were highly susceptible to the growth of RMA-S tumors, indicating that the increased susceptibility of IFNAR-deficient mice was not due to conditional effects of gene targeting or genetic background (Fig. 2, *B* and *C*).

Type I IFN acts early in protection from RMA-S challenge

We next used the IFNAR1-blocking Ab to define when type I IFNs act to promote clearance of RMA-S. WT mice were treated with anti-asGM1 to deplete NK cells or anti-IFNAR1 to block type I IFN signaling, starting either 1 day before or 3 days after challenge with 1×10^4 RMA-S cells, a dose sufficient to cause tumors in 100% of IFNAR-deficient mice, but not in untreated WT mice. Depletion of NK cells 1 day before tumor challenge rendered WT mice susceptible to tumor growth, with all (10 of 10) NK cell-depleted mice developing tumors (compared with 0 of 10 PBS-treated WT mice) (Fig. 2*F*). Interestingly, when NK cell depletion was delayed until 3 days posttumor challenge, only 1 of 10 of the WT mice succumbed to tumor growth, demonstrating that NK cells act rapidly in this model to clear tumor cells. These kinetics were matched when mice were treated with an IFNAR1-blocking Ab; when treatment was commenced before tumor challenge, 5 of 5 mice grew tumors, whereas none (0 of 10) of the WT mice treated with blocking Ab starting on day 3 succumbed to tumor growth (Fig. 2*F*). To ensure that type I IFN signaling was completely blocked in WT mice, the experiment was repeated using a higher dose of mAb (2.5 mg/mouse, given as a single dose on days -1 or $+3$ relative to tumor challenge). Using this dose, WT mice treated with MAR1–5A3 on day -1 succumbed to RMA-S tumor growth, whereas mice treated with MAR1–5A3 on day $+3$, or the control mAb GIR-208 were resistant (data not shown). These results demonstrate that NK cells and type I IFN act early in the antitumor immune response to RMA-S.

NK cell proportions, phenotype, and function in IFNAR-deficient mice

Given the sensitivity of both IFNAR1$^{-/-}$ and IFNAR2$^{-/-}$ mice to RMA-S tumor growth, we next investigated the proportions, phenotype, and function of NK cells in IFNAR-deficient mice. Flow cytometry analysis demonstrated that the proportion of NK1.1$^+$TCR$^-$ cells in the spleen of both strains of IFNAR-deficient mice was significantly decreased ($p = 0.0026$, WT vs IFNAR1$^{-/-}$; $p < 0.0001$, WT vs IFNAR2$^{-/-}$, by Mann-Whitney rank-sum test) (Fig. 3*A*). NK cells were further analyzed for expression of the NK cell surface markers CD122, CD11b, NKG2D, CD94, and Ly49 C/I. No striking alteration in the expression of the tested markers was detected, however IFNAR-deficient mice expressed slightly lower levels of CD122, CD11b, and Ly49 C+I

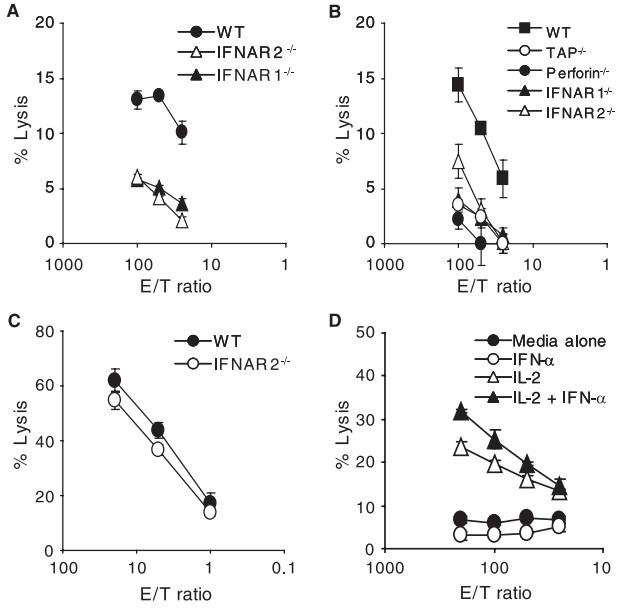

FIGURE 4. Natural cytotoxicity in IFNAR-deficient mice. *A* and *B*, Splenocytes from mice of the indicated genotypes were tested for ex vivo cytotoxicity against Yac-1 targets in a standard 4-h ^{51}Cr release assay. *C*, NK cells were sorted from the spleen of WT and IFNAR2$^{-/-}$ mice, and then tested for cytotoxicity against Yac-1 targets. *D*, Splenocytes from WT mice were incubated overnight with IL-2 and IFN-α as indicated in *Materials and Methods*, and subsequently tested for cytotoxic activity against RMA-S targets. Each data point is the mean of triplicate wells $\pm$ SD for each E:T ratio.

(Fig. 3, *B* and *C*). This reduction was not significant in the case of IFNAR1$^{-/-}$ mice, however in the case of IFNAR2$^{-/-}$ NK cells, the reduction in CD122 ($p = 0.007$), CD11b ($p = 0.005$), and Ly49 C+I ($p = 0.001$) was statistically significant, although the percentage of IFNAR2$^{-/-}$ NK cells staining positive for these markers was variable between mice. The natural cytotoxicity of IFNAR-deficient splenocytes was tested against the classical NK cell target Yac-1. Killing by ex vivo splenocytes from both IFNAR1- and IFNAR2-deficient mice was typically reduced by half or more (Fig. 4*A*), to levels similar to that observed for perforin and TAP$^{-/-}$ mice (Fig. 4*B*), which have defective NK cell cytotoxicity against Yac-1 targets (26, 37). As NK cell proportions were decreased in IFNAR-deficient mice, the cytotoxic activity of sorted NK cells against Yac-1 was tested to determine whether the decreased killing was due to the number of NK cells present or a defect in cytotoxic function. In two independent experiments, NK cells sorted from IFNAR2$^{-/-}$ mice were able to kill Yac-1 targets in a similar fashion to WT NK cells (Fig. 4*C*), demonstrating that resting IFNAR2-deficient NK cells from B6 mice express the necessary recognition and effector molecules to induce cell death, at least in the case of Yac-1 targets. RMA-S cells are poor targets for naive NK cells (data not shown), however cytokine activated NK cells kill these targets efficiently. Culture of WT splenocytes in 100 U/ml IL-2 overnight stimulated efficient killing of RMA-S, and this killing was enhanced significantly ($p < 0.05$ at all except the lowest E:T ratio, by Student's t test) by the addition of IFN-α (Fig. 4*D*). IFN-α alone was unable to induce cytotoxic activity against RMA-S, as it was unable to support NK cell survival in vitro (Fig. 4*D* and data not shown). These data demonstrate that type I IFN is required to maintain optimal numbers of NK cells during homeostasis and can significantly enhance killing induced by cytokine treatment, even though it is not essential for cytotoxic activity against some NK cell targets.

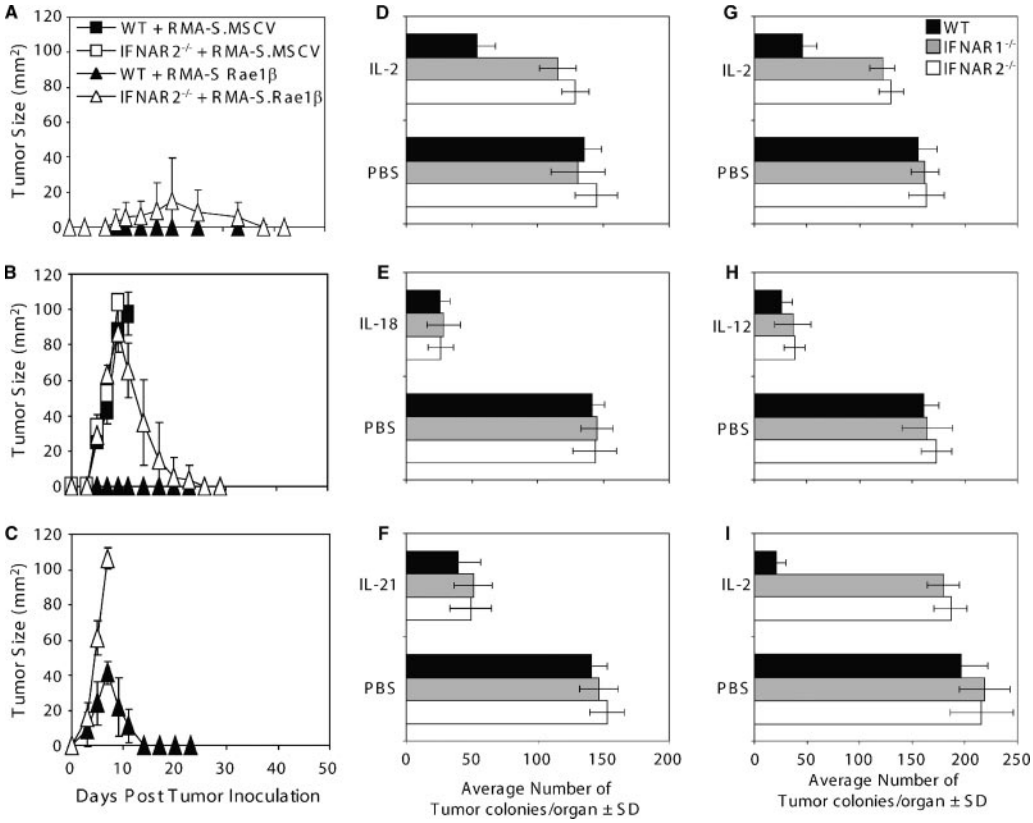

FIGURE 5. NK cell antitumor responses induced in IFNAR2-deficient mice. WT and IFNAR2$^{-/-}$ mice were challenged s.c. with either 5×10^4 (*A*), 5×10^5 (*B*), or 5×10^6 (*C*) RMA-S MSCV (■, WT mice and □, IFNAR2$^{-/-}$ mice) or RMA-S Rae1β cells (▲, WT mice and △, IFNAR2$^{-/-}$ mice) as indicated and subsequently monitored for tumor growth. Data are presented as tumor area ± SD ($n = 4–6$ mice/group). *D–H*, WT (black bars), IFNAR1$^{-/-}$ (gray bars), and IFNAR2$^{-/-}$ mice (white bars) were injected i.v. with 5×10^5 3LL (*D–F*) or B16F10 (*G* and *H*) tumor cells and treated with either PBS or the indicated cytokines as described in the *Materials and Methods*. *I*, WT, IFNAR1$^{-/-}$, and IFNAR2$^{-/-}$ mice were injected intrasplenically with 5×10^5 B16F10 tumor cells to establish liver metastases, and mice were treated with IL-2 as described in the *Materials and Methods*. Lungs or livers were harvested on day 14, fixed in Bouin's solution and tumor colonies were counted under a dissecting microscope. Data are depicted as mean tumor colonies/organ ± SD (n = 5–10 mice/group). The key depicted in *G* applies to all plots *D–I*.

Induction of antitumor NK cell responses in IFNAR2-deficient mice

Ectopic expression of the NKG2D-ligand Rae1β by RMA-S cells leads to NK cell-mediated tumor rejection through the perforin pathway (38). As IFNAR-deficient mice were highly susceptible to growth of the parental RMA-S tumor line (which does not express any NKG2D ligands), we investigated whether expression of Rae1β would be able to induce rejection of RMA-S tumors in IFNAR2$^{-/-}$ mice. WT and IFNAR2$^{-/-}$ mice were challenged with graded doses of RMA-S Rae1β and monitored for tumor growth or rejection. RMA-S Rae1β cells were rejected in WT mice, with no palpable tumors detected when mice were challenged with 5×10^4 (Fig. 5*A*) or 5×10^5 (Fig. 5*B*) cells. In contrast, two of six IFNAR2$^{-/-}$ mice developed tumors even at the lowest dose, and all IFNAR2$^{-/-}$ mice developed tumors when challenged with 5×10^5 RMA-S Rae1β cells. At this dose, tumors in IFNAR2$^{-/-}$ mice grew to 86.7 ± 5.6 mm^2 (mean ± SD) on day 9, after which they regressed completely (Fig. 5*B*). WT mice developed tumors only at the highest dose of RMA-S Rae1β tumor cells tested (5×10^6), and these tumors grew to 41.2 ± 6.1 mm^2 on day 7 before regressing (Fig. 5*C*). When IFNAR2$^{-/-}$ mice were challenged with the same dose of cells, they developed progressive tumors that grew to 106.6 ± 6.1 mm^2 by day 7, at which point the mice had to be sacrificed (Fig. 5*C*). The increased growth of RMA-S Rae1β in IFNAR-deficient mice demonstrates that although tumor rejection via the NKG2D pathway can be induced in IFNAR2-deficient mice, rejection occurs with slower kinetics compared with that seen in WT mice.

Requirement for endogenous type I IFN in cytokine immunotherapy

Rejection of lung metastasis following treatment with the recombinant cytokines IL-2, IL-18, IL-12, and IL-21 is mediated largely by NK cells. We therefore investigated the requirement for endogenous type I IFN for the treatment of 3LL or B16F10 lung metastasis with cytokine therapies. Treatment of lung metastases with IL-2 was significantly compromised in both IFNAR1- and IFNAR2-deficient mice. In WT mice, treatment with IL-2 reduced the lung tumor burden by 60.1% ($p < 0.005$, compared with PBS treated controls) for 3LL (Fig. 5*D*) and 70.5% ($p < 0.005$) for B16F10 (Fig. 5*G*) tumors. IL-2, however, failed to induce a significant reduction in 3LL lung colonies in IFNAR1$^{-/-}$ and IFNAR2$^{-/-}$ mice (11.8 and 11.3% reduction respectively, p = 0.20 and 0.09), and was less effective at reducing B16F10 metastasis (25.1 and 20.4% reduction). Similar results were also seen in the liver, where IL-2 treatment induced a 89.6% reduction in B16F10 liver metastases in WT mice, but only 17.9 and 13.5% reductions in tumor colony number in IFNAR1$^{-/-}$ and IFNAR2$^{-/-}$ mice, respectively (Fig. 5*I*). In contrast, IL-12 (Fig. 5*H*), IL-18 (Fig. 5*E*), and IL-21 (Fig. 5*F*) were able to induce significant ($p < 0.005$ in all cases, when cytokine-treated mice

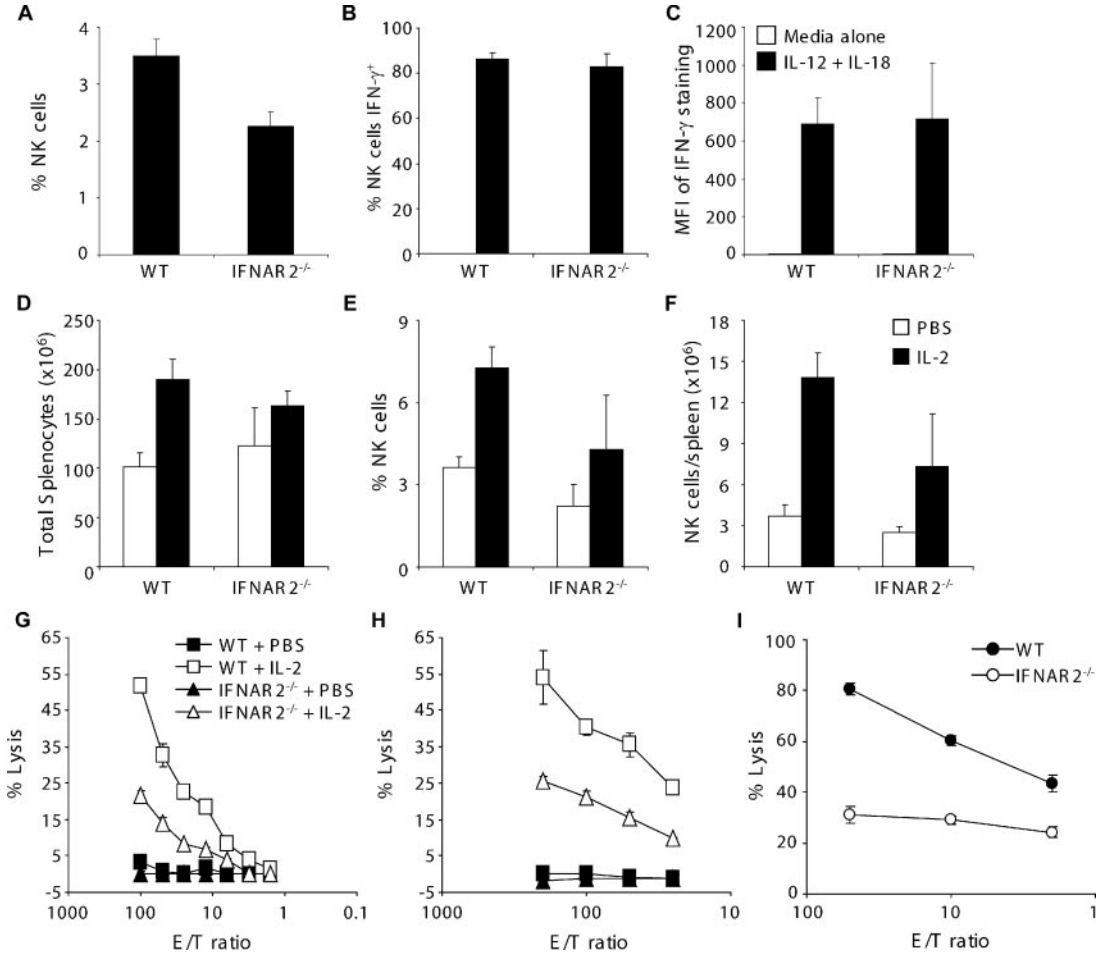

FIGURE 6. Cytokine-induced responses by IFNAR2-deficient NK cells. Splenocytes were harvested from WT and IFNAR2$^{-/-}$ mice and incubated in medium alone, or stimulated in vitro for 6 h with 100 ng/ml each of IL-12 and IL-18. Following incubation, NK cell proportions (*A*) and the percentage of NK cells staining positive for IFN-γ (*B*) were assessed by intracellular cytokine staining. The mean fluorescent index for NK cell IFN-γ staining is shown in *C*. The key depicted in *C* applies to both *C* and *D* (□ for cells culture in medium alone; ■, for cytokine-treated cells), however open columns are not visible as no IFN-γ production was observed by cells cultured in medium alone. Data for *A–C* are pooled from two independent experiments and are depicted as the mean ± SD (total of six mice/group). *D–H*, WT and IFNAR2$^{-/-}$ mice were treated i.p. with 1×10^5 U of IL-2 for 5 consecutive days and splenocytes were harvested the day after the final dose. Total splenocytes (*D*), NK cell proportions (*E*), and NK cell numbers (*F*) were determined by trypan blue counts and flow cytometry. The key depicted in *F* applies to *D–F* (□, PBS-treated mice; ■, IL-2-treated mice). Splenocytes were harvested and analyzed from six individual mice/treatment/group (pooled from two independent experiments) and data are presented as the mean ± SD for each parameter. Splenocytes (*G*) and liver mononuclear cells (*H*) from each treatment group were pooled and tested for ex vivo cytotoxicity against RMA-S targets in standard 4-h ^{51}Cr release assays. The key depicted in *G* applies to *G* and *H*. *I*, NK cells were sorted from the spleens of WT and IFNAR2$^{-/-}$ mice and cultured for 10 days in medium containing 500 U/ml IL-2, and then tested for cytotoxic activity against RMA-S MSCV targets. For cytotoxicity assays (*G–I*), each data point is the mean of triplicate wells ± SD for each E:T ratio.

were compared with PBS-treated controls of the same genotype) antimetastatic immune responses in both WT and IFNAR2$^{-/-}$ mice, suggesting that there is a specific defect in IL-2-driven immune responses in IFNAR2$^{-/-}$ mice.

Cytokine-induced functions of IFNAR-deficient NK cells

Because IL-12 and IL-18 were able to induce effective antitumor immune responses in IFNAR2$^{-/-}$ mice, while IL-2 was unable to do, so we investigated their ability to induce NK cell effector functions. IL-12 and IL-18 are primarily known as inducers of IFN-γ, so we tested the ability to of these cytokines to trigger NK cell IFN-γ secretion. Ex vivo splenocytes from WT or IFNAR2$^{-/-}$ mice were incubated with a combination of IL-12 and IL-18 for 6 h, then analyzed for IFN-γ production by flow cytometry. As observed previously, the proportion of NK cells among IFNAR2$^{-/-}$ splenocytes was reduced compared with WT (Fig. 6*A*), however the NK cells present were able to respond to IL-12 and IL-18 by secreting IFN-γ in a similar fashion to NK cells from WT mice. No significant differences in the proportion of NK cells secreting IFN-γ (Fig. 6*B*), and the mean fluorescent intensity of the IFN-γ staining (Fig. 6*C*) were observed between the WT and IFNAR2$^{-/-}$ NK cells. The ability of IL-2, which failed to induce antimetastatic activity in IFNAR2$^{-/-}$ mice, to induce NK cell responses was also further characterized. WT and IFNAR2$^{-/-}$ mice were injected with 100,000 U of IL-2 daily for 5 days, and spleens were harvested and analyzed on day 5 for NK cell proliferation and cytotoxic activity. No significant difference in spleen cellularity was observed between control WT and IFNAR2$^{-/-}$ mice (Fig. 6*D*). Following IL-2 treatment, the cellularity of the spleen increased in WT and IFNAR2$^{-/-}$ mice, however the extent of the proliferation was greater in WT mice, with a 1.9-fold increase in splenocyte number, compared with a 1.3-fold increase in IFNAR2$^{-/-}$ mice (Fig. 6*D*). After IL-2 treatment, NK cells constituted 7.28 ± 0.74% (average ± SD) of splenocytes in WT mice,

but only 4.30 ± 1.97% of IFNAR2$^{-/-}$ splenocytes, however the fold increase in NK cell proportions was similar in both genotypes (2.01-fold and 1.96-fold for WT and IFNAR2$^{-/-}$ mice, respectively) (Fig. 6*E*). The total number of splenic NK cells was decreased in IFNAR2$^{-/-}$ mice compared with WT, both before (2.47 ± 0.46 and 3.7 ± 0.81 × 10^6 per spleen, respectively) and after IL-2 (7.27 ± 3.82 × 10^6 and 13.80 ± 1.85 × 10^6) (Fig. 6*F*), and the fold expansion was slightly lower (2.95-fold and 3.73-fold increases, respectively). Although treatment with IL-2-enhanced cytotoxic activity against RMA-S in both the spleen (Fig. 6*G*) and the liver (Fig. 6*H*), IL-2 failed to induce the same level of cytotoxic activity in IFNAR2$^{-/-}$ mice compared with WT mice. We further investigated the ability of IL-2 to induce cytotoxic activity in FACS-sorted (NK1.1$^+$TCR$^-$) NK cells from WT and IFNAR2-deficient mice. Killing of RMA-S MSCV by sorted IFNAR2$^{-/-}$ NK cells cultured in IL-2 for 10 days was significantly compromised in comparison to WT NK cells (Fig. 6*I*). IFNAR2$^{-/-}$ NK cells purified by MACS sorting and cultured in the same fashion also displayed defective killing of RMA-S targets (data not shown). The compromised ability of IL-2 to induce NK cell cytotoxic activity in IFNAR2-deficient hosts correlates with the reduced capacity of IL-2 to prevent lung metastases, however it remains possible that additional defects may also diminish the IL-2-induced antitumor immune response in IFNAR2-deficient mice.

Discussion

In this study, we have examined the role of type I IFN in regulating NK cell-mediated antitumor responses, and demonstrate that these responses are significantly compromised in IFNAR-deficient mice. We also demonstrate a previously undocumented deficiency in homeostatic NK cell numbers in mice lacking endogenous type I IFN signaling. In the RMA-S model, type I IFNs were the most important factor for the elimination of tumor cells when compared with a panel of other effector molecules. Endogenous type I IFN was essential early in the NK cell-mediated antitumor immune response to RMA-S. Cytokine immunotherapy with IL-2 was significantly compromised in IFNAR-deficient mice, but IL-12, IL-18, and IL-21 were able to exert antimetastatic effects in the absence of endogenous type I IFN signaling, despite the diminished number of NK cells present.

Our findings that B6 IFNAR-deficient mice have increased susceptibility to MCA sarcomas strongly supports previous findings, demonstrating an important role for type I IFN in immunosurveillance in IFNAR1-deficient 129/Sv mice (4). The increased susceptibility of IFNAR1$^{-/-}$ and IFNAR2$^{-/-}$ mice to MCA might be (partially) due to the decreased NK cell numbers observed in IFNAR-deficient mice, however whether or not this or other defects are responsible for the increased tumor induction will require further clarification. It is interesting to note that a recent report has documented a high expression of MxA, indicative of local type I IFN production, as well as the presence of a plasmacytoid dendritic cell (pDC) infiltrate, in spontaneously regressing lesions of human melanoma patients (39), providing circumstantial evidence that a tumor immune surveillance network similar to that seen in the mouse may be at play in humans.

IFNAR-deficient mice were susceptible to an ~500-fold lower dose of RMA-S tumor than WT mice, and as few as 1000 RMA-S cells caused tumors in 100% of mice lacking type I IFN signaling. This finding would suggest that the compromised ability of IFNAR-deficient mice to clear RMA-S tumors is not simply due to the observed reduction in NK cell proportions, as while NK cells were reduced by approximately one-half, tumors could be induced by ~500-fold lower doses of tumor cells than are needed to successfully induce tumors in WT mice. The investigation of the basis for diminished NK cell-mediated immunity demonstrated that NK cell proportions were diminished in the spleen of IFNAR-deficient mice, and splenic natural cytotoxicity against Yac-1 targets by both IFNAR1- and IFNAR2-deficient B6 mice was reduced when total splenocytes were used. However, sorted NK cells from WT and IFNAR$^{-/-}$ mice had equivalent cytotoxic activity against Yac-1 targets when tested by in vitro chromium release assays. This finding demonstrated that naive IFNAR-deficient NK cells are capable of inducing Yac-1 apoptosis in an equivalent fashion to NK cells from WT animals, however we cannot exclude the possibility that the killing of other targets by resting NK cells may be defective. It has previously been reported that killing by IFNAR1$^{-/-}$ NK cells cultured in IL-2 was defective (19), and we have confirmed these findings in IFNAR2$^{-/-}$ mice. Together, these data suggest baseline cytotoxic activity is independent of endogenous type I IFN, but efficient IL-2-induced killing requires type I IFN signaling.

The reason for the diminished proportion of splenic NK cells, but normal cytotoxicity observed in unstimulated IFNAR-deficient mice, may be due to either a direct or indirect action of type I IFNs. Administration of recombinant IFN-α can stimulate NK cell proliferation, and NK cell expansion during viral infection is associated with type I IFN production, demonstrating that type I IFN itself can act as a growth factor for NK cells in vivo (40). Our data suggest that endogenous type I IFN signaling is also important for maintenance of homeostatic NK cell levels. This mechanism may be indirect, and it has been proposed that type I IFN is an important regulator of the expression of IL-15, a critical cytokine for NK cell survival and proliferation. Blocking IL-15 during IFN-α treatment prevents NK cell proliferation, but does not prevent the acquisition of cytotoxic function (41). This is reminiscent of our data that homeostatic NK cell levels are diminished in IFNAR-deficient mice, but naive NK cell killing of Yac-1 is unimpaired. Members of the type I IFN family, particularly IFN-β, are produced constitutively by a number of cell types, including fibroblasts and macrophages, and these cells may be a source of IFN for maintenance of the NK cell population. It is interesting to note that defects in NK cell development and function have been observed in mice lacking IRF-1, an important regulator or type I IFN expression and action (42, 43).

Neither IFN-α or IFN-γ was able to inhibit the proliferation of RMA-S in vitro (at up to 1000 U/ml, data not shown), so it would seem unlikely that these cytokines prevent tumor growth by a direct antiproliferative effect on tumor cells in vivo. In vitro, RMA-S is a poor target for resting NK cells (unlike other targets such as Yac-1, which are readily killed by naive NK cells), however activation of NK cells with cytokines such as IL-2 allows efficient lysis of RMA-S targets. Type I IFN can also promote the acquisition of NK cell cytotoxic activity, so one mechanism by which endogenous type I IFN may promote immunity to RMA-S in vivo could be to facilitate NK cell activation and cytotoxic activity against RMA-S. This is supported by the finding that IFN-α could enhance killing of RMA-S targets in vitro. Type I IFN was essential only within the first 3 days of RMA-S tumor challenge, during the same window in which NK cells are active. These findings suggest that, in the RMA-S model, type I IFN is necessary for the prompt activation and/or recruitment of NK cells to the site of tumor challenge. It should be noted, however, that in T cell-mediated antitumor immune responses, type I IFN is important throughout the process of tumor rejection (35). These contrasting results likely reflect the different effector populations and rejection kinetics involved in the different models.

Cytokine immunotherapy of experimental lung metastases in IFNAR2-deficient mice revealed distinct requirements for endogenous type I IFN in tumor rejection. IL-2, IL-12, IL-18, and IL-21 all promote rejection of lung metastases through an NK cell-dependent mechanism. IL-2, IL-12, and IL-21 all stimulate perforin-mediated rejection of tumor cells, whereas IL-18 promotes tumor rejection in a FasL-dependent manner (29). IL-12, IL-18, and IL-21 were able to successfully stimulate immunity to lung metastases in IFNAR2-deficient mice, despite the reduced proportions of NK cells in these mice. In contrast, IL-2 failed to induce any significant antitumor response. Analysis of the response of IFNAR-deficient mice to IL-2 demonstrated that whereas NK cells numbers were reduced in these mice, they underwent the same fold expansion as was seen in WT mice. The cytotoxic activity of IL-2-expanded IFNAR2-deficient NK cells was compromised, and this defect could not be explained simply by the number of NK cells present. This finding was further supported by data showing that liver mononuclear cells from IL-2-treated WT and IFNAR2$^{-/-}$ mice contained similar proportions of NK cells (data not shown), but NK cells derived from IFNAR2$^{-/-}$ livers had diminished cytotoxic activity compared with WT, and that sorted IFNAR2$^{-/-}$ NK cells expanded in IL-2-demonstrated defective target cell killing. The defect in IL-2 responses is seemingly independent of signaling via the common γ-chain, as IL-21, another common γ-chain-dependent cytokine, was able to successfully stimulate responses to lung metastases. Similarly, IL-15, another common γ-chain-dependent cytokine, has been reported to induce normal cytotoxic activity by IFNAR1-deficient NK cells in vitro (19). Given that IFNAR-deficient NK cells proliferate normally in response to IL-2, the defect in antimetastatic activity must be due to a failure of IL-2 to stimulate NK cell cytotoxic activity or trafficking in vivo.

An unresolved question raised in this study concerns the source of type I IFN that promotes the antitumor immune response. In the RMA-S model, our attempt to demonstrate a role for pDC in protection from tumor was inconclusive, as we were unable to demonstrate complete blocking or depletion of pDC using the mAbs 440c or 120G8 (data not shown). A 2–3-fold reduction in pDCs, achieved through administration of the 120G8 mAb, was insufficient to allow tumor development in WT mice challenged with low dose RMA-S (data not shown). Due to the broad range of cells capable of producing type I IFN, elucidating the source of this cytokine is a significant undertaking. Conventional dendritic cells, macrophages, and stomal cells, such as fibroblasts, all represent alternative cell types, in addition to NK cells themselves that could contribute to type I IFN production following tumor challenge.

Overall, endogenous type I IFN is critical for maintenance of normal NK cell numbers, but not cytotoxic activity under homeostatic conditions, and is a central regulator of NK-mediated antitumor immune responses, where it acts early to ensure prompt clearance of tumor targets. Future studies will investigate the source and species of type I IFN responsible for NK cell homeostasis and IL-2-induced cytotoxic activity.

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Disclosures

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