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# Distinct Requirements for IFNs and STAT1 in NK Cell Function<sup>1</sup>

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NK cell functions were examined in mice with a targeted mutation of the STAT1 gene, an essential mediator of IFN signaling. Mice deficient in STAT1 displayed impaired basal NK cytolytic activity *in vitro* and were unable to reject transplanted tumors *in vivo*, despite the presence of normal numbers of NK cells. IL-12 enhanced NK-mediated cytotoxicity, but poly(I:C) did not, and a similar phenotype occurred in mice lacking IFN $\alpha$  receptors. Molecules involved in activation and lytic function of NK cells (granzyme A, granzyme B, perforin, DAP10, and DAP12) were expressed at comparable levels in both wild-type and STAT1<sup>-/-</sup> mice, and serine esterase activity necessary for CTL function was normal, showing that the lytic machinery was intact. NK cells with normal cytolytic activity could be derived from STAT1<sup>-/-</sup> bone marrow progenitors in response to IL-15 *in vitro*, and enhanced NK lytic activity and normal levels of IFN- $\gamma$  were produced in response to IL-12 treatment *in vivo*. Despite these normal responses to cytokines, STAT1<sup>-/-</sup> mice could not reject the NK-sensitive tumor RMA-S, even following IL-12 treatment *in vivo*. Whereas *in vitro* NK cytotoxicity was also reduced in mice lacking both type I and type II IFN receptors, these mice resisted tumor challenge. These results demonstrate that both IFN- $\alpha$  and IFN- $\gamma$  are required to maintain NK cell function and define a STAT1-dependent but partially IFN-independent pathway required for NK-mediated antitumor activity. *The Journal of Immunology*, 2000, 165: 3571–3577.

Natural killer cells are multifunctional, non-T lymphocytes with reactivity against different infectious pathogens and tumors. In contrast to cells of the adaptive immune response, NK cell-mediated cytotoxicity does not require a preactivation process and is not restricted by Ag receptors. Because of their rapid action, NK cells are one of the essential cell types forming the first line of host defense against microbial pathogens and tumors (1, 2) and play a major effector as well as regulatory role in antiviral defense (3). The function of NK cells, like other effector cells participating in innate immunity, such as granulocytes and macrophages/monocytes, is enhanced by cytokines, including IFN (4). In fact, in the early response to infection by viruses and certain microorganisms, IFNs are usually produced coordinately at the site of infection and the kinetics of IFN production correlate with NK cell activity, peaking at 3–5 days postinfection (5, 6). Reduced NK activity has been observed in mice deficient in IFN- $\gamma$ , demonstrating a role for IFN not only as an effector of NK action but also as a modulator of NK activity *in vivo* (7).

In addition to IFNs, other cytokines have been shown to modulate NK cell differentiation and/or development, for example, IL-2, IL-12, IL-15, and IL-18 (8). IL-2 is secreted mainly by ac-

tivated CD4<sup>+</sup> T cells and is a potent inducer of perforin and granzymes, which are critical components for lysis mediated by both T cells and NK cells (9–11). IL-12, a proinflammatory and immunomodulatory cytokine produced by activated macrophages in response to infection, is also a powerful enhancer of NK cell cytotoxicity (12). Although IL-12 stimulates NK cells to produce cytokines that have been shown to participate in NK functions (IFN- $\gamma$ , GM-CSF, and TNF- $\alpha$ ), the effect on enhancement of NK activity can be independent from cytokine secretion because neutralizing Abs to IFNs or TNF- $\alpha$  are unable to inhibit IL-12-induced NK lytic activity (13). Nonetheless, mice deficient in IL-12, or its major transcriptional mediator STAT4, show reduced NK activity despite normal numbers of NK cells (14–16). In STAT4- or IL-12-deficient mice, suppressed NK activity can be restored by IL-18 treatment. The converse is observed in IL-18-deficient mice, where reduced NK activity can be rescued by IL-12 treatment. Furthermore, a profound reduction in NK activity is found in mice lacking both IL-12 and IL-18, suggesting that the roles of both cytokines in NK function are not entirely overlapping (17). IL-15, a cytokine that shares receptor  $\beta$ - and  $\gamma$ -chains and some of the biological functions of IL-2, is also produced by macrophages upon activation with LPS or IFN, and its induction following viral infection is impaired in the absence of STAT1 (18). IL-15 plays a pivotal role in the development, survival, and activation of NK cells (19) in that mice lacking IL-15R $\alpha$ , IL-2/IL-15R $\beta$ , or  $\gamma$ -chains are markedly deficient in NK cell development (20–22). Similarly, mice deficient in Jak3 or STAT5, which participate in signal transduction mediated by IL-15, or in IFN regulatory factor 1, a transcription factor required for IL-15 expression, also lack NK cells (23–25).

STAT1 is activated by multiple cytokines and growth factors, including IFNs and IL-12 (26). An essential role for STAT1 in IFN signaling was demonstrated by the IFN-resistant phenotype of cells and mice lacking STAT1 (27–29). STAT1<sup>-/-</sup> mice have also revealed roles for constitutive IFN signaling in the absence of

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pathogen infection (30), for STAT1 in regulating lymphocyte survival and proliferation (31), for IFN- $\gamma$ -dependent antitumor surveillance (32, 33), and for some IFN-independent gene responses (30). We examined the requirement for STAT1 in NK cell function *in vivo* using STAT1<sup>-/-</sup> mice and observed impaired cytotoxicity and antitumor activity compared with wild-type mice. Interestingly, although reduced NK cell activity was also observed in mice deficient in both type I and type II IFN receptors, these mice were able to reject tumors, whereas STAT1<sup>-/-</sup> mice could not. These results demonstrate an IFN-independent, but STAT1-dependent mechanism for NK cell antitumor activity.

## Materials and Methods

### Animals

Wild-type, STAT1<sup>-/-</sup> (28), IFN- $\alpha$  receptor (IFNAR)<sup>4</sup>-deficient (34), IFN- $\alpha$  receptor (IFNGR)-deficient (35), and IFN(AR+GR)<sup>-/-</sup> (30) mice were maintained on either the C57BL/6J or 129/SvEv backgrounds. RAG1<sup>-/-</sup>STAT1<sup>+/+</sup> and RAG1<sup>-/-</sup>STAT1<sup>-/-</sup> mice were C57BL/6. Identical background strains were used for comparisons between mutant and wild-type mice. Mice were housed under specific pathogen-free conditions, and all work with animals conformed to guidelines approved by the Institutional Animal Care and Use Committee of New York University School of Medicine (New York, NY).

### Flow cytometry and cytokine quantitation

Freshly isolated splenocytes from wild-type and mutant mice were stained with anti-NK cell-specific Abs DX5-FITC or NK1.1-PE (PharMingen, San Diego, CA) plus anti-CD3 $\epsilon$ -FITC or -PE (Caltag, South San Francisco, CA) for 20 min at 4°C. For intracellular staining, splenocytes were stimulated *in vitro* with IL-12 (10 ng/ml) for 24 h followed by incubation with brefeldin A (10  $\mu$ g/ml) for 4 h at 37°C to accumulate intracellular cytokine protein. Cells were stained with anti-NK1.1-PE, fixed, and permeabilized (Fix & Perm reagent; Caltag), followed by reaction with anti-IFN- $\gamma$ -FITC Ab and flow cytometric analysis. IFN- $\gamma$  secretion in culture supernatants was quantified by ELISA (PharMingen).

### In vitro NK activity assay

Splenocytes from different mice injected *i.p.* with 0.1 ml of PBS, poly(I:C) (10  $\mu$ g/mouse), or IL-12 (1  $\mu$ g/mouse) 24 h before harvest were prepared as effector cells. A total of  $1 \times 10^6$  EL-4 or YAC-1 cells labeled with 0.1 mCi of sodium [<sup>51</sup>Cr]chromate were prepared as target cells. *In vitro* NK lysis was monitored by incubating different numbers of effector cells with  $1 \times 10^4$  target cells at 37°C for 4 h. Specific lysis was calculated as follows: % lysis = [(cpm<sup>experimental</sup> - cpm<sup>spontaneous release</sup>) / (cpm<sup>maximum release</sup> - cpm<sup>spontaneous release</sup>)]  $\times$  100. Maximum release was determined following cell lysis with 1% Triton X-100. Error among triplicate measurements was <15% of the mean.

### Ab-dependent cytotoxicity assay

Splenocytes from wild-type or STAT1<sup>-/-</sup> mice were cultured *in vitro* with IL-2 (50 U/ml) for 4 days and used as effector cells. P815 cells stained with or without 2  $\mu$ g of Ab to Fc $\gamma$ RII/III (PharMingen) were labeled with sodium [<sup>51</sup>Cr]-chromate and used as target cells. Different numbers of effector cells were incubated with  $10^4$  target cells conjugated with or without Ab for 4 h. The percent lysis was calculated as described for the NK activity assay.

### RT-PCR

Total RNA was prepared from freshly isolated splenocytes of wild-type or STAT1<sup>-/-</sup> mice using Trizol reagent (Life Technologies, Gaithersburg, MD). Then, 0.3  $\mu$ g of total RNA was subject to RT-PCR, and trace [<sup>32</sup>P]dATP was included in the PCR to label the amplified fragments. The primer sets used for the PCR were as follows: granzyme A forward primer 5'-GAGACACGGTTGTCCTCAC-3', backward primer 5'-GGAAAGTATGACACCAGGC-3'; granzyme B forward primer 5'-ATGAAGATCCTCCTACT-3', backward primer 5'-CCAGGATAAGAACTCGAGA-3'; perforin forward primer 5'-CTCCAAGGTAGCCAATTTTGC-3', backward primer 5'-GCTTGGTCCCGAAGAGCAG-3'; DAP-10 for-

ward primer 5'-ATGGACCCCCAGGCTACCT-3', backward primer 5'-TCAGCCTTGCCAGGCATGT-3'; DAP-12 forward primer 5'-ATGGG GGCTCTGGAGCCCTC-3', backward primer 5'-TCATCTGTAATATT GCCTCTGTG-3'; and GAPDH forward primer 5'-ACCACAGTCC ATGCCATCAC-3', backward primer 5'-TCCACCACCCTGTTGCT GTA-3'. Following PCR, amplified DNA was resolved by 6% PAGE and detected by autoradiography using phosphorimager screens for quantitation. All quantitative data were normalized by the values for GAPDH.

### Serine esterase activity assay

Freshly isolated splenocytes were stained with mouse anti-NK1.1-PE Ab (Caltag) for 30 min at 4°C followed by two washes with PBS containing 2% BSA. Ab-conjugated cells were selected with goat-anti-mouse IgG-coated magnetic beads (DynaL, Lake Success, NY) according to the manufacturer's instructions. A total of  $5 \times 10^6$  purified NK cells or NK-depleted cells were lysed by resuspension in 60  $\mu$ l of 0.1% Triton X-100 and centrifuged at 12,000 rpm for 10 min. A total of 50  $\mu$ l of the supernatant was mixed with 0.950 ml of PBS containing 0.2 mM *N*- $\alpha$ -benzylloxycarbonyl-L-lysine thiobenzyl ester, 0.22 mM (5,5'-dithio-bis(2-nitrobenzoic acid), and 0.01% Triton X-100 before incubation at 37°C for 20 min. The reaction was terminated by addition of 0.01 ml of 0.1 M PMSF and substrate conversion was measured by absorption at 412 nm.

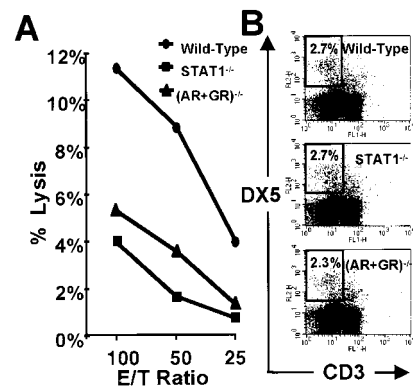
### In vivo tumor rejection

EL-4 and RMA-S tumor cells were injected *s.c.* into opposite flanks of the same mouse, and tumor growth was monitored using calipers. For *in vivo* depletion of NK cells, 0.2 ml of ascites (~0.2 mg of specific Ig) of PK136 anti-NK-1.1 Ab was injected *i.p.* into mice 1 day before tumor injection, and the effectiveness of this treatment was documented by flow cytometry. For *in vivo* enhancement of NK activity, murine rIL-12 (1  $\mu$ g/mouse) (Genetics Institute, Cambridge, MA) was injected *i.p.* 1 day before tumor injection followed by five additional daily doses of 1  $\mu$ g/mouse.

## Results

### Impaired basal NK cell activity of STAT1<sup>-/-</sup> mice

Because STAT1 is an essential signal mediator for both type I and type II IFNs, which are potent activators of NK cell activity, it was of interest to investigate NK cell activity in mice deficient in STAT1. Basal NK cell lytic activity was assessed by incubating freshly prepared splenocytes from wild-type or STAT1<sup>-/-</sup> mice with YAC-1 cells. MHC class I Ag expression is impaired in these cells, making them effective NK cell targets (36). As shown in Fig. 1A, reduced cytolytic activity was detected by splenocytes of STAT1<sup>-/-</sup> mice relative to that of wild-type mice. At an E:T ratio of 100, the activity of wild-type cells was almost three times more than that of STAT1<sup>-/-</sup> cells. Cytolytic activity of both wild-type



**FIGURE 1.** Impaired basal NK cell activity in STAT1<sup>-/-</sup> mice. **A**, Spleen cells isolated from wild-type (●), STAT1<sup>-/-</sup> (■), or IFN(AR+GR)<sup>-/-</sup> mice (▲) were analyzed for NK activity against [<sup>51</sup>Cr]-labeled YAC-1 cell targets at different E:T ratios. **B**, Normal numbers of NK cells are present in STAT1<sup>-/-</sup> mice. Splenocytes isolated from wild-type, STAT1<sup>-/-</sup>, or IFN(AR+GR)<sup>-/-</sup> mice were stained with Abs to DX5-PE and CD3-FITC followed by flow cytometry. The numbers shown in the upper left quadrant indicate the percentage of NK cells.

<sup>4</sup> Abbreviations used in this paper: IFNAR, IFN- $\alpha$  receptor; ADCC, Ab-dependent cellular cytotoxicity; C/EBP, CAAT/enhancer binding protein; IFNGR, IFN- $\gamma$  receptor.

and STAT1<sup>-/-</sup> cells was observed only with MHC class I-deficient YAC-1 targets and not with MHC class I<sup>+</sup> EL-4 cells, suggesting that lysis was specifically mediated by the NK cell population (data not shown). To examine whether the reduced NK activity of STAT1<sup>-/-</sup> mice was due to loss of IFN responsiveness accompanying STAT1-deficiency, cells from mice lacking both IFN- $\alpha$ B and IFN- $\gamma$  receptors were also tested. Indeed, NK cytolytic activity of IFN(AR+GR)<sup>-/-</sup> cells was also decreased compared with that of wild-type, although it was consistently slightly higher than that of STAT1<sup>-/-</sup> cells (Fig. 1A). These data suggested that constitutive IFN signaling was involved in the maintenance of basal NK activity.

Reduced NK cytolytic function in total splenocytes from STAT1<sup>-/-</sup> mice could result from reduced numbers of NK cells. To investigate this possibility, freshly isolated splenocytes from IFN(AR+GR)<sup>-/-</sup> and STAT1<sup>-/-</sup> mice were stained with Abs to CD3 $\epsilon$  and a NK marker, DX5, and compared with wild-type cells. Both STAT1<sup>-/-</sup> and IFN(AR+GR)<sup>-/-</sup> mice had comparable numbers of splenic DX5<sup>+</sup>CD3<sup>-</sup> NK cells compared with those of wild-type mice (Fig. 1B), suggesting that reduced NK activity was not due to reduced numbers of NK cells or impaired development of these cells in the mice lacking STAT1 or IFN receptors.

#### Restoration of NK cell activity of STAT1<sup>-/-</sup> lymphocytes by IL-12

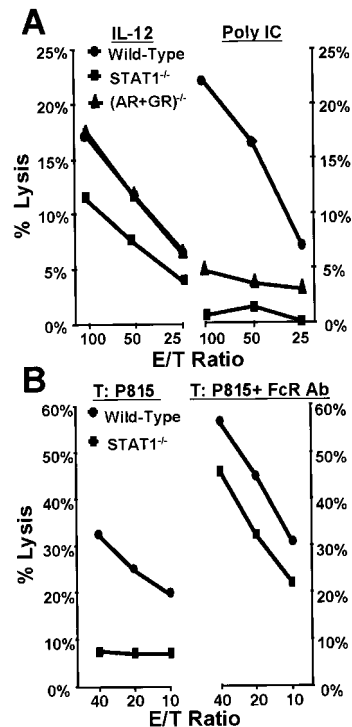
The preceding results suggested that continuous IFN and STAT1 signaling is required for maintaining basal NK activity. Because NK activity can be modulated by a variety of stimuli, such as cytokines (IL-2, IL-12, IL-18) and poly(I:C), it was of interest to determine the involvement of STAT1 in enhanced as well as basal NK activity. Wild-type or mutant mice were injected with IL-12 or poly(I:C) 24 h before cell isolation. The cytolytic activity of NK cells from both wild-type and mutant splenocytes was enhanced by treatment with IL-12 (Fig. 2A) compared with basal activity (Fig. 1A), although STAT1<sup>-/-</sup> cells still showed less activity than wild type.

In contrast to the response to IL-12, the effect of poly(I:C) on induction of NK activity was completely abrogated in both IFN(AR+GR)<sup>-/-</sup> and STAT1<sup>-/-</sup> mice (Fig. 2A, right), suggesting that poly(I:C)-induced NK activity was completely dependent on the IFN and STAT1 signaling pathway. Because poly(I:C) is an effective inducer of IFN, the involvement of individual IFN species in the poly(I:C)-mediated enhancement of NK activity was dissected using IFNAR<sup>-/-</sup> or IFNGR<sup>-/-</sup> mice. The response of IFNGR<sup>-/-</sup> mice to poly(I:C) was identical with that of wild-type mice, whereas IFNAR<sup>-/-</sup> mice failed to display increased NK cell activity (data not shown). Therefore, the effect of poly(I:C) on NK cell activity is exclusively mediated by type I IFN, consistent with previous studies using IFN- $\gamma$ -deficient mice (7).

NK cells are responsible for Ab-dependent cellular cytotoxicity (ADCC),<sup>4</sup> mediated through low affinity Fc $\gamma$  receptors. Therefore, we investigated whether ADCC was impaired by loss of STAT1. Spleen cells from wild-type and STAT1<sup>-/-</sup> mice were cultured in vitro with IL-2 for 4 days before assay for lytic function by ADCC against [<sup>51</sup>Cr]-labeled P815 cells, which had been decorated with anti-FcR Ab. As shown in Fig. 2B, both wild-type and STAT1<sup>-/-</sup> cells were capable of killing Ab-coated targets, although STAT1<sup>-/-</sup> cells were slightly less effective. The ability of STAT1<sup>-/-</sup> cells to promote ADCC demonstrates that their cytotoxic machinery is intact even though it is not manifested under basal conditions.

#### Comparable levels of effector and activation molecules are expressed by wild-type and STAT1<sup>-/-</sup> NK cells

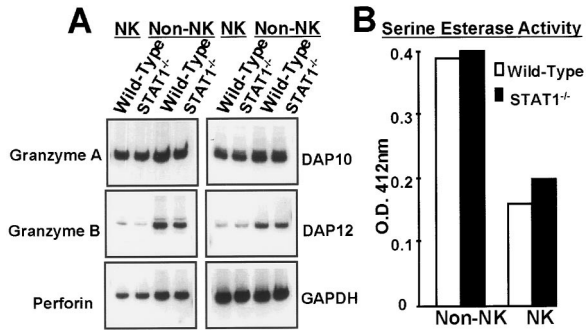
To further pinpoint possible factors that could lead to impaired cytotoxicity by STAT1<sup>-/-</sup> NK cells, the levels of different molecules known to be critical for NK lytic function were compared



**FIGURE 2.** A, Failure of poly(I:C) to stimulate NK activity in STAT1<sup>-/-</sup> mice. IL-12 (1  $\mu$ g/mouse) or poly(I:C) (10  $\mu$ g/mouse) were injected i.p. into wild-type (●), STAT1<sup>-/-</sup> (■), or (AR+GR)<sup>-/-</sup> mice (▲) 24 h before isolation of NK cells, which were assayed for lytic activity as described in Fig. 1A. B, STAT1<sup>-/-</sup> lymphocytes are competent for ADCC. Splenocytes prepared from wild-type (●) or STAT1<sup>-/-</sup> mice (■) were cultured in medium containing IL-2 (50 U/ml) for 4 days. Cultured lymphocytes were challenged with [<sup>51</sup>Cr]-labeled P815 target cells at different ratios in the absence or presence of anti-Fc receptor Ab.

between STAT1<sup>-/-</sup> and wild-type cells. NK cells were isolated by magnetic immunobead selection from freshly isolated spleen cells from both strains. Equivalent amounts of total RNA prepared from purified NK cells or from the non-NK cell population was analyzed by RT-PCR for cytotoxic effector molecules, such as granzyme A, granzyme B, and perforin, or for molecules essential for cell activation, such as DAP10 and DAP12. NK cells expressed significantly lower levels of granzyme B and DAP12 than did non-NK cells, whereas the levels of granzyme A, DAP10, and perforin were similar in both cell populations (Fig. 3A). Nonetheless, all these effector and activation molecules were expressed at comparable levels in wild-type and STAT1<sup>-/-</sup> mice in both NK and non-NK cells. Because these molecules are expressed in NK cells and T lymphocytes but are absent in B lymphocytes, the expression detected in the NK cell-depleted preparations was most likely contributed by splenic T lymphocytes.

To further confirm the RNA expression data, serine esterase activity was measured, which reflects the action of granzyme B. Total cell protein extracts prepared from equal numbers of NK and non-NK cells from wild-type and STAT1<sup>-/-</sup> mice were analyzed for serine esterase content by ELISA (Fig. 3B). The NK cell-depleted population exhibited higher enzyme activity than did the NK cells, consistent with the higher mRNA levels. However, again no significant differences were found in either NK or non-NK cells between wild-type and STAT1<sup>-/-</sup> mice. Similar results were obtained when serine esterase activity was measured in effector cells challenged with YAC-1 targets (data not shown), indicating that lack of induction of protein levels or activity is not involved in impaired killing.



**FIGURE 3.** A, Comparable levels of CTL effector molecules in wild-type and STAT1<sup>-/-</sup> mice. Total RNA prepared from NK or NK-depleted cell populations from wild-type or STAT1<sup>-/-</sup> mice was analyzed by RT-PCR using primers specific for granzyme A, granzyme B, DAP10, DAP12, and GAPDH, as described in *Materials and Methods*. B, Serine esterase levels are normal in STAT1<sup>-/-</sup> CTL. NK or NK-depleted cell populations from wild-type (□) or STAT1<sup>-/-</sup> mice (■) were isolated using magnetic beads and serine esterase content was determined as described in *Materials and Methods*.

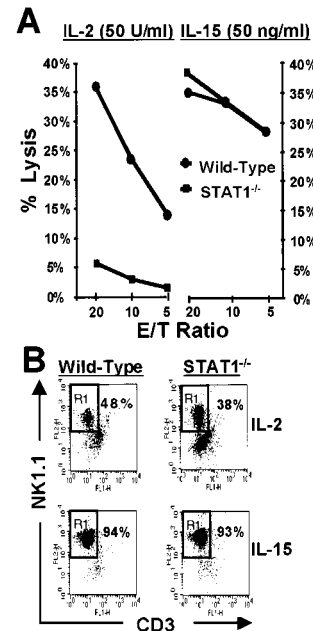
The level of perforin in NK cells from wild-type and STAT1<sup>-/-</sup> mice was confirmed by intracellular staining and by immunoblotting. Similar levels of perforin protein were found in lymphocytes from both strains (data not shown). These results collectively show that impaired NK lytic activity in STAT1<sup>-/-</sup> mice could not be accounted for by decreased expression of any of these effector or activation molecules, further demonstrating that the lytic machinery is intact in STAT1<sup>-/-</sup> NK cells.

#### IL-15-mediated restoration of NK lytic activity in vitro

Recovery of NK cells in vitro from bone marrow progenitors was compared between wild-type and STAT1<sup>-/-</sup> mice. Progenitor cells were cultured in vitro in media containing IL-2 (50 U/ml) or IL-15 (50 ng/ml) for 10 days, and the resulting lymphocytes were used as effector cells in NK activity assay against YAC-1 targets. IL-2 supported NK cell development and survival from wild-type progenitors, and these cells displayed effective cellular cytotoxicity (Fig. 4A). In contrast, STAT1<sup>-/-</sup> progenitors failed to develop lytic activity when cultured in IL-2. IL-15 was also an effective growth factor for NK cells, but in contrast to IL-2, its ability to induce NK lytic function was not limited to wild-type cells, supporting effective development of lytic activity by STAT1<sup>-/-</sup> cells. The absence of cytolytic activity displayed by STAT1<sup>-/-</sup> cells cultured in the presence of IL-2 could not be explained by failure of NK cell growth because equal numbers of NK1.1<sup>+</sup>CD3<sup>-</sup> cells were derived from wild-type and STAT1<sup>-/-</sup> progenitors (Fig. 4B). Although IL-15 was a more effective NK cell growth factor than IL-2 for both wild-type and STAT1<sup>-/-</sup> cells (lower panel), IL-2 was as effective in supporting NK cell growth from STAT1<sup>-/-</sup> cells as from wild-type progenitors.

#### Impaired NK cell-mediated tumor rejection by STAT1<sup>-/-</sup> mice

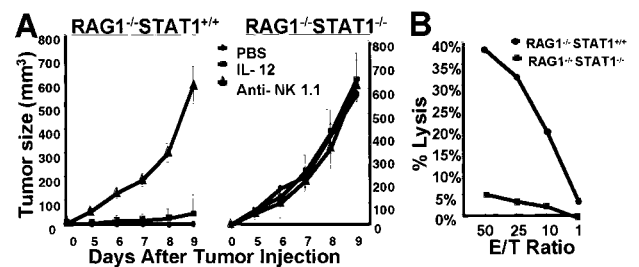
The significance of the reduced NK lytic activity observed in STAT1<sup>-/-</sup> mice was investigated by examining the ability of these mice to reject a tumor challenge. To avoid the complications of T cell-mediated cytotoxicity, we bred STAT1<sup>-/-</sup> mice onto the RAG1<sup>-/-</sup> background, resulting in deficiency in both functional T and B lymphocytes without loss of NK cells relative to their RAG1<sup>+/+</sup> counterparts. RMA-S tumor cells fail to express cell surface MHC class I Ag due to TAP deficiency (37) and therefore are sensitive to NK cell lysis. Tumor cells were injected into one flank of RAG1<sup>-/-</sup>STAT1<sup>+/+</sup> and RAG1<sup>-/-</sup>STAT1<sup>-/-</sup> mice,



**FIGURE 4.** A, STAT1<sup>-/-</sup> NK cells with normal lytic function develop in the presence of IL-15. Bone marrow prepared from wild-type (●) or STAT1<sup>-/-</sup> mice (■) were cultured in medium containing IL-2 (50 U/ml, left) or IL-15 (50 ng/ml, right) for 10 days. Lytic activity of in vitro cultured lymphocytes was measured against YAC-1 cells. B, Comparable numbers of NK cells matured from STAT1<sup>-/-</sup> and wild-type progenitors. Bone marrow cells cultured in the presence of IL-2 (50 U/ml, upper panels) or IL-15 (50 ng/ml, lower panels) were stained with anti-NK1.1-PE and CD3ε-FITC Abs followed by flow cytometry.

and, as a control, the MHC class I-expressing tumor EL-4 was injected into the opposite flank.

STAT1<sup>+/+</sup> mice rejected RMA-S tumors (Fig. 5A, left) but were incapable of rejecting EL-4 cells (not shown), a likely consequence of loss of mature T cells due to the RAG mutation. Moreover, RMA-S cells grew progressively in STAT1<sup>+/+</sup> mice that were depleted of NK cells by prior injection of anti-NK1.1 Ab, demonstrating that resistance to RMA-S was conferred by NK cells. In contrast, STAT1<sup>-/-</sup> animals were incapable of rejecting an RMA-S cell challenge, whether or not they were subjected to the NK cell-depleting regimen (Fig. 5A, right). Therefore, consistent



**FIGURE 5.** A, Impaired tumor resistance by STAT1<sup>-/-</sup> mice. PBS (●) or anti-NK1.1 Ab (0.2 mg/mouse, ▲) was administered 24 h before inoculation of 2 × 10<sup>5</sup> RMA-S tumor cells into the flank of RAG1<sup>-/-</sup>STAT1<sup>+/+</sup> (left) or RAG1<sup>-/-</sup>STAT1<sup>-/-</sup> mice (right), and tumor growth was followed by caliper measurement at the indicated days. For IL-12 stimulation, 1 μg/mouse (■) was administered 24 h before tumor inoculation, followed by five daily injections. Results shown are the average of four individual mice. B, Impaired in vitro NK lysis of RAG1<sup>-/-</sup>STAT1<sup>-/-</sup> tumor-bearing mice. 9 days following tumor injection, NK cells were prepared from RAG1<sup>-/-</sup>STAT1<sup>+/+</sup> (●) or RAG1<sup>-/-</sup>STAT1<sup>-/-</sup> mice (■) and were incubated with [<sup>51</sup>Cr]-labeled YAC-1 targets.

with the *in vitro* NK cell activity assays, STAT1<sup>-/-</sup> mice were incapable of mounting an effective NK cell response to tumor challenge *in vivo*.

To further confirm that failure to reject RMA-S tumor by RAG1<sup>-/-</sup>STAT1<sup>-/-</sup> mice was due to defective NK cell cytotoxicity, splenocytes from tumor-bearing mice were tested for lytic activity *in vitro*. Similar to the results from RAG1<sup>+/+</sup> mice, STAT1<sup>-/-</sup> cells on the RAG1<sup>-/-</sup> background were unable to kill NK target cells (Fig. 5B). STAT1<sup>+/+</sup>RAG1<sup>-/-</sup> cells displayed enhanced lytic activity, probably due to the enrichment of NK cells resulting from absence of mature conventional lymphocytes. No cytolysis was observed using MHC class I<sup>+</sup> EL-4 targets, confirming that the observed lytic activity was derived from NK cells (data not shown). The abundance of NK cells was similar between STAT1<sup>+/+</sup> and STAT1<sup>-/-</sup> mice on the RAG1<sup>-/-</sup> background as determined by flow cytometry for NK1.1<sup>+</sup> cells (~15% of total splenocytes), reinforcing the view that impaired tumor rejection resulted from a failure of NK cell lytic activity, not from a loss of NK cell development.

The ability of IL-15 but not IL-2 to support NK cell maturation *in vitro* could suggest that impaired IL-15 *in vivo* could explain the inability of STAT1<sup>-/-</sup> mice to reject tumors. Moreover, it has been reported that IFN-regulator factor 1, a STAT1 target gene, is required for IL-15 production (25). Bone marrow and splenocyte mRNA samples from RAG1<sup>-/-</sup>STAT1<sup>+/+</sup> and RAG1<sup>-/-</sup>STAT1<sup>-/-</sup> were assayed for basal IL-15 expression levels by RT-PCR. Equivalent levels of IL-15 mRNA were detected from both genotypes (not shown) despite the reduced lytic activity of STAT1<sup>-/-</sup> NK cells.

Because IL-12 was capable of enhancing NK cell cytolytic activity *in vitro* in the absence of STAT1<sup>-/-</sup> (Fig. 2A), we tested whether *in vivo* administration of IL-12 could produce effective NK cell-mediated tumor resistance. IL-12 was injected 24 h before inoculation of the tumor challenge, followed by five additional daily doses of IL-12. However, IL-12-treated RAG1<sup>-/-</sup>STAT1<sup>-/-</sup> mice were still incapable of rejecting RMA-S tumors, which grew progressively despite the IL-12 treatments (Fig. 5A, right). Therefore, the enhanced lytic activity observed *in vitro* by NK cells derived from IL-12-treated STAT1<sup>-/-</sup> mice was nonetheless insufficient for tumor rejection *in vivo*.

#### Tumor rejection is dependent of STAT1 but only partially on IFN signaling

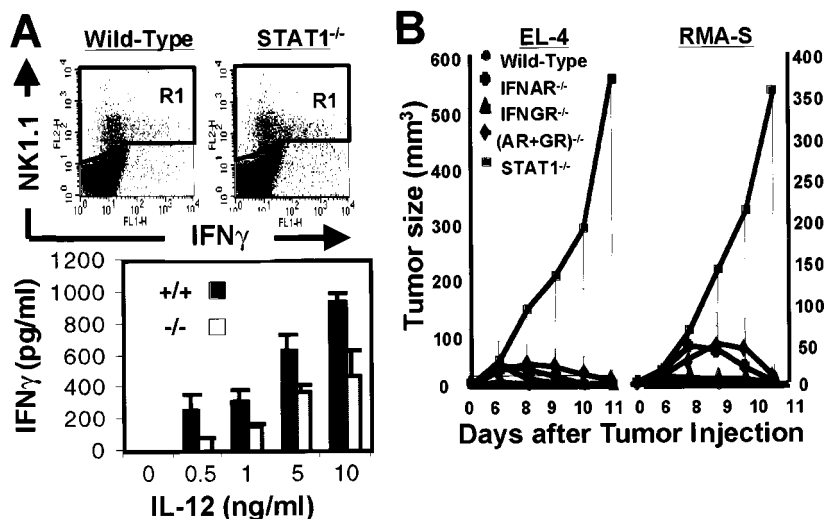
The inability of IL-12 enhancement of NK activity to rescue defective tumor killing in STAT1<sup>-/-</sup> mice could be due to additional

defects resulting from loss of STAT1. In several model systems, IFN- $\gamma$  was shown to be critical for tumor surveillance and rejection and for IL-12-mediated inhibition of tumor growth (33, 38–40). Although the ability of tumor cells to respond directly to IFN- $\gamma$  can play a major role in tumor surveillance (32), the impaired rejection of IFN-responsive tumor cells observed here would require a host defect, for instance, impaired IFN- $\gamma$  production. To address this issue, we examined IFN- $\gamma$  levels. Splenocytes from wild-type or STAT1<sup>-/-</sup> mice were cultured *in vitro* with IL-12 for 24 h, and the number of IFN- $\gamma$ -producing cells was measured by flow cytometry (Fig. 6A). By gating on the NK1.1<sup>+</sup> population (*upper panel*), it was evident that similar numbers of IFN- $\gamma$ -producing NK cells were induced in both genotypes. Non-NK1.1 cells producing IFN- $\gamma$ , which presumably represent IFN- $\gamma$ -producing TCR $\alpha\beta$ <sup>+</sup> T lymphocytes, were also present at comparable numbers in both mice, consistent with robust IFN- $\gamma$  production previously observed in STAT1<sup>-/-</sup> mice (18, 31).

While equivalent numbers of IFN- $\gamma$ -producing cells were present in STAT1<sup>-/-</sup> and wild-type animals, we tested the production of IFN- $\gamma$  in response to IL-12 stimulation. Wild-type and STAT1<sup>-/-</sup> splenocytes were cultured *in vitro* and stimulated with increasing doses of IL-12 from 0.5 to 10 ng/ml for 24 h, and the resulting culture supernatants were assayed for IFN- $\gamma$  accumulation by ELISA (Fig. 6A, *lower panel*). Both wild-type and STAT1<sup>-/-</sup> splenocytes produced IFN- $\gamma$ , although wild-type cells produced ~2-fold higher amounts. Therefore, it is unlikely that IFN- $\gamma$  production defects *per se* are sufficient to explain the impaired tumor rejection by STAT1<sup>-/-</sup> mice following IL-12 stimulation, although this weaker response may reflect the same underlying defect in STAT1<sup>-/-</sup> NK cells.

It has been shown that immune responsiveness of the host is a critical aspect of tumorigenicity in mice (39, 40), presenting another venue where lack of cytokine responsiveness of STAT1<sup>-/-</sup> mice could impact on tumor rejection. To address the potential involvement of responsiveness to IFNs, we measured tumor rejection in mice lacking receptors for IFN $\alpha$ , IFN- $\gamma$ , or both. In control experiments, wild-type mice rejected both EL-4 and RMA-S tumor challenges, whereas STAT1<sup>-/-</sup> mice were susceptible to progressive tumor growth (Fig. 6B). Similar to wild-type mice, IFN receptor-deficient mice were resistant to tumor challenge. However, tumor rejection was slower in the absence of host IFN responsiveness, particularly in the absence of IFN $\alpha$  receptors, with RMA-S tumors growing to an appreciable size before rejection (Fig. 6B, right). This result is consistent with the reduced NK cell cytolytic

**FIGURE 6.** A, Comparable levels of IFN- $\gamma$  induction by IL-12 in STAT1<sup>-/-</sup> and wild-type NK cells. Splenocytes isolated from wild-type (*left*) or STAT1<sup>-/-</sup> mice (*right*) were stimulated with IL-12 (5 ng/ml) for 24 h followed by intracellular staining and flow cytometric analysis for IFN- $\gamma$  production. B, Impaired tumor rejection requires STAT1 but not IFN responsiveness. A total of  $2 \times 10^5$  EL-4 (*left*) or RMA-S tumor cells (*right*) were injected into opposite flanks of wild-type (●), IFNAR<sup>-/-</sup> (⊕), IFNGR<sup>-/-</sup> (▲), IFN(AR+GR)<sup>-/-</sup> (◆), or STAT1<sup>-/-</sup> mice (■), and tumor growth was followed by caliper measurement at the indicated days.



activity observed *in vitro* using IFN(AR+GR)<sup>-/-</sup> mice. In contrast to IFN(AR+GR)<sup>-/-</sup> mice, however, STAT1<sup>-/-</sup> mice could not mount effective antitumor responses needed to reject either EL-4 or RMA-S tumors even though they showed only slightly lower NK activity *in vitro* (Fig. 1A). Tumor transplants were conducted over a range of initial challenge doses, from  $2 \times 10^5$  to  $1 \times 10^6$  cells per inoculation. At all doses, wild-type and IFN(AR+GR)<sup>-/-</sup> rejected tumor growth whereas STAT1<sup>-/-</sup> animals did not (data not shown), confirming that STAT1-mediated antitumor activity is partially independent of IFN signaling.

## Discussion

We have demonstrated the constitutive requirement of IFNs and STAT1 for NK cell function. Reduced NK cell lytic activity and failure to reject a NK-sensitive tumor *in vivo* were observed in mice lacking STAT1. Impairment of NK cell function was not due to abnormal growth and development of NK cells, as the number of NK cells in STAT1<sup>-/-</sup> mice was equivalent to wild-type mice. Comparable protein levels and enzyme activity of molecules involved in the effector phase of NK cell killing, including granzyme A, granzyme B, and perforin, were found in wild-type and STAT1<sup>-/-</sup> mice, suggesting that the lytic machinery was functional in cells devoid of STAT1. Indeed, normal ADCC occurred using STAT1<sup>-/-</sup> cells, suggesting that tumor recognition rather than killing may be the defect. Recently, two coreceptors, DAP10 and DAP12, were shown to transmit stimulatory signals in NK cells (41–43). The expression of these two molecules, however, was not affected in STAT1<sup>-/-</sup> NK cells, implying that neither of these proteins is responsible for impaired function. However, it remains a possibility that STAT1 is involved in transmission of signals required for NK recognition of target cells which use other molecules as yet unidentified.

Reduced NK cell lysis was also observed in mice deficient in IFN- $\alpha$  or IFN- $\gamma$  receptors, similar to defects reported previously in the absence of IFN- $\gamma$  protein (7). Interestingly, a more pronounced defect was found in mice lacking both receptors, showing that constitutive IFN signaling functions are required for maintaining full basal NK cell lytic activity and that the roles of type I and type II IFN in NK cells are not entirely overlapping. This notion was further supported by the complete restoration of NK cell activity by poly(I:C) treatment in IFNGR<sup>-/-</sup> but not IFNAR<sup>-/-</sup> mice, demonstrating that poly(I:C) functions through type I IFN. Although STAT1 is a shared signal mediator for IFN- $\alpha\beta$  and IFN- $\gamma$  pathways, greater impairment of NK cell function was found in STAT1<sup>-/-</sup> mice relative to IFN(AR+GR)<sup>-/-</sup> mice. Despite the reduced NK cell activity in both IFN(AR+GR)<sup>-/-</sup> and STAT1<sup>-/-</sup> mice, IFN(AR+GR)<sup>-/-</sup> but not STAT1<sup>-/-</sup> mice were able to reject an RMA-S tumor challenge, demonstrating a STAT1-dependent but IFN-independent requirement for this NK cell response. We have previously demonstrated a role for STAT1 outside the IFN signaling pathway, including IL-7-mediated induction of MHC class I in T lymphocytes and fibroblast growth factor-mediated inhibition of chondrocyte proliferation (30, 44). Therefore, we consider it likely that cytokines other than IFNs may participate in the STAT1-dependent maintenance of NK function.

Defective NK activity has been observed in a variety of gene-targeted mice lacking specific cytokines or transcription factors, in many cases leading to reduced NK cell number or proliferation. In contrast, absence of STAT1 did not affect NK cell development *in vitro* or *in vivo*, only the development of cytolytic activity. A similar phenotype has been observed in mice deficient in IL-12 or IL-18 or both, which resulted in reduced NK cell function without loss of NK cells themselves (14, 17). For both cytokines, loss of IFN- $\gamma$  pro-

duction appeared to be the primary defect affecting NK cell function, and both IFN- $\gamma$  production and NK cell activity could be rescued by treatment with either IL-12 or IL-18. Similarly, the reduced *in vitro* NK activity of STAT1<sup>-/-</sup> NK cells could be rescued by IL-12 treatment, demonstrating that IL-12-enhancement of NK cell activity does not require the IFN-STAT1 pathway. Although STAT1 is efficiently phosphorylated in IL12-treated cells, presumably the concomitant phosphorylation of STAT4 is sufficient for NK cell function in the absence of STAT1 protein. Recently, CCAAT/enhancer binding protein  $\gamma$  (C/EBP $\gamma$ ) was shown to be essential for NK cytotoxic activity and IFN- $\gamma$  production (45), its loss leading to impaired CTL activity but normal NK numbers. The C/EBP $\gamma$  deficiency phenotype was distinct from that caused by lack of STAT1 in that CTL activity of C/EBP $\gamma$ <sup>-/-</sup> NK cells could not be rescued by stimulation with IL-12 or IL-15, and these cells displayed severely impaired IFN- $\gamma$  production. Moreover, we detected normal levels of C/EBP $\gamma$  mRNA in STAT1<sup>-/-</sup> NK cells (data not shown). We also considered whether abnormal production of IL-18 binding protein might be involved in the STAT1<sup>-/-</sup> phenotype because this inhibitory protein must be down-modulated in response to IFN- $\gamma$  to allow IL-18 action (46). However, levels of its mRNA were also normal in STAT1<sup>-/-</sup> mice as were levels of IL-12 and IL-12 receptor (data not shown). It is possible that maturation of IL-18 could be impaired in the absence of STAT1 because IL-18 processing requires caspase I (47, 48) and levels of this enzyme are reduced in STAT1<sup>-/-</sup> splenocytes (31). However, loss of IL-18 processing capacity would lead to impaired IFN- $\gamma$  production (49), a phenotype not observed in STAT1<sup>-/-</sup> mice.

Defective NK cell activity in STAT1<sup>-/-</sup> mice could be rescued by treatment with IL-12 or IL-15, but not poly(I:C) or IL-2. However, even IL-12-enhanced NK activity was insufficient to result in tumor rejection *in vivo* and did not even alter the kinetics of tumor growth (Fig. 5), demonstrating the importance of STAT1-mediated NK activity during the antitumor response. Although IFN- $\gamma$  is key for tumor surveillance and for antitumor responsiveness of tumor cells (32, 39, 40), the different susceptibilities of STAT1<sup>-/-</sup> and IFN-nonresponsive mice define an additional mechanism of tumor resistance. The distinct activities of IL-2 and IL-15 in terms of NK cell maturation were surprising, given that these two cytokines use a common receptor signaling system and stimulate very similar intracellular pathways. However, it has been recently shown that despite these biochemical similarities, IL-2 and IL-15 produce distinct, indeed even opposing, effects on lymphocytes (50). Interestingly, we found that supraphysiological concentrations of IL-2 were capable of rescuing STAT1<sup>-/-</sup> NK cell activity *in vitro* (not shown), suggesting that signal strength rather than signal quality may be the major difference between these two similar cytokines.

In addition to cytokines, cell surface expression of MHC class I Ags affects the development of NK cells (51); therefore, the reduction in MHC class I expression in STAT1<sup>-/-</sup> mice (30) may contribute to the NK cell defect in these animals. The fact that STAT1<sup>-/-</sup> mice failed to mount an effective antitumor response to both RMA-S and EL-4 cells suggests that an essential STAT1-dependent factor for CTL activity may be shared by both NK and T cells. Indeed, a requirement for STAT1 in development of T cell-mediated antitumor immunity has recently been reported (33). Considered collectively, our data show that STAT1 plays important roles in innate antitumor immunity due to the pivotal role played by proinflammatory cytokines such as IFNs as well as to STAT1-dependent mechanisms essential for NK cell function that are independent of IFN signaling.

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