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IFN-Dependent Down-Regulation of the NKG2D Ligand H60 on Tumors[1]

Jack D. Bui,* Leonidas N. Carayannopoulos,† Lewis L. Lanier,§ Wayne M. Yokoyama,‡ and Robert D. Schreiber[2]*

In this study, we show that IFN-γ or IFN-α reduce expression of H60 on 3′-methylcholanthrene (MCA) sarcomas from 129/Sv mice. As determined by flow cytometry using either NKG2D tetramers or NKG2D ligand-specific mAb, H60 was identified as the NKG2D ligand most frequently expressed on these sarcomas, and its expression was selectively down-regulated by either IFN-γ or IFN-α in a manner that was dose- and time-dependent and reversible. Down-regulation occurred at the transcript level and was STAT1-dependent. It also had functional consequences. IFN-γ-treated MCA sarcomas with high levels of H60 were resistant to killing by IL-2-activated NK cells. Resistance was not solely dependent on enhanced MHC class I expression but rather also required H60 down-regulation. IFN-γ-treated tumor cells also displayed diminished capacity to down-regulate NKG2D on freshly isolated NK cells. Transplanted tumor cells reisolated from immunocompetent mice displayed reduced H60 expression and increased MHC class I expression compared with tumor cells that were either left unmanipulated or reisolated from mice treated with neutralizing IFN-γ-specific mAb. This report thus represents the first demonstration that certain cytokines and specifically the IFNs regulate expression of specific NKG2D ligands on murine tumors. This process most likely helps to specify the type of immune effector cell populations that participate in host-protective antitumor responses. ***The Journal of Immunology,*** **2006, 176: 905–913.**

We have shown previously that lymphocytes and IFN-γ play critical roles in mediating host immune responses against cancer. Mice lacking T, B, and NKT cells ($RAG2^{-/-}$ mice) or the ligand-binding subunit of the IFN-γ receptor ($IFNGR1^{-/-}$ mice) form more carcinogen-induced and spontaneous tumors than wild-type (WT)[3] mice (1, 2). Tumor cells have been identified as one critical target of the actions of IFN-γ. Immunogenic sarcoma cells derived from mice treated with 3′-methylcholanthrene (MCA) become nonimmunogenic when rendered IFN-γ unresponsive (3). Conversely, MCA sarcomas from IFN-γ-unresponsive mice grow in a highly aggressive manner when transplanted into immunocompetent hosts but are rejected if their IFN-γ responsiveness is restored (4). The importance of IFN-γ in enhancing tumor immunogenicity in vivo has been shown, at least in part, to be due to increased MHC class I pathway activity in the tumor targets leading to their more effective recognition by $CD8^+$ T cells. It is also known that IFN-γ decreases tumor cell recognition by NK cells in vitro (5–12). Thus, even though NK cells are known to play critical roles in regulating growth of primary and transplantable tumors (13, 14), the precise mechanisms controlling their participation in IFN-γ-dependent tumor rejection remain undefined.

The activating receptor NKG2D (15–17) expressed on NK cells and activated murine $CD8^+$ cells is thought to mediate detection of "stressed" or transformed cells which express NKG2D ligands (18). These ligands activate NK cell tumoricidal activity even when present on tumor cells that express MHC class I proteins which can engage inhibitory receptors on the responding NK cells (19, 20). The known murine NKG2D ligands include the five RAE1 molecules, H60, and MULT1 (21–23). The importance of NKG2D ligands in recognition of human or murine tumors is supported by several experimental findings. First, whereas ligand expression is rare in primary tissues, many tumor cell lines constitutively express these ligands (22–24). Second, treatment of mouse skin with carcinogens (25) and treatment of primary fibroblasts with DNA damaging agents (26) increases the transcript levels of certain NKG2D ligands. Third, enforced expression of NKG2D ligands on transplantable tumor cells converts them from ones that form progressively growing tumors into ones that are rejected in an NK cell-dependent manner (19, 20). Fourth, soluble "decoy" forms of the human NKG2D ligands MHC class I-related chain (MIC-A/B) can be detected in the serum of certain cancer patients and are associated with defective T cell function (27). Finally, impairment of NKG2D function induced by Ab blockade (28) or via constitutive expression of NKG2D ligands (29) enhances tumor formation in mouse models. These studies provide the basis for the "induced self" model of tumor recognition, wherein stress or transformation up-regulates NKG2D ligand expression, allowing for

*Center for Immunology, Department of Pathology and Immunology, †Division of Pulmonary and Critical Care Medicine, ‡Division of Rheumatology and Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, MO 63110; and §Department of Microbiology and Immunology and the Cancer Research Institute, University of California, San Francisco, CA 94143

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[2] Address correspondence and reprint requests to Dr. Robert D. Schreiber, Department of Pathology and Immunology, Washington University School of Medicine, Box 8118, 660 South Euclid Avenue, St. Louis, MO 63110. E-mail address: schreiber@immunology.wustl.edu

[3] Abbreviations used in this paper: WT, wild type; MCA, 3′-methylcholanthrene; MCMV, murine CMV; MFI, mean fluorescence intensity.

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recognition of developing tumors by NK and/or T cells (18, 30, 31).

Little is known about how expression of NKG2D ligands is regulated on tumors. Until now, cytokines have not been shown to modulate mouse NKG2D ligands, although LPS and other TLR ligands can stimulate transcriptional expression of RAE1 (but not H60) in peritoneal macrophages (32). Recently, it was shown that DNA-damaging agents enhance RAE1 and MULT1 transcript levels through an ataxia telangiectasia-related protein (ATR)/ataxia telangiectasia-mutated (ATM)-dependent pathway, although the regulation of H60 was not studied in this report (33). Other work has revealed that certain viruses such as murine CMV can down-regulate H60 expression posttranslationally through mechanisms involving proteosomal degradation of H60 proteins (34). The minor histocompatibility Ag H60 is an MHC class-I-like molecule expressed on activated splenocytes from BALB/c (35, 36) and 129/Sv (J. D. Bui and R. D. Schreiber, unpublished observations), but not C57BL/6, strains of mice and functions as a high affinity ligand of the mouse NKG2D receptor. In this report, we document that either IFN-γ or IFN-α inhibits expression of H60 and, to a lesser extent, MULT1 but not RAE1 on MCA-induced sarcoma lines from 129/Sv mice by decreasing transcript levels. This effect is dose- and time-dependent, reversible, occurs concomitantly with IFN-dependent MHC class I up-regulation, and reduces effectiveness of NK cell-dependent killing. This type of NKG2D ligand regulation is therefore distinct from that induced by viral infection or transformation and points to a critical role for the IFNs in specifying the type of protective effector functions that occur during the course of an antitumor response.

Materials and Methods

All animal protocols were approved by Institutional Review Board

Cell lines and stimulations

MCA-induced sarcomas were isolated and passaged in vitro as described (3). The F244 cell line is derived from WT 129/Sv strain mice and forms progressively growing tumors when transplanted into syngeneic WT mice (1). Recombinant murine IFN-γ and TNF-α were a gift from Genentech. Human IFN-α A/D was obtained from Hoffman-LaRoche. Unless indicated, all stimulations were performed with 1000 U/ml IFN-γ, 200 U/ml IFN-α, or 400 U/ml TNF-α for 2–4 days. Cycloheximide and actinomycin D (Sigma-Aldrich) were used at 5 and 2 μg/ml. Retroviruses containing NKG2D ligands were used to generate transductants of MCA102 and BAF as described (21).

Abs and flow cytometry reagents

Mouse NKG2D tetramers (NKG2D-tet) were prepared as described (21). Anti-K^b (AF6-88.5), anti-K^d (SF-1.1), rat IgG2a, goat anti-rat IgG, and anti-CD45 were obtained from BD Pharmingen. Anti-H60 and anti-RAE1 were from R&D Systems. The mAb to MULT1 was generated by immunizing hamsters with recombinant MULT1 protein (L. N. Carayannopoulos and W. M. Yokoyama, unpublished observations). mAb C7 was used to detect mouse NKG2D (37).

BrdU incorporation

Cells were pulsed with 25 μg/ml BrdU for 16 h and stained for surface H60 expression. BrdU incorporation was assessed using the BrdU Flow kit (BD Pharmingen). Staining with control rat IgG2a (as anti-H60 control) and mouse IgG (as anti-BrdU control) were negative.

Real-time PCR

RNA was generated using the RNA-Bee (Tel-Test) protocol. cDNA was made using the Applied Biosystems protocol. Real-time PCRs were performed using the following primers: H60 forward, 5′-GAG CCA CCA GCA AGA GCA A; H60 reverse, 5′-CCA GTA TGG TCC CCA GAT AGC T; H60 probe VIC-TTG CCT GAT TCT GAG CCT TTT CAT TCT GCT-TAMRA; RAE1 forward, 5′ATC AAC TTC CCC GCT TCC A; RAE1 reverse, AGA TAT GAA GAT GAG TCC CAC AGA GAT A; hypoxanthine phosphoribosyltransferase forward, 5′AGC CTA AGA TGA GCG CAA GT; HPRT reverse, 5′TTA CTA GGC AGA TGG CCA CA. The RAE1 primer set recognizes all RAE1 family members.

Chromium release assay

Splenocytes from C57BL/6 or 129 $\times$ B6 F_1 mice were purified using DX5 mAb-conjugated beads and a MACS MS column (Miltenyi Biotec), and NK cells were activated by culturing in medium with 1000 U/ml human IL-2 (Chiron). Day 7 IL-2-activated NK effector cells were used in a 4-h ^{51}Cr-release assay as described (21). Bars depict SE of triplicates. All experiments were done at least twice. Anti-H60 was added at 12 μg/ml. The mAb 5E6 (anti-Ly49C/I) was used at 75 μg/ml. The anti-K^b/D^b $F(ab')_2$ was obtained from Biolegend (clone 28-8-6) and used at 50 μg/ml.

NKG2D down-regulation assay

NK cells were purified from 129 $\times$ B6 F_1 splenocytes by negative selection with nylon wool, anti-CD4, anti-CD8, and anti-B220 Dynabeads (Dynal Biotech). NK cells were positively selected with MACS DX5 mAb-conjugated beads. As assessed by NK1.1 and CD122 staining, purity was 50–70% NK cells. NK cells were cultured with various tumors overnight in medium with 200 U/ml IL-2. NKG2D expression was quantitated by subtracting the mean fluorescence intensity (MFI)^y of control hamster IgG (mAb PIP) staining from the MFI of anti-NKG2D mAb C7 staining of $CD122^+CD3^-$ cells. This experiment was repeated with similar results. When there was a shift in MFI, all cells within the NK gate shifted the MFI.

Tumor transplant and ex vivo analysis

F244 sarcomas were transplanted on day 0 at 3×10^5 cells into the flank of syngeneic WT mice treated with 250 μg of anti-IFN-γ mAb H22 (38) or control PIP mAb at days -5, -2, 0, 5, and 9. On the appropriate day, tumors were excised from mice, minced, and treated with 1 mg/ml collagenase type IA (Sigma-Aldrich). Cells were filtered and stained for FACS analysis.

Results

H60 is the most commonly expressed NKG2D ligand on MCA sarcomas from 129/Sv strain mice and is down-regulated by IFN-γ

Although NKG2D ligands have been detected on many types of murine and human tumors, a systematic analysis of which ligands are expressed on a single type of tumor has not been performed. Toward this end, we characterized a panel of our murine MCA sarcomas derived from 129/Sv strain mice (1) for expression of the individual NKG2D ligands using specific mAbs and NKG2D tetramers (NKG2D-tet). As shown in Fig. 1, H60 is the most commonly detected NKG2D ligand on this representative panel of tumors. In contrast, RAE1 is highly expressed on only a minority of tumors, and MULT1 is poorly detected on all tumors. Analysis of the entire panel of 24 MCA sarcomas revealed that H60 was expressed at moderate to high levels on 70% (17 of 24) of tumors, as defined by a MFI that was at least 5-fold above control MFI (data not shown). Using the same criteria, RAE1 was highly expressed on 25% (6 of 24) of sarcomas, and MULT1 was not highly expressed on any of the 16 tumors investigated.

In previous work, we showed that IFN-γ exerts its tumor-protective effects by increasing tumor cell recognition by adaptive (i.e., $CD8^+$ T cell) immunity (1). To test whether IFN-γ increased tumor immunogenicity through its actions on NKG2D ligands, we treated the same representative panel of sarcomas described above with IFN-γ and measured NKG2D ligand expression. Surprisingly, we found that IFN-γ inhibited NKG2D tetramer staining on all tumors tested (Fig. 1). This down-regulation was detected by both NKG2D-tet and anti-H60 staining. Notably, RAE1 levels were not affected, and MULT1 expression was slightly reduced in some but not all tumors. Similar effects were observed following treatment with IFN-α (see Fig. 4).

To further document the H60 selectivity of the actions of IFN-γ, we examined the effect of IFN-γ on MCA sarcomas from C57BL/6 mice because cells from these mice do not express H60 (35). The

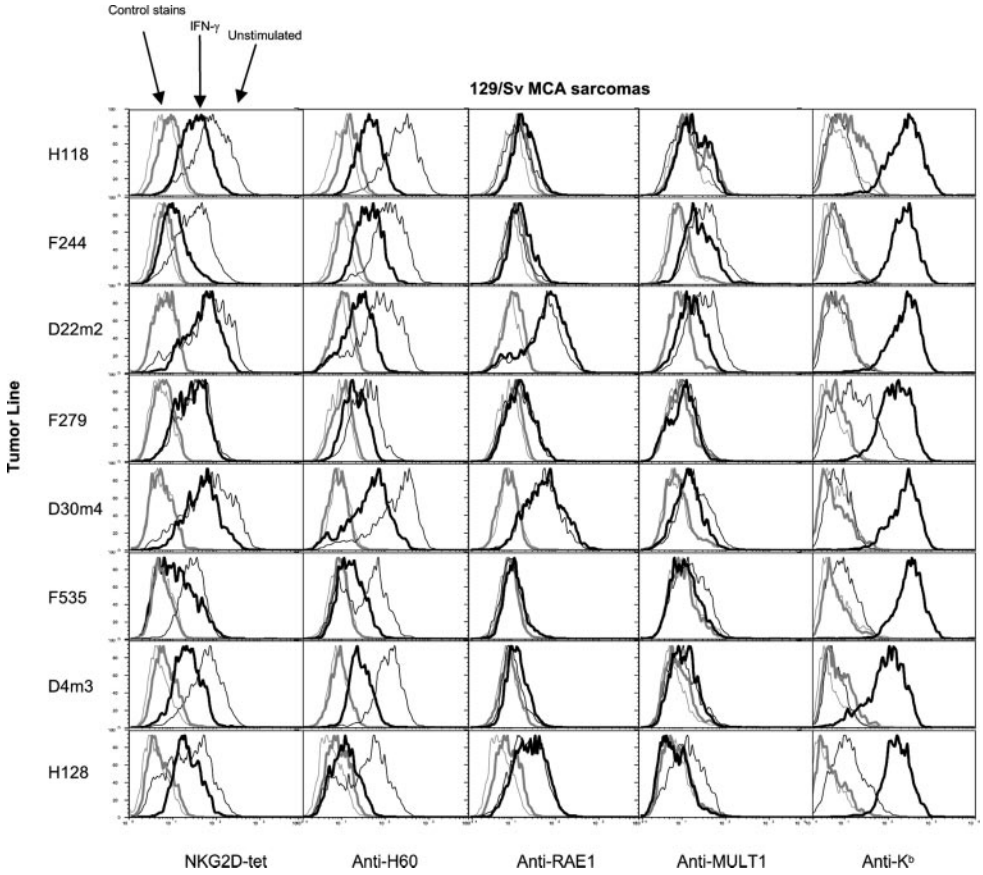

FIGURE 1. IFN-γ reduces H60, the NKG2D ligand most commonly expressed on MCA sarcomas. 129/Sv MCA sarcoma cell lines were treated with IFN-γ (thick lines) or medium alone (thin lines) and stained with the indicated reagents (black lines) or control stains (gray lines). The different tumor lines are indicated on the *y*-axis.

C57BL/6 sarcoma lines MCA106 and MCA207 displayed detectable expression of NKG2D ligands, while MCA102 did not (Fig. 2). Whereas all tumors responded to IFN-γ and up-regulated MHC class I, no change in NKG2D-tet staining was observed. Thus, the effect of IFN-γ on NKG2D-tet staining occurs predominantly through its actions on H60.

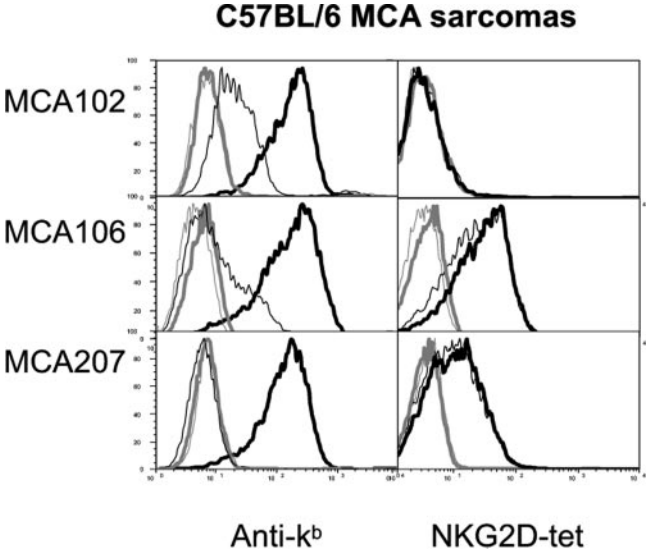

FIGURE 2. IFN-γ does not affect NKG2D-tet staining in C57BL/6 MCA sarcomas that do not express H60. C57BL/6 MCA sarcoma cell lines were treated with IFN-γ (thick lines) or medium alone (thin lines) and stained with the indicated reagents (black lines) or control stains (gray lines). The different tumor lines are indicated on the *y*-axis.

The IFN-γ-induced down-regulation of NKG2D-tet staining on 129/Sv strain MCA sarcomas was dose-dependent, occurred within 1 day of treatment, and was reversed 2 days after removal of IFN-γ (Figs. 3).

H60 expression is not dependent on cellular proliferation

Because IFNs induce an antiproliferative effect in many cell lines, we tested whether NKG2D ligand expression was dependent on the state of cellular proliferation. Toward this end, we compared NKG2D-tet staining of cells maintained in log phase, serum-starved, held at confluence, or treated with IFN-γ, IFN-α, or TNF-α. As shown in Fig. 4*A*, the down-regulation of NKG2D-tet staining occurred following treatment with either IFN-α or IFN-γ treatment. Serum starvation, confluence, and exposure to TNF-α did not decrease NKG2D-tet staining. The slight increase in NKG2D-tet staining due to starvation occurred in only two of four experiments.

We also addressed whether H60 expression was related to cell cycle status. Cells in log growth phase, serum-starved, held at confluence, or treated with IFN-γ were labeled with BrdU and expression of H60 vs BrdU incorporation was compared (Fig. 4*B*). Similar to results in Fig. 4*A*, H60 expression was decreased only in those cells treated with IFN-γ, and this decrease did not correspond to a reduction in percentage of cells which incorporated BrdU. However, BrdU incorporation was reduced in starved or confluent cells while H60 expression was not changed compared with cells in log growth phase. Furthermore, in all conditions, the expression of H60 was equivalent in $BrdU^+$ vs $BrdU^-$ cells, confirming that the effect of IFN-γ on H60 was not related to changes in cell cycle status.

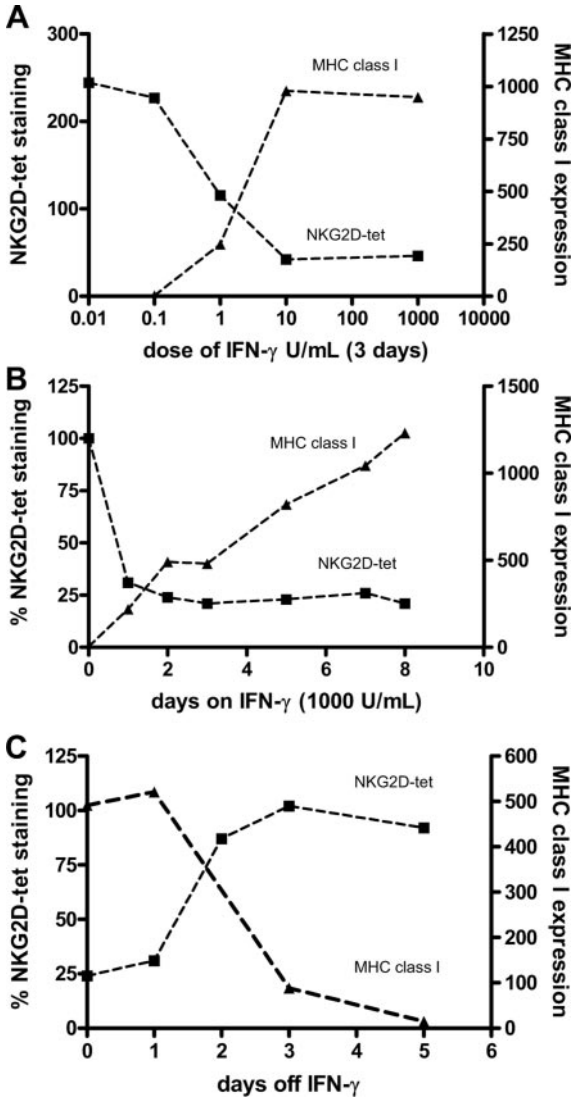

FIGURE 3. Up-regulation of MHC class I and down-regulation of NKG2D-tet occur concomitantly in response to IFN-γ treatment. The F244 sarcoma line was treated with (*A*) the indicated doses of IFN-γ for 3 days, (*B*) 1000 U/ml IFN-γ for the indicated times, or (*C*) 1000 U/ml IFN-γ for 2 days and removed from IFN-γ for the indicated times and stained with NKG2D-tet and anti-K^b. NKG2D-tet staining was quantitated by MFI (*A*) or the percent of basal MFI (*B* and *C*). Anti-K^b was quantitated by MFI.

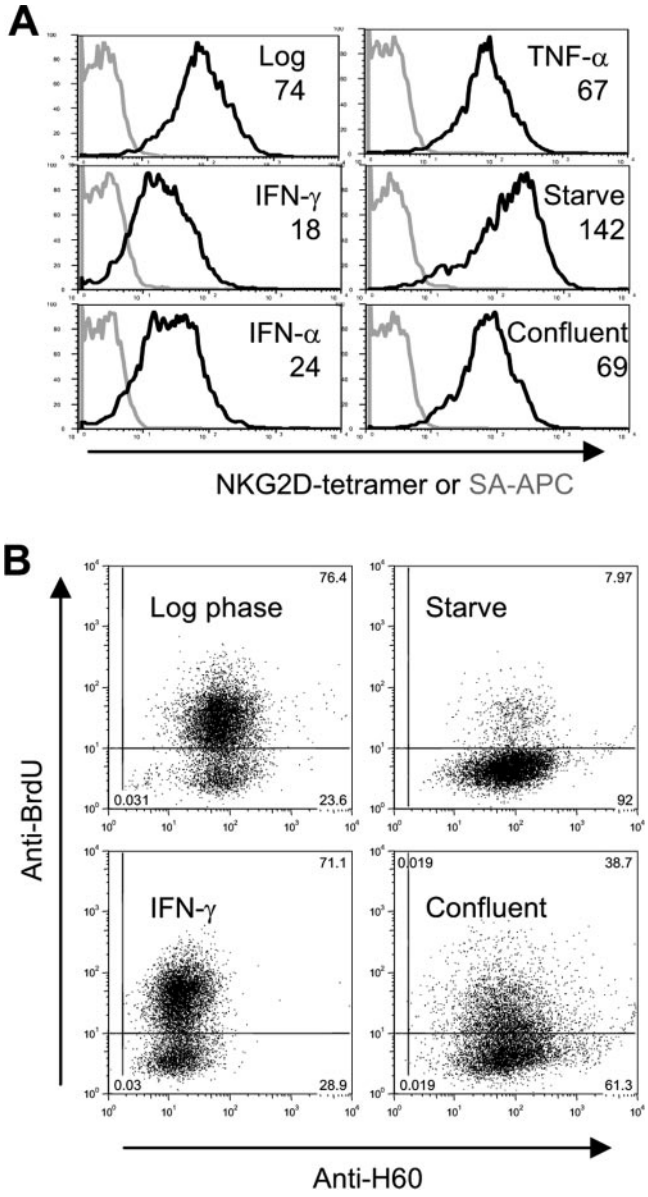

FIGURE 4. H60 down-regulation is not related to cell cycle status. The F244 sarcoma line was treated for 3 days with 1000 U/ml IFN-γ, 200 U/ml IFN-α, or 400 U/ml TNF-α, maintained in log phase with 10% FCS, starved for 2 days in 0.5% FCS, or held at confluence in 10% FCS for 3 days. BrdU was added over the last 16 h of culture. (*A*) NKG2D-tet expression or (*B*) BrdU incorporation and H60 expression is shown. The MFI is shown in *A*.

IFN-γ down-regulation of H60 occurs via a STAT1-dependent reduction in H60 transcripts

IFN-γ regulates gene expression via STAT1-dependent and -independent pathways (39, 40). To explore the mechanism of H60 down-regulation, we measured H60 transcripts in IFN-γ-treated MCA sarcomas from WT and STAT1-deficient 129/Sv mice (1). IFN-γ caused a substantial reduction in H60, but not RAE1 transcripts in STAT1-sufficient cells but not in cells lacking STAT1 (Fig. 5, *A* and *B*). A similar STAT1 dependence was observed when expression was assessed by quantitating levels of H60 protein on the cell surface using NKG2D-tet (Fig. 5*C*) or anti-H60 (data not shown). The response to IFN-γ was restored when STAT1 was retrovirally transduced into the STAT1-deficient sarcomas (Fig. 5*C*). Thus, the effect of IFN-γ on H60 occurs at the level of transcript and is manifest in a STAT1-dependent manner.

MCMV-induced H60 down-regulation occurs via a posttranslational mechanism (34). Therefore, we assessed whether IFN-γ regulation of H60 could also involve a similar mechanism. For this purpose, we expressed the different NKG2D ligands in MCA102 sarcoma cells because these cells do not display detectable levels of NKG2D ligands (Fig. 2). Surface expression of the transduced ligands, as detected by NKG2D-tet staining, did not change following IFN-γ treatment (Fig. 5*D*). This result thus demonstrates that IFN-γ regulates only endogenous H60 expression and thereby points out that its effects are mechanistically distinct from those of MCMV.

The down-regulation of H60 by IFN-γ on tumor cells compromises their recognition by NK cells

To determine whether the IFN-γ-dependent down-regulation of H60 had functional consequences, we measured two parameters of

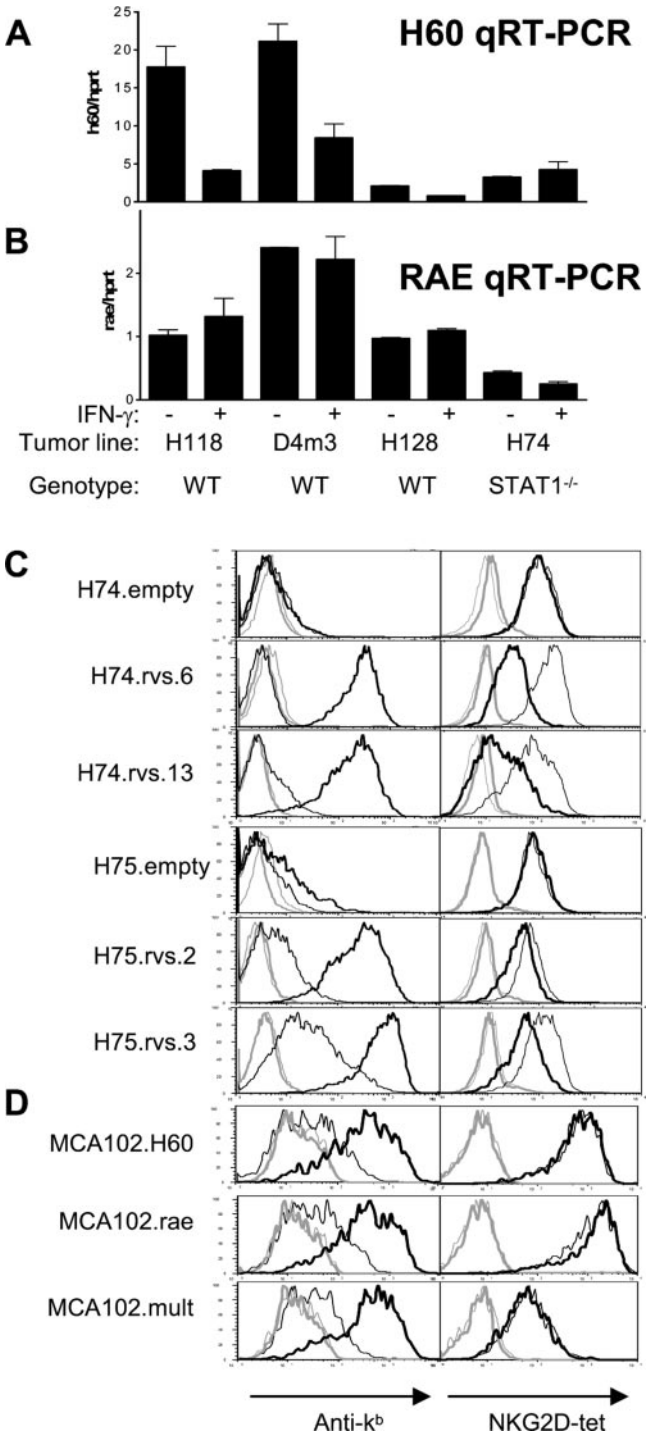

FIGURE 5. IFN-γ down-regulates H60 transcript via a STAT1-dependent mechanism and does not affect transduced H60. (*A*) H60 and (*B*) total RAE-1 transcripts were quantitated by real-time PCR from various IFN-γ-treated or unstimulated tumors as indicated on the *x*-axis. *C*, The STAT1$^{-/-}$ sarcoma lines H74 and H75 were transduced with empty retrovirus (H74.empty, H75.empty) or a STAT1-containing retrovirus (H74.rvs.6, H74.rvs.13, H75.rvs.2, H75.rvs.3). Clones were selected and treated with medium or IFN-γ, stained with the indicated reagents, and analyzed by flow cytometry. Legend is as indicated in Fig. 1. *D*, The NKG2D ligand-negative cell line MCA102 was transduced with the indicate NKG2D ligands, treated with IFN-γ, and stained with NKG2D-tet. The transduction efficiency was 30%. Only the GFP-positive population is shown for MCA102 transductants; the GFP-negative population did not stain with NKG2D-tet above background levels, and staining was not affected by IFN-γ treatment in this population. Legend is as indicated in Fig. 1.

NK cell-tumor cell interaction-tumor cell cytotoxicity and NKG2D down-regulation. Published studies have shown that IFN-γ-treatment of human and murine tumors significantly inhibits tumor killing by freshly isolated or IL-2-activated NK cells (5–12). Therefore, we monitored the capacity of IL-2-activated NK cell effectors from C57BL/6 mice to kill IFN-γ-treated or untreated F244 sarcoma cells in vitro (Fig. 6*A*). Indeed, the killing of F244 sarcoma cells was significantly diminished by pretreatment of the target cells with IFN-γ thereby confirming findings from other groups. Addition of anti-H60-blocking mAb reduced killing of unstimulated F244, demonstrating that H60 is involved in the NK cell recognition of F244. This result suggests that any event that down-regulates H60 should reduce NK cell killing of the affected target cells. The fact that recognition was not completely ablated suggests that other IFN-γ-regulated components may also play some role in the process.

Next, we assessed directly whether H60 down-regulation was required for the inhibitory effects of IFN-γ on NK cell killing. Toward this end, we used the MCA102.H60 sarcoma line that had been engineered for enforced, stable H60 expression and sorted it for NKG2D-tet staining equivalent to the F244 sarcoma line to produce the MCA102.H60.med subline. We then assessed NK cell killing of this line after treatment with IFN-γ or medium. IFN-γ treatment reduced NKG2D-tet staining of F244 but not MCA102.H60.med cells, confirming that the transduced H60 was resistant to the effects of IFN-γ (Fig. 6, FACS histogram, *inset*). MHC class I was up-regulated on both IFN-γ-treated cell lines (data not shown, but see Figs. 1 and 2 for comparison). Importantly, although IFN-γ treatment of F244 reduced killing by IL-2-activated NK cells (Fig. 6*A*), no effect was observed on the killing

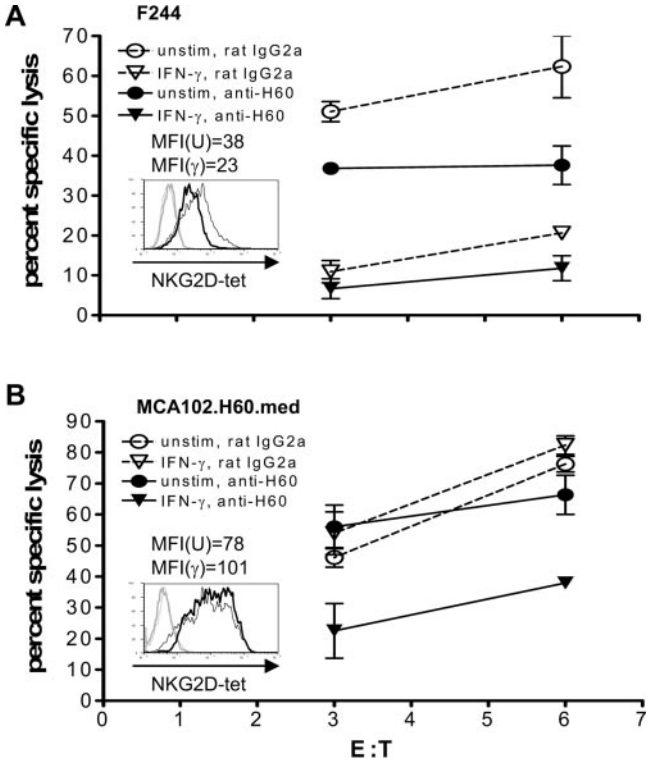

FIGURE 6. IFN-γ pretreatment of tumors inhibits IL-2-activated NK cell-mediated killing. The tumor lines F244 (*A*) or MCA102.H60.med (*B*) were treated with medium or IFN-γ and used as targets in a chromium release assay against IL-2-activated NK effector cells. Assays were performed in the presence of anti-H60 or isotype control. *Inset*, Histogram analysis of tumor lines used in killing assays. Legend is as indicated in Fig. 1.

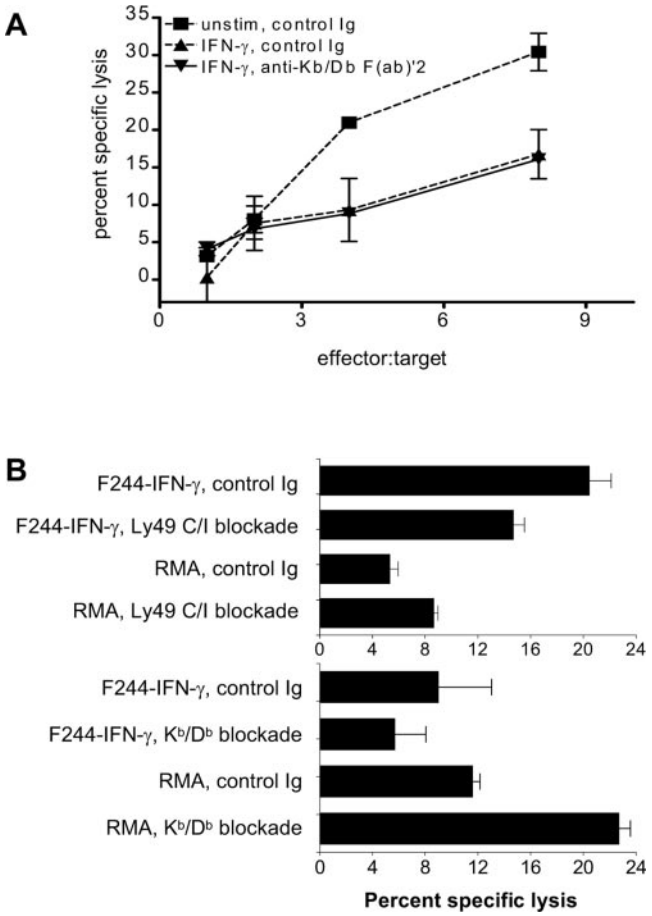

FIGURE 7. Blockade of Ly49C-MHC class I interactions does not affect IFN-γ-induced inhibition of sarcoma target recognition. *A*, The tumor line F244 was treated with IFN-γ or medium, labeled with ^{51}Cr, and used in a conventional cytotoxicity assay with day 7 IL-2-activated NK cells from (129 × B6)F_1 mice. Anti-K^b/D^b F(ab′)$_2$ was added where indicated. *B*, IFN-γ-treated F244 or untreated RMA were used as targets in chromium release assays in the presence of blocking Ab to Ly49 C/I (*top graph*) or K^b/D^b (*bottom graph*). Experiments were done on separate days at an E:T ratio of 4:1 for the *top graph* and 2:1 for the *bottom graph*. Effector cells were purified NK cells from poly(I:C)-treated mice.

of IFN-γ-treated MCA102.H60.med cells (Fig. 6*B*). These results show that enhanced MHC class I expression is not sufficient to inhibit NK cell recognition of target cells expressing substantial levels of H60–a result that confirms and extends previous observations by others (10–12). However, addition of blocking H60 mAb reduced target killing of IFN-γ-treated but not untreated MCA102.H60.med–a result that is consistent with our data described above indicating that other IFN-γ-regulated genes are also important in target recognition. The level of NKG2D tetramer staining of the transduced MCA102.H60.med cell line was within 2-fold that of F244 and was equivalent to other MCA sarcomas which still showed IFN-γ-induced inhibition of NK recognition (data not shown). It is currently unclear whether the inability of anti-H60 to block killing of unstimulated MCA102.H60.med cells is due to the presence of other recognition ligands or to the slightly higher levels of H60 on this cell line.

MHC class I recognition is not required for IFN-γ-induced inhibition of NK cell target killing

Previous reports have shown that K562 cells treated with IFN-γ are poorly recognized by NK cells through an unknown mechanism which is independent of MHC class I up-regulation (10, 11). We performed similar experiments in our system to directly address the importance of MHC class I up-regulation in mediating the inhibitory effect of IFN-γ on NK-cell target killing. In Fig. 7, Ly49C-MHC class I interactions were blocked by adding mAbs specific for Ly49C or K^b. Although both Abs were capable of augmenting the killing of RMA targets, neither Ab was able to enhance the killing of IFN-γ-treated F244 sarcomas. Thus, our studies support the hypothesis that the effect of IFN-γ on reducing target recognition by NK cells is most likely mediated by multiple factors.

Tumor cell-dependent down-regulation of NKG2D expression on NK cells is reversed when tumors are pretreated with IFN-γ

Previous work has shown that both mouse and human NK cells internalize NKG2D following exposure to NKG2D ligands (27, 32). Therefore, we examined the capacity of the MCA sarcomas to down-regulate NKG2D expression on NK cells. We incubated overnight freshly isolated NK cells with sarcomas pretreated with either IFN-γ or medium and used the C7 anti-NKG2D mAb to quantitate NK cell receptor expression. MCA sarcomas from WT 129/Sv and C57BL/6 strain mice and from STAT1$^{-/-}$ mice induced a 70–80% decrease in NKG2D expression on all NK cells (Fig. 8). Notably, IFN-γ pretreatment of WT 129/Sv tumors significantly impaired their capacity to down-regulate NKG2D on NK cells. In contrast, IFN-γ treatment of the H74.empty STAT1-deficient clone or the C57BL/6 sarcoma MCA106 did not affect their capacities to alter NK cell NKG2D expression. Thus, the down-regulation of H60 by IFN-γ on MCA sarcomas is sufficient to compromise the ability of these tumors to inhibit NKG2D expression on NK cells.

NKG2D tetramer staining is down-regulated on tumors isolated after in vivo passage

To determine whether the IFNs function to down-regulate NKG2D ligand expression on tumors in vivo, we transplanted F244 sarcoma cells into WT syngeneic mice treated with either control PIP mAb or the IFN-γ-specific H22-blocking mAb, harvested the tumors after 12 days, and assessed MHC class I and H60 expression on single tumor cell suspensions by flow cytometry. Tumors cells isolated from control mAb-treated mice (Fig. 9*A*) displayed up-regulated staining for MHC class I and down-regulated anti-H60 or NKG2D-tet staining compared either to tumor cells from mice

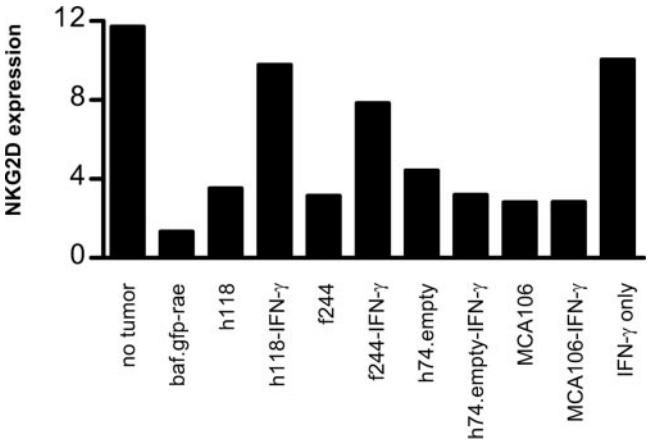

FIGURE 8. Ligand-induced NKG2D down-regulation is inhibited by IFN-γ treatment of the ligand-expressing cells. Purified NK cells were cocultured with tumors pretreated with IFN-γ or left unstimulated, and NKG2D expression on the NK cells was quantitated by flow cytometry.

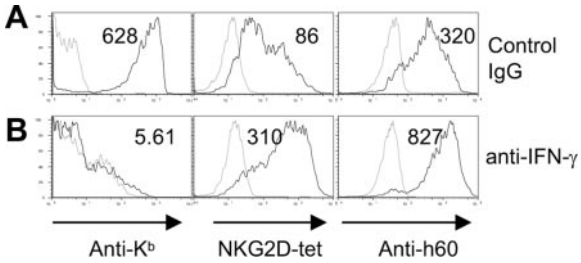

FIGURE 9. IFN-γ-dependent down-regulation of NKG2D-tet staining in vivo. The tumor line F244 was transplanted into syngeneic mice pretreated with (*A*) control mAb PIP or (*B*) anti-IFN-γ mAb H22. At 12 days posttransplant, tumors were harvested and stained with control reagents (gray lines) or anti-K^b, anti-H60, or NKG2D-tet (black lines), and subjected to FACS analysis. Shown are CD45-negative, propidium iodide-negative tumor cells.

treated with blocking Ab to IFN-γ (Fig. 9*B*) or to unmanipulated tumors (data not shown). Similar results were found when F244 cells were analyzed on days 7, 13, and 16 posttransplant (data not shown). Thus, IFN-γ is produced at sufficient doses in vivo to elicit the same effects seen on tumor targets as were observed in the in vitro studies.

Discussion

The signals that induce and/or regulate NKG2D ligand expression on murine tumors are only now being identified, and "stress" is thought to be a common inducing signal (18, 24, 30, 31, 33, 41). In the specific case of H60, previous studies have reported increases in cellular H60 message following carcinogen exposure or splenocyte activation (25, 35). The studies presented in this report indeed confirm that H60 expression is regulated at the level of transcript, but we present the novel finding that H60 transcripts are decreased in cells exposed to the IFNs. Whether this reduction is the result of decreased gene expression or degradation of the H60 message requires additional experiments. Nevertheless, the diminished H60 expression displays an obligate requirement for STAT1, a result that establishes a mechanistic basis for the effect. IFN-mediated transcriptional repression has been described for several genes, but the molecular basis is still poorly understood (42). In these studies, IFN-γ activation site elements are required but not sufficient for mediating gene repression. Additional experiments are required to identify the basis of STAT1-dependent H60 down-regulation.

We have also shown that the surface expression of H60 varies considerably between histologically similar tumors. In preliminary studies, we also found substantial differences in H60 transcript levels in these tumors. Further studies will need to examine whether differences in H60 surface expression are the result of alterations in STAT1 function, mechanisms involving posttranscriptional regulation, or epigenetic changes in the *H60* locus. The variability in H60 expression could also come as a result of the cancer immunoediting process itself (1, 2, 43, 44). Indeed, one might expect that if H60 is a critical recognition molecule that promotes immunosurveillance, then H60 levels might be expected to vary in tumors that have been edited by different immunologic processes.

Based on the data presented in this report, we speculate that H60 down-regulation is one of many IFN-γ-dependent effects which ultimately lead to inhibition of target recognition. One pertinent question is why should it be necessary to modulate expression of both an inhibitory ligand (MHC class I) and an activating ligand (H60). It should be noted that whereas only 30% of NK cells from C57BL/6 mice express Ly49C and thus can be inhibited by up-regulation of target cell MHC class I, all NK cells express NKG2D. Thus, one teleologic reason for the dual effects of IFN-γ on tumor targets is that these effects allow for a more global inhibition of target cell recognition by NK cells. At the level of the NK cell, inhibition of NKG2D function would lead to a more general blockade of NK function than inhibition via engagement of inhibitory receptors whose expression is variable on NK cells. In contrast, whereas all tumors we have tested can up-regulate MHC class I, only 70% of fibrosarcomas have moderate to high levels of H60. Thus, the combination of enhancing MHC class I and down-regulating H60 would serve as a more global means of inhibiting NK cell target recognition.

A second reason for the dual modulation relates to the observation that overexpression of NKG2D ligands on targets can lead to NK cell killing even in the presence of high levels of MHC class I (19, 20). Indeed, we have found that when H60 is transduced into a tumor at high levels and is not down-regulatable, IFN-γ no longer inhibits tumor cell recognition, even though MHC class I is highly expressed. However, anti-H60 blockade restores the ability of IFN-γ to block NK cell recognition. Thus, we propose that H60 down-regulation is an important part of IFN-γ-dependent inhibition of target recognition, especially in cases where H60 is highly expressed and might overcome inhibition by MHC class I.

We expect that more IFN-γ-regulated genes will be found to play a role in inhibiting NK cell target recognition. Other inhibitory ligands such as Qa-1 and clr-b should be examined further for their IFN-γ-dependent regulation on tumor cells. Moreover, because H60 is not expressed on all tumors and is not functional in C57BL/6 mice, one would expect that other activating ligands might be regulated by IFN-γ as well. Indeed, the failure of anti-H60 to fully block NK cell recognition of our C57BL/6 tumor line transduced with H60 suggests that there are other recognition ligands not detected by NKG2D-tet present on this target cell. Finally, whereas our discussion centers on the role of the IFNs in target recognition by NK cell surface receptors, it should be mentioned that other studies have shown that the IFNs can decrease target killing mediated by complement or cytotoxic granules (6).

Considering the pleiotropic effects of IFN-γ on tumor cells, it is difficult to ascertain the role of IFN-γ-regulated MHC class I/Ly49 interactions in determining the outcome of NK cell-tumor cell interactions. Some studies have shown that MHC class I up-regulation is required for the IFN-γ-dependent attenuation of NK cell-target cell interaction (45, 46), whereas other studies have shown that MHC class I is dispensable (11, 12). Based on our data, we would expect that in tumors which have little to no expression of H60 such as those from the C57BL/6 strain and some of our 129/Sv strain MCA sarcomas, MHC class I would play a larger role in mediating the inhibitory effects of IFN-γ on target recognition. Future experiments are required to define more precisely the role of IFN-γ-dependent MHC class I up-regulation in our panel of tumors, although the data presented herein suggest that MHC class I up-regulation is not required to mediate the effects of IFN-γ on at least a portion of MCA sarcomas with high levels of H60 (Fig. 7*B*).

We have previously reported that IFN-γ enhances tumor recognition by the immune system via up-regulation of tumor cell MHC class I resulting in enhanced tumor recognition by $CD8^+$ T cells, components of the adaptive immune system. Based on the data presented in the current report, we now speculate that IFN-γ may promote a functional switch from innate to adaptive mechanisms of tumor immunity. We know that during an antitumor response, IFN-γ acts on both tumor target cells and host cells (1–4, 47). By inhibiting H60 expression on host tissues, IFN-γ could protect

these tissues from bystander NK cell killing. In contrast, the concomitant down-regulation of H60 and up-regulation of MHC class I proteins on tumor cells would effectively convert them from NK cell targets to $CD8^+$ T cell targets. Although some studies have shown a positive role for murine NKG2D receptor-ligand interactions in costimulating $CD8^+$ T cell proliferation and cytotoxicity (17, 48), other studies have found no role for this ligand-receptor system in affecting T cell function (49) and some even report that certain NKG2D ligands can inhibit $CD8^+$ T cell function in an NKG2D-independent manner (50). Indeed, our preliminary finding is that IFN-γ treatment of the F244 sarcoma line enhanced its recognition by a T cell line while inhibiting its recognition by NK cells (J. D. Bui and R. D. Schreiber, unpublished observations). The IFN-dependent inhibition of NK cell recognition of tumors described here should be tested against models in which NK cell mediated antitumor responses are enhanced due to IFN-dependent up-regulation of TRAIL-death receptor pathways (51). At this time, it is unclear what advantage there would be in turning off the innate immune system during an antitumor response. However, NK cell proliferation is known to spontaneously cease during an antiviral response, although the molecular underpinnings of this phenomenon are still under investigation (52). We propose that the IFN-mediated down-regulation of H60 that we describe here might play a role in the broader regulation of innate and adaptive immunity by the pleiotropic actions of the IFN system.

Although it is clear that enough IFN-γ is produced at the tumor site to modulate both MHC class I and H60 (Fig. 9), it is unclear which host immune cells (i.e., NK or $CD8^+$ T cells) are producing the IFN-γ during an antitumor response. Preliminary studies show that when MCA sarcomas were transplanted into $RAG2^{-/-}$ mice, there was no tumor cell up-regulation of MHC class I or down-regulation of H60, suggesting that without adaptive immunity, the NK cells present in $RAG2^{-/-}$ mice did not produce enough IFN-γ to have effects on tumor cell recognition (J. D. Bui and R. D. Schreiber, unpublished observations). Furthermore, our preliminary studies in WT mice show that while both NK cells and $CD8^+$ T cells infiltrated MCA sarcomas, the kinetics of $CD8^+$ T cell infiltration more closely correlated with tumor cell MHC class I up-regulation and H60 down-regulation (J. D. Bui and R. D. Schreiber, unpublished observations). Future studies are needed to determine whether $CD8^+$ T cells are required for the modulation of tumor cell MHC class I and H60 and whether this modulation can be exploited to enhance tumor cell eradication by the intact immune system.

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Disclosures

The authors have no financial conflict of interest.

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