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# A Central Role for Death Receptor-Mediated Apoptosis in the Rejection of Tumors by NK Cells<sup>1</sup>

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NK cells provide a line of defense against tumors and virus-infected cells that have lost the expression of one or more MHC class I isoforms. Here, we investigate whether inhibitors of apoptosis can block the rejection of tumors mediated by NK cells, by introducing the long form of Fas-associated death domain-like IL-1 $\beta$ -converting enzyme-associated inhibitory protein (FLIP<sub>L</sub>) and poxvirus cytokine response modifier A (CrmA) into the MHC class I-deficient T lymphoma cell line RMA-S. RMA-S cells do not normally express Fas *in vitro*, and it was previously postulated that the rejection of these tumors by NK cells is strictly perforin dependent. We show that perforin-deficient NK cells directly mediate Fas up-regulation on RMA-S cells and thereafter kill the cells in a Fas-dependent manner, and that RMA-S FLIP<sub>L</sub> and RMA-S CrmA are protected from such killing. When injected in immunocompetent recipients, RMA-S cells up-regulate Fas, rendering *in vivo*-passed mock-transduced cells sensitive to Fas-mediated apoptosis. Moreover, RMA-S FLIP<sub>L</sub> and RMA-S CrmA cells establish aggressive tumors, in contrast to RMA-S mock cells that are rejected. These results demonstrate that FLIP<sub>L</sub> and CrmA function as tumor progression factors by protecting MHC class I-deficient tumors from rejection mediated by NK cells. Moreover, our data indicate that death receptor-mediated apoptosis has a more prominent role in the clearance of NK-sensitive tumors than previously suggested. *The Journal of Immunology*, 2001, 167: 2068–2073.

**T**umors that have down-regulated the expression of MHC class I molecules are able to evade the immune response mediated by CTLs, but are efficiently rejected by NK cells (1–4). NK cells receive inhibitory signals through the binding of specific receptors to MHC class I molecules, and cells that lack or have down-regulated MHC class I fail to deliver the inhibitory signals that block the cytotoxic activity of NK cells (5–7).

Cytotoxicity is mainly mediated through the release of granules containing perforin and granzymes or through the induction of death receptor-mediated apoptosis. In the perforin-dependent pathway, the entry of granzymes into the target cell leads to the activation of both caspase-dependent and caspase-independent apoptotic pathways (8). In death receptor-mediated apoptosis, death ligands on effector cells, such as Fas ligand (FasL<sup>3</sup>; CD95, Apo1), TNF, or TNF-related apoptosis-inducing ligand (TRAIL), bind to death receptors expressed on the surface of the target cell. Upon death receptor ligation, intracellular adapter molecules are recruited, and these molecules can, in turn, associate with initiator caspases through death effector domain (DED) or caspase recruitment domain interactions (9) leading to their

activation, thereby starting the caspase cascade with the final demise of the cell as a result (10).

Several proteins, of both cellular and viral origin, are able to block apoptosis at different levels. For example, the long form of Fas-associated death domain-like IL-1 $\beta$ -converting enzyme-associated inhibitory protein (FLIP<sub>L</sub>), a member of the Fas-associated death domain-like IL-1 $\beta$ -converting enzyme inhibitory protein family, prevents the association of the initiator caspases 8 and 10 with the adapter molecule Fas-associated death domain through DED-DED interactions, thereby blocking caspase activation triggered by death ligands (11, 12). Another example is the cytokine response modifier A (CrmA), an orthopoxvirus-encoded protein that has been shown to block death receptor-mediated apoptosis by preventing the activation of caspase-8 (13).

Previously, we and others have shown that inhibition of death receptor-mediated apoptosis by FLIP in murine tumor models confers resistance to rejection by conventional T cells, leading to uncontrolled tumor progression (14, 15). In this study we use MHC class I-deficient tumors that are normally rejected by NK cells to test the hypothesis that the inhibitors of death receptor-mediated apoptosis FLIP<sub>L</sub> and CrmA can act as tumor progression factors preventing the cytotoxic activity of NK cells *in vitro* and *in vivo*.

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<sup>3</sup> Abbreviations used in this paper: FasL, Fas ligand; sFasL, soluble FasL; TRAIL, TNF-related apoptosis-inducing ligand; FLIP<sub>L</sub>, long form of Fas-associated death domain-like IL-1 $\beta$ -converting enzyme-associated inhibitory protein; CrmA, cytokine response modifier A; PKOB, perforin-deficient C57BL/6 mice; CHX, cycloheximide; DED, death effector domain.

## Materials and Methods

### Cell lines and mice

The mouse T lymphoma cell lines RMA and RMA-S and the human retroviral packaging cell line Phoenix-Ampho were grown as previously described (14, 16). RMA-S cells (2, 3) have a mutation in the *TAP-2* gene that impairs peptide trafficking into the endoplasmic reticulum (17), resulting in the expression of low levels of unstable MHC class I molecules at the cell surface (18). Sex- and age-matched (4- to 6-wk-old) inbred C57BL/6 mice were obtained from Charles River (Uppsala, Sweden). FasL mutant mice (*gld*) bred on the C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in the animal facility at Stockholm University. Perforin-deficient C57BL/6 mice (PKOB) (19) were maintained in the animal facility at Karolinska Institute.

### Expression vectors and cell transduction

Human FLIP<sub>L</sub> was amplified by PCR from PBMC, using the oligonucleotides hFLIP5'*Mfe* (5'-ACT TAG CAA TTG CCA CCA TGT CTG CTG AAG TCA TCC AT-3') and hFLIP3'*Mfe* (5'-ATT CAC AAT TGT TAT GTG TAG GAG AGG ATA AGT TTC-3'). The fragment was inserted into the *EcoRI* site of the retroviral expression vector pLXIN (Clontech, Palo Alto, CA). CrmA was amplified by PCR from the CrmA/pRK5 expression vector (a gift from Dr. D. Göddel, Tulank, San Francisco, CA) using the oligonucleotides CrmA5'*Eco* (5'-TTG GCG AAT TCA CAC AAC AAA ATC GAT TGC CAT G-3') and CrmA3'*Eco* (5'-GTC AGG AAT TCG AAT GTG ATT TAA TTA GTT GTT GGA GAG-3') and inserted into the *EcoRI* site of the retroviral expression vector pLXIN. All vectors were then separately used to transiently transfect the Phoenix-Ampho packaging cell line (provided by Dr. G. P. Nolan, Stanford University, Stanford, CA). Supernatants containing recombinant viral particles were used for transduction of RMA-S cells, and stable G418-resistant clones were obtained. mRNA expression was verified by RT-PCR, and the presence of helper virus was excluded by PCR amplification of viral *env* using the primers 5'-ACCTGGAGAGTCACCAACC-3' and 5'-TACTTTG GAGAGGTCGTAGC-3'.

### Flow cytometry

Cells were washed twice in PBS containing 2% FCS and 0.1% NaN<sub>3</sub>, and incubated with an anti-H-2K<sup>b</sup> mAb (AF6-88.5), an isotype control mAb (G235-2356), or an anti-Fas mAb (Jo2), in the presence of anti-CD32/CD16 (Fcblock; all Abs were obtained from PharMingen, San Diego, CA). Propidium iodide was added to discriminate dead cells. Fresh C57BL/6 splenocytes were used as a positive control for MHC class I staining, and A20 cells were used as a positive control for Fas staining. Cells were analyzed in a FACScan (BD Biosciences, San Jose, CA), gating for living cells.

### Apoptosis and limiting dilution assays

Sensitivity to Fas-induced apoptosis was assessed by treating  $5 \times 10^5$  cells with 100 ng/ml of the anti-mouse Fas mAb Jo2 (PharMingen) for 24–72 h at 37°C. Alternatively, mouse soluble FasL (sFasL) was added at a 1/2 dilution (FasL-hCD8 $\alpha$ /pSG5 vector, a gift from Dr. H. Yagita, Juntendo University, Tokyo, Japan). In some assays, 130 or 13 U/ml IL-2 or 10-fold dilutions (from 100 to 1 U/ml) of IFN- $\gamma$  (Genzyme, Cambridge, MA) were added together with sFasL or the agonistic Jo2 mAb. The sensitivity to TNF-induced apoptosis was assessed by treating  $5 \times 10^5$  cells with 10-fold dilutions of mouse recombinant TNF (Genzyme) starting from 10<sup>4</sup> U/ml in both the absence and the presence of 10-fold dilutions of cycloheximide (CHX; Sigma, St. Louis, MO) from 10  $\mu$ M to 1 nM for 24–72 h at 37°C. CHX-induced apoptosis was assessed by treating  $5 \times 10^5$  cells with 10 or 1  $\mu$ M CHX for 3, 6, and 24 h at 37°C. Etoposide-induced apoptosis was assessed by treating  $5 \times 10^5$  cells with 10-fold dilutions of VP16 (Sigma) from 10  $\mu$ g/ml to 1 ng/ml for 24–42 h at 37°C. TRAIL-induced apoptosis was assessed by treating  $5 \times 10^5$  cells with 100–600 ng/ml recombinant human soluble TRAIL (Alexis, San Diego, CA) in the presence of 2  $\mu$ g/ml enhancer, as recommended by the manufacturers, for 24–72 h at 37°C. Cells were stained with propidium iodide, and apoptosis was monitored by flow cytometric analysis. For limiting dilution assays, cells were plated at different cell inputs, starting from 32 to 0.25 cells/well in 100  $\mu$ l medium. Cells were seeded in 48 wells for each cell input. The frequency of clonal growth was determined by visual inspection on day 12 and was calculated as previously described (20).

### Generation of effector cells

IL-2-activated NK cells were prepared according to the following protocol:  $25 \times 10^6$  erythrocyte-depleted splenocytes were resuspended in  $\alpha$ MEM containing 10 mM HEPES,  $5 \times 10^{-5}$  M 2-ME, and 10% FCS (all reagents from Life Technologies, Paisley, U.K.) supplemented with 1000 U/ml human rIL-2 (PeproTech, Rocky Hill, NJ) and cultured in 25-cm<sup>2</sup> tissue culture flasks in 10% CO<sub>2</sub> at 37°C. When NK cells were generated from perforin-deficient mice, an additional purification step was introduced at the beginning of culture, using an anti-DX5 Ab linked to MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instruction. After 5 or 6 days, IL-2-activated NK cells were resuspended by pipetting and scraping and were used for cytotoxicity assays.

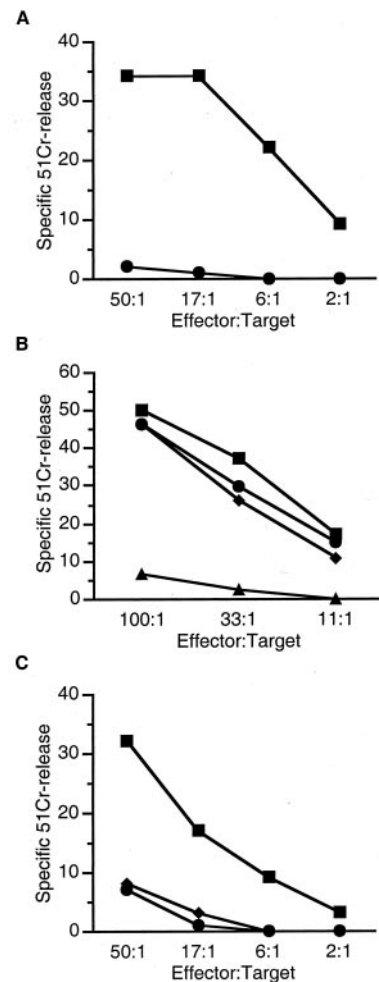
### Cytotoxicity assay and Fas up-regulation assay

Effector cells were washed once, resuspended, and used as effectors in standard 4- or 18-h <sup>51</sup>Cr release assays. Briefly, target cells were labeled with 10–20  $\mu$ l 10 mCi/ml Na<sup>51</sup>CrO<sub>4</sub> for 1 h at 37°C and then washed. Cells ( $5 \times 10^3$ ) were incubated with titrated numbers of effector cells in

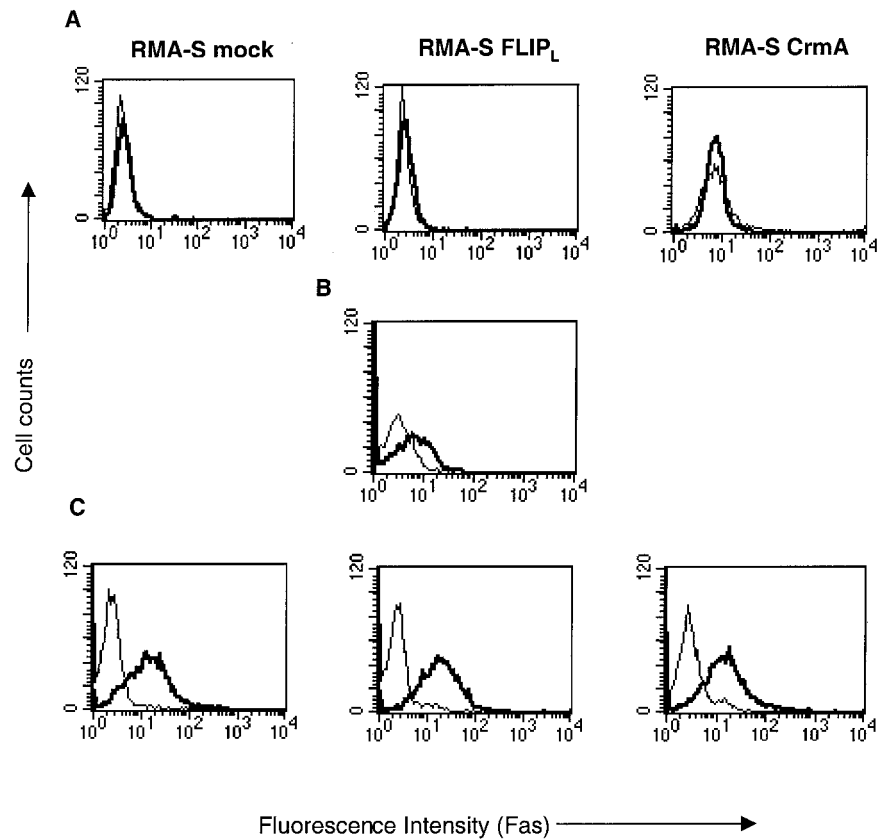
round-bottom 96-well plates for 4 or 18 h at 37°C in 10% CO<sub>2</sub>. In some experiments, anti-mouse FasL mAb (MFL3; PharMingen) was added to the culture at 10  $\mu$ g/ml. After incubation, released radioactivity was measured, and specific lysis was calculated according to the formula: % specific release = [(experimental release – spontaneous release)/(maximum release – spontaneous release)]  $\times$  100. In the Fas up-regulation assay, effector cells were incubated with  $1 \times 10^5$  target cells at a 50:1 ratio for 18 h at 37°C in 10% CO<sub>2</sub>. Cells were then analyzed in a flow cytometer by staining cells with an NK-specific mAb (2B4; PharMingen), an isotype control (G235-2356), or an anti-Fas mAb (Jo2) in the presence of anti-CD32/CD16 (Fcblock). 2B4-positive cells were then excluded from the analysis for Fas expression.

### Injection of mice with tumor cells

Groups of five to eight mice were injected s.c. in the interscapular region with  $1 \times 10^5$ ,  $1 \times 10^4$ ,  $1 \times 10^3$ , or  $1 \times 10^2$  RMA, RMA-S, and RMA-S-transduced cells. In experiments performed with NK cell-depleted mice,



**FIGURE 1.** Cytotoxicity is mediated through both the perforin and the Fas/FasL pathway. Cytotoxicity was monitored by means of specific lysis in a <sup>51</sup>Cr release assay, as described in *Materials and Methods*. *A*, NK cells generated from PKOB splenocytes were mixed with <sup>51</sup>Cr-labeled RMA-S mock cells in the absence (■) or the presence (●) of 10  $\mu$ g/ml anti-FasL mAb, and lysis was measured after 18 h. *B*, NK cells generated from C57BL/6 splenocytes were mixed with <sup>51</sup>Cr-labeled RMA (▲), RMA-S FLIP<sub>L</sub> (●), and RMA-S CrmA (◆) cells, and lysis was measured after 4 h. RMA cells were used as control targets to exclude any contribution by CD8<sup>+</sup> CTLs to the measured cell lysis, as splenocytes from C57BL/6 mice were not sorted to obtain an enriched NK cell population. *C*, NK cells generated from PKOB splenocytes were mixed with <sup>51</sup>Cr-labeled RMA-S mock (■), RMA-S FLIP<sub>L</sub> (●), and RMA-S CrmA (◆) cells, and lysis was measured after 18 h. Results shown are representative of a series of at least three experiments.



**FIGURE 2.** Fas expression is up-regulated by NK cells *in vitro* and after *in vivo* passage. Flow cytometric analysis was conducted as described in *Materials and Methods*, using a Fas-specific Ab (thick line) and an isotype-matched control (thin line) on cells *in vitro* (A), after 18-h incubation with NK cells (B), and after *in vivo* passage (C).

animals were injected i.p. with 100  $\mu\text{g}$  anti-NK1.1 2 days before tumor grafting, then 2 days, 1 wk, and 2 wk after tumor challenge. Tumors were monitored every second day by palpation, for 8 wk. Mice were killed with  $\text{CO}_2$  when tumors had reached  $\sim 1 \text{ cm}^3$ , as recommended by the Stockholm ethical committee for animal experiments or when the experiment was terminated. Tumor samples were obtained by surgical excision and were used for immunostaining and apoptosis assays.

## Results

### *In vitro* NK cell-mediated cytotoxicity is elicited through both death receptor- and perforin-mediated apoptosis

The murine tumor model, comprising RMA T lymphoma cells and the MHC class I-deficient variant RMA-S, has previously been used in many studies, contributing to the knowledge of NK cell-mediated rejection of MHC class I-deficient tumors (2–4, 16, 21, 22).

We, like others (16, 22), have observed that NK cells generated from wild-type mice readily kill RMA-S cells in a specific  $^{51}\text{Cr}$  release assay, whereas NK cells generated from PKOB fail to kill RMA-S cells after short term incubations (4 h). Interestingly, NK cells generated from PKOB induced specific lysis in RMA-S cells after lengthy incubation (18 h), as shown in Fig. 1A. Moreover, when an anti-FasL Ab was present during the incubation with NK cells under similar conditions, cytotoxicity against RMA-S cells was efficiently blocked (Fig. 1A). These results indicate that Fas-mediated apoptosis is used as a mechanism for cytotoxicity, as well as granzyme B-mediated apoptosis.

### *FLIP<sub>L</sub>* and *Crma* protect the cells from cytotoxicity by perforin-deficient NK cells

Mock-, *FLIP<sub>L</sub>*-, and *Crma*-transduced RMA-S cells were mixed with NK cells, and specific lysis was measured by means of  $^{51}\text{Cr}$  release. As shown in Fig. 1B, the mock-transduced RMA-S cells along with the *FLIP<sub>L</sub>*- and *Crma*-transduced cells were readily killed by NK cells after 4-h incubation. Similar results were ob-

tained when NK cells from *gld* mice were used (data not shown). NK cells generated from PKOB also killed RMA-S mock cells after long-term incubation (18 h), whereas RMA-S *FLIP<sub>L</sub>* and RMA-S *Crma* cells were protected against cytotoxicity, as shown in Fig. 1C. The cytotoxicity mediated through the perforin-dependent pathway is fast and is most likely masking the effect of Fas-mediated apoptosis. However, the contribution of the Fas-mediated pathway is considerable and enough to kill the cells, as shown by the percentage of specific  $^{51}\text{Cr}$  release in Fig. 1, A and C. These findings suggest that the perforin-independent cytotoxic activity of NK cells is mediated through the Fas/FasL pathway, and that it can be blocked by inhibitors of death receptor-mediated apoptosis.

### NK cells mediate the up-regulation of Fas on RMA-S cells

RMA-S cells do not normally express detectable levels of Fas when grown *in vitro*, as shown in Fig. 2A. As a consequence, they are insensitive to Fas-induced apoptosis when sFasL or an agonistic anti-Fas mAb is added (Table I). Neither IL-2 nor IFN- $\gamma$ , alone or in combination, could induce Fas up-regulation. IL-3 or supernatant from Con A-stimulated splenocytes also failed to induce Fas up-regulation (data not shown). RMA-S cells were also insensitive to other death ligands, such as TNF- $\alpha$  and TRAIL, although apoptosis could be induced by other agents, such as CHX and the DNA-damaging agent VP16 (Table I). Interestingly, NK cells were able to directly mediate the up-regulation of Fas on RMA-S cells, as shown in Fig. 2B. This finding adds further explanation to the observation that perforin-deficient NK cells need longer incubation periods with target cells to induce Fas-mediated apoptosis. These results suggest that the cytotoxic activity of NK cells against Fas-negative targets is a two-step process in which NK cells actively induce the expression of this death receptor and subsequently kill the targets by apoptosis.

Table I. *RMA-S are insensitive to FasL when grown in vitro*<sup>a</sup>

	CHX (high doses)	VP16	TNF ± CHX	Jo2 ± IL-2	sFasL ± IL-2	Jo2 ± IFN-γ	sFasL ± IFN-γ	TRAIL
RMA	+	+	-	-	-	-	-	-
RMA-S	+	+	-	-	-	-	-	-
RMA-S mock	+	+	-	-	-	-	-	-
RMA-S FLIP <sub>L</sub>	+	+	-	-	-	-	-	-
RMA-S CrmA	+	+	-	-	-	-	-	-

<sup>a</sup> +, >80% apoptosis as detected by propidium iodide incorporation; -, <15% apoptosis. A20 cells were used as positive control for Fas-induced apoptosis, Jurkat cells for TRAIL-induced apoptosis, and WEHI164 cells for TNF-induced apoptosis.

### *Fas expression is up-regulated in vivo in RMA-S tumors*

Mock-, FLIP<sub>L</sub>-, and CrmA-transduced RMA-S cells were injected s.c. in immunocompetent syngeneic C57BL/6 recipients, and tumor samples were analyzed for Fas expression. As shown in Fig. 2C, all tumors had up-regulated Fas after in vivo passage. Tumor cells were then tested for sensitivity to Fas-induced apoptosis. As shown in Fig. 3, mock-transduced tumor cells were readily killed by sFasL, whereas FLIP<sub>L</sub>- and CrmA-transduced tumor cells were protected. These findings show that Fas is up-regulated in vivo, suggesting that death receptor-mediated apoptosis may play an important role in the rejection of tumors by NK cells.

### *FLIP<sub>L</sub> and CrmA mediate tumor progression in vivo*

Mock-, FLIP<sub>L</sub>-, and CrmA-transduced RMA-S cells were injected s.c. in immunocompetent syngeneic C57BL/6 recipients to investigate whether FLIP<sub>L</sub> or CrmA would enhance tumor progression in vivo. RMA cells were used as a positive control for tumor take and RMA-S cells were used as a control to exclude that retroviral transduction could interfere with tumor rejection. The inoculum of injected cells ranged from 10<sup>2</sup> to 10<sup>5</sup> cells. When 10<sup>5</sup> cells were injected, all transduced cells formed tumors that quickly reached the maximally allowed size of 1 cm<sup>3</sup> (Table II). At lower cell doses (≤10<sup>4</sup> cells), differences in tumor take were seen between RMA-S mock cells and the RMA-S FLIP<sub>L</sub> or RMA-S CrmA cells (Table II). At 10<sup>3</sup> cells, the rejection frequency was higher for the mock-transduced cells, and at 10<sup>2</sup> cells, 96% of mock tumors were rejected, while a large proportion of the mice that had been injected with FLIP<sub>L</sub>- or CrmA-transduced cells developed aggressive tumors that grew uncontrolled (Table II). In Fig. 4 the percent tumor take for the mock-transduced cells at a 10<sup>2</sup> cell inoculum is compared with that for FLIP<sub>L</sub> (Fig. 4A) and CrmA-transduced tumor cells (Fig. 4B). RMA-S FLIP<sub>L</sub> and RMA-S CrmA cells generated tumors quickly after injection (days 12–14), and by day 30 all mice

having tumors had been euthanized. To the contrary, the only mock tumor was detectable on day 24 and had not attained the size of 1 cm<sup>3</sup> on day 34, when it was taken for ex vivo experiments. These findings indicate that the inhibitors of death receptor signaling FLIP<sub>L</sub> and CrmA can act as tumor progression factors and promote tumor survival.

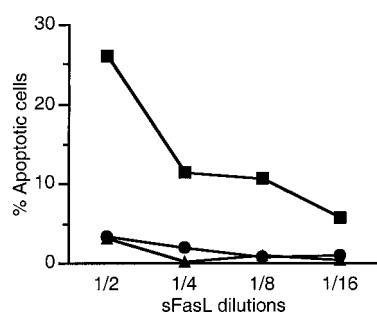
### *FLIP<sub>L</sub> and CrmA exert their tumor-progressive activity by protecting the tumors from NK cell-mediated cytotoxicity in vivo*

RMA-S cells were injected in mice depleted of NK cells to directly test the hypothesis that FLIP<sub>L</sub> and CrmA act by protecting the tumor cells from NK cell-mediated rejection. When injected into NK cell-depleted recipients, mock-, FLIP<sub>L</sub>-, and CrmA-transduced RMA-S cells grew as fast and to the same extent, as shown in Fig. 4, C and D, demonstrating the strict dependence on NK cells for the rejection process. These results also demonstrate that FLIP<sub>L</sub> and CrmA do not confer any proliferative or survival advantage to the cells in vivo in the absence of NK cells, in line with limiting dilution analysis in vitro, in which all RMA-S cells showed similar frequencies of clonable cells (mock, 1/1.9; 95% confidence interval, 1/1.6 to 1/2.8; FLIP<sub>L</sub>, 1/1.8; 95% confidence interval, 1/1.5 to 1/2.3; CrmA, 1/1.5; 95% confidence interval, 1/1.3 to 1/1.9). As an additional control, tumor samples were extracted by surgical excision and analyzed for MHC class I expression to exclude the unlikely possibility that FLIP<sub>L</sub> or CrmA could lead to the up-regulation of MHC class I, thereby rendering the tumors insensitive to NK cell-mediated cytotoxicity in vivo. As shown in Fig. 5, the RMA cells expressed MHC class I on their surface, whereas all transduced RMA-S cells stained negatively for this Ag.

## Discussion

Our data show that the expression of either FLIP<sub>L</sub> or CrmA enables RMA-S to raise tumors even at an inoculum of cells that is normally rejected. This observation indicates that several inhibitors of death receptor-mediated apoptosis can protect tumor cells from NK cell-mediated cytotoxicity, thereby enhancing tumor progression.

Previous results by Smyth et al. (22) suggest that Fas- and TNF-induced apoptosis seem to be dispensable in this tumor system, as mice defective in either FasL (*gld*) or TNF can reject RMA-S tumors to the same extent as wild-type mice. Our results show that Fas is up-regulated in vivo, and that RMA-S cells are killed in a Fas-dependent manner by perforin-deficient NK cells in vitro. These data argue for a more prominent role of death receptor-mediated apoptosis in the clearance of MHC class I-deficient tumors than previously thought. The slower kinetics seen in the in vitro experiments with perforin-deficient NK cells are due to the fact that Fas needs to be up-regulated on RMA-S cells before they can be killed. Indeed, RMA-S cells do not show detectable expression levels of Fas in vitro, whereas they up-regulate Fas after being exposed to NK cells. The induction of Fas expression in



**FIGURE 3.** RMA-S FLIP<sub>L</sub> and RMA-S CrmA tumors are protected from Fas-induced apoptosis. The sensitivity to Fas-induced apoptosis of RMA-S mock (■), RMA-S FLIP<sub>L</sub> (●), and RMA-S CrmA (▲) tumors was assessed by treating the cells with sFasL as described in *Materials and Methods*. The percentage of apoptotic cells was calculated by means of propidium iodide incorporation in a flow cytometric assay.

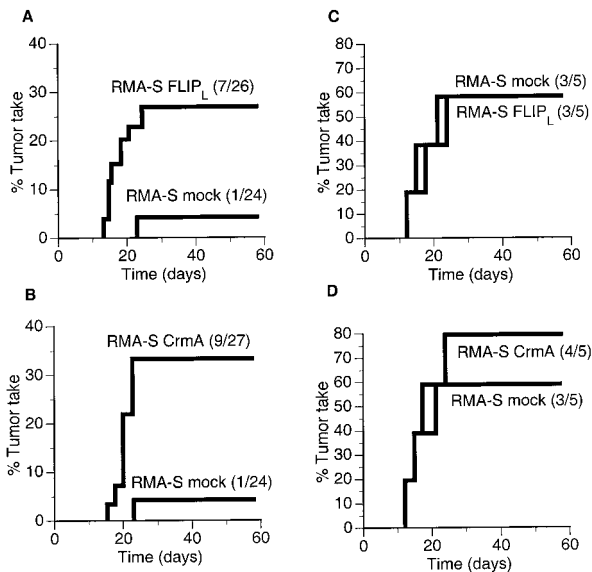
Table II. *FLIP<sub>L</sub>* and *CrmA*-transduced RMA-S cells establish tumors at a higher frequency than mock-transduced cells<sup>a</sup>

	No. of Tumor Cells Injected			
	10 <sup>5</sup>	10 <sup>4</sup>	10 <sup>3</sup>	10 <sup>2</sup>
RMA	100 (5/5)	100 (10/10)	86.6 (13/15)	79.2 (19/24)
RMA-S mock	80 (4/5)	60 (6/10)	40 (6/15)	4 (1/24)
RMA-S FLIP <sub>L</sub>	100 (5/5)	90 (9/10)	60 (9/15)	26.9 (7/26)
RMA-S CrmA	100 (5/5)	70 (7/10)	66.6 (10/15)	33.3 (9/27)

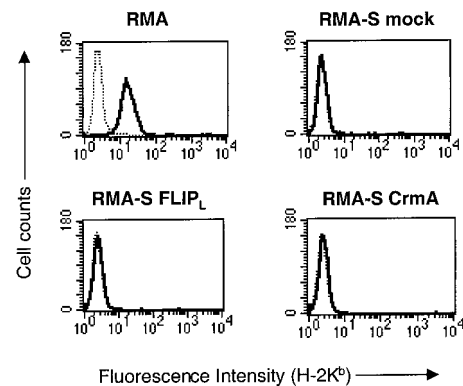
<sup>a</sup> The final percentage of tumor take is shown, along with the absolute number of mice raising tumors in each group. The results from the groups injected with RMA-S are not shown, as they were similar to those obtained with the mock-transduced RMA-S cells. All experiments were repeated at least twice with groups of 5–8 mice per cell type, except for the experiment in which 10<sup>5</sup> cells were injected that was only performed once. In all experiments, tumor appearance was not seen before day 10 after injection. Moreover, no mice that were tumor-free at day 34 developed any tumor throughout the rest of the experiment (8 wk).

target cells seem to be a general mechanism for cytotoxic lymphocytes, as a recent report shows that CTLs mediate Fas up-regulation on target cells. Moreover, the induction of Fas expression occurs regardless of the origin of the target cell, as both lymphoid and nonlymphoid targets up-regulate Fas when they encounter CTLs (23, 24). It is likely that Fas is up-regulated *in vivo* at an early time point, as a recent study indicates that Fas expression is detectable as soon as 4 days after *in vivo* grafting (23). More studies are needed to examine the mechanism through which NK cells induce Fas expression *in vitro*, although preliminary results obtained from double-chamber experiments indicate that NK cells need direct contact with target cells to induce Fas up-regulation. Recently, it was shown that NK cells express TRAIL, and that liver NK cells induce apoptosis through this death ligand during the rejection of tumor metastasis (25, 26). It would be interesting to investigate whether NK cells can also mediate the up-regulation of TRAIL receptors on target cells.

Several groups have argued that the perforin-dependent pathway is predominantly used for the rejection of RMA-S tumor cells *in vivo*, as shown by experiments in perforin knockout mice (16, 22). Our experiments in normal mice show that both pathways are responsible for the clearance of RMA-S tumor, as FLIP<sub>L</sub>- and CrmA-expressing cells are protected from NK cell-mediated rejection and can establish aggressive tumors. It is not known whether FLIP<sub>L</sub> can block the perforin-dependent pathway *in vivo*, although *in vitro* studies exclude this possibility (15, 27). It has also been debated whether CrmA can block the perforin-dependent pathway *in vivo*. In fact, all reports to date indicate that CrmA is unable to protect from the perforin-dependent pathway induced by CTLs despite the fact that CrmA is a substrate and inhibitor of granzyme B in a cell-free system (28). Nevertheless, it is noteworthy to mention that PI-9, a cellular homologue to CrmA that inhibits granzyme B, is up-regulated in CTLs. It is hypothesized that PI-9 protects these lymphocytes against autocrine and paracrine-like killing when cytotoxic granules are released (29). Our *in vitro* data indicate that neither FLIP<sub>L</sub> nor CrmA can protect target cells from the perforin-dependent pathway, whereas they efficiently inhibit Fas-mediated apoptosis. In fact, it has previously been shown that FLIP<sub>L</sub> and CrmA protect cells from death receptor-mediated apoptosis by blocking caspase-8 activation (11–13, 30). As a reasonable deduction it can be argued that these two inhibitors of apoptosis exert their tumor-progressive activity by blocking the immune response induced through death receptor-mediated apoptosis. FLIP<sub>L</sub> and CrmA can only block one pathway of cytotoxicity mediated by NK cells. Moreover, NOs (inducible NO synthase) produced by NK cells can also be involved in cell lysis (31),



**FIGURE 4.** FLIP<sub>L</sub> and CrmA promote tumor establishment and progression *in vivo* by preventing NK cell-mediated rejection. Groups of six to eight mice were injected s.c. in the interscapular region with 10<sup>2</sup> cells. The occurrence of tumors was monitored for 8 wk. The percentage of mice developing tumors when injected with RMA-S mock is compared with that of mice injected with RMA-S FLIP<sub>L</sub> (A) and RMA-S CrmA (B). The cells were also injected in NK cell-depleted mice, as described in *Materials and Methods*. The percentage of mice developing tumors when injected with RMA-S mock is compared with that of mice injected with RMA-S FLIP<sub>L</sub> (C) and RMA-S CrmA (D).



**FIGURE 5.** The transduced cells do not up-regulate MHC class I *in vivo*. Flow cytometric analysis was conducted as described in *Materials and Methods*, using a H-2K<sup>b</sup>-specific Ab (thick line) and an isotype-matched control (thin line).

and it is unclear whether inhibitors of apoptosis can interfere with this type of cell death. Nevertheless, the presence of an inhibitor of death receptor-mediated apoptosis is enough to confer an advantage to tumor cells and to protect them from the immune response.

Taken together, the data presented here complement our previous results (14, 15) and emphasize the pivotal role of inhibitors of death receptor signaling in tumor progression by a mechanism of immune evasion regardless of the lymphocyte population that is responsible for the clearance of tumors. Moreover, these results underscore the role of death receptor-induced apoptosis in tumor clearance mediated by NK cells by a multistep mechanism that includes the induction of Fas expression on Fas-negative targets and the subsequent induction of apoptosis through this death receptor.

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