HHS Public Access

Author manuscript

*J Immunol*. Author manuscript; available in PMC 2018 December 30.

Published in final edited form as:

*J Immunol*. 2009 March 15; 182(6): 3398–3405. doi:10.4049/jimmunol.0803006.

# ERK5 Knockdown Generates Mouse Leukemia Cells with Low MHC Class I Levels That Activate NK Cells and Block Tumorigenesis

**Seyma Charni**[#*], **Juan Ignacio Aguilo**[#†], **Johan Garaude**[#*], **Geoffroy de Bettignies**[*], **Chantal Jacquet**[*], **Robert A. Hipskind**[*], **Dinah Singer**[‡], **Alberto Anel**[†], and **Martin Villalba**[3,*]

[*] Institut de Génétique Moléculaire de Montpellier, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 5535, Université Montpellier 1 and 2, Montpellier, France

[†] Departamento de Bioquímica y Biología Molecular y Celular, Universidad de Zaragoza, Zaragoza, Spain

[‡] Experimental Immunology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

[#] These authors contributed equally to this work.

## Abstract

Tumor cell-based vaccines are currently used in clinical trails, but they are in general poorly immunogenic because they are composed of cell extracts or apoptotic cells. Live tumor cells should be much better Ags provided that they are properly processed by the host immune system. We show herein that stable expression of a small hairpin RNA for ERK5 (shERK5) decreases ERK5 levels in human and mouse leukemic cells and leads to their elimination by NK cells in vivo. The shERK5 cells show downregulation of MHC class I expression at the plasma membrane. Accordingly, ectopic activation of the ERK5 pathway induces MHC class I gene expression. Coinjection of shERK5-expressing cells into the peritoneum diminishes survival of engrafted wild-type tumor cells. Moreover, s.c. injection of shERK5-expressing cells strongly diminishes tumor development by wild-type cells. Our results show that shERK5 expression in leukemia cells effectively attenuates their tumor activity and allows their use as a tumor cell-based vaccine. *The Journal of Immunology,* 2009, 182: 3398–3405.

---

Recent observations strongly suggest that an efficient immune response can effectively treat cancer in the clinic: the quandary is how to generate it (1, 2). While tumor-associated Ags would seem ideally suited for this purpose, they have proven to be weak immunogens. To solve this problem, most efforts aim to genetically engineer effector cells (e.g., dendritic cells (DC)[4]) to effectively present tumor-associated Ags. Another possibility, albeit less

[3] Address correspondence and reprint requests to Dr. Martin Villalba, Institut de Génétique Moléculaire de Montpellier, 1919 route de Mende, 34293 Montpellier cedex 5, France; martin.villalba@igmm.cnrs.fr.

Disclosures

M.V.G., J.G., C.J., S.C., G.B., and R.A.H. have filed a patent application in France (FR 08/02809) related to the research presented in this paper.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0803006

explored, is altering tumor cells to make them strong immunogens. One way would be to enhance the number of tumor cells undergoing apoptosis and thereby induce an improved immune response. In fact, certain early clinical trials used irradiated autologous tumor cells to boost the antitumoral immune response (3). Unfortunately, these ex vivo apoptotic cells also proved to be weak immunogens (4) or even immunosuppres-sive (5). In light of this, living tumor cells might be much better Ags, provided that they are properly processed by the host immune system.

NK cells, which have a critical role in antitumor immunity (6, 7), spontaneously kill tumor cells while sparing normal cells. In particular, they target tumor cells that have evaded the control of CTL by down-regulating MHC class I (MHC-I) expression. Nevertheless, NK cell-activating receptors recognize ligands on distressed cells and therefore can eliminate MHC-$I^+$ cells (7, 8). In vivo, NK cells can be recruited locally by inoculation of tumor cells, preferably those lacking appropiate MHC-I expression (9). Notably, NK cell-mediated cytotoxicity does not require immunization, in contrast to that involving cells of the adaptive immune system, for example, T cells. This versatility makes NK cells an attractive target for cancer therapy in the clinic (10, 11), where one drawback is that resting human or mouse NK cells show poor effector function (12) and require priming for full activation. Despite this, the abundance of NK cells in the peripheral blood and spleen may be of particular benefit in blood-borne cancers, such as leukemias and lymphomas. Consistent with this idea, recent observations suggest that NK cell immunotherapy can be used in combination with stem cell transplantation (6). Interestingly, NK cells also generate an adaptive immune response by killing tumor cells and allowing tumor Ag presentation via interactions with DCs or by boosting DC maturation and/or activation (7).

The ERK5 MAPK cascade mediates survival and proliferative signaling (13), particularly in transformed cells of hematopoietic origin. In myeloma cells, inhibition of ERK5 blocks proliferation and facilitates apoptosis induced by dexamethasone (14). ERK5 expression is essential for survival of leukemic cells expressing Bcr/Abl (15). Hodgkin lymphoma cells show constitutive activation of the ERK5 pathway (16). Finally, expression of the microRNA miR-143 shows an inverse correlation with that of ERK5 (17), and the level of miR-143, along with miR-145, is decreased in most B cell malignancies, including chronic lymphocytic leukemias, B cell lymphomas, EBV-transformed B cell lines, and Burkitt lymphoma cell lines. These observations suggest that ERK5 plays an important, albeit poorly characterized, role in oncogenesis. Our results (18) show that ERK5 mediates the activation of the antiapoptotic transcription factor NF-κB in human and mouse leukemic T cells. ERK5 knockdown, mediated through expression of a small hairpin RNA (shERK5), reduces cell viability and sensitizes cells to death receptor-induced apoptosis. Importantly, expression of shERK5 in the murine T lymphoma cell line EL4 does not affect proliferation in vitro but compromises the induction of s.c. tumors in mice. These data show that ERK5 is essential for survival of both human and mouse leukemic T cells in vivo and led us to hypothesize that shERK-expressing cells might be useful for cancer immunotherapy.

[4]Abbreviations used in this paper: DC, dendritic cell; β2-m, β2-microglobulin; MHC-I, MHC class I; sh, small hairpin; wt, wild type.

To further this hypothesis, we have investigated the mechanism responsible of elimination of shERK5-expressing tumor cells. These cells are highly sensitive to NK cell-mediated killing in vitro and in vivo. They show strongly lowered levels of MHC-I at the cell surface, reflecting a role for the ERK5 pathway in transcriptional regulation of the MHC-I promoter programmed death-1 (PD-1). Importantly, injection of shERK5 tumor cells in syngenic mice activates NK cells and induces an immune response against wild-type (wt) tumor cells. Thus, our results show that shERK5 expression in murine leukemia cells effectively attenuates their tumor activity and allows their use as a tumor cell-based vaccine.

## Materials and Methods

### Reagent and Abs

The Abs anti-H-2K$^b$-FITC, anti-H-2K$^d$-PE, anti-NK1.1-allophycocyanin, and anti-Fas-FITC were from BD Pharmingen. The Abs against ERK5 and $\beta$-actin were from Cell Signaling Technology. The anti-mouse IgG-Alexa Fluor 647 was from Molecular Probes. Donkey anti-rabbit or sheep anti-mouse IgG abs were obtained from Amersham. CFSE was from Sigma-Aldrich. The anti-human HLA mAb W632 was a generous gift of Dr. Valerie Pinet. The c-FLIP Ab was from Alexis Biochemicals. The anti-FasL clone MFL3 was from BD Pharmigen, and the anti-CD3 (clone 145.2C11) obtained from hybridoma 145.2C11 was a kind gift from Dr. Anne-Marie Schmitt.

### Cell culture

The leukemic T cell lines EL4 and Jurkat and the murine leukemic B L1210 cell line were grown in RPMI 1640-GlutaMAX (Invitrogen) supplemented with 5% FBS. BM3.3 cells were grown in RPMI 1640-GlutaMAX supplemented with 5% FBS, 1 mM sodium pyruvate, 10 mM HEPES, 50 $\mu$M 2-ME, and 10% EL4.C16 PMA-activated supernatant. In certain experiments they were activated 3 h with 10 ng/ml PMA plus 666 nM ionomycin.

### Mice

C57BL/6 or BALB/c mice were grown at the Institut de Génétique Moléculaire de Montpellier animal facility. All experiments involving mice were performed according to the guidelines and regulations of the Centre National de la Recherche Scientifique, France (J. Garaude, S. Charni, C. Jacquet, and M. Villalba have the official degree for animal experimentation delivered by the Ministère de l'Agriculture (France)).

### Plasmids

The pcDNA expression vectors for ERK5 and a constitutively active MEK5 mutant (S313D/T317D, termed MEK5D) were a generous gift from Dr. Axel Ullrich (15). Expression vectors for $\beta$-galactosidase, the pSUPER expression vector for GFP alone or GFP plus shERK5 and the pSIRENRetroQ-puro (BD Biosciences) retroviral vectors for shERK5 and control have been described (18). The expression vectors for the nonphosphory-latable ERK5 mutant (ERK5AEF: the activating TEY motif mutated to AEF) in pcDNA and the catalytic inactive ERK5 mutant (ERK5KM: K84 mutated to M) in pLZR were generous gifts of Drs. Axel Ullrich and Atanasio Pandiella, respectively. The MHC-I promoter construct is derived from the swine class I gene PD-1 (19).

### Transient transfection and stable cell line generation

Jurkat cells in logarithmic growth phase were transfected with the indicated amounts of plasmid by electroporation (18). In each experiment, cells were transfected with the same total amount of DNA by supplementing with empty vector. Cells were incubated for 10 min at room temperature with the DNA mix and electroporated at 260 mV, 960 μF in 400 l of RPMI 1640. Stable cell lines were generated as described previously (18). Briefly, cells are plated at $1.5 \times 10^6$ cells/ml. One milliliter of a supernatant from 293T cells expressing the retroviral vectors for shERK5 or control sequence was added after plating. Three days later, cells were cultured with 2.5 μg/ml puromycin (Sigma-Aldrich). After 1 wk, surviving cells were isolated and kept on selection medium until used.

### Reporter assay

In all experiments, cells were transfected with a β-galactosidase reporter plasmid (18). Transfected cells ($10 \times 10^6$) were harvested after 2 days and washed twice with PBS. Cells were lysed in 100 μl of luciferase lysis buffer (Promega) and luciferase assays (40 μl) performed according to the manufacturer's instructions (Promega) using a Berthold luminometer. For β-galactosidase assays, 40 μl of lysate was added to 200 μl of β-galactosidase assay buffer (50 mM phosphate buffer (pH 7.4), 200 μg of 2-nitrophenyl α-D-galactopyranoside, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol) and the absorbance was measured at 405 nm. The results are expressed as luciferase units normalized to the corresponding β-galactosidase activity. The expression level of the transfected proteins was routinely controlled by immunoblot analysis.

### CFSE labeling and peritoneal clearance

Cells were labeled with CFSE as follows. One microliter of a stock solution (5 mM) was diluted into 10 ml of PBS. Five million cells were washed once in PBS, resuspended in 1 ml of the CFSE/PBS solution for 2 min at room temperature, washed once with 10 ml of PBS, and resuspended at the indicated concentration. The indicated amounts of cells were injected i.p. in 150 μl of PBS. Mice were sacrificed 3 days later and peritoneal cells were collected, washed in PBS, and analyzed on a FACSCalibur flow cytometer (BD Biosciences). In some experiments mice were injected with 50 μl of polyclonal rabbit anti-asialo GM1 serum (Wako Chemicals) or the same amount of rabbit serum control at days 2 and 0 before injecting $CFSE^{high}$-labeled L1210 shERK5 (5 μM CFSE) and $CFSE^{low}$-labeled L1210 shLuc cells (1 μM CFSE). Peritoneal exudates cells were obtained 2 days later and $CFSE^+$ cells were analyzed by flow cytometry.

### Measure of cytotoxic activity

Spleen NK cells were isolated by positive selection using anti-DX5 magnetic beads (Miltenyi Biotec), according to the manufacturer's instructions. The purity of the collected $DX5^+$ cells was typically 98% (data not shown). Cytotoxicity assays were performed essentially as described in (20). Target cells were cultured overnight at $2 \times 10^5$ cells/ml and labeled with 5 μCi/ml [$^3$H]thymidyne (GE Healthcare) at 37°C. Then, target cells were washed twice with fresh medium and cultured at $10^5$ cells/ml in RPMI 1640 with 5% heat-inactivated FCS (cytotoxicity medium). Once isolated, NK cells were incubated in

cytotoxicity medium with target cells at the corresponding E:T ratios in a V-bottom 96-wells plate, in a final volume of 200 μl. After incubating for 4 h at 37˚C, 25 μl of a solution containing 2% Triton X-100, 80 mM Tris-HCl (pH 7.5), and 8 mM EDTA was added to each well, incubating then for 15 min at 37˚C. Then, plates were centrifuged 15 min at 1400 rpm and 50 μl of supernatant was transferred to scintillation vials, adding 2 ml of scintillation liquid per vial (GE Healthcare). In EL4 cells [$^3$H]thymidyne labeling was not totally satisfactory, and we therefore used the classical $^{51}$Cr-release assay, in which we labeled target cells with 100 Ci of $Na_2{}^{51}CrO_4$ for 1 h. Afterwards cells were treated like [$^3$H]thymidyne-labeled cells. Spontaneous release was determined by the cpm present in the supernatants in the absence of effector cells, and total labeling was obtained by measuring radioactivity in target cells directly. Each point was measured by triplicate, and the percentage of specific target cell death induced by NK cells was determined by the following expression: % specific lysis = (sample cpm  spontaneous cpm/total cpm  spontaneous cpm) × 100. Spontaneous release was always <10% in the case of $^{51}$Cr release and <25% with [$^3$H]thymidyne. All experiments were performed in triplicate.

### Tumor progression in vivo

For s.c. tumor formation, EL4 cells were washed in PBS and resuspended at 1.5 × $10^6$ cells/ml. A total of 2.5 × $10^5$ cells was injected s.c. in the flank of C57BL/6 mice for pretreatment with EL4 shERK5 cells and for inoculation of the EL4 shLuc cells. Every 2 days tumors were measured, and the volume was calculated following the formula: $V \times$ length (weight)$^2$/2.

### Flow cytometry

Peritoneal cells, spleen cells (3 × $10^6$), or cell lines (2 × $10^5$) were stained for 20 min at room temperature with indicated FITC-, PE- or allophycocyanin-conjugated Abs in 200 μl of PBS. For human MHC-I staining, cells were incubated with W632 mAb before incubation with anti-mouse Alexa Fluor 647. Cells were washed and analyzed on FACSCalibur flow cytometer using CellQuest Pro software (BD Biosciences).

### Immunobloting

Cells were washed with PBS and lysed in SDS-Laemmli buffer. Proteins were separated by SDS-PAGE on minigels and processed for immunoblot analysis as previously described (18).

### RNA isolation and quantitative RT-PCR experiments

RNA from 10 × $10^6$ cells was isolated with the GenElute Mammalian Total RNA Miniprep kit as described by the supplier (Sigma-Aldrich). Total RNA was subjected to DNase treatment using a RQ1 RNase free DNase (Promega). One microgram of each sample was reverse transcribed into cDNA using a SuperScript III reverse transcriptase (Invitrogen) according to the supplier's specifications. cDNAs were amplified using the SYBR Green PCR Master Mix from Invitrogen. Amplification products were detected by real-time PCR using the Multiplex quantitative PCR system Mx3000P (Stratagene) according to the manufacturer's specifications. Reactions were conducted (5 min at 95˚C followed by 40

cycles of 30 s at 95˚C, 30 s at 64˚C, and 30 s at 72˚C). S26 was used as housekeeping gene for arbitrary unit calculation for every tested gene. Primers were designed and selected using the Primer 3 program (version 0.4.0) and were the following: S26: 5′-GAACATTGTAGAAGCCGCTGCTGTC-3′and 5′-AACCTTGCTATGGATGGCACAGCTC-3′; $\beta_2$-microglobulin ($\beta_2$-m): 5′-CAGTCTCAGTGGGGGTGAAT-3′ and 5′-ATGGGAAGCCGAACA TACTG-3′.

RT-PCR data for $\beta_2$-m mRNAs were calculated with the Multiplex quantitative PCR system Mx3000P (Stratagene) according to the manufacturer's specifications and were normalized to the values of the housekeeping gene S26.

### Statistical analysis

The statistical analysis of the difference between means of paired samples was performed using the paired *t* test. The results are given as the confidence interval (*p*). All of the experiments described in the figures were performed at least three times with similar results.

## Results

EL4 cells expressing a small hairpin RNA for ERK5 (EL4 shERK5) fail to induce tumors when injected s.c. in syngenic C57BL/6 mice (18). They also did not induce tumors in the peritoneum, because they were eliminated in <72 h (Fig. 1*A*). In contrast, EL4 cells expressing a small hairpin RNA for luciferase (EL4 shLuc) were recovered at similar levels to wt cells (Fig. 1*A*), which agreed with our observations in s.c. tumors (18). To extend this observation, we stably transfected the murine leukemia B cell line L1210 with these shRNA expression vectors. L1210 shERK5 and control L1210 shLuc cells were loaded with different CFSE concentrations and injected into the peritoneum of syngenic BALB/c mice. The numbers of L1210 shERK5 cells were vastly diminished 48 h later, unlike those of the L1210 shLuc cells (Fig. 1*B*).

The innate immune response, specifically NK cells, seemed the most likely mediator of the rapid clearance of shERK5-expressing tumor cells. Consistent with this, splenocytes from syngenic mice were capable of lysing EL4 shERK5 cells (Fig. 2*A*), but not EL4 wt or shLuc cells (data not shown and Ref. 18). Moreover, purified NK cells were more efficient in this assay, and their depletion from the splenocyte population strongly diminished lysis (Fig. 2*A*). These NK cells killed allogenic L1210 shERK5 cells with the same efficiency as syngenic BALB/c NK cells, showing that L1210 shERK5 cells were recognized as non-self by both NK cell haplotypes (Fig. 2*B*). In contrast, syngenic NK cells did not kill L1210 shLuc cells (Fig. 2*B*). Intraperitoneal injection of anti-asialo GM1 antiserum, which depletes NK cells in vivo (Ref. 21 and data not shown), led to survival of L1210 shERK5 cells (Fig. 1*B*), confirming the in vitro results (Fig. 2).

NK cells use two mechanisms to eliminate their targets: the granzyme/perforin pathway, which requires $Ca^{2+}$ and is thus blocked by EGTA, and the expression of FasL to engage the death receptor Fas. To test their relative contributions here, we performed the same assays in the presence of EGTA, anti-FasL to inhibit Fas activation, or both of them (Fig. 2*C*). The two treatments decreased killing of L1210 shERK5 cells by syngenic NK cells with similar

efficiency, implicating both pathways in this process. As expected, blocking of both pathways totally abolished killing. This also indicated that naive murine NK cells express some functional FasL, as others have observed (22) and as we have confirmed (data not shown). Although quiescent L1210 cells do not express Fas (23, 24), NK cells clearly eliminated L1210 shERK5 cells by a Fas-dependent pathway (Fig. 2*C*). This paradox was resolved when we found that quiescent L1210shERK5 cells, but not control L1210 shLuc cells, express low levels of Fas at the cell surface (Fig. 2*D*). However, the deficient NF-κB activity found in these cells makes them extremely sensitive to Fas-induced apoptosis (see below and Ref. 18).

Naive NK cells preferentially target and eliminate cells that lack MHC-I at the cell surface, and MHC-I-deficient tumor cells recruit NK cells to the site of their injection (9, 25). Given our results (Figs. 1 and 2), we analyzed MHC-I expression and NK recruitment by L1210 shERK5 cells. Indeed, compared with L1210 shLuc cells, L1210 shERK5 cells showed very low surface levels of two class I molecules, H-2D$^d$ and H-2K$^d$ (Fig. 3*A*), and considerably increased the number of NK cells recruited to the peritoneum upon injection (Fig. 3*B*). This increase was statistically significant relative to the moderate level seen with the control cells (Fig. 3*B*). A recombinant NKG2D-Fc chimera bound to L1210 shLuc and L1210 shERK5 cells, showing that both expressed ligands for the NK-activating receptor NKG2D (data not shown). NKG2D ligands induce NK cell recruitment, even to MHC-I-expressing tumor cells (26). This explains why L1210 shLuc cells recruited NK cells, although the lack of surface MHC-I expression clearly led to a more pronounced effect. Notably, the NK cells recruited by L1210 shERK5 cells expressed higher levels of granzymes, showing that they were more strongly activated (Fig. 3*C*).

The ERK5 cascade is implicated in gene regulation (13), which led us to test if it regulates MHC-I expression at the transcriptional level. We transfected Jurkat cells with a reporter gene controlled by the promoter of the MHC-I gene PD-1, together with expression vectors for wt or kinase-dead ERK5 and a constitutively active mutant of its upstream kinase MEK5 (MEK5D). Wild-type and the two ERK5 mutants, namely catalytically inactive ERK5 KM and the activation loop mutant ERK5 AEF, were expressed (supplemental Fig. 1),[5] but they were unable to activate the MHC-1 reporter gene on their own (Fig. 4*A*). In fact, the two mutants decreased its basal activity (Fig. 4A). When cotransfected with MEK5D, wt ERK5 strongly activated the PD-1 reporter (Fig. 4, *A* and *B*), whereas the two ERK5 mutants did not. Notably, this reporter gene was also activated, albeit less strongly, when cotransfected with MEK5D and ERK5 in HeLa cells (Fig. 4*D*). Taken together, these data revealed that ERK5 pathway was involved in the constitutive activation of the PD-1 promoter.

IFNs induce expression of MHC-I (27) and accordingly activate the PD-1 reporter gene driven by the PD-1 promoter (Fig. 4*B*, control +). The level of activation was increased in the presence of ectopically activated ERK5 (Fig. 4*B*); this augmentation was not seen with either ERK5 mutant (Fig. 4*B*). As previously noted (15), ERK5 KM had a stronger inhibitory effect than did ERK5 AEF, significantly diminishing IFN-driven activation of the reporter gene (Fig. 4*B*). The same level of inhibition was seen with the expression vector for

[5]The online version of this article contains supplemental material.

shERK5 (Fig. 4*B*); these data indicate a role for the ERK5 cascade in PD-1 promoter activation by IFN. Importantly, transient transfection of MEK5D and ERK5 led to increased expression of MHC-I at the plasma membrane in Jurkat cells (Fig. 4*C*), showing the physiological relevance of our results.Moreover, we did not detect MHC-I expression in L1210 shERK5 permeabilized cells, showing that class I molecules were not produced in these cells and were not accumulated in their endoplasmic reticulum (supplemental Fig. 3).

Class I molecules consist on one H chain (45 kDa), encoded within the MHC region, and an L chain, $\beta_2$-m. L1210 shERK5 cells, unlike wt and shLuc L1210 cells, showed reduced expression of endogenous $\beta_2$-m mRNA (Fig. 5*A*). Consistent with this, Jurkat cells transfected with ERK5 and MEK5D in Jurkat cells showed a significant increase in $\beta_2$-m mRNA levels (Fig. 5*B*). Therefore, ERK5 down-regulation controlled expression of both chains of class I molecules, explaining why L1210 shERK5 cells showed almost no surface expression of MHC-I.

IFNs released by CTLs increase Fas and MHC-I expression on target cells, which sensitizes them to CTL activity. Figs. 3 and 4 showed the role of ERK5 in MHC-I expression; we next investigated Fas regulation in shERK5-expressing cells. PMA/ionomycin-induced activation of the CTL clone BM3.3 up-regulates functional FasL expression and facilitates the Fas-based cytotoxicity (28). This also leads to production of IFNs that induce Fas expression in Fas-negative L1210 cells (23, 24). In a 1-h incubation, activated BM3.3 cells induced a 6-fold increase in Fas expression by L1210 shLuc cells and a striking 14-fold increase in surface levels of Fas on L1210 shERK5 cells (Fig. 6*A*). We previously found that shERK5-expressing cells fail to activate the survival factor NF-κB, which renders them more sensitive to death receptor engagement (18). Consistent with this, L1210 shERK5 cells expressed lower amounts of both isoforms of the antiapoptotic protein c-FLIP (supplemental Fig. 2), whose expression requires NF-κB activity (29). Indeed, L1210 shERK5 cells, like EL4 and Jurkat shERK5 cells (18), were highly sensitive to Fas-mediated killing (Fig. 2). Furthermore, the rapid increase in Fas expression induced by activated CTLs, together with the reduction in c-FLIP isoforms, should enhance the sensitivity of L1210 shERK5 cells to Fas-based cytotoxicity. Consistent with this, cytotoxic lymphocytes like BM3.3 (Fig. 6*B*) or NK cells (Fig. 2) killed L1210 shERK5 cells, but not shLuc cells, in short-term assays even in the presence of EGTA, which blocks the perforin/granzyme pathway (Figs. 2 and 6*B*). This effect was maximal when BM3.3 cells were activated with PMA plus ionomycin (Fig. 6*B*). Taken together, these data also support the physiological relevance of our observations.

Thus, shERK5-expressing leukemic cells present features that suggest their potential usefulness for tumor vaccination: they proliferate in vitro, whereas in vivo they are rapidly eliminated by NK cells (Figs. 1 and 2) that they recruit to the site of injection (Fig. 3).

Since elimination of tumor cells by NK cells induces an immune response against MHC-I$^+$ tumor cells in some mouse models (6, 7, 26, 30, 31), we investigated if our shERK5-expressing leukemic cells induced an immune response against wt cells. L1210 cells are not tumorigenic in immune-competent syngenic BALB/c mice. In contrast, EL4 cells induce tumors when injected in syngenic C57BL/6 mice. EL4 shERK5 cells, like L1210 shERK5 cells (Fig. 3*B*), recruited NK cells to the site of injection, in this case the peritoneum,

whereas EL4 shLuc cells, which express MHC-I, did not (Fig. 7*A*). Moreover, coinjection of EL4 shERK5 and shLuc cells strongly reduced survival of the latter in the peritoneum (Fig. 7*B*), suggesting that EL4 shERK5 cells induced an immune response that was also directed against EL4 shLuc cells. This led us to test if previous injection (i.e., vaccination) with EL4 shERK5 cells would affect tumorigenesis by EL4 shLuc cells. Syngenic mice were injected s.c. with EL4 shERK5 cells, and then with EL4 wt cells at the same site 2 or 6 wk later. This blocked tumor formation in 20% of the mice and significantly reduced tumor development in the remaining 80% (Fig. 7*C*). These data show that ERK5-expressing cells could induce an immune response that strongly restricts tumorigenesis by wt cells.

## Discussion

Tumor cell-based vaccines are an interesting alternative to peptide or cDNA immunization. Currently they generally include apoptotic cells or cell extracts, which are not strong immunogens (4) or can be immunosuppressive (5). In viral vaccination the best vaccines are those that originate with attenuated virus. We expect living tumor cells to be better immunogens than apoptotic cells due to their killing and processing by immune cells. Clearly, these tumor cells must be quickly and efficiently eliminated by the host immune system that, in consequence, will be strongly activated, thereby leading to an antitumoral immune response. In our previous work (18), we found that decreasing ERK5 levels in leukemic cells sensitizes them to death receptor-induced apoptosis. Moreover, these cells, “attenuated” by the expression of a shRNA that decreases ERK5 levels, failed to induce tumors in vivo. Here we show that these shERK5 leukemic cells are targeted by NK cells, which in turn become activated. Moreover, the shERK5 cells induce an immune response that blocks tumorigenesis by wt cells. Thus, shERK5-expressing cells can be used for NK cell priming in vivo and in vitro, which has potential relevance for therapies that activate NK cells (6, 7, 32). Moreover, an epidemiologic survey showed that high cytotoxic activity of peripheral-blood lymphocytes is associated with reduced cancer risk, suggesting a role for natural immunological host defense mechanisms against cancer (33).

The rapid elimination of injected shERK5-expressing cells rules out a role for an adaptive immune response. Instead, our data show that this is mediated by NK cells, for which the shERK-expressing cells are ideal targets, due to their loss of MHC-I expression and their extreme sensitivity to cytotoxic lymphocyte-induced cell death. Additionally, shERK5-expressing cells recruit NK cells. Several groups have proposed a simple explanation (9, 25). The interaction between MHC-I-deficient cells and NK cells triggers NK cytotoxicity but also NK activation, as well as production of cytokines such as IFN-$\gamma$and TNF-$\alpha$, which attracts new NK cells to the site. Additionally, inflammation will activate other cells (i.e., macrophages) that could produce cytokines to attract more NK cells (i.e., TNF-$\alpha$ or IL-12). Mocikat and colleagues (34) obtained an adaptive immune response against tumor cells when they stimulated the NK cell response with cells expressing low levels of MHC-I. We find that injection of leukemic cells rendered MHC-I deficient through shERK5 expression strongly inhibited tumorigenesis induced by wt cells, which very likely arises from an immune response against the latter. Activated NK cells can eliminate tumor cells expressing MHC-I (7, 8), and thus one possible explanation is that shERK5-expressing cells activate NK cells, which then directly eradicate wt tumor cells. Consistent with this scenario, we find

that wt leukemic cells are eliminated when coinjected with their shERK5-expressing counterparts. In a second scenario, NK cell activity generates an adaptive immune response, either through direct activation of DCs or via killing of tumor cells to allow presentation of tumor-associated Ags by DCs (7). This would explain how previously injected shERK5-expressing leukemic cells lead to a decrease in tumor formation by wt cells in long-term experiments (Fig. 7*C*). It is possible that both scenarios occur in our system.

Previous work has shown that tumor cells, engineered to express ligands that activate NK cells, were sensitive to NK cell-mediated cytotoxicity (26, 30). Nevertheless, tumor cells avoid the immune system by secreting NK cell-activating ligands, which block their receptors on NK cells (35, 36). We achieve the same effect by reducing expression of ERK5 and thereby that of MHC-I, which generates cells sensitive to both perforin/granzymes and death receptor activation. Additonally, down-regulation of ERK5 diminishes survival of hematopoietic tumor cells (14–17). Thus, shERK5-expressing tumor cells can be eliminated by the host immune system at different levels, an important point for potential therapeutic applications.

We find that shERK5-driven down-regulation of MHC-I expression in mouse leukemic cells took more than 3 mo (data not shown). However, it is possible to down-regulate MHC-I by other mechanisms, using shRNAs for $\beta_2$-m or HLA molecules (37, 38) or expression vectors for the adenoviral protein Gp19, which blocks class I molecules in the endoplasmic reticulum (39). Therefore, it is feasible to imagine a therapeutic approach in humans based on leukemic cells attenuated by expression of shERK5 and down-regulation of MHC-I. Whether a similar strategy might work with solid tumors or metastatic cells remains to be investigated. Nevertheless, our observations suggest the possibility of developing tumor vaccines via the attenuation of live tumor cells, thus replicating the strategy used to develop vaccines against viral diseases.

## Acknowledgments

We thank Drs. Javier Hernandez, Fernando Rodriguez, Pilar Lauzurica, and members of the Intracellular Signaling and Gene Regulation group for fruitful discussions. The authors gratefully acknowledge the invaluable expertise of Dr. Karim Chebli and other members of the Animal Facility at the Institut de Génétique Moléculaire de Montpellier. We thank the Montpellier RIO imaging platform and C. Duperray for help on cell sorting, and Dr. Gilles Uze for providing IFN-$\alpha$ and IFN-$\beta$.

This work was supported by the Institut National du Cancer/Cancéropôle Grand Sud-Ouest (to M.V.), La Ligue Nationale Contre le Cancer: Comité de la Lozère (to M.V.), the Association pour la Recherche sur le Cancer (to R.A.H.), the Spanish Ministerio de Educación Grant SAF2007-65144 (to A.A.), as well as by fellowships from La Ligue Nationale Contre le Cancer: Comité Nationale (to J.G.), the Région Languedoc-Rousillon (to S.C.), and a Formación de Personal Investigador fellowship associated with Grant SAF2004-03058 from the the Ministerio de Educación y Ciencia/ Fondo Social Europeo (to J.I.A.). This research was also supported in part by the Intramural Research Program of the National Institutes of Health National Cancer Institute, Center for Cancer Research.

## References

1. Dunn GP, Koebel CM, and Schreiber RD 2006 Interferons, immunity and cancer immunoediting. Nat. Rev. Immunol 6: 836–848. [PubMed: 17063185]
2. Nguyen van Binh P, and Duc HT 2006 Analyses and perspectives in cancer immunotherapy. Biomed. Pharmacother 60: 621–628. [PubMed: 16978826]

3. Berd D, Maguire HC, Jr., McCue P, and Mastrangelo MJ 1990 Treatment of metastatic melanoma with an autologous tumor-cell vaccine: clinical and immunologic results in 64 patients. J. Clin. Oncol 8: 1858–1867. [PubMed: 2230873]
4. Ronchetti A, Rovere P, Iezzi G, Galati G, Heltai S, Protti MP,Garancini MP, Manfredi AA, Rugarli C, and Bellone M 1999 Immunogenicity of apoptotic cells in vivo: role of antigen load, antigen-presenting cells, and cytokines. J. Immunol 163: 130–136. [PubMed: 10384108]
5. Dzik WH 2003 Apoptosis, TGF$\beta$ and transfusion-related immunosuppression: biologic versus clinical effects. Transfus. Apher. Sci 29: 127–129. [PubMed: 12941349]
6. Terme M, Ullrich E, Delahaye NF, Chaput N, and Zitvogel L 2008 Natural killer cell-directed therapies: moving from unexpected results to successful strategies. Nat. Immunol 9: 486–494. [PubMed: 18425105]
7. Vivier E, Tomasello E, Baratin M, Walzer T, and Ugolini S 2008 Functions of natural killer cells. Nat. Immunol 9: 503–510. [PubMed: 18425107]
8. Lanier LL 2008 Up on the tightrope: natural killer cell activation and inhibition. Nat. Immunol 9: 495–502. [PubMed: 18425106]
9. Glas R, Franksson L, Une C, Eloranta ML, Ohlen C, Orn A, and Karre K2000 Recruitment and activation of natural killer (NK) cells in vivo determined by the target cell phenotype: an adaptive component of NK cell-mediated responses. J. Exp. Med 191: 129–138. [PubMed: 10620611]
10. Hallett WH, and Murphy WJ 2006 Positive and negative regulation of natural killer cells: therapeutic implications. Semin. Cancer Biol 16: 367–382. [PubMed: 16934486]
11. Wodnar-Filipowicz A, and Kalberer CP 2006 Function of natural killer cells in immune defence against human leukaemia. Swiss Med. Wkly 136: 359–364. [PubMed: 16874940]
12. Bryceson YT, March ME, Ljunggren HG, and Long EO 2006 Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. Blood 107: 159–166. [PubMed: 16150947]
13. Wang X, and Tournier C 2006 Regulation of cellular functions by the ERK5 signalling pathway. Cell. Signal 18: 753–760. [PubMed: 16376520]
14. Carvajal-Vergara X, Tabera S, Montero JC, Esparis-Ogando A, Lopez-Perez R, Mateo G, Gutierrez N, Parmo-Cabanas M, Teixido J, San Miguel JF, and Pandiella A 2005 Multifunctional role of Erk5 in multiple myeloma. Blood 105: 4492–4499. [PubMed: 15692064]
15. Buschbeck M, Hofbauer S, Croce LD, Keri G, and Ullrich A 2005 Ablkinase-sensitive levels of ERK5 and its intrinsic basal activity contribute to leukaemia cell survival. EMBO Rep. 6: 63–69. [PubMed: 15608616]
16. Nagel S, Burek C, Venturini L, Scherr M, Quentmeier H, Meyer C, Rosenwald A, Drexler HG, and Macleod RA 2007 Comprehensive analysis of homeobox genes in Hodgkin lymphoma cell lines identifies dysregulated expression of HOXB9 mediated via ERK5 signaling and BMI1. Blood 109: 3015–3023. [PubMed: 17148583]
17. Akao Y, Nakagawa Y, Kitade Y, Kinoshita T, and Naoe T 2007 Downregulation of microRNAs-143 and −145 in B-cell malignancies. Cancer Sci. 98: 1914–1920. [PubMed: 17892514]
18. Garaude J, Cherni S, Kaminski S, Delepine E, Chable-Bessia C, Benkirane M, Borges J, Pandiella A, Iniguez MA, Fresno M, et al. 2006 ERK5 activates NF-$\kappa$B in leukemic T cells and is essential for their growth in vivo. J. Immunol 177: 7607–7617. [PubMed: 17114430]
19. Howcroft TK, Weissman JD, Gegonne A, and Singer DS 2005 A T lymphocyte-specific transcription complex containing RUNX1 activates MHC class I expression. J. Immunol 174: 2106–2115. [PubMed: 15699141]
20. Pardo J, Balkow S, Anel A, and Simon MM 2002 Granzymes are essential for natural killer cell-mediated and perf-facilitated tumor control. Eur. J. Immunol 32: 2881–2887. [PubMed: 12355441]
21. Kasai M, Yoneda T, Habu S, Maruyama Y, Okumura K, and Tokunaga T1981 In vivo effect of anti-asialo GM1 antibody on natural killer activity. Nature 291: 334–335. [PubMed: 7231554]
22. Wallin RP, Screpanti V, Michaelsson J, Grandien A, and Ljunggren HG2003 Regulation of perforin-independent NK cell-mediated cytotoxicity. Eur. J. Immunol 33: 2727–2735. [PubMed: 14515256]

23. Simon MM, Waring P, Lobigs M, Nil A, Tran T, Hla RT, Chin S, and Mullbacher A 2000 Cytotoxic T cells specifically induce Fas on target cells, thereby facilitating exocytosis-independent induction of apoptosis. J. Immunol 165: 3663–3672. [PubMed: 11034370]
24. Mullbacher A, Lobigs M, Hla RT, Tran T, Stehle T, and Simon MM 2002 Antigen-dependent release of IFN-$\gamma$ by cytotoxic T cells up-regulates Fas on target cells and facilitates exocytosis-independent specific target cell lysis. J. Immunol 169: 145–150. [PubMed: 12077239]
25. Esplugues E, Sancho D, Vega-Ramos J, Martinez C, Syrbe U, Hamann A,Engel P, Sanchez-Madrid F, and Lauzurica P 2003 Enhanced antitumor immunity in mice deficient in CD69. J. Exp. Med 197: 1093–1106. [PubMed: 12732655]
26. Diefenbach A, Jensen ER, Jamieson AM, and Raulet DH 2001 Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. Nature 413: 165–171. [PubMed: 11557981]
27. Aptsiauri N, Cabrera T, Garcia-Lora A, Lopez-Nevot MA, Ruiz-Cabello F, and Garrido F 2007 MHC class I antigens and immune surveillance in transformed cells. Int. Rev. Cytol 256: 139–189. [PubMed: 17241907]
28. Pardo J, Buferne M, Martinez-Lorenzo MJ, Naval J, Schmitt-Verhulst AM, Boyer C, and Anel A 2003 Differential implication of protein kinase C isoforms in cytotoxic T lymphocyte degranulation and TCR-induced Fas ligand expression. Int. Immunol 15: 1441–1450. [PubMed: 14645153]
29. Kreuz S, Siegmund D, Scheurich P, and Wajant H 2001 NF-$\kappa$B inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. Mol. Cell. Biol 21: 3964–3973. [PubMed: 11359904]
30. Kelly JM, Darcy PK, Markby JL, Godfrey DI, Takeda K, Yagita H, and Smyth MJ 2002 Induction of tumor-specific T cell memory by NK cell-mediated tumor rejection. Nat. Immunol 3: 83–90. [PubMed: 11743585]
31. Suck G 2006 Novel approaches using natural killer cells in cancer therapy.Semin. Cancer Biol 16: 412–418. [PubMed: 16914327]
32. Ruggeri L, Mancusi A, Burchielli E, Aversa F, Martelli MF, and Velardi A2007 Natural killer cell alloreactivity in allogeneic hematopoietic transplantation. Curr. Opin. Oncol 19: 142–147. [PubMed: 17272987]
33. Imai K, Matsuyama S, Miyake S, Suga K, and Nakachi K 2000 Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11 year follow-up study of a general population. Lancet 356: 1795–1799. [PubMed: 11117911]
34. Mocikat R, Braumuller H, Gumy A, Egeter O, Ziegler H, Reusch U,Bubeck A, Louis J, Mailhammer R, Riethmuller G, et al. 2003 Natural killer cells activated by MHC class $I^{low}$ targets prime dendritic cells to induce protective CD8 T cell responses. Immunity 19: 561–569. [PubMed: 14563320]
35. Groh V, Wu J, Yee C, and Spies T 2002 Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature 419: 734–738. [PubMed: 12384702]
36. Salih HR, Antropius H, Gieseke F, Lutz SZ, Kanz L, Rammensee HG,and Steinle A 2003 Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. Blood 102: 1389–1396. [PubMed: 12714493]
37. Figueiredo C, Horn PA, Blasczyk R, and Seltsam A 2007 Regulating MHC expression for cellular therapeutics. Transfusion 47: 18–27. [PubMed: 17207226]
38. Figueiredo C, Seltsam A, and Blasczyk R 2006 Class-, gene-, and groupspecific HLA silencing by lentiviral shRNA delivery. J. Mol. Med 84: 425–437. [PubMed: 16520945]
39. Schowalter DB, Tubb JC, Liu M, Wilson CB, and Kay MA 1997 Heterologous expression of adenovirus E3-gp19K in an E1a-deleted adenovirus vector inhibits MHC I expression in vitro, but does not prolong transgene expression in vivo. Gene Ther. 4: 351–360. [PubMed: 9176522]

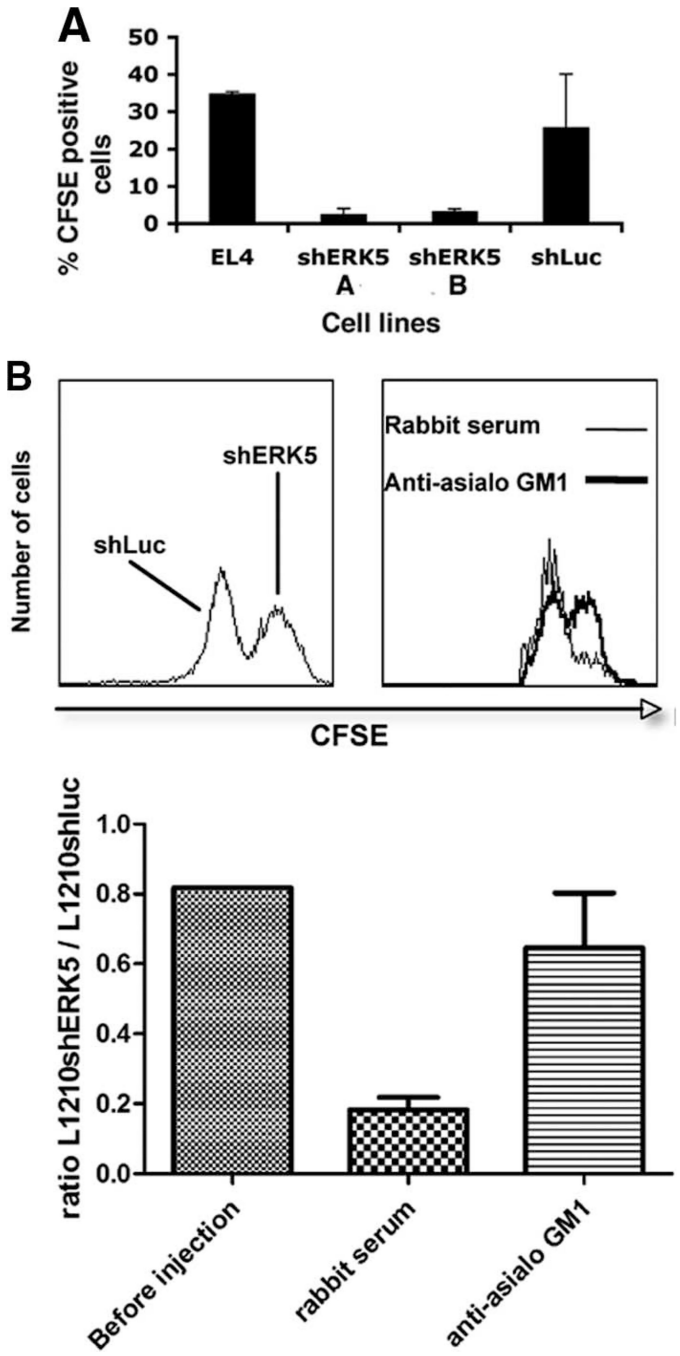


**FIGURE 1.**

Leukemia cells expressing shERK5 are eliminated within 3 days of peritoneal injection in syngenic mice. *A*, One million EL4 wt, shERK5 (two independent cell lines: A and B), and shLuc cells labeled with 1 $\mu$M CFSE were injected in the peritoneum of syngenic C57BL/6 mice. Peritoneal cells were recovered after 3 days and $CFSE^+$ cells were analyzed by FACS. The percentage positive cells was determined relative to the total number of cells recovered. *B*, Five million L1210 shLuc or shERK5 cells were labeled with 1 $\mu$M (shLuc) or 5 $\mu$M (shERK5) CFSE (*upper left panel*) and injected in syngenic BALB/c mice treated with either

anti-asialo GM1, which eliminates NK cells, or control rabbit serum. Two days later, $CFSE^+$ cells in the peritoneal wash, along with the input cells, were analyzed by FACS (*upper right panel*), and the ratio between shERK5 and shLuc cells was calculated (*lower panel*).

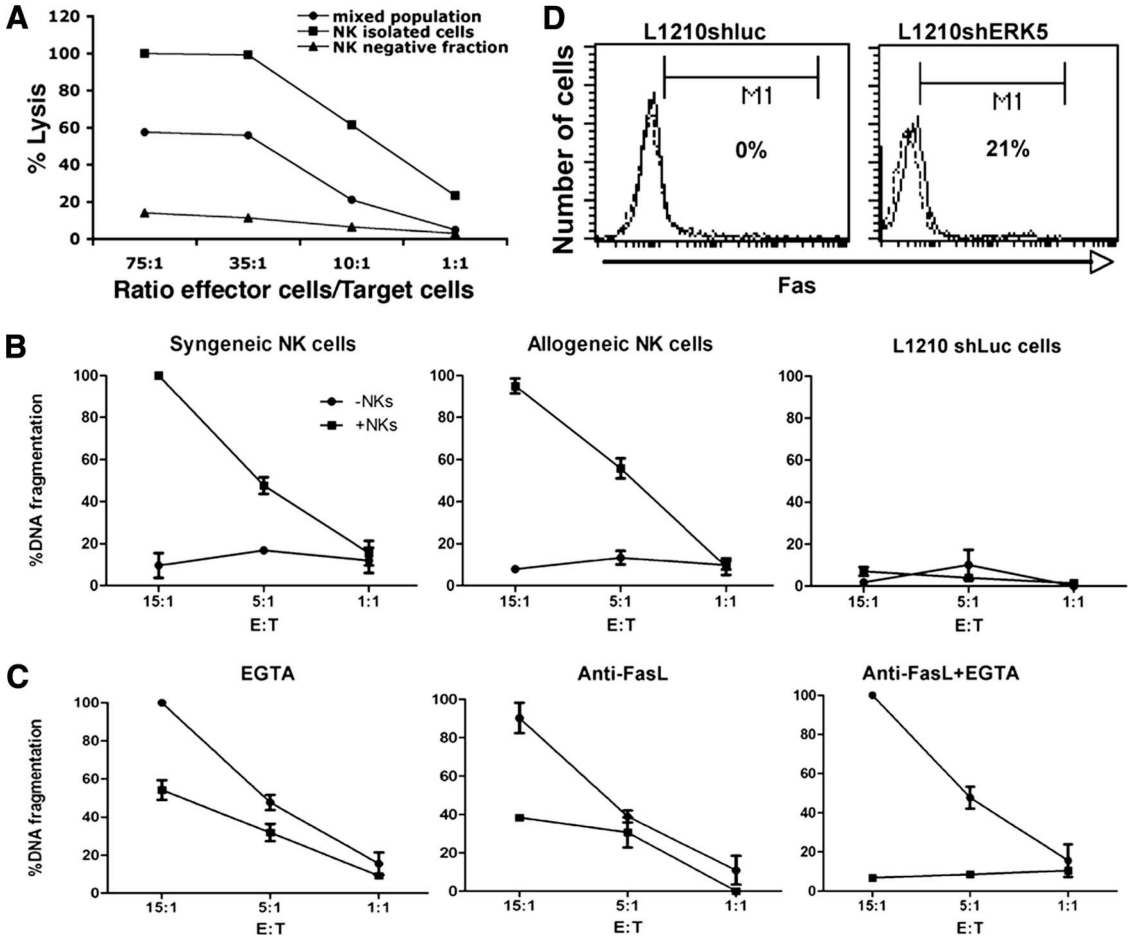


**FIGURE 2.**

NK cells target shERK5-expressing leukemia cells in vitro. *A*, EL4 shERK5 cells loaded with $^{51}$Cr were incubated 4 h with total splenocytes, purified NK cells, or splenocytes depleted of NK cells at different ratios. Cytolytic activity was measured using the $^{51}$Cr released into the cell supernatants. *B*, L1210 shERK5 cells (*left* and *middle panels*) or shLuc cells (*right panel*) were labeled with [$^{3}$H]thymidine and incubated at different ratios with effector cells and either purified NK cells (+NK) or splenocytes depleted of NK cells (NK) from syngenic (*left* and *right panels*) or allogenic (*middle panel*) mice. *C*, NK cells use both the perforin/granzyme and the Fas/FasL pathway to kill L1210 shERK5 cells in vitro. Different ratios of purified NK cells (+NKs, E = effector) were incubated with $^{51}$Cr-labeled L1210 shERK5 cells (T = target) for 4 h. Two millimolar EGTA and/or 1 $\mu$g/ml anti-FasL were added to the media during incubation. *D*, Fas expression in L1210 cells, either shLuc or shERK5, was analyzed by FACS. The dashed lines represent staining with a nonspecific IgG, and the brackets above the curves indicate the Fas-positive cells.

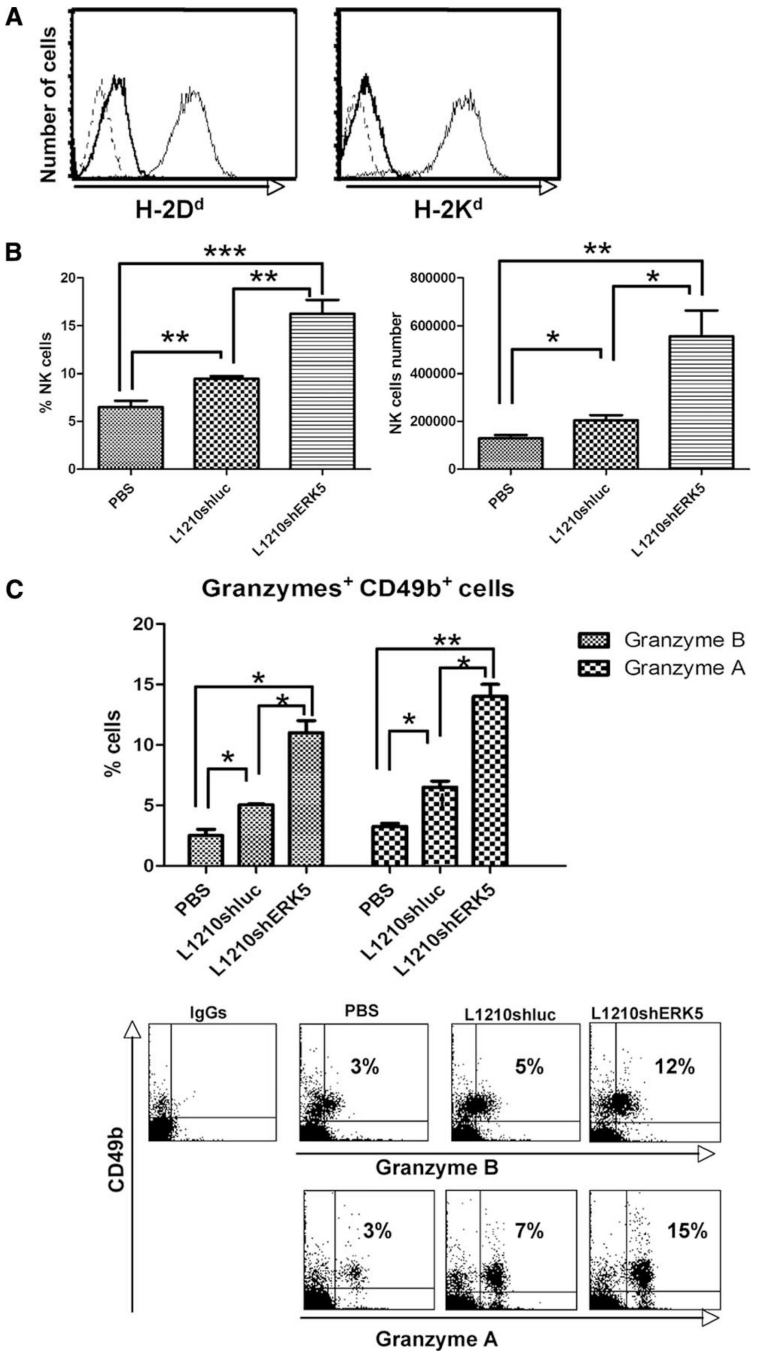


**FIGURE 3.**

L1210 shERK5 cells recruit and activate NK cells. *A*, L1210 shLuc (thin line) and L1210 shERK5 cells (thick line) were stained with anti-H-2D$^d$ (*left panel*) or anti-H-2K$^d$ (*right panel*) Abs to visualize MHC-I expression and analyzed by FACS. The dash line showed the unspecific binding of a control IgG Ab. *B*, Five million cells, either L1210 shLuc or L1210 shERK5, or PBS was injected into the peritoneum of syngenic BALB/c mice. Forty-eight hours later, cells were recovered from the site of injection and stained with an Ab to CD49b. The percentage (*left panel*) and the number (*right panel*) of NK cells were measured by

FACS analysis. The data were evaluated using Student's *t* test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *C*, Cells from *B* were stained with anti-granzyme A or anti-granzyme B Abs and analyzed as described in *B* to evaluate NK cell activation. The bars (*upper panel*) show the percentage of cells staining positive for both CD49b and granzyme, and they represent the average ± SD of three independent experiments. The lower set of panels shows the data from a typical experiment. The data were evaluated using Student's *t* test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

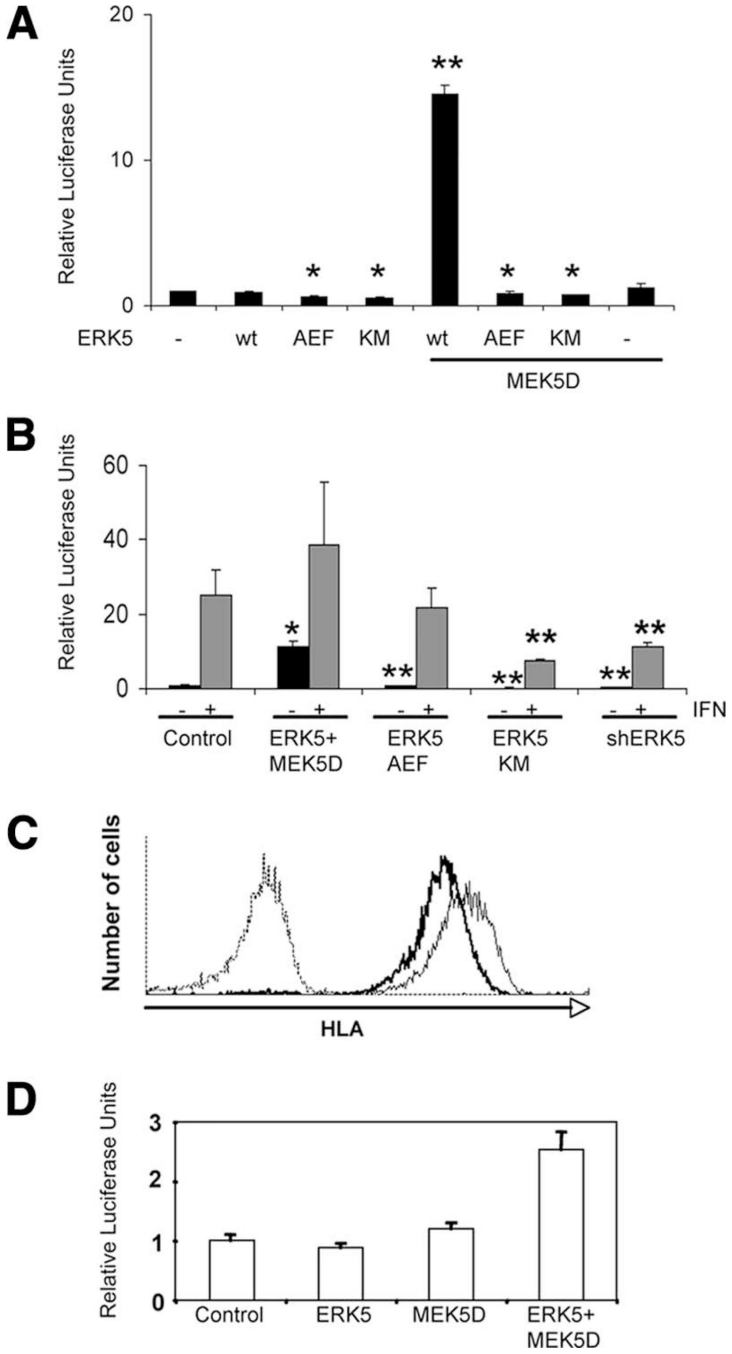


**FIGURE 4.**

ERK5 catalytic activity is required for the induction of a MHC-I reporter gene in Jurkat cells. *A*, Ten million Jurkat cells were transfected with the following: 5 $\mu$g of expression vectors for either wt ERK5, ERK5 AEF, or ERK5 KM and/or constitutively active MEK5 (MEK5D), 2.5 $\mu$g of the reporter gene PD-1-Luc, and 1 $\mu$g of the $\beta$-galactosidase expression vector. Forty-eight hours later, lysates were prepared and analyzed for luciferase and galactosidase activity. The relative luciferase units represent the ratio of luciferase/ galactosidase. *B*, ERK5 is essential for IFN-induced MHC-I up-regulation. Cells transfected

as in *A* were unstimulated (black bars) or stimulated with 1 nM each IFN-$\alpha$ and IFN-$\beta$ (gray bars) for 3 days. The data are presented as the mean ± SD of at least three independent experiments, and they were evaluated using Student's *t* test: *, $p < 0.05$ and **, $p < 0.005$ compared with control cells. *C*, Fifty million Jurkat Tag cells were transfected with with 3 $\mu$g of a GFP expression vector and either 10 $\mu$g of pcDNA3 or 5 $\mu$g each of expression vectors for ERK5 and MEK5D. Two days later, GFP cells were isolated by FACS and MHC-I expression analyzed in cells cotransfected with empty vector (thick line) or MEKD ERK5 (thin line) using an Ab to human HLA. The dotted line shows background staining in the absence of anti-HLA. *D*, HeLa cells were transfected with the following: 1 $\mu$g of expression vectors for wt ERK5 and/or constituively active MEK5 (MEK5D), 2.5 $\mu$g of the reporter gene PD-1-Luc, and 1 $\mu$g of a TK-*Renilla* luciferase reporter gene. For each transfection, a constant amount of DNA was transfected. The data were normalized to the TK-*Renilla* internal control and expressed as fold activation relative to the empty vector. The bars represent the mean ± SE of two experiments performed in duplicate.

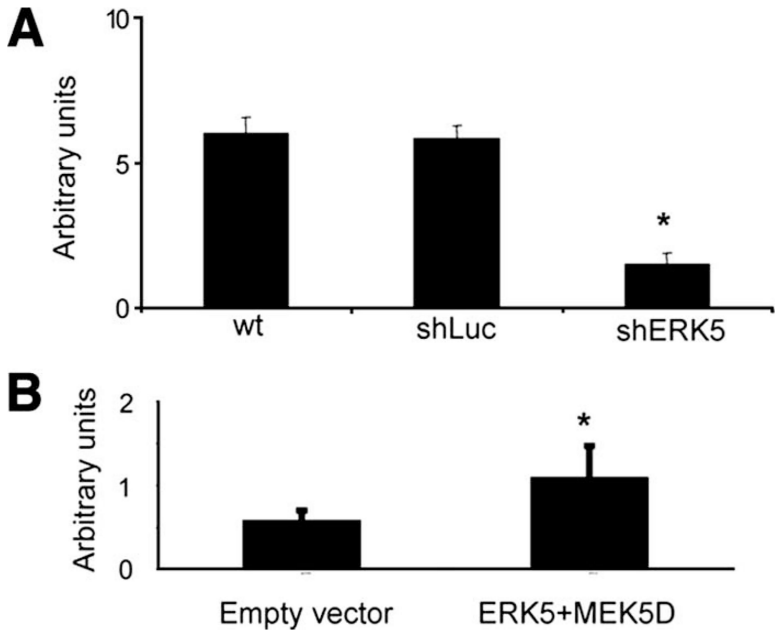


**FIGURE 5.**

The ERK5 pathway controls transcription of the endogenous $\beta_2$-m gene. *A*, RNA was isolated from $10^7$ resting L1210 wt, shLuc, or shERK5 cells and analyzed by quantitative RT-PCR for $\beta_2$-m and the housekeeping gene S26. The ratio between the two was used to calculate the arbitrary units. The data represent the mean ± SD of at least three independent experiments, which were evaluated using Student's *t* test: *, $p < 0.05$. *B*, Twenty million Jurkat cells were transfected with 3 $\mu$g of a GFP expression vector and either 5 $\mu$g each of expression vectors for ERK5 and MEK5D or 10 $\mu$g of empty vector. Two days later, $GFP^+$ cells were isolated by FACS, and RNA was isolated and analyzed as described in *A*. The data represent the mean ± SD of at least three independent experiments, which were evaluated using Student's *t* test: *, $p < 0.05$.

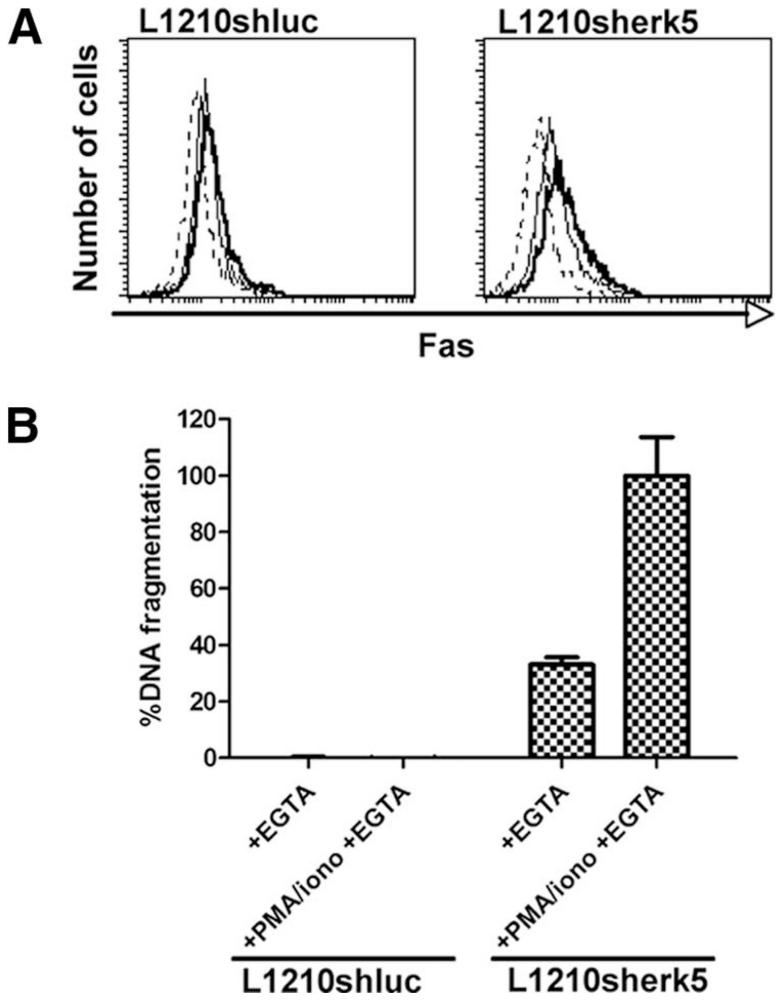


**FIGURE 6.**

L1210 shERK5 cells are highly sensitive to killing by cytotoxic lymphocytes. *A*, L1210 shLuc or shERK5 cells (dashed line) were incubated with resting (thin line) or activated (thick line) BM3.3 cells for 1 h and Fas expression in the CD8 population was analyzed by FACS. *B*, Resting or PMA/ionomycin-activated BM3.3 cells were incubated with L1210 shLuc or shERK5 cells for 2 h in the presence of 2 mM EGTA before measuring DNA fragmentation as described in *Materials and Methods*.

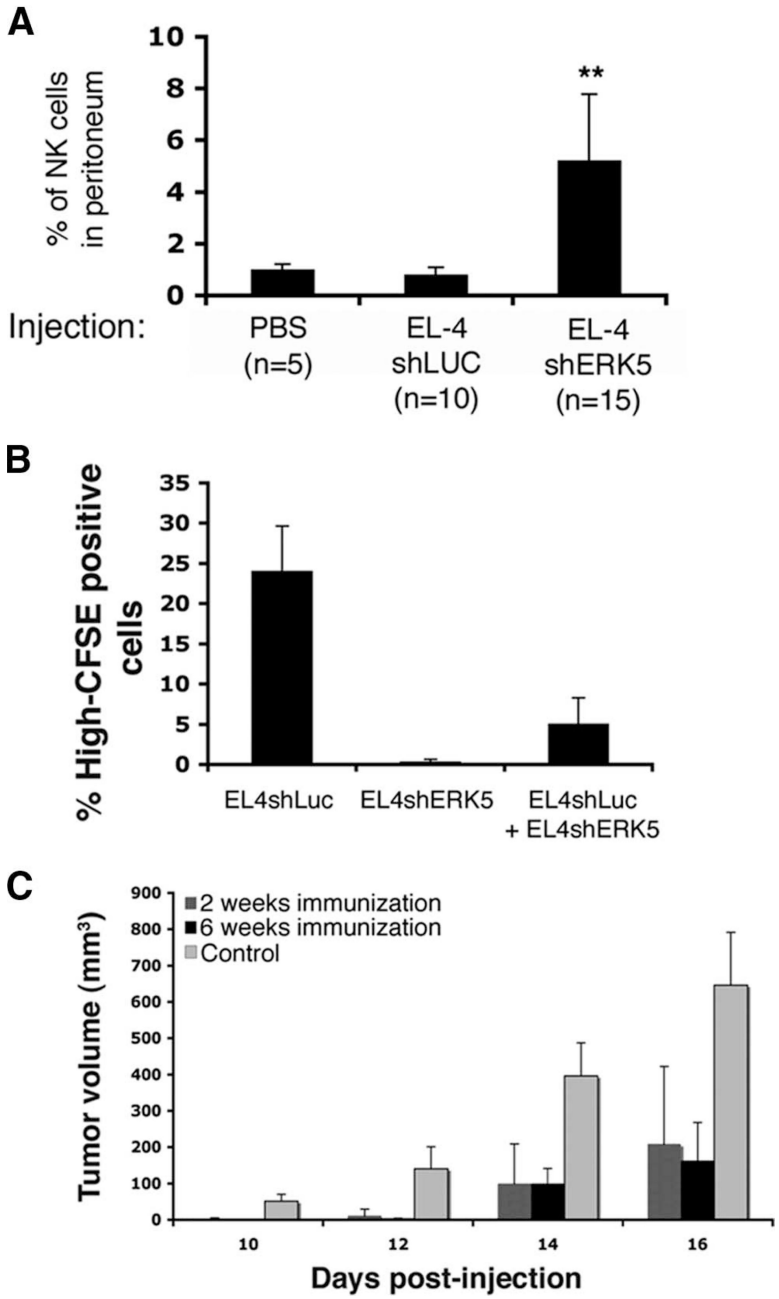


**FIGURE 7.**

EL4 leukemia cells expressing shERK5 block tumorigenesis by wt cells. *A*, Five hundred thousand EL4 cells were injected in the peritoneum of syngenic C57BL/6 mice. Three days later, cells were washed from the site of injection and the number of NK cells was determined by FACS using an Ab to NK1.1. The percentage of NK cells was calculated relative to the total number of cells. *B*, EL4 shLuc loaded with CFSE were injected alone (left bar) or together with nonlabeled EL4 shERK5 cells (right bar, $5 \times 10^5$ of each) in the peritoneum of syngenic mice ($n = 3$ mice/group). The same number of EL4 shERK5 cells loaded with CFSE were injected as a control (middle bar). After 3 days, cells were washed from the site of injection and the number of $CFSE^+$ cells was determined by FACS. The

percentage of $CFSE^+$ cells was calculated relative to the total number of cells. *C*, Syngenic C57BL/6 mice were injected with $2.5 \times 10^5$ EL4 shERK5 cells or PBS ($n = 4$) and then injected s.c. with $2.5 \times 10^5$ EL4 wt cells 2 ($n = 4$) or 6 ($n = 5$) weeks later. Tumor size was measured at the indicated times afterward.