Interleukin 12 Gene Therapy of MHC-negative Murine Melanoma Metastases1

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ABSTRACT

Immunological gene therapy of cancer relies heavily on the activation of T cells, but tumors with defects in MHC gene expression are not recognized by MHC-restricted T cells. To investigate the potential of cytokine genes for the therapy of MHC-negative tumors, we transduced B78H1, a class I-negative murine melanoma clone, with a polycistronic vector carrying murine interleukin (IL-12) genes. The clones produced 400–25,000 pg/ml IL-12; their in vitro growth properties were similar to those of parental cells. A complete inhibition of growth was observed in vivo both after s.c. and i.v. administration of all IL-12 clones. IL-12-transduced cells were also used as a therapeutic vaccine in mice bearing micrometastases by nontransduced parental cells. A significant (80–90%) reduction in the number of lung nodules was observed. Immunohistochemical analysis and studies in immunocompromised hosts showed that T cells and natural killer cells had a significant role in the elimination of IL-12-releasing cells. In situ hybridization with cytokine probes detected a strong increase in the proportion of leukocytes positive for IFN-γ, tumor necrosis factor α, IL-1β, and IFN-inducible protein 10 at the site of rejection of IL-12-engineered tumor cells. However, it was clear that the loss of in vivo growth was also due to T-cell- and natural killer cell-independent factors, possibly related to the antiangiogenic properties of IL-12. In conclusion, tumor therapy based on IL-12 gene transduction was effective on a MHC-negative metastatic tumor, suggesting a possible application to MHC-defective human neoplasms.

INTRODUCTION

The use of recombinant cytokines and of cells transduced with cytokine genes, in association with the cloning of specific tumor rejection antigens, has considerably increased the possibilities to induce or to boost an antitumor immune response based mainly on T lymphocytes. On the other hand, detailed studies of MHC gene products revealed that defects in class I MHC expression are widespread among human tumors. In fact, according to some estimates, most human tumors fail to express one or more class I MHC glycoproteins on their membrane (1, 2).

A causal link between specific MHC class I loss and immunoselection by T lymphocytes has not yet been established; however, it is certain that the absence of a given MHC gene product prevents the recognition by T lymphocytes of the antigenic peptides for which the MHC product represents the restriction element (1).

The high prevalence of tumors with class I MHC defects indicates that specific strategies should be devised to cope with tumors that cannot be efficiently killed by MHC-restricted CTLs. A good candidate for this type of application is IL-12,1 which is able to activate both specific TH1 responses and nonrestricted NK effectors (3).

IL-12 is a heterodimeric cytokine produced by antigen-presenting cells, phagocytes, and granulocytes (3). A strong antitumor efficacy of rIL-12 or of cells engineered to produce IL-12 has been reported in a great variety of tumors (4–11). Antitumor activity of IL-12 is thought to be mediated mainly by in vivo induction of IFN-γ, given that the therapeutic efficacy of IL-12 is reduced in mice treated with anti-IFN-γ antibodies (5). The IFN-inducible chemokine IP10 has been indicated in turn as a further mediator of the antitumor activity of IL-12 (7).

In this paper, we show that IL-12 gene transduction of a MHC-negative murine melanoma clone results in a complete loss of malignancy, and that IL-12-transduced cells can be used as a therapeutic vaccine against metastases produced by nontransduced MHC-negative parental cells.

MATERIALS AND METHODS

Cells. B78H1 is an amelanotic clone of B16 melanoma (12). B78H1 cells were cultured in DMEM (Life Technologies, Inc., Milan, Italy) supplemented with 10% fetal bovine serum (Life Technologies, Inc.).

Gene Transduction. IL-12-producing B78H1 cells were obtained by transduction with the polycistronic Lp40IpSN retroviral vector coding for both p35 and p40 murine IL-12 subunits as described previously (10). Single G418-resistant colonies were isolated, expanded into cell lines, and subjected to further analysis. The IL-12 concentration was determined by a two-site sandwich ELISA using 9A5 mAb to p70 and peroxidase conjugate mAb 5C3-POD to p40, kindly provided by Dr. Luciano Adorini (Roche Milano Ricerche, Milan, Italy).

Mice. Eight-week-old C57BL/6NCrlBR (referred to as C57BL/6) male mice and 5-week-old nu/nu male mice on Swiss CD1 background were purchased from Charles River Laboratories (Calco, Italy) and treated according to European Community guidelines. To obtain NK-depressed animals, we injected some groups of mice i.v. with 0.4 ml of a 1:25 dilution of anti-asialo GM1 antiserum (WAKO, Dusseldorf, Germany) 24 h before cell injection. Some mice were treated every 3 days with anti-asialo GM1 at the concentration described above and with 100 μg of anti-IFN-γ mAb (AN18 hybridoma; kindly provided by Dr. G. Garotta, Roche, Basel, Switzerland).

Tumor Growth and Metastasis. Mice were challenged s.c. with 0.2 ml of a single-cell suspension containing 5 × 103 (C57BL/6) or 106 (nude mice) tumor cells; cell doses were chosen to obtain tumors with a similar incidence and growth curve in C57BL/6 and in nude mice. The incidence and the growth of tumors were evaluated twice weekly. Neoplastic masses were measured with calipers; tumor volume was calculated as w/6 × (r/2 × a × b)3, in which a and b are two perpendicular major diameters. Experimental metastases were evaluated 28 days after the injection in a lateral tail vein of 5 × 105 (C57BL/6), 106 (nude mice), or 2.5 × 105 (anti-asialo GM1-treated C57BL/6) tumor cells suspended in PBS. Lung nodules were contrasted with black India ink; all metastasis counts were performed on dissected lung lobes under a stereoscopic microscope.

Therapeutic Vaccination. Therapeutic vaccination started 1 day after B78H1 parental cell challenge and consisted of seven injections of 106 viable or mitomycin C (80 μg/ml at 37°C for 45 min; Sigma)-treated cells 3–4 days apart.

Administration of Exogenous Mouse IL-12. Murine rIL-12, kindly provided by Dr. Maurice Gately (Hoffmann LaRoche, Nutley, NJ), was injected (0.1 μg/day diluted in PBS containing 100 μg/ml murine serum albumin) i.p. or at the site of s.c. vaccination. Starting 1 day after i.v. injection of tumor cells, mice received two courses of five daily i.p. injections with a 2-day

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3 The abbreviations used are: IL, interleukin; rIL, recombinant IL; IP10, IFN-inducible protein 10; TNF, tumor necrosis factor; NK, natural killer; mAb, monoclonal antibody.

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IL-12 Gene Therapy of MHC-negative Tumor

Morphological Analysis. Mice were treated s.c. with 10^6 B78H1 or IL-12 gene-transduced cells; at least two mice from each group were euthanized at 24, 48, 72, and 120 h after injection. Tumor fragments were embedded in OCT compound (Miles Laboratories, Elkart, IN), snap-frozen in liquid nitrogen, and stored at −80°C. Analysis was performed as described previously (13). Briefly, 5-μm cryostat sections were fixed in acetone and immunostained with antimouse mAb against leukocytes (CD45, M/19.3.4.HL2 hybridoma, T200), CD8 (53.6.72 hybridoma Lyt2), CD4 (GK1.5 hybridoma L3T4), Mac-1 (M1/70.15.11.5.HL hybridoma), Mac-3 (M37/86.6.34 hybridoma; all from American Type Culture Collection, Rockville, MD), CD3 (Boehringer Mannheim, Mannheim, Germany), granulocytes (clone RB6-8C5), dendritic cells (DEC205), endothelial cells (CD34 and CD31; Pharmingen, San Diego, CA), and NK cells (anti-asialo GM, serum; WAKO). Eosinophils were identified by the endogenous peroxidase.

Sections were preincubated with rabbit serum and sequentially incubated with optimal dilutions of primary antibodies, rabbit antirat IgG (Zymed Laboratories, Inc., San Francisco, CA), and rat peroxidase antiperoxidase (Abbott Laboratories, North Chicago, IL). Each incubation step lasted 30 min and was followed by a 10-min wash in Tris-buffered saline. Sections were then incubated with 0.03% H_2O_2 and 0.06% 3,3′-diaminobenzidine (BDH Chemicals, Poole, United Kingdom) for 2–5 min, washed in tap water, and counterstained with hematoxylin. The number of immunostained cells was determined by light microscopy at ×400 magnification in five fields on a 1-mm^2 grid and is given as cells/mm^2 (mean ± SE).

In Situ Hybridization. The presence of cytokine mRNA was investigated using in situ hybridization with cDNA probes for IL-18 [500 bp from pIL-1 130; Ref. 14; obtained from Dr. P. Lomedico (Hoffman-La Roche)], TNF-α [860-bp Pst-EcoRI fragment from pUC19 plasmid containing a mouse TNF-α complementary cDNA insert; Ref. 15; courtesy of Dr. A. Cerami (Rockefeller University, New York, NY)], IFN-γ [1.3-kb BamHI fragment from pT7/T3-19; Ref. 16; obtained from Dr. S. Landolfo (University of Turin, Turin, Italy)], and a PCR-amplified fragment of murine IP10 (17). Cryostat sections were harvested on RNA grade slides; air-dried and fixed in 4% buffered paraformaldehyde for 10 min; dehydrated in ethanol; sequentially rehydrated in PBS and 50 mM MgCl_2; washed in 200 mM Tris-HCl glycine; acetylated in 2× SSC (1× SSC, 0.15 M NaCl and 15 mM sodium citrate), 0.1 M triethanolamine, and 0.5% acetic anhydride (pH 8.0); washed in 2× SSC; and dehydrated in ethanol. Slides were then prehybridized for 10 min at 70°C with 2× SSC and 50% formamide and hybridized overnight at 42°C with 32P-labeled specific cDNA probe, 1.5 × 10^6 cpm/section, 2× SSC, 500 µg/ml yeast RNA, 5× Denhardt’s solution, 10 mM DTT, and 10% dextran sulfate. Unbound and nonspecifically bound probes were removed by sequential washes in 2× SSC, 50% formamide, and 1× SSC and 50% formamide at 45°C and in 0.1× SSC at room temperature. Slides were then dehydrated in ethanol, dipped in autoradiographic emulsion NTB (Eastman Kodak Company, Rochester, NY), exposed for 5 days at 4°C in a light-tight box, developed in D19 (Eastman Kodak Co.), fixed in Rapid Fixer (Eastman Kodak Co.), and counterstained with hematoxylin. RNA-specific binding was controlled by previous digestion with 100 µg/ml RNase A and 10 units/ml RNase T (Sigma, Poole, United Kingdom). PstI-digested pUC9 plasmid fragments were used as negative controls. Cytochromes of lipo polysaccharide and phytohemagglutinin plus immunocyt-stimulated peripheral blood lymphocytes were inserted as positive controls in each experiment. Cells were considered positive when the number of black grains in the cell was at least five times the mean number of grains present in the negative control slides. The number of positive cells was evaluated at ×400 on a 1-mm^2 grid and reported as cells/mm^2 (mean ± SD).

Cytofluorometric Studies. The membrane expression of H-2 glycoproteins was determined by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA) after indirect immunofluorescence as described previously (12). Mouse mAbs recognizing H-2K^d (clone 5041.16.1) and Ia^b (clone 28–16-85) were from Cedarlane (Hornby, Ontario, Canada). FITC-conjugated goat antimouse immunoglobulin was purchased from Technogenetics (Milan, Italy). Results shown are representative of at least three independent experiments.

Mixed Lymphocyte Tumor Culture and Cell-mediated Lympholysis Assays. Mixed lymphocyte tumor culture was performed in RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) supplemented with 10% FCS (Hyclone, Logan, UT) using a modification of a previously reported procedure (18). Responder splenocytes were stimulated either with IL-2 (20 units) alone or with IL-2 and γ-irradiated (20,000 rad) class I-transfected B78H1 cells (12). Responders and stimulators were suspended to 2.5 × 10^5 and 2.5 × 10^6 cells/ml, respectively, and mixed in a total volume of 2 ml in 24-well plates (Costar, Cambridge, MA). Cultures were incubated in a humidified atmosphere of 5% CO_2 in air. The induction of a CTL response was assessed in standard 4-h ^51Cr release assays as described previously (19). In CTL assays, B78H1 and class I transfected B78H1 cells were the specific targets; YAC-1 cells were used as controls for NK cell mediated-lysis.

RESULTS

IL-12 Gene Transduction in MHC-negative B78H1 Cells. B78H1 is a clone of B16 melanoma that does not express either class I or class II MHC gene products (Fig. 1; furthermore; B78H1 cells remained MHC-negative after in vitro treatment with IFN-γ (Fig. 1; Ref. 12).

IL-12 genes were transduced in B78H1 cells by means of a polycistronic retroviral vector. IL-12 released by the resulting clones ranged from 128 pg to 25 ng/ml/2 × 10^6 cells/48 h. Three clones named IL-12^400, IL-12^16,000 and IL-12^25,000 were chosen for further studies; the exponent in the name of each clone represents its IL-12 release in pg/ml.

In vitro growth properties of IL-12 clones, including cell morphology, growth rate, and MHC antigen expression, were similar to those of parental cells (data not shown).

IL-12 Release Abolishes B78H1 Tumorigenicity and Metastatic Ability. B78H1 cells and IL-12 clones were injected s.c. into immunocompetent C57BL/6 mice. B78H1 gave rise to rapidly growing tumors, but no tumor growth was ever observed in mice treated with IL-12-secreting cells (Fig. 2). These tumor-free mice, however, did not develop resistance to a subsequent challenge with B78H1 parental cells performed 60 days after the injection of IL-12 transfectants.

Similar results were obtained after i.v. injection of tumor cells: no metastases were ever observed in mice inoculated with high IL-12-secreting clones (Fig. 2).

The results indicate that IL-12 gene transduction abolished the in...
vivo growth of MHC-negative tumor cells. We then asked whether IL-12-transduced cells could be used as a therapeutic vaccine to inhibit the growth of nonengineered parental cells.

**Therapy of Lung Metastases.** Mice bearing B78H1 micrometastases were repeatedly vaccinated s.c. with live IL-12 clones. Both IL-12<sub>16,000</sub> and IL-12<sub>25,000</sub>, but not IL-12<sub>400</sub>, caused a significant (80–90%) reduction in the number of lung metastases (Table 1). No tumor was induced at the vaccination site; however, to perform a meaningful comparison with tumorigenic parental cells, vaccines were also pretreated in vitro with mitomycin C to block cell proliferation. Also, vaccination with mitomycin-treated IL-12<sub>16,000</sub> and IL-12<sub>25,000</sub> strongly inhibited the development of lung metastases (Table 1).

The efficacy of rIL-12 administration in the treatment of local or metastatic lesions was also evaluated. A 50% reduction in tumor volume was observed in mice bearing 7-day tumors of nonengineered B78H1 cells after two 5-day courses of rIL-12 given i.p. (data not shown). The antimetastatic efficacy of IL-12 gene-transduced cells was compared to the i.p. or s.c. administration of exogenous rIL-12. Both types of treatment seemed to reduce the metastatic load to a similar extent (Table 1).

**Tumor and Metastasis Growth in Immunocompromised Hosts.** To analyze the immune response involved in the rejection of IL-12 clones, we studied their growth in immunocompromised hosts (Fig. 3). The s.c. injection of IL-12<sub>400</sub> in athymic nude mice gave rise to tumors with growth kinetics much slower than parental B78H1 tumors. Only a small proportion of nude mice receiving IL-12<sub>25,000</sub> cells s.c. developed tumors. Experimental metastasis of IL-12 clones in nude mice was not enhanced in comparison to euthymic mice.

An increase in the number of lung nodules was observed in NK-depleted euthymic and athymic mice receiving IL-12<sub>400</sub> cells i.v.; however, the highest IL-12 producer, IL-12<sub>25,000</sub>, was completely nonmetastatic in all types of host (Fig. 3). It should be noted that IL-12<sub>25,000</sub> cells are capable of in vivo growth, because progressive tumors were sporadically observed in nude mice (Fig. 3). In euthymic mice subjected to repeated administrations of anti-asialo GM<sub>1</sub> and anti-IFN-γ antibodies, we observed progressive tumor growth in two of five mice and an initial growth followed by regression in the remaining three of five mice. Tumor-free mice were protected from a subsequent challenge with parental B78H1 cells administered 2 months later.

**Immunohistochemical Analysis of Tumor Rejection.** The complete lack of tumor growth by IL-12-transduced B78H1 cells hints at a rapid elimination of tumor cells in vivo. We performed a quantitative immunohistochemical study of normal host cells infiltrating the injection site from 1–5 days after a s.c. administration of parental B78H1 cells or IL-12 clones.

A dramatic increase in specific infiltrating leukocyte populations was induced by IL-12-secreting cells, whereas other populations were attracted both by parental cells and by IL-12-engineered cells (Fig. 4). In particular, we observed an increase in the number of CD8<sup>+</sup> and NK cells, proportional to the amount of IL-12 secreted by tumor cells. dendritic cells and eosinophils showed a threshold effect, being exclusively elicited by the high producer IL-12<sub>25,000</sub>, whereas the low producer, IL-12<sub>400</sub>, was similar to parental B78H1.

The antitumoral activity of IL-12 can also be exerted via the inhibition of neoangiogenesis (20); therefore, we examined the tumor cell injection sites, particularly the tumor growth front, for the presence of microvessels, as revealed by CD34 and CD31 staining. We found that microvessels were very scarce at the site of IL-12<sub>25,000</sub> growth, both 3 and 5 days after cell injection, whereas parental B78H1

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Table 1  **Therapy of B78H1 metastases with IL-12-transduced cells**

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<tr>
<th>B78H1 cells injected i.v.</th>
<th>Cell vaccine&lt;sup&gt;a&lt;/sup&gt; or rIL-12 treatment&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Treatment of vaccine</th>
<th>B78H1 lung metastases</th>
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<td>rIL-12 i.p.</td>
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<sup>a</sup> Therapeutic vaccinations with 5 x 10<sup>5</sup> untreated cells or 2 x 10<sup>6</sup> mitomycin-C-treated cells were administered to immunocompetent C57BL/6 mice six times at 3–4-day intervals starting on the day after the i.v. challenge with B78H1 cells. Mice were euthanized 4 weeks after challenge.

<sup>b</sup> See "Materials and Methods."

<sup>c</sup> Significantly different from untreated or B78H1-vaccinated mice, P < 0.05 at least by Wilcoxon’s rank sum test.
cells attracted a higher number of microvessels after 3 days, and a further increase was observed on the day 5 (Fig. 5). We also examined a macroscopic IL-12^{25,000} tumor grown in a mouse treated with anti-asialo GM₁ and anti-IFN-γ antibodies (see above). It showed microvessel numbers, evaluated by CD34 and CD31 staining, of 37 ± 6 and 41 ± 8, respectively. These values were comparable to those observed in B78H1 tumors of similar volume (41 ± 11 and 37 ± 6, respectively). These values were comparable to those observed in B78H1 tumors of similar volume (41 ± 11 and 37 ± 6, respectively). These values were comparable to those observed in B78H1 tumors of similar volume (41 ± 11 and 37 ± 6, respectively, B78H1 tumors. We chose IL-12 because it is able to activate both MHC-restricted and non-MHC-restricted antitumor responses (3). IL-12 is a potent antitumor agent; however, adverse effects were reported when the cytokine was administered systemically in mice, primates, and humans (21-24). A number of gene transfer therapeutic model systems have been developed to achieve activation of a systemic immune response by local delivery of IL-12, avoiding the toxic effects related to the systemic administration of the cytokine (8-10, 25-28).

The results reported here demonstrate that IL-12 gene transduction abolishes the tumorigenic and metastatic capacity of a MHC-negative tumor system, rather than from an intrinsic inhibition of the angiogenic capacity of IL-12^{25,000} cells.

**In Situ Hybridization with Cytokine Probes.** The presence of infiltrating cells expressing mRNA for some cytokines known to be modulated by IL-12 was examined at the site of tumor cell injection (Fig. 6). IL-12^{25,000} induced a significant increase over B78H1 in the number of infiltrating leukocytes expressing IL-1β, TNF-α, IFN-γ, and IP10 mRNA. IL-12^{25,000} tumor grown in a mouse treated with anti-asialo GM₁ and anti-IFN-γ antibodies showed a cytokine pattern similar to that found in B78H1 tumors.

**In Vitro Cell-mediated Cytotoxicity.** To test in vitro the cytolitic activity of lymphocytes from mice injected with IL-12^{25,000}, splenocytes were cultured in vitro with either IL-2 (20 units) alone or IL-2 plus H-2Kb- and H-2Db-transfected B78H1 cells. B78H1 cells were lysed regardless of the expression of class I at the same level as YAC-1 cells (Fig. 7, A and B). Furthermore, splenocytes from CD4- and CD8-depleted mice, after a short-term in vitro expansion with rIL-2, lysed the same targets used above (Fig. 7C), suggesting that NK cells were the functional effectors.

**DISCUSSION**

MHC class I loss or down-regulation is a widespread phenomenon in tumor biology. Several human tumor types exhibit a complete loss of expression of HLA antigens in a proportion of cases variable from 10 to 50%. An even higher proportion of HLA-deficient cases was reported for some tumor histotypes, for example, up to 88% in tumors of epithelial origin (1). Furthermore, the frequency of abnormalities in class I expression seems to have been underestimated in the past, because most of the published studies used mAbs that do not recognize selective losses of HLA class I alleles (2).

Defects in class I molecules may influence natural history of the tumor and may strongly counteract the potential benefit of immunotherapy based on T cells. Immunotherapy based on non-MHC-restricted mechanisms could result more efficient on MHC-negative tumors. We chose IL-12 because it is able to activate both MHC-restricted and non-MHC-restricted antitumor responses (3). IL-12 is a potent antitumor agent; however, adverse effects were reported when the cytokine was administered systemically in mice, primates, and humans (21-24). A number of gene transfer therapeutic model systems have been developed to achieve activation of a systemic immune response by local delivery of IL-12, avoiding the toxic effects related to the systemic administration of the cytokine (8-10, 25-28).

The results reported here demonstrate that IL-12 gene transduction abolishes the tumorigenic and metastatic capacity of a MHC-negative tumor system, rather than from an intrinsic inhibition of the angiogenic capacity of IL-12^{25,000} cells.
tumor. Therapeutic administration of IL-12-transduced cells to mice bearing nontransduced MHC-negative lung metastases resulted in a significant reduction of the metastatic load.

Multiple inflammatory and immune cells were preferentially attracted by MHC-negative tumor cells releasing IL-12. A selective increase in eosinophils, NK cells, dendritic cells, and CD8 lymphocytes was observed in the infiltrate. In turn, secondary pleiotropic cytokines were produced by infiltrating cells activated by IL-12, in particular, IFN-γ and TNF-α, which are also known inhibitors of B78H1 cell proliferation (29), and IP10, which is one of the effectors of the antiangiogenic activity of IL-12 (30).

Tumorigenic and metastatic ability of IL-12 clones was increased in nude and/or NK-depleted mice. In association with immunohistochemical and in situ results in immunocompetent mice, these data indicate that multiple mechanisms contributed to the inhibition of IL-12-secreting cells implanted in vivo. T cells were involved in the control of IL-12-secreting tumors, as evidenced by the growth of some IL-12 clones in nude mice, and NK cells played a role in the control of lung metastases. However, it must be noted that the growth of IL-12 clones was greatly inferior to that of parental cells in nude, NK-depleted, and NK-depleted nude mice. Therefore, IL-12 acted mostly through mechanisms independent of T and NK effectors. We found that in the first few days of in vivo growth, IL-12-producing cells displayed a lower angiogenic potential than parental tumors, and a strong local increase of cytokines involved in the antiangiogenic effects of IL-12, such as IFN-γ and IP10, was observed. In summary, inhibition of neoangiogenesis could be involved in the lack of in vivo growth of MHC-negative IL-12 clones.

The antitumor mechanisms activated by our MHC-negative IL-12 gene-transduced cells seem similar to those reported in other MHC-positive tumor systems (3-11, 20, 21). They include T cells, NK cells, IFN-γ, TNF, and IP10. T cells involved in our MHC-negative model were probably endowed with non-MHC-restricted activity or with immunoregulatory activity on NK function, as evidenced by in vitro cytotoxic studies.

In conclusion, tumor therapy based on gene transduction of IL-12 was effective on a MHC-negative metastatic tumor, indicating that this approach should be further pursued to devise more effective and less toxic therapies that are also applicable to MHC-defective human neoplasms.

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