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The combined actions of NK and T lymphocytes are necessary to reject an EGFP+ mesenchymal tumor through mechanisms dependent on NKG2D and IFNγ

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Better understanding of the mechanisms that mediate spontaneous immune rejections ought to be important in the quest for improvements in immunotherapy of cancer. A set of intraperitoneal tumors of mesenchymal origin that had been chemically induced in ubiquitously expressing EGFP transgenic mice provided a model in which both T and NK cells were absolutely required for tumor rejection. Tumor cells were traceable because of being fluorescent and readily grafted in RAG1$^{-/-}$ immunodeficient mice, whereas they were rejected in a majority of syngeneic C57BL/6 and EGFP-transgenic mice. Tumor-cell clones with the highest EGFP expression tended to be rejected, but a direct involvement of EGFP as the antigen recognized for the immune rejections was ruled out. Rejections were absolutely dependent on NK cells as well as on CD4$^+$ and CD8$^+$ T lymphocytes according to selective depletion studies. Furthermore, CD8$^+$ and CD4$^+$ T lymphocytes as well as NK cells were detected in the inflammatory infiltrate that mediates tumor rejection along with some DC. The effects of IFNγ, produced at the tumor site by T and NK lymphocytes, were only required at the malignant cell level and were necessary for tumor eradication. NK recognition of tumor cells was mediated by the NKG2D-activating receptor and blocking its function *in vivo* partially interfered with rejection. Therefore, complete rejection of these mesenchymal tumors requires a concerted set of activities including direct tumor-cell destruction and IFNγ production that are mediated by both NK and T cells.

Key words: NK cell; NKG2D; CD8$^+$ T cell; CD4$^+$ T cell; IFNγ

NK cells have been found to be capable of killing tumor cells with neither specific recognition nor memory.[1,2] Several lines of evidence in mouse transplantable tumors suggest a role for NK cells in tumor control.[2–5] In addition, effects mediated by NK cells have been shown in various experimental immunotherapies that include approaches based on cytokines,[6] costimulatory molecules,[7] dendritic cells,[8] and immunostimulating monoclonal antibodies.[9] The involvement of NK cells in therapy is most prominent when tumor cells are transfected to express IL-12,[6] IL-15,[10] IL-21,[11] CD70[12] or CD80.[7] Treatment with anti-CD137 agonistic mAb also relies on the function of NK cells.[13,14] In addition, intravenous adoptive transfer of autologous lymphokine-activated lymphocytes that include NK cells have been used in clinical trials for melanoma and other tumors with some objective benefit.[15] The role of NK cells in prophylaxis of malignancies as suggested by the immunosurveillance hypothesis is a matter of debate, but it has gained experimental support in the recent past.[16,17]

The cytolytic mechanisms of NK cells are activated by surface receptors that interact with molecules detected on the surface of target cells.[5] Many of such receptors have been identified in the human and in the mouse system.[5] NKG2D is a molecule expressed on NK and T cells that controls this cytolytic function both in murine and human NK cells.[18] It interacts with MHC class I-like molecules induced under stressful conditions for the target cell. In fact, gene transfection of these stress molecules such as H60 or Rae1 to experimental tumors leads to tumor regression and effective vaccination against untransfected variants.[19] Upon engagement, NKG2D activates the DAP-10 and, in some instances, DAP-12 adaptor molecules to turn on activation signaling pathways.[18] Other authors have gathered evidence in cancer patients and mice to sustain that chronic exposure to NKG2D ligands downregulates NKG2D[20] and desensitizes NK cells for their immunosurveillance mission.[21,22] Expression of NKG2D ligands in tumor cells may be the result of the activation of the DNA damage response,[23] an event that occurs early in tumorigenesis, and may involve epigenetic changes regulated by histone deacetylation.[24]

NK cytotoxicity has been proposed as the result of the balance of the action of sets of activatory receptors, such as NKG2D, and inhibitory receptors tightly controlling this function.[5] Mouse NK cells preferentially lyse allogeneic target cells since the members of the Ly49 set of clonally distributed NK-inhibitory receptors fail to recognize autologous MHC class I molecules, not expressed on allogeneic cells, that would otherwise downregulate cytotoxicity. The system is believed to be set in such a fashion that each single functional NK cell would express at least one inhibitory receptor recognizing a self MHC class I allele or become anergic during ontogeny.[25] Recent provocative experimentation has also involved mouse "memory" NK cell subpopulations in mediating contact hypersensitivity by selective recognition of certain haptens through still undefined molecular mechanisms.[26]

Immune effects of T cells on mouse tumors have been studied in more detail. However, the precise train of events that leads to optimal priming and activation of the immune system against the malignancy is still incompletely understood.[27] There is circumstantial evidence for the involvement of dendritic cells presumably cross-presenting antigens from malignant cells and the functions of other lymphocytes such as NK,[5] NKT[28] and suppressor T cells[29] are also crucial for the final outcome of the antitumor immune response in some instances. Tumor recognition by effector T cells implies tumor infiltration and recognition of tumor antigens presented on MHC class I molecules. Th1 cells can also be helpful by recognizing antigens on MHC class II molecules expressed by the stromal cellular component of the tumor.[30] A role for NK cells in the *in vitro* T-cell priming against mouse tumor cells with a requirement for IL-12 and IL-18 has also been reported.[31]

A number of research lines have demonstrated a reciprocal regulation of NK and DC.[32] DC contribute to NK activation by

Abbreviations: CTLs, cytolytic T lymphocytes; DC, dendritic cells; EGFP, enhanced green fluorescent protein; IFN, interferon; IL-, Interleukin-; LN, lymph node; 3-MCA, 3-methylcholantrene; NK, natural killer.

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paracrine secretion of IL-12[8,33] and possibly other cytokines, while NK cells can both promote DC maturation for T cell priming or the elimination of DC by cytolytic mechanisms.[34,35] The interaction of NK cells and DC could be very important to initiate and sustain antitumor T cell responses, in as much as DC are known sources of IL-2 and IL-15, that are well studied T and NK activators.[36] Recent intravital microscopy experiments support the idea of this relationship showing close interactions of DC and NK cell pairs that reside in lymph nodes.[37] Indirectly, early tumor-cell debris produced by NK-mediated tumor lysis is a potential source of antigen for DC-mediated crosspresentation. There is evidence that tumor-cell apoptotic bodies[38] or peptides chaperoned by heat shock proteins[39] can be sources of antigen transferred from the malignant cell into the professional APC. Other investigations have shown that arrival of tumor cells to LN is an important event for T-cell immunization[40] either by direct priming of CTL precursors or through intralymph-node crosspresentation by DC subsets resident in the LN.[41,42] IFNγ production by NK cells at the sentry lymph node seems to be critical for the recruitment of T cells in high numbers into the responding node, as well as for Th1 differentiation.[43] NK cells also crosstalk to DC reaching lymph nodes to upgrade their T-cell-activating functions.[36]

In this study, we describe a mouse transplantable tumor obtained in EGFP transgenic mice in pure C57BL/6 background. This tumor was found to be under a rejecting immune pressure that was mediated in part by NK cells activated through the NKG2D receptor. Experiments in this tumor system clearly show that only when the concurrent orchestrated activities of NK and T cells are present, can rejection of the tumor take place.

Materials and methods

GIPT tumors obtention

About 8×8 mm^2 spongostan (Codman, Piscataway, NJ) plugs were soaked in a 20 mg/ml 3-MCA (Sigma-Aldrich, St. Louis, MO) suspension in corn oil for ~2 min before being sewn into the pancreas of 15 female, EGFP-transgenic mice in order to obtain green fluorescent tumors. Mice were palpated and weighed weekly starting approximately 2 months postimplantation of the 3-MCA plugs, as described.[44] All of these mice developed intraperitoneal tumors in the period of time between 1.5–3 months following surgery. Tumor explants were obtained after animal sacrifice, minced to 1–2 mm cubes and plated in 6-well plates. A cell line derived from such tumor explants (GIPT) was chosen for *in vivo* experimentation, and cloned by limiting dilution for the obtention of the GIPT^{high} and GIPT^{low} variants. The GIPT^{relapse} cell line was derived from an explanted GIPT^{high} tumor that grew in a C57BL/6 mouse. Sections of tumors stained with H&E were analyzed under visible and U.V. light. Cell culture was performed in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 10% fetal bovine serum (Invitrogen, Carlsbad, CA).

Mice and in vivo *experiments*

C57BL/6 mice were obtained from Harlan (Barcelona, Spain) and were used between 6 and 14 weeks of age. RAG1$^{-/-}$ mice from The Jackson Laboratory (Bar Harbor, ME) were bred in our animal facility under pathogen-free conditions. EGFP-transgenic mice[45] were a generous gift from M. Okabe (Genome Information Research Center, Osaka University, Japan). Those transgenic mice were backcrossed 10 times to C57BL/6 mice and then brother-to-sister crossed to achieve homozygous transmission of the transgene. Mutant mice deficient in IFNγ receptor were obtained from the Jackson Laboratory, and CD1-deficient mice were kindly provided by Van Kaer and coworkers.[46] OT-1 TCR-transgenic mice, expressing a TCR recognizing the dominant H-2^b-restricted OVA epitope (SIINFEKL)[47] were kindly supplied by F. Carbone (Melbourne, Australia). All the transgenic strains were in C57BL/6 background and were bred in our animal facility. Experiments were performed in agreement with our institution's Committee on Animal Research and Ethics guidelines (study approval number 003/02). For *in vivo* tumor-growth experiments, 5×10^5 GIPT, GIPT^{high}, GIPT^{low} or GIPT^{relapse} cells were injected subcutaneously in the right flank of C57BL/6, RAG1$^{-/-}$, EGFP transgenic, CD1$^{-/-}$ or IFNγR$^{-/-}$ mice. Tumor growth was monitored twice a week by measuring two perpendicular diameters using a Vernier calliper. For NK1.1$^+$, CD4$^+$ and CD8$^+$ T-cell depletion experiments and *in vivo* IFNγ neutralization, NK1.1, CD4, CD8 and IFNγ-specific rat anti-mouse antibodies were obtained as ascitic fluid of the PK136, GK1-5, 53.6.72 and R4-6A2 hybridomas (ATCC), respectively, in nude mice (Harlan). In all cases, antibodies were administered at 100 μl/dose at days −1/0, 7 and 14 (day 0 being the day of tumor inoculation) except for anti-NK1.1, which was admistered as 150 μl/dose at days 0, 2, 7, 11, 14, 17 and 21. Depletion of NK cells was also accomplished by the administration of a rabbit anti-asialo GM1 antiserum (Wako, Neuss, Germany) at days 0, 7 and 14 (Fig. 3*a*) or with a single dose per mouse at days 0, +4 or +8 as indicated in Figure 3*c*. MI-6 anti-NKG2D antibody[48] was purified from ascitic fluid by affinity chromatography in sepharose protein-G columns (Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer's instructions. For *in vivo* NKG2D-blocking experiments, 200 μg of purified MI-6 mAb or 200 μl of ascitic fluid were i.p. administered at day −1 and then every 3–4 days for 3 weeks. Hydrodynamic injections of a reported plasmid encoding mouse Flt3-L were given with a volume of 100 ml/kg using a 27-gauge needle at a rate of 0.4 ml/s as previously described.[49]

NK and CTL cultures and cytotoxicity assays

Activated NK cells used as effectors were prepared from C57BL/6 splenocytes cultured in the presence of 6,000 IU/ml human IL-2 (Chiron, CA) for 6 days before being used in cytotoxicity assays, as described.[50] Otherwise, they were magnetically purified from the spleens of mice treated with polyI:C (200 μg, i.p.) 18 hr before or injected with human IL-2 (2×10^5 IU/dose) at days −4 and −2. *In vivo* activated or resting C57BL/6 NK cells were positively selected from spleen cells by using DX5 mAb-coupled magnetic beads with the corresponding column system (AutoMACS, Miltenyi Biotec, Germany). CTL from OT-1 mice were obtained as described.[51] Briefly, splenocytes of OT-1 transgenic mice were stimulated *in vitro* with the synthetic SIINFEKL peptide for 5 days before being used as effectors. The ^{51}Cr release assays were performed using ^{51}Cr-labeled targets, as previously described.[52] EL-4 tumor targets were used as negative control for the killing (data not shown). When stated, purified MI-6 anti-NKG2D (eBioscience, San Diego, CA) and control Rat IgG Abs were used at a final concentration of 20 μg/ml and incubated with effector cells for 30 min before adding targets. Anti-H-2K^b/D^b-blocking antibody (28-8-6, BD Pharmingen) and control mouse IgG2a were used at 10 μg/ml. Target cells were preincubated for 30 min before being added to the effectors, and the same concentration of Ab was maintained during the assay. In some experiments, target cells were preincubated with 1,000 IU/ml of recombinant murine IFNγ (Peprotech, Rocky Hill, NJ) for 36–48 hr before being used for the ^{51}Cr release assay. For the *in vitro* killing experiment shown in Figure 5*a*, 4×10^5 GIPT^{high} cells were seeded in 6-well plates and 16 hr later C57BL/6-IL-2-activated NK cells from C57BL/6 mice were added at a 40:1 E:T ratio. Fluorescence microscopy pictures showing target progressive detachment and death were taken at the indicated time points.

Immunization against EGFP

To assess the immunogenicity of EGFP protein in C57BL/6 background, 6–8-week old females were immunized by different protocols with no or very little success. The computer programs developed by Parker *et al*. (http://bimas.dcrt.nih.gov/molbio/hla_bind) and F. Borras laboratory, the latter based on the data published by H.G. Rammensee, (http://www.syfpeithi.de/scripts/MHCServer.dll) were consulted for the selection of potential MHC-I-binding peptides. The 8 EGFP-encoded peptides with the

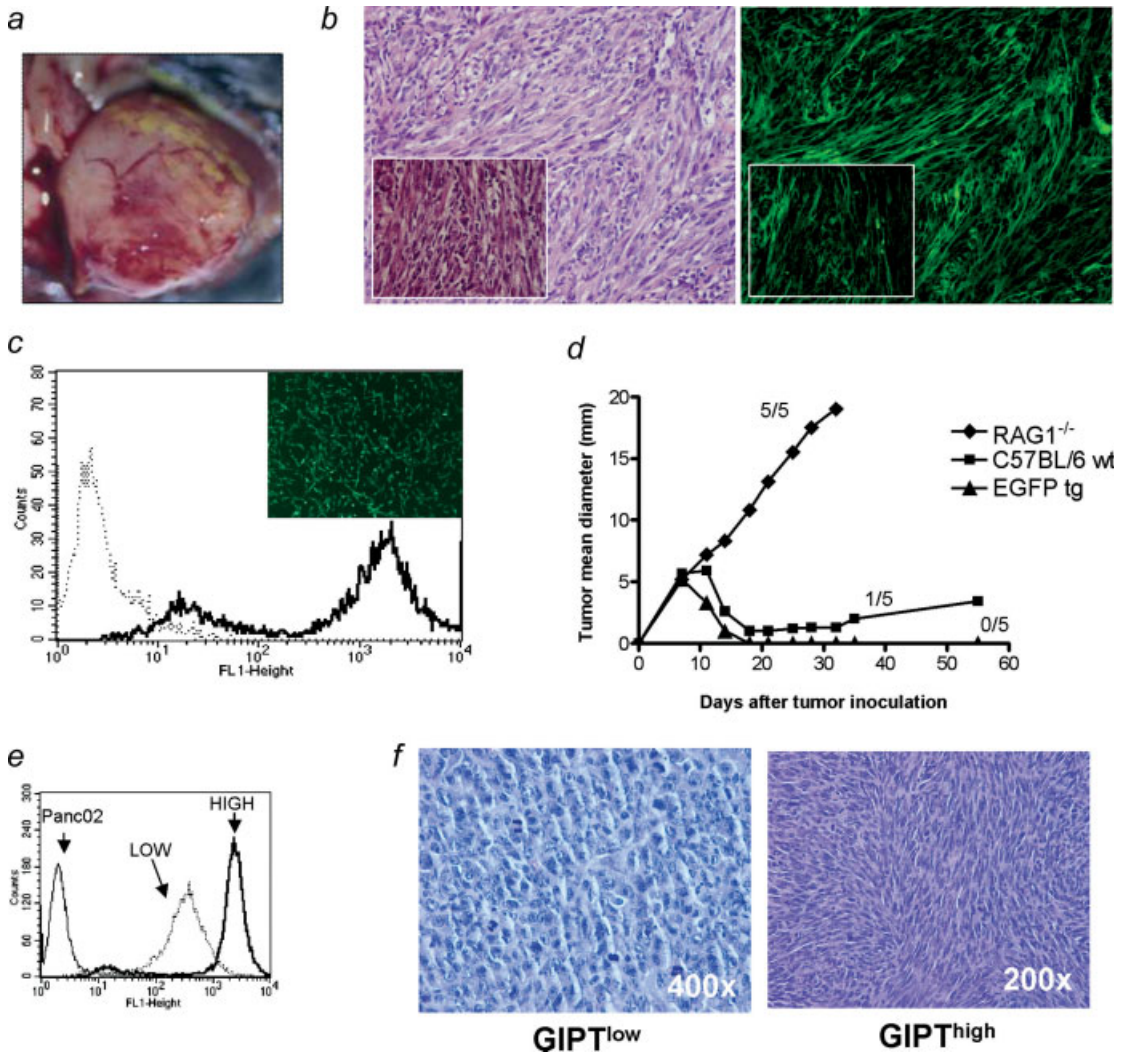

FIGURE 1 – 3-MCA induction of mesenchymal intraperitoneal tumors from EGFP transgenic mice in C57BL/6 background that are rejected in immunocompetent mice. (*a*) Picture of the dissected intraperitoneal cavity of an EGFP-transgenic female mouse showing a 2-cm diameter single tumor nodule. (*b*) Two magnifications of a microscopy field (×200 and ×400) from tumor samples under visible and UV light. (*c*) FACS analysis of a cell line named GIPT derived from the EGFP transgenic tumor showing high intensity of fluorescence (solid line) when compared with nonfluorescent Panc02 cells (dotted line). The inset shows the aspect of the cell line in culture by UV microscopy. (*d*) *In vivo* grafting of GIPT cells injected subcutaneously into the flank of the indicated mouse strains in pure C57BL/6 background, including EGFP transgenic mice, shown as the follow-up of the mean diameter of the tumor of 5 mice per group. The fraction on top of each line shows the relative number of mice developing progressive lethal tumors at the end of the experiment. This experiment is representative of 2 separately performed. (*e*) Limiting dilution of GIPT original explanted tumor-cell line rendered cells with different levels of expression of EGFP as detected by FACS, which were named $GIPT^{high}$ and $GIPT^{low}$. (*f*) H&E staining of sections from tumors derived from subcutaneous inoculation of $GIPT^{high}$ and $GIPT^{low}$ cells in $RAG1^{-/-}$ mice.

highest predicted binding affinities for H-$2D^b$ and K^b were synthetized as in Ref. 53. The selected peptides, (YNSHNVYIM, GVVPILVEL, DTLVNRIEL, AQASNSAVD, TAAGITLGM for D^b and EGDATYGKL, KLPVPWPTL, KFICTTGKL for K^b, respectively), were first evaluated for their ability to stabilize the expression of MHC-I molecules on the surface of TAP-deficient cell line RMA-S. The 3 peptides with the highest binding to RMA-S (GVVPILVEL, DTLVNRIEL, KLPVPWPTL) were used to immunize 3 C57BL/6 mice/peptide by s.c. injection at the base of the tail and footpads with a mixture containing 50 nmol of each peptide plus 50 nmol of helper peptide PADRE[54] emulsified in complete Freund's adjuvant. Fourteen days later, animals were sacrificed and their LN and spleens were removed. After 2 rounds of *in vitro* peptide restimulation, CTL activity was tested in a standard ^{51}Cr release assay using EL-4 pulsed with each peptide with no detectable cytotoxicity as a result. Other protocols of immunization (s.c. injection of mature/immature DC or total splenocytes obtained from EGFP transgenic mice, and recombinant EGFP protein) were also unsuccessful in producing an anti-EGFP response, as measured by cytotoxic activity or IFNγ production (data not shown).

Immunofluorescence and flow cytometry

Indirect and direct immunofluorescence and flow cytometry were performed as described.[55] The mAbs and reagents used were anti-CD107a-FITC, antiCD4-FITC, antiCD8-PE, antiNK1.1-PE, antiCD11c-APC, antiCD45.2-PerCP, antiIFNγ-PE, purified anti-mouse CD119 (IFNγR α chain), anti-DX5-biotin, anti-CD11c-biotin, anti-H-$2K_b$/H-$2D_b$-biotin and their respective isotype-

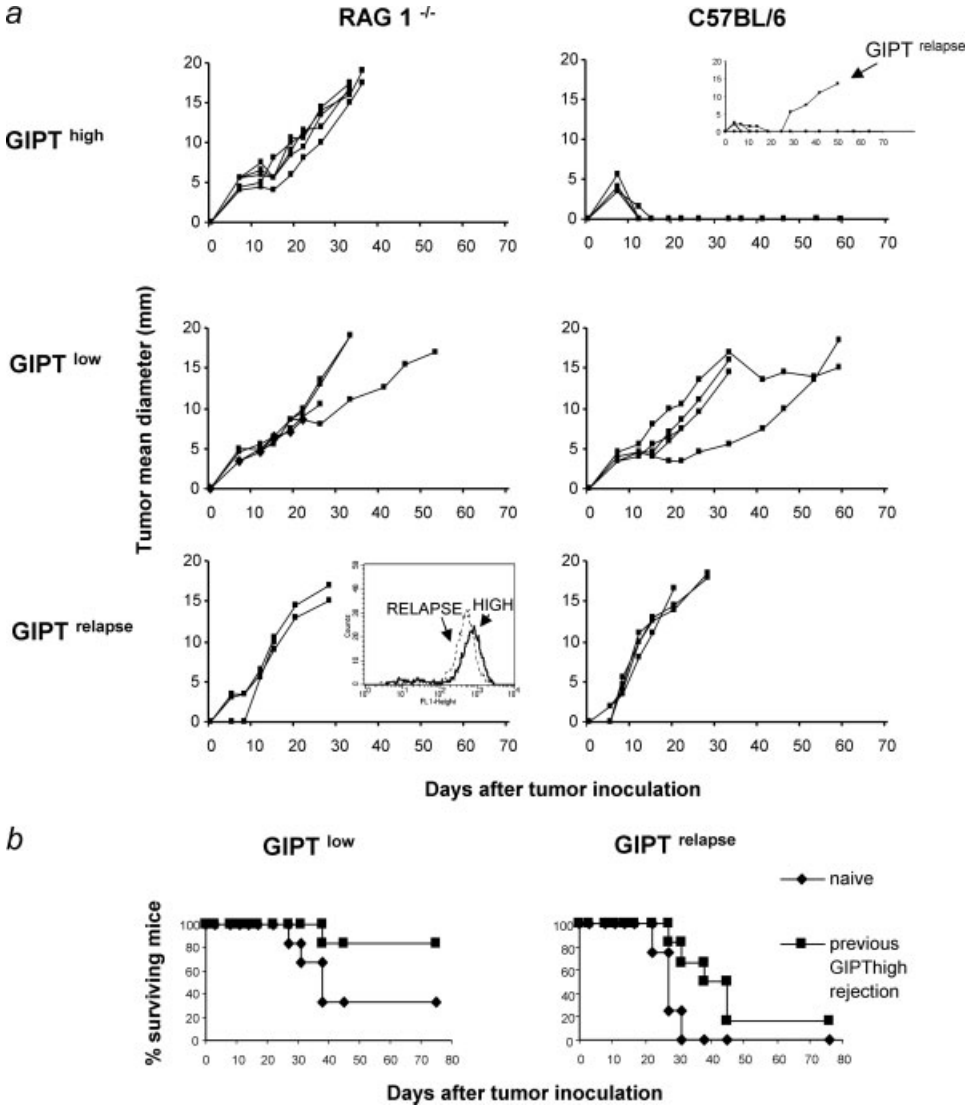

FIGURE 2 – Heterogeneous immunogenicity of clonal variants of the GIPT tumor. (*a*) Observation of the *in vivo* grafting of the GIPT clonal variants by s.c. injection of tumor cells in 5 mice/group that are individually represented. GIPT^{high} tumors progressed in RAG1$^{-/-}$ mice but were rejected in C57BL/6 after transient growth. The inset shows a tumor that relapsed in a parallel experiment with C57BL/6 mice after a 3-week control of its progression. GIPT^{low} cells progress both in RAG1$^{-/-}$ and in C57BL/6. A cell line, named GIPT^{relapse} was derived from the GIPT^{high} progressing tumor. GIPT^{relapse} showed similar fluorescence intensity to GIPT^{high} (inset) and gave rise to fast growing tumors in RAG1$^{-/-}$ ($n = 3$) and in wild type C57BL/6 ($n = 4$). Similar results were obtained in three independent experiments. (*b*) Survival of mice that had rejected GIPT^{high} tumors ~9 months prior to the experiment when they were subcutaneously challenged with GIPT^{low} ($n = 6$) and GIPT^{relapse} ($n = 6$), in comparison with gender and age matched control naïve mice ($n = 6$ and $n = 5$, respectively).

matched control antibodies (BD Biosciences, San Jose, CA); streptavidin-cy-chrome, -PE or -APC-cy7 (BD); anti-CD3-PE-cy7 and -APC, anti-NK1.1-biotin (eBioscience); anti-rat Ig-biotin (SouthernBiotech, Birmingham, AL), goat anti-rat IgG-FITC (Caltag, Burlingame, CA); anti-Rae1-APC and purified anti-Mult1 (R&D, Minneapolis, MN). For *ex vivo* tumor analysis, minced tumor fragments were digested at 37°C for 15 min with a mix containing collagenase D (Roche, Basel, Switzerland) 400 Mandl/ml and DNase I (Roche) 10 mg/ml, and passed through a 70-μm cell strainer. Blocking of Fc receptors was ensured by incubating cells with anti-CD16/32 mAb (BD). To perform FACS analysis on GIPT^{high} cells obtained from growing tumors in C57BL/6, and in analysis of tumor infiltrates, TOPRO-3 (Invitrogen) or 7-AAD (BD) were added to discard dead cells. Expression of H-2K^b/H-2D^b was assessed in the FL2 channel in TOPRO-3$^-$, CD45.2$^-$ and EGFP$^+$ cells. For detection of intracellular IFNγ in tumor infiltrating lymphocytes, CD45.2$^+$ cells were FACS-sorted from tumor infiltrates and were then incubated for 4 hr with Brefeldin-A (Sigma) in RPMI 10% FBS. After surface staining, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen). Finally, intracellular cytokine staining was performed. A FACScalibur flow cytometer and Cell Quest software, or a FACSAria and DiVa software (BD Biosciences), were used to collect and analyze the data.

GIPT^{high} cells detection in the LN

In order to follow tumor-cell migration to the lymphoid tissue, 5×10^5 GIPT^{high} cells were injected s.c. in the flanks and footpads of 3 C57BL/6 mice. After 7 hr, mice were sacrificed and their LN and spleens analyzed by flow cytometry. Tumor cells were easily detectable using forward and side-scatter electronic gating and taking advantage of their high FL-1 fluorescence intensity.

In vitro *production of IFNγ by NK and T cells*

In the experiments to determine IFNγ production by NK cells, GIPT^{high} cells were seeded at 3×10^4 cells/well in 48-well plates. After overnight incubation, 3×10^5 RAG1$^{-/-}$ splenocytes/well were added. Control wells received either targets or effectors only or C57BL/6 splenocytes with ConA 10 μg/ml (positive control). Recombinant human IL-2 at 0, 100, 250, 1000 and 6000 IU/ml and murine IL-12 (Peprotech) at 60 ng/ml were added to some wells, as indicated. All conditions were tested in triplicate. Forty-eight-hours supernatants were collected and analysed for IFNγ production using ELISA (BD PharMingen), according to the manufacturer's instructions. When testing IFNγ production by T cells, 2×10^5 irradiated GIPT^{high} cells and 8×10^5 splenocytes from mice that had rejected GIPT^{high} tumors or control naïve mice were plated per well in serum-free HL-1 medium (Biowhittaker,

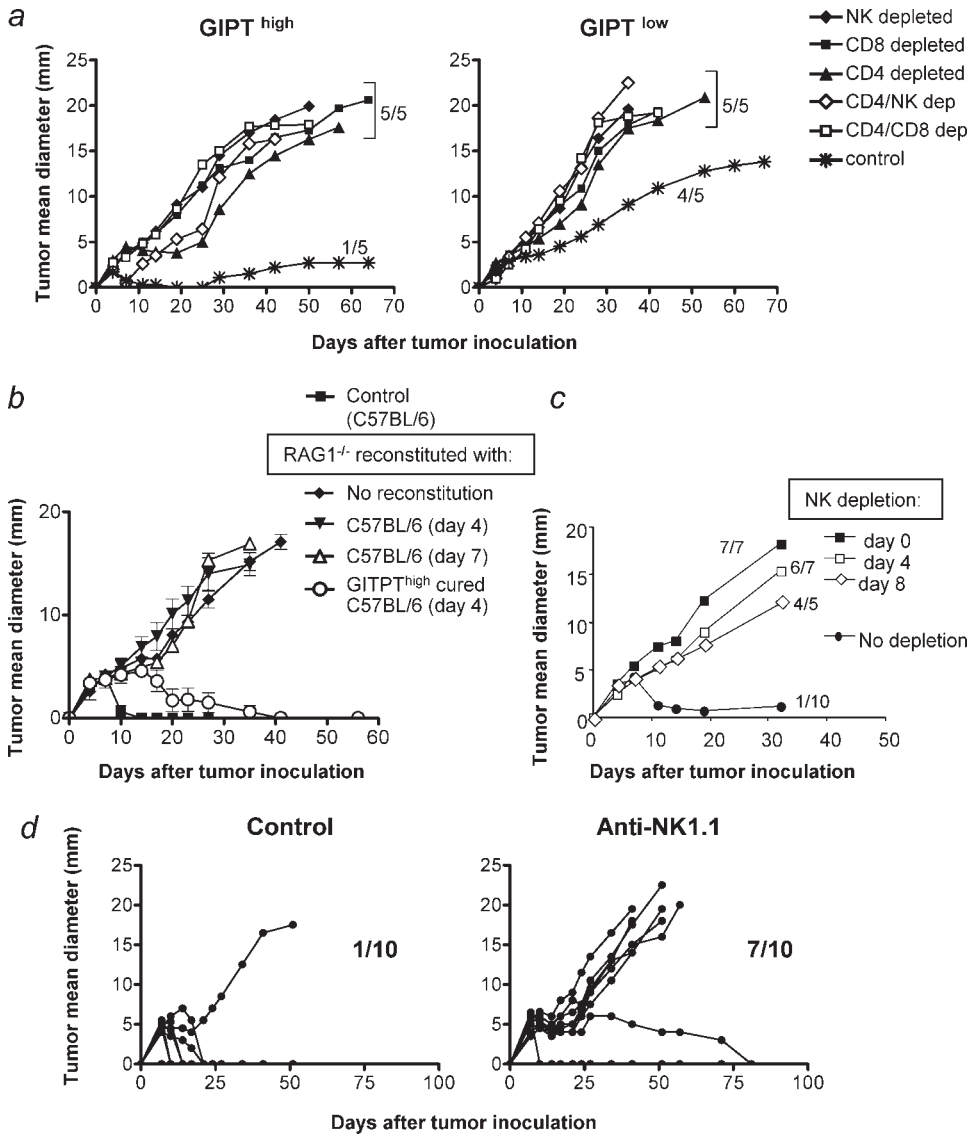

FIGURE 3 – Depletion of NK, CD4 or CD8 lymphocytes leads to tumor progression of GIPT tumors in immunocompetent syngeneic mice. (*a*) Depletions with anti-asialoGM1 antiserum, anti-CD8 mAb and anti-CD4 mAb or their combinations in mice injected with $GIPT^{high}$ (left) or $GIPT^{low}$ (right) cells. Data represent the mean diameter of the tumors with 5 mice/group. This experiment has been repeated 3 times with comparable results. Next to each graph the fraction of mice developing lethal tumors is depicted. (*b*) $GIPT^{high}$ tumor cells were inoculated in $RAG1^{-/-}$ mice and the mean diameter of 5 mice/group was followed. In some groups (triangles), mice received i.v. injections of 60×10^6 splenocytes from healthy C57BL/6 mice to reconstitute their immune system at the indicated time points. The group represented by empty circles received 60×10^6 spleen cells/mouse from donor C57BL/6 mice, which had rejected $GIPT^{high}$ tumors 1 month before becoming donors for adoptive transfer. (*c*) Effect of NK cell depletion at different time points (days 0, 4 and 8 after tumor-cell injection) in the rejection of $GIPT^{high}$ tumors in C57BL/6 mice. The fraction of mice developing progressive tumors is depicted for each group. Pooled data of 2 experiments separately performed are presented. (*d*) Individual follow-up of tumor mean diameters from mice injected s.c with 5×10^5 $GIPT^{high}$ cells that were subsequently injected with depleting anti-NK1.1 mAb admistered i.p. at days 0, 2, 7, 11, 14, 17 and 21 (right panel) compared to untreated control mice (left panel). The fraction of mice developing progressive lethal tumors is provided.

Walkersville, MD). Where indicated, those splenocytes were magnetically depleted of $CD4^+$, $CD8^+$ T cells or both, using mouse CD4 and CD8 magnetic beads (Miltenyi) and AutoMACS technology according to the manufacturer's instructions. In this case, 72-hr supernatants were collected.

NK degranulation and $EGFP^+$ material uptake by DC

For the NK *in vitro* degranulation and fluorescent material uptake by DC experiments, $2–3 \times 10^5$ $GIPT^{high}$ cells were seeded in 6-well plates. C57BL/6 splenocytes ($20–40 \times 10^6$) per well were added, and after 24-hr, NK/CD8 T-cell degranulation was measured as percentage of cells positive for the surface expression of CD107a granular membrane protein.[56] This experiment was read by electronically gating on the $DX5^+$ and $CD8^+$ populations. Similarly, uptake of fluorescent material by DC was detected at 24 hr as $EGFP^+$ cells inside the $CD11c^+$ gated cell population. In this case, splenocytes were obtained from mice injected with a plasmid encoding for human Flt3-L When working with purified populations (Fig. 7*b*), DC, NK and T cells were consecutively obtained from splenocytes of mice equally treated, using CD11c-, DX5- and CD90-specific microbeads (Miltenyi) and AutoMACS. Purity of fractions was checked by FACS. The remaining negative fraction was also kept. DC: $GIPT^{high}$ ratio was 5:1 and this experi-

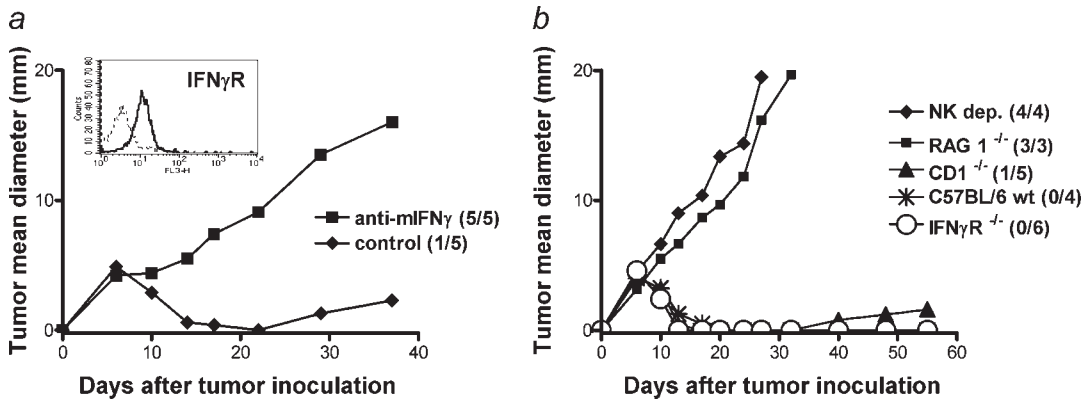

FIGURE 4 – IFNγ is required for GIPT^{high} rejection whereas expression of IFNγR by the host mouse or the presence of NKT cells are dispensable. (*a*) Tumor growth of GIPT^{high} tumors in C57BL/6 mice treated with a mAb neutralizing IFNγ or a control mAb. Inset: expression of IFNγR by GIPT^{high} cell line observed by FACS (solid line). Dashed line represents isotype matched control. (*b*) Tumor growth of GIPT^{high} cells in mice of the indicated mutated mouse strains in C57BL/6 background. For these experiments, the fraction of mice developing lethal tumors in each group is provided.

mental condition gave the maximum of EGFP intake by DC. The effect of the presence of NK and/or T cells was studied by adding cells of each population to the coculture (the same number as DCs) and matching the total number of cells/well with the negative fraction.

Statistical analysis

We analyzed the effect of CD4 and/or CD8 T cells depletion on IFNγ production by mice splenocytes by nonparametric Kruskal-Wallis test. Subsequent 2 by 2 comparisons were performed with Mann-Whitney *U* test with the correction of Bonferoni. Significant differences in tumor grafting were determined by the Fisher exact test. *P* values less than 0.05 were considered significant. All statistical analysis was carried out with SPSS v14 (SPSS).

Results

An EGFP$^+$ cell line from a methylcholantrene-induced mesenchymal tumor that is rejected in syngeneic mice

EGFP transgenic mice under the β-actin promoter express this protein in most if not all organs during development and adult life.[45] To generate EGFP$^+$ tumors, 15 of such mice in pure C57BL/6 background were surgically implanted with gauze plugs saturated with 3-MCA that were sewn to the peritoneum covering the pancreatic tissue. Seven tumors were excised from euthanized mice and explanted to give rise to continuously growing tumor cell lines. A tumor-cell line, named GIPT for green intraperitoneal tumor, was derived from the ball-shaped intraperitoneal tumor shown in Figure 1*a*, with histological features of fibrosarcoma and whose cells were brightly fluorescent under UV microscopy (Fig. 1*b*). Focal immunostaining with vimentin and lack of staining for cytokeratins and calretinin further supported the notion that GIPT tumor was indeed a fusocellular mesenchymal tumor (data not shown). Minced pieces of explanted tumor were seeded in tissue culture and gave rise to intensely fluorescent plastic-adherent cells that proliferated very rapidly (Fig. 1*c*). About 5×10^5 cells of this tumor-cell line were injected s.c. in 5 RAG1$^{-/-}$ mice that developed fast progressing tumors, whereas those tumors were rejected in every case (5 of 5) in EGFP transgenic mice and in 4 of 5 C57BL/6 mice, after a transient phase of palpable tumor nodules (Fig. 1*d*). These results indicate that GIPT cells were sufficiently immunogenic to be rejected after transient growth in syngeneic animals. Tumor rejections in ubiquitously expressing EGFP transgenic mice that should be tolerant to EGFP as an autologous antigen, indicated that EGFP is not the relevant rejection antigen.

Clonally heterogeneous behavior of GIPT cells with regard to immunogenicity that is unrelated to EGFP expression

In a series of grafting experiments, we noticed some heterogeneous behavior with some cases of tumor progression in immunocompetent mice. To homogenize the phenotype of the GIPT cell line, clones were obtained by limiting dilution and analyzed for EGFP expression. Several tumor subcultures were tested. Two clones with characteristically different fluorescence intensities were chosen and named GIPT^{high} and GIPT^{low} (Fig. 1*e*). The histopathological aspect of the malignancies derived from the inoculation of GIPT^{high} and GIPT^{low} was different. Although GIPT^{high} maintained the fusocellular organization in bundles, GIPT^{low} was much less differentiated showing a higher number of mitoses and more prominent vascularization (Fig. 1*f*).

GIPT^{high} cells showed rapid progression as s.c. solid tumors in RAG1$^{-/-}$ but were rejected after transient growth in C57BL/6 mice (Fig. 2*a*) as well as in most EGFP transgenic mice (data not shown). In contrast, GIPT^{low} progressed in immunocompetent and immunodeficient mice (Fig. 2*a*). An extensive series of experiments confirmed these results. In a minority of cases, GIPT^{high} cells were able to progress after 2–3-week delay in tumor growth in syngeneic mice (inset in Fig. 2*a* upper right). A cell line derived from one of such escape tumors was named GIPT^{relapse}. It showed similar fluorescence intensity when compared with the original GIPT^{high} cells (inset in Fig. 2*a* lower left), although it was not rejected at all in C57BL/6 (Fig. 2*a*) in spite of comparable levels of EGFP and MHC class I molecules (data not shown).

We wondered whether EGFP could be a relevant tumor rejection antigen. However, published evidence in the gene therapy literature clearly showed that EGFP was virtually invisible for CTLs in H-2^b mice,[57,58] while there was potential recognition by CTLs of a 9mer peptide presented by H-2K^d (HYLSTQSAL).[59] In addition, as detailed in Materials and methods section, we synthesized 8 peptides predicted by algorithms to fit motifs for the H-2K^b and H-2D^b molecules. All of these stabilized class I expression in RMA-S binding assays, but none was recognized by CTLs from mice that had rejected GIPT^{high} tumors (data not shown). Moreover, several attempts to immunize mice with peptides in adjuvant, pulsed onto DC, DC from EGFP transgenic mice or repeated injection of a recombinant adenovirus encoding EGFP produced negative results (Huarte *et al*., unpublished observations). These observations in conjunction with the fact that GIPT tumors showed similar grafting kinetics in EGFP transgenic mice (that should be tolerant for EGFP because of thymic expression of the transgene) (Fig. 1*d*), indicate that in fact, EGFP is not detected

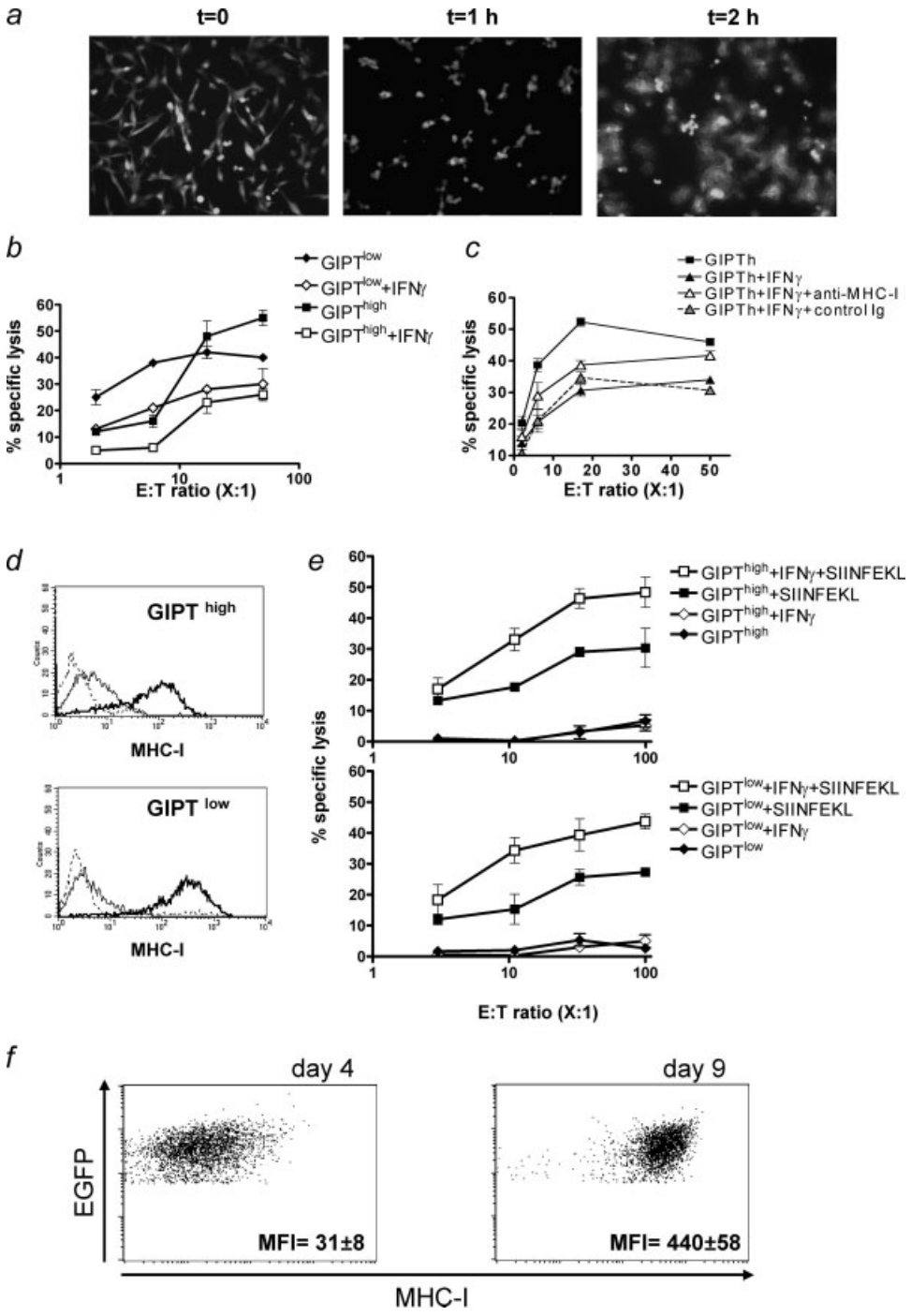

FIGURE 5 – GIPT tumor cells are highly sensitive to NK cell cytolytic activity *in vitro*. Exposure to IFNγ decreases sensitivity to NK lymphocytes but increases susceptibility to lysis by activated CTL via upregulation of surface MHC-I. (*a*) Inverted microscopy pictures (×200) under UV light of cocultures of IL-2 activated NK from C57BL/6 mice and GIPT^{high} cells (40:1 ratio) at the marked time points. (*b*) 5-hr standard ^{51}Cr release assay using as effectors IL-2-activated NK cells and GIPT^{high} or GIPT^{low} cells as targets. Empty symbols indicate that GIPT^{high} and GIPT^{low} cells were preincubated with murine rIFNγ 1000 IU/ml for 48 hr. (*c*) Experiment as in B performed with GIPT^{high} as target cells in which a blocking anti-MHC-I mAb or an irrelevant control antibody were preincubated with the target cells and maintained during the assay. (*d*) H-2K^b/ H-2D^b expression of GIPT^{high} and GIPT^{low} cells before (light line) and after (heavy line) 36 hr treatment with rIFNγ 1000 IU/ml. Isotype-matched control antibody is depicted in dashed line. (*e*) 5-hr ^{51}Cr release assay using as effectors specific CTL obtained from OT-1 transgenic mice and GIPT^{high} and GIPT^{low} pulsed and unpulsed with SIINFEKL peptide as targets. When indicated, GIPT^{high} and GIPT^{low} cells were preincubated with rIFNγ 1000 IU/ml for 36 hr. (*f*) FACS analysis of direct immunofluorescence staining with anti-H-2K^b/ H-2D^b gated on CD45$^-$ EGFP^{bright} cells on cell suspensions from excised GIPT^{high} tumors on days 4 and 9 after tumor-cell inoculation. The mean fluorescence intensity (MFI) ± SD of 4 mice/time point is provided within the dot plot.

as a tumor rejection antigen in this system. In addition, mice that had rejected GIPT^{high} showed no *in vivo* cytotoxicity against splenocytes from EGFP transgenic mice that express MHC class II, class Ia and Ib antigen presenting molecules (data not shown).

Nonetheless, other cellular antigens generated during mutagenic tumorigenesis could account for the immune-mediated rejections. These antigens were shown to induce some degree of memory. In fact, mice that had rejected GIPT^{high} tumors became immune to tumor challenge with GIPT^{low} cells in 80% of cases and showed a clear delay to lethal progression if challenged with the GIPT^{relapse} variant, indicating that the tumor-cell lines shared antigens recognizable by the immune system of these preimmunized mice but not by control naïve mice (Fig. 2*b*).

In addition, tumor-cell doses were important for immune rejection. A dose of 2×10^6 GIPT^{high} cells/animal gave rise to lethal tumors in approximately half of the mice, whereas all the mice developed lethal tumors when the dose was increased to 10×10^6 cells/animal (data not shown). This indicates that the observed immune rejection can be overiden by high numbers of malignant cells in the original inoculum.

As a whole, these data indicate that GIPT tumor growth *in vivo* is hampered by the immune system by means of recognition of antigens other than EGFP.

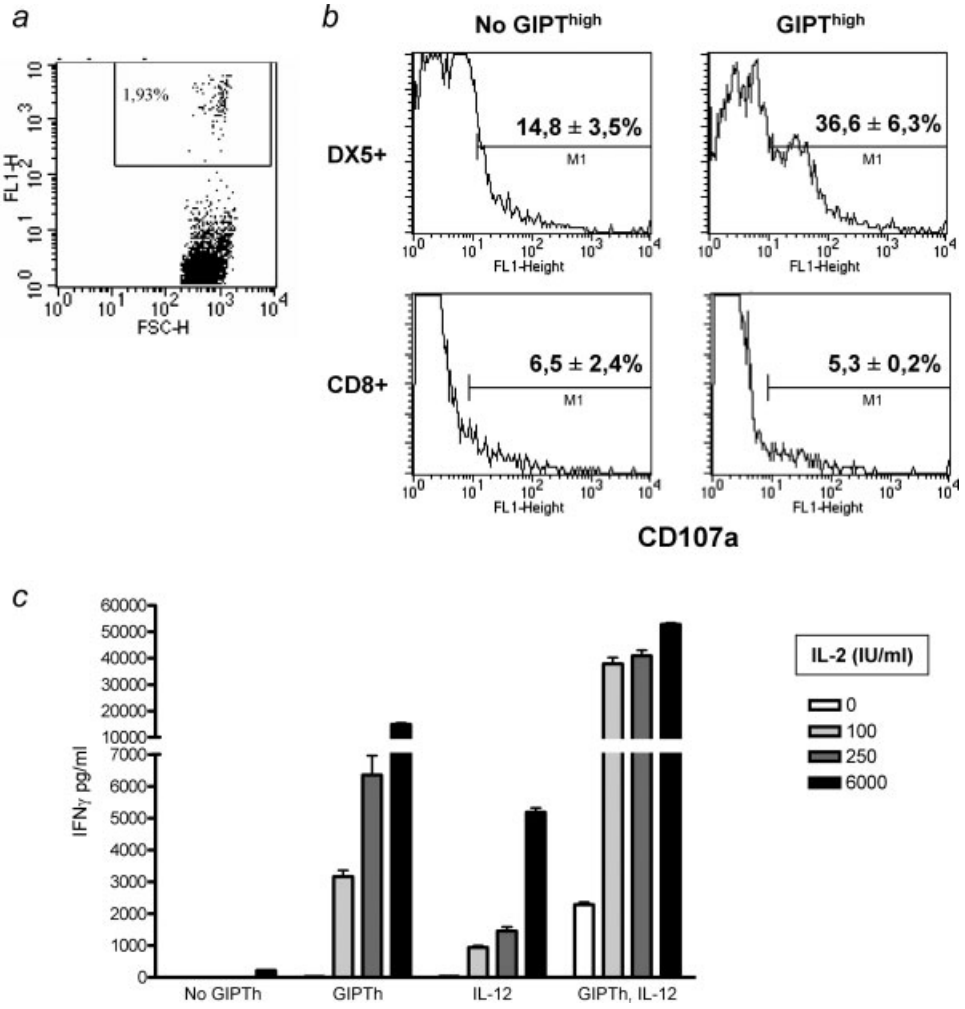

FIGURE 6 – GIPT tumor cells reach draining lymph node when injected as cell suspensions and induce degranulation and IFNγ production by splenic NK cells *in vitro*. (*a*) FACS analysis of cell suspensions from pooled inguinal LN of 3 mice injected s.c. into the 2 flanks with GIPT^{high} cells. The gated highly fluorescent cells are the GIPT^{high} cells detected in the LN. (*b*) Degranulation of spleen NK cells in 24-hr coculture with GIPT^{high} cells at a 10:1 ratio. NK were gated as DX5^{+} cells and degranulation inside that gate was measured by the acquisition of surface CD107a. The upper left histogram shows NK cells without GIPT^{high} cells and the upper right histogram shows NK in coculture with them. The lower panels show the lack of CD107a surface staining of the CD8^{+} cells gated on the same cultures. (*c*) IFNγ concentration in the 48 hr supernatants of cultures of RAG1$^{-/-}$ splenocytes with or without GIPT^{high} cells at a 10:1 ratio. When indicated in the figure, hIL-2 (0-6000 IU/ml) and/or mIL-12 (60 ng/ml) were added to the cells for the duration of the experiment.

GIPT tumors are rejected or delayed by the combined activities of NK, CD8^{+} T cells and CD4^{+} T cells

Selective depletion of lymphocyte subsets with antibodies was used to study the cellular requirements of the phenomena that lead to GIPT^{high} rejection and the delay in the *in vivo* growth of GIPT^{low} tumors. Mice depleted of NK cells with anti-asialo GM1 antiserum showed fast *in vivo* progressing courses of their tumor nodules both when injected subcutaneously with GIPT^{high} or GIPT^{low} cells. These experiments show that NK cells are critically involved in the process of rejection or in the growth retardation of GIPT^{low} (Fig. 3*a*). A similar effect was observed when CD4 or CD8 lymphocytes were eliminated *in vivo*. The rate of growth after T-lymphocyte depletion was as fast as that achieved by NK cell depletion indicating that depletion of NK cells did not leave behind any observable antitumor effects mediated by the innate immune system, as also indicated by combined depletions (Fig. 3*a*). Reconstitution of RAG1$^{-/-}$ mice, which have normal NK cells, with healthy C57BL/6 splenocytes was not able to restore rejection even if these "empty" mice were reconstituted as early as 4 days after tumor-cell injection, indicating that critical steps for the rejection process take place soon after tumor challenge. However, if reconstitution was carried out with spleen cells from C57BL/6 mice that had previously rejected GIPT^{high} cells, the tumors were rejected in 4 out of 4 cases (Fig. 3*b*). This set of results shows that the integrated function of NK and T cells is required for the rejection of this tumor model through mechanisms that needed an operational cellular immune system before the malignant tissue is established. However, as can be seen in Figure 3*c*, depletion of NK cells as late as 8 days after inoculation of GIPT^{high} cells resulted in tumor progression in 4/5 mice, which support a role for NK lymphocytes also at the effector phase of the immune response.

AsialoGM1 can be expressed on macrophages[60] and activated cytotoxic T lymphocytes[61]; therefore the conclusions of depletion with anti-asialoGM1 antisera are potentially misguiding. To further support the critical involvement of NK cells, depletions were induced by repeated injections of an anti-NK1.1 mAb that also prevented rejection in the majority of cases (Fig. 3*d*).

IFNγ is required for GIPT^{high} rejection acting on IFNγR on tumor cells but not on host mouse cells

A monoclonal antibody that neutralizes *in vivo* the bioactivity of IFNγ was able to completely eliminate the spontaneous rejection of GIPT^{high} tumors in C57BL/6 mice in all cases (Fig. 4*a*). Two potential nonmutually exclusive sites of action for this blocking antibody could be envisaged: the malignant cell shown to express IFNγR on its surface (Fig. 4a inset) or IFNγR as expressed on cells of the host mouse. Interestingly, GIPT^{high} tumor cells grafted onto IFNγR$^{-/-}$ mice failed to progress as they did in RAG1$^{-/-}$ and NK depleted mice (Fig. 4*b*). This observation showed that the critical site of IFNγ action was the tumor cell. However, *in vitro* culture of GIPT^{high} cells with up to 1000 IU/ml of IFNγ did not cause apoptosis or decrease the proliferation of the cell line (data not shown).

GIPT^{high} tumors were also completely rejected in CD1$^{-/-}$ mice that are deficient in NKT cells (Fig. 4*b*). Therefore CD1 as an antigen-presenting molecule and NKT cells are dispensable for the observed rejections, although they could still be performing immune activities that in their absence are taken over by NK cells or other subpopulations of T cells.

GIPT cells are sensitive to NK-mediated cytolysis in vitro*, but IFNγ decreases NK cell sensitivity through induction of MHC-I expression on tumor target cells*

IL-2-activated NK cells from the spleen of C57BL/6 mice can rapidly (in less than 2 hr) achieve destruction of fluorescent GIPT^{high} cells when seeded at 40:1 ratio in flat bottom culture dishes as observed by UV microscopy (Fig. 5*a*). These data were confirmed in standard 5 hr ^{51}Cr release assays that showed that IL-2-activated NK cells can lyse GIPT cells *in vitro* (Fig. 5*b*). In these assays, similar levels of tumor lysis were observed for GIPT^{high} and GIPT^{low} cells. However, IFNγ pretreatment of target cells clearly decreases sensitivity to cytolysis by activated NK cells (Figs. 5*b* and 5*c*). IFNγ readily induced MHC class I expression on GIPT^{high} and GIPT^{low} tumor cells (Fig. 5*d*) and these molecules are known to protect targets from NK lysis. Indeed, we could observe that the inhibition of NK cytolysis attained by incubation of the target cell with IFNγ can be partly reverted by a blocking mAb recognizing H2-K^b and H2-D^b (Fig. 5*c*).

In contrast, IFNγ-mediated increases of MHC-I expression augmented the sensitivity of GIPT^{high} and GIPT^{low} cells to lysis by antigen-specific CTLs. This observation was made by using activated TCR transgenic OT1 CD8$^+$ cells against GIPT tumors preincubated with the synthetic SIINFEKL cognate peptide presented by H-2K^b as a surrogate antigen (Fig. 5*e*). Our data show the ability of NK cells to destroy these tumors and the double-edged-sword effect of IFNγ: protecting target cells from NK lysis while at the same time sensitizing them for the attack of CTLs.

When explanting GIPT^{high}-derived tumors from C57BL/6 mice on days 4 and 9 after tumor-cell inoculation, it was observed that there was a marked increase in the surface levels of MHC class I molecules as assessed by immunofluorescence staining on gated viable EGFP^{bright} cells from the explants (Fig. 5*f*). This result shows that MHC class I upregulation also takes place *in vivo*.

Injected GIPT^{high} cells reach draining lymph nodes

When tumors are induced by inoculation of cell suspensions, some cells can find their way to local LN. The bright fluorescent nature of GIPT^{high} cells was ideally suited to track these events. In fact, GIPT^{high} cells reached inguinal draining LN when injected under the flank skin (Fig. 6*a*) or the popliteal LN when injected into the footpad (data not shown) within the first 7 hr. EGFP cells are no longer detectable in LN after 48 hr (data not shown). Early arrival of tumor cells to LN could potentially be involved in series of immune-promoting events.

GIPT^{high} cells induce degranulation and IFNγ secretion from NK cells

We found that DX5$^+$ NK cells obtained from lymphoid tissue degranulate upon interaction with GIPT^{high} cells, as detected by measuring the induction of surface expression of the granule molecule CD107a on NK cells, but not on CD8$^+$ T lymphocytes (Fig. 6*b*). Incubation of NK cells with GIPT^{high} cells induced the release of IFNγ in synergy with IL-2 and IL-12 (Fig. 6*c*). The effect of IL-2 and IL-12 on the GIPT^{high}-induced production of IFNγ by NK cells is relevant because conceivably these 2 cytokines are produced during a cellular immune response *in vivo*.

EGFP-transfer from GIPT^{high} cells to dendritic cells takes place in vitro *and* in vivo

We found in spleen cell cultures from mice enriched on DC by treatment with Flt3-L that the addition of GIPT^{high} cells resulted in some fluorescent transfer into the CD11c^{high} gated dendritic cells (Fig. 7*a*). It was conceivable that NK cells could play a role providing malignant cell debris for DC antigen upload. However, the addition of purified NK cells to cocultures of GIPT^{high} cells and purified splenic DC did not increase, or even slightly lessened, the transfer of fluorescent material into DC (Fig. 7*b*). As can be seen, addition of T cells to the cocultures did not increase EGFP transfer either. Moreover, CD11c^{high} DC present inside tumor

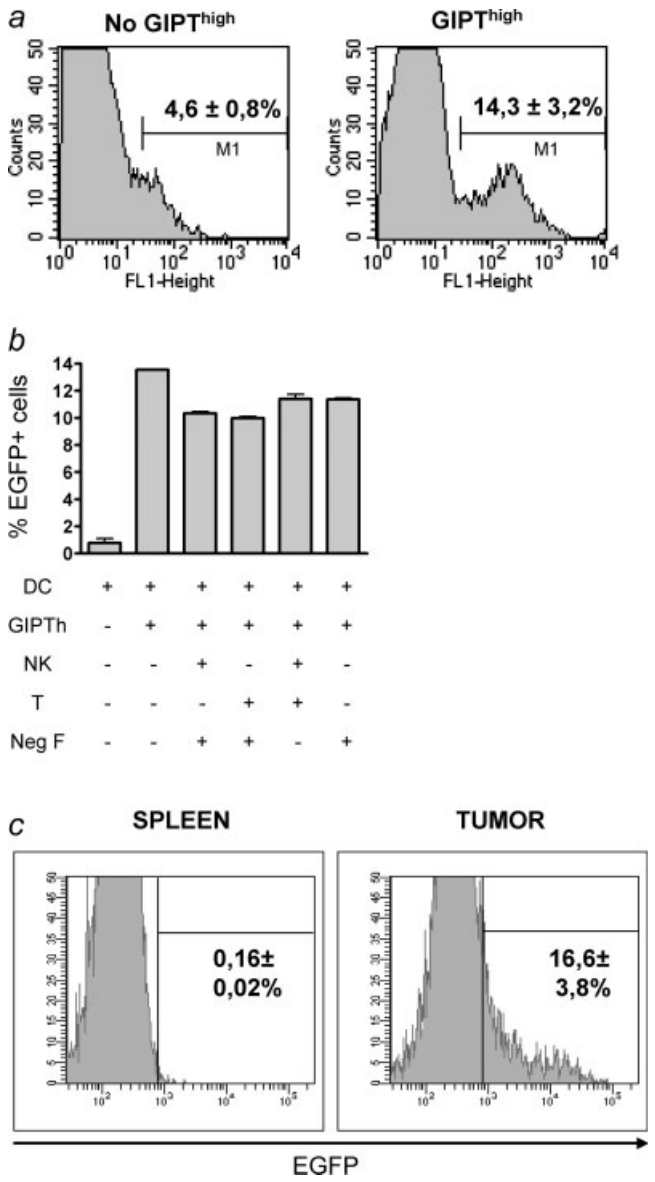

FIGURE 7 – Transfer of EGFP from tumor cells to DC takes place *in vitro* and *in vivo*. (*a*) FACS analysis of the green fluorescence in CD11c$^+$ gated cells from the spleen of mice that had been enriched in CD11c cells by hydrodynamic gene transduction of the liver with a plasmid encoding for mouse Flt3-L. Those splenocytes were cultured without (left) and with GIPT^{high} cells (right) for 24 hr before FACS data acquisition. (*b*) Quantitative analysis of DC associated fluorescence as in (*a*), in 24-h cocultures set-up adding immunomagnetically sorted CD11c^{high} (DC), DX5 (NK), CD90$^+$(T) and/or the remaining negative fraction from splenocytes taken from mice treated as in (*a*). Results are presented as the percentage of EGFP$^+$ cells within the CD11c^{high} gate in each coculture. (*c*) Percentage of green fluorescent DC analyzed on gated CD11^{high} cells in cell suspensions from spleen and tumor nodules of mice s.c. grafted with GIPT^{high} cells 8 days before. A representative histogram is shown and the mean (%) ± SD of 3 mice is provided for each case.

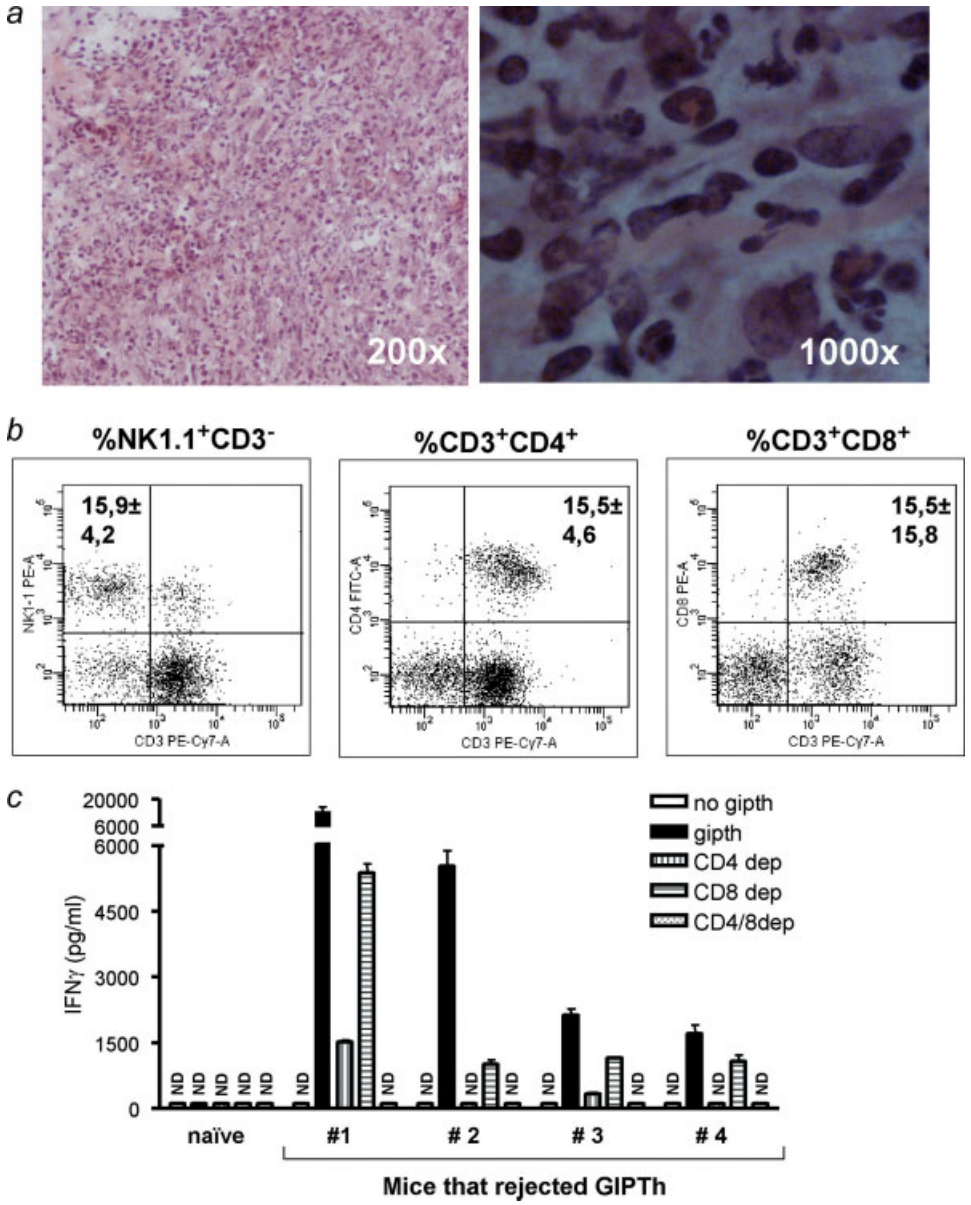

FIGURE 8 – Abundance of NK and T cells in the infiltrate of GIP-T^{high} tumors. (*a*) Light microscopy image of H&E-stained 10 day GIPT^{high} tumors showing a peritumoral mononuclear leukocyte infiltrate (left). Eosinophils were also dtected with higher magnification (right). (*b*) Immunofluorescence staining of GIPT^{high} tumors explanted at days 7–10. Tumor and dead cells (7-AAD-stained) were gated out. Lymphocytes were double-stained with combinations of anti-CD4-FITC, CD8-PE, NK1.1-PE and CD3-PE-CY7 as indicated. Percentages of NK1.1$^+$CD3$^-$, CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ cells given as the mean ± SD of 8 tumor infiltrates and a representative dot plot for each population is shown. (*c*) IFNγ concentration in the culture supernatants of 8×10^5 spleen cells and 2×10^5 irradiated GIP-T^{high} cells per well (each condition was tested in triplicate). Data represent mean ± SD from individual mice that had rejected GIPT^{high} tumors 2–5 months prior to the experiment. A control naÿve mouse is also shown. Where indicated CD4 or/and CD8 cells were immunomagnetically depleted from total splenocytes. Differences between depleted and nondepleted conditions were found significant ($p < 0.05$) according to Mann-Whitney *U* test. ND, not detectable.

nodules developed in syngeneic mice showed uptake of fluorescent material, indicating that this phenomenon also takes place *in vivo* (Fig. 7*c*). Under these conditions, fluorescent material is not found inside DC from draining lymph nodes or spleen (Fig. 7*c* and data not shown). Furthermore, *in vivo* NK depletion either with anti-asialoGM1 or anti-NK1.1 did not significantly affect the intake of fluorescent material by DC inside the tumor (data not shown).

Active NK and T cells in the tumor rejecting infiltrate: recognition of tumor cells by CD4 and CD8 T lymphocytes

The involvement of NK cells at the effector phase of tumor rejections was suspected from the data on NK depletion performed 4 and 8 days after tumor inoculation (Fig. 3*c*). GIPT^{high} tumors approximately on day 7 start to lose volume. When explanted, they show a peritumoral infiltrate mainly composed of mononuclear cells with scattered eosinophils (Fig. 8*a*). Disaggregated tumor tissue revealed the presence of CD3$^-$ NK1.1$^+$ NK cells as well as CD3$^+$CD8α$^+$ T cells (Fig. 8*b*) and CD3$^+$CD4$^+$ T cells. A minority (2.5% ± 1.14%) of CD11c^{high} DC (Fig. 7*c*) and NKT cells (Fig. 8*b*) was also observed. Recognition of tumor antigens *in vitro* by both CD8$^+$ and CD4$^+$ T cells was documented by assessing the production of IFNγ by splenocytes from mice that had rejected GIPT^{high} tumors incubated with irradiated GIPT^{high} cells (Fig. 8*c*). Depletion of either CD4 or CD8 cells from splenocytes decreased IFNγ production while simultaneous depletion completely abolished the specific IFNγ output into the culture supernatant. Naïve mice were used as a control. Similar results were obtained by adding blocking anti-CD4 or anti-CD8 mAbs to the cultures (data not shown). In spite of the contribution of CD8$^+$ T cells to tumor rejection and IFNγ production, our extensive attempts to in vitro restimulate tumor-specific CTLs active in chromium release assays from mice that had rejected GIPT^{high} tumors rendered negative results (data not shown).

To address whether NK and T cells become activated *in vivo* as to produce IFNγ, experiments of intracelullar staining of this cytokine were performed on leukocytes isolated from GIPT^{high} tumors explanted on day 8 after tumor-cell inoculation. As shown in Figure 9, both NK (CD3$^-$ NK1.1$^+$) and T (CD3$^+$ NK1.1$^-$) cells were synthesizing IFNγ without any need for *in vitro* restimulation, while their splenic counterparts were not. The percentage of

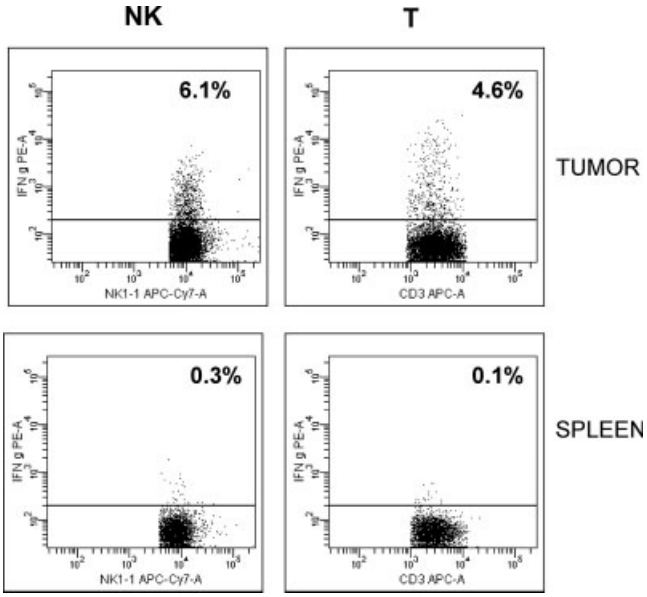

FIGURE 9 – Synthesis of IFNγ by NK and T cells in the tumor rejecting infiltrates. FACS analysis of intracellular IFNγ in cell suspensions obtained from the tumor lesions or the spleen of mice s.c. inoculated with GIPT^{high} cells 8 days before. Data are from pooled tumor nodules from 3 mice compared to spleens. Percentage of positive cells is provided inside each dot plot in which NK cells were gated as CD3$^-$NK1.1$^+$ and T cells as CD3$^+$NK1.1$^-$ lymphocytes.

IFNγ$^+$ cells was comparable in the spleens of tumor-bearing and naïve mice (data not shown).

NKG2D is involved in the recognition of GIPT tumor variants by NK cells

GIPT cells express the NKG2D-ligands Rae1 and Mult1 on their plasma membrane (Fig. 10*a*). In a series of experiments to ascertain the NK-activation receptor involved in triggering GIPT cytolysis by NK cells, it was found that anti-NKG2D-blocking mAb almost completely inhibited the lysis of GIPT^{high}, GIPT^{relapse} and GIPT^{low} cells by DX5$^+$ purified splenic NK cells from naïve C57BL/6 mice (Fig. 10*b*). DX5$^+$ NK cell killing of GIPT cells from mice that had been treated i.p with two doses of rhIL-2 (2×10^5 IU/dose) or polyI:C 200 μg to upregulate NK functions, was also inhibited by anti-NKG2D-blocking mAb. As predicted by the NKG2D-ligand expression data (Fig. 10*a*), GIPT^{high}, GIPT^{relapse} and GIPT^{low} were similarly susceptible to NKG2D-mediated cytolysis and therefore the different tumor outcomes *in vivo* cannot be attributed to this feature.

In vivo blockade with high doses of the MI-6 NKG2D-blocking mAb led to GIPT^{high} tumor progression in 5 of 11 mice in comparison with only 1 of 17 in the control group (Fig. 8*c*). These data reflect the contribution of NKG2D in the tumor rejections.

Discussion

The described experimentation definitively shows the absolute need for distinct nonoverlapping roles played by NK and T cells in order to achieve an orchestrated immune rejection of a particular transplanted tumor. These conclusions are drawn from a series of observations made from a transplantable tumor-cell line established from an intraperitoneal mesenchymal tumor chemically induced in transgenic mice ubiquitously expressing the EGFP reporter gene. Despite the lack of use of EGFP as a model tumor antigen, EGFP present at high levels permits selective tracking of fluorescent cells both for *in vivo* and *in vitro* experimentation, and offers a clear utility for these novel cell line. As opposed to stable EGFP transfectants, these tumor cells offer a series of clear advantages. They have undergone tumorigenesis in the presence of the transgene, they obviate the need for foreign enzyme genes for drug selection and they lack the propensity to change levels of transgene expression upon *in vitro* or *in vivo* passage. The 7 GIPT tumors induced were of mesenchymal origin rather than epithelial tumors of the pancreas. This is due to the fibrosis around the 3-MCA plugs as shown with other 3-MCA-intraabdominal tumors[44] and in subcutaneous 3-MCA-induced sarcomas. Local fibrosis exposes mesenchymal cells to the oncogenic mutagenesis of 3-MCA.[62]

GIPT tumor cells express EGFP, but contrary to our original expectations, EGFP proved to be invisible for T cells with the set of antigen-presenting molecules available in the H-2^b haplotype. This has been described in the literature,[57,58] and confirmed by our own repeated failure to induce anti-EGFP T cells in C57BL/6 mice. Further support came from rejections observed in EGFP transgenic mice that ought to be tolerant for EGFP as a self-antigen.[45] EGFP is a protein originally cloned from an evolutionary distant jellyfish without homology to mouse proteins. This could be taken as an example of potentially ignored heavily mutated tumor antigens if there is lack of presentation by the proper set of antigen-presenting molecules of the MHC, as previously suggested for viral antigens in human cancer.[63]

Spontaneous rejection of GIPT^{high} tumors raised questions about the cellular requirements to destroy the transiently grafted tumor nodules that progressed in T and B cell deficient RAG1$^{-/-}$ mice. It was found that there was an absolute requirement of NK cells, CTL precursors and T helper cells but not NKT cells. All these lymphocyte subsets have been involved in antitumor immune surveillance[16] and in the immunotherapy against experimental malignancies. However, this report shows that tumor rejection is only achieved if the 3 lymphocyte subsets are operating together either sequentially or simultaneously. Dissecting out the train of events leading to immune rejection, we found that GIPT cells were highly sensitive to NK lysis *in vitro*, a phenomenon that was triggered in most part through NKG2D-mediated activation upon interaction with NKG2D ligands expressed on the surface of GIPT cells. NKG2D signaling through DAP-10 is known to be involved in governing cytotoxicity and cytokine production by NK cells[18] that can under certain conditions override the inhibition posed by Ly49 inhibitory receptors.[64] Transfected NKG2D ligands at sufficient level of expression are capable of inducing tumor rejection and effective vaccination as a result of systemic T-cell immunity against tumor cells not transfected with those ligands.[19] Our observations of immune memory from tumor-rejecting mice support this point.

NK involvement in the final phase of tumor debulking can be inferred from the presence of NK cells in the rejecting infiltrates. Altogether our data indicate a dual role for NK cells that appear to be key players in the induction and execution of the rejecting immune response at least in this tumor model. Recent direct evidence for a role of NKG2D in immunosurveillance has been generated in mice treated with chemical carcinogens while under chronic treatment with an anti-NKG2D neutralizing monoclonal antibody.[17] Our results further extend the importance of the NKG2D pathway to cell lines naturally expressing ligands of NKG2D. It should be taken into consideration that NKG2D is also expressed on T cells and this pathway may further activate CD8$^+$ T cells involved in the observed tumor rejections.[65] Both NK and T cells infiltrating the tumor lesion could be detected producing IFNγ, which indicated their state of effector activation. IFNγ was shown to be necessary for immune rejection by operating on tumor cells as deduced from experiments in IFNγR$^{-/-}$ mice and in mice treated with anti-IFNγ-blocking mAbs. NK cells can be early sources of IFNγ[43] upon encounter with GIPT cells if in the proper milieu of cytokines: the induction of IFNγ on NK cells by GIPT^{high} cells is clearly costimulated by IL-12 and IL-2 acting in a concerted fashion. However, IFNγ by itself is not toxic for

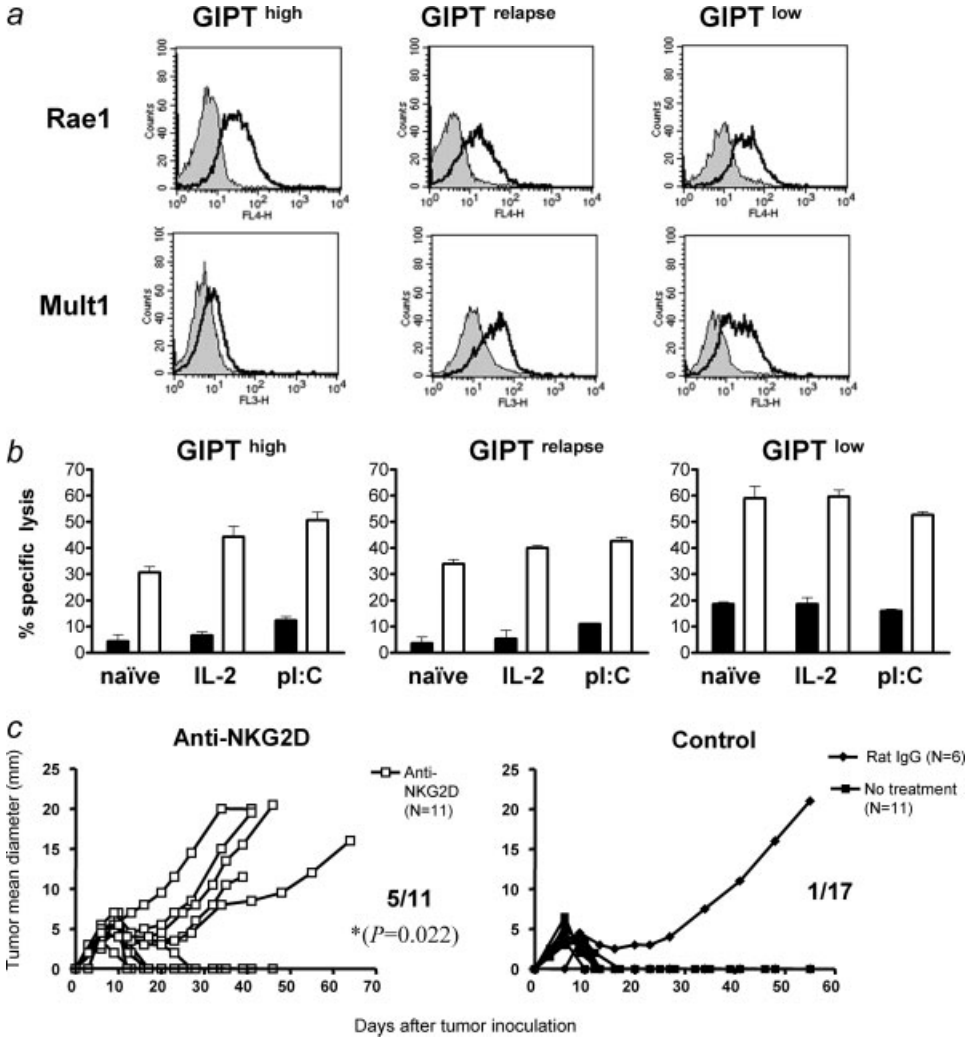

FIGURE 10 – NKG2D role in NK cell killing of GIPT^{high} cells and in tumor rejection. (*a*) FACS analysis histograms showing surface expression of the NKG2D ligands Rae1 and Mult1 on the 3 GIPT clonal variants. (*b*) 5-hr ^{51}Cr release assays with DX5 magnetic bead-purified NK cells from the spleens of C57BL/6 mice either naive or preinjected with of IL-2 or PolyI:C as indicated. NK cells were confronted with GIPT^{high}, GIPT^{relapse} or GIPT^{low} cells at 100:1 effector:-target ratio, in the presence of control antibody (white bars) or a blocking mAb against NKG2D (MI-6) at 20 μg/ml (black bars). (*c*) Individual tumor diameter follow-up of mice not treated ($N = 11$) or treated with control rat IgG ($N = 6$) or MI-6 mAb ($N = 11$), on day -1 before GIPT^{high} tumor inoculation and after that every 3–4 days during 3 weeks. The relative number of mice with progressing lethal tumors is shown as a fraction in the graph ($p = 0.022$ using a Fisher exact test).

GIPT^{high} cells in culture. This suggests that IFNγ acting on tumor cells elicits indirect mechanisms of immune destruction that are operational in the *in vivo* setting. The role of NK cells in initiating and shaping the early immune response has been shown by Martin-Fontecha *et al.*,[43] who clearly demonstrated an involvement of NK cells in the differentiation of Th0 to Th1 cells as well as in the dramatic increase in the number of lymphocytes inside the responding lymphoid tissue.[66] IFNγ secreted by NK cells is at least one of the factors involved in those effects.[66] In our system, the IFNγR is needed on tumor cells but not on cells of the host mouse. Our data are fully consistent with the proposed role of IFNγ in immunosurveillance, shown to operate on the nascent malignant cells,[67] but not on the immune system cells.

Taking advantage of the fluorescence of GIPT cells, we show that DC acquire fluorescent material from GIPT cells both *in vivo* and *in vitro*. Surprisingly, NK killing of tumor cells was dispensable for this *in vitro* transfer of biological material from tumor cells to splenic DC, as previously shown by other authors with human DC and viable tumor cells.[68] In this study, we show that CD11c^{high} cells can be detected at the tumor infiltrate carrying EGFP fluorescence uptaken from GIPT^{high} cells. Furthermore, *in vivo* depletion of NK cells did not impair the uptake of EGFP by DC, thus confirming that generation of tumor-cell debris by NK-mediated cytotoxicity was not required for these DC loading events (data not shown). The intense fluorescence of tumor cells was also exploited to demonstrate that GIPT^{high} cells reach LN soon after inoculation of tumor cells suspensions as previously described for other tumor cells.[40]

The final execution of tumor rejection, at least in syngeneic mice, is most likely mediated by T cells and NK cells, which are also located in the inflammatory infiltrate of the about-to-be rejected tumor. The contribution of NK cells is clearly needed during the effector phase, since NK depletion as late as on day 8 results in lethal tumor growth. Resting NK cells are capable to degranulate *in vitro* in the presence of GIPT^{high} cells while CD8$^+$ cells are not. This suggests an involvement of NK cells at early points of time in the orchestration of tumor rejection. The *in vivo* role of CD8$^+$ CTL could include direct tumor-cell destruction enhanced by IFNγ that upregulates MHC-I on tumor cells. The role of NK cells in favoring the priming of CTLs has been previously observed *in vitro*.[31] CD4$^+$ cells could act as helpers for CD8 immunity but could also be important for inflicting local damage on the malignant tissue when activated by stromal cells expressing MHC class II,[30] as recently observed in other tumor models.[69] In addition, we can detect eosinophils at the tumor infiltrates and their role is being currently explored, since implication of eosinophils in tumor rejections has been described for tumors undergoing interleukin-4-based immunotherapy.[70]

This study supports the notion that *in vivo* rejection of mesenchymal tumors requires the combined action of NK and T cells

and is dependent on NKG2D ligand recognition and production of IFNγ. The use of NK cells in cancer immunotherapy has received a boost as a result of studies into haploidentical bone marrow transplantation[71] and adoptive transfer of activated NK cells.[72] Knowledge showing direct involvement of NK cells in orchestrating the induction of a curative immune response in our system offers grounds for protocols of gene transfection of NKG2D-ligands before adoptively infusing NK cells. NK cells operating by their direct activity on tumor cells, their crosstalk with DC and/or their interplay with T lymphocytes all need to be considered when designing immunotherapy strategies for cancer.

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