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Effect Of Human Natural Killer and γδ T Cells on the Growth of Human Autologous Melanoma Xenografts in SCID Mice

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ABSTRACT

Natural killer (NK) cells were first identified for their ability to kill tumor cells of different origin *in vitro*. Similarly, γδ T lymphocytes display strong cytotoxic activity against various tumor cell lines. However, the ability of both the NK and γδ cells to mediate natural immune response against human malignant tumors *in vivo* is still poorly defined. Severe combined immunodeficient (SCID) mice have been successfully engrafted with human tumors. In this study, the antitumor effect of local as well as of systemic treatments based on NK cells or Vδ1 or Vδ2 γ/δ T lymphocytes against autologous melanoma cells was investigated *in vivo*. The results show that all three of the populations were effective in preventing growth of autologous human melanomas when both tumor and lymphoid cells were s.c. inoculated at the same site. However, when lymphoid cells were infused i.v., only NK cells and Vδ1 γ/δ T lymphocytes could either prevent or inhibit the s.c. growth of autologous melanoma. Accordingly, both NK cells and Vδ1 γδ T lymphocytes could be detected at the s.c. tumor site. In contrast, Vδ2 γδ T lymphocytes were only detectable in the spleen of the SCID mice. Moreover, NK cells maintained their inhibitory effect on tumor growth even after discontinuation of the treatment. Indeed they were present at the tumor site for a longer period.

These data support the possibility to exploit NK cells and Vδ1 γδ T lymphocytes in tumor immunotherapy. Moreover, our study emphasizes the usefulness of human tumor/SCID mouse models for preclinical evaluation of immunotherapy protocols against human tumors.

INTRODUCTION

Both natural killer (NK) and γ/δ T cells display strong cytotoxic activity against various tumor cell lines (1–3). How NK cells can discriminate between normal and tumor cells has now been clarified thanks to the discovery of the surface receptors and the molecular mechanisms involved in their function. It is now clear that the effector function of NK cells is regulated by a balance between opposite signals delivered by the MHC class I-specific receptors and by the activating receptors responsible for NK cell triggering. The existence of different MHC-class I-specific inhibitory receptors allowed for the explanation of why NK cells spare normal cells, whereas they kill cells that have lost the expression of MHC class I molecules as a consequence of tumor transformation (4, 5). More recently, the triggering receptors responsible for positive NK cell stimulation have been identified (6, 7). The receptors mainly involved in the tumor cell lysis are represented by the NKp46, NKp30, and NKp44 (collectively called natural cytotoxicity receptors; NCRs), all belonging to the immunoglobulin superfamily, and by the C-type lectin molecule NKG2D (7–9). The expression of NCRs is confined to NK cells, whereas NKG2D is expressed also in CD8+ α/β T lymphocytes and in γ/δ T lymphocytes. Whereas NKG2D is homogeneously expressed on these cell types, the surface density of the three NCRs is characterized to be coordinately varying in different individuals and also in NK cells isolated from a given individual (7). The cellular ligands recognized by NCR are still undefined, whereas the NKG2D ligands are represented by the stress-inducible MHC class I-related chains A and B and by the glycosylphosphatidyl inositol-linked proteins UL16-binding proteins (8, 10, 11). The involvement of NKG2D in NK cell-mediated cytotoxicity strictly correlates with the expression and the surface density of MHC class I-related chain A and UL16-binding protein on tumors (9, 12).

The majority of human peripheral γδ T cells belong to the Vγ9/Vδ2 subset, whereas a minor subset expresses Vδ1 paired with different Vγ genes (13, 14). Vδ1-expressing T cells, on the contrary, are more frequent in intestinal epithelium and other epithelial sites, thymus, and cord blood (15). Engagement of NKG2D provides a costimulatory signal for Vγ9/Vδ2 γδ T cells, allowing amplification of TCR-mediated triggering on recognition of undefined ligand(s) on tumor cells (16). On the other hand, in some Vδ1 γδ T cells, MHC class I-related chain molecule seems to deliver both the T-cell receptor-dependent signal 1 and the NKG2D-dependent costimulatory signal 2 (17).

Various experimental evidences indicate that NK cells play a role in the control of tumor growth and metastasis dissemination (18, 19). Both the activation of endogenous NK cells in a tumor-bearing host and the adoptive transfer of *ex vivo* activated NK cells may be therapeutically beneficial. The few Phase I/II clinical trials with activated NK cells performed in cancer patients do not yet allow any firm conclusion, except to ascertain the feasibility and the lack of toxicity of this therapy (20, 21). However, the clinical trials aimed either at the activation of NK cells or at the direct treatment with activated NK cells have been largely confined to patients with advanced metastatic disease. In these cases, tumor-induced immunosuppression may play a crucial role in neutralizing the potential benefits of the immune therapy (20, 22). Thus, an improved understanding of NK cell biology in cancer patients may help to design novel clinical trials based on new approaches using adoptive transfer of NK cells for cancer therapy. In this context, a recent major breakthrough highlighted the possible role of NK cells in the cure of acute myeloid leukemias. In patients undergoing haploidentical hemopoietic transplantation the occurrence of KIR-HLA-class I mismatched ("alloreactive") NK cells had important consequences in the clinical outcome, preventing severe complications such as leukemic relapses, graft *versus* host disease, and graft rejection (23, 24).

Although γδ T cells have been reported to display a role in the immunosurveillance against certain tumor cells (3, 25), their ability to control tumors *in vivo* is still controversial. However, recent work emphasized the role of Vδ1 γδ T cells in tumor cell recognition (12, 26–28).

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Mice lacking the adaptive immune response have largely been used as models to investigate the possible *in vivo* effects of adoptive immunotherapy against human cancer. Because the first report indicating that severe combined immunodeficient (SCID) mice could be successfully engrafted with human tumors, this model has been widely applied (29). Using this model, various potentially effective immunotherapeutic approaches against human tumors have been tested (29). In particular, reports would suggest that the therapeutic efficacy of NK cells can be assessed in SCID mice transplanted s.c. with human tumors (30–34). On the other hand, little information is available on the efficacy of the adoptive immunotherapy in these models using *in vitro* established human γδ T lymphocytes (35, 36).

In this study, using the SCID mice model, we analyzed the antitumor effect of local or systemic treatment with human NK or Vδ1 or Vδ2 γδ T lymphocyte populations against autologous melanoma cells. The data indicate that all three of the cell subpopulations were effective in preventing the growth when coinoculated s.c. with autologous human melanomas (referred to as Winn assay; Ref. 37). On the other hand, by infusing lymphoid populations i.v., only NK cells and Vδ1 γδ T lymphocytes were able to prevent or inhibit the s.c. growth of autologous melanoma. These data strongly suggest that both NK cells and Vδ1 γδ T lymphocytes should be exploited in adoptive immunotherapy in cancer patients. Moreover, our study emphasizes the usefulness of the human tumor/SCID mouse models for the preclinical evaluation of immunotherapy protocols against human tumors.

MATERIALS AND METHODS

Animals and Human Cells. CB.17 SCID/SCID female mice (Harlan, S. Pietro al Natisone, Italy) were used at 4–5 weeks of age and were kept under specific pathogen-free conditions. SCID mice were housed in microisolator cages and all of the food, water, and bedding were autoclaved before use.

The tumor cells were represented by the human melanoma cell line MEL15392 derived from a metastatic tumor lesion (38) cultured and expanded in RPMI 1640 plus 10% FCS. This melanoma cell line, also termed MEL15, had been already analyzed in other studies for HLA class I expression, NKG2D ligand expression, and susceptibility to NK cell lysis (9, 39, 40). NK and γδ T lymphocytes were derived from the same patient (donor MEL15392). Both human melanoma cells and the autologous PBL were from the Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy.

Purification and Generation of Polyclonal NK and γ/δ Cell Populations. To obtain enriched NK cells, peripheral blood lymphocyte was incubated with anti-CD3 (JT3A), anti-CD4 (HP2.6), and anti-HLA-DR (D1.12) monoclonal antibodies (mAbs; 30 min at 4°C) followed by goat antimouse IgG-coated Dynabeads (Dynal, Oslo, Norway; 30 min at 4°C) and immunomagnetic depletion (40). On the contrary, a positive selection using Dynabeads was used to purify the two different γ/δ cell subsets, Vδ1 and Vδ2, by the use of the specific mAbs, *i.e.,* A13 (anti-Vδ1) and BB3 (anti-Vδ2; Refs. 41, 42), following the manufacturer's instructions.

The $CD3^-$ 4^- DR^-, the $A13^+$, and the $BB3^+$ cells were separately cultured on irradiated feeder cells in the presence of 100 units/ml recombinant interleukin 2 (Proleukin; Chiron Italia srl, Milan, Italy) and 1.5 ng/ml phytohemagglutinin (Life Technologies, Inc., Paisley, Scotland) to obtain polyclonal NK or γ/δ cell populations. These culture conditions allowed us to greatly expand, in recombinant interleukin 2-containing medium, the purified subpopulations. Usually, cells were collected after 3–4 weeks of culture to be injected into the mice.

Cytolytic Assay. NK, Vδ1, and Vδ2 γδ cell populations were tested for cytolytic activity against the autologous melanoma cell line, MEL15392, and the HLA-class I negative melanoma cell line FO-1, in a 4-h ^{51}Cr release assay as described previously (40). The E:T ratios are indicated in the text.

Transplantation and Growth of Human Tumors in SCID Mice. Mice were injected s.c. into the right flank with 2×10^6 cells/mouse of the human melanoma cellular suspensions (43). The cells were resuspended in 0.2 ml RPMI 1640 containing 10% FCS. NK and γδ cells were injected s.c. simultaneously to autologous melanoma cells, with a methodological approach similar to the classical Winn assay (37), at doses ranging from 2×10^6 to 10×10^6 cells/mouse. Otherwise, NK and γδ cells were injected i.v. at the dose of 2×10^6 cells/mouse simultaneously to the s.c. melanoma cell injection or at the tumor take at days 10, 15, and 20 after the tumor cell transplantation. In some additional experiments, the i.v. treatment with the NK and γδ cells was performed each week beginning from the day 10 after the tumor cell injection until the mouse sacrifice. When the cells were administered i.v., SCID mice were also treated i.p. with 600 IU/ml IL-2. Tumor growth was monitored by measuring maximal and minimal diameters by caliper, three times a week, and tumor weight was estimated with the formula: tumor weight (mg) = length (mm) × width^2 (mm)/2, as described previously (44).

Flow Cytometry. Before injection into the mice, the *in vitro* expanded lymphocyte subpopulations were phenotypically characterized using a panel of mAb. The following mAbs were produced in our laboratory: JT3A (anti-CD3, IgG2a), c218 (anti-CD56, IgG1), c127 (anti-CD16, IgG1), A13 (anti-Vδ1, IgG1), BB3 (anti-Vδ2, IgG1), BAT221 (anti-NKG2D, IgG1), A76 (anti-NKp30, IgG1), BAB281 (anti-NKp46, IgG1), Z231 (anti-NKp44, IgG1), BAT9 (anti-Vγ9, IgG2b), and T61/7 (anti-CD44, IgG1; Refs. 9, 40–42). Leu3a (anti-CD4, IgG1), Leu2a (anti-CD8, IgG1), Leu18 (anti-CD45RA, IgG1), UCHL1 (anti-CD45RO, IgG2a), and M-A251 (anti-CD25, IgG1) were from Becton Dickinson (Mountain View, CA). Cells were stained with the appropriate mAb followed by phycoerythrin-conjugated AffiniPure $F(ab')_2$ goat antimouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA).

To evaluate the presence of the injected lymphocytes at the tumor site, the mice were sacrificed and the melanomas were excised. To obtain single cell suspension tumors were carefully minced, mechanically disrupted with the blunt end of a 5-ml syringe, and filtered. The single cell suspensions were washed twice in RPMI 1640 and stained with c127, A13, and BB3 mAb. The second reagent was Alexa Fluor 488 goat antimouse IgG $F(ab')_2$ fragment purchased from Molecular Probes, Inc. (Eugene, OR). After staining cells were fixed with 2% paraformaldehyde and analyzed on a FACSORT cytometer (Becton Dickinson) equipped with a 488 nm argon laser. Data were recorded and analyzed by using LYSIS II software (Becton Dickinson). The analysis was performed by gating *ex vivo* cells using forward and side scatter characteristics.

PCR for Human DNA in SCID Mice Organs. At 24 and 48 h after the i.v. inoculation of human NK and γδ T cells SCID mice were sacrificed and the spleen, lymph nodes, liver, and bone marrow were obtained. Single cell suspensions were prepared from spleen, lymph nodes, and liver by mechanical disruption with the blunt end of a 5-ml syringe. Bone marrow specimens were obtained by flushing of the marrow of both the femurs of the SCID mice. Amplification and detection of the HLA-DQα gene fragment were performed with GH25-GH27 primer pair and the corresponding probe RH54. The sensitivity of the assay was tested by amplifying DNA, prepared from a reference cell line (8E5 T-cell line), which was serially diluted into SCID mouse cell DNA (44).

Immunohistochemistry. For histological analysis, human tumor tissues were either fixed in 7% neutral-buffered formalin and embedded in paraffin wax, or embedded in OCT and snap frozen. For OCT snap frozen specimens 4-μm thick sections of embedded tissues were cut, air-dried, acetone-fixed, and immunostained with an anti-CD16 or anti-CD56 mAb (Dako, Glostrup, Denmark) for the NK cells, and A13 and BB3 mAb for γδ cells, using the PAP method (Dako) and counterstained with Mayer's hematoxylin, as described previously (43). For formalin-fixed and paraffin-embedded specimens, 4-μm-thick tissue sections were deparaffinized through graded series of ethanol passages and rehydrated in distilled water. Endogenous peroxidase was inhibited by a 30-min incubation in methanol containing 0.3% H_2O_2. To optimize immunodetection of CD16, nonenzymatic antigen unmasking was performed by heating tissue sections at 95°C for 6 min in an autoclave in 5 mM citrate buffer (pH 6). After cooling, sections were incubated with normal goat serum (1:50; DAKO Corp.) diluted in PBS containing 1% BSA for 30 min. Incubation with the anti-CD16 mAb was performed overnight at 4°C. Sections were subsequently rinsed three times in PBS plus Triton X-100 and treated with biotinylated goat antimouse immunoglobulin (1:100; DAKO Corp.) for CD16 staining. The slides were then covered with streptavidin-horseradish peroxidase (1:300; DAKO Corp.) for 30 min and finally visualized with the use of red 3-amino-9-ethylcarbazole (Sigma Chemical Co.) in 0.05 M acetate buffer containing 0.015% H_2O_2 or 3-amino-9-ethylcarbazole (DAKO Corp.), as

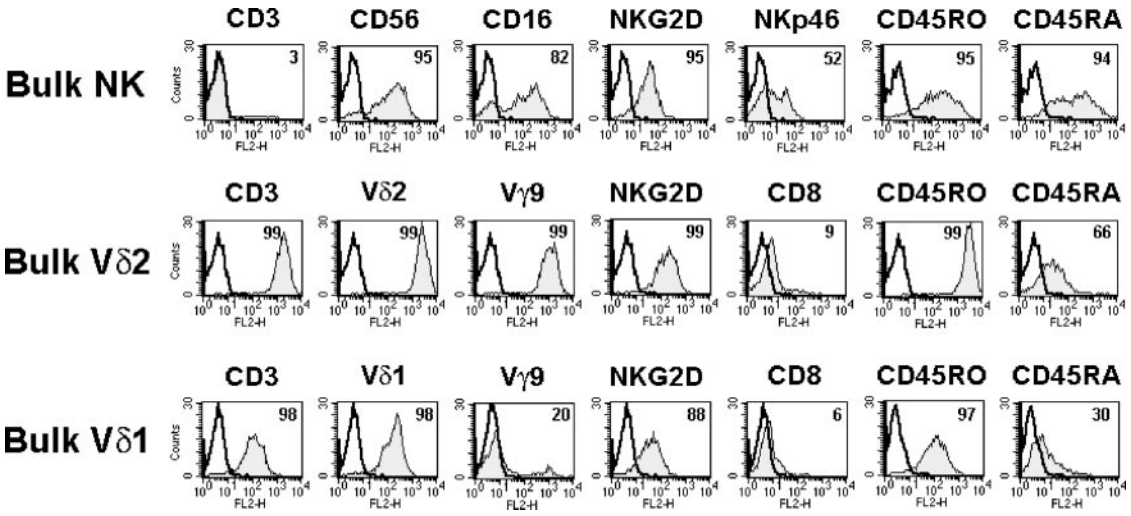

Fig. 1. Phenotypipic analysis of natural killer (NK) and γδ T cells. Polyclonal NK, Vδ1, and Vδ2 γδ cell populations, derived from donor MEL15392, were analyzed by immunofluorescence and fluorescence-activated cell sorter analysis using JT3A (anti-CD3), c218 (anti-CD56), c127 (anti-CD16), BAT221 (anti-NKG2D), BAB281 (anti-NKp46), A13 (anti-Vδ1), BB3 (anti-Vδ2), BAT9 (anti-Vγ9), Leu2a (anti-CD8), Leu18 (anti-CD45RA, IgG1) and UCHL1 (anti-CD45RO, IgG2a) followed by phycoerythrin-conjugated AffiniPure $F(ab')_2$ goat antimouse IgG. *Filled profiles* represent the monoclonal antibody (mAb) -reacting cells; *open profiles* refer to cells incubated with the second reagent alone. The percentages of mAb reacting cells are indicated.

appropriate. Sections were then counterstained with hematoxylin and mounted with glycerine-gelatin 4. Tissue sections subjected to the same treatment but without incubation with primary antibody were used as negative controls.

Statistical Analysis. The statistical difference between each group was determined by unpaired *t* test.

RESULTS

Characterization of NK and γδ T Lymphocytes. Purified NK and γδ cell subsets were expanded *in vitro* for almost 1 month under appropriate culture conditions. The various cell populations were phenotypically characterized by fluorescence-activated cell sorter analysis to verify their purity, which always exceeded 90%. Fig. 1 shows the surface expression of specific markers on the three different populations derived from the melanoma patient in a representative experiment. NK cells were pure because they were 95% $CD3^-$ $CD56^+NKG2D^+$; CD16 was expressed on 82% of cells. The NK-specific NCR (see the representative profile of NKp46) displayed a bimodal distribution with the evidence of the presence of both NCR^{dull} and NCR^{bright} NK cells (7, 40). Also the γδ cell populations appeared to be highly pure and characterized by a typical phenotype. Indeed, all of the Vδ2 expressing cells were also $V\gamma9^+$, whereas the Vδ1 cell population appeared to have 20% of $V\delta1/V\gamma9^+$ cells; both γδ subpopulations reacted with anti-CD3 and anti-NKG2D mAb. As expected, in all three of the lymphocyte populations, CD4 was completely absent (data not shown) and CD8 was scarcely represented (see the profiles for the γδ T cells). Consistent with a state of long-term activation, both NK and γδ T lymphocytes were brightly stained with anti-CD44 (data not shown) and anti-CD45RO mAb, whereas the expression of CD45RA was weaker, at least on T lymphocytes (Fig. 1); moreover, although not shown, only a small percentage of cells expressed CD25.

Both NK cells and the two subpopulations of γδ T cells were assessed for their *in vitro* cytolytic effect against the autologous melanoma cell line (MEL15392; Fig. 2). We had described previously that MEL15392, although characterized by the expression of a complete set of HLA class I alleles, was highly susceptible to lysis by the autologous NK cells (39). This susceptibility was explained by the low amounts of HLA class I molecules expressed on melanoma cells and, thus, by inefficient KIR/HLA-I interactions. In another study we had also analyzed MEL15392 (termed MEL15) for expression of NKG2D ligands and we showed that it only expressed low levels of MHC class I-related chains A, whereas UL16-binding proteins were absent (9). Indeed, NKG2D played a marginal role on inducing NK cell lysis, which almost exclusively depended on NCR, in particular NKp30 (40). As shown in Fig. 2, not only NK cells but also the two subpopulations of γδ T cells significantly lysed the autologous melanoma cells, although with different efficiency. To additionally substantiate that the lytic activity of both NK and γδ T cells was typically non-MHC restricted, we show that these populations were also capable to lyse the HLA-class I negative melanoma cell line FO-1 (Fig. 2).

Simultaneous s.c. Inoculation of NK and γδ cells Together with the Autologous Melanoma Cells. We first performed experiments aimed at evaluating the effect of the local treatment with either NK or γδ cells on the *in vivo* growth of the autologous melanoma. Thus, we inoculated s.c. 2×10^6 cells/mouse of the human melanoma cellular suspensions together with 2×10^6 cells/mouse of the NK and γδ cells. This approach (often referred to as the Winn assay) has been used successfully to monitor and quantify cell-mediated tumor immunity in animals for ~40 years (37) and more recently applied to human xenograft in SCID mice (reviewed in Ref. 29). The results clearly

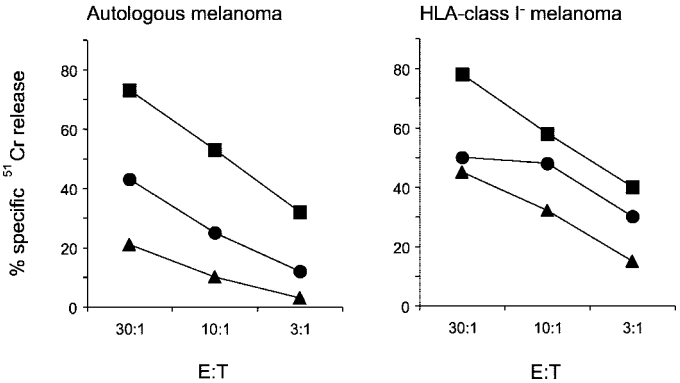

Fig. 2. Cytolytic activity of natural killer (NK) and γδ T cells. NK (■), Vδ1 (▲), and Vδ2 (●) cell populations were analyzed for cytolytic activity against the autologous melanoma cell line and the allogeneic HLA-class I negative melanoma cell line FO-1, as indicated, at different E:T cell ratios. Data from a representative experiment are shown.

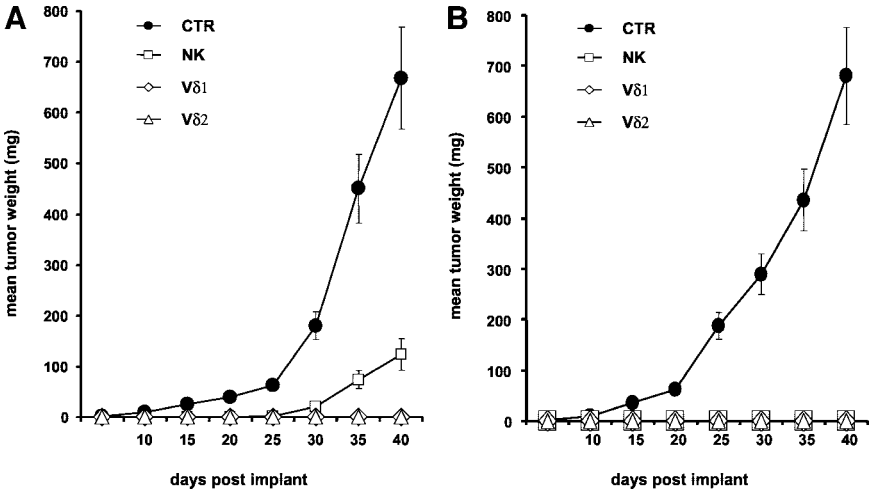

Fig. 3. SCID-Winn assay of human melanoma cells and natural killer (NK) and γδ T cells s.c. coinoculation. The figure shows the growth of a human melanoma cell line injected s.c. in SCID mice without (●) and with local coinjection of autologous NK, Vδ1 γδ T cells, or Vδ2 γδ T cells at the concentration of 2×10^6 cells/mouse (*A*) and 5×10^6 cells/mouse (*B*). Tumor growth was measured every 5 days post implant, and tumor weight was calculated as described in "Materials and Methods." The histograms represent mean of tumor weight in 20 animal/point in four different experiments; *bars*, ±SD.

showed that both subpopulations of γδ T cells were able to entirely abrogate the growth of the autologous melanoma, whereas NK cells could only slow the growth of the tumor in SCID mice when used at 2×10^6 cells/mouse concentration ($P < 0.001$ as compared with untreated animals; Fig. 3*A*). In fact, repeating the experiments at 5×10^6 cells/mouse, NK cells proved to entirely abrogate the growth of the s.c. autologous tumors in SCID mice (Fig. 3*B*). At additional time points, over the 40 days shown in the figure, no detectable tumors were found in the treated SCID mice (data not shown). This set of experiments showed that the local treatment with both NK cells and the two subpopulations of γδ T cells was able to prevent the growth of the autologous human melanoma in SCID mice.

Circulation of NK and γδ Cells into the SCID Mouse and Their Targeting to the Autologous Melanoma. We then performed experiments aimed at evaluating the tissue distribution of each human immune cell subpopulation in SCID mice, and their possible *in vivo* targeting to the autologous tumor after systemic i.v. inoculation. We first inoculated i.v. SCID mice with 10×10^6 of either NK cells or the two subsets of γδ T cells in the absence of the autologous s.c. melanoma. One and 2 days after the i.v. inoculation of the human immune cells, various SCID mice organs were collected and assessed for the presence of human sequences by DNA-PCR (44) to evaluate the presence of human cells also in SCID mouse organs where only few human cells could be obtained. The results indicated that at 24 h NK cells or one or another subset of γδ T cells were detected only in the spleen of SCID mice, whereas at 48 h only the SCID mice injected with human NK cells showed detectable human sequences in the examined tissues (Fig. 4*A*). To evaluate the presence of live human cells in the SCID mouse organs we analyzed by flow cytometry the amount of either NK cells or the two subsets of γδ T cells in the spleen of SCID mice at 24 and 48 h after the i.v. administration of the human cells. The results showed that from the spleen of the treated animals a detectable amount of human NK cells and the two subsets of γδ T cells was obtained at 24 h, whereas only human NK cells were detectable at 48 h (Fig. 4*B*). These results were highly consistent with those obtained with the analysis of human DNA sequences, confirming that human NK cells survived in SCID mice longer than the γδ T-cell subsets. We next explored the *in vivo* targeting of the various human immune cells to the autologous melanoma. To this purpose, SCID mice bearing detectable s.c. melanomas (10 days after the s.c. inoculation) were treated i.v. with either autologous NK cells or the two γδ T-cell subpopulations. One day after i.v. cell inoculation, animals were sacrificed, and fluorescence-activated cell sorter analysis was performed on *ex vivo* single cell suspensions obtained from the resected tumors. To identify NK cells we used anti-CD16 mAb, which reacted with 70–80% (range in the different experiments) of the NK cells before injection; the anti-CD56 mAb could not be used because it stained melanoma cells as well (data not shown). The results showed that human NK cells reacting with the anti-CD16 mAb were detectable in both the mouse spleen and the human autologous mel-

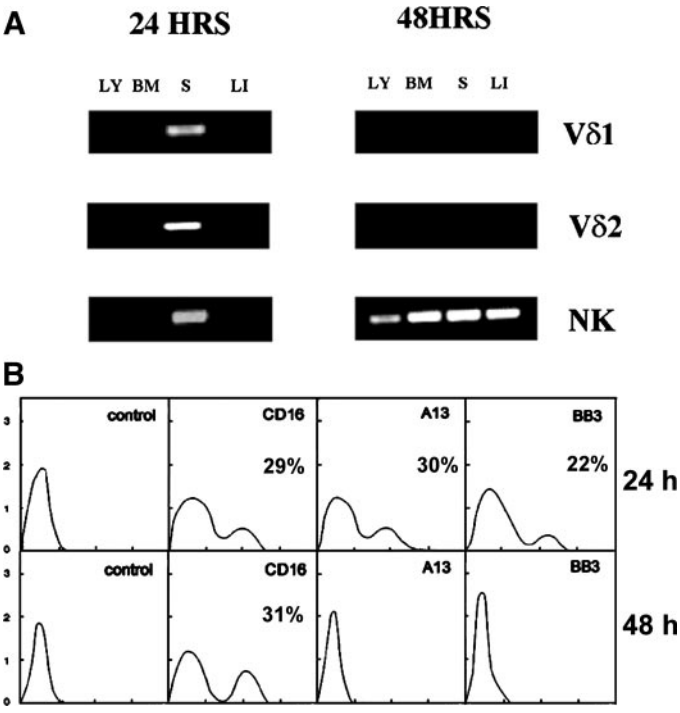

Fig. 4. DNA-PCR analysis of human sequences and flow cytometric analysis of human cells in the SCID mouse organs. *A*, DNA-PCR analysis of human sequences in the SCID mouse organs. Detection by PCR analysis of human DNA in organs of SCID mice inoculated i.v. with human natural killer (NK) cells or γδ T-lymphocyte subpopulations, sacrificed at 24 and 48 h after reconstitution. DNA was extracted by standard procedures, and HLA-DQα sequences were amplified as described previously (44). The detection limit under our experimental conditions was 1 copy of cellular equivalents per 1.5×10^5 genomes. The SCID mice organs examined were: lymph nodes (*LY*), bone marrow (*BM*), spleen (*S*), liver (*LI*). Three mice per group were analyzed in two different experiments. *B*, fluorescence-activated cell sorter analysis of NK and γδ T cells in the spleen of SCID mice at different time points after i.v. inoculation. The figure shows the flow cytometric analysis of the percentages of human CD16+, A13+, and BB3+ human cells in the *ex vivo* single-cell suspensions obtained from the spleen of the SCID mice 24 and 48 h after the i.v. inoculation of human NK cells, Vδ1, or Vδ2 γδ T lymphocytes in the SCID mice. The results shown are representative of data obtained in three mice in two different experiments.

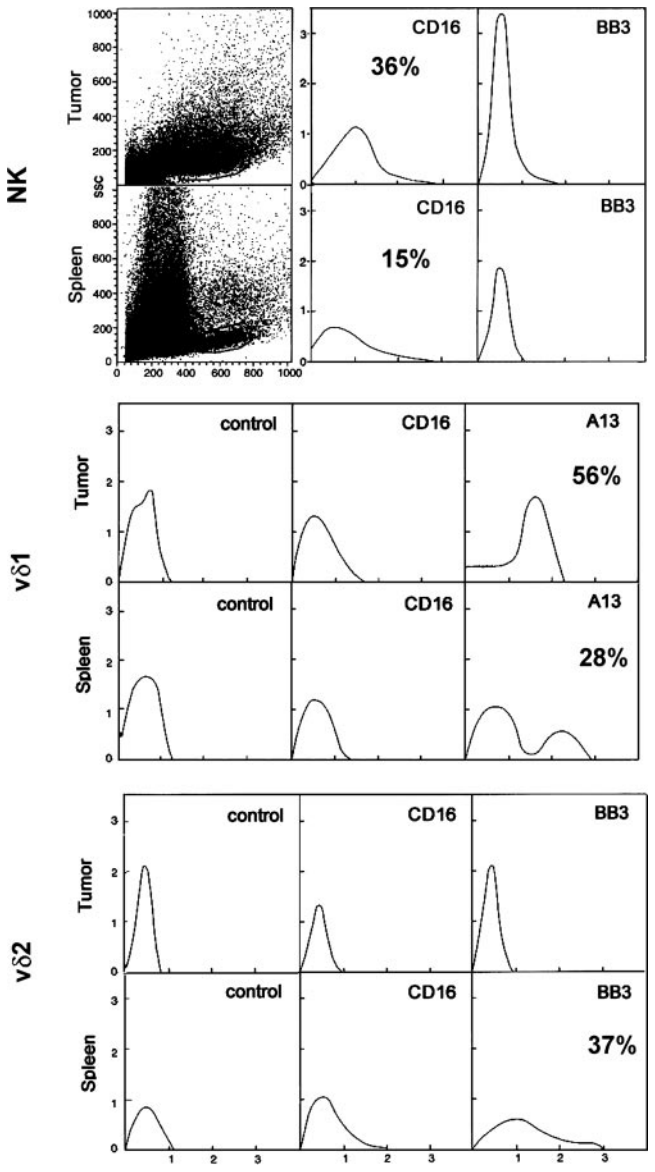

Fig. 5. fluorescence-activated cell sorter analysis of natural killer (NK) and γδ T cells targeting to the autologous melanoma in SCID mice. The figure shows the flow cytometric analysis of the percentages of CD16+, A13+, and BB3+ human cells in the *ex vivo* single cell suspensions obtained from either the s.c. human tumors or the spleen of the SCID mice 24 h after the i.v. inoculation of human NK cells, Vδ1, or Vδ2 γδ T lymphocytes autologous to the melanoma cells injected s.c. 10 days before. In the *top left*, the physical parameters-established gates where the analysis was performed either in the tumor or spleen preparations are shown. The results shown are representative of data obtained in 12 mice in three different experiments.

anoma of the treated animals (Fig. 5*A*). Regarding γδ T cells, the Vδ1$^+$ γδ T cells (recognized by the A13 mAb) were detected in both the spleen and the tumor of the cell-injected animals (Fig. 5*B*), whereas the Vδ2 γδ T cells (recognized by the BB3 mAb) were found in the spleen, but were virtually undetectable within the tumor mass (Fig. 5*C*). This set of results suggested that only NK cells and Vδ1 γδ T cells targeted to the autologous melanoma in SCID mice, whereas Vδ2 γδ T cells were trapped into the spleen of the animals.

Systemic Treatment of Melanoma-Inoculated SCID Mice with Autologous NK and γδ Cells. The following experiments were performed with the aim of exploring the effects of the systemic i.v. inoculation of the various lymphocyte subsets on the growth of autologous melanoma in SCID mice. We first evaluated the effectiveness of the i.v. inoculation of NK cells or γδ T cells simultaneously to the s.c. injection of autologous melanoma cells in preventing the growth of the tumors. In contrast with the data obtained under the condition of the simultaneous s.c. inoculation, the results of this set of experiments showed a clear-cut difference in the activity of the three immune cell subpopulations. In fact, treatment with NK cells and Vδ1 γδ T cells significantly delayed the growth of autologous melanoma ($P < 0.001$ as compared with untreated animals), whereas the inoculation of Vδ2 γδ T cells had no significant effect on the tumor growth, as compared with the untreated controls (Fig. 6*A*).

We then carried out experiments to evaluate the efficacy of the systemic immunotherapy on established autologous melanoma (10 days after s.c. inoculation) in the SCID mice model. Three therapeutic protocols were tested: (*a*) single i.v. treatment; (*b*) three treatments every each 5 days; and (*c*) weekly treatment until sacrifice. Single treatments did not show any significant inhibition of the human melanoma growth (Fig. 6*B*). The protocol based on three repeated treatments showed only a partial but significant inhibition of the autologous melanoma growth (Fig. 7*A*). Notably, the effect in delaying the tumor growth was significant from day 25 to day 35 postimplant for the treatment with Vδ1 cells ($P < 0.005$ as compared with both untreated animals and animals treated with Vδ2 cells), whereas for NK cells the effect was maintained until the end of the experiment ($P < 0.005$). Consistently with the experiments of the simultaneous inoculation of the lymphocytes i.v. and the tumor cells s.c. as described in Fig. 6*A*, NK cell treatment proved to significantly inhibit the tumor growth, whereas of the two γδ T-cell subpopulations only the Vδ1 cells proved effective in delaying the autologous melanoma growth, as compared with the untreated controls (Fig. 7*A*).

The weekly treatment showed again a marked (50–70%) inhibition of the tumor growth with both NK cells and Vδ1 γδ T cells ($P < 0.003$ at the end of the experiment as compared with untreated controls), whereas Vδ2 γδ T-cell treatment did not prove to affect the growth of the autologous melanoma (Fig. 7*B*).

The results of the three repeated treatments have shown that after termination of the treatment, NK cells appeared to maintain an inhibitory effect on the tumor growth, whereas in SCID mice treated with the Vδ1 γδ T-cell subpopulation, melanoma regained their uncontrolled growth reaching volumes comparable with those of the untreated mice at the end of the experiments (see Fig. 7*A*). To additionally investigate these results we evaluated by immunohistochemistry the presence of either human NK cells or γδ T-cell infiltrates in the autologous tumors of the treated animals after the stop of the treatment. The results of immunohistochemical analysis showed that the CD16+ cells were detectable within the tumors of the NK cell-treated animals, both as intratumoral aggregates (Fig. 8, *A* and *B*) and as perivascular infiltrates (Fig. 8*B*), whereas neither A13+ nor BB3+ cells were detectable at the same time intervals in γδ cell-treated animals (data not shown). These results suggested that the human NK cells were able to survive within the human tumor mass for a longer period of time as compared with both the γδ T-cell subpopulations.

DISCUSSION

Our present study shows that: (*a*) both NK cells and Vδ1 and Vδ2 γδ T cells can prevent tumor growth in a classical Winn assay, *i.e.*, the s.c. coinoculation at the same site of lymphocytes and tumor cells (37); (*b*) only NK cells and the Vδ1 γδ T cells inhibited s.c. tumor growth when infused i.v., reflecting the ability of these populations to traffic at the tumor site; and (*c*) the inhibitory effect of NK cells (but not of Vδ1 γδ T cells) was long-lasting, as these cells were detectable at the tumor site after a longer time interval after the termination of treatment.

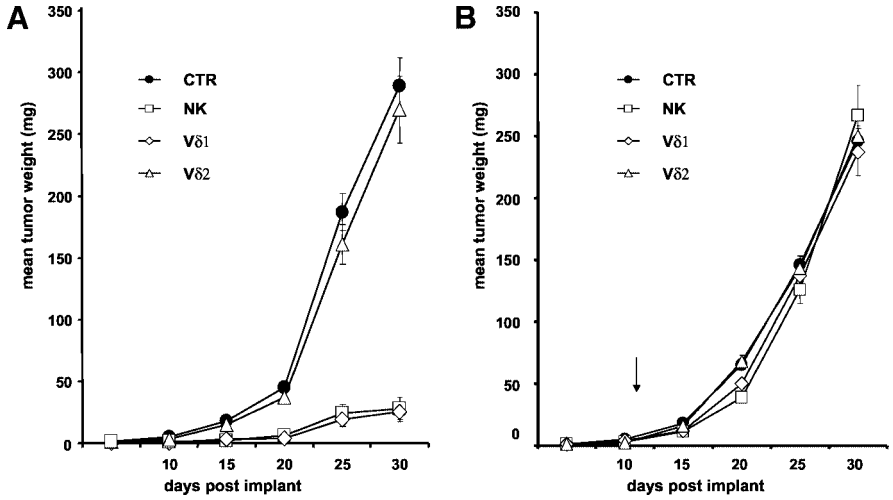

Fig. 6. Effects of systemic human natural killer (NK) and γδ T cells i.v. inoculation on the growth of the autologous melanoma in SCID mice. *A,* effects of systemic human i.v. inoculation of either human NK or γδ T cells contemporary to the s.c. injection of the autologous melanoma in SCID mice. The figure shows the growth of a human melanoma cell line injected s.c. in SCID mice without and with single i.v. injection of the autologous NK, Vδ1 T cells or Vδ2 γδ T cells, contemporary to the s.c. injection of the tumor cells. Tumor growth was measured every 5 days post implant, and tumor weight was calculated as described in "Materials and Methods." The histograms represent mean of tumor weight in 24 animals/point in six different experiments; *bars,* ±SD. *B,* effects of systemic NK and γδ T cells single i.v. inoculation regimen on the s.c. take of the autologous melanoma in SCID mice. The figure shows the growth of a human melanoma cell line injected s.c. in SCID mice without and with single i.v. inoculation of the autologous NK, Vδ1 T cells, or Vδ2 γδ T cells, at the tumor take (10 days after the s.c. implant). Tumor growth was measured every 5 days post implant and tumor weight was calculated as described in "Materials and Methods." The histograms represent mean of tumor weight in 15 animals/point in five different experiments; *bars,* ±SD.

The antitumor effect of the various human immune cells has been evaluated previously in SCID mice coengrafted s.c. with human peripheral blood lymphocytes and viable human lung tumor cells (SCID-Winn assay; Ref. 45). In this model, a key role for both NK cells and CD8+ T cells has been demonstrated. In our study, the capability of NK cells and of two different γδ T-lymphocyte subpopulations of preventing or inhibiting the growth of autologous melanoma in SCID mice has been evaluated. The local treatment with either NK cells or γδ T lymphocytes prevented the growth of the autologous melanoma in a SCID-Winn assay. Experiments aimed at defining the autologous tumor targeting by the various immune cells showed that only NK cells and Vδ1 γδ T lymphocytes were detectable within the autologous tumor mass, whereas Vδ2 γδ T lymphocytes were mostly observed in the spleen but not in the tumor tissue of the treated animals. Consistent with these results, when the treatment with NK cells and γδ T lymphocytes was performed systemically, some important differences could be detected. In fact, the simultaneous injection of the melanoma cells s.c. and the autologous cells i.v. showed that only NK cells and Vδ1 γδ T lymphocytes prevented growth of s.c. melanoma, whereas the Vδ2 γδ T lymphocytes had no effect. These data were in part surprising because the antitumor *in vitro* cytotoxicity of Vδ1 T cells is lower than that of Vδ2 T cells (Fig. 1; Refs. 13, 46). However, data in human tumor/SCID mouse may

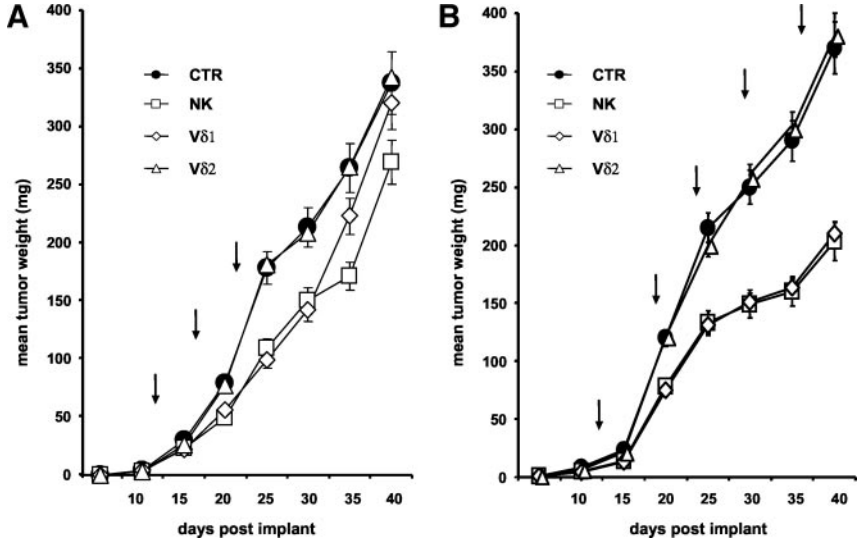

Fig. 7. Effects of repeated systemic human natural killer (NK) and γδ T cells i.v. inoculation on the growth of the autologous melanoma in SCID mice. *A,* effects of systemic NK and γδ T cells regimens on the s.c. take of the autologous melanoma in SCID mice (three times every 5 days). The figure shows the growth of a human melanoma cell line injected s.c. in SCID mice without and with three repeated i.v. injections (each 5 days) of the autologous NK, Vδ1 T cells, or Vδ2 γδ T cells. Tumor growth was measured every 5 days post implant, and tumor weight was calculated as described in "Materials and Methods." The histograms represent mean of tumor weight in 24 animals/point in six different experiments; *bars,* ±SD. *B,* effects of systemic NK and γδ T cells regimens on the s.c. take of the autologous melanoma in SCID mice (each week). The figure shows the growth of a human melanoma cell line injected s.c. in SCID mice without and with weekly i.v. injections of the autologous NK, Vδ1 T cells, or Vδ2 γδ T cells. Tumor growth was measured every 5 days post implant, and tumor weight was calculated as described in "Materials and Methods." The histograms represent mean of tumor weight in 16 animals/point in four different experiments; *bars,* ±SD.

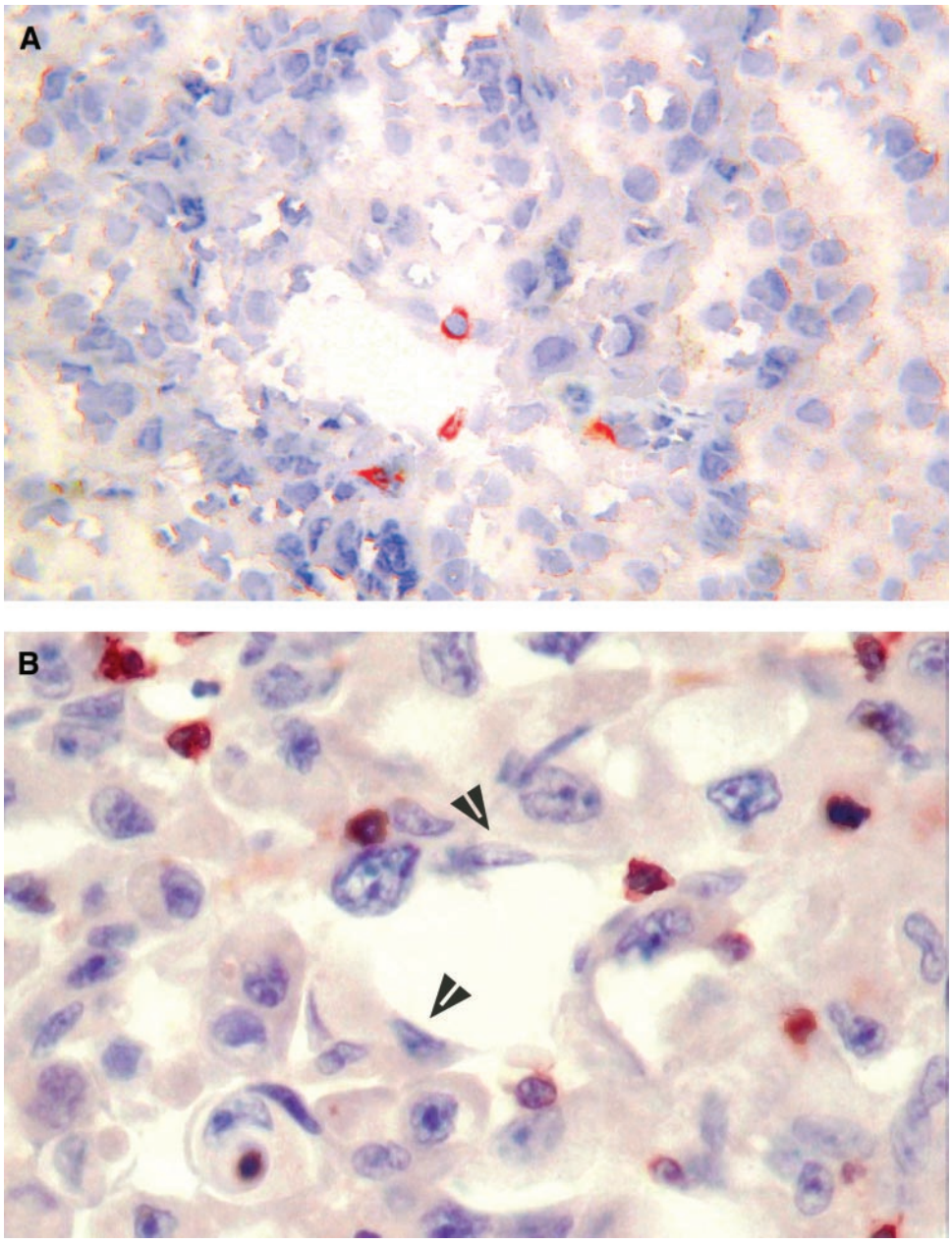

Fig. 8. Human natural killer (NK) cell infiltrates within the autologous melanoma after the stop of the systemic treatment. Consecutive sections of either frozen or paraffin-embedded human melanoma grown in SCID mice and treated i.v. with autologous NK cells were subjected to immunohistochemical staining with anti-CD16. *A*, immunohistochemical analysis of consecutive sections from frozen tissues. A frozen section showing an infiltrate of human CD16+ cells within the tumor mass (magnification, ×550). *B*, immunohistochemical analysis of consecutive sections from formalin fixed and paraffin-embedded specimens. An infiltrate of human CD16+ cells scattered within the tumor mass and localized in a perivascular region. *Arrows* indicate morphologically recognizable endothelial cells (original magnification, ×850). 3-amino-9-ethylcarbazole (DAKO Denmark) were used as chromogens, and Mayer's hematoxylin for the counterstaining.

better reflect the microenvironment conditions occurring *in vivo* in humans. In fact, Vδ1 γδ T cells are the prevalent γδ T-cell population in human tissues including intestinal epithelium and skin (13, 25, 46–48). The localization of Vδ1 γδ T lymphocytes within epithelia suggests that these cells may be effective against epithelial malignancies. Notably it has been shown recently that mice lacking γδ T cells are highly susceptible to chemical induction of skin tumors and that skin-associated γδ T cells can kill *in vitro* cutaneous carcinomas by a mechanism involving NKG2D (28).

The follow up of human melanoma-SCID mice treated weekly with NK and γδ T cells revealed that the inhibitory effect on tumor growth lasted longer in mice treated with NK cells and that infiltrating NK cells could be detected within the autologous melanoma after treatment discontinuation. These data underscore the potential, relevant role of NK cells in the immunotherapy of human melanomas. It is of note that the MEL15392 melanoma cells analyzed in this study expressed low levels of MHC class I-related chain A and lacked UL16-binding proteins. Therefore, despite the effectiveness of the treatment, it is likely that results may represent an underestimate of data that might be obtained using melanomas expressing high levels of the NKG2D ligands. The same consideration can be reported to γδ T cells, because also in these cells NKG2D plays an important role in inducing tumor cell lysis (16, 17).

Recent results obtained by our group in human melanoma-SCID mice have suggested that, although the treatment with an expanded antigen-specific T-cell clone generated *in vitro* can be highly efficient in abolishing the tumor growth when the target antigen is fully expressed, it may lead to immunoselection of antigen-loss variants in the presence of suboptimal levels of antigen expression (49). The results of the present study suggest that adoptive immunotherapy protocols against tumors should include treatments with NK and Vδ1 γδ T cells. Because conventional CTL and NK or γδ T cells use different mechanisms to kill tumor cells, their combined use may result complementary and may provide a tool to reduce the risk of tumor escape.

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