Antitumor Activity of γδ T Cells Reactive against Cytomegalovirus-Infected Cells in a Mouse Xenograft Tumor Model

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Abstract

γδ T cells recognize stress-induced autoantigens and contribute to immunity against infections and cancer. Our previous study revealed that Vδ2-negative (^{neg}) γδ T lymphocytes isolated from transplant recipients infected by cytomegalovirus (CMV) killed both CMV-infected cells and HT29 colon cancer cells *in vitro*. To investigate the antitumor effects of Vδ2^{neg} clones *in vivo*, we generated hypodermal HT29 tumors in immunodeficient mice. Concomitant injections of Vδ2^{neg}clones, in contrast to Vδ2^{+} cells, prevented the development of HT29 tumors. Vδ2^{neg} clones expressed chemokine C-C motif receptor 3 (CCR3) and migrated *in vitro* in response to chemokines secreted by HT29 cells, among which were the CCR3 ligands macrophage inflammatory protein-1δ and monocyte chemoattractant protein-4. More importantly, a systemic i.p. treatment with Vδ2^{neg} clones delayed the growth of HT29 s.c. tumors. The effect of *in vivo* γδ T-cell passive immunotherapy on tumor growth could be reverted by addition of a blocking anti-CCR3 antibody. γδ T-cell passive immunotherapy was dependent on the cytotoxic activity of the γδ effectors toward their targets because Vδ2^{neg} clones were not able to inhibit the growth of A431 hypodermal tumors. Our findings suggest that CMV-specific Vδ2^{neg} cells could target *in vivo* cancer cells, making them an attractive candidate for antitumor immunotherapy. [Cancer Res 2009;69(9):3971–8]

Introduction

The crucial role of T lymphocytes bearing T-cell receptor (TCR) γ and δ chains (i.e., γδ T cells) in protection of the host against viral infections and tumors is increasingly being recognized. γδ T cells account for 1% to 5% of CD3^{+} peripheral T lymphocytes but constitute a substantial fraction (>30%) of T cells in intestinal epithelia. In humans, most of the peripheral blood γδ T cells express Vδ2/Vγ9 TCRs. In contrast, intraepithelial γδ T cells use Vδ2^{neg} segments that can associate with various Vγ elements. Vδ2Vγ9 T cells recognize phosphoantigens that were characterized recently (1). The antigen specificity of Vδ2^{neg} γδ T cells was less explored, although it was shown that Vδ1^{+} intraepithelial lymphocytes recognize the stress-inducible major histocompatibility complex class I–related proteins A and B (MICA and MICB) on epithelial cells (2). γδ T cells do not require major histocompatibility complex–presenting molecules in the antigen recognition process, making them complementary immune candidates for new protocols of immunotherapy (3, 4).

The concept for a protective role of γδ T lymphocytes against cancer was highlighted in TCRδ$^{-/-}$ mice that displayed an increased susceptibility to cutaneous malignancy (5). In a separate study, chimeric mice in which γδ T cells could not produce IFNγ had a higher tumor incidence after skin exposure to methylcholanthrene (6).

In humans, the potential contribution of γδ T cells to antitumor immune responses was investigated through the analysis of tumor infiltrating lymphocytes. Antitumor γδ tumor infiltrating lymphocytes from epithelial tumors can belong to the resident Vδ1 population (7–10) but also to the Vδ2Vγ9 subset (11, 12). Activated γδ T cells isolated from peripheral blood mononuclear cells of healthy donors display a potent cytotoxic activity toward different cancer cells *in vitro* (13). In agreement with their potential role in tumor host defense *in vivo*, γδ T cells have been found with an increased frequency in peripheral blood mononuclear cells from disease-free survivors of leukemia after allogeneic bone marrow transplantation (14, 15). It was also reported that the reactivity of peripheral γδ T cells against nasopharyngeal carcinoma was impaired in cancer patients, whereas the deficit was restored among survivors after successful treatment (16).

Immune-suppressed transplant recipients are at high-risk for cancer development (17, 18). At the same time, these individuals have weaker defenses against infectious agents such as cytomegalovirus (CMV). A few years ago, we showed an increase of peripheral blood γδ T lymphocytes in allograft recipients infected by CMV (19). A protective anti-CMV role for γδ T cells was suggested by the concomitant resolution of viral infection (20). Among the increased γδ T cells, Vδ1^{+}, Vδ3^{+}, and Vδ5^{+} cells were predominantly found, conversely to Vδ2Vγ9 cells (21). Interestingly, Vδ2^{neg} lines and clones specifically killed CMV-infected fibroblasts as well as epithelial tumor cells. In particular, anti-CMV Vδ2^{neg} clones 4-13 and 4-29 showed a perforin-dependent cytotoxic activity against colon cancer cells (Caco2 and HT29) in contrast to normal epithelial cells. When cocultured with HT29 and Caco2, anti-CMV Vδ2^{neg} clones produced tumor necrosis factor α and IFNγ. Cancer cell killing and cytokine release by Vδ2^{neg} clones involved TCR engagement that was independent of major histocompatibility complex molecules recognition (22).

The present study was undertaken to evaluate the *in vivo* antitumor reactivity of anti-CMV Vδ2^{neg} clones, including their ability to inhibit tumor growth as well as their migratory potential toward cancer cells. To this aim, we used immunodeficient Rag$^{-/-}$γc$^{-/-}$ mice and xenografted HT29 cells under the skin. First, we show that local treatment with Vδ2^{neg} clones is able to arrest tumor growth. Next, we show a C-C motif receptor 3 (CCR3)-dependent migration of 4-29 clones *in vitro* and identify MIP-1δ

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and monocyte chemoattractant protein-4 as putative ligands involved in this process. Finally, 4-29 clones injected at distance from the tumor site were able to delay HT29 tumor growth and addition of a blocking anti-CCR3 antibody revert this effect. These findings establish a pivotal role for γδ T cells at the boundaries between anticancer and anti-infectious situations, and help to understand the *in vivo* antitumor reactivity of $V\delta2^{neg}$ γδ T cells, which can be of significant relevance for transplant recipients who are more susceptible to develop malignancies than normal individuals.

Materials and Methods

Animals and human cells. $Rag^{-/-}\gamma c^{-/-}$ mice were a gift from Dr. James Di Santo (INSERM U 668, Institut Pasteur, Paris, France; ref. 23). They were used at age 7 to 10 wk, housed in an appropriate animal facility (Université de Bordeaux 2), and kept under pathogen-free conditions.

The colon carcinoma (HT29) and skin carcinoma (A431) cell lines were from the American Type Culture Collection. They were cultured in DMEM (Life Technologies) supplemented with 8% heat-inactivated fetal bovine serum (PAA Laboratories GmbH). $V\delta2^{neg}$ clones 4-29 and 4-13 expressed the same Vγ9Vδ5 TCR (22). The Vδ2 cell line was established from a healthy donor in our laboratory. Human γδ T cells were expanded with phytohemaglutinin and irradiated allogeneic peripheral blood mononuclear cell as described previously (22). γδ T cells were cultured 2 to 3 wk before use in RPMI 1640 (Life Technologies), 10% human serum, and 1,000 IU/mL recombinant human interleukin-2 (rhIL2; Chiron) at 37°C in 5% CO_2. They were tested negative for *Mycoplasma* contamination.

Flow cytometry. γδ cells were incubated with the following monoclonal antibodies (mAb) against human chemokine receptors: phycoerythrin (PE)-conjugated anti-CXCR1 (clone 5A12), PE anti-CXCR4 (clone 12G5), PE anti-CCR5 (clone 2D7), PE anti-CXCR3 (clone 1C6), or PE anti-CCR6 (clone 11A9; BD Biosciences), or PE anti-CXCR2 (clone 242216), PE anti-CCR2 (clone 48616), or PE anti-CCR7 (clone 150703; Beckman Coulter). Additional staining was performed by incubation with anti-CCR1 (clone 53504), anti-CCR3 (clone 61828), or anti-CCR9 (clone 112509), anti-MICA (clone 159227), anti-MICB (clone 236511), anti–ULBP1 (clone 170818), anti–ULBP2 (clone 165903), anti-ULBP3 (clone 166510; R&D Systems), anti–HLA-ABC (clone W6/32; Dako) followed by incubation with PE or FITC anti-mouse IgG (Beckman Coulter). Appropriate isotype-matched control mAbs were included and the samples were analyzed on a FACSCalibur or a FACSCanto apparatus (BD Biosciences).

Cellular cytotoxicity assay. The cytolytic potential of $V\delta2^{neg}$ and $V\delta2^{+}$ γδT cells was measured by using the flow cytometry-based CD107a assay (24). Six-hour cocultures of 5×10^4 γδ T cells and 5×10^4 HT29 were carried out in the presence of PE anti-CD107a mAb (clone H4A3; BD Biosciences). After 1 h of coculture, brefeldin A was added. Cells were harvested, stained with PC5 anti-TCR pan-γδ (clone IMMU 510; Beckman Coulter), and analyzed by flow cytometry.

Detection of chemokines from tumor lysates and culture supernatants. HT29 cells (1×10^5) were injected s.c. into the right flank of a

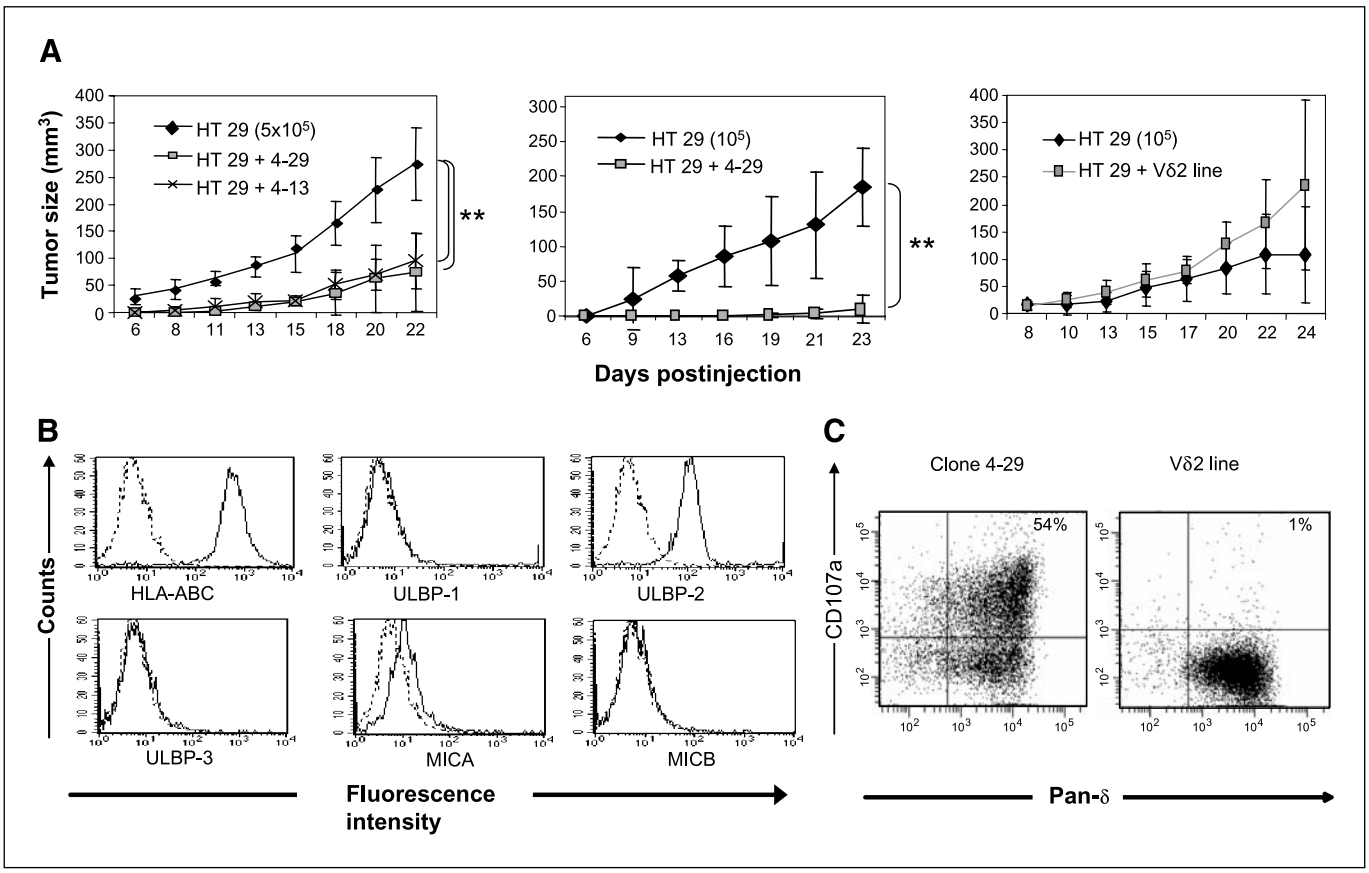

Figure 1. Local antitumor effects of anti-CMV $V\delta2^{neg}$ γδ T cells *in vivo*. (*A, left*) Tumor growth in 4 to 10 mice receiving s.c. injections of 5×10^5 HT29 cells in the absence (*HT29*) or presence of 2×10^6 4-29 (*HT29 + 4-29*) or 4-13 (*HT29 + 4-13*) $V\delta2^{neg}$ T lymphocytes. (*A, middle* and *right*) Tumor growth in 4 to 6 mice receiving s.c. injections of 1×10^5 HT29 cells in the absence or presence of 2×10^6 4-29 T cells (*middle*) or $V\delta2^{+}$ T cells (*right*). Tumor volumes are expressed in cubic millimeters; *points,* mean; *bars,* SD. **, $P < 0.003$. *B,*; HT29 cell surface expression of HLA A, B, and C, ULBP-1, ULBP-2, ULBP-3, MICA, and MICB (*bold lines*) measured by flow cytometry compared with control isotypic antibody (*dotted lines*). *C,* cytolytic activity of 4-29 T cells (*left*) and $V\delta2^{+}$ T-cells (*right*) measured using the CD107a flow cytometry assay. Data are representative of experiments repeated twice to thrice.

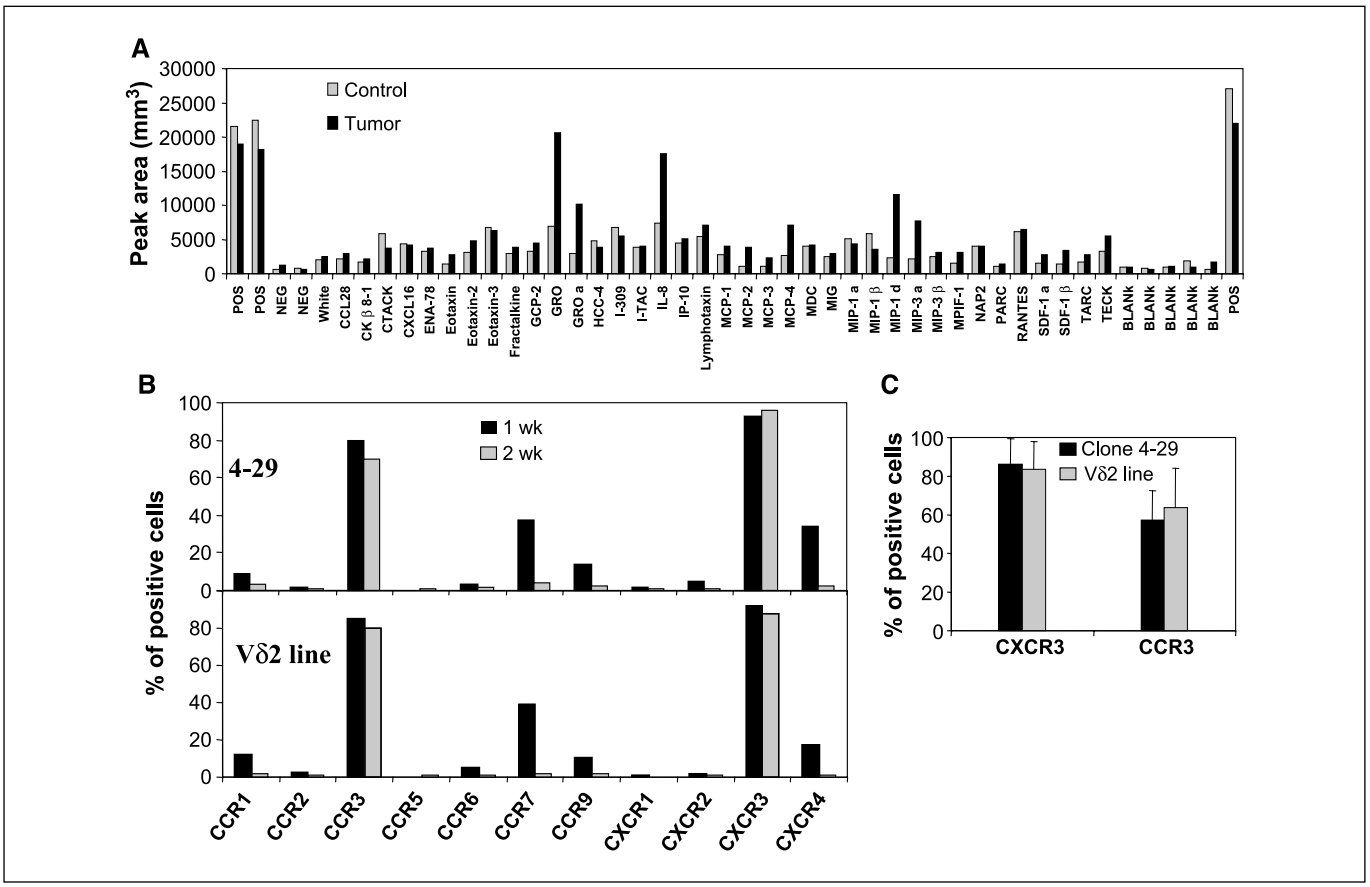

Figure 2. Chemokines secreted by HT29 solid tumors and chemokine receptors expressed on anti-CMV Vδ2^{neg} γδ T cells. *A,* chemokine microarrays were incubated with HT29 solid tumor (*tumor*) or healthy skin (*control*) extracts excised from mice. The spots on the membranes were scanned and the peak areas (*histograms*) obtained for each chemokine are shown. Experiments were repeated twice. *B* and *C,* expression of chemokine receptors on Vδ2^{neg} and Vδ2^{+} T cells were analyzed as described in Materials and Methods. *B,* one example of chemokine receptor expression at 1 (*black bars*) and 2 wk (*gray bars*) poststimulation of 4-29 T cells (*top*) and Vδ2^{+} T cells (*bottom*). The kinetic was carried out twice. *C,* cells were stained with anti–CXCR3-PE or anti–CCR3-PE antibodies. *Columns,* mean percentages of positive cells obtained from 5 to 12 different analyses performed 1 to 3 wk postactivation; *bars,* SM.

mouse. After 5 wk, the solid tumor was excised and minced in 1× lysis buffer (RayBiotech). Protein levels were quantified using BCA Protein Assay kit (Pierce). HT29 supernatants were isolated after 1 wk of culture in DMEM supplemented with 8% fetal bovine serum. Detection of chemokines was performed using the RayBio Human Chemokine Antibody Array 1 (RayBiotech) according to the manufacturer's instructions. Images were processed with ImageJ 1.39a software (NIH). Briefly, each point on the dot plot is a grayscale (8 bit) image (0–255, where 0 is black, 255 is white). Background gray levels were subtracted, and a box was drawn onto the first lane and used as a frame for the other lanes to obtain densitometric data from equal areas. The area of the peak ("peak area") was outlined and calculated.

***In vitro* chemotactic assay.** The chemotactic potential of 4-29 clones and Vδ2^{+} γδ T cells was assayed using a double-chamber system (BD Falcon). γδ Cells (5×10^5) in their culture medium (RPMI, 10% human serum, 1,000 U/mL rhIL2) were added to the upper inserts (3-μm pore size) of a 24-well transwell plate. HT29 or A431 supernatants were isolated after 1 wk of culture and added into the lower well. To test MIP-1δ–dependent migration, the lower wells were filled with DMEM 8% fetal bovine serum containing MIP-1δ (R&D Systems) at different concentrations. Where indicated, 4-29 clones were preincubated at 37°C for 90 min or overnight, in RPMI 1% FCS supplemented with 1,000 IU/mL rhIL2, in the absence or presence of pertussis toxin (SigmaAldrich) at 500 ng/mL. For blocking experiments, 4-29 clones were preincubated with either anti-CCR3 mAb (10 μg/mL) or isotype-matched control ($IgG2_a$) mAb (10 μg/mL, clone 20102; R&D Systems). Migration was allowed to proceed for 6 h at 37°C. Cells that migrated into the lower wells were counted microscopically. All assays were performed in triplicate.

Transplantation and growth of human tumors in mice. Mice received 100 μL of different inocula of HT29 cells s.c. into the right flank. Local s.c. injections of γδ cells were performed by the simultaneous inoculation of 100 μL of culture medium (RPMI, 10% human serum, and 1,000 IU/mL rhIL2) containing 4-29 Vδ2^{neg} cells or Vδ2^{+} cells. When a systemic treatment was applied, mice received HT29 cells s.c., and 4 i.p. injections of 4-29 T cells or Vδ2^{+} cells in 100 μL of culture medium at day 0, 2, 4, and 7. When mice received increased amounts of rhIL2, they were given daily from day 0 to day 7, 5,000 IU of rhIL2 i.p. alone, or in combination with 4-29 T cells that were injected at day 0, 2, 4, and 7. For blocking migration experiments, mice received 4 injections of 4-29 T cells in 100 μL of culture medium containing 100 μg/mL of anti-CCR3 or control $IgG2_a$ mAb (R&D Systems). In experiments where i.p. γδ T-cell injections were delayed, mice received 4 injections of 4-29 T cells at day 7, 9, 11, and 14. In other control experiments, mice were inoculated with A431 epithelial tumor cells and given 4 injections of 4-29 T cells i.p at day 0, 2, 4, and 7. Tumor growth was monitored by measuring the maximal and minimal diameters with a caliper thrice a week. Tumor volume was estimated using the formula: tumor volume (mm^3) = [length (mm) × width^2 (mm)]/2.

Histologic analysis. HT29 solid tumors excised from mice were fixed in 10% formalin and embedded in paraffin. Serial tissue sections (4-μm thick) were mounted on glass slides and dried at 56°C before dewaxing in xylene and rehydration in alcohols. Sections were stained with hematoxylin eosin saffranin according to standard histologic procedures. Photographs were taken with the Coolscope (Nikon) with a ×2 objective.

Statistical analysis. Data were analyzed with STAT Xact-8 with Cytel Studio (Cytel Statistical Software). For *in vivo* studies, we applied a

nonparametric permutation exact test described in detail in Siegel S. and Castellan NJ., 1988, *Nonparametric statistics for the behavioral sciences* (second edition McGraw-Hill, New York). This test is adapted for a finite data sample (<30 mice per group). For *in vitro* studies, we applied the Kruskal-Wallis test. For all experiments, a P value of <0.05 was considered significant.

Results

Concomitant s.c. inoculation of anti-CMV Vδ2^{neg} γδ T cells and HT29 colon carcinoma cells delays the development of hypodermal HT29 tumors. To test the antitumor activity of anti-CMV Vδ2^{neg} γδ T cells *in vivo*, γδ T-cell clone 4-29 was inoculated s.c. concomitantly with HT29 cells. As shown in Fig. 1*A* (*left*), a focal coinjection of both tumor and 2×10^6 4-29 T cells significantly ($P = 0.002$) delayed the appearance of the solid tumors that developed from 5×10^5 HT29 cells (effector/target ratio of 4:1). Similar results were obtained with clone 4-13 ($P = 0.003$; Fig. 1*A, left*). The consequence of local treatment with 2×10^6 4-29 T cells was proportional to the effector/target ratio, and no detectable tumors were found after 3 weeks when mice received 10^5 HT29 cells ($P = 0.002$; effector/target ratio of 20:1; Fig. 1*A, middle*). Tumor growth was not significantly affected ($P > 0.5$) by local coinjection of a Vδ2$^+$ T cell line (Fig. 1*A, right*). Respectively, 92% and 75% of Vδ2^{neg} and Vδ2$^+$ γδ T cells expressed NKG2D (data not shown), whose binding to specific ligands could be involved in cancer cells killing (25). NKG2D ligands, ULBP-2, and to a lesser extent, MICA, were found expressed on HT29 cells (Fig. 1*B*). However, in contrast to Vδ2^{neg} T cells, Vδ2$^+$ T cells did not show any cytotoxic activity against HT29 cells, as measured by the induced membrane expression of CD107a only on 4-29 T cells (Fig. 1*C*). These results suggest that, as we have previously observed *in vitro* (22), anti-CMV γδ Vδ2^{neg} T cells display TCR-dependent cytotoxic activity toward HT29 cells *in situ*, leading to the consequent inhibition of HT29 tumor growth.

HT29 tumors produce high amounts of inflammatory chemokines. To investigate whether HT29 cells were able to attract γδ T lymphocytes, we determined the pattern of chemokines produced by HT29 solid tumors isolated from Rag$^{-/-}$γc$^{-/-}$ mice. Figure 2*A* shows the results of a representative experiment. Groα (as well as other Gro family members) and IL-8 were the most abundantly expressed of the 38 chemokines analyzed, as was the case for the *in vitro* cultured counterpart (data not shown; Fig. 2*A*). MIP-3α, monocyte chemoattractant protein-4, and M1P-1δ were also easily detected, whereas other chemokines were found in much lower proportions (Fig. 2*A*). Thus, HT29 cells are able to produce inflammatory chemokines, and this production is only slightly affected by the mouse environment.

Preferential expression of CXCR3 and CCR3 by anti-CMV Vδ2^{neg} clones. We then examined which chemokine receptors were expressed by the Vδ2^{neg} 4-29 clone after 2 weeks of *in vitro* activation and culture (at which time they were used for *in vivo* experiments). Figure 2*B* depicts the results of one kinetic study conducted concomitantly on Vδ2^{neg} and Vδ2$^+$ T cells. At each time point, <10% of γδ (Vδ2^{neg} and Vδ2$^+$) T cells expressed CCR1, CCR2, CCR5, CCR6, CXCR1, and CXCR2 (Fig. 2*B*). At 1 week postactivation, CCR7 was found on 37% of 4-29 cells and 39% of Vδ2$^+$ cells, CXCR4 was found on 34% of 4-29 cells and 18% of Vδ2$^+$ cells, and CCR9 was expressed by 14% of 4-29 cells and 11% of Vδ2$^+$ cells. At 2 weeks postactivation, however, CCR7, CXCR4, and CCR9 were found on <15% of Vδ2^{neg} and Vδ2$^+$ cells (Fig. 2*B*). On the other hand, CXCR3

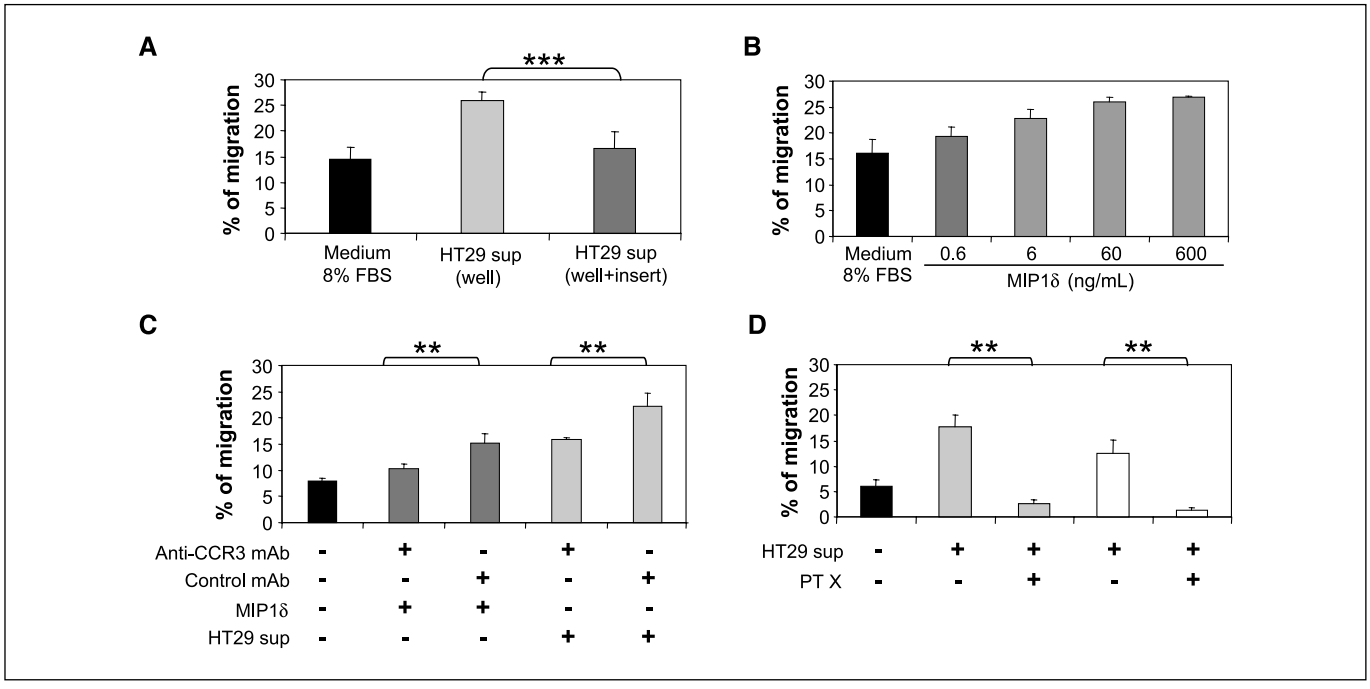

Figure 3. Migration potential of anti-CMV Vδ2^{neg} γδ T cells. *Columns,* percentages of cells that have migrated from the upper well ($n = 3$ wells) from one representative experiment of three; *bars,* SD. *A,* 4-29 T cells were assayed for their chemotactic response to HT29 supernatant (Materials and Methods). Background migration was measured with medium containing 8% fetal bovine serum in the lower well, whereas HT29 supernatant placed in the lower and upper wells (*well+insert*) reflected chemokinesis. *B,* MIP-1δ dose-dependent migration of 4-29 T cells. *C,* 4-29 T cells were preincubated with anti-CCR3 or isotype control mAb as described in Materials and Methods, and placed in the upper well. MIP-1δ (60 ng/mL) or HT29 supernatant (*HT29 sup*) was added (+) or not (−) into the lower well. *D,* 4-29 T cells were preincubated or not with pertussis toxin (*PTX*) overnight (*gray bars*) or for 90 min (*open bars*). **, $P < 0.005$; ***, $P < 0.0005$.

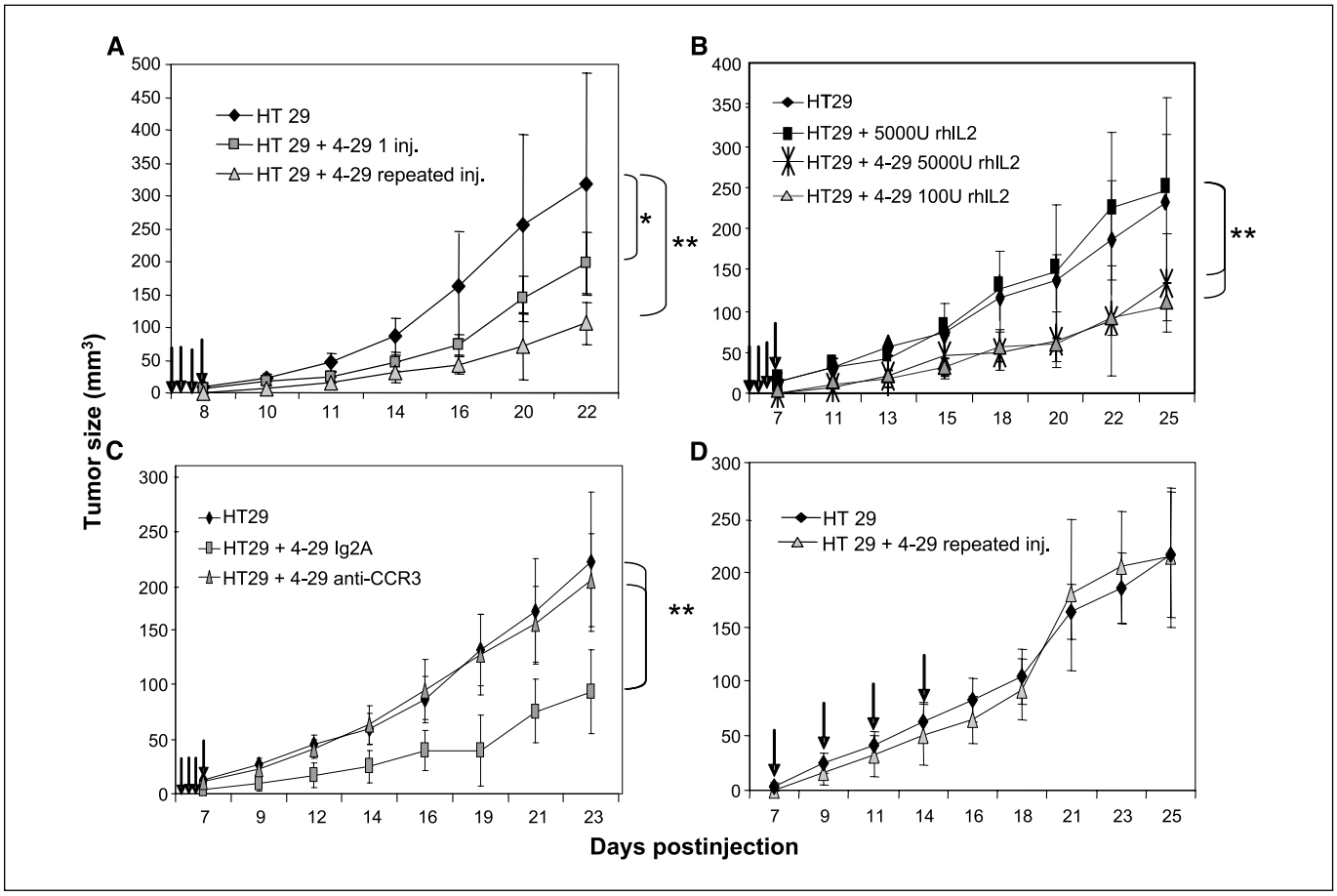

Figure 4. Repeated i.p. injections of anti-CMV Vδ2^{neg} γδ T cells delay the development of HT29 tumors. Tumor growth in mice receiving s.c. injections of 1×10^5 HT29 cells in the absence or presence of single (at day 0; *1 inj.*) or repeated (*repeated inj.*) i.p. injections of 2×10^6 4-29 T cells. *Arrows,* the 4-29 T cells were given 4 times every 2 d from day 0 to day 7 (*A, B*, and *C*), or day 7 to day 14 (*D*). *B,* when indicated, mice were given daily from day 0 to day 7, 5,000 IU of rhIL2 i.p. alone (*HT29 + 5,000 IU rhIL2*) or in combination with 2×10^6 4-29 cells (*HT29 + 4-29 5,000 IU rhIL2*) that were injected at day 0, 2, 4, and 7. *C,* mice receiving 4 repeated i.p. injections of 2×10^6 4-29 T cells preincubated with anti-CCR3 or control $IgG2_a$ mAbs. Experiments were done twice; *points,* mean tumor size of 7 to 10 mice (*bars,* SD; *, $P < 0.05$; **, $P < 0.003$).

and CCR3 were found on a large fraction of 4-29 and Vδ2$^+$ cells whenever the analysis was performed (1–3 weeks poststimulation; Fig. 2*B* and *C*). Thus, after *in vitro* expansion, anti-CMV Vδ2^{neg} and Vδ2$^+$ T cells express a comparable pattern of chemokine receptors among which CCR3, and are therefore potentially able to respond to monocyte chemoattractant protein-4 and macrophage inflammatory protein (MIP)-1δ, two CCR3-ligands produced by HT29 cells.

The chemokines secreted by HT29 cells induce *in vitro* migration of anti-CMV Vδ2^{neg} clones. To discover whether the chemokines secreted by HT29 cells were able to attract anti-CMV Vδ2^{neg} clones, we used a transwell assay. We found that HT29 culture supernatant significantly induced the motility of ~10% of 4-29 T cells (P = 0,0001; Fig. 3*A*). This effect was not simply due to chemokinesis because it was not observed when HT29 supernatant was added to both the upper and lower wells. Exogenous MIP-1δ triggered a dose-dependent migration of 4-29 cells (Fig. 3*B*). A blocking anti-CCR3 mAb abrogated MIP-1δ–dependent migration of Vδ2^{neg} γδ T cells (P = 0,004) and partially inhibited HT29 supernatant–mediated migration when compared with an isotype control mAb (P = 0,008; Fig. 3*C*). For this reason, we then tested the effect of the addition of pertussis toxin to the assay on HT29 supernatant–induced migration, which is intended to block the chemokine-mediated migration nonspecifically. As shown in Fig. 3*D*, HT29 supernatant–dependent migration was abrogated by pertussis toxin (P = 0,004). In conclusion, anti-CMV Vδ2^{neg} clones are able to migrate in response to a set of chemokines secreted by HT29 cells, among which CCR3-binding ligands may play a major role.

Distant and repeated injections of anti-CMV Vδ2^{neg} γδ T cells significantly delay the development of HT29 hypodermal tumors. Because anti-CMV Vδ2^{neg} cells migrated *in vitro* in response to chemokines secreted by HT29 cells, we tested whether distant (i.p.) injections of 4-29 clones could influence the growth of HT29 hypodermal tumors. As shown in Fig. 4*A*, a single i.p. injection of 2.10^6 4-29 T cells had a small but significant (P = 0.045) consequence on tumor growth. Notably, repeated i.p. injections of 4-29 T cells every other day for 1 week significantly (P = 0.003) improved the efficiency of the systemic immunotherapy. In treated mice, the appearance of the tumors was delayed, and the tumors were smaller throughout the experiment. The difference in tumor size between control and treated mice was obvious on hematoxylin eosin saffranin–stained tumor sections (Fig. 5); γδ-treated mice showed small and disorganized tumors 4 hours after the last inoculation of γδ

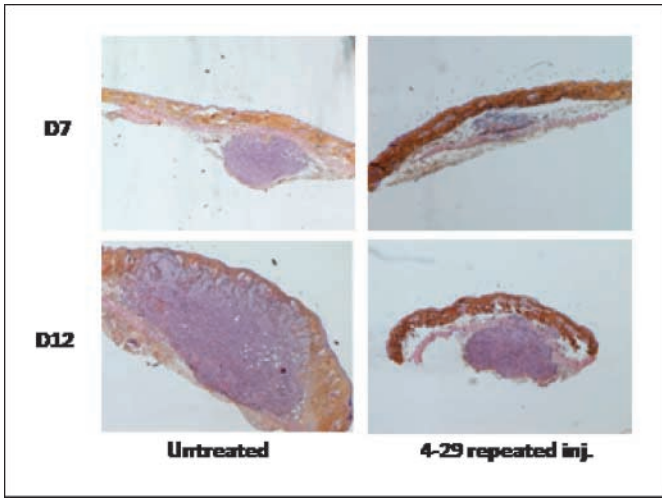

Figure 5. Mice treated with Vδ2^{neg} 4-29 T-cells show small and disorganized tumors. Histologic examination of HT29 tumors from untreated mice or mice treated as described in Fig. 4*A*, by repeated injections of 4-29 T-cells. Tumors were excised at day 7 (4 h after the last injection) or day 12 postinoculation of HT29 cells.

cells (Fig. 5, *top*). Supplementation with higher doses of rhIL2 did not enhance the antitumor activity of 4-29 clones (Fig. 4*B*). Preincubation of 4-29 clones with blocking anti-CCR3 mAb abrogated their inhibitory effect on tumor growth ($P < 0.003$; Fig. 4*C*). Figure 4*D* shows the results obtained with one group of mice given 4 i.p. injections of 2×10^6 4-29 T cells every 2 days, beginning when the tumor mass was measurable. Infusion of γδ T cells did not have any effect on subsequent tumor growth, which was similar to that of control mice. Thus, in our xenograft tumor model, anti-CMV Vδ2^{neg} γδ cells could influence tumor growth by efficiently limiting the initial tumor cell load.

The inhibitory effect of systemic γδ T-cell infusion on tumor growth correlates with the cytotoxic potential of anti-CMV Vδ2^{neg} γδ T cells. To test whether the inhibitory effect on HT29 tumor growth was specific to anti-CMV Vδ2^{neg} γδ cells, similar experiments were performed with Vδ2^{+} γδ T lymphocytes. As observed in Fig. 6*A*, repeated i.p. injections of Vδ2^{+} cells did not affect the growth of HT29 hypodermal tumors. This might be due to their inability to migrate toward the tumor cells, although *in vitro* expanded Vδ2^{+} γδ T cells also expressed CCR3 (Fig. 2*C*). To test whether HT29 culture supernatant could induce the migration of Vδ2^{+} γδ cells, we used the *in vitro* transwell assay. Approximately 4% of Vδ2^{+} γδ cells were able to migrate in response to chemokines secreted by HT29 cells (Fig. 6*B*).

We next wondered whether i.p. injection of anti-CMV Vδ2^{neg} clones could be cytostatic on cancer cells without being cytotoxic against them. We used A431 skin cancer cells against which 4-29 T cells did not display any significant cytotoxic activity (data not shown). As depicted in Fig. 6*C*, 4-29 clones were not able to inhibit the growth of A431 hypodermal tumors. However, such as HT29

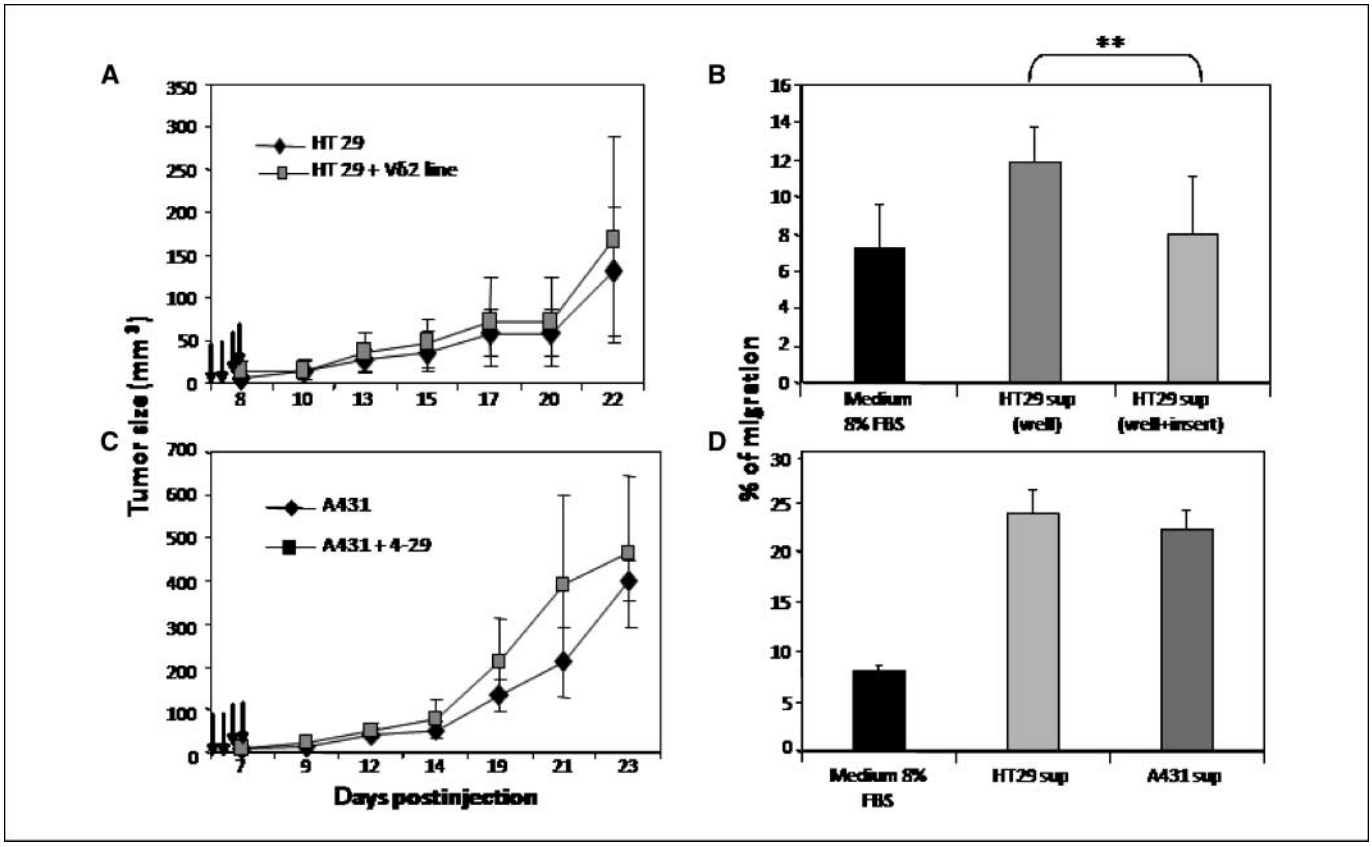

Figure 6. The inhibitory effects of γδ T-cell treatment on tumor growth correlates with the cytotoxic potential of anti-CMV Vδ2^{neg} γδ T cells. *A,* HT29 cells (1×10^5) were inoculated s.c. in mice in the absence or presence of repeated i.p. injections of Vδ2^{+} T cells. *B,* migration of noncytolytic Vδ2^{+} T cells using the transwell assay. *C,* mice received 1×10^5 A431 tumor cells s.c. in the absence or presence of repeated i.p. injections of 4-29 T cells. *D,* A431 tumor cells' supernatant-dependent migration of 4-29 T cells. *Points,* mean of tumor sizes; *bars,* SD (*A* and *C*). The experiments were reproduced twice on groups of five mice, and representative data from one experiment are shown. Differences between control and γδ-treated mice were not significant. *Columns,* percentages of migrating cells are represented ($n = 3$); *bars,* SD (*B* and *D*). Migration tests were done twice. **, $P < 0.005$.

cells, A431 cancer cells produced chemotactic factors that attracted 4-29 clones *in vitro* using the transwell assay (Fig. 6*D*). From all these analyses, we inferred that the effects of systemic *in vivo* γδ T-cell passive immunotherapy on tumor growth is dependent on the cytotoxic activity of the γδ effectors toward their targets, and that their migratory potential is probably necessary but not sufficient for their antitumor activity to be effective.

Discussion

Because severe combined immunodeficient mice can be successfully engrafted with human cells, they were used to evaluate γδ T-cell–based immunotherapy of cancer. Zheng and colleagues (26) described a partial arrest of the growth of hypodermal nasopharyngeal carcinomas when mice received i.v. injections of Vδ2Vγ9 T cells. Vδ2Vγ9 T cells transferred i.p. into severe combined immunodeficient mice induced increased survival after i.p. injections of various cancer cell lines (3, 27). Whereas most of these studies analyzed the antitumor potential of human Vδ2Vγ9 T cells from healthy donors, Lozupone and colleagues (28) showed that both Vδ2Vγ9 and Vδ1$^+$ cells expanded *ex vivo* from peripheral blood mononuclear cells of melanoma patients could prevent the growth of autologous tumors when coinoculated s.c. with cancer cells into severe combined immunodeficient mice. When γδ T cells were infused i.v., however, only Vδ1 cells could migrate toward s.c. implanted cancer cells and inhibit tumor growth (28).

The present study was carried out to evaluate the antitumor potential of CMV-induced Vδ2^{neg} γδ T cells *in vivo*, including their capacity to migrate in response to chemokines secreted by colon cancer cells as well as their ability to inhibit tumor growth. A complete analysis of the chemokines produced by HT29 cells had not been done previously. Here, we showed preferential production of inflammatory chemokines by HT29 cells, including the CCR3 ligands MIP-1δ and monocyte chemoattractant protein-4. In parallel, we provided evidence for CCR3-dependent migration of Vδ2^{neg} γδ T cells *in vitro* and *in vivo*. More importantly, we showed for the first time that virally induced Vδ2^{neg} γδ T cells can delay the growth of colon cancer cells *in vivo*, and that the antitumor effect is dependent on the specific activity of the γδ effectors toward their targets.

We showed that most of the *in vitro* expanded Vδ2^{neg} and Vδ2$^+$ γδT cells expressed CXCR3 and CCR3, and that their expression was stable postcellular activation. CXCR4, CCR7, and CCR9 were also found on Vδ2^{neg} and Vδ2$^+$ γδ cells, but their presence was transient in culture. Our results are in line with those of Glatzel and colleagues (29) who showed that CXCR4, in contrast to CXCR3, was down-regulated on phytohemaglutinin-activated Vδ2Vγ9 T cells. Although the physiologic relevance of CXCR3/CCR3 steady-state expression during γδ T-cell expansion is unclear at present, it is worth noting that those receptors bind so-called inflammatory chemokines, and thus could trigger γδ T-cell migration toward CMV-infected cells, as was recently reported for CXCR3$^+$ CMV-specific CD8$^+$ T cell (30), and colon cancer cells (the present study).

We showed that a systemic i.p. treatment with Vδ2^{neg} γδ T cells inhibited the growth of HT29 hypodermal tumors xenografted into immunodeficient mice. Repeated injections of γδ T cells increased the antitumor activity, as was shown in other xenograft models (26, 28). Vδ2^{neg} γδ T cells preincubated with anti-CCR3 mAb lost their ability to delay tumor growth, suggesting that CCR3-mediated migration is required for γδ antitumor activity; thus, even though some 4-29 clones had reached the tumor site in response to other chemokines than the CCR3 ligands, they were likely to be not sufficient to delay tumor growth.

The antitumor effect was short lived because once formed, the tumors developed with equivalent rates in untreated and γδ-treated mice. *In vitro*, HT29 cells were not sufficient per se to induce the proliferation of Vδ2^{neg} γδ T cells.[4] Moreover, the Rag$^{-/-}$γc$^{-/-}$ mouse environment is depleted of human growth factors important for the maintenance of human γδ T cells. Our attempt to improve γδ immunotherapy by increasing the concentration of rhIL2 was unsuccessful; this was probably not sufficient for long term maintenance of human γδ cells as already reported for peripheral Vδ2Vγ9 T cells in monkeys (31). This could clarify why 4-29 T cells only act at the initial steps of tumor development, when the effector/target cell ratio was in their favor. Indeed, systemic treatment with Vδ2^{neg} T cells had no effect on tumor growth when repeated injections were applied once the tumor formed.

Importantly, the antitumor activity was specific to anti-CMV Vδ2^{neg} γδ cells because Vδ2$^+$ had no effect on HT29 tumor growth. Moreover, 4-29 T cells could not inhibit A431 tumor growth, suggesting that the antitumor potential of anti-CMV Vδ2^{neg} T cells depends on their capacity to recognize their targets. The fact that Vδ2^{neg} γδ T cells exert their antitumor activity soon after the injection of HT29 cells makes it difficult to find Vδ2^{neg} γδ T cells within hypodermal tumors in the mouse model. We were not able to show unequivocally the presence of human CD3$^+$ cells by immunohistochemistry from day 3 and day 7 tumors (data not shown). Nevertheless, it seems very likely that 4-29 T cells had reached the s.c. tumor for the following reasons: (*a*) the mouse environment had little influence on the pattern of chemokine production by HT29 cells, which were shown to attract 4-29 clones *in vitro*, (*b*) the antitumor activity of 4-29 T cells was abrogated *in vivo* by addition of a blocking anti-CCR3 mAb, and (*c*) the inhibitory effect on tumor growth correlated with the capacity of γδ cells to recognize their target and be cytolytic against it.

Finally, our findings that CMV-reactive Vδ2^{neg} γδ cells are able to inhibit the growth of colon adenocarcinomas *in vivo* might be of relevance for transplant recipients. Indeed, these patients are at high risk for CMV reactivation soon after transplantation and often develop cancer. Effector memory CMV–reactive Vδ2^{neg} γδ T cells could then target the gut and exert their antitumor potential through their dual recognition ability. This might be relevant for different organs because we showed recently that epithelial cancer cells from different origins could be killed by CMV-reactive Vδ2^{neg} clones.[5] Vδ2^{neg} γδ cells recognize both CMV-infected cells and tumor cells in a TCR-dependent manner, which suggests the recognition of common antigens. Systemic treatment via autologous injections of Vδ2^{neg} γδ T cells might be tricky to envisage in humans. Once specific Vδ2^{neg} TCR ligands are identified, one could think of a way to activate Vδ2^{neg} γδ T cells *in vivo* as was recently shown with Vγ9Vδ2 cells (15, 32, 33).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

[4] Unpublished results.
[5] Unpublished results.

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