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Tumor-Specific Tc1, But Not Tc2, Cells Deliver Protective Antitumor Immunity[1]

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We investigated whether secretion of multiple cytokines by CD8$^+$ T cells is associated with improved protection against tumor challenge. We show that antitumor immunity induced by immunization with dendritic cells and a MHC class I-binding tumor peptide are dependent on secretion of IFN-γ but not IL-4 or IL-5 by host cells. To further address the role of IL-4 and IL-5 in antitumor immunity, tumor-specific TCR-transgenic CD8$^+$ T cells were activated in vitro to generate cytotoxic T (Tc) 1 cells that secrete high IFN-γ and no IL-4 or IL-5 or Tc2 cells that secrete IL-4, IL-5, and some IFN-γ. Both cell types killed target cells in vitro. Tc1 and Tc2 cells were adoptively transferred into syngeneic hosts, and their ability to protect against tumor challenge was compared. Tc1 cells were able to significantly delay tumor growth, whereas Tc2 cells or Tc2 cells from IFN-$\gamma^{-/-}$ donors had no effect. This was due to neither the inability of Tc2 cells to survive in vivo or to migrate to the tumor site nor their inability to secrete IL-4 and/or IL-5 in the presence of limiting amounts of anti-CD3. However, IFN-γ secretion by Tc2 cells was triggered inefficiently by restimulation with Ag compared with anti-CD3. We conclude that the ability to secrete "type 2" cytokines, and cytotoxic ability, have a limited role in antitumor immune responses mediated by CD8$^+$ T cells, whereas the capacity to secrete high amounts of IFN-γ remains the most critical antitumor effector mechanism in vivo. *The Journal of Immunology,* **2001, 167: 6497–6502.**

Tumor rejection can be successfully mediated by a variety of immune mechanisms. Tumors that have been genetically modified to constitutively secrete different cytokines become infiltrated by immune effector cells and are eventually rejected (1, 2). Depending on the specific cytokine secreted, the infiltrate may consist of cells as diverse as macrophages, eosinophils and dendritic cells (DC)[3] (2), thus indicating that there is redundancy in the effector mechanisms that are available to the immune system to mediate tumor rejection.

In physiological situations, infiltration of the tumor mass by effector cells is dependent on recognition of tumor Ags by specific T cells. Both CD4$^+$ and CD8$^+$ T cells have been demonstrated to play critical roles in this process (3). Removal of CD8$^+$ T cells causes enhanced tumor growth (4); conversely, the adoptive transfer of antitumor CD8$^+$ T cells results in delayed tumor growth (5–7). In addition, immunization procedures that activate CD8$^+$ T cell immune responses, e.g., DC vaccination, induce protection against tumor growth (8–10). The antitumor effect of CD8$^+$ T cells is presumably mediated through the ability of these cells to lyse tumor cells and to secrete cytokines upon recognition of Ag on tumor cells. CD4$^+$ T cells are also critical to the antitumor effect (11–15). Indeed, due to their capacity to secrete multiple cytokines, CD4$^+$ helper T cells may have a better ability to activate antitumor effector mechanisms. Both Th1 cytokines such as IFN-γ and TNF-α and Th2 cytokines such as IL-4, IL-5, and GM-CSF can contribute to the antitumor effect (16).

It has become apparent in recent years that CD8$^+$ cytotoxic T (Tc) cells are also heterogeneous in their ability to secrete cytokines. Similarly to Th cells, Tc cells can also be subdivided on the basis of the cytokines they secrete into Tc1, which secrete IFN-γ and TNF-α, and Tc2 which secrete IL-4, IL-5 and IL-10 (17–19). There is variability in the degree to which Tc2 cells can be polarized away from the default Tc1 phenotype of IFN-γ secretion and cytotoxic activity (17–19). In contrast, acquisition of the capacity to secrete IL-4 and IL-5 appears to be induced easily and reproducibly through in vitro culture in the presence of IL-4 (17–19). We took advantage of this observation to investigate whether Tc cells with a multiple cytokine secretion capacity also have an improved ability to mediate tumor rejection. We report here that whereas tumor-specific Tc2 cells have little or no effect on tumor growth, tumor-specific Tc1 cells have a strong antitumor effect.

Materials and Methods

Animals

Female and male C57BL/6J mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME). The "line 318" mouse strain, transgenic for a TCR specific for H-2D^b + fragment 33–41 of the lymphocytic choriomeningitis virus (LCMV) glycoprotein (LCMV$_{33-41}$) was kindly provided by Dr. H. Pircher (Institute of Medical Microbiology, University of Freiburg, Freiburg, Germany) (20). IL-4 and IL-5 gene knockout mice (21, 22) were obtained from Dr. M. Kopf (Basel Institute of Immunology, Basel, Switzerland). IFN-γ gene knockout mice (23) were purchased from The Jackson Laboratory and were backcrossed twice to line 318 mice to obtain IFN-$\gamma^{-/-}$ 318 progeny for experimental use.

All animals were maintained by brother × sister mating at the Biomedical Research Unit of the Wellington School of Medicine (Wellington, New Zealand). All in vivo experiments were approved by the Wellington School of Medicine Ethics Committee and performed according to institutional guidelines.

Tumor cell lines and in vitro culture medium

The tumor cell line LL-LCMV is a derivative of the Lewis lung carcinoma LLTC (C57BL/6J, H-2^b) that has been modified to express a minigene encoding LCMV$_{33-41}$ under the control of a CMV promoter (24). Unless

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[3] Abbreviations used in this paper: DC, dendritic cells; JAM, just another method; Tc, cytotoxic CD8$^+$ T lymphocyte; LCMV, fragment 33–41 of the lymphocytic choriomeningitis virus glycoprotein (LCMV$_{33-41}$).

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otherwise stated, all cultures were in IMDM (Life Technologies, Auckland, New Zealand) containing 2 mM glutamine, 1% penicillin-streptomycin, 5×10^{-5} M 2-ME (all from Sigma, St. Louis, MO), and 5% FCS (Life Technologies). LL-LCMV tumor cells were maintained in culture medium containing 0.5 mg/ml G418 (Life Technologies). The synthetic peptide $LCMV_{33-41}$ (KAVYNFATM) was obtained from Chiron Mimotopes (Clayton, Australia).

DC immunization and tumor challenge experiments

Bone marrow cells from C57BL/6J mice were cultured in medium containing 20 ng/ml IL-4 and 20 ng/ml GM-CSF as described (25). DC were harvested on day 7, loaded with peptide by incubation in medium containing 10 μM $LCMV_{33-41}$ for 2 h at 37°C, and washed three times in IMDM before in vivo injection. For in vivo tumor challenge experiments, 1×10^5 DC that had been loaded with peptide or left untreated were injected s.c. into the left flank on day 0. Mice were challenged with 5×10^5 tumor cells injected into the opposite flank on day 7. Mean tumor size was calculated as the mean product of bisecting tumor diameters as described (24).

In vitro generation of Tc1 and Tc2 cells and adoptive transfer

Single-cell suspensions from spleen and lymph nodes of line 318 mice or IFN-$\gamma^{-/-}$ 318 mice were washed in incomplete medium and resuspended at 2×10^6/ml. Cells were incubated for 2 h at 37°C to deplete adherent cells, and $CD4^+$ T lymphocytes and B lymphocytes were removed by Ab treatment (GK1.5-biotin) and magnetic depletion using Dynabeads conjugated to streptavidin or rat anti-mouse IgG (Dynal, Victoria, Australia). Recovered cells were 90–98% $CD8^+$ as determined by FACS staining.

The purified $CD8^+$ T cells were activated in six-well plates (Falcon, Oxnard, CA) that were coated overnight at 4°C with 5 μg/ml mouse anti-Armenian hamster IgG and 2 μg/ml mouse anti-Syrian hamster IgG (both from BD PharMingen, San Diego, CA) in 0.15 M NaCl, 0.015 M boric acid (pH 8.5). Plates were washed three times in PBS before purified anti-CD3 Ab (145-2C11) was added in PBS for 2 h at 37°C; then plates were washed three times in PBS. For Tc1 cell generation, naive $CD8^+$ T cells (1×10^6/ml) were added to the plates with 10 U/ml recombinant human IL-2, 10 ng/ml IL-6, and 2% 37.51 hybridoma supernatant (anti-CD28). For Tc2 cell generation, IL-4-containing supernatant was also added at 2000 U/ml. Cultures were maintained for 5 days, with replacement of medium and cytokines on days 2, 3, and 4. On day 5, cells were harvested and cultured in fresh six-well plates in medium containing 100 U/ml recombinant human IL-2 for 48 h, with replacement of IL-2 after 24 h. Cells were washed in medium before use and were at least 75% $V\alpha2^+V\beta8^+$ by FACS analysis.

C57BL/6J mice received up to 7×10^6 in vitro-generated $V\alpha2^+V\beta8^+$ Tc1 or Tc2 effector $CD8^+$ T cells by i.v. injection in the lateral tail vein. Tumor challenge took place 24 h later.

Cytotoxicity assay

The cytotoxic activity of Tc1 and Tc2 cells was determined using the just another method (JAM) test (26). Briefly, 5000 [^{3}H]thymidine-labeled EL4 cells were incubated in 96-well U-bottom plates with or without 0.01 μM $LCMV_{33-41}$ peptide for 1 h. Cultured $CD8^+$ effector T cells were added at different ratios and plates incubated for a further 3.5 h. Percent cytotoxicity was calculated from the mean of triplicate wells as described (24).

Cytokine secretion by Tc cells

Tc1 and Tc2 cells were restimulated at 1×10^6/ml in 96-well plates (Nunc, Roskilde, Denmark) coated with 10 μg/ml anti-CD3 or in the presence of 1×10^5 irradiated spleen cells and 10 μM $LCMV_{33-41}$ in complete medium for 48 h. Control cultures received no spleen cells. Cytokine-containing supernatants were harvested and analyzed by cytokine-specific ELISA using the appropriate Ab pairs (TRFK5 and TRFK4-biotin for IL-5; 11B11 and BVD6-24G2-biotin for IL-4; AN18 and XMGD6-biotin for IFN-γ) and mouse anti-IL-10 mAbs (BD Labware, Mountain View, CA) as described (27). Standard curves were generated using recombinant cytokines from hybridoma cultures: IL-4 (25 ng/ml), IL-5 (300 ng/ml), and IFN-γ (60 ng/ml) or IL-10 (5 ng/ml; BD Labware). Cytokine secretion by T cells or stimulator cells in medium alone was undetectable.

Flow cytometry

Anti-FcγRII (2.4G2), anti-CD8-FITC (2.43), and anti-Vβ8-biotin (KJ16) mAb were affinity purified from tissue culture supernatants using protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden) and conjugated to FITC or biotin. Anti-Vα2-PE was obtained from BD PharMingen. Cells were stained in PBS containing 2% FCS and 0.01% sodium azide as described (25) and analyzed on a FACSort (BD Biosciences, Mountain View, CA). Live cells were gated on the basis of forward and side scatter profiles.

CFSE labeling and adoptive transfer

In vitro-activated cells were washed in PBS and resuspended at 2×10^7/ml in PBS. CFSE (Molecular Probes, Eugene, OR) was used at 1.2 μM in PBS and added to cells for 8 min at room temperature. For migration and survival studies, lymph nodes, spleen, or s.c. tissue corresponding to the tumor site were taken from animals at the indicated times after tumor challenge. Single-cell suspensions were obtained and analyzed by flow cytometry.

Results

Antitumor immune responses induced by immunization with DC and MHC class I-binding tumor peptides require IFN-γ, but not IL-4 or IL-5

Immunization with DC loaded with tumor peptide is an effective method to induce antitumor $CD8^+$ T cell responses (8, 9, 28). We have previously shown that immunization with DC loaded with $LCMV_{33-41}$ induces protective immunity against challenge with the $LCMV_{33-41}$-expressing tumor cell line LL-LCMV (24). We used this experimental system to investigate the role of T cell-derived cytokines in the antitumor immune response. C57BL/6J control mice and IFN-$\gamma^{-/-}$, IL-$4^{-/-}$, and IL-$5^{-/-}$ mice were immunized with either C57BL/6J DC or DC loaded with $LCMV_{33-41}$ peptide or were left nonimmunized. One week later, all mice were challenged by s.c. injection of LL-LCMV tumor cells. All mice that received no DC immunization or that were immunized with DC only developed tumors that appeared and progressed with similar kinetics. As previously reported, C57BL/6J mice immunized with peptide-loaded DC showed delayed growth of LL-LCMV tumors as compared with mice injected with DC alone or nonimmunized mice (Fig. 1 and Ref. 24). In contrast, no delay in tumor

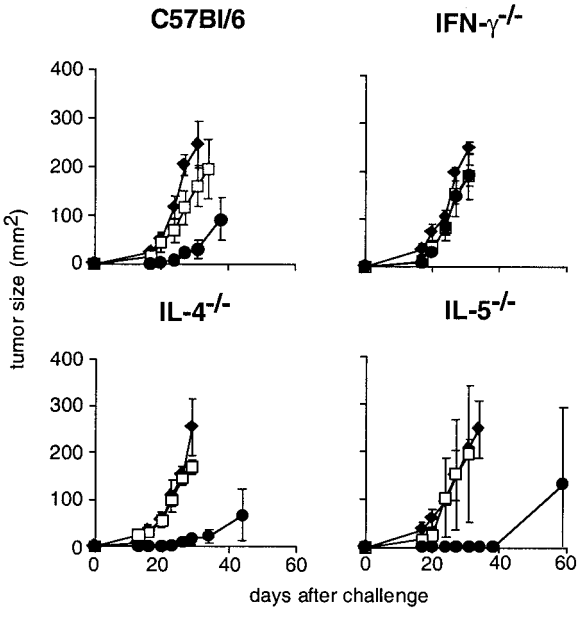

FIGURE 1. Immunization with DC and tumor Ag does not induce protective antitumor immune responses in IFN-$\gamma^{-/-}$ mice, whereas IL-$4^{-/-}$ and IL-$5^{-/-}$ mice mount good antitumor responses. C57BL/6J, IFN-$\gamma^{-/-}$, IL-$4^{-/-}$ and IL-$5^{-/-}$ mice were injected s.c. in the flank with 1×10^5 DC from C57BL/6J bone marrow cultures (□) or $LCMV_{33-41}$-loaded DC (●) or were left uninjected (◆). After 7 days, mice were challenged with 5×10^5 LL-LCMV tumor cells injected s.c. in the opposite flank. Tumor size is expressed as the mean product of bisecting tumor diameters ± SEM. Three to five mice were used in each experimental group. The experiment was repeated at least three times with similar results. Data from one representative experiment are shown.

growth could be demonstrated in IFN-$\gamma^{-/-}$ mice that had been immunized with peptide-loaded DC (Fig. 1), indicating that IFN-γ secretion by host cells is essential for the antitumor effect induced by DC immunization. Similar results have also been reported by Zitvogel et al. (11). Delayed tumor growth was observed in IL-$4^{-/-}$ and IL-$5^{-/-}$ recipient mice (Fig. 1), indicating that IL-4 and IL-5 were not essential to the antitumor immune response induced by DC immunization. In three separate experiments, tumor growth was always delayed further in IL-$4^{-/-}$ and IL-$5^{-/-}$ mice than in C57BL/6J mice, suggesting that these cytokines had an inhibitory effect on the antitumor immune response.

Generation of effector CD8$^+$ T cell populations secreting different cytokines

The apparent lack of involvement of IL-4 or IL-5 in DC-induced antitumor immune responses could indicate that IL-4- or IL-5-secreting T cells had not been induced by the immunization protocol. Furthermore, it was unclear from the DC immunization experiments whether IFN-γ played a role during the priming of antitumor T cells, or in the effector phase, or both. To resolve these issues, we examined the antitumor effect of T cells activated in vitro.

T cells were obtained from line 318 mice, which carry a transgenic TCR specific for D^b plus $LCMV_{33-41}$. The same Ag is also expressed on the LL-LCMV tumor cell line. We have previously shown that T cells from line 318 mice can proliferate in the presence of tumor cells in vitro and that their activation in vivo correlates with antitumor immunity (24, 29). Tc1 cells were generated by activating purified line 318 CD8$^+$ T cells in vitro with cross-linked anti-CD3 and anti-CD28 in the presence of IL-2 and IL-6; Tc2 cells were generated using identical conditions, but with the addition of IL-4. These conditions were chosen after extensive preliminary experiments in which the effect of cytokines such as IL-12 and neutralizing Abs to IL-4 and IFN-γ were tested and found to have little effect on the phenotype of the resulting cells (R. A. Kemp, manuscript in preparation). After 7 days of culture, T cells were harvested and restimulated with anti-CD3 to induce cytokine production. Fig. 2 shows that Tc1 cells generated from these cultures secreted IFN-γ, but no IL-4 or IL-5, and only small amounts of IL-10. In contrast, Tc2 cells secreted considerable quantities of IL-4, IL-5, and IL-10 but less IFN-γ than Tc1 cells. No culture conditions generating Tc cells that did not secrete IFN-γ were identified. Thus, to evaluate the role of IFN-γ in tumor immunity more directly, Tc2 cells were generated from line 318 mice that had been backcrossed to IFN-$\gamma^{-/-}$ mice. As shown in Fig. 2, Tc2 cells from these mice produced no IFN-γ but secreted larger amounts of IL-4, IL-5, and IL-10 than did Tc2 from IFN-$\gamma^{+/+}$ mice, suggesting that IFN-γ may have an inhibitory effect on the development of cells producing type 2 cytokines. In summary, we generated three populations of effector CD8$^+$ T cells, each with distinct cytokine secretion profiles: a "classical" Tc1 population, secreting IFN-γ but no IL-4 or IL-5; a Tc2 population, secreting IL-4, IL-5, and some IFN-γ; and an IFN-$\gamma^{-/-}$ Tc2 population secreting IL-4 and IL-5 but no IFN-γ.

To further characterize the functional properties of the three CD8$^+$ T cell populations, we investigated their in vitro cytotoxic activity. Labeled EL4 tumor cells were pulsed with $LCMV_{33-41}$ peptide, or left unpulsed and mixed with titrated numbers of Tc1, Tc2, or IFN-$\gamma^{-/-}$ Tc2 cells from in vitro culture. EL4 cells that had been left unpulsed were not killed by any of the cell types (Fig. 3). In contrast, EL4 cells loaded with specific Ag were killed by each of the three CD8$^+$ T cell populations to a similar extent, indicating that in vitro cytotoxicity was independent of the cytokine secretion potential.

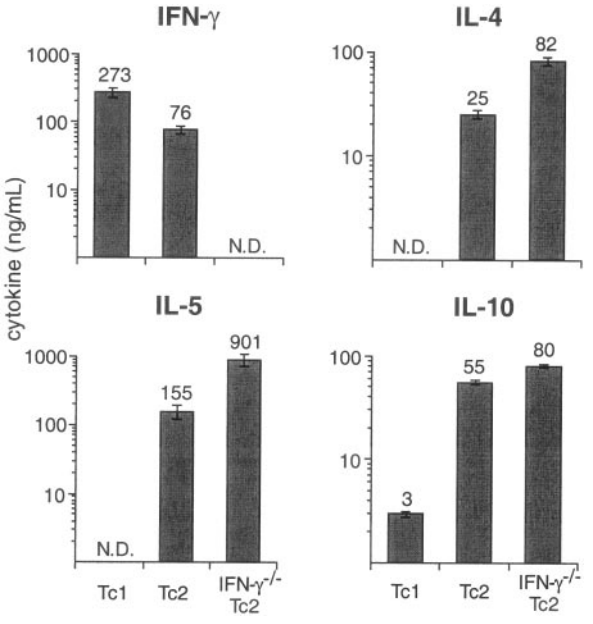

FIGURE 2. In vitro cytokine secretion by Tc1 cells, Tc2 cells, and IFN-$\gamma^{-/-}$ Tc2 cells. Tc1, Tc2, and IFN-$\gamma^{-/-}$ Tc2 cells were generated by culturing purified CD8$^+$ T cells from line 318 mice or IFN-$\gamma^{-/-}$ line 318 mice on anti-CD3-coated plates for 5 days in the presence of IL-2, IL-6, and anti-CD28. Tc2 cultures also contained IL-4. At the end of the 5-day culture, cells were harvested, expanded for 48 h in the presence of IL-2, and then restimulated for 48 h on plates coated with anti-CD3 at 10 μg/ml. Culture supernatants were assessed for cytokine content using a cytokine-specific sandwich ELISA. Results are expressed as nanograms per milliliter $\pm$ SD. N.D., Not detected. The experiment was repeated at least four times with similar results. Data from one representative experiment are shown.

Tc1 cells, but not Tc2 or IFN-$\gamma^{-/-}$ Tc2 cells, can transfer antitumor protective immunity in vivo

To evaluate the antitumor potential of in vitro-activated Tc1, Tc2, and IFN-$\gamma^{-/-}$ Tc2 cells, we adoptively transferred each of these cell types into C57BL/6J mice. Control mice received no adoptive transfer of T cells. All mice were challenged the next day with LL-LCMV tumor cells, and tumor growth was measured twice

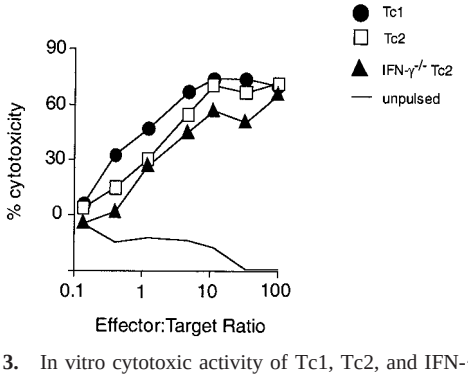

FIGURE 3. In vitro cytotoxic activity of Tc1, Tc2, and IFN-$\gamma^{-/-}$ Tc2 cells. Tc1, Tc2, and IFN-$\gamma^{-/-}$ Tc2 cells were cultured in vitro for 5 and 2 days as described in the legend to Fig. 2. At the end of the culture, cells were harvested, counted, and tested for cytotoxic activity on EL-4 targets using the JAM test. ● and ▲, EL-4 targets plus $LCMV_{33-41}$; no symbol, EL-4 target cells left unpulsed. The result from only one of the three unpulsed controls is shown because all three gave overlapping profiles. The experiment was repeated at least four times with similar results. Data from one representative experiment are shown.

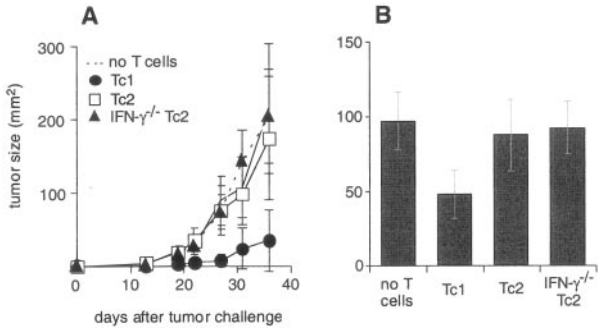

FIGURE 4. Tc1 cells protect against tumor challenge, whereas Tc2 and IFN-$\gamma^{-/-}$ Tc2 cells do not. *A*, Tumor growth in adoptive hosts of Tc1, Tc2, and IFN-$\gamma^{-/-}$ Tc2 cells. Tc1, Tc2, and IFN-$\gamma^{-/-}$ Tc2 cells were activated in vitro for 5 and 2 days as described in the legend to Fig. 2. At the end of culture, cells were harvested, and 7×10^6 Vα2$^+$Vβ8$^+$ cells were injected i.v. into C57BL/6J recipients. One day later, mice were challenged with 5×10^5 LL-LCMV tumor cells s.c. in the flank. Tumor size is expressed as the mean product of bisecting tumor diameters ± SEM in groups of three to seven mice. This experiment was repeated at least five times with similar results. Data from one representative experiment are shown. *B*, Cumulative results from five different experiments. Average tumor size ± SEM as measured at 25–27 days after tumor challenge.

weekly thereafter. Fig. 4*A* shows that mice that had received adoptive transfer of Tc1 cells had delayed tumor growth compared with control mice. In contrast, mice that had received Tc2 cells or IFN-$\gamma^{-/-}$ Tc2 cells showed no delay in tumor growth. Combined data from several experiments are shown in Fig. 4*B*. These results suggest that the production of IL-4 and IL-5 by CD8$^+$ T cells was not sufficient to mediate an antitumor effect. Surprisingly, Tc2 cells also failed to cause a significant delay in tumor growth (Fig. 4*A*) despite their in vitro cytotoxic activity and their ability to produce some IFN-γ as well as IL-4 and IL-5.

Tc1 and Tc2 cell populations have similar capacity to survive and recirculate in vivo

The results shown in Fig. 4 indicate that, unlike Tc1 cells, Tc2 cells were ineffective in controlling tumor growth. To address whether Tc1 and Tc2 cells differed with respect to their in vivo survival potential, numbers of CFSE-labeled Tc1 or Tc2 cells in the spleen, lymph node, and tumors were evaluated by FACS analysis at different times after adoptive transfer. As shown in Table I, the numbers of CFSE-labeled Tc1 or Tc2 cells recovered from each recipient mouse tissue were similar and remained relatively constant until day 13 after transfer. The numbers of CFSE$^+$ cells recovered at the tumor injection site were similar for Tc1 and Tc2 cells, indicating that both cell populations had the capacity to migrate to the tumor site. We conclude that the poor ability of Tc2 cells to mediate tumor protection was not due to their failure to survive in vivo or to reach the tumor site.

Secretion of cytokines by Tc1 and Tc2 cells requires similar degrees of TCR cross-linking and is dependent on the type of TCR ligand

We hypothesized that the inability of Tc2 cells to mediate an antitumor effect could be due to the requirement for a higher degree of TCR cross-linking to secrete cytokines. To test this hypothesis, Tc1, Tc2, and IFN-$\gamma^{-/-}$ Tc2 cells were stimulated in vitro in the presence of increasing concentrations of plate-bound anti-CD3, and their secretion of IFN-γ, IL-4, and IL-5 was compared. Fig. 5 shows that IFN-γ could be detected when Tc1 or Tc2 cells were cultured on plates coated with 0.1 μg/ml anti-CD3. Progressively higher IFN-γ secretion could be demonstrated when the amount of anti-CD3 was increased. As expected, IFN-γ secretion by Tc1 cells was higher than for Tc2 cells at each anti-CD3 concentration tested, and no IFN-γ could be demonstrated in cultures of IFN-$\gamma^{-/-}$ Tc2 cells. Secretion of IL-4 and IL-5 by Tc2 cells was also detected upon stimulation with 0.1 μg/ml anti-CD3, and at even lower concentrations in the case of IFN-$\gamma^{-/-}$ Tc2 cells. Together, these results do not support the possibility that Tc2 cells or IFN-$\gamma^{-/-}$ Tc2 cells may require higher degrees of TCR cross-linking than Tc1 cells to secrete cytokines.

To further address the lack of antitumor effect of Tc2 cells, we also compared cytokine secretion after restimulation with Ag or anti-CD3. Tc1 and Tc2 cells were generated as described and restimulated in the presence of anti-CD3 Abs or LCMV$_{33-41}$ and APC. The concentration of anti-CD3 used in these experiments was adjusted so that comparable secretion of IFN-γ by Tc1 cells could be obtained with both types of stimulation. As shown in Fig. 6, Tc1 cells secreted comparable amounts of IFN-γ whether restimulated with anti-CD3 or with LCMV$_{33-41}$. In contrast, Tc2 cells secreted ~14-fold less IFN-γ in the presence of LCMV$_{33-41}$ than anti-CD3. This finding was reproducible over many experiments; on average, the amount of IFN-γ secreted by Tc2 cells was 37 ± 9% of the amount produced by Tc1 after anti-CD3 stimulation and 8 ± 3% for stimulation with Ag and APC. Secretion of IL-4 and IL-5 by Tc2 cells was also compared in these experiments. As shown in Fig. 6, there was only a 2- to 3-fold decrease in the amount of IL-4 and IL-5 secreted by Tc2 cells after Ag stimulation, as compared with anti-CD3 stimulation. Addition of peptide alone resulted in undetectable amounts of secreted cytokine.

We conclude that the lack of antitumor activity of Tc2 cells could be due to their limited capacity to secrete IFN-γ after Ag-specific stimulation in vivo.

Table I. *Similar numbers of Tc1 and Tc2 cells are recovered from the spleens, lymph nodes, and tumor injection site of adoptive hosts at different times after transfer.*[a]

Time (days)	Tc1 Lymph Node	Tc2 Lymph Node	Tc1 Spleen	Tc2 Spleen	Tc1 Tumor Site	Tc2 Tumor Site
3	11.92	9.29	31.17	34.89	46.47	26.27
8	16.235 ± 1.619	16.085 ± 5.819	33.395 ± 16.08	37.355 ± 6.23	25.615 ± 11.915	25.115 ± 1.799
13	18.495 ± 4.603	13.523 ± 3.37	36.97 ± 8	29.487 ± 3.34	17.88 ± 8.457	9.39 ± 2.73

[a] Tc1 and Tc2 cells were activated in vitro for 5 and 2 days as described in the legend to Fig. 2 and labeled with CFSE; 7×10^6 Vα2$^+$Vβ8$^+$ cells were adoptively transferred into C57BL/6J hosts. One day later, recipient mice were challenged with 5×10^5 LL-LCMV tumor cells s.c. in the flank. Mice from each group were sacrificed at days 3, 8, and 13 after tumor challenge, and cell suspensions were prepared from spleen, lymph node, and s.c. tissue corresponding to the tumor site. The percent of CFSE$^+$ cells at each site was determined by FACS analysis. Data from one mouse (day 3) or average ± range from two mice (days 8 and 13) are shown. The experiment was repeated three times with similar results. Data from one representative experiment are shown.

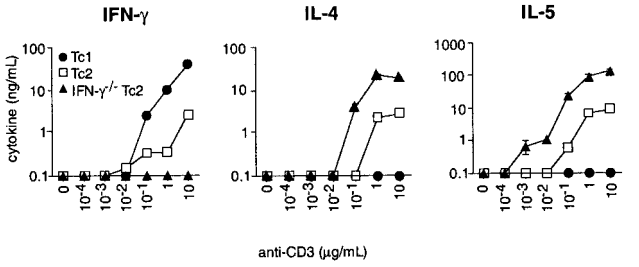

FIGURE 5. Tc1, Tc2 and IFN-$\gamma^{-/-}$ Tc2 cells require similar degrees of TCR cross-linking to secrete cytokines. Tc1 and Tc2 cells were activated in vitro for 5 and 2 days as described in the legend to Fig. 2 and then restimulated in vitro for 48 h on microtiter plates coated with decreasing concentrations of anti-CD3. Supernatants were tested for IFN-γ, IL-4, and IL-5 using cytokine-specific sandwich ELISA. Results are expressed in nanograms per milliliter ± SD. The experiment was repeated twice with similar results. Data from one representative experiment are shown.

Discussion

In this paper, we address the role of $CD8^+$ T cell-derived cytokines in tumor rejection. A number of reports have described how several cytokines (including IL-4, IL-5, and GM-CSF) can mediate antitumor effects in vivo through their ability to recruit other cell populations such as eosinophils, neutrophils, and DC (1, 2). In this study, we have generated in vitro tumor-specific $CD8^+$ T cells that have the ability to secrete multiple cytokines. Somewhat surprisingly, we observed that the ability to provide tumor protection correlated exclusively with the ability to secrete large amounts of IFN-γ, whereas the secretion of other effector cytokines such as IL-4 or IL-5 did not appear to contribute to the antitumor effect. We also observed that secretion of IFN-γ by Tc2 cells was not sufficient to mediate protective antitumor immunity in vivo.

The lack of antitumor effect of Tc2 cells could not be due to poor survival in vivo, because similar numbers of Tc1 and Tc2 cells were recovered from recipient mice (Table I). In support of our observation, data by other authors also show that T cells activated in the presence of IL-4, and presumably endowed of a Tc2 cytokine secretion pattern, survive and maintain their cytotoxic activity after in vivo transfer (30, 31).

"Type 2" cytokines, such as IL-4 or IL-5, have been reported to mediate a powerful antitumor effect in vivo (1, 2, 16, 32, 33). The adoptive transfer of tumor-specific Tc2 cells with cytokine secretion profiles similar to the Tc2 cells used here had clear effects on tumor growth in vivo (34). In addition, tumor cells producing IL-4 were readily rejected through a Th2/Tc2-dependent mechanism (32, 33, 35, 36). We were surprised to find that, in our system, Tc2 cells and IFN-$\gamma^{-/-}$ Tc2 cells did not appear to have an antitumor effect in vivo, despite their ability to secrete considerable amounts of IL-4 and IL-5 and lyse tumor target cells in vitro. It is possible, although unlikely, that the type of tumor used in our experiments is especially resistant to the action of type 2 cytokines. Alternatively, the amount of Ag expressed by our tumor cell line may be insufficient to attract a response from Tc2 cells. In support of this possibility, the LL-LCMV tumor cell line used in our studies was selected to express very low amounts of the $LCMV_{33-41}$ epitope recognized by the adoptively transferred Tc cells (24), because this was considered to represent a more physiological model of tumor antigenicity. In contrast, other studies have used tumor cells known to express high amounts of Ag (34, 37) or for which the expression of tumor Ags had not been characterized (32, 33).

In the course of our experiments, we detected no evidence that Tc2 cells may require especially high TCR cross-linking to secrete cytokines. Tc1 and Tc2 cells required stimulation with similar concentrations of anti-CD3 to secrete their typical cytokine pattern, whereas cytokine secretion by IFN-$\gamma^{-/-}$ Tc2 was detected at even lower amounts of anti-CD3. Because Tc1 cells were clearly able to mediate an antitumor effect, and this was presumably due to IFN-γ secretion, we propose that the conditions at the tumor site were likely to be conducive to cytokine secretion from not only Tc1 cells but also all types of Tc cells. However, it is not known whether recognition of tumor Ags by T cells occurs directly on the tumor cells, as is generally assumed, or on local APC that have taken up tumor Ag and present it to T cells. If recognition on local APC was required for any of the T cells used in this study, it is very unlikely that the low level of $LCMV_{33-41}$ epitope expressed by LL-LCMV would be sufficient to remain stimulatory after cross-presentation by local APC.

We do not know whether the antitumor effect of adoptively transferred Tc1 cells was due to their ability to kill tumor cells or to their ability to secrete cytokines at the tumor site. DC immunization experiments showed that IFN-γ secretion was critical to the antitumor effect (Ref. 11 and Fig. 1); thus, we presume that the effect of Tc1 cells was mediated, at least in part, via IFN-γ secretion. Although IFN-γ treatment significantly up-regulates MHC class I expression on LL-LCMV tumor cells in vitro (data not shown), it does not show a direct effect on tumor cell viability as has been reported in other systems (38). Thus, a synergistic effect between IFN-γ secretion and cytotoxic function may best explain our observations. Additional Tc1-derived cytokines may also contribute to the antitumor effect.

The Tc2 cells used in our study acquired the ability to produce IL-4 and IL-5 but also retained the capacity to secrete IFN-γ. Therefore, it was unexpected that Tc2 cells had no antitumor effect when, in our system, IFN-γ had such a dramatic role in tumor rejection (Fig. 1). This finding is probably explained by the observation that Tc2 cells had a much lower capacity to secrete IFN-γ when stimulated on Ag and APC than anti-CD3 (Fig. 6) and therefore were probably unable to secrete sufficiently high amounts of IFN-γ in the tumor context. The differential response to Ag or anti-CD3 stimulation was observed only for Tc2 cells and IFN-γ and did not extend to Tc1 cells or to other functions of Tc2 cells such as secretion of type 2 cytokines. We did not attempt to clarify the basis of this finding, but two possibilities appear especially likely. The first possibility is that secretion of higher amounts of the inhibitory cytokines IL-10 by Tc2 cells may regulate APC function in culture. Indeed, IL-10 has been reported to act in an APC-dependent manner to selectively inhibit IFN-γ secretion by $CD4^+$ and $CD8^+$ T cells, without affecting cytokine secretion by

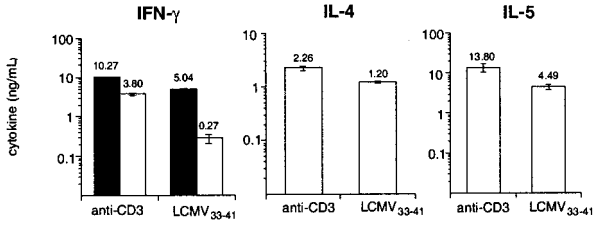

FIGURE 6. Secretion of IFN-γ, but not IL-4 or IL-5, by Tc2 cells is dependent on the type of TCR stimulus. Tc1 and Tc2 cells were activated in vitro for 5 and 2 days as described in the legend to Fig. 2 and then restimulated in vitro with 10 μg/ml anti-CD3 or with 10 μM $LCMV_{33-41}$ + 1×10^5 spleen cells for 48 h. Cytokine containing supernatants were tested for IFN-γ, IL-4, and IL-5 using cytokine-specific sandwich ELISA. Results are expressed in nanograms per milliliter ± SD. ■, Cytokines produced by Tc1 cells; □, cytokines produced by Tc2 cells. The experiment was repeated at least three times with similar results. Data from one representative experiment are shown.

Th2 clones (39). This effect obviously would not be observed when Tc2 cells are stimulated with anti-CD3, as was the case in our experiments. A second possibility is that changes in Ag recognition may result in the selective loss of some T cells responses, but not others, by Tc2 cells. A similar situation has been reported for the recognition of altered peptide ligands by T cell clones (40). Clearly, further analysis is required to assess whether secretion of IL-10, or differences in TCR signaling, are at the basis of our observation. Lastly, the lack of antitumor activity of Tc2 cells might not be due to insufficient production of IFN-γ, but to an inhibitory effect of type 2 cytokines on IFN-γ function at the tumor site (41, 42).

In conclusion, we propose that the production of IFN-γ by tumor-specific $CD8^+$ T cells is critical, although not sufficient, for antitumor immunity. Production of IL-4 and IL-5 is less critical, and their importance may depend on the tumor model used. Thus, our data indicate that antitumor immunotherapies should aim at inducing maximal Tc1 immune responses, even if this may compromise the ability to secrete other cytokines.

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