Decreased Tumor Surveillance after Adoptive T-Cell Therapy

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Abstract

The effect of cancer immunotherapy on the endogenous immune response against tumors is largely unknown. Therefore, we studied immune responses against murine tumors expressing the glycoprotein (GP) and/or nucleoprotein of lymphocytic choriomeningitis virus (LCMV) with or without adoptive T-cell therapy. In nontreated animals, CTLs specific for different epitopes as well as LCMV-GP–specific antibodies contributed to tumor surveillance. Adoptive immunotherapy with monoclonal CTLs specific for LCMV-gp33 impaired the endogenous tumor-specific antibody and CTL response by targeting antigen cross-presenting cells. As a consequence and in contrast to expectations, immunotherapy enhanced tumor growth. Thus, for certain immunogenic tumors, a reduction of tumor-specific B- and T-cell responses and enhanced tumor growth may be an unwanted consequence of adoptive immunotherapy. [Cancer Res 2007;67(15):7467–76]

Introduction

Adoptive transfer of large numbers of tumor-specific CTLs \((10^5–10^{13} \text{ cells per infusion})\) is currently being developed to treat cancer by changing the relative balance between tumor load and the immune response (1, 2). Clinically relevant responses after adoptive immunotherapy have resulted in disease stabilization or even in regression of solid tumors (1–3). These tumor responses are the proof of principle that adoptive immunotherapy in cancer patients may be efficacious. However, in a large proportion of patients, tumors progress despite adoptive immunotherapy (2–5). Loss of antigen or MHC class I expression on tumor cells, induction of T-cell anergy, and a limited survival of the transferred CTLs are well defined mechanisms that explain the lack of therapeutic efficacy in some situations (6–9). In addition, the adoptive transfer of high numbers of tumor-specific CTLs may also influence the endogenous tumor-specific immune response, but this has not been analyzed yet.

The idea that the immune system is involved in the control of tumors dates back to the 1950s when Burnet and Thomson formulated the immunosurveillance hypothesis (10). More recent experiments analyzing the spontaneous tumor development showed a role of the adaptive immune system and of IFN-\(\gamma\) in the control of solid tumor formation (11). Although various effector mechanisms of the immune system are involved in tumor control, most experimental data support the notion that eradication of solid tumors rests predominantly on CD8+ T lymphocyte activity (3, 12). Tumor-specific CTLs are induced directly either by tumor cells that migrate to secondary lymphoid organs or by dendritic cells cross-presenting tumor antigens on MHC class I molecules (13–15). Both mechanisms may be inefficient during early tumor development; therefore, antigenic solid tumors may grow outside secondary lymphoid organs due to immunologic ignorance (16, 17).

In the present study, we analyzed the immunologic control of lymphocytic choriomeningitis virus (LCMV)-glycoprotein (GP)–expressing and/or LCMV-nucleoprotein (NP)–expressing tumor cells and found that antibodies and CTLs are involved in the control of tumors transplanted as solid fragments. We therefore studied the effects of adoptively transferred monoclonal LCMV-gp33–specific CTLs (P14) on tumor-specific immune responses and tumor growth. Adoptive immunotherapy using P14 cells was therapeutically efficacious against tumors when applied to tumor-bearing immunodeficient RAG-1\(^{-/-}\) mice. In contrast, adoptive immunotherapy increased tumor growth in immunocompetent C57BL/6 (BL/6) mice by reducing the endogenous tumor-specific immune response. Our results suggest that in situations where a polyspecific endogenous immune response is necessary for tumor control, adoptive immunotherapy against a single epitope of a tumor antigen may enhance tumor progression.

Materials and Methods

Mice. BL/6 and (BL/6 \(\times\) BALB/c) \(F_1\) mice were from Harlan. RAG-1\(^{-/-}\), H8 (18), P14 TCR-tg (line 318; ref. 19), and Jj\(^{-/-}\) (20) mice were from the Institute for Laboratory Animals (Zurich, Switzerland). RAG-1\(^{-/-}\) \(\times\) OT-1 mice were from C. Mueller (Immunopathology, University of Berne, Berne, Switzerland). All animal experiments were approved by the local Animal Committee.

Cell lines and constructs. B16-gp33 cells have been described (21). MC57G (MC; ref. 13), B16.F10 (B16; ref. 21), and D2 cell lines (14) were transfected either with a plasmid expressing a codon-optimized LCMV-GP open reading frame (ORF) under control of elongation factor 1\(\alpha\) promoter (M369, -GP\(_{\alpha}\)). The codon usage of the LCMV-GP was optimized for high-level expression in mammalian cells and the corresponding cDNA synthesized by the company GeneArt. The construct has been deposited under DQ886924 at Genbank. Alternatively, an otherwise identical construct was used containing a leucine to proline mutation at position 110 of the LCMV-GP ORF (pDP12, -GP\(_{\alpha}\)). pDP12 was generated with standard two-way PCR-based site-directed mutagenesis using M369 as a template. Gene transfection was carried out using Superfect (Qiagen).

Tumor growth in vivo. Tumor cells (2 \(\times\)10\(^6\)) were injected s.c. in the flank of RAG-1\(^{-/-}\) mice. Solid tumor fragments were explanted, cut into pieces of 2 \(\times\) 2 mm, and transplanted s.c. in the flank of recipient mice. Growth was measured with a caliper and the volume was calculated by the formula \(V = \pi \times abc / 6\), where \(a, b, \text{ and } c\) are orthogonal diameters. Single-cell suspensions \(ex vivo\) were prepared by cutting solid tumors into small
Cancer Res 2007; 67: (15). August 1, 2007 7468 www.aacrjournals.org

pieces and smashing through a stainless steel grid. The suspension was then depleted from cell clumps by a quick-spin centrifugation. Single-cell suspensions of *in vitro* cultures were generated after incubation in nonenzymatic cell dissociation solution (Sigma-Aldrich).

**Activation of P14 cells.** *In vitro* activated P14 cells were generated as described (21) and purified for CD8\(^+\) T cells using MACS (Miltenyi Biotec). P14 CTLs were activated in *vivo* by injecting 10\(^6\) naive P14 splenocytes to BL/6 mice i.v. followed by an injection with 10\(^5\) plaque-forming units LCMV-WE. Eight days after, infection-activated P14 CTLs were used directly in a \(^{51}Cr\) release assay.

**Dendritic cells.** Dendritic cells were generated as described (22). For immunization, matured dendritic cells were labeled with gp33 and/or ng996 peptides (10\(^{-6}\) mol/L) for 1.5 h at 37°C. Pulsed dendritic cells (2 \(\times\) 10\(^5\)) were injected i.p. or into foot pad of BL/6 mice. In certain experiments, peptide-pulsed dendritic cells were labeled with 1 \(\mu\)mol/L CFSE in PBS for 10 min at 37°C at a cell concentration of 2 \(\times\) 10\(^4\) cells/mL.

\(^{51}Cr\) release assay and intracellular IFN-\(\gamma\) staining assay. Primary \(^{51}Cr\) release assays without restimulation or secondary \(^{51}Cr\) release assays after *in vitro* restimulation for 5 days were done as described earlier (21, 23). For intracellular staining, lymphocytes (10\(^6\)) per well were stimulated for 5 h with the relevant peptide (10\(^{-10}\) mol/L/well) in the presence of 5 \(\mu\)g/mL brefeldin A (Sigma-Aldrich) and recombiant 25 units/mL interleukin-2 (IL-2; Sigma-Aldrich) in 96-well round-bottomed plates (Carl Roth). Cells were stained for surface molecules and fixed with 4% paraformaldehyde and cell membranes were permeabilized with Perm buffer (PBS, 2% FCS, 5 mmol/L EDTA, 0.1% saponin, 0.2% Na\(_3\)). Flow cytometry with the mAb KL25 (Fig. 1A). LCMV-GP peptide expression at the cell surface was tested by flow cytometry with the mAb KL25 (Fig. 1A). LCMV-GP peptide presentation on MHC class I molecule was tested by analyzing intracellular IFN-\(\gamma\) production of LCMV-immune splenocytes after *in vitro* stimulation with the relevant tumor cell line (Fig. 1A).

We transplanted MC-GP\(_{i/e}\) and B16-GP\(_{i/e}\) tumor fragments of \(\sim 2 \times 10^6\) to \(5 \times 10^6\) tumor cells s.c. into the flank of BL/6 mice. MC cells transfected with the empty puromycin resistance vector (MC-vector) or parental B16 tumor cells served as controls. We then analyzed tumor growth, LCMV-GP-specific CTL, and antibody induction. MC-GP\(_{i/e}\) tumor fragments did not grow in immuno-competent BL/6 mice, whereas fragments of MC-vector-transfectants grew (Fig. 1B). Similarly, growth of B16-GP\(_{i/e}\) tumors was delayed when compared with nontransfected parental B16 tumors (Fig. 1C). Both MC-GP\(_{i/e}\) and B16-GP\(_{i/e}\) tumors efficiently induced LCMV-gp33-specific CTLs in BL/6 mice (Fig. 1B and C). Moreover, CTL responses against LCMV-gp276 were induced (Fig. 1B). In addition, LCMV-GP–binding antibodies were mounted in MC-GP\(_{i/e}\) and B16-GP\(_{i/e}\) tumor-bearing mice (Fig. 1B and C).

Earlier experiments using MC LCMV-GP transfectants with a nonoptimized plasmid under a cytomegalovirus promoter indicated that solid tumors grew in the absence of a CTL response due to inefficient cross-presentation of the antigen by professional antigen-presenting cells (APC; refs. 13, 14, 16). Our experiments now show that MC and B16 transfectants with our novel optimized LCMV-GP construct efficiently primed CTLs, even if tumors were transplanted as solid fragments (Fig. 1B and C). We therefore analyzed if tumor cell transfectants with the optimized LCMV-GP construct are able to induce LCMV-GP-specific CTLs via cross-priming. We tested cross-priming in (BALB/c \(\times\) BL/6) F1 mice immunized with H-2\(^d\)-positive D2 fibrosarcoma cells transfected with LCMV-GP. D2-GP\(_{i/e}\) (H-2\(^d\)) cells injected as single-cell suspension i.p. induced a potent H-2\(^d\)-gp33-restricted CTL response (Fig. 1D). Similarly, the transplantation of D2-GP\(_{i/e}\) tumor fragments to (BALB/c \(\times\) BL/6) F1 recipient mice induced gp33-restricted CTLs (data not shown).

Taken together, our results indicate that LCMV-GP–transfected B16 and MC tumors efficiently induce specific antibodies and CTLs that may be involved in the control of tumor development.

**Immunologic control of MC-GP\(_{i/e}\) and B16-GP\(_{i/e}\) tumor formation.** The immunologic effector mechanisms involved in tumor control of MC-GP\(_{i/e}\) tumors were analyzed after transplantation to RAG-1\(^{-/-}\) mice, BL/6 mice either untreated or depleted of CD8\(^+\) T cells, and B-cell-deficient JH\(^{-/-}\) mice either left untreated or depleted of CD8\(^+\) T cells (Supplementary Fig. S1A). MC-GP\(_{i/e}\) tumors regularly grew in the absence of B and T cells in RAG-1\(^{-/-}\) mice but not in immunocompetent BL/6 mice. Fifty percent of tumors grew in BL/6 mice when compared with RAG-1\(^{-/-}\) mice. MC-GP\(_{i/e}\) tumors did not grow in JH\(^{-/-}\) mice. However, 100% of tumors grew in CD8\(^+\) T-cell–depleted BL/6 mice but tumor growth kinetics were slower than in RAG-1\(^{-/-}\) mice. MC-GP\(_{i/e}\) tumors did not grow in JH\(^{-/-}\) mice. However, 100% of tumors grew in CD8\(^+\) T-cell–depleted BL/6 mice but tumor growth kinetics were slower than in RAG-1\(^{-/-}\) mice. Similarly, growth of B16-GP\(_{i/e}\) tumors was enhanced in RAG-1\(^{-/-}\) mice (Supplementary Fig. S1B). Growth of B16-GP\(_{i/e}\) tumors was slightly enhanced in JH\(^{-/-}\) mice when compared with BL/6 mice. B16-GP\(_{i/e}\) tumors grew faster in CD8\(^+\) T-cell–depleted BL/6 mice and the most rapid tumor formation was observed in CD8\(^+\) T-cell–depleted JH\(^{-/-}\) mice (Supplementary Fig. S1B). Therefore, specific CTLs and antibody-producing B cells are involved in the control of MC-GP\(_{i/e}\) and B16-GP\(_{i/e}\) tumors. To analyze if tumor rejection is predominantly mediated by CTL...
responses against the immunodominant epitope gp33 or if other CTL specificities are also involved in tumor control, tumors were transplanted to H8 mice. These mice ubiquitously express a COOH-terminal truncated version of LCMV-GP (amino acids 1–50) and gp33-specific CTLs are absent due to thymic deletion (18). All MC-GP<sup>ie</sup> tumor fragments were rejected when transplanted to H8 mice, indicating that other CTL specificities against LCMV-GP than gp33 were sufficient for tumor rejection (Supplementary Fig. S1A).

Adoptive immunotherapy in the presence or absence of endogenous immune control of tumors. Recent experiments have suggested that due to more efficient in vivo activation, proliferation, and survival, transferred naive, early effector, or central memory CTLs are most efficient in tumor control (28–30). We therefore first studied the consequences of adoptively transferred naive P14 CTLs on tumor growth. MC-GP<sup>ie</sup> tumors regularly grew when transplanted to RAG-1<sup>−/−</sup> mice. The adoptive transfer of 10<sup>7</sup> splenocytes from naive P14 mice containing ~10<sup>6</sup> LCMV-gp33-specific CTLs at the day of tumor transplantation to RAG-1<sup>−/−</sup> mice delayed or even prevented tumor growth (Fig. 2A). Surprisingly, if naive P14 splenocytes were transferred to BL/6 mice at the day of MC-GP<sup>ie</sup> tumor transplantation, most transplanted tumors grew (Fig. 2A). Titration experiments revealed that MC-GP<sup>ie</sup> tumor growth was observed in 7 of 10 tumors when 10<sup>7</sup> naive P14 splenocytes were transferred, in 3 of 6 tumors with 10<sup>6</sup> P14

![Figure 1](https://www.aacrjournals.org/7469.png)

**Figure 1.** MC-GP<sup>ie</sup> and B16-GP<sup>ie</sup> tumor cells elicit B- and T-cell responses. A, MC-GP<sup>ie</sup> (filled curve) and MC-GP<sup>i</sup> (empty curve) or B16-GP<sup>ie</sup> (filled curve) and B16-GP<sup>i</sup> (empty curve) tumor cells were analyzed for surface expression of LCMV-GP by flow cytometry. Isotype control (dotted line). Splenocytes of LCMV-immune BL/6 mice were stimulated for 5 h in vitro with different numbers of MC-GP<sup>ie</sup> or MC-GP<sup>i</sup> and B16-GP<sup>ie</sup> or B16-GP<sup>i</sup> tumor cells. Results are shown as percentage of IFN-γ<sup>+</sup>CD8<sup>+</sup> T cells after stimulation with relevant cell lines. Stimulation with gp33 peptide-pulsed splenocytes was taken as 100%. B, fragments of MC-GP<sup>ie</sup> or empty vector-transfected MC tumors were transplanted s.c. to BL/6 mice and tumor growth was followed. C, similarly, B16-GP<sup>ie</sup> or parental B16 fragments were transplanted s.c. to BL/6 mice and tumor growth was followed. Fourteen days after transplantation (MC-GP<sup>ie</sup> in B; B16-GP<sup>ie</sup> in C), splenocytes were analyzed in an intracellular IFN-γ assay after restimulation with the relevant peptide. Results are given as numbers ± SE of three to four mice per group. Values in parentheses show percentage of IFN-γ<sup>+</sup>CD8<sup>+</sup> T cells of nonpeptide-pulsed cultures. Thirty days after transplantation, LCMV-GP binding antibodies in the serum were measured in an ELISA (BL/6 mice with MC-GP, fragments or naive BL/6 mice served as controls; B and C). D, (BALB/c × BL/6) F1 mice were immunized twice (days 0 and 7) i.p. with 2 × 10<sup>7</sup>D2-GP<sup>ie</sup> tumor cells. Splenocytes were tested in a 5<sup>1</sup>Cr release assay against gp33-labeled EL-4 target cells 15 d later. LCMV-immune and naive (BALB/c × BL/6) F1 mice served as controls. Points, mean of six transplanted tumors per group; bars, SE (B and C). Numbers in (B and C) indicate total number of transplanted tumors at this experimental condition, summarized from two to six experiments. *, P < 0.05.
splenocytes, and in 0 of 6 tumors when $10^5$ P14 splenocytes were transferred (Fig. 2A). Ten of 12 transplanted MC-GP$_{1e}$ tumors grew when transplanted s.c. to naive P14 mice (Fig. 2B). In contrast, if a comparable number of MC-GP$_{1e}$ tumor cells ($2 \times 10^6$) was injected s.c. as single-cell suspensions in BL/6 or P14 mice, tumor cells were regularly controlled by the immune system and no tumor formation was observed (Fig. 2B; data not shown). Adoptive immunotherapy with tumor-specific CTLs has been reported to be most efficient in lymphopenic hosts, mainly due to homeostatic proliferation of tumor-specific CTLs (28). To test if P14 CTLs are most efficient in lymphopenic hosts, mainly due to homeostatic proliferation, adoptive immunotherapy with tumor-specific CTLs has been reported to be effective in lymphopenic hosts, mainly due to homeostatic proliferation, adoptive immunotherapy with tumor-specific CTLs in vivo. In these mice, the CD8$^+$ T-cell compartment is filled by ovalbumin-specific CTLs. It has been shown before that homeostatic proliferation of adoptively transferred TCR transgenic T cells is abrogated in RAG-1$^{-/-}$ × OT-1 mice (31). Because RAG-1$^{-/-}$ × OT-1 mice possess only ovalbumin-specific CTLs, transplanted MC-GP$_{1e}$ tumors were not controlled by the endogenous ovalbumin-specific immune system (Fig. 2C). Adoptive transfer of $10^5$ naive P14 splenocytes at the day of tumor transplantation reduced but did not control tumors in RAG-1$^{-/-}$ × OT-1 mice. This indicates that the transfer of tumor-specific P14 CTLs was also effective in a nonlymphopenic host, which lacks tumor-specific B and T cells.

Growing MC-GP$_{1e}$ tumors were explanted at the end of the experiment and tumor cells were analyzed for expression of LCMV-GP epitopes. MC-GP$_{1e}$ tumor cells isolated from RAG-1$^{-/-}$ mice efficiently stimulated IFN$\gamma$ production by splenocytes of LCMV-infected memory mice (Fig. 2D). In contrast, tumor cells isolated from RAG-1$^{-/-}$ mice after transfer of naive P14 CTLs (Fig. 2D) or isolated from P14 mice (data not shown) restimulated LCMV memory responder cells less efficiently. This indicated that CTL escape variants developed in the presence of P14 T cells.

In clinical protocols, adoptive immunotherapy is often done with in vitro activated CTL clones or lines (2, 3). We therefore activated P14 CTLs in vitro in the presence of gp33 peptide and IL-2 for 3 days. To mimic a clinically more relevant situation, treatment with activated P14 CTLs was done in mice with established tumors.

Figure 2. Fibrosarcoma growth after adoptive T-cell therapy. A to C, MC-GP$_{1e}$ tumor fragments were transplanted s.c. to RAG-1$^{-/-}$ (A), BL/6 (A), P14 (B), and RAG-1$^{-/-}$ × OT-1 (C) mice. Mice either received naive P14 splenocytes i.v. at the day of transplantation or were left untreated (A and C). MC-GP$_{1e}$ tumors were either transplanted as tumor fragments or injected as single-cell suspensions s.c. (2 × $10^5$ cells) to P14 mice (B). Black arrows, day of adoptive immunotherapy. Tumor growth was measured at the indicated days after transplantation. Points, mean of six to eight transplanted tumors; bars, SE. Numbers indicate total number of transplanted tumors at this experimental condition, summarized from one to two experiments. D, MC-GP$_{1e}$ tumor cells were explanted 31 d after transplantation from untreated RAG-1$^{-/-}$ mice and from RAG-1$^{-/-}$ mice treated with naive P14 CTLs at the day of transplantation. Single-cell suspensions of these explanted tumors were used as stimulators in an IFN$\gamma$ secretion assay of LCMV-immune splenocytes. Numbers ± SE indicate percentage of CD8$^+$ T cells producing IFN$\gamma$. Stimulation with gp33 peptide-pulsed splenocytes was taken as 100%. Values in parentheses indicate percentage of IFN$\gamma$+CD8$^+$ T cells after stimulation with nontransfected MC tumor cells. *, $P < 0.05$; **, $P < 0.01$. NS, not significant.
Mice with s.c. transplanted B16-GP§e tumor fragments were treated with 10⁶ activated P14 CTLs when tumor diameter reached ~0.3 cm in RAG-1⁻/⁻ (day 22 after transplantation) and BL/6 mice (day 32 after transplantation). Again, activated P14 CTLs efficiently reduced growth of established tumors in RAG-1⁻/⁻ mice but enhanced tumor growth in BL/6 mice (Fig. 3B). Similarly to the results observed after transfer of naive P14 CTLs (Fig. 2C), activated P14 CTLs efficiently controlled B16-GP§e tumors in the absence of homeostatic proliferation in RAG-1⁻/⁻ × OT-1 mice (Fig. 3B).

Most known tumor-associated antigens (TAA) are intracellular proteins and are not expressed on the cell surface (32). Therefore, we analyzed the influence of adoptive transfer of P14 CTLs on growth of tumors with intracellular LCMV-GP expression (B16-GP§). Similarly to the results obtained with tumors expressing LCMV-GP on the cell surface, tumor growth was enhanced after transfer of activated P14 CTLs to B16-GP§-tumor bearing BL/6 mice (Fig. 3C).

As shown above, LCMV-GP transfectants induced CTLs specific for various epitopes (Fig. 1B) as well as antibodies against LCMV-GP (Fig. 1B and C). To analyze if adoptive immunotherapy may be effective if targeted against a tumor that expresses only a single immunogenic epitope, B16 tumors transfected with LCMV-gp33 epitope were analyzed (21). The parental B16 tumor cells are poorly immunogenic and usually few cells injected s.c. as single-cell suspension are sufficient for tumor formation in immunocompetent hosts (33). The adoptive transfer of naive 10⁷ P14 splenocytes delayed B16-gp33 tumor formation in immunodeficient RAG-1⁻/⁻

Figure 3. Melanoma growth after adoptive T-cell therapy. A, B16-GP§e tumor fragments were transplanted s.c. to RAG-1⁻/⁻ and BL/6 mice. Mice received 10⁷ naive P14 splenocytes i.v. where indicated. Black arrows, day of adoptive immunotherapy. B16-GP§e tumor cells were explanted 36 d after transplantation from untreated BL/6 mice and from BL/6 mice treated with 10⁷ naive P14 splenocytes. Single-cell suspensions of these explanted tumors were used as stimulators in an IFNγ secretion assay of LCMV-immune splenocytes. Numbers ± SE indicate percentage of CD8⁺IFNγ⁺ T cells. Stimulation with gp33 peptide-pulsed splenocytes was taken as 100%. Values in parentheses show percentage of IFNγ⁺CD8⁺ T cells after stimulation with nontransfected B16 cells. B16-GP§e tumors were either transplanted as tumor fragments or injected as single-cell suspension (2 x 10⁶) s.c. to P14 mice (A). B, B16-GP§e tumor fragments were transplanted s.c. to RAG-1⁻/⁻, BL/6, and RAG-1⁻/⁻ × OT-1 mice. Mice either received 10⁷ in vitro activated P14 splenocytes i.v. after tumor was already established or were left untreated. C, B16-GP§ tumor fragments were transplanted s.c. to BL/6 mice. Mice either received 10⁷ in vitro activated P14 CTLs i.v. to untreated or were left untreated. D, B16-gp33 tumor fragments were transplanted s.c. to RAG-1⁻/⁻ and BL/6 mice. Mice either received 10⁷ naive P14 splenocytes i.v. or were left untreated. Tumor growth was measured in (A–D) at the indicated days after tumor transplantation. Points, mean of 4 to 16 transplanted tumors; bars, SE. Numbers indicate total number of transplanted tumors at this experimental condition, summarized from one to two experiments. *, P < 0.05.
mice but also in immunocompetent BL/6 mice (Fig. 3D). Importantly, B16-gp33 and B16-GPi+e transfectants were lysed similarly in vitro, suggesting that the observed differences in tumor growth are not due to differences in P14-mediated tumor cell lysis (Supplementary Fig. S2B).

Adoptive immunotherapy impairs endogenous tumor-specific immune responses. Our results revealed that the same immunotherapy protocol controlled tumor growth in the absence of a tumor-specific immune response but enhanced tumor growth in the presence of an endogenous immunosurveillance. This prompted us to analyze the effect of the adoptive transfer of gp33-specific P14 T cells on endogenous CTL and antibody responses. To reach high frequencies of specific CTLs after immunization, MC-GPi+e tumor cells were injected i.p. and the resulting CTL response was analyzed in an intracellular IFNγ assay (Fig. 4A). MC-GPi+e tumor cells induced an LCMV-gp33- and gp276-specific immune response. To analyze if and how adoptive immunotherapy influenced the endogenous immune response to a second nonrelated tumor antigen, MC-GPi+e/NP tumor cells were cotransfected with the NP of LCMV (MC-GPi+e/NP). MC-GPi+e and MC-GPi+e/NP tumor cells were pulsed before immunization with gp33 or gp276. MC-GPi+e or with MC either treated with P14 CTLs or left untreated were tested in a 51Cr release assay 21 days after tumor injection. Compared to nonpeptide-pulsed target cells, empty symbols, gp33-pulsed target cells, or gp276-pulsed target cells, Filled symbols, B16-GPi+e tumor fragments were transplanted s.c. to BL/6 mice and were either treated with 10^7 naive P14 splenocytes at the day of transplantation or left untreated. Thirty days after transplantation, LCMV-GP binding antibodies in the serum were measured by ELISA. BL/6 naive mice served as negative control. Points, mean of three mice per group; bars, SE.

Adoptive immunotherapy against tumors expressing different model tumor antigens. To analyze if and how adoptive immunotherapy against one tumor antigen influences the endogenous immune response to a second nonrelated tumor antigen, MC-GPi+e tumor cells were cotransfected with the NP of LCMV (MC-GPi+e/NP). MC-GPi+e and MC-GPi+e/NP tumor cells were lysed comparably in vitro by activated P14 CTLs (Supplementary Fig. S2A). MC cells transfected only with LCMV-NP served as controls (MC-NP). MC-GPi+e/NP tumor fragments did not grow after s.c. transplantation to BL/6 mice (Fig. 5A). Surprisingly, growth of MC-GPi+e/NP tumors was enhanced in BL/6 mice if naive P14 splenocytes were transferred at the day of tumor transplantation (Fig. 5A).
The influence of adoptively transferred P14 CTLs on the endogenous immune response against LCMV-GP and LCMV-NP was analyzed in an intracellular IFNγ assay after i.p. injection of MC-GPi+/e/NP tumor cells as single-cell suspension. BL/6 control mice mounted CTL responses against LCMV-gp33, LCMV-gp276, and LCMV-<i>n</i>p396 (Fig. 5D). In contrast, CTL responses to LCMV-gp276 and LCMV-np396 were markedly reduced in mice treated with naïve (data not shown) or activated P14 CTLs (Fig. 5D). Similarly, the transfer of naïve P14 CTLs to mice transplanted with solid MC-GPi+/e/NP tumor fragments reduced the np396-specific CTL response when compared with nontransfected controls (data not shown). In contrast, adoptive immunotherapy with LCMV-gp33–specific CTLs did not enhance growth of MC-NP single transfected control tumors (Fig. 5B) nor reduced np396-specific CTL response (Fig. 5C). This excludes a nonantigen-specific immunosuppression after transfer of monoclonal CTLs. Thus, adoptive immunotherapy with CTLs specific for one epitope of a tumor antigen impaired the induction of CTL responses directed against other epitopes of the same antigen but also against other nonrelated antigens.

**Adoptive immunotherapy targets antigen-presenting dendritic cells.** To discriminate between effects of adoptively transferred P14 CTLs on the tumor itself and on antigen cross-presenting cells, D2-GPi+/e tumor cells were transplanted to (H-2<sup>d</sup> x H-2<sup>b</sup>) F1 mice and then treated with CTLs from (BALB/c x P14) F1 mice. In this experimental system, H-2<sup>b</sup> gp33–restricted P14 CTLs will not recognize LCMV-GP epitopes presented on D2-GPi+/e tumors (H-2<sup>d</sup>) but they will recognize gp33 epitopes cross-presented on host APCs (H-2<sup>b</sup> x H-2<sup>d</sup>). The adoptive transfer of naïve 10<sup>7</sup> (BALB/c x P14) F1 CTLs markedly enhanced growth kinetics of D2-GPi+/e tumors (Fig. 6A). This experiment indicates that adoptively transferred tumor-specific CTLs enhance tumor growth by influencing antigen cross-presenting dendritic cells rather than the tumor itself.

We next analyzed the influence of adoptively transferred naïve P14 T cells on induction of CTLs after immunization with LCMV-gp33 and LCMV-np396 peptide-pulsed dendritic cells. Peptide-pulsed dendritic cells (2 x 10<sup>5</sup>) were injected i.p., and 8 days later, CTL responses were analyzed in an intracellular IFNγ assay. Dendritic cells efficiently induced LCMV-gp33– and LCMV-np396–specific immune responses in BL/6 control mice (Fig. 6B). Adoptive transfer of naïve P14 CTLs slightly enhanced the frequency of LCMV-gp33–specific CTLs but reduced the frequency of LCMV-np396–specific CTLs (Fig. 6B). We further analyzed the effect of adoptively transferred P14 CTLs on the presence of gp33–expressing dendritic cells in local lymph nodes. Dendritic cells were derived from BL/6 mice and were pulsed with gp33 and np396 or only np396, labeled with the fluorescent dye CFSE, and...
injected s.c. into the foot pad of the hind legs of BL/6 mice. At the same time, one group of mice received $2 \times 10^6$ in vitro activated P14 CTLs. Twenty hours later, the number of CFSE$^+$ dendritic cells in the draining popliteal lymph node was analyzed (Fig. 6C). The number of CFSE$^+$ dendritic cells in lymph nodes of control mice was significantly higher (absolute number, 725 in the draining popliteal lymph node was analyzed (Fig. 6C). The number of CFSE$^+$ dendritic cells in lymph nodes of control mice was significantly higher (absolute number, 725

Discussion

To characterize the immunosurveillance of fibrosarcomas and melanomas and to analyze the effect of adoptively transferred tumor-specific CTLs, we established novel tumor transfectants with high expression of LCMV-GP as model tumor antigen. Transfected tumor cells efficiently induced CTLs against different epitopes of LCMV-GP as well as antibodies binding to LCMV-GP. Both effector arms contributed to immunosurveillance of transplanted melanomas and fibrosarcomas.

The surprising and unexpected finding of our study was that adoptive immunotherapy targeting a single epitope of an antigen enhanced tumor growth of solid immunogenic tumors by reducing the endogenous immune response to antigens or epitopes not targeted by the adoptive immunotherapy. The adoptively transferred P14 CTLs acquired effector function comparable with the endogenous gp33-specific CTLs, as indicated by IFN$\gamma$ production and lysis of peptide-pulsed target cells ex vivo. Adoptive immunotherapy of H-2$d$ tumors with BALB/c $\times$ P14 CTLs in (H-2$^d$ $\times$ H-2$^b$) F1 mice showed that tumor growth was enhanced due to a direct effect of adoptively transferred T cells on antigen cross-presenting cells. Earlier experiments using minor histocompatibility antigens or epitopes of ovalbumin and LCMV have shown that CD8$^+$ T cells can cross-compete for different epitopes if presented by the same APC (34–36). We confirmed these earlier results using dendritic cells pulsed with gp33 and np396. In addition, we showed that adoptive transfer of monoclonal CTLs reduced the polyspecific endogenous immune response against a tumor to the epitope specificity of the transferred CTLs. The nature of this cross-competition has been proposed to be either of steric nature, competition for cytokines in the local environment, or inactivation of the APC via cell killing (37). Similarly to our study, it has been shown before that the CD8$^+$ T-cell–dependent elimination of dendritic cells injected for vaccination limited the induction of antitumor immunity (35, 38, 39). Here, we report that after adoptive transfer of monoclonal CTLs, mechanisms of cross-competition on antigen-presenting dendritic cells may in some situations abrogate the
induction of an endogenous tumor-specific CTL response and, as a consequence, enhance tumor growth.

It is currently ill defined how therapeutically manipulating one arm of the immune system affects the anticancer or cancer-promoting properties of the other. However, it has been reported that antibodies and/or B cells may in some situations reduce CTL-mediated tumor control and enhance tumor growth (40). In addition, Siegel et al. (41) have shown that active immunization with an oncoprotein mutant in cancer-prone mice resulted in enhanced tumor growth, probably by inducing oncoprotein-specific antibodies. An enhanced tumor growth after adoptive immunotherapy has not been reported thus far. Earlier studies have documented that adoptive immunotherapy is effective when applied before or simultaneously with a tumor challenge with tumor cells injected as single-cell suspension (21, 42, 43). Similarly, in our study, MC-GP<sub>1</sub> and B16-GP<sub>1</sub> tumors cells injected in suspension s.c. were regularly rejected by tumor-specific P14 T cells. In contrast, eradication of established tumors by adoptive T-cell therapy is difficult and dependent on tumor size and the number of transferred CTLs (42, 44). Therefore, cancer cells in solid tissue may be much more difficult to reject by the immune system.

Poorly immunogenic tumors expressing only weak CTL epitopes and tumors that are MHC class I negative may grow largely uncontrollably by the endogenous immune system. For example, poorly immunogenic B16 tumors grew similarly in pme1 TCR transgenic and control mice (8). If poorly immunogenic tumors are transfected with a single immunodominant epitope, targeting this epitope by monospecific CTLs results in tumor control. We showed in the present study that adoptive transfer of P14 CTLs improved the immunologic control of solid tumor fragments of B16 tumor cells transfected with the single immunodominant epitope of LCMV-GP (B16-gp33) in both immunodeficient RAG-1<sup>−/−</sup> and immunoprophilic BL/6 mice. Similarly, P14 T cells have been shown to cause regression of 3LL-A9-gp33 and B16-gp33 tumors (21, 45). However, even cancer cells transfected with a strong antigen as entire protein have been shown to grow in immuno-competent hosts without evidence of T-cell activation (14, 46, 47).

Consequently, transplantation of these tumors directly in TCR transgenic mice or the transfer of large numbers of tumor-specific CTLs will neither reduce immunosurveillance nor enhance tumor growth (46).

Therefore, earlier studies analyzing adaptive T-cell therapy focused on tumors that grow largely independent of the endogenous immune system. In none of these earlier experimental models, an enhanced tumor growth after adoptive immunotherapy has been reported. These poorly immunogenic tumor models may be representative for a large group of human tumors expressing low immunogenic differentiation and tissue-specific TAA (32). In contrast, in the present study, we analyzed adoptive immunotherapy against a highly immunogenic tumor that is controlled by endogenous immunosurveillance. This experimental system may be more comparable with human tumors expressing highly immunogenic TAAs, such as cancer-testis antigens or viral-tumor antigens (32).

In summary, the net therapeutic effect of adoptively transferred tumor-specific CTLs against the tumor is determined by effects not only on the tumor itself but also on tumor antigen cross-presenting cells. A therapeutic benefit is seen in situations where transferred CTLs are sufficient to eliminate the tumor completely. This situation has rarely been reached in clinically manifested tumors thus far, especially not in more advanced disease. However, protocols combining adoptive T-cell therapy with nonmyeloablative lymphodepletion, antigen-specific vaccination, and cytokine therapy or the repetitive transfer of large numbers of monospecific CTLs have been shown to cause regression of established tumors (8, 48). If the solid tumor is not eliminated completely by the adoptively transferred CTLs, the consequences of the transferred CTLs on antigen cross-presenting dendritic cells gain importance. Here, the positive therapeutic effect on the tumor may be reduced or in some situations even reversed by the reduction of the endogenous immunosurveillance to nontargeted tumor antigens. Therefore, adoptive transfer of tumor-specific CTLs may influence the delicate equilibrium between the immune control and tumor growth, depending on the effect of the therapy on the endogenous tumor immunosurveillance.

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