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Efficient Eradication of Subcutaneous but Not of Autochthonous Gastric Tumors by Adoptive T Cell Transfer in an SV40 T Antigen Mouse Model

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In stomach cancer, there is a need for new therapeutic strategies, in particular for the treatment of unresectable tumors and micrometastases. We investigated the efficacy of immunotherapy in an autochthonous model of gastric cancer, the CEA424-SV40 T Ag (TAg) transgenic mice. Treatment efficacy against both the autochthonous tumors and s.c. tumors induced by the derived cell line mGC3 were assessed. In wild-type mice, a dendritic cell vaccine loaded with irradiated tumor cells combined with CpG oligonucleotides induced efficient cytotoxic T cell and memory responses against mGC3 s.c. tumors. In contrast, neither s.c. nor autochthonous tumors responded to vaccination in CEA424-SV40 TAg mice, indicating tolerance to the SV40 TAg. To examine whether tumors in these mice were principally accessible to immunotherapy, splenocytes from immune wild-type mice were adoptively transferred into CEA424-SV40 TAg transgenic mice. Treated mice showed complete regression of the s.c. tumors associated with intratumoral infiltrates of CD8 and CD4 T cells. In contrast, the autochthonous gastric tumors in the same mice were poorly infiltrated and did not regress. Thus, even in the presence of an active anti-tumoral T cell response, autochthonous gastric tumors do not respond to immunotherapy. This is the first comparison of the efficacy of adoptive T cell transfer between transplanted s.c. tumors and autochthonous tumors in the same animals. Our results suggest that in gastric cancer patients, even a strong anti-tumor T cell response will not efficiently penetrate the tumor in the absence of additional therapeutic strategies targeting the tumor microenvironment. The Journal of Immunology, 2010, 185: 2580–2588.

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omach cancer is one of the major causes of cancer-related deaths worldwide, with a mortality rate second only to that for lung cancer (1). Survival has only moderately improved during the last half-century, despite advances in diagnosis and therapy (1). The most effective treatment for gastric cancer is radical gastrectomy, but the overall 5-yr survival rate is low, ranging from 5 to 15%. There is a need for new therapeutic strategies, especially for the treatment of unresectable tumors and for the prevention of recurrences through micrometastases (2). One approach is the use of cancer vaccines that aim to enhance the immune response against defined tumor-associated Ags or mixtures of tumor epitopes. Alternatively, in the presence of cancer-related immune suppression, therapeutic strategies delivering passive immunity may be attempted, such as the adoptive transfer of anti-tumor T lymphocytes or of specific Abs.

Few immunological approaches for the treatment of gastric cancer have been reported to date, all of them with limited success. A meta-analysis of studies investigating the efficacy of adjuvant treatment with the immune modulator polysaccharide K suggested a small survival benefit for patients after gastric cancer resection (3). Furthermore, a combination of chemotherapy and immunotherapy with polyadenyl:polyuridylic acid improved survival of resected patients compared with chemotherapy alone in a phase III study (4). Active immunization has been attempted in patients with advanced gastric cancer using vaccinations with different tumor-associated peptides (5, 6). In many cases, cellular and humoral immune responses against tumor Ags could be detected in PBMCs or serum of patients, but clinical responses were rarely observed. Thus, monitoring of immunological parameters in the blood is not predictive of the clinical success of immunotherapy. In contrast, there is evidence that increased cytotoxic T cell infiltration in the tumor positively correlates with improved survival in gastric cancer patients (7–9). However, since most immunotherapy trials have been performed in patients with either resected or unresectable tumors, little is known about the extent and functional importance of intratumoral lymphocyte infiltration induced by these therapies. Relevant preclinical models are therefore essential to investigate the effects of immunotherapy in gastric cancer within the tumor itself. CEA424-SV40 TAg transgenic mice (CEA424-TAg) spontaneously develop gastric tumors at an early age, allowing for the study of the in situ effects of immunotherapy (10). These mice, which express the SV40 large T Ag under the control of the carcinoembryonic Ag (CEA) promoter, develop gastric tumors in the pyloric region of the stomach by the age of ~40 d, with a penetrance of 5 to 15%. There is a need for new therapeutic strategies, especially for the treatment of unresectable tumors and micrometastases.
of 100%. The tumors continue to grow invasively until the age of 100–130 d, when mice die due to pyloric obstruction (10). With the gradual progression from premalignant lesions to invasive carcinoma, this model reflects the development of stomach cancer in humans. A number of tumorigenic cell lines have been derived from CEA424-TAg gastric tumors; these can be injected s.c. to induce tumors. This allows a stepwise optimization of immunotherapeutic strategies from s.c. to autotochonous gastric tumors (11). In contrast to frequently used transplantable tumor cell lines, such as the Panc-02 pancreas tumor (12) or the C26 colon carcinoma (13), these gastric tumor lines have comparatively slow growth rates that more closely mirror the development of a spontaneous tumor.

To study the influence of local factors and tumor site on the efficacy of immunotherapy, we have developed a dendritic cell (DC) vaccination combined with TLR9 activation to induce a strong anti-tumoral response against s.c. tumors in wild-type mice. We transferred splenocytes from immunized wild-type mice into CEA424-TAg transgenic mice and additionally induced s.c. CEA424-TAg tumors in the same mice. Immunotherapy of mice simultaneously bearing s.c. and autochtuous gastric tumors allowed direct comparison of the efficacy of a tumor-specific T cell response at different tumor sites.

Materials and Methods

Mice and cell line

Female C57BL/6 mice were purchased from Harlan-Winkelmann (Borchen, Germany). CEA424-TAg mice, described previously (10), were bred heterozygously from transgenic male mice. Animal studies were approved by the local regulatory authority (Regierung von Oberbayern, Munich, Germany). The murine cell line mGC3 (11) was maintained in RPMI 1640 medium supplemented with 10% FCS Gold (PAA Laboratories, Coelbe, Germany), 2 mM L-glutamine, and 100 μg/ml streptomycin. For tumor induction, 3 × 105 tumor cells were injected s.c. into the flank unless indicated otherwise. The mGC3 cell line was passaged for >6 mo.

Application of CpG

The fully phosphorothioate-modified CpG-A oligodeoxynucleotide 2336 (5′-GGGGACGACGTCGTGGGGGGG-3′) or CpG-B oligodeoxynucleotide 1826 (5′-TCCATGACGTTCCTGACGTT-3′) (Coley Pharmaceutical Group, Westchester, IL) was administered twice or orally. For oral administration, 50 μg of CpG in 50 μl of saline solution was administered twice weekly by gavage.

Generation of bone marrow-derived DCs

Bone marrow cells were harvested from murine femur and tibia of C57BL/6 mice, and erythrocytes were lysed with ammonium chloride buffer (BD Biosciences, Heidelberg, Germany). Cells were cultured in complete medium (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 μg/ml streptomycin, and 1 U/ml penicillin) supplemented with 20 ng/ml GM-CSF. After maturation, nonadherent cells were collected, suspended in PBS, and before vaccination, 3 × 106 DCs were added to induce DC maturation. After maturation, nonadherent cells were collected, suspended in PBS, and 2 × 105 cells were injected s.c. together with 100 μg of CpG-B into the flank opposite the tumor unless indicated otherwise. An additional 100 μg of CpG-B was injected peritumorally around established tumors.

Adoptive transfer

Successfully immunized donor mice were sacrificed and splenocyte suspensions prepared by mechanical tissue disruption and subsequent erythrocyte lysis in ammonium chloride buffer. Total splenocytes from each donor mouse (75–150 × 106/mouse) were transferred i.v. and i.p. in equal amounts to two recipients (one wild-type, one CEA424-TAg recipient).

NK cell activation, intracellular IFN-γ staining, and in vivo cytotoxicity assay

For analysis of NK cell activation, splenocytes from non-tumor-bearing mice were isolated 22 h after CpG-A injection, stained with anti-NK1.1-PerCP and anti-CD69-PE (BD Biosciences), and analyzed by flow cytometry. For assessment of intracellular IFN-γ production by CDS T cells, 106 splenocytes were stimulated overnight with 5 μg/ml of a 1:1 mixture of the immunodominant SV40 peptides T1 (H-2Db) and T4 (H-2Kb) (Prom- mune, Oxford, UK). Intracellular IFN-γ was quantified in CDS T cells by flow cytometry using an IFN-γ secretion assay kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the instructions of the manufacturer. For the in vivo cytotoxicity assay, splenocytes from untreated C57BL/6 mice were stained with 1 μM CFSE (Molecular Probes, Eugene, OR) and were pulsed for 1 h with 5 μg/ml each of the SV40 peptides T1 and T4 (CFSElow). A reference population of unpulsed cells was stained with 0.1 μM CFSE (CFSElow). Both cell populations were injected at a ratio of 1:1 into untreated mice or mice vaccinated with DC/CpG-B. Twenty-four hours later, CFSE-labeled target cells from the spleen were analyzed by flow cytometry. Specific lysis was calculated as follows: specific lysis (%) = 100% × [1 - (CFSEhigh cells/CFSElow cells)/(CFSEhigh cells in naive mice/CFSElow cells in naive mice)].

Immunohistology

Tumors were excised with the surrounding tissue and skin and were snap frozen in liquid nitrogen. To permit the evaluation of highly standardized tissue areas, all frozen s.c. tumor samples were bisected perpendicularly to the skin at their widest diameter prior to cutting with the cryostat. Gastric tumors were cut sagittally at the pylorus at the widest apparent diameter. Five-micrometer sections were fixed with acetone and blocked with 10% donkey serum (Chemikon International, Temecula, CA). Rat anti-mouse CD4 (BioLegend, San Diego, CA), rat anti-mouse CD8, and Syrian hamster anti-mouse CD3ε (BD Biosciences, Heidelberg, Germany) were used as primary Abs. Biotin F(ab′)2 donkey-anti-rat IgG, biotin F(ab′)2 goat anti-Syrian hamster IgG, and alkaline phosphate streptavidin (Jackson ImmunoResearch Laboratories, Newmarket, U.K.) were used as detection reagents. Tissues were counterstained with hemalaun. The number of infiltrating cells was assessed by systematically counting nonoverlapping visual fields of the whole area of the tumor section using a Zeiss Axiosvert 200 (Oberkochen, Germany) light microscope equipped with a ×40 oil immersion objective. One visual field covered a surface of 0.25 mm2. Necrotic areas and blood vessels were excluded from the evaluation. Five-micrometer sections from tumors and additionally excluding necrotic and partially differentiated tissue, were assessed histologically on hemalaun-stained sections using AxioVision software (Zeiss).

Statistical analysis

All data are presented as means ± SEM and were analyzed as appropriate by an unpaired Student t test or by the Mann-Whitney U nonparametric test. Survival was analyzed by a log-rank test. Statistical analysis was performed using SPSS (Chicago, IL) software. Mean tumor sizes are shown until the death of more than two mice per group. The last measured value for a dead mouse was included in calculation of the mean for remaining time points.

Results

DC vaccination combined with immunostimulatory CpG oligonucleotides prevents growth of s.c. CEA424-TAg tumors

We have previously developed and in a succession of steps optimized a protocol for DC-based therapy in the s.c. C26 tumor model. DCs are loaded in vitro with irradiated tumor cells and are activated by CpG-B oligonucleotides prior to administration to enhance the anti-tumor response (14). The tumor-specific CD8 immune response is additionally increased by coincubating CpG-B oligonucleotides together with the DCs (15, 16). Injection of CpG-B peritumorally around established tumors further improves the efficacy of the vaccine (17). In this study, we used the syngeneic tumor cell line mGC3 derived from CEA424-TAg gastric tumors (11) to investigate whether CEA424-TAg tumor cells can be targeted by this effective DC vaccination protocol. Additionally, CpG-A oligonucleotides were given to stimulate NK cells to maximize treatment efficacy. CpG-A injections have been shown to reduce tumor growth through the induction of anti-tumoral NK responses (18).

Wild-type mice were pretreated by weekly injections of mature DCs pulsed with irradiated mGC3 cells, combined with the immunostimulatory CpG-B oligonucleotide 1826 (DC/CpG-B). After the third of four vaccinations, s.c. tumors were induced with either
low (5 × 10^5 cells) or high (3 × 10^6 cells) numbers of tumor cells. Additionally, vaccination groups received injections of the CpG-A oligonucleotide 2336, and two booster vaccinations with Ag-pulsed DCs and CpG-B were administered. In both vaccination groups we observed a striking improvement in survival over a period of 8 mo (Fig. 1A). Indeed, whereas all untreated animals developed large tumors, in both vaccination groups tumor onset was either prevented entirely or delayed for >40 d.

We then examined the relative contribution of NK cell–activating CpG-A injections to the efficacy of the DC vaccination. Upon treatment with CpG-A injections alone, tumor growth was partially delayed, but all treated mice developed a tumor, and survival was only moderately increased (Fig. 1B). In contrast, treatment with DC/CpG-B was highly effective, as seven of nine mice remained tumor-free for >240 d. Although NK cells were activated by CpG-A treatment (Fig. 1C), the addition of CpG-A to the DC/CpG-B vaccination did not improve treatment efficacy, suggesting that NK cells were not the main anti-tumor effector cell type. To characterize the T cell response triggered by DC/CpG-B vaccination, intracellular IFN-γ production was measured in CD8 T cells from vaccinated mice. We observed a clear increase in IFN-γ production after stimulation with the immunodominant SV40 TAg peptide epitopes I and IV (Fig. 1D). Furthermore, the specific lysis in vivo of target cells loaded with the SV40 peptides reached nearly 20% in vaccinated mice (Fig. 1E), indicating that a CD8+ cytotoxic T cell immune response against the SV40 TAg can be triggered by DC vaccination. Because irradiated tumor cells represent the Ag source in the DC vaccine, it is probable that in addition to the anti-SV40 TAg response, immune responses against other tumor Ags are induced. Thus, DC/CpG-B vaccination can generate prophylactic immunity against slow-growing s.c. tumors.

**FIGURE 1.** DC vaccination combined with immunostimulatory CpG oligonucleotides prevents growth of s.c. CEA424-TAg–derived tumors. A, Wild-type mice received four weekly injections of mature DCs pulsed with irradiated mGC3 tumor cells and CpG-B starting on day −14 prior to tumor challenge (arrows). Animals were challenged s.c. on day 0 with either 5 × 10^5 or 3 × 10^6 mGC3 cells. The mice received additional injections of CpG-A on days −2, 0, 3, 7, 14, 21, 28, and 38 after tumor induction and two additional DC/CpG-B vaccinations on days 77 and 84 or were left untreated. Survival and mean tumor sizes are shown (n = 5 for all groups). Vaccination significantly improved survival (p = 0.002 for 5 × 10^5 mGC3 cells and 3 × 10^6 mGC3 cells compared with untreated groups). Tumor growth was reduced by vaccination (5 × 10^5 mGC3 cells, p < 0.0001 from day 31; 3 × 10^6 mGC3 cells, p < 0.008 from day 14). B, Mice were treated with either four weekly vaccinations of DC/CpG-B or CpG-A or a combination of both treatments as in A (n = 5). On day 0 mice were challenged with 1 × 10^7 mGC3 cells. Treatment improved survival compared with untreated group (CpG-A, p = 0.005; both groups with DC/CpG-B, p = 0.002). Tumor growth was significantly reduced by DC/CpG-B vaccination (p = 0.001 or smaller from day 24) but not by CpG-A. C, Wild-type mice (n = 5) received three injections of CpG-A every 2 d. One day after the last injection, CD69 expression on splenic NK1.1+ NK cells was examined by FACS analysis. D and E, Wild-type mice were vaccinated as in A with 4 weekly DC/CpG-B injections. D, Intracellular IFN-γ staining of CD8+ splenocytes restimulated with TAg-specific peptides before analysis. Triangles represent values from individual mice (no therapy, n = 5; DC/CpG-B, n = 8). E, In vivo cytotoxicity assay showing specific lysis of Ag-pulsed target cells. Triangles represent values from individual mice (n = 4).

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DC/CpG-B vaccination elicits a transferable memory immune response

To investigate whether mice cured from mGC3 tumors by DC/CpG-B vaccination had acquired a long-term protection against the tumor, these mice were rechallenged s.c. with mGC3 tumor cells. Five of seven mice rechallenged 157 d after the last vaccination remained tumor-free for >100 d and all mice survived (Fig. 2A). At this time, all untreated animals had succumbed to the s.c. tumors. An additional group of mice rechallenged 303 d after the last vaccination also showed 100% survival (Fig. 2A). Tumors in vaccinated mice developed later, if at all, and progressed more slowly than in mice without previous therapy. The adoptive transfer of splenocytes from these mice to naive animals without previous exposure to the mGC3 tumor Ags protected the recipients against an mGC3 tumor (Fig. 2B). Transfer recipients showed enhanced survival and delayed tumor growth (Fig. 2C). Thus, mice immunized against mGC3 tumors develop an efficient memory response that protects them from a tumor rechallenge 5 m o after the last Ag exposure. Additionally, the immune response against mGC3 tumors induced by DC/CpG-B vaccination can be transferred to naive hosts.

DC/CpG-B vaccination inhibits growth of established CEA424-TAg s.c. tumors

We next examined the efficacy of DC/CpG vaccination for the treatment of established s.c. tumors in wild-type mice. Mice with palpable s.c. tumors (day 14 after tumor induction) were treated four times by DC/CpG-B vaccination. Tumor growth was significantly retarded, and the survival of vaccinated mice was improved compared with untreated mice (Fig. 3). Thus, DC/CpG-B vaccination triggers an immune response capable of retarding tumor growth not only in a prophylactic setting but also in a therapeutic setting.

FIGURE 2. DC/CpG-B vaccination elicits a transferable memory immune response. A, Wild-type mice vaccinated as in Fig. 1A with DC/CpG-B that had rejected a first tumor were rechallenged with $3 \times 10^6$ mGC3 cells 157 ($n = 7$) or 303 d ($n = 3$) after the last vaccination. Survival of rechallenged mice was significantly improved ($p = 0.001$) compared with untreated animals ($n = 6$). B and C, On day 290 after the primary tumor challenge, splenocytes from vaccinated mice were adoptively transferred into naive wild-type mice before challenge with $3 \times 10^6$ mGC3 cells. The time course for the adoptive transfer is depicted in B. C, Survival and mean tumor sizes of adoptive transfer recipients and untreated animals are shown ($n = 5$). Survival of adoptive transfer recipients was improved ($p = 0.002$) and tumor growth was reduced ($p < 0.032$ from day 17) compared with untreated mice.

DC/CpG-B vaccination does not improve the survival of CEA424-TAg mice

To investigate the efficacy of DC/CpG-B vaccination in CEA424-TAg mice, animals were vaccinated as in Fig. 1A with DC/CpG-B before s.c. challenge with mGC3 tumors. In contrast to the significant reduction in tumor growth seen in wild-type mice (Figs. 1, 2), we observed no effect on tumor progression in CEA424-TAg transgenic mice (Fig. 4A). We further examined whether any detectable clinical benefit on the progression of the autochthonous gastric tumors could be achieved in CEA424-TAg mice by activating local immunity through oral application of CpG-B (19), since we have previously shown that local application of CpG-B alone can reduce growth of s.c. tumors (17). Repeated oral applications of CpG-B, however, did not improve survival of the transgenic mice (Fig. 4B). Also, no increase in survival was seen in transgenic animals vaccinated weekly from day 32 of age with DC/CpG-B.

Adoptive transfer of splenocytes from immunized mice results in an effective anti-tumor response in CEA424-TAg mice

Immunization with a potent TAg-expressing modified vaccinia Ankara virus vaccine did not lead to a CD8 response in CEA424-TAg mice (R. Kammerer, unpublished data), suggesting tolerance against SV40 TAg epitopes in these mice. This prompted us to assess the efficacy of adoptive cell transfer. We transferred splenocytes from DC/CpG-B-vaccinated wild-type mice into young CEA424-TAg transgenic mice aged 38–43 d. At this age, the autochthonous gastric tumors are barely detectable by microscopy ($<1$ mm$^2$). These mice were inoculated with s.c. mGC3 tumors 2 d after adoptive transfer to directly monitor the anti-tumoral immune response. In all adoptively transferred CEA424-TAg mice, s.c. tumors were palpable at day 7, then regressed completely, and in two of five mice no recurrence was palpable (Fig. 5A). In the remaining two mice, tumor growth was significantly retarded. All mice were sacrificed on day
CD3+ T cells was seen (Fig. 5) bearing established s.c. mGC3 tumors, an increase in infiltrating gastric tumors (Fig. 6). Tumors regressed completely upon adoptive transfer in these mice, whereas many submucosal tumors from adoptively transferred animals compared with untreated animals, although the total size and number of necrotic areas in gastric tumors of transfer recipients was improved ($p < 0.006$ from day 33) compared with untreated mice.

32 after tumor challenge to perform a histological examination of the s.c. and the autochthonous gastric tumors. Untreated s.c. tumors were infiltrated throughout with low numbers of CD8+ cells, and no intratumoral CD4+ T cells were detected. Adoptive transfer significantly enhanced infiltration by both CD8+ and CD4+ T cells (Fig. 5B). Furthermore, when the adoptive transfer was performed in mice bearing established s.c. mGC3 tumors, an increase in infiltrating CD3+ T cells was seen (Fig. 5C). Thus, adoptive transfer in CEA424-TAg mice leads to an efficient anti-tumoral response against s.c. tumors associated with infiltration of CD8+ and CD4+ T cells throughout the tumor.

In autochthonous gastric tumors from untreated mice, few CD8+ cells were found in the central area of the tumor. Following adoptive transfer, CD8+ T cells were only slightly increased in the central tumor area, in striking contrast to the strong lymphocytic infiltration seen in s.c. tumors in the same mice (Fig. 6A–C). In submucosal tumor areas, the number of CD8+ cells was increased in adoptively transferred animals compared with untreated animals, although the total numbers remained lower than in s.c. tumors (Fig. 6C). Although s.c. tumors regressed completely upon adoptive transfer in these mice, there was no evidence for a reduction in the size of the autochthonous gastric tumors (Fig. 6D). However, we did observe a difference in the size and number of necrotic areas in gastric tumors of transfer recipients in comparison with untreated animals (Fig. 6E). Submucosal tumor areas from untreated mice were dense with only rare necrotic areas, whereas many submucosal tumors from adoptively transferred mice displayed large necrotic areas with a loose tissue structure. In summary, we observed a striking contrast in tumor response and T cell infiltrations between s.c. and autochthonous gastric tumors in the same animals. CD8+ T cell infiltrates in s.c. tumors were associated with an efficient anti-tumor response.

Discussion

The CEA424-TAg transgenic mouse strain and s.c. tumors induced from CEA424-TAg–derived gastric cancer lines represent valuable models for the stepwise optimization of immunotherapeutic approaches toward clinical development. The tissue-derived gastric cancer cell line mGC3 is characterized by a heterogeneous composition and a slow growth rate, with tumors reaching a size of only 50 mm2 after 40–50 d (11). Immunotherapy of the s.c. mGC3 tumors proved more difficult than in rapidly growing tumor models. Using a DC-based tumor vaccine combined with immune activation by CpG, we achieved, both prophylactically and therapeutically, retardation of tumor growth and in some cases complete rejection of mGC3 tumors in wild-type mice. A tumor cell–specific cytotoxic immune response was evoked, and immunological memory could be transferred to naive animals. We have previously achieved nearly complete protection and efficient eradication of large tumors with analogous approaches in s.c. tumors induced with the C26 colon carcinoma and B16-OVA melanoma models (14, 15). Thus, the CEA424-TAg gastric tumor–derived cells can be targeted by immunotherapy, but to a noticeably lesser extent than rapidly growing clonal cell lines, such as C26 or B16-OVA. Slow tumor growth facilitates the formation of an immunosuppressive microenvironment, including the presence of stromal cells and infiltration by immunosuppressive cell types, such as regulatory T cells and myeloid-derived suppressor cells (20). Indeed, certain subsets of myeloid-derived suppressor cells require $>40$ d to accumulate in detectable numbers in tumor-bearing animals (21). In contrast, the rapid growth of many cloned tumor cell lines precludes the formation of an immunosuppressive tumor environment (22, 23). Thus, slow-growing tumors, such as the s.c. mGC3 model, may more closely resemble clinically relevant solid tumors.

Having established an immunotherapeutic protocol in the s.c. induced mGC3 tumors in wild-type mice, we applied this treatment protocol in transgenic CEA424-TAg mice bearing both induced s.c.
tumors and developing spontaneous gastric tumors. This experimental setup allows direct comparison of the anti-tumor response against the induced, s.c. tumor and the autochthonous tumor in the same animals. However, DC vaccination did not yield a clinical benefit in these mice, even when started at an early age when these mice present only precancerous lesions in the gastric mucosa (10).
This suggests that CEA424-TAg mice develop an immunological tolerance toward tumor-associated Ags. Indeed, there is evidence that CEA424-TAg mice are tolerant to the major SV40 TAg epitopes that are expressed at an early age in these animals (R. Kammerer, unpublished observations). Both peripheral and central tolerance to SV40 TAg have been described in other SV40 TAg transgenic mouse models (24–27). In some of these models, tumor growth could be effectively contained by vaccination before the onset of TAg expression (28–30). For example, a lifelong control of pancreatic tumors was achieved in rat insulin promoter–TAg transgenic mice by an early immunization with the immunodominant SV40-TAg epitope IV. Tumor control was achieved by an equilibrium between immunosurveillance and oncogene-driven tumorigenesis (30). In the present study, whole apoptotic tumor cells were selected as a source of Ag. With this approach, other tumor-associated Ags, in addition to the immunodominant SV40 TAg, may be recognized. The absence of clinical efficacy of the DC/CpG-B vaccination in CEA424 TAg mice suggests that these mice display a broad immune tolerance toward tumor-associated epitopes. This condition is of clinical relevance since tolerance toward multiple tumor Ags is frequently observed in cancer patients.

To investigate whether anti-tumoral responses in autochthonous tumors in CEA424 TAg mice are principally accessible to immunotherapy, we adopted a passive immunotherapy approach. A single adoptive transfer of splenocytes from immune wild-type mice resulted in the complete regression of s.c. mGC3 tumors in CEA424-TAg mice, demonstrating an effective anti-tumoral response in these mice. We could, therefore, in this model investigate the influence of tumor site by comparing the efficacy of immunotherapy on s.c. and gastric tumors in the same animal. We observed a striking difference in the pattern of infiltrating T lymphocytes following adoptive transfer. Whereas s.c. tumors were homogeneously...
infiltrated after transfer, most T cells in gastric tumors remained re-
stricted to the submucosal tumor-infiltrated areas underlying the
muscularis mucosae. The central tumor area, which was essentially
free of lymphocytes in untreated animals, was only infiltrated by
a small number of CD8+ cells following adoptive transfer. Tumor
localization seems to play an important role for the efficacy of adop-
tive T cell transfer: in an SV40 TAg transgenic model of hepatic
cancer, transfer of tumor-specific CD8+ T cells improved survival
(31). In contrast, in an SV40 TAg model of pancreatic cancer, the
transfer of tumor-specific CD8+ T cells did not lead to tumor influ-
tration and did not result in clinical benefit (32). In this system, the
cotransfer of activated, tumor-specific CD4+ T cells facilitated accu-
mulation of CD8+ T cells and improved anti-tumor efficacy (33). In
our model, CD8 penetration of gastric tumors was poor despite the
transfer of total splenocytes from immune mice, including both
CD4+ and CD8+ T cells that efficiently infiltrated s.c. tumors. Thus,
the efficacy of adoptive T cell transfer may depend on the site of the
tumor. Additionally, the stage of tumor development at the time of
adoptive transfer may play a role for the efficacy of immunotherapy
(34). Indeed, T cell infiltration in s.c. tumors was lower when the
adoptive transfer was performed in mice with established tumors than
when the transfer was performed before tumor induction. The auto-
tochthonous gastric tumors, however, were very small (<1 mm2 at
40 d of age in CEA424-TAg mice) at the time of adoptive transfer,
suggesting that the tumor stage did not play a major role in the
infiltration seen within these tumors. Our results therefore suggest
that in patients with stomach cancer, even early treatment with im-
munotherapy protocols that induce strong tumor-specific immune
responses may not be clinically effective.

Additional strategies may be necessary to enhance penetration of
gastric tumors by effector T cells. Potential treatment strategies
include irradiation of the tumor, systemic application of other TLR
ligands, or neutralization of the endothelin B receptor that prevents
inflammation and tumor growth (35). In a CEA424-TAg model of hepatic
cancer infiltrating lymphocytes (TILs) in gastric cancer patients. Hepatogastro-
enterology 53: 634–638.

Hyakudome, M., T. Matushita, R. Hyakudome, T. Yamamoto, S. Kinugasa,
A. Kogata, M. Katayama, T. Takaoka. 2008. Increased expression of fibrin,
talkine is correlated with a better prognosis and an increased number of both
Oncol. 15: 1775–1782.

Thompson, J., T. Epting, G. Schwarzkopf, A. Singhofen, A. M. Eades-Perner,
H. van Der Putten, and W. Zimmermann. 2000. A transgenic mouse line that
develops early-onset invasive gastric carcinoma provides a model for carcino-

Wurzenberger, C., V. H. Koelzer, S. Schreiber, D. Anz, A. M. Vollmar,
and C. Kammerer. 2006. Characterization of gastric adenocarcinoma cell lines
established from CEA424/SV40 T antigen-transgenic mice with or without a hu-

C57BL/6 mice. Cancer Res. 44: 717–726.

Immunotherapy targeting tumors of the gastrointestinal tract faces an additional challenge: a local tolerogenic environment has evolved to prevent deleterious immune responses in in-
nocuous dietary Ags and commensal microorganisms. Local re-
 sponses depend on Ag presentation by DC of the gastrointestinal
tract and include the induction of regulatory T cells and myeloid-
derived suppressor cells that promote immune tolerance (38).
Thus, site-specific tolerogenic mechanisms may contribute to the
inability of CEA424-TAg mice to suppress gastric tumors despite the
presence of an anti-tumoral response that effectively targets s.c.
tumors. Additional immunostimulatory approaches targeting the
local environment may therefore be required to overcome toleriz-
ing mechanisms. This may be achieved by selectively activating
APCs through the local delivery of TLR ligands that may be applied endoscopically (39). Indeed, we have recently shown that
TLR7 activation can block regulatory T cell–mediated suppres-
sion (40). Immunostimulatory agents may also be targeted to the
tumor using tumor-homing peptides (37, 41) or to macrophages
using β-glucan particles as oral delivery vehicles (42).

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References
2007. Efficacy of adjuvant immunochemotherapy with polysaccharide K for
56: 905–911.
4. Jeung, H. C., Y. W. Moon, S. Y. Rha, N. C. Yoo, J. K. Roh, S. H. Noh, J. S. Min,
B. S. Kim, and H. C. Chung. 2008. Phase III trial of adjuvant 5-fluorouracil and
5-fluorouracil plus adriamycin versus 5-fluorouracil, adriamycin, and polyadenylic-polycytidylic
acid (poly A:U) for locally advanced gastric cancer after curative surgery: final
5. Keno, K., A. Takahashi, H. Sugai, H. Fuji, A. R. Choudhury, R. Kesseling,
and Y. W. Moon. 2002. Dendritic cells pulsed with MHC II-restricted peptides
Res. 8: 3394–3400.
6. Sato, Y., H. Shomura, Y. Maeda, T. Mine, Y. Ume, Y. Akasaka, M. Kondo,
treatment and did not result in clinical benefit (32). In this system, the
cotransfer of activated, tumor-specific CD4+ T cells facilitated accumu-
lation of CD8+ T cells and improved anti-tumor efficacy (33). In
our model, CD8 penetration of gastric tumors was poor despite the
transfer of total splenocytes from immune mice, including both
CD4+ and CD8+ T cells that efficiently infiltrated s.c. tumors. Thus,
the efficacy of adoptive T cell transfer may depend on the site of the
tumor. Additionally, the stage of tumor development at the time of
adoptive transfer may play a role for the efficacy of immunotherapy
(34). Indeed, T cell infiltration in s.c. tumors was lower when the
adoptive transfer was performed in mice with established tumors than
when the transfer was performed before tumor induction. The auto-
chthonous gastric tumors, however, were very small (<1 mm2 at
40 d of age in CEA424-TAg mice) at the time of adoptive transfer,
suggesting that the tumor stage did not play a major role in the
infiltration seen within these tumors. Our results therefore suggest
that in patients with stomach cancer, even early treatment with im-
munotherapy protocols that induce strong tumor-specific immune
responses may not be clinically effective.

Additional strategies may be necessary to enhance penetration of
gastric tumors by effector T cells. Potential treatment strategies
include irradiation of the tumor, systemic application of other TLR
ligands, or neutralization of the endothelin B receptor that prevents
T cell homing to tumors by inhibiting endothelial adhesion (32, 35,
36). Furthermore, it was recently shown that ablature of the reg-
lulator of G protein signaling 5 (Rgs5) results in pericute matu-
rature, normalization of tumor vasculature, and significant infil-
tration of tumor parenchyma by CD8+ T cells (37).

Immunotherapy targeting tumors of the gastrointestinal tract faces an additional challenge: a local tolerogenic environment has evolved to prevent deleterious immune responses in in-
nocuous dietary Ags and commensal microorganisms. Local re-
 sponses depend on Ag presentation by DC of the gastrointestinal
tract and include the induction of regulatory T cells and myeloid-
derived suppressor cells that promote immune tolerance (38).
Thus, site-specific tolerogenic mechanisms may contribute to the
inability of CEA424-TAg mice to suppress gastric tumors despite the
presence of an anti-tumoral response that effectively targets s.c.
tumors. Additional immunostimulatory approaches targeting the
local environment may therefore be required to overcome toleriz-
ing mechanisms. This may be achieved by selectively activating
APCs through the local delivery of TLR ligands that may be applied endoscopically (39). Indeed, we have recently shown that
TLR7 activation can block regulatory T cell–mediated suppres-
sion (40). Immunostimulatory agents may also be targeted to the
tumor using tumor-homing peptides (37, 41) or to macrophages
using β-glucan particles as oral delivery vehicles (42).

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Disclosures
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