Difficulties and dangers of CEA-targeted immunotherapy against colorectal cancer

Bos, Rinke

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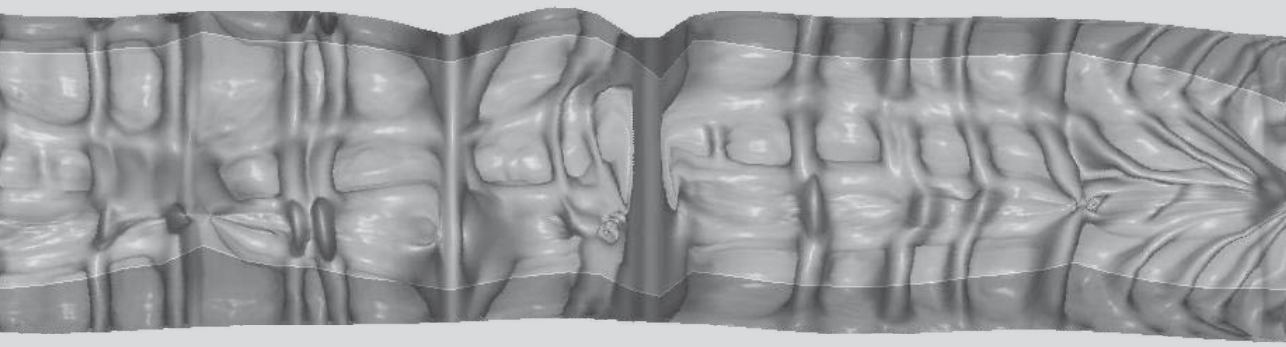

Tumor regression and autoimmunity induced by immunotherapeutic modalities targeting carcinoembryonic antigen

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Abstract – Previous experiments demonstrated that CEA-tg mice exhibit central tolerance for CEA and fail to reject CEA-positive tumors. We examined whether reconstitution of the CEA-specific T-cell repertoire would result in effective anti-tumor immunity. Therefore, CEA-reactive T-cell populations from non-tg mice were adoptively transferred into CEA-tg mice, and their impact on CEA-positive tumors versus normal tissues was analyzed. Adoptive transfer into sublethally (4.5 Gy) irradiated CEA-tg mice had only a modest anti-tumor effect, indicating the presence of peripheral regulatory mechanisms that inhibit the CEA-specific immune response in CEA-tg mice. Circumvention of this suppression, either by a combination of 4.5 Gy total body irradiation (TBI) and IL-10 receptor blockade or myeloablative irradiation, markedly increased anti-tumor efficacy of the CEA-specific immune response. However, this treatment was invariable associated with severe colitis, which was inflicted by CEA-specific T cells. Strikingly, if adoptive transfer was combined with 4.5 Gy TBI and depletion of CD25+ T-regulatory cells from the recipient, effective tumor eradication was observed in the absence of autoimmune pathology in the intestine. Our data indicate that depletion of CD25+ cells, although showing CEA-specific anti-tumor immunity, also resulted in the enhancement of other immune mechanisms that control tumor growth. This treatment regimen was also tested in a spontaneous tumor model, where we found that the average number of tumors and the average surface area of the tumors per mouse after treatment were significantly lower in the group of APC^{1638N} × CEA-tg mice compared to the group of APC^{1638N} single-tg mice. These data were supported by in vitro analysis

of T-cell responses and immunohistochemical analysis on T-cell infiltration in tumors of $APC^{1638N} \times$ CEA-tg and APC^{1638N} single-tg mice. Together, our experiments show that CEA-specific immunity in CEA-tg mice is limited by both central and peripheral tolerance. Reconstitution of the CEA-specific T-cell repertoire by adoptive transfer in combination with immune modulation can result in efficient eradication of CEA-positive tumors. However, in order to prevent hazardous CEA-specific autoimmune reactions, the choice of the right immune modulation protocol is critical.

Introduction

Important for the development of immunotherapies against cancer is a well-considered choice of a target antigen. Since carcinoembryonic antigen (CEA) was one of the first reported tumor-associated auto-antigens and a potential target for cancers of epithelial origin, many different immunotherapies targeting CEA have been intensively studied. Most of these studies have been performed in pre-clinical mouse models but data were also obtained from clinical trials. Although CEA-specific T-cell immunity has been observed in clinical trials, evidence for striking clinical anti-tumor efficacy has so far not been reported. In several CEA-tg mouse models, CEA-specific immunizations have been shown to delay, and in some cases prevent, outgrowth of CEA positive tumors. Although these mice have comparable, or even higher, CEA expression levels than humans, no autoimmune pathology towards CEA-positive tissues, in particular the intestine, was observed. This is rather puzzling in view of other reports in which expression of target antigens on healthy tissues led to the induction of auto-immunity after antigen specific vaccination [1,2]. Because of this paradox we initiated a study in which we performed a detailed analysis of the effect of a strong CEA-specific T-cell response on CEA positive tumors compared to the effect on other CEA expressing tissues in a CEA-tg mouse model. We have shown previously that CEA is expressed by human mTEC and that expression by mTEC in CEA-tg mice leads to a limited CD4+ T-cell repertoire in the periphery [3]. We were therefore not able to strongly activate the endogenous CEA-specific T-cell repertoire in CEA-tg mice. This problem might be overcome by reconstituting the T-cell repertoire of CEA-tg mice with CEA-specific T cells. We therefore adoptively transferred the T-cell repertoire of CEA-immunized wild-type mice into tumor-bearing CEA-tg mice. Our data indicate that, in addition to central tolerance also peripheral tolerance limits the CEA-specific T-cell repertoire in CEA-tg mice. Notably, most modalities in which such tolerance is overcome show anti-tumor efficacy that is accompanied by severe autoimmune pathology, which is manifested as colitis. Nevertheless, we found that not all effective treatment schedules inflicted severe autoimmune pathology. We have critically evaluated these different treatment schemes in the context of, in particular, the type and specificity of the immune responses involved, as well as the possibilities for implementation in human cancer patients.

Materials and Methods

Mice. C57BL/6 Kh (B6, H-2^b), Thy1.1 (H-2^b), CEA-tg and APC^{1638N} mice were bred in our own facilities (Leiden, The Netherlands). CEA-tg mice were originally obtained from Dr. John Thompson, Freiburg, Germany. APC^{1638N} mice were originally obtained from Dr. Ricardo Fodde, Rotterdam, The Netherlands. Mice were analyzed for CEA genotype by PCR analysis as described previously [4]. The experiments were approved by the animal experimental commission (UDEC) of Leiden University.

Immunizations and tumor challenge experiments. Mice were vaccinated twice i.m. with a 2-week interval with 100 μg of plasmid pGT64 CEA B7-1 [5] dissolved in 100 μl PBS. 4 days prior to each immunization, 80 μl 10 μM cardiotoxin was injected i.m. Two weeks after the last vaccination, spleens were isolated for in vitro tests or mice were used for tumor challenge experiments where 150.000 MC38-CEA tumor cells were injected s.c. in 200 μl PBS/0.5% bovine serum albumin. MC38-CEA cells (obtained from Dr. James Primus, Nashville, TN, USA) were cultured as described previously [3].

Irradiation and adoptive transfer. Donor mice were vaccinated twice i.m. with a 2-week interval with 100 μg of DNA dissolved in 100 μl PBS. 4 days prior to each immunization, 80 μl 10 μM cardiotoxin was injected i.m. When CD4 or CD8 depletion was performed, donor mice were injected i.p. with respectively 25 μg GK1.5 or 100 μg 2.43 antibody at day –3 and –1 before adoptive transfer. Spleen cells were isolated two weeks after the last vaccination and depleted of erythrocytes. Recipient mice were reconstituted by i.v. injection of 5×10^7 spleen cells suspended in 200 μl PBS. Recipient mice received either 4.5 Gy total body irradiation 1 day before reconstitution with spleen cells or 9.5 Gy total body irradiation 5 days before reconstitution. 9.5 Gy total body irradiation was followed by bone marrow transplantation 1 day later. Mice treated with immunomodulatory agents received either 250 μg IL10 receptor blocking antibody that was injected weekly i.p., starting at the day of the tumor challenge or 80 μg of CD25-specific antibodies that were injected i.p. 6 days before adoptive transfer. Immunization of recipient mice started 1 day after adoptive transfer. Mice were vaccinated weekly with DNA i.m., 4 days prior to each immunization, 80 μl 10 μM cardiotoxin was injected i.m. 150.000 MC38-CEA tumor cells were injected s.c. 1 day after adoptive transfer.

Histological analysis. Tissues were snap frozen in isopentane and stored at –80°C. Cryosections (4 μm) were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin (H&E). To compare the histopathologic changes that occured in the intestine of CEA-tg and wild-type mice, a scoring system was established using the following parameters: *A*. degree of inflammatory cell infiltrate in epithelia and stroma, giving a score ranging from 0-6; *B*. mucin depletion, giving a score ranging from 0-3; *C*. Crypt elongation and hyperplasia, giving a score ranging from 0-3; and *D*. crypt destruction, giving a score ranging from 0-3. The severity of the inflammatory changes in the intestine was based on the sum of the scores reported for each parameter listed above. The higher the score, the greater the inflammatory changes.

Immunohistochemistry. Cryosections (4 μm) were fixed for 10 min. with ethanol-acetone at RT. Subsequently, sections were incubated with primary antibody Thy1.1 biotin (clone HIS51, BD Pharmingen, Alphen aan den Rijn, The Netherlands), followed by horseradish peroxidase-labeled (HRP) secondary antibody (streptABCcomplex/HRP, DAKO). HRP activity was revealed by incubation in diaminobenzidine and counterstained with hematoxylin.

Cell preparation and cytokine analysis. Spleens, mesenteric lymph nodes, small intestines and colons were isolated at 1, 3 and 5 weeks after adoptive transfer. Single cell suspensions of spleen and lymph nodes were prepared by mechanical disruption. Splenocytes were depleted of erythrocytes and lamina propria lymphocytes (LPL) from the colon were isolated as described previously [6]. 2×10^5 T cells were incubated with 2×10^4 D1 cells in the presence of 5 μg/ml peptide (a mix of T-helper epitopes 1-5 [3]), or CTL peptide 571-579). After 1 hour incubation, 10 μg/ml Brefeldin A was added and 3 hours later cells were fixed. Fixation and staining procedures were done as described previously [7].

Results

Reconstitution of the CEA-specific T-cell repertoire through adoptive transfer

Our previous studies indicated that CEA-specific activation of the endogenous T-cell repertoire in CEA-tg mice is severely limited by central tolerance [3]. A clinically relevant approach to induce CEA-specific immunity in vivo could involve gene transfer of a CEA-specific TCR into autologous lymphocytes [8]. To test whether reconstitution of the CEA-specific T-cell repertoire could result in effective immunity against CEA-expressing tumors, we adoptively transferred the T-cell repertoire of CEA-immunized wild-type mice into tumor-bearing CEA-tg mice. As shown in Fig. 1, two sequential immunizations of wild-type donor mice with a CEA-specific DNA vaccine induced strong CEA-specific CD4+ and CD8+ T-cell immunity. This response was transferred to naïve CEA-tg or wild-type recipient mice by infusion of 5×10^7 donor splenocytes.

The anti-tumor efficacy of this adoptively transferred immune response was analyzed by challenging the recipient mice with a tumorigenic dose of 1.5×10^4 MC38-CEA cells one day after splenocyte infusion. In order to sustain the transferred immune response, recipient mice received weekly doses of the CEA-specific DNA vaccine. Remarkably, this treatment regimen failed to prevent, or even delay, tumor outgrowth in CEA-tg mice (Fig. 2A). In contrast, this regimen did efficiently protect wild-type mice against tumor outgrowth (Fig. 2B). These data indicate that the adoptively transferred CEA-specific T-cell response is negatively regulated in CEA-tg hosts. Therefore, the anti-tumor efficacy of the CEA-specific T-cell response in CEA-tg mice is not only restricted by central tolerance [3], but also by peripheral tolerance.

Elimination of regulatory mechanisms involved in peripheral tolerance

Others have shown that the anti-tumor efficacy of an adoptively transferred T-cell response can be significantly enhanced if the recipient mice are irradiated before lym-

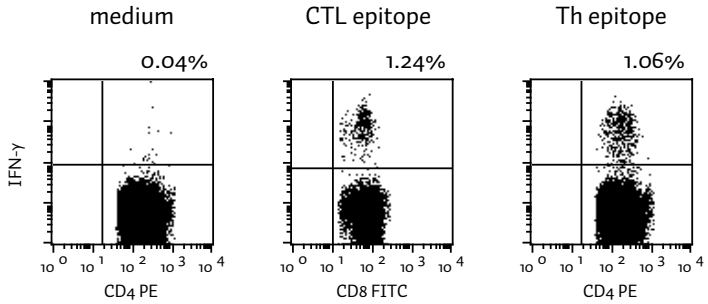


FIGURE 1. CEA-specific immune response in C57BL/6 mice induced by immunization with B7.1-CEA DNA. Mice were vaccinated twice with a two-week interval with 100 μg B7.1-CEA DNA. 4 days prior to each immunization, 80 μl 10 μM cardiotoxin was injected i.m. Two weeks after the last vaccination we isolated spleens and measured IFN-γ production by CD4+ and CD8+ T cells after peptide specific stimulation (mix of Th epitopes 1-5 [3] and CTL peptide $CEA_{571-579}$). IFN-γ production was measured direct ex-vivo by intracellular cytokine staining.

phocyte infusion. This pre-treatment was proposed to improve the performance of adoptively transferred T cells through elimination of immunoregulatory lymphocyte subsets and by creating a lymphoid environment that is more supportive of homeostatic T-cell proliferation [9]. We therefore applied our treatment regimen to CEA-tg and wild-type mice that had received 4.5 Gy total body irradiation (TBI) before adoptive transfer. Pre-treatment with 4.5 Gy TBI resulted in a marked improvement of the efficacy of the adoptive therapy, in that 35% of the CEA-tg mice did not develop tumors (Fig. 2A), while tumor growth was delayed in the other mice (Fig. 2C). 4.5 Gy TBI did not further improve treatment efficacy in wild-type recipient mice (Fig. 2B), indicating that this intervention particularly alleviates the suppression of the adoptively transferred CEA-specific T-cell response that takes place in CEA-tg recipient mice. Furthermore, 4.5 Gy TBI alone had no effect on the tumor outgrowth in either CEA-tg or wild-type mice (Fig. 2A, B), showing that the adoptively transferred T-cell response was primarily responsible for the anti-tumor effects observed. We have previously reported that the anti-tumor effect of the CEA-specific DNA vaccine in wild-type mice depends on both CD4+ and CD8+ T-cell subsets (chapter 3). The protective effect of the adoptively transferred lymphocytes similarly depends on these two T-cell subsets, both in CEA-tg and wild-type recipients, in that depletion of the adoptively transferred lymphocytes of either CD4+ or CD8+ T cells abolishes anti-tumor efficacy of the treatment (Fig. 4).

Although treatment involving 4.5 Gy TBI of the CEA-tg recipient mice improved the anti-tumor effect of the adoptively transferred lymphocytes, the majority of the mice eventually developed progressive tumor growth (Fig. 2A, C). This treatment was successful in protecting wild-type mice from tumor growth, suggesting that it was insufficient in overcoming peripheral immune regulatory mechanisms in the CEA-tg host. To further curtail these mechanisms, we modified the treatment in three different ways of which we know they are proficient in inhibiting/depleting known regulatory mechanisms. In addition to 4.5 Gy TBI, we suppressed the action of the key regulatory cytokine IL-10 [10], by injecting IL-10 receptor (IL-10R) blocking antibodies [11]. Alternatively, we increased the dose of radiation to a myeloablative dose of 9.5 Gy TBI, because this is known to more rigorously

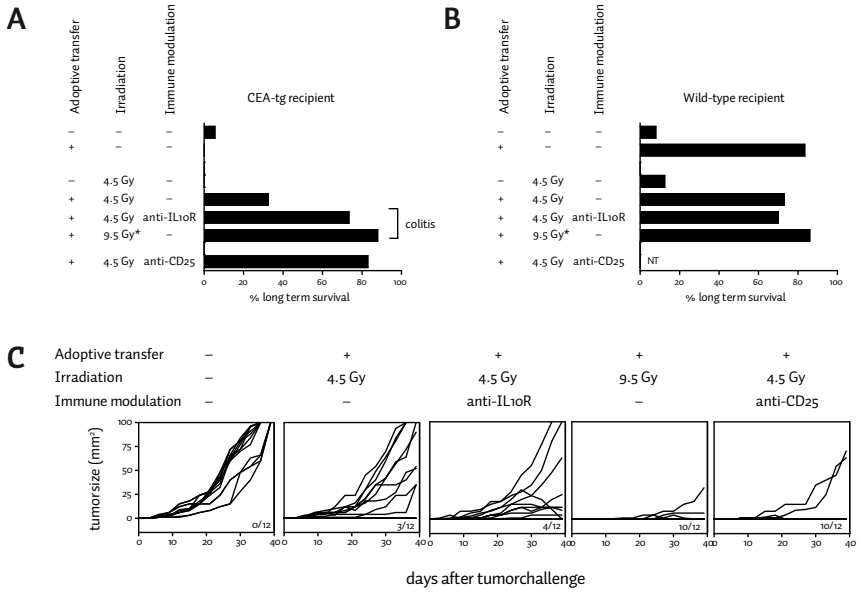


FIGURE 2. Survival after CEA-specific treatments combining irradiation with adoptive transfer in CEA-tg and wild-type mice. CEA-tg (**A**) or wild-type mice (**B**) were non-treated (n=37, n=15), only infused with immunized wild-type donor lymphocytes (n=10, n=6), irradiated with 4.5 Gy total body irradiation (n=10, n=8) or infused with immunized wild-type donor lymphocytes combined with 4.5 Gy total body irradiation (n=37, n=26). Immune modulation was added to this treatment by injecting CD25-specific antibodies (n=35, n=0) or IL10 receptor blocking antibody (n=19, n=10). *Mice treated with adoptive transfer in combination with 9.5 Gy total body irradiation were also receiving bone marrow transplantation one day after irradiation (n=17, n=14). All mice were challenged s.c. with a lethal dose of 1.5×10^5 MC38-CEA cells one day after adoptive transfer. Tumor size was measured every 3 days and mice were sacrificed when tumor size exceeded 100 mm^2. Depicted is the long-term survival percentage. Long term survival = tumorsize < 10 mm^2 at day 40 after tumor challenge. Data are cumulative of at least 2 experiments. **C**. Tumor growth of 12 mice per group. CEA-tg mice received no treatment, adoptive transfer of immunized wild-type lymphocytes combined with 4.5 Gy irradiation with or without anti-CD25 or IL10R blocking antibody or mice received 9.5 Gy irradiation combined with BMT and adoptive transfer of immunized wild-type lymphocytes. The horizontal line represents one or more mice in which no tumor outgrowth was detected. The numbers below these lines indicate the fraction of mice in each group that are tumor free at the end of the experiment.

deplete regulatory cells from the periphery and enhance homeostatic proliferation [9]. In order to preserve viability of the irradiated mice, their haematopoietic system was reconstituted through bone marrow transplantation (BMT) with syngeneic bone marrow cells. The third modification involved injection of PC61 CD25-specific antibodies into recipient CEA-tg mice that are known to deplete CD25+ cells, in particular the CD4+CD25high regulatory T cells [12]. We found all three modified treatments to improve the anti-tumor efficacy of the adoptively transferred lymphocytes in CEA-tg mice (Fig. 2A). In particular the modalities involving 9.5 Gy TBI or 4.5 Gy TBI in combination with CD25-depletion were effective, in that the majority of the mice were capable of clearing their tumors (Fig. 2C).

Taken together, our data demonstrate that the failure of the CEA-specific T-cell response in CEA-tg mice is a result of both central and peripheral tolerance and that these hurdles can be overcome by respectively reconstitution of the CEA-specific T-cell repertoire and suppression of immune regulatory mechanisms.

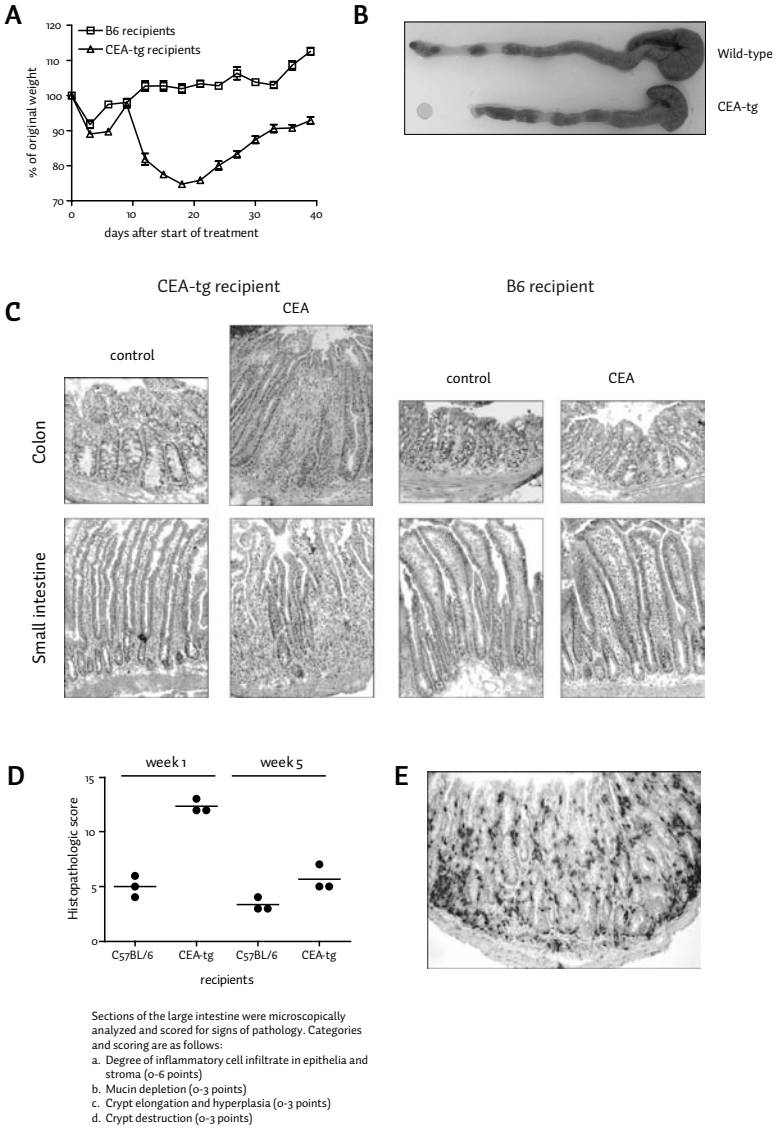
Association between effective anti-tumor treatment and autoimmune colitis

Importantly, two of the modalities, although resulting in improved anti-tumor efficacy, were accompanied by symptoms that are typical for experimental colitis [13-15], including severe weight loss, which occasionally resulted in death (Fig. 3A). Weight loss started around one week after adoptive transfer, mice started to regain weight 2 weeks later, while full recovery of the surviving mice took 6-8 weeks. Treatment involving the combination of 4.5 Gy TBI and IL10R blocking antibody resulted in severe weight loss in 50% of the cases, whereas this occurred in 100% of the mice if treatment involved 9.5 Gy TBI. These symptoms were not observed in any of the wild-type recipients receiving these treatments (Fig. 3A), suggesting that they were caused by the immune response against the CEA-positive intestinal epithelia in CEA-tg mice. In accordance with this notion, weight loss was accompanied by significant colon shortening and thickening (Fig. 3B). Histological examination of the intestine showed loss of goblets cells, crypt elongation, crypt abscesses and strong infiltration in colon and small intestine (Fig. 3C).

Pathology in the colon was more profound than in the small intestine (Fig. 3C), in correspondence with the higher CEA-expression levels in the colon [data not shown; 16]. Furthermore, the histopathological changes and increased lymphocyte infiltration, although also observed in wild-type recipients, were much more severe in CEA-tg recipients (Fig. 3C, D). To show that the adoptively transferred CEA-specific T-cell response was involved in colitis, Thy1.1+ donor lymphocytes were adoptively transferred into Thy1.2+ CEA-tg recipient mice. Histopathological analyses showed massive infiltration of the intestinal epithelium by Thy1.1+ cells (Fig. 3E). Finally, adoptive transfer of lymphocytes from CEA-tg donor mice, which display a greatly reduced CEA-specific T-cell response [3 and unpublished data], neither resulted in tumor-clearance, nor in colitis (Fig. 4A, B), indicating that the more potent CEA-specific T-cell response in wild-type donor cells was responsible for both effects. The role of the adoptively transferred T-cell response in tumor control and colitis was further dissected by using donor lymphocyte preparations depleted of either CD4+ or CD8+ T-cell subsets. Treatment with CD4-depleted donor lymphocytes neither resulted in tumor control, nor in colitis (Fig. 4B). Treatment with CD8-depleted donor lymphocytes similarly failed to control tumor growth, but did still cause colitis (Fig. 4A). These findings are in line with our previous results showing an important role for both CD4+ and CD8+ T cells in clearance of MC38-CEA (Fig. 4, [3]), as well as with work by others that indicated a pivotal role for CD4+ T cells in inducing experimental colitis [15,17,18].

CEA-specific immunity associated with colitis

In view of the impact of the adoptively transferred T-cell response on both CEA-positive tumors and CEA-expressing normal intestine, we analyzed this response in more detail. Adoptively transferred T cells from a Thy1.1+ donor were isolated from spleen, mesenteric lymph nodes and lamina propria from the colon of Thy1.2+ recipient mice. IFN-γ production by these freshly isolated T cells was analyzed in a 3-hour intracellular cytokine staining assay. One week after adoptive transfer, at the time when mice began to display weight loss (Fig. 3A), high numbers of CEA-specific, IFN-γ-producing CD4+ and CD8+ T cells could be found in CEA-tg mice, in particular in the intestinal epithe-



Sections of the large intestine were microscopically analyzed and scored for signs of pathology. Categories and scoring are as follows:

a. Degree of inflammatory cell infiltrate in epithelia and stroma (0-6 points)
b. Mucin depletion (0-3 points)
c. Crypt elongation and hyperplasia (0-3 points)
d. Crypt destruction (0-3 points)

FIGURE 3. Induction of colitis in CEA-tg mice by adoptive transfer of CEA-specific lymphocytes. **A**. Weight changes of wild-type (□) and CEA-tg (△) mice after 9.5 Gy total body irradiation in combination with BMT and adoptive transfer of immunized wild-type splenocytes. Donor cells were isolated from immunized wild-type mice and RBL was performed. 5×10^7 cells were injected i.v. on day 5 after irradiation. Recipient mice were weighed on the day of irradiation and every 3 days thereafter. The results represent the mean percentage ± SD of the original weight over time of 5 mice per group. **B**. Photograph of a representative colon from a wild-type and a CEA-tg mouse at day 7 after adoptive transfer. **C**. Histological analysis of the colon and small intestine in wild-type and CEA-tg mice. Control groups received cells from wild-type mice that were immunized s.c. with 100 µg Moloney Th peptide. The colons and small intestines in mice restored with lymphocytes from Moloney immunized donor mice were histological normal; however CEA-tg mice receiving 9.5 Gy irradiation in combination with BMT and CEA-specific cells developed severe colitis and inflammation of the small intestine. **D**. Histopathologic score of the colon from wild-type and CEA-tg recipients 1 and 5 weeks after adoptive transfer. **E**. Cryosection of the colon of a CEA-tg mouse, stained with antibodies to Thy1.1 marker that is expressed on cells from the donor.

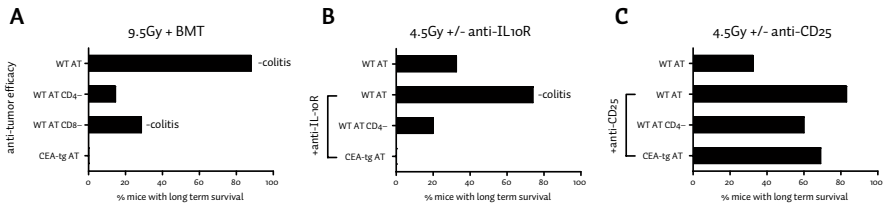


FIGURE 4. Long term survival of CEA-tg mice after different treatment regimens. A. CEA-tg recipient mice received 9.5 Gy TBI in combination with BMT and adoptive transfer of immunized wild-type cells, CD4/CD8 depleted wild-type donor cells or immunized CEA-tg donor cells. **B**. CEA-tg recipient mice received 4.5 Gy TBI and adoptive transfer of immunized wild-type cells. Adoptive transfer was combined with IL10R blocking antibody or donor cells were in addition depleted for CD4+ cells. Another group of CEA-tg recipients received adoptive transfer of immunized CEA-tg donor cells. **C**. CEA-tg recipients received 4.5 Gy TBI, adoptive transfer of wild-type donor cells combined with CD25-specific antibodies, adoptive transfer of CD4 depleted donor cells and anti-CD25 or adoptive transfer of immunized CEA-tg cells and CD25 depletion.

lium and in the draining lymph nodes. This reactivity was only found in the Thy1.1+ donor-derived population, whereas the recipient-derived Thy1.2+ cells showed no CEA-specific reactivity (Fig. 5A). Both the CEA-specific T-cell reactivity and the numbers of donor T cells gradually declined over time (Fig. 5B, C), a development that correlated with the subsidence of colitis-associated symptoms (Fig. 3A), supporting the direct involvement of the donor-type CEA-specific T cells in autoimmune colitis. Furthermore, only weak CEA-specific reactivity could be found when wild-type mice were used as recipients, and this reactivity was primarily found in spleen rather than the intestine or mesenteric lymph nodes (Fig. 5A).

Our data indicate that the CEA-specific T-cell response, when transferred into CEA-tg mice, is initially boosted by the presence of the CEA-expressing intestinal epithelium, causing strong accumulation of these T cells in intestine and draining lymphoid tissue, after which this response is gradually suppressed by a potent negative feedback. To examine whether the re-emergence of regulatory T-cell subsets from recipient origin, educated in a CEA-positive environment, might play a role in this immune control, 9.5 Gy TBI-treated CEA-tg recipient mice were reconstituted with bone marrow from RAG knockout mice. Adoptive transfer of CEA-specific lymphocytes into these mice resulted in colitis that, with respect to onset, severity and recovery, did not differ from the disease pattern observed in CEA-tg mice that were reconstituted with wild-type bone marrow cells (supplementary data 1). Therefore, regulatory T- (or B-) cell subsets of recipient origin do not play a significant role in suppression of the adoptively transferred CEA-specific T-cell response. A hint towards a more likely mechanism of immune suppression came from our finding that the lymphoid compartment of treated CEA-tg mice displayed striking numbers of IL-10 producing cells (Fig. 6). Interestingly, the numbers of these cells peaked around one week after adoptive transfer, at the time when also the CEA-specific T-cell response peaked, and gradually declined over the following week, again in correspondence with the CEA-specific T-cell response (Fig. 6A). Preliminary analysis of the phenotype of these IL-10 producing subset showed that these cells do not express the T-cell markers CD3, CD4 or CD8, nor the B-cell markers

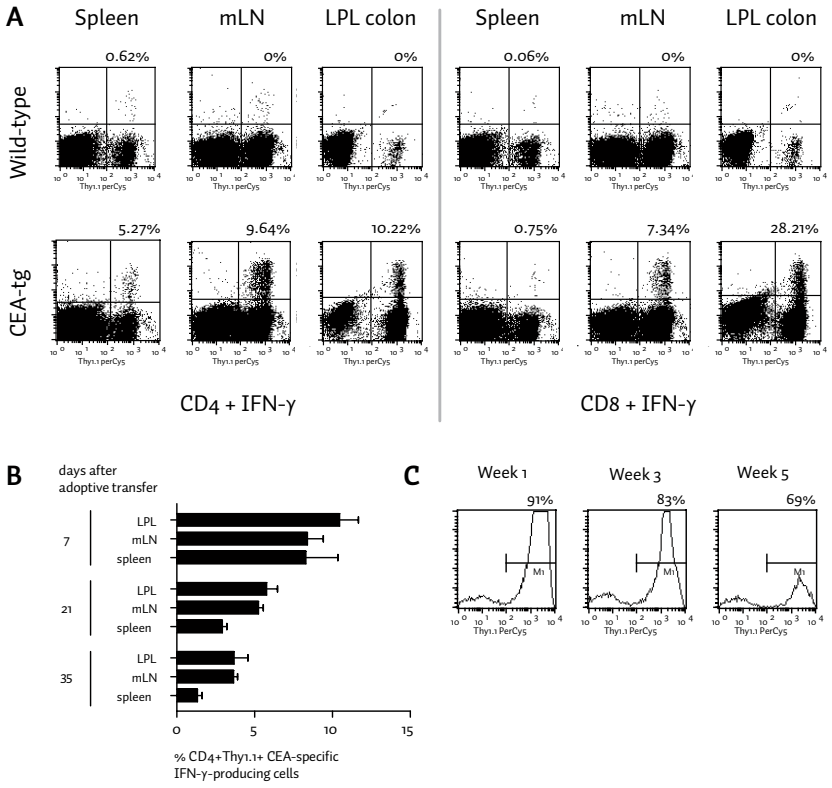


FIGURE 5. IFN-γ production by donor T cells from mice treated with 9.5 Gy TBI, BMT and adoptive transfer. A. CEA-specific IFN-γ production by CD4+Thy1.1+ and CD8+Thy1.1+ cells isolated from colon, mLN and spleen. Cells were isolated 7 days after adoptive transfer from wild-type or CEA-tg recipients. Cells were incubated with D1 cells and a mix of CEA Th epitopes or CTL peptide $CEA_{571-579}$ for 3 hours and analyzed for IFN-γ production by intracellular cytokine staining. Percentages shown are corrected for background values. **B**. CEA-specific IFN-γ production by CD4+Thy1.1+ cells isolated from colon, mLN and spleen. Cells were isolated 7, 21 and 35 days after adoptive transfer. Each bar represents the mean percentage ± SEM of IFN-γ production of 4-5 mice from two different experiments. **C**. Percentage of donor T cells recovered from mesenteric lymph nodes of CEA-tg recipients 1, 3 and 5 weeks after adoptive transfer.

CD19 and CD11b, nor the neutrophil marker Gr-1 (Fig. 6B), suggesting that we may be dealing with a myeloid suppressor cell [19]. Because the presence and activity of these cells coincide with that of the CEA-specific T cells, they seem to represent an emergency break to the pathological T-cell response, rather than a classical suppressor cell population. In the latter case, one would rather expect a suppressor population that gradually builds up as the autoimmune response subsides. Importantly, the action of the IL-10 producing cells does not prevent the CEA-specific T-cell response from clearing MC38-CEA in at least the majority of mice. Unfortunately, these cells also do not prevent colitis, although it is conceivable that this disease would be exaggerated, and even lethal, in the absence of these immune regulators.

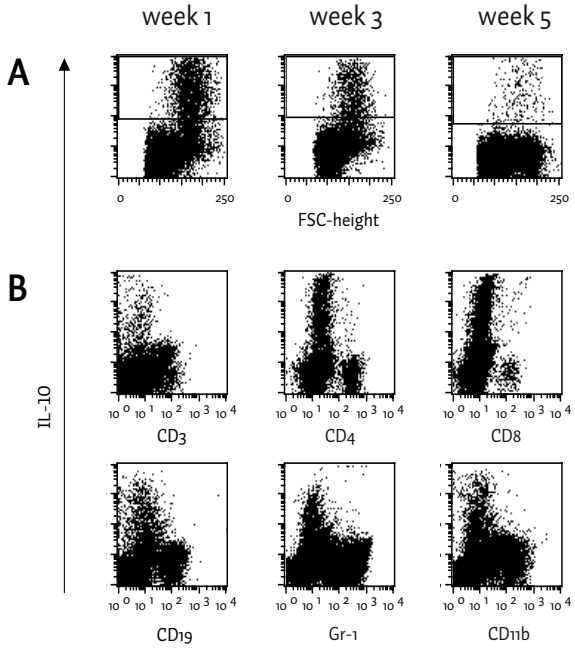


FIGURE 6. IL-10 production by cells isolated from mesenteric lymph nodes from CEA-tg recipient mice. CEA-tg received 9.5 Gy total body irradiation in combination with BMT followed by adoptive transfer of immunized wild-type splenocytes. **A**. Mesenteric lymph nodes were isolated 1, 3 and 5 weeks after adoptive transfer. Single cell suspensions were prepared and cells were plated in complete medium for 3 hours. IL-10 production was measured by intracellular cytokine staining. **B**. Mesenteric lymph nodes were isolated 3 weeks after adoptive transfer. IL-10 production was measured by intracellular cytokine staining and cells were stained for CD3, CD4, CD8, CD19, CD11b and Gr-1.

CD25 depletion allows for tumor-clearance in the absence of colitis

Intriguingly, if CEA-tg recipient mice were treated with 4.5 Gy TBI in combination with CD25-specific antibodies, the great increase in anti-tumor efficacy of the adoptive transfer was not paralleled by any signs of colitis (Fig. 2A, C, data not shown). This dissociation between anti-tumor immunity and colitis implied that CEA-specific T-cell immunity could play a less prominent role in this setting as compared to the other two modalities. We therefore compared the anti-tumor efficacy of adoptively transferred lymphocytes from CEA-vaccinated wild-type and CEA-tg donors. In contrast to what we found for the other two modalities, adoptive transfer of CEA-tg lymphocytes did have a clear anti-tumor effect when applied to 4.5 Gy CD25-depleted recipient mice (Fig. 4). Notably, adoptive transfer of lymphocytes from CEA-vaccinated wild-type donors was still more effective (Fig. 7), indicating that CEA-specific T-cell responses did play a role in tumor-eradication, but it is likely that in this setting the CEA-specific response is complement by additional effector mechanisms of which the efficacy is enhanced by CD25 depletion. Because the tumor cell MC38 is know to express a CD8+ T-cell epitope derived from an endogenous retroviral gene product of Murine Leukemia Virus (MulV) [20], we examined the CD8+ T-cell response against this epitope in mice that had successfully been treated through 4.5 Gy TBI and CD25 depletion. Indeed, these

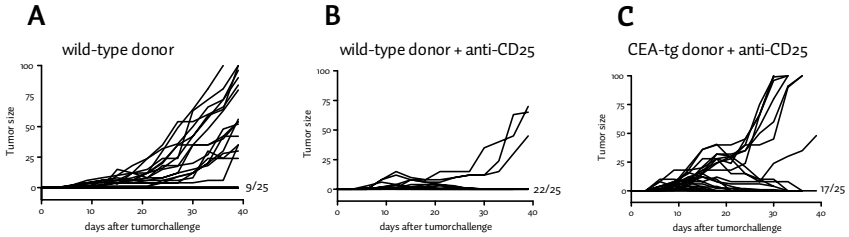


FIGURE 7. Tumorgrowth in CEA-tg mice treated with anti-CD25. A. CEA-tg mice were treated with 4.5 Gy TBI and adoptive transfer of immunized wild-type donor cells. **B**. CEA-tg mice received in addition anti-CD25 6 days before adoptive tranfer. **C**. CEA-tg mice received anti-CD25 and adoptive transfer of immunized CEA-tg donor cells. Data are cumulative from at least 3 experiments. The horizontal line represents one or more mice in which no tumor outgrowth was detected. The numbers at the right of these lines indicate the fraction of mice in each group that are tumor free at the end of the experiment.

mice showed a strong CTL response against this epitope, whereas such responses were not observed if treatment did not involve CD25 depletion (Fig. 8). These data suggest that depletion of CD25+ cells of the CEA-tg recipient mice resulted in antigen spreading of the anti-tumor T-cell response towards a non-autologous tumor specific T-cell epitope, explaining why the anti-tumor effect neither relies on a full CEA-specific T-cell repertoire, nor was associated with autoimmune colitis.

Efficacy of adoptive transfer regimens against spontaneous intestinal tumors

In the case of most transplantable tumor models, including the one employed in our studies, the tumor develops at a site distinct of that of natural carcinogenesis. This difference may greatly affect anti-tumor efficacy of the treatment, as well as the balance between efficacy and associated autoimmune pathology, in particular in cases where the target antigen is shared by tumor and normal surrounding tissue. Another critical difference may relate to the expression of additional, foreign antigens by the transplanted tumors, such as the retroviral epitope discussed above. In view of these considerations, we analyzed the impact of the previous described regimens in a model for spontaneous intestinal carcinogenesis. APC^{1638N} mice [21] were bred with CEA-tg mice resulting in APC^{1638N} × CEA-tg, which display the same CEA expression pattern as CEA-tg mice [16] and spontaneously develop CEA-overexpressing intestinal lesions [22]. When left untreated, tumor development was highly comparable between APC^{1638N} and APC^{1638N} × CEA-tg mice, in that average number and size of the tumors were the same (supplementary data 2). Therefore, comparison of these two strains, which develop CEA-negative and CEA-positive tumors respectively, will permit assessment of the efficacy of CEA-targeted immune interventions. We first tested adoptive transfer of lymphocytes from CEA-vaccinated wild-type donor cells combined with 9.5 Gy TBI and BMT. Treatment of APC^{1638N} × CEA-tg and APC^{1638N} single-tg mice was started at an average age of 8 months, when in untreated controls tumors become detectable macroscopically. Approximately one week after adoptive transfer of CEA-specific lymphocytes, all APC^{1638N} × CEA-tg mice showed severe weight loss to 65% of their original weight and the severity of the colitis eventually resulted in death of all the mice (10/10 data not shown).

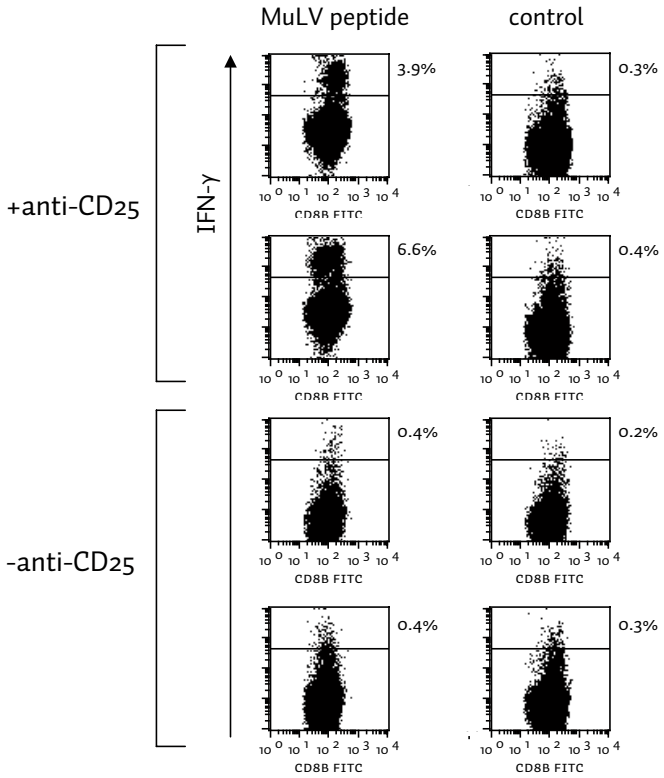


FIGURE 8. IFN-γ production by CD8+ T cells against M8 epitope expressed by MC38. CEA-tg mice were treated with 4.5 Gy TBI and received adoptive transfer of immunized wild-type donor cells with or without anti-CD25. One day after adoptive transfer mice were challenged with 1.5×10^5 MC38-CEA cells. One month later tumor-free mice from both groups were challenged with 1.5×10^5 MC38. After 28 days spleens were isolated and cells were restimulated for one week with an M8 expressing cell line (771). Recovered cells were tested for M8 specific IFN-γ production after 3 hour incubation with the M8 epitope with intracellular cytokine staining. Two representative examples per group are shown.

APC^{1638N} single-tg mice that underwent the same treatment did not show these symptoms and all survived. These data indicate that the risk for antigen specific auto-immunity is greatly increased in this model for spontaneous intestinal carcinogenesis, as compared to the transplantable tumor model, which may be due to the fact that tumor and normal intestinal tissue, sharing the target antigen, are co-localized. Because the treatment regiment involving IL-10R blockade also induced autoimmune pathology in our transplantable tumor model (Fig. 2A, Fig. 4), we did not test this in the $APC^{1638N} \times$ CEA-tg mouse model. Importantly, treatment involving CD25-depletion was not associated with autoimmune pathology in the transplantable tumor model. Therefore, this modality was applied on $APC^{1638N} \times$ CEA-tg and APC^{1638N} single-tg mice. In accordance with our experience with this treatment (Fig. 4), the $APC^{1638N} \times$ CEA-tg mice did not develop any signs of colitis. Treatment was started at an average age of 9 months and mice were sacrificed and examined 8 weeks later. Interestingly, the average number of tumors and the average surface area of the tumors per mouse after treatment were signifi-

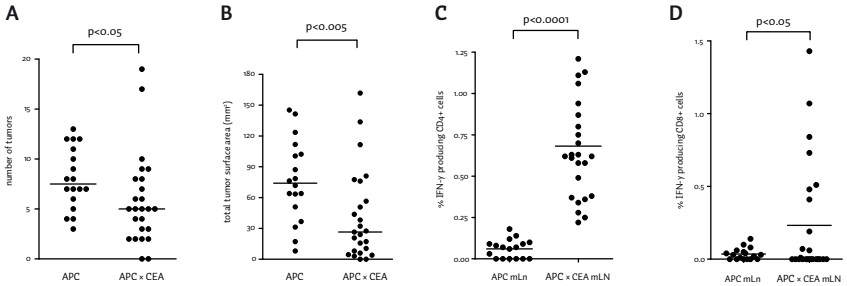


FIGURE 9. Tumorgrowth in APC^{1638N} and APC^{1638N} × CEA-tg mice after CEA-specific immunotherapy. APC^{1638N} and APC^{1638N} × CEA-tg mice received CD25-specific antibodies, 4.5 Gy TBI and adoptive transfer of immunized wild-type donor cells. Treatment was started at an average age of 9 months and mice were vaccinated every 2 weeks with B7.1-CEA DNA. Intestines were analyzed 8 weeks later for the number of tumors (**A**) and the size of the tumors (**B**). Mesenteric lymph nodes were isolated and analyzed for CEA-specific CD4+ and CD8+ T cells by intracellular IFN-γ staining after an overnight incubation with Th epitopes 1-5 (**C**) or CTL epitope 571-579 (**D**).

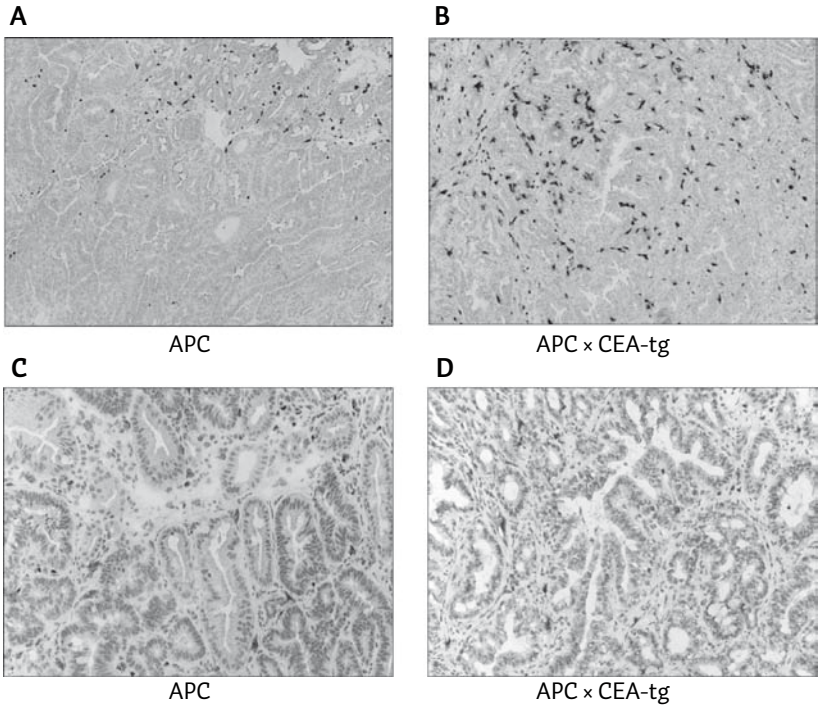


FIGURE 10. Immunohistochemical analysis of endogenous tumors. APC^{1638N} and APC^{1638N} ×CEA-tg mice received CD25-specific antibodies, 4.5 Gy TBI and adoptive transfer of CEA-immunized wild-type donor cells (**A**, **B**) or canarypox virus immunized wild-type donor cells (**C**, **D**). Cryosections (4 µm) of endogenous tumors, isolated 8 weeks after the start of the treatment from APC^{1638N} and APC^{1638N} ×CEA-tg mice, were stained for Thy1.1+ infiltrating cells. A representative example for each group is shown.

cantly lower in the group of APC^{1638N} × CEA-tg mice compared to the group of APC^{1638N} single-tg mice (Fig. 9 A, B). The notion that CEA-specific immunity suppressed tumor development in the APC^{1638N} × CEA-tg mice was supported by in vitro analyses of T-cell responses. Although, systemic CEA-specific IFN-γ production by splenocytes did not differ between the two groups (data not shown), CEA-specific IFN-γ production by T cells isolated from the mesenteric lymph nodes was very high in the APC^{1638N} × CEA-tg mice, while such responses were only barely detectable in mesenteric lymph nodes from APC^{1638N} single-tg mice (Fig. 9C, D). Moreover, hardly any infiltrating Thy1.1+ donor-type T cells were detected in the CEA-negative tumors of APC^{1638N} single-tg mice, whereas large numbers of such cells were found to concentrate in the CEA-positive tumors of APC^{1638N} × CEA-tg mice (Fig. 10A, B). The absence of infiltrating donor-type T cells in CEA-negative tumors suggested that these tumors were less penetrable by the adoptively transferred T cells than the CEA-positive tumors. To examine whether this would be related to the presence of the target antigen in the latter tumors, or would be due to a more general effect of CEA on T cells, adoptive transfer was also performed with lymphocyte populations from mice that had been vaccinated with canarypox virus, an immunogen that elicits potent T-cell responses against this virus (chapter 3). Immunohistochemical analysis of intestinal tissues indicated that this treatment resulted in comparably increased infiltration of donor-type T cells in tumors in APC^{1638N} × CEA-tg and APC^{1638N} single-tg mice (Fig 10C, D). Therefore, both CEA-negative and CEA-positive tumors appear equally penetrable by activated T lymphocytes, indicating that the increased infiltration of CEA-positive tumors after adoptive transfer of CEA-reactive T-cell populations is directly related to the presence and recognition of this target antigen. This latter point is supported by the fact that infiltration of CEA-positive tumors after transfer of CEA-reactive lymphocytes is much more extensive than after transfer of ALVAC-reactive lymphocytes (Fig. 10B, D).

In conclusion, reconstitution of the CEA-specific T-cell repertoire in CEA-tg mice can suppress the development of spontaneous, CEA-expressing intestinal tumors, in the absence of autoimmune pathology to the normal intestinal epithelium, provided that this treatment involves 4.5 Gy TBI and treatment with CD25-specific antibodies.

Discussion

The present study was designed to investigate CEA-specific anti-tumor efficacy in relation to the risk for autoimmune pathology. We found striking differences in this balance between different treatment regimens. When peripheral immune regulation was suppressed either by a combination of 4.5 Gy TBI and IL-10 receptor blockade or myeloablative irradiation (9.5 Gy TBI) combined with reconstitution of the haematopoietic system through bone marrow transplantation, anti-tumor efficacy was invariably accompanied with autoimmune colitis. Interestingly, circumvention of peripheral immune regulation by 4.5 Gy TBI in combination with CD25 depletion resulted in tumor eradication in the absence of autoimmune pathology.

Autoimmune colitis in CEA-tg mice was induced by CEA-specific T cells that infil-

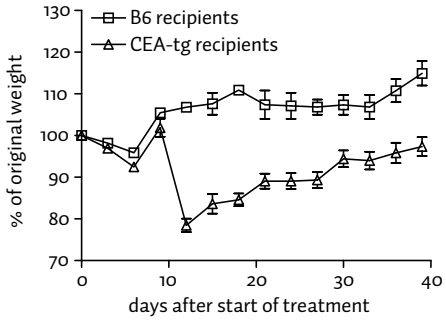
trated colonic tissue. One week after adoptive transfer, donor cells isolated from the colon and lymphoid compartments produced high amounts of IFN-γ in response to CEA peptides. Notably, colitis was transient and all mice recovered after 4-6 weeks. We showed that both the effectiveness and the number of donor cells reduced over time, but it is still not entirely clear what the mechanism is for this phenomenon. IL-10 producing cells could play an important role, as IL-10 production is very high at the time when mice have severe colitis. This could be a stress reaction on the ongoing immune response, as IL-10 is known to be a regulatory cytokine. The suppressive role that IL-10 might play in this situation is similar to the findings of others [23], and also correlates with our own data, as we have shown that IL-10 receptor blockade resulted in colitis. However, the role for IL-10 at the time of intestinal damage is mainly linked to the function of CD25+ regulatory T cells [24,25]. Intriguingly, in our model, T or B cells are not responsible for the enormous IL-10 production. Because these IL-10 producing cells seem to play an important role during severe inflammation, it is very interesting to further investigate this IL-10 producing cell population.

Wild-type mice that received lymphocyte infusion did not develop colitis and levels of IL-10 production were much lower compared to CEA-tg mice. However, irradiation is known to damage intestinal tissue [18,26,27] and we indeed also observed intestinal inflammation in wild-type mice after lethal irradiation. To reduce the harmful effects of lethal irradiation, suppression of immune regulation could also be accomplished by alternative treatments that possibly result in a better balance between anti-tumor immunity and auto-immunity. Such an alternative might be chemotherapy instead of radiation or the use of additional drugs that prevent or reduce the side effects of myeloablative irradiation like neuropeptides [28,29].

Our study also investigated the relative importance of CEA-specific immune responses in anti-tumor efficacy in different treatments. After myeloablative irradiation, tumor eradication was strictly dependent on the CEA-specific CD4+ T-cell response. When CD4+ cells were depleted from the donor, no colitis was induced and also no tumor clearance occurred. In this respect, treatment with CD25 depletion and CD4 depletion of the donor showed only a small reduction of the anti-tumor efficacy. Also adoptive transfer of cells from CEA-tg origin resulted in an effective anti-tumor response. We have shown that this is most likely the result of the induction of immune responses against other tumor antigens such as retroviral antigens. This is also likely to happen in human beings as a result of multiple different gene mutations in tumors. Notably, the observation of CTL immunity against a viral CTL epitope does not rule out that additional effector mechanisms, such as exerted by innate immune cells, contribute to the anti-tumor efficacy of this regimen. In fact, depletion of CD25+ cells was shown to also enhance NK-immunity [30]. This raises the question whether the response after CD25 depletion is only selective for tumor tissue and does not induce colitis because the CEA-specific response plays no role. This is not the case, as our data show that mice receiving adoptive transfer of CEA-tg cells do have initial tumor development. Thus, CEA-specificity is important in the initial anti-tumor response but due to CD25 depletion other reactivities can take over. The strongest argument for the role of CEA-specific immune responses comes from experiments performed in $APC^{1638N} \times$ CEA-tg mice

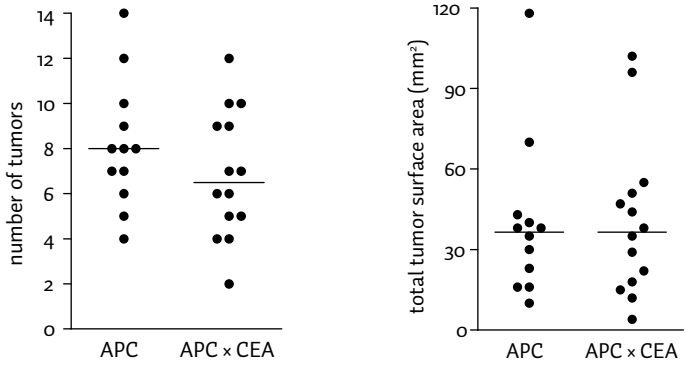
that show massive infiltration of CEA-specific cells in tumor tissue of APC^{1638N} × CEA-tg mice and not in APC^{1638N} single-tg mice. The number and size of tumors in treated APC^{1638N} × CEA-tg mice was also significantly lower than in treated APC^{1638N} mice. This effect was only partial as tumors were still present, but these data are highly relevant because these mice have endogenous instead of transplantable tumors. Importantly, treatment of APC^{1638N} × CEA-tg mice did result in anti-tumor efficacy, but this was not accompanied by autoimmune pathology. CEA expression levels in APC^{1638N} × CEA-tg mice are higher compared to human beings, so actually this model represents a worse case scenario, but still no autoimmune reactions were observed. This model is therefore more reliable than other CEA-tg mouse models in which CEA expression is much lower compared to humans and will consequently lead to an underestimation of the risk for auto-immunity [31].

We have shown that mainly CEA-specific CD4+ T-cell responses are of danger for intestinal damage and these findings have been confirmed by pilot experiments with TCR gene transfer of a CEA-specific CD4 TCR into CEA-tg splenocytes. TCR gene transfer resulted in a potent CEA-specific CD4+ T-cell response that induced colitis in CEA-tg mice but was so far not efficient in tumor eradication. Chances for exploitation for immunotherapy of CEA-positive tumors are selective application of CEA-specific CD8+ T-cell responses that will most likely result in anti-tumor efficacy without the induction of autoimmune pathology. We are currently testing whether selective induction of CD8+ T-cell responses in our available mouse models will lead to implications for the use of TCR gene transfer in clinical applications.



SUPPLEMENTARY DATA 1

Weight changes after 9.5 Gy TBI, BMT derived from RAG k.o. mice and adoptive transfer. Weight changes of wild-type (□) and CEA-tg (△) mice after 9.5 Gy total body irradiation in combination with BMT derived from RAG k.o. mice followed by adoptive transfer of immunized wild-type splenocytes. The results represent the mean percentage ± SD of the original weight over time of 5 mice per group.



SUPPLEMENTARY DATA 1

Tumorgrowth in APC^{1638N} and APC^{1638N} × CEA-tg mice without treatment. Intestines of APC^{1638N} and APC^{1638N} × CEA-tg mice were analyzed for the number of tumors and the size of the tumors at an average age of 8 months.

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