Induction of CD4⁺ and CD8⁺ anti-tumor effector T cell responses by bacteria mediated tumor therapy

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Facultative anaerobic bacteria like *E. coli* can colonize solid tumors often resulting in tumor growth retardation or even clearance. Little mechanistic knowledge is available for this phenomenon which is however crucial for optimization and further implementation in the clinic. Here, we show that intravenous injections with *E. coli* TOP10 can induce clearance of CT26 tumors in BALB/c mice. Importantly, re-challenging mice which had cleared tumors showed that clearance was due to a specific immune reaction. Accordingly, lymphopenic mice never showed tumor clearance after infection. Depletion experiments revealed that during induction phase, CD8⁺ T cells are the sole effectors responsible for tumor clearance while in the memory phase CD8⁺ and CD4⁺ T cells were involved. This was confirmed by adoptive transfer. CD4⁺ and CD8⁺ T cells could reject newly set tumors while CD8⁺ T cells could even reject established tumors. Detailed analysis of adoptively transferred CD4⁺ T cells during tumor challenge revealed expression of granzyme B, FasL, TNF- α and IFN- γ in such T cells that might be involved in the anti-tumor activity. Our findings should pave the way for further optimization steps of this promising therapy.

Introduction

First studies demonstrating an effect of bacterial infections on human solid tumors were reported >150 years ago.¹ Since then, numerous bacterial species like *Salmonella* spp. and *Escherichia coli* were observed to accumulate in tumors and induce shrinkage of the neoplasia.^{2–6} Phase I clinical trials using highly attenuated *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) could demonstrate a safe administration of this therapeutic to human patients. However, tumor colonization and anti-tumor effect was very limited in this studies most likely due to the strong attenuation of the applied strain.^{7,8} Therefore, developing a strain with an ideal attenuation level is one of the major goals to generate an applicable anti-tumor bacterium. The auxotrophic *S. Typhimurium* A1-R strain (leu/arg-dependent) is one such example

of a strain that has been shown to be very effective against human tumors in T cell deficient mouse models.^{9–17}

How bacteria invade tumors is not entirely clear. An active participation of *S. Typhimurium* was suggested.¹⁸ However, motility mutants of *Salmonella* were not hampered to invade tumors.¹⁹ Thus, Leschner *et al.* suggested an alternative mechanism.²⁰ Upon application of the bacteria, a massive systemic release of TNF- α is induced. This disrupts the already leaky pathological blood vessels of the tumor. The induced hemorrhage might carry the bacteria passively into the tumor. Hypoxic conditions induced by the interrupted blood flow result in a large necrotic area in which the bacteria thrive.

The presence of bacteria and the developing necrosis might result in a strong inflammation inducing a robust immune response. A number of studies have shown a connection between an inflammatory state within the tumor and an anti-tumor response. This could be achieved by irradiation,²¹ radiofrequency ablation,²² by injecting bacterial products like CpG²³ or direct injection of facultative anaerobic bacteria.²⁴ We also have provided indirect evidence that bacteria-induced tumor clearance might be due to the induction of an anti-tumor immune response.²⁵ On this basis, tumor specific CD4⁺ and CD8⁺ T cells were examined in more detail in the present study.

We employed the syngeneic murine colon carcinoma CT26 in BALB/c mice and administered *E. coli* TOP10 as soon as the tumors had reached 0.5 cm in diameter. TOP10 was employed because these bacteria induced tumor clearance but represented a low health burden for the mice. By depletion and adoptive transfer experiments, we could demonstrate that CD8⁺ as well as CD4⁺ T cells were responsible for tumor clearance under these conditions.

Key words: *E. coli* Top10, CD4⁺, T cells, CD8⁺, T cells, tumor necrosis, tumor antigen presentation

Additional Supporting Information may be found in the online version of this article.

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What's new?

Certain types of bacteria naturally home to and invade tumors, where they accumulate and ultimately induce tumor shrinkage. How bacteria act to reduce tumors, however, is still unknown. The work presented here shows that intravenous administration of *E. coli* to mice bearing CT26 colon carcinomas results in the induction of an immune response involving tumor-specific cytotoxic CD4⁺ and CD8⁺ T cells. CD4⁺ and CD8⁺ T cells effected neoplasia clearance. The findings could have implications for the design of therapeutic strategies that enhance the immune-inductive, cancer-fighting potential of bacteria.

Our findings clearly show that besides the known role of CD8⁺ effector T cells also CD4⁺ T cells could be major effectors in immunological responses against tumors. This knowledge should help to improve therapies that employ tumor targeting bacteria but also help to explain anti-cancer immune responses in general.

Material and methods**Mice, bacteria and cell lines**

BALB/c mice were purchased (Janvier, France). Rag1^{-/-} mice were originally purchased from The Jackson Laboratory (C.129S7(B6)-Rag1^{tm1Mom/J}). Igα^{-/-} mice were obtained from Michael Reth, Freiburg, Germany (Cd79 a^{tm2Pld}). Rag2γc^{-/-} mice (C.hybrid-Rag2^{tm1Cgn} IL-2Rgc^{-/-18}; generated at the AMC, Amsterdam). All recombinant mice were bred at the HZI. All experiments were performed with female, 8- to 12-weeks-old mice if not stated differently and under approval of LAVES (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit). Permission: 33.9-42502-04-12/0173.

S. Typhimurium SL7207 (hisG46, Δ407[aroA544::Tn10]¹⁹ and *E. coli* TOP10 were grown on LB agar²⁰ with 30 μg ml⁻¹ streptomycin at 37 °C.

CT26 (ATCC CRL-2638) cells were cultured in IMDM supplemented with 10% FCS, 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin, 50 μM 2-mercaptoethanol, 2 mM L-glutamine and maintained at 37 °C and 5% CO₂. The F1A11 (H-2^d) cell line is a murine fibrosarcoma that expresses β-galactosidase (β-gal) and was obtained by transduction of spontaneously transformed BALB/c fibroblast cell line F1 with the LBSN retroviral vector.²⁶

Tumor growth and infection

A 5 × 10⁵ cells in 100 μl PBS were injected subcutaneously (s.c.). Growth was monitored by caliper. Volume was calculated: $V = 4/3 \times \pi \times (h \times w^2)/8$; h = height and w = width. For intravenous infection (i.v.), bacteria from glycerol stocks were cultivated on streptomycin LB plates overnight. Single colonies were re-suspended in PBS and adjusted to 5 × 10⁶ bacteria in 100 μl PBS. For analysis, organs were homogenized in 0.1% (v/v) TritonX-100/PBS and homogenates were plated on streptomycin LB plates.

Depletion of immune cells

T cell depletion: anti-CD4 (150 μg in 100 μl PBS, 5-day interval, clone GK1.5) or anti-CD8 (100 μg in 100 μl, 5-day

interval, clone 53-6.7) were administered i.p./i.v. starting 2 days before infection. Granulocyte depletion: 25 μg anti-Gr1 (RB6-8C5) i.p. 1 day before infection and 1 day thereafter. Depletion was controlled 2 days after first treatment by flow cytometry.

Isolation of immune cells

Spleens were flushed with IMDM and erythrocytes were lysed in ACK buffer. CD8⁺ or CD4⁺ T cell were purified using negative isolation kits (Dynabeads Untouched Mouse CD8/CD4 Cells, Invitrogen). Purity controlled by flow cytometry was always >96%. For adoptive transfers 3 × 10⁶ cells were injected i.v.

Tumors were cut into small pieces and incubated twice in 1.5 ml collagenase/dispase solution (Roche) with gentle shaking. Cell containing supernatant was removed from tumor debris and transferred into PBS/EDTA. Cells were treated with ACK and washed twice.

Noninvasive in vivo imaging

E. coli TOP10 were transformed with the plasmid pHL304 encoding the bioluminescent *luxCDABE* operon (*lux*) from *Photobacterium luminescens*.²⁷ Bacteria were injected i.v. into CT26 bearing mice. Using the IVIS-200 system, bioluminescence emitted from the bacteria was monitored over a time period of 7 days every 24 hrs.

Statistical analysis

All displayed curves for tumor growth in Figures 1–5 represent the mean values and error bars represent the standard deviation of the mean. Curves in Figure 6 represent the median and error bars represent the mean deviation.

An extended Material and methods section can be found in Supporting Information.

Results**Comparison of *S. Typhimurium* SL7207 and *E. coli* TOP10**

We first compared *S. Typhimurium* (SL7207) that we had extensively used before and the laboratory strain *E. coli* TOP10 with regard to efficacy of tumor clearance and impact on general health of the mice. In some of the experimental animals we also depleted neutrophilic granulocytes by anti-Gr1 antibody injection, as described previously.²⁵

Figure 1a summarizes the results of three independent experiments (curves for individual mice are shown in Supporting Information Fig. S1). The total rate of tumor clearance (21/27 mice: 77.8%) was best in neutrophil depleted

Figure 1. Comparison of *S. Typhimurium* SL7207 and *E. coli* TOP10. (a) BALB/c mice were s.c. challenged with CT26 cells. After the tumor was established, mice were i.v. infected with SL7207 or TOP10. In one group of mice the neutrophilic granulocytes had been depleted additionally (+α-Gr1).²⁵ Tumor clearance and survival of mice is plotted. Data represent summarized results of three independent experiments with a total of 20–27 mice per group. (b) Bacterial colonization of tumor, spleen and liver 48 hrs after SL7207 or TOP10 infection with and without additional neutrophil depletion.

SL7207 infected mice. However, only one third of such mice survived. Thus, the overall efficacy dropped to 22.2% (6/27) of mice that had cleared the tumor and survived the infection. Similarly, 70.4% (19/27) of naive, SL7207 infected mice cleared the tumor, but only roughly 60% of them (12/27) survived the treatment (overall efficacy of 44.4%).

In contrast, *E. coli* TOP10 infected mice did not show symptoms of morbidity and survived to 100%. Tumor clearance rates in the non-depleted Group (9/20 mice: 45%) were comparable to non-depleted SL7207 infected animals. The number of *E. coli* infected mice which cleared the tumor after granulocyte depletion were even higher (12/21 mice: 57.1%) and all mice survived. Thus, the use of *E. coli* as tumor therapeutic agent might be advantageous compared to SL7207.

Bacterial colonization of tumor and organs

To analyze colonization, we infected CT26-bearing mice with both bacterial strains and quantified tissue colonization after

48 hrs. Both types of bacteria colonized tumors to the same extent (Fig. 1b). However, in contrast to the high bacterial loads in liver and spleen observed for SL7207 infected mice, no single *E. coli* could be recovered from these organs (Fig. 1b). This suggests that *E. coli* might be a valid alternative to *S. Typhimurium*.

Re-challenge of mice

The induction of an anti-tumor immune response was suggested by our previous results.²⁵ To test whether a similar reaction is elicited by *E. coli*, mice that had cleared the tumors were challenged with CT26 and contra-laterally with F1A11 as control (Fig. 2a). The re-challenge was carried out 100 days after complete clearance of the original CT26 tumor. Under these conditions, CT26 tumors grew only during a short period of 3–4 days and all such tumors were cleared by Day 6 (Figs. 2a and 2b). In contrast, growth of none of the control tumors was obstructed. These results are

Figure 2. Anti-tumor immune response after intravenous application of TOP10. (a) BALB/c mice that cleared the CT26 tumor after *E. coli* TOP10 infection were rechallenged s.c. 100 days later with CT26 again (left flank) and in addition with the independent control tumor F1A11 (right flank). (b) Individual growth curves of CT26 and F1A11 tumors on naïve BALB/c mice and on mice that initially had cleared CT26 after infection and were now re-challenged with CT26 and F1A11. (c) Rag1^{-/-} mice were challenged s.c. with CT26 cells and i.v. infected with TOP10 upon establishment of the tumor. In one group, granulocytes (α-Gr1) were depleted in addition. (d) Igα^{-/-} mice were challenged s.c. with CT26 cells and i.v. infected with TOP10 upon establishment of the tumor. WT BALB/c mice with the same treatment are plotted for comparison. All data represent one of two independent experiments consisting of five mice each group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

highly suggestive for a specific immune reaction against the CT26 tumor induced by bacterial application.

Tumor therapy in recombinant mice

To confirm this notion, we repeated the experiments in tumor-bearing Rag1^{-/-} mice lacking T and B cells. In both neutrophil depleted and non-depleted mice, tumor growth was only shortly retarded by infection (Fig. 2c). However, tumors of Rag1^{-/-} mice were well colonized. High numbers of bacteria could be observed until mice had to be sacrificed due to tumor outgrowth (Supporting Information Fig. S2). Interestingly, colonization of the healthy organs like spleen and liver of Rag1^{-/-} mice appears to be transient. Only in the initial phase of the infection, bacteria can be detected in such organs. A similar pattern could be observed for the organs of the WT control. The tumors are initially colonized in such mice but as the tumors get cleared, so does the colonizing bacteria along with the tumor (Supporting Information Fig. S2). Thus, T and/or B cells are crucial players in the anti-tumor response against CT26 after bacterial infection

whereas the innate immune response is sufficient to clear bacteria from their normal target organs.

To distinguish between requirements for T or B cells, the experiment was repeated in Igα^{-/-} mice containing normal T cells but no B cells. As shown in Figure 2d, tumor growth and clearance in Igα^{-/-} mice was comparable to that in WT mice. Apparently, B cells are not necessary for a successful anti-tumor response induced by bacterial infection.

T cell depletion during induction phase

To characterize the specific anti-tumor T cell response in more detail, we first depleted the CD4⁺ and CD8⁺ T cell subsets. Interestingly, tumor growth in uninfected wild type mice depleted of both CD4⁺ and CD8⁺ T cells is enhanced as compared to non-depleted mice (Supporting Information Fig. S3). Similar observations were made with Rag1^{-/-} mice (Fig. 2c) and for WT mice that received the anti-CD8 antibody treatment alone (Supporting Information Fig. S3). Possibly, the naïve adaptive immune system by itself is already interfering with tumor growth to some extent.

Figure 3. Depletion of CD4⁺ and CD8⁺ T cells in bacteria mediated tumor therapy. (a) *E. coli* TOP10 were applied to CT26-bearing BALB/c mice. To deplete CD4⁺ or/and CD8⁺ T cells, anti-CD4 or anti-CD8 antibodies or a mixture of both was applied in 5-day intervals starting 2 days before the infection (d -2). As control, rat IgG was injected. Tumor volume was measured and plotted. Note the different scales in case of tumor rejection. (b) BALB/c mice that cleared the initial CT26 tumor after a TOP10 infection were re-challenged 100 days later with CT26 cells and F1A11 as a control cell line, after the depletion of different T cell subsets. Depletion schedule was as described in (a). Tumor volume was measured and plotted. Curves represent tumor growth curves from individual mice. Data represent one of three experiments with similar results.

Importantly, tumors grew unobstructed when *E. coli* were applied after treatment with anti-CD8 (Fig. 3a). This suggests an essential role of such T cells. To our surprise, depletion of

CD4⁺ T cells did not interfere with the clearance of the tumor after bacterial infection. The pattern of individual mice was not different from mice that were untreated (Fig.

Figure 4. Adoptive T cell transfer of immune CD8⁺ and CD4⁺ T cells. (a) T cells from BALB/c mice that cleared the CT26 tumor after a TOP10 infection were adoptively transferred into Rag1^{-/-} mice. Those Rag1^{-/-} mice were s.c. challenged with CT26 cells at the same time as the transfer took place. Tumor volume was measured and plotted. Every group shows the mean of 5 individual mice. The data represent one of three independent experiments. (b) CD4⁺ or total T cells from BALB/c mice that cleared the CT26 tumor after a TOP10 infection were adoptively transferred into Rag1^{-/-} or Rag2γc^{-/-}. Tumor volume was measured and plotted. Every group represents the mean of five individual mice. The results are representative for one of three independent experiments. (c, d) T cells from BALB/c mice that cleared the CT26 tumor after a TOP10 infection were adoptively transferred into Rag1^{-/-} mice with established CT26 tumors. Purified T cell subsets were transferred 4 days (c) or 6 days (d) after tumor cell inoculation. (e) Tumor growth curves of single mice from (d). The results are representative for one of two independent experiments.

Figure 5. Cytotoxic effector molecules in tumor reactive CD4⁺ T cells. CD4⁺ T cells were isolated from BALB/C mice that had cleared the CT26 tumor after TOP10 infection and transferred into Rag1^{-/-} mice. At the same day, mice received a s.c. challenge of CT26 cells. Seven days later CD4⁺ T cells were isolated from tumor draining inguinal lymph nodes (tdLN) and stained for effector molecules (a). Comparison of GrB (granzyme B), FasL (Fas ligand), TNF-α (Tumor necrosis factor-α) and IFN-γ expression in recipients with or without tumor challenge (b).

3a). This suggests that in case of bacteria-mediated induction of the primary anti-tumor immune response, CD4⁺ T cells have no essential role.

T depletion in CT26-immune mice

We next repeated depletion experiments under re-challenge conditions by injecting antibodies 3 days before tumor cells. All tumors in naïve control mice grew (data not shown). Undepleted mice that were immune against CT26 due to an *E. coli* induced tumor clearance successfully rejected CT26 tumor cells (Fig. 3b) but accepted F1A11 as shown before. As expected, an almost unobstructed tumor growth was observed when both T cell subsets were depleted. Tumors grew in four out of five mice. In contrast, all mice rejected tumors in which CD4⁺ T cells were depleted and thus CD8⁺ T cells remained as the effectors. Interestingly, similar results were obtained when CD8⁺ T cells were depleted and thus CD4⁺ T cells remained unaltered. Apparently, in such mice CD8⁺ as well as CD4⁺ memory T cells exist that are able to reject CT26 tumors. However, importantly the depletion of CD8⁺ T cells in 7- to 9-weeks-old mice consistently eliminated >96% of the cells while in older mice (~6 month) depletion was only successful to 82.1%. Therefore, a contribution of CD4⁺ T cells to the rejection of re-challenging CT26 tumors needed additional confirmation.

Adoptive T cell transfer into Rag1^{-/-} mice

To confirm the tumor-specific T cell response, purified CD4⁺ and CD8⁺ T cells from mice that had cleared CT26 tumors were adoptively transferred into Rag1^{-/-} mice. At the same day, the mice received subcutaneously CT26 tumor cells. The T cells were isolated from mice 100 days after tumor clearance to ensure that the immune system was again at steady state conditions. Blood samples of the recipient mice were tested for the T cell populations 18 days after transfer. As shown in Supporting Information Figure S4, only the desired T cell subsets could be found.

CD8⁺ T cell and the mixture of CD4⁺/CD8⁺ T cells cleared the tumor after allowing growth for a short period of time (Fig. 4a). Importantly, also the CD4⁺ T cells were able to clear the tumors with the same kinetics (Fig. 4a). A contribution of contaminating CD8⁺ T cells to these results was excluded by the purity of the T cells found in the recipients (Supporting Information Fig. S4). These results confirm that not only CD8⁺ but also CD4⁺ tumor specific effector T cells are induced during bacteria-mediated tumor therapy.

Adoptive T cell transfer into Rag2^{-/-}γc mice

NK cells could be the cell population indirectly responsible for the effector function of the CD4⁺ T cells resulting in tumor rejection.²⁸ Therefore, the transfer of tumor-immune

Figure 6. Adoptive T cell transfer from CT26-bearing uninfected mice into Rag1^{-/-} mice. T cells were isolated from uninfected mice that carried a CT26 tumor for 12 days and adoptively transferred into Rag1^{-/-} mice. At the same time, mice were inoculated s.c. with CT26 tumor cells (a). CD4⁺ and CD8⁺ T cell subsets were isolated from uninfected CT26 tumor bearing mice 7 days after tumor cell inoculation. T cells were adoptively transferred into Rag1^{-/-} mice. At the same day mice were inoculated s.c. with CT26 tumor cells (b). The data represent one of two independent experiments.

CD4⁺ T cells was repeated using Rag2γc^{-/-} mice as recipients since they additionally lack NK cells. Independent of the presence or absence of NK cells, CD4⁺ T cells were able to reject CT26 tumors (Fig. 4b). Thus, NK cells are apparently not required for tumor rejection under these conditions.

Adoptive T cell transfer into Rag1^{-/-} mice with established CT26 tumors

To test whether such T cells are also able to reject already established tumors, Rag1^{-/-} mice were injected with tumor cells and after 4 or 6 days of growth 3x10⁶ of either CD4⁺, CD8⁺ T cells or a mixture of both was adoptively transferred. At this time points, the tumors were palpable or visible, respectively.

After receiving the mixture of CD4⁺ and CD8⁺ T cells or CD8⁺ T cells alone, all mice with 4 day old tumors underwent complete regression (Fig. 4c). With CD4⁺ T cells only, tumor growth was retarded but complete tumor clearance had never been observed (Fig. 4c). Apparently, the therapeutic

potential of CD4⁺ T cells is much lower compared to CD8⁺ T cells.

This was even more apparent in mice with 6-day-old tumors. Transfer of the mixture of CD4⁺ and CD8⁺ T cells resulted in a complete rejection although the kinetic was delayed compared to day 4 tumors (Fig. 4d). Similarly, some of the mice that had received CD8⁺ T cells were still able to clear the tumors (3/5). Transfer of CD4⁺ T cells showed no influence on tumor growth anymore (Fig. 4d). This suggests that CD4⁺ T cells might have an inferior therapeutic potential compared to CD8⁺ T cells.

Expression of cytotoxic effector molecules by tumor reactive CD4⁺ T cells

CD4⁺ T cells as cytotoxic effector cells have been described before.^{29–32} Several mechanisms can account for it.²⁹ To figure out which mechanism enabled CD4⁺ T cells to reject CT26 tumors in our case, we tested CD4⁺ T cells 7 days after adoptive transfer assuming that the effector function should be most apparent at this time point. When investigating the tumor draining LNs, several direct effector molecules like granzyme B and FasL that might be involved in direct killing of tumor cells were upregulated in such CD4⁺ T cells as compared to CD4⁺ T cells from the equivalent lymph node of mice without a CT26 tumor or from mice that had received naïve T cells (Fig. 5). Similarly, TNF-α and IFN-γ that might be acting indirectly were also upregulated in such T cells (Fig. 5). Thus, apparently under the influence of the bacterial infection tumor specific CD4⁺ T cells acquire a status that provokes differentiation into cytotoxic CD4⁺ T cells upon re-challenge with the CT26 tumor.

Adoptive T cell transfer from CT26-bearing uninfected mice into Rag1^{-/-} mice

The tumor-specific T cells that were observed in the present work might be induced *de novo* as soon as the bacteria are introduced into the animals. Alternatively, they might be already present in tumor bearing mice before bacterial infection and for yet unknown reasons not effective. To investigate these possibilities, we isolated T cells from uninfected BALB/c mice that carried a CT26 tumor for 12 days and adoptively transferred them into Rag1^{-/-} mice. At the same time, mice were inoculated s.c. with CT26 tumor cells. Dramatic growth retardation was observed when mice received T cells from uninfected tumor-bearing mice compared to mice that received no T cells or cells of non-tumor bearing WT mice (Fig. 6a).

The experiment was corroborated with isolated CD4⁺ and CD8⁺ T cells. In both cases tumor growth was retarded compared to control and with naïve CD4⁺/CD8⁺ T cells reconstituted Rag1^{-/-} mice (Fig. 6b). Like in Figure 4c, the effect of such CD4⁺ T cells is assumed to be not as persistent as with CD8⁺ T cells.

Thus, already in uninfected tumor bearing mice tumor-specific T cells are present, but not able to influence tumor

development. Only the changes in tumor environment and immune response elicited by the bacteria render such T cells effective.

Discussion

Although the possibility to treat solid tumors with bacteria is known since a considerable time, the use in clinics never went into routine, most likely due to the severe uncontrollable side effects. Also in the present work, problematic safety aspects of this treatment option became apparent. The *Salmonella* strain SL7207 is supposed to be a safe vaccine carrier strain. When given intravenously to tumor-bearing mice, it was very efficient in clearing the CT26 tumor. At the same time, many of the mice succumbed to the infection. This was quite in contrast to results when a laboratory strain of *E. coli* was employed. All of the infected mice survived the infection and still many mice had cleared the tumor. This argues in favor of usage of the *E. coli* strain. However, this might be only true for our particular model system. Other transplantable tumors usually re-grow after an initial phase of shrinkage (data not shown). Thus, stronger bacterial pathogenicity and immunogenicity might be required for such tumors.

When employing *E. coli* TOP10, we first confirmed the induction of a specific immune response after application of the bacteria. Mice that had cleared the original CT26 tumor reacted specifically when re-challenged with CT26 and a control tumor. Only the original CT26 tumor was rejected. In addition, colonized tumors in lymphopenic Rag1^{-/-} mice showed just a short period of tumor growth retardation. This demonstrates the involvement of the adaptive immune system in tumor clearance. The employment of mice that lack B cells indicated the essential role of T cells in this reaction.

As expected, by depletion experiments, CD8⁺ T cells could be shown to be involved in the clearance of the original, primary tumor after bacterial infection. In contrast, no effect of CD4⁺ T cell depletion could be observed in this phase of tumor therapy. Apparently, no CD4⁺ T cell help is required for the induction of the anti-tumor response although the presence of such tumor specific CD4⁺ T cells could be revealed. This is consistent with the present idea of T cell licensing. Normally, CD4⁺ T helper cells are required for the induction of CD8⁺ T cells.³³ However, under strong inflammatory conditions, as they are most likely found after an intravenous application of a high number of *E. coli*, CD8⁺ T cells could be activated without CD4⁺ T cell help.³⁴

Nevertheless, the tumor specific CD4⁺ T cells are activated under conditions of bacterial infection. Mice depleted

of CD8⁺ T cells still rejected the CT26 tumors. In addition, lymphopenic mice with transferred CD4⁺ T cells also rejected the challenging CT26 tumors. Thus, when *E. coli* TOP10 was applied to the CT26 tumor-bearing mice, the specific CD4⁺ T cells had been activated and acquired cytotoxic effector function. The absence of an effect of CD4⁺ T cell depletion during the induction phase might be due to the comparatively late appearance of cytotoxic CD4⁺ effector T cells.

That CD4⁺ T cells are able to differentiate not only into helper cells but also into cytotoxic effector cells is now well established.^{30,31,35,36} During chronic as well as acute infection conditions, CD4⁺ T cells acquire granzyme B or FasL dependent killer functions. In addition, IFN- γ and TNF- α production by such T cells was detected and a similar phenotype has been observed for CD4⁺ T effector cells in tumor models.³⁰ This is consistent with the CD4⁺ T cell phenotype perceived in our experiments.

The therapeutic potency of such CD4⁺ T cell population is apparently more limited than the potency of anti-tumor CD8⁺ T cells. It became obvious when the isolated T cell populations were tested against established tumors. Tumors that were grown for 4 days were all rejected by CD8⁺ T cells whereas the CD4⁺ T cells were only able to strongly retard tumor growth. Even more obvious was the inferior therapeutic power of the CD4⁺ T cells with tumors established for 6 days.

The reason for the differential effect could simply be due to a lower frequency of CD4⁺ effector T cells in the donor population. On the other hand, the effector mechanism of the CD4⁺ T cells might be weaker than that of the CD8⁺ T cells. This phenomenon will still require extensive molecular characterization in the future.

However, the results shown here demonstrate the importance of T cells in bacteria mediated tumor therapy. A variety of bacteria has been tested for their anti-tumor capacities. In many cases, these experiments could show a more or less growth retarding effect. In some model systems like the one presented here, it was even possible to gain high tumor clearing rates. This study obviously shows that an improvement of bacterial design for a more efficient cancer treatment always needs to go hand in hand with immunological approaches. The link between infection and effector T cells is clearly one important part in the mosaic of a successful bacteria mediated tumor therapy.

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