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The immune system prevents recurrence of transplanted but not autochthonous antigenic tumors after oncogene inactivation therapy

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Abbreviations: Tag: SV40 large T, TagLuc: fusion protein consisting of SV 40 large T and firefly luciferase, dox: doxycycline, BL: Bioluminescence, RLuc: Renilla luciferase, ATT: adoptive T cell therapy, T_E cells: monospecific $CD8^+$ T cells directed against Tag epitope I

Article category: Cancer Therapy and Prevention

What's New:

Previous tumor transplantation studies in mice showed that endogenous T cells can prevent tumor recurrence after oncogene-inactivation therapy. But, these experiments neglected the long-term, usually tolerogenic, interaction of *de novo* malignancies with the immune system. Here, we found that during autochthonous tumor regression surviving tumor cells were not eradicated by endogenous T cells and regrew after therapy cessation. However, adoptively transferred T cell targeting the cancer-driving oncogene eradicated relapsed tumors showing a successful treatment strategy.

Abstract

Targeted oncogene inactivation by small molecule inhibitors can be very effective but tumor recurrence is a frequent problem in the clinic. Therapy by inactivation of the cancer-driving oncogene in transplanted tumors was shown to be augmented in the presence of T cells. However, these experiments did not take into account the long-term, usually tolerogenic, interaction of *de novo* malignancies with the immune system. Here, we employed mice, in which SV40 large T (Tag) and firefly luciferase (Luc) as fusion protein (TagLuc) could be regulated with the Tet-on system and upon activation resulted in tumors after a long latency. TagLuc inactivation induced profound tumor regression, demonstrating sustained oncogene addiction. While tumor relapse after TagLuc inactivation was prevented in immunocompetent mice bearing transplanted tumors, autochthonous tumors relapsed or recurred after therapy discontinuation indicating that the immune system that coevolved with the malignancy over an extended period of time lost the potency to mount an efficient anti-tumor immune response. By contrast, adoptively transferred $CD8^+$ T cells targeting the cancer-driving oncogene eradicated recurrent autochthonous tumors, highlighting a suitable therapy option in a clinically relevant model.

Introduction

Small molecule inhibitors, designed to inactivate specifically a cancer-driving oncogene have been proven to be highly effective against a variety of cancers in the clinic, e.g. imatinib against BCR-ABL-driven leukemia [1]. Despite the revolutionary success of small molecule inhibitors, drug resistance and, hence, tumor recurrence, is a frequently observed problem in the clinic. Various preclinical animal models revealed that abrogation of the cancer-driving oncogene provokes tumor cell apoptosis, necrosis, senescence, autophagy, and/or differentiation, collectively proving oncogene addiction [2-9]. These cell autonomous mechanisms were long thought to be the primary mechanism responsible for tumor regression. However, recently, it was discovered in a c-myc-driven tumor transplantation model that $CD4^+$ T cells contribute to sustained tumor regression upon oncogene inactivation by release of chemokines like thrombospondins that remodel the tumor microenvironment, showing that cell extrinsic mechanism, namely cells of the adaptive immune system, contribute to the therapeutic effect of oncogene inactivation [10].

The finding that cancer cell-targeting drugs are not solely tumor cell autonomous but involve T cells was already described for cancer cells equipped with suicide genes that conferred cell death in vivo after application of the corresponding prodrug. T cell-competent mice rejected transplanted cancer cells following prodrug treatment, while tumors relapsed in T cell-deficient mice [11]. Later, it was found that cancer cell death was immunogenic when induced by certain chemotherapeutic drugs, e.g. anthracyclines, and that so treated cancer cells induced T cell-dependent tumor immunity in transplantation models [12, 13]. We showed in a model of large established transplanted tumors that therapeutic efficacy of oncogene inactivation was increased when immunodeficient hosts were reconstituted with T cells [5].

So far, the therapeutic effect of drug-driven anticancer therapies and the contribution of the immune system were mainly analyzed in experimental models with transplanted tumors. These models do not recapitulate the clinical situation because transplanted tumors,

although histologically not to discriminate from autochthonous tumors [14], grow in a short time to large tumors so that the interaction between cancer and immune cells is limited to few weeks. Mouse transplantation models do therefore not recapitulate the long-lasting interplay between cancer and the immune system as it occurs during autochthonous tumor development, which is usually sporadic, clonal and may last for decades in humans. Importantly, the process of cancer cell inoculation is associated with an acute inflammatory response facilitating T cell activation as it unlikely occurs during autochthonous tumor development. It remains therefore questionable if the results obtained from mice transplanted with cancer cells are predictive for cancer patients presenting in the clinic. Here, we established a model in which autochthonous tumors can be induced and treated by oncogene inactivating drugs. With this model we wanted to answer the question whether the immune system eradicates drug resistant cancer clones during oncogene inactivation therapy and prevents cancer recurrence after therapy discontinuation.

Material and Methods

Mice

Rag-1$^{-/-}$ or Rag-2$^{-/-}$ (Rag$^{-/-}$) mice and TCR-I mice, which are transgenic for the H2-D^{b}-restricted Tag epitope I-specific (Vβ7) T cell receptor [15], were obtained from The Jackson Laboratory. LoxP-TagLuc-pA mice [16], TRE^{loxP}stop^{loxP}TagLuc mice [5], CAG-rtTA mice expressing the reverse transactivator rtTA2^{S}-M2 [17], TCR-I x ChRLuc mice [18], all generated on a C57Bl/6 genetic background, and Tyr::Cre mice [19], were described elsewhere. Homozygous TRE^{loxP}stop^{loxP}TagLuc mice were crossed to Tyr::Cre$^{+/-}$/CAG-rtTA$^{+/-}$ mice to obtain TTC mice that were heterozygous for all three transgenes. To obtain mice with albino phenotype, mice were bred to C57BL/6-Tyr^{c-Brd} mice [20]. All animal experiments were conducted in accordance with institutional and national guidelines and regulations, after approval by the Landesamt für Gesundheit und Soziales (Berlin).

Cancer Cell Lines

Cells were cultured in Dulbecco's modified eagle medium (GIBCO), supplemented with 10% heat-inactivated fetal calf serum (PAN, Biotech) and 50 µg/ml gentamicin (GIBCO). For in vitro analyzes, cancer cell lines from primary tumors grown in TTC triple transgenic mice were established by collagenase digestion (type II, 1mg/ml, Invitrogen) of small tumor fragments. Obtained cell lines were cultured in the presence of dox (1 µg/ml). Additionally, the mouse melanoma cell line B16 was used to detect tyrosinase transcripts by RT-PCR. For tumor challenge experiments, $1x10^6$ Tet-TagLuc tumor cells or 50 to 100 µl fragments (about 1 x 1 x 1 mm cubes) derived from Tet-TagLuc tumors grown in $Rag^{-/-}$ mice were injected subcutaneously into indicated mice. Tumor growth and regression, respectively, were analyzed by BL imaging and determination of tumor volume by caliper measurement according to the formula (xyz)/2.

Doxycycline Treatment

Dox (0.2 mg/ml; Sigma) was administered by light-protected drinking water supplemented with 5% sucrose changed twice a week, or 1 µg/ml dox was added to the cell culture medium twice a week.

Bioluminescent Detection

Mice were anesthetized by isofluran inhalation using the XGI-8 Gas Anesthesia System (Xenogen) and received 3 mg of D-luciferin (Biosynth) intravenously dissolved in PBS (30 mg/ml) to detect TagLuc (firefly luciferase) tumor signal or received intravenously 100 µg coelenterazine (Biosynth), dissolved in 30% DMSO (Sigma-Aldrich) and 70% PBS ($MgCl_2$ and $CaCl_2$ free (Invitrogen)) to detect RLuc T cell signal. Mice were directly subjected to BL imaging (IVIS 200, Xenogen). The exposure time for BL image acquisition was 1 or 60 second, depending on the signal strength. The BL imaging data were analyzed with Living

Image software (Caliper Life Science). BL signal was quantified by placing a region of interest (ROI) around individual tumors.

Adoptive T Cell Transfer

Spleen cells of TCR-I x ChRLuc mice were isolated seven days after immunization with $1x10^7$ Tag^+ 16.113 cells [21] and spleen cells containing 5 x 10^6 $Vb7^+$ cells were injected intravenously into mice that were irradiated with 4 or 5 grey on the same day.

Flow cytometry and antibodies

TCR-I x ChRLuc transgenic spleen cells were stained with following anti-mouse antibodies before transfer at a final concentration of 1:100: CD3 (145-2C11, Phycoerythrin (PE), BioLegend), CD8 (53-6.7, allophycocyanin (APC), BioLegend), and Vb7 (TR310, fluorescein isothiocyanate (FITC), BioLegend). Blood samples were stained with H2-Db tetramers loaded with peptide I (SAINNYAQKL) (PE, MBL), CD8 (53-6.7, APC, BioLegend) and CD3 (17-A2, FITC). Erythrocytes from blood samples and spleen cells were lysed by BD™ Lysing solution (BD) and ammonium chloride treatment, respectively. Samples were washed in PBS and acquired using FACSCalibur (BD). Data analysis was performed using FlowJo software (TreeStar, Ashland, OR, USA)

Histology

H&E staining of sections from paraffin embedded tumors were performed according to standard techniques. Pathological analyses were performed by ECVP certified veterinary pathologists (OK, ADG).

PCR

To determine recombination status of tumors developed in TTC mice, the following primer pairs were used: #1 forward primer: 5'-CGAGGTAGGCGTGTACGGTGG-3', #1 reverse primer: 5'-GCAAATTTAAAGCGCTGATGATCC-3',
#2 forward primer: 5'-CGAGGTAGGCGTGTACGGTGG-3', #2 reverse primer: 5'-CGGATGAGCATTCATCAGGCGGG-3'.

RT-PCR

RNA was isolated using the RNA isolation kit from Stratec. After DNase treatment, cDNA was reverse transcribed using SuperScriptIII reverse transcriptase and oligo (dT) primer (life technology). To detect tyrosinase transcripts, the following primers (exon spanning) were used: forward primer: 5'-TGTACAGAGAAGCGAGTCTTGA-3'; reverse primer: 5'-ACAAATGATCTGCCAGGAGGA-3'. Beta actin cDNA was amplified using following primer pair: forward primer: 5'-ACCACACCTTCTA-CAATGAG-3', reverse primer: 5'-GTAGATGGGCA-CAGTGTGGG-3'.

Results

Prevention of transplanted tumor relapse after oncogene inactivation in immunocompetent mice

Tet-TagLuc is a fibrosarcoma cell line derived from a $TRE^{loxP}stop^{loxP}$TagLuc transgenic mouse (see below) and has been described (5). Tet-TagLuc cells express a fusion protein of SV40 large T (Tag) and firefly luciferase (TagLuc) as cancer-driver under tetracycline promoter control, which can be regulated by doxycycline (dox). Previously, we observed that the frequency of tumor relapse after oncogene inactivation therapy could be reduced in the presence of an intact immune system. [5]. To extend the data, we exploited LoxP-TagLuc-pA [16] x CAG-rtTA [17] double transgenic mice to enable growth of Tet-TagLuc cells, a

regressor cancer cell line in wildtype mice. Although LoxP-TagLuc-pA x CAG-rtTA mice are tolerant for TagLuc and transactivator antigens, Tet-TagLuc cells injected subcutaneously as single cell suspension were rapidly rejected (Figure 1A), indicating the presence of at least one additional transplantation rejection antigen. Transplantation of Tet-TagLuc tumor fragments into LoxP-TagLuc-pA x CAG-rtTA mice prevented tumor rejection and tumors grew progressively in all mice. After an average tumor growth period of 28 days (range 22 to 34 days), tumors reached a size of at least 500 mm^3 and TagLuc inactivation by dox application was started (Figure 1B and C). TagLuc inactivation resulted in rapid decline of bioluminescence (BL) signal and tumors regressed as followed by BL imaging and caliper measurement, respectively (Figure 1B and C). Tumors of all $Rag^{-/-}$ mice initially regressed but then relapsed around 1 month after therapy. In contrast, tumors from 6 out of 7 immunocompetent LoxP-TagLuc-pA x CAG-rtTA mice did not relapse, although, a small, soft lump at the tumor side remained but did not progress. These results suggested that therapeutic efficacy of oncogene-inactivation therapy depends on the presence of an intact adaptive immune system in a tumor transplantation model, similar as has been reported by Rakhra et al. [10]. Furthermore, tumors in LoxP-TagLuc-pA x CAG-rtTA mice, although grown for more than one month in the presence of the adaptive immune system, seemed not to have induced tolerance of endogenous tumor-reactive T cells.

TTC mice develop autochthonous tumors

The previous experiment utilized transplantation of cancer cells under the skin that progress in a relatively short period to full-blown tumors. To analyze whether our observations can be extended to tumors initiated and evolved in a primary host with a much longer coevolution between cancer and T cells, we utilized $TRE^{loxP}stop^{loxP}$TagLuc transgenic mouse lines described previously [5]. Like Tet-TagLuc cells, $TRE^{loxP}stop^{loxP}$TagLuc transgenic mice contain the TagLuc fusion gene under control of the Tet-promoter, but due to a loxP-flanked stop cassette located between the Tet-promoter and the TagLuc transgene, and the requirement of an active transactivator, expression was prevented in $TRE^{loxP}stop^{loxP}$TagLuc

single transgenic mice (Figure 2A). We aimed to achieve TagLuc expression under control of the Tet-on system in a melanocyte-restricted fashion to establish an autochthonous melanoma model. To this end, we crossed TRE^{loxP}stop^{loxP}TagLuc mice derived from two founder lines (F7 and F10) to CAG-rtTA and Tyr::Cre [19]transgenic mice, which express the rtTA and Cre transgenes under the CAG and tyrosinase promoter, respectively (Figure 2A). TagLuc expression was not detectable by BL imaging in the absence of dox in TRE^{loxP}stop^{loxP}TagLuc single (T), TRE^{loxP}stop^{loxP}TagLuc x CAG-rtTA double (TC), and TRE^{loxP}stop^{loxP}TagLuc x CAG-rtTA x Tyr::Cre triple transgenic (TTC) mice of both founder lines, as shown for F7 in Figure 2B. Dox administration via the drinking water (200 µg/ml) resulted in profound induction of TagLuc expression in TTC triple transgenic mice (Figure 2B and C). Low TagLuc expression was detected in TC double transgenic mice (mean average radiance TC F7 vs TTC F7, 5.17E+03 ± 1.48E+03 vs 3.79E+04 ± 3.62E+04; TC F10 vs TTC F10, 3.91E+03 ± 6.3E+02 vs 6.56E+05 ± 1.74E+06), most likely due to weak leakiness of the stop cassette. No tumor formation was observed in TC double transgenic mice with one exception for each founder line (F7: 1 out of 16 observed mice; F10: 1 out of 13 observed mice; average observation period: 42 and 36 weeks, respectively) (data not shown). TTC triple transgenic mice treated with dox since birth developed primarily non-pigmented skin tumors and visceral tumors (Figure 2D) with an average tumor latency of 42 weeks (F7) and 35 weeks (F10), respectively (Figure 2E). Histological analysis of 14 tumors isolated from eleven TTC mice revealed that the majority of tumors (8/14) were spindle cell tumors, but also pancreatic exocrine adenocarcinomas (n=2), mammary adenocarcinomas (n=2), papilloma (n=1) and a round cell tumor were identified (Figure 2F and Table 1). In all analyzed tumors, deletion of the stop cassette was detected (Figure 2G), however, none of the isolated tumors showed tyrosinase promoter activity as analyzed by detection of tyrosinase expression (Figure 2H). These results suggest that tumors arose from progenitor cells that deleted the stop cassette due to transient tyrosinase promoter activity but these cells did not commit to the melanocyte lineage. We assume that cells from these progenitor

cells transformed with higher penetrance than melanocytes so that mice succumbed to non-melanoma tumors before melanomas could develop.

Autochthonous tumors regress long-term following oncogene inactivation but dormant tumor cells regrow after therapy discontinuation

Tumor-bearing TTC mice with one (n=6), two (n=3) or more tumors (n=1) were subjected to oncogene inactivation therapy by withdrawal of dox-containing drinking water. The average age of mice at the time point of treatment was 298 days (range 166 to 584 days) (Table 2). Dox cessation resulted in a rapid decrease of BL signal in all treated mice (n=10), declining to less than 2% (range 0.009 to 10.5 %) of the original signal strength after an average observation period of eight days as shown for seven mice (Figure 3A and B and Figure S1). Decline of BL signal was followed by tumor regression, suggesting abundant cell death (Figure 3C). From in vitro analysis of cancer cell lines derived from the transgenic mice we know that TagLuc inactivation results in apoptotic cell death in a high proportion of cells (up to more than 50%) (unpublished observation). To analyze whether TagLuc inactivation in autochthonous tumor-bearing mice resulted in complete tumor cell eradication, eight of the ten mice with stably low BL signal over time (average observation period 83 days; range 19 to 203 days) were subjected again to dox-containing drinking water. Surprisingly, BL signal increased rapidly in all mice after dox reapplication and tumors developed at the primary tumor site as shown for six mice in Figure 3D-F and Figure S1. Together, oncogene inactivation in autochthonous tumor-bearing hosts resulted in relatively durable tumor regression, but, more importantly, in all treated mice some tumor cells survived and remained dormant for several weeks and up to six months, able to resume proliferation as soon as TagLuc was reactivated. These results suggest that massive tumor cell death induced by oncogene inactivation does not elicit an immune response potent enough to prevent tumor progression upon oncogene reexpression.

Adoptively transferred T_E cells eradicate autochthonous tumors that relapsed after oncogene inactivation

To analyze antigenicity of dormant cancer cells that persisted after oncogene inactivation in the autochthonous host, two tumor-bearing TTC transgenic mice were subjected to TagLuc inactivation therapy (Figure 4A). A relatively low but stable Tag-Luc signal at the former tumor site was detectable more than one month after TagLuc inactivation indicating the presence of dormant tumor cells. These mice were irradiated (5 grey) and received monospecific $CD8^+$ T cells ($5x10^6$ cells) directed against Tag epitope I (T_E cells) isolated from immunized TCR-I x ChRLuc double transgenic mice. Expansion and migration of transferred T cells were followed by detection of Renilla luciferase (RLuc, which can be distinguished from firefly luciferase in TagLuc) T cell signal by BL imaging over time (Figure 4A). Two days after T_E cell transfer, an RLuc T cell signal was localized predominantly in cervical lymph nodes and between day 5-16 also at the previous tumor site indicating that dormant tumor cells were antigenic and recognized by T_E cells in the primary host.

Regularly, we observed mice with reappearing BL signal despite continuous oncogene inactivation therapy indicating tumor relapse due to drug resistance. To ask whether transferred T_E cells could eradicate autochthonous tumors that escaped oncogene inactivation therapy, we irradiated nine mice with drug resistant tumors and injected $5x10^6$ T_E cells. Tumor rejection by transferred T_E cells was monitored by detection of TagLuc tumor signal over time. A reduction in TagLuc activity at the tumor site was observed starting at day four after T_E cell transfer and no tumor TagLuc signal was detectable after 12 days in all mice (Figure 4B and C and Figure S2). Irradiation alone did not result in tumor eradication (n=1, data not shown). Tumor rejection was accompanied by robust T_E cells expansion, reaching T_E cell counts of up to 89 % of the total T cell population at day ten after transfer (average 46.2 %) as shown in Figure 4D (n=5). While one mouse died seven days after T_E cell transfer and decline of TagLuc tumor signal, the remaining eight mice were observed long-term (54 to 180 days) and tumors did not relapse (Figure S2). Together, dormant tumor cells are antigenic and recognized by transferred T_E cells. Furthermore, T_E cell therapy proved to be

effective to eradicate tumor variants that escaped destruction by oncogene inactivating therapy in primary tumor-bearing hosts. In conclusion, immunogenic cell death after oncogene inactivation therapy, if at all operative, could neither prevent eventual tumor relapse nor tumor progression after therapy discontinuation in autochthonous tumor bearing hosts.

Discussion

In the present study, we analyzed the role of the host immune system for sustained tumor regression after oncogene inactivation therapy. In mice with transplanted tumors we found a substantial improvement of the therapeutic outcome of oncogene inactivation therapy dependent on the presence of an intact immune system, confirming earlier studies (5, 10). Since the mice were tolerant for TagLuc and rtTA and Tet-TagLuc cancer cells were rejected as single cell suspension but grew as fragments [22], we can postulate additional, yet unknown, tumor transplantation rejection antigen(s). These data strongly indicate that cancer cell death induced by oncogene inactivation can be immunogenic under special conditions (the artificial process of cancer cell injection causing acute inflammation) and elicits T cells that reject transplanted cancer cells. However, when the same therapeutic approach was analyzed against autochthonous tumors, tumors frequently relapsed under therapy or recurred after therapy discontinuation. This contrasting effect might be due to the different time period the immune system interacted with the tumor and its microenvironment in the transplanted versus the autochthonous tumor model. The period was relatively short in mice with transplanted tumors but long during autochthonous tumor development. An extended period of time in which T cells encounter their cognate antigen can result in T cell exhaustion that might have hampered the contribution of T cells to the therapeutic effect of oncogene inactivation in mice with autochthonous tumors. This assumption is supported by clinical data and an autochthonous cancer model, demonstrating therapeutic effects by so-called checkpoint inhibitors like anti-PD-1 (ligand) antibodies that unleash tumor-reactive T cells from suppression [24-26]. In addition, the inflammation created by tumor cell injection in the

transplanted tumor model might have increased the number of pre-existing tumor infiltrating T cells facilitating anti-cancer activity after oncogene inactivation in an artificial manner.

Like for other oncogenes including ras, myc, bcr-abl [2, 4, 27], the growth and survival of cancer cells in the current model remained long-term dependent on the expression of TagLuc. These data are at variance with previous data suggesting that tumors at advanced stage progress independent of Tag expression [28]. However, in this model a version of the transactivator was used that did not allow as tight regulation as rtTA used here. Also in common with other oncogenes, TagLuc inactivation resulted in complete regression of large tumors, yet some cancer cells escaped elimination in a dormant state and could rapidly be reactivated upon drug discontinuation [2, 8, 29] or became drug-resistant, e.g. through mutations in the rtTA conferring dox-unresponsiveness [5, 30]. Identifying cancer specific point mutations in tumor samples taken prior oncogene inactivation and engineering T cells with receptors that target these mutations might be a promising approach to combat relapsing tumors after oncogene inactivation therapy in the clinic. In conclusion, in a clinically relevant model, adoptive T cell therapy but not therapy targeting a cancer-driving oncogene can prevent autochthonous tumor recurrence occurring spontaneously or upon treatment discontinuation.

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Figure 1. Sustained regression of transplanted tumors after oncogene inactivation therapy in immunocompetent hosts. (A) LoxP-TagLuc-pA x CAG-rtTA mice (n=5) were injected with Tet-TagLuc cancer cells and BL signal was followed over time. Data represent a single experiment. (B) LoxP-TagLuc-pA x CAG-rtTA mice (n=7) or $Rag^{-/-}$ mice (n=6) were injected with Tet-TagLuc tumor fragments, and, when tumor reached a size of >500 mm^3, mice were treated with dox or were left untreated (n=1). FLuc tumor signal was followed over time by BL imaging. (C) Tumor growth kinetics of mice shown in (B). Data in (B) and (C) are combined from three experiments.

Figure 2. Autochthonous tumor model allowing oncogene-inactivation therapy. (A) Scheme of the breeding strategy to generate mice that conditionally express TagLuc fusion protein in the presence of dox. (B) Mice transgenic for $TRE^{loxP}stop^{loxP}$TagLuc (T) (n=5), $TRE^{loxP}stop^{loxP}$TagLuc and CAG-rTA (TC) (n=7), or $TRE^{loxP}stop^{loxP}$TagLuc, CAG-rtTA and Tyr::Cre (TTC) (n=16) were subjected to BL imaging before and after induction by dox (acquisition time 60 seconds). One representative example for each genotype is shown. (C) Average light signal of TC and TTC mice of founder line 7 and 10 before (white circle) and after induction (black triangle) by dox. Light signals of B6 mice denote background. Each symbol indicates an individual mouse. Mice with albino phenotype are indicated with hatched symbols in the “after dox” situation. (D) Photographs of seven tumor bearing TTC mice. In one mouse (top left), the abdominal wall was opened to reveal the visceral tumor (white arrow) that shows firefly luciferase activity (adjacent picture). (E) Tumor-free survival of TTC mice of founder line 7 and 10 after dox administration. Mice were counted as tumor bearing when a locally increasing signal was detectable by BL imaging with an exposure time of one second. Crosses indicate mice that were censored. (F) Light microscopy of H&E stained sections from tumors located at the snout (left) or pancreas (right). Scale bar 50 µm. (G) Tumors of TTC mice were analyzed for deletion of the loxP site-flanked stop cassette using two different primer pairs (#1 and #2). Primer location and expected band size are indicated. Two representative results (tumor #6653 and #5276) are shown. Exemplary PCR results of a

tumor with (Tet-TagLuc) and without recombination (TC 200.09) of the stop cassette are shown. (H) RT-PCR to analyze tyrosinase expression in cell lines (n=5) derived from tumors grown in TTC mice. Melanoma tumor cell line B16 served as positive control. Beta actin was detected as internal control.

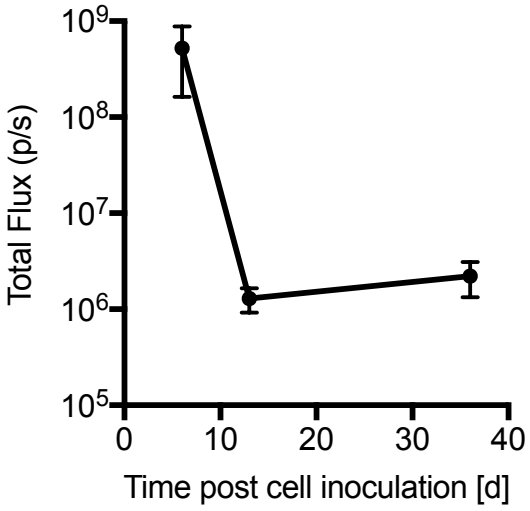
Figure 3. Autochthonous tumor regression following oncogene inactivation and regrowth of dormant cells after therapy discontinuation. **(A)** Primary tumor-bearing TTC mice (n=10) receiving dox for an average period of 298 days (range from 166 to 584) were subjected to oncogene inactivation therapy (dox off). TagLuc expression was followed by BL imaging over time (one second acquisition time). (B) Quantified BL signal from mice shown in (A). (C) Photograph of a tumor-bearing TTC mouse shown in the lower panel in (A) before and 27 days after dox cessation. The same mouse is also shown in Figure 2D. (D) A TTC mouse with two primary tumors was monitored over time by BL imaging after dox cessation and reapplication 203 days later. (E) Quantified BL signal over time of tumor located at the snout (white filled circle) and abdominal cavity (black filled circle) from mouse shown in (D). A red filled circle indicates time point of dox reapplication. (F) Photographs of the snout tumor from mouse shown in (D and E) before and after dox reapplication. See also related supplementary Figure S1 and Table 2.

Figure 4. Adoptively transferred T_E cells recognize dormant tumor cells and eradicate tumors that relapsed during target drug therapy. (A) Primary tumor-bearing TTC mice (n=2) were subjected to oncogene inactivation therapy and tumor regression was monitored by detection of tumor TagLuc signal before and after dox cessation (left panel). Mice were irradiated, injected with $5x10^6$ $RLuc^+$ TCR-I T_E cells and RLuc T cell signal was monitored over time by BL imaging (right panel). (B) Primary tumor-bearing TTC mice were treated by dox cessation and tumor regression was followed by BL imaging. When BL signal was increasing despite continued dox cessation that indicated tumor relapse, mice (n=9) were irradiated and

received a single injection of T_E cells. Kinetic of tumor eradication by T cells was monitored by BL imaging of tumor TagLuc activity. (C) Quantified TagLuc signal of mice shown in (B). Time point of dox cessation and T cell therapy are indicated by blue circle and black arrow, respectively. Two representative examples from nine treated mice are shown in (B) and (C). (D) Percentage of T_E cells in blood of immunized C57BL/6 (n=2) or tumor bearing TTC mice (n=5), respectively, ten days after T cell therapy. One representative dot blot is shown (left) and all data are summarized (right). See also related supplementary Figure S2 and Table 2.

Figure 1

A


Total Flux (p/s)
10^9
10^8
10^7
10^6
10^5
0
10
20
30
40
Time post cell inoculation [d]

B

C

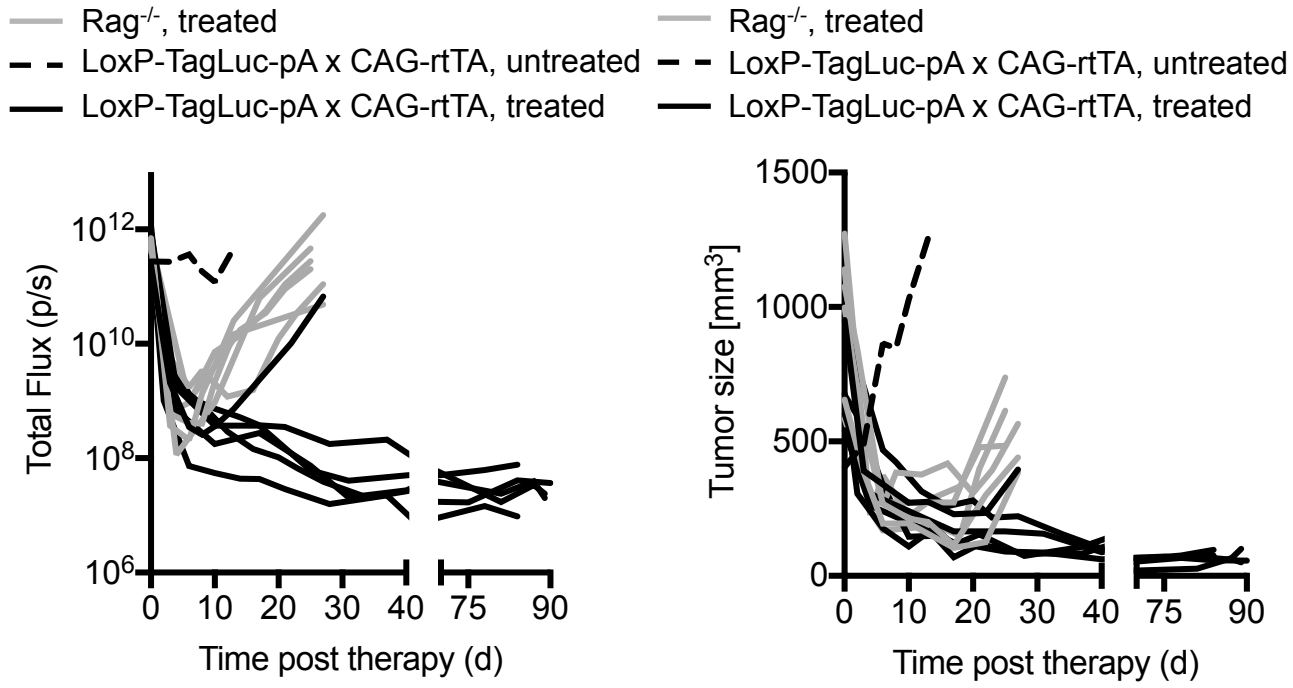
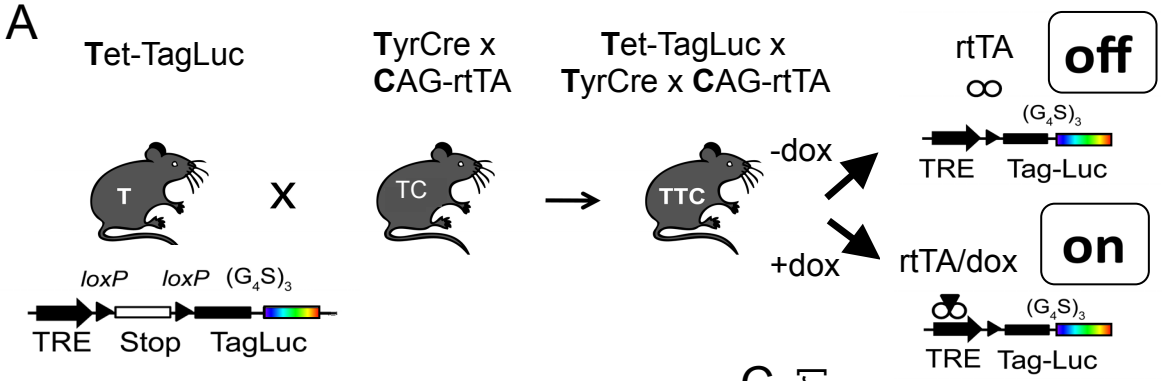
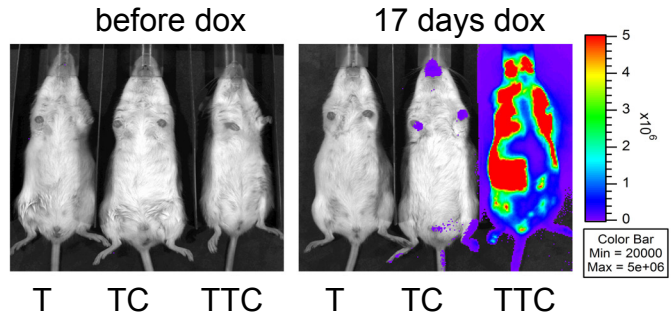
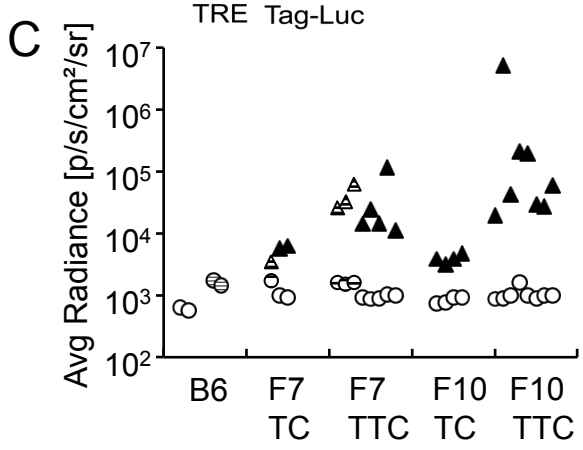
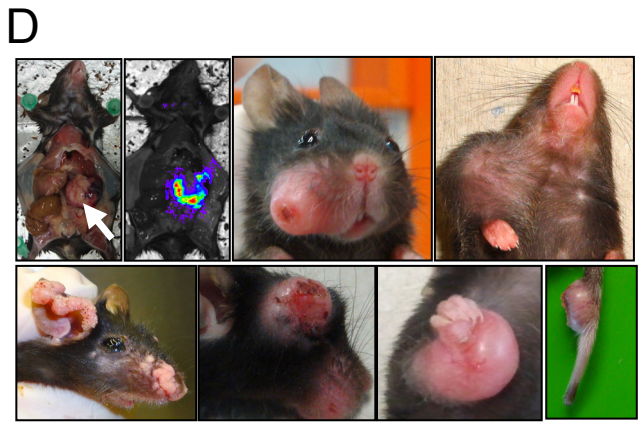

Rag-/-, treated
LoxP-TagLuc-pA x CAG-rtTA, untreated
LoxP-TagLuc-pA x CAG-rtTA, treated
Total Flux (p/s)
10^{12}
10^{10}
10^8
10^6
0
10
20
30
40
75
90
Time post therapy (d)
Tumor size [mm3]
1500
1000
500
0

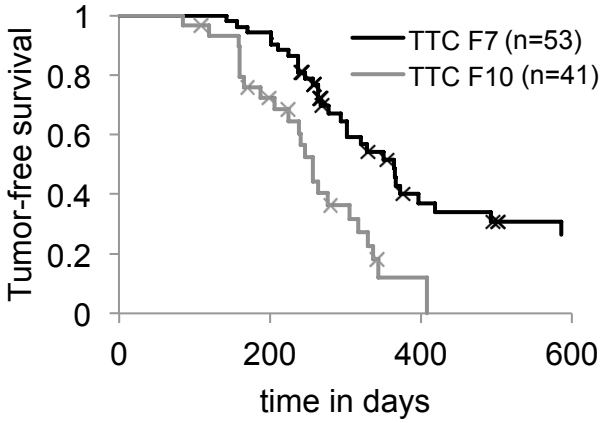
Figure 2

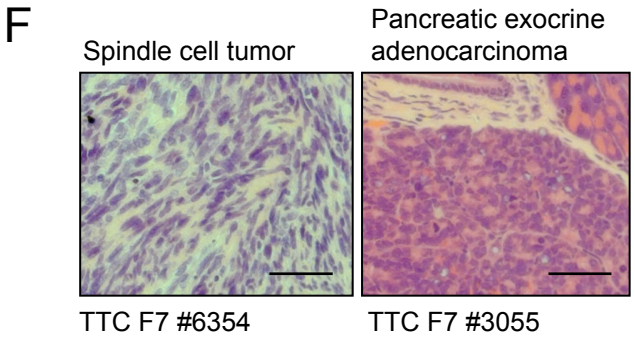

A
Tet-TagLuc
TyrCre x CAG-rtTA
Tet-TagLuc x TyrCre x CAG-rtTA
T
TC
TTC
x
loxP loxP (G4S)3
TRE Stop TagLuc
-dox
+dox
rtTA ∞
off
TRE Tag-Luc
rtTA/dox
on
TRE Tag-Luc

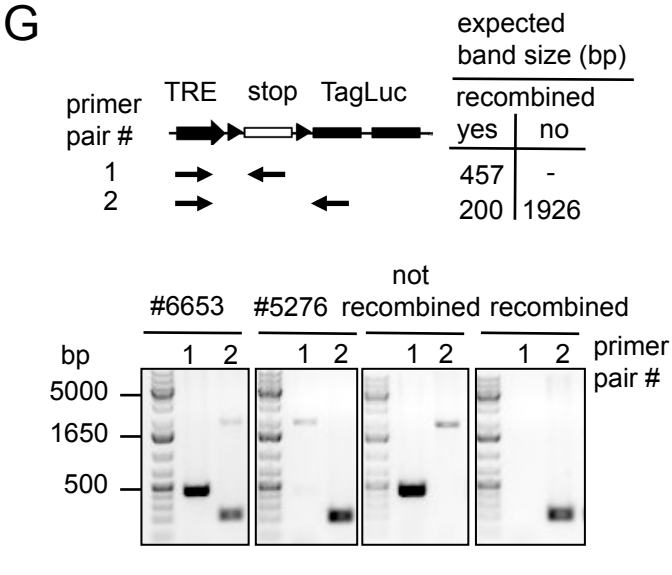

B
before dox
17 days dox
Color Bar
Min = 20000
Max = 5e+06
T TC TTC T TC TTC


C
Avg Radiance [p/s/cm²/sr]
B6
F7 TC
F7 TTC
F10 TC
F10 TTC


D


E
Tumor-free survival
TTC F7 (n=53)
TTC F10 (n=41)
time in days


F
Spindle cell tumor
Pancreatic exocrine adenocarcinoma
TTC F7 #6354
TTC F7 #3055


G
primer pair #
TRE stop TagLuc
expected band size (bp)
recombined
yes no
1 457 -
2 200 1926
not recombined
#6653 #5276
bp
5000
1650
500
primer pair #

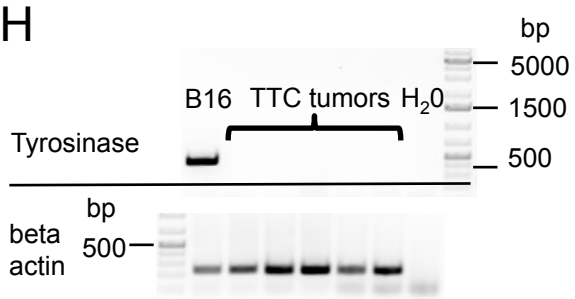
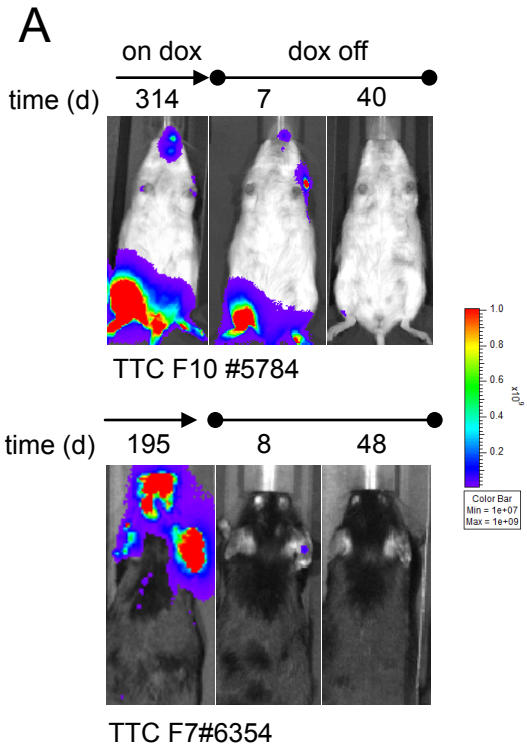
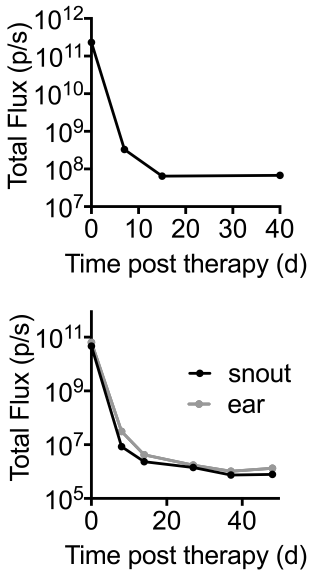
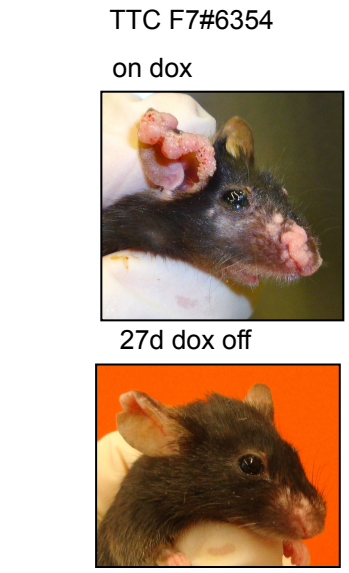
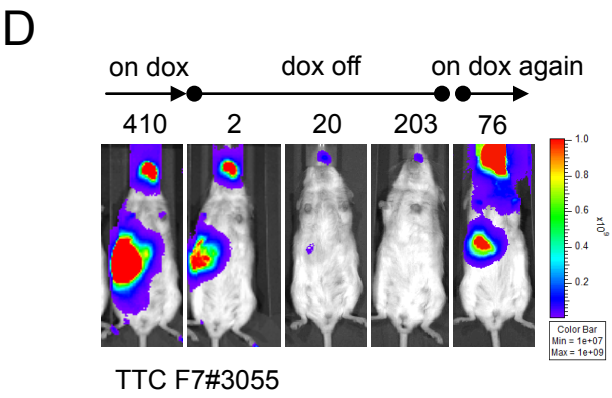

H
bp
5000
1500
500
B16 TTC tumors H2O
Tyrosinase
beta actin
bp
500

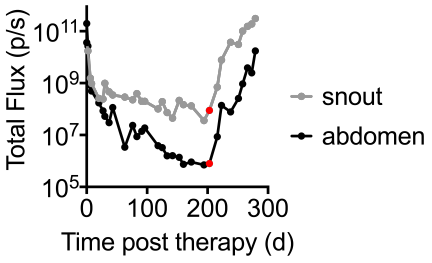
Figure 3


A
on dox
dox off
time (d)
314
7
40
TTC F10 #5784
time (d)
195
8
48
TTC F7#6354
Color Bar
Min = 1e+07
Max = 1e+09


B
Total Flux (p/s)
Time post therapy (d)
snout
ear


C
TTC F7#6354
on dox
27d dox off


D
on dox
dox off
on dox again
410
2
20
203
76
TTC F7#3055
Color Bar
Min = 1e+07
Max = 1e+09


E
Total Flux (p/s)
Time post therapy (d)
snout
abdomen

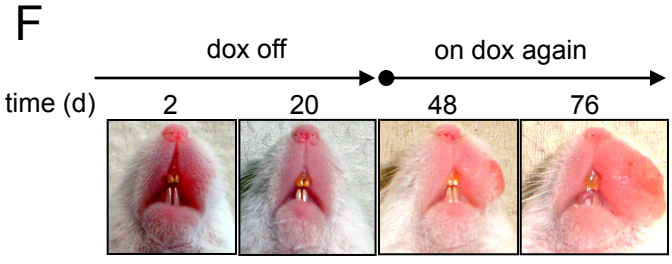

F
dox off
on dox again
time (d)
2
20
48
76

Figure 4

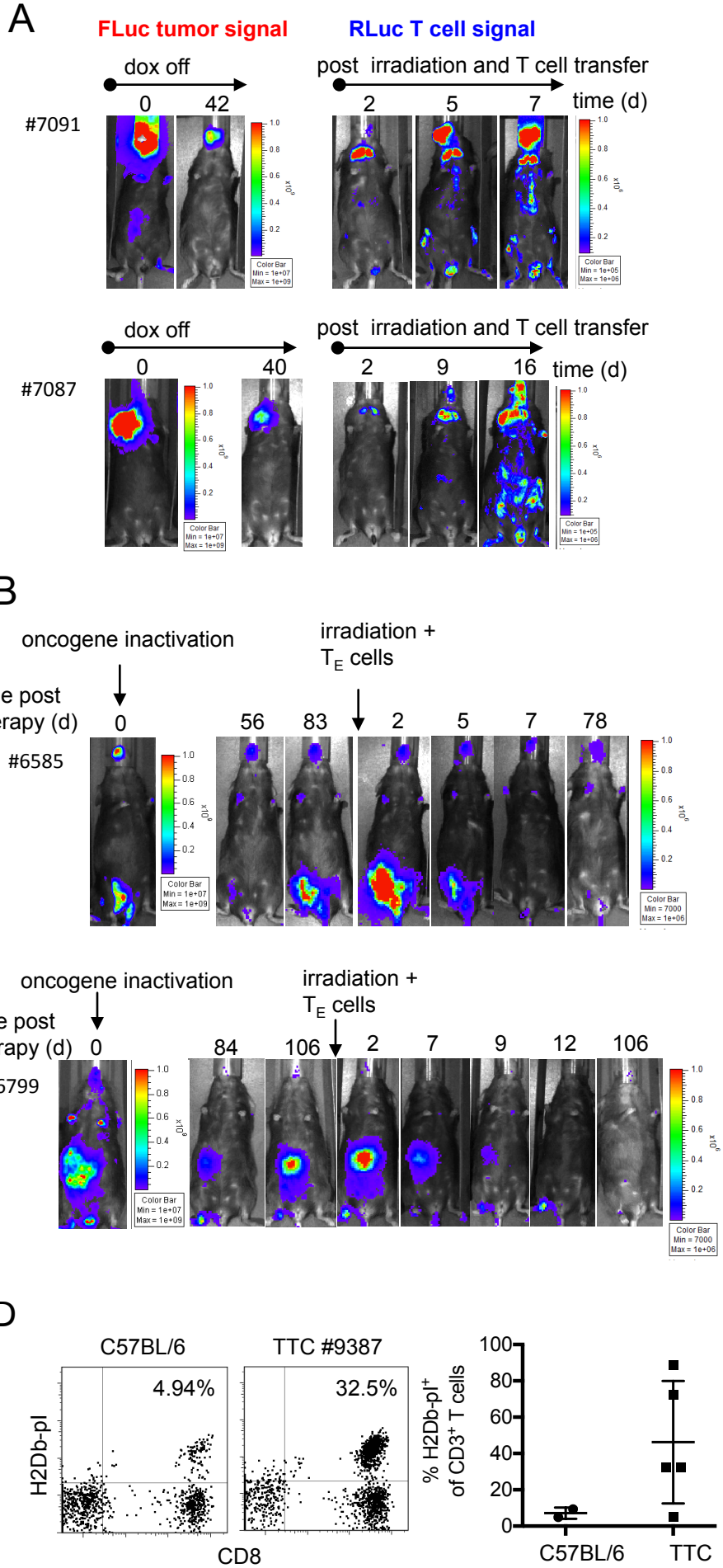

A
FLuc tumor signal
RLuc T cell signal
dox off
post irradiation and T cell transfer
#7091
0
42
2
5
7
time (d)
#7087
0
40
2
9
16
time (d)
B
oncogene inactivation
irradiation + T_E cells
time post therapy (d)
#6585
0
56
83
2
5
7
78
#6799
0
84
106
2
7
9
12
106
C
Total Flux (p/s)
Time post therapy (d)
irradiation + T_E cells
D
C57BL/6
TTC #9387
4.94%
32.5%
H2Db-pI
CD8
% H2Db-pI+ of CD3+ T cells
C57BL/6
TTC

Figure S1

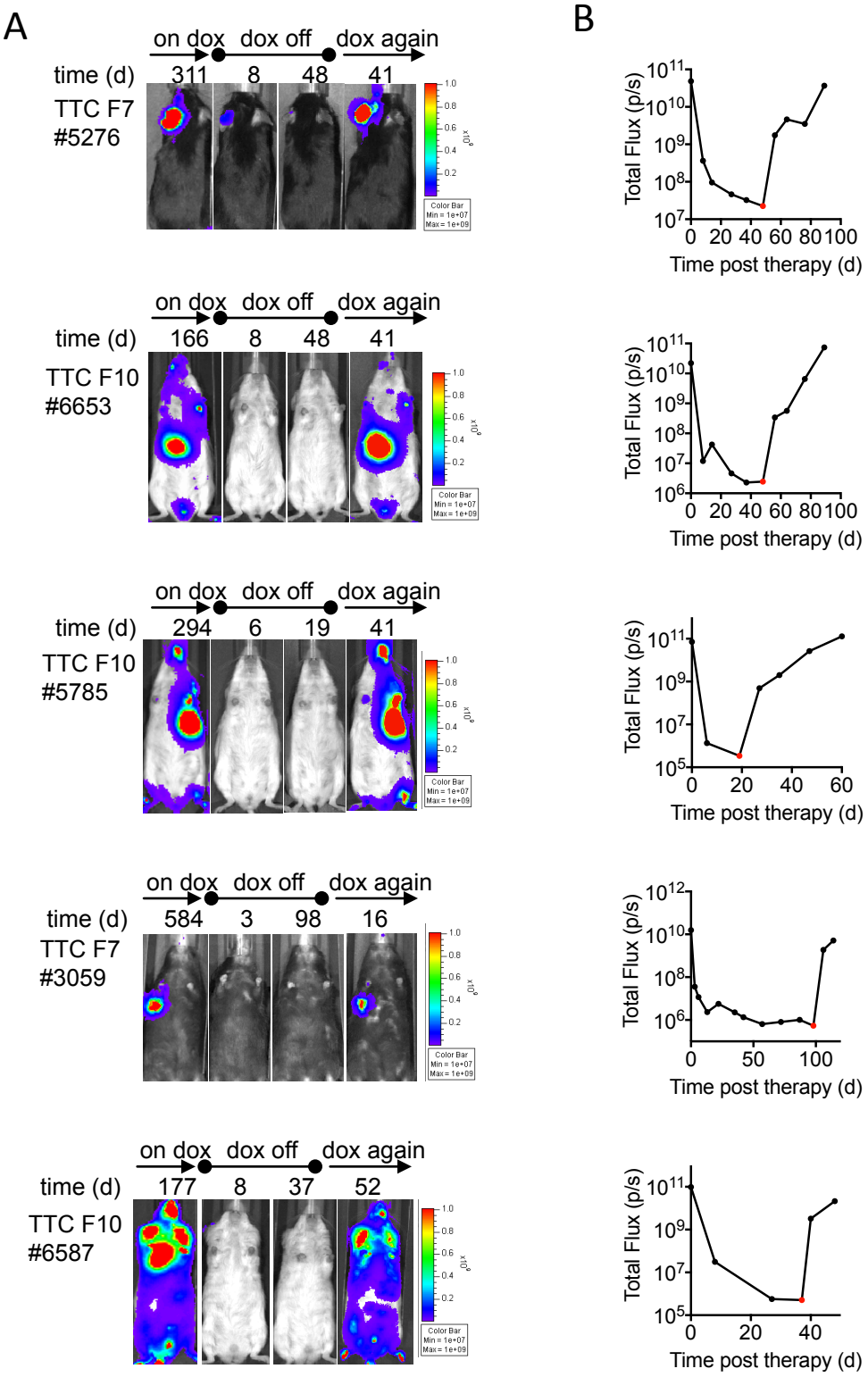


Figure S1. (A) TTC mice were subjected to oncogene inactivation therapy by dox cessation and tumor regression was monitored by BL imaging. Mice with stably low BL signal were exposed again to dox containing drinking water. (B) Quantified BL signal of mice shown in (A). Time point of dox re-administration is indicated by a red filled circle. Related to Figure 3.

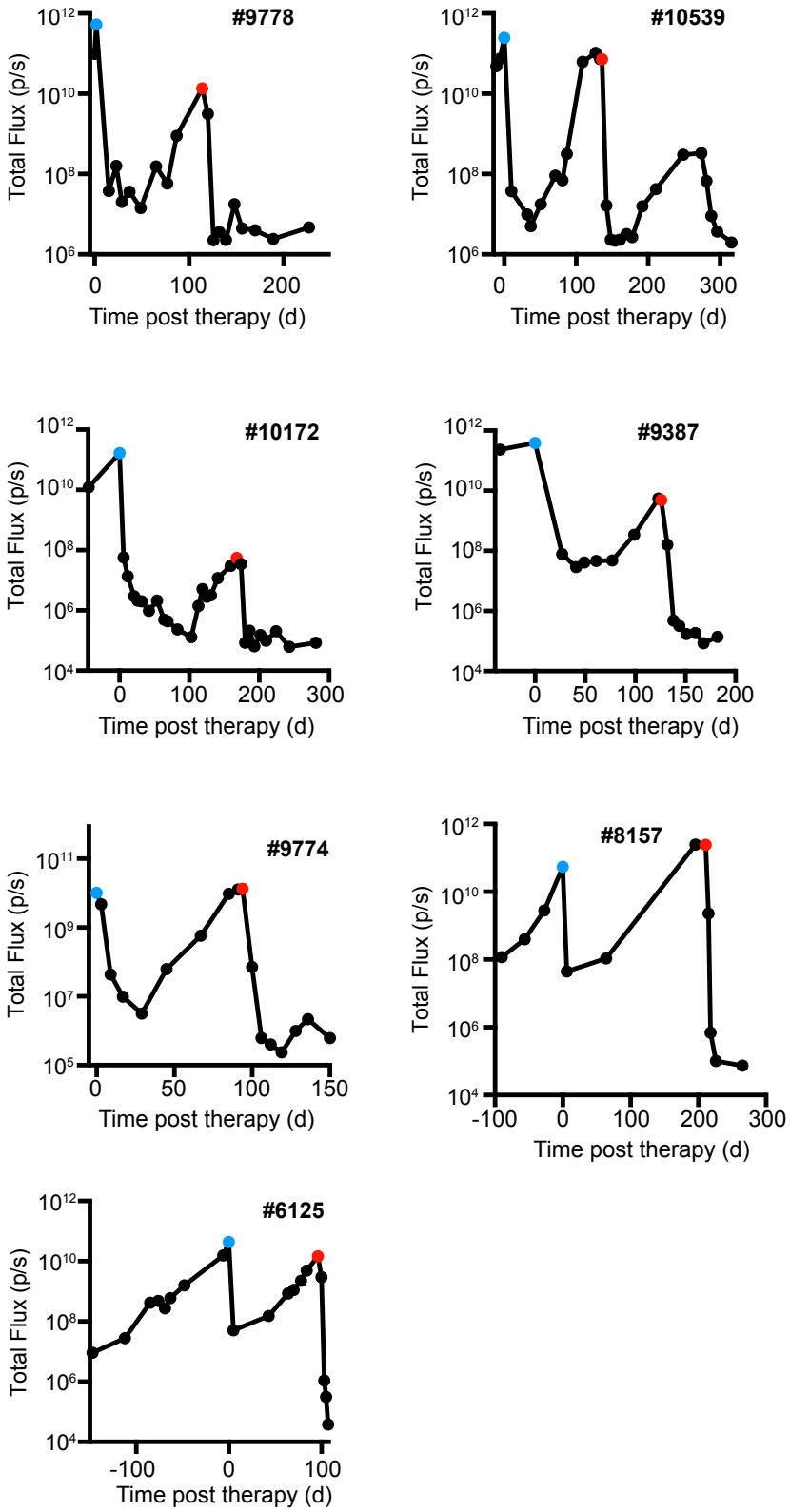


Figure S2. Autochthonous tumor development in TTC mice was monitored by BL imaging. Mice with tumors received oncogene inactivation therapy (blue circle). After recurrence of light signal despite continuous dox cessation, mice were irradiated and received T_E cells (red circle). Seven individual mice are shown. Mice #10539 and #9774 received dox-containing drinking water four days before T cell therapy. Related to Figure 4.

Table 1: Tumors developed in TRE^{loxP}stop^{loxP}TagLuc /CAG-rtTA/Tyr::Cre (TTC) transgenic mice

#	Tumor number	Tumor location	Origin	Mitotic rate	Local invasion	Diagnosis
1	TTC #7496	Rectum	M	++	+	Spindle cell tumor[A)]
2	TTC #7679a	Abdomen	E	+++	-	Mammary adenocarcinoma
3	TTC #7679b	Subcutanous	E	+++	-	Mammary adenocarcinoma
4	TTC #6653	Abdomen	E	+++	+	Pancreatic exocrine adenocarcinoma
5	TTC #3055a	Snout	M	++	+	Spindle cell tumor[A)]
6	TTC #3055b	Abdomen	E	+	+	Pancreatic exocrine adenocarcinoma
7	TTC #3059	Armpit	M	++	+	Spindle cell tumor[A)]
8	TTC #5785	Abdomen	M	+++	+	Spindle cell tumor[A)]
9	TTC #6354a	Snout	M	++	+	Spindle cell tumor[A)]
10	TTC #6354b	Ear	E	-	-	Papilloma
11	TTC #5276	Ear	M	++	+	Spindle cell tumor[A)]
12	TTC #6412	Head, cutanous	Round cell	+	+/-	Desmoplastic small-round cell tumor
13	TTC #5650	Rectum	E	+++	+	Spindle cell tumor[A)]
14	TTC #6654	Limb	E	+++	+	Spindle cell tumor[A)]

[A)] Most likely differentials: Peripheral nerve sheath tumor, leiomyosarcoma or fibrosarcoma

M = mesenchymal morphology

E = epithelial morphology

Table 2: ATT but not oncogene inactivation therapy result in tumor eradication

#	Mouse	Age at oncogene inactivation therapy (d)	Time before oncogene on again (d)	Time until spontaneous relapse (d)	Tumor reappearance after dox cessation	Age at ATT (d)	Outcome of ATT (observation period)
1	TTC F7 #5276	311	48	-	yes	-	-
2	TTC F10 #6653	166	48	-	yes	-	-
3	TTC F7 #6354	195	48	-	yes	-	-
4	TTC F10 #5785	294	19	-	yes	-	-
5	TTC F7 #3059	584	98	-	yes	-	-
6	TTC F10 #6587	177	37	-	yes	-	-
7	TTC F10 #5784	314	40	-	yes	-	-
8	TTC F7 #3055	410	203	-	yes	-	-
9	TTC F7 #7087	265	-	-	-	307	T cell signal
10	TTC F7 #7091	265	-	-	-	307	T cell signal
11	TTC F10 #6125	366	-	43	-	434	BL decrease but death
12	TTC F7 #6585	289	-	78	-	400	Rejection (96 d)
13	TTC F7 #6799	213	-	84	-	352	Rejection (154 d)
14	TTC F10 #10172	262	-	113	-	430	Rejection (113 d)
15	TTC F10 #10539	248	-	51	-	384	Rejection (180 d)
16	TTC F10 #9978	342	-	65	-	456	Rejection (113 d)
17	TTC F10 #9774	350	-	45	-	486	Rejection (56 d)
18	TTC F7 #9387	422	-	99	-	548	Rejection (56 d)
19	TTC F10 #P8157	223	-	64	-	434	Rejection (54 d)

Tabelle 2:

Mouse 1-8: group of mice was subjected to oncogene-inactivation therapy and received dox again after indicated time period.

Mouse 9-10: group of mice was subjected to oncogene-inactivation therapy and received T_E cells prior developing tumor relapse. RLuc T cell signal was followed.

Mouse 11-19: group of mice was subjected to oncogene-inactivation therapy and received T_E cell therapy after developing drug-resistant tumors. Mouse 15 and 17 were set on dox again starting four days prior T cell therapy.