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J Immunol 2010; 184:2930-2938; Prepublished online 8 February 2010;
doi: 10.4049/jimmunol.0900893
http://www.jimmunol.org/content/184/6/2930

Supplementary Material http://www.jimmunol.org/content/suppl/2010/02/05/jimmunol.0900893.DC1

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In Vivo Imaging of an Inducible Oncogenic Tumor Antigen Visualizes Tumor Progression and Predicts CTL Tolerance

Christian Buschow,* Jehad Charo,† Jehad Charo,* Kathleen Anders,‡ Christoph Loddenkemper,‡∥ Ana Jukica,§ Wisam Alsamah,§ Cynthia Perez,§ Gerald Willinsky,*∥ Thomas Blankenstein*†

Visualizing oncogene/tumor Ag expression by noninvasive imaging is of great interest for understanding processes of tumor development and therapy. We established transgenic (Tg) mice conditionally expressing a fusion protein of the SV40 large T Ag and luciferase (TagLuc) that allows monitoring of oncogene/tumor Ag expression by bioluminescent imaging upon Cre recombinase-mediated activation. Independent of Cre-mediated recombination, the TagLuc gene was expressed at low levels in different tissues, probably due to the leakiness of the stop cassette. The level of spontaneous TagLuc expression, detected by bioluminescent imaging, varied between the different Tg lines, depended on the nature of the Tg expression cassette, and correlated with Tag-specific CTL tolerance. Following liver-specific Cre-loxP site-mediated excision of the stop cassette that separated the promoter from the TagLuc fusion gene, hepatocellular carcinoma development was visualized. The ubiquitous low level TagLuc expression caused the failure of transferred effector T cells to reject Tag-expressing tumors rather than causing graft-versus-host disease. This model may be useful to study different levels of tolerance, monitor tumor development at an early stage, and rapidly visualize the efficacy of therapeutic intervention versus potential side effects of low-level Ag expression in normal tissues. The Journal of Immunology, 2010, 184: 2930–2938.

S V40 large T Ag (Tag) transgenic (Tg) mice have been widely employed as cancer-prone mice because Tag is a reliable strong oncogene. This was demonstrated in a number of Tg mice that developed, depending on the promoter used, tumors in various organs including brain, kidney, pancreas, salivary gland, bone, skin, liver, mammary gland, and prostate (1–10). Tag expression initiates malignant transformation by inactivating tumor suppressors p53 and Rb and probably through other biological activities (11). Several H2Kb class I-restricted Tag epitopes have been identified, of which the H2-Kb-restricted epitope IV is dominant (12). In most Tg models, Tag is expressed by a strong tissue-specific promoter, resulting in early CTL tolerance. However, in some Tag-Tg mice, including mice developing sporadic tumors in a stochastic fashion, functional epitope IV-specific CTLs were retained (10, 13–16).

Bioluminescent imaging (BLI) is a sensitive tool to noninvasively visualize gene expression of luciferase in vivo (17). In tumor transplantation models, BLI can detect between 100 and 1000 cells and monitor tumor growth (18, 19). Only recently, cancer-prone mice were generated that expressed the luciferase gene selectively in autochthonous tumors. This was achieved by Cre recombinase-mediated activation of an oncogene or inactivation of a tumor suppressor gene simultaneously with activation of the luciferase gene in a tissue-specific fashion (20–23). Although these models are convenient, it cannot be excluded that Cre-loxP site-specific recombination is incomplete, because two unrelated genes have to be (in)activated in the same cell, so that reporter gene expression does not necessarily reflect tumor growth. More importantly, these models do not allow the direct visualization of oncogene/tumor Ag expression, because time point, level, and stability of expression may differ from that of the luciferase gene.

Some putative tumor Ags, such as p53, WT1, survivin, or telomerase, are ubiquitously expressed in very low amounts in normal cells but overexpressed in cancer cells. Detection of a very low level of Ag expression by normal tissue cells is difficult and laborious. Therefore, it is poorly understood how low amounts of ubiquitous self-Ag expression translate into tolerance that might impact on immunotherapy (24–29). For these reasons, methods to directly visualize low-level Ag expression in vivo are desirable. In this study, we generated Tg mice that conditionally express a fusion protein of Tag and firefly luciferase (TagLuc), allowing the direct visualization of oncogene/tumor Ag expression and Cre recombinase-mediated tumor induction. This mouse model showed that BLI detects very low levels of leakiness of the stop cassette, the amount of which predicted the level of CTL tolerance. Furthermore, we show that low level of TagLuc expression by normal cells prevented Tag-specific tumor rejection.

Materials and Methods

Cells

Murine embryonic fibroblasts (MEFs), 293-derived Plat-E cells, and Tet-TagLuc fibroblasts (in vitro immortalized cells expressing the TagLuc fusion protein; K. Anders, C. Buschow, C. Loddenkemper, J. Charo, and T. Blankenstein, unpublished observation) were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (PAN Biotech, Aidenbach, Germany) and 50 μg/ml gentamycin (Invitrogen). The Tag-expressing cancer cells 16.113 and 9.27 were derived from LoxP-Tag mice (10, 16) and were cultured in RPMI 1640 Glutamax-I containing 10% FBS and 50 μg/ml gentamycin.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0900893
Plasmids

To generate the gene encoding the TagLuc fusion protein, a BglII and NcoI-digested 2895-bp SV40 Tag-encoding DNA fragment, derived from plasmid CMVTagOri (30), was ligated into the identically digested pSVLuc-NF plasmid (Promega, Madison, WI). The resulting plasmid was subsequently digested with XhoI and BstEII to replace the stop codon of the Tag gene and the start codon of the luciferase gene with an identically digested DNA fragment encoding the 3′ end of Tag and the 5′ end of luciferase connected by a polypeptide linker containing a glycine-serine polypeptide linker ([G4S]9) sequence, which was generated by PCR using the oligonucleotides Tag-PalI (5′-gccgaaacagctgccaggattt-3′) and TagLuc (5′-gccggtccgatcctgccacctcctcagacgacctcacaacctgcgttttcaggttcagggggagg-3′) and the Tag-encoding plasmid CMVTagOri as template. The polypeptide linker sequence was confirmed by DNA sequencing. The resulting plasmid contained the TagLuc fusion gene and was named pSPTagLuc. From this plasmid, the TagLuc fusion gene was excised with KpnI and EcoRV and ligated in the identically digested plasmid pcDNA3 (Invitrogen) resulting in pcDNA3TagLuc. To obtain the constructs for the Tg mice, the previously described pCAG-CAT-Tag- (10) construct was digested with SacI and SapI to insert downstream of the CAG promoter and the 3′ UTR of the Tag gene. The CAG-Tag–transfected cells were passaged for a further six passages in DMEM (Invitrogen) with 10% FBS without antibiotic selection. The pcDNA3TagLuc plasmid was digested with SacI and SapI to insert downstream of the CAG promoter and the 3′ UTR of the Tag gene. The CAG-Tag–transfected cells were passaged for a further six passages in DMEM (Invitrogen) with 10% FBS without antibiotic selection.

Function al analysis of Loxp-TagLuc- and Loxp-TagLuc-pA plasmids

The 293-derived flat-E cells, which were grown to full confluency in a six-well plate, were transfected with 3 μg Loxp-TagLuc-pA and Loxp-TagLuc-pA plasmids together with 1 μg MSCV-nlsCre (J. Charo and T. Blankenstein, unpublished observation) or MSCV-ΔsRed (31) as control plasmid in equimolar ratio using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). Forty-eight hours later, luciferase activity was determined by adding N-luciferin (Biosynth, Staad, Switzerland) at a final concentration of 100 μg/ml to the culture medium. Light intensity was quantified using a Mithras LB 940 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Analysis of TagLuc function

A total of 3 μg plasmids CAG-Tag or CAG-TagLuc were cotransfected with 1 μg phMGFP (for transfection control) into MEFs of passage 10 using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). Transfected and untransfected MEF cells were passaged for a further six passages in DMEM (Invitrogen) with 10% FBS without antibiotic selection. For determination of growth kinetics, 5 × 104 cells were plated in six-well plates. Cell numbers were determined in triplicate over a period of 8 days by cell counting (trypan blue exclusion). For the determination of cell number on day 8, transfected cells were transferred from the six-well plate on day 6 to a 10-cm culture dish. To analyze luciferase activity, cells from passage 4 posttransfection were imaged using Xenogen IVIS 200 (Caliper Life Sciences, Hopkinton, MA) for 3 min at binning 8 by adding D-luciferin at a final concentration of 100 μg/ml to the medium. CAG-Tag–transfected cells were used as negative control for imaging.

Western blot analysis

Murine bifibrasts that express the TagLuc fusion gene under the control of a doxycycline gene expression system (K. Anders, C. Buschow, C. Loddenkemper, J. Charo, and T. Blankenstein, unpublished observation) and Tag-expressing 16.113 cells were lysed using mammalian cell lysis kit (Sigma-Aldrich, St. Louis, MO), and 40 μg protein extract was mixed with 5 μl loading buffer. The mixture was loaded onto a NuPage Tris-acetate 3–8% SDS-PAGE (Invitrogen) and blotted on a polyvinylidene fluoride membrane (Amersham Biosciences, Piscataway, NJ) using a XCell II blot module (Invitrogen). After blocking with 5% dried skim milk, the membrane was incubated first with anti-Tag peptides (1μg/ml) and then with anti-

Bioluminescent imaging

Unless otherwise indicated, mice received an i.n. injection with 300 μg/g body weight n-luciferin (Biosynth) dissolved in PBS. Imaging was performed using a Xenogen IVIS 200 (Caliper Life Sciences). To analyze TagLuc expression in tumors and individual organs, mice received n-luciferin i.p. 300 μg/g body weight and additionally 150 μg/g body weight i.v. to ensure substrate saturation. After 6 min, mice were sacrificed, and the indicated organs were transferred into a 24-well plate and BLI was analyzed. All data were analyzed using Living Image analysis software (Caliper Life Sciences).

In vivo CTL and intracellular cytokine staining assays

For immunization, mice received 1 × 107 Tag-expressing 16.113 regressing tumor cells i.p (10). Seven days later, C57BL/6 splenocytes were incubated in the presence or absence of H2-Kb–restricted Tag-IV peptide (VVYDFKL) at a final concentration of 1 μM in PBS at 37˚C for 15 min and subsequently labeled with CFSE (Invitrogen) using two different concentrations (CFSE99; 1 μM for peptide-loaded spleen cells and CFSE243; 0.1 μM for control spleen cells) for 15 min at 20˚C. Cells were washed once in RPMI 1640 medium with 10% FBS and twice with PBS. A total of 2 × 107 cells at a 1:1 ratio were injected i.v. into the indicated mice. After 18 h, CFSE-labeled cells in the spleen were analyzed by flow cytometry. Specific killing was calculated as 1 − (ratio of control mouse/ratio of immunized mouse) × 100, where ratio is defined as the percentage of CFSE243/percentage of CFSE99. Alternatively, splenocytes were incubated overnight with Tag-IV peptide or a control H2-Kb–restricted OVA peptide (SIINFEKL) in the presence of GolgiPlug (BD Pharmingen, San Diego, CA). Cells were then stained for CD8α using FITC-conjugated rat anti-mouse Ab (53-6-7, BD Pharmingen). Intracellular staining of the cells was performed using the Fix/Perm kit (BD Pharmingen) with an APC-conjugated rat anti-mouse IFN-γ Ab (XMG1.2, BD Pharmingen) using the manufacturer’s protocol.

ELISA

For detection of anti-Tag Abs, serum samples from indicated mice were collected and applied onto ELISA plates coated with SV40 Tag protein as described previously (10, 16). As a reference, mouse anti-SV40 large Tag Ab (Pab 100, BD Pharmingen) was used.

Adoptive T cell transfer

A total of 0.5–1 × 107 Tag-expressing 9.27 tumor cells (10) were injected s.c. On day 6, mice were irradiated with 400 or 500 rad. On day 7, 1 × 107 splenocytes from C57BL/6 mice immunized i.p. with 1 × 107 16.113 cells
Results

Construction of the TagLuc fusion protein

The SV40 Tag sequence was fused to the luciferase gene by inserting a sequence encoding a polypeptide linker consisting of (G4S)3 repeats. Western blot analysis of cells expressing the gene encoding the fusion protein TagLuc or Tag alone demonstrated that the fusion protein had the expected size of around 150 kDa compared with the native Tag protein of around 91 kDa (Fig. 1). To evaluate whether the TagLuc fusion protein is able to induce proliferation, MEFs expressing the fusion protein TagLuc or Tag alone were transfected with plasmids encoding either the TagLuc or the Tag gene controlled by the constitutively active CAG promoter. After a total of 16 passages, TagLuc-transfected MEFs proliferated comparably to Tag-transfected MEFs, whereas nontransfected MEFs showed almost no proliferation (Fig. 1B). Additionally, the TagLuc- but not Tag-expressing MEFs exhibited a BLI signal, demonstrating that the TagLuc fusion protein had luciferase activity (Fig. 1A) and that the TagLuc gene is separated from the CAG promoter by the LoxP-flanked stop cassette encoding for the chloramphenicol-acetyltransferase gene.

Histology and immunohistochemistry

Serial sections (4 μm) of formalin-fixed, paraffin-embedded liver tissue were mounted on slides and stained with H&E. For immunostaining, sections were subjected to a heat-induced epitope retrieval step. Slides were rinsed in cool running water, washed in TBS (pH 7.4) before incubation with primary Abs against SV40 large Tag, small Tag (Pab 108, mouse IgG2a, dilution 1:200; BD Pharmingen), Ki-67 (TEC-3, rat IgG2a, dilution 1:500; DakoCytomation, Carpenteria, CA), and luciferase (LUC-1, mouse IgG1, dilution 1:500; Sigma-Aldrich) for 30 min. For detection, biotinylated donkey anti-rat (Dianova, Hamburg, Germany), the streptavidin-AP kit (K5005, DakoCytomation), or the streptavidin-PO kit (K5001, DakoCytomation), each for use with mouse/rabbit primary Abs, were applied. For Ki-67 and SV40 large Tag staining, alkaline phosphatase was revealed by Fast Red as chromogen, and for anti-luciferase staining, peroxidase was developed with a highly sensitive diaminobenzidine chromogenic substrate. Negative controls were stained similarly but omitting the primary Ab.

FIGURE 1. In vitro immortalization and luciferase activity of the TagLuc fusion gene. A, Western blot analysis shows the expected size of the TagLuc fusion protein of 150 kDa and the native Tag protein of 91 kDa. Lane 1: Lysate from murine fibroblasts that express the TagLuc fusion protein. Lane 2: Lysate from the Tag-expressing tumor cell line 16.113. B, The TagLuc fusion protein induces proliferation of MEFs similar to the native Tag protein. MEF cells were transfected with plasmids CAG-TagLuc or CAG-Tag. Transfected and untransfected MEF cells were cultured for a total of 16 passages. For determination of the growth kinetics, 5 × 10⁵ cells from each culture were plated in six-well plates. Cells were counted in triplicate over a period of 8 d by trypan blue exclusion. C, The TagLuc fusion protein has luciferase activity. TagLuc-expressing cells from passage 14 were imaged with the Xenogen IVIS 200 (Caliper Life Sciences) for 3 min with binning 8. CAG-Tag–transfected cells were used as negative control for imaging. Colored scale bar shows the luminescent data as photons/s/cm²/steridian. CAG-Tag–transfected cells were used as negative control for imaging.

FIGURE 2. BLI detects TagLuc expression due to leakiness of the stop cassette that results in CTL tolerance in LoxP-TagLuc-pA mice. A, Plasmid construct used for the generation of LoxP-TagLuc-pATg mice. B, BLI of one representative mouse out of five mice from each founder line after 60 s exposure using binning 8. C, Objective BLI measurement of Cre recombination-independent TagLuc expression. A region was drawn over the whole mouse, and light signal intensity was calculated. Each bar represents the average BLI intensity of five mice at the mean age of 24 ± 3.5 (F1), 25 ± 15 (F2), 13 ± 9 (F3), and 14 ± 16 (F5) wk expressed as photons/s/cm²/steridian. The low SD demonstrates stable BLI signals at different ages. D, Five- to fourteen-week-old mice were immunized i.p. with 1 × 10⁷ Tag-positive 16.113 regressor tumor cells or left untreated. After 7 d, a mixture of 1 × 10⁷ non–peptide-loaded, CFSElow labeled and 1 × 10⁷ Tag-specific peptide IV-loaded, and CFSEhigh-labeled splenocytes was injected into the indicated mice. The ratio between both cell populations was determined by flow cytometry of spleen cells 18 h later. A representative result from one out of two experiments per founder line is shown. Percentage in histograms of immunized mice (bottom panels) indicates the specific killing of the peptide IV-loaded population compared with naive controls (top panels). C, control; pIV, peptide IV-loaded population; stop, stop cassette (TagLuc gene is separated from the CAG promoter).
promoter by a LoxP-flanked stop cassette. By adding a polyadenylation (polyA) sequence downstream of the TagLuc gene (Fig. 2A), we aimed to enhance the expression level after removal of the stop cassette with the intention to decrease the long tumor latency that we previously experienced in another Tg mouse line carrying a conditionally expressed Tag (10). Five Tg founders were obtained with the LoxP-TagLuc-pA construct from which four founder lines (F1, F2, F3, and F5) on a C57BL/6 genetic background were established. In principle, these mice should not express TagLuc due to the stop cassette. Surprisingly, mice of all founder lines revealed a substantial BLI signal, albeit at different levels, distributed over the whole body (Fig. 2B). The objective measurement of the average BLI signal intensity varied between $5 \times 10^4$ and $5 \times 10^5$ photons/s/cm$^2$/steradian compared with $3 \times 10^2$ photons/s/cm$^2$/steradian measured for wild-type mice (Fig. 2C). We investigated by an in vivo kill assay how this leakiness of the stop cassette was detected by BLI. A similar plasmid construct as in Fig. 1A but lacking a polyA tail was used for the generation of LoxP-TagLuc Tg mice. B, BLI of one representative mouse out of five analyzed mice from each founder line with black coat color after a 60 s exposure using binning 8 (top panel). Note that the acquisition parameters are the same as in Fig. 1B. The BLI signal in F4 is visible with increased imaging sensitivity (Supplemental Fig. 1). Objective BLI of Cre recombination-independent TagLuc expression (bottom panel). A region was drawn over the whole mouse, and BLI intensity was calculated. Each bar represents the average BLI intensity of five mice at the age of 24 ± 13 (F1), 11 ± 7 (F2), 30 ± 19 (F3), and 12 ± 13 (F4) wk expressed as photons/s/cm$^2$/steradian. The low SD demonstrates stable BLI signals at different ages. C, BLI of one representative mouse out of five analyzed mice from each founder line with albino coat color (top panel). Note that the acquisition parameters are the same as in B. Objective BLI of Cre recombination-independent TagLuc expression (bottom panel). Each bar represents the average BLI intensity of five mice at the age of 15 ± 9 (F1), 11 ± 5 (F2), 9 ± 4 (F3), and 14 ± 7 (F4) wk expressed as photons/s/cm$^2$/steradian. The low SD demonstrates stable BLI signals at different ages. D, Cre-independent expression of the TagLuc gene in different organs. Mice were injected with D-luciferin i.p. (300 μg/g body weight) and i.v. (150 μg/g body weight) to ensure that the substrate was equally distributed throughout the mouse. Six minutes later, mice were sacrificed, and the indicated organs were removed, transferred into a 24-well plate, and imaged for 60s at binning 16. One of three experiments per founder line with similar results is shown, and all data are summarized in Supplemental Fig. 3.
cassette translated into immunological tolerance. Young mice from each founder line were immunized with Tag\textsuperscript{a} 16.113 tumor cells (10). After 7 d, immunized and naive control mice were injected with a 1:1 mixture of CFSE\textsuperscript{low}-labeled splenocytes without peptide and CFSE\textsuperscript{high}-labeled, epitope IV-loaded splenocytes. In contrast to non-Tg mice, all immunized Tg mice failed to eliminate the epitope IV-loaded cells (Fig. 2D). Evidently, the Cre recombinase-independent TagLuc expression in LoxP-TagLuc-pA mice resulted in CTL tolerance toward the dominant epitope of Tag.

Partial Tag-specific CTL tolerance in LoxP-TagLuc mice with very low, BLI-detectable TagLuc expression

The CTL tolerance in LoxP-TagLuc-pA mice prompted us to generate a second series of Tg mice, LoxP-TagLuc, that lack the polyA signal (Fig. 3A). This construct resembled that previously used for Tg mice, in which we observed the development of sporadic cancer and an epitope IV-specific CTL response before tumor onset (10). Four founder lines (F1–F4) were established and analyzed for Cre recombinase-independent TagLuc expression by BLI. LoxP-TagLuc mice that were of comparable age as LoxP-TagLuc-pA mice shown in Fig. 2B revealed a low TagLuc signal from lines F1–F3 (Fig. 3B). The hardly detectable BLI signal in the founder F4 mouse became better visible with increased imaging sensitivity (Supplemental Fig. 1). Compared to LoxP-TagLuc-pA mice (Fig. 2), BLI signals from LoxP-TagLuc mice were ∼10-fold lower. This indicates that the addition of a polyA sequence at the 3’ end of the TagLuc gene enhanced TagLuc expression resulting from the leakage of the stop cassette. The black coat color of C57BL/6 mice absorbs light signal. Therefore, mice of LoxP-TagLuc F1–F4 were backcrossed to C57BL/6-Tyr\textsuperscript{c-BR} mice that carry a spontaneous mutation in the tyrosinase gene locus and are albino in coat color (32). On average, the sensitivity for TagLuc detection increased ∼10-fold in LoxP-TagLuc mice with white compared with black coat color (Fig. 3B, 3C). The higher sensitivity of TagLuc detection in albino LoxP-TagLuc mice revealed a significant BLI signal also in founder line F4. No significant change in the detected BLI intensity was observed in LoxP-TagLuc F3 mice that were analyzed between 2 and 15 mo of age (Supplemental Fig. 2) and in the other LoxP-TagLuc mice between 1 and 13 mo of age (Fig. 3B).

Individual organs from each founder of LoxP-TagLuc and LoxP-TagLuc-pA mice were analyzed by BLI. Cre recombinase-independent TagLuc expression was detected in all analyzed organs of the different LoxP-TagLuc-pA lines (Fig. 3D). The different LoxP-TagLuc Tg lines differed in spontaneous TagLuc expression. Line F1 expressed TagLuc in all analyzed organs. Lines F2 and F3 showed lower TagLuc expression in individual organs, particularly in the thymus, where a BLI signal was barely detectable. In LoxP-TagLuc F4 mice, a BLI signal was detected only in heart, lung, and skeletal muscles. A weak signal observed in the liver of line F4 was probably unspecific, because the liver from a C57BL/6 control mouse similarly gave a low BLI signal. Overall, the TagLuc signal from organs of LoxP-TagLuc mice was lower compared with that of LoxP-TagLuc-pA mice (Supplemental Fig. 3). Transfection of Plat-E cells with LoxP-TagLuc and LoxP-TagLuc-pA plasmids confirmed a lower luciferase activity of LoxP-TagLuc compared with LoxP-TagLuc-pA transfected cells, both before and after Cre recombinase-mediated excision of the stop cassette (Supplemental Fig. 4).

To investigate whether the low Cre recombinase-independent TagLuc expression in LoxP-TagLuc mice prevented CTL responses against epitope IV, in vivo kill assays were performed. Significant CTL activity was detected in most mice of LoxP-TagLuc lines F2–F4 but not F1, despite variability among mice of each founder line (Fig. 4A). Compared to wild-type mice, CTL activity in LoxP-TagLuc F2–F4 mice was reduced, indicating partial tolerance. The average in vivo cytotoxicity appeared to inversely correlate with Cre recombinase-independent TagLuc expression. This can best be illustrated by comparison of LoxP-TagLuc-pA versus LoxP-TagLuc mice, but also when comparing LoxP-TagLuc F1 to F2–F4 mice. Analyzing the capacity of T cells from LoxP-TagLuc-pA and LoxP-TagLuc mice to produce IFN-γ in an epitope IV-specific fashion revealed an even higher level of tolerance than that seen with the in vivo kill assay (Fig. 4B). Because anti-Tag IgG Abs occurred spontaneously in the previously described LoxP-Tag mice older than 6 mo of age and indicated spontaneous premalignant
lesions that finally developed to sporadic cancer (16), we analyzed whether LoxP-TagLuc or LoxP-TagLuc-pA mice developed anti-Tag Abs upon aging. However, none of the analyzed mice at 5–24 mo of age revealed significant levels of anti-Tag IgG Abs in the serum (Supplemental Fig. 5).

Hepatocellular carcinoma development in LoxP-TagLuc × Alb-Cre mice

To analyze whether LoxP-TagLuc mice developed tumors upon Cre recombinase-mediated deletion of the stop cassette and activation of TagLuc, we crossed all four LoxP-TagLuc lines to Alb-Cre Tg mice, which express the Cre recombinase under the liver-specific albumin promoter. LoxP-TagLuc × Alb-Cre mice of all founder lines developed liver tumors over time, as determined by the BLI intensity of the liver region. An example of liver tumor development visualized by increasing TagLuc signals from day 55 of age until day 166, the time of end-stage tumors, is shown in Fig. 5A. BLI was performed with reduced sensitivity so that TagLuc expression due to the leaky stop cassette was not visible. Dependent on the founder line and the sex of the mice, large tumors were observed between days 120 and 220 (Fig. 5B, 5C). The ex vivo analysis of tumor tissue showed multiple hepatocellular carcinomas with a strong BLI signal (Fig. 5D,

FIGURE 5. Hepatocellular carcinoma development in LoxP-TagLuc × Alb-Cre mice visualized by increasing bioluminescence signal. A, Time course of tumor development in a LoxP-TagLuc × Alb-Cre female mouse (F2) and a single Tg LoxP-TagLuc mouse as control. All images were acquired with binning 4. Acquisition times were 60 s for day 55, 10 s for days 76 and 105, and 1 s for days 131 and 166. Signal from leakiness (see Fig. 3B) is not detected with these parameters. B, A representative BLI of one LoxP-TagLuc × Alb-Cre mouse of each founder line (F1–F4) at end-stage of tumor development (imaged on the day indicated below each image). Acquisition parameters: 1 s, binning 4 for F1–F3 and 1 min, binning 16 for F4. Signal from leaky TagLuc expression is not detected with these parameters. C, Kinetics of BLI intensity from the liver region during tumor development expressed as photons/s/cm²/steridian. Each line represents an individual mouse (solid lines = male mice, dashed lines = female mice; F1, n = 5; F2, n = 3; F3, n = 4; F4, n = 3). D, Upper panel: Photograph of a liver tumor of founder F3 at day 129 of age (left) and ex vivo BLI (right); acquisition parameters: 1 s, binning 4. One representative example out of five analyzed mice is shown. Lower panel: Immunohistology of a liver specimen with multiple nodules of hepatocellular carcinoma. Top left: H&E staining (HE; scale bar, 500 μm). High-power magnification of the boxed area indicated in the H&E image shows anti–Ki-67, anti-Tag (streptavidin-AP/Fast Red with red/nuclear labeling) and anti-luciferase (streptavidin-PO/diaminobenzidine with brown/nuclear labeling) Ab staining of the hepatocellular carcinoma (scale bar, 100 μm). One representative out of five analyzed tumors is shown. E, Deletion of the stop cassette in liver carcinoma cells of LoxP-TagLuc × Alb-Cre mice. For analysis of Cre-mediated LoxP-recombination, genomic DNA from tail biopsy and liver tumor tissue were isolated and subjected to Cre recombination-specific PCR with primers adjacent to LoxP sites. A PCR product of 1834 bp indicates no recombination, and a 114-bp PCR product indicates deletion of the stop cassette.
Immunohistochemistry of consecutive tumor tissue sections demonstrated positive staining with Abs against Tag, luciferase, and the proliferation marker Ki-67 (Fig. 5D, lower panel). Cre recombinase-mediated deletion of the stop cassette in cells of the tumor tissue was confirmed by PCR (Fig. 5E). Together, the TagLuc fusion protein can induce bioluminescent carcinomas, the development of which can be monitored over time.

**Low ubiquitous TagLuc expression prevents rejection of Tag-expressing tumors**

Unlike C57BL/6 mice, which rejected transplanted Tag-expressing 9.27 tumor cells, LoxP-TagLuc mice were unable to reject this challenge (Fig. 6A). It is not clear how a low level of ubiquitous tumor Ag expression by normal cells influences T cell therapy (e.g., causing toxicity or impeding therapeutic effects). Therefore, we analyzed whether adoptively transferred T cells from Tag-immunized wild-type mice rejected transplanted Tag-expressing tumors in LoxP-TagLuc (F2–F4) and LoxP-TagLuc-pA (F3/F5) mice. It is known that Tag-specific immunization primarily expands epitope IV-specific CTLs that eliminate tumors in mice with tissue-specific Tag expression in combination with sublethal irradiation (33). Mice were treated with immune spleen cells 7 d postchallenge with 9.27 tumor cells (10), and tumor growth was followed. None of the LoxP-TagLuc-pA or LoxP-TagLuc mice rejected the tumor, regardless of whether they received immune spleen cells, irradiation, or both treatments (Fig. 6B). No measurable decrease of TagLuc signal detected by BLI was observed (data not shown). In contrast, Rag-deficient mice rejected 9.27 tumors after immune spleen cell transfer, but not if treated with splenocytes.
from control OT-I Tg or from naive C57BL/6 mice (Fig. 6B, Supplemental Fig. 6). Thus, the low ubiquitous TagLuc expression inhibited T cell-mediated rejection of small Tag-expressing tumors.

**Discussion**

We described Tg mice, which express a transforming fusion protein, consisting of SV40 large T Ag and luciferase, after site-specific Cre-loxP recombination. This strategy allowed direct visualization of oncogene expression and noninvasive imaging of tumor growth in a spatiotemporal manner. Surprisingly, in mice of all LoxP-TagLuc-pA founder lines, a ubiquitous and Cre recombination-independent expression of the TagLuc gene was detected by BLI. This leakiness might have resulted from a bicistronic mRNA due to reading through the polyA signal 3’ of the stop cassette. Consequently, CTLs in LoxP-TagLuc-pA mice were tolerant toward the dominant Tag epitope. Because BLI detected Cre recombination-independent TagLuc expression in the thymus, T cell deletion by central tolerance is likely (34). LoxP-TagLuc mice that lacked the polyA signal sequence 3’ of the TagLuc gene showed on average a 10-fold lower Cre recombination-independent TagLuc expression. Because the two transgene DNA constructs differed only by this polyA signal sequence, the decreased TagLuc expression in LoxP-TagLuc mice was likely the result of reduced stability of a bicistronic mRNA transcript and/or reduced translation efficiency. The different leakiness of the stop cassette in the different LoxP-TagLuc founder lines was probably due to different copy number and/or chromosomal integration of the transgene. The decreased leakiness in LoxP-TagLuc compared with LoxP-TagLuc-pA mice resulted on average in less CTL tolerance. The inverse relation of the level of leaky TagLuc expression and CTL responses was also obvious between LoxP-Tag F1 versus F2–F4, but not among LoxP-TagLuc F2–F4 lines. This indicates that a certain level of leaky TagLuc expression is tolerated without complete CTL tolerance (F2 and F3), whereas very low levels are sufficient to induce partial tolerance (F4). The variations among mice of the same founder line that were indistinguishable by light imaging can be the result of two alternative mechanisms of tolerance. In the first case, the T cells in the nonresponding mice might have been deleted by central tolerance while spared from that in the responding mice due to the stochasticity of a low level Ag expression in the thymus (35). Alternatively, peripheral tolerance mechanisms prevented the Tag-specific T cells from being primed in the mice that did not respond.

LoxP-TagLuc × Alb-Cre mice developed hepatocellular carcinomas, similar to that previously described in mice expressing Tag by the albumin promoter (36). The line F4 with the lowest level of leaky TagLuc expression also expressed the lowest level of the TagLuc gene after liver-specific deletion of the stop cassette, which could explain why tumors in LoxP-TagLuc F4 × Alb-Cre mice grew with the longest latency. Similarly, it was shown that the degree of leakiness of the stop cassette in cell lines predicted the gene expression level after Cre-LoxP site-mediated deletion of the stop cassette (37). None of the Tg mice described in this study developed spontaneous tumors as previously described in another Tg mouse line carrying a dormant Tag gene (10, 16). Notably, the constructs in LoxP-Tag and LoxP-TagLuc Tg mice are identical apart from the linker and the firefly luciferase fused to the Tag in the LoxP-TagLuc lines reported in this paper. Nonetheless, spontaneous tumors did develop in the LoxP-TagLuc mice following the crossing of these mice to Alb-Cre mice, excluding the possibility that the fusion protein is not transforming in vivo. Therefore, it is likely that the sporadic Cre recombineinase-independent tumor development, which we observed in LoxP-Tag but so far not in LoxP-TagLuc mice, is due to differences in the integration site of the different constructs in the different founders.

In LoxP-Tag, anti-Tag Abs spontaneously occurred and indicated the development of sporadic tumors. In LoxP-TagLuc mice, no such Abs were detected, which is probably due to the fact that we did not observe sporadic, Cre recombineinase-independent tumors that induce the B cell response thus far. However, because LoxP-TagLuc mice have developed, in contrast to LoxP-Tag mice, substantial Tag-specific CTL tolerance already at a young age, we cannot exclude B cell tolerance against Tag.

LoxP-TagLuc mice may serve as a model for tumor Ags that are ubiquitously expressed at a low level in normal cells but often overexpressed in cancer cells. Such Ags like p53, telomerase, WT1, or survivin (38) may serve as target for T cell therapy, provided a therapeutic window can be detected, in which T cells selectively eliminate the cancer but not normal cells. It has been shown that p53-specific T cells can eliminate p53-overexpressing cancer cells in wild type mice without detectable toxicity (24). Similarly, in hemagglutinin (HA)-Tg mice, low-avidity T cells protected against challenge with HA-expressing cancer cells without causing autoimmunity (25). Whether a low amount of ubiquitous Ag expression by normal cells interferes with T cell therapy has not been well investigated. It was shown that transfer of naive wild-type T cells into HA-Tg mice that were tolerant due to ubiquitous low transgene expression could protect from a subsequent tumor challenge if the mice were immunized and challenged shortly after T cell transfer (28). However, the transferred T cells were rapidly tolerated by the Tg host without immediate immunization. Our data showed that effector T cells transferred with the immune spleen cells into irradiated LoxP-TagLuc mice were unable to reject a small tumor challenge. Rag-deficient mice that might mimic the phenotype of irradiated LoxP-TagLuc mice rejected the same tumor after T cell transfer, demonstrating that the transferred T cells, in principle, could reject the tumor. Currently, we do not know whether the transferred effector T cells were rapidly tolerated or deleted in LoxP-TagLuc mice or whether the low TagLuc expression in other organs impaired tumor infiltration. Our data contrast those obtained in the p53 tumor model (27), for which the nature of the Ag, its expression level, or distribution may be responsible. Together, we described Tg mice that allow BLI of oncogene expression in Cre recombineinase-inducible tumors and at a low level in normal tissues. This model offers the opportunity to analyze and visualize therapeutic versus autoreactive effects if the targeted Ag is ubiquitously expressed at a low level.

**Acknowledgments**

We thank A. Bradley (Cambridge, U.K.) for providing C57BL/6-Tyr<sup>−/−</sup> mice, C.H. Contag (Stanford, CA) for advice on bioluminescence imaging, and R. Naumann (Dresden, Germany) for oocyte injection to obtain Tg mice. We also thank M. Rösch and M. Hensel for excellent technical assistance.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


