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The use of light in cancer immunotherapy

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Chapter 6

A dual-color bioluminescence reporter mouse for simultaneous *in vivo* imaging of T cell localization and activation

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In revision

Abstract

Non-invasive imaging technologies to visualize the location and functionality of T cells are of great value in immunology. Here, we describe the design and generation of a transgenic mouse whose T cells contain both a constitutively expressed green-emitting click-beetle luciferase (CBG99) and a T cell activation-dependent red-emitting firefly luciferase (PpyRE9), allowing multicolor bioluminescence imaging of T cell location and activation. This dual-luciferase mouse, which we named TbiLuc, showed a high constitutive luciferase expression in lymphoid organs such as lymph nodes and the spleen. Ex vivo purified lymphocytes showed functional expression in both CD8 and CD4 T cells, whereas B cells showed no detectable signal. We cross-bred TbiLuc mice to T cell receptor-transgenic OT-I mice to obtain luciferase-expressing naïve CD8 T cells with defined antigen-specificity. TbiLuc*OT-I T cells showed a fully antigen-specific induction of the T cell activation-dependent luciferase. In vaccinated mice, we visualized T cell localization and activation in vaccine-draining lymph nodes with high sensitivity using two distinct luciferase substrates, D-luciferin and CycLuc1, of which the latter specifically reacts with the PpyRE9 enzyme. This dual-luciferase T cell reporter mouse can be applied in many experimental models studying the location, expansion and functional state of T cells.

Introduction

Bioluminescence imaging (BLI) is an optical molecular imaging technique based on the emission of light produced by luciferase enzymes expressed in cells or whole animals. It has been extensively used to study gene expression, using genetic constructs in which expression of the luciferase is driven by the promoter of the gene of interest (1). A common application of BLI is the monitoring of tumor cell growth using luciferase-expressing tumor cells (2). Besides functioning as a quantitative measure for cell number, BLI also provides information on cell viability as the light-producing reaction mediated by firefly or click beetle luciferases requires ATP, oxygen, and Mg^{2+} , thus the context of a living cell (3,4). Commonly, retroviral or lentiviral constructs harboring the luciferase gene coupled to a specific promoter are designed to integrate the luciferase gene into cells by viral transduction. Alternatively, luciferase-transgenic reporter mouse strains can be developed using similar constructs, requiring more time and effort but becoming a source of luciferase-expressing cells without the need for further modification. Such transgenic models are especially valuable if the cells of interest are to be studied in their natural, unmodified state.

BLI technology is highly suited to analyze the immune system *in vivo*, because it allows real-time visualization of the typically strong dynamics of many immune cells, which often change location and expand or contract in number over short periods of time. These characteristics are particularly true for T lymphocytes, also known as T cells, which are crucial effector cells in the cellular arm of the immune system. In particular, T cells are responsible for the clearance of viral infections and the eradication of tumors. T cells are found in high numbers in lymphoid organs such as the spleen and lymph nodes, but also circulate in the bloodstream to patrol the body and enter peripheral tissues in case these harbor their target. These properties make T cells an attractive target for BLI. Thus far, many attempts to create luciferase-expressing T cells have used viral transduction of T cells. However, T cells in their untouched natural state, immunologically referred to as 'naïve T cells', are practically impossible to transduce. Therefore, in order to allow their transduction, T cells are first artificially activated *in vitro* which facilitates their transduction, and often rested for several days before use in an experiment (5–9). However, the transition of a T cell from the naïve to the activated state is not fully reversible, as T cell activation starts transcriptional programs that cannot be reversed. Hence, although commonly ignored, the results obtained with BLI of such transduced T cells cannot be directly compared to the natural situation in which new T cell responses start with naïve T cells. These drawbacks have led to the production of a number of T cell luciferase-transgenic mouse models to allow the tracking of T cells

(10–12). While definitely a step forward from using transduced T cells, these single-luciferase transgenic models have the limitation that they only provide information on the location of T cells, but not their functional state.

We have previously shown that the click-beetle green luciferase mutant CBG99 and the red-emitting firefly mutant PpyRE9 can be efficiently combined for multicolor *in vivo* bioluminescence imaging of transplanted cells previously transduced with a single luciferase, using the substrate D-luciferin (13). In this study, we show the design and generation of a transgenic mouse model called TbiLuc, which contains the genes of the two abovementioned luciferases, expressed in T cells, allowing dual color visualization of T cell location and activation. In TbiLuc, naïve T cells constitutively express CBG99 luciferase driven by the CD2 promoter, while activated T cells will also express PpyRE9 luciferase driven by the NFAT promoter. We show that luciferase expression is restricted to T cells, and that antigen-specific or non-specific activation of T cells successfully induces the expression of the activation-dependent luciferase. However, the expression level of the two luciferases influences the ability to efficiently separate the two light signals *in vivo* using a single substrate. Therefore, we combined the recently developed luciferase substrate CycLuc1(14) as a specific substrate for firefly luciferases (such as PPyRE9) with D-luciferin as a substrate for the CBG99 enzyme. Our finding that CycLuc1 is not an efficient substrate for CBG99 allowed us to efficiently separate light signals *in vivo* using TbiLuc T cells. We cross-bred TbiLuc mice to T cell receptor-transgenic OT-I mice and subsequently localized vaccine-specific CD8 T cells in lymphoid organs and visualize their activation upon vaccination and addition of the two distinct luciferase substrates.

In summary, we have developed a dual-luciferase T cell reporter mouse which allows live bioluminescence imaging of T cell location and activation, which has numerous possible applications in many experimental models where T cells play a central role.

Materials and Methods

Mice and cell lines

The following construct was designed for the generation of the TbiLuc transgenic mouse. The PpyRE9 gene (*Photinus pyralis* red-emitting luciferase 9, a kind gift of Prof. Branchini (29)) was cloned downstream of 3 NFAT repetitive elements and minimal promoter derived from the pGL4.30 plasmid (Promega). The sequence of the human CD2 promoter was cloned upstream of the CBG99 green click beetle luciferase gene (15,25). These two cassettes were cloned to form a bidirectional construct, separated by an insulator. TbiLuc mice were generated by injection of the

bicistronic construct into pronuclei of fertilized oocytes of CBA**C57BL/6* mice. In the pups born the presence of the transgene was determined by a specific PCR using genomic DNA from tail biopsy and its activity/ function was measured by evaluation of light emission from tail-vein blood after addition of the luciferase substrate D-luciferin. Mice were back-crossed to the *C57BL/6* strain for several generations before use in experiments. Albino B6 mice (tyrosinase-deficient immunocompetent *C57BL/6* mice), TbiLuc mice (dual T cell luciferase transgenic mice), OT-I mice (T cell receptor-transgenic mice carrying the CD45.1 congenic marker) and crossed TbiLuc*OT-I mice were bred in the animal breeding facility of the Leiden University Medical Center, the Netherlands. All experiments were approved by the animal ethical committee of Leiden University. D1 is a GM-CSF-dependent immature dendritic cell line derived from *C57BL/6* mice, and B3Z is an OVA-specific CD8 T cell hybridoma carrying the lacZ reporter gene induced by NFAT (30,31). Cell lines were assured to be free of rodent viruses and Mycoplasma by regular PCR analysis. Cells were cultured as previously described (32).

Bioluminescence imaging (BLI)

Cell samples were prepared for in vitro BLI analysis in sterile black-walled flat-bottom 96-wells plates (Greiner). Cells were suspended in 100 μ L PBS containing 1 mM D-luciferin potassium salt (SynChem) or 1 mM CycLuc1 (Aobious), incubated for 5 minutes at 37°C. For in vivo BLI, mice were injected with 150 mg/kg D-luciferin or 7,6 mg/kg CycLuc1 intraperitoneally, left for 5 minutes and anaesthetized by isoflurane inhalation. In experiments using both substrates, the substrates were injected with a 3-hour interval. BLI imaging was performed using an IVIS Spectrum small animal imager (PerkinElmer) that measured the light signal using open filter and a series of 20 nm wavelength band filters from 500 nm to 700 nm, with an acquisition time of 30 seconds. Accompanying LivingImage 4.2 software (Perkin Elmer) was used for spectral unmixing of the full-spectrum measurement to identify individual signals in vitro. Signal quantification in specific regions of interest (ROI) was performed by using fixed-size ROIs throughout the experiments.

Isolation of organs and immune cells

Organs from TbiLuc mice were taken out, weighed and homogenized before BLI analysis. Immune cells were obtained from the spleen by mashing on 70 μ m cell strainers (BD Biosciences) to create single-cell suspensions, followed by erythrocyte lysis. Then, CD4 and CD8 T cells were isolated separately by negative magnetic selection (BD IMag enrichment kits), B cells by positive magnetic selection of B220+ cells (BD), while NK cells were obtained by FACS-sorting for NK1.1+ cells.

T cell activation in vitro

T cells isolated from spleens of TbiLuc or TbiLuc*OT-I mice were stimulated overnight with 100 ng/mL Phorbol 12-myristate 13-acetate (PMA) + 1500 ng/mL ionomycin (both Sigma-Aldrich), with agonistic anti-CD3 and anti-CD28 antibodies (BD Biosciences) pre-coated at 1 µg/mL at 37°C for 30 minutes, or with 50,000 D1 dendritic cells pre-loaded with OVA immune complexes. OVA immune complexes were formed by incubating a 1:300 mass ratio of OVA protein (Worthington) and anti-OVA antibody (LSBio) for 30 minutes at 37°C, after which the immune complexes were added to D1 dendritic cells and incubated overnight. Unloaded D1 cells were incubated overnight in parallel to serve as control cells.

Adoptive transfer and vaccination

Adoptive transfer consisted of 1 million purified OT-I CD8 T-cells (unless stated otherwise) isolated as described above, injected intravenously in 200 µL PBS in the tail vein. Vaccination consisted of subcutaneous injection of 1 million D1 cells pre-loaded with OVA immune complexes, or unloaded control D1 cells, in 50 µL PBS in the tail-base region.

Western Blot

Expression of PpyRE9 luciferase by activated T cells was confirmed by Western Blot. T cells were isolated and stimulated overnight as described above. Then, 3×10^6 cells were lysed and the total protein content of each sample was determined by a Pierce BCA protein assay kit (Thermo Scientific). Next, 20 µg of total cell extract was applied to a 10% SDS-PAGE and transferred onto a nitrocellulose membrane. After washing, the membrane was incubated overnight with rabbit anti-luciferase polyclonal antibody in TPBS at 1/500 dilution (Fitzgerald). The GAPDH antibody (Cell Signaling Technology) was used to correct for the amount of total protein. The blots were washed, exposed to an HRP-conjugated secondary antibody for 1 hour, and detected using enhanced chemiluminescence (ECL) reagents (Thermo Scientific). Detection of ECL signals was performed with the IVIS Spectrum and quantification of bands using Living Image Software 4.2.

Flow cytometry

Before adoptive transfer of CD8 T cells, their CD8-purity and naïve phenotype was assessed by flow cytometry. In short, spleen cells were suspended in FACS buffer (PBS with 0.5% FCS and 0.02% sodium azide), surface-stained with antibodies against CD8β, CD44, CD45.1 and CD62L (BioLegend) and analyzed on a BD Accuri C6 flow cytometer. Analysis was performed on FlowJo software (FlowJo).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 software. Data are shown as the mean \pm SEM for each group, and comparison of groups was performed by two-tailed Student's t-test. Statistical differences were considered significant at $p < 0.05$.

Results

Design and development of the dual-luciferase transgenic TbiLuc mouse

We designed a bicistronic vector containing the click beetle green luciferase (CBG99) sequence under the control of the human CD2 (hCD2) promoter and the red-emitting firefly luciferase (PpyRE9) sequence under the control of the nuclear factor of activated T cells (NFAT) minimal promoter (**Figure 1A**). NFAT expression is absent in naïve T cells and increases strongly after T cell activation, making it an ideal promoter to visualize activated T cells. The hCD2 promoter is expressed in both T cells and B cells in humans, but is T cell-specific when used in mice (15). This bicistronic vector was used to generate the TbiLuc transgenic mouse on a C57BL/6*CBA mixed background (F1). Founders were selected based on high light emission in blood and further back-crossed towards C57BL/6 background. As expected, the CD2-driven CBG99 luciferase was constitutively expressed in lymphoid organs, which was evident both in live in vivo BLI and in isolated spleen, lymph nodes and thymus (**Figure 1B, C**).

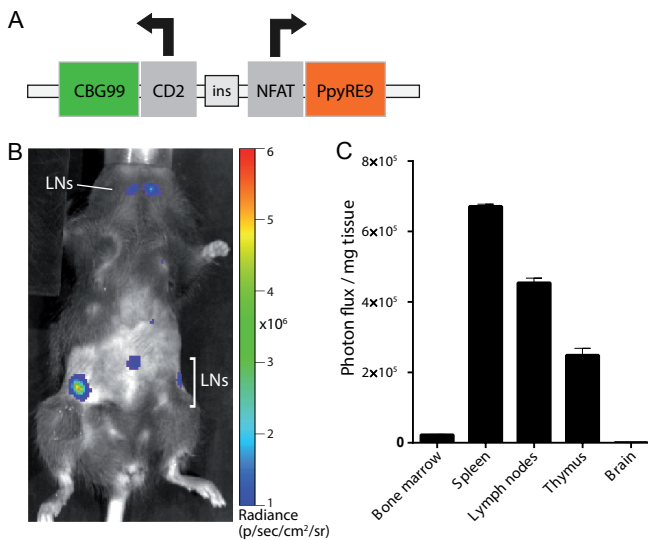


Figure 1. Design and development of the dual-luciferase transgenic TbiLuc mouse. (A) Simplified schematic overview of the dual-luciferase reporter bicistronic vector. NFAT = nuclear factor of activated T cells, CBG99 = click beetle green luciferase 99, PpyRE9 = Photinus pyralis red-emitting luciferase 9. (B) Bioluminescence measurement of a TbiLuc mouse, showing luciferase expression in secondary lymphoid organs. The abdomen is shaved to reduce signal absorption. Cervical, mesenteric and inguinal lymph nodes (LNs) are indicated. (C) Quantification of luciferase signals in several organs of TbiLuc mice (n=3), shown as photon flux per mg of tissue (mean + SEM).

Luciferase expression is restricted to T cells

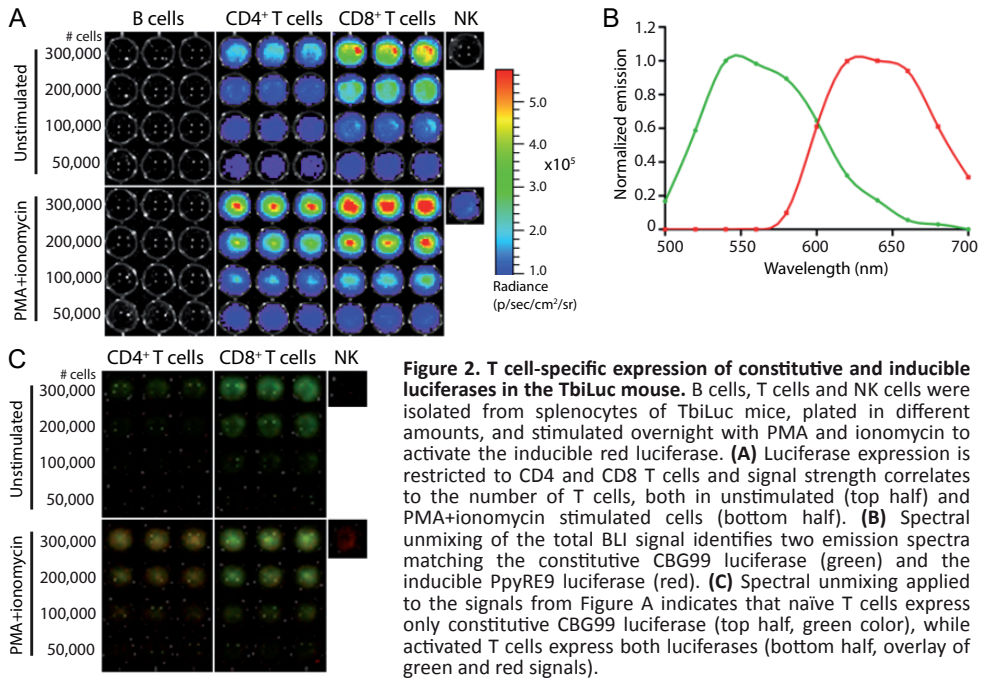
Next, we analyzed in more detail which cells in the lymphoid organs express luciferase by isolating B cells, CD4 T cells, CD8 T cells and NK cells from the spleens of TbiLuc mice. Constitutive expression of CBG99 luciferase in unstimulated cells was completely restricted to T cells, as B cells and NK cells showed no detectable bioluminescence signal (**Figure 2A**, top half). CD8 T cells produced a two-fold higher luciferase activity than CD4 T cells. The amount of the luciferase activity was proportional to the number of T cells. In parallel, we tested the validity of the dual-luciferase construct by stimulating these isolated immune cells with PMA and ionomycin, which are chemical compounds often used in combination to trigger NFAT by activating the protein kinase C (PKC) pathway and increasing intracellular levels of calcium, respectively. Also after PMA/ionomycin treatment, strong luciferase activity was observed only in T cells, except for a weakly detectable signal in NK cells (**Figure 2A**, bottom half).

T cell activation results in NFAT-induced luciferase expression

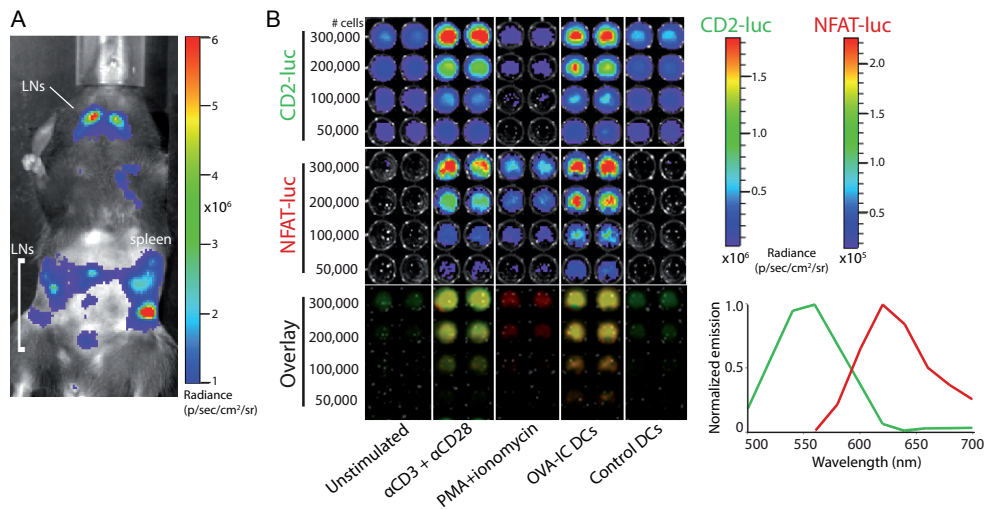
We next analyzed if the increased light signal after PMA/ionomycin treatment was mediated by the NFAT-driven PpyRE9 luciferase. A spectral unmixing algorithm was applied to separate the independent emission spectra within the sample and quantify these spectra separately. Two distinct emission patterns were identified corresponding to the emission spectra of the green CBG99 and the red PpyRE9 luciferases (**Figure 2B**). By representing the unmixed signals in artificial green and red colors, the emission spectra of the two luciferases can be assessed per single sample. Unstimulated naïve T cells expressed only the constitutive hCD2-driven CBG99 luciferase, while PMA/ionomycin treatment induced the expression of NFAT-driven red PpyRE9 luciferase, resulting in a yellow color based on overlaying green and red colors (**Figure 2C**). The low signal observed in NK cells after PMA/ionomycin treatment was confirmed to be NFAT-induced, as shown by the red color. The induction of PpyRE9 luciferase expression by PMA/ionomycin treatment was further analyzed by Western Blotting, showing a clear presence of PpyRE9 luciferase protein band after stimulation of TbiLuc CD8 T cells with PMA/ionomycin (**Supplemental Figure 1A**).

Dual-luciferase imaging of antigen-specific T cells

In order to study dual-bioluminescent T cells with known antigen specificity, the TbiLuc mouse was crossed to T cell receptor-transgenic OT-I mice whose CD8 T cells recognize the SIINFEKL epitope of chicken ovalbumin (OVA) as presented in H-2Kb MHC class I molecules. The resulting TbiLuc*OT-I mice showed constitutive luciferase expression in lymphoid organs, identical to their parental TbiLuc mice (**Figure 3A**). To study NFAT-luciferase induction in an antigen-specific manner, we purified CD8 T cells from the spleens of TbiLuc*OT-I mice by magnetic selection. This procedure yielded >90% pure CD8 T cells with a naïve phenotype (**Supplemental Figure 1b**).



6



The isolated CD8 T cells were then co-incubated in vitro with dendritic cells presenting the SIINFEKL epitope processed from the OVA protein after uptake of immune-complexes (OVA-IC), which we have previously reported as an efficient CD8 T cell vaccine (16). This led to a strong induction of NFAT-luciferase, which was completely antigen-specific as control dendritic cells did not induce any expression, identical to unstimulated naïve T cells (**Figure 3B**). Again, Western Blot analysis showed the presence of PpyRE9 protein (**Supplemental Figure 1C**). Although the use of a single D-luciferin substrate was sufficient for dual color imaging in vitro, the weak NFAT-induced red light signal did not allow efficient in vivo detection of T cell activation (data not shown). Therefore, we tested a new luciferin substrate, CycLuc1, that was specifically designed to improve light emission from firefly luciferases such as our NFAT-induced PpyRE9 red luciferase (14). By using T cell hybridomas expressing either one of the two luciferases also used in TbiLuc, we show that CycLuc1 is an inefficient substrate for CBG99 green luciferase, as indicated by the 30-fold lower signal at the emission peak as compared to D-luciferin (**Supplemental Figure 2**). This allows specific detection of PpyRE9 luciferase in TbiLuc T cells using the CycLuc1 substrate. Moreover, these data indicate that substrates given in combination compete for the active site of the enzymes, as shown by the 1.6 times lower average photon flux measured for CBG99 emission with D-Luc + CycLuc1 as compared to D-Luc alone.

Next, we investigated whether dual color imaging of TbiLuc*OT-I T cell activation could be performed using the two substrates, D-luciferin and CycLuc1. Addition of a single substrate per sample allowed efficient detection of the constitutive signal from CBG99 green luciferase by D-luciferin, and the activation signal from PpyRE9 red luciferase by CycLuc1 (**Figure 4A**). Unlike in naïve T cells, in activated T cells expressing both luciferases, simultaneous addition of the two substrates produces a spectrum with no isolated peaks of emission (**Figure 4B**). Therefore, separate addition of substrates is warranted for efficient separation of light signals in activated T cells. The validity of using CycLuc1 for specific visualization of activation-induced PpyRE9 luciferase was also found in CD4 and CD8 T cells from parental TbiLuc mice (data not shown).

Visualization of T cell localization and activation in vaccinated mice

We set out to investigate whether antigen-specific target recognition by T cells could be visualized in vivo, using an adoptive T cell transfer system. First, we visualized the fate of T cells after adoptive transfer. Intravenously injected TbiLuc*OT-I CD8 T cells homed efficiently to the lymphoid organs of recipient Albino B6 mice, and the number of transferred T cells correlated to the signal strength (**Supplemental Figure 3**). Next, mice were vaccinated subcutaneously in the tail-base with dendritic cells

(DCs) pre-loaded with OVA protein immune complexes, containing the specific T cell antigen recognized by OT-I T cells, while control mice received unloaded DCs. Based on previous in vitro results, we adopted an in vivo imaging protocol with separate administration of the two substrates with a time interval to allow clearance of the first substrate, measuring CBG99 luciferase activity using D-luciferin and measuring PpyRE9 luciferase activity using CycLuc1. Periodical bioluminescence imaging was then performed, focusing on the vaccine-draining inguinal lymph nodes, and the green and red light signals were quantified. In the first week after vaccination, OVA-vaccinated mice showed a sharp increase in both the constitutive green light signal and the activation-induced red light signal in the lymph nodes, peaking at day 8 after injection (**Figure 5A**).

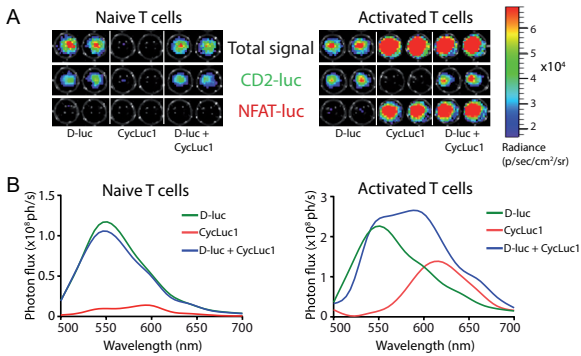


Figure 4. Efficient visualization of NFAT-induced PpyRE9 luciferase using the CycLuc1 substrate. (A) CD8 T cells from TbiLuc*OT-I mice left untreated ('Naive') or stimulated O/N with OVA immune complex-loaded dendritic cells ('Activated') were imaged using the substrate D-luciferin (D-luc), CycLuc1, or both. Total signal (top), the constitutive CD2-CBG99 green luciferase (middle) and the activation-induced NFAT-PpyRE9 red luciferase (bottom) are shown separately, on the same scale. (B) Emission spectra of naive and activated T cells (from Figure A) using different substrates, showing strong constitutive CBG99 signals using the D-luciferin substrate, and specific activation-induced PpyRE9 signals using CycLuc1.

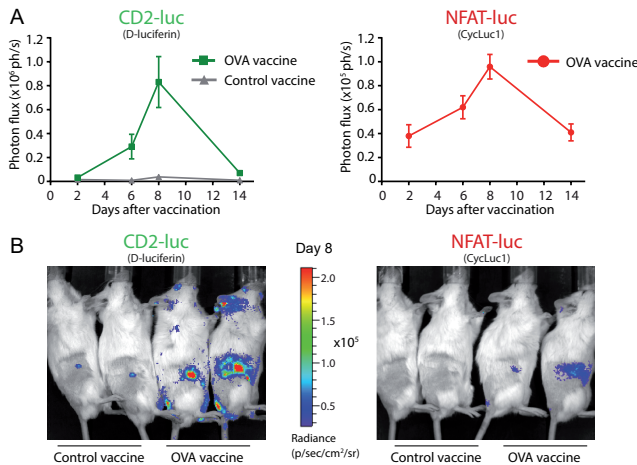


Figure 5. Visualization of T cell localization and activation in vaccinated mice. (A) Constitutive CD2-luciferase (left) and NFAT-luciferase (right) signals from vaccine-draining inguinal lymph nodes of vaccinated mice. On day 0, mice received adoptive transfer of TbiLuc*OT-I CD8+ T cells and were vaccinated with OVA immune complex-loaded dendritic cells or with unloaded control dendritic cells (n=4 mice per group). CD2-CBG99 and NFAT-PpyRE9 luciferases were imaged using the D-luciferin and CycLuc1 substrates, respectively. Mice receiving the control vaccine showed no detectable NFAT-luc signal. (B) Representative pictures of OVA-vaccinated and control-vaccinated mice on day 8, showing T cell proliferation (left) and activation (right) in OVA-vaccinated mice.

In contrast, control mice had a comparable constitutive green light signal on day 2 as OVA-vaccinated mice, but did not show a strong T cell expansion and had undetectable red light signal, suggesting that the DC-OVA vaccine specifically induced activation and expansion of TbiLuc*OT-I T cells. Two days after vaccination, T cell activation could be detected by means of PpyRE9 luciferase activity, while

T cell expansion as measured by CBG99 luciferase activity started later in time. The dramatic increase in luciferase activity in OVA-vaccinated mice, and the lack of activation-dependent red luciferase activity in control mice, can be seen in representative images from day 8 (**Figure 5B**).

Discussion

In this study, we report the design and development of a dual-luciferase T cell transgenic mouse, called TbiLuc, as a novel tool for non-invasive imaging of T cells. The dual-luciferase construct allows live visualization of both the location and the activation status of T cells, which we established in antigen-specific T cell activation studies both *in vitro* and *in vivo*. The TbiLuc mouse model has numerous applications both in fundamental T cell biology and in preclinical translational studies on the many diseases in which T cells play a role. For instance, since it has been established that T cells are crucial in the spontaneous or therapeutically-induced clearance of malignant cells, the TbiLuc mouse can be used to test the efficacy of cancer vaccines, immunomodulatory antibodies and other treatment modalities that depend on T cells. Disease models of viral infection and autoimmunity involving T cell effector cells are other possible applications of the TbiLuc mouse (17,18).

To introduce luciferase-encoding genes into cells, researchers commonly use retroviral transduction. However, naïve T cells cannot be efficiently transduced unless they are pre-treated by either TCR stimulation with cognate antigen or with cytokines such as IL-7, both of which trigger downstream signaling pathways in the T cells (6–9,19,20). This makes studies on truly naïve T cells impossible, although T cell ‘rested’ after activation and transduction are sometimes considered to represent naïve T cells; a perhaps more pragmatic rather than immunological interpretation. Instead, integration of a luciferase-encoding gene into murine oocytes, resulting in a luciferase-transgenic mouse strain, is a time-consuming but scientifically much more attractive alternative that allows the isolation of luciferase-expressing naïve T cells from the lymphoid organs of the mouse. Indeed, several groups have created luciferase reporter mice for T cell imaging and showed the potential of BLI for T cell tracking (10–12). However, these single-luciferase reporter mice only provide information on the location of T cells, without additional information on their activation state. The feasibility of creating dual-luciferase transgenic mice has been shown before, but never using cell type-specific expression (21,22). We describe the first dual-luciferase T cell transgenic mouse, which offers an important advantage in allowing simultaneous visualization of T cell location and activation.

Luciferase T cell imaging, and optical imaging in general, is especially well-suited for visualization of superficial light signals, as the signal from deeper locations in the body will be influenced by absorption and scattering of photons in the tissues. In this view, radio-imaging provides more accurate information on the location of T cells (23,24). However, optical imaging strategies avoid the undesired use of radioactive material, and BLI in particular allows the creation of reporter mice where luciferase expression can be restricted to the cell type of choice by choosing a cell-specific promoter. Our choice of the human CD2 minigene as the promoter driving the constitutively expressed CBG99 luciferase is based on earlier studies showing T cell-specific transgene expression (15,25). To our knowledge, we are the first to add a second luciferase in the creation of a T cell reporter mouse and to optimize an *in vivo* imaging protocol for measuring the activity of both luciferases. The NFAT response element driving PpyRE9 luciferase expression allows a straightforward readout of T cell activation, which is valuable extra information besides the location of the T cell. Our optimized *in vivo* imaging protocol involves separate injection of the two substrates such that the first substrate is cleared before the second substrate is administered, measuring CBG99 activity using D-luciferin and measuring PpyRE9 activity using CycLuc1. This setup provides the maximal light emission for each luciferase and avoids both biochemical interference from the other substrate and spectral interference from the other luciferase. An *in vivo* vaccination study visualized the expansion and activation of adoptively transferred T cells in vaccine-draining lymph nodes following typical kinetics of T cell responses upon vaccination (26–28). Finally, although a weak NFAT-driven luciferase activity was observed in NK cells after TCR independent PMA-ionomycin treatment, these cells can be distinguished from T cells because of the absence of green CD2-driven luciferase emission. Moreover, in models using adoptive T cell transfer, NK cells are efficiently excluded during the T cell isolation process.

Our data have shown that dual-luciferase reporter mice can be easily crossed to other (T cell-) transgenic mouse strains, which brings many possibilities to further fine-tune the reporter system for the biomedical experimental model of interest. Taken together, this proof-of-concept manuscript introduced the TbiLuc dual-luciferase T cell transgenic mouse that allows to track activation and expansion of T cell populations in naturally organized lymphoid tissue, with full retention of T cell naivety and antigen-specific functionality. Many biomedical research fields can potentially benefit from this advanced live T cell imaging model.

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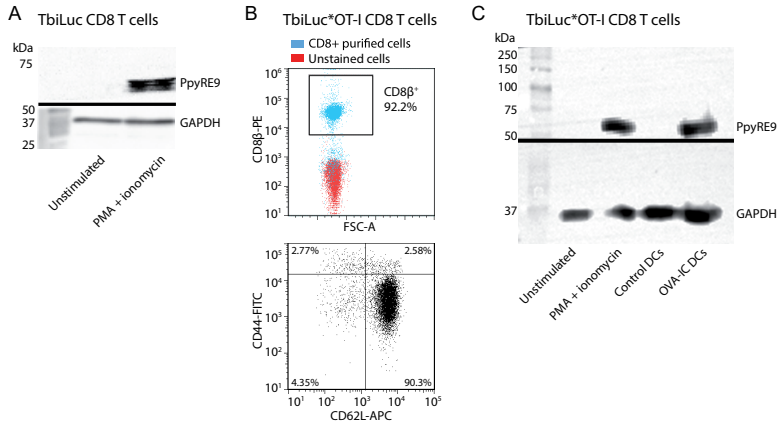
References

1. Contag CH, Bachmann MH. Advances in in vivo bioluminescence imaging of gene expression. *Annu Rev Biomed Eng.* 2002;4:235–60.
2. Kaijzel EL, van der Pluijm G, Löwik CWGM. Whole-body optical imaging in animal models to assess cancer development and progression. *Clin Cancer Res.* 2007;13:3490–7.
3. Coombe DR, Nakhoul AM, Stevenson SM, Peroni SE, Sanderson CJ. Expressed luciferase viability assay (ELVA) for the measurement of cell growth and viability. *J Immunol Methods.* 1998;215:145–50.
4. Karimi MA, Lee E, Bachmann MH, Salicioni AM, Behrens EM, Kambayashi T, et al. Measuring Cytotoxicity by Bioluminescence Imaging Outperforms the Standard Chromium-51 Release Assay. Amendola R, editor. *PLoS One.* 2014;9:e89357.
5. Na I-K, Markley JC, Tsai JJ, Yim NL, Beattie BJ, Klose AD, et al. Concurrent visualization of trafficking, expansion, and activation of T lymphocytes and T-cell precursors in vivo. *Blood.* 2010;116:e18-e25.
6. Kim D, Hung C-F, Wu T-C. Monitoring the Trafficking of Adoptively Transferred Antigen-Specific CD8-Positive T Cells *In Vivo* , Using Noninvasive Luminescence Imaging. *Hum Gene Ther.* 2007;18:575–88.
7. Rabinovich BA, Ye Y, Etto T, Chen JQ, Levitsky HI, Overwijk WW, et al. Visualizing fewer than 10 mouse T cells with an enhanced firefly luciferase in immunocompetent mouse models of cancer. *Proc Natl Acad Sci U S A.* 2008;105(38):14342-6.
8. Hailemichael Y, Dai Z, Jaffarzar N, Ye Y, Medina MA, Huang X-F, et al. Persistent antigen at vaccination sites induces tumor-specific CD8+ T cell sequestration, dysfunction and deletion. *Nat Med.* 2013;19:465–72.
9. Patel MR, Chang Y-F, Chen IY, Bachmann MH, Yan X, Contag CH, et al. Longitudinal, Noninvasive Imaging of T-Cell Effector Function and Proliferation in Living Subjects. *Cancer Res.* 2010;70:10141–9.
10. Chewning JH, Dugger KJ, Chaudhuri TR, Zinn KR, Weaver CT. Bioluminescence-based visualization of CD4 T cell dynamics using a T lineage-specific luciferase transgenic model. *BMC Immunol.* 2009;10:44.
11. Azadniv M, Dugger K, Bowers WJ, Weaver C, Crispe IN. Imaging CD8+ T cell dynamics in vivo using a transgenic luciferase reporter. *Int Immunol.* 2007;19:1165–73.
12. Charo J, Perez C, Buschow C, Jukica A, Czeh M, Blankenstein T. Visualizing the dynamic of adoptively transferred T cells during the rejection of large established tumors. *Eur J Immunol.* 2011;41:3187–97.
13. Mezzanotte L, Que I, Kaijzel E, Branchini B, Roda A, Löwik C. Sensitive Dual Color In Vivo Bioluminescence Imaging Using a New Red Codon Optimized Firefly Luciferase and a Green Click Beetle Luciferase. Herrera-Estrella A, editor. *PLoS One.* 2011;6:e19277.
14. Evans MS, Charette JP, Adams ST, Reddy GR, Paley MA, Aronin N, et al. A synthetic

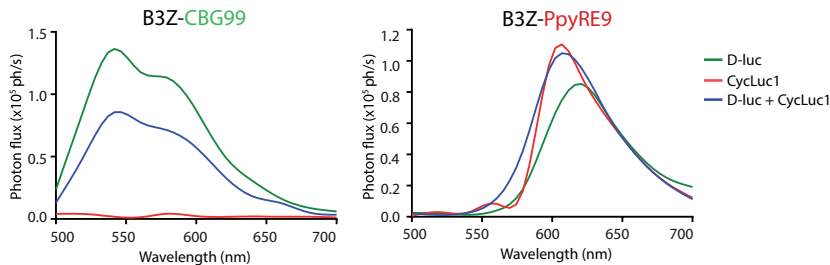
- luciferin improves bioluminescence imaging in live mice. *Nat Methods*. 2014;11:393–5.
15. Lang G, Wotton D, Owen MJ, Sewell WA, Brown MH, Mason DY, et al. The structure of the human CD2 gene and its expression in transgenic mice. *EMBO J*. 1988;7:1675–82.
 16. Schuurhuis DH, Ioan-Facsinay A, Nagelkerken B, van Schip JJ, Sedlik C, Melief CJM, et al. Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific CD8+ CTL responses in vivo. *J Immunol*. 2002;168:2240–6.
 17. Hutchens M, Luker GD. Applications of bioluminescence imaging to the study of infectious diseases. *Cell Microbiol*. 2007;9:2315–22.
 18. Luker KE, Luker GD. Bioluminescence imaging of reporter mice for studies of infection and inflammation. *Antiviral Res*. 2010;86:93–100.
 19. Cavalieri S, Cazzaniga S, Geuna M, Magnani Z, Bordignon C, Naldini L, et al. Human T lymphocytes transduced by lentiviral vectors in the absence of TCR activation maintain an intact immune competence. *Blood*. 2003;102:497–505.
 20. Takada K, Jameson SC. Naive T cell homeostasis: from awareness of space to a sense of place. *Nat Rev Immunol*. 2009;9:823–32.
 21. Tehrani AM, Hwang S-K, Kim T-H, Cho C-S, Hua J, Nah W-S, et al. Aerosol delivery of Akt controls protein translation in the lungs of dual luciferase reporter mice. *Gene Ther*. 2007;14:451–8.
 22. Noguchi T, Michihata T, Nakamura W, Takumi T, Shimizu R, Yamamoto M, et al. Dual-Color Luciferase Mouse Directly Demonstrates Coupled Expression of Two Clock Genes. *Biochemistry*. 2010;49:8053–61.
 23. Tavare R, McCracken MN, Zettlitz KA, Knowles SM, Salazar FB, Olafsen T, et al. Engineered antibody fragments for immuno-PET imaging of endogenous CD8+ T cells in vivo. *Proc Natl Acad Sci*. 2014;111:1108–13.
 24. Leech JM, Sharif-Paghaleh E, Maher J, Livieratos L, Lechler RI, Mullen GE, et al. Whole-body imaging of adoptively transferred T cells using magnetic resonance imaging, single photon emission computed tomography and positron emission tomography techniques, with a focus on regulatory T cells. *Clin Exp Immunol*. 2013;172:169–77.
 25. Zhumabekov T, Corbella P, Tolaini M, Kioussis D. Improved version of a human CD2 minigene based vector for T cell-specific expression in transgenic mice. *J Immunol Methods*. 1995;185:133–40.
 26. Knudsen ML, Ljungberg K, Kakoulidou M, Kostic L, Hallengård D, García-Arriaza J, et al. Kinetic and phenotypic analysis of CD8+ T cell responses after priming with alphavirus replicons and homologous or heterologous booster immunizations. *J Virol*. 2014;88:12438–51.
 27. Song X-T, Turnis ME, Zhou X, Zhu W, Hong B-X, Rollins L, et al. A Th1-inducing adenoviral vaccine for boosting adoptively transferred T cells. *Mol Ther*. 2011;19:211–7.
 28. Blattman JN, Cheng LE, Greenberg PD. CD8+ T cell responses: it's all downhill after their prime ... *Nat Immunol*. 2002;3:601–2.
 29. Branchini BR, Ablamsky DM, Davis AL, Southworth TL, Butler B, Fan F, et al. Red-emitting luciferases for bioluminescence reporter and imaging applications. *Anal Biochem*. 2010;396:290–7.
 30. Winzler C, Rovere P, Rescigno M, Granucci F, Penna G, Adorini L, et al. Maturation Stages of Mouse Dendritic Cells in Growth Factor-dependent Long-Term Cultures. *J Exp Med*. 1997;185.
 31. Karttunen J, Shastri N. Measurement of ligand-induced activation in single viable T cells

- using the lacZ reporter gene. *Proc Natl Acad Sci.* 1991;88:3972–6.
32. Rahimian S, Kleinovink JW, Fransen MF, Mezzanotte L, Gold H, Wisse P, et al. Near-infrared labeled, ovalbumin loaded polymeric nanoparticles based on a hydrophilic polyester as model vaccine: In vivo tracking and evaluation of antigen-specific CD8⁺ T cell immune response. *Biomaterials.* 2015;37.

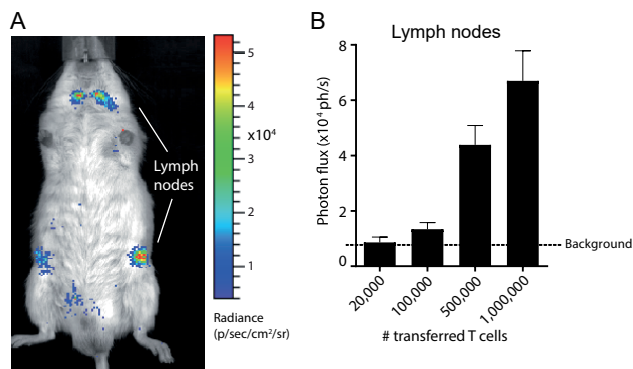
Supplementary Information



Supplementary Figure S1. (A) Western blot for the activation-induced PpyRE9 luciferase. CD8 T cells from TbiLuc mice stimulated with PMA+ionomycin, but not unstimulated cells, produce PpyRE9 luciferase protein. (B) Representative flow cytometry plots showing the efficient purification of CD8 T cells (top), which have a naïve phenotype (CD44^{lo}, CD62L^{hi}, bottom) from TbiLuc*OT-I mice. (C) Western blot for the activation-induced PpyRE9 luciferase. CD8 T cells from TbiLuc*OT-I mice stimulated with PMA+ionomycin or OVA immune complex-loaded dendritic cells (OVA-IC DCs), but not unstimulated cells or control DC-stimulated cells, produce PpyRE9 luciferase protein.



Supplementary Figure S2. Comparison of the efficacy of D-luciferin (D-luc), CycLuc1, or mixed substrates using B3Z T cell hybridoma cells transduced to constitutively express either green CBG99 (left) or red PpyRE9 (right) luciferases, as indicated. D-luciferin is the optimal substrate for CBG99, and CycLuc1 is the optimal substrate for PpyRE9, but mixing the two substrates reduces signal strength.



Supplementary Figure S3. Bioluminescence imaging of TbiLuc*OT-I CD8 T cells after adoptive transfer into recipient mice. (A) Adoptively transferred T cells home to the secondary lymphoid organs of recipient mice. Representative image, the cervical and inguinal lymph nodes are indicated. (B) Quantification of the signals in inguinal lymph nodes showing that signal strength correlates to the number of transferred T cells.

