# Bioluminescence Imaging of Adoptively Transferred Lymphocytes During Allogeneic Tumor Rejection

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**Abstract.** *Aim: The aim of the present study was to analyze the survival, spatial distribution and proliferation of adoptively transferred lymphocytes in allogeneic tumor rejection. Materials and Methods: Transgenic β-actin-luc mice that express luciferase were sensitized against SL2 tumors and were used as lymphocyte donors to study the anti-tumor effect in SL2 tumor-bearing lymphocyte-deficient RAG$^{-/-}$ mice. Whole-body bioluminescence images of recipient mice were obtained to track the adoptively transferred lymphocytes. Proliferation of lymphocytes was estimated by quantification of photon emission. Results: T lymphocytes sensitized against allogeneic SL2 tumors cured the majority of SL2 tumor-bearing RAG$^{-/-}$ mice. Bioluminescence imaging showed that transferred T lymphocytes survived in the spleen and lymph nodes. Tumor rejection was associated with lymphocyte proliferation and migration to the tumor site. Conclusion: Sensitized T lymphocytes from transgenic β-actin-luc mice reject allogeneic SL2 tumors in RAG$^{-/-}$ mice and can be tracked in vivo using bioluminescence imaging.*

The ability of T lymphocytes to destroy allogeneic tumor cells is well-established. Hematological malignancies can be cured by allogeneic bone marrow transplantation, in which the main cell type responsible for the curative effect is T lymphocytes. Patients with solid tumors can also be treated with allogeneic T lymphocytes, although responses are less frequent (1).

Proliferation of T lymphocytes has been reported as a variable associated with tumor rejection (2). Trafficking of T cells to the tumor microenvironment is also regarded critical for the success of adoptive cellular immunotherapy (3). Results from clinical trials have suggested that cancer regression is positively associated with survival and persistence of adoptively transferred T cells *in vivo* (4).

Optical imaging has been the newest modality used for dynamic non-invasive imaging of biological phenomena in small animals (5). Bioluminescence imaging has been used to image tumor growth and metastasis and to evaluate new therapies in living animals (6-10). *In vivo* imaging may reveal characteristics of lymphocytes required for effective anti-tumor response. However, studies on cell imaging of the immune system during anti-tumor response are scarce (11, 12).

In the present study, we analyzed survival, spatial distribution and proliferation of sensitized adoptively-transferred lymphocytes during rejection of allogeneic tumors in immunodeficient hosts.

## Materials and Methods

*Mice and tumors.* Transgenic luciferase-expressing B6, FVB β-actin-luc female mice were purchased from Taconic (Germantown, NY, USA). These mice at the age of 7-9 weeks were used to generate T lymphocytes sensitized against allogeneic SL2 tumors. Transgenic β-actin-luc mice carry a 14-kb fragment of the murine β-actin promoter isolated from genomic DNA, a chimeric intron and modified firefly luciferase cDNA. Spleen lymphocytes from transgenic β-actin-luc mice were transferred to 10-18-week- old SL2 tumor-bearing RAG$^{-/-}$ female mice that lack T and B lymphocytes. RAG-deficient (RAG1$^{-/-}$ or RAG2$^{-/-}$) mice were a kind gift from Prof. Reinhard Voll, University Erlangen-Nürnberg, and were bred at local breeding facility at the State Research Institute Centre for Innovative Medicine, Vilnius, Lithuania. Experimental research on animals was conducted according to guidelines of the Lithuanian Ethics Committee for the Laboratory Animal Use.

SL2 lymphoma cells were maintained by weekly intraperitoneal passage in syngeneic DBA/2 mice and subsequently frozen. Prior to use in experiments, SL2 cells were thawed, washed and resuspended in RPMI-1640 medium. Solid

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*Key Words:* murine tumor model, T lymphocytes, adoptive transfer, bioluminescence imaging.

tumors in RAG$^{-/-}$ mice were induced by subcutaneous injection on the right side of the chest of $10^5$ SL2 cells in phosphate buffered saline (PBS). At different time points after rejection of the primary tumor, these mice were re-challenged with $10^6$ or $10^7$ SL2 tumor cells in PBS subcutaneously on the left side of the chest. Transgenic β-actin-luc mice were sensitized against SL2 tumor by subcutaneous injection on the right side of the chest of $10^7$ SL2 cells in PBS.

*Isolation and transfer of spleen lymphocytes*. Eighteen days after implantation of SL2 tumors, transgenic β-actin-luc mice were sacrificed by cervical dislocation, spleens were removed and single-cell lymphocyte suspensions were prepared by enzymatic digestion using a Spleen Dissociation Kit in combination with mechanical dissociation using GentleMACS Dissociator (both from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). For separation of T lymphocytes, transgenic β-actin-luc mice were sacrificed 15 days after implantation of SL2 tumors. T lymphocytes from spleen cell suspension were separated using magnetic bead-mediated negative selection using Pan T Cell Isolation Kit II, LS columns, and MidiMACS separator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Spleen cell suspensions ($3\times10^7$ cells per mouse) or separated T lymphocytes ($1.5\times10^7$ cells per mouse) were transferred intravenously to SL2 tumor-bearing RAG$^{-/-}$ mice *via* retro-orbital injection on day 11 after tumor implantation.

*Bioluminescence imaging*. Prior to imaging, mice were shaved and sprinkled with flour to minimize the amount of light absorbed by black fur. Mice were injected intraperitoneally with 200 mg/kg body weight of D-Luciferin (PerkinElmer, Billerica, MA, USA) solution in PBS 10 min prior to imaging. Three percent mixture of isoflurane (Abbott Laboratories, West Berkshire, UK) in oxygen was used for introductory anesthesia and 1.5% mixture for maintenance anesthesia. Bioluminescence measurements were performed using ''IVIS® Spectrum'' equipment (PerkinElmer, USA). Data were acquired and analyzed using Living Image® software, version 4.3.1 (PerkinElmer).

Two-dimensional (2D) *in vivo* bioluminescence imaging was performed using the auto-exposure mode. The auto-exposure mode automatically sets the exposure time, size of the camera lens aperture and binning to keep the signal within an optimal range for quantification.

Filters of 580, 600, 620 and 640 nm wavelength were used for three-dimensional (3D) imaging. A 3D reconstruction of luminescent light source distribution inside the mouse was performed using Diffuse Luminescence Imaging Tomography (DLIT) technique and a 3D region of interest was drawn on the whole mouse. Using the region of interest analysis of images, light emission from lymphocytes expressing firefly luciferase was quantified and expressed as total flux of photons per second. The total flux of photons per second is proportional to the number of living lymphocytes *in vivo* and can be, thereby, used to estimate lymphocyte proliferation and death (5).

*Statistics*. Survival of RAG$^{-/-}$ mice was analyzed by the Kaplan–Meier method and log-rank test. The differences between total flux of photons at different time points were evaluated using the non-parametric sign test for dependent samples. The level of significance was set at $p=0.05$.

## Results

*Effect of adoptively transferred lymphocytes on growth of allogeneic SL2 tumors in lymphocyte-deficient RAG$^{-/-}$ mice*. Kaplan-Meier curves of survival of RAG$^{-/-}$ mice after implantation of SL2 tumors are shown in Figure 1. As can be seen in Figure 1, all non-treated RAG$^{-/-}$ mice die within one month after implantation of SL2 tumors. Adoptive transfer of spleen lymphocytes from transgenic β-actin-luc mice sensitized against allogeneic SL2 tumor cured 6 out of 9 SL2 tumor-bearing RAG$^{-/-}$ mice. The surviving mice were able to reject $10^6$ and $10^7$ SL2 tumor cells after re-challenge at 144 and 253 days respectively.

To prove that rejection of SL2 tumors is mediated by T lymphocytes, the experiment was repeated with isolated spleen T cells. Adoptive transfer of isolated T lymphocytes sensitized against SL2 tumor cured 6 out of 6 SL2 tumor-bearing RAG$^{-/-}$ mice. These results confirm that T lymphocytes are responsible for antitumor effect after adoptive transfer.

*Bioluminescence imaging of transferred lymphocytes during allogeneic tumor rejection. In vivo* bioluminescence imaging of lymphocytes transferred from spleens of transgenic β-actin-luc mice was performed in RAG$^{-/-}$ mice after second rechallenge with SL2 tumor cells. Overlay of luminescent and light photograph images from 6 RAG$^{-/-}$ mice at 1, 6 and 10 days after tumor rechallenge is shown in Figure 2. These images demonstrate, that on the first day after tumor rechallenge lymphocytes are distributed in various parts of the mouse body, similarly to pre-challenge period (image not shown), while on day 6 lymphocytes preferentially accumulate at the tumor re-challenge site. Ten days after tumor rechallenge lymphocytes again spread throughout the mouse body.

To identify in which organs of the mouse lymphocytes accumulated, mice were killed 4 months after T lymphocyte transfer. Luciferin was injected 10 min before sacrificing the mice. Spleen, lymph nodes, liver, kidney and intestines of the mouse were placed in separate wells of a six-well plate and imaged using auto-exposure mode. Figure 3 shows, that the greatest bioluminescence can be detected in the spleen and lymph nodes. Some luminescence can be seen in the intestines (probably gut-associated lymphoid tissue) whereas no light emission can be detected in the liver and kidneys. Thus, the majority of transferred lymphocytes persist in the secondary lymphoid organs of the mouse, *i.e.* in the spleen and lymph nodes. Bioluminescence could still be detected after 15 months after lymphocyte transfer (image not shown), suggesting that lymphocytes persist in recipient RAG$^{-/-}$ mice indefinitely.

Total flux of photons emitted from the whole mouse body at different time points after rechallenge with SL2 tumor is shown in Figure 4. This figure shows that total photon flux almost doubles from days 0-1 to day 6 and then again returns

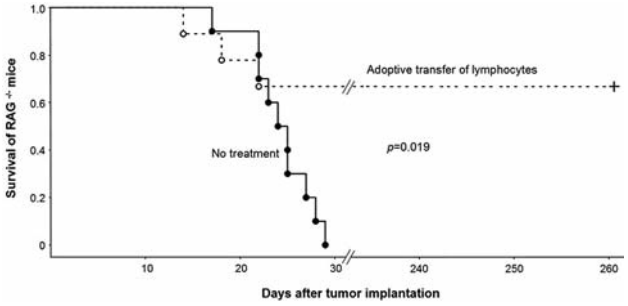


Figure 1. *Kaplan-Meier survival curves for SL2 tumor-bearing RAG$^{-/-}$ mice after adoptive transfer of spleen cells and for control SL2 tumor-bearing RAG$^{-/-}$ mice. Thirty million spleen cells from transgenic β-actin-luc mice sensitized against SL2 tumors were transferred intravenously to SL2 tumor-bearing RAG$^{-/-}$ mice via retro-orbital injection on day 11 after tumor implantation.*

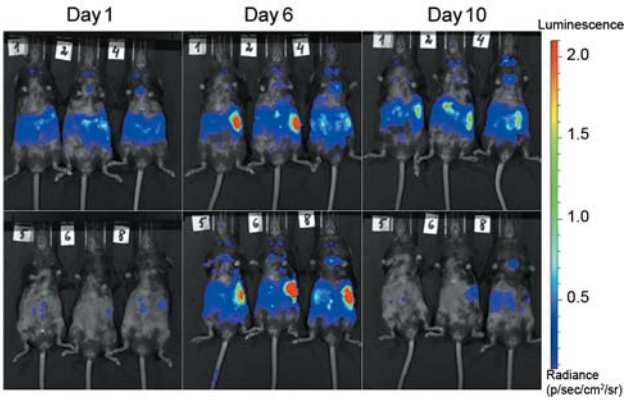


Figure 2. *Overlay of luminescent and light photograph images from 6 RAG$^{-/-}$ mice at days 1, 6 and 10 after re-challenge with SL2 tumor cells. RAG$^{-/-}$ mice had rejected primary SL2 tumors after adoptive transfer of 3×10$^7$ spleen cells from transgenic β-actin-luc mice sensitized against SL2 cells. On day 253 after implantation of the primary tumor, mice were re-challenged with 10$^7$ SL2 tumor cells on the left side of the chest. Images show accumulation of transferred spleen cells at tumor site on day 6 after re-challenge.*

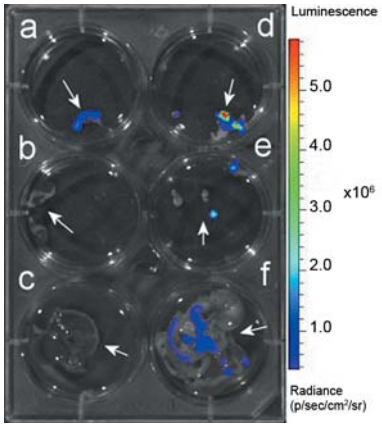


Figure 3. *Bioluminescent signals from various mouse organs 4 months after adoptive transfer of T lymphocytes: (a) spleen, (b) kidney, (c) liver, (d, e) lymph nodes, (f) intestines. The mouse was sacrificed and organs imaged 10 min after intraperitoneal injection of 200 mg/kg body weight of D-Luciferin. The majority of transferred lymphocytes accumulate in secondary lymphoid organs of the mouse, i.e. in the spleen and lymph nodes.*

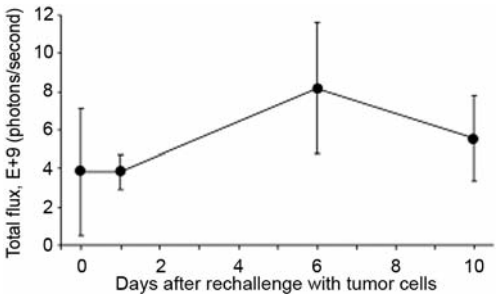


Figure 4. *Kinetics of bioluminescent signal from the whole body of RAG$^{-/-}$ mice after re-challenge with SL2 tumor cells. RAG$^{-/-}$ mice had rejected primary SL2 tumor after adoptive transfer of 3×10$^7$ spleen cells from transgenic β-actin-luc mice sensitized against SL2 cells. On day 253 after implantation of the primary tumor, mice were rechallenged with 10$^7$ SL2 tumor cells subcutaneously. Bioluminescent signal intensity is significantly higher at day 6 compared to days 0 or 1 (p=0.041). Data are from 6 mice per data point and are presented as mean±SD.*

to initial levels. This suggests that lymphocyte proliferation reaches the peak at the time of lymphocyte accumulation at the tumor site (Figure 2).

## Discussion

Our results add to a limited number of studies on imaging of cells of the immune system in tumor rejection. Kim *et al*. (11) used CD8$^+$ T cells specific against HPV-encoded oncogenic protein E7. Firefly luciferase-expressing E7-specific CD8$^+$ T-cells were generated with retrovirus carrying the luciferase gene. These lymphocytes preferentially migrated to the E7-expressing tumor site but not to the E7-negative control tumor site, and increased in number at the tumor site over time.

Charo *et al*. established a transgenic bioluminescence murine model for detection of transferred T-cells *in vivo* (12). The authors visualized the dynamic of adoptively transferred T lymphocytes during the rejection of chicken ovalbumin-expressing murine EL-4 tumor (EG.7) and SV40 large T antigen-expressing murine MCA-205 fibrosarcomas (MCA-205 P1 and MCA-205 P4). Transferred T lymphocytes expanded and preferentially accumulated within antigen-positive tumors.

We used transgenic β-actin-luc mice commercially available from Taconic (Germantown, NY, USA). These mice proved to be a convenient source of luciferase-expressing lymphocytes that can be tracked with bioluminescence *in vivo* imaging after transfer to tumor-bearing lymphocyte-deficient $RAG^{-/-}$ mice.

SL2 tumor cells are of DBA/2 origin (MHC $H\text{-}2^{d}$ haplotype). Transgenic β-actin-luc mice contain contributions both from the B6 (MHC $H\text{-}2^{b}$ haplotype) and FVB (MHC H2q haplotype) inbred strains. Our results show that similarly to rejection of foreign antigen-expressing tumor cells, rejection of allogeneic tumor cells is associated with lymphocyte proliferation and migration to the tumor site. Long-term protection from allogeneic tumor cells is mediated by persistence of transferred T lymphocytes. It remains unclear, however, whether proliferation of T lymphocytes and their migration to the tumor site are sufficient prerequisites for tumor rejection. Challenging questions still exist regarding bio-distribution and functionality of the T-cells following adoptive transfer (13). The model described in this paper is quite simple. We believe that this model could be used to highlight differences between lymphocyte proliferation and migration in effective immune response against allogeneic tumors and ineffective response against syngeneic tumors.

## Acknowledgements

The Authors would like to thank Dr. R. Koop, of PerkinElmer, for his invaluable help with *in vivo* imaging. This research was funded by a grant (No. MIP-046/2013) from the Research Council of Lithuania.

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*Received September 15, 2015*
*Revised October 7, 2015*
*Accepted October 21, 2015*