Imaging CD8⁺ T cell dynamics in vivo using a transgenic luciferase reporter

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Abstract

After activation, populations of antigen-specific T cells flow between sites of antigen expression, local lymphoid structures and other lymphoid and non-lymphoid organs. In this study, we documented the in vivo dynamics of a CD8⁺ T cell response to antigen delivered using herpes simplex virus amplicon vectors and revealed several unexpected features. First, the T cells localized to the site of vector injection, as well as the draining lymph node within 24–48 h. Second, the major site to which T cells later redistributed were intra-abdominal lymphoid organs, including milky spots, mesenteric and lumbar lymph nodes. We determined the relationship between bioluminescent signal and antigen-specific T cell numbers in various lymphoid organs, and concluded that bioluminescent signal is a valid surrogate measure of T cell abundance in superficial lymph nodes, but not in deeper structures such as the spleen.

Introduction

Initial T cell activation involves the interaction of the T cells with antigen-expressing tissue cells (direct priming) or with specialized antigen-presenting cells (APCs) that acquired the antigen through diverse uptake mechanisms (cross-priming) (1). The most effective APC for naive T cells, DC, migrate through tissues in an immature form, then upon antigen uptake are induced to differentiate, which is accompanied by their migration to lymphoid structures, and in particular to the draining lymph nodes (2). After the priming period, the activated T cells enter the circulation and migrate to many tissues, including other secondary lymphoid organs and non-lymphoid tissues (3,4). However, the dynamics of this process are incompletely understood. In particular, it is not clear whether the major sites of primed T cell accumulation are lymphoid or extralymphoid. Analysis of this issue is complicated by the fact that, whereas lymphocytes are readily isolated from lymph nodes and the spleen, their isolation from solid tissues depends on the use of tissue-dissociating enzymes, the efficiency of which is difficult to determine. Whole-animal sectioning can reveal snapshots of the in vivo distribution of T cells at different time points, but the reconstruction of T cell dynamics using this approach is difficult and laborious.

Recently, the serial snapshot approach has been augmented by in vivo imaging of several kinds. Supravital microscopy permits the resolution of cell–cell interactions within a small volume of tissue, and has revealed many feature of immune interactions that were previously inaccessible (5). However, supravital microscopy is difficult to use in the context of long-term analysis of immune responses in individual animals. To meet this need, non-destructive imaging is required. Among the options for the repeated imaging of immune responses over an extended time period is the expression of Luciferase in antigen-specific T cells (6). In this study, we used a Luciferase transgene expressed in lymphocytes to non-destructively image the time-course and in vivo redistribution of a population of antigen-specific T cells after priming with a herpes simplex virus (HSV)-based amplicon vector (7). Activated T cells were evident first in the local lymph node, and then at the site of HSV vector injection. After this, we observed systemic dispersal of the T cells. However, a high proportion of the cells remained within the
abdomen. These cells were found in mesenteric lymph nodes and in milky spots in the mesentery; other structures including the spleen were less strongly illuminated. These experiments revealed a very strong propensity of antigen-primed CD8+ T cells to migrate from a peripheral priming site to the mesenteric nodes.

Methods

Animals

Non-transgenic C57BL/6J-TyrC2J/J Albino mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and housed in a pathogen-free environment in compliance with the University of Rochester animal care guideline. The hCD2-Luciferase transgenic mice were originally made on a B10.WR background, but for these experiments were backcrossed to the C57BL/6J B6 background for three generations, and selected for H-2b homozygosity using flow cytometry. A colony of OT-1 transgenic mice was backcrossed to the CD45.1 congenic B6 strain to obtain CD45.1 homozygous mice, which were then crossed to homozygous H-2b Luciferase transgenic mice, creating double-transgenic mice in which CD8+ T cells were specific for the SIINFEKL peptide derived from ovalbumin reporter gene by placing 50

Luciferase under the control of the human CD2 promoter (terming hCD2-Luc/OT-1, mice). Male or female mice were selected for H-2b homozygosity using flow cytometry. Mice were typed for the Luciferase reporter gene. These mice, termed hCD2-Luc/OT-1, were CD45.1 heterozygous, so that the expression of CD45.1 could be used to identify their T cells. Mice were typed using the OT-1 transgenic TCR using FACS. Male or female mice were used for the experiments. The University of Rochester Animal Care and Use Committee approved all procedures.

Luciferase reporter mice

The Luciferase-expressing transgenic mice express firefly Luciferase under the control of the human CD2 promoter (termed hCD2-Luc mice; the construction of these mice is described in a separate paper). Mice were typed for the Luciferase reporter gene by placing 50 μl of whole blood in a 96-well microtiter plate and adding 20 μl of d-luciferin substrate. After 5 min, the plate was imaged (see below) to identify mice in which the blood cells expressed a high level of Luciferase.

Bioluminescence imaging

Mice were immobilized using an intra-peritoneal (i.p.) injection of Avertin (2,2,2-tribromoethanol; 240 mg kg−1 body weight), and then given an i.p. injection of the substrate, d-luciferin (214 μg g−1 body weight; Xenogen–Caliper Corp., Alameda, CA, USA). After 5 min, the mice were placed in the imaging chamber of the Xenogen in vivo imaging system (IVIS-100), which consists of a cooled CCD camera mounted in a light tight chamber, a camera refrigeration unit and a computer for data collection and analysis. A gray-scale image of the mouse was initially acquired by using a 10-cm field of view, a 0.2-s exposure time, an f/16 f/stop aperture and an open filter. Next, bioluminescence data were acquired. Mice were positioned supine to image the ventral surface or their right side to reveal the spleen. The acquisition time was 5 min, and bioluminescence data were acquired using two machine settings: high sensitivity (with maximum binning) and medium binning (with higher resolution but less sensitivity).

Analysis of bioluminescence data

Relative intensities of emitted light were presented as pseudocolor images ranging from red (most intense) to blue (least intense). Gray-scale photographs and the corresponding pseudocolor images were superimposed with Living Image (Xenogen–Caliper) and Igor (WaveMetrics, Lake Oswego, OR, USA) image analysis software. Signal emitted by regions of interest (ROI) was measured, and data were expressed as photon flux, quantified as photon s−1 cm−2 sr−1. Steradian (sr) refers to the photons emitted from a unit solid angle of a sphere. Data were represented as mean ± SEM. The machine background was subtracted electronically, both from the images and from the measurements of photon flux.

HSV-1 amplicon vector generation

Helper-free herpes simplex virus type 1 (HSV-1) amplicon vector that encodes the test antigen, ova, was described previously (7). HSV-1 amplicons are plasmid-based viral vectors that are packaged into HSV-1 capsids, but do not have viral coding sequences and are packaged using a helper-free packaging system (8). Amplicon stocks were titered as described previously (9). Viral pellets were re-suspended in PBS and stored at −80°C until use. Recipient C57BL/6J-TyrC2J/J Albino mice were inoculated with 1 × 106 expressing particles. HSV amplicon expressing ova (termed: HSVova) by intramuscular (i.m.) injection in the right quadriceps femoris, 24 h before adoptive transfer of CD8+ T cells from hCD2-Luc/OT-1 double-transgenic mice. In some experiments, we used a control HSV amplicon vector expressing β-galactosidase instead of ova (termed: HSVlac).

Adoptive transfer and flow cytometric analysis

Spleen and peripheral lymph node cells were isolated from hCD2-Luc/OT-1 mice on a CD45.1 background. RBCs were depleted using Lympholyte®-M (Cedarlane Laboratories, Ontario, Canada). Leukocytes were pooled from lymph nodes and spleen single-cell suspensions and CD8+ T cells were enriched by depletion of non-CD8+ T cells using MACS (Miltenyi Biotech, Auburn, CA, USA). In most experiment, 5 × 106 of the hCD2-Luc/OT-1 cells (>90% pure) were injected intravenously (i.v.) into recipient C57BL/6J-TyrC2J/J Albino mice. The OT-1 T cells were identified based on expression of the allotypic marker CD45.1, TCR Vα2 and CD8, using a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA); all antibodies were obtained from BD PharMingen (San Diego, CA, USA). For cell-surface staining, single-cell suspensions were incubated with antibodies in HBSS containing 5% fetal bovine serum (Cellgro, Mediatech Inc, Herndon, VA) for 20 min on ice. Subsequently, cells were washed with PBS and fixed with 1% PFA. Data were analyzed using CellQuest software (Becton Dickinson).

Statistical analysis

Data were pooled from multiple experiments and expressed as mean ± SEM. The Student’s t-test (paired) was used to measure the significance of differences observed between the experimental groups.
Results and discussion

HSV-based amplicon vectors are a promising avenue for vaccination. The vectors carry a maximum genetic payload of ~130 kb, and can transduce a variety of tissues including neural cells and muscle (10). The vectors commonly generate strong T cell immunity and memory, and we therefore used the immune response against ova encoded by an HSV amplicon vector to reveal the shifting patterns of localization of ova-specific T cells. In this vector, expression of the ova gene was driven by the HSV-derived immediate-early 4/5 promoters (Fig. 1A). Antigen-specific, Luciferase-expressing T cells were obtained by crossing the OT-1 line of TCR transgenic mice (11) with a novel line of mice in which Luciferase is expressed under the control of the human CD2 promoter (to be described in detail elsewhere, K. Dugger et al., in preparation). The double-transgenic T cell donors are termed hCD2-Luc/OT-1. These T cells were injected i.v. into congenic Albino B6 recipients.

Mice were injected with the HSVova in the quadriceps femoris muscle, and then 1 day later given an i.v. injection of hCD2-Luciferase transgenic, OT-1 transgenic T cells. At various time intervals, mice were anesthetized, given an injection of the luciferin substrate and visualized using a Xenogen IVIS imager. Figure 1(B) shows four mice, which from left to right were as follows: a mouse given HSVova i.m. and imaged at 2 days; a mouse given HSVova i.m. and imaged at 5 days; a mouse given the control HSVlac vector i.m. and imaged at 5 days and a mouse given an i.m. injection of PBS. All mice were given hCD2-Luc/OT-1 cells i.v. on day 0 and luciferin substrate immediately prior to imaging. In the mouse on the left, ellipses defining ROI were positioned around the brightly luminescent inguinal lymph node (rostral) and injection site (caudal). In the next mouse, also given HSVova, a marker enclosing the entire abdominal region was used to define the total bioluminescent signal from intra-abdominal structures. The same marker was used to measure the much smaller signal from the HSVlac control mouse. All three markers were positioned to detect the faint signals from the PBS control mouse. The false-color images from the HSVova mice show the intensity of photons emitted from the injection site in the left quadriceps femoris, or the inguinal lymph node, or several ‘hot spots’ in the abdomen. In contrast, both the HSVlac control and the PBS control mice showed faint signals from the cervical lymph nodes, inguinal nodes and the abdomen, but there was no signal from the injection site.

ROI could be defined, and the photon flux measured using proprietary Living Image software. Thus, in the HSVova mouse shown in Fig. 1(B), on the left, the injection site was emitting $1.782 \times 10^5$ photons s$^{-1}$ cm$^{-2}$ sr$^{-1}$ while the central abdominal region was emitting $1.324 \times 10^7$ photons s$^{-1}$ cm$^{-2}$ sr$^{-1}$. Elliptical ROI, similar in size to those used in Fig. 1(B) and located either around bioluminescent structures (the inguinal nodes or the injection site) or spanning the abdominal region from the sternal notch to the base of the tail and across the full width of the mouse, were used to harvest numerical data throughout this study. In some experiments, we used PBS as the control and in others, the HSVlac control vector.

Since the Luciferase-based imaging technique was non-destructive, it was possible to visualize the double-transgenic T cells in the same mice at different time points. Figure 2 shows a series of images of an HSVova mouse (left) and

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Fig. 1. (A) HSV amplicon vectors structure (modification from Bowers et al, reference 9). The amplicon consisted of a eukaryotic expression plasmid modified by the addition of an HSV origin of replication (ori) and a cleavage/packaging sequence. (B) Living Image software displays a false-color map of photon emission and allows the superimposition of elliptical regions, within which photons are measured.
a PBS control (right), both injected with $10^6$ hCD2-Luc/OT-1 T cells. On day 1 after adoptive transfer of the T cells, both mice revealed bioluminescence in the cervical lymph nodes and in inguinal lymph nodes. In addition, the HSVova mouse (left) had a detectable signal from the injection site on the mouse’s left hindlimb (at lower right on the image). On day 2, the strongest signal was from the injection site, but the signal from the local inguinal lymph node was already clearly stronger than from the other lymph nodes. On day 3, the signal was strongest in the local lymph node, but by day 5 was clearly localized over the injection site again. On day 7, foci of bioluminescence began to appear in the abdomen and by day 12 these were the strongest source of signal.

These data reveal two surprising features of the in vivo CD8$^+$ T cell response to the HSVova vector. First, while it is believed that the priming of naive T cells generally occurs in lymph nodes, we observed a clear signal from the injection site as early as 24 h after T cell transfer. Second, as the signal declined in the local, inguinal lymph node, the main focus of bioluminescence moved to the central abdominal region. The exact location of the signal varied between mice (data not shown), consistent with the hCD2-Luc/OT-1 T cells homing to anatomically mobile structures. One feature of the Living Image software is that the pseudocolor used to represent different bioluminescence intensities will ‘autoscale’, adjusting to the brightest pixel in the image. This results in apparently stronger signals in the control mice on day 1, before the immune response builds up in the HSVova experimental mouse on the left in each image, and on day 12, when the response has largely abated. The software allows the option to manually override this autoscaling feature; however, in a direct comparison of HSVova and control mice, this resulted in images where the experimental mouse was mostly crimson and anatomical details were lost. To obtain the most detailed anatomical information from both experimental and control groups, we imaged them separately, as in Fig. 7.

The capacity to measure the photon flux from the entire abdominal region (injection site, inguinal node plus central abdomen) allowed us to visualize the kinetics of this immune response and determine the effect of varying the number of precursor cells on the kinetics. Using $10^6$ hCD2-Luc/OT-1 T cells, we observed an exponential increase in bioluminescent signal with a peak on day 5, followed by a steady decline (Fig. 3, diamonds). When instead we employed $10^5$ or
The kinetics and magnitude of the responses were almost identical, with an increase to a plateau of bioluminescent signal spanning days 5, 7 and 9, and a decline thereafter (Fig. 3, triangles and closed circles, respectively). Mice that received $10^3$ hCD2-Luc/OT-1 T cells also made a detectable response, with a peak in the bioluminescent signal at day 7 followed by a slow decline. This shows that we can readily detect the immune responses of CD8$^+$ T cells, both a supraphysiological numbers ($10^6$–$10^4$) and at near-physiological numbers of precursors ($10^3$).

One interesting feature of this experiment was the effect of the number of input cells on the magnitude of the bioluminescent signal. Thus, $10^6$ T cells produced the largest signal, while $10^5$ and $10^4$ cells produced equivalent signals. The smallest cell number used, $10^3$ cells, gave rise to a smaller signal. We do not know the reason for this dose–effect relationship, but we can propose a speculative hypothesis. Thus, we consider it possible that $10^6$ OT-1 T cells function in a helper-independent manner, while smaller numbers do not. Therefore, in vivo $10^6$ OT-1 T cells are not limited by the availability of T cell help. With $10^5$ and $10^4$ OT-1 T cells, we speculate that CD4$^+$ T cells help is essential, but
it is limiting for the magnitude of the response; while CD8+ T cell help may not be limiting; instead the magnitude of the OT-1 T cell response may be limited by CD8+ T cell numbers. The analysis of such relationships may be an interesting use of bioluminescent imaging in the future.

The capacity to detect the immune response generated by 10^3 precursor cells or fewer is important, since the biological properties of the T cells we are using, derived from the OT-1 TCR transgenic mouse, exhibit different properties after adoptive transfer, depending on the cell number. Thus, in an immune response to a transgenic antigen expressed in the pancreas, these CD8+ T cells were helper-independent at high cell numbers, but below 5 x 10^5 cells per mouse revealed a need for CD4+ T cell help (12). Other cell number-dependent effects have been reported, though it is too soon to know whether these apply to all CD8+ T cells under all priming conditions (13). In our experiment, the PBS control mice that received 10^6 hCD2-Luc/OT-1 T cells emitted a background signal of -2 x 10^2 photons s^-1 cm^-2 sr^-1 (Fig. 3, open circles), while the peak signal from HSVOva mice that received 10^3 hCD2-Luc/OT-1 cells was nearly 2 x 10^6 photons s^-1 cm^-2 sr^-1, 10-fold higher. Since this signal is not clearly evident until day 7, this must represent many cycles of proliferation; we are not looking at 10^3 cells but at the product of their clonal expansion. On the basis that 10^3 input T cells elicit a 10-fold signal to noise ratio, we conclude it should be possible to image the distribution of expanded clones derived from <10^3 HSVOva-primed hCD2-Luc/OT-1 T cells, possibly from as few as 200. This number is significant, because estimates for the number of endogenous antigen-specific CD8+ T cells in a mouse are in this range (14).

To identify the abdominal structures responsible for the bioluminescent signal, a mouse was imaged under anesthesia and then dissected and re-imaged. Figure 4(a) shows the mouse with several strong intra-abdominal signals, as well as weak bilateral foci in the cervical region. Upon dissection, the cervical signals were confirmed to be cervical lymph nodes (Fig. 4b, marked 1). A bioluminescent signal was detected from the tip of the spleen (Fig. 4b, marked 2) but the strongest signals came from gut-associated structures. Thus, there was signal from the mesenteric milky spots (15) (Fig. 4b, marked 3), but the strongest signal arose from the mesenteric lymph node chain (Fig. 4b, marked 4). In addition, the deeply buried lumbar lymph nodes (Fig. 4b, marked 5) gave a discrete signal. A digital image of the dissected mouse is shown in Fig. 4(c) to identify the anatomic structures. To determine the correspondence between bioluminescent signal and traditional cell identification and enumeration by flow cytometry, hCD2-Luc/OT-1 T cells on a CD45.1 genetic background were injected into host mice that were CD45.2. At various time points, mice were imaged and the photon flux from the inguinal lymph node measured; these mice were subsequently sacrificed and the number of CD45.1+ OT-1 T cells in the lymph node was determined by flow cytometric analysis. Figure 5A shows that there was a -10-fold increase in the number of OT-1 T cells in the inguinal lymph node of HSVOva mice over PBS control mice.
on day 3, and this was also clearly the maximal time point for the bioluminescent signal. Both the cell count and the bioluminescent signal had decreased on day 7, and both were again less on day 12. Thus, in this superficial structure, there was good correspondence between the OT-1 T cell number determined by flow cytometry and the bioluminescent signal.

A similar analysis of the bioluminescent signal from the injection site and from the abdominal region using an ROI as defined in Fig. 1(B) showed that in both cases, the signal was maximal at day 5, with a steady decrease thereafter (Fig. 5B). In line with the appearance of the bioluminescent images themselves (Fig. 2), the loss of signal was less complete over the abdomen than at the injection site. To further analyze the basis for the abdominal signal, the mesenteric lymph node chain and the lumbar node were analyzed by flow cytometry at three time points. Unlike the local, draining inguinal node (Fig. 5A), the mesenteric and lumbar nodes (Fig. 5C) contained more OT-1 T cells at day 7 than at day 3, consistent with our interpretation that OT-1 T cells had redistributed from the local lymph node to the other lymphoid structures. In contrast to the inguinal lymph node, the spleen was difficult to image in the context of this response. With the mice imaged from the left side, the spleen was clearly outlined in both the HSVova experimental mouse (left) and the PBS control mouse (right) on day 1 (Fig. 6). Later in the response, the spleen sometimes gave a clear bioluminescent signal.

**Fig. 6.** Bioluminescence over the spleen in mice given HSV-OVA in the right quadriceps, then hCD2-Luc/OT-1 CD8+T cells. The left mouse in each image is an HSV-OVA mouse and the right mouse is a control. Mice were imaged with the left side uppermost to reveal the spleen. On day 1, the bioluminescent signal was comparable in the spleens of experimental and control mice. From day 2 onwards, the spleen of the experimental mouse was brightly bioluminescent, compared to control. The lateral image also reveals cervical lymph nodes, and the signals from the injection site and the abdomen.
signal distinct from that in the central abdomen (Fig. 6, the HSVova mice on days 3, 5 and 12). In contrast, at other times (Fig. 6, the HSVova mice on days 2 and 7), the spleen signal was either juxtaposed to the central abdominal signal or indistinct. This may have been because both the spleen itself and the brighter mesenteric lymph nodes are mobile structures; the exact disposition of the abdominal viscera during the 5-min imaging process may have revealed or obscured the discrete splenic signal.

The strong relocation of bioluminescent signal to the abdominal region led us to conclude that primed T cells in our HSVova model had different bio-distribution from unprimed cells. To directly test this, we represented the signal from hCD2-luc/OT-1 T cells in a mouse given $2.5 \times 10^5$ T cells and either control HSVlac vector or HSVova (Fig. 7). We imaged the experimental and control mice separately to avoid losing the weak signal from the control mice due to autoscaling. The scale bars, shown alongside each image, indicate the false-color spectra that were used. In the absence of antigenic stimulation (i.e. in the HSVlac-transduced mice), we observed bioluminescent signal mostly in the cervical lymph nodes and also the oral mucosa; notably, the abdomen was not emitting signal. In contrast, the anatomical distribution of bioluminescence from HSVova-primed cells included the cervical nodes but was mostly from intra-abdominal structures, as noted in other experiments. We conclude that priming does not simply alter the abundance of the hCD2-Luc/OT-1 T cells, but also modulates their homing properties.

**Concluding remarks**

This experiment reveals some unexpected features of the CD8+ T cell response to an HSV amplicon vector-encoding ova delivered into the quadriceps femoris muscle. We documented the strong recruitment of bioluminescent T cells to the injection site at 24 h, ahead of the maximal accumulation in the local inguinal lymph node. We also observed that toward the end of the primary response (days 7–12) the principal focus of bioluminescence was in the abdomen, due to homing of the T cells to mesenteric and lumbar lymph nodes. This study reveals the potential of bioluminescent imaging to reveal lymphocyte dynamics in vivo and to draw attention to cells localized to sites not always analyzed (such as the site of priming itself). Our cross-correlation of bioluminescent signal with cell counting reveals that bioluminescence is a good surrogate measure for cell count in the inguinal lymph node, due to its surface location and isolation from other lymphoid structures. However, issues of signal superimposition make imaging of the spleen unreliable. The newest generation of bioluminescent imaging instruments feature tomographic reconstruction of the source of the signal within the mouse; this may resolve this problem and allow non-destructive, quantitative imaging of all sites of immune activity. However, the current versions of three-dimensional imaging require longer scanning time and suffer from a decreased signal to noise ratio. Due to these limitations, larger cell numbers are needed in order to measure immunological process in vivo. However, this technology is developing rapidly and can be expected to converge with
in vivo microscopy to allow the precise imaging of small populations of responding T cells over time.

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**Abbreviations**

- APC: antigen-presenting cell
- hCD2-: double-transgenic mice expressing Luciferase under the human CD2 promoter and the ovalbumin-specific OT-1 transgenic TCR
- HSV: herpes simplex virus
- HSVlac: herpes simplex-based amplicon vector encoding β-galactosidase
- HSVova: herpes simplex-based amplicon vector-encoding ovalbumin
- HSV-1: herpes simplex virus type 1
- i.m.: intramuscular
- i.p.: intra-peritoneal
- i.v.: intravenously
- ova: ovalbumin
- ROI: regions of interest
- sr: steradian

**References**