

# Understanding Immune Cell Trafficking Patterns Via In Vivo Bioluminescence Imaging

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**Abstract** Cell migration is a key aspect of the development of the immune system and mediating an immune response. There is extensive and continual redistribution of cells to different anatomic sites throughout the body. These trafficking patterns control immune function, tissue regeneration, and host responses to insult. The ability to monitor the fate and function of cells, therefore, is imperative to both understanding the role of specific cells in disease processes and to devising rational therapeutic strategies. Determining the fate of immune cells and understanding the functional changes associated with migration and proliferation require effective means of obtaining in vivo measurements in the context of intact organ systems. A variety of imaging methods are available to provide structural information, such as X-ray CT and MRI, but only recently new tools have been developed that reveal cellular and molecular changes as they occur within living animals. We have pioneered one of these techniques that is based on the observations that light passes through mammalian tissues, and that luciferases can serve as internal biological sources of light in the living body. This method, called in vivo bioluminescence imaging, is a rapid and noninvasive functional imaging method that employs light-emitting reporters and external photon detection to follow biological processes in living animals in real time. This imaging strategy enables the studies of trafficking patterns for a variety of cell types in live animal models of human biology and disease. Using this approach we have elucidated the spatiotemporal trafficking patterns of lymphocytes within the body. In models of autoimmune disease we have used the migration of “pathogenic” immune cells to diseased tissues as a means to locally deliver and express therapeutic proteins. Similarly, we have determined the tempo of NK-T cell migration to neoplastic lesions and measured their life span in vivo. Using bioluminescence imaging individual groups of animals can be followed over time significantly reducing the number of animals per experiment, and improving the statistical significance of a study since changes in a given population can be studied over time. Such rapid assays that reveal cell fates in vivo will increase our basic understanding of the molecular signals that control these migratory pathways and will substantially speed up the development and evaluation of therapies. *J. Cell. Biochem. Supp.* 39: 239–248, 2002. © 2002 Wiley-Liss, Inc.

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Directed migration of immune cells to target organs and tissues is a key component of immune cell maturation and effective responses to insults. At all developmental stages of immune cell maturation, from the stem cells to the effector phase, movement throughout the body determines the nature of cellular function. Therefore, it is essential that we are able to reveal the spatiotemporal distribution of immune cells and the molecular signals that control specific migration patterns in the context of intact organs in the living body. Our ability to monitor the selective recruitment, time of arrival and departure of specific immune

cells in health and pathogenesis is imperative to both, understanding the role of specific cells in disease processes and to devising rational therapeutic strategies.

For decades, the migration of immune cells has been studied using a “black box”-like approach, where the body of the test subject is opaque to real time investigation. In these approaches blood and tissue samples are collected and analyzed by flow cytometric and histologic techniques at selected times after the initiation of an immunologic event. While flow cytometry is now a mainstay approach in counting transferred cells throughout the body, it does not provide information about the precise location of the analyzed cells within the organ. Conversely, microscopic analysis of tissue sections in combination with immunostaining methods allows for the analysis of spatial distribution of cells in the absence of significant temporal information. Each of these methods is subject to sampling limitations where data from selected time points and tissues may not adequately represent the immunologic event. However, valuable insights have been gained from “static” studies and they provide a solid basis for extending these analyses to those that access dynamic changes. Recent technological advancements have allowed more “dynamic” studies of immune orchestration and have given us new insights pertaining to dynamic molecular changes (e.g., at the immunological synapse [Krummel and Davis, 2002]), and immune cell interactions within a tissue. The latter analyses were conducted *in situ* by subjecting relevant tissues to intravital microscopy. True *in vivo* immune cell trafficking in live animals using whole body imaging modalities have also recently been described. In this review we will briefly discuss several recently developed imaging techniques that allow for the elucidation of dynamic immune cell migration patterns *in vivo*, and will primarily focus on the method of *in vivo* bioluminescence imaging (BLI).

### INTRAVITAL MICROSCOPY

Intravital microscopy (IVM) can be used to visualize cellular interactions in real-time as they occur in tissues that have been exposed for interrogation. Generally, experimental animals are anesthetized and the organ of interest is exposed surgically and positioned under a fixed stage intravital microscope. Subsequently, fluorescently labeled cells and/or contrast dyes

are injected intravenously and immune cell trafficking patterns are monitored shortly after transfer. Recently, transgenic mice have been generated that express reporter transgenes with optical signatures, such as enhanced green fluorescent protein (GFP), in selected lymphocyte populations, circumventing the *ex vivo* labeling step of investigated cells [Manjunath et al., 1999].

Two IVM approaches can be generally distinguished. The first is brightfield or epifluorescence intravital microscopy. This technique enables the study of immune cell interactions with micro vessels under real-time conditions at a single cell level and is extensively used to visualize lymphocyte trafficking from blood into tissues (reviewed by von Andrian and M’Rini (1998)). The second type of approach utilizes multi-photon or single-photon confocal illumination/detection IVM. Multi-photon laser scanning microscopy provides the ability to track fluorescently labeled cells over time within light scattering tissue to depths of several hundred microns ( $\mu\text{m}$ ) below the organ surface. The advantages of multi-photon IVM are that the problems of bleaching and phototoxicity associated with conventional fluorescence microscopy are largely avoided [Denk et al., 1990], while enabling the study of cell movement in as many as six dimensions by measuring time, space, fluorescent wavelength, and fluorescence intensity [von Andrian, 2002]. However, IVM methods are limited by the accessibility of the tissue, depth within the body, and are subject to motion artifacts due to respiration and cardiac function. IVM is an extremely useful tool for analyzing lymphocyte interactions and visualizing cell migration within living tissues. However, there remains a need for methods of rapid whole body analyses that are noninvasive and permit animals to be studied over the duration of days or weeks, which is not easily accomplished using IVM approaches.

### SINGLE-PHOTON EMISSION TOMOGRAPHY AND POSITRON EMISSION TOMOGRAPHY

The radionuclide-based imaging technologies of single-photon emission tomography and positron emission tomography (SPECT and PET) are sensitive modalities capable of detecting trace amounts of  $\gamma$  and  $\beta^+$ -emitting radionuclides, respectively, within the body. Thus, radioactive imaging modalities are well suited for tracking and mapping systemic biodistribution

of cells. In fact, single-photon imaging of leukocytes with  $^{111}\text{In}$  or  $^{99\text{m}}\text{Tc}$  is utilized clinically for the detection of occult infections and inflammatory sites (reviewed by Rennen et al. [2001]). The  $\gamma$ -emitters used for SPECT are generally more readily available and have longer half-lives ( $t_{1/2}$  hours to days) compared to the  $\beta^+$ -emitters ( $t_{1/2}$  minutes to days) that are used for PET. PET cameras allow electronic rather than mechanical collimation of incoming photons by recording the coincidence of simultaneous pairs of annihilation photons (511 keV per photon) at opposite detectors. Consequently, the sensitivity of PET (on the order of  $10^{-11}$ – $10^{-12}$  M) can be 1–2 orders of magnitude greater than SPECT systems (approximately  $10^{-10}$  M). The acquisition of higher count statistics is particularly valuable for detecting the fewest possible cells per unit volume with the least amount of radioactivity. The deep penetration of high-energy emission from radionuclides with direct application to clinical imaging are significant advantages of PET and SPECT, and three-dimensional reconstruction is often desirable, even for small laboratory animals. SPECT and PET-imaging systems have been described that are tailored specifically for the imaging of small laboratory animals [Chatziioannou et al., 2001; Tai et al., 2001; Blankenberg and Strauss, 2002].

The limitations of imaging radionuclide emission of exogenously labeled cells include the relatively short half-lives of some of the isotopes currently used for PET (e.g.,  $t_{1/2}^{18\text{F}} = 110$  min and  $t_{1/2}^{11\text{C}} = 20$  min), constraints in radiochemistry, high efflux of label from cells, as well as loss of signal due to dilution of the labeled compound after cell division. Cell labeling agents that have longer half-lives (such as  $^{64}\text{Cu}$ -PTSM) are currently being tested for cell tagging. As  $^{64}\text{Cu}$  has a reasonably long half-life ( $t_{1/2}^{64\text{Cu}} = 12.7$  h) labeled lymphocytes could be tracked for up to 20 h in living mice, which could potentially have utility in human imaging [Adonai et al., 2002].

To overcome the problem of dilution and the limitations due to labeling efficiency several reporter gene systems have been described that are designed to irreversibly “trap” radiolabeled compounds in the expressing cells (reviewed by Blasberg [2002]). Ponomarev et al. developed a retroviral vector expressing such a reporter gene (thymidine kinase from herpes simplex

virus; TK), which is under the control of an artificial nuclear factor of activated T cells (NFAT) sensitive promoter (Cis-NFAT/TK-GFP, GFP is expressed as a second reporter gene). This vector was then used to transduce a transformed human T cell line, and TCR-induced NFAT-mediated activation of subcutaneous Jurkat cell implants ( $5 \times 10^6$  cells in matrigel) could be monitored non-invasively by PET (using  $^{124}\text{I}$  FIAU as tracer) and fluorescent imaging (OPI) [Ponomarev et al., 2001].

### MAGNETIC RESONANCE IMAGING

Magnetic resonance imaging (MRI) has also been adapted for small animal models. MRI does not use high-energy radiation but relies on the atomic properties (nuclear resonance) of protons in tissues when they are scanned with radio frequency radiation. MRI provides detailed three-dimensional soft tissue images. The low sensitivity of MRI contrast agents, however, has hampered the use of MRI to efficiently monitor in vivo lymphocyte trafficking. The development of superparamagnetic particles (such as CLIO-particles (see Josephson et al. [1999]) to address the relatively poor sensitivity of MRI has enabled the detection of single cells and suspensions of cells by MRI. However, sufficient labeling of cells relies on the diffusion of contrast agent through the cell membrane, which is highly inefficient in most cell types. To overcome this limitation, a labeling method that uses particles coupled to HIV tat-peptide, as a transmembrane permeabilization agent, has been investigated [Lewin et al., 2000]. The results from this study indicated efficient uptake of particles into lymphocyte precursors. Dodd et al. [2001] have further demonstrated that this labeling method can be successfully used to follow migration of T cells in vivo. Analyses of several activation-, adhesion-, and homing-markers showed that the superparamagnetic particles did not interfere with normal T cell function and responsiveness. These studies suggest that superparamagnetic tat-derived particles may provide a feasible ex vivo method for labeling cells such that T cell homing events can be monitored in vivo. However, long-term responses cannot be monitored since the label is lost over time due to cell division. The use of this technique to label other immune cell subsets has yet to be evaluated.

## OPTICAL IMAGING USING BIOLUMINESCENCE

Optical imaging techniques take advantage of the fact that mammalian tissues, while relatively opaque, permit transmission of light in the visible and near infrared region of the spectrum. BLI is an optical imaging modality that is based on the fact that light emitted from internal biological sources can be detected externally using sensitive low-light imaging systems usually based on sensitive charge-coupled cameras (CCD). Reporter genes that encode bioluminescent enzymes (e.g., firefly luciferase (*luc*)) have been used as internal biological light sources and have been applied broadly to study laboratory animal models of human biology and disease [Contag et al., 1998; Burns et al., 2001; BitMansour et al., 2002; Contag and Ross, 2002; O'Connell-Rodwell et al., 2002].

In contrast to fluorescent reporter proteins (e.g., GFP and related proteins), luciferases are enzymes that produce light in the presence of substrate and thus do not require external excitation. Since the input for luciferase reactions are chemical substrates that do not produce a signal, there is essentially no background from the reporter. For this reason biochemical assays based on bioluminescence are extremely sensitive [Nicolas, 1994; Rice et al., 2001]. The high signal to noise ratio of bioluminescent assays is also apparent *in vivo*, since mammalian tissues do not emit significant amounts of light. Although a multitude of luciferases have been described, the best characterized, and broadly used luciferase is derived from the North American firefly, *Photinus Pyralis*. For the study of laboratory animals, the instrumentation required for BLI is relatively inexpensive and easy to operate compared to that used in other, non-optical, imaging modalities. Since BLI does not require the use of radionuclides, some of the precautions and expenses associated with nuclear medicine methods are avoided. The configuration of the lens and CCD detectors in most imaging systems used for BLI allow simultaneous whole body imaging of several mice, accelerating the study of multiple treatment groups. As noninvasive assays, such as BLI, allow single groups of animals to be followed over time, pretreatment data points can serve as internal controls, which can be used to improve the statistical significance of

the experimental design and reduce the number of animals required to achieve statistical significance.

## BIOLUMINESCENCE IMAGING

BLI is straightforward and can be performed by operators with a modest amount of training. Briefly, 15 min before imaging the animals are anesthetized and the substrate, luciferin, is injected intraperitoneally (i.p.) at a concentration of 150 mg/kg body weight. The substrate has been shown to distribute in saturation levels broadly to tissues throughout the body and can cross the blood–brain barrier [Rehemtulla et al., 2000] and placental barrier [Lipshutz et al., 2001]. The animal is then placed in a light-tight chamber equipped with a cooled CCD camera and appropriate lenses. A gray scale reference image of the study subject is acquired under weak illumination, and subsequently, an image of the bioluminescent emission is acquired in the absence of an external light source. A pseudocolor image representing light intensity is then superimposed over the reference image. Integration times depend on the strength of signal and can vary from less than 1 sec to 5 min. Discussions of technical details of BLI, such as optical properties of tissues, detector technologies, and new developments of reporter genes is beyond the scope of this article and have been recently reviewed elsewhere [Rice et al., 2001; Contag and Bachmann, 2002].

## SPATIOTEMPORAL MONITORING OF TARGET AND EFFECTOR CELLS—IMPLICATIONS FOR THE USE OF BLI IN DIAGNOSIS AND TREATMENT OF DISEASE

In regard to studies of *in vivo* cell trafficking, BLI generally employs reporter genes that are integrated into the genome and, therefore, are replicated with each cell division such that long-term studies can be conducted, even if reconstitution is attempted using small numbers of cells. Because of the relative ease of integrating reporter genes into tumor cell genomes, animal models of neoplastic diseases were among the first models where luciferase-based *in vivo* imaging was tested [Edinger et al., 1999; Sweeney et al., 1999; Rehemtulla et al., 2000]. When human HeLa cells were engineered to express luciferase their growth could be monitored both, externally and quantitatively after injection into immunodeficient SCID mice [Sweeney

et al., 1999]. The fact that as few as 1000 tumor cells were detectable in the peritoneal cavity indicated that BLI might be ideal for the detection and evaluation of minimal residual disease (MRD). In a study by Wetterwald et al. using a different mouse model (human mammary carcinoma, MDA-231), bone marrow metastases of  $\sim 0.5 \text{ cm}^3$  in volume (approximately  $2 \times 10^4$  cells) could be detected by BLI using an intensified CCD detector. At this size metastatic lesions elude radiographic imaging methods that rely on osteolysis for a detectable signature [Wetterwald et al., 2002]. This study further emphasized the utility of BLI for detecting small numbers of cells in living animals. Intensified CCD cameras are generally more sensitive in the blue region of the spectrum than other regions, and are therefore less efficient at collecting the more penetrating red light of the luciferase reaction. In the study by Wetterwald and coworkers, the authors may have reported greater sensitivity of detection had a cooled CCD detector been used—cooling preserves the relatively broad spectral sensitivity of the CCD.

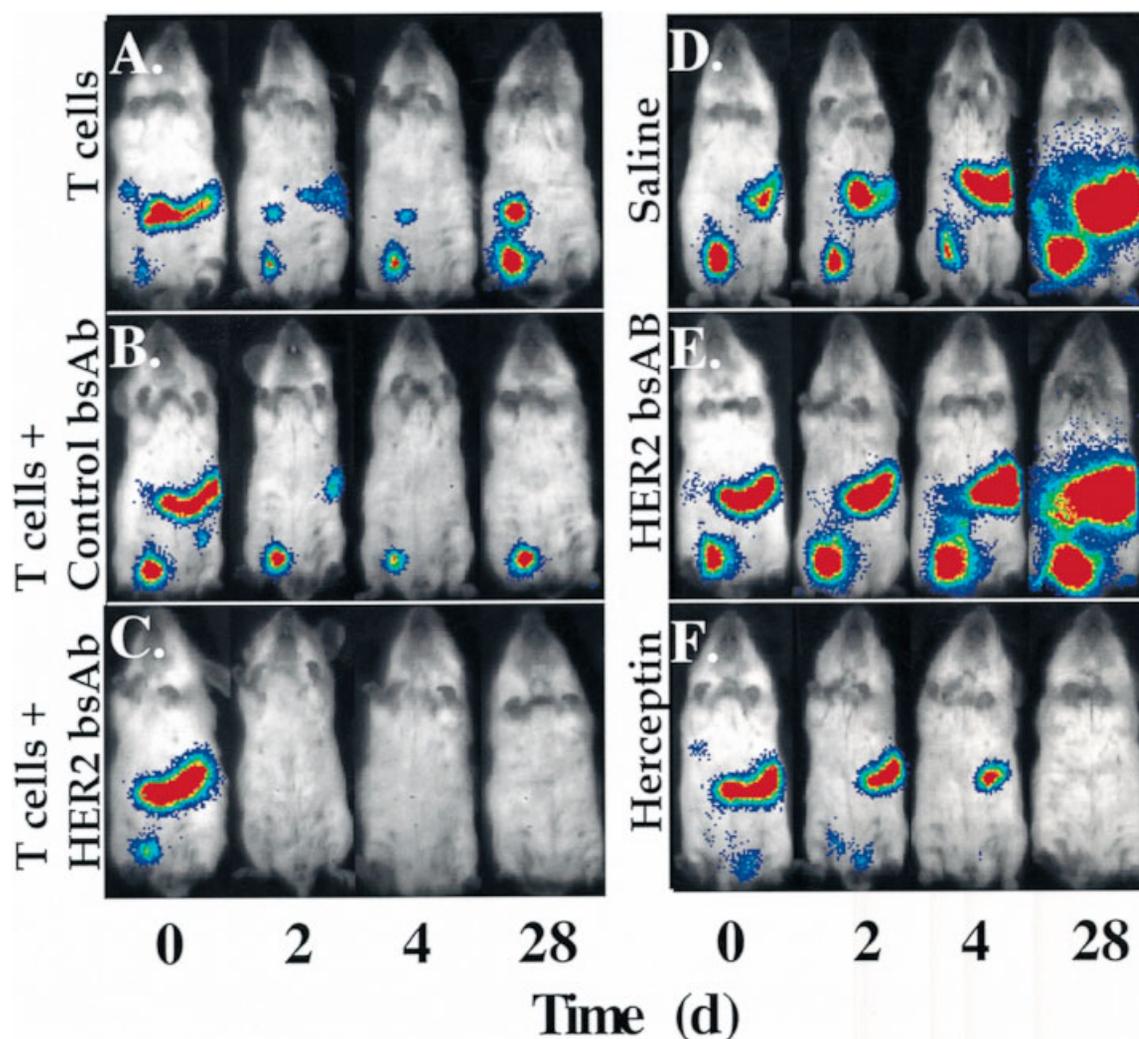
In animal models of malignancy, BLI has been shown to be useful for early detection of relapse and of micro-metastasis, and the *in vivo* data are useful for directing tissue sampling. Directed sampling of tissues for analysis by histological techniques allows the investigator to acquire samples with more information about distribution in the body. While most of the published reports using BLI have utilized tumor cells that were engineered *ex vivo* to express luciferase, other studies have used *in vivo* gene transfer as a means of identifying metastatic lesions. In a study by Adams and colleagues, a vector designed to express the firefly luciferase from an enhanced prostate-specific antigen promoter was used to transduce cells in the primary lesion *in vivo*. Imaging of animals with large primary subcutaneous lesions revealed detectable signal at sites that were distant from the primary lesion [Adams et al., 2002]. This demonstrated that it might be possible to locate metastatic lesions noninvasively by monitoring the distribution of signatures from reporter genes delivered *in vivo*.

Based on the sensitivity of detection observed in tumor models, BLI appeared to be a promising tool for the detection of circulating lymphocytes and other immune cell populations that may be present only in small numbers. Immune cell labeling and tracking to date has been con-

ducted using immune cell populations that can be expanded *ex vivo* and these have included the study of activated lymphocytes and antigen-specific hybridomas.

The effects of anti-cancer immunotherapy using *ex vivo* expanded and activated  $\text{CD3}^+\text{CD56}^+$  cells (Natural Killer-T cells; NK-T cells) were initially examined with BLI using labeled tumor targets. The cytokine enrichment protocol for obtaining these cells, referred to as cytokine induced killer cells (CIK cells), is based upon culturing mouse splenocytes or human peripheral mononuclear cells (PBMC) in the presence of  $\text{IFN-}\gamma$ , IL-2, and anti-CD3 monoclonal antibody. CIK cells show cytolytic activity against a broad array of tumor targets, including multidrug resistant cell lines and autologous fresh isolates both in culture and more importantly *in vivo* [Sweeney et al., 1999]. Sweeney et al. demonstrated that human CIK cells reduce tumor burden in mice inoculated with HeLa cells transfected with a modified firefly luciferase gene. There are some tumor targets that are not efficiently recognized by the CIK cells and Scheffold et al. have enhanced this recognition using a bispecific antibody,  $(\text{F}(\text{ab}')_2\text{HER2} \times \text{CD3})$ , which was designed to recognize markers on both the tumor (HER2 expressing ovarian carcinoma cell line transduced to express luciferase), and the effector CIK cells (Fig. 1). This study revealed that the cytotoxic activity of CIK cells could be substantially augmented in culture as well as *in vivo* using this approach [Scheffold et al., 2002]. Imaging the outcome of these studies in live mice allowed multi-parameter analyses despite the complexity of evaluating combination therapies that involve biological and cell-based therapies. A significant contribution of the recent developments in this field of *in vivo* cellular and molecular imaging may indeed be the ability to rapidly conduct studies on novel and complex therapeutic strategies using accurate *in vivo* readouts.

Understanding the distribution of the effector cells relative to the tumor target and normal organs would greatly enhance our ability to develop cell-based therapies. Therefore, Edinger and coworkers have labeled and tracked the CIK effector cells. Precursor cells were labeled using retroviral transduction of mouse splenocytes using a vector engineered with the GFP-Luc dual-function reporter gene (for review see Contag and Bachmann [2002]). These cells were

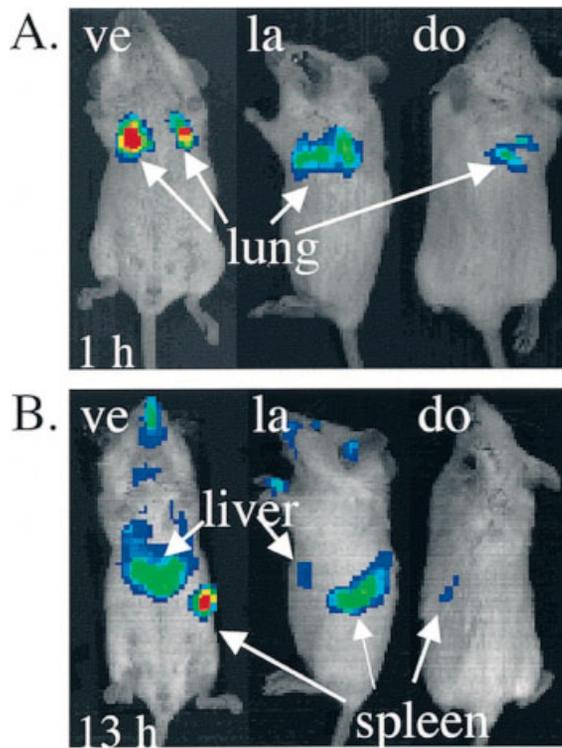


**Fig. 1.** Imaging of labeled tumor targets as a guide for the development of novel immune cell therapies. The ability of biospecific antibodies to redirect CIK cells to tumor targets was rapidly evaluated using BLI. The test group and five control groups were studied [Scheffold et al., 2002]. Luciferase-expressing SKOV3 cells were injected into SCID mice and the effects of six different treatment conditions on tumor growth

were evaluated. Groups of animals were given (A) NK-T cells alone, (B) NK-T cells along with a control bispecific antibody, (C) NK-T cells along with a bispecific antibody targeting HER2/NEU and CD3, (D) Saline only, (E) the control bispecific antibody alone, and (F) Herceptin. Signal reduction was observed in groups C and F, however the kinetics of signal reduction differed. [From *Cancer Res*, 62:5785–5791, 2002, with permission.]

then expanded under the CIK enrichment conditions. The dual function reporter genes coding for a fluorescent marker (e.g., GFP or yellow fluorescent protein (YFP)) and a luciferase [Day et al., 1998] allow both, *in vivo* imaging using bioluminescence and subsequent *ex vivo* sorting of labeled cells using the fluorescent marker [Costa et al., 2001]. In this study of immunotherapy with CIK cells, effector cell survival was monitored over time and the tissue distribution patterns were revealed relative to tumor eradication. As expected,  $luc^+$  CIK cells could be detected in the lungs early (30 min to 1 h) after intravenous injection (Fig. 2A). Sub-

sequently, CIK cells distributed to other sites in the body and could be clearly localized in the liver and spleen within the next 13 h (Fig. 2B). By 72 h, a clearly defined population of labeled effector cells infiltrated subcutaneous lesions of lymphoma cells and the signal was detectable at this site for an additional period of 12 days; during this time course the tumor completely regressed [Edinger et al., 2002]. The ability to visualize effector cell trafficking patterns in immunotherapies is a major advancement that will allow the rapid optimization of protocols and help to reveal some of the key molecular mechanisms of homing and tumor lysis that



**Fig. 2.** Trafficking patterns of NK-T cells. Labeled NK-T cells were injected into mice and the animals were imaged at (A) 1 h and (B) 13 h post injection. Cells migrated from the lungs (A) to the liver and spleen (B). The acquisition of multiple images in different positions, ventral (ve), lateral (la), and dorsal (do) reveals the need for three-dimensional representation of this type of data.

will lead to the development of more effective approaches to immunotherapy.

These experiments are not restricted to immunotherapy of neoplastic disease alone and can be used for understanding cell migration patterns in many physiological and pathophysiological conditions. This was demonstrated using CD4<sup>+</sup> T cell hybridoma and primary T cells specific for known peptide antigens, which were transduced with retroviral vectors that encoded the multifunctional reporter genes. The labeled cells were followed as they migrated to lymphatic tissues and were eventually retained at locations determined by their antigenic specificity. This was first demonstrated for lymphocyte trafficking in mouse models of human autoimmune diseases. Using a mouse arthritis model Nakajima and coworkers showed that T cell hybridomas and primary T cells from transgenic mice expressing a T cell receptor specific for a type II collagen peptide (CII), home to the inflamed joints. Selection of labeled cells was accomplished using the GFP signal and FACS, and the tempo of localization

of luc<sup>+</sup> T cells to the inflamed joints was observed by BLI [Nakajima et al., 2001]. Using a T cell hybridoma with a different specificity these studies also revealed that lymphocytes will nonspecifically migrate to sites of inflammation, but will only be retained in areas where they encounter “relevant” antigen. In another study the migratory patterns of auto-antigen-reactive CD4<sup>+</sup> T cells specific for myelin basic protein (MBP) were monitored in experimental autoimmune encephalitis (EAE), a mouse model for multiple sclerosis in humans. Understanding the trafficking patterns of immune cells is clearly crucial to revealing disease mechanisms, however, this information can also be used to optimize immune cell therapies by using tissue-specific immune cell trafficking as a “Trojan horse” to deliver therapeutic molecules. This approach has successfully been used to reduce clinical disease in mice in collagen-induced arthritis (CIA) [Nakajima et al., 2001] and EAE models [Costa et al., 2001]. In both studies GFP/luc expressing antigen-specific T cell hybridomas were retrovirally transduced to express the p40 subunit of interleukin-12 (IL-12 p40). IL-12 p40 acts as a Th1 cytokine antagonist and the local expression of this molecule using adoptive transfer of transduced antigen-specific CD4<sup>+</sup> T cells prevented disease development. In vivo tracking of the bioluminescent lymphocytes demonstrated that the induced responses were specific in that they required the expression of the immune modulatory molecule as well as the appropriate antigen-specific T cell receptor. At the termination of the study, the fluorescent moiety encoded in the vector-enabled confirmation of tissue localization of labeled cells using fluorescent microscopy. The use of antigen-dependent homing for the delivery of local gene therapy is an exciting new approach for the treatment of autoimmune diseases (reviewed by Tarner and Fathman [2002], but understanding the trafficking patterns of the genetically engineered cells is imperative to the successful development of this type of immunotherapy.

Because of the extremely high sensitivity, BLI is the method of choice in understanding and detecting very small numbers of cells in rodent models, and virtually all cells of the immune system can be engineered to express luciferase. Recently, we have transduced ex vivo expanded dendritic cells (DC) to express GFP and luciferase. Subsequently, the labeled DCs

were injected i.v. into mice that had received an allogeneic bone marrow transplant (BMT). Early data indicate, that these cells homed to different organs and could so far be followed for at least 42 days. (Schimmelpfennig and coworker, ASH abstract: #1585, Vol. 100, p 408.). Despite the potential for this technology, ex vivo labeling of primary cells can be inefficient, and thus we have initiated a program to generate transgenic mice as sources of labeled cells for trafficking studies [Contag and Bachmann, 2002]. Such approaches will greatly simplify the study of cell trafficking and engraftment, especially as related to stem cell biology.

The ability to follow hematopoietic stem cells with optical reporter genes will greatly facilitate the examination of trafficking and expansion of precursor cells after bone marrow transplantation, providing unprecedented possibilities for analysis. Furthermore, the use of knock out-animals lacking molecules important for immune cell function (e.g., cytokines, chemokines, or adhesion molecules) both, as donors and recipients of bioluminescent lymphocytes will reveal key molecular mechanisms of normal lymphocyte function and dysregulation, especially in regard to their influence on lymphocyte homing and proliferation. Bridging migration studies with activatable luciferase proteins will add functional information to the spatiotemporal resolution of BLI. Such modified luciferase genes that encode silenced enzymes can be activated by proteases and will be useful in dissecting pathways of cell migration and programmed cell death. Functional information could also easily be acquired by placing the luciferase gene under the control of promoters of T cell activation or of effector functions (e.g., NFAT or JAK-STATs, granzymes). Additionally, the development of luciferases with different emission spectra, and different chemistries, as reporters might soon make it possible to track two or more different cell populations in the same animal [Bhaumik and Gambhir, 2002]. Further improvements include development of signal amplification strategies, luciferase-based sensors for caspase activity and protein-protein interactions [Ray et al., 2002] as well as advancements in hardware and software for BLI, which may allow for reconstruction of three-dimensional images. The data in Figure 2 indicate the resolution that is currently possible with planer images. The dorsal, ventral, and lateral views were obtained in this study to

reveal the potential of multiview data sets—note the three views of the spleen at the later time point. Three-dimensional reconstruction of data sets from animals such as these would be beneficial for understanding the distribution of labeled cells.

## CONSIDERATIONS

Several factors have to be taken in consideration when using reporter genes. One concern is that the overexpressed gene could be potentially toxic to the cell or could be immunogenic. In both cases, this would result in the elimination of the labeled cell. Immunogenicity of GFP has been described previously [Stripecke et al., 1999; Gambotto et al., 2000]. In our hands, however, we have not yet detected any enhanced toxicity or immune recognition of labeled cells but more thorough studies are needed. A second concern is the effect of gene integration into the genome. Depending on the promoter used for transgene expression and positional effects of transgene insertion, this can lead to differential gene expression as compared to the parental cell. Therefore, potential altered outcomes due to gene expression effects have to be analyzed through comparisons to unlabeled cells. In addition, using populations of cells with multiple and random integration sites of the reporter construct circumvent the problems associated with using a clonal population with a single integration site.

## CONCLUSIONS

These experiments are only the foundation for a bright future of BLI and immune cell trafficking. Given the high sensitivity of in vivo cell detection with BLI combined with the molecular tools available to date, BLI offers tremendous opportunities for the cell scientist and immunologist to understand cell function in the complex environment of intact organ systems and circulation in the living animal. Imaging strategies in general will provide invaluable insight into immune cell trafficking under normal circumstances as well as in models of autoimmunity, tumor immunology, transplantation biology, and infectious diseases.

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## REFERENCES

- Adams JY, Johnson M, Sato M, Berger F, Gambhir SS, Carey M, Iruela-Arispe ML, Wu L. 2002. Visualization of advanced human prostate cancer lesions in living mice by a targeted gene transfer vector and optical imaging. *Nat Med* 8:891–897.
- Adonai N, Nguyen KN, Walsh J, Iyer M, Toyokuni T, Phelps ME, McCarthy T, McCarthy DW, Gambhir SS. 2002. Ex vivo cell labeling with  $^{64}\text{Cu}$ -pyruvaldehyde-bis(N4-methylthiosemicarbazone) for imaging cell trafficking in mice with positron-emission tomography. *Proc Natl Acad Sci USA* 99:3030–3035.
- Bhaumik S, Gambhir SS. 2002. Optical imaging of Renilla luciferase reporter gene expression in living mice. *Proc Natl Acad Sci USA* 99:377–382.
- BitMansour A, Burns SM, Traver D, Akashi K, Contag CH, Weissman IL, Brown JM. 2002. Myeloid progenitors protect against invasive aspergillosis and *Pseudomonas aeruginosa* infection following hematopoietic stem cell transplantation. *Blood* 100(13):4660–4667.
- Blankenberg FG, Strauss HW. 2002. Nuclear medicine applications in molecular imaging. *J Magn Reson Imaging* 16:352–361.
- Blasberg R. 2002. Imaging gene expression and endogenous molecular processes: Molecular imaging. *J Cereb Blood Flow Metab* 22:1157–1164.
- Burns SM, Joh D, Francis KP, Shortliffe LD, Gruber CA, Contag PR, Contag CH. 2001. Revealing the spatiotemporal patterns of bacterial infectious diseases using bioluminescent pathogens and whole body imaging. *Contrib Microbiol* 9:71–88.
- Chatziioannou A, Tai YC, Doshi N, Cherry SR. 2001. Detector development for microPET II: A 1 microl resolution PET scanner for small animal imaging. *Phys Med Biol* 46:2899–2910.
- Contag CH, Bachmann MH. 2002. Advances in in vivo bioluminescence imaging of gene expression. *Annu Rev Biomed Eng* 4:235–260.
- Contag CH, Ross BD. 2002. It's not just about anatomy: In vivo bioluminescence imaging as an eyepiece into biology. *J Magn Reson Imaging* 16:378–387.
- Contag PR, Olomu IN, Stevenson DK, Contag CH. 1998. Bioluminescent indicators in living mammals. *Nat Med* 4:245–247.
- Costa GL, Sandora MR, Nakajima A, Nguyen EV, Taylor-Edwards C, Slavin AJ, Contag CH, Fathman CG, Benson JM. 2001. Adoptive immunotherapy of experimental autoimmune encephalomyelitis via T cell delivery of the IL-12 p40 subunit. *J Immunol* 167:2379–2387.
- Day RN, Kawecki M, Berry D. 1998. Dual-function reporter protein for analysis of gene expression in living cells. *Biotechniques* Nov 25:848–850, 852–854, 856.
- Denk W, Strickler JH, Webb WW. 1990. Two-photon laser scanning fluorescence microscopy. *Science* 248:73–76.
- Dodd CH, Hsu HC, Chu WJ, Yang P, Zhang HG, Mountz JD, Jr. Zinn K, Forder J, Josephson L, Weissleder R, Mountz JM, Mountz JD. 2001. Normal T-cell response and in vivo magnetic resonance imaging of T cells loaded with HIV transactivator-peptide-derived superparamagnetic nanoparticles. *J Immunol Methods* 256:89–105.
- Edinger M, Sweeney TJ, Tucker AA, Olomu AB, Negrin RS, Contag CH. 1999. Noninvasive assessment of tumor cell proliferation in animal models. *Neoplasia* 1:303–310.
- Edinger M, Cao YA, Verneris MR, Bachmann MH, Contag CH, Negrin RS. 2002. Revealing lymphoma growth and the efficacy of immune cell therapies using in vivo bioluminescence imaging. *Blood*.
- Gambotto A, Dworacki G, Cicinnati V, Kenniston T, Steitz J, Tuting T, Robbins PD, DeLeo AB. 2000. Immunogenicity of enhanced green fluorescent protein (EGFP) in BALB/c mice: Identification of an H2-Kd-restricted CTL epitope. *Gene Ther* 7:2036–2040.
- Josephson L, Tung CH, Moore A, Weissleder R. 1999. High-efficiency intracellular magnetic labeling with novel superparamagnetic-Tat peptide conjugates. *Bioconjug Chem* 10:186–191.
- Krummel MF, Davis MM. 2002. Dynamics of the immunological synapse: Finding, establishing and solidifying a connection. *Curr Opin Immunol* 14:66–74.
- Lewin M, Carlesso N, Tung CH, Tang XW, Cory D, Scadden DT, Weissleder R. 2000. Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. *Nat Biotechnol* 18:410–414.
- Lipshutz GS, Gruber CA, Cao Y, Hardy J, Contag CH, Gaensler KM. 2001. In utero delivery of adeno-associated viral vectors: Intraperitoneal gene transfer produces long-term expression. *Mol Ther* 3:284–292.
- Manjunath N, Shankar P, Stockton B, Dubey PD, Lieberman J, von Andrian UH. 1999. A transgenic mouse model to analyze CD8(+) effector T cell differentiation in vivo. *Proc Natl Acad Sci USA* 96:13932–13937.
- Nakajima A, Seroogy CM, Sandora MR, Tarner IH, Costa GL, Taylor-Edwards C, Bachmann MH, Contag CH, Fathman CG. 2001. Antigen-specific T cell-mediated gene therapy in collagen-induced arthritis. *J Clin Invest* 107:1293–1301.
- Nicolas JC. 1994. Applications of low-light imaging to life sciences. *J Biolumin Chemilumin* 9:139–144.
- O'Connell-Rodwell CE, Burns SM, Bachmann MH, Contag CH. 2002. Bioluminescent indicators for in vivo measurements of gene expression. *Trends Biotechnol* 20:S19–S23.
- Ponomarev V, Doubrovin M, Lyddane C, Beresten T, Balatoni J, Bornman W, Finn R, Akhurst T, Larson S, Blasberg R, Sadelain M, Tjuvajev JG. 2001. Imaging TCR-dependent NFAT-mediated T-cell activation with positron emission tomography in vivo. *Neoplasia* 3:480–488.
- Ray P, Pimenta H, Paulmurugan R, Berger F, Phelps ME, Iyer M, Gambhir SS. 2002. Noninvasive quantitative imaging of protein-protein interactions in living subjects. *Proc Natl Acad Sci USA* 99:3105–3110.
- Rehemtulla A, Stegman LD, Cardozo SJ, Gupta S, Hall DE, Contag CH, Ross BD. 2000. Rapid and quantitative assessment of cancer treatment response using in vivo bioluminescence imaging. *Neoplasia* 2:491–495.
- Rennen HJ, Corstens FH, Oyen WJ, Boerman OC. 2001. New concepts in infection/inflammation imaging. *Q J Nucl Med* 45:167–173.
- Rice BW, Cable MD, Nelson MB. 2001. In vivo imaging of light-emitting probes. *J Biomed Opt* 6:432–440.

- Scheffold C, Kornacker M, Scheffold YC, Contag CH, Negrin RS. 2002. Visualization of effective tumor targeting by CD8+ natural killer T cells redirected with bispecific antibody F(ab')(2)HER2xCD3. *Cancer Res* 62:5785–5791.
- Stripecke R, Carmen Villacres M, Skelton D, Satake N, Halene S, Kohn D. 1999. Immune response to green fluorescent protein: Implications for gene therapy. *Gene Ther* 6:1305–1312.
- Sweeney TJ, Mailander V, Tucker AA, Olomu AB, Zhang W, Cao Y, Negrin RS, Contag CH. 1999. Visualizing the kinetics of tumor-cell clearance in living animals. *Proc Natl Acad Sci USA* 96:12044–12049.
- Tai C, Chatziioannou A, Siegel S, Young J, Newport D, Goble RN, Nutt RE, Cherry SR. 2001. Performance evaluation of the microPET P4: A PET system dedicated to animal imaging. *Phys Med Biol* 46:1845–1862.
- Tarner I, Fathman C. 2002. The potential for gene therapy in the treatment of autoimmune disease. *Clin Immunol* 104:204.
- von Andrian UH. 2002. Immunology. T cell activation in six dimensions. *Science* 296:1815–1817.
- von Andrian UH, M'Rini C. 1998. In situ analysis of lymphocyte migration to lymph nodes. *Cell Adhes Commun* 6:85–96.
- Wetterwald A, van der Pluijm G, Que I, Sijmons B, Buijs J, Karperien M, Lowik CW, Gautschi E, Thalmann GN, Cecchini MG. 2002. Optical imaging of cancer metastasis to bone marrow: A mouse model of minimal residual disease. *Am J Pathol* 160:1143–1153.