Immunity
Commentary

Veni, Vidi, Vici: In Vivo Molecular Imaging of Immune Response

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"I came, I saw, I conquered," Julius Caesar proclaimed, highlighting the importance of direct visualization as a winning strategy. Continuing the "From the Field" series (see Editorial [2007] *26*, 131), Gross et al. summarize how modern molecular imaging techniques can successfully dissect the complexities of immune response in vivo.

In Vivo Imaging and Immunology: A Perfect Match

The vertebrate immune system is extremely complex and highly dynamic. This complexity provides the basis for its capacity to recognize, respond, and remember pathogenic assaults, thereby providing the host with remarkable surveillance and protection mechanisms in an exogenously and endogenously hostile environment (i.e., pathogenic infections and malignant transformations, respectively). The vast majority of cells comprising our immune system are motile, migrating between the bone marrow (BM), bloodstream, secondary lymphoid organs, and affected tissues or organs. In addition, immune cells undergo tightly regulated, interaction-dependent activities (clonal expansion and depletion, transdifferentiation, and chemotaxis) that are aimed at the production, maintenance, and resolution of a proper immune reaction.

Surprisingly, knowledge of the immune system has largely originated from static endpoint experiments that provide "snapshots" of this complex activity (e.g., cytometry, histology, and solution biochemistry) (Germain et al., 2006; Negrin and Contag, 2006). Although important information has been gathered from these types of experiments, detailed and accurate studies of the regulatory dynamics of the immune system in real-time are lacking and require the capacity to temporally and spatially resolve specific immune reactions within an intact animal. Furthermore, until not long ago, our understanding of the factors that regulate migration and trafficking of immune cells had been obtained from cultured cells, likely under-representing the influences of the complex milieu in which immune reactions take place in vivo (e.g., transendothelial trafficking, endocrine and paracrine regulation, stromal interactions, circulation, oxidative state, etc.). Recent advances in cellular and molecular biology combined with noninvasive imaging technologies and strategies hold great promise for providing immunologists with new insights into the systemic regulation and dynamics of the immune machinery in both health and disease.

Brief Overview of Imaging Modalities and Strategies

Historically, radiology and imaging sciences were developed for diagnostic purposes, providing the clinical practitioner with an eyepiece to noninvasively detect anatomical disorders. However, today's imaging strategies play a much broader role and are applied to basic research paradigms, discovery, development and optimization of novel therapeutics, as well as to translational research and clinical diagnostics (Gross and Piwnica-Worms, 2005b; Tsien, 2003). Development of novel probes, genetically encoded reporters, and biomarkers extend the ability to track a particular biological event (molecular, cellular, or physiological, not only anatomical) by means of noninvasive imaging and open a whole new arena to investigate complex biological processes in vivo.

Imaging modalities provide valuable information that differ in temporal (milliseconds to hours) and spatial (microns to centimeters) resolution, as well as in other technical aspects (tissue penetration, biochemical sensitivity, signal-to-noise, tomographic [cross-sectional] potential, throughput, cost, ease of operation, and clinical translatability). Therefore, experiments should be carefully designed to best match the biological question of interest with the appropriate technology. The various noninvasive molecular imaging modalities can be categorized as optical, nuclear, and magnetic resonance imaging (MRI), each of which can be combined together in fusion imaging strategies or combined with X-ray computed tomography (CT) or ultrasound imaging (i.e., multimodal imaging) to merge molecular function, sensitivity, and spatial resolution. In brief, macroscopic optical modalities (whole-body bioluminescence or fluorescence imaging) exhibit excellent biochemical sensitivity and throughput and allow enzyme-mediated probe activation strategies and multicolor multiplexing, but show limited spatial resolution. Microscopic optical modalities (e.g., intravital two-photon microscopy, intravital fluorescence microscopy) can provide extraordinary spatial resolution (single cell, micron) and temporal resolution (subsecond rate constants), but can be somewhat invasive, limited to superficial structures, demand highly sophisticated instrumentation and qualified personnel, and are highly susceptible to motion

artifacts. Nuclear modalities, such as positron emission tomography (PET) and single-photon emission computed tomography (SPECT), offer exceptional biochemical sensitivity (i.e., the capacity to detect picomolar concentrations of target upon injection of minimal amounts of radiotracer), are quantitative and inherently tomographic, but suffer from limited spatiotemporal resolution and generally require on-site synthesis of radiolabeled tracers with the need for committed personnel. MRI offers multiplanar tomographic display, high spatial resolution at high magnetic fields, and (like nuclear modalities) can be directly translated to clinical applications, but is hindered by low sensitivity to biochemical and molecular processes, low throughput, and high cost. Commonly, instruments scaled for animal studies (microPET, nanoSPECT, microCT, etc.) are generally available to investigators at major research institutions. The advantages, limitations, technical challenges, and enhancements under investigation for each of the various imaging modalities are addressed in several in-depth reviews (Gross and Piwnica-Worms, 2005b; Negrin and Contag, 2006; Tsien, 2003) and are summarized in Table 1. Herein, we will briefly review examples of molecular imaging strategies, focusing on injectable probes and genetically encoded reporters in the context of immunological paradigms, with the intent to inspire future innovations to visualize and conquer open questions in immunology.

Imaging Innate Immunity

Innate immune defenses consist of cellular (leukocyte) mechanisms as well as chemical and humoral (inflammation and complement system) responses, each of which present viable targets for imaging. Genetic or chemical labeling of leukocytes (in vivo or ex vivo), followed by transfer to recipient animals, allows the dynamic monitoring of trafficking and accumulation of leukocytes into inflammatory sites. For example, Swirski and colleagues used fluorescence and nuclear imaging techniques to monitor infiltration of monocytes at atherogenic foci by adaptive transfer of monocytes from GFP-expressing mice and by ex vivo labeling with ^{111}In-oxine, respectively (Swirski et al., 2006). In another example, Nakamichi et al. transferred Mac1^{+} macrophages from luciferase pan-expressing mice to hemin-treated recipients and demonstrated that hemin activates heme-oxygenase 1 (HO-1) in peritoneal macrophages, thereby inducing macrophage recruitment to the pancreas and conferring protection against pancreatitis (Nakamichi et al., 2005).

Imaging strategies can also resolve inflammation-associated enzyme activities in vivo at various spatial scales depending on the modality and probe design. One prototypic design involves activatable optical imaging agents, which typically exist in a basal "off" state mediated by resonance energy transfer between a donor fluorophore and an acceptor, resulting in inter- or intramolecular quenching. If an enzyme (protease) acts on a cleavable moiety synthesized between the donor and acceptor, release of the fluorophore "signals" the presence of the target enzyme. These activation strategies (and an analogous process with MRI contrast agents) can theoretically reduce background noise and produce sensitive and specific images of enzyme activities in vivo. For example, in a recent study, Jaffer and colleagues imaged elastolytic activity of the extracellular cysteine protease cathepsin K (CatK) in an atheroma inflammation model by using a near-infrared fluorescence (NIRF) imaging agent consisting of the CatK peptide substrate GHPGGPQGKC-NH2 linked to an activatable fluorogenic polymer (Jaffer et al., 2007). As applied in this model, multiwavelength intravital fluorescence microscopy allowed detection of CatK activity and a spectrally resolved intravascular agent within intact exposed atherosclerotic vessels at submillimeter resolution. Similar strategies with fluorescently labeled, cell-permeable activatable peptides were also employed in whole-body fluorescence imaging to monitor regional caspase-3 activity during parasite-induced apoptosis in colon xenograft and liver abscess mouse models (Bullok et al., 2007). In another innovative example, Chen et al. were able to image by MRI at subcentimeter resolution LPS-induced myeloperoxidase activity in a murine myositis model by low-molecular-weight probes that polymerized upon peroxidation. This in vivo polymerization strategy increased paramagnetic relaxivity and enhanced protein binding to generate MRI contrast in response to acute inflammation (Chen et al., 2006).

Another viable strategy is to label, inject, and image chemotactic and chemokinetic compounds that are expected to accumulate at sites of inflammation. These bioactive substances include cytokines, prostaglandins, leukotrienes, or synthetic antagonists or partial agonists of such ligands. For example, it was demonstrated that scintigraphic imaging after intravenous administration of an ^{111}In- or ^{99m}Tc-labeled leukotriene B4 antagonist reveals acute infectious and inflammatory lesions in various rabbit models (Figure 1A; van Eerd et al., 2004). In another recent study, Kaufmann et al. utilized contrast-enhanced ultrasonic imaging to visualize microbubbles conjugated to monoclonal antibodies directed against vascular cell adhesion molecule-1 (VCAM-1) to noninvasively detect and quantify inflammatory, VCAM-1-positive atherosclerotic plaques in mice (Kaufmann et al., 2007).

Noninvasive imaging also allows study of gross physiological changes associated with inflammatory processes. For example, MRI can detect regional changes in water diffusion as a result of inflammation-induced edema (Lazovic et al., 2005), and ^{18}F-deoxyglucose (FDG) PET can point to regions with cellular infiltrates and increased metabolic activities. Indeed, although nonspecific, FDG PET is clinically sensitive and useful for localizing sources of fever of unknown origin (FUO), monitoring progression and response to treatment in cases of sarcoidosis and vasculitis, and diagnosing osteomyelitis (for a review, see Love et al., 2005).

Imaging Adaptive Immunity

Molecular imaging offers powerful strategies to investigate complex activities mediated by the adaptive immune system, sometimes providing

Table 1. Brief Comparison of Various Noninvasive Imaging Modalities Useful for Immunological Studies In Vivo

	Spatial Resolution	Temporal Resolution	Biochemical Sensitivity	Tomographic Potential	Biochemical S/N	Background/ Noise Sources	Cost	Clinical Translatability
Fluorescence (microscopic)	micron	ms-min	++++	yes, but for less than 1 mm depth	++++	auto-fluorescence, photobleaching, movement artifacts	+++	yes, but confined to superficial tissues
Fluorescence (macroscopic)	100 micron-mm	s-hr	+++	yes, to some extent	++	auto-fluorescence, photon scattering, photobleaching	++	future promise with NIRF probes
BLI	mm	s-hr	++++	possible	++++	almost none, food-derived phosphorescence	++	no
Nuclear (PET, SPECT, gamma scintigraphy)	mm-cm	min-hr	++++	yes	++	metabolic uptake by liver, kidneys, and thyroid; excretion pathways via gut and urine	++++	yes
MRI	100 micron-cm	s-min	++	yes	++	patient/animal movement, physiological motion, susceptibility artifacts, blood flow	++++	yes
US	mm-cm	s-min	+	yes	+	subcutaneous fat, bone interfaces, calcium	++	yes
X-ray CT	100 micron-cm	s-min	+	yes	+	patient/animal movement, metallic objects	+++	yes

Biochemical sensitivity (the ability to detect minute quantities of target biomolecules in vivo) and biochemical signal-to-noise (strength of the target signal over background) are estimated in the context of a given instrument used with an appropriate contrast agent or imagable probe. BLI, bioluminescence imaging; PET, positron emission tomography; SPECT, single photon emission computed tomography; CT, computed tomography; US, ultrasound; MRI, magnetic resonance imaging; S/N, signal to noise; ms, millisecond; mm, millimeter.

unique, dynamic information unattainable by conventional techniques. For example, provocative studies utilizing microPET imaging to follow injected, radiolabeled arthritogenic autoantibodies in mice surprisingly revealed immediate localization of the probe to commonly affected joints, indicating that the antigen likely pre-existed in an accessible form at the inflamed joints (Figure 1B; Wipke et al., 2002). This further suggested that a tissue-specific immune response can be initiated against a ubiquitously expressed antigen. Additional imaging studies with this labeled antibody have demonstrated the necessity of innate immunity factors (mast cells, neutrophils, immune complexes) for permitting access of autoantibodies to the joints, where they subsequently bind their cognate antigen and initiate inflammation (Wipke et al., 2004).

Both in vivo and ex vivo imaging of luciferase-labeled immune cells have been utilized to study graft versus host disease (GVHD). Luciferase-expressing allogeneic splenocytes were transplanted along with BM cells to induce acute lethal GVHD; bioluminescence imaging (BLI) could comprehensively monitor donor cell expansion, migration, and infiltration into GVHD target tissues in vivo (Beilhack et al., 2005). Ex vivo BLI permitted even more precise localization of donor cell infiltrates, which in turn enabled specific sampling and analysis (by FACS and histology) of BLI-positive tissues. In this case, Peyer's patches that had disappeared macroscopically (due to host animal irradiation prior to transplantation) were rendered accessible to further analysis by BLI-guided tissue sampling, thereby enhancing analysis beyond conventional approaches.

On a different scale, two-photon intravital microscopy has been utilized to study the behavior of single immune cells in intact or explanted lymph nodes in vivo. This technique was recently applied in groundbreaking studies aimed at deciphering the dynamics, motility, and cell-cell interactions of B cells and T cells in germinal centers of lymph nodes (Allen et al., 2007), thereby elucidating the complex behavior of these cells in an

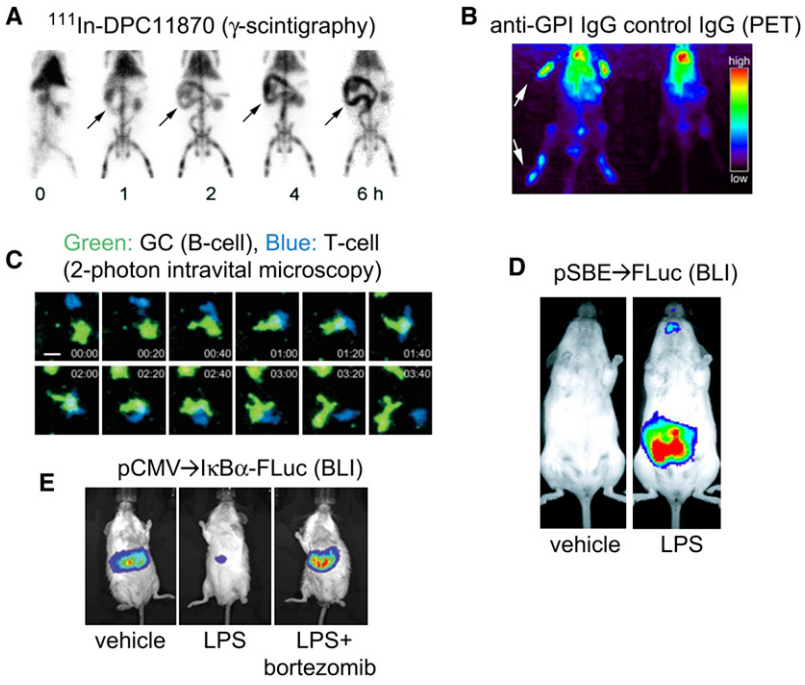

Figure 1. Selected Examples of Molecular Imaging Strategies to Investigate Immune Response In Vivo

(A and B) Imaging innate and adaptive immunity with injectable probes.

(A) γ-scintigraphic images acquired at the indicated times after injection of ^{111}In-DPC11870 (radiolabeled leukotriene antagonist) in a rabbit with acute colitis. Note the strong intestinal signal emitted as soon as 1–2 hr after administration of the probe (arrows) (reprinted by permission of the Society of Nuclear Medicine from van Eerd et al., 2004).

(B) PET imaging after injection of ^{64}Cu-labeled anti-glucose-6-phosphate-isomerase (GPI) IgG (left) or control IgG (right). Note the substantial accumulation of anti-GPI IgG in the joints (arrows) (adapted by permission from Macmillan Publishers Ltd: *Nature Immunology* [Wipke et al., 2002]).

(C) Dynamic imaging of single immune cell interactions in vivo with fluorescent reporters and 2-photon intravital microscopy. Time-lapse images of a transient interaction between a GFP-tagged B cell and a CFP-tagged T cell in the dark zone of the germinal center (GC) in an intact lymph node in vivo (from Allen et al., 2007; reprinted with permission from AAAS).

(D) Visualizing transcriptional activity in vivo by expression of an imaging reporter gene. By bioluminescence imaging (BLI), global Smad2 and Smad3 transcriptional activity was measured in pSBE→FLuc pan-expressing mice upon treatment with LPS (reprinted from Lin et al., 2005).

(E) Imaging immune-mediated signaling cascades in real-time with post-translationally modified fusion reporters (induced protein degradation and stabilization). BLI of bortezomib-mediated modulation of LPS-induced IKK activity in vivo. A reporter of IKK activity (IκBα-FLuc) was delivered to hepatocytes by hydrodynamic somatic gene-transfer techniques. Mice were then imaged after the indicated treatments. Note that bortezomib not only abrogated LPS-induced activation of IKK (degradation of IκBα-FLuc), but actually promoted reporter accumulation, consistent with inhibition of both ligand-induced degradation and basal turnover of IκBα (reprinted by permission from Macmillan Publishers Ltd: *Nature Methods* [Gross and Piwnica-Worms, 2005a]).

antigen-dependent manner within their normal environment (Figure 1C).

Imaging Host-Pathogen Interactions

The molecular imaging toolbox offers researchers noninvasive means to characterize host-pathogen interactions within living animals. For example, bioluminescent viral strains can be utilized to follow viral replication and localization in individual intact animals and to investigate viral and host factors that govern pathogenesis. A recombinant, fully virulent vaccinia virus expressing firefly luciferase (Vac-FLuc) was generated to investigate the extent to which interferons regulate replication and dissemination of vaccinia in vivo (Luker et al., 2005). The authors found that replication of vaccinia virus was substantially greater in mice lacking type 1 interferon receptors (*Ifnar1*$^{-/-}$) and that type 1 interferon affects dissemination of vaccinia from the respiratory system to systemic sites such as liver and spleen. BM transplants from wild-type animals to *Ifnar1*$^{-/-}$ animals showed substantially higher vaccinia infections compared to wild-type animals transplanted with *Ifnar1*$^{-/-}$ marrow, indicating that the protective effects of type 1 interferons are mediated primarily through parenchymal rather than hematopoietic cells. Thus, use of BLI in this study uniquely allowed for (1) facile quantification of the spatial and temporal progression of vaccinia infection in the context of different genetic backgrounds of the host and (2) detection of unexpected patterns of viral dissemination in vivo in individual animals.

A second means of investigating host-pathogen interactions is by the use of transgenic mice that express a genetically encoded imaging reporter driven by a pathogen-responsive promoter. This strategy bypasses the need to construct reporter pathogens (which may be attenuated compared to their parental strain), allowing facile examination of a variety of pathogenic strains. This approach was utilized to study the spatial and temporal progression of infection and the relative degree of virulence when three variants of herpes simplex virus 1 (HSV-1) were introduced into the cornea or flank of mice expressing FLuc under control of the HSV-1 thymidine kinase promoter (Luker et al., 2006).

Imaging Immunity-Related Signal Transduction

Numerous signaling pathways are implicated in activation and suppression of both innate and adaptive immunity, and therefore, improper regulation of these signaling cascades may lead to pathological immune responses. Integration of imaging, signal transduction, and use of animal models of immunity, inflammation, or immunological disorders is already enhancing our understanding of specific signaling events in normal and pathological immune responses.

Specifically, signaling events can be visualized in live immune cells by placing an "imagable" reporter gene downstream of a stimulus-specific inducible promoter such that observed outputs are a function of

transcriptional activity of the examined promoter and therefore reflect activity of the associated upstream signaling cascades. For example, gene expression can be visualized by a β-lactamase reporter that can hydrolyze a membrane-permeant fluorogenic substrate, changing the substrate fluorescence from green to blue (Zlokarnik et al., 1998). This system was used for quantitative analysis of M_1 muscarinic receptor agonist-induced NF-AT transcriptional activity in intact T cells.

Further, temporal and spatial patterns of signaling can be studied in vivo with transgenic mice expressing an imagable reporter. For example, to characterize Smad2- and Smad3-dependent signaling in intact animals, a transgenic mouse was generated wherein FLuc was driven by a Smad-responsive element (pSBE→FLuc) (Figure 1D; Lin et al., 2005). With BLI, the authors were able to noninvasively assess global and organ-specific changes in TGFβ and Smad2- and Smad3-dependent signaling upon systemic administration of lipopolysaccharide (LPS) or induction of traumatic brain injury, respectively, and revealed important but different responses to LPS in specific organs. Similarly, spatial and temporal changes in response to sepsis, acute arthritis, and contact hypersensitivity were monitored by BLI in transgenic mice with a serum amyloid A-driven luciferase reporter (pSAA→FLuc) (Zhang et al., 2005). When used in this way, note that BLI is useful for gross organ localization of responses, but not for the fine mapping that connects the response to a cell type or even a region with limited cell types. For this reason, under development are second-generation reporter mice that express luciferase-GFP fusion proteins, thereby enabling rapid, noninvasive regional analysis by BLI and concordant cell type-specific analysis by fluorescence microscopy or FACS.

To monitor organ-related changes in the activity of NF-κB, a transcription factor that regulates many inflammatory and immune reactions, a transgenic mouse has been generated wherein FLuc is expressed under the regulation of an NF-κB response element (pNF-κB→FLuc) (Carlsen et al., 2002). With this animal model, the researchers were able to analyze gross spatial changes in NF-κB activity before and at different times after administration of classical stressors, such as tumor necrosis factor α (TNF-α), interleukin-1α (IL-1α), LPS, UV-induced genotoxic stress, and induction of a chronic inflammatory reaction resembling rheumatoid arthritis. In this way, organ-specific responses could be mapped and analyzed on an individual basis in cohorts of mice over time.

Imaging post-transcriptional events such as translational regulation, protein-protein interactions (PPI), protein processing, or protein degradation is primarily obtained by fusing the reporter gene, a partial reporter fragment, or an upstream transactivator to the protein of interest, thereby generating a molecular sensor that activates (or deactivates) the reporter in response to a given protein interaction or modification. For example, we recently demonstrated that an IκBα-FLuc fusion reporter can be used to directly monitor in vivo the activity of IKK, a central regulator of NF-κB activity (Figure 1E; Gross and Piwnica-Worms, 2005a). Here, activation of the canonical NF-κB pathway is dependent on IKK-induced phosphorylation of IκBα, an inhibitory protein that under normal circumstances sequesters NF-κB in the cytoplasm. Phospho-IκBα is then polyubiquitinylated and degraded by the 26S proteasome, allowing nuclear translocation of NF-κB to regulate transcription of its target genes. Indeed, after hepatocellular delivery of the reporter, a decrease in bioluminescence was observed in real-time in response to LPS-mediated activation of IKK in vivo. Applying this approach to a tumor xenograft model expressing the IκBα-FLuc fusion reporter, robust time- and dose-dependent pharmacodynamic characterization of a novel IKK inhibitor (PS-1145) was characterized with a minimal number of animals.

Protein-protein interactions (PPI) play a pivotal role in signal transduction and are now recognized as an attractive target for pharmacological intervention. Over the last five years, several strategies have been developed for imaging PPI in vivo including (1) PPI-dependent reporter gene transactivation or repression (two-hybrid systems; recruitment of signal transduction cascades), (2) energy transfer techniques such as Förster or bioluminescence resonance energy transfer (FRET or BRET, respectively), and (3) reporter complementation, achieved by fusing inactive reporter fragments to interacting proteins, which upon association bring the fragments in close proximity and restore reporter activity (Gross and Piwnica-Worms, 2005b). Recently, an optimized FLuc protein fragment complementation system was developed by screening incremental truncation libraries of N- and C-terminal fragments of FLuc (Luker et al., 2004). With this system, quantitative assessment of PPI was enabled in intact cells and in vivo, exhibiting robust inducibility. Indeed, drug-specific induction of bioluminescence reached 1200-fold over background, exceeding currently available systems. This property enabled monitoring of low-affinity PPI and allowed analysis of the presence and extent of interferon γ- and Janus kinase-1-independent homodimerization of STAT1. In fact, in agreement with several subsequent reports, the existence of a large pool of nonphosphorylated STAT1 homodimers was identified with this imaging strategy.

Overall, these innovative strategies offer a means to accurately analyze the dynamic nature of cell-cell, cell-antigen, and cell-humoral factor interactions, as well as a means to detect intracellular signaling and host-pathogen interactions, all within the complex immunologic environment of the intact animal. Integration of smart probes and reporters with animal models of immune disorders will enable immunologists to address complex paradigms such as regulation of hematopoiesis, trafficking and activation of immune cells, differentiation of stem cells, and interaction between immune cells and stromal cells on microscopic and macroscopic scales and in four dimensions.

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