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Imaging—and Imaging—Immune Responses to Infections:

Laser-scanning confocal microscopy pinpoints responses of immune system components to bacterial infections in situ

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After Antonie van Leeuwenhoek viewed “animalcules” from the human oral mucosa during the 17th century, microscopy became an increasingly important tool for diagnosing infectious diseases and following host responses. Microscopy continues to advance technically, even during the past few years. For example, whereas traditional microscopy was used mainly to examine fixed or frozen tissues, recent innovations now allow us to visualize complex processes surrounding microbial pathogenesis and localized host responses.

In particular, laser-scanning confocal microscopy enables us to scan excised tissues and to follow infections in situ, identifying localized innate and adaptive immune system components. Even more exciting is using multiphoton microscopy to visualize the immune system in tissues of live animals, providing movies of on-going events during infections. We also expect that additional improvements in microscopy, better fluorescent dyes, and alternative transgenic reporter systems will lead to even sharper views of the microbial and immunological landscape. Deepening our understanding of how anatomy, cellular movement, microbial pathogenesis, and immune responses are linked will help us to make better decisions regarding the development of vaccines and treatments for infectious diseases.

Imaging Immune Responses

Microscopy with use of immunofluorescence or immunochemical markers provides a reliable means for monitoring T and B cell responses. Typically, researchers use simple antigens to determine how T cells, B cells, and antigen-presenting cells interact, and they may also label T cells and antigen-presenting cells to improve sensitivity. However, in earlier studies, investigators typically had to add large numbers of cells, thereby introducing artifacts and undermining the reliability of their findings.

The development of reagents that are based on T cell antigen receptors (TCR) for identifying antigen-specific T lymphocytes proved a major advance. Thus, for example, biotinylated major histocompatibility complex (MHC) molecules are used to bind correct antigenic peptides, form tetramers when exposed to avidin molecules that carry a fluorescent marker, and then bind to TCRs of the correct specificity expressed by CD4 or CD8 T cells. In the case of a pathogen-derived antigen whose peptide sequence and MHC restricting element are both known, such reagents can be used to measure the T cell immune response. Moreover, the responding cells can be phenotypically and functionally analyzed.

MHC tetramers are also used in confocal microscopy to identify endogenous CD8 T cells responding to viral or bacterial infections in mice. Such experiments revealed the anatomical steps that initiate immune responses in vivo.

Multiphoton microscopy further expands our ability to monitor immune responses in vivo. In using this technique, a mouse is placed on the microscope stage before fluorescently labeled cells in its popliteal or inguinal lymph nodes are imaged. Instead of live animals, experimenters may look at tissue explants mounted in oxygen and CO₂-perfused tissue chambers.

This pioneering work is enabling immunologists to observe early immune responses to infectious agents at the level of the lymph nodes and spleen. Although later events, including the attack and clearance of pathogens from infected tissues, are more difficult to observe, investigators are beginning to scan anatomic sites, including bone marrow, liver, brain, lung, and intestine, where some of those secondary immune responses occur and where early innate immune responses rapidly develop.

Tracking Immune Responses to Infections In Situ

Although we learned a lot from imaging simple antigens, efforts to observe immune response to live, replicating organisms typically add several layers of complicated challenges. For example, consider the highly orchestrated and complex process that occurs when granulomas form in response to a mycobacterial infection. After mycobacteria are introduced into the host, they are taken up by Kupffer cells in the liver. They next recruit, reorganize, and change the shape of several myeloid cell types. During these steps, the infected Kupffer cells appear not to move, perhaps serving as nucleation sites for other cell types to begin forming granulomas.

In part because of the relative ease of imaging lymph nodes, investigators in this field focus considerable attention on pathogens that enter this site. For instance, subcapsular sinus (SCS) macrophages in lymph nodes acquire virus particles and then present them to virus-specific B cells. In other experiments, dendritic cells deep in the medulla of lymph nodes acquire inactivated influenza virus particles and then present them to B cells, thereby inducing their responses. Similarly, SCS macrophages acquire live vaccinia or vesicular stomatitis viruses, whereas antigen-specific CD8 T cells interact only with infected dendritic cells adjacent to the SCS layer.

When *Toxoplasma gondii*, an intracellular parasite, invades host cells within the SCS, neutrophils quickly swarm to draining lymph nodes, providing another stunning example of the power of this microscopy technique. The swarming begins when parasites exit infected cells, apparently recruiting neutrophils.

Two-photon microscopy is enabling us to expand our long-held, two-dimensional picture of the microbial world into a more fully three-dimensional view in which hallmark anatomical features reveal underlying immune responses to infections. Enhanced imaging systems, along with other sophisticated tools—for example, transgenic systems that report intracellular signal transductions—are leading to ever-improved anatomical descriptions of cellular and subcellular events surrounding infections.

Imaging *Listeria monocytogenes* Infections

The gram-positive intracellular pathogen *Listeria monocytogenes* (LM) is acquired mainly from contaminated foods cause infections that can lead to miscarriages in pregnant women and is problematic among immunocompromised individuals. LM first infects epithelial cells lining the bowel and then spreads directly from cell to cell. It also can spread via the blood to the spleen and liver, likely moving within migrating phagocytes. Intracellular LM depends on several proteins, including the pore-forming listeriolysin O, to escape phagocytic vacuoles. In terms of host immune responses, initially, many different types of innate

immune cells respond to this pathogen. However, CD8 T cells are required for sterilizing immunity.

LM is considered a model for other intracellular pathogens. One technical complication in such research is that, because so many LM strains are human derived, they infect mice via the oral route only poorly. This difficulty is due mainly to the inability of the bacterial internalin A protein to bind a key receptor, E-cadherin, on mouse intestinal epithelial cells. However, to overcome this obstacle, researchers recently developed LM strains with a mouse-adapted internalin A protein as well as transgenic mice that express “humanized” E-cadherin.

Meanwhile, other researchers are analyzing how cells in the host spleen handle LM infections. The spleen is compartmentalized, making it adept when faced with blood-borne particulates, including bacterial pathogens. The major anatomical regions of the spleen include the white pulp (WP), red pulp (RP), and marginal zone (MZ) (Fig. 1). WP contains B cell follicles that surround T cell-containing zones, also known as the peri-arteriolar lymphoid sheath (PALS), which in turn surrounds the central splenic arteriole.

The RP contains red blood cells and dendritic cells as well as migrating T and B lymphocytes. The MZ, which demarcates an area between the RP and the WP, contains a specialized subset of B cells and a double layer of macrophages. Close to the RP are ERTR9⁺ marginal zone macrophages (MZM) and underlying these and separated by the marginal sinus are the MOMA⁺ marginal zone metallophilic macrophages (MMM) (Figure 1B). These macrophage subsets are positioned to capture viruses and bacteria entering via the bloodstream.

MZM pick up LM cells that are introduced intravenously into mice. Nine hours after localizing in the MZ, CD11c⁺ cells carry LM into the PALS, according to imaging analysis (Fig. 2). The appearance of LM in the PALS triggers several events that can be visualized with static and 2P microscopy. LM cells activate dendritic cells (DC) 6–24 hours after infection, and this leads neutrophils and natural killer (NK) cells to cluster in the PALS. The NK cells produce interferon- γ (IFN- γ), which induces nearby monocytes to mature, while T cell-antigen presenting cell (APC) interactions in the PALS activate CD8 T cells. The mRNA signature of those activated CD8 T cells is drastically altered 24 hours after infection. Thus, specific components at particular sites within both the innate and adaptive immune systems rapidly respond to LM infection.

Factors regulating pathogenesis also affect the immune response to LM. For example, neither a mutant of LM that is deficient in listeriolysin O nor heat-killed LM (HKL), which cannot escape intracellular vacuoles, can drive production of IL-12, which is needed to produce IFN- γ . These LM strains apparently no longer trigger cytosolic sensing mechanisms that activate innate immune responses. In addition, immunizing mice with such mutants destroys their MZM via a TNF-dependent mechanism, indicating the immune response to these LM forms is defective. Such findings raise safety questions regarding use of these LM strains to develop vaccines.

Visualizing CD8 T Cell Responses to LM

An individual mouse carries from 50–1,000 CD8 T cells that are specific for any particular antigen—about 2–3 orders of magnitude too low to detect individual cells. Thus, to visualize the early events surrounding CD8 T activation, we need to use adoptive transfer techniques to introduce trackable T cells. After LM cells are introduced but before T cells divide, CD8 T cells cluster next to antigen-bearing dendritic cells. Clustering intensifies depending on LM antigen levels and the extent of the initial inflammatory response. Thus, a weak

antigenic stimulus induces little or no clustering and leads to only meager development of long-term protective memory. In contrast, live LM cells trigger robust clustering and strong memory development.

Within 3 days, the CD8 T cell numbers expand substantially, and endogenous MHC class I tetramer+ cells can be detected within the spleen by confocal microscopy. Using thick sections or whole-mounted tissues enables us to visualize these tetramers more readily, perhaps because this approach better maintains three-dimensional structure within this tissue. Well after T cells are activated, we continue to see T cell receptors (TCR) reorganize and also see them forming synapses with dendritic cells in the PALS and RP.

By the fourth day, CD8 T cells are plentiful within the PALS with solitary T cells scattered throughout the T cell zones. However, on the next day, the responding CD8 T cells again cluster with DC in the PALS. These late antigen-specific interactions appear to be important for full expansion of the CD8 T cells and may also reflect CD8 T cells exerting effector functions. By day 6, CD8 T cells enter the bridging channels (BC) that serve as conduits from the white pulp to the red pulp (Fig. 3).

The bridging channels are also the entry point for naïve T cells migrating into the WP. Thus, the BC, which is often associated with the central arterioles, is a two-way path. The peak of the CD8 T cell response occurs 9–10 days after the LM infection begins, by which time most CD8 T cells relocate to the RP and other tissues. Thus, for the first time, we see the orchestrated events surrounding the CD8 T cell response to a bacterial pathogen.

We are also gaining insights by learning where memory CD8 T cells go and by following other changes that appear to be part of the secondary response to LM infections. For instance, one month after infection, we find that substantial numbers of memory cells are located in B cell zones, while smaller numbers of memory cells are in the RP. However, after another few months, all the memory CD8 T cells relocate to the RP and WP. Why memory CD8 T cells migrate in this way is not known, but it might point to a novel interaction with B cells.

If we challenge such animals again with LM, we do not see any immediate proliferation of CD8 T cells. Instead, they migrate into the PALS before expanding and then begin moving through the BC into the RP and the bloodstream. These steps occur much more rapidly than those during the primary response. Thus, the secondary response is rapid, releasing large numbers of effector cells from the priming site to meet the invading pathogen.

Conclusions

The use of sophisticated microscopy continues apace in analysis of infection and the induction of immune responses. Continued technological improvements in microscopy, fluorescent dyes, and transgenic reporter systems will allow constant improvements of our view of the microbial and immunological landscape. Understanding how anatomy, cellular movement and microbial pathogenesis are linked to the outcome of the immune response will allow us to make informed decisions regarding vaccine formulations and treatments for infectious diseases and their sequelae.

Summary

Laser-scanning confocal microscopy with labeled or fluorescent reporter molecules can track microbes and localized host immune responses to infections.

Imaging ongoing viral, parasitic, and bacterial infections in lymph nodes reveals novel microbial behaviors and immune responses.

Following *Listeria monocytogenes* infections via confocal and intravital microscopy provides a step-by-step view of how immune-cell responses are orchestrated and provides unexpected insights into candidate vaccine safety issues.

Microscopy coupled with MHC class I tetramer staining enables us to track CD8 T cell responses to *L. monocytogenes*, including their movements within specific tissues in the spleen.

Lefrançois: Mr. Fix-it Probes Immunological Memories, Ascends Mountains

Leo Lefrançois suffered a near-fatal heart attack on 13 September 2007. Sixteen months and eight stents later, he was on top of the world. “A friend had been prodding me to climb Kilimanjaro with him, and from my ICU bed, I decided to do it,” he says. He trained by hiking a volcano in Chile and a mountain in Colorado with a summit above 14,000 feet, and by spending a lot of time in the gym. On 29 January 2009, Lefrançois and his wife, Lynn Puddington, reached the peak of Mount Kilimanjaro in Tanzania. “It’s so cliché, but the heart attack has really made me wake up to a larger world and family view,” he says. “This year, we climbed through the Andes to Machu Picchu. The Himalayas may be next!”

Lefrançois, 54, is director of the Center for Integrative Immunology and Vaccine Research and a professor in the Department of Immunology at the University of Connecticut (UC) Health Center in Farmington, Conn. His research focuses on T cell immunology and immunological memory. “Precisely how memory is generated at the cellular and molecular levels has been a long-standing question in the field and somewhat of a holy grail,” he says. “My lab is focused on several aspects of CD8 T cell memory. We use various microbial pathogens to study the immune response, including *Listeria monocytogenes*, vesicular stomatitis virus, and influenza virus.”

By working with several pathogens, he and his collaborators can better detect differences in immune responses to each infection, identifying key controlling elements. The goal is to develop vaccines that will induce the most protective type of memory. “Vaccines geared toward inducing protective CD8 T cell responses have yet to fully come to fruition, although such vaccines could be important in protecting against intracellular bacterial infections, HIV, and cancer,” he says.

Lefrançois grew up in Connecticut, 13 miles from where he lives now. He received his B.S. in microbiology and medical technology from Colorado State University in 1978, and his Ph.D. in immunology from Bowman Gray School of Medicine at Wake Forest University in 1982. He worked as a research scientist in cell biology at the Upjohn Company between 1986 and 1991 and, earlier, was a research associate at the Scripps Clinic and Research Foundation in La Jolla, Calif. He returned to his home state and joined academia in 1991. “It was important for me to have our children spend some quality time with my parents, both of whom are now deceased,” he says.

Neither of his parents was educated beyond elementary school. “Nevertheless, my dad was one of the smartest people I have known,” he says. “He was self-employed most of his life, first owning a tavern and then an appliance/TV store from the 50’s on, and worked his tail off to put his kids through school. We always had the best TVs, and the first remote control.” His mother, a homemaker, raised five children who—from oldest to youngest—are separated by 20 years. “My brother and I loved to play with our chemistry set as kids, making gunpowder and whatever other potentially dangerous formulas we

could find,” he says. “When I got a real job as a scientist, I told him I was now getting paid to play with my chemistry set.”

As a youth, Lefrançois experimented with electronics and became a TV repairman in high school. Later, his lab colleagues called him “Mr. Fix-it,” a nick-name that his wife and children continue to use. Growing up in the 1960s and 1970s “was an incredible time—the moon landing, Jacques Cousteau, *Wild Kingdom*, *Star Trek*,” he recalls. “We were a TV family, of course, and these events and shows helped foster my interest in science. I still dabble in astronomy, and I am appalled at the way NASA is being treated . . . Wonderment and human discovery seem to have been trumped by budgets.”

Two of his undergraduate college professors, Robert Tengerdy and Barbara K. Joyce, fueled his interest in microbiology and immunology. He has modeled some of his teaching style on Joyce’s, calling her “one of the toughest teachers I ever had.” Tengerdy taught basic immunology, Lefrançois’s first real exposure to the field. “I became enthralled,” he says, and went on to do research with Tengerdy. “I first started by cleaning chicken cages,” he says. “Later, my project was to use polypropylene sheets impregnated with *Pseudomonas aeruginosa* to remove plutonium from cultures, a model. . . [for cleaning up] effluent water from nuclear plants.”

Lefrançois’ wife Puddington, also an immunologist, is an associate professor at the UC Health Center. Their son, 16, is a high school junior, and their daughter, 20, attends college. Besides mountain trekking, Lefrançois likes golf, skiing, and cooking with his wife, who makes the desserts, although “finding the time to be really creative in making a meal is often difficult,” he says. Although his writing is confined to grant proposals and papers, he loves to write. “I have always wanted to write a novel,” he says. “I even have a brief outline. It’s science fiction, and I hope to write it before too long.”

Marlene Cimon

Marlene Cimon lives and writes in Bethesda, Md.

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Figure 1.

The structure of the spleen. Spleen sections were stained with the indicated antibodies. The white pulp of the spleen contains the T cell zones (PALS; blue), which is surrounded by follicular B cells (B; green). The MOMA+ macrophages line the inner boundary of the Marginal zone (B, right panel; MZ). The MZ is also populated with the IgM^{hi} marginal zone B cells. The cells exit and enter the T cell zones of the white pulp by using the bridging channels (BC). The central arteriole (CA) is often associated with the BC, shown here in green (CD31; right panel)

Figure 2.

After LM is transported from the marginal zone, the bacteria replicates rapidly in the T cell zones (PALS) of the splenic white pulp. Mice were infected with LM and at 24, 48 and 72 hrs later frozen sections of the spleen were stained for B220 (green; B) and Listeriolysin-O (red; to visualize bacteria). Note that LM is primarily localized in the T cell zones (PALS) at 24 hrs, the bacterial burden increases significantly between 24 and 72 hrs.

Figure 3.

Localization of antigen-specific CD8 T cells at 5 days PI. Thick spleen sections were stained with the K^b-OVA tetramer (red), B220 (green; B) and CD31 (green; to visualize the central arteriole; CA and the associated branches). A 30-μm merged z-stack is shown. Note the Clusters of antigen-specific CD8 T cells localize along the border of the T and B cell zones (B). The image shows the bridging channel (BC) through which the antigen specific CD8 T cells (red) exit the T cell zones (PALS) into the marginal zone (MZ) and red pulp (RP).