Illuminating emergent activity in the immune system by real-time imaging

Matthew F Krummel

The imaging of tissues and organs as it is now practiced will seem primitive in the coming decade, yet use of this technology will define the origin of emergent activities and drive an era of system integration.

"…like the billowing dancing figures in a brightly lit ballroom that you gaze into from outside in the dark—and from a distance so great that you can no longer hear the music… the turning and twisting movement of the couples seems senseless."

Gustav Mahler, on the third movement of his second symphony

At present, immunologists peer into imaging space and observe the movements and activities of individual components of the immune system with only a vague understanding of what motivates each cell. How the relationships between cells fully account for variations in system-wide output after an immune insult is still very often unclear. Real-time imaging has the potential in this decade to provide clarity about how collections of cells of the immune system produce emergent activities such as tolerance or autoimmunity. Although static end-point measurements are suited to determining the present state of a system, it is time-lapse observations that demonstrate how the states are connected. Submicrometer-resolution real-time imaging allows tracking of the state of a system at the subcellular level, a level at which molecular assemblies integrate signals from the environment.

For advances to be made, one-photon and two-photon imaging of tissues and organs will improve in its ability to collect and track larger numbers of unique cells and carefully account for all aspects of information dissemination among them over time and space. Concurrently, advances in biosensor probe design and implementation will provide real-time feedback about each cell's responses to the information it has received. Most importantly, the application of such technology to truly diseased tissue will guide studies so that the focus remains firmly on understanding the systems that have the most information to provide. This is perhaps the most highly anticipated aspect of the developing science.

Matthew F. Krummel is in the Department of Pathology, University of California at San Francisco, San Francisco, California, USA.
e-mail: matthew.krummel@ucsf.edu

The era of system integration

In 1998, the eminent biochemist Dan Koshland wrote that the field of biochemistry had entered its "third phase," an era of "pathway integration"[1]. According to this view, in the first phase, individual steps in biochemical pathways were identified; in the second, the control of pathways through feedback was determined; and in the third, the quantification of pathways would calculate the rates at which reactants and products would be generated in complex subcellular compartments.

For immunologists, the coming decade represents a similar shift in emphasis: having identified many of the cell types that are important and their component receptor-ligand signaling interactions, researchers are now poised to begin to truly consider the immune system as a system. In this, the challenge has always been to determine how describable emergent activities (for example, tolerance or autoimmunity) are reached by a vast collection of cells. Each member responds in varying ways to a complex immunological insult that is transmitted largely by information transduced through the other cells of the system. In this Commentary, I will argue that an era of system integration is on the horizon, one that may be guided mostly by real-time imaging. In the simplest terms, this is mainly because not all possible emergent activities need to be modeled and studied, and in complex systems, there is great benefit in focusing first on those that actually occur.

Information theory immunology

Before delving into the question of how imaging may take immunologists to such heights, I would like to propose that the language of information theory will increasingly infiltrate the science. The immune system is essentially an information system that receives input about an insult in one syntax (for example, pattern-recognition receptor triggers, foreign proteins and peptides) and then converts that information into many other forms (for example, the upregulation and downregulation of surface proteins, chemotactic molecules and receptors necessary for the mobilization of cells to the site, the production of neutralizing antibodies and so on). The information content at the time of insult is the collection of signals that trigger a great many immune and host cell types. For that, it is important that timing and location of these individual triggers is also information that the system may use, and the same signal expressed in an alternative context may result in considerably different outcomes. For example, transforming growth factor-β, which may be expressed in many contexts *in vivo*, inhibits the survival of effector T cell but promotes survival in memory T cell populations[2]. The cell type or signaling context may explain the difference in response to similar information.

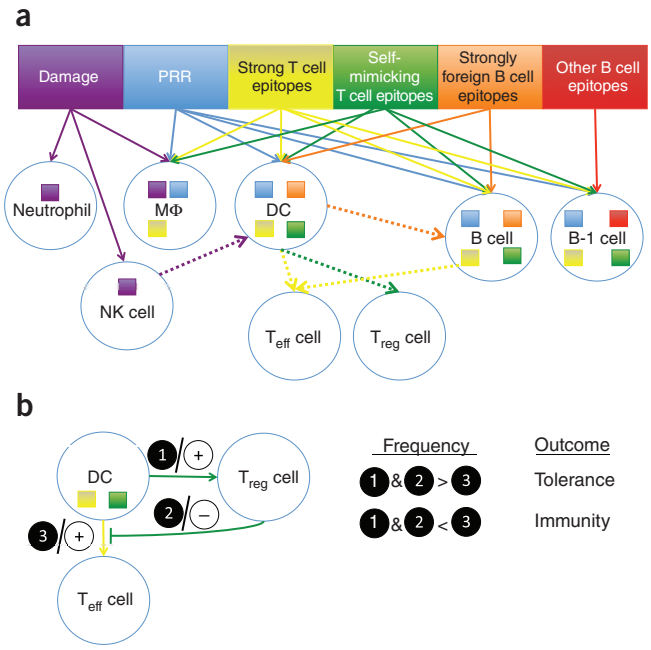

Figure 1 The immune system is an information system. (**a**) First-order information flow into the immune system. In this example, an immune insult (bar at top) delivers signals to multiple cell types (colors of boxes in cells match insult colors above). Solid lines indicate direct information flow (signaling) to specific cell types; dotted lines indicate the passage of that information to additional cell types, further influenced by integration of signals from other sources. PRR, pattern-recognition receptor; MΦ, macrophage; NK cell, natural killer cell; T_{eff} cell, effector T cell. (**b**) Information integration. A frequency-of-contact model is proposed to integrate signals amongst an array of cell types. In this example, a DC bearing both peptides recognized by T_{reg} cells (green box) and those recognized by high-affinity effector T cells (yellow box) is subject to information integration whereby the amount of each peptide and the number of each type of T cell integrate via the frequency of cells engaging in information exchange to determine whether the outcome results in T cell regulation and tolerance (dominance of pathways 1 and 2) or overcomes regulation (dominance of pathway 3). Plus and minus symbols indicate common conceptions about these signals (that is, interactions positively (+) or negatively (–) affect the target).

Many types of signals may be presented to the immune system, for example, in the form of a pathogen (**Fig. 1a**). Such a collection, although incomplete, includes innate pattern-recognition receptors such as lipopolysaccharide, a broad class of damage indicators, such as those that would trigger neutrophils and natural killer cells, as well as the classical adaptive determinants—epitopes for B cells and T cells. As understanding has emerged that mimics of self peptides may modulate regulatory T cells (T_{reg} cells), just as foreign peptides trigger effector T cells, information theory dictates that every peptide from the insult (that is, all major histocompatibility complex–binding peptides of a pathogen) provide information content. The sum of the information content that imaging integrates is then the sum of these triggers as they hit the collection of host cells of the immune system. The beginnings of a second level of transduction in processed peptides may be presented to helper T cells or T_{reg} cells, resulting in their activation, or information about tissue damage may be processed in natural killer cells, resulting in further activation of dendritic cells (DCs)[3] (**Fig. 1a**, dotted lines).

Because almost all cells of the immune system are motile, information rapidly disseminates and transforms as each triggered cell makes thousands of contacts with other cells. Notably, each cell is now a carrier of its recent experience and, because of the changes it has undergone, can now act as an agent to transmit (transduce) part of the signal to other cells via contacts and cytokine releases. As for the former, each of those contacts or secretion events is now akin to a binary decision tree in a hierarchical database structure. Is salient information that alters the next cell transferred or not? Additionally, information can both diverge and then converge as partners that have or have not both received parallel information streams may meet up physically.

The idea proposed above suggests that quantification of contact frequencies will be as important in determining the outcome as traditional parameters, such as antigen concentration. One very small-scale distillation of this idea is a broad view of a tolerance-versus-immunity decision based on frequency-of-contact competition between T_{reg} cells and effector T cells (**Fig. 1b**). In this example, activation of an effector T cell to produce memory versus tolerance may be skewed at multiple points by the presence or absence of positive or negative streams of information via T_{reg} cells, DCs or other effector T cells. Imaging of the encounter of helper T cells with DCs has been demonstrated to be regulated by T_{reg} cells[4,5], with subsequent imaging data suggesting that T_{reg} cell–DC interactions may be lytic, at least in the context of an ongoing tumor response[6]. Interestingly, activation of T_{reg} cells might generally occur faster than that of naive T cells[7], thus providing the need for profound activation of multiple effector T cells (for example, a strong agonist on a large number of DCs and a relatively large precursor frequency of effectors) to overcome the repression.

Given the need to elucidate the spatial and temporal dynamics and further characterize these pathways, what are the specific areas in which present and emerging technologies will aid understanding of the sequences and methods of activity in the system, and where will focus be best applied?

Identification of functional cell subsets

Many of the cell types that mediate immune function are now known, as are their component proteins and signaling pathways. Yet this may be an oversimplification, as rare but important subsets continue to emerge. One very useful contribution of imaging technologies is to highlight the different activities that seemingly similar cells can undertake. For example, the first real-time view of T cell activation in lymph nodes was based largely on slower motility or full arrest achieved on sites that later were shown to be DCs[8]. Static imaging or flow cytometry analysis of a tissue will identify differences in functional activities if they are extreme (such as the presence or absence of gross expression of a known protein). Where differences are profound, flow cytometry and/or static analysis may prove more than adequate in identifying such subsets.

Real-time imaging takes a more subtle approach—cells that act differently (for example, are more motile or less motile or have transient although still important interactions with one another) emerge from the analysis (**Fig. 2**). Some of these differences may be related to spatial domains in the tissue (for example, cells act differently because of their location in the tissue). But careful correlation of real-time activities and the fate of cells after such encounters may also represent functional differences of the cells before their encounters. As an example, real-time imaging has demonstrated that activating T cells have a tendency to interact and form synaptic contacts with one another in lymph nodes, reflective of their activated status. In contrast, cells that do not respond to the

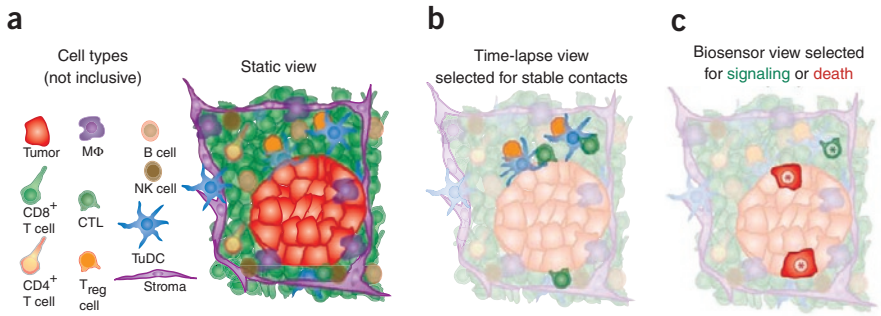

Figure 2 Emergent activity of collections of cells revealed by real-time analysis. (**a**) In this static image of a tumor microenvironment, a collection of cells of various types is observed in a crowded milieu. CTL, cytotoxic T lymphocyte; TuDC, tumor DC. (**b**) Time-lapse data of the tumor microenvironment in **a** can be displayed on the basis of persistent fluorescence intensity in a region; this can highlight contacts that are long-lived. Some, but not all, cells may adopt a particular motility or morphology phenotype or induce motile activities in adjacent cells. This would suggest either spatially organized niches for these cells or the presence of subsets in the population. (**c**) Biosensors allow further refinement and highlight cells that are actively receiving signals. Sensors are not necessarily biased toward motility arrest, but instead may 'read out' a larger collection of more subtle changes.

stimulus have much shorter interaction times[9]. This effect mostly does not depend on whether a DC seems to be central in the contact or not and is observed in many places in the lymph node. This type of activity criterion (differences in interaction time) can then be applied to determine further details of both subsets of cells involved, as well as the signals that may be transmitted at the contact.

The type of analysis described above will certainly be assisted by microscopes equipped with larger detection arrays that can achieve spectral detection. In a truly complex system, it is likely that tens or even hundreds of cells and types of cells should be identified over time. Although this sounds far fetched, at least that number of neurons can be identified by combinations of just three genetically encoded fluorophores and subsequently decoded via quantitative imaging in slices of live brain[10]. Such tools, however, will increase the need for software for careful tracking of cells and quantification of the dynamics of contacts. At this point, biosensors become a key requirement, and actually visualizing signaling represents the most profound ability to observe the different responses of cell subsets to stimuli, spatially and temporally and *in situ*.

Real-time assessments of functions?

Biosensors are poised to substantially enhance the ability to recognize meaningful information exchange as cells make contact. In the many cases in which there is good information about the requisite signaling pathways in cells of the immune system, it will be important to determine where and under what circumstances the pathway receives a signal. As the informational content of an immune insult disseminates through multiple cell types, it is important to discern the critical spaces and times at which signals are integrated *in situ*. The visualization of fluorescent fusion proteins of T cell antigen receptor signaling chains[11] and downstream kinases[12] has provided tremendous advances in the understanding of signaling dynamics in cells of the immune system in live cells *in vitro*. Such fusions allow tracking of receptors from a pre-engaged state through, in many cases, downregulation of the response. Similar approaches in small, mostly transparent organisms such as zebrafish[13] have provided additional insight by allowing analysis of the physiological context as a force in shaping signaling events. There is now evidence that the same types of fusions will provide information *in vivo*[14]. However, these remain relatively crude, and the requirement for rapid readouts with high yields of light make the field ripe for innovation. Of particular interest are fluorophores that are either activated or deactivated enzymatically, such that a small change in a specific signaling pathway gives rise to large changes in the intensity of signals. One intriguing class of these sensors is simply green fluorescent protein (GFP) or its derivatives used as tags on proteins with specific ubiquitinylation domains that are subject to degradation only after a very specific stimulus.

Tracking antigens

Returning to the question of information integration, a major goal for the next 10 years will be tracking information flow from the source all the way to the adaptive cells that respond to the peptides. Some success in this area has been provided by GFP-labeled bacteria or GFP-labeled target tissues such as the pancreas that allow visualization of phagocytic cells in contact with T cells[4,15]. However, this method is often short lived as a result of GFP degradation in the phagocyte, which leads to loss of signal. More stable labeling methods will allow the other cells that impinge on antigen-presenting cells to be followed more carefully.

Imaging of human models

What is observed depends on where the observer looks. Nowhere will this be more important than in this emerging era of system integration. Given the diversity of possible responding cells, many possible types of homeostatic or pathogenic cell-cell interactions are possible. It should not be a high

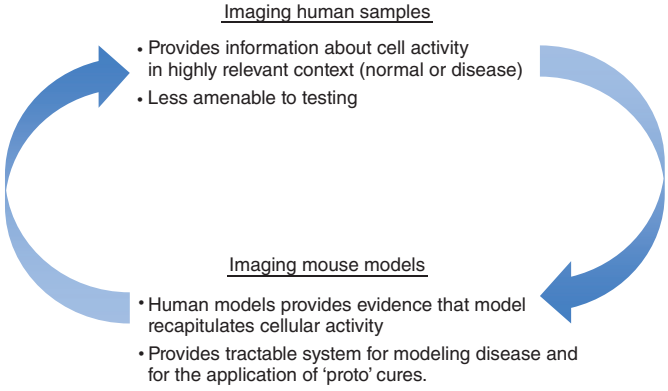

Figure 3 Real-time imaging–based feedback between confirmation and study of the nature of emergent activities. The establishment of imaging in human biopsy samples will permit cellular activities that are observed in mouse models to be confirmed as being relevant in a cohort of humans with that disease. Failure to recapitulate the results may provide reasons for adapting the model to more faithfully represent a true disease state. Further study leads back to confirmation, and indeed therapies may be first applied to mouse imaging model, then to a human biopsy model, as a prelude to a clinical trial. This model flow chart is presented for future studies in which the imaging of human samples provides corroborative data for mechanistic studies in mouse models. Subsequently, key mechanisms in mouse models can be applied and tested directly in normal or diseased human tissues.

priority at first to study immune disorders that are manufactured. A deficit in much of the live imaging that is undertaken at present is the absence of corroborating data for diseased human tissues. Thus, it remains unclear how the activities observed in immunized mice or mice that are described as models of human disease are related to the activities of such cells in actual human disease (ideal relationship, **Fig. 3**).

There are numerous reasons to think the situation will change. First, many excised organ tissues seem to survive very well *ex vivo*, notably the lungs[16] and skin[17]; thus, human biopsies may become targets for parallel studies in mice and men. Although the use of genetically encoded fluorescent proteins is restricted to mice, the viral delivery of fluorophores and the use of nonstimulatory antibodies to label tissues will go a long way in identifying key cell types to determine if the organization of the immune system in mouse models of a disease is truly reflective of the disease itself.

Conclusions

Although the tasks ahead are by no means trivial, there has already been outstanding success in areas that seemed insurmountable just 10 years ago. It will be important in the next period to focus on the most informative data sets and push the science into realms of complexity that might seem daunting at present.

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1. Koshland, D.E. Jr. *Science* **280**, 852–853 (1998).
2. Filippi, C.M. *et al. Diabetes* **57**, 2684–2692 (2008).
3. Gerosa, F. *et al. J. Exp. Med.* **195**, 327–333 (2002).
4. Tang, Q. *et al. Nat. Immunol.* **7**, 83–92 (2006).
5. Tadokoro, C.E. *et al. J. Exp. Med.* **203**, 505–511 (2006).
6. Boissonnas, A. *et al. Immunity* **32**, 266–278 (2010).
7. O'Gorman, W.E. *et al. J. Immunol.* **183**, 332–339 (2009).
8. Miller, M.J., Wei, S.H., Parker, I. & Cahalan, M.D. *Science* **296**, 1869–1873 (2002).
9. Sabatos, C.A. *et al. Immunity* **29**, 238–248 (2008).
10. Livet, J. *et al. Nature* **450**, 56–62 (2007).
11. Krummel, M.F., Sjaastad, M.D., Wulfing, C. & Davis, M.M. *Science* **289**, 1349–1352 (2000).
12. Yokosuka, T. *et al. Nat. Immunol.* **6**, 1253–1262 (2005).
13. Jontes, J.D., Emond, M.R. & Smith, S.J. *J. Neurosci.* **24**, 9027–9034 (2004).
14. Azar, G.A., Lemaitre, F., Robey, E.A. & Bousso, P. *Proc. Natl. Acad. Sci. USA* **107**, 3675–3680 (2010).
15. Egen, J.G. *et al. Immunity* **28**, 271–284 (2008).
16. Delmotte, P. & Sanderson, M.J. *Am. J. Respir. Cell Mol. Biol.* **35**, 110–117 (2006).
17. Lammermann, T. *et al. Nature* **453**, 51–55 (2008).