Imaging, Interpretation and Modeling in Modern Immunology

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1 Overview of the Field of the Meeting

Advances in microscopic imaging techniques have revolutionized our knowledge of biological cellular and subcellular dynamics in vitro and in vivo. Nowhere are these advances clearer than in modern immunology, where we have newfound abilities to chart cellular interactions in living tissues, and to identify and track individual molecular players at dynamic intercellular interfaces.

Fluorescence microscopy allows us to label individual objects with a fluorescent tag and watch them move. The objects can be individual immune cells, navigating between, and interacting with, other immune cells present in a lymph node during an immune response. At the single cell scale, we can label populations of cell surface receptor molecules and observe how they are redistributed as that cell responds (via surface receptor signaling) to a molecular stimulus present on another cell or in solution. By labeling low numbers of receptors, it is also possible to track the motion of an individual labeled receptor, and potentially infer the details of its interactions with neighboring molecules and cell surface features.

These experiments usually generate time-lapse data, representing a time-course of spatial information, and often many videos are generated during a single experiment. In order to properly interpret the wealth of spatio-temporal data, and generate quantitative and predictive models describing how immune cells behave, advances from the mathematical modeling community are needed. The main modeling tools that have been employed to date are deterministic and stochastic PDE, and agent based or lattice based simulations, often based on the Gillespie or cellular Potts model frameworks. In each case, parameter identification/estimation and fitting to complex spatiotemporal data (beyond the eyeball metric) has proven challenging and this was a particular focus of the meeting.

This BIRS workshop was structured around three complementary and interwoven themes, which are described here:

1. Two-photon microscopy of immune cell interactions in living tissues. Two-photon microscopy is a fluorescence microscopic technique that allows cells in living tissue to be imaged. The penetration depth into the tissue is much greater than with standard confocal microscopy and allows, for instance, in vivo imaging of individual fluorescently labeled immune cells moving within lymph nodes. The technique yields three-dimensional videos of cell motion that reveal details of intracellular motion and cellular interactions. Modeling of these experiments has been performed using various approaches, with considerable success. At this workshop, modellers presented their recent work on immune cell migration and inferring parameters of cellular interactions while experimental scientists outlined their new experimental approaches.

2. Molecular organization and signaling at cell-cell interfaces. T and B cells of the immune system can become activated by interacting closely with antigen-presenting-cells that carry particular antigenic molecules on their surfaces. Activation follows the establishment of a specialized cell-cell interfacial region that has been termed the immunological synapse (IS). Since the late 1990s, fluorescence microscopic experiments have revealed complex patterns of molecular reorganization at immunological synapses. Almost simultaneously, mathematical models were developed, seeking to explain the patterns. These models, usually based on PDE or stochastic simulations, have also been coupled with models of surface receptor signaling following receptor engagement by antigen. The eventual goal of experimentalists and theoreticians alike is to develop a consistent physical model of surface receptor signaling and cellular reorganization that explains the formation and function of immunological synapses. We invited recognized experimental and theoretical experts to discuss their recent work with a special focus on collaborative work.

3. Single particle techniques in cellular immunology. By labeling a single surface molecule with a fluorescent tag or fluorescent bead, we are able to track its motion across an immune cell surface, obtaining a time series of position data. By carefully analyzing the data, we should be able to learn a great deal about its motion (Brownian, corralled, directed motion, transient binding, etc) and also about the environment it moves in. There has been a great deal of theoretical work in this direction in recent years, much of it applied to immune cell surface proteins. However, a consensus on the best methods for analysis has not emerged. The current strengths and limitations of single particle tracking in immunology were discussed at the workshop, from both the experimental and theoretical perspectives.

Our specific objectives for the meeting were linked to the three main themes:

- comprehensively review previous theoretical developments in modeling two-photon data in immunology, and determine the most important new directions for modelers to take in this rapidly developing field.
- critically analyze some of the existing models related to cell signaling and organization of immunological cell-cell interfaces (immune synapses); determine how modeling and experiments can work best together.
- establish the most important mathematical/statistical problems in single particle tracking data analysis with special regard to immunological questions.

A secondary goal was to find similarities and crossovers between theoretical work developed for each of the main themes, and in particular between single-cell and single-particle tracking analysis techniques.

2 Selected Presentation Highlights

2.1 Theme One: Two-photon microscopy: experiment and analysis

Summary: Various statistical methods have been put forward to analyze the motion of immune cells moving through lymphoid and other tissues. A key classification of the methods, which became apparent at the meeting, is the difference between *track-based* and *step-based*. Track-based methods are global in the sense that the characteristics of the whole track are used to make inferences about the cell behaviour. Step-based methods are local, and are generally simpler to understand, because the track is broken up into its constituent steps (jumps in cell position between frames of the microscopic video) and the steps are then analyzed individually (to obtain displacement information) or in pairs (to obtain turning-angle information).

The obvious advantage of track-based methods is that the longer-term behaviour of the cells can be consistently analyzed, and changes in behaviour over time and space can be sought. However, a very serious disadvantage of the track-based methods is that the experimentally-observed volume is limited in the z-direction (vertical direction) and so cells commonly exit the field of view during the experiment. For this reason, very many tracks from a typical experiment are only a few frames in duration. There can also be issues with extracting reliable three-dimensional cell tracks if the cells are insufficiently sparse that they can be independently resolved. Additional experimental issues that have to be overcome include the relative lack

of precision in the z-direction compared to the x-y plane (due to the z-stack method of acquisition), significant background tissue drift over the course of the experiment, and the potential for labelled cells to have different levels of activation.

At the meeting, we heard from experimental and theoretical scientists, often working together, to resolve these issues and improve our basic understanding of immune cell motion and behaviour *in vivo*.

2.1.1 Selected Presentation Summaries:

Joost Beltman (Utrecht University) – "Analysing immune cell migration". The migration of cells between the so-called dark and light zones of the B cell germinal centre is important in our understanding of antigen-induced selection and proliferation of B cell clones. Two photon microscopy has been applied to studying the motion of cells and Joost presented analysis of the motion. Much of the talk was concerned with avoiding errors: and in particular errors arising near the boundaries of the observed region. Since cells that are near the boundaries cannot be confidently tracked (they may leave the observed region and be replaced by other cells migrating in, confounding tracking), such cells were left out of the analysis. Joost demonstrated a basic issue of analysis of mean square displacement, in the presence of immobile or very slow-moving cells - these cells dominate the displacement plots and give the impression that the trajectories can be confined. Therefore, he advocated for a more sophisticated model to understand if all the tracks available are consistent with a single model or if multiple models (for different cell behaviour types) are required. Joost performed a directed motion analysis based on turning angles between steps, and determined that migrating cells have a 0.2 μm/min movement bias toward the light zone. Certain tracks that are straighter than usual show an increased mean motion towards the light zone at 0.5 μm/min. This small, but significant motion bias has the potential to be very important in allowing dark zone cells to reach the light zone, and indicates that small hidden directed-motion components can be extracted from motion datasets via careful analysis. A final note is that the determined rate of directed motion is comparable to the background tissue drift in the experiments, which was around 0.17 μm/min. Establishing the rate of directed motion in the face of this significant effect shows particular utility of careful analysis.

Steven Kleinstein and Gur Yaari (Yale University) – "Method development and analysis of intravital two-photon data from the germinal center response". Continuing in the theme of understanding two-photon data from the germinal centre, Steve and Gur described work joint with the Haberman lab at Yale. Two-hour movies were taken at the boundary of the germinal centre, during the early stages of the germinal centre formation. Naive B cells, and antigen-specific T and B cells were imaged and the analysis sought to measure the levels of cell confinement, mobility, and directed motion. It was noted that activated (antigen-specific) B cells became more directed in their motion (compared to non-activated B cells) starting on day 2 after immunization. From a methodological standpoint, some of the most interesting discussions centred around the use of the mean of individual mean-square-displacement curves, versus the interpretation of each individual track displacement. A serious question that is raised is whether traditional MSD-based approaches are over-interpreting the noisy data and giving false positives for directed motion. A second part of this work looked at the motion of pairs of B and T cells (where operationally, pairs are defined as being within 20μm of each other). It was then possible to look at mean square displacement curves for pairs and for individual cells, separately. Relatively simple analysis indicates that a biological hypothesis that B and T cells interact and then enter the follicle, is not supported by the data. Beyond this point, it leads to the theoretical question of how pair-wise cell motions might best be analyzed to determine directional correlations and/or apparent pairwise interactions.

Johannes Textor (University of Lübeck) – "Defining the quantitative limits of intravital two-photon lymphocyte tracking". Johannes Textor addressed the following very basic question: *Are we not detecting directed motion that is biologically meaningful in the motion of naïve T cells?* He distinguished between different types of motion that could be described as directed: (i) orthotaxis (fixed advection direction), (ii) topotaxis (biased towards turning in one particular direction), (iii) klinotaxis (biased run length - like the run and tumble model for bacterial motion). In this talk he advocated for the use of Hotellings T2 test (a multivariate version of the familiar t test) to provide a theoretical basis for reliable detection of taxis. This

was demonstrated by comparison with mean-square-displacement methods. An application to chemotaxis in the rat mesenteric lymph node was presented.

Silvia Ariotti (Netherlands Cancer Institute) - "Skin patrol by tissue-resident memory T cells" Silvia, an experimentalist, described skin patrol by resident memory cells and showed very nice movies of cell movement in response to an antigen stimulus. The crawling behaviour or resident memory cells allows a population of T cells to cover a skin section with hours – cells crawl about 1 micron per minute with apparent persistent motion, and overall the cell tracks cover the area imaged, quite thoroughly, within 4hr. On the other hand, skin-resident antigen-presenting cells (langerhans cells) in the same region hardly move over 4hr. By micro-injecting peptide into patrolled area (either specific for T cells or not), T cells were observed to respond to specific antigen by rounding up and standing still immediately (and out to 12h post injection) - but the control peptide induced no change to the basic patrolling behaviour of the T cells. The fine time dynamics of patrolling were probed using a DNA vaccine that encodes a fluorescent tag and a T cell epitope. Specific T cells were observed to stop and round up before the fluorescence was detectable.

Frederik Graw (Los Alamos National Laboratory) – "Influence of the fibroblastic reticular network on cell-cell interactions in lymphoid organs" The question here is how does the anatomical structure of immune organs support the search of a naïve T cell for cognate antigen? A nice example is that the conduit system within the lymph node is destroyed by LCMV clone 13 infection, possibly for its own benefit? Frederik presented extensive simulation work where T cell movement was simulated in sparse and dense networks of follicular reticular cells (forming the framework of the tissue which T cells may migrate along). This on-lattice model incorporated a follicular reticular network of cells, connected by filaments, static and/or mobile dendritic cell targets for mobile T cells, and contained tens of thousands of simulated cells. In a dense network of cells, T cell behaviour parameterized from experiments, is approximately optimal, but the efficiency of the search for dendritic cells is less efficient when the network becomes less dense. However, the dependence on the details of the network was found to be weak on the whole. This leads to some interesting questions: experimentally, to image early persistent infection, to detect changes in cell-cell contact, as well as to establish assays for altered follicular reticular network experiments. Mathematically, the question of how to generate 3D networks with desired properties is an important one for this kind of study.

Thomas Kepler (Duke University) This late-night talk took us back to basics, presenting a model for cell dynamics in the germinal centre based on equations of motion (the Langevin equation approach). Defining position X, velocity implies $dX = Vdt$. The application of Newton's second law gives $dV = -\nu V + \phi P dt + F dt$ where orientation P follows $dP = -kPdt + \sqrt{\nu}dW + Gdt$ for rate of change of orientation ν, persistence k, thermal noise z and external forces F and G. This is a general model that can then be applied through a likelihood-based approach to experimental data for cell tracks. The approach uses more of the data than the traditional MSD plot, although it was noted again that track-based methods such as this run into trouble due to the tight confines of the experimentally observed box in the tissue. In particular, this talk was a reminder that many of the other issues we are now facing have a prehistory in physics that should be exploited.

2.2 Theme two: Spatial Organization and Immune Cell Signaling

Summary: This was probably the most varied sub-theme of the meeting, likely because the basic science of ligand detection and activation via antigen is under very rapid development and this has led to many different kinds of mathematical modelling. Additionally, the other two topics (two-photon microscopy and single particle methods) are very much concerned with fitting to a well-defined experimental data set. In this topic, however, we are often more concerned with qualitative fitting and even conceptual models. In terms of experimental talks, we heard a lot about high-resolution fluorescence microscopy, linking to other assays to measure signalling in chemical terms. From the theorists, we saw differential equation and simulation-based approaches to the fundamental questions: how does ligand binding and unbinding lead to signaling, and what physical mechanisms best explain surface organization of receptors and signalling players?

2.2.1 Selected Presentation Summaries:

Ken Jacobson (University of North Carolina, Chapel Hill) - "C-type lectins in domains on surfaces of dendritic cells" This talk showcased how different light-microscopic experimental techniques can reveal a lot of information about particular cell surface molecules at the scale of 10s of nanometers. C-type lectins, including DC-SIGN (a role of which is attachment of HIV particles to cells) are expressed in nanoscale domains on the surface of dendritic cells. The nano domains were estimated to have a median size of 615nm, and are found to be independent of cytoplasmic domains or an intact actin cytoskeleton. Using fluorescence recovery after photobleaching experiments, DC-SIGN is found to be immobile (no recovery is observed). However, scanning fluorescence correlation spectroscopy confirms this result – DC-SIGN is again found to be almost immobile, whereas hemagluttinin monomers diffuse freely within the domain. Particle tracking experiments with quantum dots further confirm that DC-SIGN is barely diffusing. Blink microscopy reveals that 40nm-200nm clusters of DC-SIGN are observed, but careful observation of bleaching in TIRF shows that most of the domains contain less than 10 DC-SIGN molecules (maybe there are 1-3 tetramers per domain) and thus perhaps the overall domain is built from several elemental domains? All this work leads to deep questions about the structure and mechanics of nano clusters and DC-SIGN organization therein: What is the true dimension of the nano domain? What is the scaffold that organizes and stabilizes nanodomains? Does pathogen binding permit local lateral mobilty and clustering of the pathogen? Solutions to these problems will speak to function of the DC-SIGN molecule in disease, for instance pathogen cargoes bound to DC-SIGN can undergo fast (microtubule-driven?) retrograde transport. Does the stability and diffusional immobility of the domains increase effective avidity and binding stability of pathogens?

Rajat Varma (National Institute of Allergy and Infectious Disease) – "TCR micro clusters" In this talk, Rajat reviewed the pre-history of TCR micro clustering, leading up to his own work where it was shown that (on supported planar bilayers doped with cognate antigen), clusters of TCR form and migrate to the centre of the nascent synapse, with cluster formation and motion continuing for many minutes as the immune synapse develops. This analysis, facilitated by TIRF microscopy, was extended to examine central vs peripheral signalling and the role of actin. Microclusters are shown to exclude the membrane phosphatase CD45, their formation is actin dependent but resistant to treatment with Src kinase inhibitors (such as PP2). However, application of latrunculin after clusters have formed reveals that the existence of clusters is now actin independent (modulo loss of migratory capacity). Rajat also showed experiments with labelled pMHC on bilayers, finding that at low densities there is typically only about one MHC per microcluster, and some go to the centre, indicating that they are trapped and transported in the TCR clusters. The talk finished with some interesting discussion on the etiology of clusters – are they simply frustrated endocytic vesicles? – and to what extent central clusters retain any signalling potential at all.

Omer Dushek and Anton van der Merwe (University of Oxford). In this double-act presentation, Omer and Anton examined the experimental and theoretical evidence for the kinetic segregation model of TCR signalling. In this model, the close juxtaposition of membranes achieved by a pioneering TCR-pMHC bond leads to an exclusion of the membrane phosphatase CD45 from the bond area. The change in the kinase-phosphatase balance then promotes signalling in a defined area of the immune synapse. This had previously been experimentally probed by generating CD45 mutants with small ectodomains. From a theoretical point of view, Omer presented an elastic membrane model with showed how an initial single bond would be subject to very high force at initial formation, possibly causing a high off-rate, but this effect is mitigated over time as long surface molecules (such as CD45 or CD43) are forced away from the close contact induced by TCR-pMHC binding. A summary of tested predictions of the kinetic segregation model was made, including: (1) Long molecules such as CD45 or CD148 should segregate away from contact points. This was experimentally demonstrated by Bunnell and Varma. (2) Truncated CD45/148 should inhibit triggering and not be excluded from signalling micro clusters. New data was presented to show that this is true. (3) By artificially lengthening the TCR-pMHC complex dimensions (Choudhuri 2005, 2009)), signalling is inhibited. Overall, we can be certain that kinetic segregation effects are playing an important role in TCR signalling.

Nigel Burroughs (University of Warwick) Theoretical models of segregation have been based on elastic sheets interacting by the formation and breaking of elastic bonds with different rest lengths. Based on detailed

image analysis of imaged cell interactions, and comparison with this type of model, Prof. Burroughs argued convincingly that a prototype 'small' molecule bond (KIR-HLA) is happiest (most favoured) at a length of 12nm, while a longer surface molecule – ICAM-1 – is happy with an 18nm separation between the membranes. By further careful analysis of the imaging, it was shown that the elastic constant (stiffness) of the deformable ICAM-1 molecule was smaller than that of the KIR-MHC bound pair. This leads into stability analysis of the related PDE system, which yields an interesting onset criterion for pattern formation in the two-species system, that is consistent with imaging data under the obtained parameter estimates.

Daniel Davis (Imperial College, London) Dan Davis presented recent super-resolution imaging of natural killer cell synapses, investigating the underlying physical mechanisms of signalling and functionality. These cells are a branch of the immune system that kill virus- or bacteria-infected cells, transformed tumour cells, etc that are missing the MHC-I molecule. MHC-I actually inhibits the signalling of the KIR receptor on the NK cell surface. Using TIRF microscopy of cells on a supported planar bilayer, the Davis lab investigated the formation and motion of signalling microclusters. The observation is that the micro clusters assemble into rings through which secretion of labelled lytic granules occurs. This leads to a good mechanical question – how do lytic granules pass through the actin meshwork that is presumably present at the hole in the ring of micro clusters? It had been thought that actin is cleared out from the centre of the ring. To clear this up, super-resolution microscopy using structured (STED-like) illumination was applied. This technique allows sub optical resolution of 100nm or less to be achieved in the xy plane of imaging. The results were surprising – rather than completely clearing, the actin mesh appeared to have been partially cleared, leaving holes of areas from 0.08 to 0.2 μm^2. This measurement has to be considered relative to the estimated size of 200-250nm diameter of a lytic granule (from EM studies). Using careful image analysis, it was shown that up to 4% of the synapse becomes potentially penetrable by a lytic granule, and that these regions are clustered together in the central part of the immune synapse. Further imaging indicates that actin mesh gaps and lytic granules colocalize, under the requirement for LFA-1 engagement by ICAM-1 in the bilayer. Summarizing, the periodic mesh structure of actin defines secretory domains in the central synapse, allowing lytic granules to dock and exocytose.

2.3 Theme three: Single particle tracking

Summary: Single particle tracking (SPT) is the experimental technique of labelling a single molecule and then following its motion, with the goal of inferring information about its interactions with the environment. In the context of immunology, particle tracking has been extensively applied to the motion of cell surface receptors, with the goal of understanding the relationship between receptor mobility and signalling. Understanding particle mobility through SPT requires the application of a mathematical model and is rather similar to the problem of understanding cell motility from two-photon microscopy, described above in theme three, although with a rather different set of experimental constraints.

2.3.1 Selected Presentation Summaries:

Raibatak Das (University of British Columbia) Using SPT data on LFA-1 and CD45 obtained by the lab of Chris Cairo, Raibatak presented a Markov-process approach to track heterogeneity. Essentially, in both cases the diffusing labeled protein is assumed to have a relatively immobile, unlabelled binding partner. Upon binding, the labeled protein slows down, regaining its mobility only upon unbinding. This yields a four-parameter model (the four parameters are the two diffusivities for bound and free protein, as well as first-order kinetic rates for the binding transition). Together with the data, this makes a hidden Markov model, where we are able to observe the particle positions, but not its binding state. To fit the data, we can use techniques originally designed for signal processing to efficiently perform maximum likelihood over the parameter space. Using simulated data, the method is benchmarked and shown to be effective provided the two states are reasonably distinguishable. Biologically, the method can now be used to make quantitative comparisons between protein behaviour on control cells versus when the cells are treated with drugs. In particular, treating the cells with a receptor-independent activator, PMA, decreases the diffusivity of LFA-1 while increasing the rate of transitions, perhaps implying that activation increases the number of binding locations for the receptor. Similarly, CD45 on Jurkat T cells is observed to increase its mobility when the

cells are treated with cytochalasin, a cytoskeletal inhibitor, but decrease on PMA treatment. Raibatak also showed developing work to identify directed motion from these kinds of datasets.

Gerda de Vries (University of Alberta) In this talk, Gerda showed how methods that have previously been used to study large animal motion in ecological settings can be adapted to address protein motion on the surface of live cells. To begin, we can verify that a motion is not simply diffusive by shuffling the step lengths and turning angles from a given walk, and determining that the motion now has a different macroscopic diffusion constant. This analysis was applied to the motion of LFA-1 on the surface of Jurkat T cells, to show that the motion is not simply diffusive. From the distribution of turning angle and step length, the mean square displacement over time of the labelled LFA-1 is shown (for most of the trajectories) to be much smaller than would be predicted by the correlated random walk. A possible explanation for this is the presence of transient confinement zones fro the LFA-1 on the cell membrane. Here, an analysis of the Variance First Passage time for the particle, can be applied. This approach is summarized as follows: fix a radius r and place the circle at one point of the trajectory. Then count the number of steps before the trajectory exits the circle. This can then be averaged over all possible index points, to give the distribution of observed first passage times for a given r. Summary statistics such as the mean and variance of the distribution, can then be plotted against r. In ecological applications, the variance has been used. A peak in the plot of variance first passage time is interpreted to correspond to a possible confinement zone radius. In the case of LFA-1, confinement zones on the scale of < 200nm are detected, generally in line with the presentation of Raibatak Das. Although somewhat ad-hoc in application, this method seems to be a powerful approach to the detection and characterization of transient confinement in SPT data.

Michael Saxton (University of California - Davis) This talk, by one of the leading theoretical experts on single particle tracking, described some of the main issues confronting practitioners in the area of SPT interpretation. A key statement that was made is "the basic principle is that the pure random walk is the control and null hypothesis. Therefore, if you claim that something happens, you must evaluate the probability that it occurs by chance in a pure random walk." Compounding this issue is that humans are very good at detecting apparent patterns in randomness. Furthermore, MSD plots for a few truly random trajectories can reveal periods of apparent confinement, directed motion, etc. Some practical advice was offered: At the start of an analysis, generate a bunch of truly random walks with the same number of steps as in the experiment. Then subject both sets of tracks to the exact same analysis. This would resolve a key, recurring issue in over interpretation of data. Prof. Saxton also provided some pointers for future work by experimentalists and theorists: (i) to establish a set of control tracking experiments and standardized techniques for analysis, to allow inter-lab verification. (ii) (related) to establish a common control for anomalous sub diffusion. (iii) to establish a clean control for clustering / transient confinement detection.

3 Continuing developments

At the meeting we identified some important features of particle or cell tracking that merit further consideration:

- The basic classifications of parametric vs nonparametric and stepwise vs trajectory-wide analysis. Both stepwise and trajectory methods are used by membrane biophysicists but they're treated simply as alternatives. Some labs choose one and some choose the other. The meeting emphasized the differences between them and showed that both are needed.

- A list of characteristic times in particle tracking: (i) correlation time for motion; (ii) frame rate; (iii) total measurement time. All of these are important, and it's useful to think in terms of the list. Additionally, for confined motion, there is also the characteristic time for observing motion in a corral (2D or 3D) of radius r, that is equivalently, the "bend time" in a plot of MSD vs t, cR^2/D, where c depends on corral geometry. There are also two much longer characteristic times, readily defined for confined random walks on lattices: the time to visit each site, and the time to visit each site uniformly. In general, for each method of analysis, there should be a characteristic time for detecting confinement

(of a specified size, at a specified probability...) In addition there is the escape time from a permeable corral, which sets limits on the total measurement time for a single corral.

- How scattered and disjoint the single-particle literature is. The literature doesn't even have a single keyword. Michael Saxton has argued in a Nature Methods news article for consistent use of "single-particle tracking", to improve searchability.

A general focus of much discussion at the meeting was the question "How do cells move?" or, rather, "What model is appropriate to describe cell movement?". The discussion ranged from the purely theoretical (Which models are possible?) to the recurring practical question of when can it be said that a model is validated, or ruled out, based on a set of data.

4 Outcome of the Meeting

The meeting was exciting and memorable for attending experimental and theoretical immunological scientists alike, and there was broad enthusiasm for a follow-up meeting in 2-3 years, either at BIRS or as a FASEB meeting.