Unravelling the signal-transduction network in B lymphocytes

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The Alliance for Cellular Signaling has chosen the mouse B lymphocyte as a model system to understand basic principles that govern cellular signalling. Progress to that end has focused initially on establishing a reproducible experimental cell system and characterizing essential signalling responses. Although unravelling this complex network will take years, findings revealed in the interim will prove immensely useful to the scientific community at large.

The B lymphocyte has an essential role in host defence and maintains a large complement of cell-surface receptors that regulate its many immunological functions. B lymphocytes travel through the circulation and migrate constantly in and out of the spleen and lymph nodes, ready to act when they encounter antigen, cytokines, bacterial endotoxin, T-cell surface molecules or other stimuli[1–3]. Migration is directed by a cooperative set of chemokines, and survival is promoted by other environmental stimuli[4–6]. They may proliferate and differentiate into antibody-secreting plasma cells when stimulated by antigen, and undergo programmed cell death in the absence of stimulation[7]. Scientists have already begun to unlock the key steps in signalling pathways that direct these important functions.

The Alliance for Cellular Signaling (AfCS) chose to study resting B lymphocytes because they possess interesting biological properties regulated by interacting signalling systems. But our goal is not to recapitulate findings that have stimulated immunologists for decades. Rather, we want to collect data from complex signalling pathways in a carefully controlled and defined cell system to address questions about the basic principles underlying signalling mechanisms.

Characterizing the cells

Achieving reliable, experimentally derived measurements from these cells requires the development of a standardized procedure for isolating and purifying splenic B lymphocytes. Borrowing from established methods[8], investigators at AfCS laboratories devised and refined a reproducible procedure for isolating splenic cells and studying their responses to ligands *in vitro*. As detailed in the first AfCS research report (see www.signaling-gateway.org/reports/v1/BC0001/BC0001.htm), the isolation procedure produced a cell population that is >95% B lymphocytes, of which >80% are resting, mature B2 cells. Cells isolated in this manner maintain their viability over short-term culture and demonstrate reproducible functional responses *in vitro* to an array of 'sentinel ligands', which include anti-immunoglobulin M (anti-IgM) antibody, terbutaline, anti-CD40 antibody, stromal cell-derived factor-1α (SDF-1α) and interleukin (IL)-4.

The short lifespan of splenic B lymphocytes in culture limits application of many tools essential for studying signalling networks, including expression of foreign DNA and retroviral strategies for expressing RNA-interference constructs. For this reason, we used the WEHI-231 cell line as an experimental surrogate. Although no cell line is a perfect substitute, WEHI-231 cells respond to many ligands that act on primary B lymphocytes and are readily amenable to transient and stable transfection of recombinant constructs[9–12]. So far we have demonstrated robust signalling responses in WEHI-231 cells that are similar to those observed for splenic B lymphocytes. WEHI-231 cells also migrate in response to chemokines and undergo apoptosis in response to B-cell receptor (BCR) stimulation.

We expect WEHI-231 cells to serve as useful adjuncts to our studies of signalling networks of B lymphocytes and as a primary experimental system for the 'Focus on PIP3 modules' (FPM) project, which is designed to accelerate analysis of signalling networks by investigating the relatively small subset of the network that regulates concentrations of the membrane lipid, phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3 or PIP_3). However, these cells will impose certain limits on our ability to study desired signalling pathways. For example, the functional response to BCR stimulation in WEHI-231 (apoptosis) differs from that of splenic B lymphocytes (proliferation)[13,14]. Although the basic organization of most of the WEHI-231 signalling network will probably resemble that of splenic B lymphocytes, analysis of specific pathways may require us to turn to other cells. Accordingly, we are exploring other possibilities, including using splenic B lymphocytes whose life in culture is extended by stimulation with agents that promote survival, such as BAFF or CD40 ligand[5,6]. Alternatively, we can isolate cells from mice that specifically express the human Bcl-2 transgene in their B-lymphocyte population to promote survival[15]. If such cells prove amenable to transfection, they will allow us to study portions of the B-lymphocyte network in addition to those that are retained from the resting state.

Estimating the complexity of the system

Having developed a relatively well-defined system for study, we are now beginning to examine the complexity of the B-lymphocyte signalling network, including identifying the components that participate in the network, identifying interactions that might occur among them, and characterizing the breadth of signalling responses.

Gene-expression profiles

To gain initial insight into the spectrum of signalling components that are expressed in our population of resting B lymphocytes, we have established a collaborative effort with the Genomics Institute of the Novartis Research Foundation[16]. A profile of gene expression has been

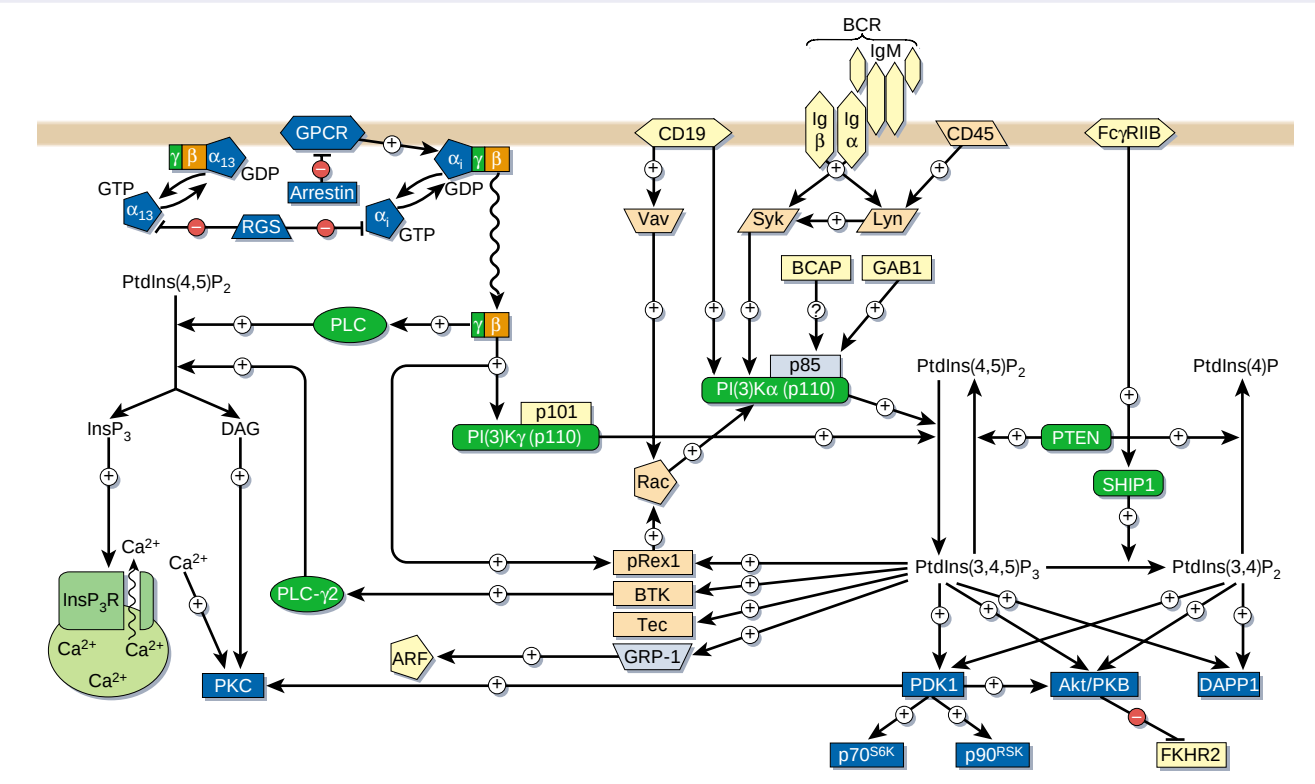

Figure 1 Graphical representation of the PIP_3 signalling module in B lymphocytes.

made using mouse Affymetrix GeneChip arrays which has estimated expression patterns of some 12,000 transcripts. The entire profile can be viewed online at www.signaling-gateway.org/reports/v1/BC0001/SupplGNFArray.xls. These data will supplement additional large-scale transcriptional profiles being compiled within the AfCS laboratories. More detailed analysis of this collection will allow us to focus attention on signalling proteins that are likely to participate in the signalling network of the B lymphocyte.

Protein–protein interactions

In collaboration with Myriad Genetics, we are beginning to compile information on protein–protein interactions from a high-throughput yeast two-hybrid screening system. So far, ~160 signalling proteins have been selected and over 600 bait–DNA-binding-domain hybrids have been prepared for screening. Currently, ~317 interactions from 33 baits have been identified and listed on our web site. This small sample already includes several well-established interactions, including those of phosphatidylinositol-3-OH kinase (PI(3)K) p110α catalytic subunit with the p85α regulatory subunit[17], and the serine/threonine kinase Pak with the small G protein Rac[18]. Several new interactions were identified, including those of phospholipase Cγ with the lipid phosphatase SHIP1, and of the tyrosine kinase Btk with the protein phosphatase calcineurin. This ongoing project promises to provide us with a wealth of new information on protein interactions.

Identifying signalling pathways

The alliance's most extensive experimental effort so far has been identifying some of the multiple signalling pathways stimulated in splenic B lymphocytes in response to each of 32 soluble stimulating ligands. The ligands, identified from surveying the literature, include chemokines, a BCR stimulus, lipids, biogenic amines, and multiple interleukins and cytokines that are known or suspected to have effects on B lymphocytes. The goal of this initial screen was to determine which ligands give functionally unique responses across a panel of signalling assays. Experimental responses measured include: changes in intracellular concentrations of calcium and cyclic AMP, immunoblots of antiphospho antibodies against ten distinct signalling proteins, and expression of approximately 10,000 mouse genes using an Agilent complementary DNA microarray. These assays were chosen to sample a broad range of signalling pathways present in these cells; to these we will add analysis of ligand-induced changes in concentrations of glycerophospholipids and we will expand the panel of antiphospho antibodies.

So far, 18 of 32 ligands showed a positive measurable response in one or more assays, which is consistent with published studies describing the signalling behaviour of these ligands. As expected, ligands known to act primarily on G_s-coupled receptors induced increases in cAMP (with some kinetic differences), but showed no effect on calcium concentrations. Chemokines, which stimulate G_i, all produced kinetically similar increases in intracellular calcium that were accompanied by proportional increases in phosphorylation of Erk1/Erk2 and other targets. A transient increase in cAMP, previously reported for some chemotactic factors[19], was also observed with most of the chemokines that act on B lymphocytes. Changes in gene expression, some substantial, have been detected with virtually all ligands, but more experimentation and analysis will be necessary to distinguish small changes from background noise.

Anti-IgM, CD40 ligand and IL-4 produced many changes in gene expression that are concordant with their varied biological effects on resting B lymphocytes, including proliferation, survival, differentiation and cytokine release. Chemokines produced relatively fewer changes, which is also consistent with their more focused role in directing cell migration. Among the 14 ligands that did not elicit detectable responses in our panel of assays is BAFF, which shows clear proliferative and pro-survival effects on cultured B lymphocytes. BAFF acts on a group of receptors related to tumour-necrosis factor (TNF) receptor, which stimulate the TRAF family of signalling components[20]. Signalling through this class of receptors typically results in activation of nuclear factor-κB and/or Jnk[3,20]. Thus our panel of assays is failing to detect signalling pathways triggered by BAFF (and

perhaps by other related ligands, such as TNF-α). The importance of these ligands in B-lymphocyte biology warrants the addition of screening assays and antiphospho antibodies that may permit detection of such signalling responses.

Despite the small number of assays and phosphoproteins examined so far, the overall data set is information-rich, and more extensive analysis will enhance its value. We are just beginning the next experimental step in assessing signal complexity, that of measuring responses to combinations of ligands. The goal will be to detect responses to pairs of ligands that are not geometrically additive sums of responses to the individual ligands. We hope to discover new synergies or inhibitory interactions between ligands, in addition to confirming those already documented. The level of complexity is likely to be great and it will take time to amass the data necessary to begin modelling an entire cellular signalling system. Within the next two years, however, focusing our resources on a more limited and well-defined portion of the signalling network offers a more practical approach to the challenge of network analysis.

A small-scale, detailed analysis of PIP_3 signalling networks

We decided to focus on PIP_3 to recapitulate on a smaller scale the experimental aims of the AfCS (see introductory article on pages 703–706), in part because PIP_3 offers opportunities to monitor signalling processes not only temporally, but also spatially (at the level of individual cells and subcellular compartments)[22]. This approach is more informative than the measurements made with cell populations, which offer only averages of many individual events.

To bring the network to an experimentally manageable size, we choose to assess only short-term responses (<20 min) to two stimuli (a chemokine, SDF-1α or BLC, and a BCR stimulus) and to analyse only the signalling network upstream of PIP_3 accumulation (except that phosphorylation of PIP_3-regulated downstream proteins such as Akt/protein kinase B will serve as quantitative readouts for PIP_3 accumulation). Although these stringent limitations will prevent the FPM from considering potentially critical feedback loops downstream of PIP_3, they do define a target network of 100–200 proteins (Fig. 1). This is a small enough network to be manageable and large enough to make it clear that the FPM is not competing with individual laboratories. The proposed FPM project will be carried out in WEHI-231 cells.

Identifying a focal point within the vast cellular network of B lymphocytes provides a basis for prioritizing and directing development of specific 'Molecule Pages', acquiring reagents and extending experiments already under way (for example, yeast two-hybrid screens). The alliance will progress towards its larger objectives while gathering data necessary to implement the FPM project. Our literature-based parts list, for example, has been focused to highlight key players in the chemokine and BCR signalling cascades. The list presently includes proteins that synthesize or degrade phospholipids, as well as receptors, adapters, scaffolds, G-protein components, phosphoproteins and other signalling molecules.

On the experimental front, the FPM project will aim to develop assays that directly measure PIP_3 activity. Additionally, because models of network behaviour depend critically on measuring flow of information through network nodes in time and space, one of the main concerns of the FPM will be to develop assays for activities of intermediate proteins within the PIP_3 signalling network.

These strategies will include the following four analyses. First, phosphorylation of key network proteins will be assessed by immunoblotting with phospho-specific antibodies. In collaboration with Cell Signaling Technology, AfCS researchers have begun testing dozens of such antibodies, directed (for example) against enzymes that synthesize or degrade PIP_3 (including the p85 regulatory component of the PI(3)Ks, PTEN and SHIP1). Second, protein–protein associations will be analysed in response to ligand stimulation. AfCS investigators are constructing retrovirally encoded pulldown baits for proteins in the PIP_3 network. Associated proteins will be detected by two-dimensional gel analysis and mass spectroscopy, and by immunoblotting. This class of intermediate responses will also help expand the parts list and modify the virtual epistasis map. Third, using green-fluorescent protein tags, the subcellular distribution of all FPM proteins will be assessed. Robust ligand-dependent translocation of a protein between compartments will serve as a useful intermediate endpoint. Finally, fluorescence resonance energy transfer (FRET) probes, which can measure associations of proteins in time and space, will target FPM proteins whose activities are especially critical for information flow through the network.

Whither from here?

Like most good experiments, our exploration of the B-lymphocyte signalling network requires considerable preparation and will take longer than we had hoped. It is too early to predict the experiment's outcome, but the first steps augur well. The project has: devised a convenient experimental cell system that produces reproducible results; begun to assess complexity of outputs to multiple ligands, alone and in combination; begun to develop a comprehensive 'parts list' for signalling in B lymphocytes; and laid groundwork for constructing epistasis maps and assessing intermediate endpoints. Finally, we have embarked on a focused but nonetheless ambitious effort to delineate and dissect a small portion of the signalling network of B lymphocytes. Scientists will enjoy continuing access to every aspect of the experiment on the web and will, we hope, profit from its results. □

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