

Multi-pathway network analysis of mammalian epithelial cell responses in inflammatory environments

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Abstract

Inflammation is a key physiological response to infection and injury and while usually beneficial it can also be damaging to the host. The liver is a prototypical example in this regard because inflammation helps resolve liver injury but it also underlies the etiology of pathologies such as fibrosis and hepatocellular carcinoma. Cells sense their environment, including the inflammatory environment, through the activities of receptor-mediated signal transduction pathways. These pathways are organized in a complex interconnected network, and it is becoming increasingly recognized that cellular adaptations result from the quantitative integration of multi-pathway network activities, rather than isolated pathways causing particular phenotypes. Therefore, comprehending liver cell signalling in inflammation requires a scientific approach that is appropriate for studying complex networks. Here we review our application of systems analyses of liver cell signalling in response to inflammatory environments. Our studies feature broad measurements of cell signalling and phenotypes in response to numerous experimental perturbations reflective of inflammatory environments, the data from which are analyzed using Boolean and fuzzy logic models and regression-based methods in order to quantitatively relate the phenotypic responses to cell signalling network states. Our principal biological insight from these studies is that hepatocellular carcinoma cells feature uncoupled inflammatory and growth factor signalling, which may underlie their immune evasion and hyperproliferative properties.

1 **Introduction**

2 Inflammation is a mechanism for preserving homeostasis in response to noxious stimuli such as infection
3 and injury. Cells of the innate immune system drive the inflammatory response, which typically begins
4 when proinflammatory stimuli activate macrophages residing in the affected tissue to produce
5 chemoattractants that recruit neutrophils to the site of inflammation. Neutrophils are white blood cells that
6 sense and eliminate pathogens. They carry out their functions in part by release of oxygen radicals and
7 degradative enzymes whose leakage can cause collateral tissue damage [1]. Once the inflammatory
8 stimulus is cleared, the inflammatory response is damped by a process called resolution, which is marked
9 by the transition from neutrophil to monocyte recruitment. Monocytes are blood-borne macrophages that
10 differentiate into macrophages once inside tissue. They serve to clear debris and promote tissue repair.
11 The inflammatory response is therefore characterized by processes that cause both damage and repair.
12 The damaging processes must be sufficiently strong to eliminate the inflammatory stimulus but not too
13 strong so as to cause excess tissue damage. A maladapted inflammatory response can lead to chronic
14 inflammation, which is a hallmark of many complex diseases such as cancer, fibrosis, cardiovascular
15 disease and diabetes. A better understanding of inflammation could therefore improve therapeutic
16 approaches to acute and chronic diseases.

17
18 The liver plays a central role in maintaining homeostasis through its functions in metabolism,
19 detoxification and inflammation. The liver is a key participant in the initial systemic response to
20 inflammation, called the acute phase response, because it synthesizes acute phase proteins such as C-
21 reactive protein, serum amyloid A and fibrinogen [2]. Conversely, components of the inflammatory
22 response are important in liver physiology and pathophysiology. The cytokine interleukin-6, for example,
23 serves to protect the liver when it is injured and promotes liver regeneration [3]. Inflammation can
24 contribute to pathological states of the liver, perhaps best exemplified by chronic inflammation due to viral
25 infection (e.g., hepatitis B and C), toxic substance exposure (e.g., aflatoxin-B1), or steatosis serving as a
26 precursor to hepatocellular carcinoma [4]. Hepatocellular carcinoma is the fifth most prevalent cancer
27 worldwide and is notoriously difficult to treat, which underlies its status as the third most lethal type of
28 cancer [5]. The considerable burdens of liver disease and diseases linked to chronic inflammation
29 emphasize the need for investigating the interplay between the liver and inflammation.

30
31 Cells adapt to their environments by the activities of receptor-mediated signalling pathways. The
32 biochemical activities of these signalling pathways regulate gene expression, metabolism and/or cell
33 structure in order to modify cell physiology. For example, in the case of hepatocytes during the acute
34 phase response, IL-6 released by macrophages and stromal fibroblasts at the site of inflammation acts
35 hormonally on hepatocytes by binding and activating a receptor complex leading to phosphorylation and
36 dimerization of the signal transducers and activators of transcription 3 (STAT3) [6, 7]. STAT3 is a
37 transcription factor that regulates the transcription of a number of genes including many involved in the

38 acute-phase response. The communication between cells acting at the site of inflammation and those of
39 the liver exemplify how the cells of multi-cellular organisms communicate in order to maintain
40 homeostasis.

41
42 The activities of individual pathways such as IL-6-STAT3 have been well studied in liver cells. However,
43 studies of isolated pathways have limited applicability to the *in vivo* situation in which cells are continually
44 exposed to multiple extracellular molecules leading to the simultaneous activity of many signalling
45 pathways. In inflammation, for example, cells are exposed to multiple pro- and anti-inflammatory
46 cytokines (e.g., tumour necrosis factor- α (TNF- α), interleukin-1 (IL-1), IL-4, IL-10, etc.), growth factors,
47 hormones and other molecules. Intracellular signalling pathways crosstalk with each other, effectively
48 forming a network, but it is poorly understood how intracellular signalling networks processes the
49 combinatorial action of multiple environmental cues. Making progress in this area will improve our
50 understanding of complex physiological responses such as inflammation and help guide the development
51 of better therapeutics. Here we review our studies of the intracellular signalling systems of hepatocytes
52 and hepatoma cells in response to inflammatory environments using a systems approach. We begin by
53 explaining what a systems approach to biology means followed by reviewing three studies in which we
54 used this approach to investigate liver cell signalling and physiology in inflammatory contexts.

55

56 **Scientific approach: Cue-signal-response experiments and mathematical modelling**

57 A hallmark of engineering practice is applying a systems approach to the design process. In this context,
58 a “systems approach” denotes studying a system by applying diverse inputs to the system and measuring
59 the outputs. Mathematical models are then used to model the relationship between input and output. The
60 parameters of the mathematical model are tuned such that the desired outputs are obtained from the
61 inputs expected under operating conditions. The engineer then modifies the design to reflect these
62 optimal parameter values.

63

64 We adopt a similar engineering approach in investigating biological systems, with a difference being that
65 we seek to “reverse engineer” the system by using input-output relationships and selected measurements
66 of the system to constrain a model of the intracellular signalling network. We implement an experimental
67 paradigm called “cue-signal-response” that reflects this input-system-output relationship [8]. The input
68 consists of molecules in the cell’s environment, the system is the cell signalling network and the output is
69 the behaviour that the cell executes to adapt to the input. In practice, a cue-signal-response paradigm
70 applied to hepatocyte physiology during inflammation involves applying inflammatory cytokines and
71 growth factors (cues) to cultured hepatocytes or hepatoma cells, perturbing intracellular signalling by
72 inhibiting kinases with small-molecule inhibitors, assessing intracellular signalling by multiplexed
73 measurement of phospho-protein levels (signals), and measuring the secretion of cytokines (responses).
74 Specific cues include inflammatory cytokines such as IL-6 and IL-1 and inhibitors target kinases such as

75 mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK),
76 phosphoinositide 3-kinase (PI3K) and p38 MAPK. The cells are lysed at specific time points and
77 multiplexed bead-based flow cytometric immunoassays based on Luminex xMAP technology are used to
78 measure the levels of ~15 phosphorylated proteins including Akt, ERK and signal transducer and
79 activator of transcription 3 (STAT3). Similar assays are also used to measure the levels of ~50 secreted
80 proteins in the cell culture media.

81
82 The resulting dataset features thousands of data points, which makes it challenging to interpret.
83 Mathematical tools are therefore used for downstream analyses. Classically, ordinary-differential-
84 equations have been the method of choice for analyzing cell signalling systems because they make direct
85 use of biochemical rate equations that describe the kinetics of enzyme-catalyzed reactions, protein-
86 protein interactions and transport processes. Disadvantages of ODE models include their critical
87 requirement for firm specification of network topological interactions, and their reliance on adjustable
88 parameters that must be robustly estimated in order to effectively represent the system. As the size of the
89 model grows, so does the uncertainty in the topology and the corresponding number of parameters,
90 which in turn increases the demand for more comprehensive biological knowledge and intensive
91 experimental data (as well as computational power, although that is a lesser challenge at this point).
92 While studying signalling from one or two pathways with ODEs is feasible, the networks that we study are
93 too uncertain and large for ODEs to be practically useful. We therefore use modelling techniques that
94 represent the system in a coarser grained fashion. In doing so, we require less data to obtain quantitative
95 insight into the system, albeit less than could be obtained with ODEs.

96
97 Our studies feature two types of mathematical frameworks, regression-based methods and logic-based
98 methods. Our implementation of these techniques has been reviewed in detail elsewhere [9-11]. Briefly,
99 regression models, such as multiple linear regression and partial-least-squares regression (PLSR), are
100 useful for quantifying the correlation between variables in context of one another. Regression models do
101 not incorporate information beyond the data itself, except that the variables included in the model are
102 specified by the modeller. This prior specification makes the models supervised but they are also strictly
103 empirical. Logic modelling, by contrast, involves translating prior knowledge or hypotheses about the
104 system structure or function into computable language. In this way, logic models are capable of bringing a
105 network diagram to operational function. We investigate cell signalling networks using logic modelling by
106 first constructing a diagram of the network based on published data (which we call a “prior knowledge
107 network”, or PKN), then collecting a cue-signal-response dataset devoted to perturbing and measuring
108 aspects of the network, followed by using optimization algorithms to identify and quantify the connections
109 in the hypothetical network that are most important for explaining the data (Figure). The resulting fitted
110 models can then be used for simulation or analysis purposes. Irrespective of the modelling approach,
111 model predictions are experimentally validated. We have used this workflow to obtain considerable

112 insights into the systems-level operation of cell signalling networks in effecting phenotypic responses in
113 diverse contexts, which we discuss in the following section.

114

115 **Liver cell information processing during inflammation**

116 We applied our interdisciplinary approach to the issue of epithelial cell signalling in inflammation by
117 devising logical and statistical modelling methods and applying them to data from cultured liver cells
118 exposed to inflammatory conditions. In the first paper from these studies, Saez-Rodriguez et al. extended
119 Boolean logic methods previously used to study biological networks from a theoretical standpoint to allow
120 the model to directly interface with experimental data [12]. Specifically, their algorithm translates a prior
121 knowledge network, in this specific example a database-derived PSN map, into a Boolean logic model
122 and optimizes the model topology to best fit experimental data. In this example, the data comprised
123 phospho-protein levels of intracellular signalling intermediates in HepG2 cells exposed to inflammatory
124 cytokines and inhibitors of several kinases. Interestingly, they found the resulting optimal models
125 consisted of substantially fewer connections than found in the PKN. Remarkably, an empty model, that
126 contained nodes but no edges connecting them, fit the data better than the PKN [12]. This result implies
127 that the comprehensive protein-protein interaction network maps and PSNs commonly used to depict
128 biological networks do not necessarily reflect networks operating in a specific cell type under specific
129 conditions. This lack of predictivity stems from two apparently paradoxical sources: 1) The networks
130 include too many interactions, presumably because they are typically curated from multiple sources, cell
131 types, time points and experimental conditions and 2) The networks lack interactions that are present and
132 functional in the network under study due to imperfect databases or incomplete understanding of the
133 biology. Indeed, by examining the data points that the original optimal model failed to adequately fit,
134 Saez-Rodriguez et al. tested new interactions to see which ones best improved the fit [12]. The existence
135 of two candidate interactions, one linking TNF-receptor associated factor 6 and MEK and another linking
136 ERK and insulin receptor substrate-1, was supported by published evidence.

137

138 An alternative modelling approach was used by Alexopoulos et al., who performed a comparative
139 analysis of the intracellular signalling networks of healthy and cancerous liver cells [13]. A cue-signal-
140 response dataset was generated in which inflammatory cytokines and growth factors were applied to
141 primary human hepatocytes and HepG2 hepatocellular carcinoma cells in concert with small-molecule
142 inhibitors targeting seven kinases from different signalling pathways. Multiple linear regression analysis
143 was used to estimate the strength of relationships between the cytokines and signals, the inhibitors and
144 signals, and the signals and secreted cytokines. The networks were defined by the relationships featuring
145 the highest regression coefficient magnitudes. The networks for the primary hepatocytes and the HepG2
146 cells were then compared, revealing that HepG2 cells displayed reduced responsiveness to inflammatory
147 stimuli but increased responsiveness to progrowth stimuli, relative to the primary hepatocytes. In
148 particular, alterations of NF- κ B signalling in HCC cells had profound phenotypic consequences because

149 primary hepatocytes secreted a number of cytokines that the HCC cells did not. These cytokines are
150 thought to be involved in recruiting cells of the innate immune system, which implies that HCC cells
151 modify the secretion patterns in order to avoid detection and elimination by the immune system.

152
153 The above studies clearly demonstrate the utility of Boolean logic and regression methods in exploring
154 how normal and healthy liver cells differentially process environmental information. However, both
155 modelling methods have important limitations. Specifically, Boolean logic describes the activity of each
156 node as either “on” or “off”, which ignores potentially important graded activity, and regression models do
157 not incorporate potentially valuable prior knowledge. Morris et al. addressed these limitations by
158 developing a novel logic-based modelling method called “constrained fuzzy logic” (cFL), which
159 incorporates prior knowledge in the same manner as Boolean logic but also models quantitative
160 behaviour [14]. Specifically, this method converts an input value from an upstream node into a continuous
161 value between 0 and 1 for the downstream node through a sigmoidal transfer function [14]. This capability
162 facilitates the ability of the resulting trained models to fit weak responses. The quantitative relationships
163 between proteins are also estimated, allowing for modelling of dose-response data, which could prove
164 valuable for pharmacological applications.

165
166 CFL was applied to the same dataset to which the BL approach had been previously applied [12]. CFL
167 was able to capture interactions that were missed by BL [14]. This included the moderate levels of
168 phosphorylation of c-Jun N-terminal kinase (JNK) and c-Jun by TGF- α stimulation. This interaction was
169 the only instance of growth factor pathway crosstalk with inflammatory pathways observed in
170 measurements of HepG2 cells, which the previous studies failed to detect [12, 13]. Furthermore,
171 stimulating HepG2 cells with IL-6 led to moderately increased phosphorylation of several species,
172 including Akt, MEK and p70 S6 kinase, in addition to strong phosphorylation of its canonical downstream
173 STAT3 pathway [14]. The PKN did not include links from the IL-6 receptor that allowed for the observed
174 moderate phosphorylation levels [14]. In the case of BL, the resulting lack of fit of these data points did
175 not adversely affect the overall fit, presumably because a similar absolute deviation resulted between the
176 intermediate levels of the measured phosphorylations and the model outputs of 0 or 1. In contrast, the
177 cFL model was sufficiently sensitive to this error that the model was deemed to inadequately fit this data
178 [14]. Morris et al. followed up this result by seeking to distinguish the pathway that most likely caused the
179 phosphorylations. To do so, they tested PKNs with new interactions either between the IL-6 receptor
180 (IL6R) and PI3K or IL6R and Ras. Most of the resulting fitted models contained the IL6R-Ras link, thus
181 indicating that the Ras-Raf-MEK pathway and not a PI3K-downstream pathway likely mediated the
182 phosphorylations [14]. This result was validated with dedicated experiments.

183

184 **Conclusions and future directions**

185 We have performed a series of studies in which mathematical models of proteomic data revealed
186 important insights into the signal transduction networks of healthy and cancerous liver cells in
187 inflammatory environments. Our principal biological insight is that hepatocellular carcinoma cells feature
188 both decreased responsiveness to inflammatory stimuli and increased responsiveness to growth factors
189 relative to normal hepatocytes, which could promote immune evasion and increased proliferation. Our
190 principal mathematical advances include devising methods for formally fitting Boolean logic models to
191 data and creating a fuzzy logic method useful for making quantitative models. For relatively small
192 networks such as the one studied here (i.e., downstream of ~5-7 receptors), cFL is a powerful approach.
193 Given the higher computational burden of cFL, Boolean logic will still be needed to model larger networks
194 until more efficient algorithms are developed. We note, however, that larger networks do not necessarily
195 provide additional predictive power because maximal predictivity was observed with models featuring
196 substantially reduced numbers of edges from the initial prior knowledge network. This finding supports the
197 use of our functional biochemistry approach because it provides data on components of the system that
198 actually carry out the cellular response. A distinctive feature of our approach is that it requires broad
199 sampling of network states, which is achieved by applying diverse experimental conditions (in our case,
200 cytokines and inhibitors, but could also include other treatments such as small interfering RNAs).
201 Attempting to process samples from hundreds of independent experiments with other proteomic
202 techniques such as mass spectrometry is currently unfeasible owing to technical limitations and time and
203 fiscal costs. Our approach therefore represents a rational, efficient and informative means to elucidating
204 epithelial cellular signalling and physiology in inflammatory contexts.

205
206 Going forward, we emphasize that systems-level approaches can be effectively used *in vivo*. A recent
207 study from our lab successfully extended previous systems-level analyses of data collected from colon
208 cancer cells *in vitro* [15-17] by applying PLSR modelling to signalling measurements taken from the
209 intestines of mice treated systemically with TNF- α [18]. Determining the biological effects of TNF- α is not
210 straightforward because stimulation of TNF- α receptors increases the activity of multiple downstream
211 signalling pathways, the quantitative integration of which determines the ultimate biological outcome. In
212 the case of mouse intestinal epithelial cells *in vivo*, for example, TNF- α was found to promote apoptosis in
213 cells of the proximal part of the small intestine, but not in the distal part, with the timing of apoptosis being
214 dose-dependent [18]. TNF- α administration also affected cell proliferation in a region-specific manner.
215 PLSR modelling of signalling and phenotype data revealed that the differential sensitivity of apoptosis
216 was due to quantitative differences in MAPK signalling kinetics between the two intestinal regions and
217 that growth arrest was related to c-Jun and activating transcription factor activation as well as MAPK
218 signalling kinetics [18]. Subsequent experiments validated the hypotheses generated from the original
219 dataset and model. This study demonstrates that our systems-level approach can be successfully applied
220 to *in vivo* contexts, despite their added complexity compared to *in-vitro*-cell-culture-based experiments.

221

222 We contend that our approach works irrespective of the experimental system because cells integrate
223 complex contextual information into biochemical activities of signalling pathways that form the basis for
224 phenotypic decisions. The cell signalling network is complex but manageable such that by measuring
225 selected nodes across this network and using mathematical models to infer the network output, we are
226 able to predict the ultimate biological outcome. We therefore anticipate systems-level approaches
227 becoming broadly applicable to the study of cellular signalling.

228

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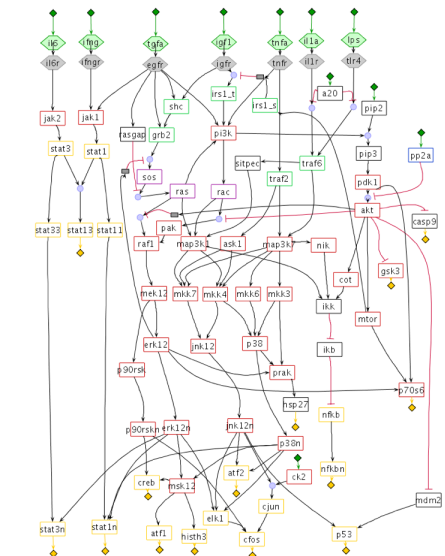
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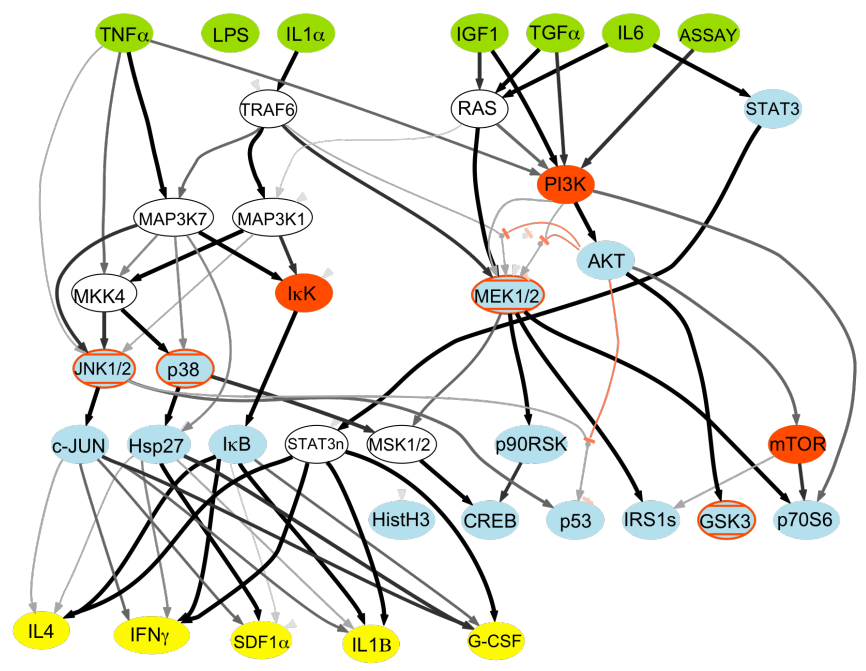
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278

279 **Figure legend**

280 Logic modelling workflow. A prior knowledge network is derived from literature, databases and/or existing
281 data. Experiments are conducted to systematically perturb and/or measure nodes distributed throughout
282 the network. The data is then used by a model optimization (i.e., data fitting) algorithm to tune the model
283 topology and if applicable, the model parameters, to minimize the discrepancy between the model output
284 and data. The resulting model is then analyzed to derive insight into the biology of the system. The figure
285 is adapted from figures contained in reference [14].

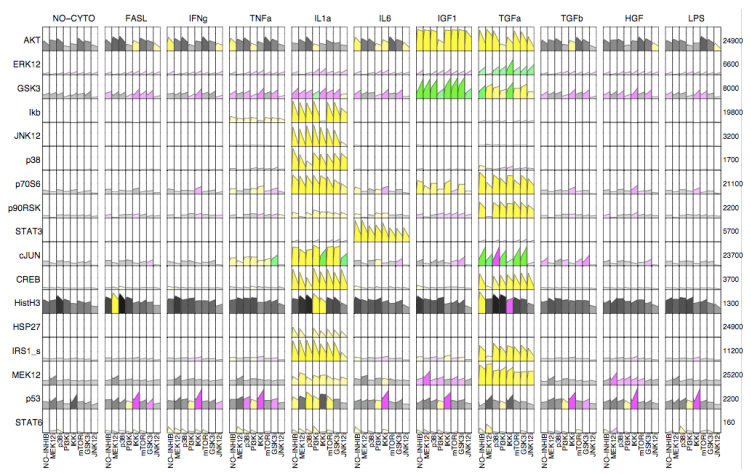


Prior knowledge network

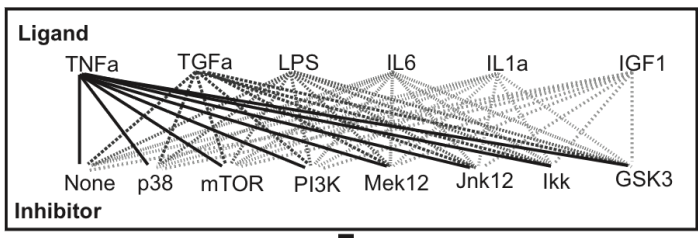


Computable logic model

Dedicated empirical data



Experimental design



Measured species' phosphorylation

Akt	Jnk12	p90RSK	CREB	IRS1s
GSK3	p38	Stat3	HistH3	Mek1/2
Ikb	p70s6	cJun	Hsp27	p53

Measured cytokine release

IL4	IL8	GCSF	IFNγ	SDF1a
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