Use of microarrays to investigate mechanisms of immunotoxicity

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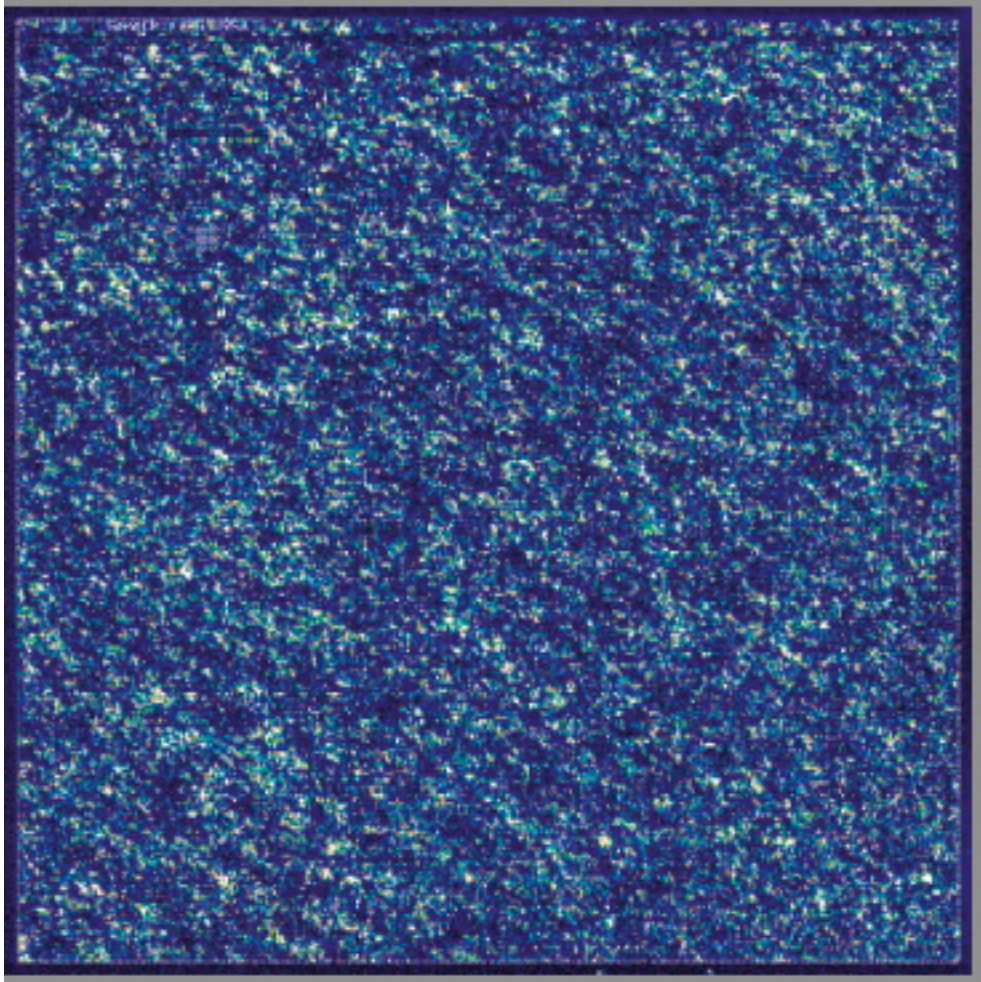
Outline

I. Selection of genomics platform
II. Data analysis for mechanistic studies in comparison to other types of studies
III. A case study: Mechanisms of Ethanol Immunotoxicity
IV. Discerning the whole elephant-Systems Biology

I. Selection of a genomics platform

a. Compatibility with software and Minimum Information About a Microarray Experiment (MIAME) submission
b. Stability and reproducibility of platform
c. “Market share” (determines the degree to which issues such a normalization or sensitivity will have been addressed)
d. Genes represented (dependent on purpose of study)



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II. Experimental considerations

a. Mixed vs. pure cell populations (changes in cell ratio/number vs. changes in gene expression)

For example, chemicals that induce a neuroendocrine stress response often selectively deplete $CD4^+CD8^+$ thymocytes and elevate neutrophils and decrease lymphocytes in the blood. Genes that are selectively expressed by these cell types would be detected as expressed differently solely due to changes in cell population ratios.

II. Experimental considerations

b. Basal vs. stimulated immune parameters as the reference point for evaluating effects of toxicants

Ethanol at 6 g/kg in mice causes greater than 2-fold increases or decreases in expression of 330 genes (Pruett, et al., unpublished) and 205 genes that were regulated more than 2-fold by lipopolysaccharide in peritoneal macrophages were altered an additional 2-fold or more by ethanol at 6 g/kg (*J. Immunol*. 173:2715, 2005).

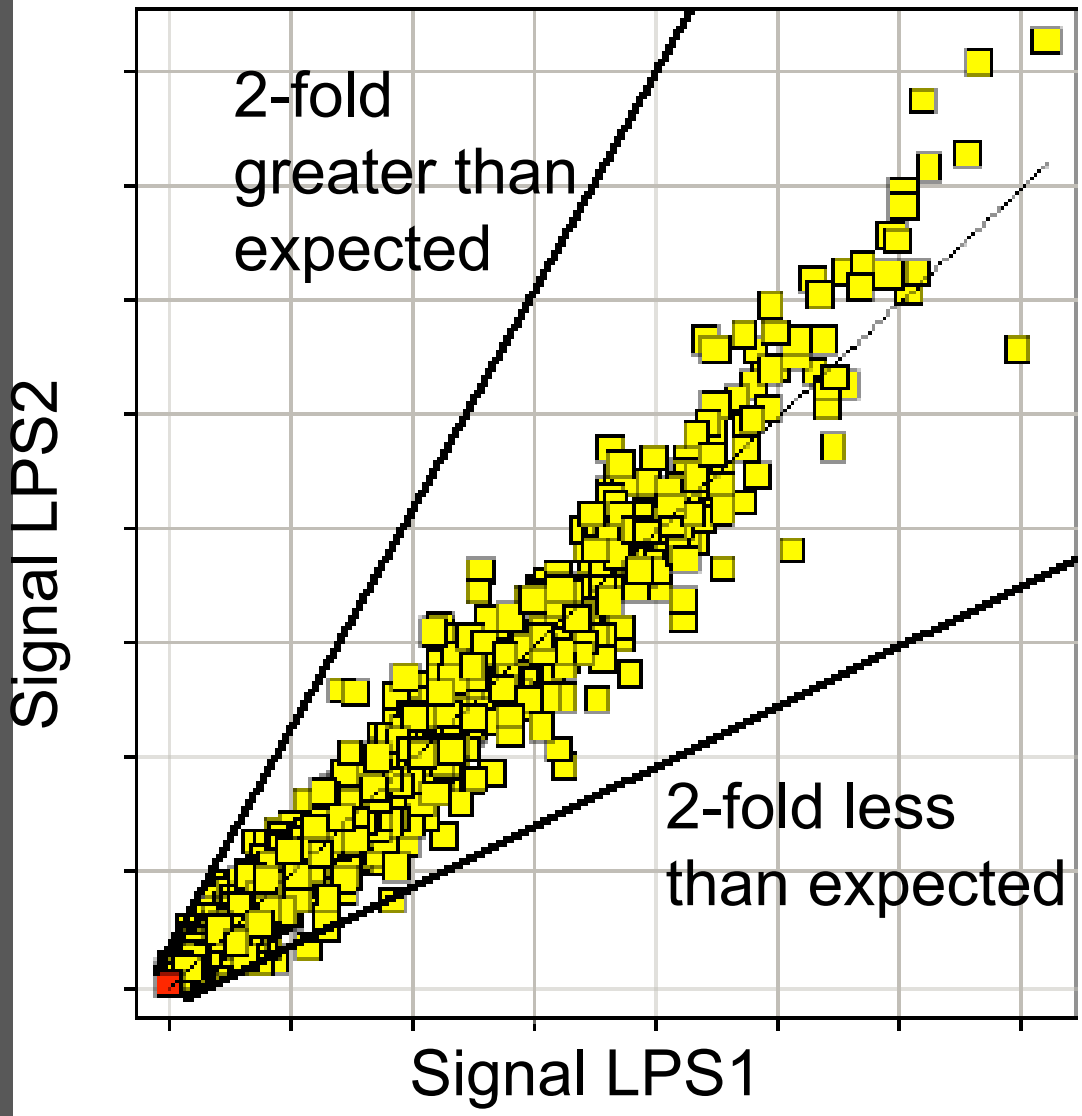
However, the functional relevance of the genes induced by LPS was generally much clearer than the genes expressed at basal levels.

II. Experimental considerations

c. Group size

The general considerations are not fundamentally different from those for experiments involving other end points. A group size of at least 4 is necessary for the use of ANOVA, which is generally an appropriate method of statistical analysis for experiments with multiple groups. However, useful data can be obtained with a smaller group size.

Comparison of Results of Duplicate Samples of Peritoneal Cells from Mice Treated with LPS



When duplicate samples yield highly concordant results (e.g., in two of our recent studies, the greatest number of genes between duplicates that were discordant was 22 out of a total of 12,000), the results can be used to indicate possible mechanisms of action, if changes in expression of key genes is confirmed by other methods.

Can genomic microarrays used in a screening (hazard identification) mode be used to elucidate mechanisms of action?

Based on limited and anecdotal experience, data from immune system cells that have not been challenged to respond in some way may be difficult to interpret, but can nevertheless yield useful mechanistic hints. For example, Boverhof et al. demonstrated that TCDD induces the activation of Suppressor of Ctyokine Signaling -2 (SOCS-2) (Mol. Pharmacol. 6:1662-1670)

A screening system using mice challenged to respond may also give clear indications regarding mechanisms (to be discussed).

II. Experimental considerations

d. Quality assurance

Variation can occur at every step of the experimental process, so Quality Assurance should be considered at each step.

1. Experimental treatments
2. RNA extraction/quality
3. Generation of cRNA, application to chip, and reading results.
4. Normalizing for background signal and differences in signal between chips.
5. Selective confirmation of results by other methods.

II. Experimental considerations

a. Should samples from multiple animals be pooled to avoid identifying genes for which expression is altered only in one animal?

This seems a reasonable approach, even though it obviously diminishes statistical power.

However, a recent study suggests that pooled samples reveal substantially fewer changes in gene expression (*Physiological Genomics* 22:346, 2005)

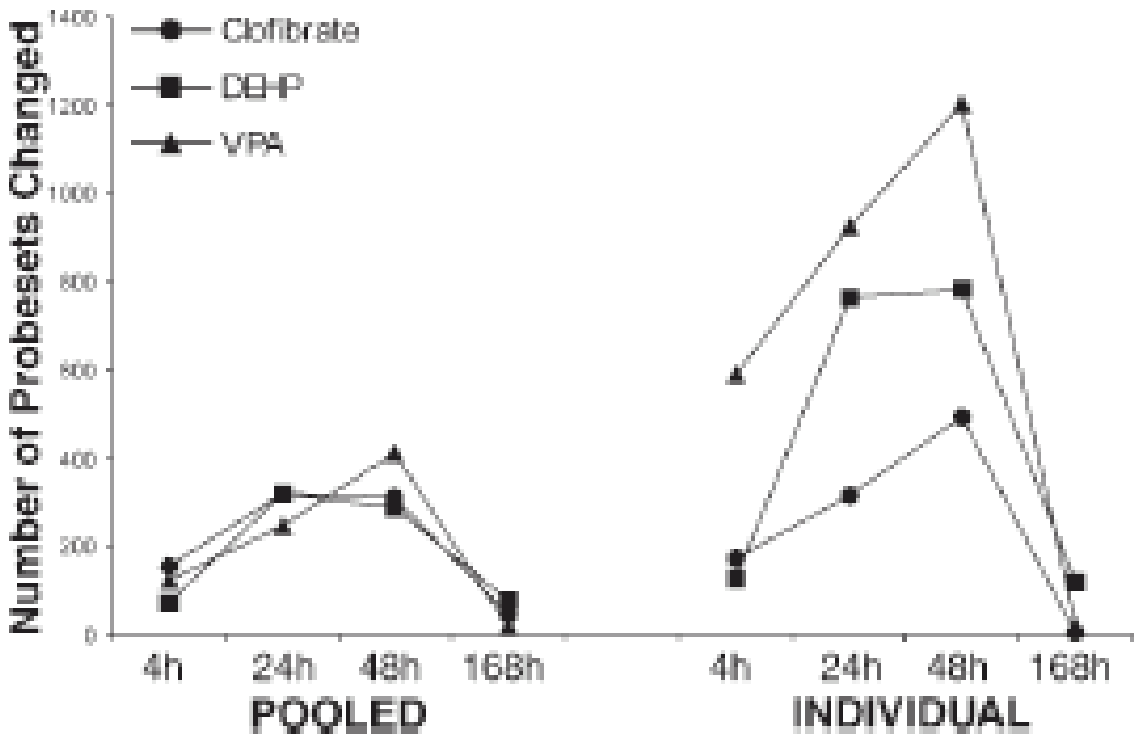
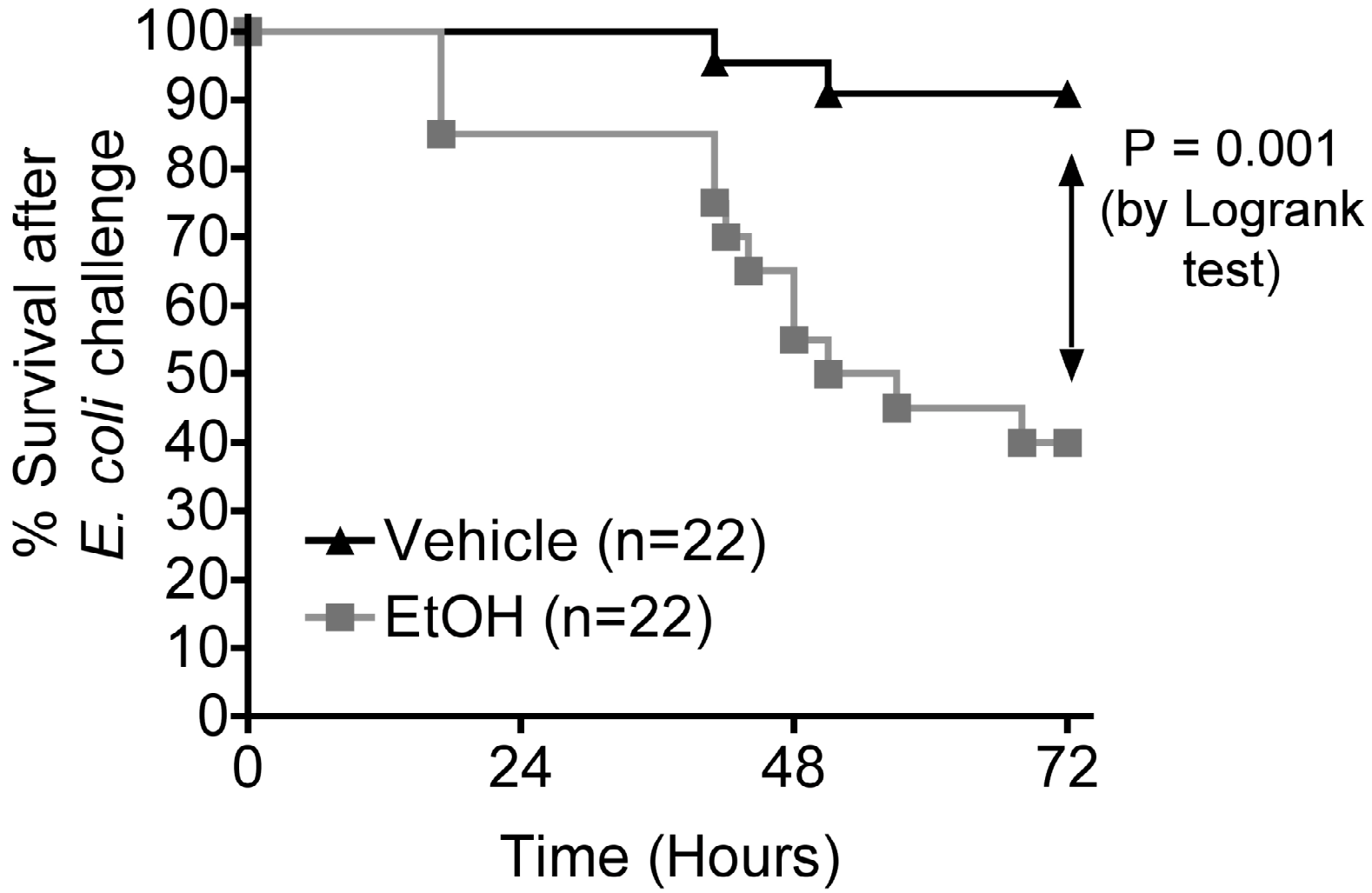


Fig. 1. No. of transcripts changed when samples were pooled or run individually. Graph represents the no. of probe sets on the chip that pass the filtering criteria for each time point postadministration of the toxicant. For filtering criteria for both approaches, see MATERIALS AND METHODS. DEHP, diethylhexylphthalate; VPA, valproic acid.

III. A case study: Mechanisms of Ethanol Immunotoxicity

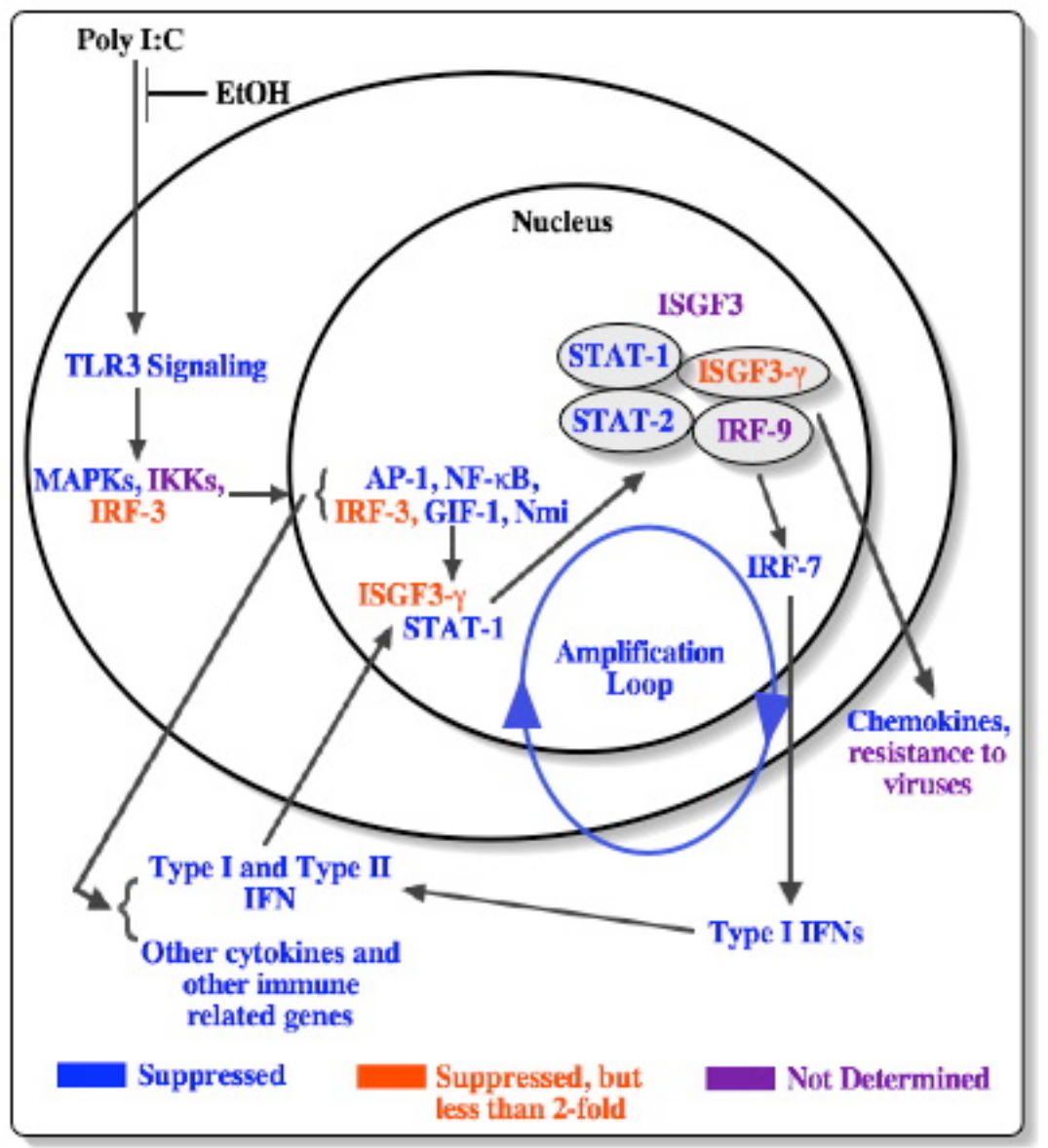
Ethanol suppresses innate resistance to bacterial peritonitis, what is the mechanism?



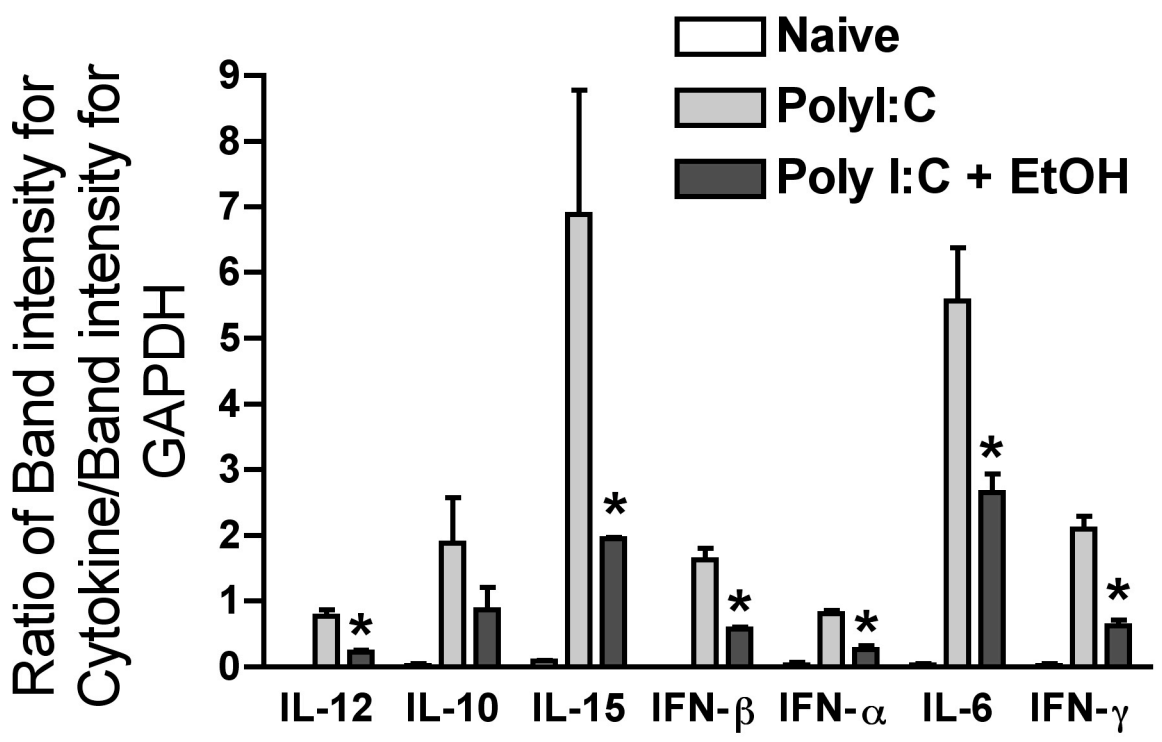
Peritoneal cell genes downregulated 2-fold or more (for at least 3 of 4 comparisons) by EtOH.					
	Fold Decrease in EtOH-Treated Mice				P1 Relative
	P1 vs E1*	P1 vs E2	P2 vs E1	P2 vs E2	to Naive
Cytokines and Chemokines					
Interleukin 1-beta	2.17	3.48	1.68	2.81	58.89
Interleukin 1 receptor type II (decoy receptor)	2.43	2.36	2.01	2.30	13.09
Interleukin 1 receptor antagonist	2.71	3.10	2.89	3.39	135.30
Interferon-beta	2.19	2.14	2.48	2.38	4.63
Interferon-gamma	3.01	26.17	1.51#	7.36#	5.39
Interleukin 15†	1.95	2.19	1.93	2.23	11.63
C-C chemokine ligand 7 (CCL7)†	2.00	2.68	2.33	2.83	276.28
C-C chemokine ligand 12 (CCL12)†	2.97	2.33	4.47	2.71	14.52
C-X-C chemokine ligand 9 (CXCL9)†	3.10	3.36	3.94	4.59	98.36
Signaling Related and Transcription Factors					
STAT 1†	2.58	2.95	2.85	3.36	3.32
N-Myc and Stat interactor (Nmi)	2.14	2.45	2.39	2.83	2.53
Interferon regulatory factor 7 (IRF7)†	2.03	2.22	2.57	2.60	16.68
GIF transcription factor†	4.17	6.59	4.89	6.92	0.24
RhoB	2.60	2.27	2.91	2.50	0.97
Inhibitor of DNA binding 1	3.41	5.31	3.66	5.82	1.52
Immune Function Related					
iNOS†	2.53	1.62	3.51	2.51	5.66
Prostaglandin E synthase†	2.43	3.27	2.68	3.66	24.42
Interferon-inducible GTPase (MX-1)†	3.27	3.58	4.17	4.53	32.45
dsRNA specific adenosine deaminase†	2.31	2.25	2.55	2.45	5.78
Calcium binding protein MRP8†	4.72	5.66	3.58	3.86	83.29
Calcium binding protein MRP14	5.54	6.23	3.71	4.56	280.14
Granzyme B	3.78	2.30	2.97	2.27	9.71
IgG Fc RI (high affinity)†	2.53	2.75	3.97	4.17	6.32
VCAM-1	2.91	3.76	2.55	3.27	73.52
Others					
Tissue inhibitor of metalloproteinaase (TIMP-1)	2.48	2.19	2.38	2.14	4.63
Heat shock protein 1B	4.86	4.72	4.14	3.84	2.75
Matrix metalloproteinase 1	2.14	2.64	5.58	7.52	2.77
Interferon stimulated protein (Igs20)†	2.38	2.07	2.79	2.48	4.43

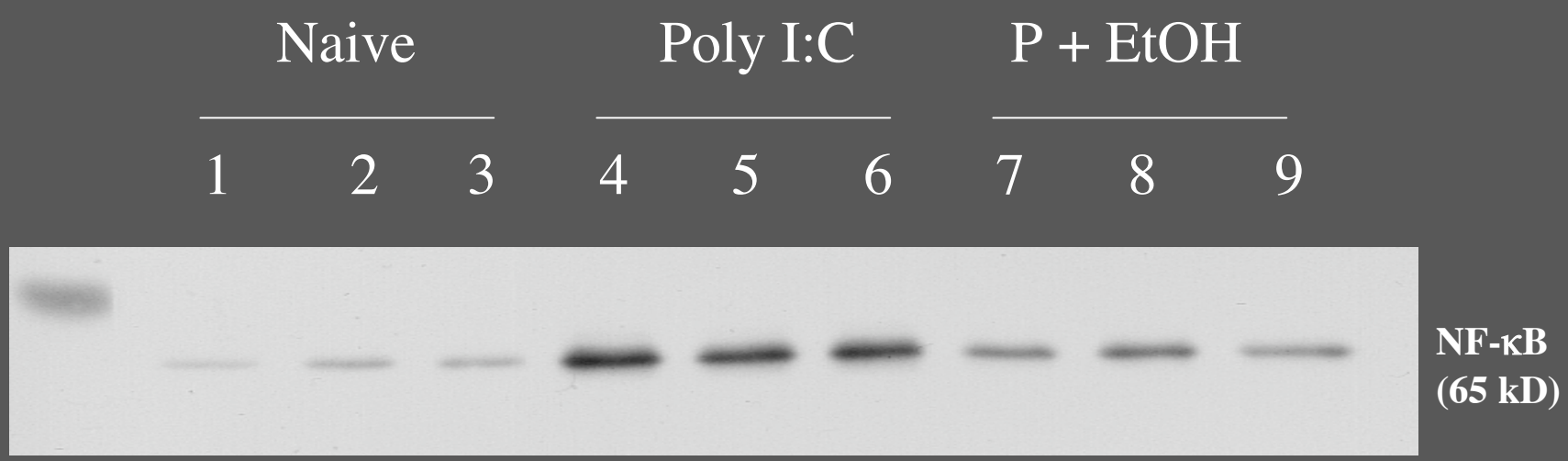
Gene expression in peritoneal macrophages increased two-fold or more by EtOH.

	Fold Increase in Gene Expression				P1 Relative
	P1 vs E1*	P1 vs E2	P2 vs E1	P2 vs E2	to Naive
MAP kinase-interacting serine/threonine kinase 2	3.16	3.03	3.51	3.86	0.16
Ets-1 (transcription factor)	2.11	2.01	2.91	2.87	0.19
Ceramide Kinase	2.79	2.69	3.29	2.95	0.19
GTPase activating protein GAPIII	2.07	2.19	2.83	3.03	0.26
MAPK 14 (p38b)	2.79	2.45	3.46	2.79	0.30
Coronin-actin binding-phagocytosis related	2.51	2.39	2.33	2.11	0.30
Kruppel-like factor 9	3.05	4.03	3.43	5.98	0.37
Spred-1 (Inhibitor of Ras)	2.38	2.93	2.01	2.57	0.65
FK506 Binding Protein 51 kD	2.99	3.14	3.58	3.16	NC
Cathepsin E	2.01	2.48	2.01	2.48	1.56
T cell death associated gene (TDAG) 51	2.83	3.18	4.03	3.36	2.91
Adrenomedullin	3.73	3.12	3.86	3.46	5.28
GADD45	2.64	2.04	3.61	2.91	15.78
4-1BB ligand for TNF receptor superfamily member 9	3.16	3.58	3.34	3.76	24.59


Poly I:C
EtOH
Nucleus
ISGF3
STAT-1
ISGF3-γ
STAT-2
IRF-9
TLR3 Signaling
MAPKs, IKKs,
IRF-3
AP-1, NF-κB,
IRF-3, GIF-1, Nmi
ISGF3-γ
STAT-1
IRF-7
Amplification
Loop
Chemokines,
resistance to
viruses
Type I and Type II
IFN
Other cytokines and
other immune
related genes
Type I IFNs
Suppressed
Suppressed, but
less than 2-fold
Not Determined

EtOH (6 g/kg) suppresses poly I:C-induced cytokine gene expression- Values are means for 1 sample of purified (FACS) macrophages and 2 samples of ficoll isolated peritoneal cells (~90% macrophages) by RNAse protection assay, which generally confirms microarray results.

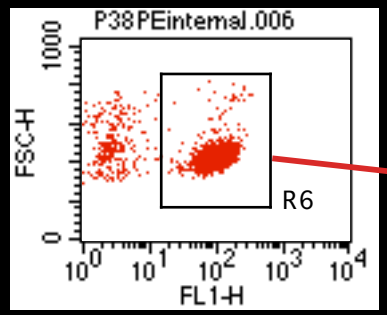


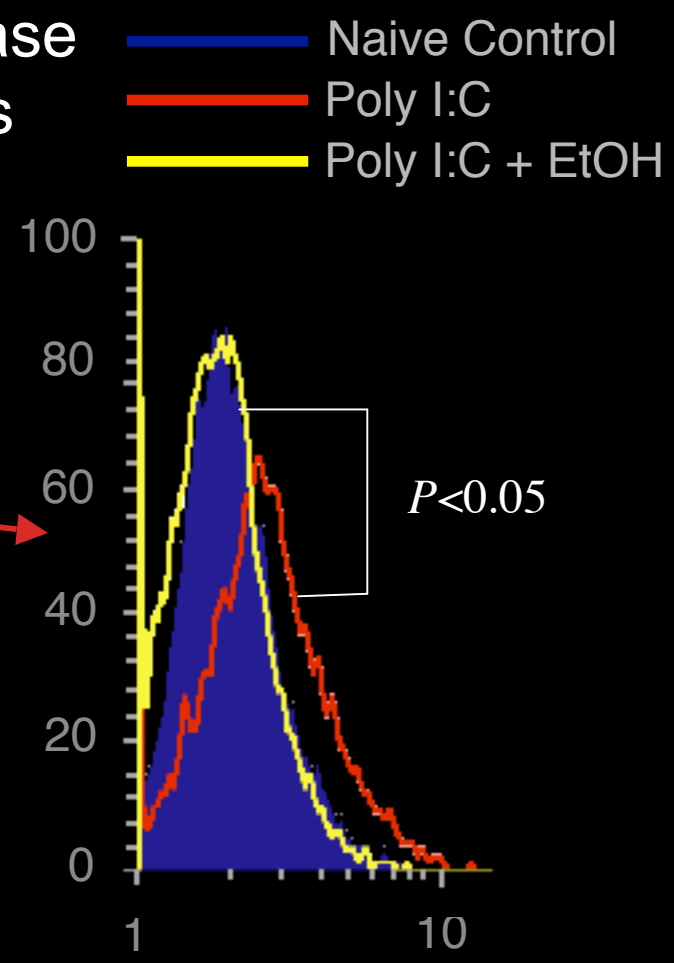


Mice were treated with poly I:C or poly I:C plus EtOH at same time, peritoneal cells were collected 30 min later, and nuclear proteins were extracted and analyzed by Western Blot.

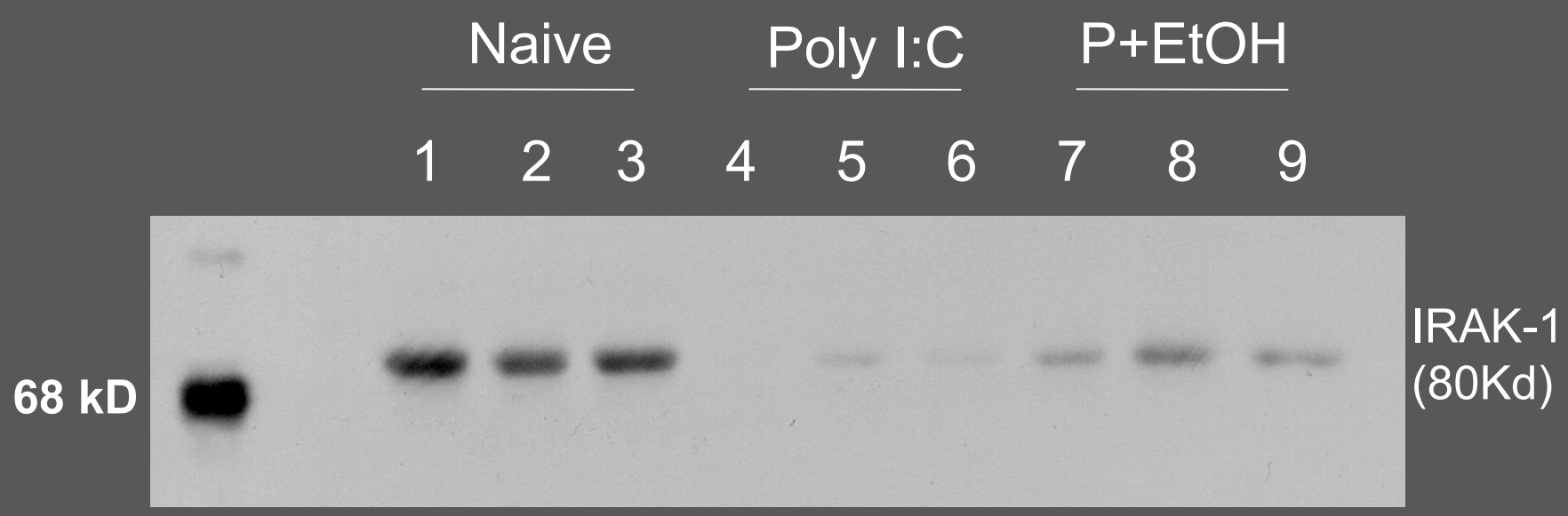
EtOH Inhibits Poly I:C-Induced Phosphorylation of the MAP Kinase p38 in peritoneal macrophages

Cells surface labeled for F4/80 were then evaluated for pp38





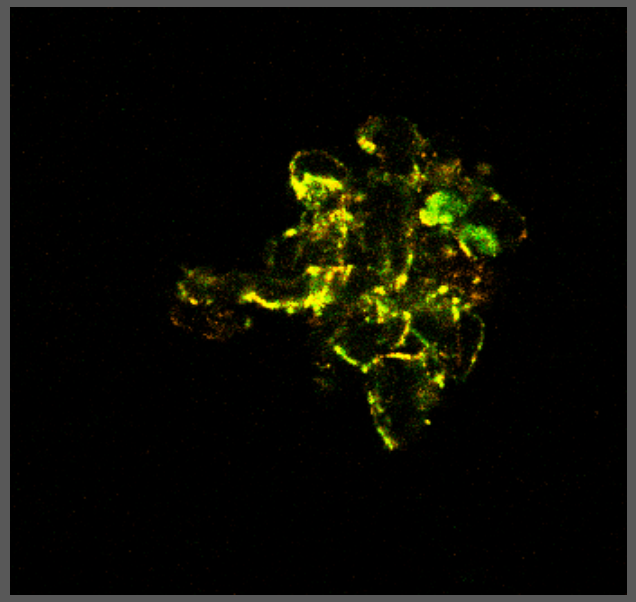
Internal pp38 labeling of F4/80 positive cells



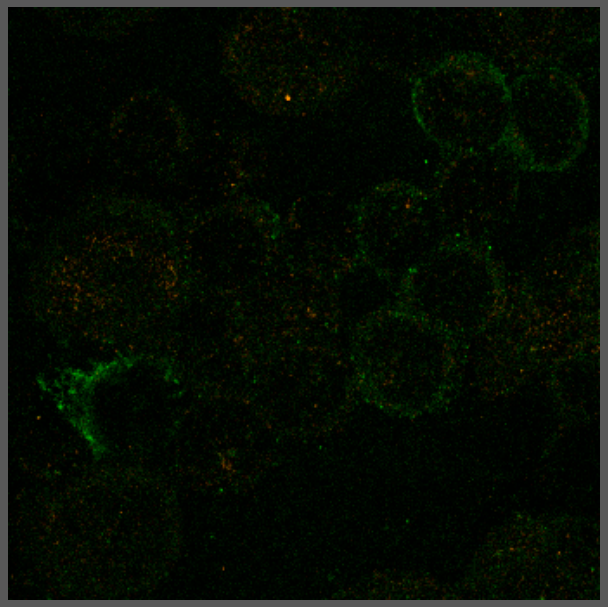
Mice were treated with poly I:C or poly I:C plus EtOH at same time, peritoneal cells were collected 1 hr later, whole cell proteins were extracted and analyzed by Western Blot.

Ethanol inhibits toll-like receptor signaling by preventing initial receptor clustering: A mechanism that might not have been considered without microarray results

Peritoneal Macrophages
15 min after LPS iv

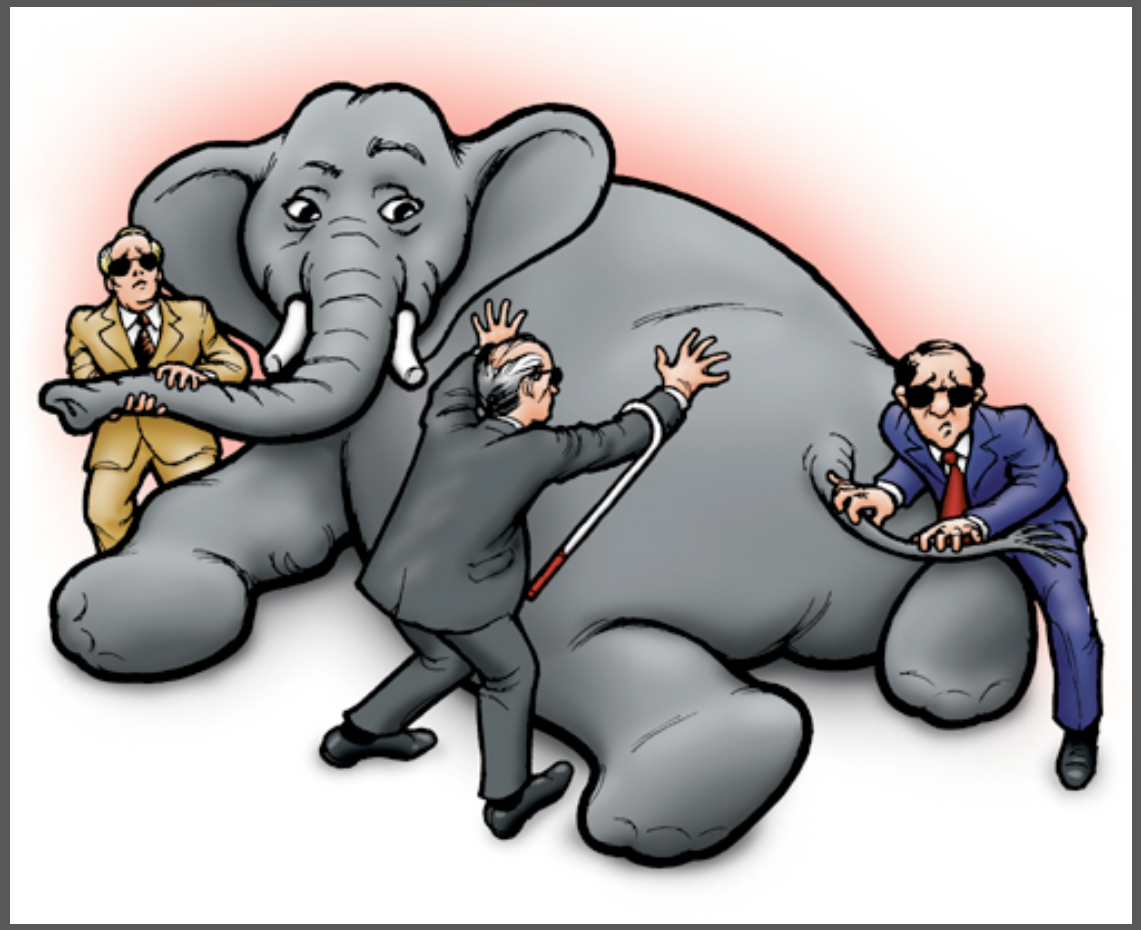


Peritoneal Macrophages
15 min after LPS iv +
Ethanol by gavage



Red = TLR4
Green = CD14
Yellow = co-localization

IV. Discerning the whole elephant-Systems Biology



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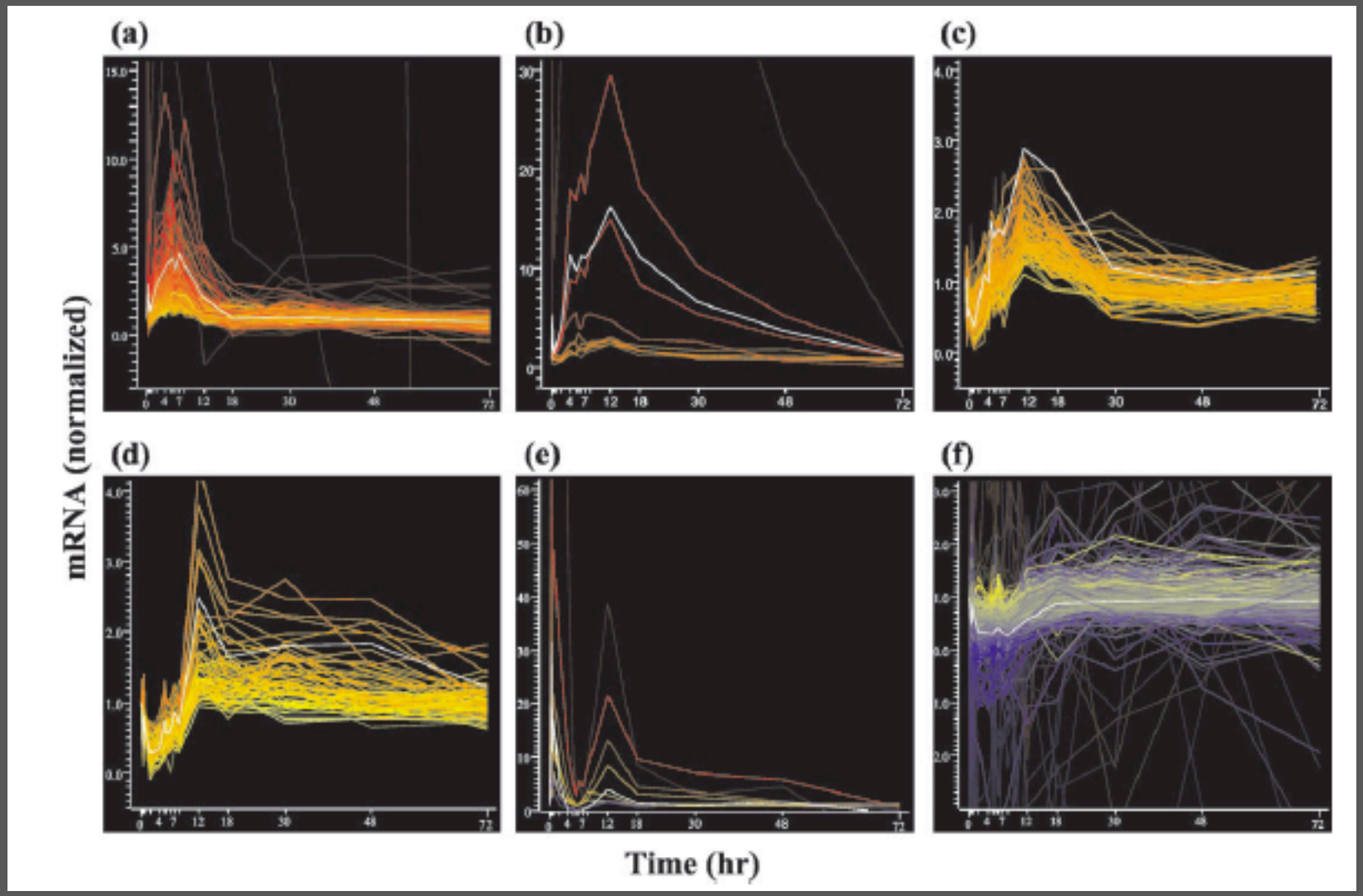
“Systems biology studies biological systems by systematically perturbing them (biologically, genetically, or chemically); monitoring the gene, protein, and informational pathway responses; integrating these data; and ultimately, formulating mathematical models that describe the structure of the system and its response to individual perturbations”.

Annual Review of Genomics and Human Genetics 2:343, 2005

Changes in gene expression over time in the liver after administration of a synthetic glucocorticoid indicate shared patterns for gene regulation



JPET 307:93, 2003

Systems Biology may be the only approach that will lead to comprehensive understanding of mechanisms of immunotoxicity.

Microarrays will be an integral part of that effort.