Collaborative Assay Development and Design

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Research projects that address complex responses need efficient assays

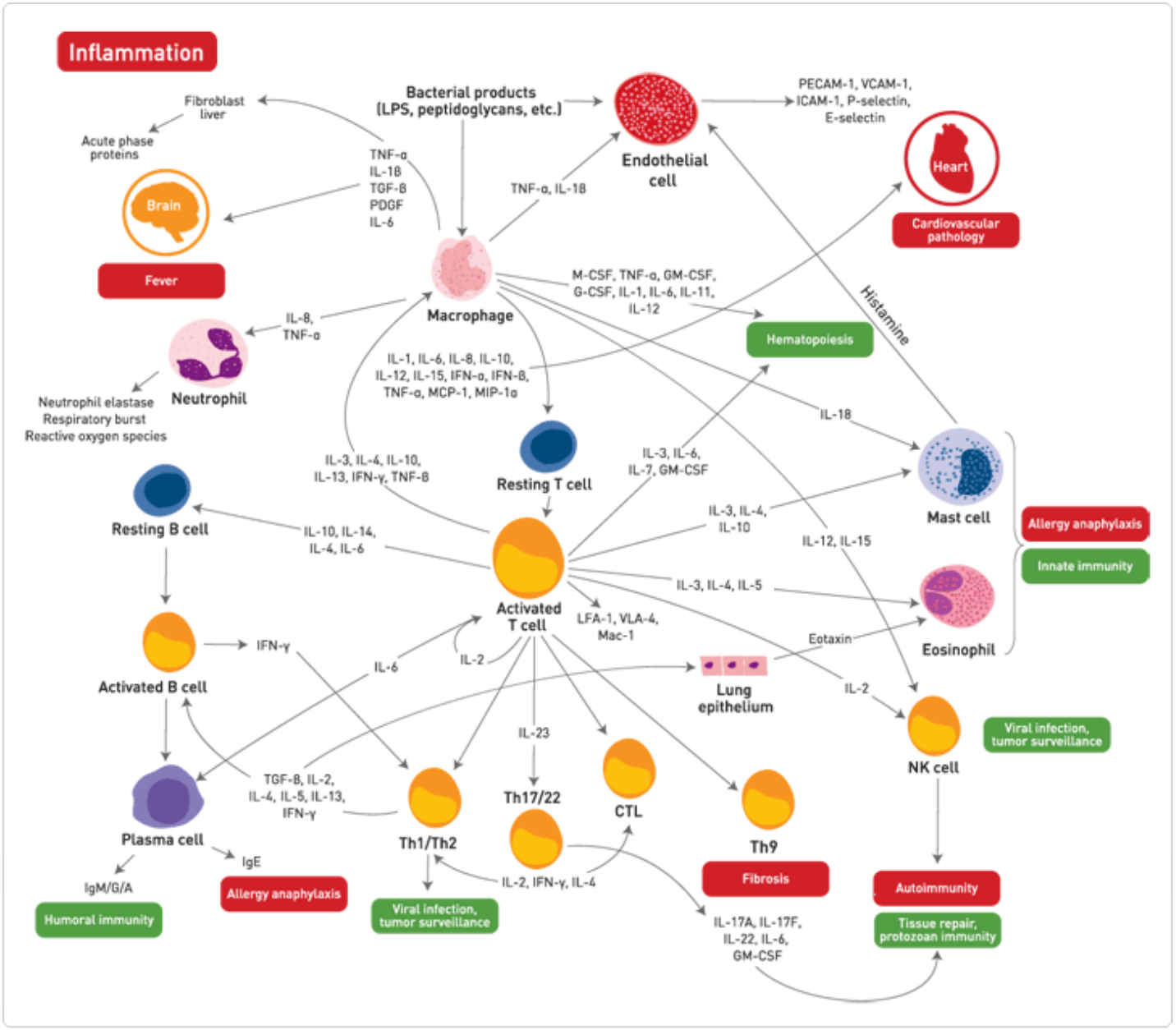
1. The effects of traumatic injuries on the immune system

    - Complex response that involves multiple immune cell types and mediators
    - Mouse and human studies

2. Treatments that enhance immunity and restore immune homeostasis after traumatic injuries and severe infections (sepsis).

    - Immuno-monitoring by profiling immune cell phenotypes by flow cytometry and cytokine profiling.
    - *In vivo* response to injury, infection, and treatments.

Increased knowledge requires increased data



From Life Technologies Website

"Old school" single-mediator assays are good, but multiplexing is more in tune with current scientific needs

1. Difficult to interpret findings and publish results without including more than one mediator or biomarker in dataset.

    - Inflammatory responses and T-cell immune regulation for example

2. More financial demands on research funding requires increasing efficiency and data collection strategies.

3. We need to gather a much information as possible from expensive mouse models and limited amounts of human samples – NIH calls this **Sample Sparing Technologies**.

History: ELISA *to* Flow Cytometry Bead Assays *to* Luminex Assays

1. ELISA – basic principle behind all immunoassays
    - Plate-bound antibody – antigen – antibody sandwiches with color absorbance detection
    - Usually a single analyte approach
2. Flow cytometry-based bead assays
    - Same principle as ELISA but on a bead with a specific detection character for gating on a flow cytometer
    - Limited number of beads can be run as multiplex
    - Not automated or customizable
3. Luminex bead assays
    - Same principle as ELISA using beads with a specific detection character
    - High number of specific beads for multiplexing
    - Customizable and automated

Why do multiplex assays enhance research efficiency?

1. ELISA requires large sample volumes – 50 uL for single single detection.
   - More samples needed to generate data
   - More reagents needed
   - Need to do multiple wells if more than one analyte
   - If assay does not work, less material for repeat

2. Multiplexing requires much less sample volume for multi-analyte detection.
   - Less sample needed – 20 uL or less
   - Less reagents needed
   - Not necessary to do multiple wells
   - Capability to run multiple assays if needed and for future assays from stored samples

Reasons for developing "in-house" multiplex assays

1. Purchased Luminex instrument in 2007 and found discrepancy in results between older technology (BD CBA) and Luminex assays.
   - Several cytokines that were detected by ELISA or CBA were not detected in Luminex assays.

2. Tested several different available kits and found inconsistencies in cytokine detection.
   - Not surprising since assays are likely built with different specific antibody pairs.

3. Some kits detected recombinant cytokines, but not natural cytokines

More reasons for developing "in-house" Luminex assays

1. Confidence that results will be accurate.

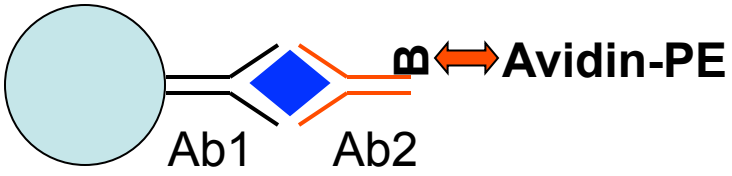
2. Reduce overall costs of performing Luminex assays for our immuno-phenotyping studies.

   - Kit costs were $100-$200/cytokine/96-well plate.
   - Discovered that making in-house assays in bulk would be less expensive for high-throughput studies.

3. Flexibility for future expansion of panels and for other multiplex assay development – opens up capabilities to make a variety of different biomarker assays.

Assay development principles and variables

Immuno-assays based on sandwich ELISA principles

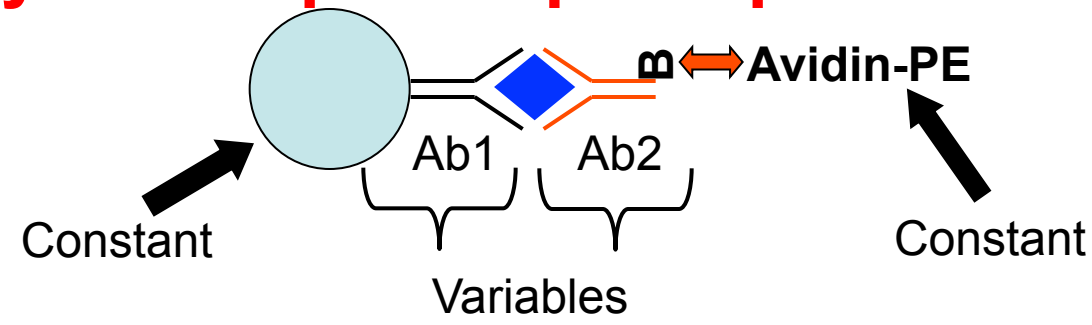


- High specificity due to dependence of signal on bi-molecular binding reagents

- Quality and specificity of assay reaction depends on the nature of Ab1 and Ab2.

- Antibodies remain the most important component of these and other types of immunoassays

Assay development principles and variables



1. Bead coupling reaction – Ab1 Concentration, reaction buffers and chemicals, reaction time, centrifugation washes, blocking buffers

2. Antibody pairing – bead-bound (Ab1) versus detection (Ab2), trial and error.

3. Detection Ab and Avidin-PE – Titration optimization to minimize reagent use and prevent non-specific background

4. Standards - Recombinant versus natural cytokine detection

Steps to building multiplex assays

1. Identify target analytes – Done by hypothesis testing and intellectual design.

2. Search for the needed reagents – Ab1, Ab2, standard.

3. Bead coupling optimization – Covalent crosslinking of Ab1 to microplex or magplex bead.

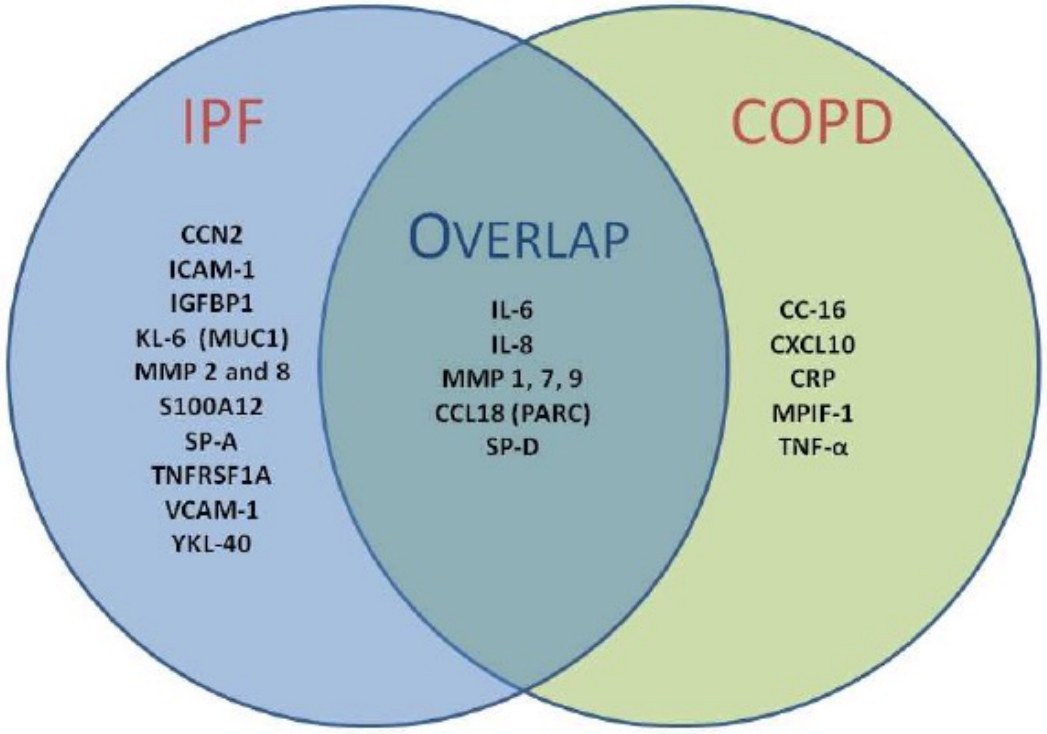
4. Test and optimize assay conditions variables – Screen for detection of standard with Ab2.

5. Validate assay by optimization of assay conditions and sample testing.

Biomarker development project for lung disease diagnosis – COPD vs IPF

NIH development project to construct biomarker sets to distinguish patients with chronic obstructive pulmonary disease (COPD) vs. interstitial pulmonary fibrosis (IPF).

Ivan Rosas and Fernanda Golzarri, BWH Pulmonary Critical Care and Lovelace Respiratory Research Institute



Steps to building the COPD/IPF panel

1. Identify target analytes – list from literature

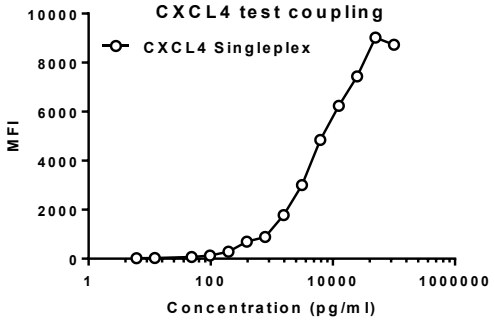
2. Search for the needed reagents – Ab1, Ab2, standard
   - Antibody search using biocompare or labome websites or trusted suppliers
   - Find Abs that have been used in ELISA before
   - Look for biotinylated Abs to save time and effort
   - Monoclonal on bead, polyclonal as Ab2 is okay

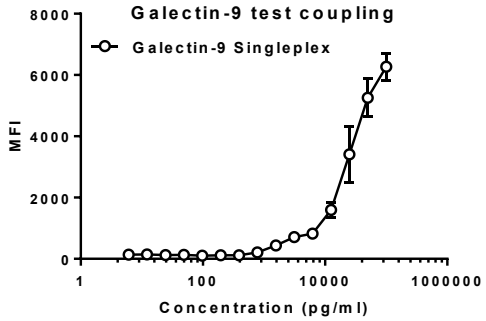
3. Find standard
   - Can be difficult for less studied proteins or complex proteins – *e.g.* surfactant protein A (SP-A)
4. "micro-batch" testing to optimize bead coupling reaction

5. Biotinylation optimization for Ab2, if needed

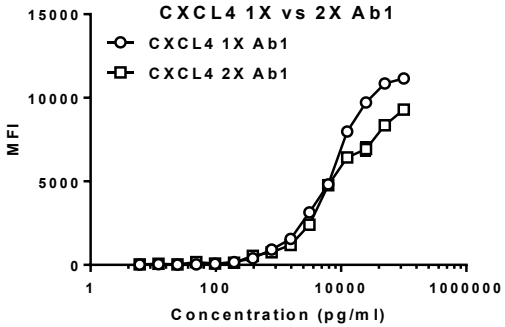
Assay development is a step-wise process: Assays developed with Cambridge Biomedical

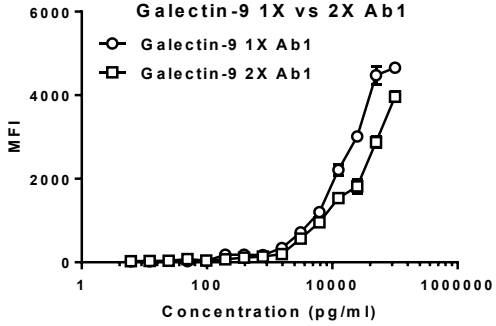
Step 1



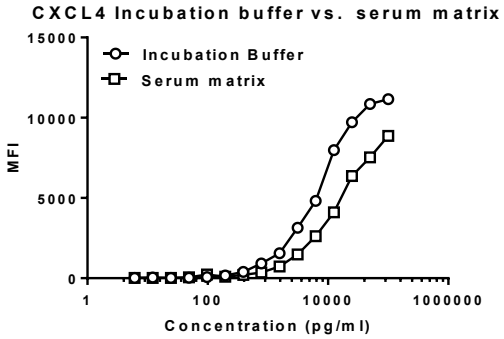


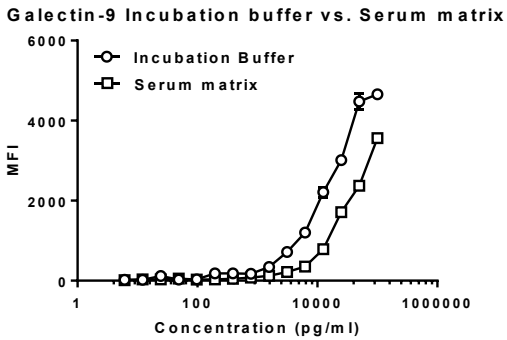
Step 2





Step 3





List of validated mouse and human cytokine and other factor assays

Mouse

IL-1α
IL-1β
IL-2
IL-4
IL-5
IL-6
IL-7
IL-10
IL-12(p40)
IL-12(p70)
IL-23
IL-13
IL-17
IL-18
IL-21
IL-33
IFN-α
IFN-γ
TNFα
G-CSF
GM-CSF
M-CSF
FLT3L
SCF
MCP-1
MIP-2
KC
CXCL15

Human

IL-1α
IL-1β
IL-1ra
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-8
IL-9
IL-10
IL-12/23(p40)
IL-12(p70)
IL-13
IL-17A
IL-18
IL-21
IL-32
FGF-2
G-CSF
GM-CSF
IFNγ
MCP-1
MIP-1α
MIP-1β
NGF
RANTES
TNFα
TNFβ
TREM-1
TWEAK
sTNF RI
sTNF RII

Collaborative consumption approach

- On campus Luminex assay collaborations with reagent replenishment support.
- An ongoing need for on-campus Luminex assay development and services – Human Immunology Center, Harvard Catalyst, or BWH-BRI

Dr. Andrew Lichtman, BWH and HMS – mouse models of T cell mediated inflammation

Dr. Charles Serhan, BWH and HMS – inflammation research in mice and man

Dr. Gerry Pier, BWH, Channing Lab – vaccine and infectious disease projects

Dr. Pedram Hamrah, MGH – eye inflammation and infections

Dr. Rachel Clark, BWH and HMS – human T cell biology in the skin

Dr. Arlene Sharpe, HMS – mouse models of basic T cell activation mechanisms

Dr. Mark Perrella, BWH and HMS – mouse stem cells and sepsis responses

Examples of how to use of Luminex multiplexing to increase research efficiency

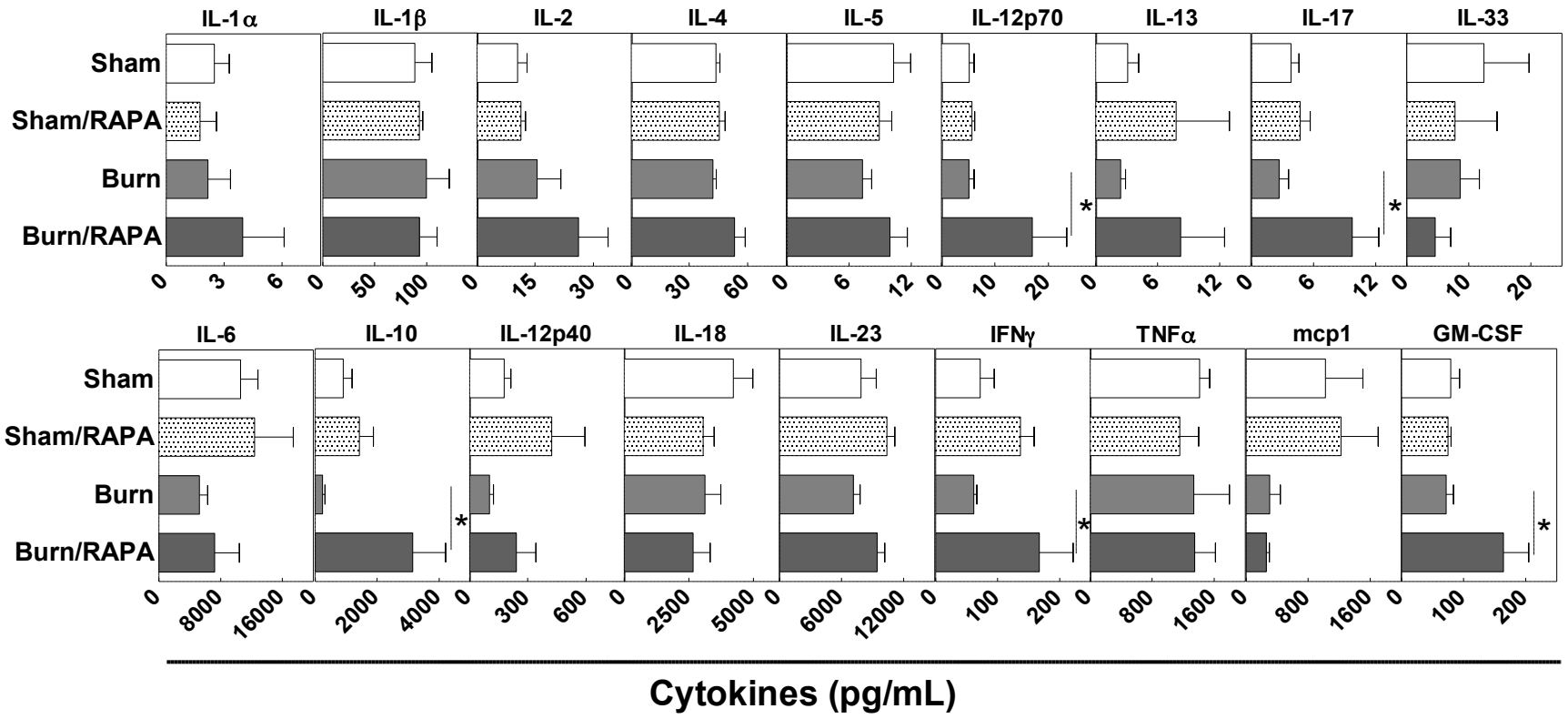
1. Micro-size experiments to gain more information from less cells

2. Test multiple stimulation conditions

3. Develop more efficient assays to save on reagent costs and sample cost – *e.g.* antibody-isotyping of vaccine assays by multiplex

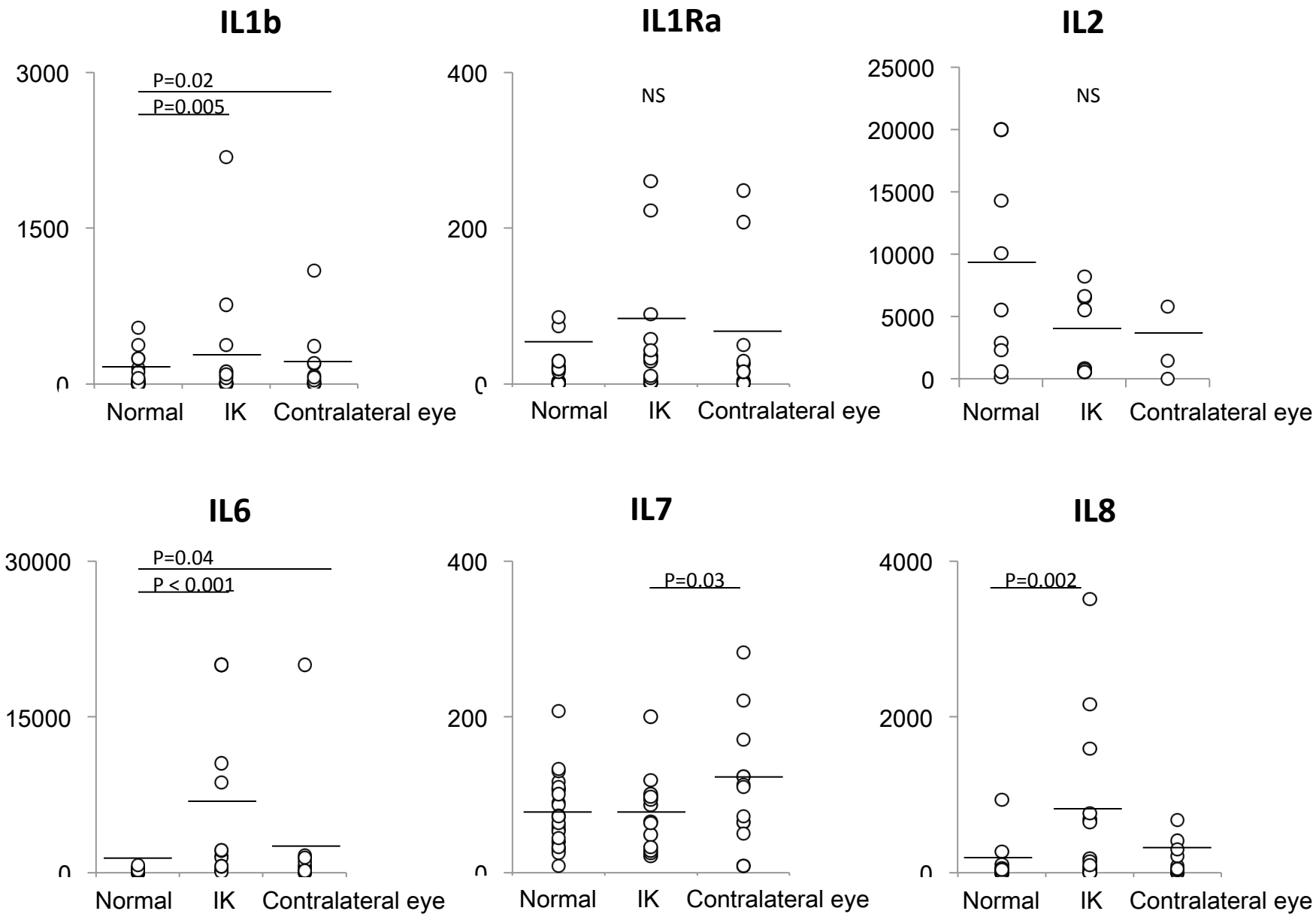
4. Potential to assay any type of cell or tissue extract – *e.g.* human tears, organ extracts, exhaled breath condensate, etc…

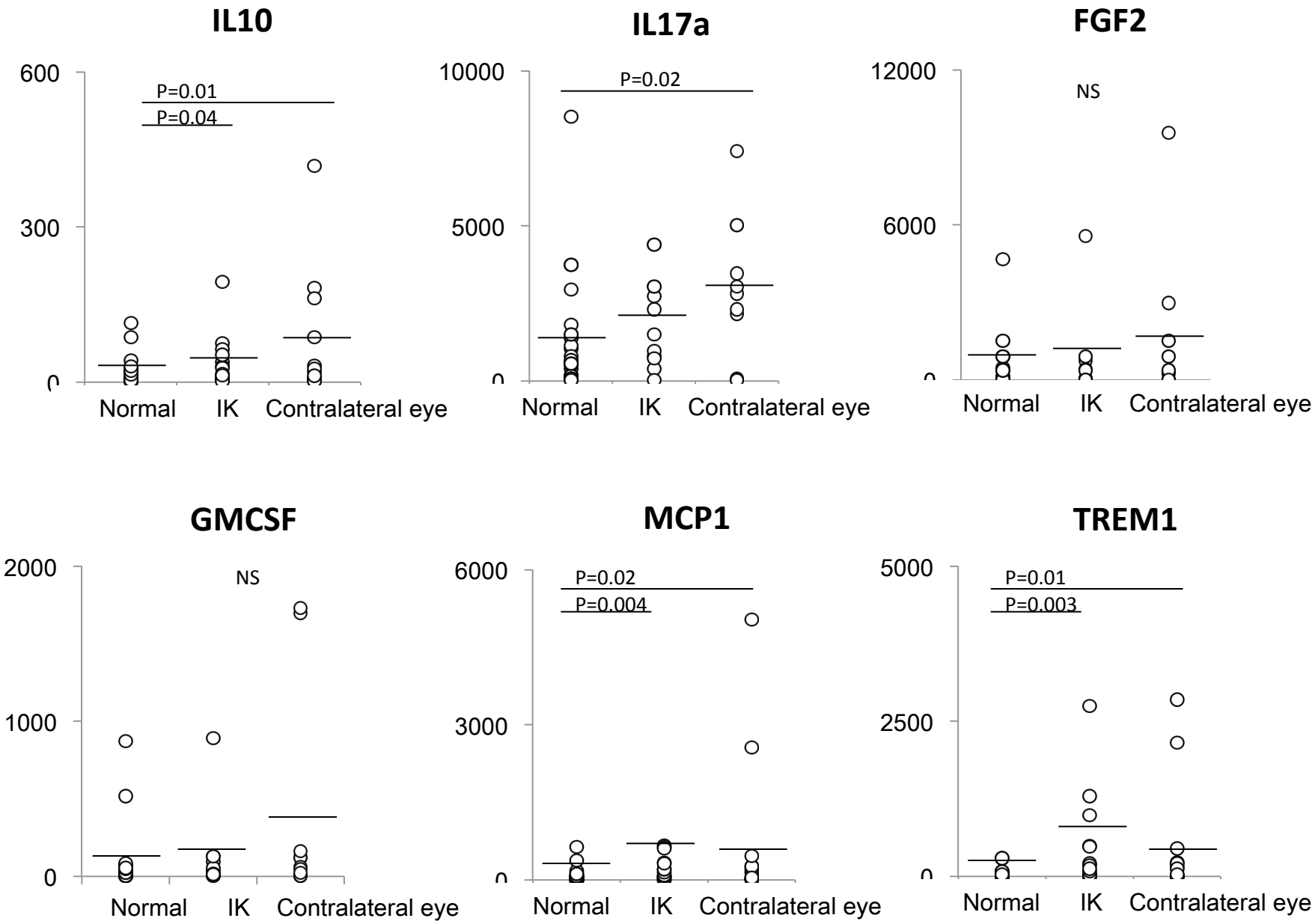
Example: Mouse study to test the influence of mTOR inhibitor treatment on the response to sepsis



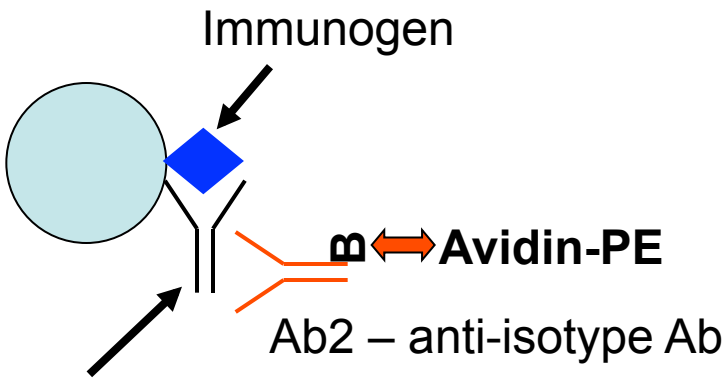
Example: Cytokine Levels in Tear Are Correlated with the Corneal Nerve Density and Dendritic Cell Counts in Eyes with Infectious Keratitis

Takefumi Yamaguchi, Bernardo Cavalcanti, Pedram Hamrah
Mass Eye and Ear Infirmary
Shizu Ishikawa, Akinori Osuka, Kentaro Shimizu, James Lederer
Department of Surgery, Brigham and Women's Hospital

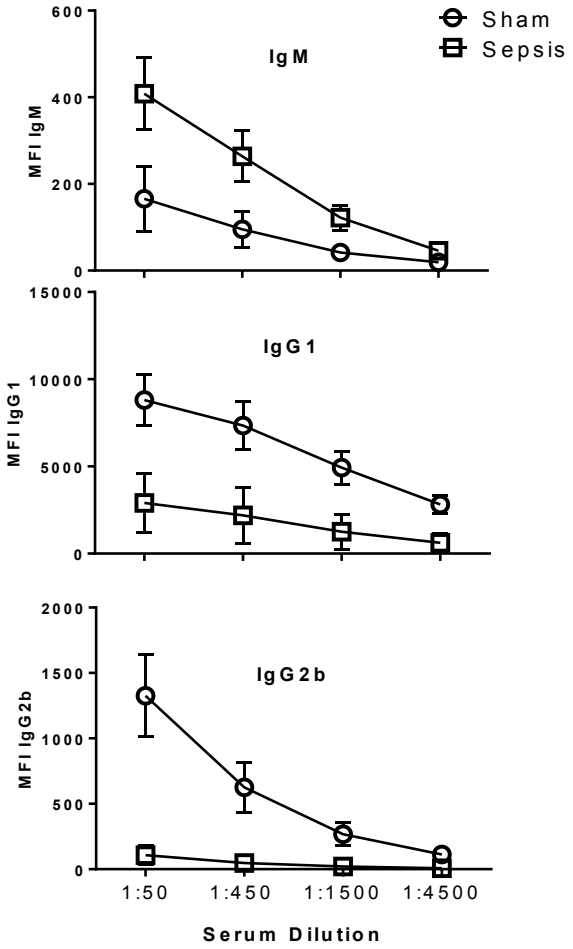

IL1b
3000
1500
0
P=0.02
P=0.005
Normal
IK
Contralateral eye
IL1Ra
400
200
0
NS
Normal
IK
Contralateral eye
IL2
25000
20000
15000
10000
5000
0
NS
Normal
IK
Contralateral eye
IL6
30000
15000
0
P=0.04
P < 0.001
Normal
IK
Contralateral eye
IL7
400
200
0
P=0.03
Normal
IK
Contralateral eye
IL8
4000
2000
0
P=0.002
Normal
IK
Contralateral eye


IL10
600
300
0
P=0.01
P=0.04
Normal
IK
Contralateral eye
IL17a
10000
5000
0
P=0.02
Normal
IK
Contralateral eye
FGF2
12000
6000
0
NS
Normal
IK
Contralateral eye
GMCSF
2000
1000
0
NS
Normal
IK
Contralateral eye
MCP1
6000
3000
0
P=0.02
P=0.004
Normal
IK
Contralateral eye
TREM1
5000
2500
0
P=0.01
P=0.003
Normal
IK
Contralateral eye

Example: Multiplexing antigen- or immunogen-specific antibody assays for vaccine testing



Plasma or serum sample from immunized mouse (or human)



Summary

1. Development of custom multiplex assay is feasible and cost effective through careful optimization and validation.

2. Provides an opportunity to develop new assays that are not commercially available.

3. Opens up opportunities for collaboration.

4. Developed of **VeloceBio** as a collaborative biomarker assay development and design company.

5. Collaboration with Cambridge Biomedical