High Throughput Technology and Predictive Immune Monitoring

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Cancer & the Immune System

- Cancer can evade and modulate the host immune response
  - Regulatory T cells are increased within the tumor microenvironment, tumor-draining lymph nodes, and blood
  - anti-tumor T cells develop but are dysfunctional
  - Other immune cell types also altered
- Immune status may predict cancer patient prognosis and guide therapy
- Immune markers may serve as surrogates for efficacy of cancer immunotherapy
- High throughput methods to systematically assess host immune function are needed

Some High Throughput Methods for Immune Analysis

- Enumeration of immune cell populations and subtypes: FACS, pMHC tetramers, ELISPOT
- Immune cells biology
  - Gene expression, microRNA, epigenetics: microarrays
  - Functional responses: CFC, phosflow, Luminex, qPCR
- Soluble factors: proteins, lipids, small molecules

Gene Expression Profiling of Lymphocytes from Melanoma Patients

12 Melanoma Patients: Stage IV, resected, no recent systemic therapy
12 Healthy donors: age- and gender-matched

PBMCs sorted by FACS into:
CD8 T cells, CD4 T cells, B cells and NK cells (>99%)

Total RNA, amplified (with amino-allyl labeling)

Hybridized onto Agilent Human microarrays (22K)
with Total Lymphocyte Reference RNA

Entrez Gene Symbol	Entrez Gene Name	Adjusted P value	↑or↓ in melanoma
IFIT3 *	interferon-induced protein with tetratricopeptide repeats 3	0.00125	↓
RSAD2 *	radical S-adenosyl methionine domain containing 2	0.00125	↓
LOC129607	hypothetical protein LOC129607	0.00125	↓
IFI44L *	interferon-induced protein 44-like	0.00125	↓
IFIT1 *	interferon-induced protein with tetratricopeptide repeats 1	0.00125	↓
IFIT2 *	interferon-induced protein with tetratricopeptide repeats 2	0.00125	↓
OAS3 *	2'-5'-oligoadenylate synthetase 3, 100kDa	0.00125	↓
FREQ	frequenin homolog (Drosophila)	0.00125	↑
OAS1 *	2',5'-oligoadenylate synthetase 1, 40/46kDa	0.00200	↓
STAT1 *	signal transducer and activator of transcription 1, 91kDa	0.00200	↓
IFI44 *	interferon-induced protein 44	0.00250	↓
ISG15 *	ISG15 ubiquitin-like modifier	0.00250	↓
SAMD9L	sterile alpha motif domain containing 9-like	0.00385	↓
PARP9	poly (ADP-ribose) polymerase family, member 9	0.00400	↓
CXCL11 *	chemokine (C-X-C motif) ligand 11	0.00400	↓
GBP1 *	guanylate binding protein 1, interferon-inducible, 67kDa	0.00688	↓
CXCL10 *	chemokine (C-X-C motif) ligand 10	0.02278	↓
MX2 *	myxovirus (influenza virus) resistance 2 (mouse)	0.02278	↓
EIF2AK2 *	eukaryotic translation initiation factor 2-alpha kinase 2, IFN-inducible	0.02526	↓
LAMP3	lysosomal-associated membrane protein 3	0.02950	↓
USP18 *	ubiquitin specific peptidase 18	0.03143	↓
SAMD9	sterile alpha motif domain containing 9	0.03545	↓
PLSCR1	phospholipid scramblase 1	0.03565	↓
BIRC4BP	XIAP associated factor-1	0.04333	↓
IFI27 *	interferon, alpha-inducible protein 27	0.04440	↓

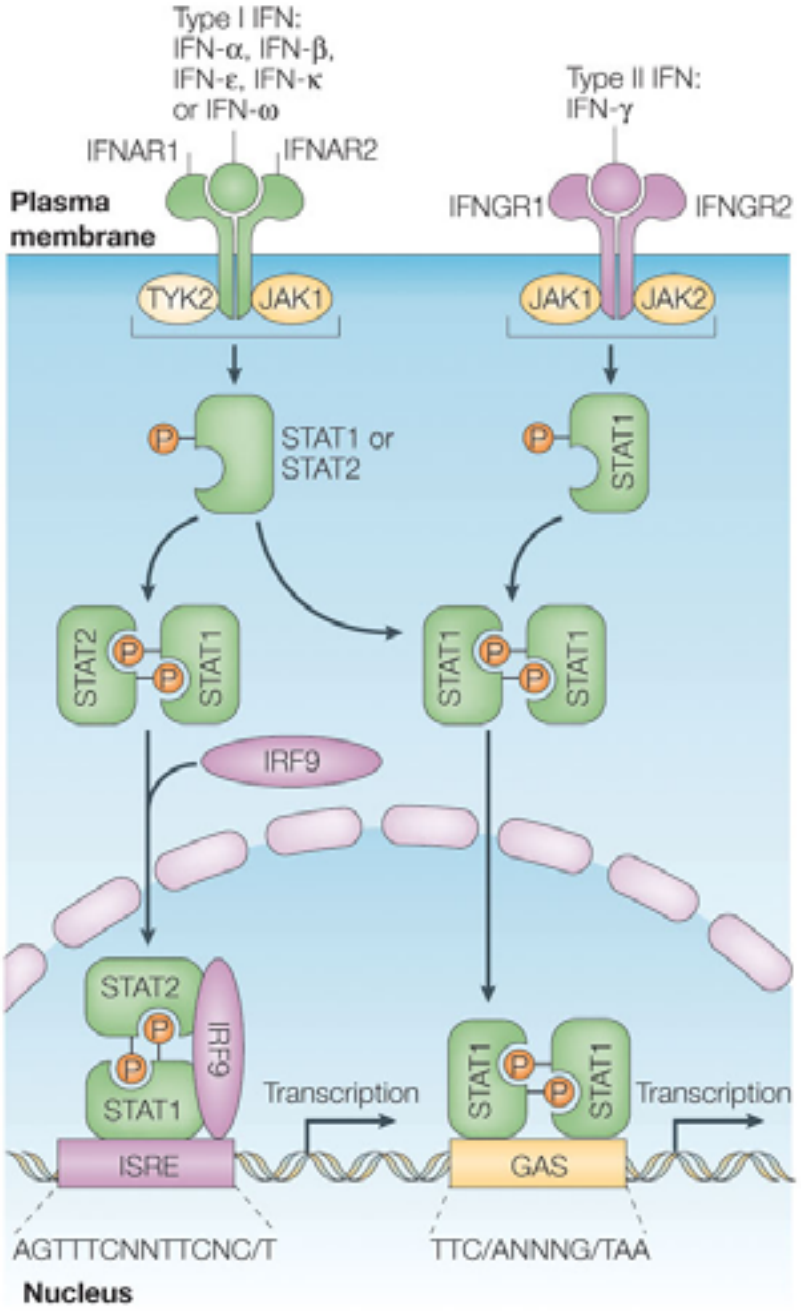
* Interferon-stimulated gene

Critchley-Thorne et al. PLoS Med. 2007 May;4(5):e176

Interferon signaling pathways

- STAT1 pY701 common to both type-I and –II IFN signaling pathways

- Anti-STAT1 pY701 validated for Phosflow analysis

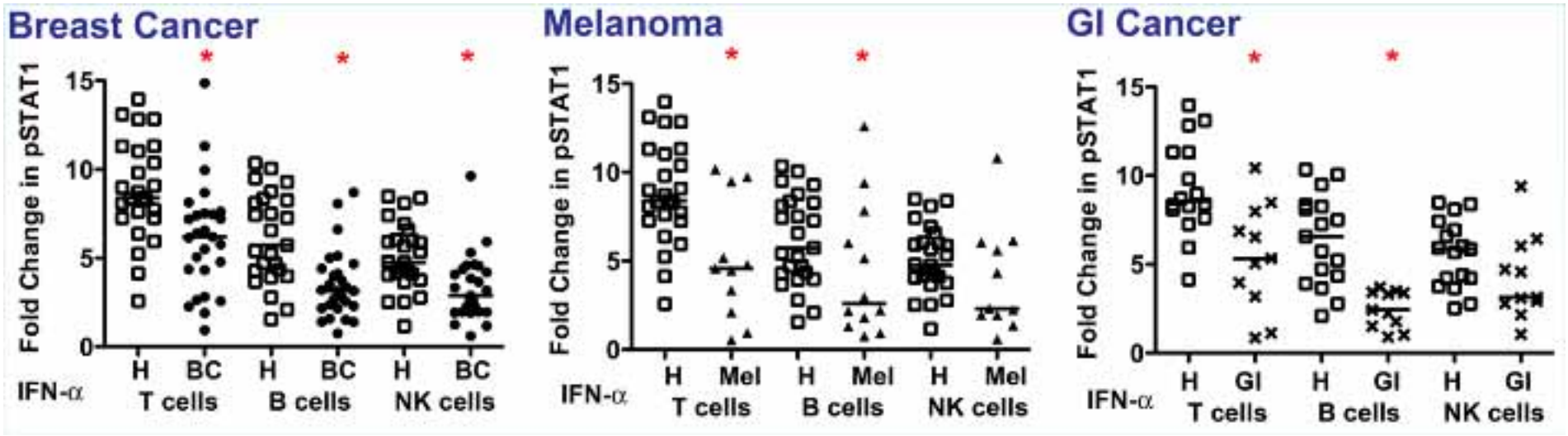


Platanias LC. Nat Rev Immunol. 2005 May;5(5):375-86.

Phospho-flow cytometry for high-throughput immune monitoring

- Analysis of signaling capacity of immune cell populations on a single-cell basis
- Intracellular staining of phosphorylated signaling molecules after stimulation with various cytokines
- Example: pSTAT1 after IFN-α or IFN-γ stimulation

IFN-α-induced pSTAT1-Y701 is reduced in T cells, B cells and NK cells from cancer patients

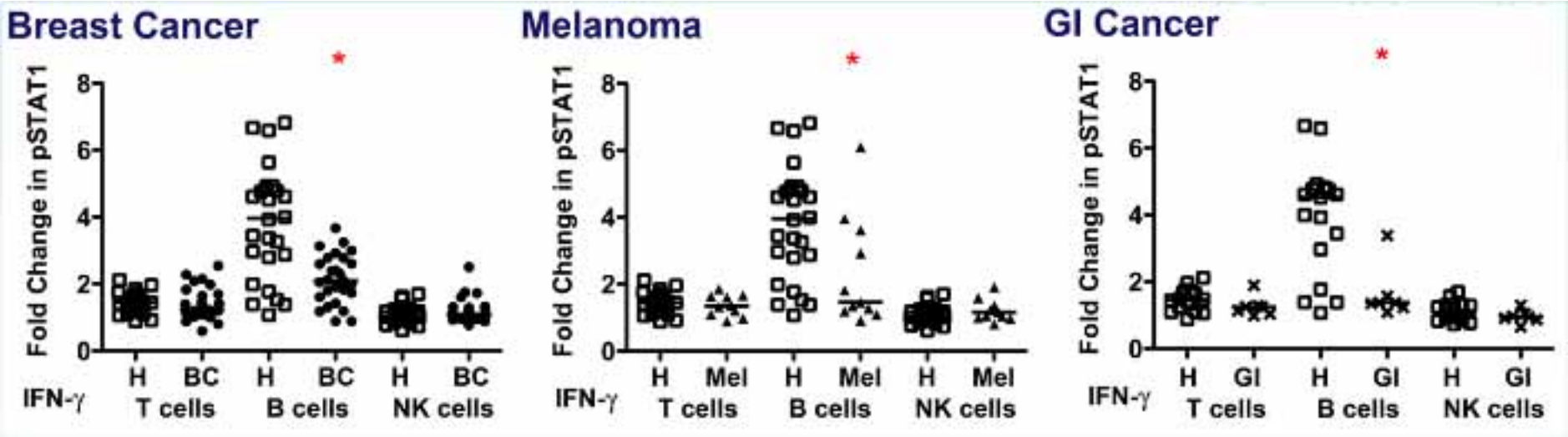


* p-value < 0.05

- Lymphocytes were stimulated with 1000 IU/mL IFN-α and pSTAT1 was measured in T cells, B cells and NK cells
- Fold change in pSTAT1: MFI pSTAT1 in IFN-stimulated cells/MFI pSTAT1 in unstimulated cells

Critchley-Thorne et al. PNAS 2009 Jun 2;106(22):9010-5

IFN-γ-induced pSTAT1-Y701 is reduced in B cells from cancer patients

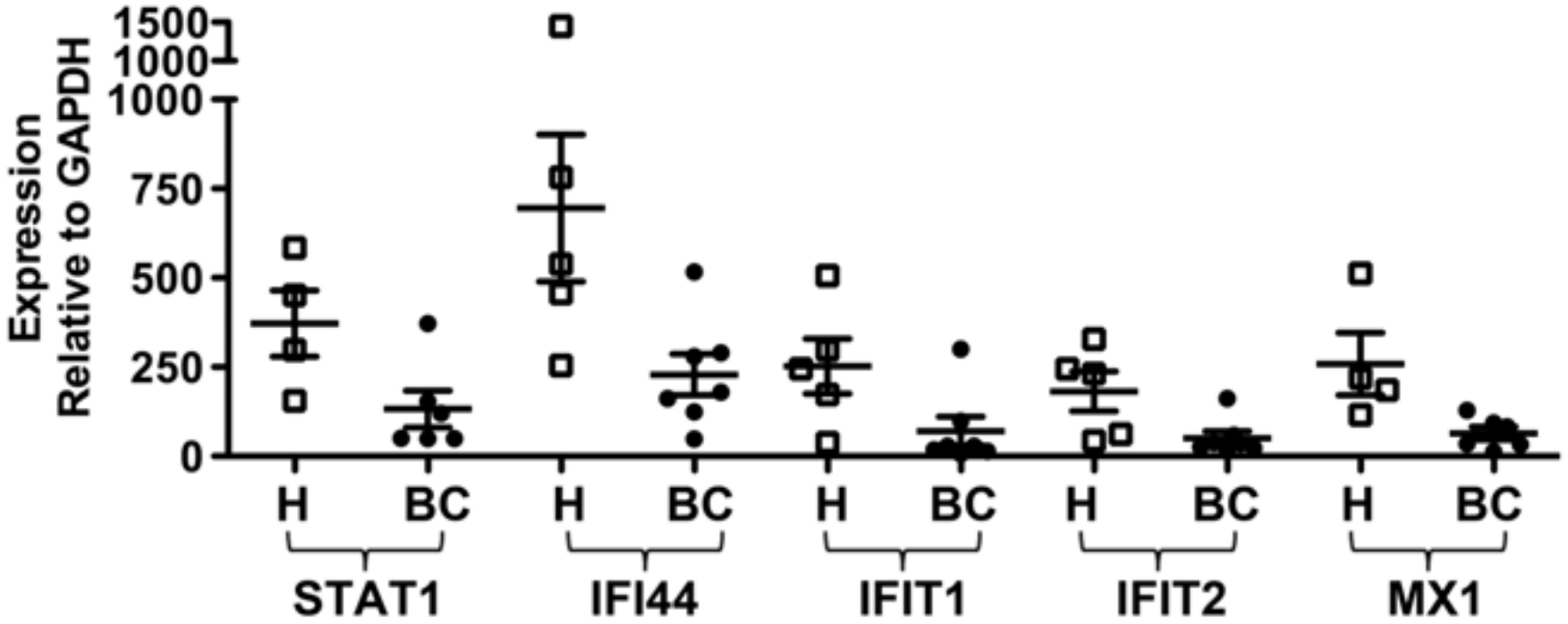


* p-value < 0.05

- Lymphocytes were stimulated with 1000 IU/mL IFN-α and pSTAT1 was measured in T cells, B cells and NK cells
- T cells and NK cells from healthy donors and cancer patients show minimal phosphorylation of STAT1 in response to IFN-γ

Critchley-Thorne et al. PNAS 2009 Jun 2;106(22):9010-5

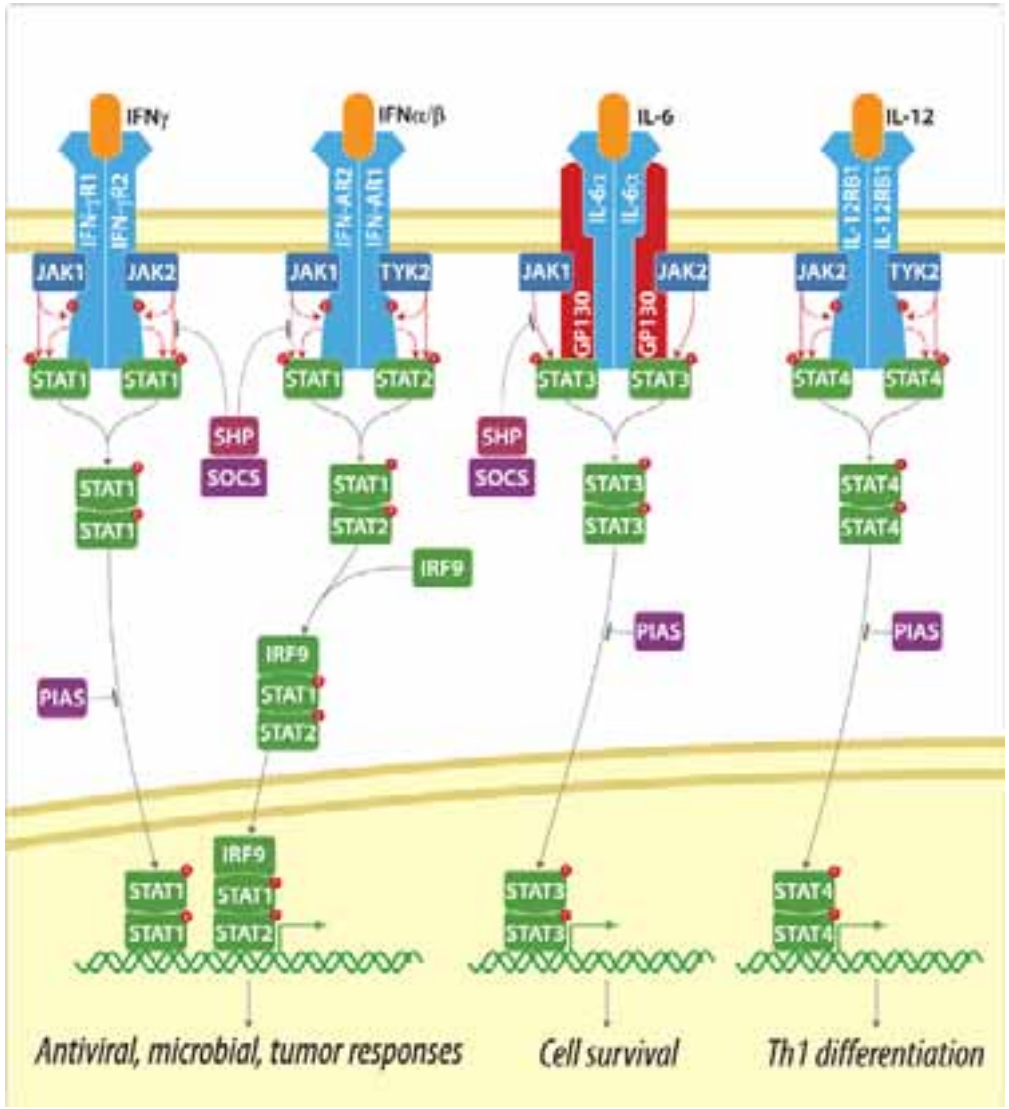
ISG Expression is Reduced in Lymphocytes from Breast Cancer Patients



ISGs measured directly *ex vivo* by rQ-PCR in unstimulated lymphocytes

Critchley-Thorne et al. PNAS 2009 Jun 2;106(22):9010-5

Other cytokine signaling pathways



Ligands		Jak kinases	STATs
IFN family[a]			
IFN-αs[b]/β/ω/Limitin		Tyk2, Jak1	**Stat1**, **Stat2**, (Stat3, Stat4, Stat5)
IFN-γ		Jak1, Jak2	**Stat1**, (Stat5)
IL-10		Tyk2, Jak1	**Stat3** **Stat1**
IL-19		?	?
IL-20		?	**Stat3**
IL-22		?	**Stat3**, (Stat5)
gp130 family			
IL-6	**Tyk2**	Jak1, Jak2	**Stat3**, Stat1
IL-11	**Tyk2, Jak2**	Jak1	**Stat3**, Stat1
OSM	**Tyk2**	Jak1, Jak2	**Stat3**, Stat1
LIF	**Tyk2**	Jak1, Jak2	**Stat3**, Stat1
CNTF	**Tyk2**	Jak1, Jak2	**Stat3**, Stat1
NNT-1/BSF-3		Jak1, Jak2	**Stat3**, Stat1
G-CSF	**Jak3**	Jak1, Jak2	**Stat3**
CT-1	**Tyk2**	Jak1, Jak2	**Stat3**
Leptin		Jak2	**Stat4**
IL-12		Tyk2, Jak2	**Stat4**
IL-23		?	**Stat4**
γC family			
IL-2	**Jak2**	Jak1, Jak3	**Stat5**, (Stat3)
IL-7		Jak1, Jak3	**Stat5**, (Stat3)
(TSLP)[f]		?	Stat5
IL-9		Jak1, Jak3	**Stat5**, Stat3
IL-15		Jak1, Jak3	**Stat5**, (Stat3)
IL-21		(Jak1), Jak3	Stat3, **Stat5**, **(Stat1)**
IL-4		Jak1, Jak3	**Stat6**
(IL-13)[f]	**Jak2, Tyk2**	Jak1	**Stat6, (Stat3)**
IL-3 family			
IL-3		Jak2	**Stat5** **Stat3**
IL-5		Jak2	**Stat5** **Stat3, Stat1**
GM-CSF		Jak2	**Stat5**
Single chain family			
EPO		Jak2	**Stat5**
GH		Jak2	**Stat5**, (Stat3)
PRL		Jak2	**Stat5**
TPO	**Tyk2**	Jak2	**Stat5**
Receptor tyrosine kinases			
EGF		(Jak1, Jak2)	Stat1, Stat3, Stat5
PDGF		(Jak1, Jak2)	Stat1, Stat3
CSF-1		(Tyk2, Jak1)	Stat1, Stat3, Stat5
HGF		?	Stat1, Stat3
G-protein coupled receptors			
AT1		Jak2	Stat1, Stat2

Invitrogen

Kisseleva et al, Gene. 2002 Feb 20;285(1-2):1-24.

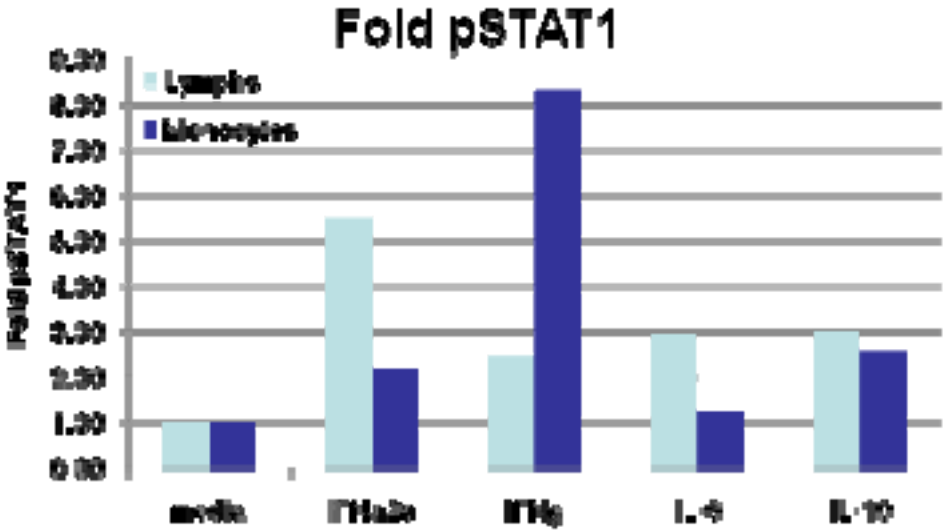
Expanded Phosflow Panels

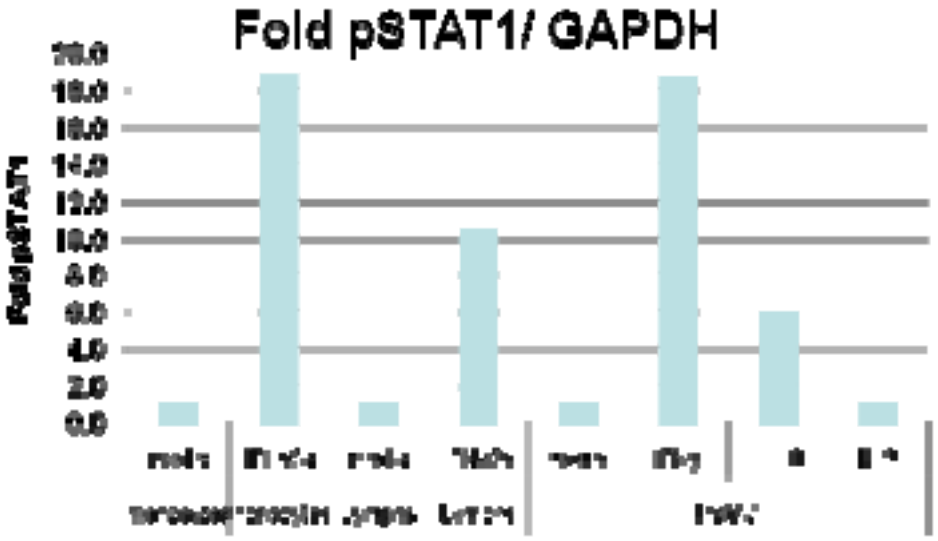
- Examine multiple immune cell types: CD4 T, CD8 T, B, NK, monocytes
- Assess additional cytokine signaling pathways beyond IFN: IL-2, IL-4, etc.
- Measure multiple signaling molecules: JAK, STAT, etc.
- Limited by available phospho antibodies and overlapping spectra of fluorophores

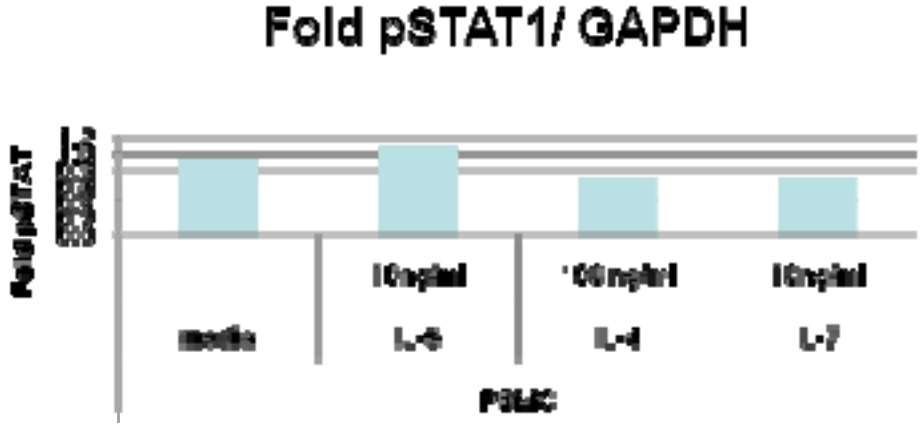
Luminex for Multiplex Analysis of Phospho-Proteins

- Multiplex analysis of up to 100 analytes from a single sample
- Allows comprehensive analysis of entire signaling networks
- High sensitivity allows signaling analysis from small sample sizes (<10 ug of cell lysate)
- Limited by inability to analyze cells on single-cell level
  - First need to separate different immune cell populations

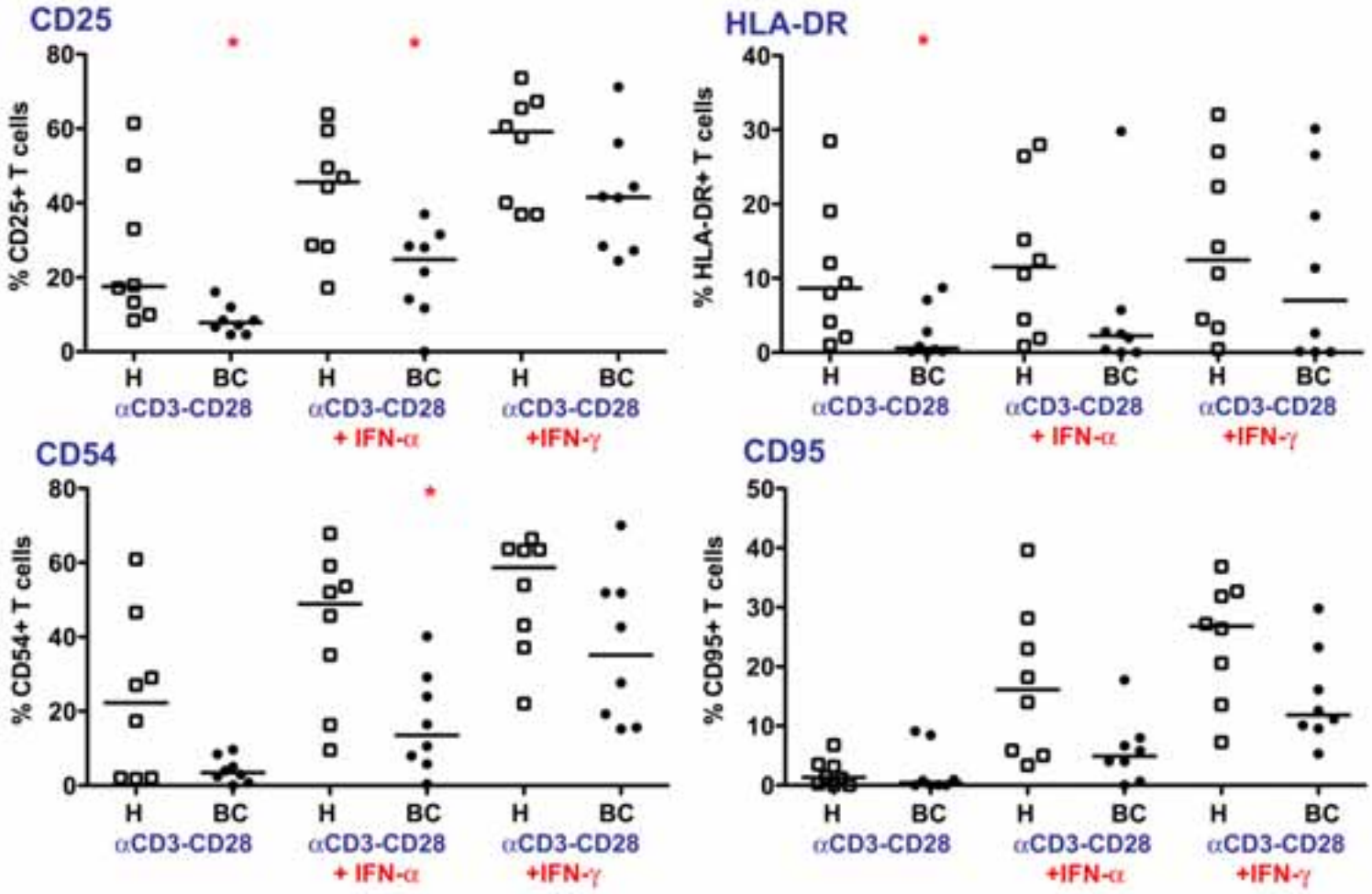
Phosflow vs. Luminex







Defects in downstream IFN functional responses in T cells from breast cancer patients



Multivariate analysis of CD25, HLA—DR, CD54 and CD95: the expression levels of these activation markers were significantly reduced in T cells stimulated with anti-CD3/CD28 alone (p=0.021) and in combination with IFN-α (p=0.038) in breast cancer patients vs. healthy controls

Summary

- IFN signaling defects develop in lymphocytes from patients with three major cancers: melanoma, breast, and GI
  - IFN-α in T, B, and NK cells
  - IFN-γ in B cells
- Downstream functional defects include reduced activation, proliferation, and increased apoptosis
- Signaling in other cytokine pathways in different immune cell types being assessed via expanded phosflow and Luminex
- Each high throughput method has advantages and limitations

Current and future directions

- Understanding global cytokine signaling patterns in different immune cell populations at different times (pre-tx, remission, relapse) will provide snapshots of immune function in cancer
- Specific immune defects may provide prognostic information or predict relapse
- Strategies to correct specific cytokine signaling defects may be useful as standalone or adjuvant therapy for cancer

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