Using Phosphoflow to Dissect Alterations in Cytokine-Induced Activation of Jak/STAT Pathway in Rheumatoid Arthritis

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The Jak/STAT Pathway

Expression of Interferon Receptors indicates presence and severity of RA

- Rheumatoid Arthritis
  - Increased IFNGR1 expression in RA patients compared to controls
  - Increasing IFNGR2 expression indicates radiographic damage
  - No change in serum levels of IFN-γ indicating that the limiting factor is the expression of receptors

Tang Q, et al., 2015
IL-2 is an important regulator of disease

- Nuetralization of IL-2 in mouse model inhibits the proliferation of CD25+CD4+ regulatory T cells, causing autoimmunity.¹
- T reg proliferation driven by IL-2 suppresses differentiation of Th17 cells.
- Suppression of Tfh populations cause a decrease in germinal center formation.²

1. JEM, 2005, Setoguchi
2. Immunity, 2012, Ballesteros-Tato
Advantages of Multi-parameter Phospho Flow Cytometry

- Evaluate activation-dependent post-translational changes in 3 or more STATs and/or other signaling molecules simultaneously
- Ability to evaluate changes in expression/activation in subpopulations within a mixed population. This is not feasible by Western blot due to cell number limitations.
- Ability to examine multiple patients/controls at the same time.
- Reproducible, sensitive and with high specificity.
Gating Strategy

Lymphocytes

FSC

SSC

Single Cells

FSC-A

FSC-H

Live Cells

SSC

Live/Dead

Lymphocytes

Single Cells

Live Cells

CD4

CD8

CD45RA

CCR7

CD25

CD4
Phosphoflow Analysis

Central Memory CD4

unstim

IFN-γ

IL-2

p-STAT1

p-STAT5

Treg

unstim

IFN-γ

IL-2

p-STAT1

p-STAT5
RADAR

• 350 patients currently enrolled
  • ~93% of patients have agreed to routine venipuncture for obtaining blood for IRB-approved studies.
• Database includes the following patient information
  • Current medications
  • Number of tender or swollen joints from 28 evaluated joints (CDAI, DAS28 score)
  • Clinical evaluation by doctor or nurse practitioner

Saved for multiplex cytokine analysis

Frozen at -80° in order to normalize results
Activation of STAT1 by IFN-γ

Central memory

Naïve CD4

Treg

Fold change of isotype
Activation of STAT5 by IL-2

Central memory

Naïve CD4

Treg

Fold change of isotype

RA

HC

Gout

Fold change of isotype
Role of Phosphatases

- Phosphatases (PTPs) are critical regulators of many signaling pathways.
- Many PTPs may be associated with autoimmunity.
- A single nucleotide polymorphism in the Ptpn22 gene is strongly associated with RA and other autoimmune diseases.\(^1\)
- PTPN22 is a direct target of FoxP3 and could be pathogenic if mutant form is overactive in Tregs.\(^2\)

1. Seminars in Immunology, 2006, Bottini
Phosphatase inhibitor increases IL-2 stimulated p-STAT5

PAO = phosphatase inhibitor
Total STAT5 decreased with phosphatase inhibitor

PAO = phosphatase inhibitor
Summary

• IL-2 is a known regulator of autoimmune disease
• There is phosphatase activity specific to RA and p-STAT5
• Attenuation of IL-2 activation of STAT5 could be pathogenic in RA
Do circulating levels of cytokines contribute to differences in cytokine-induced activation of the Jak/STAT pathway?
LEGENDplex: using flow cytometry for multiplex cytokine analysis

- Ability to evaluate up to 13 cytokines simultaneously
- In-plate assay allows for up to 80 samples to be run at one time
- Customizable panels for mouse and human
LEGENDplex assay

**Beads A**
- IL-9
- IL-13
- IL-10
- IL-6
- IL-2
- IL-5

**Beads B**
- IL-22
- IL-21
- IL-17A
- IL-17F
- IFN-γ
- TNFα
Plasma cytokine levels are not significantly different in RA vs Controls

Potential biomarkers for treatment may include the cellular response to cytokines in different sub-populations of T cells
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