Simultaneous correlation of cytokine production with Treg and Th17 cell proliferation

Jurg Rohrer, PhD
Director, R&D
BD Biosciences
Overview

• T helper (Th) cell overview
• Experimental setup
• Data analysis
• Conclusions
Th17 cells

- Developmentally distinct from Th1 and Th2 cells
- Immunity against bacterial and fungal infections
- Play a key role in autoimmune diseases (tissue injury)
- Controlling Th17 activity could aid in the treatment of autoimmune diseases
- TGF-β, IL-6, IL-21, IL-1β, and IL-23 appear to drive Th17 development
- Produce IL-17A, IL-17F; also IL-21, IL-22, IL-26, and less TNF and IL-6
Treg cells

- Actively suppress T cell proliferation, crucial for T cell homeostasis
- FoxP3, transcription factor is a specific marker for Treg
- FoxP3 is necessary for both development and function of Treg
- nTreg develop in the thymus, iTreg require TGFβ, IL-2 and RA
- Produce TGFβ and IL-10 and express high levels of CD25 and low levels of CD127
- Dampening Treg activity could improve anti-tumor responses and responses to vaccinations and chronic infections
- Boosting Treg activity could be useful in the treatment of T cell induced diseases
Experimental model

- Enrich Balb/c splenocytes by positive selection via CD4⁺ panning
- Load isolated cells with VPD450 1μM, 10 minutes
- Set up cultures as follows:
  - CD3/CD28
  - CD3/CD28/IL-6/IL-1β
  - CD3/CD28/IL-6/IL-1β/TGFβ
  - CD3/CD28/IL-6/IL-1β/TGFβ/IL-23
- Harvest cells at 1, 2, 3, and 4 days
- Fix/perm and stain cells for IL-17A, Foxp3, IL-4, IL-2, and interferon-γ (IFN-γ)
Experimental setup

Harvest Spleen  ->  CD4 cells enriched by panning

Cells loaded with VPD450 and washed

BD Cytofix/Cytoperm™ buffer
Cytokines

+ Monensin

FoXP3 Fix/Perm buffer
Foxp3 and some cytokines

- Monensin

Harvest Stimulate with PMA and Ionomycin for 4–5 hours + or – Monensin

Supernatants: Analysis with BD™ CBA Flex Sets
Cells: BD™ Phosflow Fix and Perm buffer

Day 1, 2, 3, 4
Fluorescein Diacetate Derivative

ARM = amino-reactive moiety
ECM = esterase-cleavable moiety
MFM = masked fluorophore moiety
IACB = Intracellular amino-containing biopolymer

VPD450 Dye
Non-Fluorescent
Enters cells, esterases cleave ECM to give fluorescent product
Reacts with cell components to give VPD450 adducts retained inside cells

Fluorescent
Fluorescent and Cell-retained
Spleen CD3/28 Day 2 – [VPD450]

1 μM

DNA

2.5 μM

DNA

5 μM

DNA

BrdU

1x10^7/ml

0 μM

Count

Count

Count
Spleen CD3/28 Day 2 – [Cell]

1 x 10^7 cells/ml

1 x 10^6 cells/ml

2 x 10^7 cells/ml

1 μM BrdU

DNA

BrdU

DNA

BrdU

DNA

BrdU

V450-A

V450-A

V450-A

V450-A
Human PBMC PHA Stimulation [VPD450]

Cells Only 1 μM VPD450 10 μM VPD450

Day 3

BrdU DNA VPD450

20.9% 20.6% 4%

Day 5

BrdU DNA VPD450

6% 14.5% 41%

1 x 10^6 cell/ml

BD
Condition: CD3/CD28 + IL-1β/IL-6 + TGF-β + IL-23

Day 1

Day 2

Day 3

VPD450
Which conditions for which cytokines

- All conditions result in proliferation of cells to essentially equal extents.

- Which cytokines are being produced under which conditions?

- Which cell types are producing which cytokines?
VPD450 vs IL-2 data

Condition: CD3/CD28 + IL-1β/IL-6 + TGF-β + IL-23

Day 1

Day 2

Day 3
VPD450 vs IFN-\(\gamma\) data

<table>
<thead>
<tr>
<th>Condition</th>
<th>CD3/CD28</th>
<th>+IL-1(\beta)/IL-6</th>
<th>+TGF-(\beta)</th>
<th>+IL-23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>Specimen_001-B3</td>
<td>Specimen_002-C3</td>
<td>Specimen_003-D3</td>
<td>Specimen_004-E3</td>
</tr>
<tr>
<td>Day 2</td>
<td>Specimen_005-F3</td>
<td>Specimen_006-G3</td>
<td>Specimen_007-H3</td>
<td>Specimen_008-A9</td>
</tr>
<tr>
<td>Day 3</td>
<td>Specimen_009-B9</td>
<td>Specimen_010-C9</td>
<td>Specimen_011-D9</td>
<td>Specimen_012-E9</td>
</tr>
</tbody>
</table>
## VPD450 vs IL-17A data

**Condition:**
- **CD3/CD28**
- **+IL-1β/IL-6**
- **+TGF-β**
- **+IL-23**

**Day 1**

<table>
<thead>
<tr>
<th>VPD450</th>
<th>IL-17A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen_001-B2</td>
<td>Specimen_002-C2</td>
</tr>
</tbody>
</table>

**Day 2**

<table>
<thead>
<tr>
<th>VPD450</th>
<th>IL-17A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen_003-D2</td>
<td>Specimen_004-E2</td>
</tr>
</tbody>
</table>

**Day 3**

<table>
<thead>
<tr>
<th>VPD450</th>
<th>IL-17A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen_005-F2</td>
<td>Specimen_006-G2</td>
</tr>
</tbody>
</table>

**Legend:**
- VPD450: Vertical Position Distribution 450
- IL-17A: Interleukin-17A
Cytokine co-expression

- IL-2 is expressed under all conditions
- IFN-γ is produced more under condition 1
- TGF-β is required for expression of IL-17A
- Which cytokines are co-expressed?
Co-expression of IL-17A vs IL-2

Condition: CD3/CD28 +IL-1β/IL-6 +TGF-β +IL-23

Day 1

Day 2

Day 3
Co-expression of IL-17A vs IFN-γ

Condition: CD3/CD28 +IL-1β/IL-6 +TGF-β +IL-23

Day 1

<table>
<thead>
<tr>
<th>Condition</th>
<th>Specimen</th>
<th>IL-17A</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3/CD28</td>
<td>001-A3</td>
<td>1.2%</td>
<td></td>
</tr>
<tr>
<td>+IL-1β/IL-6</td>
<td>002-B3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+TGF-β</td>
<td>003-C3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+IL-23</td>
<td>004-E3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Day 2

<table>
<thead>
<tr>
<th>Condition</th>
<th>Specimen</th>
<th>IL-17A</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3/CD28</td>
<td>005-E3</td>
<td>4.5%</td>
<td></td>
</tr>
<tr>
<td>+IL-1β/IL-6</td>
<td>006-F3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+TGF-β</td>
<td>007-G3</td>
<td>2.1%</td>
<td></td>
</tr>
<tr>
<td>+IL-23</td>
<td>008-H3</td>
<td>1.2%</td>
<td>3.2%</td>
</tr>
</tbody>
</table>

Day 3

<table>
<thead>
<tr>
<th>Condition</th>
<th>Specimen</th>
<th>IL-17A</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3/CD28</td>
<td>009-A9</td>
<td>7.5%</td>
<td></td>
</tr>
<tr>
<td>+IL-1β/IL-6</td>
<td>010-B9</td>
<td>1.9%</td>
<td></td>
</tr>
<tr>
<td>+TGF-β</td>
<td>011-C9</td>
<td>1.7%</td>
<td>1.9%</td>
</tr>
<tr>
<td>+IL-23</td>
<td>012-D9</td>
<td>1.1%</td>
<td>5.2%</td>
</tr>
</tbody>
</table>
Co-expression of IL-17A vs IL-4

Condition: CD3/CD28 +IL-1β/IL-6 +TGF-β +IL-23

Day 1

Day 2

Day 3
Tracking FoxP3

- IL-17A expression is boosted by addition of IL-23.

- Earlier on IL-17A expressing cells co-express IL-2, but over time the two become mutually exclusive.

- IL-4 expression increases as IFN-\(\gamma\) expression decreases.

- What are the FoxP3+ cells doing?
Experimental setup

Harvest Spleen

CD4 cells enriched by panning

Cells loaded with VPD450 and washed

BD Cytofix/Cytoperm™ buffer
Cytokines

+ Monensin

BD™ Phosflow Fix and Perm buffer
Foxp3 Fix/Perm buffer
Foxp3 and some cytokines

- Monensin

Harvest Stimulate with PMA and Ionomycin for 4–5 hours + or – Monensin

Supernatants: Analysis with BD™ CBA Flex Sets
Cells: BD™ Phosflow Fix and Perm buffer

Day 1, 2, 3, 4

anti-CD3e+ anti-CD28+ IL-1β+ IL-6

anti-CD3e+ anti-CD28+ IL-1β+ IL-6+ TGF-β

anti-CD28+ IL-1β+ IL-6+ TGF-β+ IL-23
Comparison of two fix/perm protocols

<table>
<thead>
<tr>
<th>BD Cytofix/Cytoperm protocol</th>
<th>Foxp3 fix/perm protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-17A</strong></td>
<td><strong>IL-17A</strong></td>
</tr>
<tr>
<td><strong>IL-2</strong></td>
<td><strong>IL-2</strong></td>
</tr>
<tr>
<td><strong>Day4 Cytofix/Perm-4 v450/8/ε</strong></td>
<td><strong>Day4 FoxP3-4 v450/8/apc/PE</strong></td>
</tr>
<tr>
<td>4.3% Q1</td>
<td>3.3% Q1</td>
</tr>
<tr>
<td>Q4 7.7%</td>
<td>Q4 3.8%</td>
</tr>
<tr>
<td><strong>IFN-γ</strong></td>
<td><strong>IFN-γ</strong></td>
</tr>
<tr>
<td><strong>Day4 Cytofix/Perm-17Per/IFN</strong></td>
<td><strong>Day4 FoxP3-17Per/IFN F/4PE</strong></td>
</tr>
<tr>
<td>4.1% Q1</td>
<td>3.4% Q1</td>
</tr>
<tr>
<td>Q4 3.8%</td>
<td>Q4 3.9%</td>
</tr>
</tbody>
</table>
Proliferation of Treg and Th17 cells

Condition:    CD3/CD28    +IL-1β/IL-6    +TGF-β    +IL-23

Day 0

Day 1

Day 2

Day 3

FoxP3

VPD450
Co-expression of Foxp3 vs IFN-γ

Condition: CD3/CD28

Day 1
- CD3/CD28: 3.3%
- +IL-1β/IL-6: 3.2%
- +TGF-β: 2.5%
- +IL-23: 2.2%

Day 2
- CD3/CD28: 6.4%
- +IL-1β/IL-6: 3.7%
- +TGF-β: 1.9%
- +IL-23: 2.1%

Day 3
- CD3/CD28: 11%
- +IL-1β/IL-6: 3.1%
- +TGF-β: 3.9%
- +IL-23: 4.4%
Co-expression of Foxp3 vs IL-17A

Condition: CD3/CD28 + IL-1β/IL-6 + TGF-β + IL-23

Day 1
- CD3/CD28: 2.9%
- +IL-1β/IL-6: 3.5%
- +TGF-β: 3.3%
- +IL-23: 4.5%

Day 2
- CD3/CD28: 1.9%
- +IL-1β/IL-6: 1.7%
- +TGF-β: 2.5%
- +IL-23: 2.3%

Day 3
- CD3/CD28: 1.9%
- +IL-1β/IL-6: 2.3%
- +TGF-β: 2.6%
- +IL-23: 2.3%
Cytokines in culture supernatants

- FoxP3 expression maintained throughout culture period.
- FoxP3+ Treg cells divide more slowly than other CD4 t cells.
- Expression of IFN-γ and IL-17A not found in Treg.
- Does cytokine expression detected in the cells correlate with cytokine detected in culture supernatants?
Experimental setup

Harvest spleen

CD4 cells enriched by panning

Cells loaded with VPD450 and washed

BD Cytofix/Cytoperm buffer
Cytokines

+ Monensin

BD Phosflow Fix and Perm buffer
Foxp3 Fix/Perm buffer
Foxp3 and some cytokines

- Monensin

Harvest Stimulate with PMA and Ionomycin for 4–5 hours + or – Monensin

Supernatants: Analysis with BD CBA Flex Sets
Cells: BD Phosflow Fix and Perm buffer

Day 1, 2, 3, 4
IL-17A and IFN-γ production
Experimental setup

Harvest spleen → CD4 cells enriched by panning

Cells loaded with VPD450 and washed

BD Cytofix/Cytoperm buffer
Cytokines

+ Monensin

Foxp3 Fix/Perm buffer
Foxp3 and some cytokines

- Monensin

Harvest
Stimulate with PMA and Ionomycin for 4–5 hours
+ or – Monensin

Supernatants: Analysis with BD CBA Flex Sets
Cells: BD Phosflow Fix and Perm buffer

Day 1, 2, 3, 4

BD
pStat5 detection on day 4

**Unactivated:** Cells were cultured, harvested, and stained with phosphospecific Stat5 antibody.

**Activated:** Cells were cultured and activated with PMA/Ionomycin for 5 hours and then stained with phospho-specific Stat5 antibody.
Condition: CD3/CD28 + IL-1β/IL-6 + TGF-β + IL-23

Day 1

Day 2

Day 3

pStat5 in activated cells over time
pStat5 in proliferating cells

**Condition:**
- CD3/CD28
- +IL-1β/IL-6
- +TGF-β
- +IL-23

**Day 1**

- Specimen_003-C2
- Specimen_004-D2
- Specimen_005-E2
- Specimen_006-F2

**Day 2**

- Specimen_007-G2
- Specimen_008-H2
- Specimen_009-A6
- Specimen_010-E6

**Day 3**

- Specimen_011-C6
- Specimen_012-D6
- Specimen_013-E6
- Specimen_014-F6
Conclusions

• Cells proliferated equally well under all four polarization conditions.

• In vitro cultures showed that TGF-β was important for polarization of CD4 cells towards Th17.

• Initial cultures show co-expression of IL-2 and IL-17 that later become independent of each other.

• Detection of secreted cytokines (by CBA) correlated with the intracellular staining.

• Cytokine production by proliferating cells resulted in increased phosphorylation of the signal transducer Stat5.
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