Simultaneous correlation of cytokine production with Treg and Th17 cell proliferation

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Overview

- T helper (Th) cell overview
- Experimental setup
- Data analysis
- Conclusions
Introduction to Th biology
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
Enrich Balb/c splenocytes by positive selection via CD4+ panning

Load isolated cells with VPD450 1\( \mu \)M, 10 minutes

Set up cultures as follows:
- CD3/CD28
- CD3/CD28/IL-6/IL-1\( \beta \)
- CD3/CD28/IL-6/IL-1\( \beta \)/TGF\( \beta \)
- CD3/CD28/IL-6/IL-1\( \beta \)/TGF\( \beta \)/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-\( \gamma \) (IFN-\( \gamma \))
Experimental setup

CD4 cells enriched by panning

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

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-CD3e+ IL-23
Fluorescein Diacetate Derivative

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 and Cell-retained

ARM = amino-reactive moiety
ECM = esterase-cleavable moiety
MFM = masked fluorophore moiety
IACB = Intracellular amino-containing biopolymer
Spleen CD3/28 Day 2 – [VPD450]

1 μM

1x10^7/ml

2.5 μM

0 μM

5 μM

BrdU

DNA

P2

49.2%

6.9%

1.4%

DNA

7AAD-A

(x 1,000)

DNA

7AAD-A

(x 1,000)

DNA

7AAD-A

(x 1,000)

Count

V450-A

BD
Spleen CD3/28 Day 2 – [Cell]

1 x 10^7 cells/ml

1 μM BrdU

195x53 μM DNA

32.3

0.1

46.5

P2

DNA 7AAD-A (x 1,000)

Count

V450-A

BD
Human PBMC PHA Stimulation [VPD450]

Cells Only

1 μM VPD450

10 μM VPD450

1 x 10^6 cell/ml

Day 3

BrdU

DNA

VPD450

Day 5

BrdU

DNA

VPD450

1 x 10^6 cell/ml
VPD450 histograms

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

IL-2

VPD450
VPD450 vs IFN-γ data

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

Day 1

Day 2

Day 3
VPD450 vs IL-17A data

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

Day 1

Day 2

Day 3

IL-17A

VPD450
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
- Specimen_001-A2: 16% in Q1, 16% in Q2
- Specimen_002-B2: 18% in Q1, 16% in Q2
- Specimen_003-C2: 15% in Q1, 15% in Q2

Day 2
- Specimen_005-E2: 27% in Q1, 21% in Q2
- Specimen_006-F2: 16% in Q1, 1.7% in Q2
- Specimen_007-G2: 14% in Q1, 2.1% in Q2

Day 3
- Specimen_008-H2: 23% in Q1, 13% in Q2
- Specimen_009-A9: 12% in Q1, 1% in Q2
- Specimen_010-B8: 11% in Q1, 1.5% in Q2
- Specimen_011-C8: 1.9% in Q1, 4.7% in Q2
Co-expression of IL-17A vs IFN-γ

<table>
<thead>
<tr>
<th>Condition</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3/CD28</td>
<td>1.2%</td>
<td>4.5%</td>
<td>7.5%</td>
</tr>
<tr>
<td>+IL-1β/IL-6</td>
<td>2.1%</td>
<td>1.9%</td>
<td>1.9%</td>
</tr>
<tr>
<td>+TGF-β</td>
<td>1.6%</td>
<td>2.1%</td>
<td>1.7%</td>
</tr>
<tr>
<td>+IL-23</td>
<td>1.2%</td>
<td>1.9%</td>
<td>5.2%</td>
</tr>
</tbody>
</table>

IL-17A vs IFN-γ
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

IL-4

IL-17A
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

Foxp3 Fix/Perm buffer
Foxp3 and some cytokines

+ Monensin

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

- Monensin

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

Day 1, 2, 3, 4

BD
Comparison of two fix/perm protocols

BD Cytofix/Cytoperm protocol

- Day 4 Cytofix/Perm-4 v450/8/ε
  - IL-17A: 4.3%
  - Q4: 7.7%
  - IL-2

- Day 4 Cytofix/Perm-17Per/IFN
  - IL-17A: 4.1%
  - Q4: 3.8%
  - IFN-γ

Foxp3 fix/perm protocol

- Day 4 FoxP3-4 v450/8/apc/PE
  - IL-17A: 3.3%
  - Q4: 3.9%
  - IL-2

- Day 4 FoxP3-17Per/IFN F/4PE
  - IL-17A: 3.4%
  - Q4: 3.9%
  - IFN-γ
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

VPD450

FOX3
### Co-expression of Foxp3 vs IFN-γ

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<tr>
<td><strong>Day 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>3.3%</td>
<td>3.2%</td>
<td>2.5%</td>
<td>2.2%</td>
</tr>
<tr>
<td>FoxP3</td>
<td>2.8%</td>
<td>3.1%</td>
<td>3.1%</td>
<td>4%</td>
</tr>
<tr>
<td><strong>Day 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>6.4%</td>
<td>3.7%</td>
<td>1.9%</td>
<td>2.1%</td>
</tr>
<tr>
<td>FoxP3</td>
<td>2%</td>
<td>1.8%</td>
<td>2.7%</td>
<td>2.5%</td>
</tr>
<tr>
<td><strong>Day 3</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>IFN-γ</td>
<td>11%</td>
<td>3.1%</td>
<td>3.9%</td>
<td>4.4%</td>
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Co-expression of Foxp3 vs IL-17A

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<tr>
<td>+IL-23</td>
<td>3.3%</td>
<td>2.5%</td>
<td>3.2%</td>
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IL-17A

FoxP3
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

Foop3 Fix/Perm buffer
Foop3 and some cytokines

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-CD3e+ anti-CD28+ IL-1β+ IL-6+ IL-23
IL-17A and IFN-γ production

[Graphs showing IL-17A and IFN-γ production over 3 days of culture with different conditions: CD3/CD28, CD3/CD28/IL1β/IL-6, CD3/CD28/IL1β/IL-6/TGFβ, CD3/CD28/IL1β/IL-6/TGFβ/IL-23]
IL-4 and IL-2 production

![Graphs showing IL-4 and IL-2 production over days of culture with different conditions.](image)

- **IL-4**
  - Days of culture: 1, 2, 3
  - Y-axis: Pg/ml of IL-4

- **IL-2**
  - Days of culture: 1, 2, 3
  - Y-axis: Pg/ml of IL-2

Legend:
- CD3 /CD28
- CD3 /CD28/IL1β/IL-6
- CD3 /CD28/IL1β/IL-6/TGFβ
- CD3 /CD28/IL1β/IL-6/TGFβ/IL-23

BD Logo
Experimental setup

Harvest spleen → CD4 cells enriched by panning

Cells loaded with VPD450 and washed

BD Cytofix/Cytoperm buffer

Cytokines

Foxp3 Fix/Perm buffer

Foxp3 and some cytokines

+ Monensin

Harvest

Stimulate with PMA and ionomycin for 4–5 hours

+ or – Monensin

- Monensin

Supernatants: Analysis with BD CBA Flex Sets

Cells: BD Phosflow Fix and Perm buffer

Day 1, 2, 3, 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

**pStat5 detection on day 4**
pStat5 in activated cells over time

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<td>Day 2</td>
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<tr>
<td>Day 3</td>
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pStat5 in activated cells over time
### pStat5 in proliferating cells

<table>
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<th>+IL-1β/IL-6</th>
<th>+TGF-β</th>
<th>+IL-23</th>
</tr>
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<tr>
<td>Day 1</td>
<td>Specimen_003-C2</td>
<td>Specimen_004-D2</td>
<td>Specimen_005-E2</td>
<td>Specimen_006-F2</td>
</tr>
<tr>
<td>Day 2</td>
<td>Specimen_007-G2</td>
<td>Specimen_008-H2</td>
<td>Specimen_009-A6</td>
<td>Specimen_010-E6</td>
</tr>
<tr>
<td>Day 3</td>
<td>Specimen_011-C6</td>
<td>Specimen_012-C6</td>
<td>Specimen_013-E6</td>
<td>Specimen_014-F6</td>
</tr>
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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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