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
Experimental setup

- Enrich Balb/c splenocytes by positive selection via CD4$^+$ panning
- Load isolated cells with VFSE 1μM, 10 minutes
- Set up cultures as follows:
  - CD3/CD28
  - CD3/CD28/IL-6/IL-1β
  - CD3/CD28/IL-6/IL-1β/TGF-β
  - CD3/CD28/IL-6/IL-1β/TGF-β/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 continued

Harvest Spleen → Cells loaded with VFSE and washed → CD4 cells enriched by panning

BD Cytofix/Cytoperm™ buffer
Cytokines

+ Monensin

Fo xp3 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
VFSE histograms

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

Day 1
- Specimen 001-B2
- Specimen 002-B2
- Specimen 003-B2
- Specimen 004-B2

Day 2
- Specimen 005-B2
- Specimen 006-B2
- Specimen 007-B2
- Specimen 008-B2

Day 3
- Specimen 009-B8
- Specimen 010-B8
- Specimen 011-B8
- Specimen 012-B8
VFSE vs IL-2 data

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

Day 1

Day 2

Day 3
VFSE vs IFN-γ data

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

Day 1

Day 2

Day 3
VFSE vs IL-17A data

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

**Day 1**
- Specimen_001-02
- Specimen_002-D2
- Specimen_003-D2
- Specimen_004-E2

**Day 2**
- Specimen_005-F2
- Specimen_006-G2
- Specimen_007-H2
- Specimen_008-A2

**Day 3**
- Specimen_009-B2
- Specimen_010-C2
- Specimen_011-D2
- Specimen_012-E2
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

IL-17A

IL-2
Co-expression of IL-17A vs IFN-γ

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

Day 1

CD3/CD28

+IL-1β/IL-6

+TGF-β

+IL-23

Day 2

Day 3
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-17A

IL-4
Comparison of two fix/perm protocols

BD Cytofix/Cytoperm protocol

- IL-17A vs. IL-2:
  - Q1: 4.3%
  - Q4: 7.7%

- IL-17A vs. IFN-γ:
  - Q1: 4.1%
  - Q4: 3.8%

Foxp3 fix/perm protocol

- IL-17A vs. IL-2:
  - Q1: 3.3%
  - Q4: 3.9%

- IL-17A vs. IFN-γ:
  - Q1: 3.4%
  - Q4: 3.9%
Experimental setup

Harvest Spleen → CD4 cells enriched by panning

Cells loaded with VFSE and washed

BD Cytofix/Cytoperm™ buffer
Cytokines

Foxp3 Fix/Perm buffer
Foxp3 and some cytokines

+ Monensin

- 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
Co-expression of Foxp3 vs IFN-γ

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

Day 1
- CD3/CD28: 3.3% Foxp3, 2.8% IFN-γ
- +IL-1β/IL-6: 3.2% Foxp3, 3.1% IFN-γ
- +TGF-β: 2.5% Foxp3, 3.1% IFN-γ
- +IL-23: 2.2% Foxp3, 4% IFN-γ

Day 2
- CD3/CD28: 6.4% Foxp3, 2% IFN-γ
- +IL-1β/IL-6: 3.7% Foxp3, 1.8% IFN-γ
- +TGF-β: 1.9% Foxp3, 2.7% IFN-γ
- +IL-23: 2.1% Foxp3, 2.5% IFN-γ

Day 3
- CD3/CD28: 11% Foxp3, 3.8% IFN-γ
- +IL-1β/IL-6: 3.1% Foxp3, 2.4% IFN-γ
- +TGF-β: 3.9% Foxp3, 2.7% IFN-γ
- +IL-23: 4.4% Foxp3, 2.4% IFN-γ
Co-expression of Foxp3 vs IL-17A

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

Day 1

<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>2.9%</td>
<td>1.9%</td>
<td>1.9%</td>
</tr>
<tr>
<td>+ IL-1β/IL-6</td>
<td>3.5%</td>
<td>2.3%</td>
<td>2.3%</td>
</tr>
<tr>
<td>+ TGF-β</td>
<td>3.3%</td>
<td>2.5%</td>
<td>3.2%</td>
</tr>
<tr>
<td>+ IL-23</td>
<td>4.5%</td>
<td>2.9%</td>
<td>7.9%</td>
</tr>
</tbody>
</table>
Proliferation of Treg and Th17 cells

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

Day 1

Day 2

Day 3

F0x3 → VFSE
Experimental setup

Harvest spleen → CD4 cells enriched by panning → Cells loaded with VFSE and washed

- Monensin
+ Monensin

BD Cytofix/Cytoperm buffer
Cytokines
Foxp3 Fix/Perm buffer
Foxp3 and some cytokines

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

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

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

IL-17A

IFN-γ

Day 1 | Day 2 | Day 3 | Day 4
---|---|---|---
0 | 0 | 0 | 0
500 | 500 | 500 | 500
1000 | 1000 | 1000 | 1000
1500 | 1500 | 1500 | 1500
2500 | 2500 | 2500 | 2500
3000 | 3000 | 3000 | 3000
3500 | 3500 | 3500 | 3500
4000 | 4000 | 4000 | 4000

BD
IL-4 and IL-2 production

**IL-4**
- **Day 1**
- **Day 2**
- **Day 3**
- **Day 4**

**IL-2**
- **Day 1**
- **Day 2**
- **Day 3**
- **Day 4**

Legend:
- CD3/CD28
- CD3/CD28/IL-1/IL-6
- CD3/CD28/IL-1/IL-6/TGFb
- CD3/CD28/IL-1/IL-6/TGFb/IL-23
Experimental setup

Harvest spleen → CD4 cells enriched by panning

Cells loaded with VFSE and washed

BD Cytofix/Cytoperm buffer
Cytokines

+ Monensin

- 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
### 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 phosphospecific Stat5 antibody.

<table>
<thead>
<tr>
<th>Condition</th>
<th>CD3/CD28</th>
<th>+IL-1β/IL-6</th>
<th>+TGF-β</th>
<th>+IL-23</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unactivated</strong></td>
<td><img src="Specimen_018-B10" alt="Histogram" /></td>
<td><img src="Specimen_019-C10" alt="Histogram" /></td>
<td><img src="Specimen_020-D10" alt="Histogram" /></td>
<td><img src="Specimen_021-E10" alt="Histogram" /></td>
</tr>
<tr>
<td><strong>Activated</strong></td>
<td><img src="Specimen_022-F10" alt="Histogram" /></td>
<td><img src="Specimen_023-G10" alt="Histogram" /></td>
<td><img src="Specimen_024-H10" alt="Histogram" /></td>
<td><img src="Specimen_015-06" alt="Histogram" /></td>
</tr>
</tbody>
</table>
pStat5 in activated cells over time

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

Day 1

Day 2

Day 3
pStat5 in proliferating cells

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

Day 1  
Day 2  
Day 3
Conclusions

- Cells proliferated equally well under all four polarization conditions

- Invitro 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
Acknowledgments

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