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

CD4 cells enriched by panning

Cells loaded with VFSE 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

BD Cytofix/Cytoperm™ buffer
Cytokines

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 | ![Histogram](image1.png) | ![Histogram](image2.png) | ![Histogram](image3.png) | ![Histogram](image4.png)
Day 2 | ![Histogram](image5.png) | ![Histogram](image6.png) | ![Histogram](image7.png) | ![Histogram](image8.png)
Day 3 | ![Histogram](image9.png) | ![Histogram](image10.png) | ![Histogram](image11.png) | ![Histogram](image12.png)
VFSE | ![Histogram](image13.png) | ![Histogram](image14.png) | ![Histogram](image15.png) | ![Histogram](image16.png)
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

Specimen_001-B3
Specimen_002-C3
Specimen_003-D3
Specimen_004-E3

Day 2

Specimen_005-F3
Specimen_006-G3
Specimen_007-H3
Specimen_008-A9

Day 3

Specimen_009-B9
Specimen_010-C9
Specimen_011-D9
Specimen_012-E9
VFSE vs IL-17A data

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

Day 1

Day 2

Day 3
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-2
IL-17A
Co-expression of IL-17A vs IFN-γ

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

Day 1

Day 2

Day 3

1.2% 4.5% 2.1% 1.6% 1.2%
7.5% 1.9% 1.7% 1.1% 2.1% 3.2%
5.2%
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 vs IL-4 expression levels:
- Day 1:
  - CD3/CD28: Q4 area
  - +IL-1β/IL-6: Q4 area
  - +TGF-β: Q4 area
  - +IL-23: Q4 area

- Day 2:
  - CD3/CD28: Q4 area
  - +IL-1β/IL-6: Q4 area
  - +TGF-β: Q4 area
  - +IL-23: Q4 area

- Day 3:
  - CD3/CD28: Q4 area
  - +IL-1β/IL-6: Q4 area
  - +TGF-β: Q4 area
  - +IL-23: Q4 area
Comparison of two fix/perm protocols

BD Cytofix/Cytoperm protocol

Foxp3 fix/perm protocol

Day4 Cytofix/Perm-4 v450/8/ε

Day4 FoxP3-4 v450/8/apc/PE

IL-17A

IL-17A

IL-2

IFN-γ

IL-2

IFN-γ

Q1 4.3%

Q1 3.3%

Q4 7.7%

Q4 3.3%

Q2

Q2

Q3

Q3

Q4

Q4

4.1%

3.4%

3.8%

3.9%
Experimental setup

CD4 cells enriched by panning

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

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

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

BD™
Co-expression of Foxp3 vs IFN-γ

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

Day 1
- CD3/CD28: 3.3%
- + IL-1β/IL-6: 2.8%
- + TGF-β: 3.3%
- + IL-23: 4%

Day 2
- CD3/CD28: 6.4%
- + IL-1β/IL-6: 3.7%
- + TGF-β: 1.8%
- + IL-23: 2.1%

Day 3
- CD3/CD28: 11%
- + IL-1β/IL-6: 3.1%
- + TGF-β: 3.7%
- + IL-23: 2.5%
Co-expression of Foxp3 vs IL-17A

Condition: CD3/CD28

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

Day 2
- +IL-1β/IL-6: 1.9%
- +TGF-β: 1.9%
- +IL-23: 2.3%

Day 3
- +IL-1β/IL-6: 1.9%
- +TGF-β: 2.6%
- +IL-23: 7.9%
Proliferation of Treg and Th17 cells

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

Day 1

Day 2

Day 3

Fop3  
VFSE
**Experimental setup**

1. **Harvest spleen**

2. **CD4 cells enriched by panning**

3. **Cells loaded with VFSE and washed**

   - BD Cytofix/Cytoperm buffer
   - Cytokines
   - + Monensin
   - - Monensin

4. **Stimulate with PMA and Ionomycin for 4–5 hours**

   + or – Monensin

5. **Day 1, 2, 3, 4**

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

**BD**
IL-17A and IFN-γ production

**IL-17A**

- CD3/CD28
- CD3/CD28/IL-1/IL-6
- CD3/CD28/IL-1/IL-6/TGFβ
- CD3/CD28/IL-1/IL-6/TGFβ/IL-23

**IFN-γ**

- CD3/CD28
- CD3/CD28/IL-1/IL-6
- CD3/CD28/IL-1/IL-6/TGFβ
- CD3/CD28/IL-1/IL-6/TGFβ/IL-23
IL-4 and IL-2 production

**IL-4**

- **Day 1**
  - CD3/CD28
  - CD3/CD28/IL-1/IL-6
  - CD3/CD28/IL-1/IL-6/TGFβ
  - CD3/CD28/IL-1/IL-6/TGFβ/IL-23

- **Day 2**
  - CD3/CD28
  - CD3/CD28/IL-1/IL-6
  - CD3/CD28/IL-1/IL-6/TGFβ
  - CD3/CD28/IL-1/IL-6/TGFβ/IL-23

- **Day 3**
  - CD3/CD28
  - CD3/CD28/IL-1/IL-6
  - CD3/CD28/IL-1/IL-6/TGFβ
  - CD3/CD28/IL-1/IL-6/TGFβ/IL-23

- **Day 4**
  - CD3/CD28
  - CD3/CD28/IL-1/IL-6
  - CD3/CD28/IL-1/IL-6/TGFβ
  - CD3/CD28/IL-1/IL-6/TGFβ/IL-23

**IL-2**

- **Day 1**
  - CD3/CD28
  - CD3/CD28/IL-1/IL-6
  - CD3/CD28/IL-1/IL-6/TGFβ
  - CD3/CD28/IL-1/IL-6/TGFβ/IL-23

- **Day 2**
  - CD3/CD28
  - CD3/CD28/IL-1/IL-6
  - CD3/CD28/IL-1/IL-6/TGFβ
  - CD3/CD28/IL-1/IL-6/TGFβ/IL-23

- **Day 3**
  - CD3/CD28
  - CD3/CD28/IL-1/IL-6
  - CD3/CD28/IL-1/IL-6/TGFβ
  - CD3/CD28/IL-1/IL-6/TGFβ/IL-23

- **Day 4**
  - CD3/CD28
  - CD3/CD28/IL-1/IL-6
  - CD3/CD28/IL-1/IL-6/TGFβ
  - CD3/CD28/IL-1/IL-6/TGFβ/IL-23
Experimental setup

1. Harvest spleen
2. CD4 cells enriched by panning
3. Cells loaded with VFSE and washed
4. BD Cytofix/Cytoperm buffer
5. Cytokines
6. Foxp3 Fix/Perm buffer
7. Foxp3 and some cytokines
8. Harvest
   - Stimulate with PMA and Ionomycin for 4–5 hours
   + or – Monensin
9. Supernatants: Analysis with BD CBA Flex Sets
10. Cells: BD Phosflow Fix and Perm buffer
11. Day 1, 2, 3, 4
12. 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 phosphospecific Stat5 antibody.

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**Condition:**

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

**Unactivated**

**Activated**
pStat5 in activated cells over time

Condition: CD3/CD28

Day 1

Day 2

Day 3

+IL-1β/IL-6

+TGF-β

+IL-23
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-A2

Specimen_010-B2

Day 3

Specimen_011-C2

Specimen_012-D2

Specimen_013-E2

Specimen_014-F2
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
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