A step-by-step protocol for Th-17 differentiation from Naïve CD4+ mouse T lymphocytes.

In this application note is reported a protocol for the isolation of mouse naïve CD4+ T-cells and their differentiation into T-Reg and Th-17 cells.

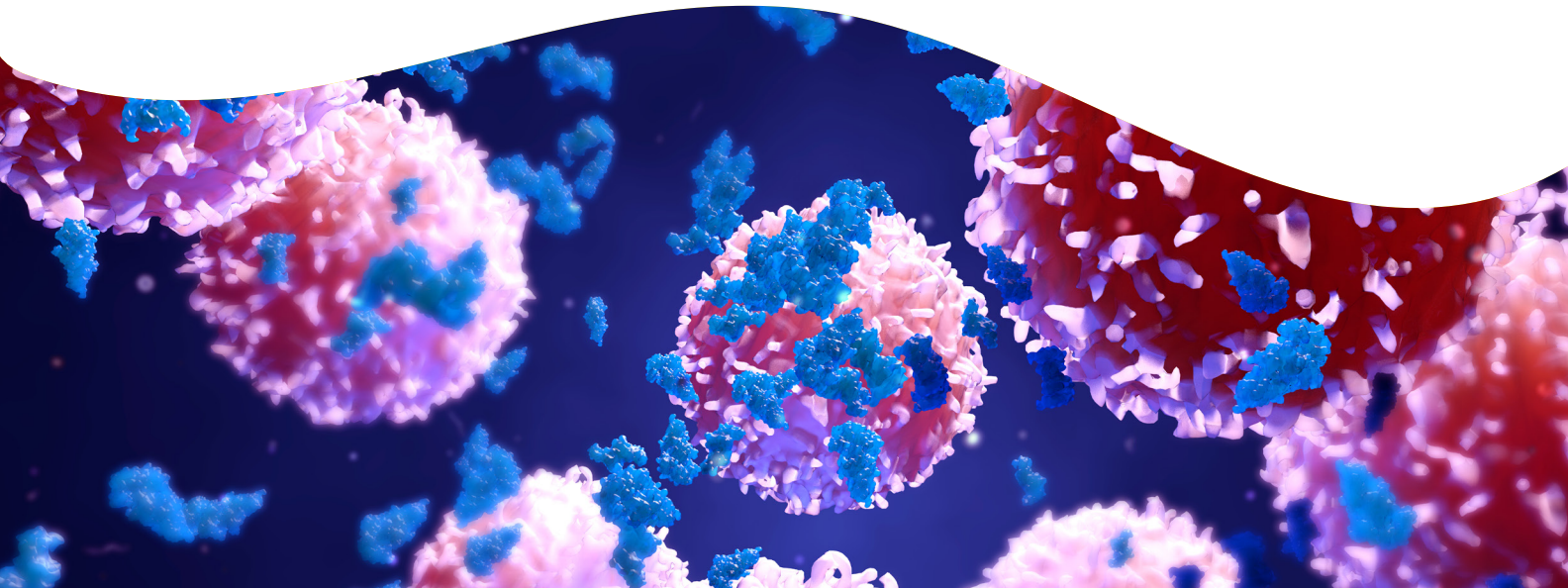
Introduction

T helper cells (Th cells), also known as CD4+ cells, play an important role in adaptive immunity. T helper cells are so named because they assist other white blood cells in the development of an immune response. Immunologists have divided Th cells into functional subsets. These include Th-17 cells which display a characteristic pattern of IL-17 secretion, a cytokine that has been shown to play a pivotal role in several diseases, in auto-immunity, in oncology, and in the brain, the heart, the intestines, and the central nervous system.

Production of IL-17 can be considered evidence of a successful differentiation of naïve cells into the Th-17 subtype, and assays capable of efficiently and precisely monitoring the production of the cytokine IL-17 have broad applications in drug discovery. Inhibitors of the differentiation of IL-17 producing Th-17 cells have shown efficacy in a number of autoimmune and inflammatory disease models.

In this application note we report a protocol for the isolation of mouse naïve CD4+ T-cells and their differentiation into T-Reg and Th-17 cells.

Cell differentiation into T-Reg can be studied by monitoring the expression of FoxP3 in FACS. Differentiation into Th-17 is detected by monitoring the expression of ROR–γt by RT-qPCR and the production of IL-17 using FACS, ELISA, and HTRF, the latter assay offering an efficient, straightforward and reliable means of detecting the pivotal cytokine.



Assay workflow

Isolation of Naïve CD4+ T-cells from mice

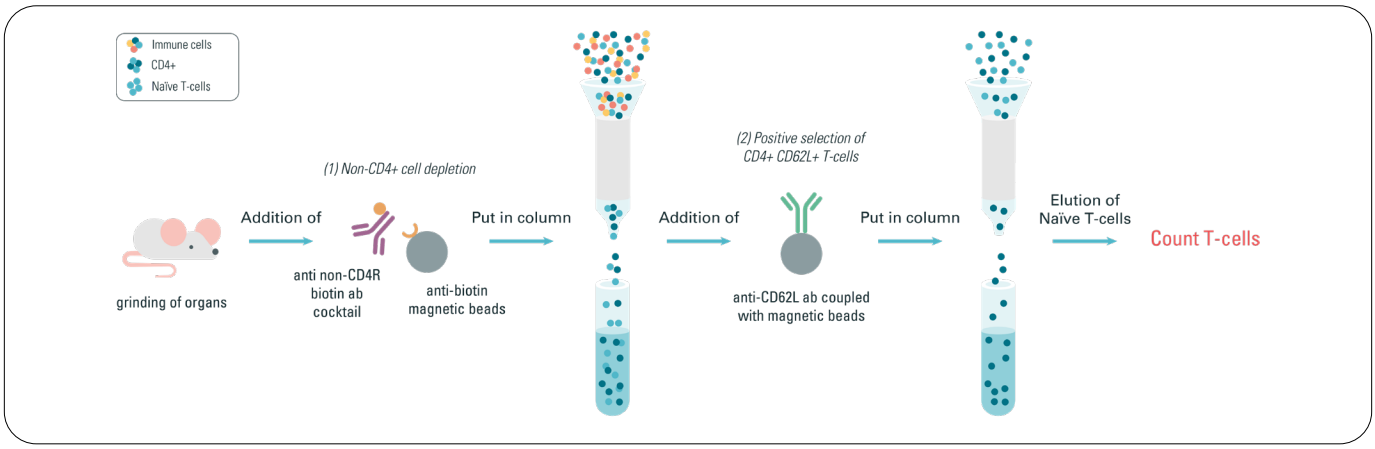


Figure 1: Assay workflow

The spleen and lymph nodes were harvested from mice and placed in a dish containing the complete medium. The spleen and lymph nodes were then mechanically dissociated. Next, naïve T-cells were isolated using a two step selection protocol following the Miltenyi Biotec recommendations (Miltenyi Biotec, CD4+ CD62L+, T-cell Isolation Kit, mouse). First step is an indirect magnetic labeling of non-CD4+ T-cells with non CD4+ T-cell biotin-antibody Cocktail* and anti-biotin MicroBeads[1]. Depletion is achieved by magnetic field separation. Second step is a direct magnetic labeling of CD4+CD62L+ T cells with CD62L MicroBeads[2].

Please refer to the appendix for a detailed procedure.

*Cocktail of biotinylated antibodies against CD8a, CD11b, CD11c, CD19, CD25, CD45R, CD49b, CD105, Ter-119, MHC class II, and TCRγ/δ.

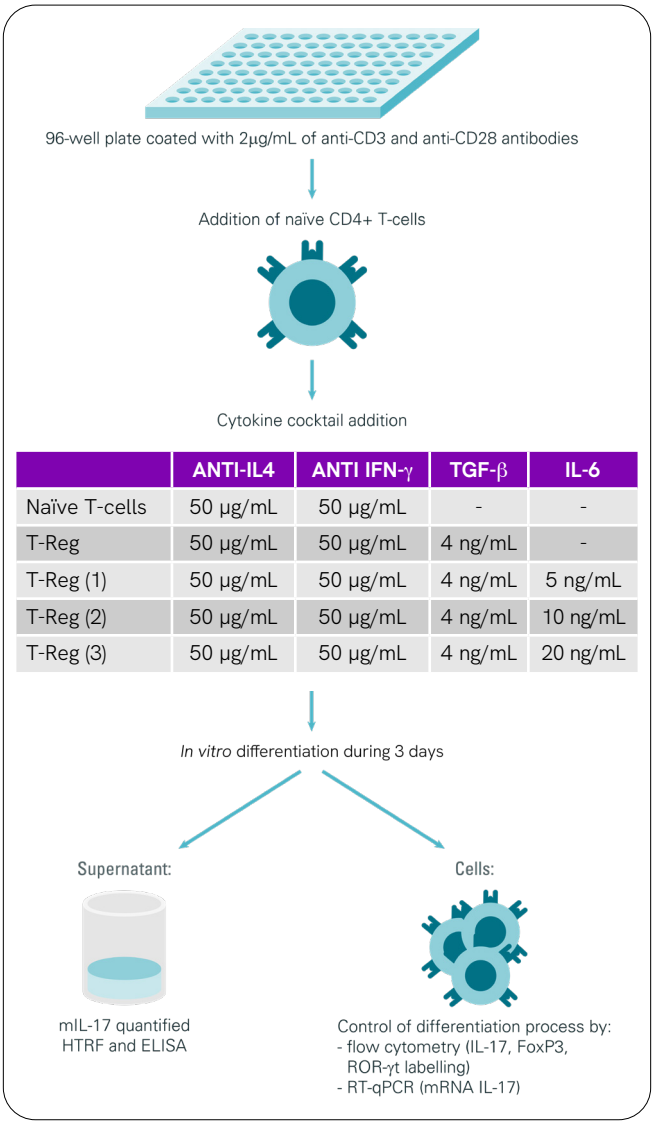
Differentiation into T-Reg and Th-17

Anti-CD3 and anti-CD28 antibodies coated onto the plate are a physiologically relevant approach to stimulate T-cells in a manner which partially mimics stimulation by antigen-presenting cells. Immobilized anti-CD3 and anti-CD28 antibodies in a 96-well plate were used for T-cell differentiation (see protocol).

T-Reg were generated by adding TGF-β at the concentrations listed in the table.

Transdifferentiation of T-Reg into Th17 was induced by supplementing the cell culture medium with 3 doses of IL6 (table above, conditions 1-3).

Blocking anti-IL-4 and anti-IFNγ antibodies prevents the differentiation of the naïve T-cells into the Th1/Tc1 and Th2/Tc2 subtypes.



	ANTI-IL4	ANTI IFN-γ	TGF-β	IL-6
Naïve T-cells	50 µg/mL	50 µg/mL	-	-
T-Reg	50 µg/mL	50 µg/mL	4 ng/mL	-
T-Reg (1)	50 µg/mL	50 µg/mL	4 ng/mL	5 ng/mL
T-Reg (2)	50 µg/mL	50 µg/mL	4 ng/mL	10 ng/mL
T-Reg (3)	50 µg/mL	50 µg/mL	4 ng/mL	20 ng/mL

Figure 2: Differentiation into T-Reg and TH-17 principle

Monitoring the differentiation process

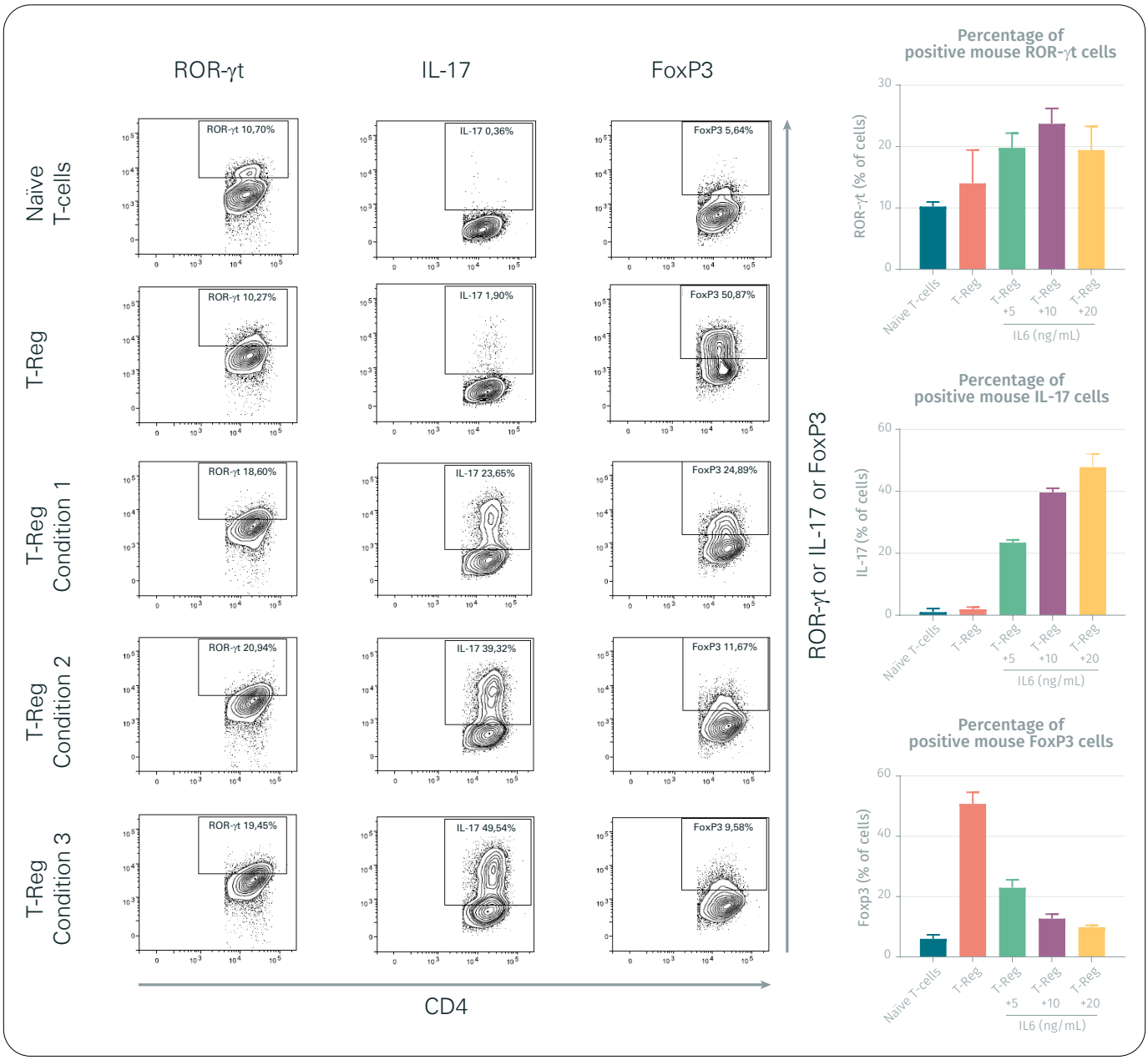


Figure 3: Monitoring the differentiation process

Flow cytometry was used to check the proper differentiation of naïve T-cells into T-Reg and Th-17 cells. The results, expressed in % of positive cells, are represented in the contour plots as well as in histograms.

Th-17 cells express a lineage-determining 'master' transcription factor, ROR-γt, which directs the production of IL-17. In the FACS experiments, Th-17 differentiation was assessed by both ROR-γt and IL-17 intracellular labelings.

FoxP3, a recognized marker of T-Reg, was used to monitor the differentiation of naïve T-cells into T-Regs.

The results show that exposure to TGFβ leads to an increase in the percentage of the T-Reg population, from 5.6% to 50.8%.

Similarly, IL-6 exposure is associated with an increase in both the IL-17 and ROR-γt labelings, indicating a successful transdifferentiation of T-Reg into the Th-17 subset.

Monitoring of differentiation using RT-qPCR, ELISA, and HTRF

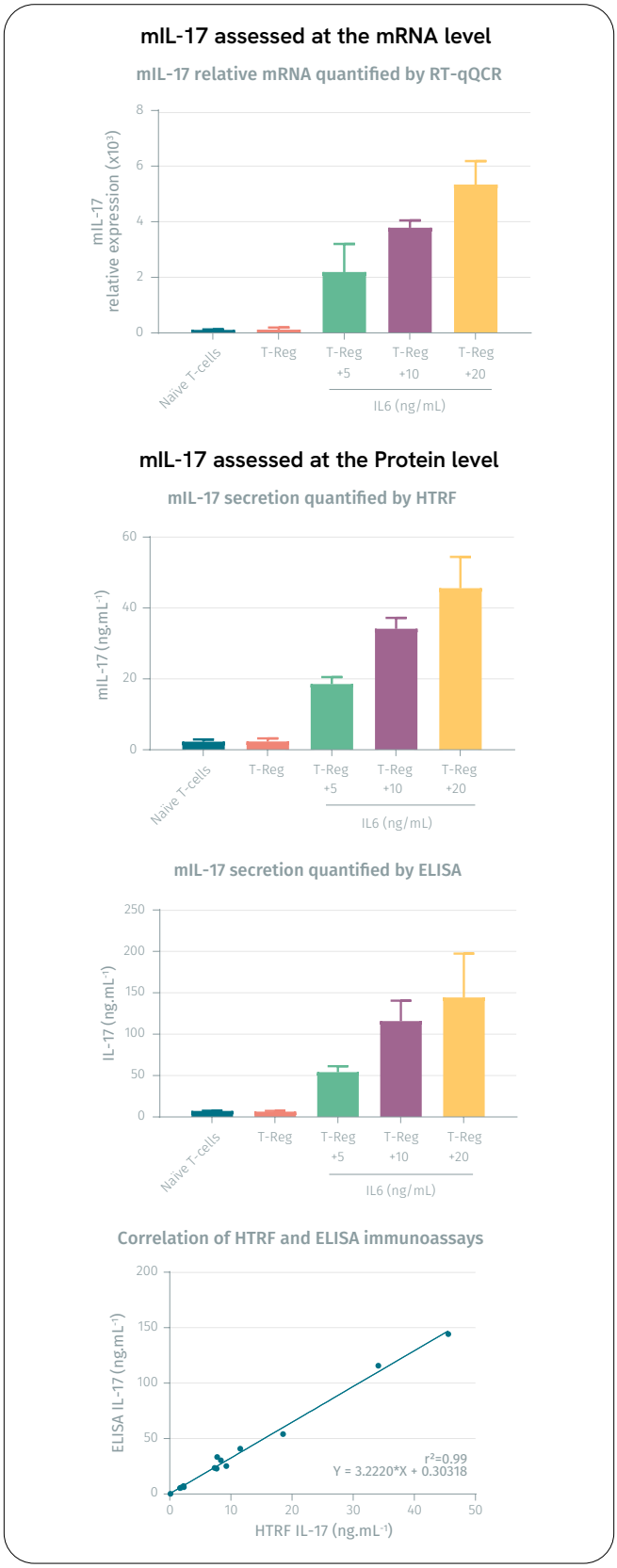


Figure 4: mIL-17 assessed at the mrnA and the protein level

IL-17 expression and production were measured at the mRNA level by RT-qPCR, as well as at the protein level using ELISA or HTRF assays.

T-Reg exposure to increasing concentrations of IL-6 is associated with an dose-dependent increase of IL-17 mRNA.

As indicated above, both ELISA and HTRF show IL-17 release in an IL-6 dose-dependent relationship, demonstrating the successful transdifferentiation of T-Reg in the Th-17 cell subset. Moreover, the HTRF mouse IL-17 assay displays an excellent correlation (R^2=0.99) with a leading absorbance ELISA for both low and high concentrations of samples.

Product references:

- HTRF mouse IL-17 kit # 62MIL17PEG (Revvity)
- CD4+ CD62L+ T Cell Isolation Kit mouse (Miltenyi Biotec)
- ELISA mouse IL-17A max standard Cat# 432503 (Biolegend)
- Anti-IFNγ Cat# 505833 (Biolegend)
- Anti-IL4 Cat# 504104 (Biolegend)

Conclusion

In this technical note we have reported a protocol for the isolation of mouse naïve CD4+ T-cells and their differentiation into T-Reg and Th-17 cells. T-Reg differentiation into Th-17 was followed by monitoring IL-17 protein using flow cytometry, ELISA, and HTRF, whereas mRNA was assessed by RT-qPCR.

While FACS analyses involve intracellular IL-17 staining, immunoassays like ELISA and HTRF are performed on cell supernatants, thereby enabling the assessment of the secretory capability of the cells. In addition, these two immunological-based methods enable absolute quantification of IL-17. Even though current assay strategies for measuring IL-17 heavily rely on ELISA methods, which involve multiple washing steps, HTRF assay offers the benefits of lower sample consumption and faster time to results.

Appendix

Isolation of Naïve CD4+ T-cells protocol

Material

Magnetic-Activated Cell Sorting (MACS) buffer

PBS 1X + 0,5% BSA + 2 mM EDTA

Complete medium

RPMI + 5 mL PSA + 50 mL SVF + 5 mL NapPyr + 5 mL Hepes

Complete differentiation medium

RPMI 10% SVF + PSA + Na pyruvate 1X + NEAA 1X

CD4+ CD62L+ T cell isolation kit II mouse,
Cat# 130-093-227, Miltenyi

References produits

- Mouse IL-17A Elisa max standard Biolegend, Cat# 432503
- Mouse IL-17A Elisa max standard Biolegend, Cat# 432501
- Anti-IFNg Biolegend, Cat# 505833
- Anti-IL4 Biolegend, Cat# 504104

Method

- Coat a 96-well plate with anti-CD3 and anti-CD28 antibodies at 2 µg/mL in 1X PBS (Bioxcell). Incubate 2 h at 37 °C
- Take the spleen and lymph nodes from the mice and place them in a dish containing the complete medium
- Prepare a 6-well plate, deposit 5 mL of complete medium and place a filter in a well. Put the organs in the sieve and grind everything with a 2.5 mL syringe plunger

Collection of CD4+ cells

- Recover the medium containing the cells and put it in a 50 mL falcon
- Add 3 mL of complete medium to rinse the wells and repeat until the medium is entirely clear
- Centrifuge for 10 min at 1000 rpm
- Resuspend the cell pellet in 800 µL of MACS buffer for 200 M cells (per mouse)
- Add 200 µL of anti-CD4- antibody for 200 M cells (per mouse)
- Incubate 10 min on the ice
- Add 600 µL of Macs Buffer for 200 M cells (per mouse)
- Add 400 µL of anti-biotin beads for 200 M cells (per mouse)
- Incubate 15 minutes on ice
- Add 10 mL of macs buffer
- Centrifuge for 5 minutes at 1250 rpm and empty the supernatant
- Resume to have 1 mL of Macs buffer (per mouse)
- Place LS columns on the magnet and then pre-separation filter 30 µL on the column
- Place a falcon 15 below the column
- Wash the column with 3 mL of Macs buffer. Once the buffer has run, place 1 mL of cell suspension column
- Wash the column twice with 3 mL of Macs buffer
- Pool the 15 mL recovery tubes and centrifuge for 5 min at 1250 rpm
- Resume in 1600 µL of buffer Macs (per mouse)
- Add 400 µL of anti-CD62L (per mouse)
- Incubate 15 minutes in the ice
- Add 10 mL of Macs buffer
- Centrifuge 5 min at 1250 rpm
- Resume the pellet with Macs buffer to pass 1 mouse on a column and 500 µL per column
- Place MS columns on the magnet
- Place a falcon 15 below the column
- Hydrate the column with 500 µL of Macs buffer
- Once the buffer has drained, dispense 500 µL of cell suspension per column
- Wash the column 3 times with 500 µL of Macs buffer
- Detach the column from the magnet and place it on a new 15 mL tube
- Place 1 mL of Macs buffer on the column and quickly push the plunger at once to detach the cells
- Repeat the flush with 500 µL

- Count cells, centrifuge and resuspend at 0.43 M cells for 50 µL in differentiation medium
- Prepare cytokine cocktail at 4X their intended final concentration in complete differentiation medium (see table below)

	Anti-IL4	Anti IFN-γ	TGF-β	IL-6
Naïve T-cells	200 µg/mL	200 µg/mL	-	-
T-Reg	200 µg/mL	200 µg/mL	20 ng/mL	-
T-Reg (1)	200 µg/mL	200 µg/mL	20 ng/mL	20 ng/mL
T-Reg (2)	200 µg/mL	200 µg/mL	20 ng/mL	40 ng/mL
T-Reg (3)	200 µg/mL	200 µg/mL	20 ng/mL	80 ng/mL

For a final 1X concentration

	Anti-IL4	Anti IFN-γ	TGF-β	IL-6
Naïve T-cells	50 µg/mL	50 µg/mL	-	-
T-Reg	50 µg/mL	50 µg/mL	4 ng/mL	-
T-Reg (1)	50 µg/mL	50 µg/mL	4 ng/mL	5 ng/mL
T-Reg (2)	50 µg/mL	50 µg/mL	4 ng/mL	10 ng/mL
T-Reg (3)	50 µg/mL	50 µg/mL	4 ng/mL	20 ng/mL

- Remove coating solutions from the wells (2 µg/mL anti-CD3 + 2 µg/mL anti-CD28)
- Dispense 100 µL of complete differentiation medium in each well followed by 50 µL of selected cytokine mix and lastly 50 µL of cell suspension to be differentiated
- Incubate 72 h at 37 °C, 5% CO_2

DAY 3: Analysis

- Retain and set aside supernatant for ELISA and HTRF IL-17 quantification
- Resuspend cells in 200 µL complete medium
- Homogenize and keep 50 µL for RT-qPCR
- Centrifuge 5min at 1400 rpm
- Add 200 µL of a solution containing 5 µL Ionomycine (1 mg/mL) + 2,5 µL PMA (100 µg/mL) + 5 µL of Golgi Stop for a final 5 mL volume of complete medium
- Incubate 4 h at 37 °C et 5% CO_2 prior to fluorescent labellings and FACS analysis

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