A complete workflow for cell preparation, isolation, polarization and analysis

T_H1, T_H2 and T_H17 polarization of naïve $CD4^+$ mouse T cells

Introduction

$CD4^+$ T helper (T_H) cells play a central role in the adaptive immune system by controlling a variety of cellular responses, defending the host against pathogens and tumor development. Their cytokine secretion suppresses or stimulates immune responses and leads to antibody production by B cells, immunoglobulin class switch, and macrophage activation, for example. Their crucial impact on immune responses and distinct role in the protection against disease make T_H cells a focus of many researchers studying immune regulation. The various T_H cell subsets can be differentiated from naive $CD4^+$ T cells *in vitro* using specific combinations of polarizing cytokines.

We show a complete workflow for reliable and efficient mouse T_H cell differentiation, starting with single-cell preparation, followed by isolation of naive $CD4^+$ T cells and *in vitro* activation and differentiation, through to comprehensive cell analysis. Furthermore we demonstrate that *in vitro* T_H cell differentiation in the presence of TexMACS Medium led to a higher expression level of the characteristic effector cytokines in the various T_H subsets compared to RPMI 1640. The reagents, instruments, and protocols are proven tools for research on T_H cell subset development and function.

Workflow

5 min	**Preparation of a single cell suspension from mouse spleen**
18 min	**Isolation of naïve $CD4^+$ T cells**
6 d	**T_H1, T_H2 or T_H17 polarization**
5 h	**Intranuclear staining of lineage-specific transcription factors**
120 min	**Restimulation of polarized T cells and brefeldin A treatment**
60 min	**Intracellular staining of lineage-specific effector cytokines**
	Flow cytometric analysis

Material

T cell cultivation

- TexMACS Medium (# 130-097-196)
- Fetal Bovine Serum (FBS)
- 100x penicillin/streptomycin stock solution
- 2-Mercaptoethanol
- 96-well plate (e.g. Gas-permeable Culture Plate # 150-000-364)

▲ **Note:** Depending on the cell number to be cultivated and differentiated per well, 6-, 12-, 24- or 48-well plates might be used. Please refer to section '*Material preparation/Plate sizes for in vitro T cell polarization*' to choose the appropriate cell culture dish.

Preparation of single-cell suspension from mouse spleen

- Pre-Separation Filters, 30 µm (# 130-041-407)
- MACSmix™ Tube Rotator (# 130-090-753)
- CO_2 incubator at 37 °C
- gentleMACS Dissociator (# 130-093-235), gentleMACS Octo Dissociator (# 130-095-937), or gentleMACS Octo Dissociator with Heaters (# 130-096-427)
- gentleMACS C Tubes (# 130-093-237, # 130-096-334)
- (Optional) ART® 1000 REACH™ pipet tips (Molecular BioProducts, Inc.) for removal of dissociated material from the closed C Tubes.

Buffer (standard wash and dilution buffer)

- autoMACS® Rinsing Solution (# 130-091- 222)
- BSA Stock Solution (# 130-091-376)

Magnetic labeling and separation

- Naive $CD4^+$ T Cell Isolation Kit, mouse (# 130-104-453)
- LS Columns (# 130-042-401)
- MACS Separator for LS columns (e.g. MidiMACS™ Separator # 130-042-302)
- MACS Multistand (#130-042-303)

Flow cytometric analysis of freshly isolated naïve $CD4^+$ T cells (optional)

- CD45-VioGreen (# 130-102-412)
- CD4-VioBlue (# 130-102-456)
- CD62L-PE (# 130-102-543)
- CD3ε-APC-Vio770 (# 130-102-306)
- CD44-FITC (# 130-102-511)
- Propidium iodide (#130-093-233)

T_H cell polarization

- T_H1 cells: CytoBox T_H1, mouse (#130-107-761)
- T_H2 cells: CytoBox T_H2, mouse (#130-107-760)
- T_H17 cells: CytoBox T_H17, mouse (#130-107-758)

Activation of naïve $CD4^+$ T cells

- T cell Activation and Expansion Kit, m (#130-093-627)

Removal of MACSiBead Particles

- MACSiMAG Separator (#130-092-168)

Restimulation and brefeldin A treatment

- Ionomycin
- Phorbol 12-myristate 13-acetate (PMA)
- Brefeldin A

Material preparation

Analysis of lineage-specific transcription factors and cytokines

- Fox P3 Staining Buffer Set (# 130-093-142)
- Inside Stain Kit (#130-090-477)
- T-bet-PE, human and mouse (# 130-098-596)
- Gata3-APC, human and mouse (#130-100-649)
- RORg(t)-APC, human and mouse (#130-103-838)
- IFN-γ-APC,mouse (#130-102-340
- IL-4-PE, mouse (#130-102-435)
- IL-17A-PE, mouse (#130-102-344)

Supplemented T cell medium

TexMACS medium supplemented with FBS (final concentration 10%), 2-Mercaptoethanol (final concentration 0.01 mM) and 100x penicillin/streptomycin stock solution (final concentration 1%).

▲ **Note:** Medium referred to as fully supplemented T cell medium (e.g. in *5. Polarization of naïve T cells* consists of supplemented T cell medium (TexMACS including 10% FBS, 0.01 nM 2-Mercapto-ethanol and 1% penicillin/streptomycin stock solution) **with the addition** of cytokines and antibodies from the **CytoBoxes**.

▲ **Note:** Addition of penicillin/streptomycin to the T cell media is optional

Buffer (standard wash and dilution buffer)

Prepare a solution of PBS, pH 7.2, 2mM EDTA and 0.5% BSA by diluting MACS® BSA Stock Solution 1:20 with autoMACS® Rinsing Solution.

Fixation and Permeabilization Solution (FoxP3 Staining Buffer Set)

To achieve the appropriate working concentration for safe fixation and permeabilization of cells, the Fixation/Permeabilization Solution 1 must be diluted 1:4 with the Fixation/Permeabilization Solution 2 (i.e. for 10^6 cells use 0.25 mL of Fixation/Permeabilization Solution 1 plus 0.75 mL of Fixation/Permeabilization Solution 2).

Permeabilization Buffer (FoxP3 Staining Buffer Set)

To achieve the appropriate working concentration for safe permeabilization of cells, the 10× Permeabilization Buffer must be diluted 1:10 with deionized or distilled water before use (i.e. 1 mL of 10× Permeabilization Buffer plus 9 mL of deionized/distilled water).

▲ **Note:** Before dilution make sure that buffer does not contain any precipitates.

Loading of Anti-Biotin MACSiBead Particles

▲ Resuspend Anti-Biotin MACSiBead™ Particles thoroughly by vortexing before use, to obtain a homogenous suspension.
▲ Anti-Biotin MACSiBead Particles are supplied without preservative. Remove aliquots under aseptic conditions.
▲ It is recommended to load Anti-Biotin MACSiBead Particles in batches of 1×10^8 Anti-Biotin MACSiBead Particles. Loaded Anti-Biotin MACSiBead Particles are stable for up to 4 months when stored at 2–8 °C.

1. Pipette 100 µL of CD3ε-Biotin and 100 µL CD28-Biotin into a sealable 2 mL tube and mix well.
▲ **Note:** This antibody combination, with a final antibody concentration of 10 µg antibody per 1 mL loaded Anti-Biotin MACSiBead Particles, is optimized for achieving maximum T cell activation.

2. Add 300 µL of buffer and mix well.

3. Resuspend Anti-Biotin MACSiBead Particles thoroughly by vortexing.

4. Remove 500 µL Anti-Biotin MACSiBead Particles (1×10^8 Anti-Biotin MACSiBead Particles) and add to antibody mix.
▲ **Note:** Anti-Biotin MACSiBead Particles can be loaded in a flexible manner with biotinylated antibodies or ligands other than those supplied. If desired, add other biotinylated antibodies or ligands at appropriate concentrations and adjust with buffer to a total volume of 1 mL accordingly.

5. Incubate for 2 hours at 2–8 °C under constant, gentle rotation by using the MACSmix Tube Rotator at approximately 4 rpm (slowest permanent run program).

6. The loaded Anti-Biotin MACSiBead Particles (1×10^8 Anti-Biotin MACSiBead Particles/mL) are now ready to use. Do not remove the loaded Anti-Biotin MACSiBead Particles from the antibody mix. Store at 2–8 °C for up to 4 months.

Calculation of cytokine concentration

In order to obtain maximal reproducibility for your T_H cell differentiation experiments, it is recommended to always dose recombinant cytokines at a defined unit dose in [U/mL]. To calculate the cell culture concentration in [ng/mL] corresponding to the concentration in [U/mL], apply the following formula:

Example for Mouse IL-12

$$\text{Final culture concentration in [ng/mL]} = \frac{60\ \text{U/mL}}{\text{biological activity in [U/mg]*}} \times 10^6$$

* Please, refer to corresponding data sheet or CoA to obtain the biological activity.

Plate sizes for in vitro T cell polarization

For T cell polarization the cells should be resuspended in culture medium at 1×10^6 cells/mL. The cells should be plated at a density of 1×10^6 cells/cm². Both the dilution and the cell density are important to assure optimal stimulation and cell growth. The following table lists culture plate sizes suitable for different cell numbers. It also indicates the appropriate amount of medium to add.

Total cell number	Medium volume to add	Culture plate	Well diameter
0.25×10^6	0.25 mL	96 well	0.64 cm
1.00×10^6	1.00 mL	48 well	1.13 cm
2.00×10^6	2.00 mL	24 well	1.60 cm
4.00×10^6	4.00 mL	12 well	2.26 cm
10.00×10^6	10.00 mL	6 well	3.50 cm

Protocol

1. Preparation of single cell suspension from mouse spleen

▲ **Note:** For details on the use of the gentleMACS Dissociators, refer to the gentleMACS Dissociator user manuals.
▲ **Note:** For cell culture experiments subsequent to tissue dissociation, all steps should be performed under sterile conditions.
▲ **Note:** The weight of one mouse spleen amounts to 80–120 mg (female BALB/c mouse, 6–7 weeks old).

1. Transfer mouse spleen into the gentleMACS C Tube containing the following amount of buffer:

- 1–2 mouse spleens: 3 mL
- 3–4 mouse spleens: 6 mL
- 5–6 mouse spleens: 9 mL

2. Tightly close C Tube and attach it upside down onto the sleeve of the gentleMACS Dissociator.
▲ **Note**: Close C Tube tightly beyond the first resistance.
▲ **Note**: It has to be ensured that the sample material is located in the area of the rotor/stator.

3. Choose and run one of the following gentleMACS Programs:

- 1–2 mouse spleens: **m_spleen_01**
- 3–6 mouse spleens: **m_spleen_04**

4. After termination of the program, detach C Tube from the gentleMACS Dissociator.

5. (Optional) Perform a short centrifugation step to collect the sample material at the bottom of the tube.

7. Resuspend sample and apply the cell suspension to a Pre- Separation Filter, 30 µm, placed on a 15 mL tube (1–2 mouse spleens per C Tube) or to an appropriate cell strainer placed on a 50 mL tube (3–6 mouse spleens per C Tube).
▲ **Note:** Dissociated tissue can be removed from the closed C Tube by pipetting through the septum-sealed opening in the center of the cap of the C Tube. Use ART 1000 REACH 1000 µL pipette tips.

8. Wash Pre-Separation Filter with 5 mL of buffer.

9. Discard Pre-Separation Filter and centrifuge cell suspension at 300×g for 10 minutes at room temperature. Aspirate supernatant completely.

10. Resuspend cells in buffer to the required volume for further applications. For example, resuspend cells in 10 mL buffer for magnetic labeling.
▲**Note:** Process cells immediately

2. Magnetic labeling

1. Prepare cells and determine cell number.

2. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.

3. Resuspend cell pellet in 400 µL of buffer per 10^8 total cells.

4. Add 100 µL of Biotin-Antibody Cocktail per 10^8 total cells.

5. Mix well and incubate for 5 minutes in the refrigerator (2–8 °C).

6. Add 200 µL of buffer per 10^8 total cells.

7. Add 200 µL of Anti-Biotin MicroBeads per 10^8 total cells.

8. Add 100 µL of CD44 MicroBeads per 10^8 total cells.

9. Mix well and incubate for 10 minutes in the refrigerator (2–8 °C).
10. (Optional) For highest recovery wash cells by adding 1–2 mL of buffer per 10^8 total cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely. Resuspend up to 10^8 cells in 500 µL of Buffer.

11. Proceed to magnetic separation

3. Magnetic separation

▲**Note**: Always wait until the column reservoir is empty before proceeding to the next step.
▲**Note**: Choose an LS Column and a suitable MACS Separator.

1. Place LS Column in the magnetic field of a suitable MACS Separator. For details refer to the respective MACS Column data sheet.

2. Prepare column by rinsing with 3 mL of buffer.

3. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells, representing the enriched naive $CD4^+$ T cells.

4. Wash column with 3 mL of buffer. Collect unlabeled cells that pass through, representing the enriched naive $CD4^+$ T cells, and combine with the effluent from step 3.
▲**Note:** (Optional) If flow cytometric analysis of the freshly isolated naïve $CD4^+$ T cells is desired, take an aliquot (up to 10^6 cells) and proceed to *4. Flow cytometric analysis of freshly isolated naïve $CD4^+$ T cells.*

5. (Optional) Remove column from the separator and place it on a suitable collection tube. Pipette 5 mL of buffer onto the column. Immediately flush out the magnetically labeled non-naive $CD4^+$ T cells by firmly pushing the plunger into the column.

4. Flow cytometric analysis of freshly isolated naïve $CD4^+$ T cells (optional)

▲ **Note:** The recommended antibody dilution for labeling of cells and subsequent analysis by flow cytometry is 1:10 for up to 10^6 cells/50 µL of buffer.
▲ **Note:** Volumes given below are for up to 10^6 nucleated cells. When working with fewer than 10^6 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 2×10^6 nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).

1. Determine cell number.

2. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.

3. Resuspend up to 10^6 nucleated cells per 25 µL of buffer.

4. Add 5 µL of each antibody:

- CD45-VioGreen (# 130-102-412)
- CD4-VioBlue (# 130-102-456)
- CD62L-PE (# 130-102-543)
- CD3ε-APC-Vio770 (# 130-102-306)
- CD44-FITC (# 130-102-511)

5. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).
▲ **Note:** Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice requires increased incubation times.
6.Wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
7. Resuspend cell pellet in a suitable amount of buffer (e.g. 500 µl) for analysis by flow cytometry or fluorescence microscopy.
▲ **Note:** Add propidium iodide to a final concentration of 1 µl per mL (e.g. 0.5 µl in 500 µl) to the cell suspension, if exclusion of dead cells via fluorescence is desired.

5. Polarization of naive T cells

1. Prepare fully supplemented T cell medium by adding cytokines and antibodies to the supplemented TexMACS Medium as follows:

For T_H1 cell polarization (CytoBox T_H1, mouse):

- 10 ng/mL (60 U/mL) Mouse IL-12
- 10 ng/mL (50 U/mL) Mouse IL-2
- 10 µg/mL Anti-IL-4 pure – functional grade

For T_H2 cell polarization (CytoBox T_H2, mouse):

- 10 ng/mL (200 U/mL) Mouse IL-4
- 10 ng/mL (50 U/mL) Mouse IL-2
- 10 µg/mL Anti-IFN-γ pure – functional grade

For T_H17 cell polarization (CytoBox T_H17, mouse):

- 20 ng/mL (10.000 U/mL) Mouse IL-6
- 10 ng/mL (40 U/mL) Mouse IL-23
- 10 ng/mL (8400 U/mL) Mouse IL-1β
- 2 ng/mL (10 U/mL) Human TGF-β1
- 10 µg/mL Anti-IL-4 pure – functional grade
- 10 µg/mL Anti-IFN-γ pure – functional grade
- 10 µg/mL Anti-IL-2 pure – functional grade

▲ **Note**: Refer to section '*Material preparation/Calculation of cytokine concentration*' for conversion from U/mL to ng/mL.

2. Resuspend loaded Anti-Biotin MACSiBead Particles thoroughly and transfer e.g. 40 µL ($4×10^6$ loaded Anti-Biotin MACSiBead Particles) per $2×10^6$ cells (bead to cell ratio 2:1) to a suitable tube.
▲ **Note:** If unloaded MACSiBead Particles shall be used for negative control experiments, replace loaded Anti-Biotin MACSiBead Particles with unloaded Anti-Biotin MACSiBead Particles.

3. Add 1 mL of culture medium to the loaded Anti-Biotin MACSiBead Particles and centrifuge at 300×g for 5 minutes.

4. Aspirate supernatant and resuspend loaded Anti-Biotin MACSiBead Particles in e.g. 1 mL of fully supplemented T cell medium including the cytokines and antibodies (from CytoBox) to a final concentration of $4x10^6$ Anti-Biotin MACSiBead Particles per mL.

5. Determine cell number of freshly isolated mouse naïve $CD4^+$ T cells.

6. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.

7. Resuspend cells at a density of $2×10^6$ cells per mL in fully supplemented T cell medium including the cytokines and antibodies (from CytoBox).

8. Transfer the desired amount of cells and the loaded Anti-Biotin MACSiBead Particles from step 4 and 7 at a ratio of 1:1 to a suitable cell culture vessel to a final density of $1×10^6$ cells per mL per cm^2 with 2 loaded Anti-Biotin MACSiBead Particles per cell (e.g. $0.25×10^6$ cells in 125 µL medium + 125 µl loaded Anti-Biotin MACSiBeads Particles per well of a 96-well plate).
▲ **Note:** Depending on the cell number to be cultivated and differentiated per well, 6-, 12-, 24- or 48-well plates might be used. Please upscale reagents accordingly and refer to section '*Material preparation/ Plate sizes for in vitro T cell polarization*' to choose the appropriate cell culture dish.

9. Incubate at 37 °C and 5–10% CO_2 for up to 6 days.
▲ **Note**: Inspect cultures daily, and add fresh fully supplemented TexMACS Medium if required.

10. At day 2, gently pipette culture up and down to break up all cell clumps.

11. Split the cell culture every two days 1:4 or 1:2, depending on the proliferation of cells, and add fresh fully supplemented TexMACS Medium.

12. After 6 days of cultivation, polarized T_H1, T_H2 or T_H17 cells can be further processed for downstream analysis, for example, intranuclear transcription factor or intracellular cytokine staining. T cells require a restimulation for further expansion or analysis of cytokine expression.

6. Removal of MACSiBead Particles

▲ **Note**: For further flow cytometric analysis it is recommended to remove the MACSiBead particles from the cell suspension.

1. Harvest cells and pool different wells of same condition
Wash empty wells with cold buffer to rinse out the remaining cells on the plate.

2. Determine cell number.

3. Wash cells with cold buffer.

4. Resuspend cells in buffer at a density of up to 2 x 10^7/ mL and vortex thoroughly.

5. Place the tube in the magnetic field of the MACSiMAG Separator.

6. Allow the MACSiBead Particles to adhere to the wall of the tube for 4 minutes.

7. Remaining the tube in the magnet, carefully remove the supernatant containing the MACSiBead- depleted cells and place in a new tube.

8. Remove the tube from the separator and add buffer to the same volume as before.

9. Vortex sample, replace tube in the MACSiMAG Separator and repeat steps 4-5.

10. Collected cells can now be further processed as required.

7. Analysis of lineage-specific transcription factors

T_H1 cells (e.g. T-bet; Anti-T-bet-PE, human and mouse; # 130-098-596)

T_H2 cells (e.g. Gata3 and T-bet; Anti-T-bet-PE, human and mouse; #130-098-596 and Anti-Gata3-APC, human and mouse; #130-100-649)

T_H17 cells (e.g. RORg(t); Anti-RORg(t)-APC, human and mouse; #130-103-838)

▲ **Note:** The recommended antibody dilution for all Anti-T-bet, Anti-Gata3 and Anti-RORg(T) conjugates is 1:11 for up to 10^6 cells/100 µL of buffer for labeling of cells and analysis by flow cytometry.
▲ **Note:** Use freshly prepared Fixation and Permeabilization Solution and Permeabilization Buffer from FoxP3 Staining Buffer Set (#130-093-142) for cell fixation and permeabilization.

1. Determine cell number.

2. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.

3. Resuspend up to 10^6 nucleated cells in 1 mL of cold, freshly prepared Fixation/Permeabilization Solution.

4. Mix well and incubate for 30 minutes in the dark in the refrigerator (2–8 °C).

5. Wash cells by adding 1–2 mL of cold buffer per 10^6 cells and centrifuge at 300×g for 5 minutes at 4 °C. Aspirate supernatant completely.

6. Wash cells by adding 1–2 mL of cold 1× Permeabilization Buffer per 10^6 cells and centrifuge at 300×g for 5 minutes at 4 °C. Aspirate supernatant completely.

7. Resuspend up to 10^6 nucleated cells in 100 µL of cold 1× Permeabilization Buffer.
▲ **Note:** For staining with several antibodies in this step, reduce the volume of 1× Permeabilization Buffer accordingly. For efficient permeabilization, the volume of 1× Permeabilization Buffer should be at least 30% of the overall staining volume.

8. Add 10 µL of each needed transcription factor antibody.

9. Mix well and incubate for 30 minutes in the dark in the refrigerator (2–8 °C).

10. Wash cells by adding 1–2 mL of cold 1× Permeabilization Buffer per 10^6 cells and centrifuge at 300×g for 5 minutes at 4 °C. Aspirate supernatant completely.

11. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry or fluorescence microscopy.
▲ **Note:** Due to fixation and permeabilization, cells are smaller than viable cells. Thus, FSC/SSC settings of the flow cytometer might need to be adjusted.

8. Restimulation and brefeldin A treatment of polarized T_H cells for downstream analysis of effector cytokines

1. Add 20 ng/mL PMA and 1µg/mL Ionomycin to cells and incubate for 3 hours at 37°C.

2. Add brefeldin A to a final concentration of 2µg/mL to the cells and incubate for another 2 hours at 37°C.

3. Harvest cells, determine cell number and proceed to next step.

9. Analysis of lineage-specific effector cytokines

T_H1 and **T_H2 cells** (e.g. IFN-γ and IL-4; Anti-IFN-γ-APC,mouse; #130-102-340 and Anti-IL-4-PE, mouse; #130-102-435).
T_H17 cells (e.g. IFN-γ and IL-17; Anti-IFN-γ-APC, mouse; #130-102-340 and Anti-IL-17A-PE, mouse; #130-102-344).
▲ **Note:** The recommended antibody dilution of Anti-IFN-g-APC,mouse, Anti-IL-4-PE, mouse and Anti-IL-17A-PE, mouse for cell labeling and subsequent flow cytometric analysis is 1:10 for up to 10^6 cells/50 µL of buffer.
▲ **Note:** Volumes given below are for up to 10^6 nucleated cells. When working with fewer than 10^6 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for $2×10^6$ nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).
▲ **Note:** T cells require a restimulation prior to analysis of cytokine expression.
▲ **Note:** After successful restimulation and brefeldin A treatment, intranuclear transcription factor and intracellular cytokine staining can be combined for certain cytokine/transcription factor combinations (see Fig. 2). Therefore please follow the protocol steps *8. Restimulation and brefeldin A treatment of polarized T_H cells for downstream analysis of effector cytokines*, *9. Analysis of lineage-specific effector cytokines* and *7. Analysis of lineage-specific transcription* in this order.
▲ **Note:** Anti-IL-4 staining cannot be combined with intranuclear staining using the FoxP3 Staining Buffer Set**.**
▲ **Note:** Use Inside Fix and Inside Perm from Inside Stain Kit (#130-090-477) for cell permeabilization and fixation.

1. Wash up to 10^6 cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.

2. (Optional) Stain cell surface antigens that are sensitive to fixation with appropriate antibodies according to the manufacturer's recommendations. Then wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.

3. Resuspend up to 10^6 cells in 250 µL of buffer.

4. Add 250 µL of Inside Fix (from Inside Stain Kit). Mix well and incubate for 20 minutes in the dark at room temperature.

5. Centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.

6. Wash cells by adding 1 mL of buffer and centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.
▲ **Note:** Fixed cells may be stored in azide containing buffer at 2–8 °C for up to 1 week.

7. (Optional) Stain cell surface antigens that are sensitive to permeabilization with appropriate antibodies from Miltenyi Biotec. Then wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely

8. Wash cells by adding 1 mL of Inside Perm (Inside Stain Kit) and centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.

9. Resuspend cells in 45 µL of Inside Perm. Add 5 µL of the antibody.
▲ **Note:** For staining with several antibodies in this step, reduce the volume of Inside Perm accordingly. For efficient permeabilization, the volume of Inside Perm should be at least 30% of the overall staining volume.

10. Mix well and incubate for 10 minutes in the dark at room temperature.

11. Wash cells by adding 1 mL of Inside Perm and centrifuge at 300×g for 5 minutes. Aspirate supernatant carefully.

12. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry or fluorescence microscopy. Store cells at 2 – 8 °C in the dark until analysis. Mix well before flow cytometric acquisition.
▲ **Note:** Samples may be stored at 2–8 °C in the dark for up to 24 hours.
▲ **Note:** Do not use propidium iodide (PI) or 7AAD staining.

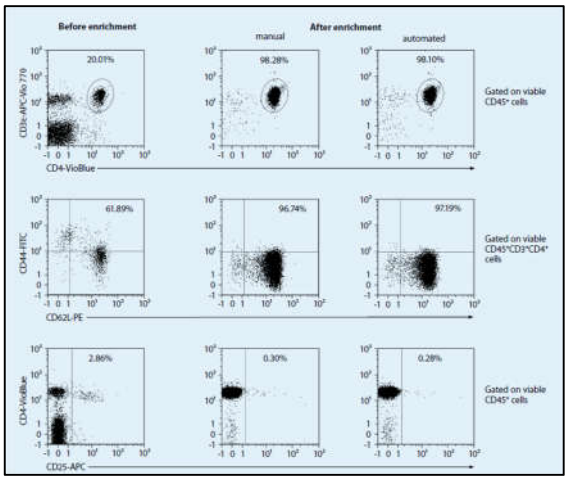

Fig. 1) Example of magnetic enrichment of naive CD4⁺ T_H cells. Naive CD4⁺ T cells were isolated from a single-cell suspension, which was prepared from a 6-week-old BALB/c mouse spleen, using the Naive CD4⁺ T Cell Isolation Kit, mouse. Cell separation was performed either manually with MACS Columns or automatically with the autoMACS Pro Separator. Cells were fluorescently stained with CD45-VioGreen™, CD4-VioBlueR, CD62L-PE, CD3ε-APC-VioR 770, CD44-FITC, and CD25-APC (all from Miltenyi Biotec) and analyzed by flow cytometry using the MACSQuant Analyzer 10. Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence, and a gate was set on CD45⁺ cells. The isolated naïve (CD44low/–CD62L⁺CD25–) CD4⁺ T cells with a purity of about 98% were used as starting material for *in vitro* polarization towards Th1, Th2, and Th17 cells.

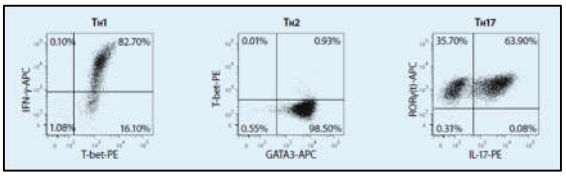

Fig. 2) Analysis of lineage-specific transcription factors in Th1, Th2, and Th17 cells. On day 5 of the *in vitro* culture, the differentiation into the various T_H cell subsets was examined by analyzing the expression of the lineage-specific transcription factors T-bet, GATA3, and RORγ(t). To this end, cells were intranuclearly stained with the respective fluorochrome-conjugated antibodies and the FoxP3 Staining Buffer Set (all from Miltenyi Biotec) and analyzed by flow cytometry on the MACSquant Analyzer 10.

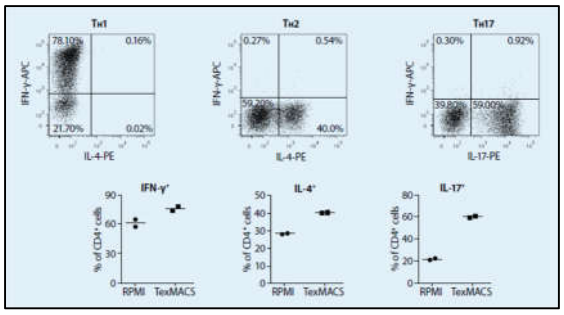

Fig. 3) Analysis of lineage-specific transcription factors in T_H1, T_H2, and T_H17 cells. (A) To determine the effector function of T cells after polarization, the capacity of the cells to produce lineage-specific effector cytokines was analyzed by flow cytometry on day 5. To this end, cells were restimulated with PMA/Ionomycin for 5 hours and stained intracellularly for the detection of IFN-γ, IL-4, and IL-17, using the respective fluorochrome-conjugated antibodies in combination with the Inside Stain Kit (all from Miltenyi Biotec). In line with the expected characteristic cytokine expression profiles, the T_H1 culture showed a high percentage of IFN-γ–producing, but no IL-4–producing cells. The T_H2 culture featured a high frequency of IL-4–, but no IFN-γ–expressing cells, whereas the T_H17 culture was characterized by a high level of IL-17–producing cells and the absence of IFN-γ–producing cells. (B) TexMACS Medium was developed specifically for T cell activation and expansion. *In vitro* T_H cell differentiation in the presence of TexMACS Medium led to a higher expression level of the characteristic effector cytokines in the various T_H subsets compared to RPMI 1640.