

Direct *ex vivo* isolation, cultivation and expansion of CD4$^+$ T cells from mouse spleen

In vitro expansion of mouse CD4$^+$ 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.

All the more it is important to provide a reliable workflow for the isolation and cultivation of CD4$^+$ T cells directly from mouse spleen that is fully compatible with your downstream application of choice.

Mouse spleens were dissociated into a single cell suspension using the gentleMACS Dissociator. Then, CD4$^+$ T cells were magnetically enriched with the CD4$^+$ T cell Isolation Kit, mouse and subsequently activated and expanded with CD3 and CD28 antibody loaded MACSiBeads (T cell Activation and Expansion Kit, mouse). Proliferation, phenotype, and expression of activation markers was assessed by flow cytometry at different timepoints. On day 7 after activation, cells were examined for cytokine expression. Therefore cells were restimulated with ionomycin and PMA, treated with Brefeldin A and intracellularly stained for cytokines. Additionally, cell culture supernatants were collected 24 h after restimulation and analyzed for cytokines using the MACSPlex Cytokine 10 Kit, mouse.

Hereby we provide a protocol for the 'untouched' isolation of mouse CD4$^+$ T cells from spleen, including their cultivation, activation and expansion for the best results subsequent experiments and analyses. Furthermore, all tools for the assessment of proliferation, phenotype, activation markers and cytokine expression were included.

Workflow

5 min	**Preparation of a single cell suspension from mouse spleen**
18 min	**Isolation of mouse CD4$^+$ T cells**
35 min	**Staining for cell proliferation analysis**
	Flow cytometric analysis of phenotypical surface markers (e.g. day 0)
7 d	**Activation and expansion of mouse CD4$^+$ T cells**
	Flow cytometric analysis of activation markers (e.g. days 1, 2)
	Analysis of cell proliferation (e.g. days 1, 3 and 7)
	Flow analysis of phenotypical surface markers (e.g. day 7)
8 min	**Removal of MACSiBeads**
4 h	**Restimulation of CD4$^+$ T cells**
	Collection of cell culture supernatants for MACSPlex cytokine analysis
60 min	**Cell harvest and intracellular staining of lineage-specific effector cytokines**
	Flow cytometric analysis MACSPlex analysis of supernatants



Material

T cell cultivation

- TexMACS Medium (# 130-097-196)
- 2-Mercaptoethanol
- Mouse IL-2 Research Grade (# 130-098-221)
- 24-well plate (e.g. Gas-permeable Culture Plate # 150-000-362)
- 96-well plate (e.g. Gas-permeable Culture Plate # 150-000-364)

Optional

- RPMI1640 medium
- 100x L-Glutamine stock solution (200 mM)
- 100x penicillin/streptomycin stock solution
- Fetal bovine serum (FBS)

Spleen dissociation

- Pre-Separation Filters, 30 µm (# 130-041-407)
- MACSmix™ Tube Rotator (# 130-090-753)
- CO2 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)

Buffer (standard wash and dilution buffer)

- autoMACS® Rinsing Solution (# 130-091- 222)
- Bovine serum albumin (BSA Stock Solution; # 130-091-376)

Magnetic cell separation

- $CD4^+$ T Cell Isolation Kit, mouse (# 130-104-454)

Magnetic enrichment

- LS Columns (# 130-042-401)
- MACS Separator for LS columns (e.g. MidiMACS™ Separator # 130-042-302)
- MACS Multistand (# 130-042-303)

T cell activation and expansion

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

Removal of MACSiBead Particles

- MACSiMAG Separator (# 130-092-168)

Analysis of cell proliferation

- Flow cytometry cell proliferation assay (e.g. CellTrace™ Violet Cell Proliferation Kit from Thermofisher, # C34557)
- Phosphate buffered saline (PBS)
- Fetal bovine serum (FBS) for blocking

Restimulation and brefeldin A treatment

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

Analysis of cytokines and surface markers

- Inside Stain Kit (#130-090-477)
- Antibodies, mouse:
  - CD3ε-APC-Vio770 (# 130-105-460)
  - CD4-VioGreen (# 130-109-413)
  - CD62L-PE (#130-102-543)
  - CD69-VioBlue (# 130-103-949)
  - CD25-VioBright FITC (# 130-108-999)
  - CD25-PE-Vio770 (# 130-108-998)
  - CD137-PE (# 130-102-568)
  - CD154-APC (# 130-102-454)
  - CD134-PE-Vio770 (# 130-109-743)
  - CD25-VioBright FITC (# 130-108-999)
  - Anti-IL-2-PE (# 130-110-154)
  - Anti-TNF-α-FITC (# 130-102-294)
  - Anti-IFN-γ–PE (# 130-102-388)



- MACSQuant Analyzer, MACSQuant Analyzer 10 (# 130-096-343), or other flow cytometers equipped with violet (405 nm), blue (488 nm) and red (635 nm) lasers able to discriminate FITC, PE, and APC fluorescence
  ▲ Note: The MACSQuant VYB cannot be used.
- MACS® Chill 96 Rack (# 130-094-459), when using MACSQuant Analyzer or MACSQuant Analyzer 10
- MACSQuant Calibration Beads (# 130-093-607), when using the MACSQuant Analyzer or MACSQuant Analyzer 10

Flow cytometric analysis of secreted cytokines in the cell culture supernatant

- MACSPlex Cytokine 10 Kit, mouse (# 130-101-740)
- Polypropylene or polystyrene reagent tubes for serial dilutions of the MACSPlex Cytokine 10 Standard as well as for preparation, dilution, and storage of unknown samples
- (Optional) Vacuum manifold or centrifuge with adapters to accommodate microtiter plates
- Orbital shaker for microtiter plates or tubes (frequency 450–1400 rpm)
- MACSQuant Analyzer, MACS® Chill 96 Rack and MACSQuant Calibration Beads (as in section '*Material/Analysis of cytokines and surface markers*')

Material preparation

T cell medium

TexMACS medium supplemented with 2-Mercaptoethanol (0.01 mM final concentration), 100x penicillin/streptomycin stock solution (1% final concentration) and 50IU/mL Mouse IL-2.
▲ **Notes:** Please see next column.

▲ **Note:** Make sure to **freshly** add IL-2 to the T cell medium for cell expansion. For some experimental procedures T cell medium **without** IL-2 has to be used (**see chapters 9., 10. and 11.**)
▲ **Note:** Optionally up to 10% FBS can be added to the TexMACS medium to further boost expansion rates.
▲ **Note:** Instead of TexMACS medium, RPMI supplemented with FBS (10% final concentration), 100x L-Glutamine stock solution (1% final concentration), 2-Mercaptoethanol (0.01 mM final concentration), 100x penicillin/streptomycin stock solution (1% final concentration) and 50U/mL Mouse IL-2 can be used for T cell cultivation and expansion.
▲ **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

Reconstitution of mouse IL-2, research grade

It is recommended to reconstitute lyophilized Mouse IL-2, research grade with deionized sterile-filtered water to a final concentration of 0.1–1.0 mg/mL in a minimal volume of 100 µL.
▲ **Note:** Further dilutions should be prepared with 0.1% bovine serum albumin (BSA) or human serum albumin (HSA) in phosphate-buffered saline.

- The ED50 is ≤0.2 ng/mL corresponding to an activity of ≥5×10^6 U/mg
- Recommended reconstitution: 0.1mg/mL by reconstituting a 100 µg vial of mouse IL-2, research grade with 1 mL deionized sterile-filtered water
- This results in a final activity of 500U/µl.

- To receive a cell culture medium supplemented with e.g. 50U/mL, freshly add 1 µl reconstituted mouse IL-2, research grade to 10 mL cell culture medium
- Prepared aliquots of mouse IL-2 can be stored at -70 °C for up to 6 month until further usage



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.

Phenotyping antibody panel

Freshly prepare the following mastermix for each sample:

- 20 µl buffer
- 5µl of each antibody:
  - CD4-VioGreen
  - CD3ε-APC-Vio770
  - CD44-FITC
  - CD62L-PE
  - CD69-VioBlue
  - CD25-PE-Vio770
- Store mastermix in the dark in the refrigerator (2−8 °C) until use. Do not store for extended periods.

Activation marker antibody panel

Freshly prepare the following mastermix for each sample:

- 15 µl buffer
- 5µl of each antibody:
  - CD69-VioBlue
  - CD3ε-APC-Vio770
  - CD25-VioBright FITC
  - CD4-VioGreen
  - CD137-PE
  - CD134-PE-Vio770
  - CD154-APC
- Store mastermix in the dark in the refrigerator (2−8 °C) until use. Do not store for extended periods.

Preparation of the MACSPlex Cytokine 10 Standard

▲**Note**: Reconstitute and dilute MACSPlex Cytokine 10 Standard with MACSPlex Buffer, or use the same media as is used for the dilution of the unknown sample.
▲ **Note**: Only use freshly prepared MACSPlex Cytokine 10 Standard solutions. Do not store or reuse reconstituted or diluted standards.
▲ **Note**: Use polypropylene or polystyrene reagent tubes. Do not use glass vials. The generation of standard curves requires eight samples: seven samples of the MACSPlex Cytokine 10 Standard, and one blank control. These samples will be measured as duplicates (refer to section *12. Analysis of secreted cytokines using MACSPlex Cytokine 10 Kit, mouse).*

1. Thaw one vial containing the lyophilized MACSPlex Cytokine 10 Standard.

2. Open the vial and add 200 µL of MACSPlex Buffer or media to the pellet. Mix gently. This is the stock solution (10, 000 pg/mL for IL-4, IL-5, IL-17A, IL-23 and 50, 000 pg/mL for IL-2, IL-10, IL-12p70, IFN-γ, TNF-α, GM-CSF).

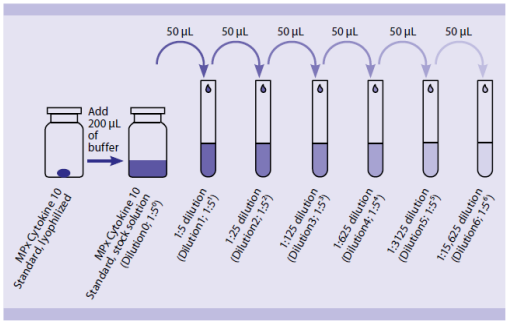
3. Label six reagent tubes and arrange them in the following order:
1:5 (1:51; 2,000 pg/mL or 10,000 pg/mL)
1:25 (1:52; 400 pg/mL or 2000 pg/mL)
1:125 (1:53, 80 pg/mL or 400 pg/mL)
1:625 (1:54; 16 pg/mL or 80 pg/mL)
1:3125 (1:55; 3.2 pg/mL or 16 pg/mL)
1: 15,625 (1:56; 0.6 pg/mL or 3.2 pg/mL)

4. Pipette 200 µL of MACSPlex Buffer or media into each tube.

5. Perform a 1:5 dilution by transferring 50 µL from the stock solution to the tube labeled 1:5 and mix thoroughly. Continue making 1:5 serial dilutions by transferring 50 µL from the tube labeled 1:5 to the tube labeled 1:25 and so on to the tube labeled 1:15,625. Mix each dilution before performing the next transfer.

6. Keep 200 µL MACSPlex Buffer or media as blank control (0 pg/mL).

Serial dilution of the MACSPlex Cytokine 10 Standard:





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.

6. 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.

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

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

9. 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. Mix well and incubate for 10 minutes in the refrigerator (2–8 °C).

9. (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.

10. 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.

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. Analysis of cell proliferation

▲ **Note:** Cell proliferation was analyzed on days 1, 3 and 7. However, please feel free to modify checkpoints according to your experimental needs.
▲ **Note:** Please make sure to have at least one additional well per condition (e.g. stimulated and unstimulated control) for analysis purposes left.
▲ **Note:** To analyze cell proliferation use e.g. CellTrace™ Violet Cell Proliferation Kit (Thermofisher, #C34557).
▲ **Note:** To assess expansion rates, please also determine cell numbers on each respective day by e.g. counting cells with a Neubauer Chamber or by using the counting function of the MACSQuant Analyzer 10.
▲ **Note:** The here recommended procedure for using the CellTrace™ Violet Cell Proliferation Kit differs from the manufacturer's instructions. If desired, follow manufacturer's instructions instead.

<u>Recommended procedure:</u>

1. Freshly prepare the 5 mM CellTrace™ Violet stock solution by adding 20 µl DMSO (Component B, provided with the CellTrace™ Violet Cell Proliferation Kit) to 1 vial of CellTrace™ Violet (Component A). Mix well.

2. Determine cell number.

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

3. For each sample resuspend up to $2x10^6$ in 1 mL PBS and transfer to a suitable tube (e.g. 15 mL tube).

4. Add 1 µl of the 5 mM CellTrace™ Violet stock solution to a final concentration of 5 µM.

5. Mix well and incubate for 5 min. at 37°C

6. Add 1 mL of FBS, mix well and incubate for 2 minutes.

7. Add 10 mL PBS, mix well.

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

9. Resuspend cells in an appropriate amount of cell culture medium (e.g. 2×10^6 cells in 2 mL/well of a 24-well plate).

10. Treat cells as other samples and proceed with cell stimulation, expansion, incubation, or analysis.

11. On measuring time points, resuspend cells directly in the well and take an aliquot (e.g. 100 µl when cultivating in a 24-well plate).

12. Add an appropriate amount of buffer to dilute cells (e.g. 500 µl buffer to an aliquot of 100 µl) and analyze proliferation by flow cytometry in an instrument with a 405 nm excitation source (e.g. MACSquant Analyzer 10)

5. T cell activation and expansion with MACSiBeads

1. Resuspend loaded Anti-Biotin MACSiBead Particles thoroughly and transfer 40 µL (4×10^6 loaded Anti-Biotin MACSiBead Particles) per 2×10^6 cells to a suitable tube.
▲ **Note:** If unloaded MACSiBead Particles shall be used for negative control experiments, replace loaded Anti-Biotin MACSiBead Particles by adding 40 µL (4×10^6 beads) of unloaded Anti-Biotin MACSiBead Particles per 2×10^6 cells.

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

3. Aspirate supernatant and resuspend loaded Anti-Biotin MACSiBead Particles in 1 mL of fresh T cell medium supplemented with IL-2.

4. Resuspend cells at a density of 2×10^6 cells per mL of T cell medium supplemented with IL-2.

5. Add the cell suspension and the prepared Anti-Biotin MACSiBead Particles from step 3 to a suitable cell culture vessel at a density of 1×10^6 cells per mL per cm2 (e.g. 2×10^6 cells in 2 mL/well of a 24-well plate).

6. Incubate at 37 °C and 5–10% CO_2 for up to 2 days.
▲ **Note:** Inspect cultures daily, and add fresh T cell medium if required.

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

8. Split the cell culture every two days 1:4 or 1:2, depending on the proliferation of cells, and add fresh T cell medium supplemented with IL-2.
(keep cells at a density of 1×10^6 cells per mL per cm2; e.g. 2×10^6 cells in 2 mL/well of a 24-well plate).
▲ **Note:** Daily inspect the culture. Depending on the expansion rate, it might be necessary to split culture more frequently than every 2 days.

9. After 6–8 days of activation, T cells are in a resting state and further expansion of T cells requires a restimulation.

6. Flow cytometry - phenotyping

▲ **Note:** Cells were phenotyped on days 0 and 7. However, please feel free to modify checkpoints according to your experimental needs.
▲ **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. For each sample resuspend up to 10^6 nucleated in 50 µl phenotyping mastermix (see *Material Preparation*, in section *Phenotyping antibody panel*).

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.
▲ **Note:** Add propidium iodide according to the manufacturer's instructions before flow analysis.

7. Flow cytometry – activation marker

▲ **Note:** Activation markers were analyzed on days 1 and 2 after activation with the T Cell Activation and Expansion Kit, m. However, please feel free to modify checkpoints according to your experimental needs.
▲ **Note:** Proceed is as *in 6. Flow cytometry – phenotyping,* but use the activation marker mastermix instead (see '*Material Preparation*', in section '*Activation marker antibody panel*').

8. 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 $2x10^7$/ mL and vortex thoroughly.

5. Place the tune 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.

9. Seeding of cells for restimulation and subsequent analyses

▲ **Note**: Restimulation of cells is performed in the absence of IL-2.

1. Determine cell number

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



3. Resuspend cells at a density of $1x10^7$ cells/ml in T cell medium without IL-2.

4. Depending on the total cell number and experimental requirements, transfer either 1 ml cell suspension ($1x10^7$ cells) per well to a 24-well plate or 100 µl cell suspension ($1x10^6$ cells) per well to a 96-well plate.

10. Restimulation and brefeldin A treatment of mouse CD4⁺ T cells

▲ **Note**: Make sure to treat samples from whom supernatants for the MACSPlex analysis are to be taken only with PMA/ionomycin and **not** with brefeldin A.
▲ **Note**: Make sure to use T cell medium without IL-2

1. Add 20 ng/mL PMA and 1µg/mL ionomycin to cells and incubate for 2 hours at 37°C.

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

3. Cells are now ready for further analyses.

▲ **Note**: For the analysis of lineage-specific effector cytokines (**see chapter 12**), cells can be further processed immediately after the brefeldin A treatment. For the collection of supernatants for MACSPlex analysis, it is recommended to stimulate cells with PMA/ionomycin up to 24 h. Analysis with MACSPlex after shorter stimulation times (e.g. 2-4 h), might still be successful, but result in lower data scores.

11. Collection of supernatant for MACSPlex analysis of secreted cytokines

▲ **Note**: Make sure to treat samples from whom supernatants are to be taken only with PMA/ionomycin and not with brefeldin A.
▲ **Note:** It is recommended to collect supernatants on day 8, 24h after restimulation with PMA/ionomycin. Analysis with MACSPlex after shorter stimulation times (e.g. 2-4 h), might still be successful, but result in lower data scores.
▲ **Note:** Keep supernatants cool after collection (2-8°C) and either process them immediately or freeze them to -70°C for further use. Frozen supernatants can be stored for up to 4 weeks at -70°C.
▲ **Note**: Handle all blood components and biological material as potentially hazardous.
▲ **Note**: If unknown samples are expected or known to contain levels >2000 pg/mL, it is recommended to dilute the samples to make sure the fluorescence values are within the dynamic range of the standard curve (see step 4.).

1. Collect 100 µl of supernatant

2. Centrifuge cell culture supernatant at 10,000×g for 10 minutes at 4 °C.

3. Transfer the supernatant into a new tube.

4. (Optional) Dilute with cell culture medium or MACSPlex Buffer.

5. Immediately analyze supernatants with MACSPlex or freeze to -70°C until use.



12. Analysis of lineage-specific effector cytokines by intracellular staining

▲ **Note:** The recommended dilution for the CD154-APC, Anti-TNF-α-FITC, Anti-IFN-γ-PE and Anti-IL-2-PE anti-mouse antibodies 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:** For the following staining panels 2 cell samples are required.

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 (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 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.

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 35 µL of Inside Perm. Add depending on the panels that is to be stained, 5 µL of each antibody:

<u>Panel 1:</u>

- CD154-APC, mouse
- Anti-TNF-α-FITC, mouse
- Anti-IFN-γ-PE, mouse

and

<u>Panel 2:</u>

- CD154-APC, mouse
- Anti-TNF-α-FITC, mouse
- Anti-IL-2-PE, mouse

▲ **Note:** For staining with even more 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. (Optional) If biotinylated antibody was used, resuspend the cell pellet in 100 µL of Inside Perm, add 10 µL of fluorochrome-conjugated anti-biotin antibody, and continue as described in steps 10 and 11.

13. 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.

13. Flow cytometric analysis of secreted cytokines using MACSPlex Cytokine 10 Kit, mouse

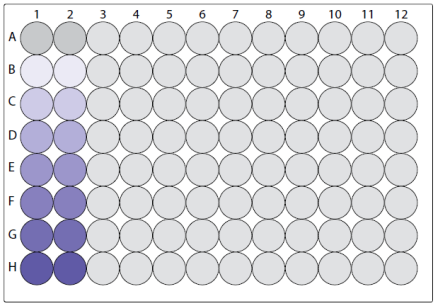
▲ **Note:** Run the assay at room temperature. Work fast and keep samples protected from light, for example, cover plate or tubes with aluminum foil, especially during incubation steps.

▲ **Note:** Unknown samples should be run in replicates, for example, in duplicates or triplicates and in different dilutions to make sure the fluorescence values are within the dynamic range of the standard curve.

▲ **Note:** The MACSPlex assay can either be performed using the MACSPlex Filter Plate with a vacuum manifold or standard polypropylene or polystyrene 1.5 mL reagent tubes. Please refer to the respective section of the protocol (12.1 or 12.2) when proceeding.

13.1 Protocol for the assay using the MACSPlex Filter Plate

Setup of the assay using a 96-well plate:



Well position	Sample	Dilution
A1/A2	Blank control	
B1/B2	MPx Cytokine 10 Standard (0.6 pg/mL or 3.2 pg/mL)	1:15,625 (Dilution6, $1:5^6$)
C1/C2	MPx Cytokine 10 Standard (3.2 pg/mL or 16 pg/mL)	1:3125 (Dilution5, $1:5^5$)
D1/D2	MPx Cytokine 10 Standard (16 pg/mL or 80 pg/mL)	1:625 (Dilution4, $1:5^4$)
E1/E2	MPx Cytokine 10 Standard (80 pg/mL or 400 pg/mL)	1:125 (Dilution3, $1:5^3$)
F1/F2	MPx Cytokine 10 Standard (400 pg/mL or 2 ng/mL)	1:25 (Dilution2, $1:5^2$)
G1/G2	MPx Cytokine 10 Standard (2 ng/mL or 10 ng/mL)	1:5 (Dilution1, $1:5^1$)
H1/H2	MPx Cytokine 10 Standard (10 ng/mL or 50 ng/mL)	Stock solution (Dilution0; $1:5^0$)
A3-H12	Add unknown samples	

Design your assay using two columns of the MACSPlex Filter Plate for the standards. Add each of the seven standard samples in duplicates next to each other. Standards should be run in order from the lowest concentration (blank control: 0 pg/mL) to the highest concentration (stock solution: 10,000 pg/mL or 50,000 pg/mL). Start with the unknown sample in the next column of the plate. For details, see *'Setup of the assay using a 96-well plate'.*

▲ Note: Place the MACSPlex Filter Plate on a non-absorbent surface during loading steps and incubation, i.e. remove any tissues from the surface, to prevent the wells from running dry. Ensure that residual drops under the plate are completely removed to prevent liquid transfer, by placing the plate briefly on a tissue.

▲ Note: Cover unused wells of the filter plate for later use with the adhesive foil provided with the kit.

▲ Note: Washing steps are described for the use of a vacuum manifold. Alternatively, a centrifuge with an adapter for microtiter plates can be used: Put the MACSPlex Filter Plate on top of a conventional 96-flatbottom microtiter plate without lid and place both into the adapter. Centrifuge at 300×g for 3 minutes at room temperature.



1. Pre-wet required wells of the MACSPlex Filter Plate with 200 µL of MACSPlex Buffer per well and aspirate off using a vacuum manifold designed to accommodate the filter plate (max. –300 mbar) until the wells are drained.

2. Place the filter plate briefly on a tissue to remove any residual liquid.

3. Add 50 µL of MACSPlex Buffer or media as a blank control, 50 µL of each dilution, and the stock solution of the MACSPlex Cytokine 10 Standard to the corresponding wells of the filter plate.

4. Add 50 µL of each unknown sample per well.

5. Resuspend MACSPlex Cytokine 10 Capture Beads by vortexing for at least 30 seconds and transfer 20 µL of MACSPlex Capture Beads to each well.

6. Incubate filter plate for 2 hours protect from light on an orbital shaker (450 rpm).

7. Apply the filter plate to the vacuum manifold and aspirate until wells are drained. Place the filter plate briefly on a tissue to remove any residual liquid.

8. Add 200 µL MACSPlex Buffer to each well and apply the filter plate to the vacuum manifold and aspirate off until wells are drained. Place the filter plate briefly on a tissue to remove residual liquid.

9. Repeat step 8.

10. Add 80 µL of MACSPlex Buffer to each well.

11. Add 20 µL of MACSPlex Cytokine 10 Detection Reagent to each well.

12. Incubate filter plate for 1 hour protect from light on an orbital shaker (450 rpm).

13. Repeat wash steps 7 and 8.

14. Add 200 µL of MACSPlex Buffer to each well.

15. For sample acquisition using MACSQuant Instruments and the Express Mode place the filter plate onto the Chill 96 Rack. To prevent liquid transfer from the wells, ensure that residual drops under the plate are completely removed by placing the plate briefly on a tissue.
▲ Note: Perform the flow cytometric acquisition on the same day, as prolonged storage of samples can result in increased background and reduced sensitivity.
▲ Note: Keep samples protected from light by using the protection lid during the flow cytometric acquisition with the MACSQuant Instrument.

13.2 Protocol for the assay using 1.5 mL reagent tubes

▲ Use polypropylene or polystyrene reagent tubes. Do not use glass vials.
▲ Standards should be run as duplicates. The order starts from the blank control (0 pg/mL) moving to the highest concentration (stock solution 10,000 pg/mL or 50,000 pg/mL).

1. Label reagent tubes for the blank control, each dilution and the stock solution of the MACSPlex Cytokine 10 Standard, and unknown samples.

2. Pipette 50 µL of MACSPlex Buffer or media as blank control, 50 µL of each dilution and the stock solution of the MACSPlex Cytokine 10 Standard into the corresponding reagent tubes. Pipette 50 µL of each unknown sample into the corresponding reagent tube.

3. Resuspend MACSPlex Cytokine 10 Capture Beads by mixing for at least 30 seconds and transfer 20 µL of MACSPlex Cytokine 10 Capture Beads to each tube.

4. Incubate for 2 hours protect from light on an orbital shaker (1400 rpm).

5. Add 0.5 mL of MACSPlex Buffer to each tube.

6. Centrifuge at 3000×g for 5 minutes.

7. Carefully aspirate off the supernatant, leave 20 µL in the tube.

8. Resuspend the MACSPlex Capture Bead pellet in each tube by adding 0.5 mL of MACSPlex Buffer and pipetting up and down.

9. Repeat steps 6 and 7.

10. Resuspend the MACSPlex Capture Bead pellet in each tube with MACSPlex Buffer to a total volume of 80 µL by pipetting up and down, e.g., add 60 µL of MACSPlex Buffer to the remaining 20 µL of supernatant (see step 7).

11. Add 20 µL of MACSPlex Cytokine 10 Detection Reagent to each tube.

12. Incubate for 1 hour protect from light on an orbital shaker
(1400 rpm).

13. Add 0.5 mL of MACSPlex Buffer to each tube.

14. Centrifuge at 3000×g for 5 minutes.

15. Carefully aspirate off the supernatant, leave 20 µL in the tube.

16. Resuspend each pellet in 0.5 mL of MACSPlex Buffer by pipetting up and down.

17. Repeat steps 14 and 15.

18. Resuspend the pellet in each tube with 200 µL of MACSPlex Buffer.

19. For sample acquisition with the MACSQuant Express Mode, transfer samples to a 96-well microtiter plate. Place the microtiter plate onto a Chill 96 Rack and measure.
▲ **Note:** Acquire cytokine standards first, beginning with the standard samples of the first dilution series in order from the blank control to the highest concentration. Then process the standard samples of the second dilution series in the same order (see also figure 2). Afterwards acquire the unknown samples.
▲ **Note:** Perform the flow cytometric acquisition on the same day, as prolonged storage of samples can result in increased background and reduced sensitivity.
▲ **Note:** Keep samples protected from light by using the protection lid during the flow cytometric acquisition with the MACSQuant Instrument.

13.3 Flow cytometer set up

The kit includes MACSPlex Setup Beads for flow cytometer set up. MACSPlex Setup Beads are not required when using the MACSQuant Analyzer or MACSQuant Analyzer 10 but for all other instruments. The kits is not suitable for use with the MACSQuant VYB.

13.3.1 Setup of the MACSQuant Instrument

Calibrate the MACSQuant Instrument using MACSQuant Calibration Beads (# 130-093-607). For details, refer to the data sheet of the MACSQuant Calibration Beads. After successfully completing the calibration, the MACSQuant Instrument is ready for measurement. No further steps are required as all necessary setup steps are performed automatically during calibration.

13.3.2 Setup of other flow cytometers

The analysis of MACSPlex Cytokine 10 Kit requires a flow cytometer with a blue (e.g. 488 nm) and a red (e.g. 635 nm) laser, which are capable of detecting FITC, PE, and APC. For the purpose of setting up these cytometers, MACSPlex Setup Beads are included in the kit. For instructions on the setup procedures of other flow cytometers, please refer to the application note "General instructions for MACSPlex Cytokine Kits" available on the product page at www.miltenyibiotec.com/130-101-740.

13.4 Flow cytometric acquisition and data analysis using the MACSQuant® Express Mode

To perform the acquisition and data analysis of the MACSPlex Cytokine 10 Kit, mouse with the MACSQuant® Instrument it is recommended to use the Express Modes "MACSPlex_Standard" and "MACSPlex_Sample" to achieve automated measurement and data analysis. For details refer to the special protocol “Data acquisition and analysis of MACSPlex Cytokine Kits using the MACSQuant® Analyzer Express Modes” available at *www.miltenyibiotec.com/130-101-740* under the Library tab. The minimum version number of the Express Mode package needed to run the assay on the MACSQuant Instrument can be found there as well. To check the version number of your Express Mode package available on your MACSQuant Instrument please select *Help> Info> expressModes* within the MACSQuantify Software. The version number of the Express Mode package is increasing with each Express Mode updates. Make sure the MACSQuant Instrument contains an Express Mode package with at least the same or higher version number than the special protocol is marked with.

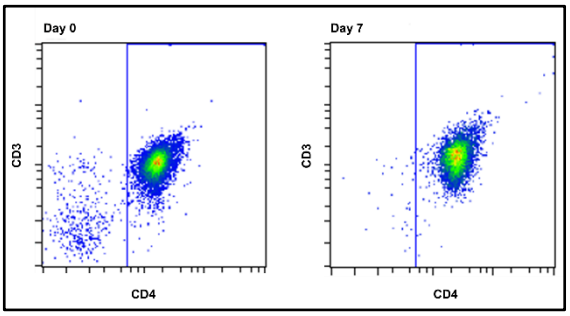


Fig. 1) Flow cytometric analysis of phenotypical surface markers of mouse CD4⁺ T cells. CD4+ T cells were isolated from a single-cell suspension, which was prepared from a BALB/c mouse spleen, using the CD4⁺ T Cell Isolation Kit, mouse. Cells were fluorescently stained with CD3ε and CD4 antibodies directly after isolation (Day 0) or after 7 days of expansion (Day 7), using the T cell Activation and Expansion Kit, mouse and TexMACS Medium. Flow analysis was performed using the MACSQuant Analyzer 10.

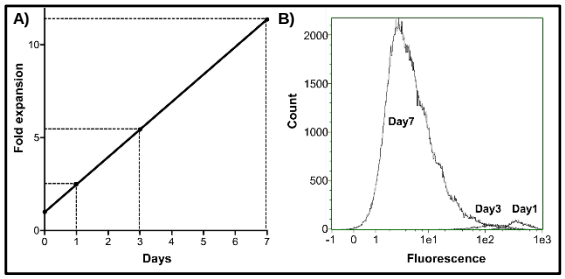


Fig. 2) Analysis of cell proliferation. (A) Cell proliferation was analyzed by counting cells directly after isolation and subsequently on days 1, 3 and 7 after activation/expansion using the T cell Activation and Expansion Kit, mouse and TexMACS Medium. Cell numbers were determined using the MACSQuant Analyzer 10. **(B)** Visualization of cell proliferation by flow cytometry using CellTrace™ Violet Cell from ThermoFisher

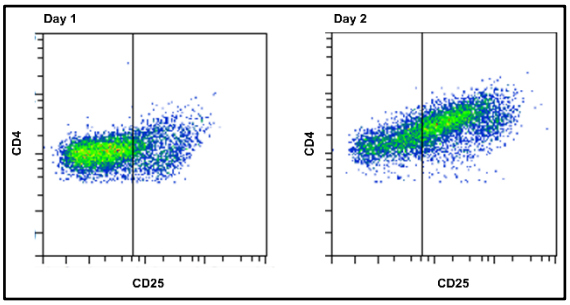


Fig. 3) Flow cytometric analysis of activation markers of mouse CD4⁺ T cells. CD4⁺ T cells were isolated from a single-cell suspension, which was prepared from a BALB/c mouse spleen, using the CD4⁺ T Cell Isolation Kit, mouse. Cells were fluorescently stained with CD4 and CD25 antibodies on days 1 and 2 after activation/expansion with the T cell Activation and Expansion Kit, mouse and TexMACS Medium. Flow analysis was performed using the MACSQuant Analyzer 10.

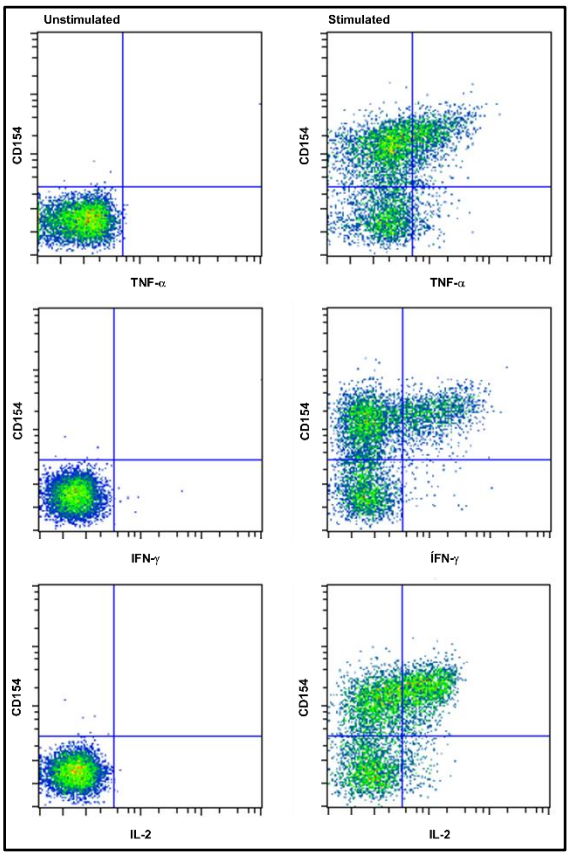


Fig. 4) Intracellular staining and flow analysis of lineage-specific effector cytokines. Mouse CD4$^+$ T cells were expanded for 7 days using the T cell Activation and Expansion Kit, mouse and TexMACS Medium. Cells were then restimulated by PMA/ionomycin and treated with brefeldin A. Subsequently surface activation marker CD154 as well as the effector cytokines TNF-α, IFN-γ and IL-2 were stained with the according antibodies and analyzed by flow cytometry using the MACSQuant Analyzer 10. Unstimulated Mouse CD4$^+$ T cells served as controls.

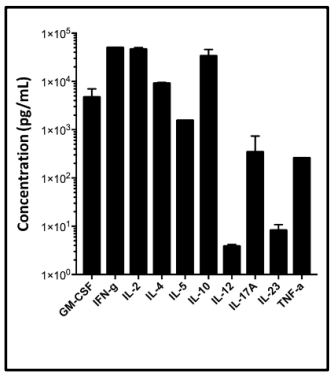


Fig. 5) MACSPlex analysis of secreted effector cytokines. Mouse CD4$^+$ T cells were expanded for 7 days using the T cell Activation and Expansion Kit, mouse and TexMACS Medium. Cells were then restimulated by PMA/ionomycin. Cell culture supernatants were collected after 4h of restimulation and analyzed via flow cytometry for cytokine secretion using the MACSPlex Cytokine 10 Kit, mouse. Flow cytometric analysis was performed with the MACSQuant Analyzer 10 using the Express mode.