Chapter 7

Generation and Expansion of T Helper 17 Lymphocytes Ex Vivo

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Abstract

CD4+ T helper (Th) lymphocytes are essential elements of the complex cellular networks regulating the initiation, development, and termination of adaptive immune responses. Different independent and specialized subsets of Th cells can be distinguished based on their dedicated transcription factor and cytokine expression profiles. Th17 lymphocytes have been described about a decade ago as CD4+ Th cells producing high quantity of IL-17A as a signature cytokine. Since their initial discovery, Th17 have drawn intense scrutiny for their dominant role in the pathogenesis of multiple autoimmune, infectious diseases and allergy. The influence of Th17 lymphocytes in cancer remains however ambiguous. The plethoric functions of Th17 may rely on the remarkable plasticity of these cells, endowed with the ability to transdifferentiate into other Th subpopulations depending on the environmental cytokine context. The possibility to generate Th17 ex vivo has facilitated the elucidation of the signals and transcription factors required for their differentiation and functions and has allowed for the evaluation of their functions following adoptive transfer in vivo. Several protocols have been developed to produce Th17 in vitro. The intent of this chapter is to provide examples of procedures for generating and expanding Th17 ex vivo.

Key words Th17 lymphocytes, Mouse, Human, Ex vivo generation

1 Introduction

CD4+ T helper (Th) lymphocytes critically contribute to the development and coordination of immune responses. They provide essential support to CD8+ cytotoxic T lymphocytes (CTLs) [1, 2], B lymphocytes [3], natural killer cells (NK) [4] and significantly participate to the recruitment and activation of innate cells such as macrophages and dendritic cells (DC) [5–7]. The activation of naïve CD4+ T lymphocytes requires the recognition of antigenic peptides in the context of MHC Class II by their specific receptor (TCR) together with co-stimulatory signals delivered by antigen-presenting cells. Different subtypes of activated antigen-experienced CD4+ T cells exhibiting distinct phenotypic and functional properties can be produced depending on the nature of the cytokines present in the microenvironment [8–10]. A growing number

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of Th lymphocyte subsets have been characterized based on their cytokine secretion profile and their dedicated differentiation programs controlled by specific transcription factors [11–13]. These Th lineages include well-characterized Th1 and Th2 cells as well as regulatory (immunosuppressive) T lymphocytes (Treg) and more recently identified Th9, Th22, T follicular, and Th17 [7, 12, 14, 15].

Cardinal features inherent to Th17 include the secretion of substantial amounts of interleukin 17A (IL-17A) and the expression of the transcription factor retinoic acid receptor-related orphan receptor gamma t (RORγt) [16, 17]. In addition, Th17 cells produce IL-21 and IL-22 [18] and, depending on the differentiation/environmental conditions, secrete variable amounts of TNFα, IFNγ, and/or GM-CSF [19–23]. Th17 represent a dominant pro-inflammatory lymphocyte subpopulation involved in the elimination of pathogens that are not cleared by Th1 or Th2 responses. These cells are also potent inducers of tissue inflammation and contribute to the pathogenesis of multiple autoimmune diseases in animals and humans [12, 21, 24]. Of note, the role of Th17 in the development of cancer remains debatable [25]. Adoptive transfer experiments have supported the antitumoral effects of Th17, highlighting their potential therapeutic interest [23, 26–29], but the significance of the presence of endogenous Th17 that develop in a context of progressing cancer is unclear [18, 30, 31]. This unstable balance between the pro- versus antitumoral function of Th17 likely stems from the high plasticity of these cells, capable of trans-differentiating into either pro-inflammatory effector cells such as Th1 [32] or immunosuppressive FoxP3$^+$Treg [33] depending on the environmental conditions [25]. In this context, the concentration of TGFβ1 present in the milieu is an important factor determining the pro- or anti- inflammatory characteristics of polarized Th17.

Many different protocols have been used to generate Th17 in vitro from naïve CD4$^+$ T lymphocytes using specific cytokine cocktails. In mice, TGFβ1, IL-6, and IL-23, in the presence of TCR and CD28 signals (antigen-presenting cells, plate-bound anti-CD3 plus anti-CD28 or anti-CD3/anti-CD28-coated microbeads) are required and sufficient to induce the differentiation of naïve CD4$^+$CD25$^-$ T cells into Th17 [25, 34]. IL-6, by inhibiting TGFβ-induced FoxP3 expression, blocks Treg differentiation, resulting in the polarization of naive T cells towards IL-17-producing RORγt$^+$ lymphocytes. However, some studies have demonstrated that pathogenic Th17, responsible for the development of experimental autoimmune encephalomyelitis (EAE), can be generated in IL-6$^{-/-}$ mice [20, 35]. These results highlight the possibility that Th17 can also develop in absence of IL-6. IL-21 has been identified as an alternative factor capable of inhibiting TGFβ-induced FoxP3 expression in the absence of IL-6 [20, 36]. IL-21 is produced in large quantities by Th17 and fosters an autocrine amplification feed-back loop increasing Th17 generation in

the absence of IL-6 [37]. IL-23 also plays an essential role in Th17 development. The IL-23 receptor (IL-23R) is composed of IL23R and IL-12Rβ2 [38]. The level of expression of IL-23R by naïve $CD4^+$ T cells is very low, while Th17 highly express this receptor. In agreement with these considerations, IL-23 does not seem required for Th17 initial lineage commitment, but this cytokine enhances Th17 expansion and promotes their survival, stabilization and pro-inflammatory properties [39]. Consistently, the number of Th17 is significantly reduced in IL-23p19-deficient mice compared to their wild-type counterparts [40, 41]. Additionally, IL-23 substantially contributes to the pathogenic functions of Th17 [40, 42]. Finally, IL-23 is a critical factor required for the generation of prolonged in vitro Th17 cultures [39]. IL-1β, by inducing interferon regulatory factor 4 (IRF4) which is a critical regulator of IL-21, has also been reported as an additional factor involved in the differentiation of Th17 cells in pro-inflammatory environments [43, 44]. In humans, the precise parameters required for Th17 differentiation remain unclear. Although some reports have argued that TGFβ1 may not be needed for human Th17 generation [45–47], others have reported that this cytokine is essential for the development of these cells [36, 48]. The possibility of producing Th17 with TGFβ plus IL-21 but not IL-6 has been proposed [36] and IL-1β alone or in combination with TGFβ has also been used to generate human Th17 [49]. Importantly and similar to the results obtained in mice, the presence of IL-23 is required for Th17 stabilization and optimal proliferation [50].

As Th17 cells have gained increasing interest in the field of autoimmunity, infectious diseases and cancer, the development of protocols allowing for the production of large number of these cells ex vivo is essential to further study their function, regulation, lineage stability and impact in different pathologies. For instance, the developmental fate of Th17 adoptively transferred to cancer-bearing hosts and the influence of the tumor environment on their lineage commitment and pro-inflammatory properties remain to be clearly elucidated. The possibility to harness the therapeutic potential of these cells against infectious agents or cancer represents another application of ex vivo-generated Th17. In this chapter, we describe examples of procedures suitable for in vitro generation of mouseand human Th17 lymphocytes from naïve $CD4^+CD25^-$ T cells.

2 Materials

2.1 Murine Th17 Generation

1. Six- to eight-week-old mice.
2. Complete medium for murine Th17 generation: RPMI 1640 supplemented with 10 % FBS, 100 U/ml penicillin, 100 μg/ml

streptomycin sulfate, 0.5× MEM nonessential amino acids, and 1 mM sodium pyruvate.

3. Naive $CD4^+CD62L^+$ T Cell Isolation Kit II, mouse (Miltenyi Biotech, catalogue # 130-093-227).
4. AutoMACS separator or LS Columns and magnets (MidiMACS or QuadroMACS (Miltenyi).
5. Anti-CD3/anti-CD28-coated (expansion/activation) microbeads (Invitrogen).
6. Magnet (DynaMag™-15 Magnet, Invitrogen) to recover washed microbeads.
7. Cytokines: mouse IL-12, IL-2, IL-7, IL-6, TGFβ (Peprotech) and IL-23 (R&D Systems).
8. Blocking antibodies: anti-mouse IFNγ and anti-mouse IL-4 antibodies (Affymetrix eBioscience).

2.2 Human Th17 Generation

1. Human PBMC.
2. Complete Medium (CM): AIM-V medium containing glutamine, nonessential amino acids, 10 mM Hepes buffer, and 1 mM sodium pyruvate.
3. Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences AB).
4. Miltenyi Human Naïve T Cell Isolation Kit II (Cat#: 130-094-131).
5. AutoMACS separator or LS Columns and magnets (MidiMACS or QuadroMACS (Miltenyi).
6. Human Anti-CD3/anti-CD28-coated microbeads (Invitrogen).
7. Magnet (DynaMag™-15 Magnet, Invitrogen) to recover washed microbeads.
8. Cytokines: Human IL-6, TGFβ1, IL-1β, IL-2 and IL-23 (Miltenyi).

2.3 Real-Time PCR

1. NanoDrop ND1000 spectrophotometer (NanoDrop).
2. Primers specific for RORγT and T-bet (ABI; Applied Biosystems), RNA extraction kit (Qiagen RNAeasy Mini kit), Reverse transcription (iScript cDNA synthesis kit), and Real-Time PCR reagent (IQ Supermix) (Bio-Rad).
3. Bio-Rad iCycler iQ real-time PCR detection system (Bio-Rad).

2.4 Flow Cytometry

1. Flow cytometry buffer (1× PBS, 1 % BSA).
2. Human FcγR (Fc-gamma receptor)-binding inhibitor (Affymetrix Ebioscience).
3. 1× Fixation/Permeabilization buffer (Affymetrix Ebioscience).

4. Anti-human/mouse RORγt-PE (clone AFKJS-9), anti-human/mouse Tbet-PE (clone eBio4B10), mouse IgG1K Isotype Control-PE (clone P3.6.2.8.1), rat IgG2a K Isotype Control-PE (Clone eBR2a) (Affymetrix Ebioscience).
5. LSRII-Fortessa flow cytometer (BD bioscience).
6. Flowjo software (Vx, Tree Star, Inc).

2.5 ELISAs

1. Human IL-17A ELISA Ready-SET-Go!® (Affymetrix ebioscience).
2. Human IFN-gamma ELISA Ready-SET-Go!® (Affymetrix ebioscience).

2.6 General Materials

1. Sterile PBS.
2. Red blood cell lysis buffer (BD biosciences).
3. Cell strainer.
4. Centrifuge tubes.
5. Hemocytometer.
6. 0.4 % Trypan blue.
7. MACS sorting buffer (0.5 % BSA, 2 mM EDTA).
8. 24-Well plates and Petri dishes.

3 Methods

3.1 Generation of Murine Th17 Lymphocytes Ex Vivo

1. Euthanize mice by carbon dioxide asphyxiation or other approved techniques and harvest spleens. Cut the spleens in small pieces with scissors and dissociate the tissues in a 40 μm cell strainer with a 5 ml syringe plunger in a Petri dish containing complete medium to obtain a single-cell suspension.
2. Collect the cell suspension in a 50 ml tube and centrifuge at 300 × *g* for 5 min at 4 °C. Remove the supernatant.
3. Resuspend the pellet in red blood cell lysis buffer and incubate for 1 min.
4. Add complete culture medium and centrifuge (400 × *g* for 5 min at 4 °C). Remove the supernatant.
5. Resuspend cells in MACS sorting buffer and determine the number of viable cells using trypan blue and a hemocytometer.
6. Isolate naive $CD4^+CD25^-CD62L^+$ T lymphocytes using a $CD4^+CD62L^+$ T cell isolation kit II strictly following the manufacturer's instructions. The isolation requires two steps: (1) Depletion of non-$CD4^+$ T cells using a biotin-antibody cocktail II and anti-biotin microbeads. Cells are separated using an AutoMACS device (program "Depletes") or LS columns and a

MidiMACS or QuadroMACS magnet and the negative fraction enriched in $CD4^+$ cells (flow-through) is collected (outlet port neg1 of the AutoMACS). (2) Positive selection of $CD4^+CD62L^+$ T cells using CD62L microbeads. Cells are separated using an AutoMACS device (program "Possel") or MS columns, and the positive fraction is collected (outlet port pos1 of the AutoMACS). Cell labeling is performed in MACS sorting buffer and incubation times are as described by the provider.

7. Centrifuge cells (400 × *g* for 5 min at 4 °C). Remove the supernatant.
8. Resuspend purified naïve $CD4^+$ T cells at a concentration of 10^6 cells/ml in complete culture medium.
9. Wash the appropriate amount of anti-mouse CD3/anti-mouse CD28-coated expansion/activation beads (*see* **step 10**) in a 15 ml tube with PBS and EDTA and recover the beads using a DynaMag™-15 Magnet as instructed by the provider.
10. Add washed expansion/activation beads at a ratio of 2 beads to 1 naïve $CD4^+$ T lymphocyte.
11. Add cytokines and blocking antibodies: 40 ng/ml IL-6, 0.5 ng/ml TGFβ1, 5 μg/ml blocking anti-IFNγ, and 5 μg/ml blocking anti-IL-4 antibodies.
12. Plate cells in 24-well plates (1 ml, 10^6 cells/well).
13. After 3 days, collect cells, remove expansion/activation beads using a DynaMag™-15 Magnet and repeat **steps 7–9**.
14. Add 40 ng/ml IL-23 and plate cells in 24-well plates (1 ml, 10^6 cells/well).
15. On day 6, cells expressing the transcription factor RORγt and producing IL-17 can be obtained (Fig. 1). The generated cells can be characterized by real-time PCR, flow cytometry or ELISA for expression of specific markers and transcription factors and cytokine production. These polarized cells can also be used for further analyses in vitro or to evaluate their therapeutic potential in vivo [26].

3.2 Generation of Human Th17 Lymphocytes Ex Vivo

1. Collect blood from healthy volunteers or patients as desired (100–300 ml).
2. Dilute blood in 1× PBS (approximately 1 to 2 dilution).
3. Pipet 15 ml of Ficoll-Paque™ PLUS in a 50 ml tube.
4. Gently layer 30 ml of diluted blood onto the Ficoll.
5. Centrifuge the samples at 930 × *g* for 20 min at room temperature with no brake.
6. Collect the peripheral blood mononuclear cells (PBMC) layer from the Ficoll gradient and transfer the cells in a 50 ml tube.

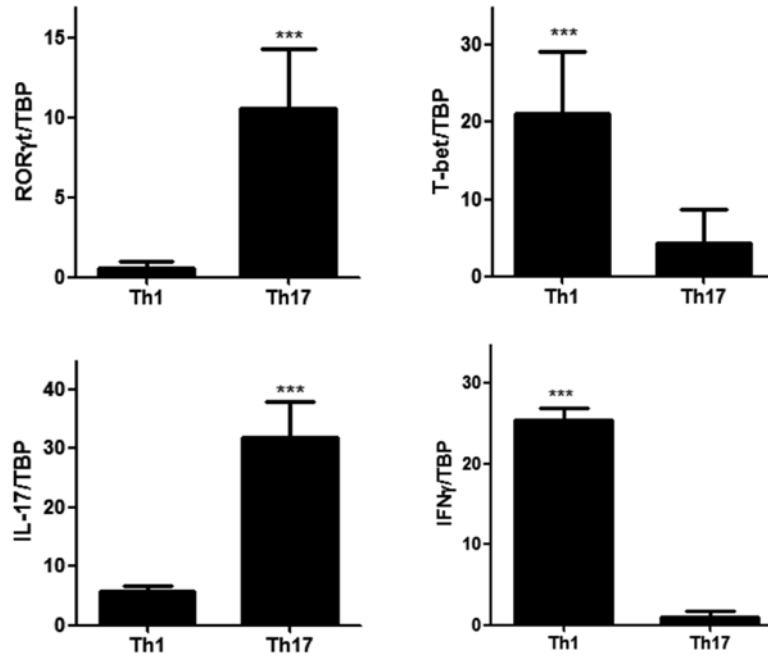

Fig. 1 Example of mouse Th17 lymphocyte generation in vitro. Mouse naïve CD4+ T cells were cultured in Th17-polarizing conditions and analyzed for RORγt and IL-17 expression. Cells were collected at the end of the differentiation period and RORγt, Tbet, IL-17, and IFNγ mRNA expression was determined by RT-PCR. Th1 cells, generated as reported [26], were used as comparison. Data are represented as mean ± SEM of three different experiments (***, $p < 0.001$)

7. Centrifuge cells (300 × *g*, 5 min, room temperature) and wash twice in PBS.
8. Resuspend the cells in MACS sorting buffer and determine cell number.
9. Perform isolation of naïve T lymphocytes using Miltenyi Human Naïve T Cell Isolation Kit II strictly following the provider's instructions. Untouched naïve CD4+ T cells are isolated by depletion of non-CD4+ T lymphocytes and of memory CD4+ T cells that are labeled with a cocktail of biotin-conjugated antibodies and anti-biotin microbeads. Cells are separated using an AutoMACS device (program "Depletes") or LS columns and a MidiMACS or QuadroMACS magnet and the negative fraction enriched in naïve CD4+ cells (flow-through) is collected (outlet port neg1 of the AutoMACS).
10. Centrifuge cells (300 × *g*, 5 min, room temperature) and determine cell number.
11. Resuspend naïve CD4+ T cells in CM (~1 million cells/ml).
12. Wash the appropriate amount of anti-human CD3/anti-human CD28-coated expansion/activation beads (*see* **step 13**)

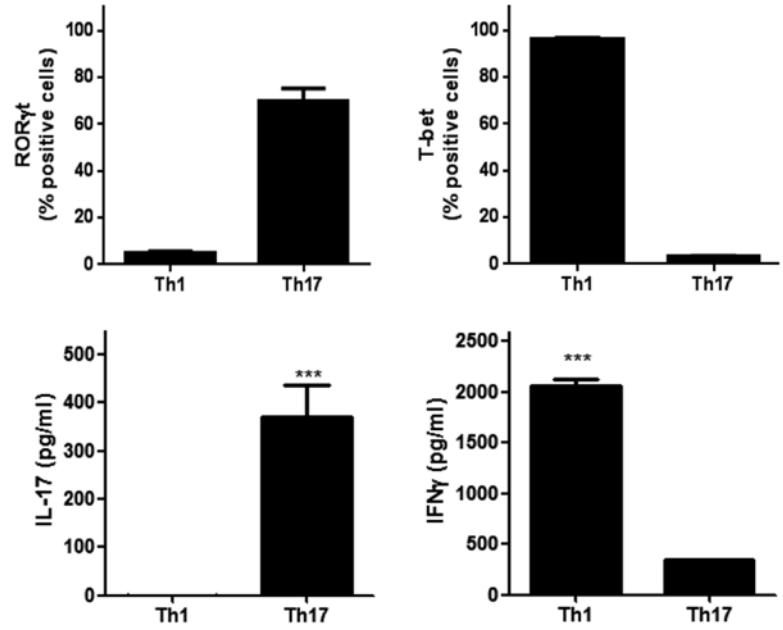

Fig. 2 Example of human Th17 lymphocyte generation in vitro.Human CD4+ T cells were cultured in Th17-polarizing conditions and analyzed for RORγT expression and IL-17 production. Cells were collected at the end of the differentiation period and the percentage of RORγt + and Tbet + cells was determined by flow cytometry. The concentration of IL-17 and IFNγ was detected by ELISA in 48 h culture supernatants (10^6 cells/ml). Th1 lymphocytes were used as comparison. Th1 were generated following the same procedures as for Th17 cells, except that (**a**) human IL-2 (20 UI/ml), human IL-12 (10 ng/ml), human IL-7 (20 ng/ml) were used, (**b**) the beads to T cell ratio was 1 to 1, and (**c**) cells were cultured for a total of 3 days (***, $p < 0.001$)

in a 15 ml tube with PBS and EDTA and recover the beads using a DynaMag™-15 Magnet strictly following the provider's instructions.

13. Add washed beads at a ratio of 2 beads to 1 naïve CD4+T lymphocyte.
14. Add cytokines: 20 ng/ml human IL-6, 5 ng/ml TGFβ1, 50 ng/ml IL-1β, 5 ng/ml IL-2.
15. Plate cells in 24-well plates (1 ml, 10^6 cells/well).
16. After 3 days, add 50 ng/ml human IL-23 in 1 ml of fresh CM per well.
17. Split cell by half on day 5 and add fresh CM to each well.
18. CD4+ T cells expressing RORγt and producing IL-17 (Fig. 2) can be collected on day 6–7 for further analyses. The generated cells can be characterized by real time PCR, flow cytometry or ELISA for expression of specific markers and transcription factors and cytokine production.

3.3 Characterization of Ex Vivo-Generated Th17 Cells

3.3.1 Real-Time PCR

1. Extract total mRNA from the collected cells using Qiagen RNAeasy Mini kit strictly following the manufacturer's protocol.
2. Measure RNA concentration using NanoDrop ND1000 spectrophotometer.
3. Run reverse transcription reaction using 250 ng of total RNA and iScript cDNA synthesis kit, following the manufacturer's instructions.
4. Real-time PCR reaction: 20 μl of the PCR reactions are set up in 96-well plates containing 10 μl 2× IQ Supermix, 1 μl TaqMan® primer/probe set, 2 μl of the cDNA synthesis reaction, and 7 μl of nuclease-free water. Reactions are run and analyzed on a Bio-Rad iCycler iQ real-time PCR detection system.
5. Analysis: Cycling parameters are determined and resulting data are analyzed by using the comparative C_t method as means of relative quantification, normalized to an endogenous reference (TATA Box Bonding Protein, TBP) and relative to a calibrator (normalized C_t value obtained from control cells), and expressed as $2^{-\Delta\Delta}C^t$ (Applied Biosystems User Bulletin #2: Rev B "Relative Quantification of Gene Expression").

3.3.2 Flow Cytometry

1. One million cells are washed in flow cytometry buffer and incubated for 15 min with human FcγR (Fc-gamma receptor)-binding inhibitor.
2. Cells are incubated with 1× fixation/permeabilization buffer at 4 °C for 30–45 min.
3. Cells are washed twice with 1× permeabilization buffer.
4. Cells are incubated with anti-human/mouse RORγt-PE, anti-human/mouse Tbet-PE or Mouse IgG1K Isotype Control-PE) or Rat IgG2a K Isotype Control-PE at 4 °C for 30 min.
5. Samples are washed twice with 1× permeabilization buffer, then once with 1× PBS.
6. Samples are resuspended in 1× PBS and analyzed using a LSRII-Fortessa flow cytometer.
7. Data are analyzed using Flowjo software.

3.3.3 ELISA

1. Generated human Th cells are cultured for 48 h (10^6 cells in 1 ml CM per well of a 24-well plate) in the presence of anti-human CD3/anti-human CD28-coated expansion/activation beads (2 beads to 1T lymphocyte) and the culture supernatant is collected and centrifuged (300×*g*, 5 min, room temperature).
2. IL-17 and IFN-γ are detected in cell-free culture supernatants by enzyme-linked immunosorbent assays using human IL-17A

ELISA Ready-SET-Go!® or Human IFN-gamma ELISA Ready-SET-Go!® reagent sets strictly following the manufacturer's instructions.

4 Notes

1. To obtained optimal cell purity, start from single-cell suspension and strictly follow the provider's instructions regarding cell number and amount of beads (Miltenyi Biotech).
2. Keep cells on ice or at 4 °C during the isolation procedures until plating. Use pre-chilled MACS buffer (Miltenyi Biotech).
3. When purifying PBMC on Ficoll monolayers, ensure that the blood does not mix with the Ficoll before centrifugation. When collecting the PBMC ring, do not mix with or collect Ficoll (Miltenyi Biotech).
4. Cell viability of purified naïve T lymphocytes before initiation of the cultures should be 90–100 % (Miltenyi Biotech).
5. Activation beads should be washed in a minimum of 1–2 ml of buffer by extensive mixing (Miltenyi Biotech).
6. Examine cell cultures every day and split cultures if necessary depending on growth rate. Cell clusters should be observed. Do not let cells overgrow (Miltenyi Biotech).
7. Cytokine reconstitution and storage should be performed according to the provider's instructions. Avoid using products that are close to their expiration date. Avoid freeze/thaw cycles (Miltenyi Biotech).

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