# Rapid, column-free two-step procedure for the enrichment of human Th17 cells from peripheral blood

M. Fairhurst, J. Yu, and T. E. Thomas
STEMCELL Technologies Inc., Vancouver, BC, Canada

## Introduction

Human interleukin 17 (IL17) producing CD4+ T helper (Th17) cells have been identified as a distinct effector T cell subset. They act as key drivers of autoimmune diseases such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease. Phenotypically characterized as CD4+ T cells expressing CCR6, CCR4, CD161, and IL-23R, they also lack expression of CXCR3. Although their cytokine profile can be heterogeneous, they typically produce IL17 cytokines, are IL17+IFN-γ− and express the lineage-specific transcription factor RORC. A major disadvantage of current isolation methods is the requirement for previous *in vitro* stimulation. We have developed a two-step EasySep™ immunomagnetic column-free method for the enrichment of CD4+CXCR3−CCR6+ T cells from fresh peripheral blood nucleated cells (PBNC). Non-CD4+ T cells and CXCR3+ cells are first targeted for depletion using a cocktail of antibody complexes and dextran-coated magnetic particles. Labeled cells are separated using an EasySep™ magnet, and pre-enriched CD4+CXCR3− T cells are poured off into a new tube. Next, CCR6+ cells are positively selected from the pre-enriched fraction. The procedure can be automated using RoboSep™. Starting with frequencies of 5 ± 2% CD4+CXCR3−CCR6+ T cells in fresh PBNC, purities of 91 ± 3% (n=16) are obtained. Enriched CD4+CXCR3−CCR6+ T cells show increased levels of IL17 cytokine production (minimal IFN-γ) as assessed by ELISA and intracellular staining. Increased RORC mRNA expression is found in the enriched CD4+CXCR3−CCR6+ T cells compared to total CD4+ or CD4+CXCR3+ T cells. Dissecting the role of human Th17 cells in the modulation of immune responses is a requirement for the development of future therapies. These studies will be facilitated by the enrichment of non-activated *in vivo* derived Th17 (CD4+CXCR3−CCR6+) cells using this easy and rapid enrichment strategy.

## Conclusions

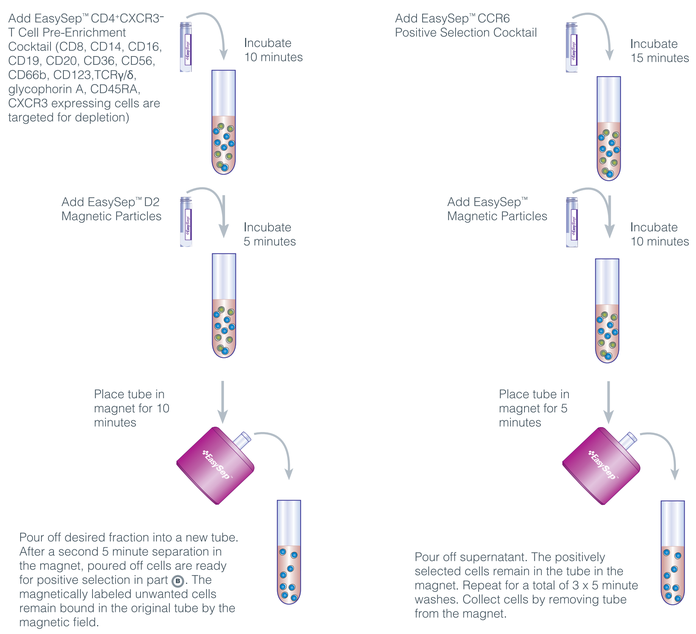
- Highly enriched Th17 (CD4+CXCR3−CCR6+) cells are obtained in 80 minutes; procedure can be automated using RoboSep™.
- Enriched Th17 (CD4+CXCR3−CCR6+) cells demonstrate the following benefits:
  - can be obtained without prior *in vitro* stimulation
  - can be expanded by culturing with soluble anti-CD3 monoclonal antibody in the presence of irradiated APC for 14 days
  - increased levels of IL17 cytokine with minimal IFN-γ (compared to total CD4+ or CD4+CXCR3− T cells) as assessed by intracellular cytokine staining (data not shown) or analysis of supernatants by ELISA
  - increased level of RORC mRNA expression compared to total CD4+ or CD4+CXCR3+ T cells

## Methods

**Preparation of Starting Cell Suspension**
A single cell suspension of mononuclear cells (PBMC) was prepared from either fresh whole blood or buffy coat suspensions of peripheral blood using Ficoll-paque PLUS. Alternatively, peripheral blood apheresis (Leucopak PBNC) cells were used following red blood cell lysis and one or more washes to remove platelets. Use of fresh (≤24 hours) cells is recommended. Typically start cells were resuspended at 5x10$^7$ cells/mL in PBS + 2% FBS and 1mM EDTA.

**Assessing Enriched Cells**
The purity of Th17 (CD4+CXCR3−CCR6+) T cells can be measured by flow cytometry after staining with fluorochrome-conjugated anti-CD4, anti-CXCR3, and anti-CCR6 antibodies. In addition, intracellular staining of IL-17 cytokine was assessed after stimulation of cells with PMA-Ionomycin. For ELISA, enriched cells were stimulated with anti-CD3/anti-CD28 beads for 24, 48, and 72 hours. Supernatants were collected and analysed for secreted cytokines.

### FIGURE 1: Two-step EasySep™ procedure for column-free enrichment of Th17 (CD4+CXCR3−CCR6+) cells from human peripheral blood



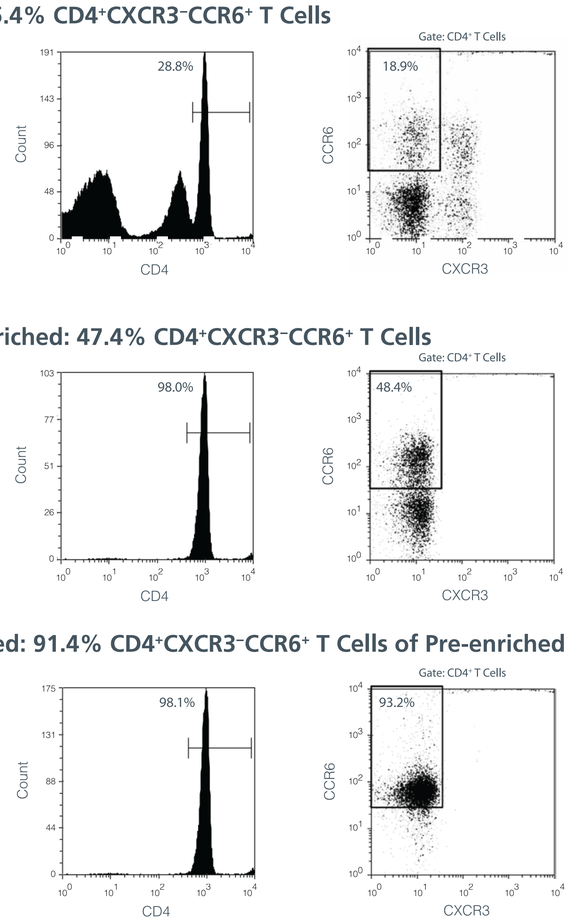

## Results

### TABLE 1: Purity and recovery of human Th17 (CD4+CXCR3−CCR6+) cells enriched from peripheral blood by manual EasySep™ or RoboSep™

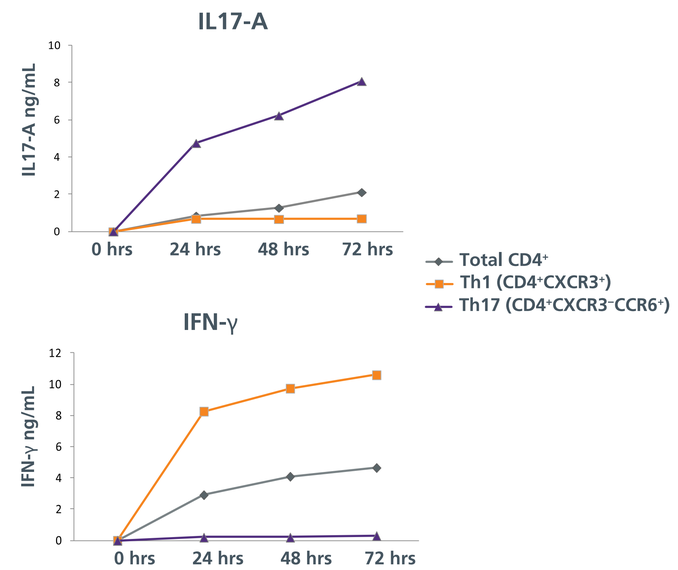
| n | % start | % purity enriched | Typical recovery of CD4+CXCR3−CCR6+ T cells from start |
|---|---|---|---|
| 16 | 4.7 ± 1.8 | 91.3 ± 3.7 | 1.5x10$^6$ CD4+CXCR3−CCR6+ T cells from 1x10$^8$ peripheral blood nucleated cells |

Purities determined by flow cytometry. Values are expressed as means ±SD.

### FIGURE 2: Phenotypic assessment of human Th17 (CD4+CXCR3−CCR6+) cells enriched using EasySep™

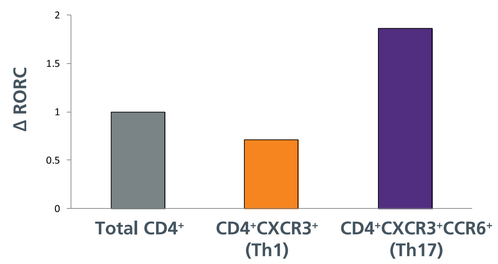



### FIGURE 3: Enriched CD4+CXCR3−CCR6+ T cells show increased levels of IL17-A secretion by ELISA with minimal IFN-γ secretion compared to CD4+CXCR3+ T cells




Freshly isolated CD4+CXCR3−CCR6+ T cells were stimulated with anti-CD3/anti-CD28 coated beads at a ratio of 8:1 (cells: beads). Supernatants were collected at 24, 48, and 72 hours and cytokines were assessed by ELISA. Results for one of three comparable experiments are shown. A similar trend was observed with intracellular staining.

### FIGURE 4: Enriched CD4+CXCR3−CCR6+ T cells show an increased level of RORC mRNA




Freshly isolated CD4+CXCR3−CCR6+ T cells were stimulated with anti-CD3/anti-CD28 beads at a ratio of 8:1 (cells: beads) for 48 hours. Expression of RORC relative to that of 18S was analysed by quantitative RT-PCR.