BECKMAN COULTER
Life Sciences

Characterizing Human CD4+ T cell Populations by Intracellular Cytokine Profile Using Flow Cytometry

Laura Maggi, PhD| Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy

Gianni Montaini, BSc | Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy

Introduction

CD4+ T cells play a central role in the function of the immune system, firstly by helping B cells to produce antibodies, but also by orchestrating CD8+ T cells and macrophage functions against a wide variety of pathogenic microorganisms. Human CD4+ Th cells can be subdivided into lineages on the basis of their immunological functions, which are supported by the expression of well-defined profiles of specific transcription factors, cytokines, and homing receptors. These lineages include effector cells, which protect from pathogens, and regulatory T cells (Treg), which protect from effector responses when they become dangerous for the host, as it happens for autoimmune responses and, in some circumstances, also for response to exogenous antigens. CD4+ effector T cell can be subdivided into three main types (Figure 1). Th1 cells producing IFN-γ, protect from intracellular pathogens and can be responsible for some organ-specific autoimmune disorders; Th2 cells producing IL-4, IL-5, and IL-13 play a protective role from helminths and are critical for the development of allergic inflammation; Th17 cells producing IL-17A and F, are involved in the protection from extracellular pathogens, and can play a role in the pathogenesis of several chronic inflammatory disorders (1, 2).

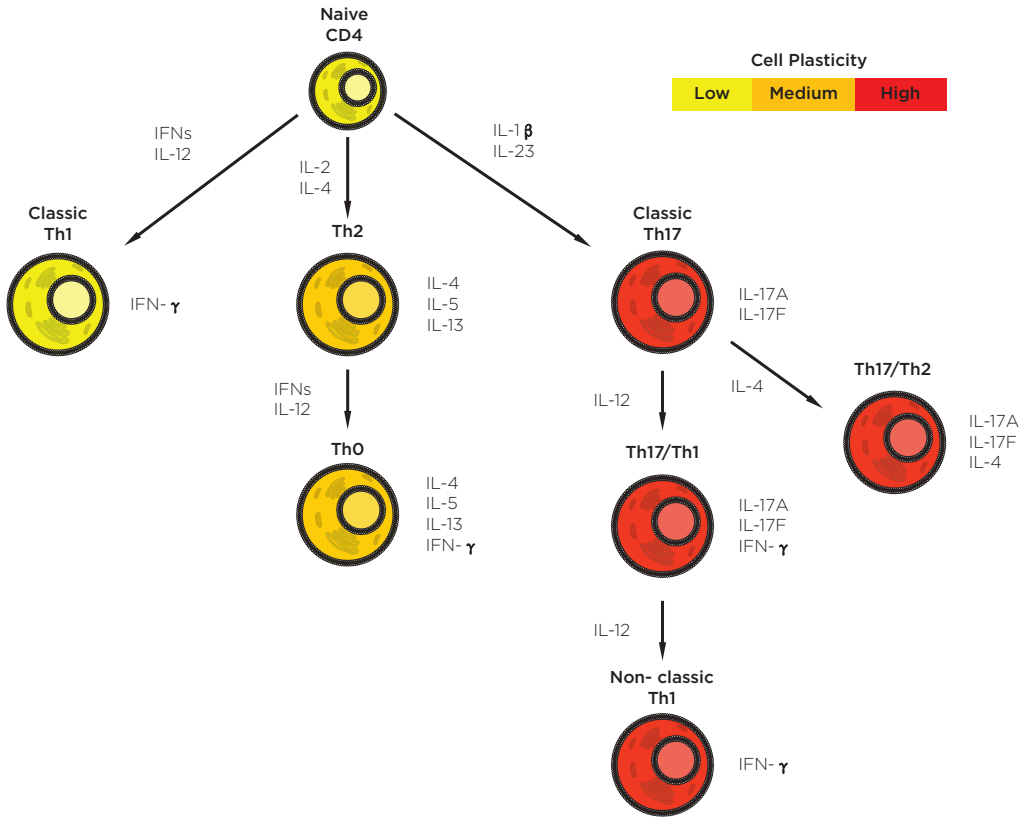

Figure 1. Human T helper cell subsets classification.

We've previously demonstrated that most human Th17 cells are contained within the CD161+CCR6+fraction of circulating and tissue-infiltrating CD4+ T cells. These cells originate from CD161^{+} precursors present in umbilical cord blood and new born thymus (3). Moreover, part of the human IL-17A-producing cells were found to produce IFN-γ (Th17/Th1). Both Th17 and Th17/Th1 exhibit plasticity towards Th1 cells in presence of the polarizing cytokines, IL-12 or TNF-α (4, 5) (Figure 1).

In this paper, we first obtained in vitro CD161^{+} and CD161^{-} T cell clones. These clones were evaluated for their cytokine production in response to PMA and Ionomycin stimulation. Cytokines produced by each clonal population were determined by intracellular Cytokine staining followed by flow cytometry analysis on the CytoFLEX S platform (CytoFLEX S flow cytometer, P/N B75408, configured as B2-R3-V4-G4 with Plate Loader B63215). Each CD4^{+}CD161^{+} T cells clone was shown to exhibit one of the following phenotypes: Th17 (producing IL-17 alone), Th17/Th1 (producing both IL-17 and IFN-γ) or non-classic Th1 clones (producing IFN-γ alone), whereas the CD4^{+}CD161^{-} CCR6- clones exhibit the following phenotype: Th2 (producing IL-4 alone) and classic Th1 clones (producing IFN-γ alone). This paper will focus on the intracellular cytokine staining and acquisition/analysis on CytoFLEX platform.

Materials

PRODUCT	MANUFACTURER	PART NUMBER
PMA	Sigma Aldrich	P8139
Ionomycin	Sigma Aldrich	I0634
Brefeldin A	Sigma Aldrich	B7651
Formaldehyde	Sigma Aldrich	F1635
Saponin	Sigma Aldrich	S7900

Tips For Success

1. To enhance cytokine detection, add Brefeldin-A (a Golgi inhibitor, final concentration: 5 µg/mL) in 20 µl/well of medium (complete RPMI 1640 plus 10% FCS), two hours after stimulation and incubate cells for a minimum of four hours following addition.

2. Optimize scatter settings for intra-cellular staining as cell size is often reduced as result of permeabilization.

Procedures

Cell culture: Isolation and Stimulation of CD4+ T-Cells

CD4+ T-Cells were isolated from the peripheral blood mononuclear cells (PBMCs) of healthy donors by immunomagnetic cell separation (Miltenyi biotech) and further divided into the CD161+CCR6+ and CD161-CCR6- fractions by flow cytometry cell sorting (FACSAria, BD Bioscience). Both cell fractions were seeded under limiting-dilution conditions (0.5 cell/well) (2) and expanded in 96-well plates for at least one month until each clone reached 6-8 wells of 96-well plate. Then one well for each clone was phenotypically and functionally evaluated for intracellular cytokine production subsequent to PMA (final concentration: 10 ng/mL) and ionomycin (final concentration: 1 µM) stimulation in presence of BFA.

Fixing and Intracellular staining

1. At the end of six hours stimulation, centrifuge the plate 500g x 3 min, discard the supernatant
2. Wash the cells with 150 µl/well of PBS, pH 7.2
3. Re-centrifuge at 500g for 3 min; discard the supernatant
4. Repeat steps 2.2 and 2.3
5. Add 100 µl/well of formaldehyde (2% in PBS, pH 7.2), ensure single-cell suspension by pipetting the cells pellet several times
6. Incubate for 15 min at room temperature.
7. Add 100 µl/well of 0.5% BSA in PBS, pH 7.2
8. Centrifuge at 500g for 3 min; discard the supernatant
9. Wash again the cells with 150 µl/well of 0.5% BSA in PBS, pH 7.2
10. Repeat step 2.8
11. Add 14 µl/well of specific fluorochrome-conjugated monoclonal antibodies cocktail in permeabilizing solution (0.5% saponin, 0.5% BSA, pH 7.2); ensure single-cell suspension by pipetting the cell pellet several times.
12. Incubate for 15 min at room temperature.
13. Add 150 µl/well of permeabilizing solution.
14. Centrifuge at 500g for 3 min; discard the supernatant.
15. Add 150 µl/well of 0.5% BSA in PBS, pH 7.2.
16. Acquire plate on CytoFLEX S Flow Cytometer.

Data acquisition on the CytoFLEX S Flow Cytometer

1. Set up plate experiment as instructed in the "CytoFLEX Series Instructions for Use" Manual (PN: B49006), Chapter 5, "Data Acquisition and Sample Analysis"
2. In Acquisition mode, create the following dot plots:

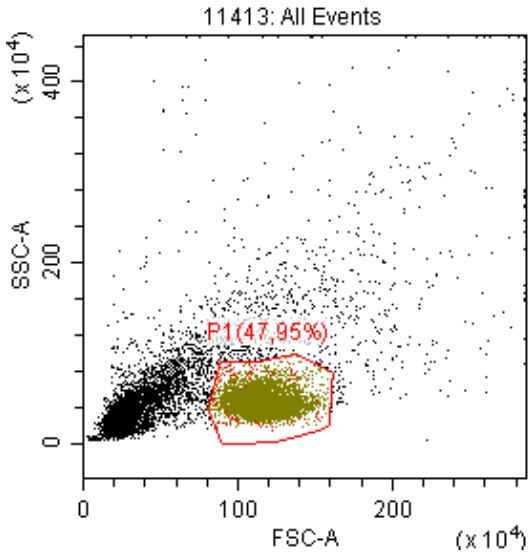

Figure 2 Identification of Lymphocyte Population. The FSC vs SSC dot plot demonstrates events collected from the cell culture. The clustering of the T cell clones (P1 gate), is clearly distinguishable from debris and feeder's dead cells.

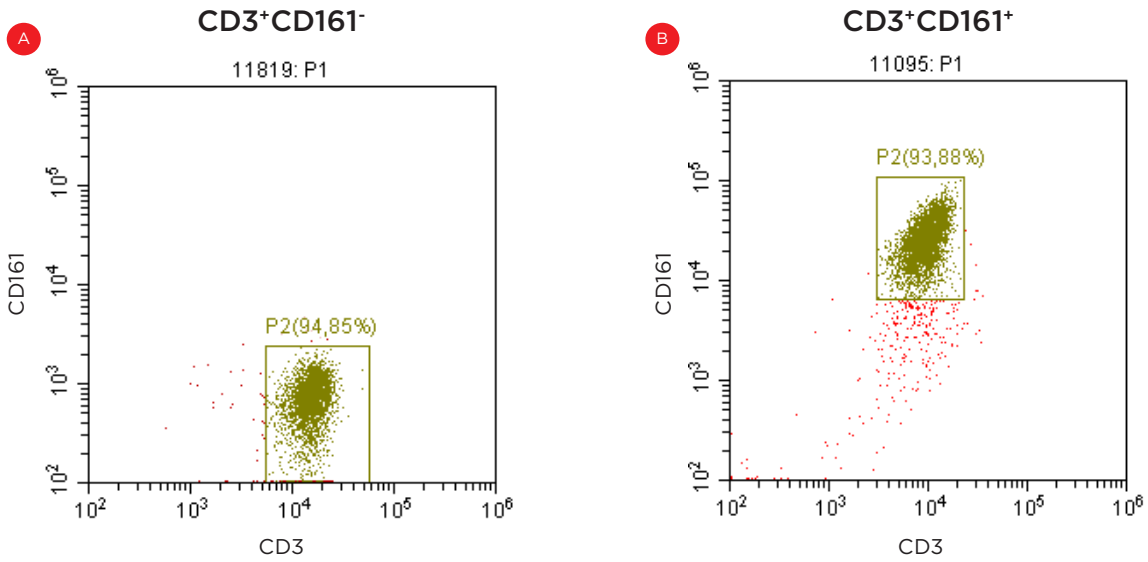

Figure 3 Preservation of T cell clone phenotypes in culture. Cells grown in culture following sorting and limiting dilution steps were analysed for their surface expression. 94.85% of sorted CD3⁺CD161⁻ population remained CD3⁺CD161⁻, while 93.88% of sorted CD3⁺CD161⁺ clones remained CD3⁺CD161⁺ as depicted the P2 gate in the left and right plots respectively.

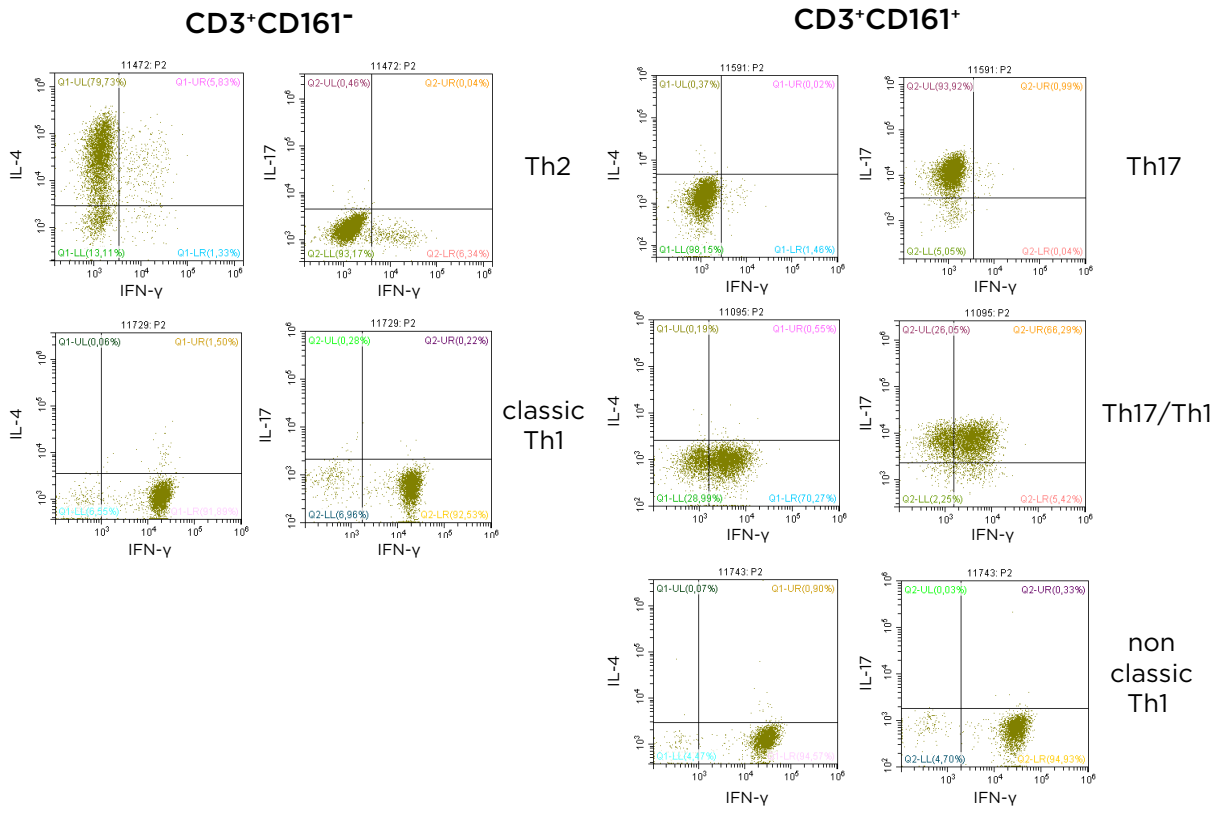

Figure 4 Evaluation of Cytokine production in each T cell clone. Each of the CD3⁺CD161⁺ or CD3⁺CD161⁻ clones were evaluated for IFN-γ, IL-4, and IL-17 cytokine production. The CD3⁺CD161⁺ clones showed three different cytokine production profiles: IL-17 only (Th17), IL-17+IFN-γ+ (Th17/Th1), and IFN-γ+ only (non-classic Th1). For all profiles in CD3⁺CD161⁺ T cell clones, IL-4 is not produced. The CD3⁺CD161⁻ clones showed two different cytokine production profiles: IL-4 only (Th2) and IFN-γ only (classic Th1). In these profiles, IL-17 is not produced.

Results

Based on the procedure described above, PBMC derived-CD4+ T cell clones were isolated based on CD3/CD161 phenotype and subsequently evaluated for their functional profiles. Each T cell clone was plated and grown in 96-well plates. Aliquots of these cells were transferred to fresh 96-well plates, stained, and analysed on the CytoFLEX S Flow Cytometer with a plate loader module. Each T cell clone was clearly distinguished from debris and dead cells in FSC vs SSC dot plot (figure 2), then each T-Cell clone was grown and analysed for its CD3/CD161 expression. 94.85% of sorted $CD3^+CD161^-$ population remained $CD3^+CD161^-$, while 93.88% of sorted $CD3^+CD161^+$ clones remained $CD3^+CD161^+$ as depicted in figure 3.

These cultured T cell clones were further subjected to stimulation with PMA and Ionomycin. Brefeldin-A was added two hours following stimulation to prevent cytokine secretion. The cells were then analysed in a plate format using the CytoFLEX S Flow Cytometer for intracellular IFN-γ, IL-4, and IL-17 production. $CD3^+CD161^+$ clones showed three distinct profiles: IL-17 positive only (Th17), IL-17+IFN-γ + (Th17/Th1), and IFN-γ+ only (non-classic Th1). For all profiles in $CD3^+CD161^+$ T-Cell clones, IL-4 is not produced. The $CD3^+CD161^-$ clones showed two different cytokine production profiles: IL-4 only (Th2) and IFN-γ only (classic Th1). In these profiles, IL-17 is not produced.

In summary, we show that $CD3^+$ T cell clones can differentiate into five T-helper subsets based on their CD161 and cytokine expression levels: $CD3^+CD161^+$ can be differentiated into three phenotypes, while $CD3^+CD161^-$ can be differentiated into two other phenotypes. These results confirm our previous findings that Th17 cells are contained within $CD3^+CD161^+$ T-Cell fraction.

Conclusions

In conclusion this method allows an easy and clear characterization of human CD4+ T cells clones when using the CytoFLEX S Plate Loader. In fact, a great number of PBMC derived-CD4+ T cell clones could be analysed for their phenotypical and functional profile because of the rapid sample acquisition procedure performed with the instrument and of the accurate sample analysis based on the possibility to clearly define positive and negative cells, for the expression of specific surface markers in combination with intracellular cytokines.

References

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