

Use of Flow Cytometry for Detection of Apoptotic Cell Death in Th17 Cells [†]

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[†] Presented at the 2nd International Cell Death Research Congress, Izmir, Turkey, 1–4 November 2018.

Published: 6 December 2018

Abstract: Flow cytometry (FC) is a powerful and reliable system for cell death studies. It allows to study the molecular changes in both the surface and cytoplasmic part of the cells. Depending on the laser range of flow cytometry, it is possible to collect a large number of information about a single cell by combining multiple labeling strategies. With these assets, flow cytometry allows scientists to accelerate their research. Flow cytometry is also widely used in clinical studies. Therefore, we used this powerful tool to study human T helper 17 (Th17) cell apoptosis. Newly discovered Th17 cells are important players of immune response regulation. They are also involved in different types of pathologies including autoimmune diseases and cancer. There are intensive data in the literature about molecules that are involved in Th17 differentiation signaling networks, but the apoptotic and survival molecular mechanisms of this cells are not fully understood yet. Therefore, apoptosis of Th17 cells were measured by Flow cytometry. Healthy human subjects have been invited to study with the informed consent which is approved by the ethics committee. Human Peripheral Blood Mononuclear Cells (PBMCs) were isolated from peripheral blood of healthy volunteers. Phenotypically characterized and sorted naïve CD4⁺ T cells from PBMC were cultured under Th17 polarizing conditions. IL-17, IL-22 or CCR6 molecules were used to monitor Th17 cells. Apoptotic cell death of Th17 cells were measured by plasma membrane changes and DNA fragmentation. During differentiation stages, plasma membrane changes were monitored by Annexin V concomitantly with 7AAD. At day 7, there were Annexin V positive cells in Th17 cells. Apoptotic cell death occurs through sequential events including caspase activation and DNA fragmentation. DNA fragmentation data showed that Th17 cells were not apoptotic compared to negative control cultures. This finding suggested that survival molecules of Th17 cells interferes with this apoptotic process.

Keywords: Th17 cells; apoptosis and flow cytometry

1. Introduction

Flow cytometry is an important technology that measures the size and content properties of cells and particles and is used in many fields. In addition, this technology allows the use of fluorescent biochemical markers to look at various cellular properties, such as expressions of proteins, metabolic activities or DNA content. In flow cytometry devices, the cells and other particles in the liquid suspension, whose dimensions are between 1 μm and 25 μm , can be analyzed. With flow cytometry analysis methods, it is possible to show the measured parameter results on a cell or particle. At the same time, the assay methods are used to establish a connection between biomolecules, cell size, intracellular structure or combination of these parameters, identified by fluorescent markers, and allows the identification of subpopulations in the sample [1]. Modern flow cytometers are typically equipped with two or more lasers and are capable of measuring 10,000 or more particles per second with six or more detectors.

In living cells with flow cytometry, many biomolecules or cellular characteristics can be measured simultaneously, which further contributes to the complete investigation of the effect of more chemical or biological agents on cellular functions. There are different strategies for collecting high-throughput biological data from a cell [1]. This strategy may include a combination of fluorescence receptor binding readings and cell number and cell viability indicator in a simple example in flow cytometry. In a more complex example, the evaluation of binding events between multiple targets on individual cells and each binding event can be identified by fluorescent markers of different colors. For instance, up to 50 separate parameters are analyzed in a single sample using various cell surface markers and intracellular cytokine labels. Another multiple data collection approach is to identify each population in a sample by pre-labeling them with fluorescent markers of different colors (barcoding) and then merging them into a single sample. Barcoding aims to label individual cells in multiple populations, combine them and then analyze each population using software.

Cell death or apoptosis can be characterized by various molecular and morphological changes. One of the earliest events in the apoptotic cascade is changes in the plasma membrane. Phosphatidylserine (PS), which is found on the inner surface of the cell membrane in the cells where apoptosis is induced, is transferred to the outer surface of the plasma membrane while maintaining the integrity of the membrane. In the advanced stages of apoptosis, plasma membrane integrity is lost. Phosphatidylserine (PS) on the outer surface of the plasma membrane can be used to monitor apoptosis [2]. Another molecular feature of apoptosis is the activation of caspases, which are inactive enzymes that must be activated during apoptosis. For example, CD95/Fas is involved in the apoptotic pathway as a cytosolic protein, caspase-8, which exhibits homology to the FADD (Fas-associated death domain) involved in the cell death pathway. The N-terminal region of caspase-8 contains an amino acid sequence called the death site, which facilitates direct caspase-8-FADD interaction. Thus, it is important to demonstrate that Caspase-8 is activated in receptor-mediated apoptosis [3]. One of the effector caspases activated during late apoptosis is caspase-3. Active caspase-3 induces a series of events that ultimately kill cells [3]. DNA fragmentation is also a sign of apoptosis. Therefore, we used this powerful technology to study human T helper 17 (Th17) cell apoptosis.

2. Materials and Methods

Ethics approval for this study was obtained from the Noninvasive Ethics Committee of Dokuz Eylül University, İzmir, Turkey. The venous blood was drawn from the healthy volunteers by health professionals at the Dokuz Eylül University Blood Bank. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation method [4]. Naive CD4⁺ T cells were sorted by magnetic sorting to have only purified-naive CD45RA⁺CD4⁺ T cells. Cells were stimulated with CD3, CD28, IL-1-beta, IL-6 and TGF-beta antibodies to differentiate naive CD4⁺ T cells into Th17 cells for up to seven days. IL-4 and IFN-gamma antibodies were used to block Th1 and Th2 polarization. Medium alone is used as negative control. Th17 cells were identified with RORC and CCR6 expression. Detection of apoptotic cells were carried out by Annexin V/7AAD labeling [5] and labeled cells were analyzed by Flow cytometry. DNA fragmentation in Th17 cells were detected by APO-DIRECT kit. Naive CD4⁺ T cells were cultured 7 days under Th17 polarization conditions. At the end of the cells culture, cells were labeled according to kit protocol. Apo-direct is a single-step staining method for labeling DNA breaks with FITC-dUTP to detect apoptotic cells by flow cytometry. In this method, exogenous TdT catalyzes a template-independent addition of brominated deoxyuridine triphosphates (Br-dUTP) to the 3'-hydroxyl (OH) termini of double- and single-stranded DNA. Then cells were stained with a FITC-labeled anti-BrdU mAb. These sites are identified by flow cytometry. Therefore, this method often referred to as "end-labeling" or "TUNEL" (terminal deoxynucleotidyltransferase dUTP nick end labeling). Camptothecin-induced human lymphoma cell line was used as positive control.

3. Results

One of the earliest events in the apoptotic cell death is changes in the plasma membrane. It can be detected by phosphatidylserine (PS) exposure on the cell surface by labeling cells with Annexin V

conjugated with fluorescence. During the later stages of apoptosis, plasma membrane integrity is lost. This can be monitored by 7-AAD labeling. Use of Annexin V concomitantly with 7AAD allows the identification of apoptotic cells by Flow cytometry. At day 7, there were Annexin V positive cells in Th17 cells. Apoptotic cell death occurs through sequential events including caspase activation and DNA fragmentation. One of the effector caspases that is activated during the apoptosis is the caspase-3. Proceeding towards to the later stages of the apoptosis, DNA fragmentation occurs. Both caspases and DNA fragmentation can be measured by Flow cytometry. DNA fragmentation data showed that Th17 cells were not apoptotic compared to negative control culture.

4. Discussion

Collected data from our experiments indicated that Th17 cells display plasma membrane changes, but there was no detectable DNA fragmentation. This finding suggested that survival molecules of Th17 cells interferes with this apoptotic process. We will continue to study Th17 cell apoptosis to understand molecules of different pathways and their interactions with each other.

Acknowledgments: This work was supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK) (Grant number: 215Z127 to Ayten Nalbant). The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

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