BioResearch

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Validation of Th17 Cell Differentiation from Peripheral Blood CD4+ T Cells Through Assessment of mRNA Expression and Cytokine Secretion Using Microplate Reading and Cellular Imaging

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Introduction

CD4+ T cells signal and regulate an immune response to pathogens after interacting with an antigen-MHC (major histocompatibility) complex. Depending upon the specific cytokine signals, transcription factors, and epigenetic modifications, the cells differentiate into distinct phenotypic and functional effector cells, collectively referred to as T helper cells (Th). One such differentiated T cell subset are the pro-inflammatory T helper 17 (Th17) cells. These cells can be beneficial to the host during infection, as they amplify ongoing inflammation by inducing expression of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α).

The potential of Th17 cells for longevity, self-renewal and durable memory could contribute to autoimmunity. Th17 cells are well known for their association with multiple inflammatory and autoimmune disorders, including the common ones like Psoriasis, Rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis. Th17 cells are uniquely characterized by production of the pro-inflammatory cytokine, IL-17. Research has shown that targeting the IL-17 pathway has attenuated disease severity in preclinical models of autoimmune diseases, which has caused a growing interest in their use as a potential therapeutic target.

In addition, Th17 cells are of interest in the growing field of cancer immunotherapy. They have been shown to have both anti-tumor as well as pro-tumorigenic activity, dependent on the cancer milieu. Recent research has focused on the stem-cell-like properties of Th17 cells in the context of their potential ability to enable long-term responses to cellular immunotherapies. Better understanding of the intricacies of development and impact of Th17 cells in tumor immunity is warranted. One hurdle is currently the ability to robustly produce Th17 cells for studies.

T cells, isolated from peripheral blood mononuclear cells and selected for CD4, are traditionally differentiated into Th17 cells using a cocktail of specific antibodies and cytokines. The procedure to create and confirm this differentiation can be labor- and time-intensive. Here, we demonstrate a validated, robust method to differentiate peripheral blood CD4+ T cells into functional Th17 cells using a bead-based activation technology. A novel cell imaging multi-mode reader can be used to image phenotypic differences between cells exposed to the antibody/cytokine cocktail and negative control cells as differentiation proceeds. Creation of fully functioning Th17 cells was then confirmed by assessing IL-17F mRNA levels using a fluorescence RNA in situ hybridization (RNA FiSH) assay, in addition to IL-17 secretion using a homogeneous, bead-based immunoassay technology. The reader previously described was able to perform all brightfield and fluorescence imaging steps, as well as laser-based excitation for the secretion immunoassay. The combination provides a comprehensive solution for the creation and validation of this important class of helper CD4+ T cells.

BioTek Instrumentation

Cytation™ 5 Cell Imaging Multi-mode Reader

Cytation™ 5 is a modular, multi-mode microplate reader that combines automated digital microscopy and microplate detection. Cytation™ 5 includes filter- and monochromator-based microplate reading, and offers laser-based excitation for Alpha assays. The microscopy module provides up to 60× magnification in fluorescence, brightfield, H&E and phase contrast. With special emphasis on live-cell assays, Cytation™ 5 features temperature control to 65°F (37°C ± 0.2°C), CO_2/O_2 gas control and dual injectors for kinetic assays. Shaking and Gen5 software are also standard. The instrument was used for image-based phenotypic differentiation monitoring, in addition to microplate reader- and image-based validation of Th17 cell creation.

Materials

Miltenyi Th17 Cell Activation/Expansion Components

The T Cell Activation/Expansion Kit was developed to activate and expand human T cells. The base kit consists of Anti-biotin MACSiBead Particles and biotinylated antibodies against human CD2, CD3, and CD28. The optimized protocol for Th17 cell differentiation recommends the following components from Miltenyi Biotec Inc. (San Diego, CA): T Cell Activation/Expansion Kit, Human (Cat. No. 130-091-441), TexMACS Medium, Research Grade (Cat. No. 130-097-196), Human IL-1β, Premium Grade (Cat. No. 130-093-897), Human IL-6, Premium Grade (Cat. No. 130-095-352), Human IL-23, Research Grade (Cat. No. 130-095-757), Human TGF-β1, Premium Grade (Cat. No. 130-095-067), anti-IFN-γ, Pure-Functional Grade, Human (Cat. No. 130-095-743), and anti-IL-4, Pure, Human (Cat. No. 130-108-049).

Microplates

Falcon® 24-well clear flat bottom not treated cell culture plate (Cat. No. 351147) and Falcon® 96-well clear round bottom not treated microplate (Cat. No. 351177) were from Corning Life Sciences (Corning, NY).

Cells

Peripheral Blood CD4+ T Cells (Cat. No. 2W-200) were from Lonza (Walkersville, MD).

Validation Assay Chemistries

AlphaLISA IL-17 Immunoassay Research Kit (Cat. No. AL219C) was from Perkin Elmer (Waltham, MA). QuantiGene® ViewRNA ISH Cell Assay Kit (Cat. No. QVC0001), ViewRNA® Probe Human IL17F (RUO) (Cat.O No. VA1-10122-01) and ViewRNA® Probe Human ACTB (RUO) (Cat. No. VA6-10506-01) were from Affymetrix Inc. (Santa Clara, CA).

Methods

Anti-biotin MACSiBead™ Particle Loading

100 µL each of CD2-Biotin, CD3-Biotin, and CD28-Biotin antibodies (100 µg/mL) were added to a 2 mL tube and mixed. 500 µL of anti-biotin MACSiBead particles (1×10^8 total particles) were then added to the antibody mix. Finally, 200 µL of PBS pH 7.2, supplemented with 0.5% human serum albumin and 2 mM EDTA, was added to the tube and mixed. The tube was placed at 4°C for two hours with gentle rotation. Following incubation the tube of loaded particles was kept at 4°C until the beginning of the differentiation procedure.

CD4+ Cell Differentiation Procedure

Cryopreserved CD4+ cells were thawed, added to TexMACS Medium, spun at 1000 RPM for 8 minutes, and then resuspended at a concentration of 1.11×10^6 cells/mL. An aliquot of MACSiBead particles sufficient for the experiment being performed was removed from the stored beads, added to 200 µL of medium, and spun at 300 × g for five minutes. The beads were then resuspended at a concentration of 5.0×10^5 beads per 100 µL of medium. Cells, beads, cytokines, and antibodies were then added together in the final concentrations, shown in the following table:

Differentiation Component	Concentration
CD4+ T cells	1.0×10^6/mL
Loaded MACSiBead particles	5.0×10^5/mL
IL-1β	20 ng/mL
IL-6	30 ng/mL
IL-23	30 ng/mL
TGF-β1	2.25 ng/mL
Anti-IFN-γ antibody	1 µg/mL
Anti-IL-4 antibody	2.5 µg/mL

Four 1 mL aliquots and four 200 µL aliquots were added to separate wells of 24-well and 96-well plate, respectively. Naïve CD4+ cells, in the absence of loaded particles, were also added to the same plates as a negative control. Plates were placed in a 37°C/5% CO_2 incubator for 7 days without medium exchange.

Validation of Th17 Cell Creation

Following the completion of the incubation period, medium aliquots were removed for cytokine secretion determination with the AlphaLISA IL-17 assay. Cells were then resuspended and transferred to an imaging plate to analyze mRNA expression using the QuantiGene® ViewRNA ISH Cell Assay.

Phenotypic Monitoring of Cell Differentiation

Image-based monitoring of Th17 cell differentiation was performed during the incubation period. 24-well and 96-well plates were placed into the Cytation™ 5, with the imaging chamber previously set to 37°C/5% CO_2. Brightfield images were captured to assess potential phenotypic differences taking place in the cells and culture.

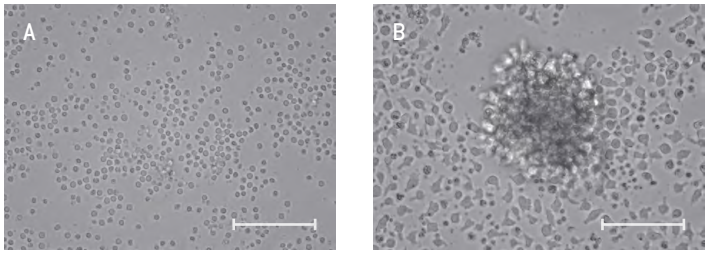

Figure 1
Brightfield images of differentiating CD4+ cells. Images captured from 24-well differentiation plate of **(A)** negative and **(B)** positive control wells using 20× objective. Scale bars: 100 µm.

Anti-biotin MACSiBead Particles loaded with biotinylated antibodies are used to mimic antigen-presenting cells and activate resting T cells. Cells are attracted to the bound antibodies, causing creation of the Th17 lineage in the presence of appropriate cytokines and additional antibodies. Therefore, a positive indication of differentiation is visible clumping of cells around the bead particles. Activated cells also demonstrate increased signs of proliferation. These phenotypes are seen in the images captured from 24-well positive control wells and 96-well positive control wells. Organization around bead particles and actively proliferating cells are visualized, which is absent from negative control wells.

Validation of hIL-17 Secretion

At the conclusion of the 7-day incubation period, two 5 µL aliquots of cell medium were removed from the four positive control wells in the 24-well and 96-well differentiation plates and transferred to a light gray 384-well AlphaPlate™ (Cat. No. 6005350), in addition to duplicate aliquots from negative control wells. A titration of hIL17 analyte was created and transferred to the same assay plate. The remaining steps of the AlphaLISA assay were then performed according to the vendor protocol.

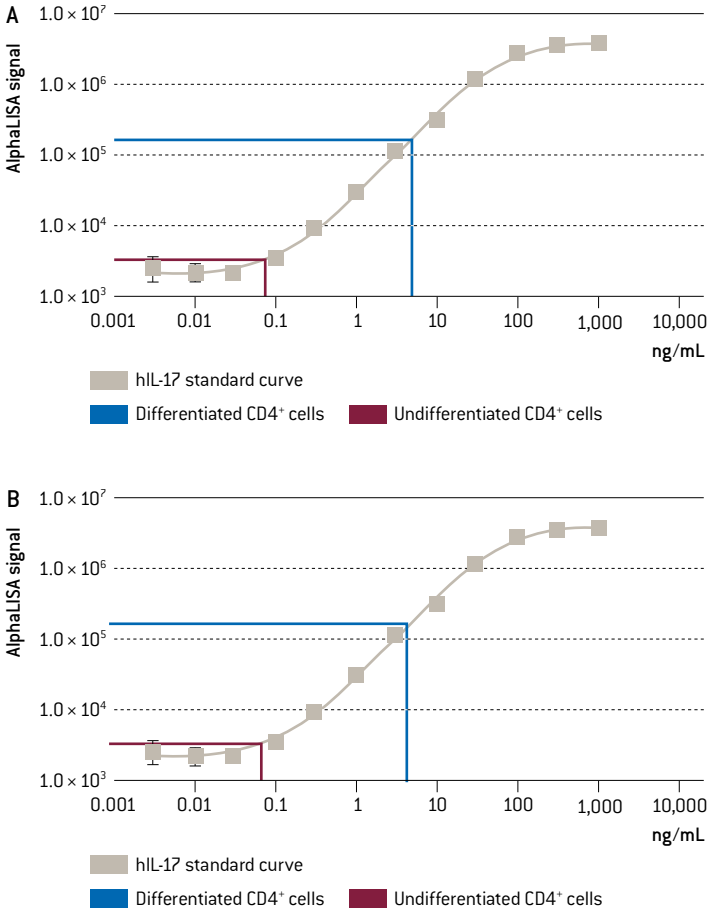

Figure 2
hIL-17 secretion determination. Average secreted cytokine concentrations from eight replicate measurements for **(A)** 24-well and **(B)** 96-well differentiation plates.

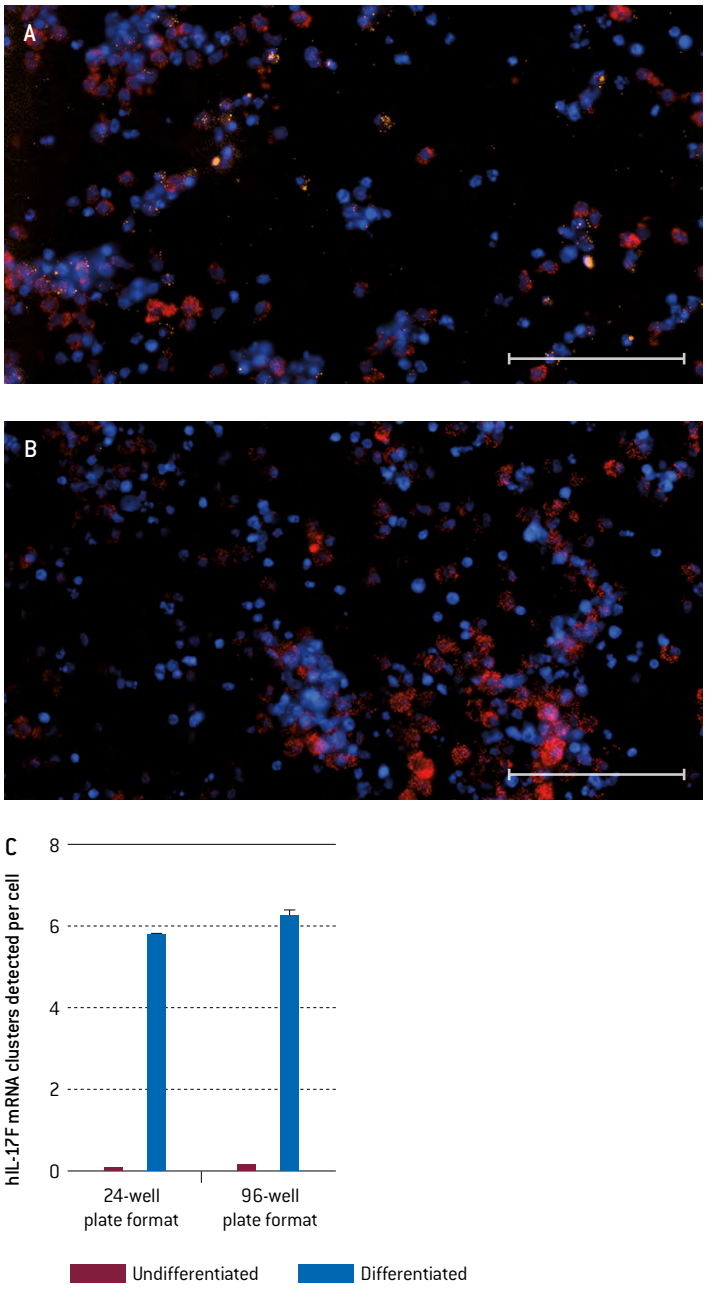

Figure 3
hIL-17F mRNA assessment. (A) Whole positive control image captured using a 20× objective from cells differentiated in 24-well format. **(B)** Whole negative control image from undifferentiated cells taken from 24-well plates. Red: ACTB mRNA expression imaged using Cy5 imaging channel; Orange: hIL-17F mRNA expression imaged using RFP imaging channel; Blue: DAPI labeled nuclei imaged using DAPI imaging channel. **(C)** Graph of differentiated and undifferentiated mRNA expression per imaged cell. Scale bars: 100 µm.

It was found that IL-17 supernatant concentrations were 4.8 ± 0.6 ng/mL in 96-well plates and 4.5 ± 0.7 ng/mL in 24-well plates. These concentrations represent a greater than 50-fold increase in IL-17 relative to negative controls. This validates that the bead-based differentiation process activates CD4+ cells into IL-17 secreting cells.

ISH-Based Analysis of IL-17F mRNA Expression

Validation of Th17 cell creation was also performed at the RNA level through incorporation of a fluorescence RNA *in situ* hybridization technique. Cells were removed from the wells of the 24-well and 96-well differentiation plates, fixed, and added to a poly-L-lysine imaging plate. The plate was baked at 50°C for 30 minutes to dry the cells and increase adherence to the plate. The remainder of the hybridization procedure was then carried out. Fluorescence imaging was then completed to ascertain levels of hIL-17F mRNA expression.

Images from wells containing differentiated cells exhibit a higher level of visible hIL-17F mRNA expression compared to wells containing undifferentiated cells (Figure 3 A–C). This is further confirmed through cellular analysis performed on each test well, where a greater than 50-fold increase in mRNA expression is seen from positive control wells.

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Conclusions

1. The bead-based protocol using MACSiBead particles is able to create activated Th17 immune cells suitable for downstream applications.
2. Cryopreserved CD4+ cells from Lonza simplify differentiation by removing isolation and propagation steps from the final procedure.
3. The AlphaLISA IL-17 assay provides an easy-to-use, responsive method to determine analyte secretion from positively differentiated cells.
4. ViewRNA fluorescent ISH cell assays allow for sensitive analyte mRNA expression detection.
5. The Cytation™ 5 Cell Imaging Multi-mode Reader provides laser excitation, in addition to PMT and CCD-based detection to enable monitoring and validation of differentiation with a single instrument.
6. The combination of differentiation procedure, cells, assessment methods and instrumentation provide a simplified, robust process for the creation and validation of activated immune cells.

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