CD4+ T cells signal and regulate an immune response to pathogens after interacting with an antigen-MHC (major histocompatibility complex). This activation causes the cells to differentiate into distinct phenotypic and functional effectors, collectively referred to as T helper cells (Th), depending on specific cytokine signaling and transcription factors and epigenetic modifications. One such differentiated T cell subset are the pro-inflammatory Th1 helper (Th1) cells. These cells can be beneficial to the host during infection, as they amplify ongoing inflammation by inducing expression of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). However, uncontrolled activation of Th1 cells is associated with multiple inflammatory and autoimmune disorders including arthritis, primary Sjögren’s syndrome (pSS), multiple sclerosis (MS), and cancer. Naïve CD4+ T cells that are stimulated into Th1 differentiation 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.

The process of attaining viable Th17 cells typically includes multiple steps. CD4+ T cells are isolated from peripheral blood mononuclear cells (PBMCs) and further selected against CD4. Purified cells are then differentiated into Th17 cells using a cocktail of specific antibodies and cytokines. The entire procedure to create and perform this differentiation can be labor and time intensive.

Here we demonstrate a validated, robust method to differentiate cryopreserved peripheral blood CD4+ T cells into functional Th17 cells using a bead-based activation technology. A novel cell imaging multi-mode reader is used to monitor phenotypic differences between cells exposed to the antibody/cytokine cocktail and negative or positive control cells as differentiation proceeds. Creation of fully functioning Th17 cells was then confirmed by assessing IL-17 mRNA levels using a fluorescence RNA in situ hybridization (FISH) assay. In addition, to IL-17 secretion using a homogenous, bead-based immunoassay technology. The reader previously described performed all brightfield and fluorescence imaging steps, as well as laser-based excitation for the detection of mRNA. The combination provides a comprehensive solution for the creation and validation of this important class of helper CD4+ T cells.

**Materials**

- Miltenyi Th17 Cell Activation/Expansion Components: The Th1 Cell Activation/Expansion Kit was developed to activate and expand human T cells. The kit consists of Anti-Biotin MACS®Bead® Particles and biotinylated antibodies against human CD2, CD3, and CD28. The optimized protocol for Th17 cell differentiation consists of the following components which were purchased from Miltenyi Biotec, Inc., San Diego, CA. Th1 Cell Activation/Expansion Kit, Human (Catalog No. 130-091-841), TexMACS™ Medium, Research Grade (Catalog No. 130-097-196), Human IL-1β, Premium Grade (Catalog No. 130-095-871), Human IL-6, Premium Grade (Catalog No. 130-095-352), Human IL-23, Research Grade (Catalog No. 130-095-757), Human TGF-β1, Premium Grade (Catalog No. 130-095-067), anti-IFNγ, Pure-Functional Grade, Human (Catalog No. 130-095-743), and anti-IL-4, Pure, Human (Catalog No. 130-108-049).
- Microplates: Falcon® 24 well polystyrene flat bottom not treated cell culture plates (Catalog No. 351147) and Falcon 96 well clear round bottom not treated microplate (Catalog No. 351177) were donated by Corning Life Sciences (Corning, NY). AlphaPlate-384, Light Gray, untreated (Catalog No. 351147) and Falcon 96 well clear round bottom not treated microplate (Catalog No. 351177) were donated by Corning Life Sciences (Corning, NY). AlphaPlate-384, Light Gray, untreated (Catalog No. 351147) and Falcon 96 well clear round bottom not treated microplate (Catalog No. 351177) were donated by Corning Life Sciences (Corning, NY). AlphaPlate-384, Light Gray, untreated (Catalog No. 351147) and Falcon 96 well clear round bottom not treated microplate (Catalog No. 351177) were donated by Corning Life Sciences (Corning, NY). AlphaPlate-384, Light Gray, untreated (Catalog No. 351147) and Falcon 96 well clear round bottom not treated microplate (Catalog No. 351177) were donated by Corning Life Sciences (Corning, NY). AlphaPlate-384, Light Gray, untreated (Catalog No. 351147) and Falcon 96 well clear round bottom not treated microplate (Catalog No. 351177) were donated by Corning Life Sciences (Corning, NY).
- Cells: Peripheral Blood CD4+ T Cells (Catalog No. 2W-202) were purchased from Lonza Group Ltd. (Basel, Switzerland).
- Validation Assay Antibodies: AlphaSta® IL-17 Immunoassay Research Kit (Catalog No. ALU19C) was purchased from PerkinElmer, Inc., Waltham, MA.
- TCR1-val85Fv mouse IgG2a, kappa isotype control (Catalog No. 130-095-056), anti-IFNγ, Pure-Functional Grade, Human (Catalog No. 130-095-743), and anti-IL-4, Pure, Human (Catalog No. 130-108-049).

**Methods**

**CD4+ Cell Differentiation Procedure:** Cryopreserved CD4+ cells were thawed, added to TexMACS Medium, centrifuged at 1000 RPM for 8 minutes, and then resuspended at a concentration of 1.1x10^6 cells/mL of MACS®Bead® particles sufficient for the experiment removed from the stored beads, added to 200 µL of medium, and centrifuged at 200g for five minutes. The beads were then resuspended at a concentration of 5x10^6 beads per 100 µL of medium. Cells, beads, cytokines, and antibodies were then added together in the final concentrations below:

<table>
<thead>
<tr>
<th>Differentiation Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ Cells</td>
<td>1.0x10^6/mL</td>
</tr>
<tr>
<td>Loaded MACS®Beads Particles</td>
<td>5.0x10^6/mL</td>
</tr>
<tr>
<td>IL-17</td>
<td>20 ng/mL</td>
</tr>
<tr>
<td>IL-6</td>
<td>30 ng/mL</td>
</tr>
<tr>
<td>IL-23</td>
<td>30 ng/mL</td>
</tr>
<tr>
<td>Anti-IFNγ Antibody</td>
<td>2.25 mg/mL</td>
</tr>
<tr>
<td>Anti-IL-4 Antibody</td>
<td>2.25 mg/mL</td>
</tr>
</tbody>
</table>

Four 1 µl aliquots and four 200 µl aliquots were added to separate wells of a 24-well and 96-well plate, respectively. 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% CO2 incubator for 7 days without medium exchange.

**Validation of Th17 Cell Creation:** Following incubation, 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 of 24- and 96-well plates and plated into the Cytation 5, with the imaging chamber pre-set to 37 °C/5% CO2 incubator with medium exchange.

**Validation of hIL-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- and 96-well differentiation plates, fixed, and added to a poly-L-lysine imaging plate. The plate was incubated at 55 °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 assess levels of IL-17F mRNA expression.

**Conclusions**

1. The bead-based protocol using MACS®Bead® particles creates 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 AlphaSta® 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 downstream applications.
6. The combination of differentiation procedure, cell assessment methods and instrumentation provides a simplified, robust procedure for the creation and validation of activated immune cells.

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