Troubleshooting Calcium Flux in CD8 T cells

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Abstract

CD8 T cells play an important role in the adaptive immune system. They include cytotoxic T cells, which are activated after binding to major histocompatibility complex (MHC) class I protein to kill viral pathogens invading the immune system. Several signaling pathways induce functional gene transcription in CD8 T cells. My project is to develop and troubleshoot a signaling protocol for a novel CD8 T cell. To achieve this goal, we evaluated published literature to identify relevant methods and protocols, determined appropriate reagents to use in the protocols, and analyzed data via flow cytometry. We first stimulated cells with ionomycin to evaluate calcium flux as calcium changes are downstream of several signaling pathways. To assess calcium flux, we loaded cells with Indo-1 which is cleaved and forms a complex upon calcium binding. We also stimulated cells with anti-CD3 plus anti-IgG to stimulate T cells through the T cell receptor (TCR) and assessed calcium responses. When cells are stimulated with ionomycin the indo-1 levels fluctuate, but poor TCR responses were achieved. Overall, we found lower indo-1 responses than expected as for ionomycin and TCR stimulation, therefore, we need to access more protocols and optimize the reagents, such as testing at varying temperatures and indo-1 concentrations.

Background

CD8 T cells are known for their cytotoxic effector function. They require extracellular stimulatory signals, mediating by T cell receptor (TCR) complexes. TCR recognizes antigens on major histocompatibility complex (MHC) class I protein with the assistance of CD8 coreceptors. After binding to MHC class I, TCR:MHC-I interactions induce cytokines, chemokines, costimulatory molecules to drive the differentiation of activated T cells into specific T cell subtypes. This results in the generation of various types of T cells with different specialized functions. One function of differentiated CD8 T cells is secretion of cytotoxins that kill an infected or a damaged cell. We focus on a novel CD8 T cell subset known as CD8 follicular T cells that maintain cytotoxicity and acquire the ability to promote B cell responses. The signaling pathway utilized by this new CD8 subset are largely unknown. Many T cell signaling pathways utilize Ras-extracellular signal-related kinase (ERK), protein kinase C, inositol triphosphate (IP_3)-Ca^{2+}-nuclear factor of activated T cells (NFAT). We begin our studies by focusing on the calcium flux specifically at the CRAC channel. Indo-1 is a calcium indicator dye commonly used in flow cytometry because it shifts in emission frequency when calcium is excited at a single wavelength. This allows us to measure the changes in intracellular calcium concentration, providing an indirect indicator of T cell activation response. We explored the calcium flux assay using ionomycin and TCR stimulation and evaluating optimal temperature and concentration of each reagent. We also utilized various analysis strategies to troubleshoot the data.

Signaling Pathway

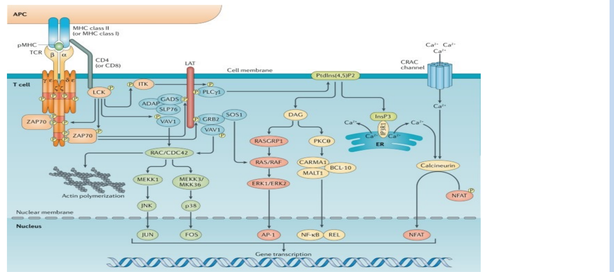

Figure 1. T cell receptor signaling pathway. Representation of the T cell receptor pathway. TCR requires extracellular stimulatory signals to recognize antigen on MHC class I protein with the assistance of CD8 coreceptors. The binding of TCR:MHC-I drives the differentiation of activated T cells into specific T cell subtypes, resulting in generation of various types of T cells into specific T cell subtypes. One function of differentiated CD8 T cells is secretion of cytotoxins that kill an infected or a damaged cell. The CRAC channel and ER of the T cell in the pathway is focused for calcium flux signaling in this experiment.

Calcium Dye Marker

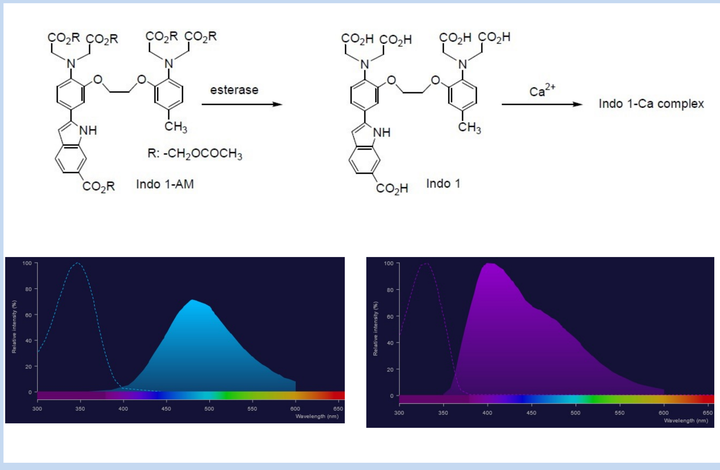

Figure 2. Indo-1-AM calcium dye. A chemical representation of calcium dye marker. It was used in the calcium flux assays to determine the emission frequency of calcium wavelength during the excitation stage when testing with flow cytometry. Esterase is a hydrolase enzyme that cleaves esters into an acid and an alcohol. This action allows the binding of calcium to Indo-1, forming an Indo-1-Ca complex. The CD8 T cells are rested for 30min at room temperature for the cleavage process. The purple region indicates the wavelength for Indo 1 Ca-bound and the blue region indicates the wavelength for Indo 1 Ca-free.

FACS Analysis of Ionomycin Stimulation

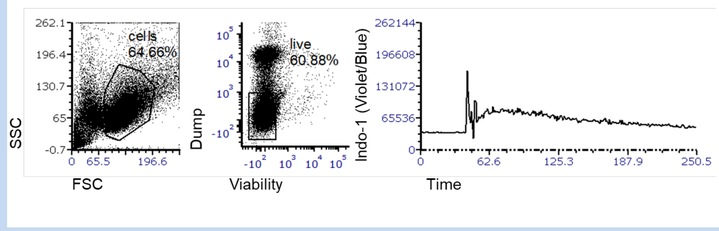

Figure 3. Ionomycin stimulation of live CD8 T cells. Gated of the cellular concentration of SSC, Dump, and FACS plot of Indo-1 versus time are shown. Leftmost diagram shows the WT lymph node live cells. Middle diagram shows viability, only gating live cells. FACS plot represents the Time on X axis and Indo-1 calcium dye on Y axis. 250ng/mL of ionomycin was used to stimulate the wild type of lymph node to see calcium flux when 1.5ug/mL of indo-1-AM concentration was used. The ratio of calcium bound to calcium free is reflected on the Indo-1 level. High Indo-1 level indicates high ratio of calcium bound.

FACS Analysis of TCR Stimulation

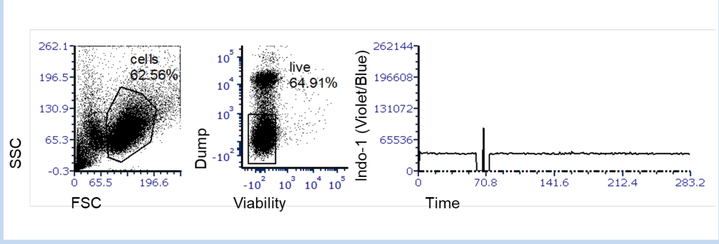

Figure 4. TCR stimulation of live CD8 T cells. Gated of the cellular concentration of SSC, Dump, and FACS plot of Indo-1 versus time are shown. Leftmost diagram shows the WT lymph node live cells. Middle diagram shows viability, only gating live cells. FACS plot represents the Time on X axis and Indo-1 calcium dye on Y axis. 20ug/mL of anti-CD3 and 50ug/mL of anti-IgG were used to stimulate the wild type of lymph node to see calcium flux when 1.5ug/mL of indo-1-AM concentration was used. The ratio of calcium bound to calcium free is reflected on the indo-1 level. Low Indo-1 level indicates high ratio of calcium free.

Conclusion

Figure 1 illustrates the signaling pathway of TCR that leads us to focus on the calcium flux at the CRAC channel and develops protocol by evaluating optimal temperature and concentration of each reagent. Figure 2 represents the chemical structure of Indo-1 calcium dye. It is crucial to understand the dye undergoes cleavage process and allows calcium molecules to bind and form complexes. This is essential in the experiment to measure the calcium emission frequency during excitation wavelength. Figure 3 and 4 shows the results of Ionomycin and TCR stimulation. By analyzing the diagram, the cells stimulated with ionomycin fluctuated the indo-1 levels, however, the cells fluctuated poorly with TCR stimulation. Therefore, more experiments must be performed, such as testing at varying temperatures and Indo-1 concentrations, to reach a definitive conclusion.

Reference

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