**ABSTRACT:**

Cytotoxic T lymphocytes (CTLs) are the principal cells of the acquired immune response for defense against viral infections and tumors. The accurate analysis of such a functional effector population is critical to the elucidation of correlates of protection for diseases involving cellular immunity. Once standardized and validated, these assays can be routinely used as surrogates of markers of efficacy in preventive and therapeutic strategies involving immune responses. Multiparametric flow cytometry has enabled the accurate identification and evaluation of targeted lymphocyte subpopulations. In the current study, the authors have evaluated the functional capacity of effector T cells using 5 color, 7 parameter flow cytometry by interrogating a combination of phenotypic and functional surfaces and intracellular markers. Peripheral blood mononuclear cells obtained from healthy donors were subjected to restricted polyclonal stimulation using Staphylococcus enterotoxin B or peptide specific stimulation for varying times ranging from 6-72 hrs. The T cell populations were analyzed using combinations of markers differentiating naïve, effector, memory, activated and proliferating subpopulations along with functional evaluation utilizing intracellular cytokine, granzyme B, and perforin expression as assessed by antibody-induced CD107 expression. Our findings indicate that there is a complex profile of effector T cells that varies with the donor and time point stimulated. The functional capacity of effector T cells is especially dependent on the “intrinsic” state of the donor PBMC population with granzyme B regulation being a striking feature of the response. Such profiles when correlated with disease outcome could enable the targeted identification of effector CTL subpopulations associated with therapeutic success thus enabling the development of “surrogate profiles” of efficacy.

**RESULTS:**

**FIGURE 1: Dual Cytokine Expression.** PBMC were cultured without CD107ab and with Biradex-A for 1 and 5 hours. Cells were prepared with the 5-color antibody combination: CD107ab-FITC/CD27-Pe/CD45RA-APC/CD8-PE/CD28-CIT. A generous lymphocyte scatter gate was used to create a CD8 gate for the CD28 vs. CD107ab vs. CD27 vs. CD45RA tomograms. The key shows how the events are sorted and lists the order of precedence (top has highest precedence).

**FIGURE 2: Activation and Cytotoxicity Expression Kinetics.** PBMC were cultured at 2x10^6 viable cells/ml, 100ul/tube (1x75 sterile Falcon) in a 37°C, 5% CO2 humidified incubator for 7.2 hours. Paired cultures: Non-stimulated and a 2ug/ml SEB. 10ul CD107ab per 100ul culture was added at the beginning of incubation period. Fixation, Lysis, and Permeabilization was performed according to the IntraPrep reagent package instruction. Staining Tubes (Antibodies were added at different times during the preparation method: culture, surface, intracellular. Antibodies are listed left-to-right below in fluorochrome order: FITC-PE-ECD-APC-P7T. The key shows how the events are sorted and lists the order of precedence (top has highest precedence).

**FIGURE 3: Memory/Naïve Cytotoxic Function.** PBMC were cultured for 1, 5, and 72 hours in the presence of CD107ab APC-BP and Biradex-A for 1, 5, or 12 hours respectively. Cells were prepared with the 5-color antibody combination: CD107ab-FITC/CD27-Pe/CD45RA-APC/CD8-PE/CD28-CIT. A generous lymphocyte scatter gate was used to create a CD8 gate for the CD107ab vs. IFNγ vs. GrnzB tomograms. The key shows how the events are sorted and lists the order of precedence (top has highest precedence).

**SUMMARY:**

- A simple model using a single assay, only 5 colors, and 3-5 tubes with automated analysis, is described here that assesses simultaneously the complex functional responses of lymphocytes.
- This model will be used to standardize and automate the method for routine use in larger scale testing.
- Cytotoxicity A is a “snapshot marker” of proliferation (only 5 & G2 phase) and it allows us to answer an opposite question than that answered by CFSE. With CFSE and Cytotoxicity A we can determine, of those that have killed, who is proliferating now?
- Discrete expression of IFNγ, effector and degranulation molecules seen here has been cited in the literature using multiple assay types to create a single result.
- Functionality diverse, polyclonal CTL, populations can be activated specifically and manifest virtually identical cytotoxic effector function, however, there are marked differences in proliferation and cytotoxic secretion. (Ju Lim)
- The method of immune activation is key to response pathways, and the high concentration of SEB used here can cause skewed responses, therefore, it’s important to develop this model further to evaluate different antigen classes at multiple doses and timepoints.

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