

Multiparametric Flow Cytometric Evaluation of Cytotoxic T Lymphocyte Populations

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RESULTS:

ABSTRACT:

Cytotoxic T lymphocytes (CTL) 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 surrogate markers of efficacy in preventative and therapeutic strategies involving immune response. 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 surface and intracellular markers. Peripheral blood mononuclear cells obtained from healthy donors were subjected to restricted polyclonal stimulation using Staphylococcal 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 naive, effector, memory, activated and proliferating subpopulations along with functional evaluation utilizing intracellular cytokine, granzyme B, perforin and degranulation as assessed by activation-induced CD107 expression. Our findings indicate that there is a complex profile of effector T cells that varies with the donor and time post stimulation. The functional capacity of effector T cells is especially dependent on the "intrinsic" state of the donor PBMC population with granzyme B up-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.

MATERIALS:

- PBMC isolated from whole blood collected in 8 ml sodium citrate CPT tubes (Becton Dickinson)
- RPMI-1640 pen/strep/glutamine with 10% FCS (in house)
- Staphylococcus Enterotoxin B (SEB) – List Biological Laboratories
- PBS (Beckman Coulter)
- Monoclonal Antibodies:
 - CD107a-APC and CD107b-APC (Southern Biotech)
 - IFN γ -FITC, IL2-PE, CD69-ECD, CD3-APC, CD8-PC7, CD45RA-ECD, CD27-PE, CD28-FITC, CD3-PC7, (Beckman Coulter catalog items used at recommended doses)
 - Custom conjugates of GRANZYME B-PE and CyclinA2-PE (Beckman Coulter Custom Design Service)
- IntraPrep Kit (Fixation, Lysis, and Permeabilization. Beckman Coulter)

METHODS:

- PBMC were isolated on Day 0 from whole blood according to the CPT tube manufacturer's instructions.
- Viability and cell count were measured using the Beckman Coulter ViCELL automated trypan blue instrument.
- PBMC were cultured at 2×10^6 viable cells/ml, 100ul/tube (12x75 sterile Falcon) in a 37°C, 5% CO $_2$, humidified incubator for 1-72 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 insert.
- Staining Tubes (Antibodies were added at different times during the preparation method: c=culture, s=surface, ic=intracellular. Antibodies are listed left-to-right below in fluorochrome order FITC-PE-ECD-APC-PC7):


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icIgG1/icIgG1/sIgG1/icIgG1/sCD3 [no CD107ab added to the culture]
icIFN $\gamma$ /icIL2/icCD69/sCD3/sCD8 [no CD107ab added to the culture]
icIFN $\gamma$ /icGranzymeB/sCD3/sCD107ab/sCD8
icIFN $\gamma$ /sCD27/sCD45RA/sCD107ab/sCD3
sCD28/icCyclinA2/sCD45RA/c107ab/sCD3 [only for later timepoints]
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- Flow cytometric analysis was performed using a Beckman Coulter Cyomics FC500 with 2 lasers, and took advantage of the totally automated instrument setup, compensation, and analysis.

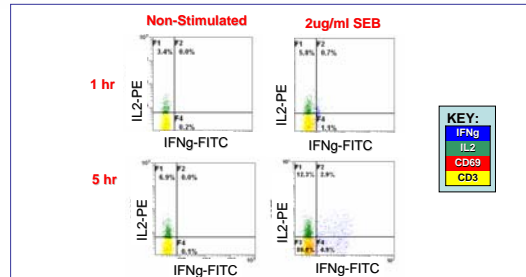


FIGURE 1: Dual Cytokine Expression.

PBMC were cultured without CD107ab and with Brefeldin-A for 1 and 5 hours. Cells were prepared with the 5-color antibody combination IFN γ -FITC/IL2-PE/CD69-ECD/CD3-APC/CD8-PC7. The CD3+CD69+ lymphocytes were mapped onto an IFN γ vs. IL2 scattergram. The key shows how the events are colored and lists the order of precedence (top has highest precedence).

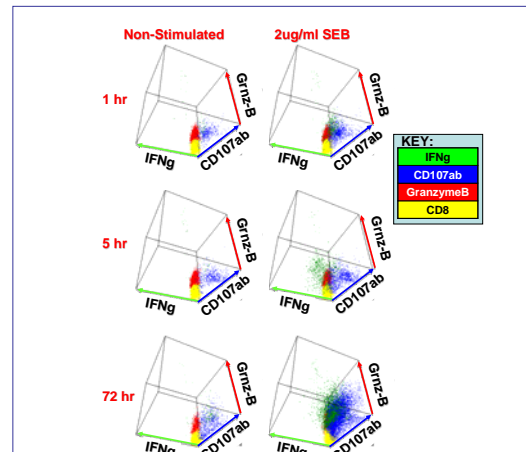


FIGURE 2: Activation and Cytotoxicity Expression Kinetics.

PBMC were cultured in the presence of CD107ab-APC for 1, 5, and 72 hours and Brefeldin-A for 1, 5, or 12 hours respectively. Cells were prepared with the 5-color antibody combination IFN γ -FITC/GranzymeB-PE/CD3-ECD/CD8-PC7. A general lymphocyte scatter gate was used to create a CD8 gate for the CD107ab vs. IFN γ vs. GrnB tomograms. The key shows the colored events and the order of precedence (top has highest precedence). Degranulation, effector molecule and cytokine expression are not coupled and the expression patterns change over time as expected.

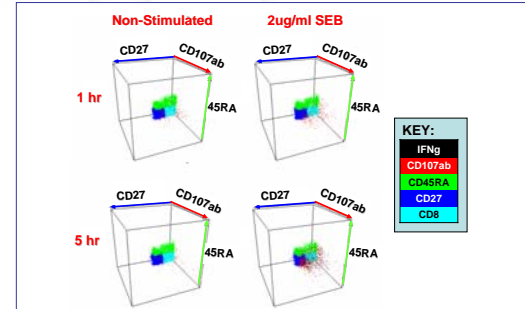


FIGURE 3: Memory/Naive Cytotoxic Functions.

PBMC were cultured for 1, 5, and 72 hours in the presence of CD107ab-APC and Brefeldin-A for 1, 5, or 12 hours respectively. Cells were prepared with the 5-color antibody combination IFN γ -FITC/CD27-PE/CD45RA-ECD/CD8-PC7. A general lymphocyte scatter gate was used to create a CD8 gate for the CD27 vs. CD45RA vs. CD107ab tomograms. The key shows how the events are colored and lists the order of precedence (top has highest precedence).

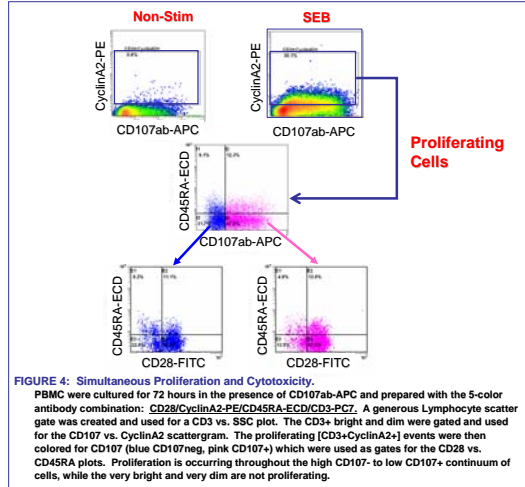


FIGURE 4: Simultaneous Proliferation and Cytotoxicity.

PBMC were cultured for 72 hours in the presence of CD107ab-APC and prepared with the 5-color antibody combination: CD28/CyclinA2-PE/CD45RA-ECD/CD3-PC7. A general Lymphocyte scatter gate was created and used for a CD3 vs. SSC plot. The CD3+ bright and dim were gated and used for the CD107 vs. CyclinA2 scattergram. The proliferating CD3+CyclinA2+ events were then colored for CD107 (blue CD107neg, pink CD107+) which were used as gates for the CD28 vs. CD45RA plots. Proliferation is occurring throughout the high CD107- to low CD107+ continuum of cells, while the very bright and very dim are not proliferating.

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 usage in larger scale testing.
- Cyclin-A2 is a "snapshot marker" of proliferation (only S & G2 phase) and it allows us to answer an opposite question than that answered by CFSE. With CD107ab and CyclinA2 we can determine, of those that have killed, who is proliferating now?
- Discordant expression of IFN γ , effector and degranulation molecules seen here has been cited in the literature using multiple assay types to create a single result.
 - Functionally diverse, polyclonal CTL populations can be activated specifically and manifest virtually identical cytotoxic effector function, however, there are marked differences in proliferation and cytokine secretion. (Ji 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.

References:

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