

Following Stimulation with Mitogens and Microbial Antigens

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Abstract

Introduction: For the diagnosis and management of immunodeficiencies, it is necessary to assess the status of T-lymphocyte immunity, antibody forming capacity, and innate immunity. Previous methods to assess T cell immunity have included detection of ATP or ³H thymidine incorporation after stimulation with a mitogen or antigen; however, these methods can suffer from a lack of specificity, a requirement for isotope use, and extended cell culture times.

Methods: We optimized and validated an intracellular cytokine assay by flow cytometry that measures the percent of T lymphocytes that are induced to express the CD69 activation marker as well as interferon-γ in response to antigens or mitogens. Informed consent was obtained from healthy donors. The assay involved stimulation of a whole blood culture overnight, and the addition of Brefeldin A. After staining of surface and intracellular markers (CD45, CD3, CD69, and IFNγ), the lymphocytes were analyzed with the use of flow cytometry, with 50,000 CD3+ cells acquired. The percent CD45+CD3+CD69+IFNγ+ in the CD3+ population was quantified, and the unstimulated control was used for positioning of gates. Stimulation of purified peripheral blood mononuclear cells (PBMCs) was compared to the whole blood stimulation assay.

Results: The validation process for the whole blood assay included measures of intra- and inter-assay precision, robustness, precision of the flow cytometry and analysis steps, and the development of a reference range with healthy adult donors. A majority of healthy donors responded to a mix of viral particle lysates as well as to a viral peptide mix with means of 0.84 and 0.17%, respectively and all or a great majority responded to Staphylococcus enterotoxin B (SEB) and mitogens (PHA and PWM). In assays with the PBMCs performed in parallel, the responses to the viral mix, the viral peptide mix, and PHA gave similar relative values to the whole blood assay.

Conclusions: This panel of assays will provide a measure of T-cell reactivity against several common infectious agents and to mitogens and a superantigen (SEB). This cell-mediated immunity assessment will have utility in the evaluation of 1) patients with a recurrent infection and a suspected immune deficiency and 2) immunosuppressed transplant patients.

Background

The response of the T cell compartment to stimulation by various antigens has been detected by different means, including by proliferation (³H thymidine incorporation, CFSE), cytokine production (Elispot, ex vivo stimulation and measurement of released cytokines), or by stimulation of activation markers (CD69, CD154), binding of Class I or II epitopes (tetramers, pentamers), or by combinations of intracellular cytokines and activation markers or tetramers by flow cytometry. Intracellular cytokine staining has been widely used to monitor presumed functional responses to vaccines in pre-clinical studies and clinical trials of vaccines, notably to HIV-1.

Materials and Methods

T cell Responses

Intra-cellular cytokine staining

Whole blood samples were co-stimulated with antibodies to human CD28 and CD49d and were cultured with a recall antigen mix “RAM” (VZV, CMV, and mumps virus), *Candida albicans*, Tetanus toxoid, a viral peptide mix “CEF” (CMV, EBV, influenza virus), pokeweed mitogen, phytohemagglutinin, Staphylococcal enterotoxin B (SEB), or serum-free AIM-V medium alone. After 2-3 hr at 37°C, Brefeldin A was added and the stimulation was continued at for an additional 16 hours. After EDTA treatment, RBC lysis, fixation, and permeabilization, cells were stained with anti-CD45-FITC, anti-CD3-PC5, anti-CD69-PE (all from Beckman Coulter) and anti-interferon gamma-PC7 (BD Biosciences). Cells were analysed on the Beckman Coulter FC500 flow cytometer, gating on side scatter and CD45, then CD3 vs. CD45, and finally on CD69 and IFNγ. A total of 50,000 CD3+ cells were acquired. The analyses were performed with the Beckman Coulter CXP software.

Intra- and inter-assay variability

These were tested by the performance of replicate cultures and flow assays by individual operators, and between operators on the same and different days. The variability of the flow analyses alone was also determined.

Reference ranges

These were determined for all of the antigens and mitogens with at least 20 healthy adult donors.

Results

The validation process for the whole blood assay included measures of intra- and inter-assay precision, robustness, precision of the flow cytometry and analysis steps, and the development of a reference range with healthy adult donors. A majority of healthy donors responded to a mix of viral particle lysates as well as to a viral peptide mix with means of 0.84 and 0.17%, respectively and all or a great majority responded to Staphylococcus enterotoxin B (SEB) and mitogens (PHA and PWM). Not all healthy donors will respond to all antigens; 7 of 24 and 8 of 20 reached the level of significance in their responses to *Candida* and Tetanus toxoid, respectively. All of the stimuli were shown by paired T-test to have results significantly different from the negative control except for *Candida* (P values all < 0.005 except for *Candida*, P=0.1035).

In assays with the PBMCs performed in parallel, the responses to the viral mix, the viral peptide mix, and PHA gave similar relative values to the whole blood assay. We are starting to compare values with the PBMC assay to those of the Immuknow assay on patient samples submitted for Immuknow testing.

T cell intracellular cytokine responses of healthy adult donors

Stimulus	Number of donors	Minimum (%)	Maximum (%)	Mean
AIM-V	24	0.008	0.030	0.0182
Candida	24	0.000	1.020	0.0894
CEF	20	0.010	0.621	0.1735
PHA	21	0.940	7.819	3.8177
PWM	21	0.029	4.013	1.0054
RAM	24	0.048	4.297	0.8395
SEB	22	0.088	13.152	4.1945
Tetanus	20	0.010	0.154	0.0513

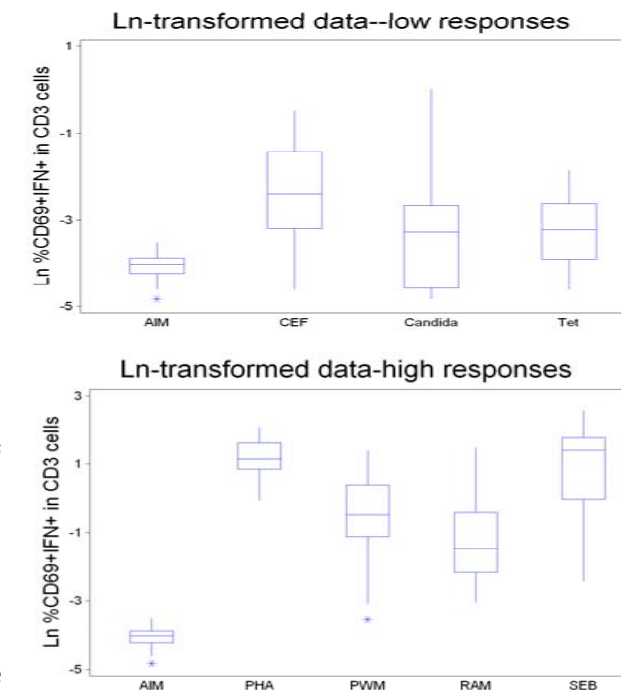


Fig. 1. Ranges of responses to various stimuli shown as natural log-transformed data. The middle line is the median, and the box encloses the middle two quartiles of the data. Note the different scale on the ordinates. A, low response antigens; B, high response antigens or mitogens.

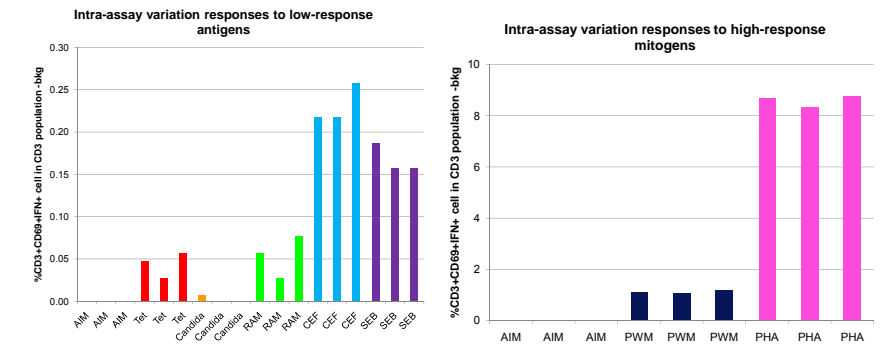


Fig. 2. Intra-assay variability was assessed by the performance on the same day of triplicate samples with analysis by a single operator. Greater variability was seen in the low response antigens. The % coefficient of variation was <20% on responses >2% CD3+CD69+IFN+ in the CD3+ population; for low responses, the values fell within the mean ± 3-fold of the mean. A, low response antigens; B, high response antigens or mitogens.

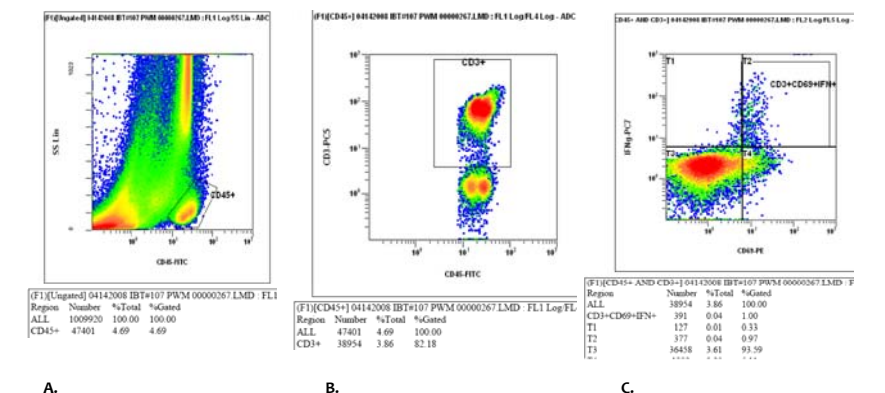


Figure 3. Intracellular Interferon-γ production (A), Lymphocytes were gated based on forward scatter and CD45 expression and (B), T cells were gated from the CD45+ population based on CD3 staining, including CD3+ downregulated cells. (C) The percent of activated T cells in the CD45+CD3+ population was gated based on CD69+ and IFN-γ+ staining for the various antigens and mitogens. Unstimulated (AIM-V medium alone) cultures were used to establish the gates.

Conclusions

This panel of assays provides a measure of T cell reactivity against several common infectious agents and to mitogens and a superantigen (SEB). The assessment of this T cell-mediated response will have utility in the evaluation of:

- patients with a recurrent infection and a suspected immune deficiency
- immunosuppressed transplant patients