ELISPOTs produced by CD8 and CD4 cells follow Log Normal size distribution

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INTRODUCTION: Each positive well in ELISPOT assays always contain spots of variable sizes that range from micrometers up to a millimeter in diameter. Therefore, when it comes to counting ELISPOTs it is critical to decide how to set the lower and the upper spot size thresholds to discriminate between non-specific background noise, spots produced by individual T cells, and spots formed by T cell clusters. If the spot sizes produced by T cells would not follow a certain statistical distribution, the size thresholds (gates) for counting would need to be set based on subjective judgment, thus leading to variability in spot counts when parameters are set by different investigators. In contrast, if the spot sizes follow a defined statistical distribution, precise predictions can be made as to the minimal and maximal spot sizes that belong to a given population. Hence, the gates could be set automatically ensuring counting results that are independent of subjective judgments.

METHODS: We studied the distributional properties of IFN-γ, IL-2, IL-4, IL-5 and IL-17 ELISPOTs elicited in PBMC of 24 healthy donors after stimulation of each donor cells with 32 individual viral peptides representing defined HLA Class I-restricted epitopes for CD8 cells (individual CEF peptides) and with inactivated CMV or EBV virions that recall CD4 cells.

RESULTS: A total of 334 CD8- and 80 CD4-positive recall responses have been analyzed so far. Invariably, for all donors, antigens, and cytokines, the spot size distributions followed a Log Normal function with significance levels over 5% according to the Kolmogorov-Smirnov test. Coefficient of Variation (CV) of mean Log spot sizes for different donor/antigen combinations in the same experiment was between 4% and 7% (Figures 2, 4, and 5) and much smaller then CV of log sizes for a single experimental distribution ~25% average (Figure 1) resulting in minimal variance in gate positions.

CONCLUSIONS: The data establish that ELISPOTs generated by CD8 or CD4 cells producing IFN-γ, IL-2, IL-4, IL-5, and IL-17 show size distributions that follow a standard known probability function, Log Normal. Therefore, size gates for counting such ELISPOTs can be set automatically by means of statistics permitting harmonization of results obtained by different investigators.

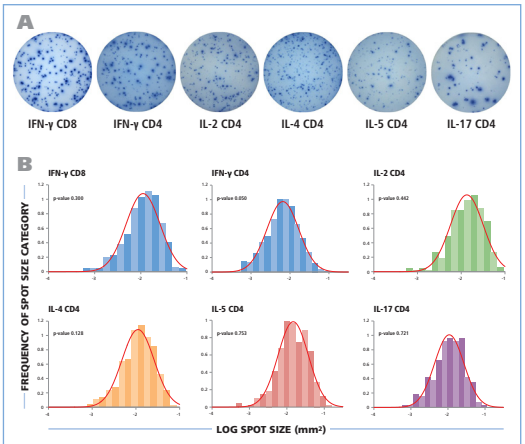

Figure 1: **ELISPOT assays produce a wide range of cytokine spot sizes by CD8 and CD4 cells. Spot size distributions for representative individual recall responses follow a Log Normal function.** (A) Representative images of ELISPOT wells are shown for cytokines produced by CD8 or CD4 cells, as specified. Due to the size range, subjective decisions on minimum spot sizes and clusters provide arbitrary spot counts. (B) The experimental size distributions of typical recall responses are shown as histograms for the specified cytokines with the theoretical Normal function indicated by the red lines. To test for the normality of the spot size distributions, the Kolmogorov-Smirnov goodness of fit test was used. The p-values are shown for reaching a target significance level of $\alpha = 0.05$.

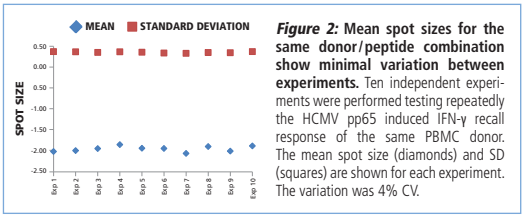

Figure 2: **Mean spot sizes for the same donor/peptide combination show minimal variation between experiments.** Ten independent experiments were performed testing repeatedly the HCMV pp65 induced IFN-γ recall response of the same PBMC donor. The mean spot size (diamonds) and SD (squares) are shown for each experiment. The variation was 4% CV.

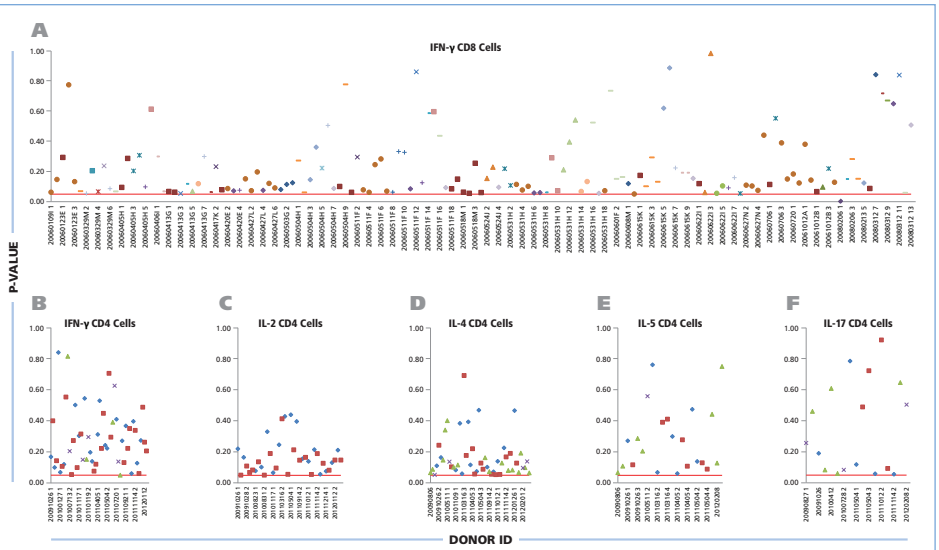

Figure 3: **Log Normal size distributions for all cytokine ELISPOTs produced CD8 and CD4 cells.** (A) IFN-γ ELISPOT size distributions were studied for a total of 334 positive recall responses induced by 32 individual CEF peptides in 24 donors. The Kolmogorov-Smirnov goodness of fit test was used to test the normality of spot size distribution for each individual positive recall response. The calculated p-value (Y axis) for the experimental distributions of the individual donors (X axis) for various peptides (different markers) are shown. The target significance level of 5% is shown by the red cutoff line. (B-F) Antigens that recall CD4 cell-inactivated CMV and EBV (shown as different markers) were tested detecting 80 positive donor/antigen combinations for IFN-γ, IL-2, IL-4, IL-5, and IL-17. As in A, the spot size distribution of each positive response was studied to fit a Log Normal distribution.

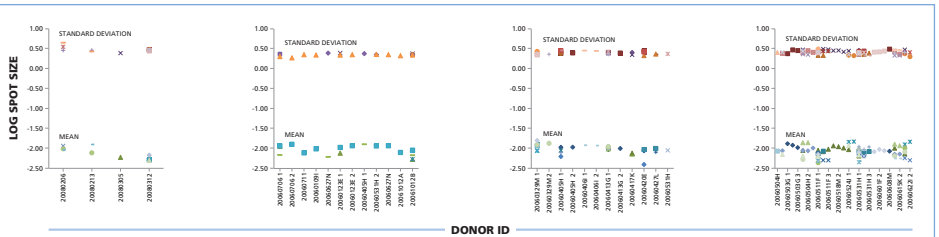

Figure 4: **IFN-γ ELISPOTS produced by CD8 cells show minimal size variation between donors.** Four experiments are shown, one in each panel. IFN-γ produced by CD8 cells in response to 32 individual CEF peptides (shown as different markers) was studied. The mean log spot size and SD for each positive response is plotted vs. the PBMC donor ID. Mean log spot size differences were in the 7.5% CV range.

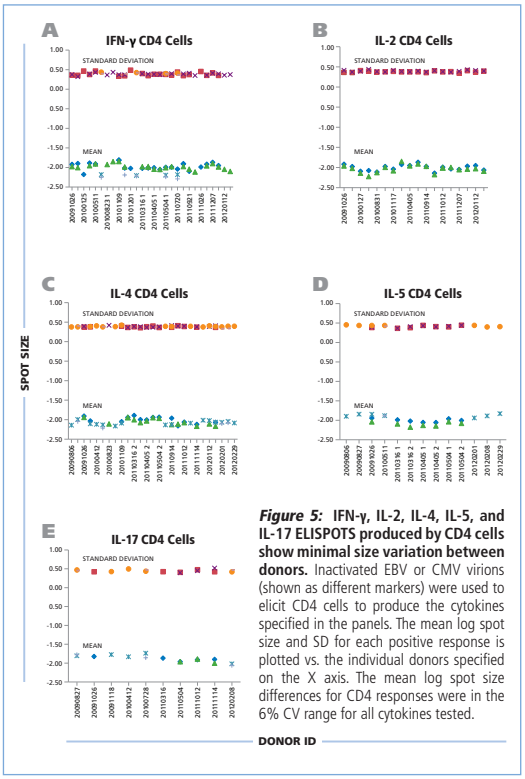

Figure 5: **IFN-γ, IL-2, IL-4, IL-5, and IL-17 ELISPOTS produced by CD4 cells show minimal size variation between donors.** Inactivated EBV or CMV virions (shown as different markers) were used to elicit CD4 cells to produce the cytokines specified in the panels. The mean log spot size and SD for each positive response is plotted vs. the individual donors specified on the X axis. The mean log spot size differences for CD4 responses were in the 6% CV range for all cytokines tested.