Characterization of the CMV-specific CD4 cell response

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INTRODUCTION: Most humans become infected with HCMV. Typically, the immune system controls the infection, but the virus persists and can reactivate in states of immunodeficiency. While the HCMV-specific CD8 cell and antibody response has been accessible to investigation, studies of the CD4 cell subsets have been limited by the low frequency of the antigen-specific CD4 cells in PBMC. In particular, there is a paucity of data on the Th2, Th17 and polyfunctional CD4 cell subclasses. Using ELISPOT that excels in low-frequency measurements, we have established the above parameters of CD4 cell immunity in a cohort of 40 healthy HCMV controllers, thereby providing reference values against which CD4 cell reactivity can be compared when the immune surveillance of HCMV fails.

METHODS: PBMC from 40 healthy human donors were studied. UV-inactivated HCMV virions were used as the antigen that recalls CD4 memory cells (Figure 2). The HCMV-induced ELISPOT count was established in 250,000 PBMC/well measuring the frequency of IFN-γ-, IL-2-, IL-4- and IL-17-producing cells, as well as those co-expressing IL-2 and IFN-γ in a double-color ImmunoSpot® assay. All cytokines were measured using the corresponding ImmunoSpot® Test Kits, and to assure low background, serum-free CTL-Test™ Medium was used. The spots were counted using an ImmunoSpot® S6 Core reader.

RESULTS: Studies of cytokine elicitation kinetics showed maxima at 24h for IFN-γ and IL-2, 48h for IL-4, and 72h for IL-17 (Figure 1). Cytokine recall responses were seen in 32 of 40 donors (80%). IFN-γ recall was seen in all of these positive donors, with the exception of 3 donors, in which IL-17 recall was detected in the absence of IFN-γ (Figure 4). While IL-4 recall occurred in 18 donors (45%), it was not seen in isolation. Seven of the donors produced IFN-γ in the absence of IL-2. For the donors that produced both IL-2 and IFN-γ, the frequency of cells that produced both IFN-γ and IL-2 was in the range of 7-45% of the IFN-γ producing cells (Figure 5).

CONCLUSIONS: **(1)** IFN-γ or IL-17 production in isolation or in combination were seen, suggesting that both Th1 and Th17 immunity – individually or jointly – is consistent with a controller status. **(2)** IL-4 production was not seen in isolation, suggesting that isolated Th2 immunity is not compatible with a controller status. **(3)** One-fourth of the IFN-γ-positive donors were IL-2 negative, and thus did not possess detectable numbers of polyfunctional CD4 cells. While polyfunctional CD4 cells could be detected in the majority of donors, they do not seem to be essential for a controller status.

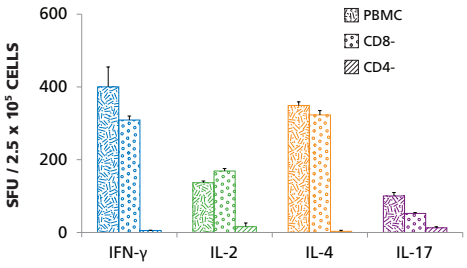

Figure 1: **IFN-γ, IL-2, IL-4, and IL-17 production by antigen-stimulated CD4 cells shows fundamentally different kinetics — simultaneous measurements are misleading.** PBMC were stimulated with inactivated HCMV virus (that stimulates CD4 memory cells, see Figure 2) in ELISPOT assays of 24, 48, and 72h duration **(A)**. Maximal number of IFN-γ spots were reached by 24h. At later time points the spots were overdeveloped **(B)**. IL-2 production also peaked at 24h. In contrast, IL-17 production barely started at 24h, and reached its peak at 72h. IL-4 showed an intermediate time course peaking at 48h. Therefore, measuring these cytokines at a single time point would be misleading. Characteristic spots for all the cytokines measured at the optimal time point are shown **(C)**.

Figure 2: **HCMV-induced cytokine spots are produced by CD4 cells.** PBMC, CD8 cell-depleted PBMC (still containing CD4 cells), and CD4 cell-depleted PBMC (still containing CD8 cells) were tested in IFN-γ, IL-2, IL-4, and IL-17 ELISPOT assays as specified at the optimal time point for each cytokine. The data showed that, unlike CD8 cell depletion, CD4 depletion abrogated the response. The results are consistent with the antigen presentation requirements of extracellular proteins that the inactivated virus represents. While ELISPOT assays inherently do not reveal whether CD4 or CD8 cells are secreting the cytokines, cell separation experiments are readily suited to provide this information.

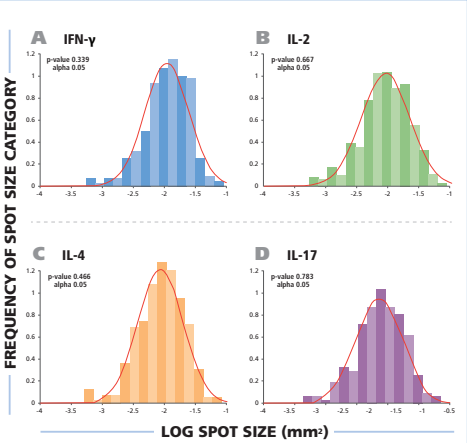

Figure 3: **HCMV-induced IFN-γ, IL-2, IL-4, and IL-17 spots produced by CD4 cells show a log-Normal distribution permitting automated counting based on statistical criteria.** While for each of the cytokines individual spot sizes varied considerably (see Figure 1C), analysis of the size distribution showed a log-Normal function for each cytokine (Panels A-D). The statistical analysis was done by the Kolmogorov-Smirnov test. The p-values are indicated for each cytokine. Because of the log-Normal distribution, statistics-based automated gating can be applied for counting of each of these cytokine spots.

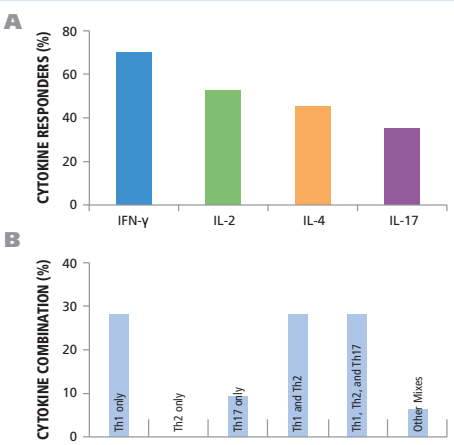

Figure 4: **Establishing the cytokine signature of CD4 cells responding to HCMV in 40 healthy human donors.** PBMC from 40 healthy human donors were tested for the HCMV-induced production of IFN-γ, IL-2, IL-4, and IL-17 at the optimal time point for each cytokine. (A) The percentage of individuals that generated a recall response for each of the cytokines is shown. (B) The percentage of individuals hosting the different CD4 effector cell lineages. Note: none of the donors responded with Th2 only and in 10% of the donors Th17 was present in the absence of Th1. The majority of donors showed a mixed response profile.

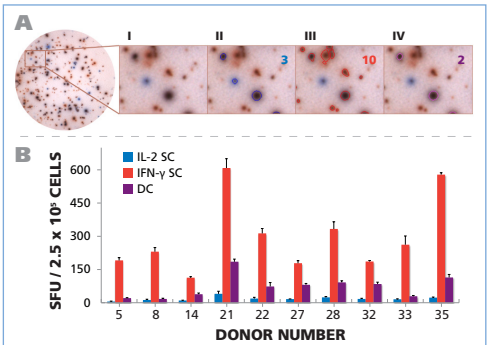

Figure 5: **Detection of polyfunctional CD4 cells by Dual-color ELISPOT.** An IL-2/IFN-γ double-color assay was performed on HCMV-stimulated PBMC of 10 donors that were found to be positive for both cytokines (Figure 4A). A representative well is shown **(A)**. Such wells were counted with the ImmunoSpot® Double-color Software that automatically discerns the color composition of the spots. The raw image is shown in A-I. Blue-colored spots detecting IL-2 are outlined by the software in A-II. Red-colored spots detecting IFN-γ are outlined by the software in A-III. Spots that were positive for both were identified and outlined in A-IV. **(B)** Double-positive, polyfunctional CD4 cells were present in all donors that were IFN-γ- and IL-2-positive, and constituted between 7% and 45% of the Th1 cells. (However, one-fourth of the IFN-γ-positive donors were IL-2-negative, and thus did not possess detectable numbers of polyfunctional CD4 cells, and 10% hosted Th17 cells only; see Figure 4.)