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Direct Visualization of Cytokine-Producing Recall Antigen-Specific CD4 Memory T Cells in Healthy Individuals and HIV Patients

Thomas Helms,*‡ Bernhard O. Boehm,† Robert J. Asaad,‡ R. P. Trezza,* Paul V. Lehmann,* and Magdalena Tary-Lehmann2*

We have used computer-assisted cytokine ELISA spot analysis to measure the frequencies, the type of cytokine, and the amount of cytokine produced by individual recall Ag-specific CD4 memory cells in freshly isolated blood. We studied the memory cells specific for tetanus toxoid and purified protein derivative in 18 healthy individuals and in 22 HIV-infected patients on highly active antiretroviral therapy (HAART). In healthy individuals, the frequency, cytokine signature, and cytokine production per cell of these memory cells were stable over time. Although it is presently unclear whether the maintenance of the memory T cell pool depends upon Ag persistence, cross-reactive Ag stimulation, or cytokine-driven bystander stimulations and expansions, our data strongly argue for a stable memory cell pool in healthy individuals. In HIV patients, however, the frequency of these memory cells was a function of the viral load. The decreased numbers of functional memory cells in patients with high viral loads might provide one mechanism behind the immunodeficient state. Although the cytokine output per cell was unaffected in most patients (20 of 24), in some patients (4 of 24) it was >100-fold reduced, which might provide an additional mechanism to account for the reduced immunocompetence of these patients. The ability to visualize directly and quantify the cytokine produced by the low frequency memory cells in freshly isolated blood that have been physiologically stimulated by Ag should aid comprehensive studies of the Ag-specific memory cell pool in vivo, in health and disease. The Journal of Immunology, 2000, 164: 3723–3732.

Immunity mediated by T cells can be comprehensively defined by three parameters: first, the clonal size (frequency) of the Ag-reactive memory cell pool; second, the type of cytokines that these T cells produce; and third, the amount of cytokine that the individual memory cells produce. Yet, measuring any of these parameters as they occur in vivo, in freshly isolated cellular material such as human blood, has been at or below the detection limits of current technology (1). Because of the \(10^{12}\) different T cell clones with unique receptors, T cells with specificity for any given Ag tend to occur at very low frequencies (<1/1000) even for clonally expanded memory cell populations and, hence, have been generally inaccessible to direct measurement.

The classic approach used to establish clonal sizes of Ag-specific T cells has been limiting dilution analysis (LDA). The results obtained by LDA, however, have recently become controversial when new flow cytometry-based technical developments (tetramer and/or intracytoplasmic cytokine staining) that can directly visualize Ag-specific T cells in the high frequency range (>1/1,000) yielded fundamentally different results. While, for example, LDA suggested precursor frequencies of virus-specific CD8 cells to be in the range of 1/4000–1/10,000 (2), tetramer staining, intracytoplasmic staining, and ELISPOT analysis, in confirmation of each other, showed that these frequencies can be as high as 10% of all CD8 cells in the acute phases of the infection (3–5). This discrepancy between the indirect LDA data on which much of our present understanding of T cell population kinetics in general is based and the results obtained by methods suited for directly measuring Ag-specific T cells raised renewed interest in the most basic parameters that define immune function and memory in vivo, the clonal sizes and cytokine effector functions of memory T cells in freshly isolated material. By permitting a new look at T cells (1), the recently gained ability to monitor memory cells directly should help to resolve many of the controversial issues about T cell function in vivo, issues at the very heart of our understanding of immunological protection and of deficiency states such as the one caused by HIV infection.

Without the ability to visualize memory T cells directly, it is still unknown whether they are generally long-lived or short-lived, whether they require Ag to persist, or whether their frequencies fluctuate as a result of cross-reactive stimulations and/or cytokine-driven bystander reactions (6, 7). Evidence for all of these conflicting scenarios has been generated in murine models. Particularly during acute viral infections like HIV, the massive expansions of virus Ag-reactive T cells could result in a compensatory decline in the number of memory cells specific for third-party Ags as a result of the homeostatic control that keeps the overall number of T cells in the body constant (8–10). The opposite is conceivable as well, that long-lived memory cells specific, e.g., for tetanus toxoid (TT) or...
purified protein derivative of *Mycobacterium tuberculosis* (PPD) expand clonally under the influence of bystander cytokine during such infections (11–13). Furthermore, to understand the various states of T cell immunodeficiency, it is critical to know whether they are the result of reduced numbers of Ag-specific memory cells (frequencies), impaired function of these cells (reduced cytokine output per cell), a change in memory cell function (Th1/Th2 switch), or possibly a combination of these (14). Cytokine ELIS-POT analysis (15) might provide a unique tool with which to address these questions.

Naive T cells do not produce type 1 or type 2 effector cytokines (such as IFN-γ or IL-5), and for Ag to induce them to differentiate into memory cells producing IFN-γ or IL-5 requires several days (16); therefore, when freshly isolated T cells contained within PBMC are challenged with an Ag and subjected to IFN-γ or IL-5 ELISPOT assays of 24–48 h duration, as we did, it should be only the memory cells that differentiated in vivo that produce the cytokine. Moreover, because the duration of such assays (24–48 h) is too brief for in vitro proliferation and/or differentiation to occur, the number of spots detected should reflect both the frequency of memory cells present in the cell isolate and the commitment to produce IFN-γ or IL-5 imprinted on them in vivo. Furthermore, with the cytokine being continuously captured around the secreting T cell for the entire duration of the assay, the size of the spots produced should reflect the total cytokine produced by the individual cell; hence, the assay directly measures the biological function of these cells. Should the above postulates hold, the cytokine ELISPOT assay would provide a powerful tool for studies that characterize memory T cells based on their frequencies in freshly isolated PBMC and the cytokine output of individual cells; this is what we set out to establish in the first set of experiments reported in this work.

We recently introduced technical improvements in ELISPOT analysis that include membranes that improve the signal-to-noise ratio (17) and computerized image analysis (18). We report in this work the first formal evidence that this modification of the assay is suited to characterize the frequency, cytokine signature, and cytokine output of individual memory cells specific for recall Ags. These advances enabled us to characterize, for the first time, the recall Ag-specific T cell memory cell pool in healthy individuals and HIV patients at the highest conceivable resolution and at the single cell level. This article is intended to establish the feasibility of monitoring Ag-specific CD4 memory in the low frequency range, not to provide a complete analysis of the range of responses exhibited by highly variable human population.

### Materials and Methods

**Subjects and sample collection**

Peripheral blood was obtained by venipuncture from 22 HIV-positive patients (14 women and 8 men; median age 37.2 years, range 23–66 years) from the Special Immunity Unit at the University Hospitals (Cleveland, OH). All patients were under HAART therapy at the time of testing. The CD4 counts ranged from 150 to 1070 cells/ml, as specified in Fig. 4C. Viral load was determined by PCR (Amplicor; Hoffmann-LaRoche, Nutley, NJ) and is shown in Fig. 4C. The patients were categorized according to the Centers for Disease Control and Prevention (CDC) classification: 14 belonged to group 1, 7 to group 2, and 1 to group 3; because there were no statistically significant differences in the data we obtained while testing their recall Ag-specific memory response, the data were pooled. The 19 healthy individuals that we tested (10 women, 9 men, between 22 and 45 years of age) were members of our laboratory and adjoining laboratories. PBMC were isolated from 10 to 30 ml of heparinized blood by standard Ficoll density-gradient centrifugation (using Isopaque-Robbins Scientific, Sunnyvale, CA; for T cell separation, 100 ml blood was processed). All studies were performed under the approval of the Institutional Review Board for Human Investigation at the University Hospitals of Cleveland.

### ELISPOT assays

ImmunoSpot plates (Cellular Technology, Cleveland, OH) were coated overnight at 4°C with capture Abs dissolved in PBS that were specific for either IFN-γ (mAb M700A-E, 6 µg/ml; from Endogen, Woburn, MA) or IL-5 (mAb TRFK5, 5 µg/ml; hybridoma was obtained from American Type Culture Collection, Manassas, VA, and the Ab was grown and purified in our laboratory). The plates were then blocked with BSA (10% v/v in PBS) for 1 h and washed three times with PBS. Cells were plated in complete RPMI medium (94% RPMI + 5% ABO serum + 1% t-glutamime). RPMI was from BioWhittaker (Walkersville, MD); ABO serum was from Gemini Bioproducts (Calmasas, CA) and was heat inactivated at 56°C for 30 min. When T cell lines were tested (see Fig. 1), the indicated numbers of T cells were plated per well along with a constant number of 300,000 autologous T cell-depleted APC (these were obtained by ELISPOT (4) or plate adherence). For the experiments shown, we used an IFN-γ-producing human-alloreactive, CD4+ T cell line (PAN) and an IL-5-producing hemagglutinin peptide-specific human CD4+ line. The APC were irradiated with 3000 rad of γ rays from a cobalt source. Similar autologous APC were used for testing purified T cell fractions (see Fig. 3): CD3+ and CD4+ cells were obtained by negative selection, passing PBMC through affinity columns Human T Cell Enrichment and Human CD4 Subset Column Kit (both from R&D Systems, Minneapolis, MN). The efficacy of enrichment was controlled by FACS analysis, staining with labeled anti-CD4, anti-CD8, and anti-CD3 Abs (all from PharMingen, San Diego, CA). The enrichment for the desired phenotype was between 87 and 94%. When unseparated PBMC were tested, 3 × 106 freshly isolated cells were plated. A constant concentration of the activation culture, Ags were added as specified in the figures. TT was from Connaught (Swiftwater, PA; Lot 1/100), PPD from Evans Medical (Langhurst, U.K.; 10 µg/ml), Staphylococcal Mite DF from Bayer (Elkhart, IN; 200 AU/ml), and PHA from Sigma (St. Louis, MO; 10 µg/ml). Control wells contained reservered PBMC with medium alone. After 24 h for IFN-γ assays, or after 48 h for IL-5 assays, the plates were washed and biotinylated detection Abs added, without substitution, for 12 h, at 4°C. The secondary Abs specific for IFN-γ were mAb M701 from Endogen (3 µg/ml) and for IL-5, mAb 18522D from PharMingen; they were in 10 g/L BSA/PBS with 0.5% Tween (Sigma). Subsequently, streptavidin-HRP (Dakopatts, Glostrup, Denmark; 1/2000 dilution) in PBS/BSA/Tween was washed and biotinylated detection Abs added for 2 h at room temperature. The spots were developed using AEC (Pierce Pharmaceuticals, Rockford, IL) solution: 10 mg/ml AEC dissolved in 1 ml N,N-dimethyl formamide (Fisher Scientific, Fine Lawn, NJ), of which 1 ml was freshly diluted into 30 ml of 0.1 M sodium-acetate buffer (pH 5). This solution was then filtered and mixed with 15 µl H2O2 to provide the final AEC development solution, of which 200 µl plated per well. The plates were developed for 10–20 min for IFN-γ assays, and 45–60 min for IL-5 assays; the reaction was stopped by rinsing with tap water when clear spots became visible macroscopically. The plates/membranes were air dried overnight before the plates were subjected to image analysis.

### Image analysis

We used a Series 1 ImmunoSpot Image Analyzer (Cellular Technology) specifically designed for the ELISPOT assay. Digitized images were analyzed for the presence of areas in which color density exceeds background by an amount set on the basis of the comparison of experimental wells (containing T cells, APC, and Ag) and control wells (containing T cells and APC only); see, e.g., Fig. 2, A vs B. After background and noise subtraction and separation of the areas formed by touching and partially overlapping spots, additional criteria of spot size and circularity are applied to gate out speckles and artifacts caused by spontaneous substrate precipitation and nonspecific Ab binding. Objects that do not meet these criteria are gated out from the total area used in analysis. The area size is then determined and areas recognized as spots and counted. The spot size distribution (see Fig. 2, D and E; Fig. 3, C and D; and Fig. 6A), a built-in function, is based on the array of spot sizes in a given well sorted according to distinct size categories, and these results were plotted in above figures.

Intracellular cytokine staining was performed as described (22). T cell lines (5 × 106/ml) were cultured with syngeneic irradiated, T cell-depleted PBMC at 5 × 105/ml in complete RPMI medium supplemented with 10% PHA (10 µg/ml) or their respective Ags for 6 days, washed, and then stained and labeled with monoclonal Abs (Becton Dickinson, San Jose, CA) was used; for staining of IL-5, FITC-labeled TRFK-5; and for CD3, peridinin chlorophyll protein-labeled anti-human CD3 Abs (Becton Dickinson) were used, and uncoated control mAbs were from Becton Dickinson. The samples were analyzed on a FACSScan flow cytometer (Becton Dickinson). As a protein transport inhibitor, we used Monensin or brefeldin A (PharMingen).

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responses were undetectable by intracytoplasmic cytokine staining performed in parallel (data not shown). Spots detected closely matched the number of cytokine-producing cells, the lowest dilution tested. Moreover, the number of cytokine plated and the spot numbers detected was seen down to 1/300,000. Says, however, a linear relationship between the cell numbers of the same type, the detection limit of intracytoplasmic staining was reached at 0.1% by ELISPOT).

The frequency of IL-5-producing T cells in freshly isolated PBMC was at the detection limit of intracytoplasmic staining, these are not shown (they were shown by red). The results of cytokine-positive cells identified by intracytoplasmic cytokine staining performed in parallel are shown as the percentage in parentheses. Because the frequency of IL-5-producing T cells in freshly isolated PBMC was at the detection limit of intracytoplasmic staining, these are not shown (they were 0.1% by ELISPOT). D. Frequency measurements by cytokine ELISPOT assays in Ag-stimulated PBMC. PBMC were first activated at 300,000 cells/well with PPD or TT, and 8 h later the specified number of cells was plated in serial dilutions in ELISPOT assays. The PPD-induced IFN-γ spot formation is shown by ○; that induced by TT, ●; TT-induced IL-5 spot formation is represented by ▲; PPD did not induce IL-5 production. These Ag-induced responses were undetectable by intracytoplasmic cytokine staining performed in parallel (data not shown).

**Statistical analysis**

The overall significance of the differences seen for HIV patients in Figs. 4 and 5 was calculated by the Mann-Whitney Rank Sum Test, and for Fig. 6, using the t test.

**Results**

IFN-γ and IL-5 ELISPOT assays measure the accurate frequencies of recall Ag-specific CD4 memory cells with a detection limit of 1/300,000 cells

Although ELISPOT analysis has frequently been performed in settings of T cell recall assays, it has not been stringently established whether the assay accurately measures the true frequency of CD4 memory T cells in freshly isolated PBMC in the low frequency range. We first tested human CD4+ T cell lines and freshly isolated PBMC activated with mitogen because the high frequency of cytokine-producing T cells under these conditions permitted us to directly compare the resolution of intracytoplasmic FACS staining and ELISPOT assays. The percentage of T cells producing IFN-γ and IL-5 was first established by intracytoplasmic FACS analysis (Fig. 1, A and B, and in Fig. 1C the numbers in parentheses). When these activated T cells were serially diluted with inactivated T cells of the same type, the detection limit of intracytoplasmic staining was reached at ~1/5,000 (data not shown). In the ELISPOT assays, however, a linear relationship between the cell numbers plated and the spot numbers detected was seen down to 1/300,000 cells, the lowest dilution tested. Moreover, the number of cytokine spots detected closely matched the number of cytokine-producing T cells plated after correction for the frequency of cytokine-producing cells (as established by intracytoplasmic staining). When, for example, 123 activated T cells of the PAN line were plated per well (of which 23% were IFN-γ positive by flow cytometry), together with 300,000 APC, then 27 ± 2 spots were detected, that is, 22% of the plated T cells generated IFN-γ spots. Although both the ELISPOT assay and intracytoplasmic cytokine staining gave equal results for detecting per cell cytokine production, the ELISPOT assay was able to detect a single cell in the presence of 300,000 bystander cells where intracytoplasmic staining could not, having reached a functional detection limit at 1 in 5,000.

We next attempted to detect recall Ag-induced cytokine production in freshly isolated PBMC. When PBMC of healthy donors were tested with intracytoplasmic cytokine staining for IFN-γ and IL-5 production induced by TT and PPD, no signal over background was seen. In contrast, very clear signals were obtained in ELISPOT assays (Fig. 2B provides an example of TT-stimulated PBMC; the spontaneous cytokine production of these PBMC is shown in Fig. 2A). By using criteria of spot size and density analogous to gating in FACS analysis, image analysis of ELISPOTS permitted the objective evaluation of the results to distinguish between background and signal, count the numbers of spots, and determine their size distribution (Fig. 2, D and E).

The spectrum of spot sizes induced by TT (or PPD) was comparable with those seen in mitogen-stimulated T cell lines or PBMC (Fig. 2, D vs E, and sample wells B vs C). After verifying with T cell lines that these spots were produced by individual T
cells (see above), the amount of cytokine produced in total and by individual T cells could be derived from the size histogram. Based on this, the similarity in the spot size distributions found in the recall Ag-activated PBMC and T cell lines suggested that the cytokine spots from the PBMC population also reflected cytokine production by individual T cells. This notion was further confirmed when the PBMC activated by TT and PPD were serially diluted, as the resulting plot of the number of PBMC plated against the number of IFN-\(\gamma\) and IL-5 spots detected conformed to a linear function that passed through the origin (Fig. 1D). These data establish that IFN-\(\gamma\) and IL-5 ELISPOT assays are suitable for direct visualization of cytokine-producing CD4 memory cells in the low frequency range (IL-4 and IL-10 data are not shown, as the production of these cytokines was not confined to memory cells).

Recall Ags induce IFN-\(\gamma\) and/or IL-5 production only in sensitized individuals

We next tested whether the IFN-\(\gamma\) and the IL-5 induced by the test Ags in PBMC are secreted by Ag-specific memory T cells. Most healthy individuals that we tested produced IFN-\(\gamma\)-Ags in PBMC are secreted by Ag-specific memory T cells. Most PPD-responsive individuals, IFN-\(\gamma\) was also unaffected by the concurrent production of the other type of cytokine (Fig. 3C and D). The findings provide additional evidence that true frequencies of cytokine-producing cells are established by the IFN-\(\gamma\) and IL-5 cytokine ELISPOT assays and

![Figure 2](image)
that they are directly visualizing the memory cells while avoiding the caveats of more complex bioassays in use for low frequency analysis. We attribute this lack of cross-inhibition to the fact that both types of memory cells are resting when plated, not secreting cytokine, such that their initial activation occurs in the absence of the potentially inhibitory cytokine of the other type. Before the induced cytokines can establish inhibitory effects, the plate-bound cytokine is developed.

The frequency of memory cells producing IFN-γ and IL-5 in healthy individuals is stable over time

Because it has been a matter of debate whether the clonal sizes of Ag-specific memory T cells in an individual are stable over time (6, 7), we repeatedly tested the recall responses to TT and PPD of four healthy donors over a period of up to 4 yr, measuring IFN-γ and IL-5 production. The frequencies of cytokine spots were within a tight range over this time period (Table II). After having established the validity of the cytokine ELISPOT approach for monitoring individual CD4 memory cells in PBMC and after having first gained insight by testing healthy individuals, we set out to study the frequency and single cell cytokine output of recall Ag-specific memory cells in HIV patients.

The frequency of TT-specific memory cells producing IFN-γ and IL-5 shows no evidence of type 2 bias in HIV patients on HAART therapy

We tested 22 HIV patients whose CD4 cell counts were between 150 and 1070 cells/ml and all of whom were on HAART therapy at the time of testing. The numbers of IFN-γ spots induced in their PBMC by TT were significantly decreased (Mann-Whitney Rank Sum Test, p = 0.006) compared with the 18 healthy donors that were tested (Fig. 4A). The apparent reduction in IL-5 production (Fig. 4B) was not statistically significant, however (p = 0.143). These data are consistent with a moderate TT-specific Th2 bias in the HIV patients (19, 20). When the production of these cytokines was measured for the entire T cell pool by stimulating cells with mitogen (PHA), the frequency of IFN-γ-producing cells was slightly increased (p = 0.083), while that of the IL-5-producing cells was in the same range (Fig. 4B). At the polyclonal level,

Table II. The frequency of memory cells producing IFN-γ and IL-5 specific for TT or PPD is stable in healthy individuals over time

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*IFN-γ and IL-5 production induced by TT and PPD was tested by ELISPOT assays on four donors for up to 4 years at the time points indicated. Means of replicate wells were shown (300,000 PBMC/well) after the subtraction of the medium control (typically 0–10 spots per well); SD for the replicate wells was <10%.

FIGURE 3. The uninhibited frequency of T cells producing IFN-γ and IL-5 is measured by ELISPOT assays. A, Freshly isolated PBMC, or B, T cells isolated from PBMC of the same donor (along with T cell-depleted autologous APC) were tested in parallel for IFN-γ (solid) or IL-5 (hatched) spot formation in the presence of PPD or DM alone, or in the presence of both Ags simultaneously (PPD + DM), as specified. The percentage of CD3-positive cells in each test cell population is indicated. Cytokine production in the medium control (<5 spots/well) has been subtracted and SD for replicate wells are shown. The data show that the cytokine-producing cells are CD3⁺, that their numbers are directly proportional to the percentage of CD3 cells present in the culture, and that the production of IFN-γ and IL-5 is mutually uninhibited. C, Size distribution of the IFN-γ, and D, IL-5 spots from the assay with the purified CD3 cells (shown in B) as induced by the Ags separately or by together.
when tested at single cell resolution, we therefore found no type 2 bias in the response of these patients.

The frequency of TT-reactive memory cells in HIV patients is inversely defined by the viral load

The patients were subdivided into groups with <10^3, 10^3–10^4, and >10^4 copies of HIV-RNA/ml of blood. The group with <10^3 copies (n = 12) had frequencies of IFN-γ-producing TT-reactive cells indistinguishable from those of healthy individuals (p = 0.615). Patients with a viral load exceeding 10^3 (n = 12), however, had considerably lower, barely detectable numbers of TT-reactive IFN-γ-producing memory cells (p < 0.001). This reduction in frequencies did not reflect an overall reduction in CD4 cell numbers in the patients because the frequencies shown (Fig. 4, A and B) have been normalized for CD4 cells and because the high viral loads did not correlate (r^2 = 8.406) with low CD4 cell numbers in patients (Fig. 4C). Moreover, the reduction in TT-reactive CD4 cells was selective, as PHA-induced IFN-γ production was essentially unaffected by the high viral load (p = 0.328).

Reduced IFN-γ and IL-5 production per cell in a subpopulation of HIV patients

ELISPOT assays measure, over the entire test period (24-48 h in our set up), the amount of cytokine secreted by individual T cells physiologically stimulated by encountering Ag; hence, where there is decreased cytokine production in immunodeficiency such as occurs in HIV, these assays can determine whether it was caused by a decrease in the frequency of the responding cells or a reduction in the cytokine output of the individual cells. In 17 of the 22 patients, the production of IFN-γ and IL-5 per cell in response to stimulation with TT, PPD, and PHA was indistinguishable from

HIV patients that are PPD skin test negative show IFN-γ responses to PPD

Only bacillus Calmette-Guérin-vaccinated healthy donors displayed IFN-γ responses to PPD (Fig. 5A, p = 0.001). Nine of the twenty-two patients showed a significant PPD recall response (Fig. 5B, p < 0.001). The frequency of the patients’ IFN-γ-producing memory cells was in the range of 70–400 cells per million CD4 cells, slightly but not statistically significantly reduced when compared with the 10 vaccinated healthy individuals that we tested (p = 0.064). While all PPD-responsive healthy donors tested positive in the PPD:Mantoux skin test, none of the four PPD-responsive patients that we succeeded to test was skin test positive (data not shown). As in the healthy donors, the PPD recall response in the HIV patients was dominated by IFN-γ-producing memory cells; two of the patients, however, in addition to showing a high frequency IFN-γ response, also had IL-5-producing memory cells, a response type that was not seen in healthy individuals. The 13 patients that did not respond to PPD fell into the full spectrum of high, intermediate, and low viral loads (data not shown). Their lack of responsiveness, therefore, could be attributed either to the immunodeficiency that was seen for TT in patients with high viral loads, or to the lack of sensitization to mycobacterial Ags. These two possibilities cannot be readily distinguished for the PPD response, because none of the HIV patients have been bacillus Calmette-Guérin vaccinated, and their sensitization to mycobacterial Ags was environmental. (This distinction can be made for the tetanus toxoid response, however, because all HIV patients have been vaccinated with TT.)
healthy individuals; however, five of the patients displayed spots of dramatically reduced, although still clearly detectable, size after TT, PPD, and PHA challenge when tested in parallel, under identical assay conditions. When studied by the $t$ test, the changes in spot sizes $<0.05 \text{mm}^2$ reached statistical significance ($p = 0.0375$), as did the reduction of spots that exceeded this size ($p = 0.0001$). Fig. 6A shows representative spot size histograms for these two response types, one from a patient who responded as healthy individuals do and one who displayed the reduced spot size. Enlarged details of wells are also shown to illustrate both response types (Fig. 6C and D; the medium control for 6C is shown in 6B). In these two groups, the frequencies of cytokine-producing cells were in a comparable range; it was the cytokine production per cell that differed. To date, this reduced cytokine production per cell was seen only in HIV patients; we encountered it neither in healthy individuals nor in immunosuppressive therapy (21), nor in other patient groups ($n > 100$ to date) that we are in the process of studying and that include individuals with type 1 diabetes, multiple sclerosis, and tumors. The lower production of cytokines in HIV-infected patients did not correlate with viral load, CD4 count, or any other observable feature (data not shown).

**Discussion**

Tetramers, intracytoplasmic cytokine staining, and ELISPOT assays are among the new techniques becoming available that will make it possible to take a closer, more direct look at memory cell populations in freshly isolated cellular material, thereby helping to redefine the most basic parameters of T cell-mediated immunity by establishing definitively the numbers, type 1/type 2 differentiation state, and population kinetics of the Ag-specific memory cells in vivo (1). ELISAs and RT-PCRs, the techniques primarily used, do not yield the frequencies of cytokine-producing cells. Although in situ PCR does measure frequencies, it does not perform well in the low frequency range and, additionally, measures mRNA (which frequently is posttranscriptionally controlled) only at a single time point. Whereas intracytoplasmic cytokine staining requires pharmacological disruption of the Golgi apparatus and usually additional signal-enhancing treatments to detect cytokine (22), the cytokine ELISPOT assay measures the biologically relevant cytokine naturally released by individual cells over the entire duration of the assay.

In previous experiments, in which tetramer technology was used to study Ag-specific CD8 responses in HIV and other viral infections, it came as a major surprise that the numbers of the virus-specific cells can rise to frequencies as high as 10% of all CD8 cells during acute infection: data obtained with both ELISPOT (23) and intracytoplasmic cytokine staining (24) confirmed these results for high frequency CD8 cells. Similar information has not yet been obtained for Ag-specific CD4 cells. Our testing of CD4 cell lines in ELISPOT and intracytoplasmic staining in parallel showed that both assays provide essentially identical frequency measurements in mitogen-stimulated cell populations. Although we found that intracytoplasmic cytokine staining, like FACS analysis in general, had a detection limit of $\sim 1/5000$ (25), we showed that the IFN-$\gamma$ and IL-5 ELISPOT approach provides accurate frequency measurements down to frequencies as low as 1 in 300,000 cells (Fig. 1C). Moreover, by performing experiments with purified cells, we established that the cytokine signals obtained when testing PBMC for recall Ag responses were derived from T cells (Fig. 3). The cytokine responses were present only in sensitized individuals (Table I), as is consistent with our cytokine ELISPOT findings in experiments comparing responses in sensitized with those of naive or control-immunized mice (17, 18). This observation is also consistent with the fact that naive T cells do not produce IFN-$\gamma$ or IL-5 (16). Importantly, while several cell lineages

**FIGURE 6.** Decreased cytokine production per cell in a subset of HIV patients. A, The size distribution of the PPD-induced IFN-$\gamma$ production is shown for one of the five patients that displayed the reduced cytokine output per cell phenotype (dashed line) and for one patient that produced the spot size distribution characteristic for the remaining HIV patients ($n = 17$, continuous line), being indistinguishable from that of healthy donors. B–D, Representative, enlarged well segments illustrating the size difference between the patient with the unimpaired (D) and the reduced (C) cytokine production per cell. The medium control for C is shown in B to illustrate that the weak spot formation is clearly distinguishable from the background. The numbers of spots over background in the entire well are shown in each panel. The frequency of the IFN-$\gamma$-producing cells was, therefore, in the normal range; only the amount of cytokine produced per cell was decreased.
other than T cells can produce IFN-γ and IL-5, no such nonspecific responses were induced by the test Ags in the recall assays or by the cytokines released by the Ag-stimulated T cells. The linear relationship between the number of primed T cells present in the cultures and of the number of spots detected (Fig. 1, C and D), despite the presence of a constant number of APC, shows that only cognate cytokine production from activated T cells was detected. The exact number of T cells that expressed these cytokines was therefore detected at all cell dilutions in the ELISPOT assay down to 1/300,000, thus providing accurate frequency measurements for type 1 and type 2 cells producing IFN-γ and IL-5, respectively (Fig. 1). Enabled by this high resolution, we report in this work the first direct visualization of the recall Ag-specific, CD4 memory cell pool in healthy individuals and in HIV patients on HAART therapy.

Memory cells reactive to TT and PPD in healthy individuals (and in most HIV patients) occurred in the frequency range 1/5,000–1/100,000 CD4 cells (Fig. 4). Despite considerable interindividual variations, which should not come as a surprise given the fully MHC-disparate genotypes and different Ag histories of the test population, the frequency of Ag-specific memory cells in healthy individuals was remarkably constant over time. We tested four donors repeatedly over time periods of up to 4 yr and found that the frequencies of memory cells reactive to TT and PPD that produced IFN-γ and IL-5 were essentially unchanged (Table II). These data might contribute to the ongoing discussions on the population kinetics of memory cells. It has been shown in animal models and proposed also for humans that, during infections, TNF-α and IL-15 cause clonal expansions in third-party, Ag-specific memory cell populations (11–13). The healthy (non-HIV-infected) donors experienced several episodes of flu-like infections during the testing period, during which they displayed high IFN-γ activity in medium control wells (data not shown). After recovery, this spontaneous production ceased and the frequencies of Ag-specific spots returned to what they were before. These data support the notion that memory cell populations, once their clonal size has established itself after immunization, remain stable in the absence of specific stimulation by the Ag.

Although the TT-specific memory cell pool in most healthy test persons comprised both cells producing IFN-γ and IL-5 (Table I, Fig. 4), the PPD-specific memory cells produced IFN-γ, but not any IL-5 (Table I, Fig. 5). Conversely, DM Ag induced a highly polarized IL-5 response, which in some donors occurred without any IFN-γ production. These polarized response phenotypes are not the result of dominant cytokine response types overriding or masking less dominant ones, but reflect the true frequencies of memory cells committed to the production of the respective cytokine (Fig. 3). Based on studies of long-term cultured T cell clones (which can lose differentiation in cell culture) and on assays that are not suited to defining the cellular sources of the individual cytokine, it has been widely held that the clear-cut polarization of T cell responses along the Th1/Th2 paradigm seen in the mouse does not hold for humans (26). Our data, obtained at single cell resolution, suggest that IFN-γ and IL-5 can be expressed by human memory T cells in a mutually exclusive manner, favoring emerging concepts that the expression of type 1 and type 2 cytokine genes is individually regulated in T cells (27–29). Indeed, when we performed two-color ELISPOT assays simultaneously detecting IL-5 and IFN-γ, we found that these cytokines were not produced by Th0-type cells that coexpress both, but that IFN-γ and IL-5 were produced by different CD4 cells (manuscript in preparation).

We applied this high resolution analysis to memory cell repertoires in HIV patients in the hope of gaining new insights into the basic parameters defining Ag-specific T cell immunity, which were still controversial. It was important to study these parameters because they could change as a consequence of both HIV infection and therapy. For example, the production of T cells is reduced in untreated patients, as is the life span of the T cells (30, 31), yet, with antiviral treatment, the generation of precursor cells increased again and exceeded the normal rate, while the 1/12 of T cells remained strongly reduced (32, 33). After treatment, there is an initial increase in the numbers of memory T cells, and only later do frequencies of T cells with a naive phenotype return to approximately the normal range, at which time CD8 cell numbers fall (34, 35). Moreover, the frequency of recall Ag-specific memory T cells in the blood may change if they are redistributed between the extralymphoid compartments and the recirculating pool, as has been postulated; additionally, depletion/exhaustion and regeneration may contribute to the overall T cell repertoire in ways that are still debated (36, 37). It is also unclear how the massive expansion of the virus-specific CD8 cells affects the CD4 memory cell pool specific for third-party Ags, including those reactive to recall Ags, as is whether a bias toward type 1 or type 2 immunity in patients exists. All of these problems emphasize the need for high resolution analysis.

Previous attempts to establish recall Ag-specific T cell memory in freshly isolated cellular material from patients relied on readout systems that do not measure the frequency of the responding cells (ELISAs, RT-PCR, proliferation); other studies utilized T cell clones and in vitro expanded cell lines, which also do not provide data on the frequencies or state of differentiation/function of Ag-specific cells in vivo (reviewed in Ref. 38). LDAs were performed (39) to determine the precursor frequency of TT-reactive IL-2-producing T cells (the limitations of which are specified above and in Ref. 1). In one study, the numbers of PPD-specific, IFN-γ-producing T cells were measured by ELISPOT (40). Most of these studies came to the conclusion that T cell reactivity declines with the progression of HIV infection, but they left open the questions of how T cell function correlates with virus load and whether this loss of function is a result of decreased frequencies or decreased cytokine output, i.e., whether it is a function of T cell exhaustion, the dilution of these cells by virus-specific CD8 cells, or their suppressed state. Although many of these changes seem to be reversible after HAART therapy (34, 35), the extent to which specific immune reactivity is restored remains unclear. All 22 patients that we studied were undergoing this therapy, so it was important for us to establish how the reactivity of individual T cells to recall Ags in these patients compares with that of healthy individuals.

All patients that had a low viral load (<10^3 copies/ml) had frequencies of TT-specific IFN-γ-producing memory cells in a range indistinguishable from healthy individuals, although they had decreased frequencies of IL-5-producing memory cells (Fig. 4). All of these patients were revaccinated with TT, as is common practice for the cohort we studied. The lack of IL-5 (type 2) memory to TT might have resulted from vaccinating/boosting concurrent with the ongoing HIV infection that, through IL-12 induction, should bias the differentiation of T cells toward the expression of type 1 cytokines.

In the patients with a viral load exceeding 10^3 copies/ml, the TT-reactive T cells producing IFN-γ occurred in ~10-fold-reduced numbers, and IL-5-producing cells were barely detectable (Fig. 4, A and B). Although reduced IFN-γ production in mitogen-stimulated PBMC has been reported in cases in which the disease has progressed (41), we observed a direct correlation between viral load and recall Ag-induced T cell function, but not to mitogen-driven cytokine production (Fig. 4, A and B). Because in patients the number of virus-specific CD8 cells can expand considerably, it is possible that the recall Ag-specific T cells have been diluted out.
Although the patients with the low viral load showed T cell response similar to healthy individuals, it might be premature to conclude that this signifies fully restored immune competence, as suggested by our data on the PPD responses in the HIV patients. Nine of the patients had PPD-reactive IFN-γ-producing memory cells (Fig. 5). Either cross-reactive stimulation with nonmycobacterial infections might prime these T cells or opportunistic infections with mycobacteria (including M. tuberculosis and Mycobacterium avium) might specifically prime these PPD-reactive memory cells; future studies will be required to distinguish between these possibilities. Irrespective of how this PPD reactivity was acquired, these patients were PPD skin test negative, while the vaccinated, and only the vaccinated, healthy individuals were skin test positive, even though some had PPD-reactive T cell frequencies equal to or lower than those of the patients. The loss of skin test reactivity, which represents a PPD Ag-specific, type 1, T cell-mediated, delayed-type hypersensitivity (DTH) reaction, in HIV infection is a known phenomenon thought to result from impaired T cell function (42). Our data show that the DTH deficiency of HIV patients to PPD neither results from the absence of PPD-reactive memory nor from impaired ability to secrete IFN-γ. Whether a counterregulatory, type 2 response contributes to this deficiency is a point that still needs to be elucidated: unlike any of the healthy donors, two of the HIV patients showed IL-5 responses to PPD. DTH is a complex inflammatory response that depends not only upon T cells secreting IFN-γ, but upon their ability to kill, to respond to chemokines, and upon the response of the nonlymphoid compartment to T cell-mediated inflammation (43), among others. Apparently, one of these downstream responses is impaired in HIV. Our data suggest that PPD ELISPOT recall assays might have diagnostic value. It could show that patients have been exposed to M. tuberculosis, a pathogen that represents a major infectious complication in HIV disease and one that presently cannot be diagnosed reliably because of the frequency of false-negative skin tests (42); if recognized, this infectious complication can readily be prevented or treated. Alternatively, it might signify infection with M. avium, which is also an infectious complication requiring treatment.

Five of the HIV patients showed a dramatic reduction in the cytokine output of individual cells (Fig. 6), which, as opposed to reduced numbers of T cells producing normal amounts of cytokine per cell, constitutes a distinct mechanism for immunodeficiency. The impaired cytokine production of individual T cells was seen after T cell activation induced by TT, PPD, and mitogen (PHA and anti-CD3). The frequency of T cells induced by these stimuli was, however, in the range of HIV patients with normal cytokine output per cell (Fig. 6). These data suggest that there is an overall immunodeficiency present in these patients that may emerge as a result of, e.g., a burst of antiviral immune response resulting in cytokine-mediated immune suppression. In the five patients that displayed this response type (which we have not seen in other states of immunodeficiencies or diseases; see above), we found no correlation with viral load, CD4 cell counts, or therapy. Studies of larger cohorts and longitudinal testing will be required to understand more closely this phenomenon in particular and T cell memory in general when taking into account the complexity of the disease afforded by the genetic variability of the host and the virus and of various environmental influences. The initial observations reported in this work demonstrate the feasibility of studying clonal sizes, cytokine signatures, and the cytokine output of individual Ag-specific T cells, and provide early insights into these issues. High resolution analysis in the low frequency range should facilitate progress in defining the function of the immune system in healthy individuals and patients. Moreover, it should be an invaluable tool with which to study with unprecedented accuracy the effectiveness of vaccination against HIV and other infectious organisms by directly visualizing the specific T cell response induced.

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