Evaluation of Antigen-Specific T-Cell Responses with a Miniaturized and Automated Method

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The evaluation of antigen-specific T-cell responses is helpful for both research and clinical settings. Several techniques can enumerate antigen-responsive T cells or measure their products, but they require remarkable amounts of peripheral blood mononuclear cells (PBMCs). Since screening numerous antigens or testing samples from pediatric or lymphopenic patients is hampered in clinical practice, we refined a miniaturized, high-throughput assay for T-cell immunity. Antigens and cells in 10-μl volumes were dispensed into 1,536-well culture plates precoated with anti-gamma interferon (anti-IFN-γ) antibodies. After being cultured, the wells were developed by enzyme-linked immunosorbent assay for bound cytokine. Miniaturization and automation allowed quantitation of antigen-specific responses on 104 PBMCs. This method was applied for epitope mapping of mycobacterial antigens and was used in the clinic to evaluate T-cell immunity to relevant opportunistic pathogens by using small blood samples. A comparison with conventional methods showed similar sensitivity. Therefore, current flow cytometric methods that provide information on frequency and phenotype of specific T cells can be complemented by this assay that provides extensive information on cytokine concentrations and profiles and requires 20- to 50-fold fewer PBMCs than other analytical methods.

Numerous methods are currently available to enumerate antigen-specific T cells and to measure their functions, most of them being based on flow cytometric analysis. Such methods have been compared and critically discussed in recent reviews (4, 17). A common feature of these methods is the requirement, on average, of 2 × 105 to 5 × 105 peripheral blood mononuclear cells (PBMCs) to test a single antigen. This imposes a limitation on the analytical antigenic breadth, which depends on the number of available PBMCs. In the case of lymphopenic subjects, the population that most frequently needs to be monitored for immunocompetence, this is particularly crucial. Also, the small volumes of pediatric blood samples may result in inadequate numbers of PBMCs for extended analysis of specific T-cell immunity. By the same token, screening of large peptide panels for epitope mapping on PBMCs from healthy donors (blood donors or vaccinees) is also limited to a few hundred peptides (16) instead of the thousands of peptides needed to cover several immunodominant proteins (14). Therefore, we felt the need to develop a miniaturized assay that makes use of limited numbers of PBMCs, thereby enhancing our screening power.

A miniaturized assay based on 384-well plates instead of conventional 96-well plates or 5-ml tubes was developed in our laboratory and named cell–enzyme-linked immunosorbent assay (cell-ELISA) (8). This method was based on a previous observation that lymphocyte cultures can be run in 96-well plates in which the wells had been precoated with an anti-cytokine antibody. The cytokine secreted by antigen-specific T cells was captured by the solid-phase antibody, and the plate was developed as a conventional ELISA plate (11). Miniaturization from 96- to 384-well plates (8) resulted in a reduction of tested lymphocytes from 2 × 105 to 5 × 105 down to 5 × 104 per single antigen, corresponding to a scaling-down factor of 4- to 10-fold.

In this report, we describe further miniaturization in 1,536-well plates by seeding 104 PBMCs per well, meaning a reduction in cell number by a factor of 20- to 50-fold over conventional assays. We also describe here the use of pathogen-specific, established CD4 and CD8 T-cell lines as possible standards for T-cell assays, the comparison of cell-ELISA with other established methods with respect to sensitivity, and the preliminary application of cell-ELISA to clinical samples and to peptide screening for epitope mapping in a miniaturized format.

MATERIALS AND METHODS

The cell-ELISA method, based on an assay originally designed for mouse splenocytes in 96-well plates (12), has been described in detail for 384-well plates (8). The adoption of 1,536-well plates required extensive automation of all procedures for antigen dispensing (proteins or peptides), for liquid and cell handling, and for plate development and scanning. The assay was performed with sterile reagents under a laminar flow cabinet.

Automated instrumentation. The four-channel MultiProbe (Perkin-Elmer, Shelton, CT) was used to transfer proteins and peptides from vials to 96-well master plates. The 96-channel Hydra II (Matrix) was used for simultaneous dispensing of antigens from 96-well master plates to 384-well and 1,536-well plates. The eight-channel MultiWell (Matrix Technologies, Hudson, NH) was used for dispensing reagents and cells in 384-well plates (catalogue no. 164688;
NUNC, Roskilde, Denmark). The eight-channel MultiDrop (Thermo OY, Finland) was used for dispensing reagents and cells in 1,536-well plates (NUNC catalogue no. 1356.13). The EL800 (Biotek, Winooski, VT) was used for 384-well plate spinning, and the Victor 3V (Perkin-Elmer) was used for 1,536-well plate spinning.

**Reagents.** Phosphate-buffered saline (PBS) and RPMI 1640 medium were purchased from BioWhittaker, Verviers, Belgium. RPMI 1640 medium was enriched with 10 mM-l-glutamine and with 5% fetal calf serum selected for low background in the cell-ELISA assay (complete medium). Antibody pairs were from Mabtech (Stockholm, Sweden). Antibodies for phenotyping and for intracytoplasmic cytokine staining (ICS) were from Becton Dickinson (San José, CA). Pentamers were from Prommune, Oxford, United Kingdom. Protein antigens, derived from different opportunistic pathogens or whole inactivated pathogen bodies, have been described previously (6, 7, 8, 9). Pathogens used as whole bodies, have been described previously (6, 7, 8, 9). Pathogens used as whole bodies or from pathogen bodies from which proteins were derived included the following: Candida albicans, Aspergillus fumigatus, Aspergillus niger, Cryptococcus neoformans, Toxoplasma gondii, Mycobacterium bovis, Mycobacterium tuberculosis var. bovis” Bergey et al. 1934 pro synon.), Mycobacterium tuberculosis (”Mycobacterium tuberculosis var. hominis” Bergey et al. 1934 pro synon.), cytomegalovirus (CMV), and human immunodeficiency virus type 1 (HIV-1). Peptides were synthesized by INBIOS, Naples, Italy, or by IPT, Berlin, Germany (6, 7, 8, 9).

Final antigen concentrations were 10 μg/ml for proteins and 1 μg/ml for peptides.

**Blood samples.** Blood was obtained from healthy donors who gave informed consent. Blood from patients was obtained after approval of the Institutional Ethical Committee and after the informed consent was signed.

**Plate coating.** Plates (1,536 wells) were coated with 5 μl of the first antigen-antibody interface (anti-IFN-γ) antibody (AbD) μg/ml in PBS. After overnight incubation at 4°C, the plates were washed twice with PBS and saturated with 10 μl PBS-1% human serum albumin (20% solution; Kedrion, Lucca, Italy) at room temperature (RT) for 1 h. After saturation, the plates were washed twice with 13 μl Hanks’ balanced salt solution and received 4 μl of complete medium.

**Antigen dispensing.** Antigens, dispensed with predefined patterns in 96-well master plates, were transferred into 1,536-well plates using the Hydra II liquid handler. One microliter of 10 μl PBS-1% human serum albumin (20% solution; Kedrion, Lucca, Italy) at room temperature (RT) for 1 h. After saturation, the plates were washed twice with 13 μl Hanks’ balanced salt solution and received 4 μl of complete medium.

**Cell-ELISA.** PBMCs obtained from heparinized peripheral blood by the conventional Ficoll gradient were brought to 2 × 10^6 cells/ml in complete medium and automatically dispensed into thawed plates at 5 μl per well. The plates were incubated for 2 days in a 5% CO₂ atmosphere. At the end of the culture, supernatants or cells could be collected for further experiments. After washing twice with PBS-Tween 20 0.05% (Tween 0.1% when PBMCs were derived from HIV-positive donors), the plates were spun upside down on filter paper at 500 g for 2 min for complete removal of washing buffer. Then, the wells received 5 μl biotinylated antibody at 1 μg/ml for 1 h. After washing twice with PBS-Tween and three times with PBS and drying by centrifugation, the wells received 5 μl alkaline phosphatase-streptavidin at 1 μg/ml. After being incubated at RT, the plates were washed as described in the previous step and the wells received 10 μl p-nitrophenylphosphate. After a 1-h incubation at RT, the plates were scanned. Results are shown as the optical density at 405 nm × 1,000 or as picograms/milliliter of cytokine, referred to as a titration curve.

**Methods for specific T cells.** ICS, enzyme-linked immunosorbent assay (ELISPOT), and pentamer staining were performed according to standard procedures, following the manufacturer’s instructions (Becton Dickinson, Mabtech, and ProImmune). Flow cytometric analysis was performed with a CyAn instrument (Dako) using Spectricle and Flow-Check Fluorospheres (Dako) for instrument setting and compensation and with standard gating practices. Staining with carboxyfluorescein succinimidyl ester (CFSE) was performed as described previously (7, 9). CD4 and CD8 T-cell lines were produced and maintained as described previously (7, 9).

**RESULTS**

Use of cell-ELISA to test antigen-specific T-cell lines. The accuracy of assays that measure T-cell responses is limited by the fact that no cellular standard is generally used. Established T-cell lines that contain 100% antigen-specific T cells represent a valuable, but not easily available, standard. We have produced CD4 and CD8 T-cell lines with different specificities (7, 9). The consistent production of IFN-γ by T-cell lines at different restimulation cycles was tested in preliminary experiments. CD8 lines specific for pp65 NV9 peptide and CD4 lines specific for tetanus toxoid (TT) or for pp65 pep30 (amino acids 117 to 131) were stimulated in 1,536-well plates. Supernatants from replicate wells were collected and tested for IFN-γ concentration by the Luminex assay (CD8 supernatants) and by the Cytomix assay (CD4 supernatants). Cytokine concentrations fluctuated within a limited range from 1,500 to 7,500 pg/ml in the T-cell lines tested between the second and seventh restimulation cycles. CD4 or CD8 T cells from established T-cell lines were cultured for 3 weeks after restimulation with antigen and APCs, followed by expansion with IL-2. Once the cells had reverted to a resting state (loss of proliferation and loss of blast-like appearance), they were used for the restimulation experiments shown here. T cells were labeled with the vital dye CFSE and mixed 1:10 with autologous PBMCs in the presence or in the absence of antigen. After a 2-h incubation, brefeldin was added overnight and the cells were finally stained for IFN-γ.

Figure 1 demonstrates that representative T-cell lines contain up to 100% specific T cells. In the case of an HIV-specific CD4 line (Fig. 1A and B), >97% of the input T cells labeled with CFSE and spiked into nonresponding PBMCs produced

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IFN-γ upon antigenic stimulation. Similarly, in the case of a CMV-specific CD8 T-cell line, both IFN-γ ICS (Fig. 1C and D) and tetramer enumeration (Fig. 1E) concurred to define that the line contained >98% specific cells. While a scattered staining for intracytoplasmic IFN-γ production is generally observed with PBMCs, it can be noted that, as shown in the plots in Fig. 1, T-cell lines were highly homogeneous with respect to IFN-γ staining. Since resting T cells overlapped with the lympho gate of PBMCs, the same gate was used for analysis of the mixtures (CFSE-labeled T cells plus IFN-γ phycoerythrin staining as shown in Fig. 1A to D). No differences were detected by physical analysis between mixtures with and without antigen when the cell mixtures were analyzed after incubation (data not shown).

In order to estimate a sensitivity threshold, the CD4 and CD8 T-cell lines were diluted into irradiated, autologous, nonresponding PBMCs (right panels). In both cases, it can be seen that as few as five specific T cells in 10^4 PBMCs per well, corresponding to a frequency of 0.05%, could be detected by cell-ELISA. Z factor scores for the two experiments were 0.63 and 0.51. Since a Z factor score between 0 and 0.5 indicates a marginal assay and a Z factor score between 0.5 and 1 indicates an excellent assay (20), a 0.05% frequency of specific T cells can be reliably detected by our method. To further demonstrate their usefulness as reference standards, T-cell lines at different restimulation cycles were challenged with antigen and IFN-γ was measured in supernatants by Luminex or Cytomix assays. IFN-γ levels fluctuate within a restricted range between 1,500 and 7,500 pg/ml, irrespective of the restimulation cycle.

**Sensitivity of cell-ELISA using PBMCs.** Since T cells from established lines may not mirror T cells from PBMCs, we used donors with steady frequencies of CMV NV9-positive, pentamer-positive CD8 cells (donor 3, 0.5%; donors 1, 4, and 5, 1%) over time. Untreated PBMCs were diluted into autologous, irradiated PBMCs as a source of APCs. Pentamer staining was taken as a reliable measure of NV9-positive CD8 T cells. As shown in Table 1, the different methods were comparable with respect to sensitivity, with around five specific T cells out of 5 x 10^4 PBMCs (0.01%) detected by the different assays. By considering the frequency of specific T cells (either assumed by pentamer staining, as in the case of PBMCs, or experimentally determined, as in the case of T-cell lines) and the amount of IFN-γ produced in the culture vessel, we can estimate the amount of cytokine produced by a single cell in a time unit. As shown in Table 2, we tested PBMCs from donors 3 and 1 that were undiluted or diluted 1:5 into autologous, irradiated PBMCs. The total amount of IFN-γ produced upon NV9 stimulation was divided by the number of
specific T cells present in the culture, showing that a single T cell produced 1.1 to 3.7 pg cytokines in 36 h. Similar results were obtained with a CD4 T-cell line serially diluted into autologous, nonresponding PBMCs, showing that one single T cell produced 1.3 to 2.7 pg IFN-γ in 36 h (Table 3). Assuming a mass of 20 kDa for human IFN-γ (2), this corresponds to approximately 3 × 10^4 IFN-γ molecules secreted by a single T cell.

**Epitope mapping by cell-ELISA.** The 1,536-well plates were used for a preliminary epitope mapping of different *M. tuberculosis* strain H37Rv proteins that have been previously reported as being immunodominant in infected or immune subjects. For initial experiments, peptide panels from small proteins (Ag85B of *M. bovis*, rdESAT6, rdESAT6-like protein 9, culture filtrate protein 10 [CFP-10], and RV 2628 from *M. tuberculosis*) were used, represented by a total number of 105 overlapping peptides. Sequence from these proteins have the following accession numbers: Ag85B, P12942; rdESAT6, CAE55648; rdESAT6-like protein 9, CAA16104; CFP-10, CAA17966; and RV 2628, NP_217144. Some of these proteins have already been mapped (1, 15, 18). RV 2628 was chosen as one of the proteins encoded by dormant operons that are particularly immunogenic in infected subjects (5).

Figure 2 shows that numerous stimulatory peptides can be

| Assay          | 3   | 1  | 4  | 5  |
|----------------|-----|----|----|----|
| ICS            | 16–40 | 2–10 | 2–10 | 2–10 |
| ELISPOT        | 1–5  | 2–10 | 2–10 | 1–5 |
| Proliferation  | 1–5  | 2–10 | 2–10 | 2–10 |
| Cell-ELISA     | 1–5  | 1–5 | 1–5 | 1–5 |

* PBMCs containing 0.5% (donor 3) and 1% (donors 1, 4, and 5) CMV NV9 pentamer-positive CD8 cells were serially diluted into autologous, irradiated, nonresponding PBMCs and tested by ICS, ELISPOT, proliferation, and cell-ELISA assays.

**TABLE 1. Comparative sensitivity of different assays**

| Donor | Dilution | No. of NV9-positive CD8 T-cells | IFN-γ (pg/ml) | IFN-γ (pg/50 μl) | IFN-γ (pg/cell) |
|-------|----------|---------------------------------|--------------|-----------------|----------------|
| 3     | 1:1      | 250                             | 10,400       | 520             | 2.1            |
|       | 1:5      | 50                              | 1,120        | 56              | 1.1            |
| 1     | 1:1      | 500                             | 32,700       | 1,610           | 3.2            |
|       | 1:5      | 100                             | 7,440        | 372             | 3.7            |

* PBMCs from donors 3 and 1 were used undiluted or diluted 1:5 into autologous, nonresponding PBMCs. Cultures stimulated with NV9 peptide were set up in 384-well plates. The number of NV9-positive CD8 cells was determined based on the pentamer assay (0.5% for donor 3; 1% for donor 1).

**TABLE 2. IFN-γ produced by NV9-specific single CD8 T-cells from PBMCs**

**TABLE 3. IFN-γ produced by single cells from an HIV gp120-specific CD4 T-cell line**

| No. of HIV-specific T cells/well | IFN-γ (pg/ml) | IFN-γ (pg/50 μl) | IFN-γ (pg/cell) |
|---------------------------------|---------------|-----------------|----------------|
| 100                            | 5,400         | 270             | 2.7            |
| 10                             | 256           | 13              | 1.3            |
| 1                              | 30            | 1.5             | 1.5            |
| 0                              | <20           | ND              | ND             |

* Different numbers of cells (100, 10, 1, and 0) from the HIV-specific T-cell line were stimulated with antigen in the presence of autologous, irradiated, nonresponding PBMCs. The IFN-γ concentration and IFN-γ amount per well were calculated to determine cytokine production per cell. IFN-γ produced by one specific CD4 T-cell ranged from 1.3 to 2.7 pg. ND, not determined.

defined. These experiments on three donors were not run to provide a thorough epitope mapping for these antigens, but rather as a proof of principle that cell-ELISA is a valuable tool to approach epitope mapping in a miniaturized format (1,536-well plates) by using a small number of PBMCs. In fact, 105 peptides could be tested in duplicate with 2 million PBMCs only.

**Suitability of cell-ELISA to analyze clinical samples.** Blood samples of 5 ml were also sufficient to test a broad panel of recall antigens to appreciate the immunocompetence of selected patients. Results are shown for three tuberculosis patients with active infection and for three HIV patients with active tuberculosis infection. We obtained extended profiles of specific responses (13 antigens in duplicate) with 1.3 × 10^4 PBMCs only (Fig. 3). It can be noted that the lack of response to the mycobacterial antigens purified protein derivative (PPD), rdESAT6, and Ag85 in HIV-positive patient donor 9 does not imply a generalized immunodeficiency. In fact, responses to CMV antigens (CMV lysate, CD4, and CD8 peptide pools), to *C. albicans*, and to *Aspergillus fumigatus* were present.

**Cytokine profiling on cell-ELISA supernatants.** Culture supernatants from cell-ELISA can be transferred to a replica plate. Supernatants from positive wells, according to cell-ELISA, can be screened later for extended cytokine profiles by using available micromethods. Figure 4 shows examples of cytokine profiles determined by Cytomix or by Luminex assays on 10 μl of supernatants from three CD4 T-cell lines specific for CMV, for an immunodominant CMV pp65 peptide (pep30; amino acids 117 to 131), and for TT and from two CD8 T-cell lines specific for the NV9 peptide of CMV pp65. The results indicate that most cytokines were produced only upon antigen stimulation and therefore were informative to characterize positive responses. In contrast, other cytokines (e.g., IL-8) with high background production in the absence of antigen (Fig. 4, black bars) were poorly informative. These cytokine profiles do not mirror profiles observed with T-cell lines specific for different antigens and with antigen-stimulated PBMCs (data not shown), suggesting that these profiles are markers of individual T-cell responses and cannot be easily predicted.

**Comparative IFN-γ concentration by cell-ELISA and conventional ELISA.** The possibility of using supernatants from cell-ELISA cultures to test IFN-γ by conventional ELISA or by other high-throughput methods is confirmed by data shown in
Fig. 5. PBMCs from donor 1 were stimulated with TT, PPD, and CMV and with pp65 immunodominant peptides 45, 62, and 128 (6). Cell-ELISA wells were developed in parallel with a conventional ELISA performed on supernatants from the same cultures. IFN-γ concentrations detected by ELISA (Fig. 5, gray bars) in the supernatants were comparable to or slightly lower than concentrations detected by cell-ELISA (Fig. 5, black bars). This demonstrates that only a small fraction of the cytokine can be sequestered by the solid-phase antibody present in the cell-ELISA plates, thereby remaining available for subsequent cytokine assays based on ELISA-like methods.

Fig. 2. Epitope mapping by cell-ELISA. PBMCs from three PPD-responsive, healthy subjects were dispensed into 1,536-well plates into which overlapping peptides of mycobacterial Ag85 protein (31 peptides), rdESAT6 protein (17 peptides), rdESAT6-like protein 9 (17 peptides), CFP-10 protein (18 peptides) and RV 2628 protein (22 peptides) had been dispensed. Plates were processed as described. Results are shown as the optical density (OD) at 405 nm at 1 h. Several peptides can be identified in all of the proteins as stimulatory for T-cell responses in the tested subjects (donors 6, 7, and 8). The dotted line indicates an arbitrary threshold set at four times the background level without antigen.

Fig. 3. Testing a broad panel of recall antigens with limited numbers of PBMCs. Thirteen antigenic preparations plus PHA were dispensed in duplicate into 384-well plates and tested with 5 × 10^4 PBMCs/well from patients with active tuberculosis with (HIV+) or without (HIV−) HIV infection. Antigens included CMV lysate, CD4 and CD8 peptide pools from CMV pp65, TT, mycobacterial proteins (PPD, rdESAT6, Ag85), fungi or protozoa (Candida albicans [Ca], Aspergillus fumigatus [Asp fum], Aspergillus niger [Asp niger], Cryptococcus neoformans [Cry], Pneumocystis carinii [Pc], and Toxoplasma gondii [Toxo]). Results are shown as IFN-γ pg/ml ± the standard deviation.
DISCUSSION

Improved methods to test T-cell immunity are highly desirable for both basic and clinical immunology. A drawback of current assays is that the number of PBMCs required to test each antigen limits the antigenic analytical breadth. This must be kept in mind, considering that patients who most often need to be examined for cellular immunocompetence are severely lymphopenic. This is also a problem for pediatric patients, from whom small blood samples are available. In addition, when proteins need to be dissected with panels of overlapping peptides to identify immunodominant sequences (19), large numbers of lymphocytes from the responding subjects are required (12, 16). Therefore, assay miniaturization can help enhance the capacity to define the specific T-cell response with the available PBMCs and to extend the analytical power of epitope mapping.

Here we described the miniaturization of the immunoenzymatic assay we have previously reported (8), which allows testing of a single antigen with as few as 10^4 PBMCs cultured in a volume of 10 μl complete medium in 1,536-well plates. The wells, precoated with a monoclonal antibody specific for a given cytokine (e.g., IFN-γ), received the antigens to be tested and were frozen for months without a loss of function. PBMCs were seeded in the thawed plates and cultured for 2 days. At the end of the culture period, the supernatants could be saved for later cytokine profiling and the wells were developed by conventional ELISA to reveal the captured cytokine produced by antigen-activated T cells.

All of the procedures based on the use of 1,536-well plates were automated by using liquid handlers and dispensers, in addition to appropriate scanners. In fact, while automated handling of reagents and cells in 384-well plates is advisable (8), it becomes mandatory in 1,536-well plates. In order to use a cell density similar to that of conventional 96-well plates, we dispensed 10^4 PBMCs in 10 μl per well. This value may represent the lowest threshold for a miniaturized T-cell assay. In fact, the frequency of antigen-specific T cells is the limiting factor (4, 11). Assuming a reasonable frequency of 1% to 0.1% of T cells specific for a given recall antigen, we need at least 10^3 PBMCs to have one specific T cell per well giving a positive signal. Since our method has a sensitivity of around five cells...
per well, we need at least $5 \times 10^5$ PBMCs per well. We may need even more cells if we consider the expected frequencies of CD4 T cells, which are generally lower than the frequencies of CD8 T cells (16).

The frequency of specific T cells in PBMCs cannot be defined with accuracy because cellular standards are not easily available. Therefore, we used established CD4 and CD8 T-cell lines as possible standards, since they contain 100% specific T cells and can be spiked into nonresponding PBMCs to test assay accuracy. In addition, established T-cell lines can also be used to compare sensitivity thresholds of different assays, as previously reported (8). These arguments have relevant implications when it comes to the definition of accuracy and precision of T-cell assays for clinical purposes.

Another point that is worth considering is whether the frequency of cytokine-producing specific T cells is more or less relevant than the overall amount of effector cytokines produced at the inflammation site. While functional in vitro assays that enumerate specific T cells, including ICS, ELISPOT, and cytokine capture assays, cannot appreciate cytokine concentrations when it comes to the definition of accuracy and precision of T-cell assays for clinical purposes.

The high-throughput approach can also be applied to epitope mapping. Automation allows simultaneous manipulation of numerous 1,536-well plates, thereby permitting us to test in principle, thousands of different peptides. To underline the analytical power of the method, we should keep in mind that a 1,536-well plate corresponds to sixteen 96-well plates. At the analytical power of the method, we should keep in mind that a 1,536-well plate corresponds to sixteen 96-well plates, thereby permitting us to reach a consensus on this issue.

In order to test broad antigenic panels with limited amounts of PBMCs, peptide pools arranged as matrices can be used (3, 13). Nevertheless, this approach requires sequential steps to define the individual immunodominant peptide(s). Alternatively, cell-ELISA can be used as a high-throughput approach for epitope mapping by testing all of the individual peptides in one shot. As a proof of principle, we have shown here that this is feasible by using small mycobacterial proteins. An additional advantage of cell-ELISA is represented by the fact that saved supernatants can be tested for extended cytokine profiles using high-throughput methods based on flow cytometry or on proteomic microarrays that are required when small volumes are available for testing.

In conclusion, extension of the cell-ELISA assay from the 384- to the 1,536-well format provides a useful tool for analysis of T-cell immunity and for epitope mapping, thanks to the small number of required PBMCs. In addition, the sensitivity of the assays to low frequencies of specific cells and the possibility of defining cytokine profiles, coupled with automation for HTS, suggest that this assay may have numerous applications as a complement to other current methods for the measurement of antigen-specific T-cell responses.

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