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Estimation of low frequency antigen presenting cells with a novel RELISPOT assay

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Abstract

Adequate presentation of self and foreign antigens is a key factor for efficient T-cell immunosurveillance against pathogens and tumors. Cells presenting foreign antigens usually comprise a rare population and are difficult to detect even at the peak of infection. Here we demonstrate a CD8⁺ T-cell-based approach that allows detection of specific antigen-presenting cells (APC) at a frequency of less than 0.0005%. When T cells are in excess, they form rosettes with rare APCs, which appear as single spots in an IFN- γ ELISPOT assay. Using this RELISPOT (Rosette ELISPOT) method we demonstrate the dynamic interplay between CD8 T cells and professional and non-professional APCs following virus challenge.

1. Introduction

The efficiency of antigen presentation by MHC molecules and the frequency of antigen presenting cells (APC) are crucial elements of a successful T-cell immune response to a pathogen. Detailed knowledge of these parameters is critical for a thorough understanding of normal immunological processes as well as for understanding and combating the subversion of these processes by certain tumors and viruses. Enumeration of professional APCs such as dendritic cells (DCs) bearing a given MHC-peptide complex is particularly difficult, since DCs comprise only a small population of leukocytes and only a minute fraction of these DCs presents a given antigenic peptide to T cells when the T-cell immune response is initiated (Porgador et al., 1998; Norbury et al., 2002). Additionally, specific MHC-peptide complexes expressed on the surface of DCs sometimes number only a few per cell (Demotz et al., 1990), which is below the level of detection of the assays currently in use. Antibody-based detection of cells expressing specific MHC-peptide complexes is possible (Porgador et al., 1997; Polakova et al., 2000; Denkberg et al., 2002), but often presents a significant challenge due to low cell-surface expression of the specific MHC-peptide complexes. Additionally, antibodies with TCR-like specificities are difficult to generate. Another option for detection of specific APCs, engineering of high avidity multivalent recombinant TCR molecules (Plaksin et al., 1997; Subbramanian et al., 2004), also requires significant effort. Both the antibody and multivalent-

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TCR methods of detection require the expression of relatively large numbers of MHC-peptide complexes on the cell surface, which is not the case for the majority of antigens.

However, in the natural immune response, both CD4⁺ and CD8⁺ T cells are capable of recognizing very small numbers of MHC-peptide complexes on the surface of the APCs (Demotz et al., 1990), and therefore the most logical choice would be to use the T cells themselves as the mechanism of detection. T-cell lines and clones as well as T cells from TCR-transgenic animals have been used for some time for the detection of APCs, using cytokine production (Belyakov et al., 2004) (Somasundaram et al., 2004) and T-cell proliferation (Somasundaram et al., 2004) as readouts for the presence of APCs presenting a specific peptide. The most sensitive method of APC detection published to date is in fact based on T cell-APC interaction (Karttunen et al., 1992). This method uses an antigenspecific T-cell hybridoma that contains a lacZ reporter construct that becomes activated upon TCR engagement, thus allowing enumeration of T cells that have contacted APCs. However, since more than one T cell can be in contact with a given APC, the number of lacZ positive cells is not likely to directly represent the number of APCs. The number of antigen presenting cells can be estimated indirectly by the generation of a calibration curve (Karttunen et al., 1992).

The common limitation of the current assays that use T cells as readout is that these assays do not allow the direct estimation of the exact number of APCs in a test sample, thus providing only relative levels. Here, we demonstrate a new, higher-sensitivity approach for detection of the actual frequency of APCs using a modified IFN- γ ELISPOT assay and an antigen-specific CD8⁺ T-cell line. When T cells are in excess, they form rosettes with the APCs presenting the cognate antigen and become activated to secrete IFN- γ . The resulting spots can provide a quantitative measure of the number of peptide-presenting APC in a given sample.

2. Methods

2.1 Mice, cell isolation and cell lines

BALB/c mice, 4–6 weeks old, were obtained from Frederick Cancer Research Facility (Frederick, MD). Mice were used following National Institutes of Health guidelines and Institutional Animal Care and Use Committee-approved animal protocols

P815 cells were grown in complete media (CM) consisting of RPMI-1640 supplemented with 10% FCS. Only adherent P815 cells were used for all assays. DCs were grown from bone marrow by cultivating in CM with 1 μ g/ml GM-CSF for 5 days. The P18-I10-specific T-cell line MHb (Derby et al., 2001a) was maintained in CM containing 10% T-Stim Culture Supplement (BD Biosciences, San Jose, CA). Every 10 days T cells were restimulated with addition 0.5 nM P18-I10 peptide and irradiated splenocytes from BALB/c mice.

2.3 Viruses and peptides

VSC8 (Chakrabarti et al., 1985) is a TK-negative attenuated vaccinia virus. vPE16 (Earl et al., 1990) expresses the gp160 IIIB determinant of HIV, which contains the H-2D^d-restricted CTL epitope RGPGRAFVTI (P18-I10) (Takahashi et al., 1988; Takahashi et al., 1989). These were kindly provided by Drs. P. Earl and B. Moss. DCs and P815 cells with greater than 99% viability were infected with sonicated viruses at a concentration of 10⁷ cells/ml in PBS/ 0.5% BSA at a multiplicity of infection of 5 pfu/cell for 30 min. Afterwards, a 10X volume of CM was added to the samples and incubated for additional 15–18h. The P18-I10 peptide from gp160 HIV IIIB was purchased from Multiple Peptide Systems (San Diego, CA) at >95% purity.

2.4 Infections and immunizations

BALB/c mice were infected intraperitoneally (i.p) or in the footpad with 2×10^7 PFU of virus in 0.1 ml phosphate-buffered saline (PBS). At different time points after virus challenge, cells from draining lymph nodes, spleens and ovaries were isolated using a solution of 5 mg/ml Collagenase D (Roche Applied Science, Indianapolis, IN) and 20 µg/ml DNase I (Sigma, St. Louis, MO) in CM. The erythrocytes in the spleen were lysed with ACK lysing Buffer, (Cambrex, Walkersville, MD).

2.5 Ex-vivo ELISPOT Assay

HTS plates (Millipore, Billerica, MA) were incubated overnight with capture anti-IFN- γ antibody (MABTECH, Cincinnati, OH). Plates were washed and blocked for 1h with CM. P815 target cells were pulsed with 1 µM P18-I10 peptide for 1h and washed twice. Target and effector cells were transferred to the ELISPOT plate. After 24h of co-cultivation, spots were developed with a biotinylated anti-IFN- γ antibody (MABTECH, Cincinnati, OH), Vectastain ABC kit (Vector Laboratories, Burlingame, CA), and an AEC, 3-amino-9-ethylcarbazole, substrate kit (Vector Laboratories, Burlingame, CA), according to the manufacturer's instructions.

2.6 RELISPOT Assay

ELISPOT plates were prepared as for the ELISPOT assay described above. Prior to the assay the MHb T-cell line was rested overnight in RPMI 1640 media with 1% FCS to prevent non-specific IFN- γ release. 3×10^5 MHb T-cells and different number of peptide-pulsed or virus antigen-presenting cells were added to each well; for initial titration experiments 20 to 3200 of P815 cells were added per well; for the detection of *in vitro*-infected P815 cells we used 10–10000 cells per well; for detection of virus-presenting cells in ovaries 500–500,000 cells per well; for lymph node dendritic cell experiment 500–50,000. All APCs were titrated down from the maximal to minimal number indicated for particular experiments. The values used for analysis of the data (except for titration experiments) were from those wells, numbers of spots in which did not exceed 100 per well. Dendritic cells were either used alive or were fixed in 0.5% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 min. at 10°C and washed 3 times in 50ml of PBS. T cells and APCs were incubated together for 18–24 h and developed as described in above.

2.7 Antibodies, tetramer assays and flow cytometry

All flow cytometry samples were analyzed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). P18-I10 complexes on the surface of infected cells were analyzed with the KP15 mAb, which specifically recognizes the H-2D^d-P18-I10 peptide complex (Polakova et al., 2000), using a three-step amplification method with biotinylated secondary antibody (BD Pharmingen, San Jose, CA) and PE-labeled Streptavidin (BD Pharmingen, San Jose, CA) as previously described (Dzutsev et al., 2007).

To measure antigen-specific T cells from ovaries, were stained with the P18-I10-H-2D^d tetramer provided by the NIAID Tetramer Core Facility and anti-CD8 α MAb (eBiosciences, San Diego, CA) in PBS with 1% BSA in the presence of anti-CD16/CD32 MAb (BD Pharmingen, San Jose, CA). These T cells were not used in the RELISPOT assay.

2.8 Microscopy

P815 cells were incubated with 1 µM P18-I10 peptide in CM for 1h and labeled with 2 µM CFSE (Molecular Probes, Carlsbad, CA) for 15 min at 37°C in PBS with 1% FCS, followed by two washes in CM. P18-I10 specific T cells were labeled with PKH26 Red Fluorescent Cell Linker (Sigma, St. Louis, MO) according to the manufacturer's instructions. Labeled cells were

mixed in different ratios to replicate conditions during ELISPOT and RELISPOT assays. Cells were transferred into a clear flat bottom plate and incubated for 1 h at 37°C. Following the incubation, undisturbed cells were visualized using a Nikon Eclipse TE300 Fluorescence microscope, and images obtained were analyzed using ImageJ software (NIH).

3. Results

3.1. Antigen-specific CD8⁺ T cells form clusters around antigen-presenting cells and appear as single spots in IFN- γ ELISPOT assay

We established the experimental system using a previously characterized high-avidity CD8⁺ T cell line, MHb (Derby et al., 2001b), which recognizes the P18-I10 epitope from gp160 HIVIIIB in complex with H-2D^d (Takahashi et al., 1988). This line was previously shown by CTL assay to detect APCs pulsed with as little as 0.1 nM of the P18I10 peptide (Derby et al., 2001b). To establish whether we could determine the frequency of APC presenting this epitope, we co-cultured 25–400 P18-I10 peptide-pulsed P815 cells with 3×10^5 MHb cells per well in a standard IFN- γ ELISPOT assay. In the presence of excess antigen-specific T cells, APCs bind to multiple T cells and form rosettes (Fig. 1a). Analysis of the plate revealed that spots are generally much larger and of higher intensity than in a traditional ELISPOT assay (Fig. 1b,c), although there is an overlap in the size distribution (Fig. 1c and f). The spots in the majority of IFN- γ ELISPOT assays range in size from, 5000 to 10000 μm^2 (Czerkinsky et al., 1983; Taguchi et al., 1990) while the average size observed in the Rosette ELISPOT (RELISPOT) assay is 10000–20000 μm^2 (Fig. 1c). Reproducing the conditions of the RELISPOT assay in a clear polystyrene plate, we could observe, using a fluorescence microscope, that multiple T cells form large rosettes with the antigen presenting cells, which likely accounts for the large size of the spots (Fig. 1a,d). Whether the T cell/APC rosettes include multiple antigen-specific CD8⁺ T cells or just a single T-cell-APC conjugate, they should appear as single spots in the IFN- γ RELISPOT assay.

We determined the accuracy and sensitivity of this assay by titrating the number of APC added per well. Until saturation is reached at 200 APC per well, we detect a number of spots that nearly exactly matches the number of APC added (Fig. 1g). Titration of the APCs is critical, due to the limited surface area of the well and the large size of the spots. We found that the most accurate determination of APC number can be obtained with ≤ 100 spots per well (Fig. 1g, h). We were able to detect as few as 2–5 specific APCs per million cells, and the same result was obtained when the total number of APC was held constant by dilution with unpulsed APC (data not shown).

3.2. Estimation of the frequency of vaccinia virus-infected P815 cells in vitro

After establishing the sensitivity of this technique, we then went on to demonstrate that the RELISPOT method can be used to determine the number of APC presenting viral antigen in a culture of vaccinia virus-infected cells. The H-2D^d-restricted P18-I10 epitope is expressed by the gp160-containing recombinant vaccinia virus vPE16 and can be detected (Dzutsev et al., 2007) using the KP15 antibody, which recognizes the P18-I10/H-2D^d complex (Polakova et al., 2000). In the culture of P815 cells 24h post infection with vPE16 all KP15 positive cells were also gp120 positive (Fig. 2b); whereas VSC8-infected cells were negative by both KP15 and anti-gp120 staining (Fig. 2a). To be able to demonstrate the accuracy of the method we infected P815 cells with a suboptimal dose of vPE16 virus and either co-cultured with MHb cells in a RELISPOT assay (Fig. 2d) or analyzed by flow cytometry (Fig. 2c). Both assays detected a similar percentage of APCs (Fig. 2c,d), demonstrating the ability of the RELISPOT assay to accurately quantify infected cells presenting specific MHC-peptide complexes.

3.3. The kinetics of antigen presentation and CD8⁺ T cell infiltration in the ovaries of vPE16 vaccinia virus-infected mice

We next determined the effectiveness of this technique for estimating the number of P18-I10-antigen-presenting cells following *in vivo* infection of mice with vaccinia virus. Mice were infected for one to ten days with either control vSC8 virus or vPE16. Ovaries were isolated and digested with collagenase, and the resultant cell suspension was analyzed with the P18-I10-specific RELISPOT assay. At day 5 post-infection we could detect multiple P18-I10 presenting cells in the ovaries of mice infected with vPE16 (Fig. 3a, left side) but not in those of vSC8-infected mice (Fig. 3a, right side), again demonstrating the specificity of the RELISPOT assay.

Antigen-presenting cells in the ovaries probably represent a mixture of infected ovarian cells and antigen-crosspresenting dendritic cells and macrophages. In order to analyze the interplay between antigen-specific CD8⁺ T-cells and virus-infected cells in the ovaries of vaccinia virus-infected mice, we performed a time course study in which we examined both the number of viral antigen-presenting cells and the number of CD8 T cells per ovary, including the number of infiltrating CD8 T-cells responding to the dominant P18-I10 epitope itself, estimated by flow cytometry, at various days post-infection. VPE16-presenting cells could be detected by RELISPOT in the ovaries (approximately 700 cells per ovary or 0.03% of total ovarian cells, which was more than 50-fold over the detection limit) as early as day 3 post-infection, a time point at which P18-I10-specific T cells were undetectable by tetramer staining (Fig. 3b). The peak number of P18-I10-presenting cells occurs at 5 days post-infection, which also corresponds to the peak viral load in the ovaries of mice infected with vPE16 (approximately 10⁸ pfu (Belyakov et al., 1998)). Interestingly, at the 5-day time point, P18-I10-presenting cells represent only 0.5% of the total ovarian cell population, a percentage comparable to that of P18-I10-tetramer positive CD8⁺ T cells present in the ovary (open circles, Fig. 3b). On day 5, at the time, when viral-presenting cells reach a peak, the number of total CD8⁺ T-cells already comprise approximately 5% of ovarian cells and exceeds the number of infected and other antigen-presenting cells by more than 5-fold. Maximum infiltration of the ovaries with CD8⁺ T-cells, 5×10⁵ per organ, was observed at day 6 (black squares, Fig. 3b); among these CD8 T-cells 10⁵ cells were P18-I10-specific (black triangles, Fig. 3b). The percent of total CD8 T-cells at day 6 reaches approximately 20% of the total ovarian cell population and exceeds the number of cells presenting viral antigens by more than 100-fold. Interestingly, on day 7 when the percentage of infected cells is down to less than 0.01%, total CD8⁺ T cells still comprise 10% of the cells in the ovaries, of which approximately 1% are specific for P18-I10.

3.4. Estimation of the frequency of antigen-presenting dendritic cells in the draining lymph nodes of mice immunized with vPE16

Dendritic cells are known to be the primary APC capable of initiating the T-cell immune response. Therefore, the ability to accurately enumerate the frequency of antigen-presenting DC is important for a thorough understanding of T-cell priming *in vivo*. To investigate this, we analyzed the number of antigen-presenting DCs in the draining lymph nodes of mice infected in the footpad with 2.5×10⁷ pfu vPE16 or VSC8 control virus. To assure quantitation of only the dendritic cell population, CD11c⁺ cells were purified from individual LNs by magnetic bead separation prior to the RELISPOT assay. We initially found that vaccinia virus-infected DCs could non-specifically activate T cells to produce IFN-γ. (Fig. 2e, black bars), possibly by secretion of cytokines as previously noted in other systems (Kambayashi et al., 2003). We subsequently performed fixation of DCs with 0.5% paraformaldehyde for 15 minutes prior to incubation with T cells, as has been utilized previously by others (Karttunen et al., 1992; Norbury et al., 2002). Fixation proved critical for background reduction, completely eliminating non-specific spots without affecting the antigen-specific response (Fig. 4a, grey bars) or the accuracy of the assay, which was tested separately using P18-I10 peptide-pulsed

and fixed bone marrow-derived DCs using the same methods described earlier for P815 cells (data not shown).

Interestingly, when we used this approach to examine various lymphoid organs for the presence of P18-I10-presenting DCs, at 18–20 hours post-infection, we found that antigen presenting DCs are concentrated mostly in the popliteal LN (500–600 P18-I10-presenting DCs per LN or approximately 0.06% of total LN cells) and are almost absent in the spleen and other draining LNs (Fig. 4b).

4. Discussion

Our study describes a new approach for easy estimation of very low frequencies of cells presenting a specific antigen-MHC complex, and further demonstrates the utility of this method for the detection of infected cells and antigen-presenting dendritic cells *in vivo* after challenge with vaccinia virus. The sensitivity of the described method (0.001–0.0005% of total cells) is much higher than any other previously reported T-cell-based approach. It also directly provides the actual number of antigen presenting cells without the need for calibration curves.

This method possesses certain advantages and disadvantages common to ELISPOT assays in general. The high sensitivity of the RELISPOT assay is coupled with an inability to directly assess the phenotype of the APCs detected. However, magnetic bead separation of different cell populations prior to the RELISPOT assay may overcome this limitation. It is important to bear in mind that the RELISPOT assay detects both types of APCs - the actively infected cells and the uninfected cells that take up and cross-present viral antigens.

Additionally, obtaining an appropriately high-avidity T-cell line that can detect low levels of peptide-MHC complexes may provide a challenge; however the availability of TCR-transgenic mice and the use of various sorting techniques (Brosterhus et al., 1999; Yee et al., 1999) may significantly improve yield and shorten the time required for the generation of new T-cell lines. For certain experimental systems, it may also be possible to use CD8⁺ T-cell-enriched cell populations from virus-immunized animals, since, during the acute phase of infection, virus-specific T cells may comprise at a large percentage of CD8⁺ T cells (Moutaftsi et al., 2006).

The potential applications of the RELISPOT method are numerous, ranging from the practical, such as estimating the efficiency of low-frequency gene transfection, to the study of more complicated immunological questions. For example, the ability of this assay to directly estimate numbers of virus-infected cells may provide a better understanding of the *in vivo* immune response to a virus infection than may be gleaned through an indirect measurement such as viral load, since CD8⁺ T cells recognize infected cells but not the viruses themselves. Using the RELISPOT approach, we observed that at the peak of a vaccinia virus infection, the ovaries contain approximately 2×10^4 infected cells, whereas the usual number of infectious viral particles detected in the ovaries is almost 4 logs higher, 10^8 pfu. This difference may reflect both the number of virus particles per infected cell, and a large amount of cell-free virus. Because the ratio of virus particles to infected cells can vary over time, the viral load cannot be used as a substitute for a measure of the number of infected cells. The RELISPOT method is advantageous in that it gives a more accurate picture of the actual number of infected cells that need to be recognized and destroyed by T cells in order to clear the infection. The RELISPOT assay could also allow one to address questions of biology and migration of dendritic cells with much higher sensitivity than has been possible to date. Since only a very small fraction of dendritic cells present a particular antigen in the lymph node, the sensitivity of this assay makes it a valuable new tool for studying DCs.

In conclusion, we believe that the very high sensitivity and precision of the RELISPOT assay make it a valuable tool for use in many studies involving antigen presentation and antigen

presenting cells. The intrinsic ability of T cells to detect only a few peptide-MHC complexes per cell and just a few cells among millions may expand our ability to study more delicate and rare events, and ultimately contribute to the comprehensive understanding of processes critical for T cell-based immunosurveillance and host defense.

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Abbreviations used

APC, Antigen-Presenting Cell; ELISPOT, Enzyme-Linked Immunosorbent Spot; RELISPOT, Rosette ELISPOT; DC, Dendritic Cell; LN, Lymph node.

FIGURE 1.

Rosettes of CD8⁺ T cells with APCs produce single spots in an IFN- γ RELISPOT assay. a) P18-I10 specific T cells (green), when added in excess of antigen presenting cells (red), form rosettes that contain the target cell and one or more T cells. b,c) The rosettes appear as large single spots. in the RELISPOT assay, in contrast to the much smaller spots observed in a traditional ELISPOT assay (e,f), in which the APCs are in excess (d). g) Estimation of the maximal number of APCs that can be added to the well without negatively affecting the result. h) Percent of total APCs detected by RELISPOT (calculation from panel g). At greater than 200 APCs per well, results are not interpretable due to the large size of the spots.

FIGURE 2.

RELISPOT can detect viral antigen-presenting APC following *in vivo* infection with recombinant vaccinia virus. a,b) KP15-positive cells (P18-I10-presenting cells) are also gp120 positive. Cells were infected either with vPE16 virus (b) expressing gp120 or control vSC8 virus (a). d) Confirmation of assay specificity. P815 cells were infected with vSC8 or vPE16 *in vitro* and analyzed by RELISPOT assay (black bars) or by staining with the KP15 mAB, which specifically recognizes the H-2Dd-P18-I10 peptide complex (grey bars).

FIGURE 3.

Kinetics of the numbers of the cells presenting viral antigens and CD8 T-cells in the ovaries during the first 10 days post-infection, a) Typical wells in a RELISPOT assay of the ovaries of BALB/c mice 5 days post-IP infection with vPE16 (a, left side) or vSC8 control virus (a, right side). b) Kinetics of the viral replication in the ovaries. Antigen presenting cells presented by open circles projected on the right Y-axis. Numbers of total CD8⁺ T-cells (black squares) and P18-I10 specific CD8⁺ cells infiltrating ovaries projected onto the left Y-axis. Representative experiment of 2.

FIGURE 4.

Estimation of the numbers of professional APCs. a) Dendritic cells require fixation prior to analysis in the RELISPOT assay. Dendritic cells were isolated by anti-CD11c magnetic bead separation from popliteal LNs of mice infected through the footpad with control vSC8 or vPE16. Without fixation DCs induced a high background (black bars), but fixation with 0.5% PF (grey bars) completely eliminated the background without affecting the results. b) Quantification of P18-I10-presenting DCs in spleen and lymph nodes 20h post-footpad inoculation with vPE16 virus.