Quantification of Antigen-Reactive T Cells by a Modified ELISPOT Assay Based on Freshly Isolated Blood Dendritic Cells

M. Schmitz,¹ J. Rohayem,² R. Paul,¹ B. Weigle,¹ A. Stein,³ and E.P. Rieber^{1*}

¹*Institute of Immunology, Medical Faculty, Technical University, Dresden, Germany*

²*Institute of Virology, Medical Faculty, Technical University, Dresden, Germany*

³*Department of Dermatology, University Hospital Carl Gustav Carus, Dresden, Germany*

The enzyme-linked immunospot (ELISPOT) assay has become a widely employed method for quantification of antigen-reactive T lymphocytes. In recent years, various types of antigen-presenting cells (APCs) have been tested as stimulator cells in ELISPOT protocols to achieve a highly sensitive and rapid assay which is not impaired by a marked nonspecific cytokine release. However, the currently available APCs still have disadvantages, such as significant background reactivities, limited sensitivity, and time-consuming preparation procedures. Recently, we succeeded in defining a novel subpopulation of circulating dendritic cells (DCs) that can easily be prepared from human blood. These M-DC8+

DCs proved to be very effective in the induction of antigen-specific T cell responses. In the present study we provide evidence that M-DC8+ DCs are particularly well suited as APCs for the detection of antigen-specific CD8+ T cells after challenge with viral or tumor peptides in ELISPOT assays. In addition, protein-loaded M-DC8+ DCs proved to be quite efficient in the presentation of MHC class II-bound peptides, thus allowing the determination of frequencies of antigen-reactive CD4+ T cells. The use of M-DC8+ DCs as stimulator cells can improve the ELISPOT assay by combining high sensitivity, rapidity, and low background reactivity. *J. Clin. Lab. Anal.* 16:30–36, 2002. © 2002 Wiley-Liss, Inc.

Key words: ELISPOT assay; T cells; dendritic cells; interferon-gamma; immune monitoring

INTRODUCTION

Over the past decade, molecular identification and characterization of human tumor antigens have led to the development of peptide-based tumor vaccines, which are evaluated in clinical trials (1,2). These approaches are based on the finding that cytotoxic T lymphocytes (CTL) recognize peptides derived from tumor-specific antigens presented on the surface of cells by MHC class I molecules (3). Numerous studies have clearly demonstrated that tumor cells are sensitive to CD8+ T cell-mediated killing, and adoptive cellular immunotherapy has been shown to be effective in the treatment of cancer (4). In addition, there is good evidence that also CD4+ T cells are involved in tumor-directed immune reactions (5). Particularly for the evaluation of vaccination trials in cancer patients, the monitoring of antigen-specific CTL and T helper cell responses by a reliable technique appears to be crucial.

Conventional methods of detecting antigen-specific T cells, such as limiting dilution analysis (LDA) and chromium release assay, are circumstantial since they require repeated in vitro stimulation by antigen, and expansion of reactive cells in the presence of growth factors (such as interleukin-2 (IL-2)) before cytotoxicity can be determined. A further drawback of these methods is that only subsets of CTL have the

capacity to proliferate in vitro. Thus, responses measured in these assays are dependent on the proliferative potential of T cells and may not exactly reflect the frequency of tumor-specific T cells in the patient's blood. Rather, they represent only a fraction of the compartment of memory T cells (6). Recently, new techniques have been developed which allow the quantification of the frequency of antigen-specific CD8+ T cells based on their capacity to specifically bind tetrameric MHC-peptide complexes (7). Alternatively, intracellular (8) or released (9) cytokines are determined at a single cell level after contact with antigen. In contrast to the limiting dilution approach, these new techniques do not require repetitive in vitro stimulation of T cells. Furthermore, the sensitivity seems to be significantly higher when compared with LDA (10).

Among the methods measuring the frequency of antigen-reactive T cells, the ELISPOT assay stands out because of its

Grant sponsor: Wilhelm Sander-Stiftung; Grant number: 99.009.1.

*Correspondence to: Ernst Peter Rieber, Institute of Immunology, Medical Faculty, Technical University of Dresden, Fetscherstr. 74, 01307 Dresden, Germany. E-mail: rieber@rcs.urz.tu-dresden.de

Received 6 August 2001; Accepted 27 September 2001

versatility and practicability (11). Basically, it determines by a capture enzyme-linked immunosorbent assay (ELISA) technique cytokines secreted by individual T cells upon recognition of peptide/MHC complexes presented on appropriate antigen-presenting cells (APCs). However, a substantial limitation of this technique is the development of antigen-independent reactions that often are hardly distinguishable from antigen-specific spots. This annoying background reactivity is particularly observed when the human leucocyte antigen (HLA)-A2.1-expressing T2 cell line is used for antigen presentation (12).

In this report we show that background reactivity can be almost completely eliminated when freshly isolated autologous dendritic cells (DCs) are used as APCs. In addition, the sensitivity of the ELISPOT assay is increased when compared with CD8⁺ T cell-depleted peripheral blood mononuclear cells (PBMCs) as APCs. DCs are known to be pivotal in the initiation of primary T cell responses against viruses, bacteria, and tumors (13–16). In addition, animal models have demonstrated that DCs are capable of inducing protective and therapeutic antitumor responses (17–19). The first clinical trials based on DCs loaded *ex vivo* with tumor antigens appeared promising (20–23). Recently, we succeeded in defining a novel DC population in human blood with the help of the monoclonal antibody M-DC8, which recognizes an epitope selectively expressed on these cells (24). With this antibody fresh DCs can be isolated at high purity from PBMCs in a one-step immunomagnetic separation procedure. The M-DC8⁺ DCs were found to be very efficient in the induction of tumor-directed CTL responses *in vitro*. In the present study, M-DC8⁺ DCs are introduced as potent APCs in the ELISPOT assay, which allow the quantitative detection of virus- and tumor peptide-specific CD8⁺ T cells and antigen-specific CD4⁺ T lymphocytes.

MATERIALS AND METHODS

Isolation of Leukocyte Subsets

Blood samples were obtained from melanoma patients and healthy HIV-seronegative donors, with informed consent. The isolation of the M-DC8⁺ DCs was performed as described previously (24). Briefly, PBMCs were prepared by Ficoll-Hypaque (Biochrom, Berlin, Germany) density centrifugation. M-DC8⁺ DCs were obtained by incubating PBMCs for 15 min at 4°C with diluted supernatant of the M-DC8 hybridoma containing 10 µg/ml of antibody. After washing with phosphate-buffered saline (PBS), 1×10^8 cells were resuspended in 500 µl of PBS and labeled with 10 µl of rat anti-mouse IgM coupled to paramagnetic microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) for another 15 min at 4°C. After washing, cells were thoroughly resuspended to avoid cell aggregation, and sorted on a VS⁺ separation column (Miltenyi Biotec). If necessary, the highly enriched cells were further purified by a second round of magnetic cell sort-

ing. Degassed, ice-cold PBS containing 1% human serum (CC pro, Neustadt, Germany) was used as the running and elution buffer. By this technique >95% pure M-DC8⁺ DCs were prepared from PBMCs containing 0.5–2% of this DC population.

CD8⁺ T cells and CD4⁺ T cells were isolated from freshly prepared PBMCs using immunomagnetic separation (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the selected CD8⁺ and CD4⁺ T cells was >95%, as determined by flow cytometry.

PBMCs were depleted of CD8⁺ T cells using magnetic cell sorting (Miltenyi Biotec) according to the manufacturer's instructions.

Peptides

The peptide GILGFVFTL, derived from influenza matrix protein (25), and the tyrosinase peptide YMDGTMSQV (26) were used in this study. The peptide ILKEPVHGV, derived from HIV reverse transcriptase (27), served as the negative control. These peptides are known to be recognized by CTL in association with HLA-A2.1. The peptides were obtained from Dr. J. Wehland, Department of Cell Biology, GBF, Braunschweig, Germany. They had been purified by reversed-phase HPLC and were further analyzed by mass spectrometry. The lyophilized peptides were solved in 100% DMSO at a concentration of 40 mg/ml, further diluted with PBS to a final concentration of 2 mg/ml, and frozen as aliquots at –20°C.

Overexpression and Purification of Recombinant Tyrosinase

cDNA coding for a 28-kDa intramelanosomal domain of human tyrosinase (aa 285–475) was generated by PCR using plasmid pcDNAI/Amp-Tyr harboring the tyrosinase full-length cDNA (kindly provided by Dr. T. Wolfel, Mainz, Germany) as template, and primers TyrN2 (5'-CACGGATCCCAT-CAGTCTTTA TGCAATGGA-3') and TyrC1 (5'-AACTGCA-GGTGATGGTATGGTATGCCA GATCCGACTCG-CTTGTT-3') (Genaxis Biotechnology, Spechbach, Germany) containing BamHI and PstI restriction sites, respectively. The PCR product was ligated into pGEM-T (Promega, Mannheim, Germany) for sequencing and then subcloned into expression vector pQE30 (Qiagen, Hilden, Germany) using the introduced BamHI and PstI sites enabling expression with N-terminal 6xHis-tag.

The recombinant tyrosinase fragment was overexpressed in *E. coli* M15[pREP4] (Qiagen), purified from cell lysates by affinity chromatography on Ni-NTA-agarose column and refolded by dialysis against buffer RFB (100 mM H₃BO₃, 10 mM Tris, pH 7.4). For use as control, a noninduced culture of *E. coli* M15[pREP4] harboring plasmid pQE30 was grown under the same conditions and processed identically.

ELISPOT Assay

Wells of MultiScreen-HA plates (Millipore, Bedford, MA) were coated with 100 μ l of mouse-anti-human interferon- γ (IFN- γ) antibody (10 μ g/ml; clone 1-D1K; Mabtech, Nacka, Sweden) in PBS. After incubation overnight at 4°C, unbound antibody was removed by six washings with PBS. The coated wells were blocked with 100 μ l RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% human serum. After 1 hr at 37°C, the blocking medium was discarded. Then 5×10^4 purified M-DC8+ DCs were plated in each well and pulsed with 100 μ g/ml of peptide or 2 μ g/ml protein in a final volume of 100 μ l/well. In addition to the tyrosinase fragment, KLH (Boehringer-Mannheim, Mannheim, Germany) and tetanus toxoid (Behringwerke, Marburg, Germany) were used in this study. After 2 hr of incubation at 37°C 2×10^5 purified CD8+ T cells or CD4+ T cells were added to each well. The cells were spun down at $150 \times g$ for 3 min and incubated for 36 hr at 37°C in a final volume of 100 μ l RPMI 1640 supplemented with 10% human serum per well. Cells were removed by six washings with PBS/0.05% Tween and 100 μ l of biotinylated detection-antibody against human IFN- γ (2 μ g/ml; clone 7-B6-1 (Mabtech)) were added per well. After 2 hr at 37°C the plates were rinsed six times with PBS/0.05% Tween. Then, 100 μ l of an avidin-biotin peroxidase complex preparation (ABC Vectastain-Elite kit, Vector Laboratories, Burlingame, CA) were added to each well at a dilution of 1/100 and incubated for 1 hr at room temperature. Unbound complexes were removed by three washings with PBS/0.05% Tween and three washings with PBS. The staining reaction was started by adding a solution of 3-amino-9-ethyl carba-

zole (Sigma, St. Louis, MO) dissolved according to the manufacturer's instructions. The reaction was stopped after 4 min by washing plates under running tap water. Spots were counted using a stereomicroscope at 40 $\times$ magnification.

RESULTS

M-DC8+ DCs as Antigen-Presenting Cells for Influenza Peptide-Reactive CD8+ T Cells

To find out whether the efficiency of antigen-specific activation of T cells in the ELISPOT assay can be increased by using freshly isolated M-DC8+ DCs as APCs, this DC subset was prepared by immunomagnetic separation in freshly drawn blood from two healthy donors expressing the HLA-A2.1 allele. The DCs were loaded with the HLA-A2.1-restricted influenza-derived peptide and then cocultured with autologous purified CD8+ T cells. As negative controls, APCs were either pulsed with HIV-derived peptide or left unpulsed. For comparison, CD8-depleted PBMCs from the same donors were used as APCs and treated identically. As can be seen in Fig. 1, T cells showed a clear-cut response against influenza peptide when M-DC8+ DCs or PBMCs were used as APCs. However, in both donors the M-DC8+ DCs induced a significantly higher rate of IFN- γ -producing CD8+ T cells when compared with PBMCs. The frequencies of CD8+ T lymphocytes secreting IFN- γ after coculture with unpulsed M-DC8+ DCs or with CD8+ T cell-depleted PBMCs varied from 1 to 5 per 2×10^5 cells (Fig. 1). The reaction of CD8+ T cells against DCs or PBMCs loaded with HIV-peptide was in the range of that observed with unpulsed APCs, thereby underlining the specificity of the assay.

Fig. 1. IFN- γ ELISPOT based on M-DC8+ DCs or CD8-depleted PBMCs for analyzing the frequency of influenza peptide-reactive CD8+ T cells: 2×10^5 purified CD8+ T cells of two healthy donors were incubated for 36 hr

with 5×10^4 of the indicated APCs, which were either pulsed with the influenza matrix peptide or an HIV peptide, or were used unpulsed. Columns represent mean values of duplicate wells; bars indicate SEM.

M-DC8+ DCs as Stimulator Cells for Tyrosinase Peptide-Reactive CD8+ T Cells

To find out whether M-DC8+ DCs are also suitable to detect antigen-specific CD8+ T cells in low frequencies, purified CD8+ T cells from two selected healthy donors were tested against a tyrosinase peptide. The donors had previously been identified as T cell responders to tyrosinase. The CD8+ T lymphocytes were cultured for 36 hr with autologous M-DC8+ DCs or PBMCs which had been pulsed with the tyrosinase peptide. HIV peptide-loaded APCs or unpulsed APCs served as negative controls. As demonstrated in Fig. 2, M-DC8+ DCs activated a substantial number of peptide-specific CD8+ T lymphocytes to secrete IFN- γ , which clearly surmounted the number of spots obtained with CD8-depleted PBMCs. None of the individuals tested showed a significant response against DCs that had been loaded with the HIV-derived peptide or left unpulsed. These results reveal the superiority of M-DC8+ DCs used as APCs in ELISPOT assays when peptide-specific CD8+ T cells occurring in low frequencies are to be determined.

M-DC8+ DCs as Antigen-Presenting Cells for Tetanus Toxoid-Reactive CD4+ T Cells

To assess the capability of M-DC8+ DCs to activate IFN- γ -secreting memory CD4+ T lymphocytes in ELISPOT assays, in vitro cocultures of protein-loaded M-DC8+ DCs with autologous CD4+ T helper cells were analyzed. The M-DC8+ DCs were purified from the peripheral blood of two healthy donors and loaded with tetanus toxoid protein or KLH protein as negative control. Autologous CD4+ T lymphocytes

were isolated by magnetic separation and were tested for reactivity against both proteins. As demonstrated in Fig. 3, a significant response against tetanus toxoid protein was detected after coculturing with M-DC8+ DCs, whereas only minimal reactivity against unpulsed or KLH protein-loaded DCs was seen.

M-DC8+ DCs as Stimulator Cells for Tyrosinase-Reactive CD4+ T Cells

To assay whether M-DC8+ DCs are also suitable as stimulator cells to detect CD4+ T cells recognizing tumor-associated antigens (TAA), a recombinant His-tagged tyrosinase protein fragment was prepared. After separation on SDS-PAGE, the purified recombinant tyrosinase fragment appeared as a single band of approximately 28 kDa as detected by Coomassie blue staining and Western blotting using anti-His antibody (Fig. 4). With the more sensitive silver staining, additional faint bands were seen (data not shown). These bands represented bacterial proteins, as they also occurred when lysates of bacteria transformed with empty pQE30 were subjected to the same expression/purification procedure. For this reason, mock preparations were included as controls when the frequency of tyrosinase-specific CD4+ T cells was assayed. The M-DC8+ DCs were purified from the peripheral blood of two melanoma patients and loaded with tyrosinase protein fragment or mock preparation. Autologous CD4+ T lymphocytes were isolated by magnetic separation and tested for reactivity against both preparations. As shown in Fig. 5, CD4+ T cells from both donors revealed a marked IFN- γ release when cocultured with tyrosinase-presenting DCs,

Fig. 2. Detection of tyrosinase peptide-reactive CD8+ T cells by IFN- γ ELISPOT based on M-DC8+ DCs or CD8-depleted PBMCs: 2×10^5 purified CD8+ T cells were incubated for 36 hr with 5×10^4 of the indicated

APCs, which were either pulsed with the tyrosinase peptide or an HIV peptide, or were used unpulsed. Each experiment was performed in duplicate. Columns represent mean values of duplicate wells; bars indicate SEM.

Fig. 3. IFN- γ ELISPOT based on M-DC8+ DCs for quantification of tetanus toxoid protein-reactive CD4+ T cells: 2×10^5 purified CD4+ T cells from two healthy donors were incubated for 36 hr with 5×10^4 of the indicated stimulator cells, which were either pulsed with the tetanus toxoid protein or an irrelevant KLH protein, or were used unpulsed. Columns represent mean values of duplicate wells; bars indicate SEM.

whereas only marginal numbers of IFN- γ -producing CD4+ T cells were observed with unpulsed DC. To exclude that the CD4+ T cell activation was mainly due to immunogenic bacterial proteins contaminating the tyrosinase preparation, DCs were pulsed with the eluate from a nickel column loaded with the lysate of mock transfected bacteria. As can be seen in Fig. 5, a distinct T cell activation was obtained against the mock eluate. However, the response was definitely lower when compared with the activation by the recombinant tyrosinase. The results indicate that the M-DC8+ DC-based ELISPOT system is quite suitable for the detection of CD4+ T cells recognizing tumor-associated proteins.

DISCUSSION

The ELISPOT assay is widely used for quantifying tumor-directed T cell responses on a single cell level in malignant diseases, and for monitoring vaccination trials. This straightforward method is characterized by its sensitivity and rapidity in the detection of memory T cells in response to antigen challenge. A close correlation between the number of spots and the number of peptide-reactive CD8+ T cells has been documented (28). In recent years, several types of APCs were assayed in ELISPOT protocols in order to achieve a highly sensitive and rapid assay that is not impaired by an inappropriately high nonspecific IFN- γ release. The commonly used TAP-deficient T2 cell line expressing HLA-A2.1 molecules proved to be suitable for loading with peptides in vitro, and to be potent in inducing spot production by single T cells

Fig. 4. Overexpression and purification of recombinant His-tagged tyrosinase. *E. coli* M15[pREP4] cells were transformed with the expression vector pQE30 containing a part of the coding region of the tyrosinase gene. To check the expression and purification of recombinant tyrosinase, cell lysates and eluates were analyzed by SDS-PAGE and Coomassie staining or Western blotting using anti-His-tag antibodies.

(29,30). However, the use of T2 cells usually results in significant background reactions which may be due to the activation of alloreactive T cells against mismatched HLA molecules (12,31). To reduce background reactivity, HLA-A2.1-transfected insect cells were generated and used as APCs in ELISPOT assays. These cells efficiently activated peptide-specific T cells and showed a significant reduction of background spots (12). However, single-cell cloning of transfected insect cells is difficult because of their strict culture requirements. Furthermore, the mean level of HLA expression on insect cells is rather low and the assay is limited to those peptides which fit into the transfected MHC class I-molecules. A further disadvantage of using MHC class I-transfected cells as APCs is the restriction to peptides recognized by CD8+ T cells, whereas CD4+ T cell reactions cannot be determined.

Recently, autologous PBMCs have been introduced as APCs which are also able to reduce unspecific reactions. Several studies demonstrated that PBMCs were effective stimulators of antigen-specific T cells in ELISPOT assays (28,32,33). More recently, DCs representing professional APCs were assayed as stimulator cells in ELISPOT assays. Monocyte-derived DCs were used for the identification of MHC class II-restricted epitopes as well as for monitoring tumor antigen-specific T cell responses during vaccination

Fig. 5. IFN- γ ELISPOT based on M-DC8+ DCs for quantification of tyrosinase-reactive CD4+ T cells: 2×10^5 purified CD4+ T cells of two melanoma patients were incubated for 36 hr with 5×10^4 of the indicated stimulator cells, which were either pulsed with the tyrosinase protein or the mock preparation, or were used unpulsed. Columns represent mean values of duplicate wells; bars indicate SEM.

trials (34,35). In addition, DCs were found to markedly increase the sensitivity of the ELISPOT technique in comparison to PBMCs (36). One major drawback of using DCs derived from peripheral blood monocytes is the time-consuming procedure required to prepare these cells. Recently, we succeeded in defining a new population of circulating DCs that can easily be isolated from PBMC preparations by the monoclonal antibody M-DC8. These cells can be used as unmodified cells which express high levels of MHC and costimulatory molecules and are capable of generating a tumor-specific CTL response in vitro. Intrigued by these observations, we designed an ELISPOT assay based on freshly isolated M-DC8+ DCs. In experiments in which purified CD8+ T cells were tested against influenza peptide, the response was found to be enhanced when peptide-pulsed M-DC8+ DCs were used as APCs, compared with PBMCs. These results confirm previous observations showing the presence of a considerable number of influenza matrix peptide-specific CD8+ T cells in the blood of normal donors (37). More interestingly, when analyzing the reactivity of purified CD8+ T lymphocytes against a well-known tyrosinase-derived tumor peptide, M-DC8+ DCs clearly increased the antigen-specific spot production when compared with CD8-depleted PBMCs. A significant background reaction against DCs without peptide or with control peptide was not seen in any of the experiments. According to these results, the ELISPOT assay based on M-DC8+ DCs proved to be very effective in detecting IFN- γ -secreting CD8+ T cells after challenge with viral or tumor peptides.

There is good evidence that tumor-specific CD4+ T cells

take over substantial immune effector functions against tumors (5). The crucial role these cells play in the activation of tumor-specific CTL has been stressed in various studies. The help provided by CD4+ T cells is effective by means of IL-2 secretion and by CD40L (CD154)-mediated stimulation of APCs, which empower them to directly activate CD8+ CTL precursors (38–40). Analysis of the frequency of CD4+ T helper cells in peripheral blood thus becomes increasingly relevant. When the M-DC8+ DC-based ELISPOT assay was applied to detect the frequency of CD4+ T cells reacting against tetanus toxoid in healthy donors, a significant response against this antigen was observed, whereas the reactivity against unpulsed or KLH protein-loaded DCs was very low. In addition, the capacity of protein-loaded M-DC8+ DCs to detect tumor antigen-specific CD4+ T cells in ELISPOT assay was evaluated for two melanoma patients. DCs were loaded with a recombinant tyrosinase fragment purified from *E. coli*. Despite an optimized purification procedure, the recombinant tyrosinase preparation contained minute contaminations of *E. coli* proteins, as detected by silver staining after separation on SDS polyacrylamide gels. Since bacterial proteins are known to be immunogenic (41), activation of CD4+ T cells by mock extracts had to be included as control. The response to the *E. coli* proteins, however, was substantially lower when compared with the activation induced by DCs loaded with recombinant tyrosinase. These results demonstrate that protein-loaded M-DC8+ DCs are very efficient in the presentation of MHC-class II bound peptides, thus allowing the quantitative determination of antigen-reactive CD4+ T cells in ELISPOT assays.

In summary, the experiments demonstrate that M-DC8+ DCs are very well suited as APCs in IFN- γ ELISPOT assays. The use of this subpopulation of circulating DCs has several advantages. Due to the dense surface expression of MHC class I and II molecules and costimulatory molecules, they are very efficient in the activation of IFN- γ -secreting T lymphocytes after challenge with peptides or whole proteins, resulting in a high sensitivity of the modified ELISPOT assay. Furthermore, these cells induce only very low background reactivities, which is favorable for the quantification of antigen-reactive memory T cells in ELISPOT assays. In addition, the M-DC8+ DCs population can easily be prepared within a few hours. These particular properties render M-DC8+ DCs attractive as APCs for the detection and quantification of antigen-specific T lymphocytes in ELISPOT assays.

ACKNOWLEDGMENTS

The technical assistance of Barbel Lobel, Karin Gunther, Antje Oehmichen, and Andreas Schwab is greatly appreciated. We thank Drs. Thomas Wolfel and Wolfgang Herr, Mainz, Germany, for assistance in the establishment of the ELISPOT technique, and Dr. Jurgen Wehland, GBF, Braunschweig, Germany, for providing peptides.

REFERENCES

- Rosenberg SA, Yang JC, Schwartzentruber DJ, et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat Med* 1998;4:321–327.
- Marchand M, Van Baren N, Weynants P, et al. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. *Int J Cancer* 1999;80:219–230.
- Townsend A, Bodmer H. Antigen recognition by class-I restricted T lymphocytes. *Annu Rev Immunol* 1989;7:601–624.
- Rosenberg SA, Yannelli JR, Yang JC, et al. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin-2. *J Natl Cancer Inst* 1994;86:1159–1166.
- Pardoll DM, Topalian SL. The role of CD4+ T cell responses in antitumor immunity. *Curr Opin Immunol* 1998;10:588–594.
- Ogg GS, McMichael AJ. HLA-peptide tetrameric complexes. *Curr Opin Immunol* 1998;10:393–396.
- Altman JD, Moss PAH, Goulder PJR, et al. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 1996;274:94–96.
- Carter LL, Swain SL. Single cell analyses of cytokine production. *Curr Opin Immunol* 1997;9:177–182.
- Taguchi T, McGhee JR, Coffman RL, et al. Analysis of Th1 and Th2 cells in murine gut-associated tissues. Frequencies of CD4+ and CD8+ T cells that secrete IFN-gamma and IL-5. *J Immunol* 1990;145:68–77.
- Murali-Krishna K, Altman JD, Suresh M, Sourdive D, Zajac A, Ahmed R. In vivo dynamics of anti-viral CD8 T cell responses to different epitopes. An evaluation of bystander activation in primary and secondary responses to viral infection. *Adv Exp Med Biol* 1998;452:123–142.
- Czerkinsky C, Anderson G, Ekre H-P, Nilsson L-A, Klareskog L, Ouchterlony O. Reverse ELISPOT assay for clonal analysis of cytokine production. I. Enumeration of gamma-interferon-secreting cells. *J Immunol Methods* 1988;110:29–36.
- Janetzki S, Song P, Gupta V, Lewis JJ, Houghton AN. Insect cells as HLA-restricted antigen-presenting cells for the interferon-gamma ELISPOT assay. *J Immunol Methods* 2000;234:1–12.
- Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 1991;9:271–296.
- Hart DN. Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood* 1997;90:3245–3287.
- Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998;392:245–252.
- Banchereau J, Briere F, Caux C, et al. Immunobiology of dendritic cells. *Annu Rev Immunol* 2000;18:767–811.
- Mayordomo JI, Zorina T, Storkus WJ, et al. Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. *Nat Med* 1995;1:1297–1302.
- Zitvogel L, Mayordomo JI, Tjandrawan T, et al. Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. *J Exp Med* 1996;183:87–97.
- Nair SK, Heiser A, Boczkowski D, et al. Induction of cytotoxic T cell responses and tumor immunity against unrelated tumors using telomerase reverse transcriptase RNA transfected dendritic cells. *Nat Med* 2000;6:1011–1017.
- Hsu FJ, Benike C, Fagnoni F, et al. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat Med* 1996;2:52–58.
- Nestle FO, Aljaghi S, Gilliet M, et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* 1998;4:328–332.
- Thurner B, Haendle I, Roder C, et al. Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J Exp Med* 1999;190:1669–1678.
- Geiger J, Hutchinson R, Hohenkirk L, McKenna E, Chang A, Mulé J. Treatment of solid tumours in children with tumour-lysate pulsed dendritic cells. *Lancet* 2000;356:1163–1165.
- Schakel K, Mayer E, Federle C, Schmitz M, Riethmüller G, Rieber EP. A novel dendritic cell population in human blood: one-step immunomagnetic isolation by a specific mAb (M-DC8) and in vitro priming of cytotoxic T lymphocytes. *Eur J Immunol* 1998;28:4084–4093.
- Bednarek MA, Sauma SY, Gammon MC, et al. The minimum peptide epitope from the influenza virus matrix protein. Extra and intracellular loading of HLA-A2. *J Immunol* 1991;147:4047–4053.
- Skipper JCA, Hendrickson RC, Gulden PH, et al. An HLA-A2-restricted tyrosinase antigen on melanoma cells results from posttranslational modification and suggests a novel pathway for processing of membrane proteins. *J Exp Med* 1996;183:527–534.
- Walker BD, Flexner C, Birch-Limberger K, et al. Long-term culture and fine specificity of human cytotoxic T lymphocyte clones reactive with human immunodeficiency virus type 1. *Proc Natl Acad Sci U S A* 1989;86:9514–9518.
- Schmitt A, Keilholz U, Scheibenbogen C. Evaluation of the interferon-gamma ELISPOT-assay for quantification of peptide specific T lymphocytes from peripheral blood. *J Immunol Methods* 1997;210:167–174.
- Herr W, Schneider J, Lohse AW, Meyer zum Buschenfelde K-H, Wolfel T. Detection and quantification of blood-derived CD8+ T lymphocytes secreting tumor necrosis factor alpha in response to HLA-A2.1-binding melanoma and viral peptide antigens. *J Immunol Methods* 1996;191:131–142.
- Herr W, Linn B, Leister N, Wandel E, Meyer zum Buschenfelde K-H, Wolfel T. The use of computer-assisted video image analysis for the quantification of CD8+ T lymphocytes producing tumor necrosis factor alpha spots in response to peptide antigens. *J Immunol Methods* 1997;203:141–152.
- Houbiers JG, Nijman HW, Van der Burg SH, et al. In vitro induction of human cytotoxic T lymphocyte responses against peptides of mutant and wild-type p53. *Eur J Immunol* 1993;23:2072–2077.
- Zier K, Johnson K, Maddux J-M, et al. IFN-gamma secretion following stimulation with total tumor peptides from autologous human tumors. *J Immunol Methods* 2000;241:61–68.
- Scheibenbogen C, Lee K-H, Mayer S, et al. A sensitive ELISPOT assay for detection of CD8+ T lymphocytes specific for HLA-class-I-binding peptide epitopes derived from influenza proteins in the blood of healthy donors and melanoma patients. *Clin Cancer Res* 1997;3:221–226.
- Jager E, Jager D, Karbach J, et al. Identification of NY-ESO-1 epitopes presented by human histocompatibility antigen (HLA)-DRB4*0101-0103 and recognized by CD4+ T lymphocytes of patients with NY-ESO-1-expressing melanoma. *J Exp Med* 2000;191:625–630.
- Meidenbauer N, Harris DT, Spitler LE, Whiteside T. Generation of PSA-reactive effector cells after vaccination with a PSA-based vaccine in patients with prostate cancer. *Prostate* 2000;43:88–100.
- Subklewe M, Chahroudi A, Bickham K, et al. Presentation of Epstein-Barr virus latency antigens to CD8+, interferon-gamma-secreting, T lymphocytes. *Eur J Immunol* 1999;29:3995–4001.
- Jager E, Ringhoffer M, Arand M, et al. Cytolytic T cell reactivity against melanoma-associated differentiation antigens in peripheral blood of melanoma patients and healthy individuals. *Melanoma Res* 1996;6:419–425.
- Bennett SRM, Carbone FR, Karamalis F, Flavell RA, Miller JFAP, Heath WR. Help for cytotoxic T cell responses is mediated by CD40 signaling. *Nature* 1998;393:478–480.
- Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* 1998;393:474–478.
- Schoenberger SP, Toes REM, Van der Voort EIH, Offringa R, Melief CJM. T help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 1998;393:480–483.
- Chaux P, Vantomme V, Stroobant V, et al. Identification of MAGE-3 epitopes presented by HLA-DR molecules to CD4+ T lymphocytes. *J Exp Med* 1999;189:767–778.