Flow Cytometric Measurement of Intracellular Cytokines Detects Immune Responses in MUC1 Immunotherapy

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ABSTRACT

The detection of tumor-specific T cells in immunized cancer patients usually relies on lengthy and difficult CTL assays; we now report on flow cytometry to detect the intracellular cytokines interleukin 2 (IL-2), IL-4, IFN-γ, and tumor necrosis factor α (TNF-α) produced by CD4+CD69+ and CD8+CD69+ activated T cells after MUC1 antigen stimulation. Peripheral blood mononuclear cells were obtained from 12 patients with adenocarcinoma injected with mannan-MUC1; cells were exposed in vitro for 18 h to MUC1 peptide in the presence of CD28 monoclonal antibody and Brefeldin; permeabilized cells were used for the expression of cytokines. After stimulation in vitro with MUC1-variable number of tandem repeats peptides, CD8+CD69+ T cells from all immunized patients generated 3–9 times higher levels of TNF-α (P < 0.038) and IFN-γ (P < 0.010) than did cells from 12 normal subjects; minor increases in IL-4 occurred. By contrast, CD4+CD69+ cells showed no overall alteration in TNF-α and IFN-γ cytokine production, although in some patients, their measurement was informative; the measurement of IL-2 was not useful in either CD4+CD69+ or CD8+CD69+ cells. We conclude that in MUC1-immunized patients, the measurement of TNF-α and IFN-γ in activated CD69+CD8+ T cells may be indicative of their immune status.

INTRODUCTION

Most immunotherapeutic studies for solid tumors are attempting to induce CD8+ CTLs rather than antibodies for antitumor affects, and they include the use of dendritic cells pulsed in culture and reinfused (1–3), targeting the mannose receptor

MATERIALS AND METHODS

Ags. Human MUC1-GST fusion protein containing five VNTR regions of the sequence PAHGVTSAPOGDSTAP was expressed in Escherichia coli, purified and chemically conjugated to mannan to form M-FP (22). GST was cleaved from the fusion protein using the site-specific protease factor Xa obtained from Roche Molecular Biochemicals (Mannheim, Germany), and the fusion protein containing the five VNTR lacking GST was labeled as VNTR. Tetanus toxoid and influenza vaccine (A/Johannesburg/82/96, A/Sydney/5/97, and B/Harbin/7/98) were used as controls.

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4 The abbreviations used are: CTLp, CTL precursor frequency; Ag, antigen; APC, aliphophycocyanin; BDIX, Becton Dickinson Immunocytometry Systems; BFA, Brefeldin A; IL, interleukin; GST, glutathione S-transferase; mAb, monoclonal antibody; M-FP, mannan-MUC1 fusion protein; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; PHA, phytohemagglutinin; TNF, tumor necrosis factor; VNTR, variable number of tandem repeats.
Antibodies. The following mAbs were obtained from BDIS (San Jose, CA) conjugated to either FITC, PE, PerCP, or APC: Leu4 (CD3; PerCP and APC), Leu3a (CD4; PerCP and APC), Leu2a (CD8; PerCP and APC), Leu23 (CD69; PE and PerCP), Leu28 (CD28; nonconjugated), IFN-γ (clone 25723.11; FITC and PE), TNF-α (clone 6401.111; FITC and PE), IL-2 (clone 5344.111; FITC and PE), IL-4 (clone 3010.211; PE), γ1 (mouse IgG1 control; FITC and PE), and γ2a (mouse IgG2a control; FITC and PE).

Cell Preparation and Antigenic Stimulation. PBMCs were obtained from whole blood that was collected from the following: (a) normal subjects; (b) subjects boosted with tetanus toxoid or influenza vaccine; or (c) patients with adenocarcinoma injected i.m. with M-FP. 1 week after their last M-FP immunization. Patients received a total of seven immunizations. Blood was collected in CPT blood collection tubes obtained from Becton Dickinson Vacutainer Systems (Franklin Lakes, NJ), and PBMCs were separated by centrifugation. PBMCs (3 × 10^7) were placed in 16 × 125-mm polystyrene tissue culture tubes, 3 μg of CD28 mAb were added, and the cultures were left for 10 min at room temperature. Ags and PHA were added at previously determined optimal concentrations (50 μg/ml M-FP, 20 μg/ml VNTR, 10 μg/ml influenza vaccine, 10 μg/ml tetanus toxoid, 2 μg/ml PHA), and the tubes were placed at a 5° horizontal slant at 37°C in a humidified 10% CO_2 incubator for 18 h, with BFA added at a final concentration of 5 μg/ml after 2 h. BFA is a potent inhibitor of intracellular transport that results in intracellular accumulation of cytokines. Because of the toxicity associated with prolonged exposure to BFA, we observed no decrease in the viability of the cells at the concentration of BFA used, which was lower than that described in other methods employing a 10-h incubation with BFA. PHA rather than PMA/ionomycin (23) was used as an indicator of positive stimulation because cells could survive with no apparent loss in viability during the incubation with PHA.

Immunofluorescence Staining. After stimulation for 18 h with Ag, cell preparations were treated with 100 μl of 20 mM EDTA (final concentration, 2 mM) for 10 min to detach adherent cells, washed with cold PBS, resuspended in 1 × FACs lysing solution at 5 ml/3 × 10^7 cells (BDIS), and left at room temperature for 10 min. Cells were washed in PBS containing 0.5% BSA and 0.1% sodium azide (buffer) and resuspended in FACs permeabilizing solution at 0.5 ml/3 × 10^7 cells (BDIS) for 10 min at room temperature. Cells were washed in buffer and stained for cell surface molecules and intracytoplasmic cytokines for 30 min at room temperature. After staining, cells were washed, fixed in 1% paraformaldehyde in PBS, and kept at 4°C until analyzed on the flow cytometer. Cytokines could only be detected when fresh PBMCs that had not been frozen were used (data not shown).

Flow Cytometric Analysis. Cells were analyzed on a FACScalibur flow cytometer equipped with a second 633-nm line diode laser (BDIS) using forward and side scatter parameters to identify lymphocytes, with FITC, PE, PerCP, and APC as the fluorescence markers. For each analysis, 40,000 events were usually acquired, gated on a logical gate of viable lymphocytes and CD3, CD4, or CD8 expression (most files required fine tuning after acquisition), and analyzed using the CELL QUEST program (BDIS) for CD69^+ cytokine-producing cells. Isotype matched antibodies were used to verify the staining specificity and as a guide for setting the markers to delineate positive and negative populations. The intra- and interassay variations were found to be <10%. The results are represented as follows: (a) net percentage positive after subtraction of background; or (b) the ratio of test Ag to the no-Ag control (Ag^+/Ag^-) in comparisons of samples stimulated with the same Ag.

RESULTS

Parameters for MUC1 T Cell Cytokine Responses. The use of the intracytoplasmic staining for cytokines has previously been demonstrated for mitogens, superantigens, and viral peptides (19, 20) but not for tumor immunotherapy. Before measuring the level of intracellular cytokines secreted by T cells after stimulation with MUC1, we examined the parameters for optimal cytokine secretion in activated CD69^+ cells. Cells from an immunized patient were stimulated with M-FP for a period of 18 h; within 6 h of stimulation, peak levels of activated cells were reached, at which 30.8% of the cells were activated, as shown by the presence of CD69^+ cells (Fig. 1A). Cytokine levels had increased by 6 h to reach a maximum by ~18 h. At that time TNF-α production was the highest, with 5.5% of CD3^+ CD69^+ containing TNF-α^+ cells versus 0.14% at 3 h (39-fold increase; Fig. 1B). CD69^+ IFN-γ^+ cells had increased 9-fold by 18 h with 1.7% CD3^+CD69^+ containing IFN-γ^+ cells versus 0.19% at 3 h, whereas IL-2-containing cells showed a 2-fold increase (1.4% CD3^+CD69^+IL-2^+ versus 0.68% at 3 h). Thus, for further studies, 18 h of stimulation with the MUC1 Ag was used.

BFA is used in studies to detect intracellular cytokines as it inhibits their extracellular transport; the time of exposure to BFA for the detection of TNF-α, IL-2, and IFN-γ in activated T cells was examined (Fig. 1C). BFA was added either 1, 2, or 3 h after the addition of M-FP and remained in the culture. When BFA was added after 1 h of culture, the ratio of CD4^+CD69^+ cytokine-producing cells (calculated as the number of CD4^+CD69^+ cytokine-producing cells stimulated with M-FP divided by the no-Ag control) was 2.5 for TNF-α, 1.2 for IL-2, and 1.2 for IFN-γ. When added after 2 h of culture, the ratio of cytokine-producing cells was 4.6 for TNF-α and 1.9 for IFN-γ but was unchanged for IL-2 (1.1). After 3 h of incubation with BFA, the ratio of cytokine-producing cells had decreased. Thus, BFA was added after 2 h of exposure to Ag, and a further 16 h of culture was performed; these did not reduce the viability of cytokine-producing cells.

Previous studies had found that the addition of the CD28 mAb enhanced the ability to detect intracellular cytokines after stimulation with Ag (24); PBMCs were therefore stimulated with M-FP in the presence and absence of the CD28 mAb (Fig. 2). The addition of the CD28 mAb increased, by 8-fold, the number of TNF-α^+ and IFN-γ^+ cells in CD8^+ CD69^+ T cells (IL-2^+ cell numbers remained low; data not shown). After stimulation of PBMCs with M-FP and CD28, several findings were apparent: (a) 1.05% CD8^+CD69^+ contained TNF-α^+ cells versus 0.12% in the absence of CD28 (Fig. 2, B and C); (b)
0.69% of CD8+CD69+ cells contained IFN-γ+ cells in the presence of the CD28 mAb versus 0.09% in its absence (Fig. 2, E and F); and (c) the CD28 mAb alone did not generate significant cytokine production (Fig. 2, A and D). Thus, all experiments used a culture period of 18 h with Ag and CD28 mAb, with BFA being added after 2 h.

**Detection of Cytokines after Stimulation with Tetanus Toxoid, Influenza Ags, or PHA.** To further validate the intracytoplasmic cytokine measurements, we sought intracellular cytokines in PBMCs: (a) in normal subjects injected with either tetanus toxoid or influenza vaccine; or (b) in normal subjects and patients with adenocarcinoma after stimulation with the mitogen PHA. In a subject immunized with influenza vaccine 60 days earlier, CD3+ cells were examined for intracellular cytokines before and after immunization (Fig. 3A). Compared with preimmune results, the ratio of CD3+CD69+ cytokine-producing cells (calculated as the number of CD4+CD69+ cytokine-producing cells stimulated with M-FP divided by the no-Ag control (M-FP+/Ag-)).
IL-2 and IL-4 in CD4+ cells received tetanus toxoid immunization within the last 3 years. Increases in the intracellular cytokines TNF-α from subjects immunized with influenza or tetanus toxoid showed measurable cytokine-producing cells, but not from normal subjects. Thus, subjects who had not been deliberately immunized but had presumably been exposed to the influenza virus the previous year showed higher levels of cytokines (mean ratio, 1.3) 2.1 days after immunization, the ratio was higher than in 12 normal subjects (mean ratio, 0.8). IL-2 and IL-4 measurements were not different from that of normal subjects. In CD4+CD69+ cells from subject 2, TNF-α did not alter, IFN-γ was higher than normal at 45 days, and, again, IL-2 and IL-4 were not different from the normal subjects. Subjects immunized with influenza or tetanus toxoid showed measurable increases in the intracellular cytokines TNF-α or IFN-γ but not IL-2 and IL-4 in CD4+ cells; similar findings occurred with CD8+CD69+ cells.

To measure the ability of PBMCs from cancer patients immunized with M-FP and nonimmunized individuals to respond to a T cell mitogen, cells were stimulated for 18 h with PHA and CD69+ cells producing TNF-α, IL-4, or IFN-γ, which was measured (Fig. 4). Both CD4+CD69+ and CD8+CD69+ T cells from patients and normal subjects produced TNF-α. However, the ratio of cytokine-producing cells (calculated as the number of CD4+CD69+ cytokine-producing cells stimulated with tetanus toxoid divided by the no-Ag control) for TNF-α was 2.2 before immunization in CD4 cells and 5.0 at 33 days after immunization. This was therefore appropriate to examine their responses after MUC1 immunization.

**Cytokine Responses to MUC1 in Several Subjects.** Intracellular cytokine production in response to a MUC1 stimulus was examined in CD4+CD69+ and CD8+CD69+ cells from patients with adenocarcinoma who had been immunized with mannan MUC1. For these experiments, PBMCs were activated in autologous plasma with M-FP or VNTR and stained with CD3 APC-, CD8 PerCP-, CD69 PE-, and FITC-conjugated cytokine mAbs (to TNF-α, IL-4, and IFN-γ). Prior to examining...
the 12 patients, several subjects were examined to determine which cytokines were the most appropriate and what was the best time to examine patients' cells after immunization. Fig. 5 shows representative three-color plots of CD8 T cells from a patient after four M-FP immunizations, whose PBMCs were cultured without Ag (Fig. 5, A, D, and G), with M-FP (B, E, and H), or with VNTR (C, F, and I). In general, better responses were seen with MUC1 VNTR than with M-FP. Thus, for TNF-α, the reactive cells after VNTR stimulation were 2.72% versus 1.10% after M-FP (no Ag, 0.63%) and for IFN-γ, 2.34% versus 0.91% after VNTR stimulation versus 0.91% after M-FP (no Ag, 0.50%) i.e., VNTR gave a greater than 2-fold increase in the number of cells detected. Furthermore, the cytokine responses seen in CD8⁺CD69⁺ cells were greater than those of CD4⁺CD69⁺ cells, as the number of TNF-α⁺ and IFN-γ⁺ cells after stimulation with M-FP and VNTR was similar to that after

Fig. 4 Mitogen stimulation to induce intracellular cytokine. PBMCs from 12 patients with adenocarcinoma immunized with MUC1 (● and ◦) and 11 normal subjects (■ and □) were stimulated with PHA. Cells were fixed, permeabilized and stained with antibodies against CD4, CD8, CD69, and TNF-α (A), IL-4 (B), and IFN-γ (C). Forty thousand events gated on viable CD4⁺ (● and ■) or CD8⁺ (◆ and □) lymphocytes were analyzed for CD69⁺ cytokine-producing cells. The results are expressed as cytokine production (y axis), calculated as the number of CD69⁺ cytokine-producing cells stimulated with PHA divided by the no-Ag control (PHA/Ag⁻). The mean for each group is shown (horizontal line).

Fig. 5 Cytokine profile of a patient immunized with M-FP. PBMCs were stimulated in vitro for 18 h either with no Ag (A, D, and G), M-FP (50 μg/ml; B, E, and H), or VNTR (20 μg/ml; C, F, and I). Cells were stained with antibodies to CD3, CD8, and TNF-α (A–C), IFN-γ (D–F), or IL-4 (G–I). Forty thousand events gated on viable CD3⁺ lymphocytes were analyzed for the presence of cytokines. The percentage of cytokine⁺ cells is indicated in each plot (CD8⁺ in the right quadrant and CD4⁺ in the left quadrant). The number of CD4⁺ cells (CD8-negative population; top left) staining for cytokines was calculated after subtracting the percentage contributed by the CD8⁺ cells.
stimulation with no Ag (note that in Fig. 5, the CD4^+ cells were calculated by subtracting the CD8^- fraction from CD3^+). Finally, neither CD8^-CD69^- nor CD4^-CD69^- cells produced significant amounts of IL-4. Thus, subsequent measurements concentrated on IFN-γ, TNF-α, and IL-4 in CD4^+CD69^- and CD8^-CD69^- cells.

Most of the samples from immunized patients were tested 1–2 weeks after their last immunization with M-FP (in 2 patients, the samples were tested 25 weeks after the last immunization). Previously, using a CTL response, we were unable to identify the optimal time needed between immunization and testing (25). However, because of the small volume of blood needed to perform flow cytometric analysis, the intracellular cytokine production could be examined soon after M-FP immunization (Fig. 6). Serial PBMC samples collected from a patient receiving immunizations with M-FP were stimulated with M-FP and CD4^-CD69^+ and CD8^-CD69^+ cells were examined for the presence of TNF-α, IL-4, and IFN-γ-producing cells. To minimize possible errors in our calculations of cytokine-producing cells, we included in each test a PHA-positive control, which indicated a <10% interassay variability (data not shown). It was of interest that CD8^-CD69^+ T cells could be shown to have increases in TNF-α and IFN-γ after four injections, whereas in this study, IL-4 increased after the first injection. Furthermore, CD4^-CD69^- T cells demonstrated an increase IFN-γ-producing cells after the third immunization, and so responses were measured 7 days after the fourth immunization.

Cytokine Responses in 12 Immunized Subjects. The intracellular cytokines present in CD4^-CD69^- and CD8^-CD69^- T cells were then examined in 12 immunized and 10 normal subjects (Fig. 7). The samples from immunized patients were tested 1–2 weeks after their last of seven immunizations with M-FP. The ratios of cytokine-producing cells (calculated as the number of CD4^-CD69^- or CD8^-CD69^- cytokine-producing cells stimulated with M-FP or VNTR divided by the no-Ag control) after stimulation with M-FP (Fig. 7, A–C) or VNTR (Fig. 7, D–F) were determined. Several findings were apparent: (a) the responses of all cytokines to VNTR peptide were greater than (M-FP); (b) CD8^-CD69^- T cells from MUC1-immunized subjects contained more cells expressing TNF-α after VNTR stimulation than those from nonimmune subjects (2.6 versus 0.76; P < 0.038); (c) the same finding occurred with CD8^-CD69^- cells and IFN-γ (4.5 versus 0.4; P < 0.01); (d) although IL-4 also increased in CD8^-CD69^- cells (2.0 versus 0.8), this was not significant; (e) CD4^-CD69^- cells stimulated with either M-FP or VNTR showed no significant differences between immunized and normal subjects for TNF-α, IFN-γ, or IL-4, although individual patients exhibited some alterations.

Thus, the responses in 12 immunized patients were statistically significant for TNF-α and IFN-γ when the VNTR peptide was used and CD8^-CD69^- cells were examined; in these cells, IL-2 (not shown) and IL-4 were not altered. There were no differences when CD4^-CD69^- cells were examined for the cytokines IL-2, IL-4, IFN-γ, and TNF-α.

DISCUSSION

Immunotherapy has potential for the treatment of some forms of cancer, in which patients are immunized with an immunogen/adjuvant combination and the effectiveness of the immunization is measured as the amplification or generation of immune responses (25, 26), as reproducible tumor responses have not been found as yet. For solid tumors it is likely that CD8^- cytotoxic T cells are those required for tumor elimination—acting by cytotoxicity and/or cytokine release—particularly the T1 cytokines IL-2, IFN-γ, and TNF-α. However, the in vitro measurement of CTL responses in patients with cancer is difficult: compared to murine studies, in which the spleen or lymph nodes are used, in humans, peripheral blood must be
The number of CD69+ cells were analyzed for CD69 (CD69 amounts in the 12 patients; clearly, the major responding cell type was CD8+, but as the study progressed, there was a modest increase in IL-4 production but not IL-2, IL-4, IL-8, or TNF-α.). Forty thousand events gated on viable CD4+ (○ and □) or CD8+ (● and ■) lymphocytes were analyzed for CD69+ cytokine-producing cells. The results are expressed as cytokine production (y axis) calculated as the number of CD69+ cytokine-producing cells stimulated with Ag divided by the no-Ag control (Ag1 / Ag1). The mean for each group is shown (horizontal line).

The results were clear: (a) CD8+CD69+ T cells from immunized patients produced, intracellularly, the T1 cytokines IFN-γ and TNF-α; (b) CD4+CD69+ cells showed the same trend, with IFN-γ and TNF-α, but the increases were not significant; (c) there was a modest increase in IL-4 production but not IL-2 production [neither one was produced in significant amounts in the 12 patients; clearly, the major responding (CD69+) cells producing cytokines were CD8+ T cells]. Optimal results were obtained in the presence of a CD28 mAb and required Brefeldin to be added after 2 h of culture with the VNTR peptide but not with the MUC1 fusion protein. On the basis of these results, we can state that detection of intracellular cytokines is a simple and reliable method of measuring T cell responses in patients with cancer. Thus, the detection of immune responses in the cells of the 12 MUC1-immunized patients required three conditions: the appropriate cells (CD8+ and CD69+, not CD4+ and CD69-), Ag in the correct form (VNTR but not M-FP), and the appropriate cytokines (IFN-γ and TNF-α but not IL-2, IL-4, or IL-8). The results in the 12 patients convincingly demonstrated that CD8+ cells were making T1 cytokines, and the method was substantially simpler to perform than the earlier CTL/CTLp and T cell proliferation studies and delayed hypersensitivity testing (25).

There are a number of technical aspects that require further comment. First, in these patients, the detected cytokines did not survive freezing and thawing of the cells; thus, tests have to be performed on the day the patient’s blood is taken, but the results are then available within 24 h. It may be desirable to perform all tests simultaneously on cells taken over the period of immunization; to avoid variations in responses, however, the variations that we found in Ag responses in normal individuals were small, and we consider this not to be a major problem. There are real advantages in having the answers within 24 h: during this time ELISA tests are done to measure antibody, and an assessment of the immune status can be rapidly determined. A second technical aspect was that the use of Brefeldin (addition after 2 h of Ag stimulation) and the culture period (culturing for a further 16 h) are different from studies in viral diseases (19, 20, 24). This is not surprising and indicates that in each disease and possibly for each peptide or antigenic system, the appropriate time of culture with Brefeldin has to be assessed. It was also apparent that improved responses were obtained in the presence of the CD28 antibody, in agreement with other studies (24). A third aspect to consider is which cytokines to measure. Initially, we examined IL-2, IL-4, IL-8, IFN-γ, and TNF-α, but as the study progressed, IL-8 was abandoned, as it was present nonspecifically in some of the patients; because it is produced by natural killer cells and neutrophils rather than T cells (28, 29), it was not examined further. Measurements of IL-2 or IL-4 were not useful measurements, which is of interest. As described previously (25), patients make a significant MUC1 antibody response to the immunizing peptide, with titers in excess of 1:10,000 by ELISA. These are presumably due to a T2 type response from CD4+ cells; and therefore, IL-4 was expected to be present, but CD4 cells making IL-4 could not be detected. Perhaps the peripheral blood is not the place to seek cytokine-producing cells for antibody responses, and a different profile may well have been present in lymph nodes or spleen.

Our findings of CD8+IFN-γ+ and CD8+TNF-α+ cells indicate a phenotypic pattern suggestive of a T1 response in patients vaccinated with MUC1. Particularly after stimulation...
with the VNTR peptide, CD8 T cells from immunized patients produced 9 times more IFN-γ-producing cells than did CD8 T cells from normal patients (30, 31); however, we note that to this extent, CD45RO<sup>high</sup> memory effector cells have such a cytokine profile (32, 33). The immunization using mannan MUC1 gives T1 responses in mice, with a cytotoxic T cell response, little antibody, and IFN-γ, TNF-α, and IL-12 secretion (4). However, in our patients, although CTLs were found in approximately ~20% (25), more patients (~60%) made antibodies—possibly a T2 response due to the cross-reaction of MUC1 peptides with antigalactosidase antibodies (34), leading to immune complex formation. Moreover, the cellular responses measured by the flow cytometric analysis of intracytoplasmic cytokines were of higher frequency than found previously, and so the measurement of cytokines may well be a more sensitive assay to measure MUC1 cellular responses (25). However, these were different patients, and unfortunately, we were not able to measure both CTLs and intracytoplasmic cytokines in the same set of patients, although it should not be surprising that both T1 and T2 responses can occur in the same patient, given the complexity of the Ag administered. [The Ag consists of a 100-mer linked to mannan, which is known to contain epitopes that can be presented by both Class I molecules (epitopes have been mapped for both murine and human MHC Class I molecules) and must also contain Class II presenting molecules (which have not been mapped, but the peptide gives rise to T-dependent high antibody responses)]. In addition, we had shown previously that the administration of oxidized and reduced mannan MUC1 together could give rise to both T1 and T2 responses (35); perhaps the same occurs in patients.

Thus, the measurement of intracytoplasmic cytokines is simpler and gives a higher frequency than the measurement of CTLs, and as the numbers are higher, one would be more inclined to accept these results than those from CTLs. However, these results highlight one of the major difficulties with cancer therapy: there is no clinical response, such as tumor shrinkage, to help decide what is the optimal type of immune response and how it should be measured. Thus, the ideal situation would be to have tumors disappearing and relate this to defined cellular assays (be they tetramer binding, intracytoplasmic cytokines, CTLs, or ELispot or cytokine secretion in plasma); only in this way can the meaning of the results of the different tests be determined.

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REFERENCES


