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Dual-Color ELISPOT Assay for Analyzing Cytokine Balance

Yoshihiro Okamoto and Mikio Nishida

Summary

A dual-color enzyme-linked immunospot (ELISPOT) assay enabled us to analyze three kinds of cytokine-secreting cells simultaneously. T helper (Th) cells can be subdivided into at least two distinct functional subsets based on their cytokine secretion profiles. The first type of clones (Th1) produces interleukin (IL)-2 and interferon (IFN)-γ but not IL-4 or IL-5. The second type of clones (Th2) produces IL-4 and IL-5 but not IL-2 or IFN-γ. Furthermore, the presence of the third type (Th0) cell, which is a precursor of Th1 or Th2 cells, has been demonstrated to produce both Th1- and Th2-type cytokines. The dual-color ELISPOT assay is developed to differentiate these three subtypes of Th cells in an identical well. In the system, the red spots corresponding to IL-2-secreting cells (Th1) were developed with horseradish peroxidase and amino-ethyl-carbazole/H_2O_2. The light blue spots corresponding to IL-4-secreting cells (Th2) were developed with alkaline phosphatase and Vector blue (chromogenic substrate for alkaline phosphatase). The mixed colored (indigo) spots corresponding to both kinds of cytokine-secreting cells (Th0 cells) were developed with both chromogenic substrates. With this system, we could detect the IL-2- and/or IL-4-secreting cells simultaneously in a murine spleen cell or human peripheral mononuclear cell preparation.

Key Words: Dual-color enzyme-linked immunospot assay; interleukin-2; interleukin-4; cytokine balance; mouse; human.

1. Introduction

The enzyme-linked immunospot (ELISPOT) assay is an efficiently sensitive technique for the enumeration of single cells secreting cytokines ***(1)***. Variations of the ELISPOT assay have been developed by some investigators, including our group ***(2–9)***. Recently, we developed a dual-color ELISPOT assay ***(4)***, which was named "Stardust Assay," by improving an ordinary ELISPOT assay. This new method enabled us to analyze three kinds of cytokine-secreting cells simultaneously.

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T helper (Th) cells can be subdivided into at least two distinct functional subsets based on their cytokine secretion profiles ***(10)***. The first type of clones (Th1) produces interleukin (IL)-2 and interferon (IFN)-γ but not IL-4 or IL-5. The second type of clones (Th2) produces IL-4 and IL-5 but not IL-2 or IFN-γ. Furthermore, the presence of the third type (Th0) cell, which is a precursor of Th1 or Th2 cells, has been demonstrated to produce both Th1- and Th2-type cytokines ***(11,12)***.

The dual-color ELISPOT assay is developed to differentiate these three subtypes of Th-cells in an identical well. In the system, the red spots, which correspond to IL-2-secreting cells (Th1-cells), were developed with horseradish peroxidase and amino-ethyl-carbazole (AEC)/H_2O_2. The light blue spots, which correspond to IL-4-secreting cells (Th2-cells), were developed with alkaline phosphatase and Vector blue (chromogenic substrate for alkaline phosphatase, Vector Laboratories, CA, USA). The mixed colored (indigo) spots, which correspond to both kinds of cytokine-secreting cells (Th0-cells), were developed with both chromogenic substrates (**Fig. 1**). A photographic profile of different colored spots resembles "Stardust." Thus we call this technique "Stardust Assay." With this system, we could detect the IL-2- and/or IL-4-secreting cells simultaneously in a murine spleen cell preparation (**Fig. 2A,B**). In the present article, the dual color ELISPOT assay enabling simultaneous detection for plural numbers of cytokines will be described.

2. Materials

2.1. Reagents and Buffers

1. Capture (first) antibody: anti-mouse IL-2 monoclonal antibody (Genzyme, Cambridge, MA) and anti-mouse IL-4 monoclonal antibody (clone BVD4-1D11: Pharmingen, San Diego, CA).
2. Detection (second) antibody: rabbit polyclonal antibody for mouse IL-2 (Bectone Dickinson, Bedford, MA) and biotinylated monoclonal antibody for mouse IL-4 (clone BVD6-24G2: Pharmingen, San Diego, CA).
3. Streptavidin-conjugated alkaline phosphatase (GIBCO BRL Co. Ltd., NY).
4. Horseradish peroxidase-conjugated F(ab'2) fragment donkey anti-rabbit IgG(H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).
5. 30% Hydrogen peroxide (H_2O_2).
6. Bovine serum albumin (BSA, globulin free).
7. Cell culture medium (e.g., RPMI 1640 containing 10% heat-inactivated fetal bovine serum [FBS]).
8. Phosphate-buffered saline (PBS): Dissolve 80 g of NaCl, 2.0 g of KCl, 11.5 g of Na_2HPO_4, and 2.0 g of KH_2PO_4 in 900 mL of distilled water (dH_2O). Check pH and adjust to 7.4 with 1 *M* NaOH if necessary. Make volume up to 1 L with dH_2O. Store at room temperature. Dilute 1 in 10 with dH_2O for use.

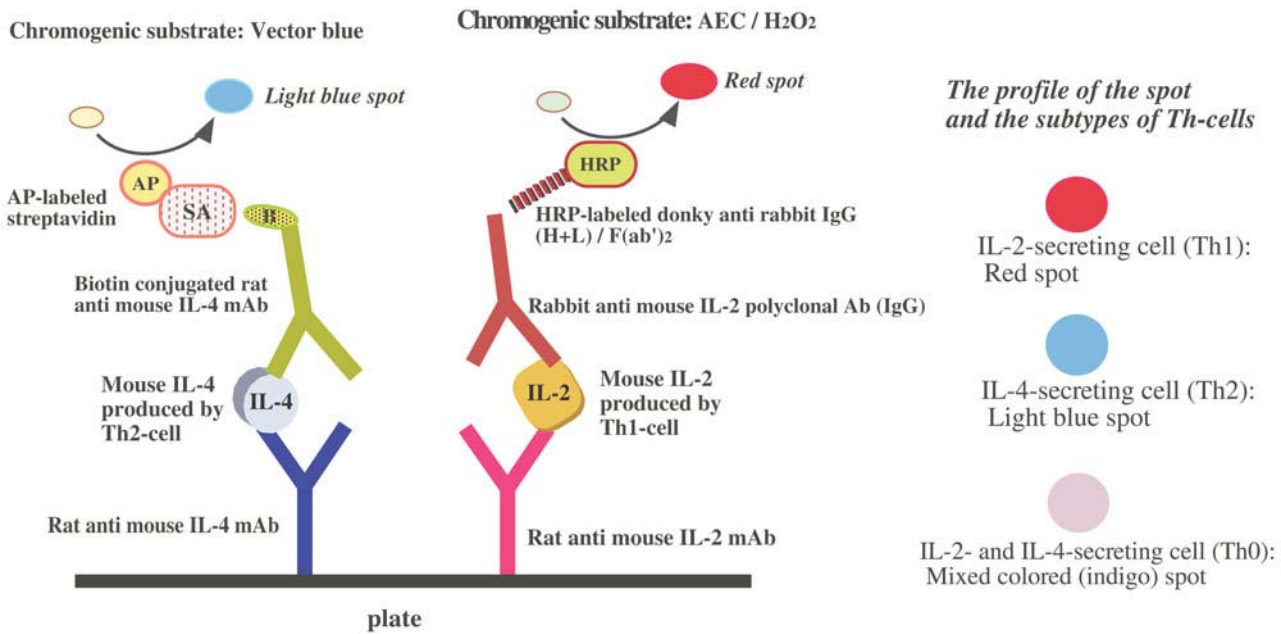
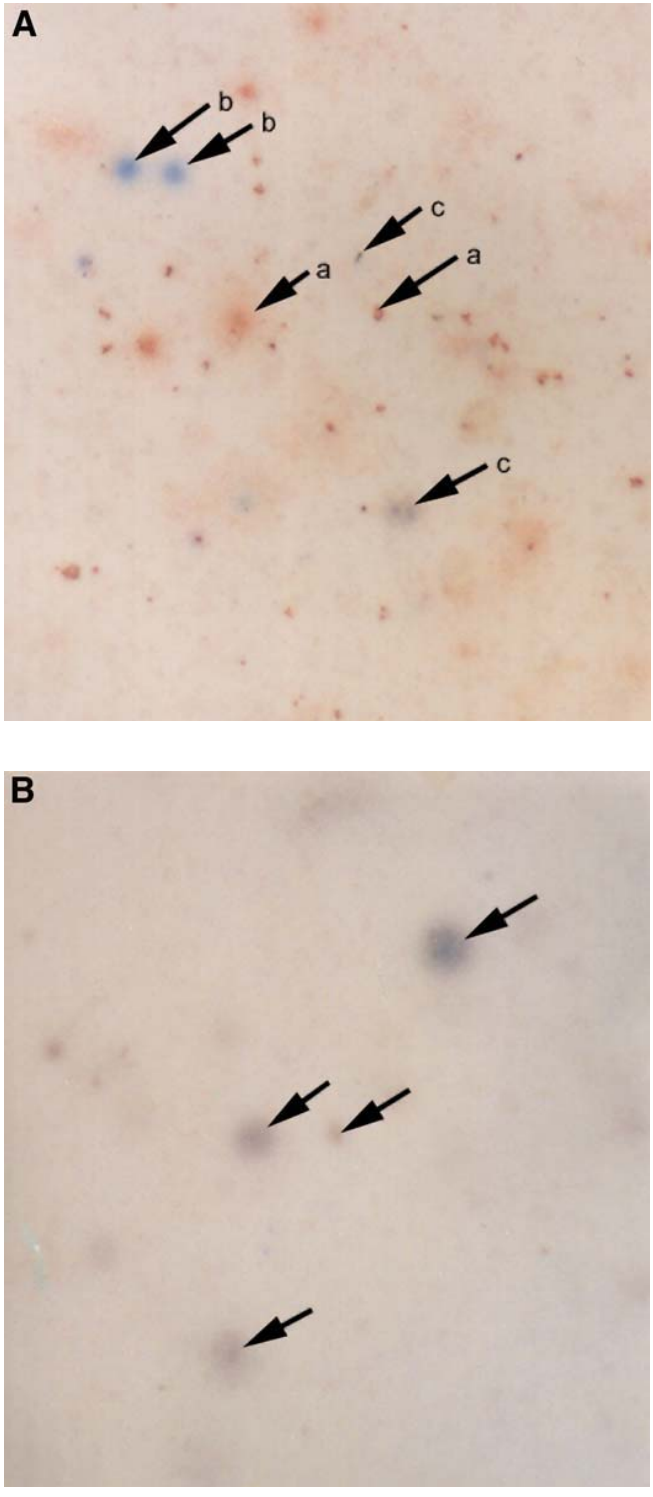


Fig. 1 The schematic representation of a dual-color detection (Stardust assay). Abbreviations: mAb, monoclonal antibody; AP, alkaline phosphatase; HRP, horseradish peroxidase; SA, streptavidin; B, biotin. (Reprinted from **ref. *4*** with permission from Elsevier.)


A
b
b
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a
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c
B

9. 0.05% Tween-20 in PBS (PBS-T): Add 0.5 mL of Tween-20 to 1 L of PBS from above.
10. Blocking solution: 5% BSA in PBS.
11. Substrate solution: 3-amino-9-ethylcarbazole (AEC) substrate kit (Vector Laboratories, CA) and Vector Blue substrate kit (Vector Laboratories, CA).

2.2. Equipment

1. 37°C CO_2 incubator: It is important that the incubator is absolutely leveled to prevent cells from rolling to one side of the well.
2. Microscope (magnification; ×10–40)
3. 96-Well nitrocellulose-backed plate (Millipore Multiscreen HA plate, Millipore, MA).
4. Plastic plate seal (Sumitomo Bakelite Co., Ltd., Tokyo, Japan).

3. Methods

1. Prepare first antibody mixture including an anti-mouse IL-2 monoclonal antibody (5 µg/mL) and an anti-mouse IL-4 monoclonal antibody (5 µg/mL) in PBS. Coat the wells of a 96-well nitrocellulose-backed plate with 100 µl of the antibody mixture per well (*see* **Notes 1** and **2**).
2. Seal the plate with plastic plate seal to prevent evaporation. Incubate overnight at 4°C.
3. Wash the plate three times with PBS-T (*see* **Note 3**).
4. Add 300 µL of blocking solution (5% BSA/PBS).
5. Incubate for 2 h at room temperature.
6. Wash the plate three times with sterile PBS.
7. Prepare cell suspension at different concentrations, for example, 1×10^5 cells/mL, 2×10^4 cells/mL, and 4×10^3 cells/mL. Add 100 µL of each cell suspension per well, in triplicate (*see* **Notes 4** and **5**).
8. Incubate at 37°C in 5% CO_2 for 18 h (s*ee* **Note 5**).
9. Wash the plate five times with PBS-T.
10. Add 100 µL of the detection antibody mixture including a rabbit polyclonal antibody for mouse IL-2 (2 µg/mL) and a biotinylated monoclonal antibody for mouse IL-4 (2 µg/mL) in PBS-T containing 1% BSA per well.

Fig. 2 (A). Typical profile of the dual color ELISPOT assay. **(A)** Crude spleen cells of normal BALB/c mice were stimulated with 1 µg/mL Concanavalin A for 18 h. After the stimulation, the cells were added to wells coated with the mixture of anti-IL-2 and IL-4 antibody, and subsequently spots were developed by the enzyme-substrate system shown in **Fig. 1**. Red spots corresponding to IL-2-secreting cells are indicated by *arrow a*, light blue spots corresponding to IL-4-secreting cells are indicated by *arrow b*, and the indigo spots corresponding to the Th0 type cells are indicated by *arrow c* (×40). **(B).** The ideal spots of Th0 cells. The mixed colored spots (indigo) are shown as the ideal profile of the spots corresponding to a Th0 cell (arrows; ×40). (Reprinted from **ref. *4*** with permission from Elsevier.)

11. Seal the plate to prevent evaporation. Incubate overnight at 4°C.
12. Wash the plate five times with PBS-T.
13. Add 100 µL of the mixture including a horseradish peroxidase-conjugated $F(ab')_2$ fragment donkey anti-rabbit IgG(H+L) (diluted 1: 5000) and a streptavidin-conjugated alkaline phosphatase (diluted 1:2000) per well.
14. Seal the plate to prevent evaporation. Incubate for 2 h at room temperature.
15. Wash the plate five times with PBS-T.
16. Expose wells to 100 µl of AEC/H_2O_2 substrate solution (Vector Laboratories, Inc., Burlingame, CA) and examine for red spots to identify IL-2. These reactions developed for 5–7 min at room temperature.
17. Wash with PBS several times to eliminate AEC/H_2O_2 substrate solution.
18. Next, 100 µL of the Vector blue substrate solution (Vector Laboratories, Inc., Burlingame, CA) was added to each well, yielding light blue spots within 10–20 min to stain IL-4. The mixed-colored (indigo) spots correspond to both kinds of cytokine-secreting cells (*see* **Note 6**).
19. Wash the plate several times with dH_2O.
20. The developed plate is dried and count the number of spots in each well under low magnification (approx ×40) with a microscope. A typical profile of the dual color ELISPOT assay is shown in **Fig. 2** (*see* **Notes 7– 9**).

Recent studies revealed that the balance of cytokines secreted by different types of cells affected the state and progression of various diseases, including infectious, allergic and autoimmune disorders ***(13)***. The present procedure provides a useful tool for quantitatively analyzing micro-levels of dynamic immune responses. Practically we analyzed the changes in cytokine balance in collagen-induced arthritic (CIA) mice as an animal experimental model of human rheumatoid arthritis using the dual-color ELISPOT assay. We could obtain the valuable results that, at the prearthritic phase Th1 cells, were dominant, and after the onset of clinical arthritis, there was a shift from a Th1-dominant to a Th2-dominant state (**Fig. 3**; **ref. *14***).

Furthermore, we have optimized a human dual-color ELISPOT assay system with replacing antibodies for murine cytokines to those for human, and evaluated the cytokine balance in a patient with juvenile rheumatoid arthritis (JRA). It was demonstrated that the frequency of both IL-2- and IL-4-secreting cells in the peripheral mononuclear cells of the patient with JRA was markedly higher than those of healthy individuals. The ratio of Th1/Th2 of the patient was lower than that of healthy subjects (**Fig. 4**; **ref. *15***).

In summary, the dual-color ELISPOT assay (Stardust assay) is an excellent method to monitor the cytokine balance in diseases and should be one of the most powerful tools for not only animal experiments but also clinical investigation.

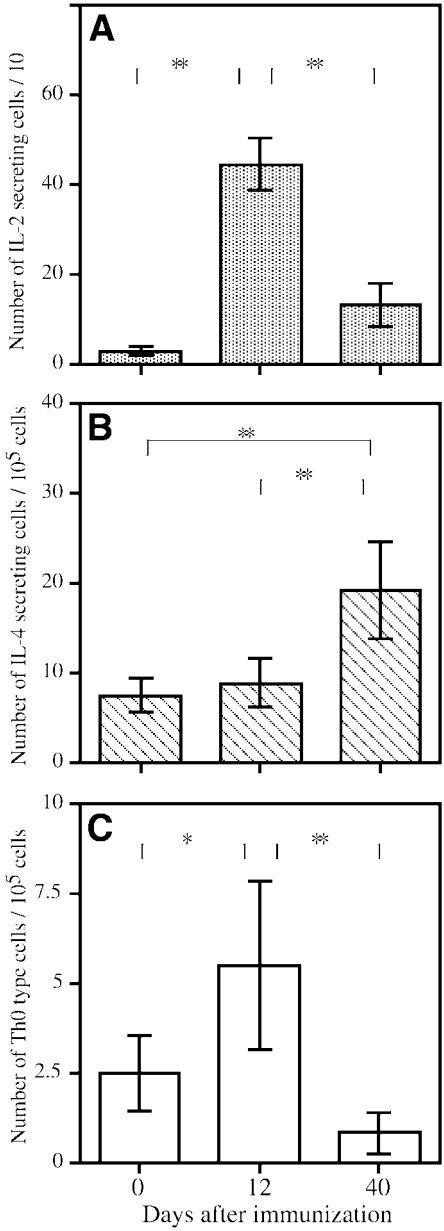

Fig. 3. The population change of Th cells in spleen of mice with CIA. Dual-color ELISPOT assay was conducted on spleen cells obtained from mice with CIA at different times after collagen type II (CII)-immunization. Cells were incubated with CII (50 μg/mL) in a well coated with the mixture of anti-IL-2 antibody and anti-IL-4 antibody for 18 h and, subsequently, spots were developed as described in Materials and Methods. **(A)** The frequency of IL-2 secreting cells (Th1 cell), **(B)** The frequency of IL-4 secreting cells (Th2), **(C)** The frequency of cells (Th0) secreting both cytokines. The results are expressed as mean ± SD of six assay wells. Significant differences were determined by Kruskal–Wallis nonparametric one-way analysis of variance and Scheffé's F test. $^{**}p < 0.01$, $^{*}p < 0.05$. (Reprinted from **ref. *14*** with permission from Mary Ann Liebert, Inc.)

4. Notes

1. Keep reagents and assay plate sterile during **steps 1** to **8**.
2. Higher concentration of coating antibody may give better results. However, optimal concentration (usually 2–10 μg/mL) should be examined in preliminary experiments.

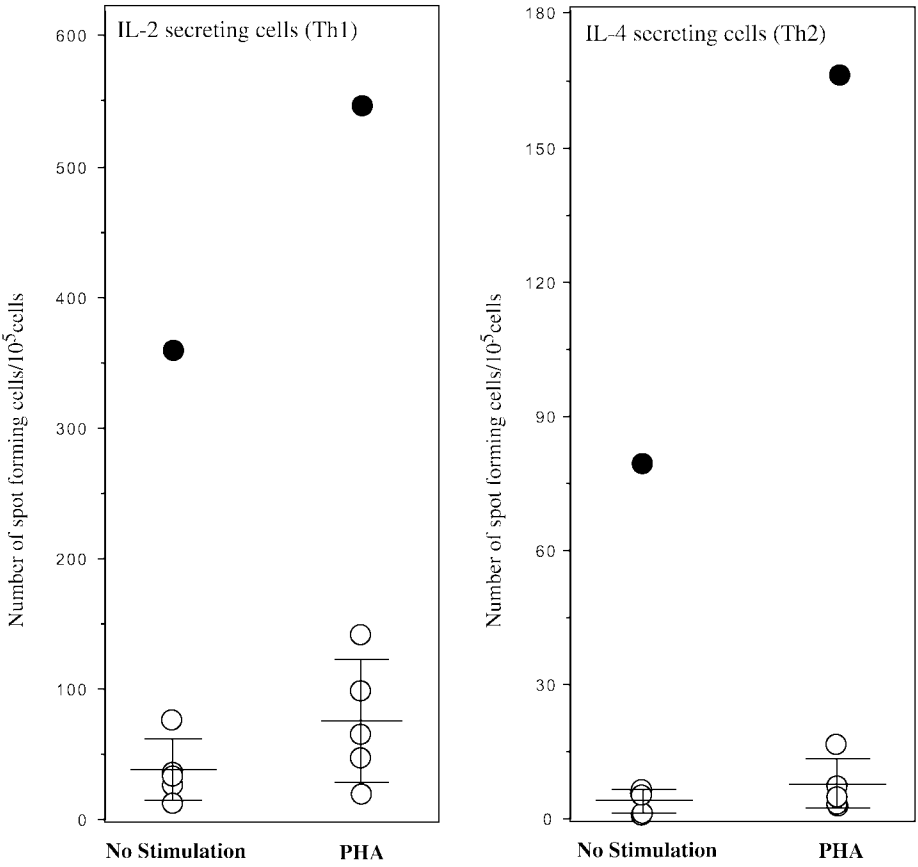

Fig. 4. Frequency of IL-2- and IL-4-secreting cells in a patient with JRA. PBMCs of each healthy volunteer (24 ± 2 yr old, range 22–33, two males and three females) and one patient with JRA (4 yr old, male) were prepared. The cells were washed with RPMI 1640 medium. Subsequently the cells were stimulated with 2 µg/mL phytohemaglutinin for 18 h. Nonstimulated cells were employed as a control. After the stimulation, the cells were applied to the dual-color ELISPOT assay. Open circles represent healthy individuals; closed circles represent the patients with JRA. Each circle represents the mean values of at least six assay wells. Each horizontal bar represents the mean values ± SD of five healthy individuals. (Reprinted from **ref. *15*** with permission from Elsevier.)

3. For each wash, fill wells with approx 300 µL of PBS(-T), soak for at least 1 min per wash, and invert plate to discard a washing solution.
4. Various types of cell specimens are applied to this assay (e.g., spleen, lymph nodes, bone marrow, or a cell-fraction purified from various sources). The cell suspension is prepared by washing cells extensively with incomplete medium, then resus-

pending the cells in medium containing 10% heat inactivated FBS. The cell specimen should be kept on ice until use. The viability of the cells should be assessed by trypan blue dye exclusion test before use to identify the number of living cells.

5. The optimal cell concentration and time of incubation will differ in individual experiment. The cell specimen is sequentially diluted to detect the appropriate number of spots in a well, and the conditions to produce 10–200 spots per well should be used to count the total number of cytokine-secreting cells per sample. It is difficult to count the number of spots precisely when more than 200 spots per well were developed.
6. To detect the cells secreting both kinds of cytokines precisely, a reference profile of the double-stained spots (indigo) should be provided in the plate. To obtain the ideal spots corresponding to the cells secreting both kinds of cytokines, the plate was incubated with biotinylated monoclonal antibody for IL-4 and followed by the two kinds of chromogenic system; the mixture of the horseradish peroxidase-labeled and the alkaline phosphatase-labeled streptavidin was added to the well after the incubation with the biotinylated monoclonal antibody for IL-4. By this procedure, we obtained a typical profile corresponding to the cells secreting both cytokines (*see* **Fig. 2B**).
7. The color depth or the size of spots depends on the amount of secreted cytokines. The strong and well-defined spots should be counted; any small or faint spots are likely to be artifacts and should not be counted.
8. The developed spots would be kept for several weeks if the plates are stored at 4°C under light protection.
9. To confirm specificity of the assay, the experiment using the wells coated with an irrelevant antibody (e.g., anti-IL-6 antibody coated well is used) as a negative control should be included.

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