

Monocyte-Derived Dendritic Cells as Antigen-Presenting Cells in T-Cell Proliferation and Cytokine Production

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Abstract

Dendritic cells (DC) are widely considered to be the major antigen-presenting cell (APC) type in immune responses. These cells are obtained from adherent cells or are purified CD14⁺ monocytes from peripheral blood mononuclear cells (PBMC) by in vitro stimulation with granulocyte, macrophage-colony-stimulating factor (GM-CSF) plus interleukin (IL)-4. They express high levels of MHC class II and costimulatory molecules, internalize Ag rapidly via Fc receptors and mannose receptors, and, by macropinocytosis, produce large amounts of IL-12 on CD40 ligation, and are potent in presenting soluble Ag and in stimulating allogeneic mixed-leukocyte reactions.

To study primary T-cell responses and cytokine production in allergy patients, we have developed an in vitro system by using highly purified T cells as responder cells and monocyte-derived DC (MDC) as the APC. MDC provide a convenient and potent APC source for T-cell response studies.

Key Words: Antigen-presenting cells; dendritic cells; T-cell proliferation; cytokines.

1. Introduction

Dendritic cells (DC) are widely considered to be the major antigen (Ag)-presenting cell (APC) type in immune responses (*1*). In human, the best studied DC type is the monocyte-derived DC (MDC) (*2,3*). These cells are obtained from adherent cells or are purified CD14⁺ monocytes from peripheral blood mononuclear cells (PBMC) by in vitro stimulation with granulocyte, macrophage-colony-stimulating factor (GM-CSF) plus interleukin (IL)-4. They express high levels of MHC class II and costimulatory molecules, internalize Ag rapidly via Fc receptors and mannose receptors, and, by macropinocytosis (*4*), produce large amounts of IL-12 on CD40 ligation (*5*), and are potent in presenting soluble Ag and in stimulating allogeneic mixed-leukocyte reactions (*2,3*). MDC

are considered immature DC (*1–3*). They can be induced to develop into mature DC with elevated MHC class II expression, decreased Ag uptake, and increased Ag presentation potency by stimulation with inflammatory cytokines such as TNF and IL-1, bacterial products such as endotoxin, and T-cell ligands such as CD40 ligand (*2,3,5,6*). MDC have been useful in studying the mechanism of Ag presentation (*1,2*) and in clinical trials in the treatment of viral infections and cancer (*3,7,8*).

In the past, human Ag-specific T-cell response studies have been severely limited by the lack of suitable potent autologous APC. The great majority of T-cell response studies utilize total PBMC and rely on macrophages and B cells in the cultures to function as the APC. However, this cell mixture can detect only strong to moderate T-cell proliferative responses. Furthermore, T-cell cytokine production is influenced by the different cell types present in the cultures, making the results difficult to interpret. MDC have provided a convenient, readily available, and potent APC source for T-cell response studies. To study primary T-cell responses in allergy patients, we have developed an in vitro system by using highly purified T cells as responder cells and MDC as the APC (*9*). This method has been successfully applied to studies on T-cell proliferation and cytokine production in response to allergens and allergen peptides and should be generally applicable to in vitro studies on T-cell and APC functions.

2. Materials

2.1. PBMC Isolation

1. Anticoagulant acid citrate dextrose (ACD-A) solution: 22 g/L trisodium citrate·2H₂O, 8 g/L citric acid·H₂O, 24.5 g/L dextrose. Sterilize by filtration (0.20- μ m filter; Nalgene, Rochester, NY) and store at 4°C (*see Note 2*).
2. Ficoll-paque, density 1.077 g/L (Amersham Pharmacia, Piscataway, NJ). Store at 4°C.
3. Ca²⁺–Mg²⁺ solution: 0.25 M CaCl₂, 0.078 M MgCl₂, sterilize by filtration.
4. Phosphate-buffered saline (PBS): 1 part endotoxin-free 10× PBS without Ca²⁺ and Mg²⁺ and 9 parts endotoxin-free H₂O (Irvine Scientific, Santa Ana, CA).

2.2. Isolation of CD14⁺ Monocytes and MDC Derivation

1. Anti-CD14 monoclonal antibody (mAb) 63D3 (ATCC, Bethesda, MD).
2. Goat-anti-mouse IgG magnetic microbeads (Miltenyi, Auburn, CA).
3. Miltenyi LS column and VarioMACS magnetic cell separation assembly.
4. Wash buffer for cell separation columns (PBS/BSA/EDTA): 0.5% bovine serum albumin (BSA), 5 mM EDTA in PBS. Store at 4°C. When ready to use, filter solution through a 0.2- μ m 115-mL filter unit (Nalgene). Leave solution in vacuum in the filter unit for 15 min at room temperature to deaerate solution and store buffer on ice.
5. Human recombinant GM-CSF (Peprotech, Rocky Hill, NJ); 1×10^7 units/mg protein. Dissolve in RPMI 1640 with 10% fetal bovine serum at 10 μ g/mL to 1 mg/mL

of GM-CSF. Aliquot into sterile freezing or Eppendorf tubes and store at -70°C (see **Note 3**).

6. Human recombinant IL-4 (Peprotech): 2×10^7 units/mg protein. Dilute and aliquot as in GM-CSF.
7. Complete medium: RPMI 1640 (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 10% heat-inactivated (56°C , 30 min), defibrinated autologous plasma (from stock 70% plasma; see **Section 3.1**, step 4).

2.3. T-Cell Purification for Ag-Specific Responses

1. Monoclonal antibodies against human CD19 (Pharmingen, San Diego, CA), CD56 (B159.5.2, Dr G. Trinchieri, Wistar Institute, Philadelphia, PA), CD13 (9), and CD83 (Coulter Immunotech, Miami, FL).
2. Goat-anti-mouse IgG magnetic microbeads (Miltenyi), LS columns, VarioMACS magnetic sorting assembly, and PBS/BSA/EDTA column washing solution as described in **Section 2.2**, step 4.

2.4. MDC Antigen Presentation in T-Cell Proliferation

Ags: recombinant Der p 2 (9) at 2 mg/mL; tetanus toxoid at 1.4 mg/mL and diphtheria toxoid at 0.5 mg/mL (Massachusetts Department of Health, Jamaica Plains, MA), dialysed against PBS and sterile filtered; and phytohemagglutinin (PHA, Wellcome Diagnostics, Dartford, England) at 9 mg/mL.

2.5. T-Cell Ag-Specific Cytokine Production

1. Recombinant IL-2 (Hoffmann LaRoche, Nutley, NJ): Dissolve IL-2 in RPMI 1640 with 10% fetal calf serum (FCS) at 10,000 U/mL. Aliquot in 2500 U/vial and store at -70°C .
2. Ag as described in **Section 2.4**.
3. Der p 2 20 mer overlapping set: A set of thirteen 20 mer Der p 2 peptides (University of Virginia Biomolecular Research Facility) with 10 amino acid overlap between adjoining peptides is dissolved in H_2O at 1 mM and stored at -20°C .

2.6. Assay of Th1 and Th2 Cytokines in Supernatants of Stimulated T Cells by ELISA

1. Immulon 4 plates, 96 well (Dynatech, Chantilly, VA).
2. Paired anti-human (hu) IFN- γ (Endogen, Woburn, MA), anti-huIL-4 (Pharmingen), and anti-huIL-5 (Pharmingen) capture and biotinylated detecting mAb. Store in aliquots at -20°C .
3. Standard recombinant huIFN- γ , huIL-4, and huIL-5 (all from Endogen): Dissolve as 0.2 $\mu\text{g}/\text{mL}$ in RPMI 1640 containing 10% FCS and store in 25- μL aliquots at -70°C . Larger aliquots (e.g., 0.5 mL) can be used as stock aliquots and later realiquoted.
4. Streptavidin-horseradish peroxidase (HRP; Pharmingen).
5. Coating buffer: 0.1 M sodium bicarbonate solution, pH 8.2.

6. Plate-blocking solution: 5% BSA in PBS and 0.05% Tween 20 (Fisher Scientific, Pittsburgh, PA). Prepare fresh and stir solution for 1–2 h to allow BSA to dissolve before use.
7. PBS/Tween plate-washing solution: 0.05% Tween 20 in PBS.
8. HRP substrate: 0.4 mg/mL *O*-phenylenediamine (Sigma Chemical Co., St Louis, MO) in citrate–phosphate buffer, pH 5.0 (24.3 mL of 0.1 M citric acid and 25.7 mL of 0.2 M dibasic sodium phosphate per 100 mL buffer), and 80 μ L H₂O₂ per 100 mL substrate solution. Prepare fresh for each measurement.
9. Stop solution: 3 N HCl.

3. Methods

The described procedure requires two donations of 200 mL of blood each with donations 7 d apart. Monocytes are purified from the PBMC of each donation for MDC generation. T-responder cells are purified from the second donation. A complete experiment spans 13 and 17 d for T-cell proliferation and cytokine production, respectively.

3.1. PBMC Isolation (see Note 4)

1. Draw 7.5 mL ACD-A into each of four 60-mL syringes with a 18-G needle.
2. Using a 19-G butterfly needle (Abbott Laboratories, N. Chicago, IL), draw 50 mL of blood in each syringe from the arm by venipuncture. Mix contents after each syringe is filled.
3. Put the blood into 50-mL conical centrifuge tubes. Spin blood at 450g for 15 min at 4°C. Pipet out plasma and pool in 50-mL conical tubes. Avoid disturbing the white buffy coat cells on top of the packed red cells. Note the volumes of plasma and total blood.
4. To the plasma, add Ca²⁺–Mg²⁺ solution (in mL) calculated by the empirical formula: $8.4 \times \text{plasma volume} / \text{total plasma plus red blood cell volume}$. Incubate plasma at 37°C. If no clotting occurs after 1 h, add increments of 0.2 mL of Ca²⁺–Mg²⁺ solution and continue the incubation. Defibrinated plasma is pipeted out, aliquoted, and stored at –20°C. Use this plasma as 70% autologous plasma (see Note 5).
5. Pool the packed cells in a 500-mL sterile bottle. Add cold PBS to 420 mL.
6. Pipet 12 mL of Ficoll-paque into each of twelve 50-mL conical polypropylene tubes (Falcon 2070, B–D). Gently overlay the Ficoll-paque with 35 mL of diluted packed cells.
7. Centrifuge at 1400g at 15°C for 20 min.
8. Pipet out interface cells (PBMC) with a cotton-plugged pasteur pipet into 50-mL conical tubes. Dilute cells at least twofold with cold PBS. Spin cells down at 200g at 4°C for 10 min.
9. Suction off supernatant. Pool all PBMC into one 50-mL conical tube with 50 mL cold PBS and spin cells down again.
10. Resuspend cells in 5 mL cold PBS. Count a 20 $\times$ diluted aliquot of cells with a hemocytometer.

3.2. Isolation of CD14⁺ Monocytes and MDC Derivation (see Note 6)

1. Transfer the washed PBMC to an 8-mL round bottom tube (Falcon 2027). Rinse the original 50-mL tube with 2 mL cold PBS and pool all the cells. Spin cells down at 200g at 4°C for 10 min.
2. Resuspend cells in 4 mL cold complete medium (see **Section 2.2.**). Add 80 µg purified anti-CD14 mAb 63D3. Rotate in the cold for 30 min (see **Note 7**).
3. Centrifuge cells down at 200g for 10 min at 4°C. Wash off free mAb in cell suspension twice with cold PBS.
4. To the cell pellet add 50 µL heat-inactivated autologous plasma (see **Note 8**), 50 µL PBS/BSA/EDTA, and 80 µL goat-anti-mouse IgG magnetic microbead. Incubate in the refrigerator for 15 min.
5. To purify CD14⁺ monocytes, set up a VarioMACS assembly in a laminar flow hood with a LS column mounted in the column holder. The column is equilibrated with 5 mL cold PBS/BSA/EDTA followed by 0.5 mL of ice-cold autologous heat-inactivated plasma to block nonspecific binding (see **Note 8**). In this and subsequent steps, maintain sterility of cells by ensuring all reagents and supplies are sterile, and work aseptically.
6. To the magnetic bead-treated cells, add 1 mL cold PBS/BSA/EDTA and load the cells to the LS column with a cotton-plugged pasteur pipet. Wash tubes by the addition of 0.5 mL PBS/BSA/EDTA solution. Wait until all the cells have drained into the column. Load the wash solution (PBS/BSA/EDTA). Collect the flow-through cells in an 8-mL tube. Repeat washing with 2-mL aliquots of PBS/BSA/EDTA solution until 6 mL of flow-through wash solution are collected.
7. Remove the column containing the magnetically selected CD14⁺ cells from the VarioMACS unit. Connect the bottom outlet of the column to a 10-mL syringe with a sterile three-way stopcock. Flush cells out by pushing through 6 mL of PBS/BSA/EDTA in 2-mL aliquots with the syringe, and collect the column washes at the column top with a pasteur pipet (see **Note 9**). Pool cells.
8. Count cells and culture CD14⁺ monocytes in complete medium supplemented with 50 ng/mL GM-CSF and 50 ng/mL IL-4 at 1×10^6 cells/mL, 4 mL/well in 6-well plates (Falcon 3046).
9. At the end of 4 d refeed cells by taking out 2 mL of medium and adding 2 mL of fresh medium containing GM-CSF and IL-4 (see **Note 10**).

3.3. T-Cell Purification for Ag-Specific Responses

In this section, Ag-specific response measurements using T cells purified from PBMC from the second donation by negative selection and MDC from the first donation are described.

1. Seven days after the first blood donation, collect another 200 mL blood from the same donor. Process cells and plasma as in the first collection (see **Section 3.1.**).
2. After fractionating the cells through the separation column into CD14⁺ monocytes and CD14⁻ PBMC (see **Section 3.2.**), the CD14⁺ cells are incubated in complete

medium (*see* **Section 2.2.7.**) containing GM-CSF and IL-4 for MDC generation for use in T-cell cytokine production studies (*see* **Section 3.5.**).

3. T cells are purified from the CD14⁻ cell fraction by negative selection. Resuspend the cells in 1 mL complete medium. Add anti-CD19, anti-CD56, anti-CD13, and anti-CD83 mAb to a final concentration of 20 µg/mL each. Rotate the cells in the cold room for 30 min.
4. Wash cells twice with PBS/BSA/EDTA and spin cells down at 200g for 10 min at 4°C. Resuspend cell pellet in 50 µL autologous heat-inactivated plasma, 50 µL PBS/BSA/EDTA, and 80 µL goat-anti-mouse IgG magnetic microbeads. Incubate in the refrigerator for 15 min.
5. Load cells in a LS column and select magnetic microbead-bound cells with a VarioMACS sorting assembly. Collect the flow-through T cells and wash columns as in **Section 3.2., step 6.** Count the T cells with a hemocytometer.

3.4. MDC Ag Presentation in T-Cell Proliferation

1. Detach MDC by washing medium over cells with a cotton-plugged pasteur pipet. Wash cells twice with cold PBS and centrifuge washed cells down at 200g, 4°C for 10 min. Resuspend in complete medium (**Section 2.2.7.**) at 1×10^6 cells/mL (*see* **Note 11**).
2. Resuspend purified T cells at 2×10^6 cells/mL in complete medium. To 96-well plates (Falcon 3072) add 100 µL of T cells into quadruplicate wells.
3. Add graded number of MDC at T:DC ratios of 1000:1, 300:1, 100:1, 30:1, 10:1 in a final volume of 50 µL complete medium. Add allergen to 10 µg/mL in 50 µL medium. The final culture volume per well is 200 µL.
4. For allergen dose–response studies, use a T:DC ratio of 10:1 and add graded amounts of allergen from 0.1 to 10 µg/mL. For the measurement of T-cell responses to overlapping Der p 2 peptides, 4 µL of 1 mM peptides was added to give a final concentration of 20 µM peptide.
5. Include negative and positive controls. Negative control wells contain no Ag. Positive control wells contain 3 µg/mL tetanus toxoid or 20 µg/mL PHA.
6. Incubate the plates at 37°C, 5% CO₂ for a total of 5 d. At the end of the incubation period, add 50 µL [³H]-thymidine (20 µCi/mL, 6.7 Ci/mmol; PerkinElmer, Boston, MA) and pulse cells for 8 h.
7. Harvest cells by a multiwell cell harvester and count [³H]-thymidine incorporation in a scintillation counter.

3.5. T-Cell Ag-Specific Cytokine Production

1. Resuspend purified T cells at 4×10^6 per mL. To 16-mm wells in 24-well plates (Falcon 3047), add 250 µL T cells.
2. Add 100 µL of 0.5×10^6 per mL 7-d MDC (**Section 3.4.1.**).
3. To each of the wells add 100-µL graded amounts of allergens (0.3–10 µg/mL final concentration) or 100 µL of 0.1 mM peptide in duplicate and add complete medium (**Section 2.2.7.**; 50 µL) to make the final volume of 500 µL. Incubate cells for 7 d to activate memory T cells to become effectors.

4. At the end of 7 d, add 1.5 mL of RPMI 1640 to each well to wash. Centrifuge the 24-well plates in microtiter plate carriers at 150g at 4°C for 5 min. Pipet out 1.6 mL supernatant carefully with a 1-mL pipeter (pipetman).
5. Repeat washing and pipet out 1.6 mL supernatant again. Add 100 μ L of 0.5×10^6 per mL fresh DC, 100 μ L of fresh Ag, and 10 μ L of rIL-2 (2500 U/mL) to induce cytokine production by effector T cells. Incubate cells for 48 h for IL-4 and 72 h for IL-5 and IFN- γ production measurements.

3.6. Assay of Th1 and Th2 Cytokines in Supernatants of Stimulated T Cells by ELISA

1. Coat Immulon 4 plates with 50 μ L of 2 μ g/mL anti-huIFN- γ , anti-huIL-4, or anti-huIL-5 capture mAb in 0.1 M sodium bicarbonate solution, pH 8.2 at 4°C overnight.
2. Wash plate three times with PBS/Tween.
3. Add 250 μ L of 5% BSA in PBS/Tween to block plates for 2 h at room temperature.
4. Spin debris in cell supernatants down in a microfuge at 12,000g for 15 min.
5. Dilute supernatants with complete medium at 3 $\times$, 10 $\times$, 30 $\times$, and 100 $\times$ for IFN- γ and IL-5 measurements. Use undiluted supernatants for IL-4 measurements.
6. Prepare standard curves for cytokines by making 200 μ L for each dilution of 3, 1, 0.3, 0.1, and 0.03 ng/mL of standard cytokine in complete medium.
7. Wash plates three times with PBS/Tween. Add 50 μ L supernatants or cytokine standard in duplicates for each dilution. Cover plates with plastic wrap. Incubate at room temperature for 2 h.
8. Discard supernatants and wash plates three times with PBS/Tween.
9. Add 50 μ L of 0.2 μ g/mL biotinylated detecting anti-huIFN- γ , anti-huIL-4, or anti-huIL-5 mAb. Cover plates with plastic wrap. Incubate at room temperature for 2 h.
10. Wash plates three times with PBS/Tween. Add 1:5,000 diluted streptavidin-HRP for 1 h.
11. Wash plates three times and add 100 μ L of substrate solution and incubate for 15 min at room temperature (*see Note 12*).
12. Add 50 μ L 3 N HCl to stop the reaction. Measure absorbance at 492 nm.

4. Notes

1. T-cell proliferative responses to the presentation of tetanus toxoid or diphtheria toxoid by MDC derived from adherent cells (*10*) or low-density Percoll fractionated monocytes (*11*) are shown in **Fig. 1**. MDC from adherent cells and Percoll-purified monocytes comprised only 70 and 85% of the total cell population, respectively. The small contaminating lymphocytes could not be purified from the MDC easily by magnetic depletion. Ag presentation of these MDC is compared with that by E⁻ cells obtained by the depletion of CD2⁺ T cells by E-rosetting of PBMC with neuraminidase-treated sheep erythrocytes followed by centrifugation over a Ficoll-paque cushion as described in **Section 3.1 (Fig. 1)** and can be compared with that by MDC from CD14⁺ monocytes as shown in **Ref. (9)**. T-cell IFN- γ or IL-5 production in response to MDC presentation of either protein Ag (Der p 2) or Ag peptides is shown in **Fig. 2**.

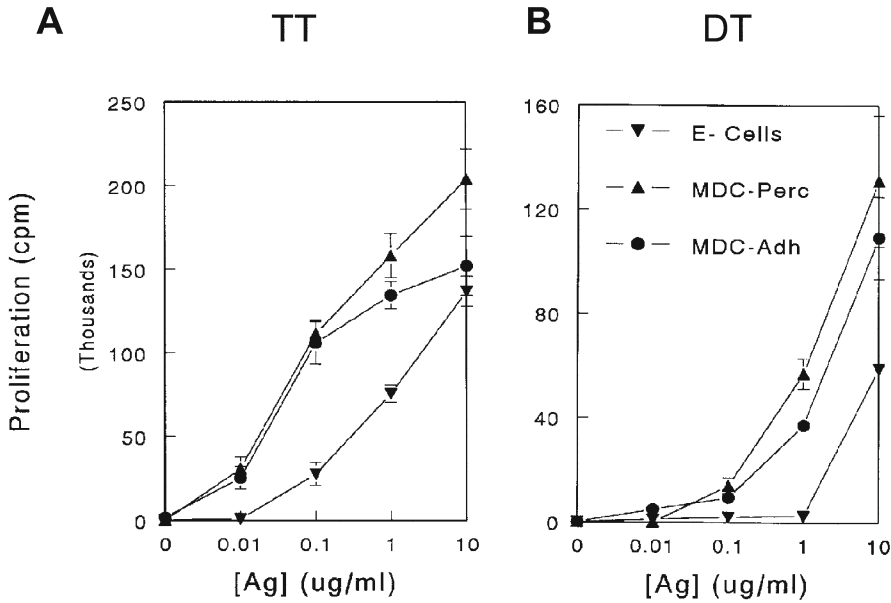


Fig. 1. T-cell proliferation in response to graded doses of Ag presented by MDC. T-cell proliferation was performed as described in **Section 3**. MDC were cultured from Percoll-purified low-density monocytes (**11**) (MDC-Perc) or from the adherent cells in PBMC (**10**) (MDC-Adh). The Ag presentation of MDC was also compared with that by sheep erythrocyte negative non-T cells (E^- cells) added at 6×10^4 cells/well. The Ags used were tetanus toxoid in (**A**) and diphtheria toxoid in (**B**).

- Human blood monocytes are exquisitely sensitive to endotoxin stimulation. Solutions are prepared from endotoxin-free water and stored in endotoxin-free containers. Glass bottles and heat-resistant caps are baked overnight at 180°C before use.
- Avoid repeated freezing and thawing of cytokines and always dilute cytokines in serum-containing solutions to avoid cytokine denaturation.
- The method described is designed for the collection of two blood donations of 200 mL each over a period of 8 d. Volunteers participating in this protocol donate blood for a maximum of eight experiments per year, with 400 mL blood donated per experiment. The method can be scaled down for less blood collected, in which case yellow cap (ACD-A) 8-mL vacutainer tubes (B-D, San Jose, CA) can be used.
- Autologous plasma is used for lower background proliferation and cytokine production. The Ca^{2+} - Mg^{2+} solution used to defibrinate the plasma should be kept at minimal amounts. Excessive amount of Ca^{2+} in plasma will lead to the precipitation of $\text{Ca}_3(\text{PO}_4)_2$ on culture.

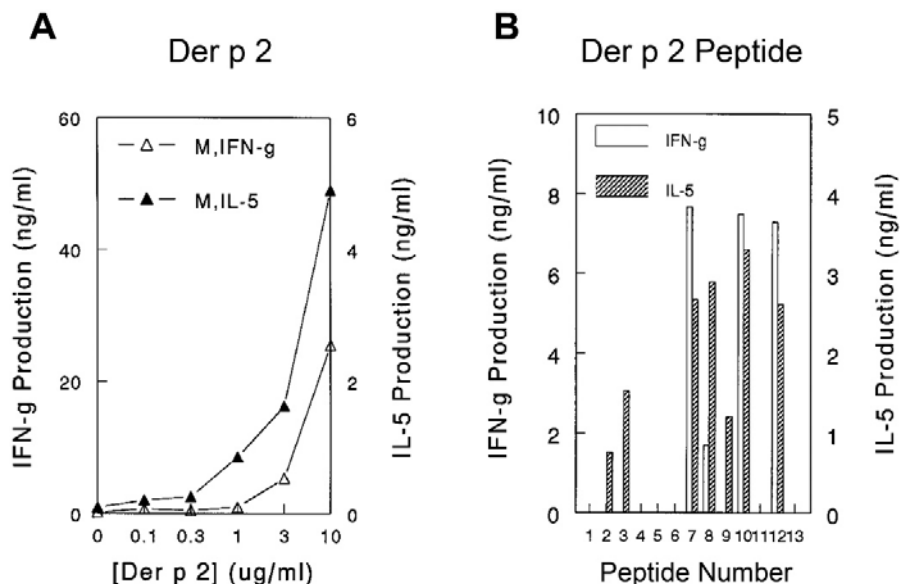


Fig. 2. IFN- γ and IL-5 production by T cells stimulated with house dust mite allergen Der p 2 or Der p 2 peptides. Purified T cells were stimulated with Der p 2 (**A**) or Der p 2 overlapping 20 mer peptides (**B**) as described in **Section 3**. MDC from CD14⁺ monocytes were used as the APC. The results are representative of four experiments from the same individual.

6. The key to the isolation of pure CD14⁺ monocytes is to block nonspecific binding on columns with plasma, keep the column washing solution cold and deaerated, and incubate the mAb-coated cells with goat-anti-mouse IgG magnetic microbead at refrigerator temperatures with minimal bead volumes of 20%.
7. Hybridoma supernatants at 1:2 dilution can be used instead of purified mAb.
8. It is important to heat-inactivate the plasma or serum used with magnetic microbead-coated cells. Otherwise the beads which are made of dextran will activate complement by the alternative pathway, resulting in cell killing. For heat inactivation, heat serum or plasma at 56°C in a water bath for 30 min.
9. It is more efficient to collect positively selected cells by pushing wash solutions from the bottom of the separation column and pipetting cells out from the top than to allow the cells to drip out from the bottom by gravity, especially when the columns are partially clogged.
10. More frequent feeding with cytokines may increase MDC yield.
11. Strongly adherent cells can be detached by incubation in PBS/BSA/EDTA on ice for 30 min followed by pipeting with a pasteur pipet.
12. The color development time can be varied so as to obtain the optimal absorbance readings.

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