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Analysis of T Cell Proliferating and Polarizing Potential of Murine Dendritic Cells in Allogeneic-mixed Leukocyte Reaction

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[Abstract] Dendritic cells (DCs) play a critical role in mounting the T cell response against different infectious agents. Nature and intensity of the induced T cell responses are defined by activation status of DCs. It is generally accepted that IL-12, IL-4/IL-5 and IL-23 producing DCs induce T_H1, T_H2 and T_H17 type of immune responses, respectively (Kumar *et al.*, 2015). Besides cytokines, levels of co-stimulatory molecules on DCs also influence the response of T cells.

The activation status of DCs can be determined by examining DC culture supernatants for different cytokines and by analyzing expression of co-stimulatory molecules on these cells. However, these approaches provide indirect information about T cell activating potential of DCs. Analysis of T cell responses in a co-culture system is a more direct approach to examine T cell proliferating and polarizing efficacy of DCs.

A protocol to analyze the T cell proliferating and polarizing potential of DCs in an allogeneic mixed leukocyte reaction (allo-MLR) is described here.

Materials and Reagents

1. Round bottom, 96-well cell culture plates (Corning, catalog number: 3799)
2. RPMI-1640 medium (HiMedia Laboratories, catalog number: AT028)
3. Dulbecco's Phosphate Buffered Saline (HiMedia Laboratories, catalog number: TS1006)
4. Heat-inactivated fetal bovine serum (Biological industries, catalog number: 04-121-1A)
5. Antibiotic-antimycotic (penicillin-streptomycin) solution, 100x (HiMedia Laboratories, catalog number: A002A)
6. Dendritic cells (derived by culturing mouse bone marrow cells in the presence of recombinant GM-CSF) (PeproTech, catalog number: 315-03)
7. Untouched $CD4^+$ and $CD8^+$ T cells from allogeneic mouse strain (isolated from spleen of Balb/c mice using CD4 T cell enrichment kit and CD8 T cell enrichment kit (BD, catalog number: 558131 and 558471, respectively)
8. Fluorochrome-conjugated FITC anti-mouse CD3, PE anti-mouse CD4 and PE anti-mouse CD8 antibodies (BD Pharmingen, catalog number: 555274, 553730 and 553032, respectively)

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9. Concanavalin A (Sigma-Aldrich, catalog number: C5275)
10. ^{3}H-thymidine (BARC)
11. Trypan Blue (Sigma-Aldrich, catalog number: T8154)
12. RPMI-10 (see Recipes)

Equipment

1. Haemocytometer
2. Humidified CO_2 incubator
3. Laminar air flow bio-safety cabinet
4. Centrifuge
5. Gamma-irradiator
6. Microscope
7. Flow-cytometer

Procedure

1. Harvest mouse bone marrow-derived dendritic cells (BMDCs) from plates by gentle pipetting, give a wash with PBS and prepare the suspensions of 1.0 x 10^5, 2.0 x 10^5 and 4.0 x 10^5 cells per ml in RPMI-10 medium.
   (BMDCs are derived by culturing mouse bone marrow cells in the presence of GM-CSF. Briefly, add 4 x 10^6 bone-marrow cells per well of 6-well plate in RPMI-10 medium supplemented with 20 ng/ml GM-CSF. Remove culture medium along with non-adherent cells on day 3 and day 5, and fresh 4.0 ml GM-CSF-supplemented medium to each well. Harvest immature DCs on day 7 by gently pipetting. After giving a wash in RPMI-10 medium, cells can be used for subsequent experiments. The purity of DCs derived following this protocol is ~85%. These cells can be used directly in allo-MLR or can be further purified.)
2. To analyze the ability of DCs to induce T-cell proliferation, add 50 µl of DC suspensions (equivalent to 0.5 x 10^4, 1.0 x 10^4 and 2.0 x 10^4 DCs) per well in a round bottom plate in triplicates.
   (It is necessary to plate the increasing number of DCs to achieve an increasing ratio of stimulator cells to responder cells. It is advised not to add DCs into outer wells of the plate because culture media tend to evaporate from these wells at higher rates. Instead, these wells can be filled with autoclaved distilled water).
3. To analyze the ability of DCs to induce T-cell polarization, similarly add 50 µl of 2.0 x 10^5 cells/ml DC suspension (= 1.0 x 10^4 cells) per well in a round bottom plate in triplicates.
4. Add the desired stimuli such as LPS or heat-killed mycobacteria to plated DCs and adjust final volume of total contents per well to 100 µl.

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[Dilute stock solution of LPS or mycobacterial suspension to required concentration in RPMI-10 medium. LPS could be used at a concentration of 0.1 to 1.0 µg/ml, whereas heat-killed bacteria (prepared by autoclaving) can be used preferably at a multiplicity of infection (MOI) of 5 to 10].

5. Keep plates in a humidified CO_2 incubator for 24 h.
6. Next day, isolate $CD4^+$ and $CD8^+$ T lymphocytes from the spleen of naïve allogeneic mice using a negative selection kit as suggested by manufacturer. Determine purity of lymphocytes using anti-mouse CD3/CD4 and CD3/CD8 antibodies by flow cytometry.
   Note: If DCs are derived from C57BL/6 mice, lymphocytes can be prepared from Balb/c mice.
7. Irradiate DCs with gamma-rays in a gamma-irradiation chamber (irradiation dose, 25 Gy). Irradiation will prevent the proliferation of DCs, which could otherwise give false results.
8. Adjust concentration of lymphocytes to 1.0 x 10^6 cells/ml. Add 100 µl of cell suspension to irradiated DCs.
9. Set positive controls by stimulating $CD4^+$ T cells and $CD8^+$ T cells with Concanavalin A (final concentration, 5 µg/ml).
10. Keep plates at 37 °C in a humidified CO_2 incubator.
11. After 72 h, add 1.0 µCi ^{3}H-thymidine per well of the plate set up with T cell proliferation assay. Keep plates back into the CO_2 incubator.
12. After 18 h, transfer plates to -20 °C. Plates can be thawed immediately or next day.
13. Harvest the cells onto a filter paper and wash them using an automated cell harvester.
14. Measure the ^{3}H-thymidine levels on filter paper using a beta scintillation counter.
15. Collect culture supernatants from plate set up with T cell polarization assay, after 96 h. Store supernatants at -80 °C or immediately analyze for T_H1, T_H2, T_H17 signature cytokines by ELISA.

Recipes

1. RPMI-10
   RPMI-1640 base medium supplemented with 10% heat-inactivated FBS and 1% antibiotic-antimycotic solution

References

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