The Fleischman Lab

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Title: Fluorescent Detection of Phagocytosis in Macrophages

Introduction

Phagocytosis of various targets can be measured in macrophages by utilizing fluorescent targets that will be detectible by conventional flow cytometry or by fluorescence microscopy. These fluorescent targets can consist of various materials, ranging from latex beads to CFSE-labeled bacteria, granulocytes, or monocytes. The macrophages then engulf the fluorescently-labeled targets and then themselves become fluorescent, allowing for relatively simple detection of phagocytic cells. This protocol includes steps for the preparation of primary human macrophages, though other macrophage sources can easily be substituted.

Materials

1. Primary human mononuclear cells
2. R10 medium (RPMI + 10% heat-inactivated FBS + Pen/Strep)
3. Macrophage medium (R10 medium + 10 ng/ml hM-CSF)
4. Serum-free medium such as X-Vivo
5. PBS
6. Serological pipettes
7. 2 ml non-barrier serological pipettes
8. Filtering flask with bleach and vacuum line
9. Micropipettes and tips
10. For flow cytometry, use non-treated 24-well plates
11. For fluorescence microscopy, use either non-treated or TC-treated 24 well plates
12. Latex beads or target cells (see protocols for CFSE staining of leukocytes and *E. coli*)
13. FACS tubes, for flow cytometry
14. Optional: PFSB (PBS + 0.5% BSA) and CD11b-PE
15. Cell Stripper or Trypsin-EDTA
16. 37°C 5% CO_2 incubator with ≥95% humidity
17. Flow cytometer or fluorescence microscope

Protocol

A. Macrophage Preparation

		Notes
1.	Resuspend freshly-isolated MNCs in warm macrophage medium at a concentration of $1x10^6$ cells per ml. This is day 0.	*Do not use frozen MNCs for macrophage preparation.*
2.	Plate 1 ml of MNCs per well into a 24-well plate and place in the incubator. Macrophages are an adherent population after 7 days.	*Use non-TC plates for flow cytometry, as macrophages will be impossible to remove from TC-treated plates. TC*
3.	On day 6 (or 1 day before the phagocytosis assay), remove media from wells and replace with 1 ml of warm R10 medium. Return to incubator.	*Be careful - cold medium can detach macrophages!*
4.	On day 7 (or the day of the phagocytosis assay), remove media from wells and replace with 500 µl of warm serum-free medium (we use X-Vivo). Return to incubator for 2 hours before beginning assay.	*Macrophages will be ready at day 7 and should be used within 1-2 days.*

	B. Phagocytosis Assay	
1.	Plate 500 µl of solution containing fluorescently-labeled targets directly on top of macrophages.	*Final volume per well should be 1 ml.*
2.	Return macrophages to the incubator for 1 to 2 hours.	
3.	Remove supernatant containing targets from macrophage wells. As a positive control, replace the targets into empty wells.	
4.	Wash macrophage wells 5x with 1 ml room-temperature PBS.	
5.	For flow cytometry, remove cells by vigorous scraping with a 1000 µl pipette containing 1 ml of PBS. a. For stubborn cells, remove PBS and cover the bottom of the wells with a small amount of Cell Stripper or trypsin/EDTA. b. Incubate for about 10 minutes to facilitate cell removal. Scrape vigorously with a 1000 µl pipette and 1 ml of PBS. Wash cells in PFSB and centrifuge at 1200 rpm for 10 minutes, then proceed to staining.	*CFSE lies in the FITC channel, so design your staining panels accordingly.*
6.	For fluorescence microscopy, cells should remain fixed to the plate. a. *Optional:* Macrophages can be stained with 2 µl CD11b PE in 500 µl PFSB at 4°C for 30 minutes. b. After staining, wash cells 1x with 1 ml PFSB and proceed to visualization on the microscope. Add 500 µl PBS to each well and visualize on the microscope.	*PE antibodies can be visualized in the Texas Red filter of the Nikon Ti-E on the first floor of Gross Hall. CFSE will be apparent in the FITC channel of the microscope.*