

Isolation of Human Placental Mast Cells

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Mast cells have been identified as resident cells of human placental tissue by immunohistological procedures, suggesting that they may play a role in pregnancy. However, the study of placental mast cells requires their isolation. Here, we describe a procedure to isolate placental mast cells from placenta of healthy women. At-term placentas were recovered, and small pieces were excised. After extensive washing, they were digested using enzyme, and cell preparations were centrifuged on a Percoll density gradient. A double positive selection was then performed using magnetic beads covered with CD117 and IgE antibodies. The purity of isolated mast cells was finally analyzed by flow cytometry, and was nearly 90%, demonstrating that our protocol was convenient to obtain fresh placental mast cells in sufficient quantity for research investigations. © 2018 by John Wiley & Sons, Inc.

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INTRODUCTION

Mast cells have been identified as resident cells of human placental tissue by immunohistological procedures, suggesting that they may play a role in pregnancy. However, the study of placental mast cells requires their isolation. Here, we describe a procedure to isolate placental mast cells from placenta of healthy women. At-term placentas were recovered, and small pieces were excised. After extensive washing, they were digested using enzyme, and cell preparations were centrifuged on a Percoll density gradient. A double positive selection was then performed using magnetic beads covered with CD117 and IgE antibodies. The purity of isolated mast cells was finally analyzed by flow cytometry, and was nearly 90%, demonstrating that our protocol was convenient to obtain fresh placental mast cells in sufficient quantity for research investigations.

PLACENTA TISSUE DISSOCIATION

1. Obtain at-term placentas from an appropriate Gynecology-Obstetrics Department after necessary approvals and informed consent according the institutional ethic committee.
2. Placentas should be stored in 0.9% NaCl within 24 hr at 4°C.
3. Please be aware of temperature control as defined in the protocol

**BASIC
PROTOCOL 1**

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NOTE: Experiments should be performed in a Biosafety P2 laboratory. All equipment and products should be sterile before use.

Materials

Hank's balanced salt solution (HBSS, Life Technologies)
1 M hydroxyethyl piperazine ethane sulfonic acid (HEPES, Life Technologies)
Magnesium sulfate (MgSO_4 ; Sigma-Aldrich)
Trypsin/EDTA (Life Technologies, cat. no. 15400054)
Deoxyribonuclease I (DNase I), lyophilized powder (Sigma-Aldrich)
Placental tissue
0.9% (w/v) NaCl
Fetal bovine serum (Life Technologies)

Laboratory scale
Scalpel
10-ml plastic culture plates (100 mm $\times$ 20 mm, CML-ID)
Scissors or razor blade
Rotating shaker
100- μm filter (BD Falcon)
50-ml conical tubes (BD Falcon)
Centrifuge

1. Prepare the working digestion solution according to the weight of placenta pieces. Below is an example for 30 g of tissue.

1 $\times$ HBSS	300 ml
1 M HEPES	7.5 ml
MgSO_4	0.3 g
Trypsin/EDTA	30 ml
DNase I I	60 mg

2. Transport placentas in 1 liter of 0.9% NaCl.
3. Weigh the placenta and place it with the maternal surface up and the fetal side, with the umbilical cord insertion point down.
4. Using scalpel, cut pieces of approximately 1.5 cm and place tissue pieces into culture plates containing 10 ml of 0.9% NaCl.
5. Chop/mince the tissues into fine pieces of 1 to 2 mm^3 with scissors or razor blade.
6. Extensively wash the tissue pieces in different culture plates using 0.9% NaCl to ensure that the tissue pieces are largely free from maternal blood.
7. Transfer the minced tissues into a culture flask containing one half of the working digestion solution (see step 1) according to the sample weight.
8. Incubate for 45 min at 37°C with moderate shaking (60 rpm) to efficiently dissociate cells from the tissue.
9. Recover isolated cells and keep them at room temperature. Add the remaining working digestion solution to the flask containing the partially digested tissue and incubate again at 37°C (60 rpm for 30 min) to completely dissociate cells from the tissue.
10. Filter cells and cell debris through a filter (100 μm) and recover isolated cells in 50-ml conical tubes containing 2 ml of FBS to inactivate the enzymes contained in the digestion solution.

11. Centrifuge 15 min at $1000 \times g$, room temperature, to pellet the cells. Remove supernatant.

Approximately 1×10^8 cells are obtained with 30 g of tissue.

12. Dilute cells in 50 ml basal (serum-free) DMEM/F12 medium and proceed to the isolation of placental cells as described in Basic Protocol 2.

ISOLATION OF PRIMARY PLACENTAL CELLS

To isolate primary placental cells, a Percoll gradient procedure must be performed.

Materials

D-glucose powder (Sigma Aldrich)
Phosphate-buffered saline (PBS; Life Technologies, cat. no. 14190250)
Percoll (GE Healthcare)
Dissociated cells (Basic Protocol 1)
Human mast cell culture medium (see recipe)

50-ml conical tubes (BD Falcon)
Centrifuge

Additional reagents and equipment for basic cell culture techniques including counting cells and determining viable cells by trypan blue exclusion (Phelan & May, 2015)

1. Prepare Percoll solution:
 - a. Prepare 1 g/liter D-glucose in phosphate-buffered saline.
 - b. Prepare a 90% Percoll solution with PBS/D-glucose.
 - c. Dilute the Percoll solution to 25% and 60% in PBS.
2. Slowly add 15 ml of 60% Percoll to a 50-ml conical tube, and then carefully add 15 ml of 25% Percoll on top of this layer.
3. Slowly deposit 15 ml of cells using a 10-ml pipet (10 ml) on top of the 25% Percoll and centrifuge 20 min at $700 \times g$, room temperature (without braking).
4. Remove tubes from centrifuge without disturbing the phases.
5. Aspirate cells located at the interface between the two Percoll density phases as depicted in Figure 1.
6. Transfer the cell phase (approximately 15 ml) to a 50-ml conical tube and add 30 ml of human mast cell culture medium.
7. Centrifuge 15 min at $1000 \times g$, room temperature, to pellet the cells.
8. Dilute the pellet in 500 μ l of human mast cell culture medium.
9. Count cells and determine cell viability by trypan blue exclusion (Phelan & May, 2015).

Approximately $2\text{--}3 \times 10^7$ cells are obtained from 30 g of tissue.

10. Dilute cell suspension in 30 ml of human mast cell culture medium and centrifuge 10 min at $700 \times g$, room temperature.

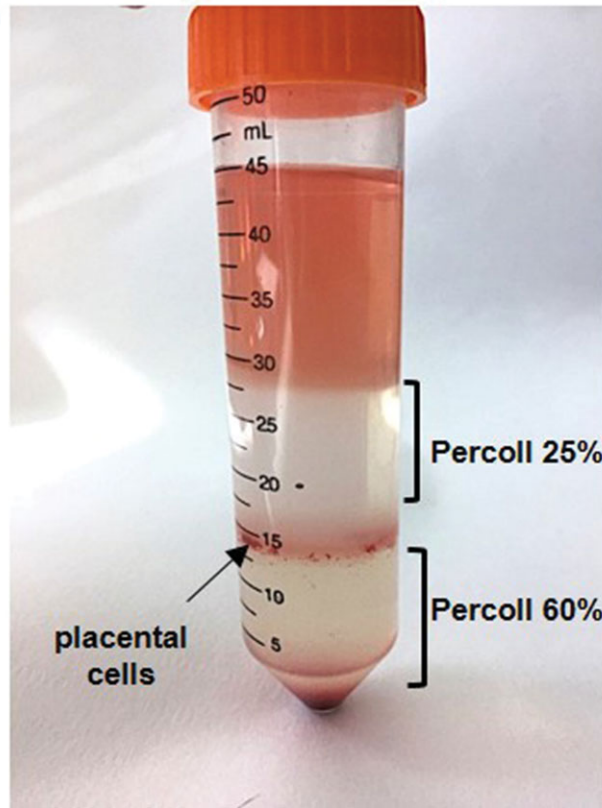
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Figure 1 Isolation of placental cells by Percoll cushion. Representative image showing placental cells between the two phases of Percoll.

BASIC PROTOCOL 3

POSITIVE SELECTION OF PLACENTAL MAST CELLS

To obtain high-purity mast cells, an enrichment with a positive selection column must be used with two specific markers CD117 and IgE. This protocol was used according to the indications of the manufacturer Miltenyi Biotec.

Materials

Placental cell pellet (Basic Protocol 2)
 Magnetic-Activated Cell Sorting (MACS) separation buffer, (Miltenyi Biotec)
 FcR Blocking Reagent (Miltenyi Biotec)
 CD117 microbeads (Miltenyi Biotec)
 Anti-IgE-phycoerythrin (PE) (Bühlmann)
 Anti-PE beads (Miltenyi Biotec)
 Human mast cell culture medium (see recipe)

Column LS (Miltenyi Biotec)
 15-ml conical tubes (BD Falcon)
 Centrifuge

NOTE: All the experiments were performed at 4°C except when otherwise specified.

CD117 selection

1. Resuspend cell pellet in MACS separation buffer (300 μ l of buffer for 1×10^8 cells).
2. Add 100 μ l of FcR Blocking Reagent to prevent nonspecific binding of antibodies

3. Add 100 μ l of CD117 microbeads and incubate for 15 min.
4. Wash the cells with 1 to 2 ml of MACS separation buffer and centrifuge 10 min at $300 \times g$, room temperature.
5. Completely remove the supernatant and resuspend the cell pellet with 500 μ l of MACS separation buffer per 1×10^8 cells.
6. Prepare MACS Columns and Separators during the centrifugation. Place an LS column in the magnetic field of the MACS separator and add 3 ml of MACS separation buffer onto the column. Use a receptacle to catch the flowthrough. Discard the flowthrough.
7. Place a 15-ml conical tube below the column and place the cell suspension onto the column.
8. Remove column from the separator and place it on a suitable 15-ml collection tube.
9. Add 5 ml of MACS separation buffer onto the column and immediately flush out the labeled cells by pushing the plunger into the column to obtain CD117⁺ placental cells.
10. Centrifuge CD117⁺ cells 10 min at $700 \times g$, room temperature.

IgE selection

11. Resuspend cell pellet in 300 μ l of MACS separation buffer per 10^8 cells.
12. Add 10 μ l of IgE-PE antibody per 10^6 cells.
13. Incubate 30 min in the dark.
14. Wash the cells with 1 to 2 ml of MACS separation buffer and centrifuge 10 min at $300 \times g$, room temperature.
15. Resuspend cell pellet in 80 μ l of MACS separation buffer per 10^7 cells.
16. Add 20 μ l of anti-PE microbeads per 10^7 cells and incubate for 15 min in the dark.
17. Wash the cells with 1 to 2 ml of MACS separation buffer and centrifuge 10 min at $300 \times g$, room temperature.
18. Completely aspirate the supernatant and resuspend the cell pellet with 500 μ l of MACS separation buffer per 10^8 cells.
19. Prepare MACS Columns and Separators during the centrifugation. Place an LS column in the magnetic field of the MACS separator and add 3 ml of MACS separation buffer onto the column. Use a receptacle to catch the flowthrough. Discard the flowthrough.
20. Place a 15-ml conical tube below the column and place the cell suspension onto the column.
21. Remove column from the separator and place it on a suitable 15-ml collection tube.
22. Add 5 ml of MACS separation buffer onto the column and immediately flush out the labeled cells by pushing the plunger into the column to obtain double positive CD117⁺/IgE⁺ cells corresponding to placental mast cells.
23. Centrifuge placental mast cells 10 min at $500 \times g$, room temperature.
24. Resuspend cell pellet in human mast cell culture medium and incubate cells at 37°C in an atmosphere containing 5% CO₂.

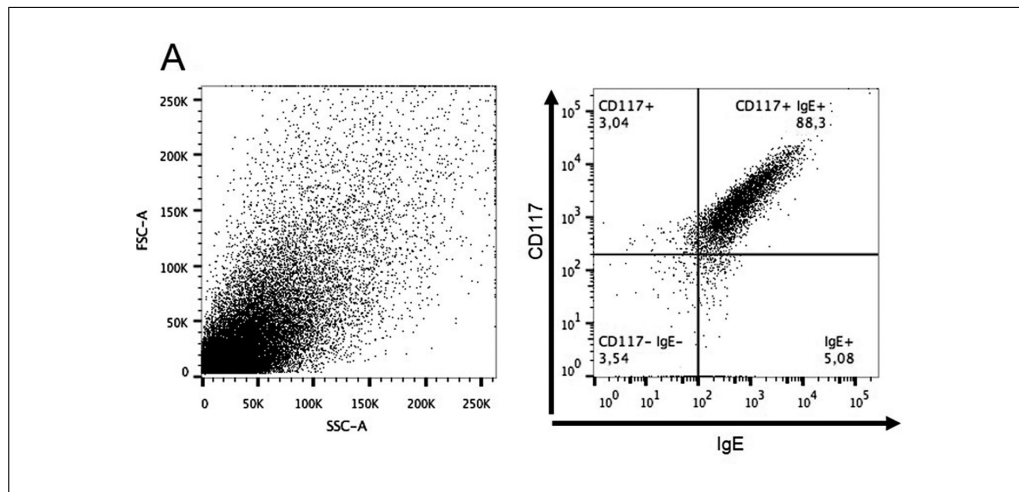


Figure 2 Characterization of placental mast cells by flow cytometry. Placental mast cells were observed (left panel) and characterized using CD117 and IgE antibodies (right panel).

BASIC PROTOCOL 4

CHARACTERIZATION OF PLACENTAL MAST CELLS BY FLOW CYTOMETRY

Verify the purity of mast cells by flow cytometry using CD117 and IgE markers and appropriate isotype controls.

Materials

CD117⁺/IgE⁺ cells (Basic Protocol 3) PBS containing 5% FBS
 Phosphate-buffered saline (PBS; Life Technologies, cat. no. 14190250)
 Anti-IgE-phycoerythrin (PE, Bülmann)
 Anti-CD117-allophycocyanin (APC, Beckman Coulter)
 Mouse IgG1-PE (Beckman Coulter)
 Mouse IgG1-APC (Beckman Coulter)
 Flow cytometer (e.g., BD Fortessa)
 FlowJo software (BD)

Additional reagents and equipment for flow cytometry (Robinson et al., 2018)

1. Centrifuge placental mast cells 10 min at $500 \times g$, room temperature, then remove supernatant. Suspend CD117⁺/IgE⁺ cells in PBS/5% FBS and incubate them with CD117-FITC, IgE-PE antibodies, or appropriate isotype controls during 1 hr at 4°C in the dark.
2. Wash labeled cells with PBS/5% FBS and centrifuge for 5 min at $300 \times g$, room temperature.
3. Resuspend cell pellet in 250 μ l of PBS
4. Analyze the expression of CD117 and IgE by flow cytometry, e.g., by using a BD Fortessa cytometer and FlowJo software.

85% to 90% of selected cells should express both CD117 and IgE as depicted in Figure 2.

Approximately $2-6 \times 10^6$ CD117⁺/IgE⁺ cells may be obtained from 30 g of tissue.

REAGENTS AND SOLUTIONS

Human mast cell culture medium

Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (DMEM/F12, Life Technologies)

10% fetal bovine serum (FBS, Life Technologies)

1 × penicillin/streptomycin (Life Technologies)

Add 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (final concentrations) to DMEM/F12 medium and filter the supplemented medium through a 0.2-µm bottle-top filter.

Supplemented DMEM-F12 can be stored for 3 to 4 weeks at 4°C in the dark.

COMMENTARY

Background Information

Described over a century ago by Paul Ehrlich (Ehrlich, 1878), mast cells are derived from pluripotent progenitor cells (CD34⁺/CD117⁺) from bone marrow (Kirshenbaum et al., 1999). They are then released into the bloodstream and migrate subsequently into various tissues, where they mature into CD117⁺/IgE⁺ cells. Two subtypes of mast cells, based on the protease composition of their secretory granules, have been described after their migration in mucosal and connective tissues (Bischoff, 2007; Enerbäck, 1966). Histological procedures show that mast cells are present in most tissues in close contact with the environment, such as lung, intestine, skin, colon, brain, and placental tissues (Bischoff, 2007). Human placenta is composed of several cell populations, including smooth muscle cells, fibroblasts, endothelial cells, syncytiotrophoblasts, and cytotrophoblasts, as well as immune cells such as lymphocytes, macrophages, dendritic cells, and mast cells (Xu, Plazyo, Romero, Hassan, & Gomez-Lopez, 2015). It has been previously shown that leukocytes are present in the placental bed during all stages of pregnancy; their number is slightly increased toward the third trimester (Kwan et al., 2014; Williams, Searle, Robson, Innes, & Bulmer, 2009). Using immunohistochemical methods, the number of placental mast cells is evaluated as 7.6×10^5 mast cells/g of wet weight tissue (Purcell & Hanahoe, 1991). The role of these placental mast cells has been poorly investigated. It has been suggested that they are involved in pre-eclampsia via their release of histamine (Szewczyk et al., 2005; Szukiewicz et al., 1999; Szukiewicz, Szukiewicz, Maslinska, Szewczyk, & Watroba, 2003). So far, only lines of human mast cells such as HMC-1 (with a non-functional IgE receptor) or mast cells generated *in vitro* from blood-cord precursors (providing a large number of pure and homogenous mast cells) are used to investigate placental mast cells (Kirshenbaum, 2000; Kirshenbaum et al., 1999). These models are likely not accurate to study the func-

tional properties of placental mast cells. Thus, we isolated placental mast cells in sufficient number and purity for research investigations.

Troubleshooting

The success of this protocol depends on a few critical steps. First, isolation of placental cells requires correct preservation of the placenta before the beginning of the procedure. For example, if the preservation of the placenta is incorrect, enzymatic digestion will be more difficult, and ultimately a low cellular yield will be obtained. Second, the isolated placental cells could be contaminated by blood cells from the mother. Thus, it is important to extensively wash the pieces of tissue to remove the maternal blood. Third, loss or activation of cells can occur during the protocol. Thus, it is important to respect the indicated temperatures, in particular concerning the enzymatic digestion (37°C), the centrifugation steps (room temperature), the magnetic separation (4°C), and the Percoll preparation, as well as the centrifugal force without braking during Percoll density centrifugation. In addition, during the dissociation of placental tissue, shaking should be performed at the indicated speed, to prevent cell death. Finally, it is important to note that the sterility of the tissue is difficult to control. Thus, it is important to use only sterile solutions and perform all work in a biosafety hood.

Anticipated Results

Generally, mast cell isolation from at-term placenta is easy to achieve with placental mast cells of reasonable purity. However, mast cell yield is dependent on the tissue samples used. Placental tissue is variable according to the donor and the weight and the quality of the tissue. From 10 g of tissue, obtained from at-term placenta, this procedure leads to a yield of approximately 7×10^6 cells with 80% to 85% of purity. Of note, the number of placental mast cells is dependent on the weight of the placenta.

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Time Considerations

The tissue dissection, enzymatic digestion, and Percoll centrifugation take approximately 3 to 4 hr. The duration of mast cell enrichment by positive selection takes approximately 2 hr. The characterization of cells by flow cytometry takes 2 hr.

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Conflicts of Interest

The authors declare no conflicts of interest.

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