TEMPLATE for PROTOCOL UNIT

Generation and analysis of human and murine osteoclasts

Running Title: Osteoclast generation and analysis

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Significance Statement

Bone integrity is maintained by a concerted action of bone forming osteoblasts and bone resorbing osteoclasts. Increased osteoclast differentiation and/ or activity result in osteopenia, osteoporosis or local bone erosions which affect a large proportion of elderly people and thereby constitute a major burden for the society. It is therefore of interest to understand how osteoclast are affected by cells, cytokines, hormones or other substances. The protocols described in this manuscript allow the *ex vivo* differentiation of murine and human osteoclasts as well as the evaluation of osteoclast differentiation and resorption activity. Together, they provide useful tools for the investigation of osteoclast regulation.

ABSTRACT

Osteoclasts are the only bone resorbing cells in the body. Together with bone forming osteoblasts they are responsible for a balanced bone homeostasis and constant bone remodeling. Aberrant activation of osteoclasts leads to bone loss as seen in postmenopausal osteoporosis or in autoimmune diseases like rheumatoid arthritis. Although, a lot of research has been performed to understand and prevent osteoclast-mediated bone loss, the mechanisms of osteoclast hyperactivation are not completely understood. This article describes several protocols for the *ex vivo* generation of murine and human osteoclasts allowing to study the effects of specific cells, cytokines or chemical substances on osteoclast formation and activity without the need of expensive and time-consuming animal experiments. In addition, we provide protocols for the specific staining of osteoclasts and the analysis of resorption activity using calcium phosphate coated surfaces or bone slices.

Keywords: osteoclast generation, PBMC, bone marrow, resorption, TRAP staining

INTRODUCTION

Osteoclasts are large, multinucleated cells that develop by cell fusion of macrophage like precursors. The main function of osteoclasts is bone resorption. In fact, osteoclasts are the only cell type capable to degrade bone and a lack of osteoclasts leads to severe osteopetrosis (Kong et al., 1999). For bone resorption, osteoclasts build up a sealing zone directly on the bone surface that surrounds an isolated cavity. In the so formed resorptive microenvironment, osteoclasts secrete protons and proteases which dissolve hydroxyapatite and the organic bone matrix, respectively (Takito, Inoue, & Nakamura, 2018). Every year up to 10 % of the body bone matrix is resorbed by osteoclasts and rebuilt by osteoblasts, a process that is necessary to remove micro damages and to preserve bone integrity. However, aberrant activation of osteoclasts e.g. because of hormonal changes or inflammatory cytokines leads to increased bone resorption causing osteopenia or osteoporosis.

Osteoclast generation and activation is mainly controlled by the cytokine receptor activator of NF- κ B ligand (RANKL) expressed by osteoblasts, osteocytes and a variety of immune cells. The discovery of this cytokine in 1998 enabled scientists to generate osteoclasts in cell culture without the need of osteoblasts or stromal cells (Yasuda et al., 1998). This was an important step making it possible to study direct effects of cytokines, hormones, cells or chemical substances on osteoclast maturation and/ or bone resorption. Since then the number of publications about osteoclast function increased every year.

This article provides optimized protocols for the *ex vivo* generation of human and murine osteoclasts from whole blood and bone marrow, respectively as well as the *ex vivo* generation of osteoclasts by co-culture with osteoblasts. In addition, we explain how to specifically visualize osteoclasts for quantification and how to assess the resorptive activity of generated osteoclasts.

BASIC PROTOCOL 1

Generation of human osteoclasts from peripheral blood mononuclear cells (PBMCs)

Introductory paragraph

The differentiation of human osteoclasts from PBMCs is an easy and cost effective way to test the effects of distinct substances on osteoclast maturation and/ or function in a human system. This protocol describes the isolation of PBMCs from human blood with a Ficoll gradient, subsequent enrichment for monocytes by plastic adherence and the differentiation into osteoclasts. This method is very gentle to the cells, but results in a mixture of cells that still contains considerable amounts of lymphocytes after monocyte enrichment. If lymphocytes might react to the substances of interest, one should consider using directly isolated monocytes as starting cells as described in the alternate protocol 1.

Materials

sterile phosphate buffered saline (PBS), pH 7.2 (without calcium and magnesium)

Ficoll

Blood from human donors containing anticoagulants (e.g. ethylenediaminetetraacetic acid (EDTA), citrate or heparin)

sterile PBS containing EDTA (1 mM) (= PBS/ EDTA)

adherence medium (= α -MEM with 1 % penicillin/ streptomycin and 1 % fetal calf serum (FCS))

osteoclast medium (= α -MEM with 1 % penicillin/ streptomycin and 10 % FCS)

recombinant human macrophage colony-stimulating factor (M-CSF) (Peprotech, #300-25)

recombinant human soluble RANKL (Peprotech, #310-01)

recombinant human transforming growth factor β (TGF- β) (Peprotech, #100-21C)

50 ml conical centrifuge tubes

centrifuge

cell counting device

cell culture plates

cell culture incubator

Protocol steps—*Step annotations*

1. Bring PBS and Ficoll to room temperature.

2. Take human blood that has been prevented from clotting with EDTA, citrate or heparin and mix it with the same amount of PBS.

You can expect 10-20 mio PBMCs from 10 ml blood, depending on the donor.

3. Put 12.5 ml of Ficoll in a 50 ml conical tube and slowly layer 25 ml of the blood-PBS mixture over the Ficoll (Figure 1).

The volumes can be extended up to 15 ml of Ficoll and 30 ml of blood-PBS mixture. You should not use less than 10 ml of Ficoll to ensure sufficient space between the individual cell layers. A correct layering is important to obtain pure PBMCs. The blood-PBS should not mix with the Ficoll, but float above.

4. Centrifuge the tubes for 25 min at room temperature and 800 g without brake!

The idea of this step is to separate PBMCs from granulocytes and red blood cells using cellular density. The centrifuge should be well balanced and operated without brake to obtain well defined cell layers.

5. Carefully take the interphase cloud (containing the PBMCs) floating on the Ficoll layer and put it into a new tube. Fill up the tube to 50 ml with cold PBS/ EDTA (1 mM).

At this step, the interphase cells from 2 tubes can be combined into 1 new tube. The total interphase volume should not exceed 25 ml to have enough space for PBS/ EDTA. Ficoll is toxic for cells, so the cells should be processed immediately.

6. Centrifuge the tubes for 15 min at 4°C and 245 g with lowered deceleration (e.g. level 4 from 9).

7. Discard the supernatant and resuspend the pelleted cells in 50 ml of cold PBS/ EDTA.

At this step, the cells from 2 tubes can be combined into 1 tube.

8. Centrifuge the tubes for 10 min at 4°C and 160 g.

9. Discard the supernatant and resuspend the pelleted cells in 50 ml of cold PBS/ EDTA.

At this step, the cells of 2 tubes can be combined into 1 tube.

10. Centrifuge the tubes for 12 min at 4°C and 100 g.

The idea of the washing steps with PBS/ EDTA is to get rid of platelets that would disturb the assay.

11. Discard the supernatant and resuspend the pelleted cells in adherence medium.

12. Count cells and plate them at a density of 3 mio PBMCs per ml using 500 µl, 250 µl and 100 µl for 24, 48 and 96 well plates, respectively.

13. Incubate cells for 1-2 h at 37°C and 5 % CO₂. Then take off the medium by flushing twice the cells to remove non-adherent cells.

During the incubation, monocytes adhere to the cell culture plate, while lymphocytes stay in suspension. For this step it is important not to have more than 1 % of FCS in the medium to facilitate the adhesion process.

14. Add osteoclast medium containing M-CSF (30 ng/ml), RANKL (2 – 10 ng/ml) and TGF-β (1 ng/ml) (1000 µl, 500 µl and 200 µl per well for 24, 48 and 96 well plates, respectively). Culture the cells at 37°C and 5 % CO₂. Exchange the medium every 3 d. After 7-9 d mature osteoclasts with multiple nuclei should have formed (Figure 2). For osteoclast staining see Support Protocol 1.

For osteoclast differentiation, the FCS is very critical and can cause enormous differences in the osteoclast yield. We strongly recommend testing FCS from several sources to ensure optimal assay performance. The amount of RANKL has to be adapted to the assay performance in the individual lab and to the needs of the researcher. Larger amounts of RANKL result in faster osteoclast differentiation and larger osteoclasts. However, for some experiments it is beneficial to differentiate osteoclasts under suboptimal RANKL concentrations.

ALTERNATE PROTOCOL 1

Generation of human osteoclasts from purified blood monocytes

Introductory paragraph

Some research questions require starting the osteoclast assay with purified monocytes to avoid effects mediated by lymphocyte contaminations. There are several techniques available to purify monocytes from human blood. Here, we describe exemplarily a method that uses direct monocyte labeling with magnetic beads from Miltenyi binding to CD14. This method results in a high purity with typically less than 5 % contaminating cells. But, the procedure is quite stressful for the cells and it is unclear if monocytes change their behavior due to the labeling (Bhattacharjee, Das, Mishra, Sahay, & Upadhyay, 2017). As an alternative, negative isolation kits are available and less stressful for the cells. However, they result in a lower monocyte purity that in our hands is comparable with the purity achieved by plastic adherence described in Basic Protocol 1.

Additional Materials (also see Basic Protocol 1)

CD14 monocyte isolation kit (for example from Miltenyi # 130-050-201)

magnetic cell separation (MACS)-buffer (see recipe)

Miltenyi columns (LS, #130-042-401)

Miltenyi magnets

Protocol steps—*Step annotations*

1. Isolate PBMCs as described in steps 1 to 10 of Basic Protocol 1.
2. Resuspend PBMCs in MACS-buffer and count the cells.
3. Centrifuge the cells for 5 min at 4°C and 300 g.
4. Remove the supernatant completely and resuspend the cells in 80 µl MACS-buffer per 10⁷ cells.
5. Add 20 µl of CD14 beads per 10⁷ cells. Mix well and incubate for 15 min at 4-8 °C.
6. Add 1-2 ml of MACS-buffer per 10⁷ cells.
7. Centrifuge the cells for 5 min at 4°C and 300 g.
8. Remove the supernatant completely and resuspend the cells in 500 µl MACS-buffer.
9. Place a LS MACS column into the respective magnet and wash it with 3 ml of MACS-buffer.

This step can be performed during the centrifugation in step 7 but you should take care that the column does not run dry. Add more MACS-buffer to the column if the cells are not ready in time. If you have less than 10⁸ cells, you can also work with MS columns.

10. Put the resuspended cells on the column. Wash 3x with 3 ml of MACS-buffer to remove unlabeled cells.

At this step all monocytes bound to the magnetic beads are kept in the column while other cells can be collected in the flowthrough.

11. Detach the column from the magnet and place it above a 15 ml conical tube. Add 5 ml of MACS-buffer on the column. Put the plunger on top and firmly press until all liquid (containing the monocytes) is in the collection tube.

We recommend determining monocyte purity using flow cytometry.

12. Centrifuge the cells for 5 min at 4°C and 300 g.
13. Discard the supernatant and resuspend the cells in osteoclast medium.

14. Count cells and plate them at a density of 375×10^3 cells per ml using 1000 μ l, 500 μ l and 200 μ l per well for 24, 48 and 96 well plates, respectively. Add M-CSF (30 ng/ml) and RANKL (2 – 10 ng/ml). Exchange the medium every 3 d. After 6-9 d mature osteoclasts with multiple nuclei should have formed. For osteoclast staining see Support Protocol 1.

For osteoclast differentiation, the FCS is very critical and can cause enormous differences in the osteoclast yield. We strongly recommend testing FCS from several sources to ensure optimal assay performance. The amount of RANKL has to be adapted to the assay in the individual lab and the needs of the researcher. Larger amounts of RANKL result in faster osteoclast differentiation and larger osteoclasts. However, for some experiments it is beneficial to differentiate osteoclasts under suboptimal RANKL concentrations.

BASIC PROTOCOL 2

Generation of murine osteoclasts from bone marrow

Introductory paragraph

When working with murine cells, bone marrow is the starting material of choice for the generation of osteoclasts. Bone marrow naturally contains macrophage-like osteoclast precursors which reflect the *in vivo* situation more accurately than blood-derived monocytes. Bone marrow is usually derived from adult mouse femurs and tibias. If more cells are needed, the humeri can be taken in addition, which increases the cell yield up to 50 %. From one mouse between 25 and 40 mio cells can be gained, depending on individual skill. Depending of the experimental settings, bone marrow derived cells of several mice can be pooled. The following protocol describes the isolation procedure of bone marrow cells and their differentiation to osteoclasts.

Materials

donor mice: common wildtype strain, e.g. C57BL/6; 6 to 14 weeks old; male or female

70 % ethanol

sterile PBS, pH 7.2 (without calcium and magnesium)

red blood cell lysis buffer (see recipe)

osteoclast medium (= α -MEM with 1 % penicillin/ streptomycin and 10 % FCS)

recombinant murine M-CSF (Peprotech, #315-02)

recombinant murine RANKL (Peprotech, #315-11)

sterile scissors and tweezers

petri dishes 10cm, sterile
23 G and 27 G sterile needles
1 ml and 5 ml syringes
50 ml conical centrifuge tubes
70 μ m cell strainer, sterile
cell counting device
cell culture plates
cell culture incubator

Protocol steps—*Step annotations*

1. Kill the mouse using CO₂ inhalation and cervical dislocation.

You can utilize other mouse strains than C57BL/6 as long as there are no known defects in osteoclast or macrophage differentiation.

2. Sterilize mouse fur with 70 % ethanol.

3. Carefully remove femurs and tibias together with the surrounding muscle tissue and put them into a sterile 10 cm petri dish.

This step can be performed outside of a cell culture bench. As long as the bones are not damaged, the risk of bacterial contamination is very low. It is possible to use the humeri in addition to obtain more cells, but they are more difficult to handle due to their smaller size.

4. Prepare femurs and tibias under sterile conditions:

a. Remove all skin, tendons and muscles as good as possible.

It might be helpful to use a sterile gaze paper and rub the remaining tissue from the bones.

b. Cut off both ends of all bones with a scalpel or a sterile scissor.

c. Flush out the bone marrow with 10 ml of osteoclast medium for all bones from one mouse using a 5 ml syringe and a 23 G needle in a fresh sterile petri dish. Discard the flushed bones.

Try to flush all bone marrow cells out of the bones. Afterwards the bones should appear white/ transparent. If needed, take more medium or flush again with the same medium.

d. Separate cell aggregates as good as possible using a 1 ml syringe and a 27 G needle by drawing the cell suspension in and out of the syringe. Then flush the cell suspension through a 70 µm nylon mesh in a 50 ml Falcon tube.

e. Flush the nylon mesh with additional 2 ml of medium.

5. Centrifuge the cells for 7 min at 4°C and 300 g.

6. Resuspend cells in 5 ml of red blood cell lysis buffer. Incubate at room temperature for 2 min and then add 30 ml of PBS for neutralization.

7. Centrifuge the cells for 7 min at 4°C and 300 g.

8. Resuspend pelleted cells in 5 ml of osteoclast medium. Plate cells for overnight adherence in a 6 well plate (1 well per cells from 1 mouse) and incubate them at 37°C and 5 % CO₂.

This step will deplete stromal cells which are adherent. It is important not to extend the incubation time too much, as osteoclast precursors will also adhere with time. It is best to start cell isolation in the afternoon and to continue the protocol the next day directly in the morning.

9. The next day, take non-adherent cells by pipetting the medium two times up and down without scraping the bottom.

10. Centrifuge the cells for 7 min at 4°C and 300 g.

11. Resuspend the cells in 5 ml osteoclast medium and count viable cells.

12. Plate cells at 1 mio cells/ml in osteoclast medium containing 30 ng/ml M-CSF and 10 ng/ml RANKL using 1000 µl, 500 µl and 200 µl per well for 24, 48 and 96 well plates, respectively.

RANKL and M-CSF concentrations may vary between mice and lab conditions and should be titrated.

13. Change the medium after 3 d. After 4-5 d mature osteoclasts with multiple nuclei should have formed (Figure 2). For osteoclast staining see Support protocol 1.

Have a look on your cells at least once daily from day 4 after cytokine addition as murine osteoclasts are very unstable after they have fully differentiated. In contrast to human osteoclasts, murine osteoclasts die very quickly after maturation.

BASIC PROTOCOL 3

Generation of murine osteoclasts from bone marrow in co-culture with murine osteoblasts

Introductory paragraph

Osteoblasts are natural regulators of osteoclastogenesis by producing the pro-osteoclastogenic cytokine RANKL and its decoy receptor osteoprotegerin (OPG). The here described co-culture assay enables to study osteoblast-osteoclast-interactions *ex vivo*. This protocol explains the isolation of murine osteoblast precursor cells from calvaria of neonatal mice and their co-culture with bone marrow cells for osteoclast differentiation.

Additional Materials (also see Basic Protocol 2)

donor mice: e.g. C57BL/6, 2 to 5 days old, male or female (e.g., Charles River Laboratories, Jackson Laboratories)

70 % ethanol

sterile PBS, pH 7.2 (without calcium and magnesium)

digestion medium *see recipe*

trypsin/ EDTA

dexamethasone (Sigma D4902) solved in ethanol

vitamin D3 (Calbiochem, 679101)

sterile scissors and tweezers

petri dishes 10 cm, sterile

15 ml conical centrifuge tubes

water bath

cell culture plates

cell counting device

cell culture incubator

Protocol steps—*Step annotations*

Osteoblast isolation

1. Decapitate 2-5 d old mice and sterilize the heads with 70 % ethanol.

Per calvaria you can expect around 1 mio cells after 2-4 d of culture in a 10 cm petri dish.

2. Carefully prepare the calvaria and remove all skin, fat and remaining tissue.

It is important to remove the remaining tissue thoroughly as it interferes with the digestion medium.

3. Wash the calvaria in PBS and leave it there until all calvarias have been prepared.
4. Put each calvaria into 1 ml digestion medium and incubate at 37°C on a shaking platform for 10 min in a water bath.
5. Take the supernatant and discard this first digestion fraction (= fraction 1).

The first fraction is often very contaminated with cell debris and therefore should be discarded.

6. Add again 1 ml digestion medium to each calvaria and incubate at 37°C on a shaking platform for 10 min in a water bath.
7. Put the liquid phase (= fraction 2) in a 15 ml conical centrifuge tube. Repeat step 6 three times and put the fractions 3-5 to the same centrifuge tube.
8. Centrifuge the combined fractions (2 to 5) for 7 min at 4°C and 300 g.
9. Resuspend the cell pellet from each calvaria in 500 µl osteoclast medium and seed the cells in a well of a 6 well plate containing 1 ml of osteoclast medium.
10. Let the cells grow until sub confluency (plate should be covered around 80%) and then plate them in a 10 cm dish. Cells can be easily detached with pre-warmed trypsin-EDTA after a PBS washing step.

The cells should be subconfluent after 1-2 d.

11. Let the cells again grow until sub confluency and then split one 10 cm dish on five 10 cm dishes.

This step can be repeated at maximum two times depending on the amount of cells required for the experiment.

Osteoblast-osteoclast co-culture

12. Seed osteoblasts in 24 well plates in osteoclast medium at 50.000 cells in 500 µl per well and let them attach overnight.
13. Isolate bone marrow cells and incubate them over night as described in Basic Protocol 2 (step 1-11).
14. The next day, remove medium from the osteoblasts and replace by osteoclast growth medium containing the bone marrow cells (1 mio cells in 1 ml per well), vitamin D3 (10^{-8} M) and dexamethasone (10^{-7} M).
15. Change the medium every 2 d.

16. Between 5 and 7 d after co-culture start, mature osteoclasts with multiple nuclei should have formed (Figure 2). For osteoclast staining see Support Protocol 1.

SUPPORT PROTOCOL 1

Tartrate-resistant acidic phosphatase (TRAP) staining

Introductory paragraph

TRAP is secreted by osteoclasts and serves in serum as a marker of bone resorption. Although TRAP is not exclusively expressed by osteoclasts, it can be used for osteoclast staining in bone tissue and in cell culture (Hayman, 2008). The here described TRAP staining is a fast, easy and relatively robust method for the visualization of pre-osteoclasts and mature osteoclasts. During the staining process, TRAP-positive cells will turn purple which facilitates the visualization and analysis of osteoclasts.

Materials

Sigma Kit #387A

paraformaldehyde (36 %)

acetone

50 % glycerin in PBS

conical tubes

microscope

Protocol steps—*Step annotations*

All volumes given (except for the recipes) are for a 48 well plate. Volumes can be scaled up and down as needed for other plate formats. All steps are performed at room temperature.

1. Prepare the fixation solution: Mix 25 ml of citrate solution (provided by the kit) with 8 ml of 36 % paraformaldehyde and 65 ml of acetone. The fixation solution can be stored at 4°C for several months.

After mixing, white crystals coagulate. This does not affect the performance of the fixation solution.

2. Prepare the staining solution: All chemicals except distilled water are provided by the kit. The volumes can be scaled up or down, as needed. The following recipe is for approximately 10 ml of staining solution.

First mix 100 µl of sodium nitrite and 100 µl of fast garnet solution in a 1.5 ml tube. Mix it thoroughly with a vortex mixer for 30 s and let it rest for 1-2 min. Put 9 ml of deionized water into a 15 ml conical tube. Put aluminum foil around the tube to protect it from light. Add 100 µl of naphthol, 400 µl of acetate and 200 µl of tartrate to the water. Add the prepared sodium nitrite – fast garnet solution to the tube and mix by inversion. The solution is stable at 4°C up to 1 week when protected from light.

The TRAP staining solution is very light sensitive and not very stable. When brown particles appear that can't be resolved by mixing, the solution should not be used anymore.

3. Take your osteoclast assay culture plates from the incubator, remove the culture medium and wash the cells with 500 µl of PBS.

4. Add 250 µl of the fixation solution to each well and incubate for 3 min. Wash 2 times with 500 µl of PBS.

Don't exceed the fixation time to ensure good enzymatic activity.

5. Add 250 µl of the staining solution and incubate for 5-15 min. Osteoclasts and mononuclear precursors will turn purple. The optimal staining duration can largely vary between assays and has to be determined with a microscope. When the cells look intensively purple (not black, so you can still see the nuclei!) stop the reaction by washing with 500 µl of PBS.

6. Add 500 µl of 50 % glycerin in PBS to each well. Count osteoclasts either under a microscope or on images taken from the wells. For evaluation, purple stained cells with at least 3 nuclei are considered as osteoclasts. Purple stained cells with 1-2 nuclei are considered as pre-osteoclasts.

Glycerin prevents the cells from drying out. Stained plates can be stored for several years at room temperature in the dark.

Osteoclasts can become very large and can contain 20 nuclei or even more. For the evaluation of an experiment it might therefore be helpful to split osteoclasts into a group with few nuclei (= small osteoclasts – e.g. 3-5 nuclei for human and 3-9 nuclei for murine osteoclasts) and a group with many nuclei (= large osteoclasts - e.g. >5 nuclei for human and >9 nuclei for murine osteoclasts).

SUPPORT PROTOCOL 2

Resorption assay using calcium phosphate coated plates

Introductory paragraph

Often, it is not only of interest to know the number of developed osteoclasts, but also if they are fully functional. Osteoclast function can be analyzed with a resorption assay using either calcium phosphate coated plates or bone slices. In this section, we describe how to evaluate bone resorption using calcium phosphate coated plates (in this example from Corning®). This method is quite robust and enables the researcher to look at his cells during the assay as the calcium phosphate coating is transparent. It is also less expensive than bone slices. For resorption evaluation, a von Kossa staining is performed. In this staining silver ions replace calcium and are photochemically converted to black silver, making calcified substances visible (Meloan, Puchtler, & Valentine, 1973).

Materials

Additional Materials:

All ingredients are dissolved in deionized water.

deionized water

5 % silver nitrate solution

5 % sodium carbonate dissolved in 25 % formaldehyde solution

5% sodium thiosulfate Solution

calcium phosphate coated plates (Corning® osteo assay surface plates)

microscope

Protocol steps—Step annotations

Volumes are given for 96 well plates and can be upscaled as needed.

1. Prepare and culture your cells as described in Basic Protocol 1, 2 or 3 in calcium phosphate coated plates instead of usual cell culture plates.

Culture your cells directly in the coated plates. Look regularly at osteoclast formation using a microscope. Osteoclasts maturation in resorption plates might take longer than in regular cell culture plates. Especially for murine osteoclast assays we recommend to let the cells grow 1-2 d longer to give the cells enough time for resorption.

2. Remove the medium and lyse the cells with 200 µl deionized water until no cells are visible anymore.

Replace the water 2-3 times. It might help to shake the plate gently on an orbital shaker to remove cells. It is important to completely remove all cells to get a good staining result. Avoid harsh pipetting to prevent damage to the coated surface.

3. Incubate the wells with 100 µl of 5 % silver nitrate solution for 30 min in the dark.

In this step silver ions react with the calcium phosphate coating on the plate.

4. Wash at least three times with deionized water.

Make sure to wash all unbound silver ions away to avoid a massive background staining.

5. Add 100 µl of a 5 % sodium carbonate solution solved in 25 % formaldehyde for 5-60 s.

A photoreaction takes place immediately and the coated wells are colored dark brownish – black.

6. Wash three times with deionized water.

7. Add 100 µl of a 5 % sodium thiosulfate solution and incubate for 2 min.

This step is required to remove the remaining unbound silver.

8. Wash three times with deionized water.

9. Dry the plate and take microscopy images for quantification of the resorbed area (Figure 3).

SUPPORT PROTOCOL 3

Resorption assay using bone slices

Introductory paragraph

Often, it is not only of interest to know the number of developed osteoclasts, but also if they are fully functional. Osteoclast function can be analyzed with a resorption assay using either calcium phosphate coated plates or bone slices. In this section, we describe how to evaluate bone resorption using bone slices. Bone slices are more expensive than calcium phosphate coated wells and the assay is more complicated. But bone slices mimic the *in vivo* situation much better, as they represent real bone.

Materials

Additional Materials:

bovine bone slices (e.g. Ids 96 well format #DT-1BON1000-96)

trypsin/ EDTA

5 % sodium hypochlorite in water

0,1 % toluidine blue

tweezer

Protocol steps—*Step annotations*

We did not achieve good osteoclast differentiation when we seeded the monocytes or bone marrow cells directly on bone slices. We thus recommend to pre-differentiate the cells in normal cell culture plates and to transfer them at day 3 (for murine cells) or day 5 (for human cells) to the bone slices.

1. Prepare and culture your cells as described in Basic Protocol 1, 2 or 3 in normal cell culture plates.
2. Put the bone slices with a small tweezer into a 96 well cell culture plate and incubate them in osteoclast medium for 24 h at 37°C.
3. After 3 d (for murine cells) or 5 d (for human cells) remove the medium and add pre-warmed trypsin/ EDTA to the cells (250 µl in a 24 well plate). Incubate the cells at least 5 min at 37°C until the cells can be detached.
4. Detach the cells, add osteoclast medium and centrifuge the cells at 300 g for 5 min at 4°C.
5. Resuspend the pelleted cells in osteoclast medium and count the cells.
6. Remove the medium from the bone slices and seed 50000 cells in 200 µl osteoclast medium containing M-CSF and RANKL as described in Basic Protocol 1, 2 and 3 on each bone slice.
7. Let the cells grow for at least 2 d until you see mature osteoclasts at the well edges.

You can't see the osteoclasts on the bone slice. To determine the best staining time point, you can either try to look at cells at the edges of the well next to the bone slice or you prepare two extra wells without bone slices. For the first experiments we recommend to test different time points for the staining as the osteoclast differentiation rate varies between different labs.

8. For staining of the resorbed pits, wash the bone slices with PBS and incubate them for 1-2 h in 5 % sodium hypochlorite at room temperature to lyse all cells.
9. Wash the bone slices 3-5 times with water.
10. Stain with 0.1 % toluidine blue for 1 h, then wash several times with water until the water does not turn blue anymore.

At this step, the edges of resorption pits will be stained blue to purple. They look like clouds (Figure 3).

11. Take pictures with a microscope from the blue to purple stained pits.

REAGENTS AND SOLUTIONS

Use deionized water for all recipes.

Digestion medium

Supplement α -MEM cell culture medium with 0.1 % collagenase from *clostridium histolyticum* (Sigma #C9891) and 2 % dispase II (Sigma #D4693-1G) followed by sterile filtration.

Always prepare fresh digestion medium! Stir the medium up to 1h to dissolve collagenase and dispase.

MACS buffer

Supplement phosphate-buffered saline (PBS), pH 7.2 with 2 % FCS and 2 mM EDTA followed by sterile filtration.

The buffer can be stored for up to 1 month at 4°C.

Red blood cell lysis buffer

Supplement water with 20 mM HEPES, 0.15 M NH_4Cl and 0.1 mM EDTA followed by sterile filtration.

The buffer can be stored up to 1 year at room temperature.

COMMENTARY

Background Information

Osteoclast differentiation assays have a long tradition. The first assays have been co-cultures of calvarial osteoblasts with spleen or bone marrow cells (Akatsu et al., 1992). The discovery of RANKL as driving factor of osteoclastogenesis in 1998 (Yasuda et al., 1998) made direct osteoclast assays without osteoblasts possible. For murine osteoclast assays, the here described protocol is the one mostly used. In some labs, bone marrow cells are cultured only

with M-CSF in the first 3 days to get rid of non-adherent hematopoietic cells before starting osteoclast differentiation. Bone marrow contains a mixture of various cell types and if these cells might interfere with the planned assay this variation is a good option. There are several protocols available for the differentiation of human osteoclasts from blood monocytes, but most of them take 14 d or longer (Abdallah et al., 2018; Susa, Luong-Nguyen, Cappellen, Zamurovic, & Gamse, 2004). We here provide a rapid protocol that takes only about 7 d.

Critical Parameters + Troubleshooting

Osteoclasts are very sensitive to many substances. Thus, FCS is the most critical factor for osteoclast assays and can suppress osteoclast development completely. We strongly recommend testing multiple FCS samples from different suppliers and lot numbers to ensure optimal conditions for osteoclast differentiation. With a suitable FCS, one should not need more than 10 ng/ml RANKL for osteoclast differentiation. If you need 100 ng/ml RANKL to see osteoclasts, you should consider changing your FCS.

For human osteoclast assays it is important to keep in mind that there is a great variability between individual donors regarding the number of osteoclasts generated. We therefore recommend testing of at least 3-4 independent donors to avoid donor specific effects. In general, human osteoclast assays are more delicate and variable than mouse osteoclast assays. To ensure a good performance, platelets should be removed and after plastic adherence, unbound cells should be washed away as good as possible.

After medium exchange, osteoclast maturation can be very fast. Especially when working with murine cells, you should look at your cells at least once daily after the medium exchange as murine osteoclasts detach and die rapidly after maturation.

Understanding Results

You should see multinucleated osteoclasts as shown in Figure 2 after 7-9 d for human assays and 4-5 d for murine assays. Co-cultured osteoclasts should be multinucleated after around 5-7d. If no TRAP positive multinucleated cells are seen, there could be a problem with the FCS or RANKL. If the cells die during culture time, check your M-CSF.

When performing resorption assays, the results should look like in Figure 3. You might need to increase the assay time to promote the formation of resorption pits.

Time Considerations

Isolation of human PBMCs should take about 3 h + 1 h incubation time and about 1 h for washing and medium exchange. The whole assay takes about 7 d.

Isolation of murine bone marrow cells takes about 2-3 h depending on sample size. The next morning it takes additional 1-2 h for cell counting and seeding. The whole assay takes about 5-6 d.

For the co-culture of osteoclasts and osteoblasts, you should plan about 2-3 h for osteoblast isolation and additional 4-5 d for cell growth. When bone marrow cells are added to the osteoblasts, it takes around 5 to 7 days until osteoclasts appear.

The TRAP staining takes about 1 h depending on sample size. For staining of the resorption assays you should plan 1-2 h for cell detachment + about 1 h for the staining process.

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FIGURE LEGENDS

Figure 1: Isolation of PBMCs with a Ficoll gradient. The left image shows the blood/ PBS layer floating on the Ficoll layer before centrifugation. The right picture shows the separated layers after centrifugation. Erythrocytes and granulocytes have a higher density than Ficoll and are collected at the bottom of the tube. PBMCs form a small ring lying between the Ficoll layer and the upper part containing a mixture of blood plasma and PBS.

Figure 2: Representative images of *ex vivo* generated osteoclasts after TRAP staining. Human osteoclasts, murine osteoclasts and murine coculture osteoclasts have been generated with Basic Protocol 1, Basic Protocol 2 and Basic Protocol 3, respectively. Mature osteoclasts appear as large outstretched cells containing at least 3 nuclei and stained purple due to the TRAP staining. TRAP positive (= purple) cells with only 1 or 2 nuclei are considered as pre-osteoclasts.

Figure 3: Representative images of resorption assays. Shown are representative images of resorption assays using calcium phosphate coated wells (see Support Protocol 2) and bone slices (see Support Protocol 3). Resorbed areas of calcium phosphate coated wells appear white on a brownish to black background. Resorption pits on bone slices have a cloud-like appearance and are surrounded by dark purple edges (marked with black arrows). While resorption assays with calcium phosphate plates are easier to perform and more stable resorption assays with bone slices allow investigating resorption pits of single osteoclasts.