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Plasma cell purification from murine bone marrow using a two-step isolation approach

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Abstract

Interest in plasma cells has increased greatly in the past decade. While several studies have examined the longevity and transcriptional control of antibody secreting cells *in vivo*, few studies have examined freshly isolated plasma cells *ex vivo*. Studies of primary plasma cells have been limited primarily due to the difficulty of isolating the large numbers of plasma cells necessary for experiments. In this protocol, plasma cells are purified from murine bone marrow in approximately 5 hours, following a two-step isolation method utilizing column enrichment and FACS-sorting based on CD138 (Syndecan-1) surface expression.

Keywords

bone marrow; plasma cell; CD138 (Syndecan-1); murine; isolation

1. Type of Research

An important facet of long-lived immunity is the continuous synthesis of antibody, provided primarily by plasma cells with an extended lifespan in the bone marrow (Benner et al., 1981; Ho et al., 1986; Manz et al., 1997; Slifka et al., 1998). Plasmablasts, precursors of plasma cells, migrate from secondary lymphoid tissue and enter the bone marrow where they complete their development into terminally differentiated plasma cells (Dilosa et al., 1991; Hargreaves et al., 2001; Wehrli et al., 2001; Hauser et al., 2002). Supported by microenvironmental stromal cells, plasma cells remain in the bone marrow for many years, perhaps for the lifetime of the animal (Manz et al., 1997; Slifka et al., 1998; Minges Wols et al., 2002). Increasing numbers accumulate in marrow with age, making the bone marrow from older mice a good source of primary plasma cells.

As the function of plasma cells is to continuously secrete antibody, plasma cells downregulate many of the molecules needed for activation and differentiation that B cells at prior stages possess. Lack of identifying cell surface molecules makes it difficult to isolate a pure population of plasma cells. One unique feature of plasma cells is the expression of relatively high levels of CD138 (Syndecan-1), which has proven to be an effective means of identifying antibody secreting cells *in vivo* in both humans and mouse (Sanderson et al., 1989; Luque et al., 1998). Using CD138 expression has been less successful for purifying plasma cells directly from bone

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marrow for use *ex vivo* because the frequency of plasma cells is rare. Our lab is interested in studying the survival needs of bone marrow plasma cells *in vitro*. Therefore, we developed a two-stage protocol to isolate pure populations of plasma cells.

2. Time Required

The entire procedure takes approximately 5 hours, in the following steps:

1. Bone extractions and bone marrow cell isolation: 1h
2. CD138 and magnetic bead staining: 1h
3. Positive selection of CD138⁺ cells using magnetic bead column depletion: 1h
4. Purification and sorting of CD138⁺ cells using FACS: 2h*

* the length of time for this step may vary depending on the sorting speed of the FACS instrument.

3. Materials

- tibias and femurs from 20–22 month old BALB/c mice (with proper approval from Institutional Animal Care and Use Committee, Loyola University Medical Center)
- scissors for dissection and bone extraction
- forceps
- 10cc syringes (Becton Dickinson, Franklin Lakes, NJ)
- 25g syringe needles (Becton Dickinson)
- culture media containing RPMI 1640 supplemented with 5% fetal calf serum (FCS) (Hyclone, Logan, UT), 2mM L-glutamine (Gibco Life Technologies, Rockville, MD), 1% penicillin/streptomycin (Gibco Life Technologies), and 5×10^{-5} M β -2-mercaptoethanol (Fisher Scientific, Pittsburgh, PA); ["LTBMC media"]
- Hanks BSS (containing Ca^{2+} and Mg^{2+}) supplemented with 5% HIFBS and 2mM EDTA (Fisher Scientific); ["SB"]
- Hanks BSS (*no* Ca^{2+} and Mg^{2+}) supplemented with 5% HIFBS and 2mM EDTA (Fisher Scientific); ["column media"]
- RPMI 1640 containing 5% FCS, L-glutamine, 0.1mg/ml gentamycin (Gibco Life Technologies), and 5×10^{-5} M β -2-mercaptoethanol; ["sort media"]
- R-Phycoerythrin (PE) conjugated rat anti-mouse CD138 (Syndecan-1) monoclonal antibody (eBioscience, San Diego, CA)
- anti-PE microbeads (Miltenyi Biotec, Auburn, CA)
- MS separation columns (Miltenyi Biotec)
- midiMACS separator magnet (Miltenyi Biotec)
- 100 μ M Nytex membrane (Sefar America, Depew, NY)
- Falcon polystyrene 5ml round bottom tubes (Becton Dickinson)
- Falcon polypropylene 5ml round bottom tubes (Becton Dickinson)
- BD FACSAria flow cytometer (Becton Dickinson, San Jose, CA). Analysis was performed with FACSDiva software (Becton Dickinson).

4. Detailed Procedure

4.1 Preparation of single cell suspension from murine tibias and femurs

- i. Trim away muscle from tibias and femurs to avoid contamination and keep the bones in LTBM media (RPMI 1640, 5% FCS, 2mM L-glutamine, 1% penicillin/streptomycin, 5×10^{-5} M β -2-mercaptoethanol) until use in step iii. [Author's note: We find that using ≥ 4 mice does not proportionately increase the plasma cell yield. We suggest using 2–3 mice/plasma cell preparation.]
- ii. Fill a 10cc syringe with LTBM media and attach a 25 gauge needle.
- iii. Using tweezers to hold the bone above an empty 15ml conical tube, insert the needle into the one end of the bone and gently “flush” out the marrow into the tube by depressing the syringe plunger. Repeat the procedure with the other end of the bone. The bone appears white all over when the majority of the marrow has been removed.
- iv. Use a 1cc syringe outfitted with a 25 gauge needle to break up marrow plugs in the conical tube by aspirating the media in and out of the needle.
- v. Centrifuge the cells at 1100 RPM, 4°C for 10min. Discard the supernatant.
- vi. Resuspend the cells in 5–10ml LTBM media/mouse and count using trypan blue exclusion.
- vii. Remove and save 1×10^6 cells to use an “unstained” control sample for FACS-sorting.
- viii. Centrifuge the cells at 1100 RPM, 4°C for 10min. Discard the supernatant.

4.2 CD138 and microbead staining

- i. Resuspend the pelleted cells with 10 μ g/ml rat anti-mouse CD138 in cold SB (Hanks BSS (Ca^{2+} , Mg^{2+}), 5% HIFBS, 2mM EDTA). The volume should be adjusted such that 50 μ l of diluted antibody is used per 1×10^6 cells.
- ii. Incubate the cells for 20 minutes on ice to prevent CD138/anti-CD138 antibody internalization. In addition, covering the cells or incubating the cells in the dark will help prevent fluorochrome quenching, necessary for FACS-sorting later in the procedure.
- iii. Add 10ml of cold SB to wash the cells. Centrifuge the cells at 1100 RPM, 4°C for 10min and discard the supernatant.
- iv. Resuspend the cells with anti-PE microbeads diluted in cold SB. Use 10 μ l beads per 10×10^6 cells in 100 μ l total volume per 10×10^6 cells.
- v. Incubate cells as indicated in step ii.
- vi. Wash cells as indicated in step iii.

4.3 Positive selection of CD138⁺ cells using magnetic column separation

- i. Resuspend the pelleted cells in 500 μ l of column media (Hanks BSS (*no* Ca^{2+} / Mg^{2+}), 5% HIFBS, 2mM EDTA).
- ii. Place an MS column into the separator magnet. Create flow resistance by adding a 25 gauge needle to the bottom of the column. Add 500 μ l cold column media to the column and discard the effluent. Take care not to let the column run dry after this point.
- iii. Add the cells to the column and collect the effluent in a 15ml conical tube positioned in ice. The effluent will contain CD138^{neg} cells.

- iv. Wash the column 3–4× with 500µl column media, collecting the effluent in the same tube as in step iii.
- v. Remove the column from the magnet and rest it inside a clean 15ml conical tube. Fill the column with 2ml column media and use the provided plunger (contained in the column package) to flush the column and remove the attached cells. The removed fraction contains CD138⁺ plasma cells.
- vi. Centrifuge the tubes containing CD138⁺ cells at 1100RPM, 4°C, for 10min. Discard the supernatant. CD138^{neg} cells can be retained and used for analysis of column efficiency if desired.

4.4 Purification of CD138⁺ plasma cells by FACS

- i. Resuspend the effluent fraction and the eluted fraction in 1.5ml sort media each (RPMI 1640, 5% FCS, L-glutamine, gentamycin, 5×10^{-5} M β -2-mercaptoethanol). Use a cotton-plugged Pasteur pipette to slowly filter the cells through the Nytex membrane into a polystyrene tube. This step ensures that cell clumps are removed and will prevent the FACS machine from clogging during the sorting procedure.
- ii. Repeat step i with the “unstained” fraction from 4.1, step vii.
- iii. Using a properly calibrated flow cytometer and the unstained and effluent fractions (CD138^{neg}) as a guide, set the parameters for sorting such that cellular debris is excluded and only CD138^{hi} cells are collected. These are plasma cells (Figure 1).
- iv. Sort the CD138^{hi} cells into a polypropylene tube containing v/v FCS + sort media. The large volume of serum increases the recovery of viable cells post-sort.
- v. Briefly vortex the sorted plasma cells and pellet by centrifugation @ 1250 RPM, 4°C for 12min.
- vi. Carefully withdraw the supernatant with a micropipettor. Resuspend the cells in desired volume for enumeration and desired experimental use.
- vii. An aliquot of the cells may be cytocentrifuged onto a slide, fixed with methanol for 10min, and stained with hematoxylin and eosin to assess the purity of the sort based on morphology.

5. Results

Plasma cells represent a rare population of the bone marrow and cannot be isolated in large quantities without several purification steps. It would be difficult to sort a pure population in a time frame necessary to maintain viability. This problem is overcome by using older mice, in which long-lived plasma cells have accumulated, and using a 2-stage procedure for isolation of CD138^{bright/hi} cells. The first step uses a magnetic bead column for enrichment of CD138⁺ cells (Fig. 1A), and the second step purifies the rare CD138^{bright/hi} population by sorting (Fig. 1B). Magnetic bead column selection is highly specific as the effluent fraction (CD138^{neg}) does not contain any detectable CD138^{bright/hi} cells (data not shown). By flow cytometry, plasma cells fall in the medium to large lymphocytes gate as determined by forward side scatter (FSC) with low side scatter (SSC) (Fig. 1C). An average of 10^5 – 2×10^5 total plasma cells/2–3 mice are obtained using this approach from a starting population of approximately 100×10^6 bone marrow cells. We found that increasing the numbers of mice to $n \geq 4$ does not yield an increased proportionate number of plasma cells. Cell viability following the protocol is always >90% by trypan blue exclusion. Sorted cells stained with H&E exhibited small, dense, eccentric nuclei; prominent Golgi; and voluminous cytoplasm, characteristic of terminally differentiated plasma cells. Greater than 95% of sorted CD138^{bright/hi} cells exhibited the

morphology of differentiated plasma cells (Fig. 1D). In addition, the protocol does not interfere with the ability of CD138^{bright/hi} plasma cells to secrete immunoglobulin, as assessed by ELISA (Table 1).

6. Discussion

Plasma cells account for <0.5% of the total bone marrow in young, non-immunized mice. Thus, isolating a pure population of plasma cells, in useful quantities, is difficult. Old mice accumulate plasma cells in their bone marrow, roughly accounting for 1–5% of the bone marrow. Without pre-enrichment, FACS-sorting directly for plasma cells would likely take 5–6 hours of sort time to sort the plasma cells. Consequently, viability of the sorted cells would decrease. By using an additional column purification step, the CD138^{bright/hi} population (representing terminally-differentiated plasma cells) is greatly enriched, will significantly shorten the sort time, and will enhance the viability of the selected population. This protocol can also be used to isolate plasma cells from immunized mice with similar success (Minges Wols et al., 2007).

When using this protocol, the most frequent problem is the occasional presence of aggregates clogging the magnetic flow column during the first purification step. There are several troubleshooting approaches one can use to help circumvent this problem. One solution is to use a larger column, for example a LS⁺ (Miltenyi Biotec) instead of an MS⁺ column (used in this protocol). The former allows for a greater free volume, which can minimize column clumping. Alternatively, the flow resistor needle can be removed for a short period to allow the flow to increase. Removing the flow resistor will not affect your enrichment. Another solution is to ensure that HBSS *without* calcium and magnesium is being used in the column buffer, as the absence of calcium and magnesium will hinder cell-cell interaction and thus aggregate formation. Finally, if the problem persists, the cells can be transferred to a new column and cells already column-bound can be gently flushed out and added to the final enriched fraction.

7. Essential References

Manz et al. (1997)

Minges Wols et al. (2002)

Slifka et al. (1998)

9. Quick Procedure

9.1 Preparation of single cell suspension from tibias and femurs

- i. Clean bones of muscle tissue and keep in LTBM media.
- ii. Fill a 10cc syringe with LTBM media and attach a 25g needle.
- iii. Hold the bone with forceps over a 15ml tube. Use the syringe to flush out the bone marrow. Repeat procedure for both ends of the bones.
- iv. Disperse bone marrow plugs in the tube with the syringe.
- v. Centrifuge the cells at 1100 RPM, 4°C for 10min and discard the supernatant.
- vi. Count the cells using trypan blue exclusion.
- vii. Save 1×10^6 to use as an unstained control sample.
- viii. Wash cells as indicated in step v.

9.2 CD138 and microbead staining

- i. Stain the cells with 10 μ g/ml mAb CD138 in cold SB (50 μ l of diluted antibody per 1 $\times$ 10⁶ cells).
- ii. Incubate the cells on ice for 20 minutes in the dark.
- iii. Add 10ml of cold SB to the cells. Centrifuge the cells at 1100 RPM, 4°C for 10min and discard the supernatant.
- iv. Resuspend the cells with anti-PE microbeads diluted in cold SB (10 μ l beads per 10 $\times$ 10⁶ cells; 100 μ l total volume per 10 $\times$ 10⁶ cells).
- v. Incubate cells as indicated in step ii.
- vi. Wash cells as indicated in step iii.

9.3 Positive selection of CD138⁺ cells using magnetic column separation

- i. Resuspend the cells in 500 μ l of column media.
- ii. Place an MS column into the separator magnet and attach a 25g needle to the bottom of the column. Add 500 μ l cold column media to the column and discard the effluent.
- iii. Add the cells to the column and collect the effluent in a 15ml conical tube set in ice.
- iv. Wash the column 3–4 $\times$ with 500 μ l column media, collecting the effluent in the same tube as in step iii.
- v. Remove the column from the magnet and insert it inside a clean 15ml conical tube. Add 2ml column media and plunge the attached cells.
- vi. Centrifuge the tubes from step iv and step v at 1100RPM, 4°C, 10min. Discard the supernatant.

9.4 Purification of CD138⁺ plasma cells by FACS

- i. Resuspend the cells in 1.5ml sort media and filter them into a polystyrene tube through using a Nytex membrane.
- ii. Sort the CD138^{hi} cells into a polypropylene tube containing v/v FCS + sort media.
- iii. Use the cytometer to collect CD138^{hi} cells.
- iv. Vortex the sorted plasma cells and pellet by centrifugation @ 1250 RPM, 4°C for 12min.
- v. Carefully aspirate the supernatant. Resuspend the cells in desired volume.
- vi. Assess purity of plasma cells using hematoxylin and eosin if desired.

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Figure 1. Purification of bone marrow plasma cells

Total bone marrow cells were stained with anti-CD138 antibody and analyzed directly by flow cytometry (A) or after enrichment using a magnetic bead column (B). The CD138 negative and positive populations were identified by both an isotype antibody control sample and an unstained control sample. (C) Dot plot comparing the FSC versus SSC of sorted CD138^{hi/bright} plasma cells (black) overlaid onto the dot plot of column-enriched cells (gray) to demonstrate that CD138^{hi/bright} cells fall in the medium to large lymphocyte gate. (D) CD138^{hi/bright} column-enriched and FACS-sorted plasma cells were cytocentrifuged onto slides, fixed, and stained with hematoxylin and eosin to determine plasma cell morphology.

Table 1Immunoglobulin isotypes represented by purified bone marrow plasma cells in culture^a

Isotype (ng/ml) ^b				
IgG1	IgG2a	IgG2b	IgG3	IgM
32.7 +/- 14.3 ^c	38.6 +/- 26.2	26.6 +/- 11.8	9.12 +/- 4.0	415.0 +/- 185.0

^a Supernatants from CD138^{hi}/bright column-enriched and FACS-sorted plasma cells were collected 24 hours following isolation. Plasma cells were co-cultured with a primary bone marrow stromal cell adherent layer.

^b Supernatants were analyzed for the presence of immunoglobulin using ELISA. Two-fold serial dilutions of isotype-specific standards were included on every test plate at a range of 1000ng/ml—1.92ng/ml.

^c Data shown are the average +/- SEM of three independent plasma cell isolations.