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Protocol

Purification of human lamina propria plasma cells by an immunomagnetic selection method

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Abstract

Plasma cells (PC) are the terminal stage of B-lymphocyte differentiation and, as such, they are dedicated to large-scale secretion of antibodies (Ab). Bone marrow (BM) and lamina propria (LP) become the final reservoirs of PC generated in response to systemic and mucosal antigen stimulation, respectively. Although the majority of human PC are held in the mucosa LP, they have received less attention than PC present in other locations. A key step for many PC studies is the design of isolation protocols to purify them. A purification procedure for LPPC has not been described as yet and, therefore, we decided to develop a protocol for this purpose, comprising three main steps: (1) dissecting LP from colonic tissue; (2) releasing LP cells by a short 15-min collagenase digestion; (3) isolating LPPC by positive immuno-magnetic selection using the distinctive expression of CD54 (ICAM-I) on LPPC. By following this protocol, a viable, highly purified LPPC fraction can be obtained in less than 2 1/2 h.

Keywords: Human; Colon lamina propria plasma cell; Isolation; Mucosal immunity; CD54 (ICAM-1)

1. Type of research

Plasma cells (PC) are the final stage of the B-lymphocyte differentiation sequence, and become cellular factories devoted to high rate synthesis and secretion of antibodies (Ab). In rodents and humans, bone marrow (BM) becomes the final PC reservoir, following the systemic humoral response elicited by antigens in distant inductive territories. Accordingly, BMPC are the cells responsible for the majority of serum Ig formation (MacMillan et al., 1972) and are considered the paradigmatic PC. In general, less attention has been paid to PC responsible for mucosal humoral immune response, despite them constituting a larger numerical population and producing larger amounts of Ig than BMPC (Tseng, 1981; Brandtzaeg et al., 1989). Similar to the BM's role in systemic response, the most prominent hom-

Abbreviations: Ab, antibody; BM, bone marrow; LP, lamina propria; mAb, mouse monoclonal antibody; PC, plasma cells; BSA, bovine serum albumin.

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ing site of mucosal PC is the intestinal lamina propria (LP) (Brandtzaeg et al., 1999). These LPPC predominantly produce Ab of the IgA isotype which are mainly secreted to the mucosal surfaces, where their function is essential for the defense against potentially invasive micro-organism (Brandtzaeg, 1994). One of the most specific features of human (Bhan et al., 1981; Stashenko et al., 1981) but not mouse (Oliver et al., 1997) PC is their high expression of CD38 ($CD38^h$). Our group is interested in the comparison of human PC genetic, phenotypic and 'in vitro' functional properties from different locations including LPPC. A key step for these analysis is to obtain highly purified viable PC. To this end, we have previously described protocols for purifying scarce PC from tonsil, blood and BM (Medina et al., 2000, 2002), confirming the $CD38^h$ phenotype as a common specific marker for human PC. Despite the abundance of PC in the human mucosa LP, a procedure for purifying viable LPPC has not been to date clearly established. The usual protocol to isolate LP cells (Fiocchi and Youngman, 1997) requires 15–18 h; this is not only time-consuming but is also a potential drawback for preserving the functionality of such fragile cells as PC. Therefore, an initial aim of the present procedure design has been to reduce the time needed to obtain the LP cell fraction. Once this step was achieved, a phenotypic screening of LP cells by using monoclonal Ab and flow cytometry, indicated CD54 (ICAM-1) as the most distinctive surface marker of LPPC. Finally, positive immunomagnetic selection via CD54 produced a purified functional LPPC fraction (Medina et al., 2003).

2. Time required

The whole procedure takes approximately 3 h, in the following steps:

1. Colonic mucosa layer dissection: 30 min.
2. Isolation of colonic LP cells by enzymatic digestion: 40 min.
3. Purification of LPPC by immunomagnetic selection: 80 min.
4. FACS staining to assess the degree of PC enrichment: 45 min.

3. Materials

- Colonic samples from normal areas of surgically resected specimens, with the proper approval from the Institutional Review Board (Comité Etico, Hospital Universitario Puerta del Mar).
- Petri dishes 15-cm diameter (Nunc, Roskilde, Denmark).
- Scissors and dispensable scalpels.
- Phosphate buffered saline (PBS).
- Collagenase-V (Sigma, St. Louis, MO).
- Shaking temperature-controlled bath.
- Culture medium consisting of RPMI 1640 supplemented with 10% fetal calf serum, 10 mM L-glutamine and 0.05 mg/ml gentamicine (Gibco, Gaithersburg, MD),
- EDTA (Pharmacia Biotech, Uppsala, Sweden).
- Bovine serum albumin (BSA) (Sigma).
- Purified mouse monoclonal antibody (mAb) and phycoerythrin (PE)-labeled mAb against CD54, PE-labeled mAb against CD19 and Cy-Chrome (CyC)-labeled mAb against CD38, and the corresponding isotypic negative controls (Becton Dickinson, San Jose, CA).
- Goat anti-mouse IgG bound to magnetic microbeads, selection columns of LS^+ type and midiMACS magnet (Miltenyi Biotec, Auburn, CA).
- FACScalibur instrument, equipped with a 488-nm emitting laser and detector photomultiplier tubes for red/orange (PE) through a 585/42-nm bandpass filter and red (CyC) through a 650-nm longpass filter (Becton Dickinson, San Jose, CA). Analysis was performed with Cellquest software (Becton Dickinson).

4. Detailed procedure

4.1. Mucosa layer dissection

(i) Place the human colonic tissue on a Petri dish and use scissors to cut it longitudinally so that epithelium faces up.
(ii) Thoroughly rinse the epithelial surface with PBS to clean away mucus and debris.
(iii) Transfer the specimen to a new Petri dish.
(iv) Use two scalpels to detach the mucosa layer (that includes lamina propria) from submucosa. The

best way to perform this step is starting at the edge of the tissue, using one scalpel to fasten it and the other to strip off the mucosa layer as if it were an adhesive paper.

(v) When detachment is advanced, change to other edge and repeat the procedure pushing the released mucosa layer towards the middle of the specimen. When all the mucosa from the periphery has been detached, the central zone can be easily released. Following this procedure, an almost continuous mucosa sheet can be obtained (Medina et al., 2003; Fig. 1).

(vi) Transfer the released mucosa layer to a 50-ml tube, wash it in cold PBS inverting repeatedly the tube and once the tissue sinks to the bottom, remove the liquid using a vacuum pump at low pressure to avoid trapping the tissue. Repeat this washing step until the supernatant is transparent.

4.2. Isolation of LP cells by enzymatic digestion

(i) Once the mucosa layer is properly washed, place it on a Petri dish containing 5 ml of cold RPMI 1640 medium and mince it in pieces of about 1 cm^2, to facilitate enzymatic digestion.

(ii) Transfer all the pieces to a 50-ml tube, add 10–15 ml of digesting solution collagenase-V (1 mg/ml in RPMI-1640) and incubate in a shaking bath (15 min, at 37 °C).

(iii) Stop the reaction by adding cold culture medium to a final volume of 50 ml.

(iv) Centrifuge the resulting cell suspension at low speed ($20 \times g$, 3 min) to break down the remaining tissue pieces and collect the supernatant that contains the released cells.

(v) Add 50 ml cold culture medium to the pelleted tissue remains, shake the tube several times to harvest additional released cells and repeat step iv.

(vi) Repeat step v.

(vii) Centrifuge the supernatants of steps iv steps v steps vi containing the released cells at $400 \times g$ for 5 min at 4 °C, pool the pelleted cells together in a single tube and wash them in 50 ml cold culture medium. This cell population will be referred as LP cells.

4.3. Purification of colon LPPC by immunomagnetic selection

(i) Resuspend the cells obtained in the previous step in cold separation buffer (2 mM EDTA, 0.5% BSA in PBS) at a maximum concentration of 100×10^6 cells/ml.

(ii) Add mAb against CD54 (10 μg/ml) and incubate for 10 min in the dark at 4 °C.

(iii) Wash twice ($400 \times g$, 5 min, 4 °C) and resuspend in the same buffer up to 200×10^6 cells/ml.

(iv) Add goat anti-mouse magnetic micro-beads (1:20 vol/vol according to the manufacturer's instructions) and incubate for 15 min in the dark at 4 °C.

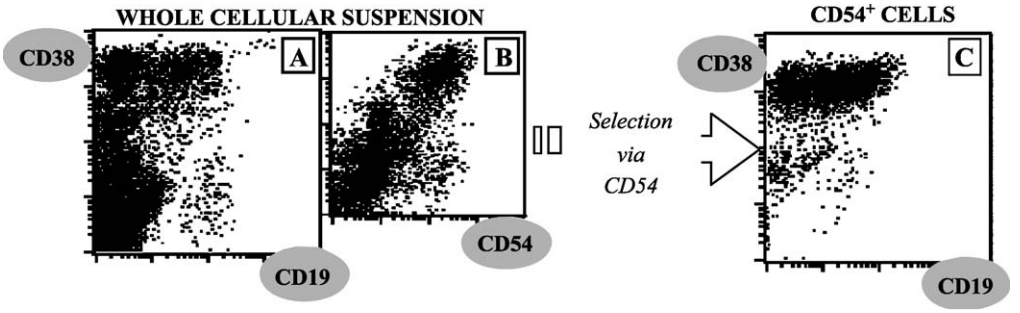

Fig. 1. Purification of PC from human colon lamina propria (LP). (A) LP cells were obtained by collagenase digestion of mucosa layer (see detailed protocol), and studied using labeled-mAb and flow cytometry analysis. A dot-plot analysis of the CD19/CD38 cell expression is shown: a cell subset of $CD38^h$ $CD19^{+/-}$ can be observed. (B) Dot-plot analysis of LP cells labeled for CD38 and CD54 showing the distinctive expression of CD54 by the LP $CD38^h$ cells. (C) Dot-plot analysis of CD38 and CD19 expression of LP CD54-selected cells showing the high degree of enrichment in $CD38^h$ cells obtained by this procedure.

(v) Wash twice (400 × *g*, 5 min, 4 °C), resuspend in the separation buffer up to 50×10^6 cells/ml and maintain in ice.
(vi) Place an LS^+ separation column in the MidiMacs magnet, apply 500 μl of separation buffer at the top of the column, allow it to run through and discard the effluent.
(vii) Pipette the stained cells (step v) onto the column, allow to run through and collect the effluent as the negative cell fraction.
(viii) Wash the column three times with 6 ml of separation buffer and include the effluent in the negative cell fraction.
(ix) Remove the column from the magnet, add 5 ml of the same buffer, flush out the cells using the plunger supplied, and collect them as the positive cell fraction (referred to as LP $CD54^+$ cells). This fraction should contain mostly PC.

4.4. Staining for standard two-color FACS to assess the degree of PC purification

(i) Deposit LP cells and LP $CD54^+$ cells in two tubes of FACS analysis per cell fraction, containing $0.1-0.5 \times 10^6$ cells in 200 μl PBS/tube.
(ii) Add isotypic negative control mAb (PE and CyC labeled) to one tube, and mAb against CD38 (CyC labeled) and CD19 (PE labeled) to the other. Incubate the cells for 20 min in the dark at 4 °C.
(iii) Wash twice in PBS (400 × *g*, 5 min, 4 °C) and resuspend in 300 μl of the same buffer.
(iv) Acquire the sample data in a properly calibrated cytometer.
(v) Analyze data using a suitable program. LPPC are detected as a conspicuous $CD38^h$ and $CD19^{+/-}$ cell subpopulation, and they are highly purified in the LP $CD54^+$ cell fraction. It is important to use an accurate forward/side scatter gating to exclude debris from the cellular population.

5. Results

After dissection and enzymatic digestion of the mucosa layer, an average of 60×10^6 cells is recovered (ranging from 28 to 100×10^6 LP cells per sample). These results represent an average recovery of 2×10^6 cells/cm^2. Cell viability is always more than 90% by trypan blue exclusion test. Under microscopy, this cell fraction consists mainly of cells of lymphoid appearance and some large cells that resemble epithelial cells. LP cell analysis by flow cytometry reveals that a subpopulation expressing a $CD38^h$ and $CD19^{+/-}$ phenotype is clearly detected (Fig. 1A). The $CD38^h$ phenotype is a common feature of human PC (Bhan et al., 1981; Stashenko et al., 1981). LP cells showing this phenotype account for $21.5 \pm 2\%$ cells (mean ± S.E.M., $n = 16$) of the total LP cell population. Taking advantage of the distinctive expression of the adhesion molecule CD54 (ICAM-1) by LP $CD38^h$ cells (Fig. 1B), a single immunomagnetic positive selection step via CD54 is enough to produce a highly purified ($94.4 \pm 1\%$; mean ± S.E.M.) $CD38^h$ cell fraction (Fig. 1C). These $CD38^h$ cells from human colonic LP are PC, as determined by Giemsa staining and optical microscopy and cytoplasmic IgA immunofluorescent staining (Medina et al., 2003).

6. Discussion

Usual protocols for isolating cells from human colon LP take up to 15–18 h, including dissection, abundant stirring washes to deplete epithelial cells and an 8-h collagenase digestion period (Fiocchi and Youngman, 1997; Panja et al., 1994). The present protocol produces an equivalent LP cell fraction in an hour and a half. A short 15-min collagenase digestion is enough to obtain a recovery similar to that reported by using longer digestion periods (Fiocchi and Youngman, 1997). This difference in time requirement could be explained by the dissection step introduced in the present protocol, since by using the detached mucosa layer, instead of the whole colonic wall, the LP area may be made more accessible to the collagenase solution. The presence of some epithelial cells in the LP cell fraction does not apparently hamper the purification of PC by immunomagnetic selection (Fig. 1C). In fact this protocol could be a suitable starting point for isolating LP lymphoid subpopula-

tions other than LPPC, which requires an immunomagnetic selection step.

The main aim of this procedure is to purify functional PC. Therefore, LP CD38^h cells were analyzed in the search for the most distinctive surface marker for this subpopulation. In human LP cells, CD54 is a molecule almost exclusively expressed by CD38^h LP cells (Fig. 1B), and as result, it is useful for purifying LPPC by positive immunomagnetic selection. When LPPC isolation was tried using CD38 immunomagnetic selection, the degree of purification obtained was worse, as cells expressing intermediate amounts of surface CD38 (Fig. 1), probably LP T lymphocytes, were also retained in the positive fraction (data not shown). Another candidate molecule for consideration in this respect was CD138 (syndecan-1) since it has already been used with good results for human BMPC purification (Medina et al., 2002), and has been detected on gastric LPPC by immuno-hystological techniques (Tanabe et al., 1999). However, CD138 is rapidly shed even at room temperature, and this would be incompatible with a 37 °C digestion step. In fact, CD138 is not detected on LPPC obtained by the present protocol. Despite PC tendency to apoptosis, neither collagenase digestion nor magnetic isolation via CD54 abrogate their main functional feature, as cultured LPPC isolated following the present protocol spontaneously secrete IgA for 2 weeks in a linear fashion similar to that described for BM PC (Medina et al., 2003).

When trying this protocol, the most frequent troubleshooting is the occasional presence of aggregates in the cellular suspension that impairs a proper isolation of PC. Sometimes the LP cell fraction (step vii, Section 4.2) contains visible fibrillar material that aggregates free cells and can retard and even block the flow through the isolation column, resulting in poor PC purification. Therefore, it is important to use LS$^+$ separation columns, larger than MS$^+$ ones, taking advantage of their greater free volume to minimize column clumping. We have tried several extra steps for removing these aggregates from the LP cell fraction: DNAse and mucolitic treatment, ficoll centrifugation and nylon mesh filtration, but with no apparent improvement. In our opinion, when aggregates appear in the LP cell fraction, the best alternative is to modify the standard procedure as follows:

(1) If supernatant of washing in step vii of Section 4.2 contains abundant visible fibrillar aggregates, discard it, resuspend the pellet in 30 ml of separation buffer (2 mM EDTA 0.5% BSA in PBS), gently mix the suspension and pass it through a sterile gauze into a 50-ml tube. In spite of some cell loss, the adherence of aggregates to the gauze results in a cleaner cell suspension. After centrifugation (400 × *g*, 5 min, 4 °C), continue the standard protocol (Section 4.3).
2) If during the first washing of the selection column to elute the negative fraction (step viii, Section 4.3), the flow becomes slow, there is an obstruction of the column lumen that will probably retain non-stained cells, and hampering the PC purification. In this case, the best option is to recover the cells from the clogged column and pass them through a new column. In detail: (a) complete the volume to 6 ml (if necessary), (b) remove the column from the magnet, (c) use the plunger to gently flush out the retained cells and collect them, (d) prepare a new separation column (as in step vi, Section 4.3), (e) add the cell suspension to the column (as in step vii, Section 4.3) and continue the standard protocol.
3) If, at the end of the standard procedure, control staining of the LP CD54$^+$ cell fraction for flow cytometry reveals a suboptimal PC purification, pass the cellular suspension through a new selection column (repeat the standard protocol from step vi, Section 4.3).

7. Quick procedure

7.1. Mucosa layer dissection

(i) Cut colonic piece on a Petri dish so that epithelium faces up.
(ii) Rinse the epithelial surface with PBS.
(iii) Change the specimen to a new Petri dish.
(iv) Use two scalpels to detach mucosa layer starting at the edge of the tissue, as if it were an adhesive paper.

(v) Repeat the procedure from the other edges until the mucosal layer can be fully released from the central zone of the specimen.
(vi) Wash the mucosa layer in cold PBS (by inversion in a 50-ml tube) until the solution is transparent.

7.2. Isolation of colonic LP cells by enzymatic digestion

(i) Mince the mucosa layer on a Petri dish into pieces of about 1 cm^2.
(ii) Transfer the pieces to a 50-ml tube, add 10–15 ml of digesting solution collagenase-V (1 mg/ml in RPMI-1640) and incubate in a shaking bath (15 min, 37 °C).
(iii) Stop the reaction by adding cold culture medium to a final volume of 50 ml.
(iv) Centrifuge the resulting cell suspension at low speed ($20 \times g$, 3 min) and collect the supernatant.
(v) Add 50 ml cold culture medium to the pelleted tissue pieces, shake the tube several times and repeat step iv.
(vi) Repeat step v.
(vii) Centrifuge the supernatant of step iv–vi ($400 \times g$, 5 min, 4 °C), pool the pelleted cells in a tube and wash again in cold culture medium.

7.3. Isolation of colonic LPPC by immunomagnetic selection through CD54

(i) Resuspend cells in separation buffer (2 mM EDTA 0.5% BSA in PBS) adjusting up to 100×10^6 cells/ml.
(ii) Incubate them with mAb against CD54 (10 μg/ml, 10 min, dark, 4 °C).
(iii) Wash twice ($400 \times g$, 5 min, 4 °C) and resuspend the cells in the same buffer up to 200×10^6 cells/ml.
(iv) Incubate them with goat anti-mouse magnetic micro-beads (1:20 vol/vol, 15 min, dark , 4 °C).
(v) Wash twice ($400 \times g$, 5 min, 4 °C), resuspend in separation buffer up to 50×10^6 cells/ml and maintain in ice.
(vi) Place an LS^+ separation column in the Midi-Macs magnet, apply 500 μl of separation buffer and discard the effluent.
(vii) Pipette the stained cells (step v) onto the column, allow cells to run through and collect as the negative cell fraction.
(viii) Wash the column three times with 6 ml of separation buffer and include the effluent in the negative cell fraction.
(ix) Remove the column from the magnet, add 5 ml of the same buffer, use the plunger to flush out the cells and collect them as the positive cell fraction (LPPC).

7.4. Staining for standard two-color FACS to assess the degree of PC purification

(i) Sample LP cells and LP $CD54^+$ cells in FACS tubes: two tubes/fraction, $0.1–0.5 \times 10^6$ cells/tube and 200 μl PBS/tube.
(ii) Add isotypic negative control mAb (PE and CyC labeled) to one tube, and mAb against CD38 (CyC) and CD19 (PE) and incubate (1:20, 20 min, dark, 4 °C).
(iii) Wash twice in PBS ($400 \times g$, 5 min, 4 °C) and resuspend (300 μl PBS).
(iv) Acquire the sample data in a properly calibrated FACScalibur cytometer (or a similar instrument).
(v) Analyze data using Cellquest program (or any adequate software). CD54+ PC population is detected as $CD38^h$ and $CD19^{+/-}$ cells.

8. Essential references

Fiocchi and Youngman (1997).
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