Primary Human Naïve B cells (IgD+) and Germinal Center B cells (CD77+)

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Goal: To obtain large numbers of high purity human primary Naïve B cells and Germinal Center B cells from fresh de-indentified human tonsillectomy specimens based on the expression phenotypic markers, such as IgD for Naïve B cells (NB), and CD77 for Germinal Center B cells (GCB). Ficoll Histopaque gradient centrifugation was used to isolate tonsilar mononuclear cells first. Individual tonsilar B cell populations were then stained with appropriate antibodies and collected by a magnetic microbeads separation system with a double positive selection setting (MACS, Miltenyi Biotec).

Reagents:

1). Ice cold RPMI+ media = RPMI + 10% FCS + 1% Pen/Strep + 5mM EDTA;
2). Ice cold wash buffer = PBS + 5mM EDTA;
3). Ice cold staining buffer = PBS + 0.5% BSA + 5mM EDTA;
4). Room temperature Ficoll (Fico-Lite H);
5). Anti-CD77 (rat IgM; Beckman Coulter);
6). Mouse anti-rat IgM, IgG1 isotype (MARM; BD);
7). Rat anti-mouse IgG1 Microbeads (MACS);
8). IgD-FITC (BD);
9). Anti-FITC Microbeads (MACS).
10). 2X Freezing media = 8 ml FCS + 2 ml DMSO

Step 1: Isolation of tonsilar mononuclear cells (MBC).

1). Place tonsil specimens from one patient in a petri dish with RPMI+ on ice, scrape cells off the specimens as much as possible;
2). Place a 70μm sieve on a 50 ml falcon tube on ice, filter RPMI+ (with cells scraped off the tonsil specimens) through the sieve;
3). Collect all remaining solid pieces in a sieve in a petri dish with RPMI+ and use a syringe stopper to mash through;
4). Filter RPMI+ through the same sieve and collect into the 50 ml falcon tube in step 2);
5). Repeat steps 3) and 4) several times until most of the cells have been retrieved (may require as many as 5 times);
6). Spin the cells down at 1200 rpm for 10 min in a pre-chilled (4°C) centrifuge;
7). Discard supernatant, resuspend cells with ice cold RPMI+;
8). Spin the cells down at 1200 rpm for 10 min in a pre-chilled (4°C) centrifuge;
9). Discard supernatant, resuspend cells with 16 ml RPMI+;
10). Add 3 ml room temperature Ficoll into a 15 ml falcon tube;
11). Slowly add 4 ml of cell suspension from step 9) on top of the Ficoll;
12). Spin at 800 g for 20 min at 18 degrees OR at 400 g for 30 min;
13). Use a Pasteur pipette to transfer top media layer and the layer of cells in between the Ficoll phase and media phase to a new 50 ml tube (try not to pick up any Ficoll);
14). Top the tube with ice cold wash buffer;
15). Spin at 1200 rpm for 5 min at 4 degrees;
16). Resuspend in 50 ml wash buffer again*, take an aliquot, dilute 1/10 in PBS, and count the number of the MBCs with trypan blue.
* If not proceed to cell separation step on the same day, MBCs can be resuspended in RPMI+ and kept overnight at 4 degrees.

Step 2: Staining/cell separation.

1). Spin cells at 1200 rpm for 5 min at 4 degrees;
2). Resuspend cells with ice cold staining buffer at $2x10^8$/ml in a 15 ml falcon tube;
3). Divide MBCs in half, add antibodies according to which cells you wish to isolate.

A. For GCBs:

i. Add 25 µl rat anti-CD77 per 10^8 cells, incubate for 10 min on ice;
ii. Add up to 15 ml staining buffer, spin at 1200 rpm for 5 min at 4 degrees, resuspend with staining buffer in initial volume;
iii. Add 5 µl mouse anti-rat IgM, IgG1 isotype (MARM) per 10^8 cells, incubate for 10 min on ice;
iv. Add up to 15 ml staining buffer, spin at 1200 rpm for 5 min at 4 degrees, resuspend with staining buffer in initial volume;
v. Add 50 µl rat anti-mouse IgG1 Microbeads per 10^8 cells, incubate for 10 min on ice;
vi. Add up to 15 ml staining buffer, spin at 1200 rpm for 5 min at 4 degrees, resuspend with staining buffer in initial volume;
vii. Filter cells, load onto autoMACS (no more than 2 ml per separation), perform PosselD isolation for better purity;
viii. Take an aliquot to count the number of GCBs;
ix. Add 2X Freezing media, freeze immediately.

B. For NBs:

i. Add 12.5 µl IgD-FITC per 10^8 cells, incubate for 10 min on ice;
ii. Add up to 15 ml staining buffer, spin at 1200 rpm for 5 min at 4 degrees, resuspend with staining buffer in initial volume;
iii. Add 50 µl anti-FITC Microbeads per 10^8 cells, incubate for 10 min on ice;
iv. Add up to 15 ml staining buffer, spin at 1200 rpm for 5 min at 4 degrees, resuspend with staining buffer in initial volume;
v. Filter cells, load onto autoMACS (no more than 2 ml per separation), perform PosselD isolation for better purity;
vi. Take an aliquot to count the number of NBs;
vii. Add 2X Freezing media, freeze immediately.