Purification and immunophenotypic characterisation of human $CD19^{+}CD24^{hi}CD38^{hi}$ and $CD19^{+}CD24^{hi}CD27^{+}$ B cells

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Abstract

Regulatory B cells (Bregs) have regulatory capacity via the production of IL-10. IL-10 expression and immunosuppression has been described in a number of human B cell subsets, two of which include the $CD19^{+}CD24^{hi}CD38^{hi}$ and $CD19^{+}CD24^{hi}CD27^{+}$ populations. In this chapter, we describe how to identify and isolate these subsets from peripheral blood B cells via flow cytometry. We also explain how to expand Bregs in culture and identify them based on intracellular expression of IL-10.

Key words

Regulatory B cells, IL-10, autoimmunity, immune regulation, phenotyping, flow cytometry

1 Introduction

Regulatory B cells (Bregs) have been described in a range of B cell subsets in mice and humans, with the primary effect of inhibiting differentiation of pro-inflammatory T cell subsets, and inducing Treg differentiation [1, 2]. While human Bregs are less well understood compared to their murine counterparts, two of the better characterised human B cell subsets in which Bregs are enriched are the $CD19^{+}CD24^{hi}CD38^{hi}$ (immature) and $CD19^{+}CD24^{hi}CD27^{+}$ subsets [1, 3]. Moreover, impaired function of Bregs within both of these subsets have been demonstrated in autoimmunity, allergy and cancer [4, 5].

The $CD19^{+}CD24^{hi}CD38^{hi}$ subset represents the immature B cell population newly emerged from the bone marrow and entering circulation [6]. Previous work by our laboratory demonstrated that this subset produces large quantities of IL-10 following TLR9 and CD40 stimulation [1]. $CD19^{+}CD24^{hi}CD38^{hi}$ Bregs have the capacity to inhibit the differentiation of naïve $CD4^{+}$ T cells into pro-inflammatory Th1 and Th17 T cells, inhibit effector $CD4^{+}$ T cell production of IFNγ and TNFα, and promote the differentiation of $CD4^{+}CD25^{+}$ regulatory T cells (Tregs) [2]. In addition to CpGC stimulation, IFNα expands Bregs from this subset; in this context plasmacytoid dendritic cells (pDCs) represent the source of IFNα following TLR9 stimulation. The $CD19^{+}CD24^{hi}CD38^{hi}$-derived Breg subset participates in a regulatory feedback loop restraining further production of IFNα by pDCs, representing an important homeostatic mechanism to prevent excessive inflammation [7]. Reduced frequencies of $CD19^{+}CD24^{hi}CD38^{hi}$ Bregs and impaired regulatory capacity has been observed in a number of conditions including systemic lupus erythematosus (SLE) [1, 7], rheumatoid arthritis [2] atopic dermatitis [8] and psoriasis [9]. In SLE, $CD19^{+}CD24^{hi}CD38^{hi}$ Bregs produce less IL-10 on a per-cell basis, and have impaired ability to suppress $CD4^{+}$ T cell pro-inflammatory cytokine production – defects which are more pronounced with high disease activity [1, 7].

The $CD19^{+}CD24^{hi}CD27^{+}$ memory B cell population is also appreciated for its regulatory potential. A rare subset of $CD19^{+}CD24^{hi}CD27^{+}$ IL-10^{+} B cells, termed B10 cells, has been described in humans [3]. B10 cells are observed in newborn cord blood at very low frequencies and in adult blood, spleen and tonsils. B10 precursors (B10pro) are enriched within the $CD19^{+}CD24^{hi}CD27^{+}$ subset; B10pro B cells acquire the capacity to express IL-10 (intracellularly and at the transcriptional level) following 48 h stimulation *in vivo* with dual

CD40 and TLR stimulation, with the phorbol ester PMA, ionomycin and Brefeldin A stimulation for the final 5 hours of culture. Unique to the $CD19^{+}CD24^{hi}CD27^{hi}$ B10 subset is the capacity to suppress monocyte TNFα production, which is IL-10 dependent [3]. In RA patients, reduced frequencies of $CD19^{+}CD27^{+}$ have been observed which is associated with impaired capacity to inhibit $CD4^{+}$ T cell IFNγ production [10]. Conversely, Breg inhibition of $CD4^{+}$ IFNγ production is detrimental in patients with gastric cancer. Frequencies of gastric tissue $CD19^{+}CD24^{hi}CD27^{+}$ Bregs are elevated in these patients, inhibiting $CD4^{+}$ proliferation and IFNγ production, and higher frequencies of these cells is associated with a poor prognosis [11].

The significance of aberrant Breg function in the pathogenesis of autoimmune disease, allergy and cancer highlights a requirement for in-depth characterisation and functional understanding of Bregs. However, the marked heterogeneity of the Breg populations in terms of surface phenotype, regulatory function and expansion stimuli is a major complicating factor in understanding their origin and immunological role. Unlike Foxp3 as the hallmark transcription factor for Tregs, no such transcription factor or surface marker has been identified for Bregs, and at present, IL-10 expression remains the defining factor in humans, emphasising a need to identify surrogate Breg markers.

Flow cytometry can be used to comprehensively phenotypically characterise B cell subsets, and to measure the intracellular expression of IL-10, thus allowing identification of Bregs. Here we illustrate how to isolate B cells from whole blood and distinguish the $CD19^{+}CD24^{hi}CD38^{hi}$ and $CD19^{+}CD24^{hi}CD27^{+}$ subsets *ex vivo*. We also describe how to prepare B cells for isolation of the $CD19^{+}CD24^{hi}CD38^{hi}$ and $CD19^{+}CD24^{hi}CD27^{+}$ subsets via fluorescence-activated cell sorting (FACS) which can be subsequently cultured to expand Bregs and investigate their developmental fate and IL-10 expression under different culture environments. Finally, we designate how to expand Bregs in culture using a range of stimuli to allow exploration of their phenotype, potentially beyond the $CD19^{+}CD24^{hi}CD38^{hi}$ and $CD19^{+}CD24^{hi}CD27^{+}$ subsets described here, and also to facilitate functional assays to assess their suppressive capacity in a range of immunological contexts.

2 Materials

2.1 General equipment

1. 5 ml round-bottom polystyrene tubes (with filter cap).
2. 5 ml round-bottom polypropylene capped tubes.
3. Cell sorter (e.g. BD FACS Aria).
4. Flow cytometer (e.g. BD LSRII).
5. Sodium heparin-containing vacutainer tubes.
6. Round-bottom 96-well culture plate.
7. 50 ml tubes.
8. 10 ml serological pipets.

2.2 Buffer and reagents

1. RPMI 1640 culture medium.
2. Complete medium: RPMI 1640 culture medium supplemented with 10% fetal calf serum (FCS) and 100 IU/ml penicillin and streptomycin.
3. Phosphate-buffered saline (PBS), with $MgCl_2$ and $CaCl_2$, sterile-filtered, pH 6.9-7.1.
4. Collection media: 50% FCS in RPMI 1640.
5. Staining buffer: 2% FCS and 0.01% sodium azide in PBS.
6. Ficoll-Paque (density 1.077 g/ml)
7. Red Blood Cell Lysing Buffer.
8. EasySep™ Human B Cell Enrichment Kit and magnet (STEMCELL) (*see* **Note 1**).

2.3 Reagents for B cell culture and flow cytometry analysis

1. CpGC ODN 2395.
2. Recombinant human IFNα.
3. Human megaCD40L.
4. PIB cocktail to maximise intracellular IL-10 production; 50 ng/ml phorbol 12-myristate 13-acetate (PMA), 250 ng/ml ionomycin and 5 μg/ml Brefeldin A diluted in complete media
5. 4',6-diamidino-2-phenylindole (DAPI) at 0.05 μg/ml prepared in sterile water (*see* **Note 2**).

6. LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit (ThermoFisher) (prepared at a 1:500 dilution of reconstituted stock in PBS) (*see* **Note 2**).
7. FcR blocking reagent.
8. Fluorescently-conjugated monoclonal antibodies (mAbs) for detection of surface molecules and intracellular cytokines (*see* Table 1).
9. Intracellular (IC) Fixation buffer.
10. Permeabilisation buffer.
11. Anti-Mouse Ig, κ/Negative Control Compensation Particles

3 Methods

3.1 Isolation of peripheral blood mononuclear cells from whole human blood

The following method is to isolate PBMC from human peripheral blood samples. For healthy donors, 50 ml whole blood yields on average approximately 6-7 x 10^7 PBMC. Directions in this section are based on whole blood volume of 50 ml. Scale up or down according to your sample volume.

1. Collect human blood in sodium heparin-containing vacutainer tubes.
2. Pool 50 ml whole blood into two 50 ml tubes (25 ml each) and dilute 1:1 with RPMI 1640 medium (*see* **Note 3**).
3. Pipette 15 ml Ficoll-Paque into three 50 ml tubes.
4. Using a 10 ml serological pipet, slowly layer diluted whole blood over the Ficoll-layer (33 ml per tube). Hold the pipette at approximately 70° to the inside of the tube to ensure full control of layering and minimal mixing of the blood and Ficoll layers.
5. Centrifuge layered tubes at 800 x g for 25 min at room temperature with minimal acceleration and brake.
6. Collect the resulting PBMC layers at the Ficoll interface into one 50 ml tube using a Pasteur pipette. Do not collect any red blood cells (*see* **Note 4**).
7. Make tube volume to 50 ml with complete medium.
8. Wash PBMC by centrifuging 500 x g for 10 min at 4°C, resuspend cell pellet in 25 ml complete medium.
9. Count PBMC and estimate sample viability (*see* **Note 5**).
10. Make tube volume up to 50 ml with complete medium, centrifuge at 500 x g for 10 min at 4°C, resuspend cell pellet in the appropriate volume for *ex vivo* staining, B cell isolation (Subheading 3.2.), cell culture (Subheading 3.3) or freezing cells for future use (*see* **Note 6**).

3.2 B cell isolation from total PBMC

The following method is to isolate CD19$^+$ B cells from PBMC samples using the STEMCELL EasySep™ Human B Cell Enrichment Kit. Refer to the provided protocol for this kit for details of all applications. Here we describe using this kit alongside the EasySep™ Magnet (single

sample). This method starts from freshly isolated PBMC but can be performed on previously frozen PBMC (*see* **Note 7**).

1. Wash PBMC in separation buffer by topping up the tube volume to 50 ml with separation buffer and centrifuging at 500 x g for 10 min at 4°C.
2. Resuspend the cell pellet at $5x10^7$ cells/ml in separation buffer (*see* **Note 8**) into a 5 ml round-bottom polystyrene tube.
3. Proceed with B cell enrichment, following manufacturer's instructions (*see* **Note 9**).
4. Count the isolated B cells (*see* **Note 5**).
5. Wash B cells by making tube volume up to 15 ml with complete medium and centrifuging at 500 x g for 10 min at 4°C.
6. Resuspend B cells at $1x10^8$ cells/ml in separation buffer for cell sorting, at $2.5x10^6$ cells/ml in staining buffer for immediate *ex vivo* staining for flow cytometry, or at 1.25-$2.5x10^6$ cells/ml in complete medium for B cell cultures (*see* **Note 10**)
7. For immediate *ex vivo* staining for flow cytometry, continue to Subheading 3.5 (steps 1-10). For setup of B cell cultures, continue to Subheading 3.4.

3.3 Preparation of B cells for isolation of CD24^{hi}CD38^{hi} and CD24^{hi}CD27^{+} B cells using FACS

The following method is to prepare B cells for isolation of the CD24^{hi}CD38^{hi} and CD24^{hi}CD27^{+} B cell subsets using FACS. Alternatively, cell sorting can be performed on total PBMC (*see* **Note 11**).

1. Wash isolated B cells twice with separation buffer by centrifuging at 500 x g for 10 min at 4°C.
2. Resuspend B cells at $1x10^8$ cells/ml in separation buffer in 5 ml round-bottom polystyrene tubes. Set aside approximately $1x10^5$ cells for a DAPI single-stained compensation sample.
3. Add anti-human CD19 BV785, CD24 APCeFluor780, CD38 PerCPeFluor710 and CD27 PE/Dazzle 594 mAbs (*see* Table 1) at the optimal concentration per tube as determined by titration (*see* **Note 12**). Incubate for 20 min on ice in the dark.
4. Wash cells twice by filling each tube with separation buffer and centrifuging at 500 x g for 5 min at 4°C.

5. Resuspend cells at 30-50 $x10^6$ cells/ml for sorting.
6. Remove cell clumps by pipetting each sample through the filter cap of a filter-top 5 ml round-bottom polystyrene tube.
7. To stain dead cells, add DAPI at 0.05 μg/ml to each sample, plus the single-stained compensation sample.
8. Sort B cells according to the expression of subset-specific markers (*see* Fig. 1A).
9. Collect sorted B cell subsets in 5 ml round-bottom capped polypropylene tubes containing 2 ml collection medium.

3.4 Culturing B cells for Breg expansion

This method describes the process for setting up a culture of isolated B cells or sorted B cell subsets in order to expand the IL-10^+ population.

1. Resuspend previously counted isolated B cells at a volume allowing to seed 2.5-$5x10^5$ B cells per well onto a 96-well plate (*see* **Note 10**) and allowing a final volume of 200 μl after the addition of stimuli (*see* **Note 13**).
2. Add required stimuli for Breg induction to relevant wells. Resuspend CpGC ODN 2395, IFN-α and megaCD40L in complete medium so that the final concentration in the B cell culture is 1μM, 1000 U/ml and 1μg/ml respectively (*see* **Note 14**)
3. Fill the empty wells surrounding samples with complete medium (*see* **Note 15**)
4. Incubate at 37°C, 5% CO_2 for 72 h.
5. During the final 5 h of culture, decant supernatants (*see* **Note 16**) and resuspend cells in 100 μl of PIB cocktail (*see* **Note 17**). Incubate for 5 h at 37°C, 5% CO_2.
6. At the end of incubation proceed to Subheading 3.5 for staining of cells for analysis by flow cytometry.

3.5 Fluorescent staining for surface markers and intracellular cytokines analysis by flow cytometry

The following method describes how to use fluorescently-conjugated mAbs to stain surface markers and intracellular cytokines for analysis by flow cytometry. For surface marker staining alone (either *ex vivo* or post-culture) follow steps 1 to 10.

1. B cells will have been prepared in 96-well plates for *ex vivo* staining as per Subheading 3.2 (step 11) or post-culture as per Subheading 3.4.

2. Wash plate twice in PBS by centrifuging at 600 x g for 5 min at 4°C.
3. Resuspend cells in 50 μl LIVE/DEAD Fixable Blue Dead Cell Stain and incubate for 20 min at room temperature in the dark.
4. Wash cells twice with staining buffer by centrifuging at 800 x g for 3 min at 4°C.
5. Resuspend cells in FcR blocking reagent at 20 μl per 10^7 cells. Incubate for 10 min in the dark at 4°C.
6. Wash cells twice in staining buffer by centrifuging at 800 x g for 3 min at 4°C.
7. Resuspend cells in 50 μl anti-human CD19 BV785, CD24 APCeFluor780, CD38 PerCPeFluor710, CD27 PE/Dazzle 595, IgM BV510, IgD BV605 mAbs at optimal concentrations determined by titration (*see* **Note 12**), diluted in staining buffer. Incubate for 30 min in the dark at 4°C.
8. Wash plate twice in staining buffer, centrifuging at 800 x g for 3 min at 4°C.
9. Resuspend cells in 100 μl Intracellular (IC) Fixation buffer. Incubate for 10 min in the dark at 4°C. Wash cells twice with staining buffer, centrifuging at 800 x g for 3 min at 4°C.
10. If staining for cell surface markers only (*ex vivo* or after culture), resuspend cells in 200 μl staining buffer in 5 ml polystyrene round-bottom tubes. Store in the dark at 4°C until analysing by flow cytometry. If additionally staining for intracellular cytokines, continue with this section.
11. Wash cells twice with permeabilisation buffer, centrifuging at 800 x g for 3 min at 4°C. Resuspend cells in 50 μl permeabilisation buffer, incubate for 5 min in the dark at 4°C.
12. On top of the 50 μl permeabilisation buffer, add 50 μl anti-IL-10 APC mAb at the optimal concentration as determined by titration (*see* **Note 12**), diluted in permeabilisation buffer. Incubate for 40 min in the dark at 4°C. For the IL-10 FMO control, add 50 μl permeabilisation mAb instead of anti-IL-10 APC mAb.
13. Wash cells twice with staining buffer, centrifuging at 800 x g for 3 min at 4°C. Resuspend cells in 200 μl staining buffer in 5 ml round-bottom polystyrene tubes. If samples are not immediately analysed by flow cytometry, store samples in the dark at 4°C prior to analysis (*see* **Note 18**).
14. Prepare Anti-Mouse Ig, κ/Negative Control Compensation Particles as per manufacturer's instructions for each fluorochrome used.
15. Proceed to flow cytometry acquisition. As per Fig. 1, gate on lymphocytes, exclude doublets and dead cells and gate on the $CD19^+$ population. Within the CD19+

population, use CD24 and CD38 expression to gate on the $CD24^{hi}CD38^{hi}$ subset, and use CD24 and CD27 expression to gate on the $CD24^{hi}CD27^{+}$ subset. Gate on the IL-10^{+} B cell population (within individual subsets or as part of the total $CD19^{+}$ population), using the unstimulated sample and fluorescence-minus-one (FMO) control as guidelines (*see* Subheading 3.6 and Fig. 2).

3.6 Guidelines for analysis of stained cells by flow cytometry

Peripheral B cell subsets can be broadly identified by their expression of the surface markers CD24, CD38 and CD27. Immature B cells are identified as $CD24^{hi}CD38^{hi}$, mature B cells as $CD24^{int}CD38^{int}$ and memory B cells as $CD24^{+}CD38^{hi}$. CD27 is typically a marker of post-germinal centre B cells and thus with CD24 expression may be used as a memory B cell marker (plasmablasts and plasma cells have low/negative CD24 expression). Note that not all memory B cells are $CD27^{+}$, for example a $CD27^{-}IgD^{-}$ double-negative memory B cell population has been described [12]. *Ex vivo*, surface expression of CD24, CD38 and CD27 is sufficient to identify the $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ subsets (*see* Fig. 1A).
Following stimulation and *in vitro* culture, delineation between individual B cell subsets is not always clear due to the spread of surface marker expression by B cells during differentiation. For example, discriminating between the $CD24^{hi}CD38^{hi}$ immature and $CD24^{int}CD38^{int}$ mature subsets is not always evident as both of these markers are expressed as a spectrum. Analysing the expression of surface IgM and IgD (*see* Fig. 1B) can validate B cell subset gate placement compared to CD24, CD38 and CD27 expression alone. For example, $CD24^{hi}CD38^{hi}$ immature B cells are $IgM^{hi}IgD^{hi}$, whereas $CD24^{int}CD38^{int}$ B cells are $IgM^{+}IgD^{+}$. Therefore, checking that within the gated $CD24^{hi}CD38^{hi}$ subset the expression of IgM and IgD is high increases confidence that placement of the $CD24^{hi}CD38^{hi}$ gate is correct. Additionally, IgM and IgD expression provides greater resolution into the memory B cell compartment by delineating between IgM^{+} non-class-switched memory and $IgM^{-}IgD^{-}$ class-switched memory.

Fig. 2 shows an example of gate positioning for IL-10^{+} cells within the total B cell, $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ populations. Also shown is the FMO control and unstimulated B cell sample. As following Breg expansion there may not be a clear delineation between IL-10^{+} and IL-10^{-} populations. Therefore we recommend using the unstimulated sample as an additional guideline for IL-10 gate placement; isolated B cells cultured for 72 h without stimulation will produce very little IL-10 (0-3%) (*see* Fig. 2)

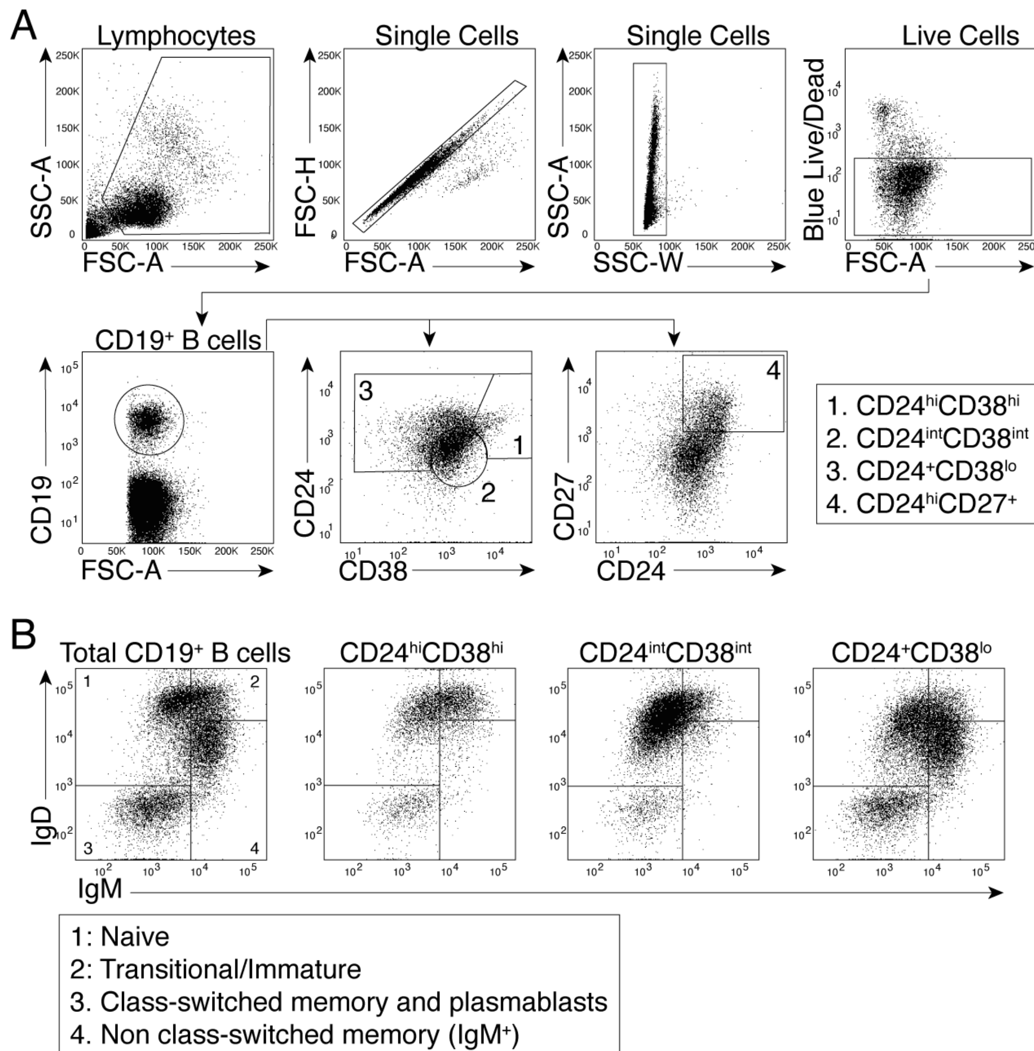

Fig. 1 Gating strategy for ex vivo identification of CD24^{hi}CD38^{hi} and CD24^{hi}CD27^{+} B cell subsets. Isolated healthy human PBMCs were stained for flow cytometry according to the protocol described in Subheading 3.5. Dot plots show gating strategies for ex vivo identification of CD24^{hi}CD38^{hi} and CD24^{hi}CD27^{+} B-cell subsets. In addition, expression of IgM and IgD can be used to validate gates set for CD24^{hi}CD38^{hi} and CD24^{hi}CD27^{+} subsets. (a) Flow cytometry dot plots show the gating strategy for ex vivo identification of live, CD19^{+}CD24^{hi}CD38^{hi} and CD24^{hi}CD27^{+} B cell subsets from a total PBMC population. PBMC sample is gated on lymphocytes, then to include single cells and exclude doublets, then to include live cells only. B cells are then identified by the expression of CD19. The markers CD24 and CD38 are used to identify CD24^{hi}CD38^{hi} B cells, while CD24 and CD27 allow identification of CD24^{hi}CD27^{+} B cells. (b) Flow cytometry dot plots show IgM and IgD expression within the total B cell population, and within the CD24^{hi}CD38^{hi}, CD24^{int}CD38^{int}, and CD24^{+}CD38^{lo} subsets. Labels 1-4 indicate the B cell phenotype within each quadrant.

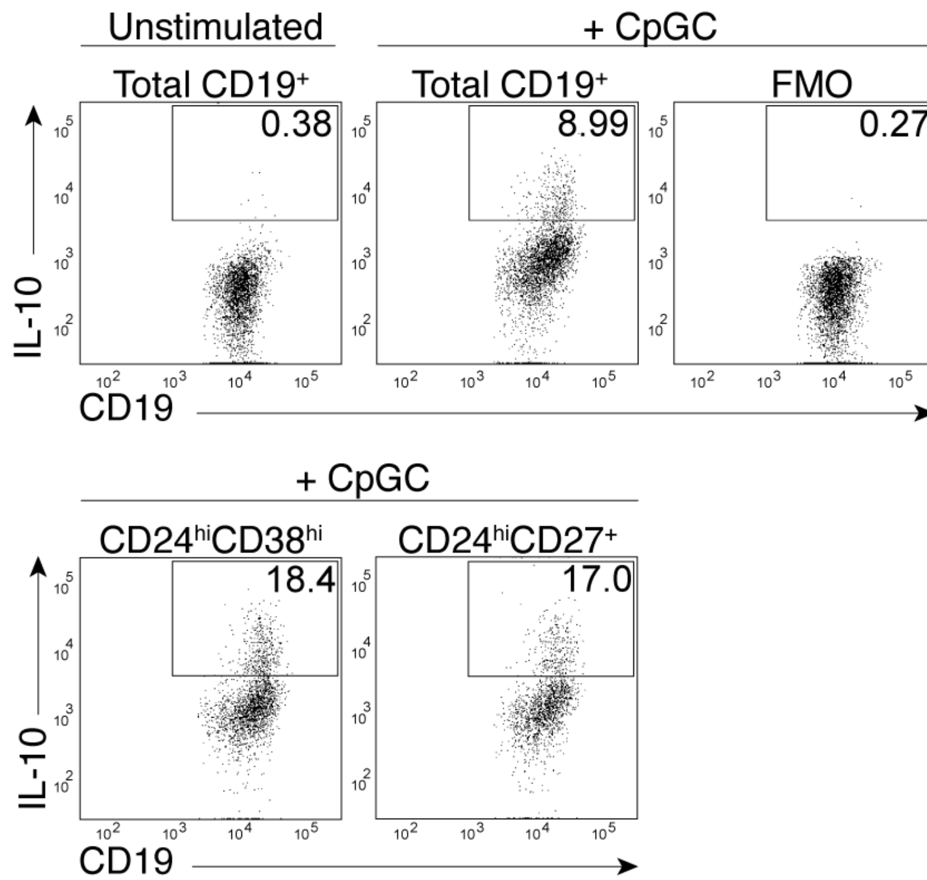

Fig.2 IL-10 intracellular staining post-CpGC stimulation in vitro. Flow cytometry dot plots show IL-10+ B cells in the total $CD19^+$ B-cell population and within the $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^+$ subsets following 72h stimulation with 1μM CpGC. Also shown is an unstimulated sample and the fluorescence-minus-one (FMO) sample to indicate true staining and inform IL-10^+ gate placement.

4 Notes

1. We recommend the STEMCELL EasySep B cell enrichment kit as the isolated B cell fraction has 95-99% purity.
2. For cell sorting we recommend using DAPI for staining dead cells as it can be added immediately prior to cell sorting thus minimising incubation times. We recommend using LIVE/DEAD Blue for staining dead cells for flow cytometry as this is compatible with cell fixation. Although other similar dyes to LIVE/DEAD Blue are available, we recommend this dye since it is excited by a UV laser and is compatible with the fluorescent mAb panel we describe in this protocol.
3. To retrieve the maximum amount of blood from the vacutainer tube use a Pasteur pipette to rinse out any remaining sample with RPMI 1640 medium.
4. Following centrifugation of harvested PBMC there may be residual red blood cell contamination (the pellet will be red in colour). In this event, resuspend the pellet in 5 ml Red Blood Cell Lysis Buffer and incubate for 5 min at room temperature before washing in complete medium and continuing with the protocol.
5. We suggest using a trypan blue exclusion assay to count cells and assess viability.
6. B cell isolation is optimally performed immediately following PBMC isolation as this avoids cell death occurring during the freezing/thawing process. However, it may be required that PBMC are stored in advance. In this case, prepare freezing medium (10% DMSO in FCS) for a volume allowing 1×10^7 PBMCs/ml. Store freezing medium on ice until use. Following cell count and centrifugation, resuspend the cell pellet in 1 ml of cold freezing medium, then pour in the remaining medium. Rapidly aliquot resuspended cells at 1 ml per cryovial. DMSO is toxic to cells so minimise the length of time cells are in contact with DMSO. Store cryovials in a Mr. Frosty™ container (ThermoFisher) with propan-2-ol in a -80°C freezer to allow freezing at -1°C per minute. Transfer cryovials to liquid nitrogen storage after no longer than 3 days at -80°C storage. If cells are left any longer cell viability declines.
7. Thaw and wash cells rapidly to minimise cell exposure to DMSO (which is toxic to cells) and to optimise viability. Minimise the length of time cryovials are on ice following removal from liquid nitrogen storage.
8. Following centrifugation, aspirate as much supernatant as possible without disturbing the cell pellet before resuspending in the appropriate separation buffer volume. This is because the efficacy of the B cell Enrichment Kit is volume-sensitive.

9. At the final step of the B cell enrichment protocol when extracting the B cell fraction, after 2-3 seconds of tube inversion there will be droplets attached to the tube edge. Do not dislodge these into the B cell fraction as these may contain non-B cells which will decrease the purity of the isolated B cell sample.
10. The number of B cells plated per well may vary depending on the numbers of B cells isolated and the number of wells intended to include per sample. We recommend plating between 2-5x10^5 B cells per well, since B cells proliferate more and tend to have higher viability at lower cell densities. Ensure number of B cells plated per well is consistent across conditions/repeats.
11. The time required to sort a sample will be greatly reduced by starting from isolated B cells rather than total PBMC.
12. While we describe in this protocol (*see* Table 1) to use 2μg/ml mAb for extracellular marker staining and 4μg/ml mAb for IL-10 intracellular staining, we suggest titrating mAbs to find the optimal concentrations for individual laboratories.
13. Include an unstimulated B cell sample, and a sample to be used as an FMO for IL-10 staining. These can both be used as guidelines for correct IL-10 gate placement (*see* Subheading 3.6 and Fig. 2).
14. Titrate stimuli to find optimum concentration for the needed application. Although our given concentrations were found to maximally induce Bregs, we recommend titrating each stimulus in order to find optimal concentrations for individual laboratories.
15. During incubation some medium may evaporate from wells and this can reduce cell viability. Filling the empty wells surrounding samples with medium is a way to limit evaporation from the samples and therefore reduce cell death.
16. Cell culture supernatants can be decanted into a 96-well plate and stored at -80°C for further analysis, such as ELISA. ELISA measures secreted cytokine in cell supernatants, and this could be of interest since flow cytometry measures the intracellular expression of cytokines, which may not be representative of secreted IL-10.
17. PMA and ionomycin activate B cells and induce cytokine production. Brefeldin A inhibits cytokine secretion so in combination with PMA and ionomycin increases intracellular cytokine levels, maximising intracellular IL-10 detection by flow cytometry.

18. To maintain optimal sample and staining quality, we recommend analysing samples by flow cytometry no longer than 48 h after surface marker staining, and no longer than 24 h after for intracellular cytokine staining. Optimally, samples should be run soon after staining. Some tandem fluorochromes (for example PE-Cy5) can degrade and separate into differentially fluorescing components.

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Table 1. Fluorescent mAbs against B cell surface markers and IL-10

Target	Fluorochrome	Clone	Concentration	Isotype
CD19	BV785	HIB19	2 μg/ml	Mouse IgG1, κ
CD24	APCeFluor780	SN3A52H10	2 μg/ml	Mouse IgG1, κ
CD38	PerCPeFluor710	HB7	2 μg/ml	Mouse IgG1, κ
CD27	PE/Dazzle	M-T271	2 μg/ml	Mouse IgG1, κ
IgM	BV510	MHM-88	2 μg/ml	Mouse IgG1, κ
IgD	BV605	IA6-2	2 μg/ml	Mouse IgG2a, κ
IL-10	APC	JES3-19F1	4 μg/ml	Rat IgG2a, κ