A two-step non-flowcytometry-based naïve B cell isolation method and its application in Staphylococcal enterotoxin B (SEB) presentation

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Summary

Background: **To study the role of human naïve B cells in antigen presentation and stimulation to naïve $CD4^+$T cell, a suitable method to reproducibly isolate sufficient naïve B cells is required.**

Methods: **To improve the purity of isolated naïve B cells obtained from a conventional one-step magnetic bead method, we added a rosetting step to enrich total B cell isolates from human whole blood samples prior to negative cell sorting by magnetic beads. The acquired naïve B cells were analyzed for phenotypes and for their role in Staphylococcal enterotoxin B (SEB) presentation to naïve $CD4^+$T cells.**

Results: **The mean (SD) naïve B cell (CD19+/CD27-) purity obtained from this two-step method compared with the one-step method was 97% (1.0) versus 90% (1.2), respectively. This two-step method can be used with a sample of whole blood as small as 10 ml. The isolated naïve B cells were phenotypically at a resting state and were able to prime naïve $CD4^+$T cell activation by Staphylococcal enterotoxin B (SEB) presentation.**

Conclusions: **This two-step non-flow cytometry-based approach improved the purity of isolated naïve B cells compared with conventional one-step magnetic bead method. It also worked well with a small blood volume. In addition, this study showed that the isolated naïve B cells can present a super-antigen "SEB" to activate naïve CD4 cells. These methods may thus be useful for further *in vitro* characterization of human naïve B cells and their roles as antigen presenting cells in various diseases.** ***(Asian Pac J Allergy Immunol 2012;30:214-23)***

Keywords: Naïve *B cell, antigen presentation, naïve $CD4^+$T cell, resetting, magnetic cell sorting, superantigen, staphylococcal enterotoxin B (SEB)*

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Introduction

Human naïve B cells are defined as mature B cells that have never been exposed to antigens. Ideally, the absence of immunoglobulin (Ig) genes' somatic hypermutation (SHM) and isotype switching are considered as absolute markers to differentiate naïve B cells from memory B cells.[1] Typically, naïve B cells can be differentiated from other B cell populations through their specific phenotypes. Early studies relied on IgD expression to identify naïve B cell (IgD^+B cells).[1] However, the use of IgD for the naïve B cells' positive selection has a major disadvantage, as the engagement of the molecule could modulate the naïve B cell's properties. In the early 1990s, as CD27 was discovered as a universal marker of human memory B cells ($CD27^+$B cells).[2,3] This discovery thus allowed negative isolation of naïve B cells ($CD27^-$B cells) from memory B cells ($CD27^+$B cells), and this method is now commonly applied in most commercial isolation kits and flow cytometric sorting processes.

Peripheral blood is a convenient sample source to study human immune cells, including naïve B cell. An example of a commercial kit generally applied for human naïve B cell isolation is the Naïve B cell

Isolation kit II (Miltenyi Biotec). The kit removes non-$CD19^+$ and $CD27^+$cells using a cocktail of specific antibodies and microbead labeling allowing negative naïve B cell isolation directly from peripheral blood mononuclear cells (PBMCs) by one-step cell depletion. Numerous studies exclusively followed this procedure for naïve B cell isolation.[4-13] However, various other combining methods have also been applied to achieve the isolation of such cells for different purposes. Typically, these combined methods consist of two major isolation steps, total B cell pre-enrichment and subsequent naïve B cell isolation. Enrichment of B cells could be achieved either through immune-magnetic beads (MACS®, Dynabeads® and StemSep®) or rosetting with red blood cells (RBCs) (RosetteSep®).[14-19] Although the use of magnetic beads is claimed to provide highly enriched B cells with a purity ($CD19^+$cells) of around 95-99%, the technique requires PBMC isolation prior to the enrichment step which extends the duration of the process and, consequently, increases the level of mortality. Flow cytometric cell-sorting of enriched total B cells theoretically should provide superior cell purity and the flexibility to simultaneously sort many cell populations of interest as compared to magnetic cell sorting. However the technique is not suitable as a routine method and not convenient for simultaneously isolation of cells from several samples. Moreover, the technique requires adequate cell marker labeling, expensive sorting machine and also expertise.

Although it is well known that the priming of naïve T cells is mainly mediated by dendritic cells' (DCs) antigen presentation, naïve B cells are also able to initiate T cell immunity as well.[20] Unfortunately, with the exception of numerous studies using mice, *the contribution of human naïve B cells in antigen presentation* to naïve $CD4^+$T cells has been poorly characterized. This is probably due to some important limitations, including the low frequency of Ag-specific naïve $CD4^+$T cells, the intrinsically high activation threshold of naïve T cell[21] and the limited Ag processing/presentation properties of naïve B cells, themselves.[11]

T cell bacterial super-antigens (BSAg) are microbial toxins capable of binding to the invariant region of major histocompatibility complex class II (MHC-II) molecules and the particular motif of the variable region of the β chain (Vβ) of T cell receptor (TCR), simultaneously. Staphylococcal enterotoxin B (SEB), a well-established BSAg, was extensively used in the characterization of various antigen presenting cells (APCs), including human naïve B cell.[22] Though the pulsation of SEB on human naïve B cells was known to stimulate total $CD4^+$T cell activation,[22] the specific priming effect on naïve $CD4^+$T cells remains to be investigated.

In this study, we evaluated a simple two-step method to improve peripheral naïve B cell's purity by the combination of a rosetting process (Human B lymphocyte enrichment cocktail, StemCell) and the Naïve B cell Isolation kit II (Miltenyi Biotec). Overall the processes were reproducible and could be performed manually without specific automated equipments for cell isolation. In addition, to assess the quality of purified naïve B cells, we have also demonstrated that the isolated naïve B cells could present the superantigen "SEB" to prime autologous naïve $CD4^+$T cells.

Methods

Antibodies and Reagents

Anti-human CD40(5C3), CD45RO(UCHL1), CD19 (4g7), CD3(ucht1), CD25(m-a251), CD45RA (hi 100), HLA-II(tu39), CD4(RFT-4G), CD11c(b-ly6) antibodies were purchased from BD Pharmingen (San Diego, CA). IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) was obtained from R&D Systems (Minneapolis, MN). Propidium iodide (PI) and PKH-26 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Staphylococcal enterotoxin B, from *Staphylococcus aureus* (SEB) was purchased from Sigma-Aldrich (Singapore). RosetteSep® human B lymphocyte enrichment cocktail and RosetteSep® Human $CD4^+$T cell enrichment cocktail were purchased from StemCell Technologies Inc. (Vancouber DC, CA). Dynabeads® CD25 was purchased from Invitrogen Dynal (AS, Oslo, Norway). A naïve B cell isolation kit II, anti-PE microbeads and anti CD14 microbeads were purchased from Miltenyi Biotec (Auburn, USA).

Human Blood samples

Blood samples were collected from healthy donors at the National Blood Centre of the Thai Red Cross Society into heparin vacutainers. All samples were collected after the donors had signed a written informed consent in accordance with the institutional reviewed board-approved protocols of the Faculty of Medicine, Chulalongkorn University. Blood samples were collected and used for different cell type preparation: 10 ml for naïve B cells, 5-10 ml for MoDCs, 5 ml for naïve $CD4^+$T cells, and 5 ml for PBMCs used in percentage yield calculation.

Isolation and culture of peripheral naïve B cells

a. One-step method

Peripheral naïve B cells were isolated from PBMCs using the Naïve B cell Isolation kit II (Miltenyi Biotec) according to the manufacturer's protocol. Briefly, PBMCs were isolated from blood samples by density-gradient sedimentation on Ficoll-Hypaque Isoprep (Robbins Scientific, Sunnyvale, CA) at 1200 g for 20 minutes at 23°C. Acquired PBMCs were then incubated with a cocktail of biotinylated antibodies specific to non-naïve B cells (CD2, CD14, CD16, CD27, CD36, CD43 and CD235a) and anti-biotin microbeads for 15 and 20 minutes. Labeled PBMCs were washed with cold MAC (2 mM EDTA 0.5% BSA PBS) buffer and then flushed through the magnetic column (MS). The flow-through fraction was collected and considered to be the naïve B cell's fraction (1.5 ml). The B cell purity (%$CD19^+$cells) was determined.

b. Two-step method

Total B cells were enriched directly from blood sample using RosetteSep® human B lymphocyte enrichment cocktail (StemCell, DC, CA). The enriched B cells were subsequently treated for naïve B cells ($CD19^+CD27^-$cells) isolation using the Human naïve B cell isolation kit. In brief, 20-minutes incubation of donor blood sample with the RosetteSep cocktail (50 µl cocktail/1 ml blood) was performed to allow non-B cells (CD2, CD3, CD16, CD36, CD56, CD66b and CD235a positive cells) to crosslink with red blood cells (RBCs). Enriched human B cells were separated from crosslinked cells by density-gradient sedimentation on Ficoll-Hypaque Isoprep Robbins Scientific, Sunnyvale, CA) at 1200 g for 20 minutes at 23°C. The enriched total B cells were then incubated with a cocktail of biotinylated antibodies specific to non-naïve B cells and anti-biotin microbeads for 15 and 20 minutes. In this study, the amount of antibodies as well as the beads was estimated based on approximation of 1×10^6 PBMCs/1 ml blood sample. Labeled total B cells were washed with cold MAC buffer and then flushed through the magnetic column. The naïve B cell fraction (flow-through fraction) was collected, and determined for B cell purity (%$CD19^+$cells), naïve B cell purity (%$CD19^+CD27^-$cells) and memory B cell purity (%$CD19^+CD27^+$cells). For 4 samples, 5 ml of blood was used to estimate the number of naïve B cells/ml blood after their separation using gradient centrifugation on Ficoll-Hypaque Isoprep (Robbins Scientific) at 1200 g, 23°C for 30 minutes. The acquired data were included for yield calculation. Enriched naïve B cells were maintained in RPMI 1640 with L-glutamine (Invitrogen GIBCO, Grand Island, NY) supplemented with 10% Fetal bovine serum (FBS) (Cambrex Bio Science Walkersville, Walkersville, MD). The cells were cultured in 96-well U-bottom plates (5 $x10^4$ cells/well) in 10% FBS RPMI at 37°C, 5% CO2 in a humidified incubator for 18, 36 and 60 hours to assess the cell viability by propidium iodide (PI) staining.

Isolation and culture of peripheral naïve $CD4^+$T cells

Naïve $CD4^+$T cells ($CD4^+CD25^-CD45RA^+$ $CD45RO^-$cells) were isolated from blood sample using RosetteSep® Human $CD4^+$T cell enrichment cocktail, Dynabeads® CD25 (Invitrogen, Oslo, Norway) and anti-PE microbeads (Miltenyi Biotec, Auburn, USA). Briefly, human $CD4^+$T cells were enriched directly from blood samples using RosetteSep® Human $CD4^+$T cell enrichment cocktail by a process similar to that of B cell enrichment. The enriched total $CD4^+$T cells were then depleted for $CD25^+$cells by Dynabeads® CD25 according to the manufacturer's protocol to collect $CD4^+CD25^-$T cells. This fraction was subsequently incubated with anti-CD45RO-PE monoclonal antibody and anti-PE microbeads for 25 and 30 minutes. Bead labeled-total $CD4^+CD25^-$T cells were flushed through the magnetic column (MS). The flow-through fraction considered to be naïve T cells was maintained in 10%FBS RPMI at 37°C, 5% CO2 in a humidified incubator lesser than 18 hours prior to co-culturing or further process.

Preparation of monocyte-derived dendritic cells (MoDCs)

To prepare monocyte-derived dendritic cells (MoDCs), collected PBMCs were subsequently isolated for $CD14^+$cells using CD14 MicroBeads (Miltenyi Biotec, Auburn, USA) according to the manufacturer's protocol. Enriched $CD14^+$cells (1-2 $x10^6$ cells/well) were cultured in the presence of 3 µg/ml IL-4 and GM-CSF in RPMI supplemented with 10%FBS for 6 days. Purity of $CD11c^+$ $CD209^+CD3^-$ MoDCs was >90%.

Staphylococcal enterotoxin B (SEB) pulsation and activation of naïve $CD4^+$T cell

Naïve B cells, MoDCs or naïve $CD4^+$T cells were pulsed with 0.5 µg SEB per 1-1.5$x10^5$ cells for an hour at 37°C with 5% CO_2. To assure the absence of cell activation of SEB pulsed naïve B cells, the

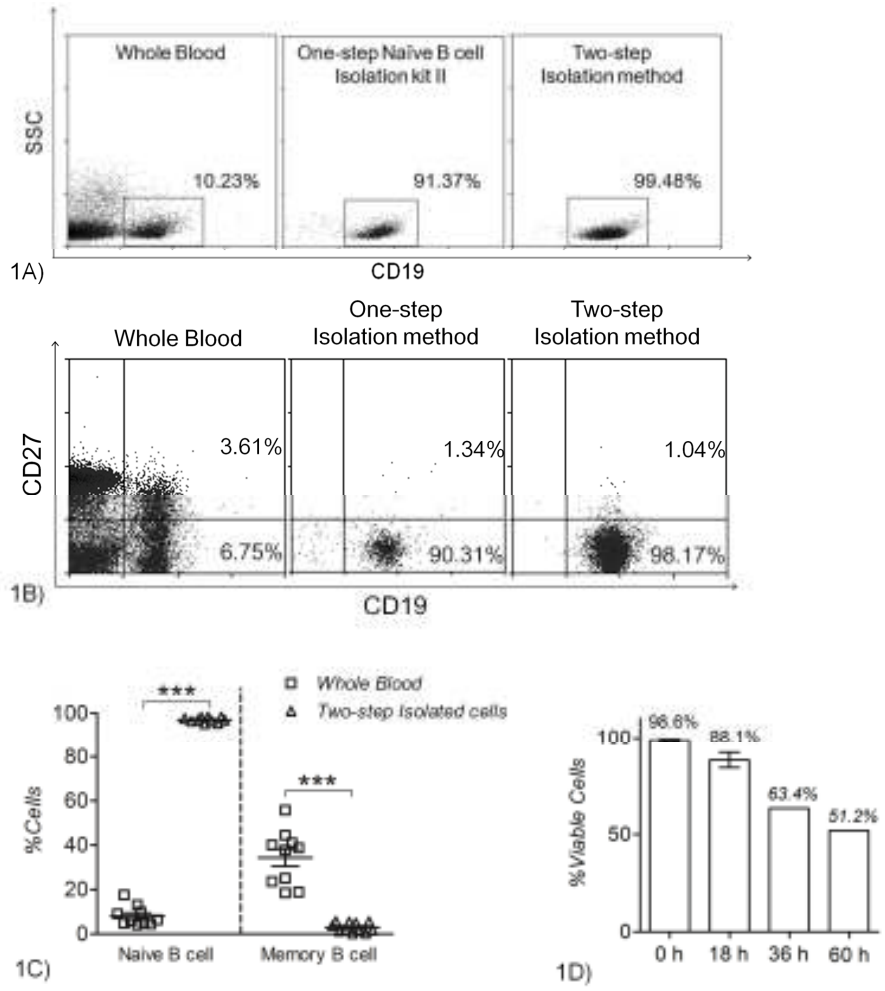

Figure 1 Purities of total B cells and naïve B cells acquired using the one-step Naïve B cell Isolation kit II (Miltenyi) and the Two-step isolation method. (A) Representative percentages of B cells ($CD19^+$cell) determined in whole blood, cells isolated using the One-step Naïve B cell Isolation kit II and those obtaind using the Two-step isolation method. (B) Percentages of naïve B cells ($CD19^+CD27^-$cell) in whole blood, cells acquired after One-step and Two-step Isolation from representative samples. (C) Comparison of naïve B cells ($CD19^+CD27^-$cell) and memory B cells ($CD19^+CD27^+$cells) in whole blood and cells acquired after Two-step Isolation (n=10) (D) Viable naïve B cells determined at 0, 18, 36 and 60 hours by Trypan blue dye (0 h) and propidium iodide (PI) (18-60 h). *Asterisk within the graph denoted significantly different values. *** indicated p <0.001.

expression of CD69, CD80, and CD86 was determined at 24 hours-post pulsation in two samples. Immediately after pulsation, autologous naïve $CD4^+$T cells were co-cultured with SEB pulsed-naïve B cells or -MoDCs in a 96-well U-bottom plate at the ratio of 1:1 for 68 hours to acquire a total of 8-10x10^4 cells. Increases of cell size (FSC) and activated T cells ($CD4^+CD25^+$cells) were then determined in each co-culture. Interfering doublets of B and T cell's complexes ($CD4^+CD19^+$cells) in gated mononuclear population was determined and found to be less than 0.5% in total cells.

Flow cytometry

Cells were incubated with the appropriate conjugated antibodies for 20 minutes at 4°C, washed and applied onto a flow cytometer (FACSCaliber, BD). To determine the cell's viability, cells were incubated with PI at a concentration of 100 ng/ml for 15 minutes at 4 °C. The data were acquired and analyzed using CellQuestPro (Becton Dickinson, San Jose, USA) and Summit program (Dako, CO, USA), accordingly.

Statistical analysis

Differences between the experimental groups were detected by One-way analysis of Variance (1-way ANOVA). To further detect significant differences among experimental groups, a Bonferroni post hoc test was performed. Significant differences between the two experimental groups were detected using an unpaired t-test. All statistic analyzes were performed using Graph Pad Prism 3.0.

Results

The purity of naïve B cells is improved by a two-step isolation method

The blood samples from healthy donors contained 11.8±5.6% total B cells ($CD19^+$cells) (Figure 1A) and 8.0±4.4% naïve B cells ($CD19^+CD27^-$cells) (n =15) (Figure 1B and 1C), respectively. Five blood samples were used to isolate naïve B cells using a one-step protocol based on the Naïve B cell isolation kit II as described in the materials and methods section. Following the use of this method, the average B cell purity ($CD19^+$cell) reached was 90% (90.6±1.2%) (Figure 1A). To improve the naïve B cell purity, we performed a two-step isolation method by the addition of a rosetting B cell enrichment step (Human B lymphocyte enrichment cocktail, StemCell) prior to naïve B cell purification. The enriched B cell purity acquired after rosetting was 80-81% (n =3) (Supplementary Figure 1). Following naïve B cell isolation, a drastic improvement in B cell's purity ($CD19^+$cells) with a mean of 99% (99.1±0.5%) (p <0.0001) (n =10) was observed (Figure 1A), while the acquired naïve B cells ($CD19^+CD27^-$cells) purities reached 97.0±1.0% (n=10) with 2.0±2.0% contaminated with memory B cells ($CD19^+CD27^+$cells) (Figure 1B and 1C). The yield determined by PBMCs' calculation was 28.2±9.7% (Supplementary Table 1). Immediate cell viability was always >95% (98.6±2.0, n=10) based on Trypan blue dye exclusion (Figure 1D). To analyze the viability of the isolated naïve B cell under resting conditions prior to any further usage, purified naïve B cells were stained with propidium iodide (PI) at 18 h, 36 h and 60 h post-isolation. The B-cell viability dropped with time from 88% to 50% within 60 h (Figure 1D) Thus, to avoid any viability issue further experiments were performed with resting B cells for 18 hours.

Naïve B cells and monocyte-derived dendritic cells (MoDCs) differentially express co-stimulatory molecules

The expression level of HLA class II (HLA-II), CD40, CD80 and CD86 in naïve B cells was compared with that of MoDCs prepared from the same blood sample (Figure 2). Naïve B cells expressed more CD40 than those on MoDCs (Figure 2A). By contrast, naïve B cells expressed much lower CD80 and CD86 when compared to MoDCs in terms of both positive cell number (p <0.001) (Figure 2A and 2B) and expression density (p <0.01) (Figure 2A and 2C). By mean fluorescence intensities (MFIs), the expression density of HLA class II on MoDCs was also higher than that of naïve B cell (p <0.05) (Figure 2C). No significative up-regulation of the co-stimulatory molecules CD69, CD80 or CD86 was observed following naïve B cell pulsation with SEB (Figure 2D and 2E).

Naïve B cells upregulated CD25 expression on $CD4^+$T cells during Staphyloccocus enterotoxin B (SEB) presentation

The typical phenotypic changes of T cell activation were analyzed by flow cytometry in populations of naïve $CD4^+$T cells co-cultured for 68 h with SEB-pulsed naïve B cells or with SEB-pulsed MoDC. Naïve $CD4^+$T cells cultured in the absence of APCs (resting naïve T cells) and SEB pulsed naïve $CD4^+$T cells were regarded as controls (Figure 3). Slight increases of T cell size and CD25 expression (p <0.05) were observed in the SEB pulsed naïve T cells when compared with those of the resting naïve T cells (Figure 3).[23] Significant T cell activation was detected in the SEB-presented samples, both by naïve B cells and MoDCs in comparison with resting samples and the direct SEB-pulsed naïve T cell controls, as indicated by the increase of both cell size (Figure 3A and 3B) and CD25 expression (Figure 3A and 3C) (p<0.001). Nonetheless, lower degrees of T cells activation were evident when the naïve T cells were SEB-presented by naïve B cells in comparison with that by MoDCs (p <0.001) (Figure 3).

Discussion

To investigate the *in vitro* interaction between human naïve B cells and naïve $CD4^+$T cells, an appropriate cell isolation protocol is required to get a sufficient numbers and purity of naïve B cells. The present study used a two-step isolation method to isolate human naïve B cell from peripheral blood samples. We demonstrated that an additional step of

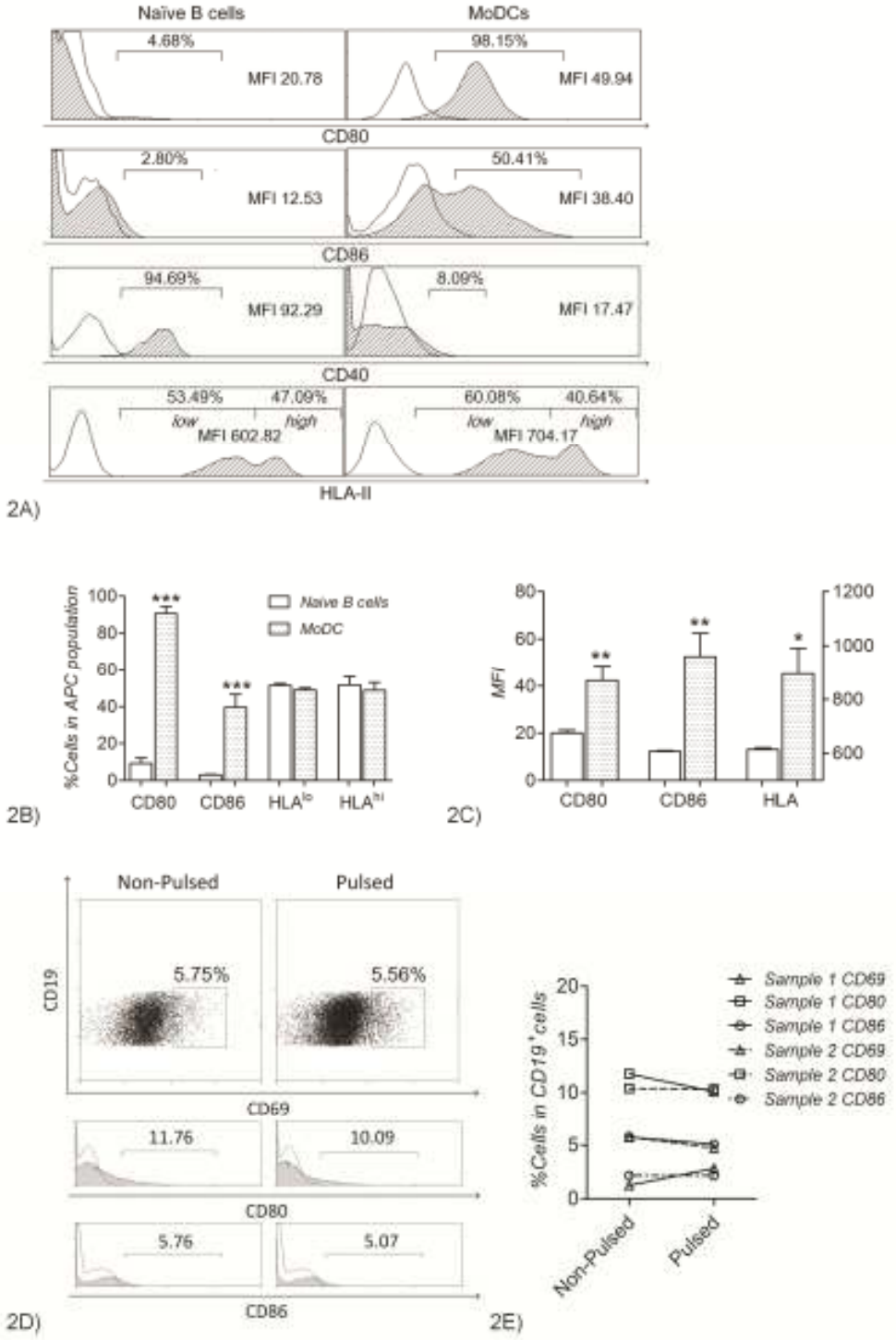

Figure 2 B7 co-stimulatory molecule and HLA class II expression on naïve B cell and monocyte derived dendritic cells (MoDCs). (A) Expression of CD80, CD86, CD40 and HLA-II of 18-hour resting naïve B cells and control MoDCs acquired from representative samples (MFI = Mean fluorescent intensities, striated curve = Stained cells and Clear curves = non-stained cell control). Comparison of (B) positive cell number and (C) MFI of CD80, CD86, CD40 and HLA-II expression between naïve B cells (n= 6) and MoDCs (n=5) (MFI of HLA-II was determined based on a right-axis scale, while the rest were based on a left-axis scale). (D) A representative sample and (E) comparison of CD69, CD80 and CD86 expressions on SEB pulsed (Pulsed) and non-pulsed naïve B cell control (Non-Pulsed) (n=2) after 24 hour culture. *Asterisk within the graph denoted significantly different values between Naïve B cells and MoDCs. * indicated $p < 0.05$, ** indicated $p < 0.01$, *** indicated $p < 0.001$.

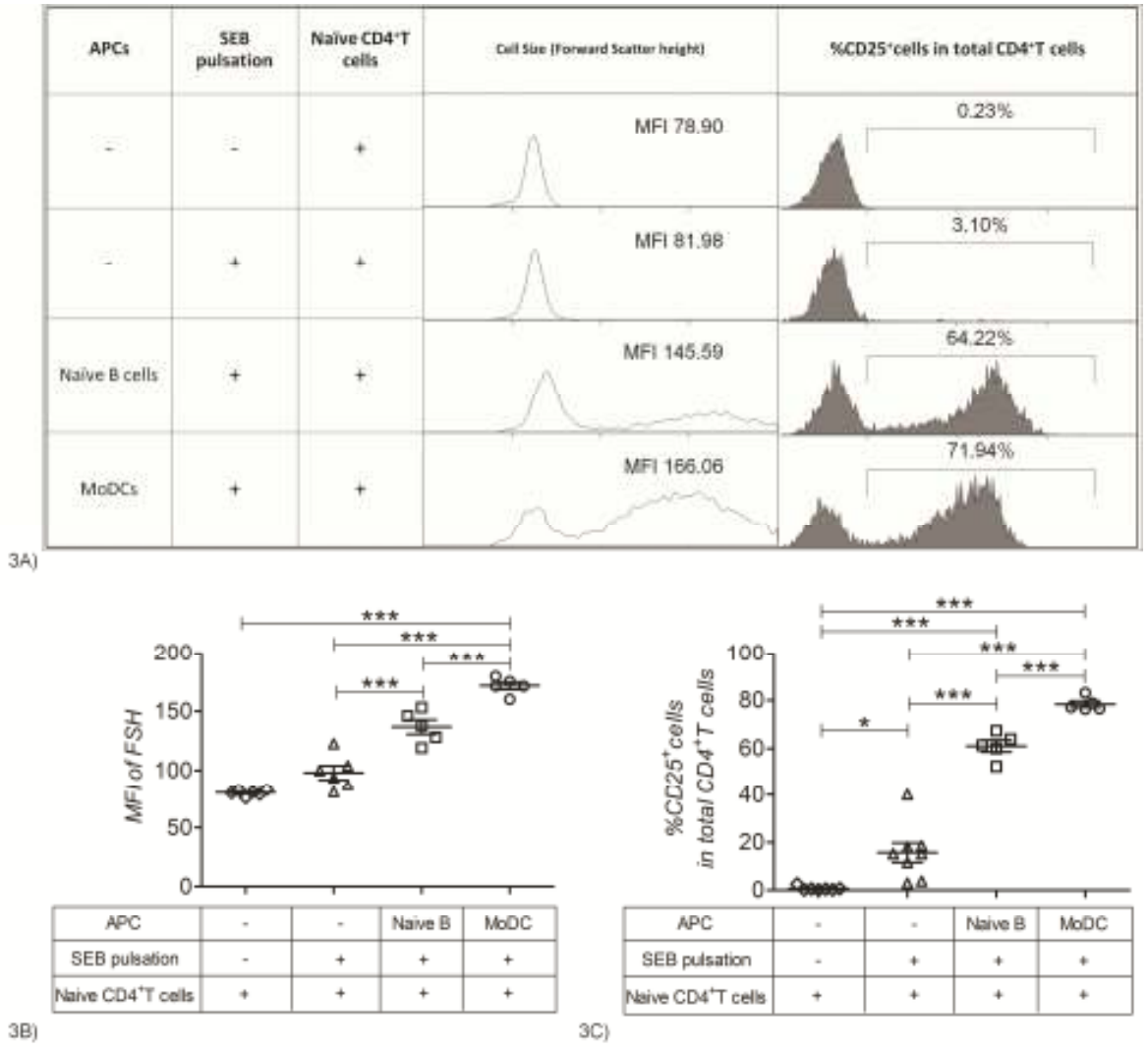

Figure 3 Determination of T cell activation after 68 hours of culture. (A) Cell size (FSC-H) and CD25$^+$T cell's number of resting naïve CD4$^+$T cells, SEB pulsed naïve CD4$^+$T cells, naïve CD4$^+$T cells co-cultured with SEB pulsed naïve B cells and naïve CD4$^+$T cells co-cultured with SEB pulsed MoDCs prepared from a same representative sample. Comparisons of (B) FSC-H and (D) CD25$^+$T cells number among all experimental groups (n=5). *Asterisk within the graph denoted significantly different values. *indicated p <0.05, **indicated p <0.01, and ***indicated p<0.001.

total B cell enrichment by rosetting prior to the naïve B cells' negative selection by a commercial magnetic cell isolation kit (Naïve B cell Isolation kit II, Miltenyi Biotec) was able to improve naïve the B cells' purity. Application of higher purity of naïve B cell should thus provide us a more reliable and reproducible experimental result.

Although the naïve B cell isolation kit II (Miltenyi Biotec) is commonly used for human peripheral naïve B cell isolation, it is interesting to note that this manufacturer did not provide any data about the yield as well as the purity of naïve B cells. According to a literature review of naïve B cell isolation using this kit, approximately 90% yield of purity was reached in most of the reports[4-6,9] (Supplementary Table 2 and 3), which is in agreement with our present results (Figure 1A and Table 1). However, two reports from the same lab stated that a 97% purity can be achieved following the same purification method.[11,13] This indicates a variability of the yield when using this one-step method. Our current two-step protocol allowed isolation of naïve human B cells with not only a higher B cell purity but also a higher reproducibility. The purity rate reached 99.0±0.5% from 10 independent experiments (Figure 1A and Table 1). In terms of the volume of

Table 1. Comparison between one-step and two-step naïve B cell purification methods used in the current study

Considerations	One-step method (Naïve B cell Isolation kit II, Miltenyi Biotec)	Two-step method (B cell Enrichment cocktail, Stem Cell & Naïve B cell Isolation kit II, Miltenyi Biotec)
Procedures[a]	PBMC isolation Naïve B cell isolation	Rosetting® B cell Enrichment Naïve B cell isolation
Approximate processing time	1 h 35 min	1 h 45 min
Acquired B cell's purities	90.6±1.2%	99.1±0.5%
Acquired naïve B cell's purities	90.0±2.2%	97.0±1.0%
Viability (immediate)	>95%	>95%

[a] PBMC isolation = 45 min, Rosetting B cell enrichment = 60 min, Naïve B cell isolation (Miltenyi Biotec) = 45 min

the samples, this two-step method was able to isolate sufficient naïve B cells from as little as 10-15 ml of whole blood. Actually a similar process has been previously reported for cell isolation from tonsils but it has not been implemented in whole blood samples.[24] Other well established different two-step methods for peripheral naïve B cells isolation are summarized and compared in Supplementary Table 3. According to our review, all of the non-flow cytometry-based two-step methods consumed much more time than conventional one-step isolation i.e., required 40 min to 1 h 40 min in addition (Supplementary Table 3), whereas approximately 10 more minutes is needed for the additional B-cell enrichment step in our proposed protocol (Table 1). Minimizing the time of the cell separation processes should reduce the risk of activation and therefore loss of healthy naïve B cells. Moreover, this two-step method allows the isolation of naïve B cells which are maintained unlabeled by any fluorescent dyes or magnetic beads, making them suitable for further culture experiments (Supplementary Table 3). The method is also cost-effective, since it requires neither special instruments nor flow cytometric sorting machine.

In this study, the proposed two-step method had given us the mean % yield of naïve B cells of 28% (range 18 - 40%). According to our knowledge, there is no information on the percentage yield of isolated naïve B cells reported in the available published papers. Although one might consider the yield is relatively low, sufficient number of naïve B cells have always been isolated from blood samples with volumes as low as 10 ml to conduct further experiments (with the need for at least 5×10^4 cells per well). This approach can therefore be used to study the role of naïve B cells in clinical settings when limited blood volume is an issue, such as in pediatrics. In addition it may allow us to do a larger scale clinically-correlated study to investigate any pathogenic role of naïve B cells in some particular disorders such as autoimmune diseases.

CD80 and CD86 are members of the B7 peripheral protein family providing a co-stimulatory signal necessary for $CD4^+$T cell activation and survival during antigen presentation via human leuckocyte antigen class II (HLA-II) by an antigen presenting cell (APC).[25] The other important molecule, CD69 is generally regarded as an early lymphocyte activation marker.[8] The purified peripheral blood naïve B cells, obtained from this study, were found to be in a resting state or in a very modest activation state, as judged by the very low expression levels of CD69, low CD80 and CD86 at baseline and up to 18 hours after the isolation. We also observed comparable findings to previous studies i.e., more CD80, CD86, HLA-II but not CD40 were detected on the control MoDCs[25, 26] (Figure 2A-2C). In order to validate the quality of our purified naïve B cell preparations, we evaluated their capacity to function as antigen presenting cells. We therefore used a superantigen "SEB" presentation system to illustrate the T cell activation with SEB pulsed-naïve B cells. After SEB pulsation, there were no significant changes in expression of the early activation marker (CD69) or B7 co-stimulatory molecules (CD80 and CD86) on the naïve B cells (Figure 2D and 2E). This indicates that SEB pulsation in this study did not significantly activate the naïve B cells. In addition, T cell activation was

detected after 68 hour of culture (Figure 3). Thus SEB pulsation could be successfully applied for the study of naïve B cells' superantigen presentation to naïve $CD4^+$T cells.

During $CD4^+$T cell activation, an increase of cell size and expression of several surface molecules, including the IL-2 receptor α chain (CD25), were commonly demonstrated.[35] The naïve $CD4^+$T cells either stimulated by SEB pulsed naïve B cells or SEB pulsed MoDCs increased both in size and CD25 expression more than those observed in directly SEB pulsed naïve T cell controls, without APCs at 68 h of culture (Figure 3). According to previously published data, the results indicated that SEB was more effective at stimulating T cells in an APC dependent way[23] (Figure 3C and 3D). Nonetheless, lesser activation of T cells primed by SEB pulsed naïve B cells was observed when compared to the results from the priming by SEB pulsed-MoDCs (Figure 3). This finding may be explained by a lower expression of CD86 on human naïve B cells compared with dendritic cells.[25]

In summary, this two-step non-flow cytometry approach can reproducibly isolate human peripheral naïve B cells from as little as 10 ml of whole blood with a higher purity and reproducibility, compared with the conventional one-step method. The rosetting step to enrich naïve B cells with required only an additional 15 minutes. The isolated naïve B cells had resting phenotypes and were able to prime naïve T cells in a superantigen "SEB" presentation system. Their efficiency in priming naïve T cells was however lower than that of MoDCs.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://apjai.digitaljournals.org

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