CHAPER II

DETERMINATION TWO-STEP NAÏVE B CELL ISOLATION METHOD AND SEB PULSATION SYSTEM

2.1 QUESTION

2.1.1 Can Rosette Sep® B cell enrichment kit (StemCell) improve purity and consistency of peripheral naïve B cells isolated from Naïve B cell Isolation Kit II (Miltenyi)?

2.1.2 Can peripheral naïve B cell present SEB and prime naïve $CD4^+$ T cell activation?

2.2 HYPOTHESIS

Application of Rosette Sep® B cell enrichment (StemCell) pior to Naïve B cell Isolation Kit II (Miltenyi) results in consistently pure naïve B cells which can be successfully used for SEB presentation to naïve $CD4^+$ T cell.

2.3 OBJECTIVES

2.3.1 To determine the consistency and purity of naïve B cell isolated by Naïve B cell Isolation Kit II (Miltenyi) in our laboratory

2.3.2 To improve the efficiency of Naïve B cell Isolation Kit II (Miltenyi) by inclusion of pre-enrichment step of total B cells by Rosette Sep® B cell enrichment kit (StemCell)

2.3.3 To determine the effect of SEB pulsation on naïve B cell activation state

2.3.4 To study SEB presentation by naïve B cells to naïve $CD4^+$ T cells

2.4 MATERIALS AND METHODS

2.4.1 ANTIBODIES AND REAGENTS

Anti-human CD40 (5C3), CD45RO (UCHL1), CD19 (4g7), CD3 (ucht1), CD25 (m-a251), CD45RA (hi100), 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) were obtained from R&D Systems (Minneapolis,

MN). Carboxyfluorescein succinimidyl ester (CFSE) was from Molecular Probes (Merelbeke, Belgium). 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). Naïve B cell isolation kit II, anti-PE microbeads, anti CD14 microbeads were purchased from Miltenyi Biotec (Auburn, USA).

2.4.2 HUMAN BLOOD SAMPLE

Blood samples were collected from healthy donors at National Blood Centre 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 preparations of various cell types: 10 ml for naïve B cells, 5-10 ml for monocyte-derived dendritic cells (MoDCs), 5 ml for naïve $CD4^+$T cells, and 5 ml for PBMCs used in percentage yield calculation.

2.4.3 ISOLATION AND CULTURE OF PERIPHERAL NAÏVE B CELLS

2.4.3.1 ONE-STEP METHOD

Peripheral naïve B cells were isolated from PBMCs using Naïve B cell Isolation kit II (Miltenyi) according to the manufacturer's protocol. Briefly, PBMCs were isolated from blood sample 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 as naïve B cell's fraction (1.5 ml). The B cell purity (%$CD19^+$cells) was determined.

2.4.3.2 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 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. Enriched total B cells were then incubated with a cocktail of biotinylated antibodiesspecific to non-naïve B cells and anti-biotin microbeads for 15 and 20 minutes. In this study, the amount of antibodies as well as 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 contaminated memory B cell (%$CD19^+CD27^+$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 1500 rpm, 23°C for 30 minutes. The acquired data was 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 plate (5×10^4 cells/well) for 18, 36 and 60 hours to assess the cell viability by propidium iodide (PI) staining.

2.4.4 PREPARATION OF MONOCYTE-DERIVED DENDRITIC CELLS

To prepare monocyte-derived dendritic cells (MoDCs), collected PBMCs were subsequently isolated for $CD14^+$cells using CD14 MicroBeads (Miltenyi, Auburn, USA) according to the manufacturer 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%.

2.4.5 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, Auburn, USA). Briefly, human $CD4^+$T cells were enriched directly from blood sample using RosetteSep® Human $CD4^+$T cell enrichment cocktail by 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 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 as 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.

2.4.6 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, expression of CD69, CD80, and CD86 were determined at 24 hour-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 total 8-10$x10^4$ 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 lesser than 0.5% in total cells (Figure 9).

2.4.7 TROGOCYTOSIS ASSAY

During antigen recognition, T cells interact with antigen-presenting cells (APCs) and normally capture plasma membrane fragments of the APCs with a whole array of components presented on them (trogocytosis) (Hudrisier et al., 2005; Joly and Hudrisier, 2003) (Daubeuf et al., 2006). To determine SEB recognition of naïve T cell on naïve B cells and simultaneously detect proliferation of T cell in the same system, naïve T cells were labeled intracellularly with CFSE ($CFSE^+$cells) by protocol previously reported (Quah et al., 2007) with a final dye concentration of 1.5 uM and naïve B cells' membranes were labeled with PKH26 Red Fluorescent Cell Linker prior to co-culture. SEB pulsed naïve $CD4^+$T cells were also included as control. Plasma membrane of naïve B cells was stained withr according to manufacturer's protocol with final dye concentration to 0.1 uM. In this study, 8×10^5 naïve B cells and 7×10^5 naïve T cells were used for cell labeling. After labeling, the viability of the cells was determined by tryphan blue dye exclusion. Labeled naived B cells were pulsed with SEB as previously described or left non-pulsed (resting) at 37°C, 5% CO2 in a humidified incubator as non-pulsed control. SEB pulsed/non-pulsed naïve B cells were co-cultured with CFSE labeled naïve $CD4^+$T cells as previously described in activation of naïve $CD4^+$T cell (2.6). Co-cultured cells at 5 minutes, 15 minutes, 60 minutes, 1 day and 5 days were collected for analyses. Naïve $CD4^+$T cells ($CFSE^+$cells) earlier interacted with naïve B cells (PKH^+cells) should capture naïve B cell's membrane fragment which was PKH positive (Daubeuf et al., 2006), and thus could be indicated as PKH^+CFSE^+cells. Interfering B and T cell's complexes ($CD4^+CD19^+$cells) in gated mononuclear population at 1 hour of co-culture which should contribute to major fault positive result was determined in separate culture. Lesser than 0.5% of complexes were presented (Figure 10).

2.4.8 FLOW CYTOMETRY

Surface molecules' fluorescence staining was performed according to standing operating procedures (SOP). Briefly, 0.5-2x104 cells of interest were separated into each well of V-shape 96-well plate. The cells were washed with cold-PBS and centrifuged at 1800 rpm for 5 minutes at 4°C prior to discarding the supernatant. All cells were incubated in 0.01% EDTA PBS for 10 minutes in a humidified CO2 incubator at 37°C with 5% CO2. After incubation, the cells were washed and spun down once more prior to adding the surface antibodies' cocktail of interest. The cells were incubated with surface antibodies' cocktail in the dark for 20 minutes at 4°C, and then washed and applied onto a flow cytometer (FACSCaliber, BD). To determine cell's viability, cells were incubated with PI at concentration of 100 ng/ml for 15 minutes at 4 ^{o}C. The data were acquired and analyzed using CellQuestPro (Becton Dickinson, San Jose, USA) and Summit program (Dako, CO, USA), accordingly.

2.4.9 STATISTICAL ANALYSIS

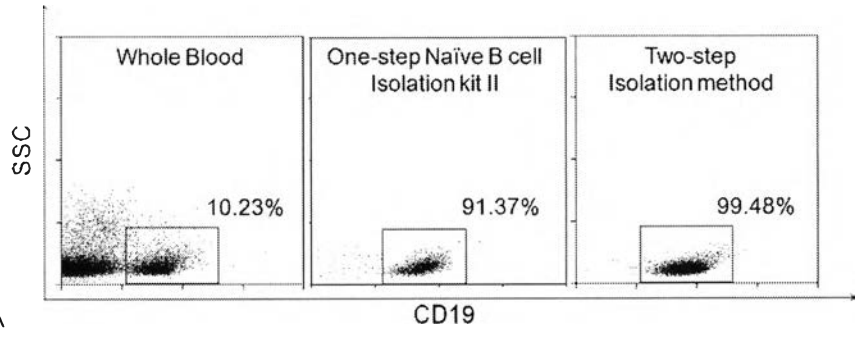
Differences among experiment groups were detected by One-way analysis of Variance (1-way ANOVA). To further detect significant differences among experimental groups, Bonferroni post hoc test was performed. Significant difference between two experiment groups was detected by Unpaired t-test. All statistic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

2.5 RESULTS

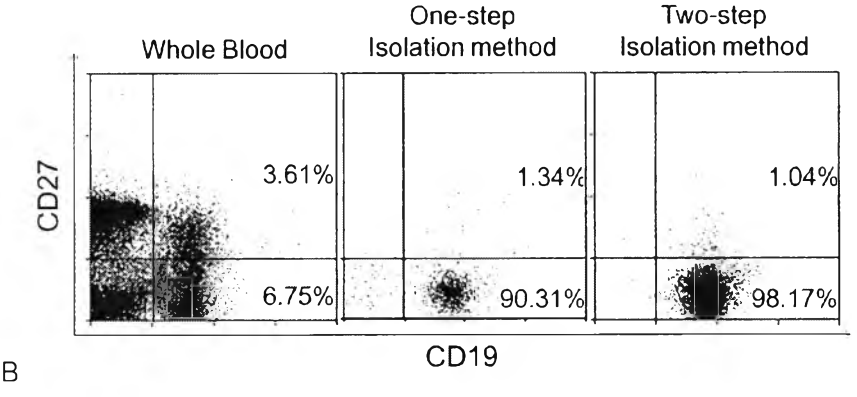
2.5.1 THE PURITY OF NAÏVE B CELLS WAS IMPROVED BY A TWO-STEP ISOLATION METHOD

Blood samples from healthy donors contained 11.8±5.6% total B cells ($CD19^{+}$cells) (Fig 5A) and 8.0±4.4% naïve B cells ($CD19^{+}CD27^{-}$cells) (n=15) (Fig 5B and 5C), accordingly. 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 material and methods. Following this method, the average B cell's purity ($CD19^{+}$cell) reached 90%

(90.6±1.2%) (Fig 5A). To improve the naïve B cell purity, we performed a two-step isolation method by the addition of rosetting B cell enrichment step (Human B lymphocyte enrichment cocktail, StemCell) prior to naïve B cell purification. Enriched B cell purity acquired after rosetting was 80-81% (n=3) (Supplementary Fig 2). After following naïve B cell isolation, a drastic improvement of B cell's purity ($CD19^+$cells) with a mean of 99% (99.1±0.5%) ($p<0.0001$) (n=10) was observed (Fig 5A), while the acquired naïve B cells ($CD19^+CD27^-$cells) purities reached 97.0±1.0% (n=10) with 2.0±2.0% contaminated memory B cells ($CD19^+CD27^+$cells) (Fig 5B and 5C). The yield determined by PBMCs' calculation was 28.2±9.7% (Table 2). Immediate cell's viability was always >95% (98.6±2.0, n=10) based on Trypan blue dye exclusion (Fig 5D). To analyze the viability of the isolated naïve B cell under resting condition 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 (Fig 5D) Thus, to avoid any viability issue, further experiments were performed with resting B cells for 18 hours.



5A



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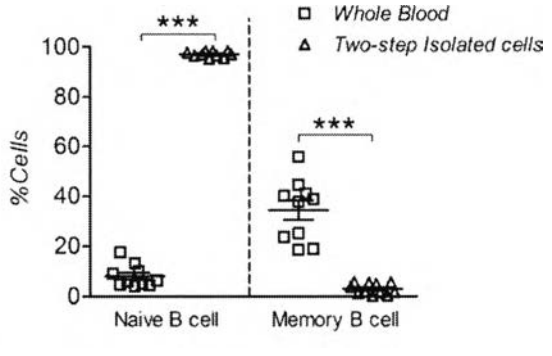

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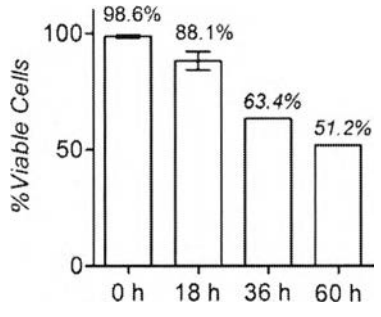


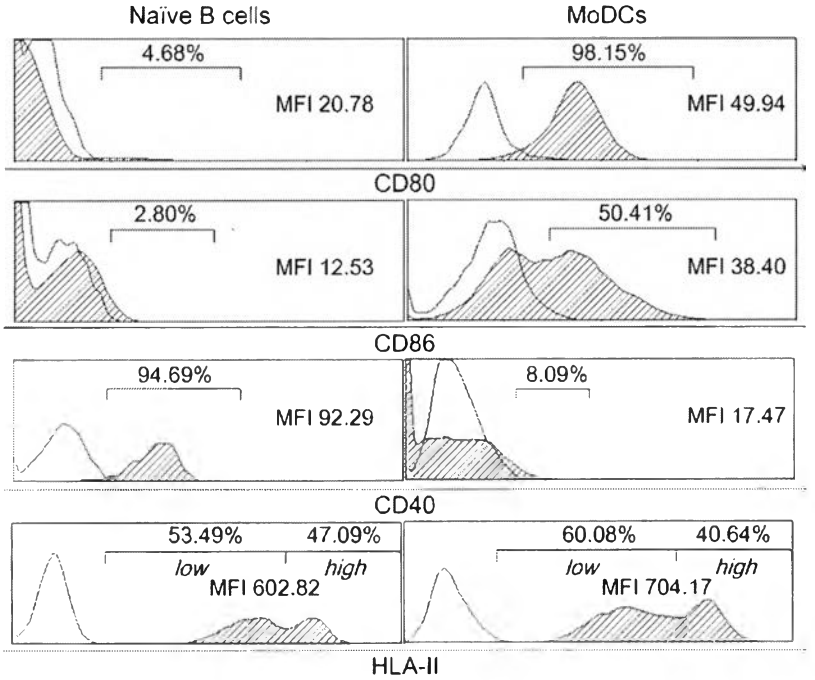
5D

Figure 5. Purities of total B cells and naïve B cells acquired from one-step Naïve B cell Isolation kit II (Miltenyi) and Two-step isolation method. (A) Representative percentages of B cells ($CD19^+$cell) determined in whole blood, cells isolated from One-step Naïve B cell Isolation kit II and those from 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 $p<0.001$.

2.5.2 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 6). CD40 was remarkably expressed on naïve B cells when compared to MoDCs (Figure 6A). In 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 6A and 6B) and expression density (p<0.01) (Figure 6A and 6C). By mean fluorescence intensities (MFIs), expression density of HLA class II on MoDCs was also higher than that of naïve B cell (p<0.05) (Figure 6C). No significative up-regulation of the co-stimulatory molecules CD69, CD80 or CD86 was observed following naïve B cell pulsation with SEB (Figure 6D and 6E).



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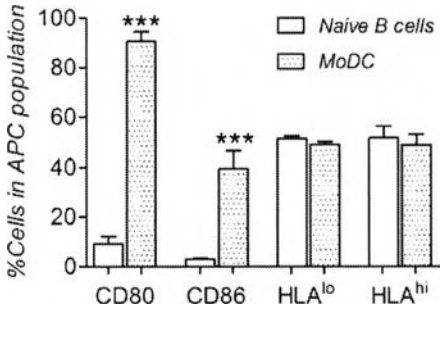

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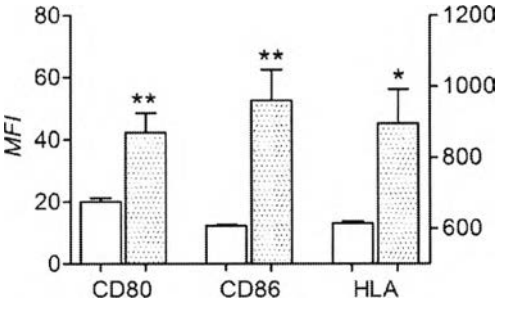

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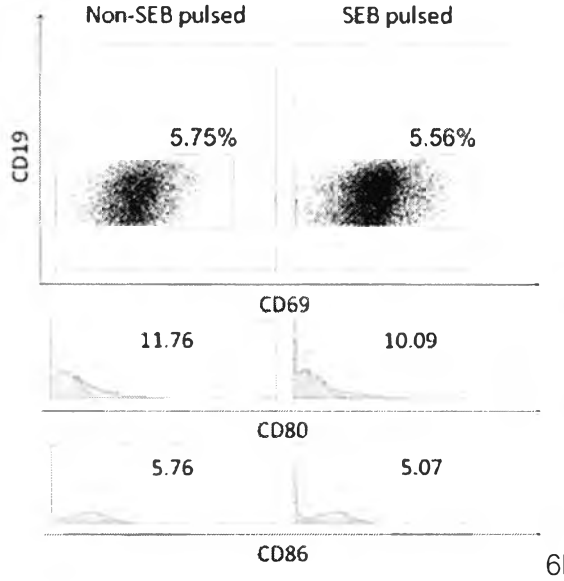

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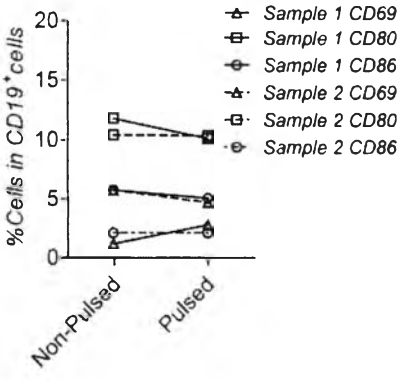
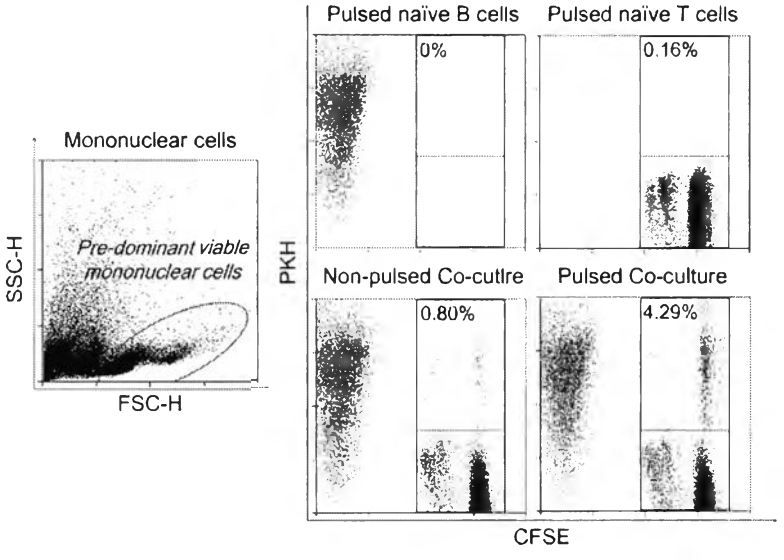


6E

Figure 6 B7 co-stimulatory molecule and HLA class II expressions on naïve B cell and monocyte derived dendritic cells (MoDCs). (A) Expressions 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 right-axis scale, while the rest were based on 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 $p<0.001$.

2.5.3 SEB PULSATION ENHANCED NAÏVE CD4$^+$T CELL'S INTERACTION WITH NAÏVE B CELLS, AND INCREASED CONSEQUENT ACTIVATION-INDUCED T-CELL PROLIFERATION.

To determine whether SEB-pulsed naïve B cells could interact with naïve T cell, a simple trogocytosis-based assay was performed. The PKH-labeled naïve B cells (PKH$^+$CFSE$^-$cells) and CFSE labeled naïve T cells ((PKH$^-$CFSE$^+$cells) were demonstrated in Figure 7A. An increase of non-healthy cells outside the gated region after labeling step should be due to toxicity from PKH. Since the presence of B and T cell's complexes in co-culture was limited (Figure 10), the presence of double positive cells (PKH$^+$CFSE$^+$cells) should represent naïve T cells previously interacted with naïve B cells and captured their membrane (Figure 7A). Due to limited numbers of acquired cells, analysis was performed only at 5 minutes, 15 minutes, 60 minutes, 1 day and 5 days after co-culturing (Figure 7B). According to ourresults, the percentage of naïve T cells that captured naïve B cells' membrane (PKH$^+$CFSE$^+$cells) were detected more in the pulsed co-culture experiment than the non-pulsed co-culture control, especially at 5-60 minutes of theco-culture period ((Figure 7 A and 7B). The fast drop of membrane capture rate at day 1 and 5 was noticeable (Figure 7B) and should be due to SEB depletion and the tremendous loss of naïve B cell viability after 1st day of culture (implied by Tryphan blue dye exclusion and Figure 5E). At day 5 of culture, CD4$^+$T cells co-cultured with SEB-pulsed naïve B cells proliferated more than non-pulsed and the direct SEB pulsed naïve T cell controls as shown by a higher shifting of CFSE intensities (Figure 7C).



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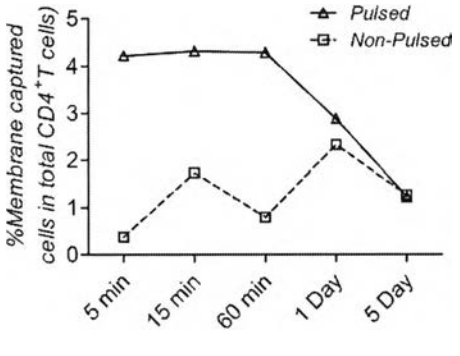

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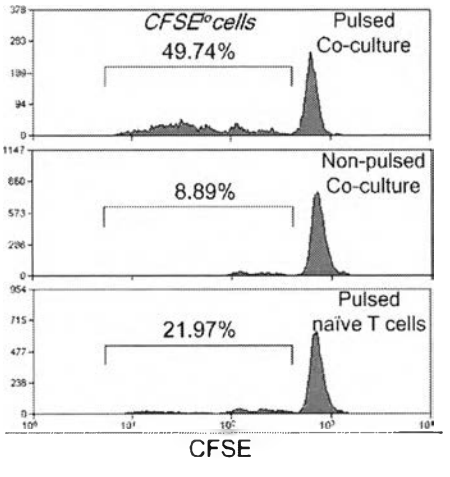
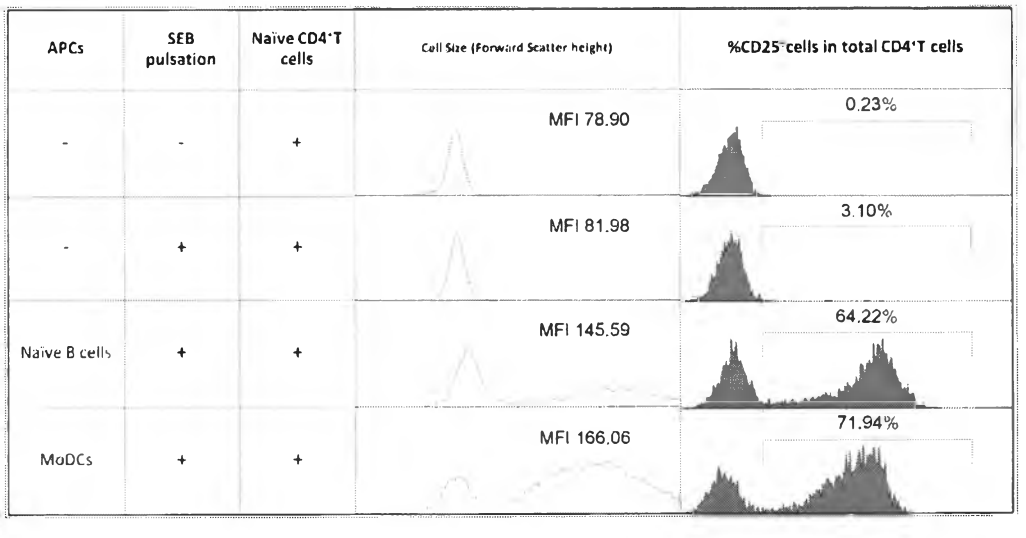


7C

Figure 7. Trogocytosis-based assay of SEB presentation by naïve B cell (A) Predominantly viable mononuclear cells of PKH^{+}SEB pulsed naïve B cells (Pulsed naïve B cells), $CFSE^{+}$SEB pulsed naïve $CD4^{+}$T cells (Pulsed naïve T cells), co-culture of $CFSE^{+}CD4^{+}$T cells with PKH^{+}non-pulsed naïve B cell control (Non-pulsed Co-culture) and with PKH^{+}SEB pulsed naïve B (Pulsed Co-culture) at 1 hour culture. Numbers of naïve $CD4^{+}$T cells that could capture naïve B cell's membrane were demonstrated by the presences of $PKH^{+}CFSE^{+}$cells. (B) Percentages of naïve $CD4^{+}$T cells capturing naïve B cell's membrane in both Non-pulsed and Pulsed Co-culture at different culture time. (C) Comparison among proliferating $CD4^{+}$T cells ($CFSE^{lo}$cells) in SEB-pulsed Co-culture, Non-pulsed Co-culture and SEB pulsed naïve T cells at day 5 of culture.

2.5.4 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 were performed. Naïve CD4^{+}T cell cultured in absence of APCs (resting naïve T cells) and SEB pulsed naïve CD4^{+}T cells were regarded as controls (Figure 8). Slight increases of T cell's 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 8A-8C) (Hewitt et al., 1992; Lee and Vitetta, 1992; Watson and Lee, 2006). Significant T cell activation were detected in the SEB-presented both by naïve B cells and MoDCs in comparison with resting and the direct SEB-pulsed naïve T cell controls as indicated by the increases of both cell size (Figure 8A and 8B) and CD25 expression (Figure 8A and 8C) (p<0.001). Nonetheless, lower degrees of T cells activation were presented when the naïve T cells were SEB-presented by naïve B cells in comparison with that by MoDCs (p<0.001) (Figure 8).



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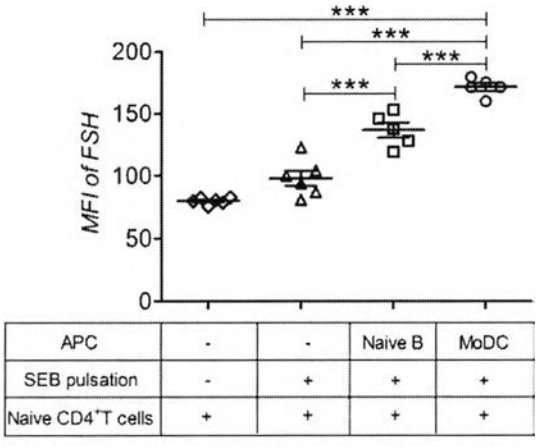

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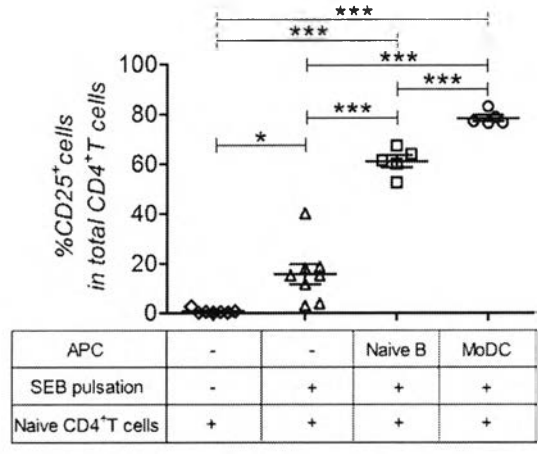

8C

Figure 8. 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 cell's number among all experiment groups (n=5). *Asterisk within the graph denoted significantly different values. * indicated $p<0.05$, ** indicated $p<0.01$, and *** indicated $p<0.001$.

2.6 DISCUSSION

To investigate *in vitro* interaction between human naïve B and naïve $CD4^+$T cells, appropriate cell isolation protocol is required to get a sufficient number and purity of naïve B cells. The present experiment has performed a simple two-step isolation method

to isolate human naïve B cell and has shown the importance of an additional step of total B cell enrichment prior to the naïve B cell negative selection by a commercial magnetic cell isolation kit (Naïve B cell Isolation kit II, Miltenyi) to improve naïve B cell's purity isolated from blood samples. Indeed, the B cell purity ($CD19^+$cells) obtained from the two-step method was 99% as compared to 90% acquired from the conventional one-step method. (Figure 5)

Although the naïve B cell isolation kit II (Miltenyi) is commonly used for the 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 deep review of studies on naïve B cell isolation using this kit (Table 1 and Table 3), typical 90% yield of purity was reached in most of the cases (Frenzel et al., ; Kaur et al., 2007; Martin et al., 2007; Pallasch et al., 2008) (Table 3), which is in agreement with our present results(Figure 5A). However, two reports from the same lab stated that a 97% purity can be achieved following the same purification method (Jiang et al., 2007; Jiang et al.). Our 2-step protocol allowed isolation of naïve human B cells with not only a higher B cell purity but also a higher reproducibility. Indeed, the purity rate reached 99.0±0.5% from 10 independent experiments (Figure 5A). Although we found no other study applying this two-step method for the naïve B cell isolation from 10-15 ml of blood sample, a similar process has been previously reported in the cell isolation from tonsils (Koelsch et al., 2007). Other well established different two-step methods for peripheral naïve B cells isolation were summarized and compared in Table 1. According to Table 1, all non-flow cytometry-based two-step methods consumed more time than conventional one-step isolation. Nevertheless, our performed protocol only slightly increased the process time around 15 minutes, while other magnetic bead based technique needed 40 min to 1 h 40 min (Table 1). Shortest duration should reduce the risk to activate and to lose healthy naïve B cells during cell isolation. Moreover, this

performed two-step method allows the isolation of naïve B cells which are maintained unlabeled by any fluorescent dyes or magnetic bead, making them suitable for further culture experiment (Table 1). The method is also convenient and may be cost-effective, since it required neither special instrument 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 - 4 0%). According to our knowledge, there is no information on the percentage yield of isolated naïve B cells reported from the available published papers. Although one might consider the yield is relatively low, sufficient number of naïve B cells has always been isolated from blood sample volume as low as 10 ml to conduct further experiments (with the need of at least 5×10^4 cells per well). Since less than 10 ml of blood can be used for the isolation and performing the *in vitro* study, this method is therefore can be applied in case when limited blood volume is an issue such as in pediatrics or some patients.

CD80 and CD86 are member of 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) (Reichardt et al., 2007a). The other important molecule, CD69 is generally regarded as early lymphocyte activation marker (Lu et al., 2009). The purified peripheral blood naïve B cells were found to be under resting state or at a very modest activation state as judged by the very low expression level of C69, low CD80 and CD86 at baseline and up to 18 hours after the isolation. Supporting other previous studies, more CD80, CD86, HLA-II but not CD40 were detected on the control MoDCs (Reichardt et al., 2007a; Watt et al., 2007) (Figure 6A-6C). In order to validate the quality of our purified naïve B cell preparations, we evaluated their capacity to function as antigen presenting cells. We thus set 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 early activation marker (CD69) or B7 co-stimulatory molecules (CD80 and CD86) on the naïve B cells (Figure 6D and 6E). This indicates that SEB pulsation in this study did not significantly activate the naïve B cells. In addition,

interaction between naïve B and T cell (Figure 7A and 7B), T cell activation (Figure 8) and T cell proliferation (Figure 7C) were clearly detected in the experiment. According to these evidences, we show that SEB pulsation could be successfully applied for the study of naïve B cells' superantigen presentation to naïve $CD4^{+}$T cells.

Table 1 Comparisons among naïve B cell purification method

Considerations	Applied commercial kits/tools for naïve B cell isolation							
	Naïve B cell Isolation kit II (Miltenyi)	B cell Enrichment cocktail (Stem Cell) & Naïve B cell Isolation kit II (Miltenyi)	B cell Isolation kit II (Miltenyi) & Naïve B cell Isolation kit II (Miltenyi)	CD19 microbeads (Miltenyi) & CD27 microbeads (Miltenyi)	Anti-CD19 microbeads (Miltenyi) & Anti-CD3 microbeads (Miltenyi) & CD27 mocrobeads (Miltenyi)	B cell Enrichment cocktail (Stem Cell) & Flow Cytometric Sorting Machine	Anti-CD19 microbeads (Miltenyi) & Flow Cytometric Sorting Machine	Human B cell enrichment kit (Stem Cell) & Flow Cytometric Sorting Machine
References	1	This study	2	3	4	5	6	7
Procedures[a]	PBMC isolation Naïve B cell isolation	Rosetting[a] B cell Enrichment Naïve B cell isolation	PBMC isolation Negative B cell isolation Naïve B cell isolation	PBMC isolation Positive CD19⁺cell isolation & bead detachment $CD27^{-/+}$ cell seperation	PBMC isolation Positive CD19⁺cell isolation & bead detachment $CD3^{+}$cell depletion $CD27^{-/+}$ cell seperation	Rosetting[a] B cell Enrichment Flow cytometric cell sorting	PBMC isolation Positive CD19⁺cell isolation & bead detachment Flow cytometric cell sorting	PBMC isolation EasySep[a] B cell enrichment Flow cytometric cell sorting
Acquired cell types	Naïve B cell	Naïve B cell	Naïve B cell	Naïve B cell Memory B cell	Naïve B cell Memory B cell	Naïve B cell Memory B cell Others	Naïve B cell Memory B cell Others	Naïve B cell Memory B cell Others
Approximate processing time	1 h 35 min	1 h 45 min	2 h 15 min	2 h 35 min	3 h 15 min	ND[b]	ND[b]	ND[b]
Requirement of naive B cell labeling	No	No	No	Yes	Yes	Yes	Yes	Yes
Requirement of sorting machine	No	No	No	No	No	Yes	Yes	Yes
Acquired B cell's purities	90% to >99%	98.56% to 99.92%	N/A	95% to >97%	N/A	>98%	98% to >99%	90% to >95%
Acquired naïve B cell's purities	90% to >98.5%	95.24% to 98.38%	>95%	85% to >95%[6]	>96%	N/A	90% to >99.99%	90% to >95%
Viability (immediate)	>95%	>95%	>95%	>95%	>95%	>95%	>95%	>95%
CD69 /$CD80^{lo}$/$CD86^{lo}$phenotype	Yes	Yes	N/A	Yes	Yes	Yes	Yes	N/A

[a] PBMC isolation = 45 min, Negative B cell isolation (Miltenyi) = 45 min, Rosetting B cell enrichment = 60 min, Naïve B cell isolation (Miltenyi) = 45 min, Positive CD19+cell isolation & bead detachment = 65 min, CD27-/+ cell seperation = 45 min, CD3 depletion = 45 min, EasySep B cell enrichment = 40-70 min

[b] Flow sorting time was not included due to variation of flow sorting machine used among studies and also the lack of total B cell yield's data prior to sorting.

References : 1-(Brown et al., 2008; Frenzel et al., ; Giordani et al., 2009; Isnardi et al., ; Jiang et al., 2007; Jiang et al., ; Kaur et al., 2007; Lu et al., 2009; Martin et al., 2007; Pallasch et al., 2008) ; 2-(Dorner et al., 2008); 3-(Bekeredjian-Ding et al., 2005; Poeck et al., 2004) ; 4-(Huggins et al., 2007) ; 5-(Herve et al., 2007; Ng et al., 2004; Weller et al., 2004; Yurasov et al., 2005); 6-(Douagi et al., 2009; Duty et al., 2009; Gujer et al.) ; 7-(Fecteau et al., 2006; Fecteau and Neron, 2003)

During $CD4^+$T cell's activation, increase of cell size and expressions of several surface molecules including IL-2 receptor α chain (CD25) were commonly demonstrated (Reichardt et al., 2007b). 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 control without APCs at 68 h of culture (Figure 8). According to previously published data, the results indicated that SEB was more effective to stimulate T cell in APC dependent way (Hewitt et al., 1992) (Figure 8C and 8D). As expected, lesser activation of T cells primed by SEB pulsed naïve B cells when compared to SEB pulsed MoDCs was also presented (Figure 8). This notice confirmed the idea that human naïve B cells represent poor APCs due to the lack of B7 co-stimulatory molecules' expressions when compared to dendritic cells (Reichardt et al., 2007a).

In summary, this study proposes a two-step non-flow cytometry approach to reproducibly isolate human peripheral naïve B cells from whole blood samples and to study their *in vitro* T-cell superantigen "SEB" presenting role to naïve $CD4^+$T cells. We have shown that adding B cell enrichment step by rosetting could consistently improve the purity of naïve B cells acquired after the subsequent magnetic cell sorting by the Naïve B cell Isolation kit II (Miltenyi Biotec). This approach has a number of advantages including consistent high cell purity with no requirement of cell labeling no requirement of expensive equipments (such as flow cytometer), and only as small as 10 ml of blood samples can be used. Consequently, this approach is particularly designed for naïve B cell isolation from limited blood volume as in the case of infectious, autoimmune, allergic or other pathological patients.

The isolated naïve B cells had resting phenotypes and were able to prime naïve T cells. The efficacy of the naïve B cells in presenting a superantigen "SEB" to naïve T cells was however lower than that of the MoDCs. To illustrate the possible unique properties naïve B cell's SEB priming effect, further characterization of naïve B cell priming naïve T cell activation in phenotypes and cytokine production was performed in the next experiment. In several autoimmune diseases (for example, SLE, type I diabetes,

EAE, etc), the role of naïve B cells as APC was merely evidenced in B cell depleted patients. Moreover, the *in vitro* Ag presentation to naïve $CD4^+$T cells by naïve B cell is still poorly characterized. Hopefully, our method will open an optional access to illustrate this issue, *in vitro*.

2.7 SUPPLEMENTARY FIGURES

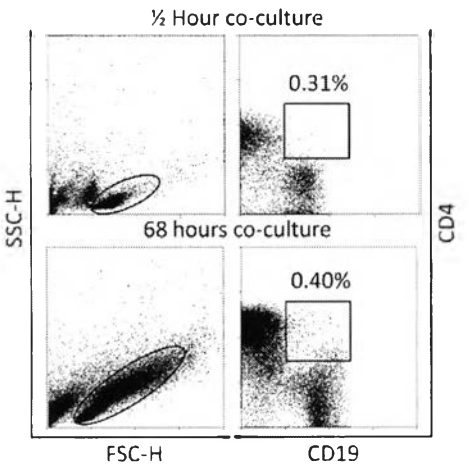

Figure 9. A representative co-culture of SEB pulsed naïve B cells and autologous naïve $CD4^+$T cells at ½ and 68 hours with determination of complexes between B and T cells ($CD3^+CD19^+$cells).

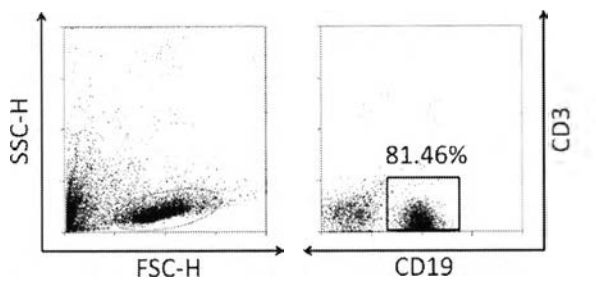

Figure 10. A representative sample of total B cell purity acquired after rosetting enrichment step.

2.8 SUPPLEMENTARY TABLES

Table 2. Naïve B cell's yield of the two-step isolation method

Sample	Naïve B cell numbers (×10^5 cells)		Yield
	[a]Total	Isolatable	
A	12.13	2.24	18.47
B	7.37	2.29	31.11
C	8.24	2.03	24.63
D	4.50	2.01	44.62

[a]Expected naïve B cell number was calculated from total blood volume used for naïve B cell isolation (15 ml) multiplying with total naïve B cell number /1 ml blood (determined in PBMCs isolated from the same sample).

Table 3. B cell purity acquired among studies applying Naïve B cell Isolation Kit II (Miltenyi)

Team	Year	Sample	Volume (ml)	Applied PBMCs for isolation (×10^7 cells)	B cell purity (%)
This study	2011	Blood	10-15	1.5-3	90
(Kaur et al.,)	2007	Blood	50	NI	90
(Pallasch et al.,)	2008	Blood	NI	NI	90
(Martin et al.,)	2007	Blood	NI	NI	90
(Frenzel et al.)	2010	Blood	NI	NI	90
(Jiang et al.)	2007, 2010	Blood	NI	NI	97
(Brown et al.,)	2008	Buffy coat	NI	60	NI
(Isnardi et al.)	2010	Buffy coat	NI	NI	95
(Lu et al.,)	2009	Buffy coat	NI	50-80	95
(Giordani et al.,)	2009	Buffy coat	NI	NI	95

NI = No information provided