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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Roles of B cells in humoral immune responses were described, while their roles in cellular immune response were more controversial (Bergmann et al., 2001; Constant et al., 1995; Di Rosa and Matzinger, 1996; Epstein et al., 1995; Fu et al., 1998; Golovkina et al., 1999; Homann et al., 1998; Joao et al., 2004; Langhorne et al., 1998; Ngo et al., 2001; Phillips et al., 1996; Shen et al., 2003). Accumulating data indicate the non-antibody-dependent effects of B cell in several T cell-mediated autoimmune diseases (Bourdette and Yadav, 2008; Cohen et al., 2006; Dorner et al., 2009; Hauser et al., 2008; Levesque, 2009; Levesque and St Clair, 2008; Liossis and Sfikakis, 2008; Pescovitz et al., 2009). In fact, B cells can either play effector or regulatory functions on T cells' cellular immune responses. To characterize B cells according to their contrast functions, the term 'effector B cell' (Be) (Harris et al., 2000) and 'regulatory B cell' (Breg) (Mizoguchi and Bhan, 2006) were introduced. "Be" cells function by enhancing and maintaining T cells' effector functions (Johansson-Lindbom et al., 2003; Menard et al., 2007; Schultze et al., 1999; Sugimoto et al., 2007; Wagner et al., 2004; Wojciechowski et al., 2009). On the contrary, Breg cells tolerize T cells' response by various mechanisms including antigen presentation (Figure 1). Direct antigen presentation by Breg cells to T cells can induce T cells' deletion, abortion of proliferation or changes in cytokine production (Bennett et al., 1998; Fuchs and Matzinger, 1992; Gilbert and Weigle, 1994; Hollsberg et al., 1996; Parekh et al., 2003; Tian et al., 2001; Townsend and Goodnow, 1998). Under proper conditions, antigen presentation by Breg cells even constituted T cells (Fiorina et al., 2008; Hu et al., 2007; Jiang and Chess, 2000; Marino et al., 2009; Noble et al., 1998b; Reichardt et al., 2007b; Saadoun et al., 2008; Sfikakis et al., 2005; Sfikakis et al., 2007; Vigna-Perez et al., 2006) with regulatory function. Recently, naïve B cells, antigen-inexperience mature B cells are considered as important Breg population, as well (reviewed in Lund and Randall).

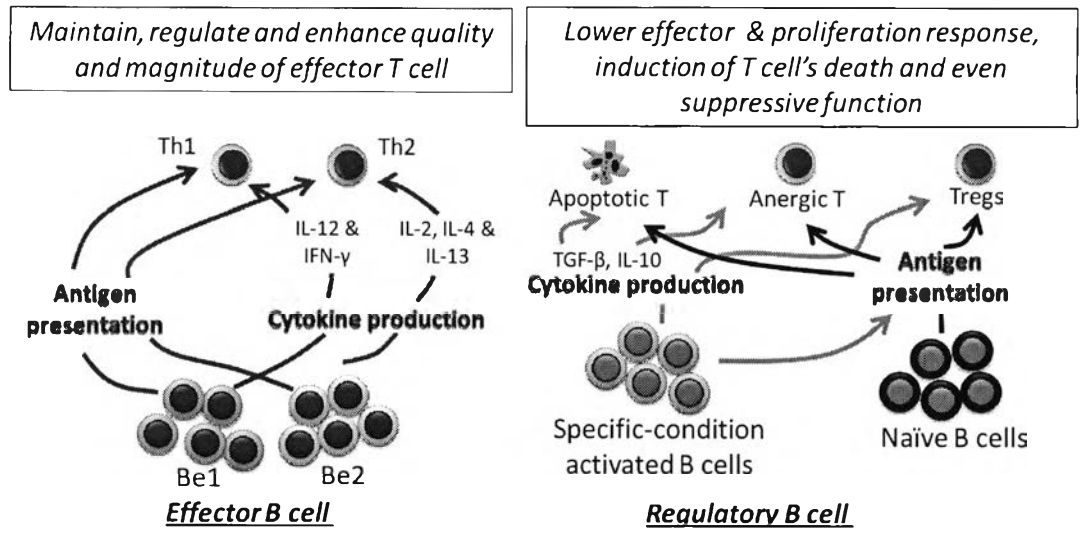

Figure 1. Effector and regulatory B cell's mode of actions: Antigen presentation and cytokine production are common mechanisms used by both effector and regulatory B cell to modulate T cell's immune responses. While effector B cells help maintaining and enhancing quality and magnitude of T helper cell type 1 (Th1) and 2 (Th2), regulatory B cell lowered both effector T cell function, cell proliferation and either induced of T cell's death or its immunosuppressive function (Ashour and Seif, 2007; Lund, 2008; Lund and Randall).

Antigen presentation is a process in which antigen presenting cells (APCs) present the antigens (Ag) to specific T cells. Interaction plane between APC and T cell formed during the process is known as Immunological synapse (IS). IS contains with several clusters of interacting molecules including MHC-TCR and other co-stimulatory molecules (Figure 2) (Friedl et al., 2005; Reichardt et al., 2007a). Of several clusters, interaction between B7 molecules (CD80 and CD86) on APCs and their ligands (CD28 and CTLA-4) on T cells can determine the outcome of antigen presentation. While B7-CD28 interaction renders positive co-signaling for T cell's activation, B7-CTLA-4 tolerizes T cell. Interestingly, CD28 and CTLA-4 prefer different types of B7 molecules. While CD28 prefers to bind with B7.1 (CD80), CTLA-4 efficiently interacts with B7.2 (CD86) (Pentcheva-Hoang et al., 2004). For this reason, professional APCs like dendritic cells (DCs) effectively prime T cells' effector functions by large amounts of B7 molecules (Sato and Fujita, 2007) with relatively high B7.2 (CD86) (Labeur et al., 1999). In contrast,

naïve B cells tolerize T cells by limited B7 co-stimulatory molecules (Eynon and Parker, 1992; Gilbert and Weigle, 1994; Kearney et al., 1995; Parker et al., 1995; Watt et al., 2007; Yuschenkoff et al., 1996) with relatively high B7.1 (CD80) (Reichardt et al., 2007a).

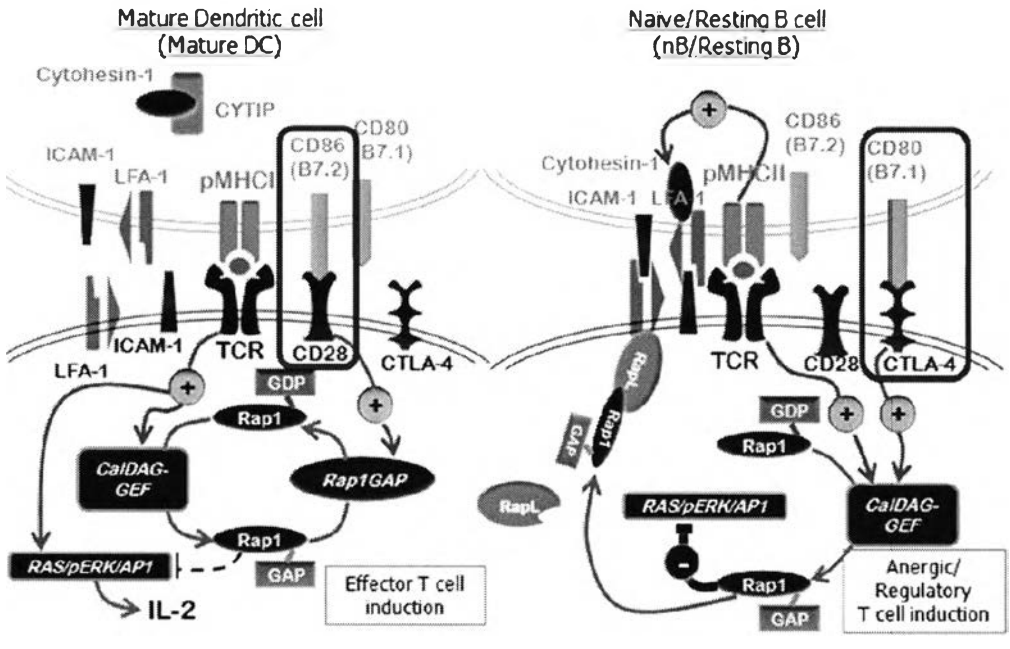

Figure 2. Differences in immunological synapse (IS) formed by mature dendritic cells (DCs) and naïve B cells: Though several interacting molecules were shared between the two synapses. Their major interacting co-stimulatory molecules presented are different. CD86 is major interacting co-stimuatory molecule on DC; while naïve B cell uses CD80. These bring about different signaling pathway via CD28 and CTLA-4 on T cell, respectively. According to this reason, DCs effectively prime effector T cell function; while naïve B cell prefers tolerization (Adapted from Reichardt et al., 2007a).

T cells tolerized by naïve B cells demonstrate unique immunoproperties. They secreted low helper-T cell type 1 (T_H1) associated cytokines (IFN-γ and IL-2), became anergy to proliferation and promoted lesser antibody production (Carpenter et al., 2009; Eynon and Parker, 1993; Gilbert and Weigle, 1994; Knoechel et al., 2005; Morris et al., 1994; Noorchashm et al., 2000; Raimondi et al., 2006a, b; Tang et al., 2009; Tsitoura et al., 2002). Interestingly, naïve B cell's antigen presentation could even differentiate $CD4^+CD25^+$regulatory T cells from naïve $CD4^+$T precursors, *ex vivo* (Reichardt et al., 2007b) and *in vivo* (Shah and Qiao, 2008; Sun et al., 2008). Evidences from Rituximab administration in T cell mediated autoimmune disease (Ahuja et al., 2007; Bouaziz et al.,

2007; Eming et al., 2008; Fiorina et al., 2008; Hamel et al., 2008; Hu et al., 2007; Marino et al., 2009; Matsushita et al., 2008; Saadoun et al., 2008; Sfikakis et al., 2005; Sfikakis et al., 2007; Stasi et al., 2007; Tamimoto et al., 2008; Vallerskog et al., 2007; Vigna-Perez et al., 2006; Xiu et al., 2008; Yanaba et al., 2007; Yu et al., 2008) support tolerization induced by naïve B cells' antigen presentation, as well. Since the drug specifically deplete mature B cells (Gong et al., 2005; Taylor and Lindorfer, 2008), it allows new repopulation of naïve B cells from non-depleted immature B cells. In accordance with T cell's tolerance induced by naïve B cells, therapeutic effects of Rituximab were demonstrated with: decrease of T cell's activation; decrease of T cell's proliferation; alteration of cytokines' productions; and increase of regulatory $CD4^{+}$T cells' numbers (Ahuja et al., 2007; Bouaziz et al., 2007; Eming et al., 2008; Fiorina et al., 2008; Hamel et al., 2008; Hu et al., 2007; Marino et al., 2009; Matsushita et al., 2008; Newell et al., ; Saadoun et al., 2008; Sfikakis et al., 2005; Sfikakis et al., 2007; Stasi et al., 2007; Tamimoto et al., 2008; Vallerskog et al., 2007; Vigna-Perez et al., 2006; Xiu et al., 2008; Yanaba et al., 2007; Yu et al., 2008).

In vitro characterization of naïve B cell's antigen presentation in human is not as simple as mouse model. These are due to two important limitations, the lack of transgenic model and the inert antigen presenting property of naïve B cells. Here, we suggest employment of bacterial superantigens (SAgs) to surpass these limitations. SAgs can bind both MHC-II molecules (Dellabona et al., 1990; Fischer et al., 1989; Fraser, 1989) and β motif of T cell receptor (TCR) (Choi et al., 1990; Fleischer and Schrezenmeier, 1988; Kappler et al., 1989; Langford et al., 1978; Peavy et al., 1970; White et al., 1989). The crosslink thus generates signals mimicking those of conventional antigen presentation. Since β motif of TCR has limited variation and can be bound with various SAgs. Up to 20% of host total T cells are expected to react with a type of SAg (comparing with only 0.001-0.01% for antigen-specific T cells). Due to this, observable T cells' responses to SAgs are comparable to those acquired from transgenic mouse models (Petersson et al., 2004). Pattern of T cells' responses are clearly demonstrated to continuous SAgs' administrations. Early polyclonal T cells' activation (Dinges et al.,

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2000; Manders, 1998; Wieneke et al., 1993), following with deletion of most activated T cells (Kawabe and Ochi, 1991; McCormack et al., 1993; Wahl et al., 1993; Webb et al., 1990; White et al., 1989) by cytotoxic T cells' function (Jiang et al., 1995; Noble et al., 1998a; Wang et al., 1998) were commonly found. The remaining reactive T cells become autonomously (Attinger et al., 2000) or environmentally tolerant to SAg stimulation (Cauley et al., 1997). The left-over tolerant $CD4^+$T cells are heterogeneous populations containing with anergic T cells, SAg specific Tregs and other $CD4^+$T cells actively suppressed by Tregs (Maeda et al., 2000; Miller et al., 1999). Of note, tolerance induction to SAg and conventional peptide antigens are very similar. For this reason, studies in SAgs' immune responses can be representatives those of common antigens (Ivars, 2007).

DCs are believed to be major APCs in immune responses to several bacterial superantigen (SAg) including Staphylococcal enterotoxin B (SEB). Not only initiating early effector functions of T cells, DCs also promote tolerance and SAg specific Tregs' development in late SAg response (Ivars, 2007). On the other hand, antigen presenting role of B cells was very controversial (Bhardwaj et al., 1993; Izcue et al., 2001; Miethke et al., 1995; Muraille et al., 1995; Stark Aroeira et al., 1997; Stohl and Elliott, 1995; Stohl et al., 1994). Participating in SAg immune responses, B cells produce SAg-specific Ig in T cell-dependent fashion (Jabara and Geha, 1996; Stohl et al., 1994). Not only demonstrating humoral immunity's role, these studies also implied us *in vivo* SAg presentation by B cells. Few evidences from early studies implied roles of B cells in SAg tolerance, as well (Florquin et al., 1996; Stark Aroeira et al., 1997). A study indicated association between B cell and persistent of TH2-type cytokines (Florquin et al., 1996) which flavored tolerance induction. The other more remarkable study demonstrated role of B cell in maintenance of anergy to SAg (Stark Aroeira et al., 1997). Though some evidences revealed us effects of SAg presentation by B cells, characterization of these B cell populations is still not broadly studied. Since immunomodulatory roles of naïve B cells are of particular interest (previously described), it's interesting to determine the cells' roles in SAg' responses, as well. Moreover, most studies about roles of B cells

were performed in mouse models (Bhardwaj et al., 1993; Izcue et al., 2001; Miethke et al., 1995; Muraille et al., 1995; Stark Aroeira et al., 1997; Stohl and Elliott, 1995; Stohl et al., 1994). We found no report about SAg presentation by human naïve B cells, and thus confirmed its value to be studied in more details.

The common sample sources to study human naïve B cells are peripheral blood, spleen and tonsil. Based on their unique characteristics, naïve B cells could be isolated by various isolation strategies. Ideally, series of surface markers (IgD, IgM, CD19, CD20, CD21, CD23, CD27, CD38, CD40, CD77, CD80 and CD86) along with detection of immunoglobulin V genes mutation will be required for detailed classification and discrimination of naïve B cells from the other B cell populations (Bohnhorst et al., 2001; Vugmeyster et al., 2004). However, only some of these markers were practically applied. Among these molecules, IgD, CD27 and CD38 ($IgD^{+}CD27^{-}/CD38^{dim}$cells) were frequently applied for peripheral, splenic and tonsilar naïve B cells (isolation (Abbas et al., 2005; Agematsu et al., 1997; Crotty et al., 2004; Frazer et al., 1997; Good et al., 2009; Klein et al., 1998; Longo et al., 2009; Pascual et al., 1994). Despite their varied isolation sites, there are some common characteristics of human naïve B cells, such as small cell size, absence of IgV mutation rate and Ig production in response to Ag.

Although several common characteristics were shared among human peripheral, splenic and tonsilar naïve B cell subsets, possible differences among the cell subsets were implied. Supporting this idea, Tonsilar naïve B were previously indicated to be more activated and expressed more recombination-activating gene (RAG) than their Per nB counterparts (Girschick et al., 2001). In addition, incomparable recirculation between peripheral and different lymphoid compartments (especially spleen when compared with other lymphoid organs) could also affect the incongruous degrees in relationship among naïve B cell subsets (Flaishon et al., 2000; Palanichamy et al., 2009). Despite these notices, variations in characteristics among human naïve B cell subsets were still limitedly studied possibly due to two major limitations: the limited number of each naïve B cell subset and the lack of candidate phenotypes/characteristics for determination. According to these, it is thus reasonable

to approach this issue with a more convenient method. An option to be considered is differential gene expression analysis which allows vast screening of genes and underlying metabolisms in a cell type of interest. Since expression data of peripheral, splenic and Tonsilar naïve B cells are available (Abbas et al., 2005; Good et al., 2009; Longo et al., 2009), differential gene expression analysis among them was thus possible.

In this study, we set a simple two-step method to improve peripheral naïve B cell's purity by the combination of rosetting process (Human B lymphocyte enrichment cocktail, StemCell) and the Naïve B cell Isolation kit II (Miltenyi). We demonstrated that the isolated naïve B cells acquire their resting normal phenotype could present the superantigen "SEB" to prime autologous naïve $CD4^+$T cells. Isolated naïve B cells expressed low MHC-II and B7 co-stimulatory molecules (CD80 and CD86), and could prime limited $CD4^+$T cell activation. The current study thus suggests tolerogenic APC role of naïve B cell similar to those illustrated in mouse models. Interestingly, differential gene expression analysis among human peripheral, splenic and tonsilar naïve B cell subsets revealed possible differences in immunoproperties between peripheral naïve B cells and those of lymphoid origins (splenic and tonsilar naïve B cells). This suggested us to be aware of generalizing knowledge acquired among naïve B cell subsets including those associated with Ag presentation.

In conclusion, the current study successfully provided us a convenient method to illustrate the role of human naïve B cell to prime naïve $CD4^+$T cell activation. In several autoimmune diseases (for example, SLE, type I diabetes, EAE, etc), the role of naïve B cell as APC was merely evidenced in B cell depleted patients and limitedly introduced in *in vitro* experiment (Lund and Randall). Hopefully, the method will open an optional access to illustrate this issue, *in vitro*. In addition, the study performed expression profile meta-analysis to determine for unknown immunoproperties' variations among human naïve B cell subsets. Generally, the lack of supporting evidence is a common obstacle to launch a sensible immunological question. The current study provided an example to apply differential gene expression meta-analysis to approach such a question. With a

similar method, we hope that other non-practical immunological studies could also be applied with the same approach.

1.2 LITERATURE REVIEWS:

Naïve B cells are characterized as mature B cells that have never been exposed to antigens. The cells can differentiate into plasma cells or memory B cells after acquiring help from helper T cells (T_H) in an antigen-dependent fashion. Murine naïve B cells are usually distinguished from memory cells according to their small size, low affinity to bind antigen and expression of IgG (McHeyzer-Williams and McHeyzer-Williams, 2005). On the contrary human naïve B cells are commonly characterized by some particular phenotypes. Human B cells can be isolated from peripheral blood (PB), tonsil or spleen with varied types and subpopulations. Early studies about human naïve B cell identified the cells by high surface IgD in absence of IgG expressions on B cells (Coffman and Cohn, 1977; Yuan et al., 1977). Among B cells residing in human tonsil, naïve B cells were primitively differentiated from memory B cells and germinal center B cells by high IgD expression in absence of CD38 (Jelinek et al., 1986; Lagresle et al., 1993; Pascual et al., 1994). The case of high IgD expression in splenic naïve B cells was also reported (Pascual et al., 1994; Zandvoort et al., 2001). These were later supported by confirming unmutated IgV region genes in IgD^{hi}cells comparing with rapidly mutated IgV genes of IgD^{-}cells (Klein et al., 1994; Kuppers et al., 1993; Pascual et al., 1994). In the early 1990s, CD27 was introduced as a new marker of human memory B cells (Agematsu et al., 1997; Crotty et al., 2004; Lagresle et al., 1993). Together with IgD expression, most $CD27^{-}IgD^{hi}$cellss representing naïve B cell population secreted very low levels of IgG in response to antigens (Agematsu et al., 1997; Maurer et al., 1992) with observable unmutated IgV genes (Klein et al., 1998; Tangye et al., 1998). According to the previously demonstrated association between the phenotypes and immunoproperties, CD27 is currently regarded as an important marker to discriminate between human naïve and memory B cell population. However, some new markers to precisely distinguish naïve B cells from other B cell populations have been constitutionally introduced. For example, ATP-binding cassette sub-family B member 1 (ABCB1) transporter protein was recently suggested as a more accurate marker to

identify human naïve B cell population (Ruprecht and Lanzavecchia, 2006; Wirths and Lanzavecchia, 2005).

Immunity to pathogens usually requires both B cells and T cells' co-operation. B cells clearly participate in humoral immune responses while T cells participate in both cellular and humoral immune responses. Inversely, the roles of B cells in cellular immune response are still controversial due to conflicting evidences indicating the importance of B cells in T cell-dependent cellular immune responses. Though some early studies showed that B cells were dispensable for the generation and maintenance of antigen-specific T cell responses (Di Rosa and Matzinger, 1996; Epstein et al., 1995; Phillips et al., 1996), the others showed contrary results (Bergmann et al., 2001; Constant et al., 1995; Homann et al., 1998; Langhorne et al., 1998; Shen et al., 2003). Moreover lacking of B cells during mouse's embryonic development can generate a number of abnormalities (Fu et al., 1998; Golovkina et al., 1999) i ncluding T cells' diversities and numbers (Joao et al., 2004; Ngo et al., 2001).

Although it is well known that the priming of naïve T cells is mainly mediated by the dendritic cells' (DCs) antigen presentation, naïve B cells are also able to initiate T cell immunity as well (Lund and Randall, ; Reichardt et al., 2007a). Unfortunately, with the exception of numerous studies using mouse, the contribution of human naïve B cells in antigen presentation to naïve $CD4^{+}$T cells were poorly characterized. This is probably due to some important limitations including low frequency of Ag-specific naïve $CD4^{+}$T cells, intrinsic high activation threshold of naïve T cell (Arstila et al., 1999; Geiger et al., 2009; Goldrath and Bevan, 1999) and the limited Ag processing/presentation properties of naïve B cells, themselves (Jiang et al.).

1.2.1 ROLE OF B CELL IN CONTROLLING T CELL MEDIATED IMMUNE RESPONSE

Various T cell's repertoires are generated in vertebrates to deal with different antigenic threats of pathogens. The diversity, though considered useful can result in autoimmunity under imbalanced immune responses (Gallegos and Bevan, 2006; Streilein, 1999). To maintain and control the homeostasis of autoimmunity, a number of

mechanisms for peripheral T cell tolerance induction exist. Recently, the roles of B cell in peripheral T cell tolerance induction are of particular interest. Emergent data indicated the benefits of B cell depletion as an effective therapy for several T cell-mediated autoimmune diseases, such as multiple sclerosis (Bourdette and Yadav, 2008; Hauser et al., 2008), type 1 diabetes (Pescovitz et al., 2009), rheumatoid arthritis (Cohen et al., 2006) and others (Dorner et al., 2009; Levesque, 2009). Furthermore, the observed clinical efficacy is unnecessarily correlated with autoantibody production (Levesque and St Clair, 2008; Liossis and Sfikakis, 2008). The antibody-independent mechanism is convinced in newly developed antibody non-secreting B cells mouse models. The overall data warrant us the important role of B cell in controlling cellular immune response.

Due to their immunomodulatory effects mentioned above, B cells play both effector and regulatory role in modulating cellular immune responses. The term effector B cell (Be) (Harris et al., 2000) and regulatory B cell (Breg) (Mizoguchi and Bhan, 2006) were then introduced to define these B cells' subsets with opposite immunomodulatory functions (Figure 1). Effector B cells (Be cells) can be further subdivided based on their functional cytokine profiles into B effector 1 cells (Be1 cells) and B effector 2 cells (Be2 cells). B effector 1 cells (Be1 cells) do not secrete considerable amounts of IL-4, IL-13 or IL-2 but can secrete IL-10, TNF and IL-6. Be1 cells are primed by T cells in the presence of antigen and/or TLR ligands in the presence of T_H1-type cytokines secrete IFNγ and the p40 subunit of IL-12 (in mice) or IFNγ and the p70 subunit of IL-12 (in humans) (Harris et al., 2005a; Harris et al., 2000). On the contrary, B effector 2 cells (Be2 cells) secrete significant amounts of IL-2, IL-4, IL-13. In some condition, Be2 cells secrete IL -10, TNF and IL-6, as well (Harris et al., 2005b; Harris et al., 2000). The cells only produce minimal amonts IFNγ and IL-12. Be2 cells are primed by T cells and antigen in the presence of T_H2-type cytokines (Harris et al., 2005b; Harris et al., 2000). Be cells can maintain, regulate and enhance the quality and magnitude of T cell mediated immune responses (Johansson-Lindbom et al., 2003; Menard et al., 2007; Schultze et al., 1999; Sugimoto et al., 2007; Wagner et al., 2004; Wojciechowski et al., 2009). Requirement of

Be cells for optimal cellular response of helper T cells (T_H cells) was evidenced *in vivo* in parasitic infestation (Menard et al., 2007) and bacterial infection model (Wojciechowski et al., 2009), as well.

In contrast with Be cells, regulatory B cells (Breg cells) are B cells with regulatory function on cellular immune response of T cells. The term was initially introduced by Mizoguchi to characterize B cell subpopulations with regulatory functions independent of immunoglobulins (Igs) secretion (Mizoguchi and Bhan, 2006). Breg cells are able to control immune responses via a number of different mechanisms in both direct and indirect ways. Direct antigen presentation by B cells to antigen specific $CD8^+$T cells can induce deletion of the cells in FAS-FASL independent fashion (Bennett et al., 1998). The mechanism was supported in abortive proliferation in presence of continuous cells' death in $CD4^+$T cells recognizing antigen presented by rare B cell population, *in vivo* (Townsend and Goodnow, 1998). Lipopolysaccharide-activated B cells (LPS-activated B cells) were reported to induce Th1 cells' apoptosis via FAS-FASL interaction and TGF-β dependent fashions (Tian et al., 2001). Apart from T cell deletion's induction, antigen presentation by B cells can constitute $CD8^+$T cells with unresponsiveness, and then subsequently anergy (Fuchs and Matzinger, 1992; Hollsberg et al., 1996; Parekh et al., 2003). Reduction of $CD4^+$T cell proliferation's capacity was reported in $CD4^+T_H$ cells undergoing antigen presentation by $F(ab')_2$ goat anti-mouse IgM treated resting B cells (Gilbert and Weigle, 1994) and LPS-activated B cells (Tian et al., 2001). Another remarkable mechanism of Breg cells is enhancement of T cells' suppressive function. Generation of $CD8^+$T cells with inhibitory function on T_H2 cells ($CD8^+$T suppressor cells) can be achieved by antigen presentation of activated B cells. The process depended on either classical or non-classical MHC-I molecules (Jiang and Chess, 2000; Noble et al., 1998b). Interestingly, numbers of reports demonstrated effects of B cells contributing to generation or mediation of regulatory $CD4^+$T cells both *in vivo* (Fiorina et al., 2008; Hu et al., 2007; Marino et al., 2009; Saadoun et al., 2008; Sfikakis et al., 2005; Sfikakis et al., 2007; Vigna-Perez et al., 2006) and *in vitro* (Reichardt et al., 2007b).

Phenotypes of B cells with suppressive functions are varied among studies. Surprisingly, both resting and activated B cells can demonstrate regulatory functions. According to mouse models, two well-characterized Breg populations are of particular interest, the IL-10 producing B cells (B10 cells) (Yanaba et al., 2008) and the resting/naïve B cells. IL-10 producing B cells (B10 cells) are B cells that primarily produce IL-10 following stimulation with combinations of antigen, CD40L and TLR ligands. B10 cells are present in both B1 and B2 lineage. The cells with different phenotypes can prevent autoimmune diseases in several mouse models (Bouaziz et al., 2008; Mauri and Ehrenstein, 2008). In human, possible B10 population was recently characterized (Noh et al.). Another well recognized Breg subpopulations in mouse models are resting or naïve B cells. Naïve B cells are defined as virgin mature B cells that never expose to Ag. Considered as poor antigen presenting cells (APCs), naïve B cells are dispensable for priming of $CD4^{+}$T cells' effector functions (Epstein et al., 1995; Fuchs and Matzinger, 1992). Interestingly, antigen presentation by naïve B cells has more important role in priming T cells' tolerance (Lund and Randall). The tolerance induction property of the cells is due to their unique characteristic of antigen presentation which will be described further in details.

In summary, activated effector B cells enhance effector T cell response and simultaneously provide unflavored environment (inflammatory milieu) for regulatory or tolerized T cell expansion (Shah and Qiao, 2008). On the contrary, naïve or appropriately activated B cells facilitate tolerance and development/expansion of Treg (Chen et al., 2009; Gros et al., 2008; Shah and Qiao, 2008; Sun et al., 2008; Tu et al., 2008). Together with other professional APCs such as dendritic cells (DCs), interactions among these cells are crucial in homeostasis of body's immune response (Figure 3). For this reason, abnormalities or distortions of the cells' interactions can be the causes of pathogenic conditions.

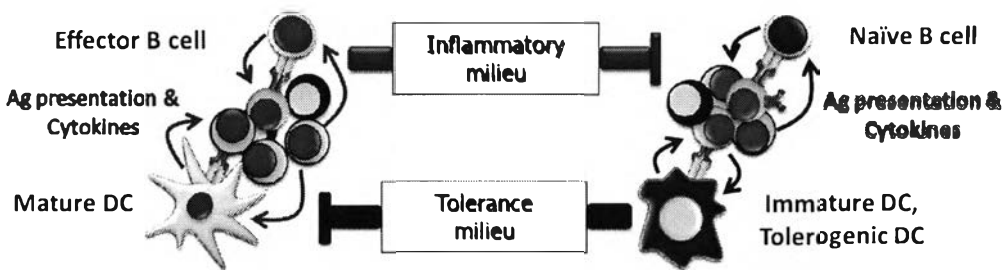

Figure 3. Schematic figure representing the control of adaptive immune response by DC and B cell Ag presentation. Ag presentation and cytokine production by effector B cell and mature DC to recognize naïve $CD4^{+}$T cell results in inflammatory milieu, while those by immature DC, tolerogenic DC and naïve B cell results in tolerance milieu. The balance between these two milieus helps controlling the outcome of immune response (Lund and Randall, ; Mahnke et al., 2007).

1.2.2 ANTIGEN PRESENTATION BY DENDRITIC CELLS AND B CELLS

Antigen presentation is a process in which antigen presenting cells (APCs) presenting the antigens (Ags) and then enable them to be recognized by antigen specific T cells. Antigen presentation is fundamental process to initiate cellular immune responses of T cells and requires physical interaction between T cells and APCs. At single-cell level, the T cell-APC interaction consists of three different phrases: contact acquisition that initiates the approximation of the cells; immunological synapse (IS) formation which is the formation of interaction plane; and, detachment of the cells that allows continuation of T cells' migration. Establishment and achievement of these three physical states have impacts on contact duration, engagement of interacting molecules and downstream signaling (Friedl et al., 2005). Several factors can affect T cell-APC interactions including type and activation status of APCs (Friedl et al., 2005; Reichardt et al., 2007a).

Dendritic cells are professional APCs capable priming effector function for T cells. The cells express various families of molecules on their cells' surfaces responsible for capturing Ags, presenting Ags and co-stimulating recognizing T cells (Sato and Fujita, 2007). During immunological synapse (IS) formation, interaction of co-stimulatory molecules on APCs and CD28 on recognizing T cells is important co-signaling to

complete T cells' activation. Unlike dendritic cells (DCs) which show significant levels of immunogenic co-stimulatory molecules on the cell surfaces, naïve B cells express limited levels of them (Eynon and Parker, 1992; Gilbert and Weigle, 1994; Kearney et al., 1995; Parker et al., 1995; Watt et al., 2007; Yuschenkoff et al., 1996). In these models, tolerance induction by naïve B cells fitted with 'Danger model' previously suggested by Matzinger in 2002 (Matzinger, 2002). According to the model, immunogenic APCs increase expression of co-stimulatory molecules in response to danger signals and thus, are capable of activating T cells (Matzinger, 2002). In absence of danger signals, co-stimulatory molecules are not up-regulated on APCs. Under this circumstance the responding T cells are prone to become tolerized not activated (Matzinger, 2002). Tolerance induction by naïve B cell's antigen presentation is suggested as the consequence of its unique pattern of immunological synapse (IS) formation by Reichardt in 2007 (Reichardt et al., 2007a). To form IS at single-cell level, multiple receptors and adhesion molecules are assembled on both APC and Ag recognizing T cell. IS consists with clusters of interacting molecules that are well or untidily segregated according to the type of T cell-APC interactions (Friedl et al., 2005). Some interacting adhesion and co-stimulatory molecules are normally demonstrated in several studies models. During IS formation, $CD4^{+}$T cells use T cell receptors (TCRs) to recognize antigen presented in groove of major histocompatibility class II (MHC-II) of APC. LFA-1 and ICAM-1 on both APC and T cells interacted with each other as surplus common adhesion molecules. B7 co-stimulatory molecules (CD80 and CD86) on APC interacted with CD28 to transmit positive signals for TCR signaling proteins. On the contrary, interaction of B7 molecules with CTLA-4 on T cells generates negative signaling for T cell's activation. Previous study of co-stimulatory signaling indicated different preferences of CD28 and CTLA-4 for B7 molecules. CD28 prefers to bind with B7.1 (CD80), while CTLA-4 chooses to interact with B7.2 (CD86) more efficiently (Pentcheva-Hoang et al., 2004). Comparing with naïve B cells, mature DCs express very high amounts of B7 co-stimulatory molecules, especially B7.2 (CD86) (Labeur et al., 1999). This allows powerful triggers of CD28 to enhance proximal signaling of TCR generating full T cell's activation. On the other hand,

naïve B cells express very low levels of B7 molecules. Moreover the relative amount of CD80 is higher than CD86. Thus, CTLA-4 is relatively more advantage over CD28 to trigger T cell and transmit negative regulatory signal to T cell (Figure 2).

1.2.3 ROLE OF NAÏVE B CELLS IN T-CELL TOLERANCE INDUCTION

Responding T cells tolerized by naïve B cells demonstrate unique immunoproperties. In response to Ag restimulation, tolerized T cells secrete low levels of T_H1 associated cytokines (IFN-γ and IL-2), become anergy to proliferation and promote lesser antibody production (Eynon and Parker, 1993; Gilbert and Weigle, 1994; Knoechel et al., 2005; Morris et al., 1994; Noorchashm et al., 2000; Raimondi et al., 2006a, b; Tang et al., 2009; Tsitoura et al., 2002). Interestingly, naïve B cell's antigen presentation was reported to induce or promote differentiation of $CD4^+CD25^+$ regulatory T cell both *ex vivo* (Reichardt et al., 2007b) and *in vivo* (Shah and Qiao, 2008; Sun et al., 2008). Several B cell depletion studies and clinical trial by administration of Rituximab (Rituxan/MabThera; Genentech/Roche/Biogen Idec) in both mouse (Ahuja et al., 2007; Bouaziz et al., 2007; Fiorina et al., 2008; Hamel et al., 2008; Hu et al., 2007; Marino et al., 2009; Matsushita et al., 2008; Xiu et al., 2008; Yanaba et al., 2007; Yu et al., 2008) and human models (Eming et al., 2008; Saadoun et al., 2008; Sfikakis et al., 2005; Sfikakis et al., 2007; Stasi et al., 2007; Tamimoto et al., 2008; Vallerskog et al., 2007; Vigna-Perez et al., 2006) suggest the T cell's tolerance induction, as well. Rituximab is mouse-human chimeric antibody specific for CD20 presented on mature B cells. Cross-linking of CD20 and Rituximab induce B cell depletion by antibody-dependent cell cytotoxicity and complement-dependent toxicity (Gong et al., 2005; Taylor and Lindorfer, 2008), and thus allowing new naïve B cells developed from left-over immature B cells to repopulate. The therapeutic effects of Rituximab were presented with: changes in T cell's activation; T cell's proliferation; decrease or altered ratio of T_H1/T_H2 associated cytokines ,and increase of regulatory $CD4^+$T cells. More evidences of naïve B cell tolerizing $CD4^+$T cells by antigen presentation were presented in human model. In alloantigen presentation, direct human naïve B cells stimulated low $CD4^+$T cells'

proliferation and immunogenic cytokine production (Carpenter et al., 2009) suggesting their fate to become tolerance (Matzinger, 2002). A recent cohort study in renal transplant patients indicated the relation between elevated peripheral blood naïve B cells and successful transplant tolerance (Newell et al.).

1.2.4 NAÏVE B CELL ISOLATION METHODS

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 (Rajewsky, 1996). 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) [2, 3]. However, the use of IgD for the naïve B cells' positive selection has a major disadvantage, as the engagement of the molecule could modulate naïve B cell's properties (Jabara et al., 2008; Pecanha et al., 1993). In the early 1990s, as CD27 was discovered as a universal marker of human memory B cells ($CD27^+$B cells) (Agematsu et al., 1997; Crotty et al., 2004; Klein et al., 1998), such discovery thus allowed negative isolation of naïve B cells ($CD27^-$B cells) from memory B cells ($CD27^+$B cells), and are now commonly applied in most commercial isolation kit and flow cytometric sorting performance.

Peripheral blood is a convenient sample source to study human immune cells, including naïve B cell. An example of commercial kit generally applied for human naïve B cell isolation is the Naïve B cell Isolation kit II (Miltenyi). The kit removes non-$CD19^+$ and $CD27^+$cells by specific antibodies' cocktail and microbead labeling allowing negative naïve B cell isolation directly from peripheral blood mononuclear cells (PBMCs) in one-step cell depletion. Numerous studies exclusively followed this procedure for naïve B cell isolation (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). However, various other combining methods were also applied to achieve the isolation of such cells for different purposes. Typically, these combined

methods consisted of two major isolation steps, the total B cell pre-enrichment and the subsequent naïve B cell isolation (Carpenter et al., 2009; Dorner et al., 2008; Douagi et al., 2009; Fecteau et al., 2006; Fecteau and Neron, 2003; Gujer et al., ; Herve et al., 2007; Huggins et al., 2007; Jahrsdorfer et al., 2005; Jiang et al., 2007; Klein et al., 1998; Lu et al., 2009; Ng et al., 2004; Poeck et al., 2004; Quach et al., ; Weller et al., 2004; Wulff et al., 2004; Yurasov et al., 2005). Enrichment of B cell could be achieved either through immunomagnetic beads (MACS®, Dynabeads® and StemSep®) or rosetting with red blood cells (RBCs) (RosetteSep®) (Herve et al., 2007; Ng et al., 2004; Quach et al., ; Weller et al., 2004; Wulff et al., 2004; Yurasov et al., 2005). Although the use of magnetic beads is claimed to provide highly enriched B cell's purity ($CD19^+$cells) of around 95-99%, the technique required PBMC isolation prior to enrichment step which extended the duration of the process and, consequently, increase the level of mortality. Flow cytometric cell-sorting of enriched total B cells theoretically should provide superior cell purity and flexibility to simultaneously sort many cell populations of interest than magnetic cell sorting. However the technique is not suitable as a routine method and not convenient to isolate simultaneously cells from several samples. Moreover, the technique requires adequate cell markers' labeling, expensive sorting machine and also required expertise.

1.2.5 THE REPRESENTATION OF IGD^+CD27^-B CELL AS HUMAN NAÏVE B CELL POPULATION

Recent studies have revealed that circulating human IgD^+CD27^-B cells were actually comprised of heterogeneous B cell populations: Transitional B cells (Bofill et al., 1985; Campana et al., 1985; Palanichamy et al., 2009; van Zelm et al., 2007), intermediate B cells (Lee et al., 2009) and absolute naïve B cells. Transitional B cells are developing B cells that will undergo 2^{nd} negative selection at spleen in order to become mature for follicular or marginal zone compartment (Chung et al., 2003). On the other hand, intermediate B cells are identified as circulating B cell populations that represent pre-stage cells prior to complete differentiation into mature naïve B cells (Lee et al., 2009). Though all cell types have IgD(+)CD27(-) phenotypes identical to those of mature

naïve B cells, variations in expression levels of CD5, CD9, CD10 and CD38 among the cells are still noticeable (Lee et al., 2009; Palanichamy et al., 2009). Transitional B cells have CD5(+)CD9(hi)CD10(hi)CD38(hi) phenotypes discriminating them from intermediate B cells which are CD5(+)CD9(med)CD10(med)CD38(med). The varied expression levels of these phenotypes make both cell types unique from mature naïve B cells, since normal mature naïve B cells show CD5(-)CD9(lo)CD10(-)CD38(lo) phenotypes.

Each peripheral $CD27^-$B cell population not only constituted itself with unique phenotype but also their immunoproperties. The unique immunoproperties of each $CD27^-$B cell population are generally correlated with the cell's nature. For example, transitional B cell represents an important stage of autoreactive B cell selection checkpoint (Carsetti et al., 1995; Chung et al., 2003). In agreement with this, the transitional B cell was known to undergo strong spontaneous apoptosis upon BCR stimulation and CD40 ligation (Lee et al., 2009; Su and Rawlings, 2002). On the contrary, intermediate B cells were identified as pre-naïve B cells (Lee et al., 2009). With the same BCR stimulation, intermediate B cells were shown to acquire naïve B cell phenotype. Moreover, intermediate B cells only partially responded to the stimulation and underwent more spontaneous apoptosis than do naive B cells (Lee et al., 2009). Another remarkable difference between intermediate and mature naïve B cell was the response to BAFF/BLyS (B cell-activating factor belonging to the TNF family) which could enhance only naive but not intermediate B cell survival (Lee et al., 2009).

In several human studies, peripheral naïve B cells were isolated by their absence of CD27 expressions (Brown et al., 2008; Carpenter et al., 2009; Dorner et al., 2008; Douagi et al., 2009; Fecteau et al., 2006; Fecteau and Neron, 2003; Frenzel et al., ; Giordani et al., 2009; Gujer et al., ; Herve et al., 2007; Huggins et al., 2007; Isnardi et al., ; Jahrsdorfer et al., 2005; Jiang et al., 2007; Jiang et al., ; Kaur et al., 2007; Klein et al., 1998; Lu et al., 2009; Martin et al., 2007; Ng et al., 2004; Pallasch et al., 2008; Poeck et al., 2004; Quach et al., ; Weller et al., 2004; Wulff et al., 2004; Yurasov et al., 2005). Based on this idea, the isolated naïve B cells were generally referred to $CD27^-$B cells

regardless of their heterogeneous populations. Based on characterization evidences, around 85% of isolated $CD27^-$ B cells were mature naïve B cells while the rest were respectively contaminated intermediate (13%) and transitional (2%) B cells.

1.2.6 HUMAN NAÏVE B CELL SUBSETS AND THEIR POSSIBLE DIFFERENCES IN PHENOTYPES AND IMMUNOPROPERTIES

The common sample sources to study human naïve B cells are peripheral blood, spleen and tonsil. Based on their unique characteristics, nB cells could be isolated by various isolation strategies. Ideally, series of surface markers (IgD, IgM, CD19, CD20, CD21, CD23, CD27, CD38, CD40, CD77, CD80 and CD86) along with detection of immunoglobulin V genes mutation will be required for detailed classification and discrimination of naïve B cells from the other B cell populations (Bohnhorst et al., 2001; Vugmeyster et al., 2004). However, only some of these markers were practically applied. Among these molecules, IgD, CD27 and CD38 ($IgD^+CD27^-/CD38^{dim}$cells) were frequently applied for peripheral, splenic and tonsilar naïve B cells (isolation (Abbas et al., 2005; Agematsu et al., 1997; Crotty et al., 2004; Frazer et al., 1997; Good et al., 2009; Klein et al., 1998; Longo et al., 2009; Pascual et al., 1994). Despite their varied isolation sites, there are some common characteristics of human naïve B cells, such as small cell size, absence of IgV mutation rate and Ig production in response to Ag.

Though several common characteristics were shared among naïve B cell subsets (peripheral, splenic and tonsilar), some possible variations were also implied. During B cell development, immature B cells formed in bone marrow emigrate and home majorly to spleen where they can mature and become naïve B cells in follicles or marginal zone (MZ) B cells in MZ area of spleen (Pillai and Cariappa, 2009; Thomas et al., 2006). Mature naïve B cells normally recirculate from spleen throughout the body entering/existing lymphoid organs where they could encounter antigen (Ag) and further differentiate into Ag specific memory B cells or plasma cells (Hardy et al., 1983; Waldschmidt et al., 1988). Several cellular factors required for further differentiation of naïve B cells were regionally provided only in lymphoid niches, but not peripheral blood (Pillai and Cariappa, 2009; Thomas et al., 2006). This unequivalence in niches should

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thus affect lymphoid nB, and likely to modulate their intrinsic properties which were absence in peripheral naïve B cells. Supporting this idea, Tonsilar nB were indicated to be more activated and expressed more recombination-activating gene (RAG) than their Per nB counterparts (Girschick et al., 2001). Additionally, incongruous degrees in relationship between peripheraland splenic naïve B cells when compared to those of other lymphoid origins were also implied. It's well established that most matured peripheral naïve B cells were actually the recirculating splenic naïve B cells (Flaishon et al., 2000; Palanichamy et al., 2009), and thus implied close relationship between the cells. On the contrary, human tonsil was not a major site for B cell maturation with limited naïve B cells' recirculation (Palanichamy et al., 2009). This implied more its more distant relationship with peripheral naïve B cells and probably higher degrees of different characteristics, as well.

Since plausible variation in some characteristics among human nB subsets was not well-established, their contributions to different immune responses observed among nB subsets were limitedly known. An investigation into nB's characteristics among nB subsets was thus necessary to clarify this notion. However, the direct approach to the comparison should face with several limitations. Accessibility to all nB sources (peripheral blood, spleen and tonsil) must meet with ethical approvals and extensive isolation of the cells also consumed considerable effort and expense. Though isolation of small nB cells' numbers from each source should be the solution of choice, this consequently allowed limited cell's characteristics able to be observed. According to this reason, it's important to perform a sensible screening method to find out these candidate characteristics.

Gene expression microarray technology is a measurement of numerous genes' expressions in a single array containing multiple probes specific to each gene transcript. The tool is very useful to screen genes and underlying metabolisms specific to cell type or a provided perturbation. Gene expression microarray was widely applied in several human B cell studies and therefore provided us several expression data of B cell population including those of Per nB, Splenic nB and Tonsilar nB (Abbas et al.,

2005; Good et al., 2009; Longo et al., 2009). By suitable analysis on the data, varied characteristics among human naïve B cell subsets based on their differential gene expressions could be screened. The comparisons not only provided us list of candidate phenotypes varied among nB subsets, but also helped predicting their possible variations in cell properties by gene categorization and performance of biological term enrichment (Huang da et al., 2009a, b).

1.2.7 LIMITATION OF HUMAN NAÏVE B CELLS' ANTIGEN PRESENTATION STUDIES

Human naïve B cell isolation can be achieved by exploiting expression of IgD, CD27 and ABCB1. Nevertheless, the currently available isolation systems used have their own limitations. For example, surface IgD labeling for cell isolation is detrimental for further culture or stimulation, since engaging of antibodies to B cell receptors (BCR) can subsequently change the B cells' immunoproperties. With the same limitation, positive naïve B cell isolations of naïve B cells with other markers are not recommended for cell culture studies, as well. As far as we can reach, current commercial product available for human naïve B cell's negative isolation is $CD19^{+}CD27^{-}$cells isolation kit. According to our preliminary study, the purities of naïve B cells solely isolated by the kit were not consistent among samples and most found contaminated cells are those of $CD3^{+}$cell (T cell) populations.

Characterization of human naïve B cell's specific antigen presentation is not convenient. The main limitations are due to the lack of transgenic model and inert antigen presentation's properties of naïve B cells, themselves. Unlike transgenic mouse models of which all body's naïve T cells are supposed to recognize specific antigen, only 0.001-0.0001% of total human's body naïve T cells are supposed to show specificity to a newly introduced antigen. To prime expansion of detectable numbers of activated T cells from naïve T cells in short-termed (2-3 days), none of common antigens is capable. Long termed stimulation with specific antigen pulsed naïve B cells to expand adequate numbers of cells for analysis is possible. However the process is not practical as it seems. Since peripheral blood contains only 5-17% of naïve B cells in total

mononuclear cells (unpublished data), it consumes large amounts of time and expenses to isolate sufficient autologous naïve B cells for repeated stimulation.

An optional solution for this might be the employment of MHC-II-dependent T cell superantigens. Superantigens (SAgs) are microbial or viral toxins capable of binding MHC-II molecules (Dellabona et al., 1990; Fischer et al., 1989; Fraser, 1989) and TCR, simultaneously (Choi et al., 1990; Kappler et al., 1989; White et al., 1989). The toxins are extreme antigens stimulating polyclonal activation and expansion of T cells. In contrast to conventional antigens, SAgs are not processed and presented as short peptides by APCs. To stimulate T cells, these superantigens directly bind the MHC-II molecules outside the groove (Dellabona et al., 1990; Fischer et al., 1989; Fraser, 1989) then the TCRs (Choi et al., 1990; Kappler et al., 1989; White et al., 1989). The cross-links between the molecules are able to generate the signals mimicking those of conventional antigen presentation, regardless of TCR specificity (Fleischer and Schrezenmeier, 1988; Langford et al., 1978; Peavy et al., 1970). The specificities of SAgs to T cells depend on their ability to bind variable β motif of TCR. Since β motif of TCR has limited variation and one type of SAg can bind with more than one type of TCR's β motif. Normally, up to 20% of host total T cells can react with one type of SAg (reviewed in Petersson et al., 2004). Common bacterial SAgs used in studies are those produced by *Staphylococcus aureus* and *Streptococcus pyogenes*. *Staphylococcus aureus* can produce more than 10 SAgs known as Staphyloccal enterotoxins (SEs) A, A, B, C1-3, D, E, G, H, I, J, K, L, M and toxic shock syndrome toxin-1 (TSST-1). SAgs produced by *Streptococcus pyogenes* are streptococcal pyrogenic exotoxins (SPEs) A1-4, C, G, H, I, J, L and M, the streptococcal mitogenic exotoxins (SMEZ) 2, 3. . .24 and the streptococcal SAG (SSA). Classification of bacterial SAgs' ability to bind MHC-II and TCR reveal some unique characteristics among these toxins. Crystal structure as well as mutational studies of SAgs reveal that some SAgs (SEB, SEC, SEG, SSA, SPE-A and TSST-1) exclusively bind α-chain of MHC-II molecules with low binding affinity (Hartwig et al., 1994; Jarraud et al., 2001; Papageorgiou et al., 1995; Papageorgiou et al., 1999; Sundberg and Jardetzky, 1999; Sundberg et al., 2002). In addition to α-chain binding, some SAgs (SPE-G, SPE-H, SPE-I

SPE-J, SPE-L, SPE-M, SMEZ and SMEZ-2) (Li et al., 1997; Li et al., 2001; Petersson et al., 2001; Proft et al., 1999; Proft et al., 2003; Zhang et al., 1998) can bind to β-chain of MHC-II, as well. The binding of SAg to α and β-chain of MHC-II on the same APC can cross-link 2 adjacent MHC-II molecules together. In case that the toxin also binds with TCR, this can generate the trimolecular complex of 2MHC-TCR molecules (reviewed in Petersson et al., 2004) and should be avoided in exploiting SAg to represent common antigen presentation.

Presence of SAgs in host body causes massive proliferation and activation of T cells. In response to SAg, activated polyclonal T cell secrete large amounts of the cytokines interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). Non-specific polyclonal activation of T cells can cause lethal diseases, such as poisoning and toxic shock (Dinges et al., 2000; Manders, 1998; Wieneke et al., 1993). Moreover, disruption of self-tolerance by SAg is involved in pathogenesis of autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis (Cole and Griffiths, 1993; Schiffenbauer et al., 1993). Though the benefits of SAgs to producing microbes are not clearly clarified, local inflammation promoted by the toxins might provide more blood and nutrient supply for the microbes (Marrack and Kappler, 1990). In contrast to acute exposure response, prolonged exposure to the toxin increases cell's deletion and anergic induction of activated T cells. The state of unresponsiveness to SAg's re-stimulation is defined as SAg tolerance. Interestingly, numbers of accumulating data indicated that expansion of specific regulatory T cell subpopulations are deeply involved with SAg tolerance induction (Ivars, 2007).

1.2.8 SUPERANTIGEN TOLERANCE INDUCTION AND ROLE OF NAÏVE B CELL

Majority of the activated T cells expanded in response of SAg are deleted in prolong exposure to SAgs (Kawabe and Ochi, 1991; McCormack et al., 1993; Wahl et al., 1993; Webb et al., 1990; White et al., 1989). Deletion of $CD4^{+}$T cells induced in Fas/FasL dependent fashion by $CD8^{+}$T cells' functions (Jiang et al., 1995; Noble et al., 1998a; Wang et al., 1998). After extensive deletion in several mouse studies, the

remaining SAg-reactive cells are anergic and tolerant to SAg exposure. These cells decrease their responses to IL-2 (McCormack et al., 1993; Miethke et al., 1994; Sundstedt et al., 1994; Sundstedt et al., 1997). SAg-induced anergy was either autonomous (Attinger et al., 2000) or depended on other non-T cells (Cauley et al., 1997). Interestingly, evidences from many studies indicate role of regulatory T cells (Tregs) in SAg tolerance induction, as well. Left-over $CD4^+$T cells from deletion phrase are heterogeneous population containing with anergic T cells, Tregs and other $CD4^+$T cells actively suppressed by Tregs (Maeda et al., 2000; Miller et al., 1999). SAg-specific Tregs are extensively characterized in mouse model. Both $CD4^+CD25^-$ and $CD4^+CD25^+$Tregs are generated in SAg-induced responses. SAg-specific $CD4^+CD25^-$ Tregs suppress immune response by secreting IL-10 and TGF-β (Feunou et al., 2003; Grundstrom et al., 2003; Grundstrom et al., 2000; Miller et al., 1999). To induce tolerance against repeated administrated SAgs, *in vivo* presence of $CD4^+CD25^+$T cells (natural Tregs) were need, as well (Feunou et al., 2003). Dominant anergic T cells, $CD4^+CD25^-$IL-10^{hi} and $CD4^+CD25^+$ IL-10^-Treg populations in SAg tolerant mice result in alteration cytokine production patterns; presented with increase of serum IL-10; but decrease of serum IL-2, IFN-γ and TNF-α concentrations (Florquin et al., 1996; Sundstedt et al., 1994; Sundstedt et al., 1997). Since natures in immune responses are shared between SAg and conventional peptide Ag, SAg can be used as the model for tolerance induction study (reviewed in Ivars, 2007).

Staphylococcal enterotoxin B (SEB) is a typical bacterial T cell superantigen (Li et al., 1998; Petersson et al., 2004) exclusively capable of cross-linking α chain of MHC-II and variable β motif of TCR (Alouf and Muller-Alouf, 2003; Kappler et al., 1989). The toxin is extensively applied for both nonprofessional and professional antigen presenting cells (APCs) studies (Collins et al., 1984; Glimcher and Kara, 1992; Lamphear et al., 1998; Lee and Vitetta, 1992). Moreover, SEB tolerance induction has been widely studied (reviewed in Ivars, 2007). Since the toxin can effectively cross-link APCs with polyclonal $CD4^+$T cells, we come to an idea to exploit it for the study of human naïve B cell's priming antigen presentation to naïve $CD4^+$T cell.

Immature dendritic cells (imDCs) are important APCs driving immune responses in both early and long-termed stages to SAgs (reviewed in Ivars, 2007). Small amounts of SAgs are needed by imDC to initiate T cells' activation (Bhardwaj et al., 1993), and a reason for this is due to high expression of B7 co-stimulatory molecules on DC surface (Muraille et al., 1995). To induce SAg-specific IL-10 producing Treg differentiation, imDCs are suggested as major APCs, as well (Grundstrom et al., 2003; Ivars, 2007; Miller et al., 1999). In contrast, antigen presenting role of B cells are very controversial. Though some of previous studies suggested B cells as unimportant APCs in SAg's responses and tolerance induction (Bhardwaj et al., 1993; Miethke et al., 1995; Muraille et al., 1995), the others contradict the idea (Izcue et al., 2001; Stark Aroeira et al., 1997; Stohl and Elliott, 1995; Stohl et al., 1994). There are some explanations about this disconcordance. Localization, dose and time can affect B cells' SAg tolerance induction (Gonnella et al., 2001; Zinkernagel et al., 1997). To demonstrate role of B cell in SAg's immune response, B-cell-deficient mouse models were usually used. Since totally depleted and reduced numbers of B cells are different, B-cell-deficient models can mask the actual roles of B cells SAg's immune response (Grundstrom et al., 2003; Miller et al., 1999).

SAg antigen's presentation by B cells to $CD4^+$T cells are important to drive B cells' activation and generation of immunoglobulin (Ig) (Jabara and Geha, 1996; Stohl et al., 1994). Apart from production of Ig in SAg's response, B cells also play particular role in SAg tolerance (Florquin et al., 1996; Stark Aroeira et al., 1997). T cell anergy induced by repeated injection of SEB in BALB/c mice was associated with persistence of B cells, possibly associated with B-T cell's interaction (Stark Aroeira et al., 1997). Moreover, B cells additionally show importance in anergy maintenance in $CD4^+$T cells to SEB, both *in vivo* and *in vitro* (Stark Aroeira et al., 1997). Though B cell can tolerize T cells in SAg response model, characterization of B cells is still not clearly clarified. Few evidences from previous studies implied us the role of naïve B cells tolerizing $CD4^+$T cell in SAg tolerance induction. For example, B cell-deficient mice were inferior in anergy maintenance of $CD4^+$T cells to SEB than wild type ones (Stark Aroeira et al., 1997). The

data slightly implied the fundamental role of naïve B cell in priming $CD4^{+}$T cells' anergy and thus may comparable to that evidenced in conventional peptide application (Figure 4).

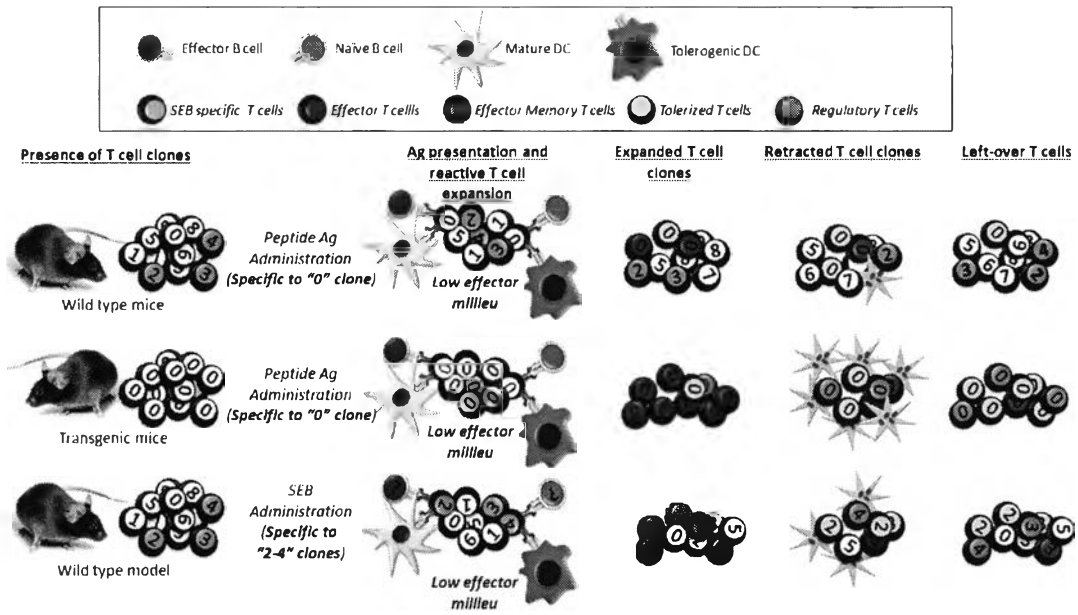


Figure 4. Review of tolerance induction mechanisms in (A) wide type mice administrated with specific peptide Ag, (B) transgenic mice administrated with specific peptide Ag and (C) wide type mice administrated with SEB. The peptide antigen was specific to T cell clone "0", while SEB was specific to multiple clones of T cells ("2-4"). All admistrations were performed in absence of immunogenic adjuvant and thus brought about impotent effector milieu during antigen presentation process. Though supported by some studies, the antigen presenting roles of both effector and naïve B cells during SEB response are still limitedly known. The lack of sufficient effector stimulation thus greatly affects T cell differentiation during retraction phrase after activated T cell's expansion. This results in higher number of left-over anergic and regulatory T cell clones specific to Ag or SEB than effector memory T cell. Due to the limited number of antigen specific T cell clones presented in wild type mice, observation of these mechanisms by applying specific peptide Ag is not practical. On the contrary, SEB can interact with 5-20% polyclonal specific T cells and thus more suitable model for observation in wild type mice.

Role of human naïve B cells in priming $CD4^+$T cells' responses to SAgs are lesser implied in human than mouse models. In conventional or alloantigen response models, naïve B cells can tolerize $CD4^+$T cells via B7 and CTLA-4, but not CD28 interaction (previously described). In a study of human T Cell-dependent B Cell differentiation, interaction of B7 and CTLA-4 was more important in SAg-induced Ig generation than in SAg-induced T cell proliferation (Stohl et al., 1994). This data at some levels supported B7-CTLA-4 model in SAg response. Direct human naïve B cells' alloantigen presentation stimulate low $CD4^+$T cells' proliferation and immunogenic cytokine production (Carpenter et al., 2009) suggesting the fate to become tolerance (Matzinger, 2002). *In vivo* evidences of human naïve B cells generating T cells' tolerance were better introduced in autoimmune patients treated by Rituximab (Eming et al., 2008; Saadoun et al., 2008; Sfikakis et al., 2005; Sfikakis et al., 2007; Stasi et al., 2007; Tamimoto et al., 2008; Vallerskog et al., 2007; Vigna-Perez et al., 2006). A recent cohort study in renal transplant patients also indicated the corelation between elevated peripheral blood naïve B cells and successful transplant tolerance (Newell et al.). Though several studies suggest tolerance induction of human naïve B cells to allo- and auto-antigens, evidence in SAg response is very limited.

In this study, we set a simple two-step method to improve peripheral naïve B cell's purity by the combination of rosetting process (Human B lymphocyte enrichment cocktail, StemCell) and the Naïve B cell Isolation kit II (Miltenyi). We demonstrated that the isolated naïve B cells acquire their resting normal phenotype could present the superantigen "SEB" to prime autologous naïve $CD4^+$T cells. To further characterize phenotype and function of naïve $CD4^+$T cells recognized SEB presented by human naïve B cells, Monocyte derived dendritic cells (MoDCs), polyinosine-polycytosine (PolyIC) stimulated MoDC and CpG oligodeoxynucleotides (or CpG ODN) stimulated naïve B cells were used as control APCs. According to their determined phenotypes, naïve B cells expressed lesser MHC-II and B7 co-stimulatory molecules (CD80 and CD86) when compared to those of other APC controls. In agreement with their poor co-stimulatory phenotypes, SEB pulsed naïve B cells could prime $CD4^-$T cells to acquire

only limitedly activating phenotypes and to produce lesser helper T cell effector cytokines when compared with those primed by other APCs. Total activated $CD4^+$T cells primed by naïve B cells were subsequently determined for their immunosuppressive property; however, no SEB specific suppression was demonstrated. In this study, we introduced a simple human peripheral naïve B cell isolation method which was reproducible and could be performed manually without specific automated equipments. Interestingly, we also demonstrated the poor naïve $CD4^+$T cell priming property of naïve B cells during SEB presentation. Since several evidences implied us the possible acquisition of anergic T cell's property subsequently after poor response priming, the current study thus suggests tolerogenic APC role of naïve B cell during both SAg and conventional Ag responses similar to those previously illustrated in mouse models.

In addition, this study also determined differential gene expressions among human peripheral, splenic and tonsilar naïve B cells. In comparison among naïve B cell subsets, variations in phenotype and cell properties were produced between peripheral naïve B cells and those of lymphoid origins (splenic and tonsilar naïve B cells) supporting the possible effect of lymphoid niches on the resident naïve B cells. Several candidate molecules differentially expressed between Peripheral and Lymphoid naïve B cells were introduced, including those associated with common B cell's phenotypes. The findings suggest that the study results generated from peripheral naïve B cells may not always be generalized for the biological activities of other lymphoid naïve B cells. This increased us awareness to imply the knowledge of naïve B cell's Ag presentation generally acquired from mouse splenic source with human peripheral naïve B cells used in this study. Along with other factors, the difference in naïve B cells' sources could be a contributor to determine the outcome of primed T cell immunoproperties. Nonetheless, further biological confirmation need to be warranted.

In conclusion, the current study successfully provided us a convenient method to study and imply the role of human naïve B cell as APC to naïve $CD4^+$T cells. In several autoimmune diseases (for example, SLE, type I diabetes, EAE, etc), the role of naïve B cell as APC was merely evidenced in B cell depleted patients and limitedly introduced in

in vitro experiment (Lund and Randall). Hopefully, the method will open an optional access to illustrate this issue, *in vitro*. In addition, the study performed expression profile meta-analysis to determine for unknown immunoproperties' variations among human naïve B cell subsets. Generally, the lack of supporting evidence is a common obstacle to launch a sensible immunological question. The current study provided an example to apply differential gene expression meta-analysis to approach such a question. With a similar method, we hope that other non-practical immunological studies could also be applied with the same approach.

1.3 QUESTIONS:

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

1.3.2 Are activated $CD4^+$T cells primed by SEB pulsed naïve B cells different from those primed by dendritic cells?

1.3.3 Are peripheral naïve B cells good representatives of lymphoid naïve B cells by differential gene expression analysis?

1.4 HYPOTHESES:

1.4.1 Human peripheral naïve B cells can constitute T cell with unique immunoproperties in the SEB presentation dependent way as compared to DCs.

1.4.2 Peripheral naïve B cells are not different from splenic or tonsilar naïve B cells by differential gene expression analysis.

1.5 OBJECTIVES:

1.5.1 To improve reliable human peripheral naïve B cell non-flow sorting isolation method

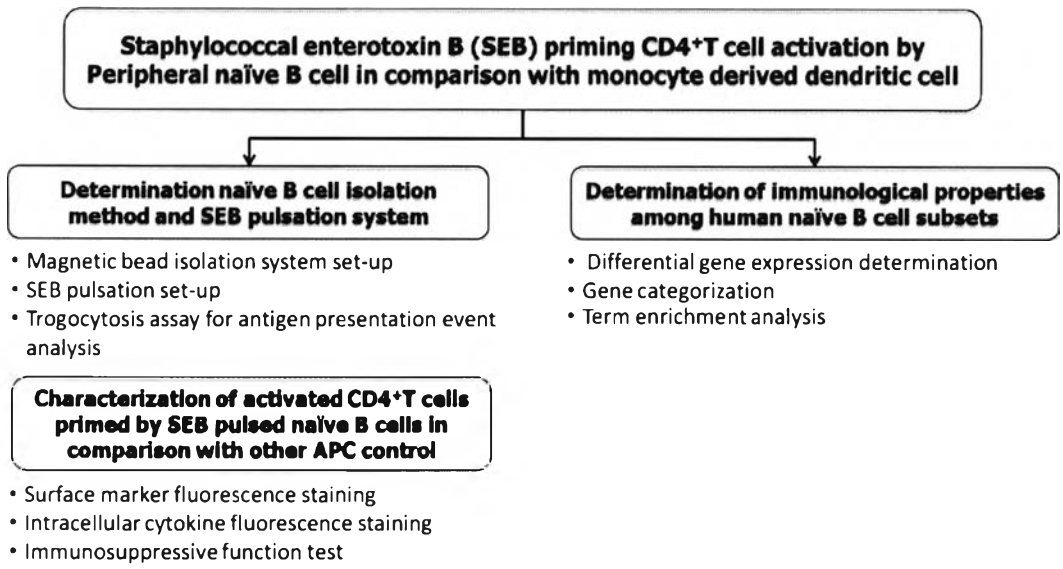
1.5.2 To explore antigen presentation event of human peripheral naïve B cell by exploiting SEB pulsation

1.5.3 To determine and compare immune properties of activated T cells expanded from naïve $CD4^+$T cells recognizing SEB on naïve human B cell with those of DCs

1.5.4 To determine differences in expression profile among human peripheral, splenic and tonsilar naïve B cell subsets

1.5.5 To imply possible different immunoproperties among peripheral, splenic and tonsilar naïve B cell subsets by their differential gene expressions

1.6 CONCEPTUAL FRAMEWORK:



1.7 METHODOLOGY

According to the conceptual framework, the study contained with three major parts; "Determination naïve B cell isolation method and SEB pulsation system", "Characterization of activated $CD4^+$T cells primed by SEB pulsed naïve B cells in comparison of other APC control" and "Determination of immunological properties among human naïve B cell subsets". Each part of the study had its specific questions, hypotheses and objectives to be archieved. Determination naïve B cell isolation method and SEB pulsation system part was prior performed to acquire reliable system for the next part, Characterization of activated $CD4^+$T cells primed by SEB pulsed naïve B cells in comparison of other APC control. To determine representatives of peripheral naïve B cells of those isolated lymphoid origins (spleen and tonsil), "Determination of

immunological properties among human naïve B cell subsets" part was additionally performed.