CHAPTER V

OVERALL DISCUSSION AND CONCLUSION

To investigate *in vitro* interaction between human naïve B cells and naïve $CD4^+$T cells, the present study has performed a simple two-step isolation method to isolate pure human naïve B cells and has applied a T cell superantigen, Staphylococcal enterotoxin B (SEB) to study the cell's role as APC to prime naïve $CD4^+$T cell activation. High B cell purity ($CD19^+$cells) was obtainable from the two-step isolation method proposed (Figure 5) and SEB pulsation system used in this study did not obviously activate naïve B cells determined by their CD69(-)CD80(lo)CD86(lo) phenotypes (Figure 6 and 9). To evaluate naïve B cell's priming role, naïve $CD4^+$T cells co-cultured with SEB pulsed naïve B cells for 68 hours were defined for their phenotypes, cytokine production pattern and cell suppressive function in comparison with those primed by other APCs (CpG activated B cells, MoDCs and Poly I:C stimulated MoDCs). Activated T cells acquired from naïve B cell priming revealed CD25(+)CD62L(hi)CD95(lo) phenotypes (Figure 12) with limited IL-2 and IL-4 production (Figure 13). However, the acquired cells showed no suppressive function (Figure 14). This suggested us that SEB presentation by naïve B cell cannot induce human regulatory T cell differentiation from naïve $CD4^+$T cell in this study. There are several different factors in culture systems that could be contributed to unsuccessful induction of suppressive function. Among these contributors, possible intrinsic variation among naïve B cells isolated from different sites is of our interest. By applying bioinformatic metaanalysis, peripheral naïve B cells were considered unique from both splenic and tonsilar naïve B cells. However, further warrant is needed.

Though the Naïve B cell isolation kit II (Miltenyi) is commonly used for the human peripheral naïve B cell isolation, 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). Our 2-step protocol allowed isolation of naïve human B cells with not only a higher B

cell purity and also higher reproducibility (99.0±0.5%) (Figure 5). Our performed protocol only slightly increased the process time from common method by naïve B cell isolation kit II (Miltenyi). The method is also convenient and may be cost-effective, since it required neither special instrument nor flow cytometric sorting machine. In addition, less than 10 ml of blood can be used for the isolation and performing the *in vitro* study. This method can therefore be applied in case when limited blood volume is an issue such as in pediatrics or some patients.

To acquire comparative APC controls, other three types of APCs were included in this study composing of CpG activated B cells, MoDCs and Poly I:C stimulated MoDCs. These cells represented TLR9 activated B cell, immature DC and mature DC induced by TLR3 stimulation, accordingly. CD80, CD83, CD86 and HLA-II expressions were determined on each APC type. Successful CpG activated B cell and Poly I:C stimulated MoDCs inductions were implied by their CD83 upregulation (Breloer et al., 2007; Prazma et al., 2007). Since CD80 and CD86 are regarded as important T cell co-stimulatory molecules (Reichardt et al., 2007a), the least immunogenic APC role of naïve B cell was demonstrated due to the lowest expression of these molecules (Reichardt et al., 2007a; Watt et al., 2007) (Figure 11B). This was in agreement with the lowest number of activated T cells ($CD25^+$T cells) and IL-2/IL-4 producing cells acquired in the co-culture between naïve $CD4^+$T cells and SEB pulsed naïve B cells. Some phenotypes of these activated T cells unique from those primed by DCs were presented. Activated T cells primed by SEB pulsed B cells had CD4(+)CD25(+)CD62L(hi)CD95(lo) phenotypes, while those primed by DCs were CD4(+)CD25(+)CD62L(lo)CD95(hi) (Figure 12A and 12B). Since CD62L downregulation (Berg et al., 1989; Cauley et al., 2000; Rajagopalan et al., 2006) and CD95 upregulation (Arens et al., 2005; Slifka and Whitton, 2000) are common during early T cell activation in response to both antigen or SEB, these further supported the inefficient effector T cell phenotypes primed by naïve B cells.

T cell differentiation is determined since early state of T cell response (Ivars, 2007; Nakata et al., 1995; Vella et al., 1995; Zhu et al.). As early as 68-hour, limited IL-2, IFN-γ and IL-4 productions of T cells induced by SEB-presenting naïve B cells were presented (Figure 13). Since early limited production of these cytokines is a common predictor of later anergic T cell's generation (Fathman and Lineberry, 2007; Reichardt et al., 2007a), the acquired result implied later SEB specific anergic T cell induction by naïve B cells. This notice thus supported tolerance inducing role of naïve B cells during bacterial superantigen response (Ashour and Seif, 2007; Raimondi et al., 2006a; Stark Aroeira et al., 1997). In contrast to that reported in transgenic mouse study (Reichardt et al., 2007b), no immunosuppressive function of activated $CD4^{+}$T cells primed by SEB presented naïve B cells was illustrated (Figure 14). This suggested that regulatory T cell's differentiation could not be induced by the SEB presentation system used in this study.

Several actors could affect regulatory T cell induction in the current study. These include the differences between *in vitro* and *in vivo* induction system, culture time (reviewed in Ivars, 2007), the intrinsic SEB's immunoproperties (Kissner et al., ; Mooney et al., 1990), naïve B cell sources (Agematsu et al., 1997; Crotty et al., 2004; Klein et al., 1998), and intrinsic differences among naïve B cells isolated from various organ sites. In this study, the intrinsic variation among naïve B cells isolated from different organ sites was of our interest. Due to the lack of knowledge and also ethical limitation, we thus approached this issue by bioinformatic metaanalysis.

According to gene expression meta-analysis performed in this study, peripheral naïve B cells were considered unique from other lymphoid naïve B subsets (splenic and tonsilar naive B cells) (Figure 16 and Table 4). Differential gene expression analysis supported the idea that splenic naïve B cells were more activated than peripheral ones possibly due to several stimuli which are absent in peripheral blood. Though these

differences seemed to show no significance on B cell's antigen presenting role, the indirect effect were still possible via their nonequivalent activation states. However, further warrant must be confirmed for the absolute conclusion.

The current study demonstrates a two-step, non-cell sorter baed method to isolate pure human peripheral naïve B cells by including rosetting B cell enrichment step (StemCell) prior to application of conventional magnetic bead based naïve B cell isolation kit (Miltenyi). In substitute for conventional peptide Ag which is not convenient in application with naïve B cell's antigen presentation study, we observed the interaction between naïve B cell and naïve $CD4^{+}$T cell by applying Staphylococcal enterotoxin B (SEB). The inefficient effector phenotype of activated T cells primed by SEB pulsed naïve B cells were illustrated. However, these cells were shown lack of immunosuppressive function, and thus different from those previously reported in mouse study (Reichardt et al., 2007b). In conclusion, two-step method by adding a resetting step onto the commercial magnetic bead-based isolation kit provided a more purity yield of human peripheral naïve B cells. The isolated naïve B cells with/without SEB pulsed had a significant lower CD80 and CD86 compared with MDCs. In this study we could not induce regulatory T cell differentiation directly from SEB-presented naïve B cells. We also found that based on the gene expression analyses, peripheral blood naïve B cells were significanlt different from the lymphoid (both tonsilar and splenic) naïve B cells. Thus further studies to see the possible roles of human lymphoid naïve B cells and other factors in Treg induction are warranted.