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B cell-associated immune profiles in patients with end-stage renal disease (ESRD)

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Abbreviations: ESRD, end-stage renal disease; HD, hemodialysis; Scr, serum creatinine

Abstract

Most of the previous studies on immune dysregulation in end-stage renal disease (ESRD) have focused on T cell immunity. We investigated B cell subpopulations in ESRD patients and the effect of hemodialysis (HD) on B cell-associated immune profiles in these patients. Forty-four ESRD [maintenance HD patients (n = 27) and pre-dialysis patients (n = 17)] and 27 healthy volunteers were included in this study. We determined the percentage of B cell subtypes, such as mature and immature B cells, memory B cells, and interleukin (IL)-10$^+$ cells, as well as B cell-producing cytokines (IL-10, IL-4 and IL-21) by florescent activated cell sorting (FACS). B cell-associated gene expression was examined using real-time PCR and B cell producing cytokines (IL-10, IL-4 and IL-21) were determined using an enzyme-linked immunosorbent assay (ELISA). The percentage of total B cells and mature B cells did not differ significantly among the three groups. The percentages of memory B cells were significantly higher in the pre-dialysis group than in the HD group ($P < 0.01$), but the percentage of immature B cells was significantly lower in the pre-dialysis group than in the other groups. The percentages of IL-10-expressing cells that were CD19$^+$ or immature B cells did not differ significantly ($P > 0.05$) between the two subgroups within the ESRD group, but the serum IL-10 concentration was significantly lower in the pre-dialysis group ($P < 0.01$). The results of this study demonstrate significantly altered B cell-associated immunity. Specifically, an imbalance of immature and memory B cells in ESRD patients was observed, with this finding predominating in pre-dialysis patients.

Keywords: B-lymphocyte subsets; kidney failure, chronic; precursor cells, B-lymphoid; renal dialysis

Introduction

Patients with end-stage renal disease (ESRD) have altered immunity compared to the general population (Girndt *et al*., 1999). These alterations are characterized by impaired defensive immune cells, despite evidence of activation of markers by immune-competent cells. Previous studies reported that impairment of T cell function is important in the development of immune-dysregulation in ESRD patients. For example, various types of effector T cells such as Th1/Th2 cells, memory T cells, and regulatory T cells are associated with altered immunity in ESRD patients (Vanholder *et al*., 1991; Haag-Weber and Horl, 1993; Alvarez-Lara *et al*., 2004; Yoon *et al*., 2006). We previously reported that IL-17-producing effector memory T cells were significantly increased in ESRD patients (Chung *et al*., 2012).

Much evidence indicates that B cell impairment is also involved in immune dysregulation in ESRD patients. For example, only 50-75% of adult ESRD patients develop protective antibodies against the hepatitis B virus surface antigen after vaccination (de Graeff *et al*., 1985), and this clinical finding is supported by experimental evidence (Meuer *et al*., 1987; Girndt *et al*., 1993). However, only a few studies investigated the effect of ESRD on B cells (Raskova *et al*., 1987; Pahl *et al*., 2010). In addition, little is known regarding the effect of hemodialysis on B cell-associated immune profiles of ESRD patients.

The aim of this study was to investigate the B-lymphocyte immune profiles of a group of ESRD patients. In addition, we evaluated the effects of maintenance hemodialysis (HD) on immune cells by comparing the immune profile of maintenance HD patients and pre-dialysis patients.

Results

Baseline and laboratory findings of the patient population

Serum creatinine (Scr), blood urea nitrogen (BUN), and intact parathyroid hormone (i-PTH) levels were significantly higher, and hemoglobin levels significantly lower, in the ESRD group compared to the healthy control group ($P < 0.05$, respectively). In contrast, leukocyte and lymphocyte counts and C-reactive protein (CRP) did not differ significantly between the two groups. In the comparison between the HD and pre-dialysis groups, only the BUN levels were slightly higher in the pre-dialysis group; differences between other parameters were not significant (Table 1).

Comparison of B cell subtypes ($CD19^+$ total B cells, memory B cells, mature B cells, and immature B cells) between the three groups

As shown in Figure 1 and Figure 2, the percentage of circulating memory B cells was significantly higher in the pre-dialysis group than in the HD group. The values were 34.6 ± 12.4 in the pre-dialysis group ($P = 0.008$ as compared with HD), 20.1 ± 7.5% in the HD group ($P = 0.007$ as compared with healthy controls), and 27.2 ± 6.2% in the healthy controls. By contrast, the frequency of $CD19^+$ total B cells did not differ significantly between the three groups (HD, 20.1 ± 7.5%; pre-dialysis, 34.6 ± 12.4%; healthy, 27.2 ± 6.2%). The frequency of immature B cells was also significantly higher in the HD group as compared with the pre-dialysis group: HD, 8.5 ± 4.2% ($P = 0.045$ as compared with pre-dialysis) and healthy controls (5.0 ± 2.3%). However, the frequency of mature B cells did not differ between the HD and pre-dialysis patients ($P > 0.05$).

Comparison of total IL-10^+ B cells, immature IL-10^+ B cells, and regulatory T cells between the three groups

As shown in Figure 3, the percentage of IL-10^+/$CD19^+$ B cells did not differ significantly between the HD group (1.2 ± 0.5%) or pre-dialysis group (1.1 ± 0.4%) as compared with the healthy controls (1.4 ± 0.4%; Figure 3A). Additionally, the percentage of IL-10^+ immature B cells and regulatory T cells (CD25high $Foxp3^+/CD4^+$) did not differ significantly between the HD group (IL-10^+ immature B cells, 4.2 ± 3.3%; regulatory T cells, 7.8 ± 1.3%) or pre-dialysis group (IL-10^+ immature B cells, 4.7 ± 2.1%; regulatory T cells, 7.0 ± 2.5%) as compared with the healthy controls (IL-10^+ immature B cells, 5.8 ± 3.2%; regulatory T cells, 9.6 ± 2.6%; Figures 3B and 3C).

Table 1. Baseline and laboratory characteristics of the patient population

	Healthy controls (n = 27)	ESRD (n = 44)	P	HD (n = 27)	Pre-dialysis (n = 17)	P
Age (yr)	35.7 ± 9.2	44.9 ± 10.4	< 0.01	46.3 ± 9.3	42.2 ± 11.9	0.19
Male (n, %)	9 (33.3)	30 (68.2)	< 0.01	19 (70.4)	11 (64.7)	0.47
BUN (mg/dl)	12.9 ± 4.1	66.2 ± 25.3	< 0.01	57.4 ± 19.3	84.0 ± 27.0	< 0.01
Scr (mg/dl)	0.9 ± 0.2	9.6 ± 2.6	< 0.01	9.9 ± 2.8	8.9 ± 2.2	0.25
Leukocytes ($\times 10^3$ cells/mm^2)	6.9 ± 1.7	6.6 ± 1.8	0.60	7.0 ± 1.6	6.0 ± 2.0	0.07
Lymphocyte ($\times 10^3$ cells/mm^2)	2.8 ± 0.7	2.4 ± 0.8	0.19	2.5 ± 0.6	2.4 ± 1.1	0.54
Hemoglobin (g/dl)	14.0 ± 2.1	9.9 ± 1.7	< 0.01	10.4 ± 1.5	8.7 ± 1.5	< 0.01
i-PTH (pg/ml)	34.4 ± 5.0	175.5 ± 201.0	0.03	159.1 ± 218.7	211.8 ± 156.0	0.44
CRP (mg/L)	0.04 ± 0.04	0.2 ± 0.3	0.15	0.22 ± 0.30	0.11 ± 0.24	0.27

ESRD, end-stage renal disease; HD, hemodialysis; BUN, blood urea nitrogen; Scr, serum creatinine; i-PTH, intact parathyroid hormone; CRP, C-reactive protein.

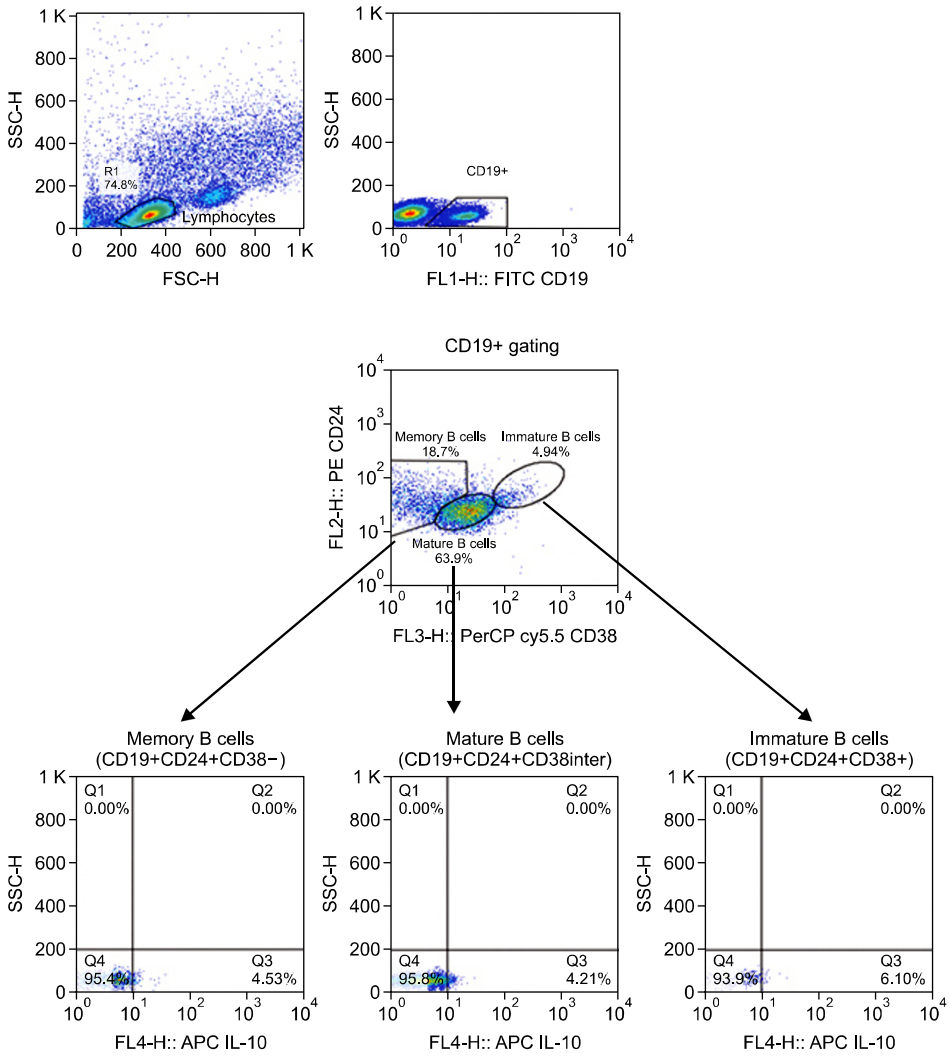

Figure 1. Flow cytometric analysis of B cell subsets. PBMCs were stained with anti-CD19 FITC, anti-CD24 PE, anti-CD38 PerCP cy5.5, and anti-IL-10 APC. CD19+ cells were gated for further analysis. B cells were divided into subpopulations according to the expression of CD24+, CD38+, and IL-10^{+}; CD19+CD24+CD38- (memory B cells), CD19+CD24+CD38inter (mature B cells), CD19+CD24+CD38+ (immature B cells).

Expression of TCL1A, MS4A1, and BLNK mRNA measured by real-time PCR in PBMCs of healthy controls and HD and pre-dialysis patients

After peripheral blood mononuclear cells (PBMCs) of the three groups were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, expression levels of TCL1A, MS4A1, and BLNK mRNA were determined using real-time polymerase chain reaction (PCR). As shown in Figure 4, BLNK mRNA levels were significantly higher in the HD and pre-dialysis groups as compared with the healthy controls: HD, 1.2 ± 0.4 (P = 0.027 as compared with healthy controls); pre-dialysis patients, 1.3 ± 0.1 (P = 0.030 as compared with healthy controls); and healthy controls, 0.6 ± 0.2. However, TCL1A and MS4A1 mRNA expression levels did not differ between HD and pre-dialysis patients ($P > 0.05$).

Comparison of cytokine production between the three groups

Within the three groups, IL-10 production was significantly lower in the pre-dialysis group (6.1 ± 1.4%) as compared with HD patients (4.4 ± 0.5%; P = 0.007; Figure 5A). As shown in Figures 5B and 5C, IL-21 production did not differ between the ESRD patients and healthy controls. IL-21 production values were as follows: HD group, 3.2 ± 1.3%; pre-dialysis group, 2.6 ± 0.4%; and healthy control group, 3.4 ± 1.0%. Within the three groups, IL-4 production was significantly higher in the HD group (101.7 ± 13%) as compared with the healthy control group (56.4 ± 15.8%; P = 0.014; Figure 5C).

Discussion

In this study, we evaluated the B lymphocyte-

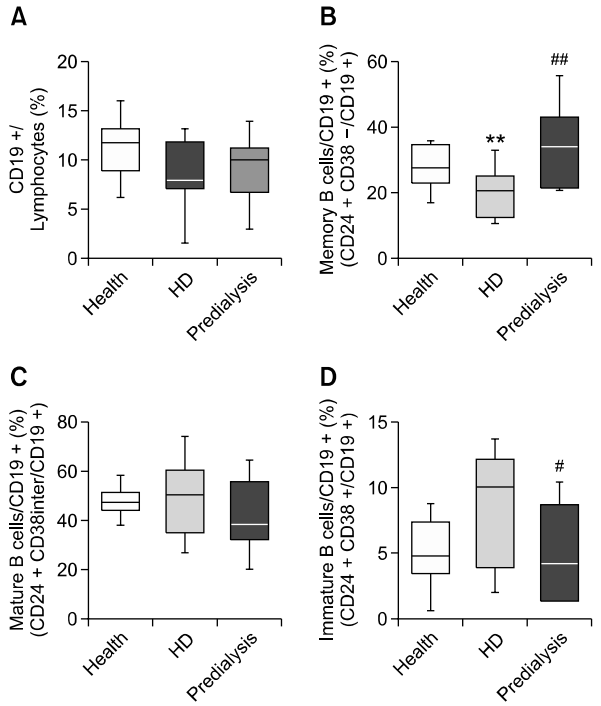

Figure 2. Distribution of CD19+ total B cells, memory B cells, mature B cells, and immature B cell subsets in the healthy control, HD, and pre-dialysis groups. PBMCs from healthy controls (n = 27), HD patients (n = 27), and pre-dialysis patients (n = 17) were stimulated for 4 h *ex vivo* with PMA and ionomycin in the presence of GolgiStop. The percentages of CD19+ total B cells, memory B cells, mature B cells, and immature B cells were measured by flow cytometry. The frequency (%) of CD19+ lymphocytes (A), memory B cells (CD24+CD38-/CD19+ cells) (B), mature B cells (CD24+CD38inter/CD19+ cells) (C), and immature B cells (CD24+CD38+/CD19+ cells) (D) in healthy controls, HD patients, and pre-dialysis patients. Bars show the mean. $^{**}P < 0.01$ versus healthy controls, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus HD.

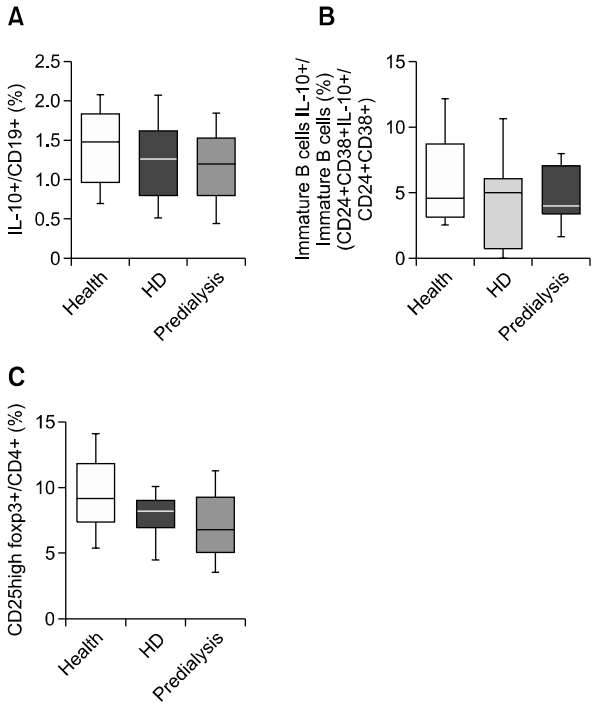

Figure 3. Distribution of total IL-10$^+$ B cells, IL-10$^+$ immature B cells, and regulatory T cells in the healthy control, HD, and pre-dialysis groups. PBMCs from all groups were treated as described in Figure 1 and the Materials and Methods section. (A) The frequency (%) of IL-10$^+$/CD19+ cells in healthy controls, HD patients, and pre-dialysis patients. (B) The frequency (%) of immature B cells IL-10$^+$ (CD24+CD38+IL-10$^+$/CD24+ CD38+) in healthy controls, HD patients, and pre-dialysis patients. Bars show the mean. (C) The frequency (%) of regulatory T cells (CD25+high Foxp3+/CD4+) in healthy controls, HD patients, and pre-dialysis patients. Bars show the mean.

associated immunological profile of ESRD patients as compared with the general population. Our results showed that the distribution of B cell subsets had significantly different patterns in each group. The most prominent finding in this study was the decrease in immature B cells and the increase in memory B cell in pre-dialysis patients.

Our finding of an imbalance between immature B cells and memory B cells in the pre-dialysis group compared to the HD group is significant. In a previous report (Pahl *et al*., 2010), reported a diffuse reduction of B cell subpopulations, including memory B cells, in maintenance HD patients in spite of an elevation in B cell growth and an increase in differentiation and survival factors (Pahl *et al*., 2010). In this study, there was a significant decrease in memory B cells in the HD group compared to the healthy control group, consistent with the previous report. However these cell types were significantly increased in the pre-dialysis group compared to the HD group, which suggests that the immune system was activated.

In addition, we investigated CD19$^+$CD24hiCD38hi B cells that have previously been identified as an immature transitional B cell (Sims *et al*., 2005; Plebani *et al*., 2007). Of note, this cell type has regulatory capacity. In previous reports, it significantly inhibited the differentiation of pro-inflammatory cytokine-expressing CD4$^+$ T cells in a dose- and contact-dependent manner (Blair *et al*., 2010). However, whether this cell type was dysregulated in ESRD patients has not been investigated.

In this study, a significant decrease in immature B cells was found in the pre-dialysis group compared to the HD group, which suggests that there is defective regulatory activity in pre-dialysis patients, potentially due to their uremic state. Resolution of uremic toxicity by hemodialysis can recover the B cell balance, thereby increasing the percentage of immature B cells in the HD group. As expected, the uremic state was more severe in the pre-dialysis group, as reflected by the higher BUN levels. Combined with the increase in memory B cells, the decrease in immature B cells may reflect the

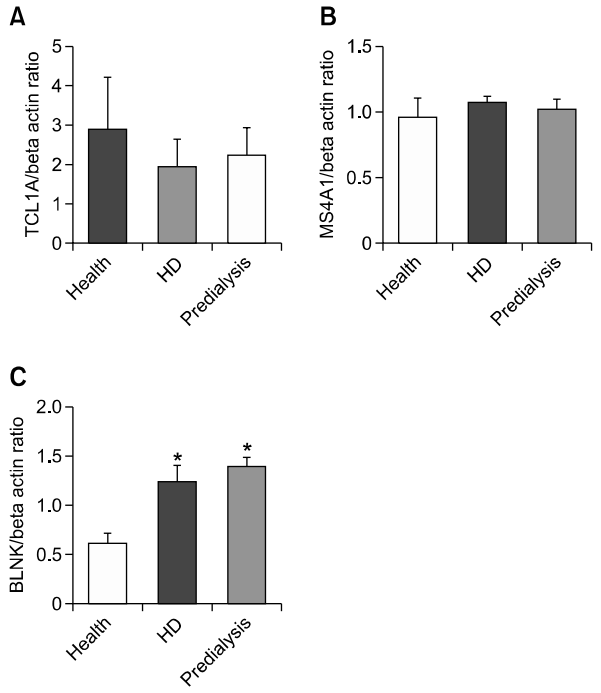

Figure 4. Expression of TCL1A, MS4A1, and BLNK mRNA measured by real-time PCR in PBMCs of healthy control, HD, and pre-dialysis groups. PBMCs from all groups were treated as described in Figure 1 and the Materials and Methods section. The expression levels of TCL1A (A), MS4A1 (B), and BLNK (C) mRNA were measured using real-time PCR. $^{*}P < 0.05$ versus healthy controls.

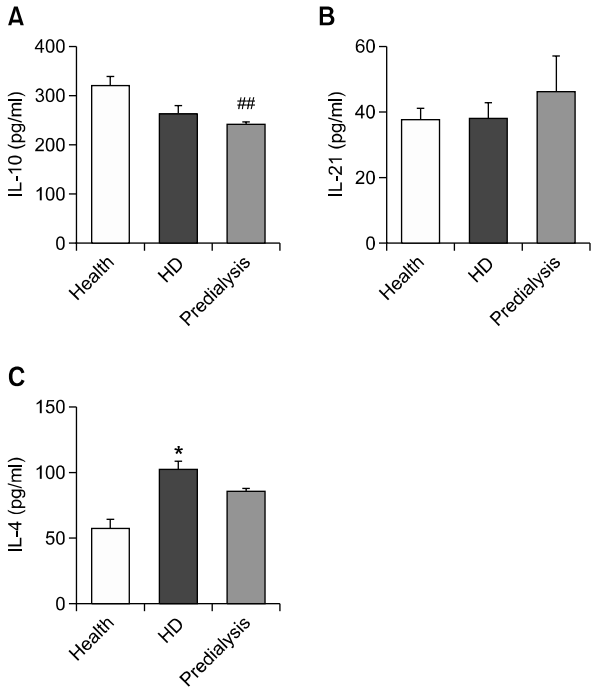

Figure 5. Expression of IL-10, IL-21, and IL-4 in the serum of healthy control, HD, and pre-dialysis groups. (A) Concentrations of IL-10 in serum samples from healthy control, HD, and pre-dialysis groups. (B) Concentrations of IL-21 in serum samples from healthy control, HD, and pre-dialysis groups. (C) Concentrations of IL-4 in serum samples from healthy control, HD, and pre-dialysis groups. The data represent the mean ± SD of three separate experiments. $^{*}P < 0.05$ versus healthy controls, $^{\#\#}P < 0.01$ versus HD.

imbalance of effector and regulatory capacity in the pre-dialysis group.

There were no significant differences in these cells between the pre-dialysis group and healthy controls. The reason for this is unclear, but we postulated that the characteristics of memory B cells and immature B cells in the pre-dialysis group may differ from those of the healthy controls. ESRD patients have non-specific immune activation in spite of susceptibility to pathogens (Vanholder *et al*., 1991; Haag-Weber and Horl, 1993). Therefore, it is possible that the extra memory B cells in the pre-dialysis group are not immune-competent cells sensitized to a pathogenic challenge, but represent non-specifically activated cells due to the severe uremic conditions.

We further investigated the transcriptional expression of markers associated with B cell development and differentiation using real time PCR. In previous reports, BLNK mRNA expression levels were highest in the spleen, with lower levels of expression in the liver, kidney, pancreas, small intestines, and colon (Fu *et al*., 1998). Analysis of BLNK protein expression levels in hematopoietic and fibroblast cell lines of human, mouse, or rat origin demonstrated their preferential expression in human and mouse B cells. While all human B cells examined expressed BLNK and BLNK-s, mouse B cells expressed only one detectable form of the BLNK protein. Hence, BLNK protein expression among hematopoietic cells appears to be limited to the B cell lineage, with human B cells expressing two alternatively spliced forms and mouse B cells expressing a single form (Minegishi *et al*., 1999; Pappu *et al*., 1999; Chiu *et al*., 2002). We found significantly increased expression of BLNK in the HD and pre-dialysis groups. BLNK regulates biological outcomes of B cell function and development. The BLNK gene encodes a cytoplasmic linker or adaptor protein that plays a critical role in B cell development. This protein bridges B cell receptor-associated kinase activation with downstream signaling pathways, thereby affecting various biological functions. BLNK is a pivotal adapter protein in signal transduction from the pre-BCR and BCR. It contains multiple tyrosine phosphorylation sites that provide binding sites for key signaling proteins, such as PLCγ, Btk, and Vav (Kurosaki and Tsukada, 2000). BLNK mutations cause a complete block in B cell development at the pro-B cell to pre-B

cell transition in humans (Minegishi *et al*., 1999; Newell *et al*., 2010). In BLNK-null mutant mice, the developmental block is partial, resulting in the accumulation of pre-BCR$^+$ large pre-B cells in the bone marrow and a reduced number of mature B cells in the periphery (Jumaa *et al*., 2005).

B lymphopenia in end stage renal disease may be partially attributed to an increased susceptibility to cell death by apoptosis that is associated with a decreased expression of Bcl-2 (Fernandez-Fresnedo *et al*., 2000).

BCR signaling leads to growth arrest and apoptosis in immature B cells, whereas it promotes survival and proliferation of mature B cells *via* activation of Rel-dependent antiapoptotic and proproliferative genes (Grumont *et al*., 1998; Tumang *et al*., 1998). Both BLNK and BCAP play important roles in BCR signaling (Tan *et al*., 2001; Simeoni *et al*., 2004). The long isoform of BLNK was also implicated in promoting BCR-induced apoptosis (Grabbe and Wienands, 2006).

As shown in Figure 4, BLNK mRNA levels were significantly higher in the HD and pre-dialysis groups as compared with the healthy controls. BCR signaling growth arrest and apoptosis in immature B cells, whereas it promotes survival and proliferation of mature B cells.

Tcl1, which is abundantly expressed in immature and IgM$^+$ memory B cells, respectively, plays a crucial role in regulating Akt activation, thereby affecting B cell survival and death *via* the Bcl-2-regulated pathway. In Tcl1-deficient mice, the number of splenic follicular, germinal center, and marginal zone B cells is reduced (Kang *et al*., 2005). CD20 belongs to the MS4A family of molecules with multiple membrane-spanning domains, and is expressed on pre-B and mature B cells, but is lost upon differentiation into plasma cells. CD20 is unlikely to have a natural ligand, but *in vitro* studies with CD20 monoclonal antibodies have demonstrated its involvement in the regulation of B cell activation and proliferation (Kuijpers *et al*., 2010). The expression of Tcl1 and MS4A1 did not differ among the three groups, which suggests that they are not involved in immune impairment in ESRD patients.

The cytokine IL-10, which is known to be associated with the function of regulatory B cells, was significantly reduced in the pre-dialysis group. However IL-10 producing immature B cells did not differ between the three groups, which suggests that the major cell population contributing to the reduced IL-10 levels in the pre-dialysis group is not B cells. IL-21, which is associated with growth and differentiation of B cells, did not differ between the three groups either (Ettinger *et al*., 2008). The effects of IL-21 on B cells vary depending on the context, e.g., whether *in vivo* or *in vitro* (Ozaki *et al*., 2002, 2004), suggesting that IL-21 influences multiple aspects of B cell differentiation. The results of this study suggest that IL-10 and IL-21 are not associated with B cell-associated immune impairment in ESRD patients. To determine which cytokine is associated with the defects in regulatory B cells found in this study, further investigations may be required. IL-4, which is a major Th2-associated cytokine, were significantly increased in the HD group. In our previous study, we reported an increase in Th2 cells in the HD group, and it was consistent with the increased levels of IL-4 found in this study (Chung *et al*., 2012).

In conclusion, this is the first report demonstrating that number of B cells with regulatory function was significantly decreased, and the expression levels of markers associated with this cell type were reduced in ESRD patients. This may explain one of the underlying mechanisms of immune dysregulation in ESRD patients. We anticipate that the results of this study may contribute to the development of therapeutic options for immune dysregulation in ESRD patients.

Methods

Patients and clinical information

The patient population was comprised of 44 ESRD patients (27 patients on hemodialysis and 17 patients with stage 5 chronic kidney disease, but not on HD) and 27 healthy controls. In HD patients, samples were taken in the morning, one day after HD. All of the laboratory and immunological results were measured in blood specimens taken at the same time. This study was approved by the Institutional Review Board of the Catholic University (KC10SISI0235), and we obtained informed consent from all patients.

Isolation of human cells

PBMCs were prepared from heparinized blood by Ficoll-Hypaque (SG1077) density-gradient centrifugation. Cell culture was performed as described previously (Alvarez-Lara *et al*., 2004). In brief, the cell suspension was adjusted to a concentration of 10^6/ml in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. The cell suspension (1 ml) was dispensed into 24-well multi-well plates (Nunc, Roskilde, Denmark). For cytokine detection at the single-cell level, PBMCs were stimulated with 50 ng/ml PMA and 1 μg/ml ionomycin for 4 h (Woo *et al*., 2011).

FACS analysis

For analysis of human intracellular cytokine production, PBMC were stimulated with GolgiStop (BD Biosciences, San

Diego, CA) added in the final 4 h, along with PMA and ionomycin. For intracellular staining, cells were stained with combinations of the following monoclonal antibodies (mAbs): CD4-PE/Cy7 (RPA-T4, IgG1; BioLegend, San Diego, CA); CD25-APC (M-A251, IgG1, κ; PharMingen, San Diego, CA); CD38-PerCP cy5.5 (HIT2, IgG1, κ; PharMingen); CD19-FITC (SJ25-C1, IgG1; SouthernBiotech, Birmingham, Alabama); and CD24-PE (ML5, IgG2a, κ; PharMingen). Cells were washed, fixed, permeabilized, and stained to detect intracellular cytokines with mAbs to Foxp3-FITC (PCH101, IgG2a, κ; eBioscience); and IL-10-APC (JES3-19F1, IgG2a, κ; PharMingen). Appropriate isotype controls were used for gate-setting for cytokine expression. Cells were analyzed on a FACS calibur flow cytometry system (Becton Dickinson Systems).

Real-time PCR

After incubation for 4 h with PMA and ionomycin, mRNA was extracted using RNAzol B (Biotex Laboratories, Houston, TX) according to the manufacturer's instructions. Reverse transcription of 2 μg total mRNA was performed at 42°C using the Superscript™ reverse transcription system (Takara, Shiga, Japan). PCR was performed in a 20 μl final volume in capillary tubes using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany). The reaction mixture contained 2 μl of LightCycler FastStart DNA MasterMix for SYBR® Green I (Roche Diagnostics), 0.5 μM of each primer, 4 mM $MgCl_2$, and 2 μl of template DNA. All capillaries were sealed, centrifuged at 500 × *g* for 5 s, and then amplified following denaturation (95°C for 10 min), followed by 45 cycles of 10 s at 95°C, 10 s at 60°C (β-actin) or 57°C (TCL1A, MS4A1, BLNK), and 10 s at 72°C. The temperature transition rate was 20°C/s for all steps. The double-stranded PCR product was measured during the 72°C extension step by detection of fluorescence associated with the binding of SYBR Green I to the product. Fluorescence curves were analyzed with the LightCycler software v. 3.0 (Roche Diagnostics). The LightCycler was used to quantify TCL1A, MS4A1, and BLNK mRNA. The relative expression level of each sample was calculated as the level of TCL1A, MS4A1, and BLNK normalized to an endogenously expressed housekeeping gene (β-actin). Melting curve analysis was performed immediately after the amplification protocol under the following conditions: 0 s (hold time) at 95°C, 15 s at 71°C, and 0 s (hold time) at 95°C. The rate of temperature change was 20°C/s, except for 0.1°C/s in the final step. The generated melting peak represented the amount of specific amplified product. The crossing point (C_p) was defined as the maximum of the second derivative from the fluorescence curve. Negative controls were also included and contained all elements of the reaction mixture except template DNA. All samples were processed in duplicate (Jeong *et al*., 2011).

Enzyme-linked immunosorbent assay

In brief, a 96-well plate (Nunc) was coated with 4 μg/ml monoclonal antibodies against IL-10, IL-21, and IL-4 (R&D Systems) at 4°C overnight. After blocking with PBS/1% BSA/0.05% Tween 20 for 2 h at room temperature (22-25°C), test samples and the standard recombinant IL-10, IL-21, and IL-4 were added to the 96-well plate and incubated at room temperature for 2 h. Plates were washed four times with PBS/Tween 20 and then incubated with 500 ng/ml biotinylated mouse monoclonal antibodies against IL-10, IL-21, and IL-4 for 2 h at room temperature. After washing, streptavidin-alkaline phosphate-horseradish peroxidase conjugate (Sigma) was added, and the plate was incubated for 2 h. The plate was washed again and incubated with 1 mg/ml *p*-nitrophenyl phosphate (Sigma) dissolved in diethanolamine (Sigma) to develop the color reaction. The reaction was stopped by the addition of 1 M NaOH, and the optical density of each well was read at 405 nm. The lower limit of IL-10, IL-21, and IL-4 detection was 10 pg/ml. Recombinant human IL-10, IL-21, and IL-4 diluted in culture medium were used as the calibration standards whose concentrations ranged from 10 to 2000 pg/ml. A standard curve was drawn by plotting optical density against the log of the concentration of recombinant cytokines and was used to calculate the IL-10, IL-21, and IL-4 concentrations in the test samples.

Statistical analysis

Statistical analysis was performed using the SPSS software (version 16.0; SPSS Inc., Chicago, IL). Continuous variables were compared using Student's t-testor one-way analysis of variance (ANOVA). For categorical variables, chi-square frequency analysis was used. The results are presented as mean ± standard deviation (SD). P values < 0.05 were considered statistically significant.

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