



Higher frequency of CD4⁺CD25^{high} Treg cells in hemophilia patients with factor VIII inhibitor

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ABSTRACT. The production of factor VIII inhibitor antibodies remains the most costly and serious complication in replacement therapy of hemophilia A. We investigated the clinical significance of CD4⁺CD25^{high} T regulatory (Treg) cells in hemophilia patients. Our trial included 6 severe hemophilia A patients with factor VIII inhibitors, 6 hemophilia patients without inhibition of factor VIII, and 6 healthy persons (controls). Plasma factor VIII: c was measured by clotting assay. Peripheral blood samples were examined using multiparameter flow cytometry with fluorescent-labeled monoclonal antibodies. Plasma levels of IFN- γ , IL-2, IL-10, and TGF- β were measured by ELISA. The frequency of CD4⁺CD25^{high} Treg cells in CD4⁺ cells was $1.07 \pm 0.38\%$ in inhibitor patients and $0.57 \pm 0.14\%$ in non-inhibitor patients. The proportion of Treg cells in healthy controls was similar to that of the non-inhibitor patients. However, there were significant differences between the inhibitor and non-inhibitor patients in levels of IFN- γ , IL-2, IL-10, and TGF- β . We conclude that the proportions of Treg cells and the concentrations of T cell cytokines in inhibitor patients are higher than those in non-inhibitor patients. The increased number of Treg cells

and increased T-cell cytokines may be related to the development and efficiency of the factor VIII inhibitor.

Key words: Regulatory CD4⁺ cells; Factor VIII; Hemophilia; Inhibitor

INTRODUCTION

Hemophilia A (HA) is a common hereditary bleeding disorder. The treatment of these patients requires infusion of exogenous FVIII. The development of FVIII inhibitor is the most serious complication (Key, 2004; Kempton et al., 2006). The incidence of inhibitors in hemophilia A patients is higher compared with any other inherited clotting factor deficiency, but for reasons that remain uncertain.

The immune process in FVIII inhibitor development is very complex, in which the body's own immune system in response to FVIII plays an important role. When exogenous FVIII is administered to a hemophiliac, some of the FVIII will be presented to (detect the) antigen-presenting cells (APCs) as antigen. Inhibitors to FVIII are antibodies, and the generation of antibodies involves a cellular cascade from APCs to T lymphocyte to B lymphocyte. Over the last several years, significant progress has been made in our understanding of the immune mechanisms that lead to inhibitor formation (White et al., 2005). However, many important immunological questions remain unanswered. The CD4⁺CD25⁺ regulatory T cells, one part of pan-T lymphocytes, may achieve their own initiative immune tolerance so as to maintain the body's own immune suppression through a cell contact mechanism or inhibitory factor-dependent mechanism (Sakaguchi et al., 1995). Th1/Th2 cells also play an important role in immune regulation and are key in the maintenance of immune induction and immune tolerance. In addition, inducing immune tolerance is a therapy for hemophiliacs with inhibitors (Brackmann and Gormsen, 1977).

CD4⁺ cells are essential for the development of an anti-FVIII antibody response. In this study, we measured CD4⁺CD25⁺ regulatory T cells, and cytokines secreted from Th1/Th2 cells in HA patients with or without FVIII inhibitors, to explore their regulatory role in the immune mechanisms that lead to the synthesis of the acquired FVIII inhibitors.

MATERIAL AND METHODS

Equipment and reagents

These included a flow cytometer (EPICS-XL α MCL, Beckman-Coulter, USA); human regulatory T cell staining kit #3 (cat #88-8995-40, lot #E029453; eBioscience, USA); CD4-FITC/CD25-PE (clone No. RPA-T4 & BC96), FoxP3-PE-Cy5 monoclonal antibody (clone No. PCH101), control rat IgG2a-PE-Cy5 (clone number: eBR2a); cell fixation membrane reagent (fixation & permeabilization) in 0.01 M phosphate buffer solution (PBS); and IFN- γ , IL-2, IL-10, TGF- β kit (CAT No. DIF50, D2050, D1000B, DB100B, R&D Systems, USA). FVIII: c concentration and FVIII antibody titers were determined by bead solidification method (STAGO, France).

Clinical cases

HA patients (FVIII < 5%) were enrolled from the clinic or hospital ward of the Anhui

Provincial Hospital. These included 6 with inhibitors (study group, Table 1), and 6 without inhibitors (control group). Another six randomly selected healthy persons were enrolled as blank controls for our study.

Table 1. FVIII: c and antibody titer results.

Case	FVIII: c (%)	Inhibitor titer (BU)
1	0.5	11.36
2	2.3	4.96
3	3.5	0.94
4	1.0	11.68
5	2.6	7.36
6	1.5	2.75

Detection of FVIII: c and antibody titers

FVIII: c concentration and antibody titer were detected by the bead solidification method using the Stago coagulometer.

CD4⁺CD25⁺ Treg cell subsets

Peripheral blood mononuclear cells (PBMC) were separated from fasting EDTA anti-coagulated venous blood, using lymphocyte separation medium. PBMC surface CD4 and CD25 and cytoplasmic Foxp3 were determined by flow cytometry in 100- μ L samples containing approximately 1×10^6 PBMC, and after direct fluorescent labeling and analyzing for the percentage positivity in samples, data were gathered using the Cellquest software.

Detection of cytokine release

Plasma concentrations of IFN- γ , IL-2, IL-10, and TGF- β in EDTA anti-coagulated specimens were determined by ELISA, following the assay kit instructions.

Statistical analysis

The SPSS 16.0 statistical software was used for statistical processing of experimental data. Data are reported as means \pm standard deviation using single-factor analysis of variance (one-way ANOVA), and pairwise comparison between the groups tested was carried out using the LSD method.

RESULTS

FVIII: c and antibody test results in hemophilia A patients with inhibitor

FVIII: c concentration and antibody titers are given in Table 1.

CD4⁺CD25^{high} Treg cells accounted for the proportion of CD4⁺ T cells

The CD25-positive and -negative cells in CD4⁺ T cells in the peripheral blood of

FVIII inhibitor- as in the samples of the positive HA patients, the formation of two different groups with clear boundaries appeared. However, in FVIII inhibitor-negative patients, CD25-positive and -negative boundaries were not obvious in the CD4⁺ T cells, appears over-type (Figure 1). The cells expressing high levels of CD25 and identified as CD4⁺CD25^{high} Treg cells, were present in a higher proportion of CD4⁺CD25⁺ cells in inhibitor patients (1.07 ± 0.38)% than in non-inhibitor patients (0.57 ± 0.14)% ($t = 3.88$, $P = 0.01$). However, the proportion of CD4⁺CD25^{high} Treg cells in non-inhibitor patient was not significantly different from that of the healthy controls (Table 2).

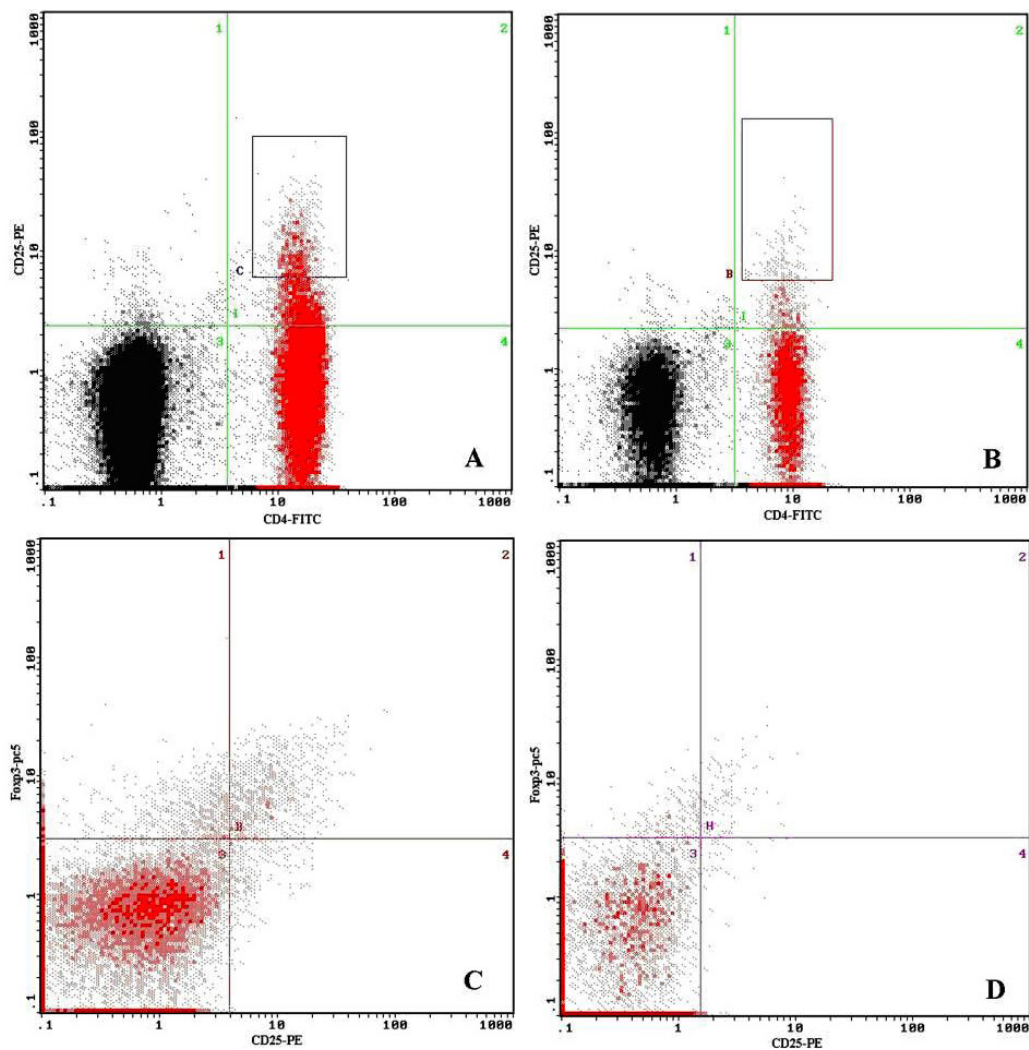


Figure 1. FCM detecting CD4⁺CD25^{high} Treg cells. **A.** CD4⁺CD25^{high} expression; **B.** CD4⁺CD25^{high} low expression; **C.** CD4⁺CD25^{high} Treg cells with high expression; **D.** CD4⁺CD25^{high} Treg cells with low expression.

Table 2. Percentage of CD4⁺CD25^{high} Treg cells in CD4⁺ T cells (means \pm SD, %).

Group	N	CD4 ⁺ CD25 ^{high} Treg/CD4 ⁺ cell (%)
FVIII inhibitor-positive	6	1.02 \pm 0.36
FVIII inhibitor-negative	6	0.66 \pm 0.20
Healthy controls	6	0.64 \pm 0.18

ELISA results

As indicated in Table 3, plasma IFN- γ and IL-2 concentrations were lower in the inhibitor patients (Group A) but higher in the non-inhibitor patients (Group B) compared to the healthy control group (Group C). IL-10 and TGF- β inhibitor concentrations were higher in the inhibitor patients and lower in the inhibitor-negative patients when compared with the control group, with the levels between (which 2 groups) being statistically significant from each other ($F_{\text{IFN-}\gamma} = 7.698$, $P_{\text{IFN-}\gamma} = 0.005$; $F_{\text{IL-2}} = 7.703$, $P_{\text{IL-2}} = 0.005$; $F_{\text{IL-10}} = 5.831$, $P_{\text{IL-10}} = 0.013$; $F_{\text{TGF-}\beta} = 6.337$, $P_{\text{TGF-}\beta} = 0.010$). The four cytokines above in inhibitor-positive and -negative patients showed statistically significant differences ($P_{\text{IFN-}\gamma} = 0.003$; $P_{\text{IL-2}} = 0.006$; $P_{\text{IL-10}} = 0.004$; $P_{\text{TGF-}\beta} = 0.007$) (Table 3).

Table 3. Cytokine concentrations between HA patients and the control group (means \pm SD, pg/mL).

Group	N	IFN- γ	IL-2	IL-10	TGF- β
FVIII inhibitor+ (A)	6	20.65 \pm 4.36	128.70 \pm 8.72	180.48 \pm 78.66	58.76 \pm 11.03
FVIII inhibitor- (B)	6	29.52 \pm 5.29	150.36 \pm 13.21	147.87 \pm 96.58	55.43 \pm 10.22
Healthy controls (C)	6	25.93 \pm 5.08	155.53 \pm 15.86	158.33 \pm 86.88	56.32 \pm 10.32
P value		$P_1 = 0.007$; $P_2 = 0.651$; $P_3 = 0.003$	$P_1 = 0.003$; $P_2 = 0.677$; $P_3 = 0.006$	$P_1 = 0.047$; $P_2 = 0.247$; $P_3 = 0.004$	$P_1 = 0.008$; $P_2 = 0.932$; $P_3 = 0.007$

* P_1 = group A vs group C; P_2 = group B vs group C; P_3 = group A vs group B.

DISCUSSION

The formation of FVIII inhibitor is a serious complication in replacement therapy in HA patients. The mechanism remains unclear, and a complex interaction of several variables likely leads to inhibitor formation in this patient group (Astermark, 2006; Reding, 2006). HA patients may produce a FVIII inhibitor because of immune balance being damaged (offset) primarily by interfering (presenting a) with antigen-presenting influence inhibitors produced with immune disorders. For extracellular proteins, such as exogenously administered FVIII in HA, processing of the peptide antigens is mediated by MHC class II molecules (Lechler et al., 1996). The immune response to FVIII is dependent on the interaction of different CD4⁺ T-cell subsets (Th1, Th2, and Th3) specific for FVIII.

Inhibitor formation in HA patients

In the present study, we found that HA patients with high FVIII antibody titers were mostly medium types (5/6), with one severe type with low FVIII: c concentration. These patients may received more amounts of and an earlier exogenous of FVIII, which is helpful for the generation of inhibitor to the FVIII (Sharathkumar et al., 2003; Lusher et al., 2004; Chalm-

ers et al., 2007; Gouw et al., 2007). At the same time, FVIII or vWF protein of these patients may exhibit abnormalities in their molecular structure or in the vision of these patients, which would increase the effect of immune recognition of T cells.

Role of CD4⁺CD25^{high} Treg cells in HA patients

Regulatory T (Treg) cells, sometimes known as suppressor T cells, are a specialized subpopulation of T cells, which suppress activation of the immune system and thereby maintain tolerance to self-antigens (Wood and Sakaguchi, 2003). The Treg cell field is further complicated by reports of additional suppressive T cell populations, including Tr1, Th3, CD8⁺CD28⁻, and Qa-1 restricted T cells. Treg cells, specifically expressing CD25 and transcription factor Foxp3, account for 5-10% of normal human peripheral blood CD4⁺ T cells (Sakaguchi, 2005). Tregs can inhibit effective T cell proliferation and cytokine production, so as to prevent CD8⁺ cells from differentiating into fully functional cytotoxic cells. Once lacking Treg cells, these inhibitory functions will return (Tang and Bluestone, 2008). *In vitro* experiments showed that only CD25^{high} T cells have immunosuppressive effects (Baecher-Allan et al., 2001), and that the number and function of Treg cells were related to pathogenesis in a variety of immune diseases.

In this study, CD4⁺CD25^{high} (as a specific molecular marker of Treg cells) was higher in inhibitor patients than in the non-inhibitor patients. However, the percentage of CD4⁺CD25^{high} Treg cells in the non-inhibitor patients was not significantly different from that of healthy controls. These results demonstrate that autoimmune hyperfunction exists in hyperthyroidism inhibitor-positive patients. The increase in the number of Treg cells may be its own feedback to the antibodies produced, and reduces the body's immune response to FVIII. At present, the negative regulation mechanism of Treg cells in the immune response is not fully understood. In our research, the proportion of CD4⁺CD25⁺ Treg cells in inhibitor-positive HA patients is high, which may be related to CD4⁺ T cells releasing immunosuppressive factors, such as TGF- β , to inhibit the production of FVIII antibodies. On the other hand, CD4⁺ T cells can differentiate into effector Treg cells secreting IL-10 or TGF- β by interacting with Treg cells (Tang and Bluestone, 2008). TGF- β is not required for Treg functionality, in the thymus, since thymic Treg from TGF- β insensitive TGF β RII-DN mice are functional. Treg cells can also play an immunosuppressive effect by the non-restricted MHC direct contact between cells, and this role in inhibitor-positive patients will eventually lead to immune dysfunction (Maloy et al., 2003). Recently, Walters et al. (2009) found that short-term and low-dose anti-CD3 increased the percentage of Treg cells and prevented the formation of anti-FVIII antibodies, confirming the negative regulatory role of Treg cells in the formation of the inhibitor. This observation requires confirmation with additional experiments.

Immune role of cytokines secreted from T cells

Antibody production by B cells is controlled by a complex interaction of different CD4⁺ subsets, each specific for the presenting antigen. Th1 cells can release pro-inflammatory cytokines such as IL-2, IL-12, and IFN- γ , which can stimulate the inflammatory immune response and activate cytotoxic T cells. Th2 cells can release anti-inflammatory cytokines such as IL-4 and IL-10, which inhibit the induction of Th1 cells and B cell activation. Th1/Th2 cell

dysfunction or imbalance is the source of a variety of autoimmune diseases. Treg cells play an inhibitory role in immune regulation, and their increase in activity likely breaks the Th1/Th2 balance in the inhibitor patient, causing a change in the Th1- and Th2-related cytokines. Th1 and Th2 cells play a role in immune regulation through their secreted cytokines. We observed that the most important inflammatory cytokines, IFN- γ and IL-2, which are secreted by Th1 cells and which can stimulate the inflammatory immune response and activate cytotoxic T cells, were decreased in inhibitor patients. Conversely, levels of the anti-inflammatory cytokine IL-10 secreted by Th2 cells and of TGF- β from Treg cells were significantly increased in inhibitor patients compared with those in non-inhibitor patients. IL-10 inhibits the proliferation and function of Th1 cells and antigen-presenting cells - the higher its level, the stronger the immune inhibitory function. Furthermore, TGF- β , which is an important immunosuppressive cytokine in immune regulation, also increased.

The inhibitory antibodies in HA patients are polyclonal IgG antibodies (Fulcher et al., 1987; Gilles et al., 1993). IgG1 and IgG2 antibodies are driven by the action of Th1 cells, while IgG4 is a Th2 cell-driven subclass. IgG4 is frequently the major component of anti-FVIII antibodies, and Th2 cells can also stimulate B cells that produce IgG4 antibody subclasses. Therefore, Th2 cells have a dual role in the immune response with both upregulatory and downregulatory effects. In summary, antibody production by B cells is controlled by a complex interaction of different CD4⁺ subsets, each specific for the presenting antigen. In both HA and acquired hemophilia patients, there is a strong positive correlation between inhibitor titer and the proportion of Th2-driven IgG4 anti-FVIII antibody (Reding et al., 2002).

CONCLUSION

In summary, the formation of hemophilia FVIII inhibitors was related to the individual's immune functional status. Treg cells are the key component of immune tolerance to self and foreign antigens (e.g., exogenous FVIII). Treg cells suppress the immune response to these therapeutic coagulation factors and protein presented and induced by B and T cells. Therefore, in-depth study of Treg cell function will be helpful for further elucidating the pathogenesis, diagnosis, and treatment of hemophilia inhibitor patients.

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