Understanding of the roles of $CD4^+CD25^+$ T cells and Tregs in development of anti-FVIII in hemophilia A: insights for inhibitor development prevention

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Hemophilia A, characterized by factor VIII (FVIII) deficiency, can be managed with factor replacement therapy. However, FVIII-neutralizing antibodies developed in people with hemophilia A (PwH) can reduce treatment efficacy. This study investigated the impact of FVIII inhibitors on immunity in PwH.

Twenty PwH (with the presence and absence of inhibitors) and 10 healthy individuals have participated. $CD4^+$, $CD4^+CD25^+$, Treg cell percentages, proliferation levels, and cytokine levels in cell-culture supernatants were evaluated. An increased $CD4^+CD25^+$ T-cell subset was noted in PwH without inhibitors. $CD4^+$ and $CD4^+CD25^+$ T cells showed increased proliferation, while Treg cells had decreased proliferation in PwH without inhibitors compared to controls. With rFVIII added to cell culture, $CD4^+CD25^+$ proliferation decreased and Treg proliferation increased in PwH with inhibitors, while it remained unchanged in other groups. IL-10 was reduced in both PwH groups. TGF-β was decreased in PwH with inhibitors compared to those without inhibitors. IL-10/TGF-β ratio was reduced in both PwH groups compared to controls. rFVIII in culture conditions significantly reduced TNF-α only in PwH with inhibitors, while TGF-β was decreased in PwH without inhibitors and healthy controls.

Monitoring T-cell immunity in PwH before anti-FVIII antibody development may improve treatment success and help prevent antibodies and related complications. ***Blood Coagul Fibrinolysis*** **36:371–380 Copyright © 2025 Wolters Kluwer Health, Inc. All rights reserved.**

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Introduction

Hemophilia A is an X-linked hereditary bleeding disorder characterized by the deficiency of factor VIII, resulting from mutations in the FVIII gene. FVIII activity can be completely absent or can range from 5 to 40% of normal levels in blood. Depending on the factor VIII activity, hemophilia A is classified as mild, moderate, or severe. Although people with hemophilia (PwH) were previously treated using only whole blood and fresh plasma for the replacement of clotting factor VIII (FVIII), current treatments include plasma-derived FVIII (pdFVIII) concentrates, monoclonal antibody-purified pdFVIII products, or recombinant FVIII proteins (rFVIII) are used for therapeutic purposes. Studies have shown that FVIII administration can impair the activation of T cells, leading to atypical regulatory T cell activity, which triggers the synthesis of inhibitor (INH) protein against FVIII in one in three PwH [1]. These inhibitory proteins against FVIII can neutralize the effects of FVIII replacement and may lead to a high risk of uncontrollable bleeding, increased morbidity, and mortality [2,3]. Inhibitor proteins against FVIII are generally directed at specific epitopes of the large FVIII molecule, primarily the A2, C1, and C2 domains [4,5]. They are typically of the immunoglobulin G1 and G4 subclasses [6], where cytokines from Th1 cells stimulate IgG1 and IgG2 subclasses, while Th2 cells stimulate the IgG4 subclass. However, studies have reported that the class switch to IgG4 occurs only in PwH with inhibitors, neither in healthy individuals nor PwH without inhibitors [7]. Therefore, it is suggested that the Th2-mediated immune responses may dominate the development of inhibitory antibodies in PwH [8]. Several factors are associated with the development of FVIII inhibitors including specific cytokine gene polymorphisms, tumor necrosis factor (TNF)-α, and interleukin (IL)-10, the type of FVIII gene mutation, the mode of FVIII protein administration, and the immunologic state of PwH [9–11].

While many studies have investigated the immunologic mechanism of inhibitory protein development, a complete understanding remains elusive. Research has demonstrated that immune responses leading to inhibitor development are controlled by a $CD4^+$ T cell-dependent process. This mechanism involves multiple steps: stimulation of $CD4^+$ T cells and B cell maturation by follicular

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$CD4^+$ T cells, class switching mediated by dendritic cells, and B cells acting as antigen-presenting cells (APCs), and production of antibodies by IgG-secreting plasma cells, and memory B cells [8,12–14]. Upon activation, $CD4^+$ T cells then differentiate into Th1 and Th2 cells, and regulatory T cells (Tregs). The cytokines secreted from Th1 and Th2 cells not only stimulate B cells differentiation into antibody-producing cells but also regulate immune system functions [15]. Specifically, Th1 cells secrete pro-inflammatory cytokines including TNF-α, interferon (IFN)-γ, and IL-2, that activate APCs, while Th2 cells generate IL-4, IL-5, and IL-13, which drive immunoglobulin class-switching to IgE [16].

In this study, we examined T cell pathology in PwH with inhibitory protein, by analyzing $CD4^+$, $CD4^+CD25^+$ (activated T cells) and $CD4^+CD25^+CD127^-$ (Tregs) in peripheral blood samples. To further characterize T cell subset functions, we evaluated their proliferative responses and pro-inflammatory/anti-inflammatory cytokine production in peripheral blood mononuclear cell (PBMC) cultures both with and without rFVIII stimulation.

Materials and methods

Participants

The study cohort included 20 PwH who were diagnosed and followed up in the Hereditary Bleeding Disorders Unit of the Oncology Institute, Istanbul University, along with 10 healthy controls. PwH were stratified according to the development of inhibitor antibodies. *Study groups;* Group 1, severe PwH with inhibitors [$n = 10$; mean age 34.7 ± 18.3 years; mean FVIII levels 0.34 ± 0.3 IU/l; inhibitor titer 20.8 Bethesda Unit (BU) (range:4–114)]. Group 2, PwH without inhibitors [$n = 10$; mean age 19.8 ± 14.8 years; mean FVIII levels 0.27 ± 0.18 IU/l; inhibitor level < 0.6 BU/ml]. Group 3, healthy controls, [$n = 10$, mean age 34 ± 7.39 years).

Clinical characteristics of the PwH are summarized in Table 1. Ten of the 20 PwH had anti-FVIII antibodies. Among inhibitor-positive patients, the mean diagnosis time was 12 months (range: 1–120 months), family history percentage in both groups varied between 50 and 60%. All participants underwent screening for Hepatitis B surface antigen (HBsAg), HIV, and hepatitis C virus (HCV) by using Architect i2000SR (Abbott Inc., Wiesbaden, Germany). Complete blood counts (CBCs) were evaluated by Coulter LH-780 (Beckman Coulter Inc., Miami, Florida, USA). The individuals who had anemia (hemoglobin levels <9.5 g/dl), hematological or immunological diseases, or seropositivity for HCV, HBs Ag, and HIV were excluded from the study. The six PwH with inhibitors were treated with rFVIII, fitusiran, emisizumab, activated prothrombin complex concentrates (APCCs), and marstazimab, PwH with treatment-related bleeding or thrombosis at the time of the blood sampling were excluded. Blood samples of the subjects were collected into heparinized sampling tubes (Vacuette tube, BD, Plymouth, UK), and transported within 15 min to Istanbul University, Aziz Sancar Institute of Experimental Medicine, Department of Immunology. The study was approved by the Local Institutional Ethics Board of Istanbul University Faculty of Medicine Clinical Studies (Committee no: 29.09.2020/165181).

FVIII and inhibitor-level measurements

Factor VIII (FVIII) levels were quantified using the one-stage clotting method with a CS-2500 coagulation analyzer (Sysmex, Norderstedt, Germany). Inhibitor levels were determined via the classic Bethesda method [17].

Flow cytometry analyses of $CD4^+$ T cell subsets

PBMCs were isolated from heparinized blood using Ficoll density centrifugation with Histopaque (Sigma, St. Louis, Missouri, USA). Freshly isolated PBMCs were washed with ice-cold staining buffer (PBS containing 2% FBS) and stained with the following surface markers:BV785-conjugated antihuman CD3, BV510-conjugated antihuman CD4, APC-Cy7conjugated antihuman CD127, and (PE)-Cy5-conjugated antihuman CD25 antibodies (all mAbs used for flow cytometry were applied at the manufacturer-recommended concentrations (5 μl), all purchased from

Table 1 Clinical characteristics of people with hemophilia A

PwH	INH(+)*	INH(-)*
Total number (*n*)	10 (F-M)	10
Age (*year*) median (Min-Max)	31.5 (4–63)	18 (4–49)
Diagnosis age (*month*) median (Min-Max)	12 (1–120)	9 (1–84)
PwH's age *(year)* of Inhibitor detection median (Min-Max)	11 (1–38)	–
Family history of Hemophilia A	6/10	5/10
Presence of an inhibitor (+) individual in the family	0/10	0/10
History of vital organ bleeding	1/10	0/10
Walking disorder	9/10	4/10
History of extensive FVIII exposure	8/10	4/10
Treatment		
Continuing current treatment	Four PwH have used fitusiran, 2 have used rFVIIa, each of the remaining PwH used emicizumab, aPCC, marstacimab, and concizumab	FVIII
FVIII level (mean ± SD)	0.34 ± 0.3	0.27 ± 0.18
Inhibitor titer median (Min-Max)	20.8 (4–114)	–

*INH(+): PwH with inhibitors. INH(−): PwH without inhibitors.

Biolegend, USA) for 20 min (min) at room temperature, in the dark. After staining, cells were washed once and then re-suspended in 500 μl of 1% paraformaldehyde in PBS before by flow cytometer acquisition.

Lymphocytes were first identified by their forward scatter (FSC) and side scatter (SSC) properties, to exclude debris and nonlymphoid populations. Unstained PBMC samples were used as controls, due to their auto-fluorescence properties. To improve the precision, singlet gating was performed by plotting FSC-A against FSC-H. Subsequently, the cells were gated based on their CD3 and CD4 expressions, to isolate T cells from other immune cells. From this population, $CD3^+CD4^+CD127^-$ regulatory T cells were identified, followed by gating on $CD3^+CD4^+CD127^-CD25^+$ cells to accurately determine the proportion of Tregs in the sample (Fig. 1) [18]. On the other hand, $CD3^+CD4^+CD25^+$ T cells were accepted as activated T cells [19]. All flow cytometry analysis in this study was performed on a NovoCyte flow cytometer, using Novo Express software (ACEA Biosciences, USA), following the Minimum information about a Flow Cytometry Experiment (MIFlowCyt) standards, to ensure reproducibility, transparency. NovoCyte systems features three lasers (e.g., 488, 561, and 640 nm), with detection of up to 16 fluorescence parameters, and uses a pressure-driven fluidics system, for enhanced stability during sample acquisition. All data were analyzed by FlowJoV10 (BD Bioscience, USA).

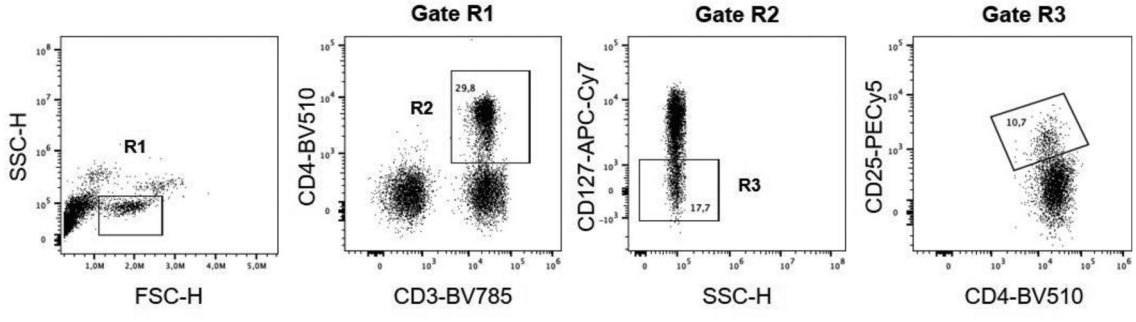
Cell cultures and proliferation assay with CFSE

PBMCs isolated from heparinized blood using Ficoll density centrifugation with Histopaque were preserved in RPMI 1640 medium (GIBCO, Grand Island, New York, USA), supplemented with 10% fetal bovine serum-FBS (Seromed, Biochrom, Berlin, Germany), 2 mmol/l L-glutamine (GIBCO), 1 : 100 diluted MEM nonessential amino acids (GIBCO), and 100 U/ml penicillin and 100 g/ml streptomycin (Both Sigma, St. Louis, Missouri, USA). Cells were labeled with 5 μmol/l final concentration of carboxyfluorescein succinimidyl ester (CFSE) (Thermo Fisher Scientific, USA) by incubation for 6 min at 4°C in the dark, and cultured for 120 h at 37°C in a 5% CO_2 in 48-well plates according to our previous studies [20,21] under three conditions: unstimulated, 1 U/ml albumin-free recombinant FVIII (rFVIII, Kogenate FS, Bayer, Bayer Corporation, Elkhart, Indiana, USA) [1,22], and 5 μg/ml phytohemagglutinin (PHA, Thermo Fisher, USA). Following 120 h of incubation, cell-culture supernatants were collected and stored at -80°C until required for ELISA analyses. Then, cells were stained with BV785-conjugated antihuman CD3, BV510-antihuman CD4, APC-Cy7-conjugated antihuman CD127 and (PE)-Cy5conjugated-antihuman CD25 antibodies (all from Biolegend, USA), analyzed on an ACEA Novocyte flow cytometer, to assess proliferation percentages of total lymphocytes, $CD4^+$ T cells, total $CD4^+CD25^+$ T cells (effector and Treg), and suppressive $CD4^+CD25^+CD127^-$ Treg cells (Fig. 2a). Spontaneous cell proliferation (unstimulated, Fig. 2b), proliferation in response to rFVIII (Fig. 2c) and phyohemaglutinin as a positive control (Fig. 2d) were evaluated. Throughout the cell cultures, cell viability and morphologic analyses were monitored daily by inverted light microscopy, and forward scatter/side scatter (FSC/SSC) gating was used to exclude dead cells/debris during analysis.

Determination of cytokines from cell culture supernatants

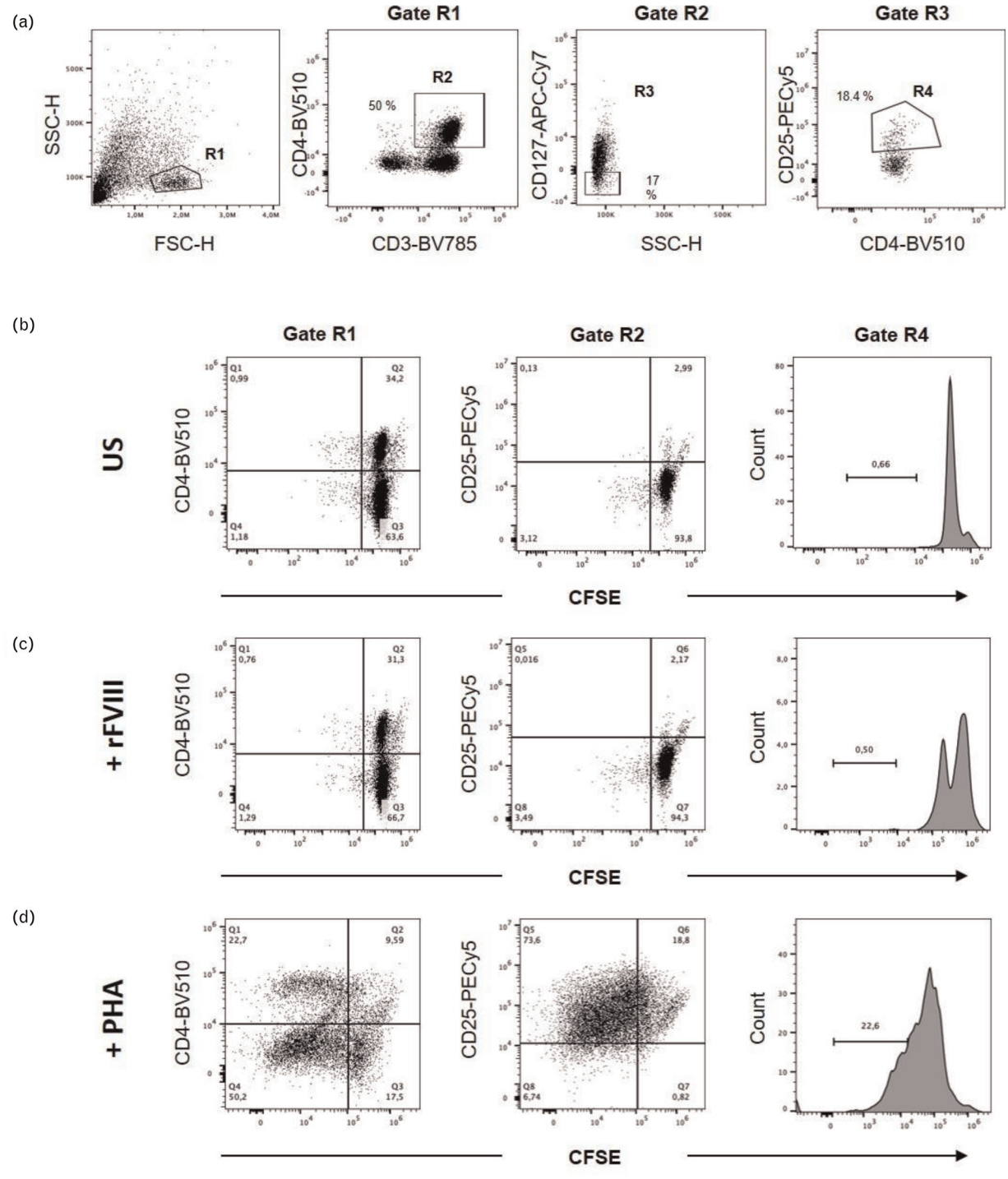
Cell supernatants from unstimulated, rFVIII stimulated (1 U/ml), and PHA (5 μg/ml, positive control) cultures were stored at -80°C. IL-10, IL-13, TNF-α, TGF-β, IL-2, and IFN-γ concentrations were measured using sandwich ELISA (Diaclone Research, Besancon, France). The detection limits were 8.6 pg/ml for TGF-β, 4.9 pg/ml

Fig. 1



Flow cytometric gating strategy for Treg cell analysis. A representative figure for the gating strategy of freshly isolated peripheral blood mononuclear cells (PBMCs), in order to evaluate the frequency of peripheral blood $CD4^+$ T cells, $CD4^+CD25^+$ activated T cells and Treg cells. (Gates; R1: lymphocytes, R2: $CD3^+CD4^+$ T cells, R3: $CD3^+CD4^+CD127^-$ T cells, R4: $CD3^+CD4^+CD25^+CD127^-$ Treg cells).

Fig. 2



Cell proliferation analysis. A representative figure for the analysis of proliferation. (a) Gating strategy for investigation of the proliferation percentages of peripheral blood $CD4^+$ T cells, $CD4^+CD25^+$ activated T cells and Treg cells. Demonstrative plots for (b) spontaneous proliferation (c) stimulation with recombinant factor VIII (1 U/ml), and stimulation with phytohemagglutinin (PHA, 5 μg/ml). Values indicate percentages of proliferating cell subsets following cell culture. (Gates; R1: lymphocytes, R2: $CD3^+CD4^+$ T cells, R3: $CD3^+CD4^+CD127^-$ T cells, R4: $CD3^+CD4^+CD25^+CD127^-$ Treg cells).

for IL-10, 1.5 pg/ml for IL-13, 8 pg/ml for TNF-α, 5 pg/ml for IFN-γ and 7 pg/ml for IL-2. IFN-γ and IL-2 levels were not detected, because they were below the detection level.

Statistical analysis

Data were analyzed using the SPSS 22 package (IBM SPSS Statistics for Windows; IBM Corp., Armonk, New York, USA). The results were expressed as mean ± standard error mean (SEM). The normality of data distribution was evaluated using the Shapiro-Wilk test. Comparison of the experimental groups was performed using one-way analysis of variance (ANOVA). Post hoc comparisons were conducted using Tukey's HSD test for variables with equal variances, and Tamhane's T2 test for variables with unequal variances, as determined by Levene's test. Bonferroni correction was applied where appropriate to adjust for multiple comparisons. Additionally, correlation analyses were performed using Spearman's test for unequal variances, and Pearson's test for equal variances. Statistical significance was defined as a P value less than 0.05. Our analyses revealed a medium-to-large effect size ($\eta^2_p = 0.204$), indicating that the findings are biologically meaningful and statistically interpretable [23,24].

Results

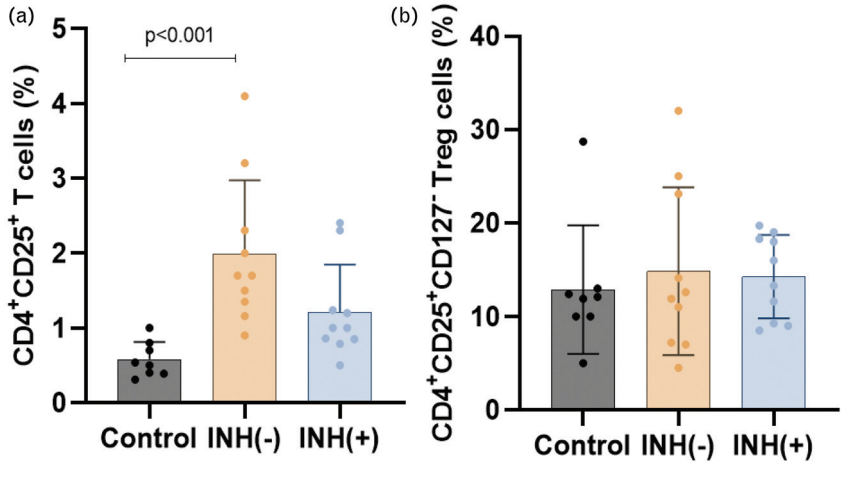
Increased expression of activated CD4$^+$ T cells

When the peripheral blood CD4$^+$ T cell subsets were investigated, the percentages of CD4$^+$CD25$^+$ T cells, were found to be higher in PwH without inhibitors, compared to healthy controls ($P < 0.001$) (Fig. 3a). The differences in CD4$^+$ T cells and CD4$^+$CD25$^+$CD127$^-$ Tregs among the study groups were nonsignificant (data not shown, in Fig. 3b, respectively).

Influence of recombinant FVIII on the proliferative responses of CD4$^+$ T cell subsets

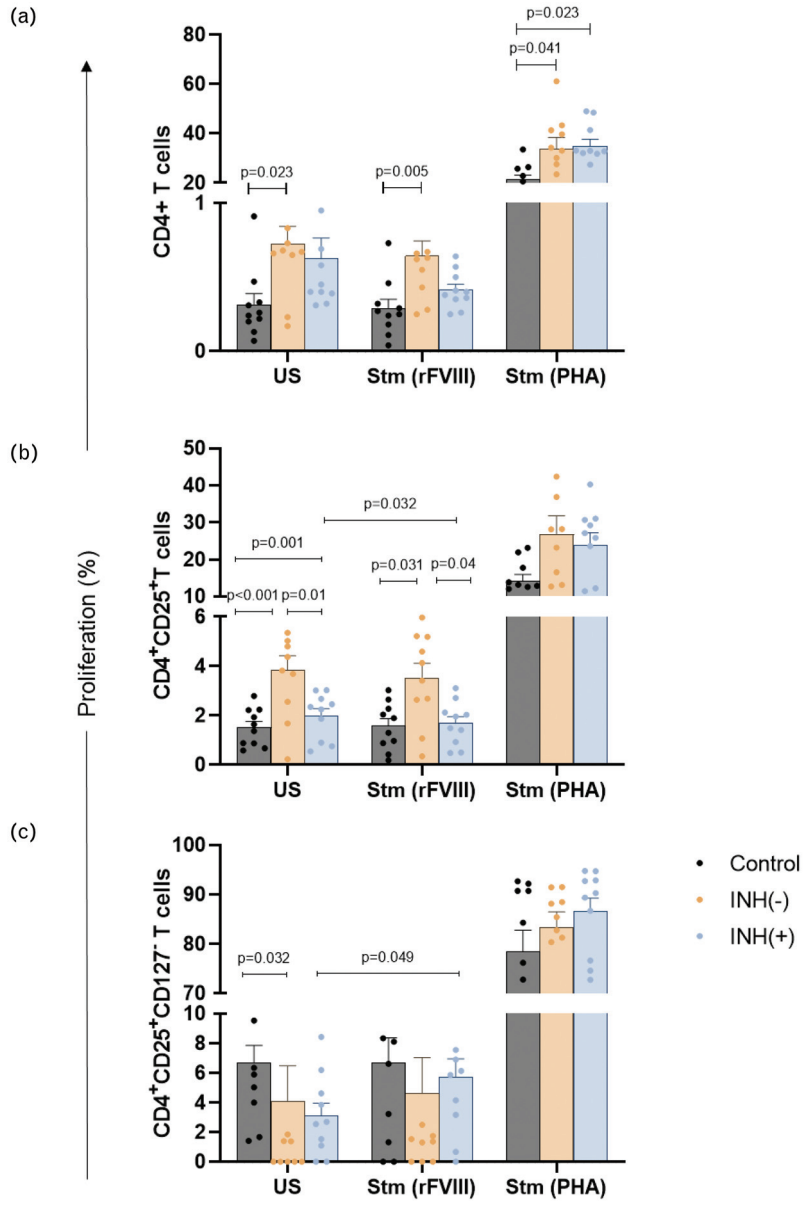
To investigate the effects of rFVIII on cell proliferation, PBMCs from PwH with or without inhibitor, and from healthy controls were cultured either with or without rFVIII and PHA. Proliferation was assessed following 120 h of cell culture. When the proliferation percentages of CD4$^+$ T cells were examined, PwH without inhibitors had significantly greater spontaneous proliferation, than healthy controls ($P = 0.023$), though proliferation levels of PwH with and without inhibitors were similar between PwH with and without inhibitors. With the addition of rFVIII, PwH without inhibitors had significantly increased proliferative responses relative to healthy controls, but the responses were similar between both PwH groups. Furthermore, no significant difference in CD4$^+$ T cell proliferation was observed in between cultures with and without rFVIII in any of the study groups. As expected, the presence of PHA triggered significant proliferation of CD4$^+$ T cells in all study groups. In both PwH groups, responses were increased relative to healthy controls ($P = 0.023$ and $P = 0.041$, respectively) (Fig. 4a). The proliferation levels of CD4$^+$CD25$^+$ T cells represent as the activated T cells [19]. There were significant differences among the PwH groups in the absence of any stimulation. Proliferation levels of PwH without inhibitors were significantly higher, than those of PwH with inhibitors and controls ($P = 0.01$ and $P = 0.001$, respectively), while, both PwH groups had significantly increased proliferation of CD4$^+$CD25$^+$ T cells, compared to healthy controls. With the addition of rFVIII, proliferation levels in the group without inhibitors were significantly stronger than the other hemophilic group and controls ($P = 0.04$ and $P = 0.031$, respectively). rFVIII

Fig. 3



The frequencies of CD4$^+$ T cell subsets in peripheral blood samples of PwH and healthy control groups. The frequencies of CD4$^+$CD25$^+$ T (a) and CD4$^+$CD25$^+$CD127$^-$ Treg cell subsets (b) present in freshly isolated peripheral blood samples of healthy controls, PwH without inhibitors and PwH with inhibitors groups. *Control: Healthy control groups ($n = 8$); INH(+): PwH with inhibitor ($n = 10$); INH(-): PwH without inhibitor ($n = 10$).

Fig. 4



The proliferation percentages of the $CD4^+$ T cell subsets following cell culture in the absence and presence of rFVIII and PHA. The proliferation percentages of $CD4^+$ (a), $CD4^+CD25^+$ T cells (b), and Treg cells (c) following 120 h of cell culture, with the absence or presence of rFVIII and PHA, were analyzed by flow cytometry in healthy controls, and in PwH without inhibitors and PwH with inhibitors groups. *Control: Healthy control groups ($n=10$); INH(+): PwH with inhibitor ($n=10$); INH(-): PwH without inhibitor ($n=10$). Stm, stimulated; US, unstimulated.

significantly reduced $CD4^+CD25^+$ T cells proliferation in PwH with inhibitors ($P=0.032$). PHA induced significant proliferation in all study groups, though no significant differences were observed between healthy controls, and either PwH group (Fig. 4b). The $CD4^+CD25^+CD127^-$ T cell subset represents Treg cells with immune regulatory potential [20,21]. The spontaneous proliferative responses of Treg cells were significantly lower in PwH without inhibitors, than in healthy controls ($P=0.032$). rFVIII induced similar proliferative responses among the study groups, but only, the increase in Treg proliferation was significant in PwH with inhibitors ($P=0.049$). PHA also triggered significantly increased responses in all study groups ($P<0.001$), though no significant differences were observed between healthy controls, and either PwH group (Fig. 4c).

Cytokine contents of peripheral blood mononuclear cell culture supernatants in relation to rFVIII triggering

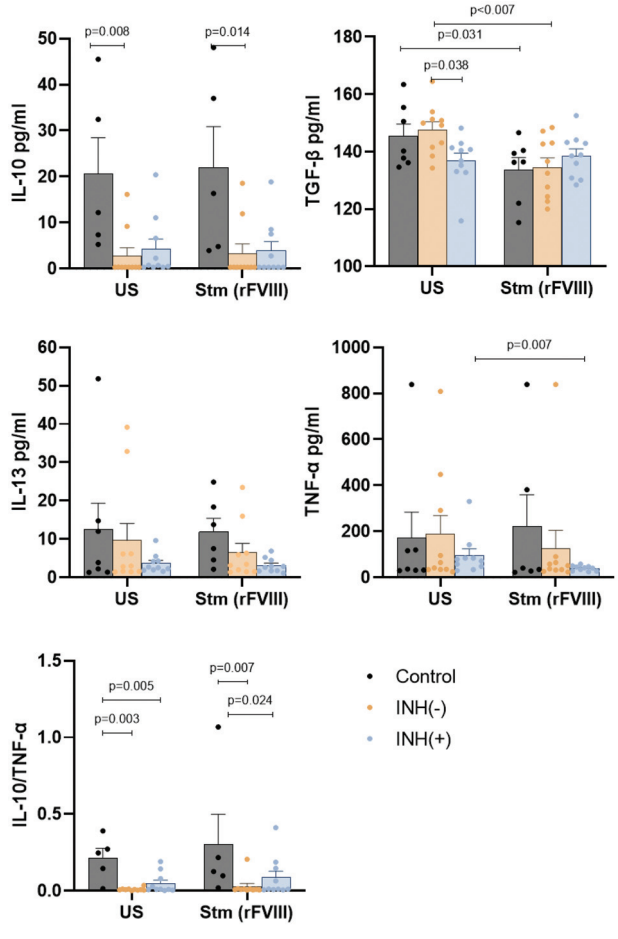
The cytokine contents of cell culture supernatants of PBMCs from both PwH groups and healthy controls,

both with and without rFVIII stimulation, were investigated. IL-10, as the most significant regulatory cytokine was reduced in both PwH groups, with PwH without inhibitors showing significantly lower levels than healthy controls ($P = 0.008$). A similar pattern was observed with rFVIII stimulation, while the presence of rFVIII in cell culture conditions did not significantly alter IL-10 levels any group (Fig. 5a). TGF-β, another important regulatory cytokine, showed significantly lower expression in PwH with inhibitors compared to PwH without inhibitors ($P = 0.038$). rFVIII stimulation decreased TGF-β levels in PwH without inhibitors ($P = 0.007$) but remained unchanged in inhibitor group (Fig. 5b). IL-13, an important Th2 cytokine, tended to be lower in PwH without inhibitor, with the lowest levels in PwH with inhibitors. No significant changes were observed among the PwH groups with or without rFVIII stimulation (Fig. 5c). TNF-α, a key pro-inflammatory cytokine showed no significant differences in spontaneous levels among groups. However, rFVIII stimulation down-regulated TNF-α in PwH with inhibitors ($P = 0.007$) (Fig. 5d). The IL-10/TNF-α ratio, reflecting anti-inflammatory status, was significantly lower in PwH groups compared to healthy controls ($P = 0.005$ and $P = 0.003$, respectively). rFVIII stimulation increased this ratio in inhibitor group compared to noninhibitor group ($P = 0.024$), and healthy controls ($P = 0.07$) (Fig. 5e).

Discussion

The development of inhibitory antibodies in PwH is a critical issue, as it interferes with treatment and leads to high morbidity and mortality. Therefore, screening for inhibitor development, is essential for comprehensive hemophilia treatment to ensure effective medical care [25]. Accordingly, it is essential to understand the mechanism of

Fig. 5



The cytokine contents of cell culture supernatants of PwH and healthy controls. Cytokine levels of IL-10 (a), TGF-β (b), IL-13 (c), TNF-α (d) IL-10/TNF-α ratio was evaluated (e), in the supernatants of PBMC cell culture in healthy controls, and in PwH with and without inhibitors following 120 h of cell culture with the absence or presence of rFVIII. *Control: Healthy control groups ($n = 7$); INH(+): PwH with inhibitor ($n = 10$); INH(-): PwH without inhibitor ($n = 10$). Stm, stimulated; US, unstimulated.

inhibitor antibody development and closely follow up PwH before inhibitor development, in terms of protecting their general health situation and life quality.

In our study, we investigated T cell subsets, and cytokine profiles in the supernatants of PBMC cultures following stimulation with rFVIII and PHA. Previous studies have highlighted the importance of $CD4^+$ T cells in inhibitor antibody development [15,26]. While effector T cells activate B cells, to differentiate into plasma cells, and produce the anti-FVIII response, Tregs suppress this response, and inhibit antibody production [27]. Although studies have demonstrated the roles of activated $CD4^+$ T cells during the initial development of antibodies against FVIII [8], in cases of multiple exposure to the same antigen, memory cells, or long-lived plasma cells take control of antibody responses [28,29]. Our results showed higher $CD4^+CD25^+$ T cell and Treg levels in PwH with or without inhibitor compared to healthy controls before incubation (Fig. 3a,b). Notably, following 120 h of incubation, both T cell types were stimulated, with levels higher in the PwH without inhibitors than inhibitor-positive group (Fig. 4a,b). Moreover, rFVIII stimulation increased Treg proliferation only in inhibitor-positive group, supporting Tregs' immunomodulatory role (Fig. 4c). These findings align with the studies demonstrating the delicate balance between effector T cells and Tregs in inhibitor development [30,31]. However, the results of several studies investigating the roles of T cell subsets in inhibitor development are conflicting. Some studies showed an increase in $CD4^+$ T cells [15], whereas others reported a decrease in Tregs, with unchanged levels of $CD4^+$ T cells in PwH with inhibitor [32]. In an experimental study by Smith *et al.* [33], it was reported FVIII-specific Tregs exhibited a stronger suppressive effect than nonspecific Tregs, and increased Treg levels were associated with decreasing inhibitor titers during the development of antigen tolerance [15,34,35].

Several studies have also demonstrated the effects of the cytokines secreted from $CD4^+$, $CD4^+CD25^+$, and Tregs in the immunoregulation of antibody responses [36–38]. Tregs exert their suppressive effects on T cells, B cells, natural killer (NK) cells, dendritic cells through humoral pathways, including IL-10, TGF-β, IL-35, granzyme B, as well as via cell-to-cell contact mechanisms through cell surface receptors such as CTL-1 [39,40]. However, the suppressive effects on cells may vary depending on the location, and the type of the immune reaction between T cells and APCs [39]. Under circumstances, the differentiation of naive T cells into Th1 and Th2 cells and the generation of Tregs are suppressed during the immunoregulatory mechanism [41,42].

In our study, IL-10, TGF-β, IFN-γ, and IL-13 concentrations were measured to assess the function of $CD4^+$ and $CD4^+CD25^+$ T cells, and Tregs. IL-10 and TGF-β are synthesized by Tregs and thereby modulate pro-inflammatory functions, and important for the generation and maintenance of Tregs. Inflammation regulation is achieved by IL-10 via inhibiting pro-inflammatory cytokines such as TNF-α and IL-6 secretions [43]. According to our results, IL-10, was lower in both PwH groups, than healthy controls, and the addition of rFVIII did not alter IL-10 levels in any group, consistent with the previous studies [10] (Fig. 5a). TGF-β is also participates in the regulatory function of Tregs [38]. TGF-β levels were also demonstrated lower expression in inhibitor-positive group compared to no PwH without inhibitors, and rFVIII stimulation reduce TGF-β levels in PwH without inhibitors and healthy controls, so that the difference between both groups disappeared with the addition of rFVIII (Fig. 5b). Based on our findings, the decreased levels of both cytokines, in the PwH with inhibitors group may indicate an insufficient T cell response against antibody development. Nevertheless, the suppression of TGF-β secretion in inhibitor-negative individuals, and controls with the addition of rFVIII, along with the lack of change in inhibitor group, may suggest insufficient TGF-β secretion during the phase of inhibitor development phase, and inducing effect of rFVIII in the generation of inhibitor antibody. Chaves *et al.* [10] also reported lower IL-10 secretion in monocytes of PwH without inhibitors and with inhibitors compared to healthy controls and demonstrated the stimulation of IL-10 secretion in all groups with the addition of rFVIII with which aligns with our findings. They also reported a higher IL-10/TNF-α ratio in the PwH with inhibitors, suggesting the dominance of a T cell-dependent pro-inflammatory cytokine response at the beginning of FVII treatment, but an anti-inflammatory/regulatory response following multiple exposures to FVIII [10]. However, the IL-10/TNF-α ratio was not altered between PwH groups based on our results. The decreased IL-10/TNFα ratio in both groups was observed compared to healthy controls (Fig. 5e), though, the addition of rFVIII in the culture medium caused an increased ratio in inhibitor group compared to the PwH without inhibitors. The decreased IL-10/TNFα in the group without inhibitors suggests a pro-inflammatory state mediated by $CD4^+CD8^+$ T cells before inhibitor development. The increased IL-10/TNF-α ratio with FVIII addition may indicate the stimulation of an anti-inflammatory response, supporting the findings of Chaves *et al.* [10].

Moreover, we demonstrated decreased IL-13 and TNF-α levels in PwH with inhibitors compared inhibitor-negative and control groups, both in unstimulated and rFVIII-stimulated conditions, consistent with results of Chaves *et al.* [10]. We observed a 58.5 and 33% suppression in TNF-α levels with the addition of FVIII in both PwH groups, respectively. In contrast to our findings, Karim *et al.* [44] reported higher TNF-α and IL-10 levels in PwH with inhibitors compared to the no-inhibitor group. However, in another study reported no changes in IL-10,

TGF-β1, and IFN-γ concentrations, with the addition of rFVIII, along with no proliferative responses [45]. The differences in cytokine results among the studies may be explained by the variations in incubation periods, the types and concentrations of FVIII used, the cytokine evaluation methods (intracytoplasmic or in supernatant), and the timing of inhibitor generation depending on the PwH's immunologic state.

A limitation of this study was that hemophilia patients who developed antibodies against FVIII could not be classified according to the time of inhibitor emergence. In future studies, it would be valuable to classify acute cases before starting immune tolerance induction (ITI) treatment and chronic cases to better investigate the immunoregulatory role of cells and cytokines.

Based on our results, increased percentage of Tregs, and lower IL-10 and TGF-β levels in PwH with inhibitor may suggest an excessive immunosuppressive effect of Tregs, which can also be detrimental in some conditions. Therefore, an aberrantly suppressed immune system may fail to prevent inhibitor antibody development.

Our findings highlight the importance monitoring the importance of monitoring pro-inflammatory and anti-inflammatory responses and assessing T cell-dependent immunity early, before anti-FVIII antibodies develop in PwH. This approach could enable successful treatment, ultimately improve the quality of life in PwH.

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The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Conflicts of interest

This study was supported by the Research Fund of the Istanbul University with the project number: 37038. The authors declare no conflicts of interest.

Artificial intelligence and artificial intelligence assisted technologies were not used in our article.

The authors confirm that they have completed the data sharing notification form.

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