HemaSphere
Powered by EHA

EHA EUROPEAN HEMATOLOGY ASSOCIATION

Article
OPEN ACCESS

Role of Regulatory Cells in Immune Tolerance Induction in Hemophilia A

Sarah J. Schep[1,2], Roger E. G. Schutgens[1], Kathelijn Fischer[1], Jan Voorberg[3], Marianne Boes[2]

Correspondence: Sarah J. Schep (s.j.schep-2@umcutrecht.nl).

Abstract

The main complication of hemophilia A treatment is the development of neutralizing antibodies (inhibitors) against factor VIII (FVIII). Immune tolerance induction (ITI) is the prescribed treatment for inhibitor eradication, although its working mechanism remains unresolved. To clarify this mechanism, we compared blood samples of hemophilia A patients with and without inhibitors for presence of immunoregulatory cells and markers, including regulatory B-cells (Bregs), regulatory T-cells (Tregs), myeloid-derived suppressor cells (MDSCs), and expression of regulatory markers on T-cells (programmed cell death protein 1 [PD1], inducable T-cell costimulator, cytotoxic T-lymphocyte-associated protein 4 [CTLA4]), by use of flow cytometry. By cross-sectional analysis inhibitor patients (N = 20) were compared with inhibitor-negative (N = 28) and ex-inhibitor (N = 17) patients. In another longitudinal study, changes in immunoregulatory parameters were evaluated during ITI (N = 12) and compared with inhibitor-negative hemophilia A patients (N = 36). The frequency of Bregs, but not of Tregs nor MDSCs, was significantly reduced in inhibitor patients (3.2%) compared with inhibitor-negative (5.9%) and ex-inhibitor patients (8.9%; $P < 0.01$). CTLA4 expression on T-cells was also reduced (mean fluorescence intensity 133 in inhibitor versus 537 in inhibitor-negative patients; $P < 0.01$). Fittingly, in patients followed during ITI, inhibitor eradication associated with increased Bregs, increased Tregs, and increased expression of CTLA4 and PD1 on CD4+ T-cells. In conclusion, inhibitor patients express significantly lower frequency of Bregs and Tregs marker expression, which are restored by successful ITI. Our findings suggest that an existing anti-FVIII immune response is associated with deficits in peripheral tolerance mechanisms and that Bregs and changes in immunoregulatory properties of CD4+ T-cells likely contribute to ITI in hemophilia A patients with inhibitors.

Introduction

The congenital bleeding disorder hemophilia A is caused by a deficit of coagulation factor VIII (FVIII), which results in spontaneous and severe bleeding. Prevention and treatment of bleeding consist of lifelong administration of FVIII. Although new therapeutic modalities have improved patient's well-being, 1 major and challenging complication in the treatment of hemophilia A remains: the development of anti-FVIII antibodies (inhibitors). These inhibitors develop in about one-third of all patients with severe hemophilia A, mostly during the first 15–20 exposure days (EDs).[1–4] Anti-FVIII antibodies render FVIII replacement ineffective, and are associated with increased risk of uncontrollable bleeding, increased morbidity, and reduced quality of life.[5,6]

So far, the most successful method to eliminate inhibitors is immune tolerance induction (ITI), in which long-term administration of high-dose FVIII successfully down-modulates the anti-FVIII antibody production in about two-third of patients.[7–10] The working mechanism of ITI is incompletely understood as few studies thus far addressed FVIII-induced immunological changes during ITI.[11–19]

Regarding inhibitor development, several risk factors are described, of which the *F8* gene mutation and age and intensity of first treatment are well established.[2,3,20–23] Polymorphisms in immune response genes also appear to contribute to the inhibitor risk, whereas the role of the FVIII product type or exposure to "danger signals" during FVIII administration is more debated.[24–30]

The strong correlation between *F8* gene mutation and the risk of inhibitor development results from the lack of central tolerance to FVIII in these patients with complete absence of FVIII. Accordingly, FVIII-reactive T-cells and B-cells are not eliminated during development, with the inherent risk to become activated after FVIII administration. Not all severe hemophilia patients, however, develop an inhibitor and also healthy controls and noninhibitor patients can harbor anti-FVIII reactivity.[31–34] Thus, peripheral tolerance mechanisms appear to play a crucial role in counteracting the FVIII-directed immune response.[32,33,35,36] Subsequently, inhibitor formation will occur as soon as this peripheral tolerance fails, either due to genetic vulnerability, exogenous immune system challenges, or a combination of both.[37]

Compared with the prevention of inhibitor development, the question of how to restore tolerance to FVIII may be even more

[1]Van Creveldkliniek, University Medical Center Utrecht, University Utrecht, The Netherlands
[2]Center for Translational Immunology (CTI), University Medical Center Utrecht, University Utrecht, The Netherlands
[3]Department of Molecular and Cellular Hemostasis, Sanquin Research and Landsteiner Laboratory, University of Amsterdam, The Netherlands
Supplemental digital content is available for this article.

HemaSphere (2021) 5:5(e557). http://dx.doi.org/10.1097/HS9.0000000000000557.
Received: 18 December 2020 / Accepted: 9 March 2021

difficult to solve, given the highly effective antigen-specific B- and T-cell memory response in a primed immune system.

The aim of this study is to improve the knowledge regarding the working mechanism of ITI. To this end, we evaluated the role of several immunoregulatory cell types and markers in ITI, which are identified as being involved in tolerance mechanisms in other (auto-)immune of inflammatory disorders. These include not only regulatory T-cells (Tregs) but also myeloid-derived suppressor cells (MDSCs), regulatory B-cells (Bregs), and tolerance markers programmed cell death protein 1 (PD1), programmed death-ligand 1 (PD-L1), inducable T-cell costimulator (ICOS), and cytotoxic T-lymphocyte-associated protein 4 (CTLA4).

The immunoregulatory function of Tregs is well-known and limited evidence suggests also a role for Tregs in inhibitor eradication in hemophilia.[13,34] MDSCs are a heterogeneous group of immature myeloid cells with a immunoregulatory function, which are mostly studied in the context of cancer given their role in the tumor immune escape but also have been described as being involved in tolerance induction in autoimmune diseases.[38–40]

Bregs comprise a subset of B cells with immunosuppressive capacity by the production of interleukin-10 (IL-10).[41] The deficit or dysregulated function of these cells has been identified as contributing factor for several autoimmune or inflammatory diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and colitis ulcerosa.[42–44]

By comparison of these immunoregulatory markers between patients with and without inhibitor and evaluation of changes during ITI, we aimed to identify potential key players in the restoration of immune tolerance to FVIII, with the ultimate goal to improve current ITI protocols for hemophilia A.

Methods

Study design

This study involves a cross-sectional and longitudinal evaluation of differences and/or changes in immune-regulatory cells and markers, that is, the immune profile, of hemophilia A patients using flow cytometry-based assays. For the cross-sectional analysis, differences in the immune profile between patients without an inhibitor, patients with an inhibitor and patients with an inhibitor in the past (ex-inhibitor patients) were evaluated. For the longitudinal analysis, changes in immune profile during the course of ITI (pre- and post-ITI) were assessed and compared with a control cohort.

Study population

The participants of this study are all hemophilia A patients treated at the Van Creveldkliniek, the hemophilia treatment center of the University Medical Center Utrecht (UMC Utrecht). The cross-sectional cohort consists of 65 patients, of which the majority (N = 37) was included between 2017 and 2018 (cohort A). Hemophilia patients were included when previously treated with FVIII concentrate (> 50 EDs) and belonging to either one of the following categories: (1) no inhibitor; (2) active inhibitor (defined as an anti-FVIII titer > 0.3 Bethesda Units [BU]/mL, as used at the UMC Utrecht); or (3) inhibitor in the past, successfully treated with ITI (ex-inhibitor patients). Exclusion criteria were the presence of anemia (hemoglobin < 6 mmol/L; < 9.67 g/dL), a diagnosis with other hematologic or immunological comorbidities, use of immunosuppressive medication, and having active infection at the moment of blood withdrawal. Of note, the cross-sectional cohort was complemented with 28 patients (cohort B) who participated in previous studies between 2001 and 2008 and from whom peripheral blood mononuclear cells (PBMCs, stored in liquid nitrogen) were available.[45,46]

The longitudinal cohort includes 12 inhibitor patients (cohort C) who underwent ITI and from who serial blood samples were stored during ITI (between 2001 and 2013; PBMCs stored in liquid nitrogen). The results of cohort C were compared with a control cohort (cohort D), consisting of 36 hemophilia patients without inhibitor, who had 2 blood samples withdrawn with a 6-month interval without clinical changes during this period (between 2017 and 2018). The study was approved by the local medical ethics committee and signed informed consent was obtained from all patients.

Data collection

From all study participants, the following data were collected: age at moment of blood withdrawal, hemophilia severity, number of EDs, anti-FVIII titer, and, if applicable, ITI treatment data (including start- and stop-date, FVIII treatment dose and regimen, maximum inhibitor titer, and date inhibitor eradication). Successful tolerance induction was defined as a negative anti-FVIII antibody titer (≤ 0.3 BU), a recovery of at least 66% and a FVIII terminal half-life of at least 6 hours.[10]

PBMC isolation

Blood was drawn into citrate tubes for inhibitor testing using the Bethesda assay and heparin tubes to isolate PBMCs using Ficoll-Paque PLUS (GE Healthcare) density centrifugation. Isolated PBMCs were resuspended in cell culture medium (RPMI 1640) 1640 containing 1% penicillin/streptomycin and 1% L-glutamine (all Gibco), 20% heat-inactivated fetal calf serum (FCS, Sigma-Aldrich, F7524), and 10% dimethyl sulfoxide (Sigma-Aldrich) and stored in liquid nitrogen until use.

Flow cytometry analysis of immune profiles

For evaluation of the immune profile, 3 different flow cytometry panels were used: (1) B-cell panel for Breg evaluation; (2) monocyte panel for MDSCs and expression of PD-L1 and HLA-DR on monocytes; and (3) T-cell panel for Treg evaluation and expression of PD1, CTLA4, and ICOS on T-cells.

B-cell panel

AF700-anti-CD3 and BV711-antiCD19 (BioLegend); PB-anti-CD20 (Sony Biotechnology); AF700-anti-CD56, PE-Cy7-anti-CD5, and PE-anti-CD24 (BD Biosciences); BV510-anti-CD1d (BD Horizon); and allophycocyanin (APC)-anti-CD38 and FITC-anti-CD27 (eBioscience).

Bregs were defined as $CD19^{+}CD24^{high}CD38^{high}$ cells[42–44,47]; the frequency of Bregs is expressed as the percentage of Bregs from the total number of $CD19^{+}$ B-cells. Previously, functional experiments were performed to establish that IL-10+ producing B-cells are enriched in this $CD19^{19}CD24^{high}CD^{high}$ B-cell fraction. For this purpose, PBMCs from healthy donors and inhibitor patients were stimulated with CpG oligodeoxynucleotides class B and phorbol myristate acetate/ionomycin for measurement of IL-10 production and differentiation between the total B-cell population and the $CD24^{high}CD38^{high}$ and $CD24^{low}CD38^{low}$ B-cell subsets (See Supplemental Figure 1, http://links.lww.com/HS/A150).

Monocyte panel

Live/dead staining 7-aminoactinomycin D, PB-anti-CD15, FITC-anti-HLA-DR, PE-Cy7-anti-PD-L1, PE-CF594-anti-CD56, and V500-anti-CD3 (BD Biosciences); BV785-anti-CD14 and BV711-anti-CD19 APC-anti-CD11b (eBioscience); and PE-anti-CD33 (Bio-Rad). Monocytes were defined as $CD3^{-}CD19^{-}CD56^{-}(Lin^{-})CD14^{+}$ cells.

MDSCs were defined as $Lin^{-}HLA\text{-}DR^{low}CD11b^{+}CD33^{+}$; the frequency of MDSCs is expressed as the percentage of MDSCs from the total number of PBMCs. HLA-DR and PD-L1 expression on monocytes was presented as mean fluorescence intensity (MFI).

T-cell panel

AF594-anti-CD3, BV785-anti-CD4, BV711-anti-CD45RO (BioLegend); APC-Cy7-anti-CD45RA (Sony Biotechnology); BV510-anti-CD27, PE-Cy7-anti-CD25, FITC-anti-CD69, PE-anti-CTLA4 (BD Biosciences); PerCP-Cy5'5-anti-PD1 (Sony Biotechnology); and APC-anti-ICOS (eBioscience). Tregs were defined as $CD4^+CD25^+FoxP3^+$ cells; the frequency of Tregs is expressed as the percentage of Tregs from the total number of $CD4^+$ T-cells. Expression of CTLA4, ICOS, and PD1 was presented as MFI.

For surface immunostaining, PMBCs were thawed, washed in PBS/2% FCS/0.1% sodium azide, and incubated with antibodies (0.5×10^6 cells, 30 min, 4°C). After staining, cells were washed twice in PBS/2% FCS. Cells of the B-cell and monocyte panel were immediately analyzed.

Cells from T-cell panel were fixed and permeabilized (Cytofix/Cytoperm; eBioscience; 30 min, 4°C) for intracellular staining. Thereafter cells were washed twice and stained with anti-FoxP3 antibody (30 min, 4°C). Cells were washed twice and resuspended in PBS/2% FCS for analysis.

Fluorescence activated cell sorting (FACS) acquisition was performed on an LSR Fortessa flow cytometer (BD Biosciences). Only samples with cell viability ≥ 95% were included, and we ensured minimal inter-test variability by measuring in sets with an equal distribution between the different groups (no inhibitor/inhibitor/ex-inhibitor); for the longitudinal cohort, all available serial samples from 1 patient were measured at once. For data analysis, FlowJo software (LLC, version 10, Ashland, OR) was used. A complete gating strategy for the 3 FACS panels is shown in Supplemental Figure 2 (http://links.lww.com/HS/A150).

Statistical analysis

Data are expressed as mean with SD and 95% confidence interval or median with interquartile range (IQR) as appropriate. Kruskal-Wallis test was used to evaluate group differences in the cross-sectional cohort. In case of statistically significant results, a post hoc analysis was performed using the Bonferroni correction. A sensitivity analysis was performed in which only patients with severe hemophilia A were included.

Data from the longitudinal ITI cohort were compared with the longitudinal control cohort in order to differentiate ITI-specific changes from aspecific time-related changes in outcome parameters. Here, for each ITI-patient, 2 timepoints were selected: pre-ITI, representing the start of ITI, and post-ITI, representing the first negative anti-factor VIII (aFVIII) titer or the time point at which the lowest aFVIII titer was reached (requiring a minimal 3-fold decrease in aFVIII titer). For each outcome, parameter relative changes pre- and post-ITI were calculated and expressed as a ratio. For the control cohort, relative changes were calculated using the 2 samples, withdrawn at a 6-month interval. Mann-Whitney *U* test was used to compare ratios of the ITI cohort and control cohort. A sensitivity analysis was performed in which only patients were included with a negative inhibitor titer at the post-ITI timepoint. A P value < 0.05 was considered as statistically significant. All analyses were performed with IBM SPSS Statistics for Windows, version 25.0.0.2 (IBM Corp, Armonk, NY). Graphs were produced with GraphPad Prism, version 8.3.0 for Windows (GraphPad Software, La Jolla, CA).

Results

Comparison of immune profile between inhibitor, no inhibitor, and ex-inhibitor patients

Patient characteristics

The cross-sectional cohort (cohorts A and B) consisted of 65 patients in 3 groups, of which 28 (43.1%) without an inhibitor, 20 (30.7%) with a current inhibitor (30.7%), and 17 (26.2%) with an inhibitor in the past, that is, ex-inhibitor patients (Table 1). Cohort A represented 37 of 65 patients (56.9%), including 20 no inhibitor patients, 7 inhibitor patients, and 10 ex-inhibitor patients. The remainder of patients from cohort B (28/65, 43.1%) consisted of 8 no inhibitor patients, 13 inhibitor patients, and 7 ex-inhibitor patients.

Median age of the cohort was 32 years (IQR, 10–53 y) and the majority of patients suffered from severe hemophilia A (81.5%), with no significant differences between subgroups. Age at inhibitor development, the maximum anti-FVIII titer and the frequency of high responders (anti-FVIII > 5 BU) were comparable between current inhibitor and ex-inhibitor patients.

In the inhibitor group, 12 of 20 (60.0%) of patients were treated with ITI at any moment during their disease course: 2 received ITI in the past (unsuccessful), 6 were on ITI at the moment of blood withdrawal, and 4 started with ITI after blood withdrawal. The 8 inhibitor patients, not treated with ITI, were mostly patients with mild hemophilia A (6/8). In the ex-inhibitor group, 14 of 17 received ITI in the past; in the other 3 patients, the anti-FVIII reactivity resolved spontaneously. These 3 patients all had low-titer inhibitors, 2 with severe hemophilia A (age 1 and 2 y) and 1 with mild hemophilia A (76 y). The time to a negative inhibitor in these patients ranged from 4 to 92 months.

Frequency of immunoregulatory cell types and markers

Using flow cytometry, the frequency of immunoregulatory cell types and markers in PBMCs was measured and compared between the 3 groups (no inhibitor, inhibitor, and ex-inhibitor; Figures 1 and 2; and Supplemental Table 1, http://links.lww.com/HS/A150).

The total number of B-cells was not significantly different among the 3 groups (inhibitor: 14.2% [IQR, 9.6%–23.0%]; noninhibitor: 10.4 [8.1–14.4]; and ex-inhibitor: 10.6 [6.5–15.4]; $P = 0.06$). The frequency of Bregs (expressed as percentage of CD19+ B-cells), however, was significantly lower in inhibitor patients compared with both noninhibitor patients and ex-inhibitor patients (respectively, 3.2% [IQR, 1.6–6.6%] versus 5.9% [IQR, 4.1–10.4%] and 8.9% [IQR, 4.6–15.6%]; $P < 0.01$; Figure 1).

Total T-cell numbers and the frequency of CD4+ T-cells were similar among all groups ($P = 0.36$ and $P = 0.72$, respectively; Supplemental Table 1, http://links.lww.com/HS/A150). Specifically, the frequency of Tregs was also not different. Regarding regulatory markers ICOS and PD1 on CD4+ T-cells and PD-L1 on monocytes, similar to Bregs, the lowest expression was observed in inhibitor patients and the highest expression in ex-inhibitor patients, although these results did not reach statistical significance (PD1: $P = 0.89$; ICOS: $P = 0.66$; PD-L1: $P = 0.14$; Figure 2; and Supplemental Table 1, http://links.lww.com/HS/A150). Also, the expression of CTLA4 on T-cells, both on effector T-cells (Teffs) and Tregs, was reduced in inhibitor patients compared with the other 2 groups. The difference between inhibitor and no inhibitor patients reached statistical significance (CTLA4 on all CD4+ T-cells: MFI 133 [IQR, 91–177] versus 537 [IQR, 450–625], respectively; $P = 0.001$). No differences were observed in the frequency of MDSCs and the expression of HLA-DR on monocytes (See Supplemental Table 1, http://links.lww.com/HS/A150).

Subanalysis, including data of severe hemophilia A patients only, confirmed these findings, showing a significant decrease in frequency of Bregs and CTLA4 in inhibitor patients and downward trends in PD1, ICOS, and PD-L1 (See Supplemental Table 2, http://links.lww.com/HS/A150).

Changes in immune-regulatory cell types and markers before and after ITI

In order to further clarify the association between immunoregulatory components and restoration of tolerance to FVIII, we

Table 1

Baseline Characteristics of Cross-sectional Cohort (Cohorts A and B).

Characteristics	No Inhibitor, N = 28 (43.1%)	Inhibitor, N = 20 (30.7%)	Ex-inhibitor, N = 17 (26.2%)	*P*
Age, y	30.9 (11.8–52.8)	43.3 (1.6–62.8)	23.2 (9.0–47.8)	0.95
Hemophilia severity				0.16
Mild	2 (7.1%)	6 (30.0%)	2 (11.8%)	
Moderate	1 (3.6%)	1 (5.0%)	0 (0.0%)	
Severe	25 (89.3%)	13 (65.0%)	15 (88.2%)	
Treatment regimen				< 0.01
On demand	7 (25.0%)	17 (85.0%)	2 (11.8%)	
Prophylaxis	21 (75.0%)	3 (15.0%)	15 (88.2%)	
FVIII product type				
pd-FVIII	2 (7.1%)	1 (5.0%)	0 (0.0%)	< 0.01
rFVIII	22 (78.6%)	9 (45.0%)	14 (82.4%)	
Bypassing agent	0 (0.0%)	9 (45.0%)	1 (5.9%)	
DDAVP	0 (0.0%)	1 (5.0%)	1 (5.9%)	
rFVIII-Fc	4 (14.3%)	0 (0.0%)	1 (5.9%)	
Bypassing agent	0 (0.0%)	17 (85.0%)	0 (0.0%)	
rFVIIa		12 (60.0%)		< 0.01
aPCC		4 (20.0%)		
DDAVP		1 (5.0%)		
Current aFVIII titer, BU	0.0 (0.0–0.0)	7.2 (3.3–18.4)	0.0 (0.0–0.0)	< 0.01
Age at inhibitor development, y		19.4 (1.4–39.8)	2.4 (1.1–14.5)	0.14
Maximum aFVIII titer, BU		26.0 (7.9–236.3)	45.0 (3.7–75.4)	0.62
High responder (aFVIII > 5 BU)		16 (80.0%)	10 (62.5%)	0.29
ITI status at blood sampling				
Before ITI		12 (60.0%)	NA	< 0.001
During ITI		6 (30.0%)	NA	
After ITI		2 (10.0%)	14 (82.4%)	
Never received ITI			3 (17.6%)[a]	
ITI success rate		8 (66.7%)	14 (100%)	0.04
ITI duration, mo		11.6 (1.2–36.0)	15.0 (6.2–28.1)	0.62

Categorical variables are presented as cases/total (percentage). Continuous data are presented as median (interquartile range). Tested with Fisher exact test (categorical variables) or Kruskal-Wallis test (continuous variables).

[a]Three patients had spontaneous resolution of inhibitor and were not treated with ITI.

aFVIII = anti-factor VIII; aPCC = activated prothrombin complex concentrate; BU = Bethesda Units; DDAVP = desmopressin; FVIII = factor VIII; ITI = immune tolerance induction; NA = not applicable; pd-FVIII = plasma-derived factor VIII; rFVIIa = recombinant activated factor VII; rFVIII = recombinant factor VIII; rFVIII-Fc = recombinant factor VIII-Fc fusion product (extended half-life product).

evaluated changes in immune profiles during the course of ITI in relation to anti-FVIII antibody titers in a longitudinal cohort of 12 ITI patients (cohort C). Results were compared with a control cohort of 36 inhibitor-negative hemophilia A patients, cohort D (See Supplemental Table 3, http://links.lww.com/HS/A150 for patient characteristics).

Clinical details of the ITI patients are summarized in Table 2. All but 1 patient (who had spontaneous resolution of anti-FVIII antibodies) underwent ITI with a median duration of 1.6 years (IQR, 0.4–3.0 y) and a success rate of 83.3% (10/12). Compared with the control cohort, in which the frequency of immunoregulatory variables remained stable over time, ITI patients showed an increase in the frequency of included immune-regulatory cells and markers post-ITI compared with the pre-ITI measurement (Figure 3).

Effective ITI was associated with a significant increase in the ratio of Bregs (1.8 versus 1.0; $P = 0.02$), the ratio of Tregs (1.5 versus 1.0; $P = 0.02$), and several Tregs markers: CTLA4 on Tregs (2.2 versus 1.0; $P = 0.03$) and PD1 on CD4+ T-cells and Teffs (1.3 versus 1.0; $P = 0.04$ and 1.3 versus 1.0; $P = 0.05$, respectively). Thus, we hereby correlate inhibitor eradication with upregulation of several regulatory cells and markers.

Next, to substantiate these results, we performed a subanalysis in only ITI patients who showed complete inhibitor eradication post-ITI (ie, anti-FVIII ≤ 0.3 BU) (Table 3). Analysis in this subgroup of 8 ITI patients (compared with the same control group) further emphasized our data: the Breg ratio and CTLA4 ratio were significantly higher the ITI group (Breg ratio: 2.0 versus 1.0; $P = 0.04$ and CTLA4 ratio on Tregs: 2.7 versus 1.0; $P = 0.01$). Increased ratios were also observed for PD-L1 on monocytes, Tregs and PD1 and ICOS on T-cells, but these data did not reach statistical significance (Table 3).

Discussion

This study aimed to improve the knowledge of ITI in hemophilia by comparing immune profiles between noninhibitor, inhibitor, and ex-inhibitor patients and evaluating changes during the course of ITI. We found the lowest frequency of immunoregulatory cells and markers, in particular of Bregs and CTLA4 on T-cells, in inhibitor patients, with evidence for reversal during ITI, supporting the role of these immunoregulatory components in restoration of tolerance to FVIII. Thus, inhibitor status may be associated with a reversible lack of peripheral immune tolerance, which can be restored by ITI treatment.

Bregs play a pivotal role in the maintenance of peripheral tolerance,[41,48] which to date is mostly ascribed to their abundant production of IL-10, a cytokine that inhibits pro-inflammatory cytokines and supports the generation and maintenance of Tregs.[48,49] In healthy persons, it was shown that Bregs possess immune-regulatory capacity by the inhibition of naive T-cell differentiation into T helper 1 (T_H1) and T_H17 cells and the conversion of $CD4^+CD25^-$ cells into Tregs.[42,50] In immune-related diseases, ranging from SLE and RA to common variable immunodeficiency and transplant patients with graft rejection, numerical or functional deficits of these cells are described.[42,44,47,50,51] Regarding hemophilia A, to date, only 1 publication cross-sectionally evaluated regulatory cells in inhibitor formation.[52] Inhibitor patients had a reduction in Bregs and

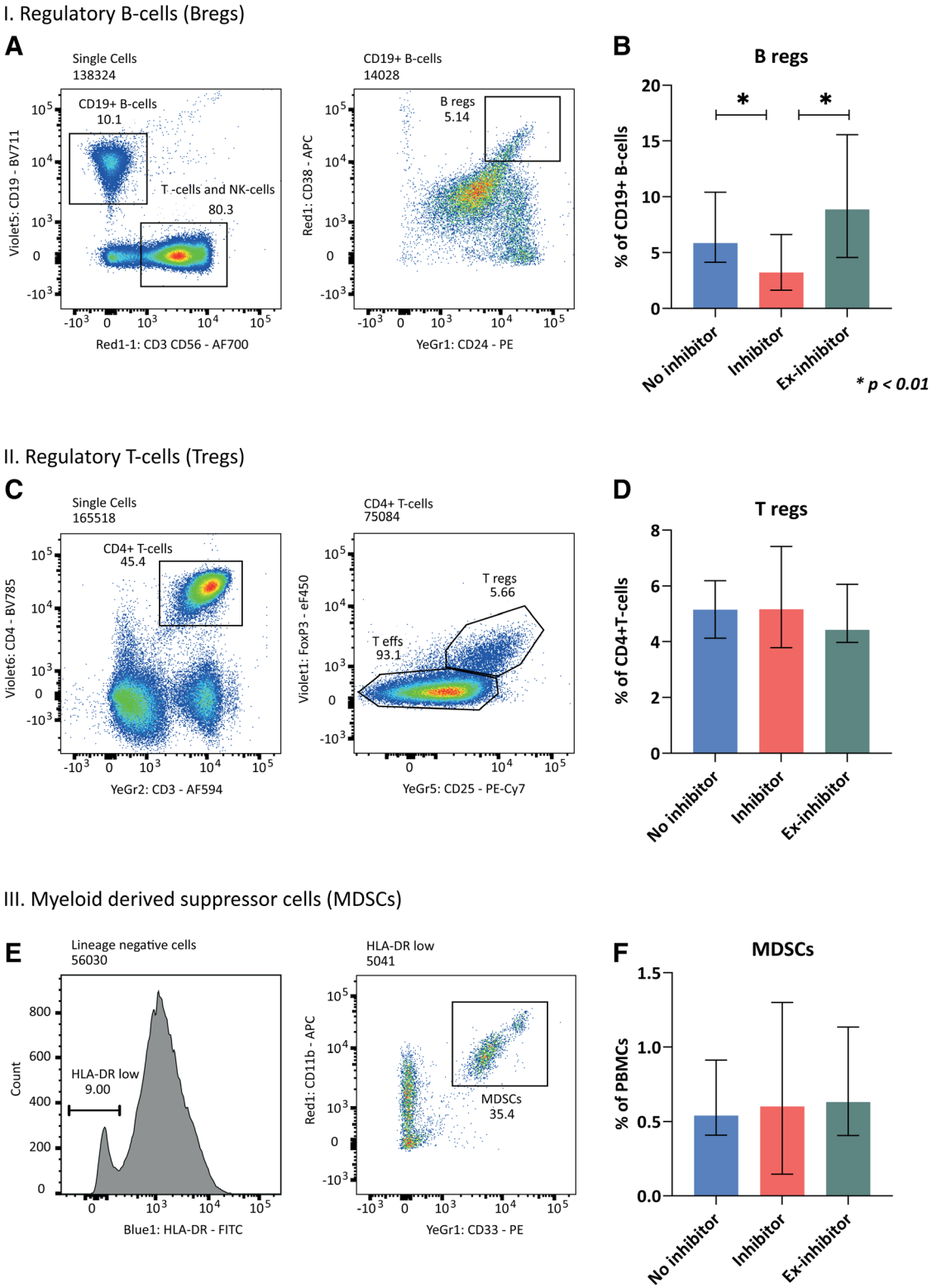

Figure 1. Frequency of immunoregulatory cells in hemophilia A patients. PBMCs of hemophilia A patients were analyzed by flow cytometry for the frequency of Bregs, Tregs, and MDSCs. (A, C, and D), The relevant gating strategy to select, respectively, Bregs ($CD19^+CD24^{high}CD38^{high}$), Tregs ($CD4^+CD25^+FoxP3^+$), and MDSCs ($Lin^-HLA\text{-}DR^{low}CD11b^+CD33^+$). (B, D, and F), The median (+ IQR) frequency of, respectively, Bregs, Tregs, and MDSCs in no inhibitor patients, inhibitor patients, and ex-inhibitor patients (patients with inhibitors in the past; successfully treated with ITI). APC = allophycocyanin; Bregs = regulatory B-cells; IQR = interquartile range; ITI = immune tolerance induction; MDSC = myeloid-derived suppressor cell; NK = natural killer; PBMC = peripheral blood mononuclear cell; Tregs = regulatory T-cells.

produced less IL-10 after activation compared with both hemophilia A patients without inhibitors and healthy controls. Our study confirmed these data and went beyond by inclusion of ex-inhibitor patients and the change in number of regulatory cells during ITI treatment. We showed that ex-inhibitor patients express the highest number of Bregs and that Bregs increase by

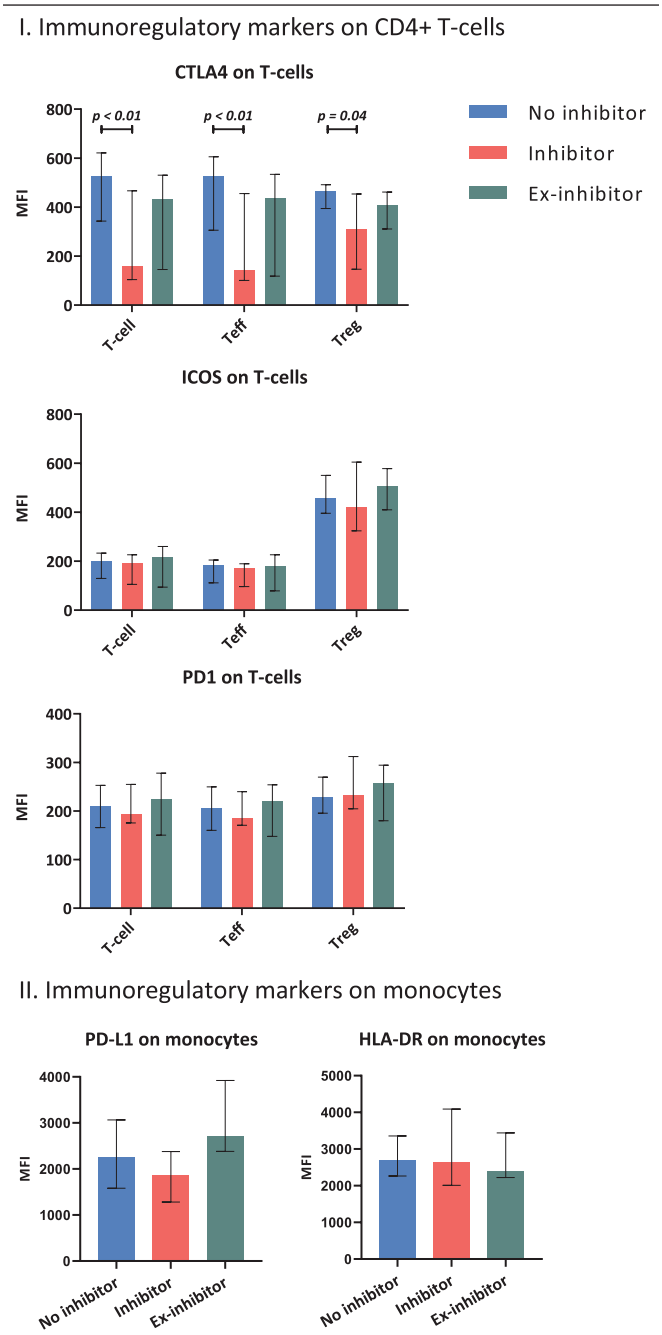

Figure 2. Frequency of immunoregulatory markers on CD4+ T-cells and monocytes in hemophilia A patients. PBMCs of hemophilia A patients were analyzed by flow cytometry for the frequency of immunoregulatory markers PD1, ICOS, and CTLA4 on CD4+ T-cells and PD-L1 and HLA-DR on monocytes, expressed as the median (+ IQR) MFI. Results are presented for all CD4+ T-cells and separately for Teffs and Tregs. Within the CD4+ T-cell fraction, the majority were Teffs (94.9%, IQR, 93.8%–96.0%) and the remainder were Tregs (5.1%, IQR 4.0%–6.2%), with no significant differences between the 3 groups (for details, See Supplemental Table 1, http://links.lww.com/HS/A150). CTLA4 = cytotoxic T-lymphocyte-associated protein 4; ICOS = inducible T-cell costimulator; IQR = interquartile range; MFI = mean fluorescence intensity; PBMC = peripheral blood mononuclear cell; PD1 = programmed cell death protein 1; PD-L1 = programmed death-ligand 1; Teffs = effector T-cells; Tregs = regulatory T-cells.

more than 100% during ITI. Taken together, the results support a potential role for Bregs in the induction of peripheral immune tolerance to FVIII treatment.

Contrary to Bregs, Treg results were more variable. In the cross-sectional cohort, no differences were found between inhibitor and noninhibitor patients, whereas we did observe a significant increase of 50% in Tregs during ITI. This is the first study evaluating the frequency of Tregs according to inhibitor status and during ITI. Given the well-described suppressive function of Tregs is it likely that these cells are involved in downregulation of the immune response to FVIII, which is also supported by earlier work of Tregs in tolerance induction to FVIII.[13,34,53]

The inhibitory Treg (qualitative) efficacy was not specifically addressed in our study, due to limitations in availability of samples. In support for a role of Tregs, inhibitor patients did express the lowest levels of regulatory receptors PD1, ICOS, and most significantly CTLA4, which also increased during ITI.

The receptor CTLA4 is mainly expressed on activated CD4+ T-cells but also constitutively on Tregs, where it inhibits T-cell activation by competing with CD28 for the binding of B7 (CD80/CD86) molecules on APCs. As a consequence of such restriction of co-stimulatory CD28/B7 signals, T-cell responses are downregulated, benefiting T-cell homeostasis and promoting peripheral tolerance.

We found that CTLA4-expression was inversely related to the presence of inhibitors and positively correlated with the induction of tolerance, underscoring earlier research in which polymorphisms in the CTLA4 gene were associated with the risk of inhibitor development.[54–57] Moreover, a hemophilic mouse model also showed a beneficial role of CTLA4 in ITI.[58] In this study, injection of CTLA4-immunoglobulin (CTLA4-Ig) prevented the primary (inhibitor) antibody response to FVIII. In addition, CTLA4-Ig also prevented or diminished further increases in aFVIII titers after FVIII administration in hemophilic mice, which already developed an aFVIII antibody response. Thus, CTLA4 appears to play a role in tolerance induction and could be a potential target for therapy against inhibitory aFVIII antibodies.

A limitation of our study pertains the restricted number of PBMCs available per patient, which allowed our evaluation to regulatory cell numbers and marker expression but unfortunately prevented functional evaluation of Bregs and Tregs or identification of FVIII-specific immune cell populations. The latter is particularly challenging given the low frequency of these specific cells in peripheral blood. Regarding human Bregs, investigators so far use markers that include a population of Bregs as based on demonstrated ability to produce IL-10.[41,48] In our study, we also used these same markers ($CD19^{+}CD24^{high}CD38^{high}$) to include Bregs.[42,44,50] Moreover, we performed functional assays to confirm that IL-10 production is enriched in the $CD19^{+}CD24^{high}CD38^{high}$ B-cell population (See Supplemental Figure 1, http://links.lww.com/HS/A150).

While we did obtain reasonable size patient cohorts, a second limitation of this study is the heterogeneity with regard to age and severity of hemophilia A of included patients. Although in the cross-sectional study, age was similar between the 3 groups and a subanalysis (including only patients with severe hemophilia A) was performed, we cannot rule out results being affected by patient heterogeneity. Indeed, patients included in our longitudinal ITI cohort were from a considerable age range, including young children. For reasons that pertain to medical-ethical considerations, it was not feasible to include age-matched controls for the very young ITI patients. We were careful to investigate whether the deficit of age-matched controls impedes interpretation of our results. However, we did not find a significant correlation between age and level of Bregs neither CTLA4 in the cross-sectional cohort that obviously takes age into consideration (Bregs: Pearson relation coefficient, –0.21; $P = 0.09$ and CTLA4: Pearson relation coefficient, 0.11; $P = 0.41$). Moreover, within the ITI cohort, an increase in immunoregulatory cells or markers was not limited to the young patients (0–2 y) but was also observed in the adult ITI patients. Thus, despite the lack of

Table 2

Clinical Characteristics of Longitudinal ITI Cohort (Cohort C).

Subject ID	Age When Inhibitor (y)	Titer Maximum (BU)	Age Start ITI (y)	Duration ITI (y)	Pre-ITI (T = 0) aFVIII Titer	Post-ITI (T = 1) aFVIII Titer
1	0.1	245.0	0.9	1.6	53.0	0.2
2	0.6	44.1	0.8	0.8	44.1	1.0
3	0.7	207.0	0.8	2.9	18.0	0.0
4[a]	1.0	3.40	na	na	3.4	0.0
5	1.0	7.7	1.1	3.1	2.5	0.1
6	1.1	67.0	1.1	1.6	43.5	3.2
7[b]	1.2	927.0	2.1	> 7	124.0	6.9
8	1.4	45.0	1.7	0.2	113.9	0.1
9	1.5	16.0	1.7	0.4	5.8	0.1
10	2.1	8.3	2.2	0.3	3.2	1.0
11	7.9	144.0	40.9	5.0	7.6	0.4
12	23.6	900.0	31.6	4.4	580.0	2.4
Total	1.1 (0.7–1.9)	56.0 (10.2–236.0)	1.1 (0.8–1.9)	1.6 (0.4–3.0)	16.4 (4.0–50.8)	0.2 (0.1–2.1)

Categorical variables are presented as cases/total (percentage). Continuous data are presented as median (interquartile range).

[a]Low-titer antibodies; spontaneous inhibitor eradication.

[b]Patient is still receiving ITI at the moment of analysis.

aFVIII = anti-factor VIII; BU = Bethesda Units; ID = identification number; ITI = immune tolerance induction; na = not applicable.

an age-matched control group, we still consider that our data provide new valuable insights into ITI in hemophilia A patients.

Considering heterogeneity, time between last FVIII exposure and blood withdrawal was also variable. Especially in inhibitor patients, this ranged from several days or months in most patients to a couple of years in 2 patients with persistent (high-titer) antibodies after unsuccessful ITI. To what extent timing of blood draw relative to FVIII infusion affects immune cell subsets and markers is not understood, and our project does not fully address this question. The high median inhibitor titer

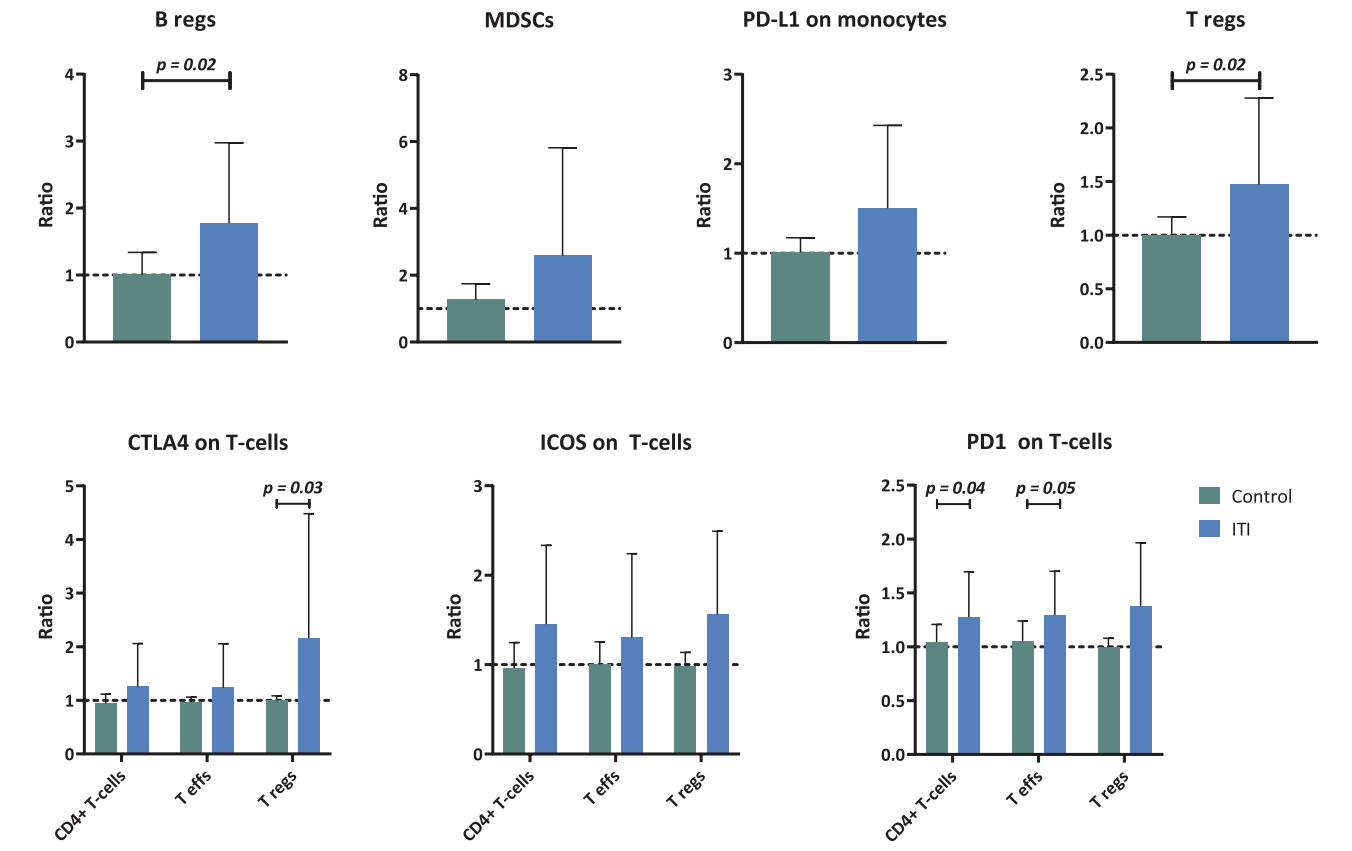

Figure 3. Relative change in immunoregulatory cells and markers before and after ITI. The relative change in frequency of immunoregulatory cell types and markers was evaluated by flow cytometry during inhibitor eradication in 13 ITI patients and compared with 36 control patients (hemophilia A patients with no inhibitor). The mean (+SD) ratios of outcome parameters express the relative change in frequency of regulatory immune cells and markers during ITI or during a 6-mo interval without any relevant clinical changes for the control cohort. The ratio as presented was calculated by dividing the cell frequency post-ITI by the frequency pre-ITI; for the control cohort, frequencies were calculated by dividing frequency at T = 6 mo with the frequency at T = 0. A ratio of 1 (dashed line) means that the frequency of the measured parameter was unchanged; a ratio above 1 means an increase in frequency; and a ratio less than 1 means a decrease in frequency. Bregs = regulatory B-cells; CTLA4 = cytotoxic T-lymphocyte-associated protein 4; ICOS = inducible T-cell costimulator; ITI = immune tolerance induction; MDSC = myeloid-derived suppressor cell; Teffs = effector T-cells; PD1 = programmed cell death protein 1; PD-L1 = programmed death-ligand 1; Tregs = regulatory T-cells.

Table 3

Relative Change in Immunoregulatory Cells and Markers Before and After Successful ITI Compared With Control Hemophilia A Patients.

Immunoregulatory Parameters	ITI Patients, N = 8 Ratio	Control Patients, N = 36 Ratio	*P*
aFVIII (BU)	0.02 (0.00-0.04)	1.0 (1.0-1.0)	< 0.01
Bregs (% of B-cells)	2.0 (0.8-3.2)	1.0 (0.9-1.1)	0.04
MDSCs (% of PBMCs)	3.1 (0.0-6.3)	1.3 (1.1-1.4)	0.87
PD-L1 on monocytes (MFI)	1.4 (0.8-2.0)	1.0 (1.0-1.1)	0.19
Tregs (% of CD4+ T-cells)	1.4 (0.7-2.0)	1.0 (0.9-1.1)	0.24
CTLA4 (MFI)			
On T-cells	1.4 (0.6-2.2)	1.0 (0.9-1.0)	0.12
On Teffs	1.4 (0.5-2.2)	1.0 (1.0-1.0)	0.18
On Tregs	2.7 (0.4-5.0)	1.0 (1.0-1.0)	0.01
PD1 (MFI)			
On T-cells	1.3 (0.8-1.7)	1.0 (1.0-1.1)	0.52
On Teffs	1.3 (0.9-1.7)	1.0 (1.0-1.1)	0.56
On Tregs	1.5 (0.9-2.0)	1.0 (1.0-1.0)	0.06
ICOS (MFI)			
On T-cells	1.0 (0.6-1.4)	1.0 (0.9-1.1)	0.89
On Teffs	0.9 (0.5-1.3)	1.0 (0.9-1.1)	0.26
On Tregs	1.4 (0.6-2.2)	1.0 (0.9-1.0)	0.51

Subanalysis including only ITI patients with a negative aFVIII titer (≤ 0.3 BU) at the post-ITI timepoint. The change in frequency of immunoregulatory cells and markers was compared between ITI patients and control hemophilia A patients. For each patient, the relative change in frequency of cells/markers was calculated (ratio) between start of ITI and the first negative inhibitor (in ITI patients) or with a 6-mo interval (in the control group consisting of inhibitor-negative hemophilia A patients). Differences in the ratio (expressed as mean + 95% CI) between the 2 groups (ITI vs control) were compared using the Mann-Whitney *U* test.

aFVIII = anti-factor VIII; Bregs = regulatory B-cells; BU = Bethesda Units; CI = confidence interval; CTLA4 = cytotoxic T-lymphocyte-associated protein 4; ICOS = inducible T-cell costimulator; ITI = immune tolerance induction; MDSCs = myeloid-derived suppressor cells; MFI = mean fluorescence intensity; PBMC = peripheral blood mononuclear cells; PD1 = programmed cell death protein 1; PD-L1 = programmed death-ligand 1; Teffs = effector T-cells; Tregs = regulatory T-cells.

in both cohorts (7.2 BU and 18.8 BU in the cross-sectional and ITI cohort, respectively), however, demonstrates that all patients had an active immune response to FVIII at the moment of blood withdrawal. Therefore, regardless of the exact timing to last FVIII exposure, we consider our data valid to evaluate differences in immune tolerance mechanisms in inhibitor and noninhibitor patients.

A final limitation of this study is the fact that *F8* gene mutational status was unknown for most patients, and this could not be taken into account when interpreting results.

The novelty of our study is that we carefully addressed the role of various immunoregulatory cells and markers in ITI in hemophilia A patients. Obtained results from the cross-sectional cohort were confirmed in the longitudinal analysis of ITI patients, which further strengthens the findings of this study.

We found the outcomes most remarkable for Bregs and CTLA4 expression on T-cells. For reconsolidation of both these findings, further experimentation is necessary. A possible outcome might be that Bregs initiate restoration of tolerance by IL-10 production, leading to upregulation of Tregs markers and thereby blocking the capacity of these T-cells to stimulate B-cell production of FVIII-neutralizing antibodies. This study contributes to the mechanistic insight of ITI by showing new pivotal targets for tolerance reinstatement. This could include administration of IL-10 agents, representing the most important effector cytokine of Bregs or CTLA4-Ig, which already showed to inhibit anti-FVIII responses in a murine hemophilia model.[58]

In conclusion, we showed that the presence of anti-FVIII antibodies is associated with lower frequencies of immunoregulatory cells and markers and that these immune-tolerogenic components increased during tolerance induction. These results suggest that an existing anti-FVIII immune response is associated with deficits in peripheral tolerance mechanisms, which can recover during the course of ITI.

Future research, preferably in a more homogeneous cohort, should validate these findings and exploit the identified key immunoregulatory cells and markers towards the development of improved ITI protocols.

Acknowledgments

We are thankful to all participants of this study and the staff of the Van Creveldkliniek and Center for Translational Immunology. We also acknowledge Paul Kaijen of Sanquin Amsterdam for processing and coordinating the storage of samples of hemophilia A patients included in this study.

Disclosures

REGS has received research support from Bayer, Baxter, CSL Behring, Novo Nordisk, Pfizer, and Sobi (funds to the institute). All the other authors have no conflicts of interest to disclose.

Sources of funding

This work was supported by Bayer.

References

1. Wight J, Paisley S. The epidemiology of inhibitors in haemophilia A: a systematic review. *Haemophilia*. 2003;9:418–435.
2. Iorio A, Halimeh S, Holzhauer S, et al. Rate of inhibitor development in previously untreated hemophilia A patients treated with plasma-derived or recombinant factor VIII concentrates: a systematic review. *J Thromb Haemost*. 2010;8:1256–1265.
3. Gouw SC, van den Berg HM, Fischer K, et al. Intensity of factor VIII treatment and inhibitor development in children with severe hemophilia A: the RODIN study. *Blood*. 2013;121:4046–4055.
4. Rota M, Cortesi PA, Steinitz-Trost KN, et al. Meta-analysis on incidence of inhibitors in patients with haemophilia A treated with recombinant factor VIII products. *Blood Coagul Fibrinolysis*. 2017;28:627–637.
5. Walsh CE, Jiménez-Yuste V, Auerswald G, et al. The burden of inhibitors in haemophilia patients. *Thromb Haemost*. 2016;116(suppl 1):S10–S17.
6. Mahlangu J, Oldenburg J, Callaghan MU, et al. Health-related quality of life and health status in persons with haemophilia A with inhibitors: a prospective, multicentre, non-interventional study (NIS). *Haemophilia*. 2019;25:382–391.
7. Lenk H, ITT Study Group. The German Registry of immune tolerance treatment in hemophilia–1999 update. *Haematologica*. 2000;85(10 suppl):45–47.
8. Mariani G, Kroner B, Immune Tolerance Study Group (ITSG). Immune tolerance in hemophilia with factor VIII inhibitors: predictors of success. *Haematologica*. 2001;86:1186–1193.
9. DiMichele DM, Kroner BL, North American Immune Tolerance Study Group. The North American Immune Tolerance Registry: practices, outcomes, outcome predictors. *Thromb Haemost*. 2002;87:52–57.
10. Hay CR, DiMichele DM, International Immune Tolerance Study. The principal results of the International Immune Tolerance Study: a randomized dose comparison. *Blood*. 2012;119:1335–1344.
11. Hausl C, Ahmad RU, Sasgary M, et al. High-dose factor VIII inhibits factor VIII-specific memory B cells in hemophilia A with factor VIII inhibitors. *Blood*. 2005;106:3415–3422.
12. Reipert BM, Allacher P, Hausl C, et al. Modulation of factor VIII-specific memory B cells. *Haemophilia*. 2010;16:25–34.
13. James EA, Kwok WW, Ettinger RA, et al. T-cell responses over time in a mild hemophilia A inhibitor subject: epitope identification and transient immunogenicity of the corresponding self-peptide. *J Thromb Haemost*. 2007;5:2399–2407.
14. Pautard B, D'Oiron R, Li Thiao Te V, et al. Successful immune tolerance induction by FVIII in hemophilia A patients with inhibitor may occur without deletion of FVIII-specific T cells. *J Thromb Haemost*. 2011;9:1163–1170.

15. Sultan Y, Kazatchkine MD, Maisonneuve P, et al. Anti-idiotypic suppression of autoantibodies to factor VIII (antihaemophilic factor) by high-dose intravenous gammaglobulin. *Lancet*. 1984;2:765–768.
16. Gilles JG, Desqueper B, Lenk H, et al. Neutralizing antiidiotypic antibodies to factor VIII inhibitors after desensitization in patients with hemophilia A. *J Clin Invest*. 1996;97:1382–1388.
17. Sakurai Y, Shima M, Tanaka I, et al. Association of anti-idiotypic antibodies with immune tolerance induction for the treatment of hemophilia A with inhibitors. *Haematologica*. 2004;89:696–703.
18. Waters B, Lillicrap D. The molecular mechanisms of immunomodulation and tolerance induction to factor VIII. *J Thromb Haemost*. 2009;7:1446–1456.
19. Reipert BM, van Helden PM, van den Helden PM, et al. Mechanisms of action of immune tolerance induction against factor VIII in patients with congenital haemophilia A and factor VIII inhibitors. *Br J Haematol*. 2007;136:12–25.
20. Astermark J, Berntorp E, White GC, et al. The Malmö International Brother Study (MIBS): further support for genetic predisposition to inhibitor development in hemophilia patients. *Haemophilia*. 2001;7:267–272.
21. Eckhardt CL, van Velzen AS, Peters M, et al. Factor VIII gene (F8) mutation and risk of inhibitor development in nonsevere hemophilia A. *Blood*. 2013;122:1954–1962.
22. Gouw SC, Van Der Bom JG, Van Den Berg HM, et al. Influence of the type of F8 gene mutation on inhibitor development in a single centre cohort of severe haemophilia A patients. *Haemophilia*. 2011;17:275–281.
23. Gouw SC, van der Bom JG, Marijke van den Berg H. Treatment-related risk factors of inhibitor development in previously untreated patients with hemophilia A: the CANAL cohort study. *Blood*. 2007;109:4648–4654.
24. Eckhardt CL, van der Bom JG, van der Naald M, et al. Surgery and inhibitor development in hemophilia A: a systematic review. *J Thromb Haemost*. 2011;9:1948–1958.
25. Franchini M, Coppola A, Rocino A, et al. Systematic review of the role of FVIII concentrates in inhibitor development in previously untreated patients with severe hemophilia a: a 2013 update. *Semin Thromb Hemost*. 2013;39:752–766.
26. Peyvandi F, Mannucci PM, Garagiola I, et al. A randomized trial of factor VIII and neutralizing antibodies in hemophilia A. *N Engl J Med*. 2016;374:2054–2064.
27. Hay CR, Ollier W, Pepper L, et al. HLA class II profile: a weak determinant of factor VIII inhibitor development in severe haemophilia A. UKHCDO Inhibitor Working Party. *Thromb Haemost*. 1997;77:234–237.
28. Astermark J, Oldenburg J, Pavlova A, et al. Polymorphisms in the IL10 but not in the IL1beta and IL4 genes are associated with inhibitor development in patients with hemophilia A. *Blood*. 2006;107:3167–3172.
29. Astermark J. Inhibitor development: patient-determined risk factors. *Haemophilia*. 2010;16:66–70.
30. Lai JD, Moorehead PC, Sponagle K, et al. Concurrent influenza vaccination reduces anti-FVIII antibody responses in murine hemophilia A. *Blood*. 2016;127:3439–3449.
31. Algiman M, Dietrich G, Nydegger UE, et al. Natural antibodies to factor VIII (anti-hemophilic factor) in healthy individuals. *Proc Natl Acad Sci U S A*. 1992;89:3795–3799.
32. Reding MT, Wu H, Krampf M, et al. Sensitization of CD4+ T cells to coagulation factor VIII: response in congenital and acquired hemophilia patients and in healthy subjects. *Thromb Haemost*. 2000;84:643–652.
33. Hu GL, Okita DK, Diethelm-Okita BM, et al. Recognition of coagulation factor VIII by CD4+ T cells of healthy humans. *J Thromb Haemost*. 2003;1:2159–2166.
34. Hu G, Guo D, Key NS, et al. Cytokine production by CD4+ T cells specific for coagulation factor VIII in healthy subjects and haemophilia A patients. *Thromb Haemost*. 2007;97:788–794.
35. Lacroix-Desmazes S, Navarrete AM, André S, et al. Dynamics of factor VIII interactions determine its immunologic fate in hemophilia A. *Blood*. 2008;112:240–249.
36. Walker LS, Abbas AK. The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat Rev Immunol*. 2002;2:11–19.
37. Varthaman A, Lacroix-Desmazes S. Pathogenic immune response to therapeutic factor VIII: exacerbated response or failed induction of tolerance? *Haematologica*. 2019;104:236–244.
38. Gabrilovich DI. Myeloid-derived suppressor cells. *Cancer Immunol Res*. 2017;5:3–8.
39. Boros P, Ochando J, Zeher M. Myeloid derived suppressor cells and autoimmunity. *Hum Immunol*. 2016;77:631–636.
40. Wegner A, Verhagen J, Wraith DC. Myeloid-derived suppressor cells mediate tolerance induction in autoimmune disease. *Immunology*. 2017;151:26–42.
41. Mauri C, Bosma A. Immune regulatory function of B cells. *Annu Rev Immunol*. 2012;30:221–241.
42. Blair PA, Noreña LY, Flores-Borja F, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity*. 2010;32:129–140.
43. Daien CI, Gailhac S, Mura T, et al. Regulatory B10 cells are decreased in patients with rheumatoid arthritis and are inversely correlated with disease activity. *Arthritis Rheumatol*. 2014;66:2037–2046.
44. Wang X, Zhu Y, Zhang M, et al. Ulcerative colitis is characterized by a decrease in regulatory B cells. *J Crohn's Colitis*. 2016;10:1212–1223.
45. van Helden PM, Kaijen PH, Fijnvandraat K, et al. Factor VIII-specific memory B cells in patients with hemophilia A. *J Thromb Haemost*. 2007;5:2306–2308.
46. van Helden PM, van den Berg HM, Gouw SC, et al. IgG subclasses of anti-FVIII antibodies during immune tolerance induction in patients with hemophilia A. *Br J Haematol*. 2008;142:644–652.
47. Barsotti NS, Almeida RR, Costa PR, et al. IL-10-producing regulatory B cells are decreased in patients with common variable immunodeficiency. *PLoS One*. 2016;11:e0151761.
48. Rosser EC, Mauri C. Regulatory B cells: origin, phenotype, and function. *Immunity*. 2015;42:607–612.
49. Carter NA, Vasconcellos R, Rosser EC, et al. Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells. *J Immunol*. 2011;186:5569–5579.
50. Flores-Borja F, Bosma A, Ng D, et al. CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. *Sci Transl Med*. 2013;5:173ra23.
51. Newell KA, Asare A, Kirk AD, et al. Identification of a B cell signature associated with renal transplant tolerance in humans. *J Clin Invest*. 2010;120:1836–1847.
52. Boulassel MR, Al-Ghonimi M, Al-Balushi B, et al. Regulatory B cells are functionally impaired in patients having hemophilia A with inhibitors. *Clin Appl Thromb Hemost*. 2018;24:618–624.
53. Schep SJ, Schutgens REG, Fischer K, et al. Review of immune tolerance induction in hemophilia A. *Blood Rev*. 2018;32:326–338.
54. Astermark J, Wang X, Oldenburg J, et al. Polymorphisms in the CTLA-4 gene and inhibitor development in patients with severe hemophilia A. *J Thromb Haemost*. 2007;5:263–265.
55. Bafunno V, Santacroce R, Chetta M, et al. Polymorphisms in genes involved in autoimmune disease and the risk of FVIII inhibitor development in Italian patients with haemophilia A. *Haemophilia*. 2010;16:469–473.
56. Marchione VD, Zuccoli JR, Abelleyro MM, et al. A prevalent CTLA4 missense variant significantly associates with inhibitor development in Argentine patients with severe haemophilia A. *Haemophilia*. 2017;23:e166–e169.
57. Abdulqader AMR, Mohammed AI, Rachid S. Polymorphisms in the cytotoxic T lymphocyte-associated protein-4 immune regulatory gene and their impact on inhibitor development in patients with hemophilia A. *J Int Med Res*. 2019;47:4981–4992.
58. Qian J, Collins M, Sharpe AH, et al. Prevention and treatment of factor VIII inhibitors in murine hemophilia A. *Blood*. 2000;95:1324–1329.