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ORIGINAL ARTICLE *Laboratory science*

A longitudinal evaluation of anti-FVIII antibodies demonstrated IgG4 subclass is mainly correlated with high-titre inhibitor in haemophilia A patients

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Summary. The development of inhibitory antibodies against factor VIII (FVIII) (inhibitor) is the major complication in haemophilia A patients. The FVIII-binding antibodies development comprises a polyclonal immunoglobulin (Ig) G response. Recent studies showed strong correlation between the presence of neutralizing anti-FVIII antibodies (inhibitors) and IgG4 subclass. The aim of this study was to evaluate anti-FVIII IgG subclasses in haemophilia A patients with inhibitor both in a cross-sectional and in a longitudinal analysis. Inhibitors were determined by Nijmegen–Bethesda assay. Anti-FVIII IgG subclasses were performed by ELISA, and samples from 20 healthy individuals were used to validate the test. We studied 25 haemophilia A patients with inhibitor, previously treated exclusively with plasma-derived FVIII concentrates or bypassing agents. The IgG subclasses distributions were evaluated in two groups of patients classified according to inhibitor response. IgG1 and IgG4 antibodies were most prominent in haemophilia A patients with inhibitors when compared with IgG2 and IgG3. This study reports for the first time the behaviour of FVIII-binding IgG1 and IgG4 subclasses in a longitudinal analysis, in a clinical setting, of high-response inhibitor haemophilia A patients, showing the correlation of IgG4 and the inhibitor titres. In spite of being considered a non-pathologic antibody subclass with anti-inflammatory properties in other situations, IgG4 is correlated with the presence of high-titre inhibitor in the haemophilia setting. The comprehension of the IgG4 role in immune response may be crucial to establish the process for designing specific tolerance to FVIII.

Keywords: haemophilia, IgG4, immunoglobulin, inhibitor

Introduction

The development of inhibitory antibodies against factor VIII (FVIII), also called inhibitors, can occur in approximately 25 a 30% of haemophilia A patients after repeated infusions of FVIII protein [1–3]. The inhibitor presence is a major complication, leading to a significant increase in the morbidity, and resulting in a negative impact in the quality of life of the haemophilia A patients [4]. This complication involves a complex immune mechanism, and hinders the replacement therapy effectiveness in haemophilia A patients [5,6]. The immune response generates antibodies against FVIII protein that neutralizes the procoagulant function of FVIII (inhibitors). In some cases, the antibodies against FVIII do not interfere in its haemostatic function, known as non-inhibitory antibodies, but may enhance FVIII removal from the plasma [2,7,8].

Clinical and experimental data indicate that FVIII inhibitor development depends on $CD4^+$ T-cell help [9]. Interactions between B cells and $CD4^+$ T cells not only initiate expansion and differentiation of B cells, but also trigger isotype switching and affinity maturation of antibodies [10]. Both inhibitory and non-inhibitory antibodies are mainly reported as immunoglobulin G (IgG) isotype [11,12]. The characterization of IgG subclasses contributes to better understand the FVIII-triggered immune response.

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Current studies demonstrated a strong correlation between high titre of anti-FVIII antibodies of IgG4 subclass with the presence of inhibitors [13] and immune tolerance induction (ITI) failure [14]. Whelan *et al.* demonstrated that non-inhibitory antibodies can be present in healthy individuals. However, IgG4 is exclusively observed in haemophilia A patients with inhibitors or acquired haemophilia A.

We describe here the distribution of IgG subclasses observed in a population of haemophilia A patients with inhibitors (>0.6 BU mL^{-1}) in two different contexts. Initially, we performed a cross-sectional analysis of the IgM and IgG subclasses presence in these patients, followed by a prospective evaluation of the IgG1 and IgG4 subclasses titrer in some high-response inhibitor patients.

Patients and methods

Ethics

This study was part of a multicentre study (*EMBIH – Estudo Multicêntrico Brasileiro de Inibidores em Hemofilia*) involving six haemophilia treatment centres from Brazil and coordinated by the haemophilia unit from the Hemocentro Unicamp in Campinas, Sao Paolo. Data collection was approved by the Institutional Review Board of the participating centres. The procedures followed were in accordance with the ethical standards and with the Helsinki Declaration.

Human plasma samples

Plasma samples from healthy individuals and haemophilia A patients were collected in 0.109 M citrated tubes as Clinical and Laboratory Standards Institute recommendation. The plasma samples were separated by 2500 g for 10 min and storage at −80°C until the assay performance. All samples from haemophilia A patients were submitted for heat treatment, 56°C by 30 min, before the assay performance in order to improve the tests sensitivity.

Detection of neutralizing antibodies against FVIII

The neutralizing anti-FVIII antibodies were detected by the Bethesda assay with Nijmegen modification [15,16]. Considering the high results variability of this assay demonstrated by different external quality control, three different dilutions for FVIII activity determination we performed [17].

Inhibitor classification

The inhibitor was classified according to the International Society on Thrombosis and Haemostasis. Low-response inhibitor was defined as a persistently ≤5 Bethesda units per millilitre (BU mL^{-1}) antibody even after repeated challenge with factor concentrate. The term high-response inhibitor was used for the cases where the inhibitor titre was >5 BU mL^{-1} at least in one measurement.

ELISA for detection of binding antibodies

ELISA method was performed using two different plasma-derived FVIII (pdFVIII) concentrates, (i) Octavi SD Optimum® (Octapharma, Lingolsheim, France), and (ii) an intermediate purity pdFVIII concentrate, 8Y® (Bio Products Laboratory, Hertfordshire, UK), which contains von Willebrand factor (VWF). On the first day, polysorp microtitre plates (Nunc® MaxiSorp Sigma-Aldrich, St. Louis, Missoure, USA) were coated with 1 μg mL^{-1} FVIII overnight at 4°C. On the second day, the plates were washed (phosphate-buffered saline PBS; pH 7.4 containing Tween 20) and non-specific binding sites were blocked by blocking buffers incubation (washing buffer with bovine serum albumin) for 1 h at room temperature (RT). In sequence, considering the second washing step, test samples and both negative (healthy individual) and positive (patient with high title of inhibitor) controls were incubated for 2 h at 37°C. The third washing was performed and then the anti-human-IgG specific for subclasses IgG1 (clone 4E3), IgG2 (clone HP6002), IgG3 (clone HP6050) and IgG4 (clone HP6025) and IgM (clone UHB) from Southern Biothechnology Inc., Birmingham, AL, USA was applied as enzyme-conjugated secondary antibodies by incubation for 1 h at 37°C. After the last washing step, the o-phenylenediamine was added and incubated at RT in the dark. The optical density (OD) for each sample was assessed using a Microplate Reader (Sunrise, Tecan, Männedorf, Zurique, Switzerland) in 492 nm. The OD of each sample was corrected for blank values.

For the ELISA method, each sample was analysed at least eight different times with an interval dilution from 1:10 until 1:640. The result was considered positive when the OD was higher than the cutoff established for blank background signal level. For all samples, a linear curve was designed to confirm the reactivity of Ig against FVIII.

Validation of ELISA for the detection of FVIII-binding antibody

Plasma samples from 20 healthy individuals (10 female, and 10 male) of different blood groups were used for the initial validation of the ELISA IgG subclass anti-FVIII. First, all samples were individually evaluated for the presence of the inhibitor.

The precision (inter-assay and intra-assay variability) of the method was evaluated and the inter-assays variability was approximately one dilution.

Statistical analysis

Statistical analysis consisted of the determination of the non-parametric coefficient of correlation according to Spearman for correlation analysis of FVIII-binding antibodies and FVIII inhibitors, and chi-square test, both using Prism 6.0 (GraphPad Software, San Diego, CA, USA).

Results

Samples from 20 healthy individuals were analysed for the presence of antibodies against pdFVIII, and presented no signal of reactivity.

We analysed samples from 25 Brazilian severe haemophilia A patients (mean age 23 years; ranging from 8 to 49 years), with 44% African-descendants. These haemophilia A patients exclusively received replacement therapy with pdFVIII concentrates. After the development of the inhibitor, they were treated with bypassing agents, either activated prothrombin complex concentrate (aPCC) or recombinant activated FVII (rFVIIa), when the inhibitor titre was higher than 5 BU mL^{-1}, or there was no clinical response to FVIII concentrate. Patients with low-titre inhibitor occasionally received pdFVIII, according to the clinical response. In this group 20 patients (80%) were classified as having high-response inhibitors, and 5 (20%), low-response inhibitors. Among the 20 high-response inhibitors patients, 5 presented a low titre inhibitor ($\leq$5 BU mL^{-1}) at the moment of the analysis.

IgG subclasses distribution among haemophilia A patients

The IgG subclasses distributions were evaluated in two groups of patients classified according to inhibitor response. IgG1and IgG4 antibodies were most prominent in haemophilia A patients with inhibitors when compared with IgG2 and IgG3. Among the patients with low-titre inhibitor at the moment of the analyses, we observed that few patients were positive for IgG2 (2 of 10, 20%), IgG3 (1 of 10, 10%), and IgG4 (2 of 10, 20%), only in the 1:10 dilution (Table 1). On the other hand, all patients in this group were positive for IgG1 (10 of 10, 100%), with dilutions ranging from 1:10 to 1:320 ($P < 0.0001$) (Table 1). Among the patients with high-titre inhibitor, all of them (15 of 15, 100%) were positive for IgG4, with dilutions from 1:10 to 1:320. Regarding IgG3 and IgG2, 2 of 15 (13%), and 6 of 15 (45%) were positive, respectively. With respect to IgG1, 12 of 15 (80%) were positive with dilutions from 1:10 to 1:320, which was similar to the result of the previous group ($P < 0.0001$) (Table 1). Interestingly, in 5 high-response inhibitor

Table 1. Evaluation of anti-FVIII IgG subclasses in haemophilia A patients with inhibitor.

			IgG1		IgG2		IGg3		IgG4	
Patients	Inhibitor titre (BU mL^{-1})	Responding inhibitor	I*	II*	I*	II*	I*	II*	I*	II*
Low-titre inhibitor (<5 BU mL^{-1})										
L1	0.7	Low	++++	–	+	–	–	–	–	–
L2	0.75	Low	++	–	+	–	–	–	–	–
L3	0.95	Low	+++	–	–	–	–	–	–	–
L4	1.4	Low	+	–	–	–	–	–	+	–
L5	2.8	Low	++	–	–	–	–	–	–	–
L6	0.9	High	–	+++	–	–	–	–	–	–
L7	1.9	High	++++	-	–	–	+	–	+	–
L8	3.15	High	+	+++	–	–	–	–	–	–
L9	2	High	++++	–	–	–	–	–	–	–
L10	4.9	High	++	–	–	–	–	–	–	–
High-titre inhibitor (>5 BU mL^{-1})										
H11	7.4	High	++++	–	–	–	–	–	++++	–
H12	8	High	+++	–	–	–	–	–	+	–
H13	8.4	High	+++	–	+++	–	–	–	+	–
H14	11	High	++++	–	–	–	–	–	++++	–
H15	11.5	High	++++	–	++++	–	–	–	++	–
H16	12	High	+	–	+	–	–	–	++	–
H17	12.5	High	+	–	–	–	–	–	+	–
H18	14.5	High	–	–	–	–	–	–	+	–
H19	16	High	++	–	+++	–	–	–	+	–
H20	25	High	++	–	–	–	–	–	+++	–
H21	35	High	–	–	–	–	++++	–	++++	–
H22	40	High	++++	–	++++	–	++	–	++++	–
H23	58	High	–	–	–	–	–	–	++++	–
H24	155	High	+	–	+	–	–	–	+++	–
H25	384	High	+	+	–	–	–	–	+	–

*Two different pdFVIII concentrates were used as target antigen in the ELISA, (I) Octavi SD Optimum® (Octapharma, France), with FVIII 100 IU mg^{-1}, (II) 8Y® (Bio Products Laboratory, UK), with FVIII 2 IU mg^{-1}, and contains high levels of VWF. The results are presented as a positive reaction obtained with the following dilutions of the tests sample: –, negative; +, 1:10; ++, 1:40; +++, 1:80; ++++, 1:320.

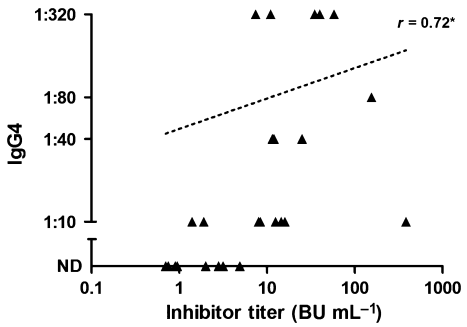

Fig. 1. Correlation between titres of FVIII-specific IgG4 and inhibitor. Correlation between titres of FVIII-specific IgG4 and titres of inhibitor in haemophilia A patients with inhibitors. The correlation was determined using the 2-tailed non-parametric correlation according to Spearman, and calculated using GraphPad Prism 6 software. The correlation is significant (*$P < 0.001$).

patients with low-titre inhibitors at the moment of the analysis, IgG4 was nearly absent, with a single patient having a positive sample in the dilution 1:10 (Table 1). IgM was not detected in any analysed sample (data not shown).

Considering that IgG4 was present almost exclusively in high-response inhibitor patients, we correlated the inhibitor and IgG4 titres, and a significant correlation was observed ($r = 0.72$; $P < 0.001$) (Fig. 1). In contrast, for IgG1, no correlation was observed between the inhibitor and IgG1 titres.

IgG1 and IgG4 subclasses distribution in a longitudinal analysis

The observation of a lower occurrence of IgG4 in high-response inhibitor patients with a low titre of inhibitor led us to perform a longitudinal analysis of 5 high-response inhibitor patients, in order to assess the behaviour of IgG1 and IgG4 titres in relation to the inhibitor titre. The observation period ranged from 14 to 31 months. All these patients were preferentially treated with bypass products, aPCC and/or rFVIIa.

Patient 1 (Fig. 2), when challenged with a pdFVIII concentrate, showed an anamnestic response with increased inhibitor titre that correlated with a higher IgG4 titre ($r = 0.910$; $P < 0.001$), and very low variation was observed in the IgG1 titre. During the 31 months of follow-up, no correlation between IgG1 and inhibitor titre was noted. A similar trend between IgG4 and inhibitor titres was also observed during the longitudinal evaluation of patients 2, 3, and 5 ($r = 0.949$, $P = 0.083$; $r = 0.803$, $P = 0.133$; $r = 0.866$, $P = 0.333$, respectively) (Fig. 3). In patient 4, we observed persistent high-titre inhibitors (13–18 BU mL^{-1}), with presence of both IgG1 and IgG4 (titres from 1:20 to 1:160), and no correlation between immunoglobulins and inhibitor titres.

Different reactivity obtained between different types of pdFVIII

In the cross-sectional analysis of the 25 haemophilia A inhibitor patients, the ELISA method for the detection of binding antibodies was performed using two different pdFVIII products, a high purity FVIII concentrate, Octavi SD Optimum® (Octapharma, 100 IU mg^{-1} of FVIII), and a low purity FVIII concentrate, 8Y® (Bio Products Laboratory, 10 IU mg^{-1} of FVIII). We observed a considerable reduction in the reactivity using the low purity FVIII concentrate, which contained VWF, in all analysed samples (Table 1). Only 3 of 25 patients presented IgG1 reactivity to 8Y® (dilutions ranging from 1:10 to 1:80), and all of them were high-response inhibitor patients. Two of these patients had low-titre inhibitor at the moment of the analysis (0.9 and 3.15 BU mL^{-1}) with positivity in the 1:80 dilution for both. The other patient had high titres of inhibitors (384 BU mL^{-1}) at the moment of the analysis, with positivity to IgG1 in the dilution 1:10 when the ELISA was performed with both products. This patient also presented positive IgG4 in the dilution 1:10, but only when the high purity product was used.

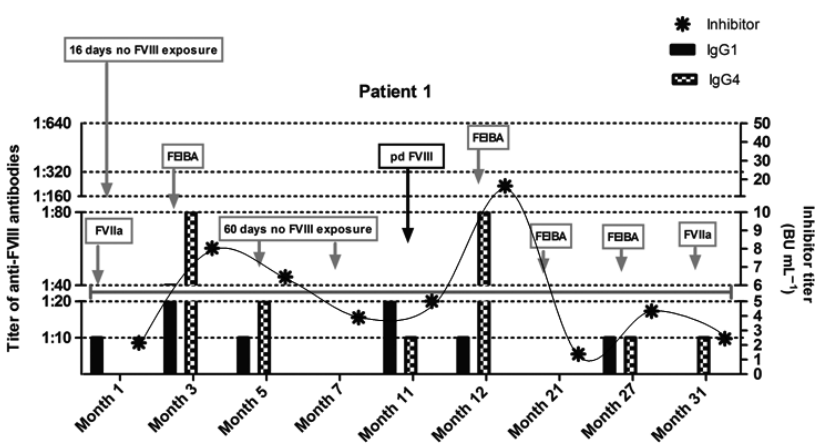

Fig. 2. Patient 1 longitudinal analysis of IgG1 and IgG4 in high-responding inhibitor patients and anamnestic response to pdFVIII with increase in IgG4. The correlation between inhibitor and IgG4 was r* 0.910 (*Spearman correlation).

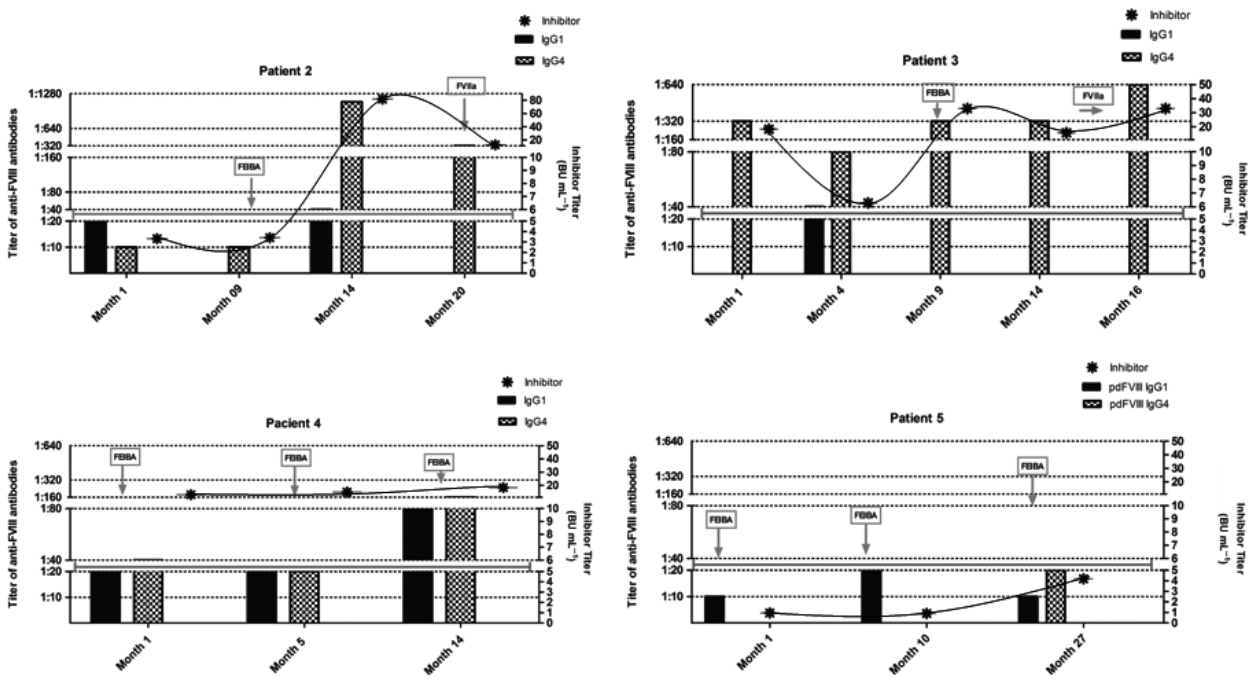

Fig. 3. Patients 2–5 longitudinal analyses of IgG1 and IgG4 in high-responding inhibitor patients. The correlation between inhibitor and IgG4 were *r** 0.949, *r** 0.803, *r** 0.866, patients 2, 3 and 5 respectively (*Spearman correlation).

Discussion

This study reports for the first time the behaviour of FVIII-binding IgG1 and IgG4 subclasses in a longitudinal analysis of high-response inhibitor haemophilia A patients, showing the correlation of IgG4 and the inhibitor titres. The characterization of the IgG1 and IgG4 subclasses in haemophilia A patients contributes to better understand the mechanism of immune response against FVIII and in early stages may provide information to indicate mechanisms leading to low and high-titre inhibitors in different inhibitor classification of patients, and also to design new clinical assays. Our results demonstrated that IgG4 subclass correlates with high-titre inhibitors, and it is nearly absent even in high-response inhibitor patients, when they present low-titre inhibitor. A similar result was observed in a study that evaluated IgG subclasses during ITI response, where in low-titre inhibitor patients, anti-FVIII antibodies consisted primarily of IgG1 subclass, whereas, anti-FVIII antibodies of IgG4 subclass were more prominent in patients with high titre inhibitors, who needed prolonged treatment or those whose ITI failed [14].

In a recent report, 18 haemophilia A patients with high-response inhibitors, and 4 acquired haemophilia A patients were evaluated for the presence of FVIII-binding antibodies using a different methodology, the surface plasmon resonance platform. Among the haemophilia A patients with inhibitor, the authors observed a predominance of IgG1 and IgG4, similar to the results reported here, although no correlation between the immunoglobulin and inhibitor titres were performed in their study [18].

In another study, in addition to evaluating Ig isotypes in haemophilia A patients with inhibitor, a large group of healthy individuals was evaluated using ELISA platform method, and it was reported a prevalence of immunoglobulin against FVIII of 19% (116 of 600). However, IgG4 was completely absent in haemophilia patients without inhibitors and in healthy individuals [13]. In our group of 20 healthy individuals no FVIII-binding antibodies were detected, and this difference can be related to the small number of analysed samples. Nevertheless, among haemophilia A patients with inhibitor included in their study, it was observed that IgG1 and IgG4 were the most frequent subclasses found, in accordance with our results.

The IgG subclasses reactivity depends on the type of product used to perform the analyses. The patients included in our study received exclusively pdFVIII and were evaluated with two different types of pdFVIII product, a high purity FVIII concentrate, and a low purity FVIII concentrate containing VWF. A marked reduction in reactivity was observed when the assay was performed with the low purity product. However, this lower *in vitro* reactivity does not result in a better haemostatic effect for the replacement therapy when this kind of product is used in patients with clinically significant inhibitors. Notwithstanding, there is a

discussion regarding the better success rates when FVIII concentrates containing VWF is used in ITI protocols [19,20].

The Nijmegen–Bethesda assay still considered the best method for the inhibitory antibody detection. However, limitations involving this assay are commonly reported, especially for the low-titre inhibitors. Alternative assays are required to accurately quantify low-titre anti-FVIII antibodies [21]. Low sensitivity of Bethesda assay to low titre inhibitor lead a misidentification and also miscorrelation with other technologies, as ELISA and Luminex [22], which detected inhibitory and no-inhibitory antibodies. Although inhibitory antibodies are the primary concern when attempting to restore haemostatic function, both inhibitory and non-inhibitory antibodies provide information about the immunological state of a patient. However, the clinical significance of the non-inhibitory FVIII-binding antibodies is controversial.

A number of sensitive immunoassays have been developed to allow the screening of clinical samples with inhibitory and non-inhibitory anti-FVIII antibodies to provide complementary information to the Bethesda assay [22]. The detection of IgG4 FVIII-binding antibody may provide a methodology with better sensitivity and specificity, without increasing the detection of antibodies with no clinical relevance.

Furthermore, the behaviour of IgG1 and IgG4 observed in our cross-sectional analysis, between low and high response inhibitor patients, was confirmed in a longitudinal analysis. In both analyses, we observed the IgG subclasses modulation to be linked to inhibitor titre and not to the type of inhibitor response. IgG4 was nearly absent in high-response inhibitor patients with low-titre, and in the longitudinal analysis IgG4, but not IgG1, titres correlated with the increase in inhibitor titre observed during the anamnestic response to FVIII. Both cross-sectional and longitudinal analysis confirm that the presence of IgG4 is mainly associated with high-titre inhibitor.

The IgG4 has long been considered a non-pathologic antibody subclass with anti-inflammatory properties [23]. It is functionally different from other IgG subclasses because of its poor ability to activate complement and Fc-receptor – expressing effector cells. However, novel characteristics of IgG4 in fibro-inflammatory diseases have been recently described [24]. Immune reactions in such diseases are characterized by the presence of a high level of IgG4 in the peripheral blood with an activation of regulatory T (Treg) cells. These findings further support the association of IgG4 with the activation of Treg cells. Understanding the behaviour of each different type of $CD4^+$ T-cells, particularly Treg cells, in the context of an environment with high levels of anti-FVIII IgG4 is important to establish the process for designing specific tolerance to FVIII, which is crucial for the prevention and treatment of inhibitors in haemophilia patients.

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Authors contribution

S.A.L.M. designed the study, performed the assays, collected and analysed the data, and wrote the manuscript; A.C.T. performed the assays, L.H.S. performed the assays, A.L.A.S. collected the data; S.S.M., analysed the data, and reviewed the manuscript; and M.C.O. designed the study, collected the data and critically revised and approved the final version of the manuscript.

Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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