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ORIGINAL ARTICLE

Severe haemophilia A patients have reduced numbers of peripheral memory B cells

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Summary. The development of inhibitors is a complication of replacement treatment in Haemophilia. Loss of factor VIII-specific memory B cells in the spleen is associated with down regulation of antibodies in mice treated with high doses of FVIII, but changes in B cell memory have not been described in haemophilic patients. The aim of this study was to evaluate the phenotype of circulating lymphocytes in severe haemophilia A. Twenty patients with inhibitors (PI), 22 without inhibitors (P), nine patients during immune tolerance induction (ITI) treatment and 20 healthy donors (HD) were included. Peripheral blood lymphocytes were examined using flow cytometry. Anti-FVIII antibodies were measured using Bethesda and flow cytometry. Percentages of T subsets and B lymphocytes were similar in all groups. In contrast, memory B cells (CD27+) were decreased in PI and P compared with HD, but the level of significance was higher in PI ($P = 0.001$) than P ($P = 0.01$). PI with high level of anti-FVIII antibodies presented the lowest B memory values. CD70 expression was also lowest in PI. Non-switched CD27+ subpopulation (IgD+) was prevalent in PI, but did not show statistical significance. When ITI failed, the percentages of CD27+ B cells after 12 months of ITI were lowest. In a longitudinal study performed in four patients, an increased percentage of CD27+ and CD70+ B cells during ITI was found. This work suggests that different peripheral lymphocyte markers, such as CD27 and CD70 on B cells, may be helpful to evaluate anti-FVIII response and to monitor the success of ITI.

Keywords: anti-FVIII antibodies, haemophilia, memory B cells

Introduction

The development of inhibitors is an important complication of replacement treatment in haemophilia A. Antibodies inhibiting FVIII activity are observed in about 25% of patients with severe disease [1]. Antibodies to FVIII that do not affect the haemostatic activity of FVIII can be also developed [2]. Why some patients develop FVIII inhibitors, whereas others do not, is a question that has not been answered as yet. Several risk factors of the host may influence the generation of inhibitory anti-FVIII antibodies [3]. Haemophilic patients develop inhibitors because replacement FVIII is seen as foreign protein that triggers an adaptive immune response. The generation of antibodies involves the participation of antigen-presenting cells, T lymphocytes and finally B lymphocytes that differentiate into plasma cells and memory B cells. Nowadays, there are available therapies for the eradication of FVIII inhibitors that include the administration of high-dose FVIII to induce specific immune tolerance (ITI) [4]. During immune response to T-dependent antigens, such as FVIII, immunological memory is established mainly via the generation of memory B cells [5]. Therefore, it is necessary to evaluate the presence of memory B cells and their variation after induction of immune tolerance.

In mice treated with high doses of FVIII, permanent loss of FVIII-specific memory cells (antibody producing cells) in the spleen is associated to down regulation of anti-FVIII antibodies [6], but changes in B cell memory have not been described in haemophilic patients.

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Human memory B cells are a heterogeneous population. Phenotypic characteristics can be used to identify B cell subsets [7]. CD27, belonging to the TNFR family, is widely used as a marker of human memory cells, because its expression correlates with the presence of somatic mutations in Ig genes [8]. However, a CD27-IgG+ B cell population expressing mutated IgG genes was also described [9]. Adult peripheral blood CD27+ can be subdivided into two discrete subtypes: IgD− and IgD+. IgD-CD27+ B cells produce IgG, IgM and IgA, whereas IgD+CD27+ B cells predominantly produce IgM [10]. During differentiation into plasma cells, B lymphocytes change their chemokine expression profile. The IgG+ plasma cell precursors that were formed in a secondary immune response against systemic antigenic challenge migrate towards ligands of the C-X-C motif chemokine receptors (CXCR3 and CXCR4). CXCR3 is important in the regulation of the localization of IgG-plasma cells formed within memory immune responses [11]. In addition to chemokine receptors, other molecules are important as markers of B lymphocytes along their differentiation process, one among them being CD70. CD70 is a type II transmembrane glycoprotein and is physiologically found on a small subset of activated memory B cells [12]. It has been reported that CD70 can deliver co-stimulatory signals in cognate T–B interactions [13] through interaction with CD27 present in T lymphocytes. Moreover, CD70+ B lymphocytes are necessary to provide signals for the retention of CD27 on viral specific memory CD8+ T lymphocytes [14,15].

In this study, we describe the immunological profile of haemophilia A patients with and without FVIII inhibitors. Flow cytometric (FC) analysis was used to quantify the percentage of total B cells (CD19+) and to discriminate B cell subsets in the peripheral blood. We found decreased CD27+ memory B cells in patients with inhibitors and these cells had low CD70 expression. The IgD+ CD27+ B subset was prevalent in these patients. After ITI, the characteristics of memory B cells resembled those of normal donors. The fact that peripheral blood memory B cells are reduced in patients with FVIII inhibitors, and that this defect can be corrected by successful ITI, suggests that these variations are associated with the generation of the inhibitors, perhaps reflecting migration of the plasma cell precursors to the secondary lymphoid tissue to give rise to mature plasma cells.

Materials and methods

Patients

We studied 22 haemophilia A patients without inhibitors (P), 20 haemophilia A patients with active inhibitors who did not receive ITI (PI), nine haemophilic A patients who received ITI (PI–ITI) and 20 healthy donors (HD). The subjects were HIV− and their age ranged between 2 and 20 years. All patients were chosen among those who received periodic assistance and control at the 'Fundación Argentina de Hemofilia'. This study was approved by the Ethics Committee of the Academia Nacional de Medicina of Buenos Aires. All donors gave informed consent.

ITI treatment

Patients who were admitted for ITI received 100 or 200 U Kg^{-1} of anti-haemophilic FVIII once a day during 18 months.

Inclusion criteria: Severe haemophilia A, inhibitor of high response (>5 BU mL^{-1}), low historical peak inhibitor titre (<200 BU mL^{-1}), low-inhibitor titre (<10 BU mL^{-1}) prior to the initiation of ITI, FVIII gene mutation, time between inhibitor diagnosis and starting ITI <5 years.

Antibodies

The following monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinin chlorophyll protein (PerCP) were used: FITC anti-CD3 (clone SK7), FITC anti-CD27 (clone L128), PerCP anti-CD4 (clone SK3) and PE anti-CD8 (clone SK1) were from Becton Dickinson (BD Biosciences, San Jose, CA, USA). PE anti-CCR4 (clone IG1), PE anti-CXCR3 (clone 1C6/CXCR3), PE anti-CD70 (clone Ki-24), PECy5 anti-CD19 (clone HIB19), FITC anti-CD45RO (clone UCHL1) and FITC anti-CD25 (clone M-A251) were from Pharmingen, BD Biosciences. FITC polyclonal rabbit anti-human IgD (Dako, Glostrup, Denmark). Appropriate isotype controls were used to define the positive populations.

Flow cytometry

Peripheral blood samples from all patients were collected on Heparin. A three colour assay was used. Aliquots of 100 μL were incubated with monoclonal antibodies according to the manufacturer's recommendations. Samples were then lysed using FACS Lysing solution (Becton Dickinson) during 10 min at room temperature. After centrifuging, cells were washed. Flow cytometric analysis of surface markers was performed on a FACScan cytometer (Becton Dickinson) equipped with a 488 nm argon ion laser and analysed with CellQuest software. Lymphocytes were selected according to size (FSC) and side (SSC) scatter profiles (R1). The percentages of B lymphocyte subpopulations were obtained from dot plots displaying fluorescence intensities. In each assay, we assessed at least 20 000 lymphocytes.

Flow cytometry anti-FVIII assay

The evaluation of anti-FVIII antibodies by flow cytometry was recently described [16]. Briefly, plasma dilutions (1/4–1/2000) were made to react with 2.5 μL of microspheres (Polysciences) coupled with rFVIII (FVIII-m) according to the manufacturer's recommendations or microspheres without rFVIII (Control-m), in a final volume of 50 μL during 2 h at 4°C. After washing, captured antibodies were detected using biotinylated goat F(ab′)2 fragment anti-Human IgG (Immunotech, Marseille, France) followed by PE-conjugated streptavidin (Vector, Ontario, Canada). Microspheres were washed and resuspended in 250 μL of FACSflow (Becton Dickinson) and analysed by flow cytometry. We used logarithmic amplification in all the parameters recorded. Beads were selected by gating (R1) according to FSC and SSC profiles. A threshold of 200 was set up on SSC parameter to eliminate unwanted events. Ten thousand gated microspheres from R1 were acquired for each determination. MFI (Mean Fluorescence Intensity) of FL-2 emission was recorded. The MFI ratio between MFI of FVIII-m and MFI of Control-m was calculated each time after the analysis. For semiquantitative results, an index was calculated multiplying the highest MFI ratio by the inverse of the corresponding plasma dilution.

Bethesda assay

Inhibitory antibodies were measured using the Nijmegen modification of the Bethesda method, as previously described [17]. We defined a positive test by the Bethesda assay as a result >0.5 BU mL^{-1}.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney test in GraphPad software (San Diego, CA, USA). Values of $P < 0.05$ were considered statistically significant.

Results

Phenotype of freshly isolated peripheral blood T and B cells in patients with and without anti-FVIII antibodies and HD

The analysis of CD3+ cells did not reveal any significant difference between the two groups of severe haemophilia A patients and HD (Table 1). We employed classic markers to evaluate the two major subsets of T cells, helper CD4+ and cytotoxic CD8+. We also studied the expression of CD25 (necessary for lymphocyte proliferation) on CD4+ cells, and the expression of CXCR3 and CCR4, representing enriched subsets in Th1 and Th2 cells respectively [18]. The results were similar in the three groups. The percentage of CD4 + CD45RO+, a memory T cell marker, was higher in the patients, but this difference was not statistical significant compared with HD. The B cells were identified by the expression of CD19. Table 1 shows that the percentages of total CD19+ cells tended to be higher in Haemophilia patients than in non-haemophilic controls, but this difference was not statistically significant. Naïve cells and memory B cells were distinguished by the absence and presence of CD27 expression respectively. Table 1 shows that CD19+CD27+ cells were decreased in PI and P compared with HD, but the level of significance was higher in PI ($P = 0.001$) than in P ($P = 0.01$). Expression of CXCR3+ on B cells was also weakly decreased in both PI and P with respect to HD. Likewise, CD70 expression on B cells was reduced in P and PI with respect to HD ($P = 0.01$ and $P = 0.001$ respectively) and was lowest in PI with respect to P ($P = 0.01$).

Table 1. Lymphocyte frequencies in normal healthy donors (HD) and severe haemophilia A patients with (PI) and without (P) inhibitors.

Subset	HD	P	PI
%CD3+	73.87 ± 8	68 ± 7.4	70 ± 5.7
%CD3+CD4+	58.8 ± 11.43	50.8 ± 11.5	56.7 ± 7.9
%CD8+	39.95 ± 10.01	47.8 ± 12.8	41.6 ± 8.7
%CD4+CD27+	82 ± 11	83 ± 15	88.4 ± 12
%CD4+CD25+	11 ± 6	10 ± 3.7	12.2 ± 4.5
%CD4+CXCR3+	40.5 ± 13.7	38.2 ± 16.5	33 ± 13.4
%CD4+CCR4+	34 ± 14	40 ± 15	38 ± 15
%CD4+CD45RO+	42.7 ± 7.1	56 ± 17	54 ± 23
%CD19+	11.33 ± 5	15.12 ± 6	14.9 ± 5.2
%CD19+CD27+	29.46 ± 10	22.16 ± 8.2*	18.7 ± 9†
%CD19+CXCR3+	22.9 ± 9.1	16.8 ± 6.9	18.6 ± 8.8
%CD19+CD70+	13.8 ± 6.6	7.8 ± 3.6*	5.8 ± 3.4†‡

Lymphocytes were analysed using flow cytometry after staining peripheral blood with monoclonal antibodies. Results are the percentages (mean ± SD) of each subset.
*P vs. HD, $P = 0.01$.
†PI vs. HD, $P = 0.001$.
‡PI vs. P, $P = 0.01$).

Focusing on the population of memory B cells (CD19+CD27+), the CD70 expression was lowest on CD27+ from PI (Fig. 1a), whereas CXCR3 was similar in the three groups (Fig. 1b). We further investigated IgD expression on peripheral blood CD27+ B cells. It is known that B cells can be separated into IgD+ (non-switched) and IgD− (switched) cells. Fig. 1c illustrates that the non-switched CD27+ subpopulation was prevalent in PI patients (62.2 ± 22.7%) with respect to P (51.41 ± 6.8%) and HD (33.8 ± 19.8%), although these differences were not significant.

Peripheral B memory cells and anti-FVIII antibodies

To analyse if there was any relationship between the proportion of CD19+ CD27+ cells of each patient and the level of anti-FVIII antibodies, we separated PI into

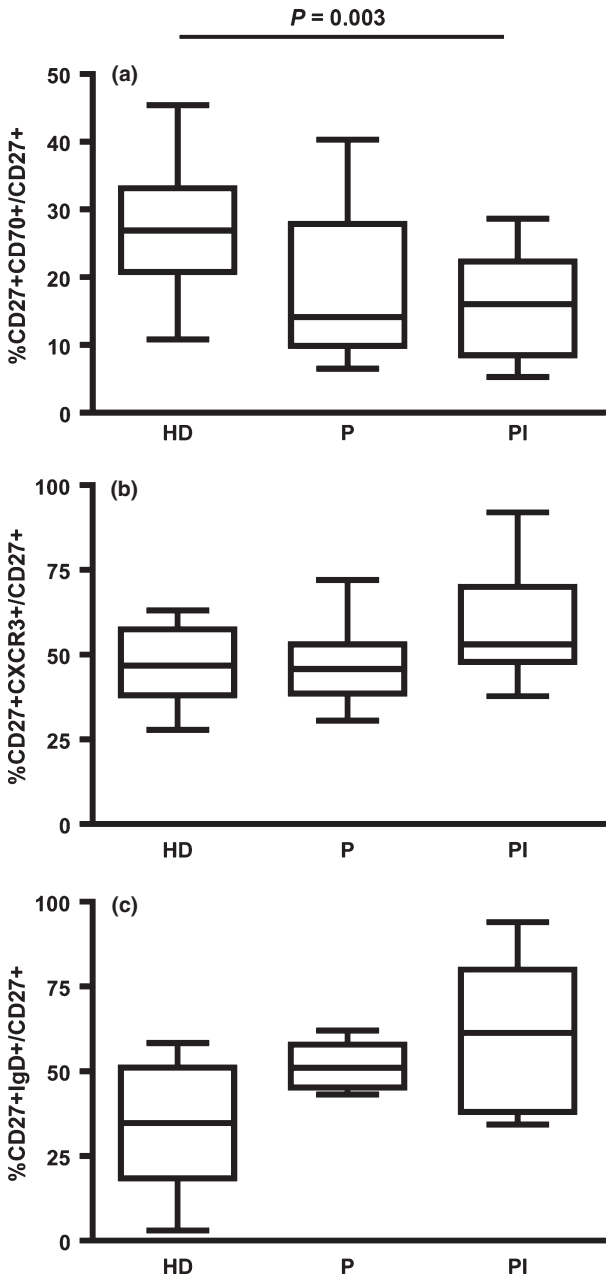

Fig. 1. Comparison of subpopulations of memory B cells in peripheral blood: The expression of CD70 (a), CXCR3 (b) and IgD (c) was studied on CD19+CD27+ cells from severe haemophilia A patients with (PI), without (P) inhibitors and healthy donors (HD). Cells were stained with monoclonal antibodies and analysed using flow cytometry. Boxes extend from the 25th percentile to the 75th percentile, with a horizontal line at the median. Whiskers extend down to the smallest value and up to the largest.

two groups: Group I – those whose anti-FVIII titre by flow cytometry was $\leq 5 \times 10^2$, and Group II – patients with high anti-FVIII titres ($>5 \times 10^2$). We found that 33% (nine patients) in Group I had a percentage of memory B cells below the normal value (mean–SD), whereas in Group II most patients (77%) had memory B cells below the normal value (mean–SD) (Fig. 2),

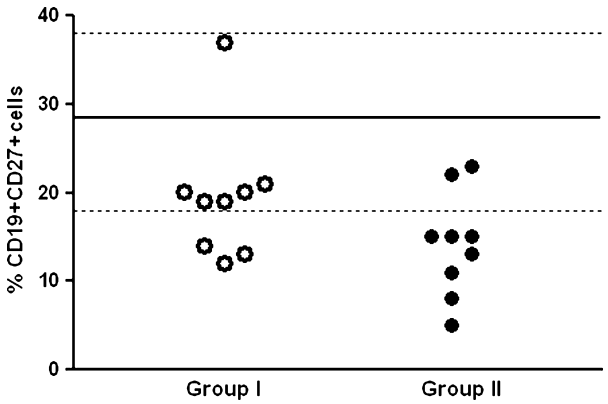

Fig. 2. Percentages of memory B cells and anti-FVIII antibodies: Peripheral CD19+CD27+ cells from severe haemophilia A patients were identified using flow cytometry after staining with monoclonal antibodies. Titres of anti-FVIII antibodies were obtained by flow cytometry employing microspheres. Control-m (without FVIII) and FVIII-m (coupled with FVIII) microspheres were incubated (2 h, 4°C) with different dilutions (1/4–1/2000) of plasmas and stained with biotinylated anti-Human IgG followed by PE-conjugated streptavidin. Indices were calculated as the highest ratio between mean fluorescence intensity (MFI) of FVIII-m and Control-m, multiplied by the inverse of the corresponding dilution. Patients were divided in two groups according to the antibody titre: Group I ($\leq 5 \times 10^2$) and Group II ($>5 \times 10^2$).The solid line indicates mean value of healthy donors and dotted lines represent the SD.

suggesting a negative correlation between the anti-FVIII titres and the percentage of circulating memory B cells.

Memory B cells during ITI

As treatment with high-dose FVIII in haemophilic mice with inhibitors had shown to reduce splenic memory cells in accordance with the reduction of the plasma antibody titre [6], we analysed the effects of ITI on the percentage of circulating memory B cells in nine haemophilic patients after 1 year of treatment. Figure 3 shows that in the two patients who failed ITI, the percentages of CD27+CD19+ cells remained below the normal values (28.5 ± 10%). In three patients in whom ITI was not successfully completed, levels of memory B cells were within the normal range. Moreover, in three-fourth of successfully treated patients, the highest CD27+CD19+ values were observed.

To examine the changes of CXCR3 and CD70 expression on peripheral CD27+ B cells during ITI, we obtained samples at different times of treatment, from four haemophilia A patients. The results shown in Fig. 4 indicate that the low CD27+ B cells values observed at the beginning of treatment, increased thereafter. The level of CXCR3 on B cells remained within the normal range, whereas CD70 expression increased as the treatment progressed. In patients A, C and D, the levels of inhibitors (Bethesda) were reduced after 12 months of the treatment, although in patient D only a partial success was obtained. Patient B is still under treatment.

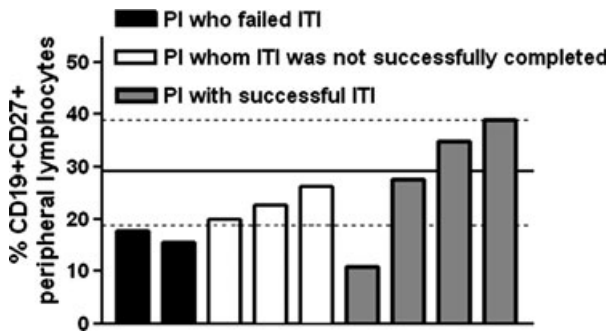

Fig. 3. Memory B cells after 12 months of starting immune tolerance induction (ITI) treatment. Peripheral blood cells from nine severe haemophilia A patients receiving ITI were stained with PECy5 anti-CD19 and fluorescein isothiocyanate anti-CD27 monoclonal antibodies and analysed using flow cytometry. Patients were separated according to outcome of ITI. The solid line indicates mean value of healthy donors and dotted lines represent the SD.

Discussion

The development of inhibitory antibodies against FVIII is the major complication for replacement treatment in these patients. Inhibitory antibodies rapidly inactivate FVIII, thereby rendering patients unresponsive to further replacement therapy. Patients presenting inhibitors are usually treated by regular infusions of high doses of FVIII to eradicate the inhibitors. This treatment (ITI) is successful in about 80% of patients with low titres of inhibitors, but in only 60% of patients with high titres. The precise mechanism of action of such therapy has not been elucidated. Our studies were carried out to examine the phenotypic characteristics of peripheral blood lymphocytes in severe haemophilia A patients who were treated with FVIII. Although no major variations in the T cell subsets were observed in patients compared with HD, altered B cell subpopulations were found. Limited knowledge is available with respect to the development and persistence of FVIII-specific memory B cells in patients with haemophilia A [19,20]. In humans, peripheral B cells that express CD27 are considered to be derived from the germinal centre reaction following specific antigenic stimulation [21]. Memory B cells play an essential role in maintaining established antibody responses [22]. We found decreased peripheral CD27+ B cells in haemophilia A patients with inhibitors. This is not surprising, as FVIII-specific memory B cells should be migrating to secondary lymphoid organs to give origin to the mature plasma cells, actively secreting anti-FVIII antibodies detected in plasma. Reipert *et al.* [19] have reported low frequency of FVIII-specific memory cells in the blood of one patient of six haemophilia A patients with inhibitors and this patient had the highest neutralizing antibody titre (Bethesda). The Bethesda method is the current standard test to evaluate anti-FVIII inhibitors, but non-inhibitory antibodies interacting with non-functional sites of FVIII are not detectable by this assay. We used an immunomethod [16] to evaluate the total humoral anti-FVIII response, as this assay detects both inhibitor and non-inhibitor antibodies.

Seventy seven per cent of the patients who presented the highest level of anti-FVIII antibodies (more than 5×10^2), also had CD27+ cells below normal values. This percentage decreased to 33% in patients with low anti-FVIII antibodies.

To better characterize peripheral memory B cells in haemophilia A patients, we studied their surface expression of IgD, CXCR3, CD70. IgD+ B cells are mainly naïve cells [8,23], but activated or switched memory B cells exhibit decreased levels of surface IgD expression [10], indicating that expression of cell surface IgD may be helpful to monitor the development of memory B cell responses. A high percentage of IgD+

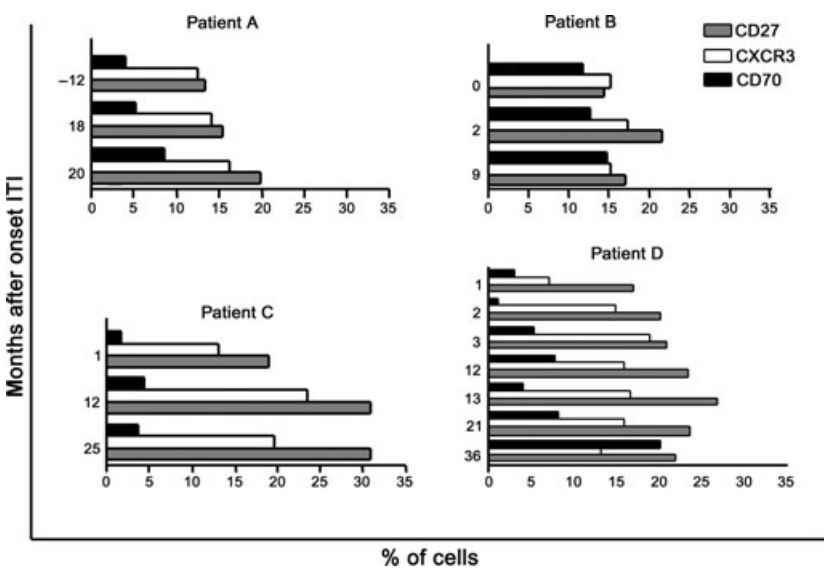

Fig. 4. Percentages of CD27+, CXCR3+ and CD70+ B cells from peripheral blood in four patients with severe haemophilia A during immune tolerance induction (ITI): Cells were analysed using flow cytometry after staining with monoclonal antibodies against CD19, CD27, CXCR3 and CD70. The number of months relative to the onset of ITI (t = 0) is depicted on the left of each panel.

expression on circulating CD27+ B cells in the PI group would indicate that non-switched B cells prevailed in this group of patients. The existence of a small CD27+IgD+IgM+ memory B subpopulation ('non-switched' memory cells) in peripheral blood has been reported [24]. Although these cells had been associated with T-independent responses [25], they can also participate in the response to T-dependent antigens. In our study, similar IgM expression on IgD+ B cells in PI, P and HD was observed (data not shown), suggesting that this subpopulation of 'non-switched' memory cells was not expanded in Haemophilia patients, whether they had FVIII inhibitors or not.

Memory B cells migrate selectively to inflamed tissues. CXCR3 is an important regulator for the homing of murine IgG-secreting plasma cells formed in memory immune responses. Ligands for CXCR3 would probably attract plasma cell precursors to sites of inflammation. CXCR3 is expressed only in a fraction of human memory B cells, and it is induced early following activation, even before these cells have divided [11]. Thus, this chemokine receptor seems to play an important role in the immune response, favouring the generation of the B cell memory. By examining the expression of CXCR3 on peripheral blood memory B cells in the three groups included in this study, we did not find differences, ruling out that CD27+ peripheral B cells in patients with inhibitors belong to non-activated lymphocytes. On the other hand, the expression of CD70, which is known to deliver co-stimulatory signals in cognate T–B interactions, was significantly decreased in CD19+ cells from haemophilia A patients with respect to HD. The lowest values were observed in PI.

It is reasonable to believe that specific memory B cells have to be eradicated or functionally inactivated during a successful ITI therapy with FVIII in patients with haemophilia A. This would lead to a reduction in the generation of mature plasma cells in secondary lymphoid organs secreting the inhibitory antibodies. The results of this study indicate that total memory CD27+ B cells that express CD70+ were initially low in the peripheral blood of patients with high inhibitor titres. As ITI progressed, the percentage of memory B cells in the blood increased, reaching normal values in patients who had responded, by reducing the plasma inhibitor levels. Treatment was partially successful in patient D (Fig. 4) whose peripheral B memory after increasing initially, showed a slow reduction associated to failure to eliminate the plasma anti-FVIII inhibitors. Initial low values of peripheral blood memory B cells in patients with high inhibitor titre indicate that both FVIII-specific and non-specific memory B cells are reduced in these patients. The reduction of FVIII-specific memory B cells after treatment could be explained by migration of these cells into the secondary organs, where they differentiate into the plasma cells that give rise to circulating antibodies, but the overall low values of CD27+ B cells in peripheral blood is difficult to understand. Nevertheless, restoration of the memory B phenotype in association with successful treatment suggests that evaluation of this parameter can be useful to monitor the effects of ITI.

Conclusion

According to the data presented in this study, peripheral CD27+ B cells are altered in severe haemophilia A patients, especially in those with inhibitors. After ITI, values were restored, suggesting that the measurement of immunological parameters would be useful to monitor anti-FVIII response.

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Authorship

MBI, MF, LP and NG performed the research. MC and RPB managed and cared for the patients with Haemophilia. MBI and NG analysed the data. MMB and NG designed the research study and wrote the paper.

Disclosures

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

References

1 Reding M. Immunological aspects of inhibitor development. *Haemophilia* 2006; **12**: 30–6.
2 Dazzi F, Tison T, Vianello F *et al.* High incidence of anti-FVIII antibodies against non-coagulant epitopes in haemophilia A patients: a possible role for half-life of transfused FVIII. *Br J Haematol* 1996; **93**: 688–93.
3 Astermark J. Why do inhibitors develop? Principles of and factors influencing the risk for inhibitor development in haemophilia. *Haemophilia* 2006; **12**(Suppl. 3): 52–60.
4 Coppola A, Di Minno M, Santagostino E. Optimizing management of immune tolerance induction in patients with severe haemophilia A and inhibitors: towards evidence-based approaches. *Br J Haematol* 2010; **150**: 515–28.
5 Waters B, Lillicrap D. The molecular mechanisms of immunomodulation and tolerance induction to factor VIII. *J Thromb Haemost* 2009; **7**: 1446–56.
6 Hausi C, Ahmad R, 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**: 3415–22.
7 Sanz I, Wei C, Eun-Hyung Lee F, Anolik J. Phenotypic and functional heterogeneity of human memory B cells. *Semin Immunol* 2008; **20**: 67–82.
8 Klein U, Rajewsky K, Küppers R. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J Exp Med* 1998; **188**: 1679–89.
9 Fecteau J, Côté G, Nerón S. A new memory CD27-IgG+ B cell population in peripheral blood expressing V_H genes with low

frequency of somatic mutation. *J Immunol* 2006; **177**: 3728–36.
10 Agematsu K, Nagumo H, Yang F *et al.* B cell subpopulations separated by CD27 and crucial collaboration of CD27+ B cells and helper T cells in immunoglobulin production. *Eur J Immunol* 1997; **27**: 2073–9.
11 Muehlinghaus G, Cigliano L, Huehn S *et al.* Regulation of CXCR3 and CXCR4 expression during terminal differentiation of memory B cells into plasma cells. *Blood* 2005; **105**: 3965–71.
12 Glouchkova L, Ackermann B, Zibert A *et al.* The CD70/CD27 pathway is critical for stimulation of an effective cytotoxic T cell response against B cell precursor acute lymphoblastic leukemia. *J Immunol* 2009; **182**: 718–25.
13 Groth C, Dräger R, Warnatz K *et al.* Impaired up-regulation of CD70 and CD86 in naive (CD27-) B cells from patients with common variable immunodeficiency (CVID). *Clin Exp Immunol* 2002; **129**: 133–9.
14 Matter M, Mumprecht S, Pinschewer D *et al.* Virus-induced polyclonal B cell activation improves protective CTL memory via retained CD27 expression on memory CTL. *Eur J Immunol* 2005; **35**: 3229–39.
15 Belmonte L, Parodi C, Baré P, Malbrán A, Ruibal-Ares B, de Bracco MM. Factors involved in the generation of memory CD8+ T cells in patients with X- linked lymphoproliferative disease (XLP). *Clin Exp Immunol* 2007; **147**: 456–64.
16 Irigoyen M, Primiani L, Felippo M *et al.* A flow cytometry evaluation of anti-FVIII antibodies: correlation with ELISA and Bethesda assay. *Haemophilia* 2011; **17**: 267–74.
17 Verbruggen B, Novakova I, Wessels H, Boezeman J, van der Berg M, Mauser-Bunschoten E. The Nijmegen modification of the Bethesda assay for factor VIII:C inhibitors: improved specificity and reliability. *Thromb Haemost* 1995; **73**: 247–51.
18 Appay V, van Lier R, Sallusto F, Roederer M. Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry Part A* 2008; **73A**: 975–83.
19 Reipert B, Allacher P, Hausl C *et al.* Modulation of factor VIII-specific memory B cells. *Haemophilia* 2010; **16**: 25–34.
20 Van Helden P, Van Haren S, Fijnvandraat K, van den Berg H, Voorberg J. Factor VIII-specific B cell responses in haemophilia A patients with inhibitors. *Haemophilia* 2010; **16**: 35–43.
21 McHeyzer-Willams L, McHeyzer-Willams M. Antigen-specific memory B cell development. *Annu Rev Immunol* 2005; **23**: 487–513.
22 Moser K, Tokoyoda K, Radbruch A, MacLennan I, Manz R. Stromal niches, plasma cell differentiation and survival. *Curr Opin Immunol* 2006; **18**: 265–70.
23 Hayakawa K, Ishii R, Yamasaki K, Kishimoto T, Hardy R. Isolation of high-affinity memory B cells: phycoerythrin as a probe for antigen-binding cells. *Proc Natl Acad Sci* 1987; **84**: 1379–83.
24 Klein U, Kuppers R, Rajewsky K. Evidence for a large compartment of IgM-expressing memory B cells in humans. *Blood* 1997; **89**: 1288–98.
25 Tangye S, Good K. Human IgM+CD27+ B cells: memory B cells or 'memory' B cells? *J Immunol* 2007; **179**: 13–9.