Cytokine production by CD4\(^+\) T cells specific for coagulation factor VIII in healthy subjects and haemophilia A patients

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Summary

Haemophilia A patients treated with human factor VIII (fVIII) may develop antibody (Ab) inhibitors to fVIII. fVIII-specific CD4\(^+\) T cells are common in haemophilia A patients, but also in healthy subjects who do not have a sustained anti-fVIII Ab response. Here, we examined the fVIII-induced IFN-\(\gamma\), IL-4- and TGF-\(\beta\)-producing CD4\(^+\) T blasts by culturing peripheral blood mononuclear cells (PBMC) from controls and patients with recombinant fVIII. fVIII exposure significantly increased IFN-\(\gamma\)- and IL-4-, but not TGF-\(\beta\)-producing CD4\(^+\) T blasts in patients with inhibitors. Patients without inhibitors had fVIII-induced IFN-\(\gamma\)- and TGF-\(\beta\)-, but not IL-4-producing CD4\(^+\) T blasts. Controls did not have IL-4-producing CD4\(^+\) T blasts. However, controls whose PMBC proliferated in response to fVIII had fVIII-induced CD4\(^+\) T blasts that produced IFN-\(\gamma\), the number of which correlated with the intensity of the proliferative response to fVIII of their PMBC, whereas controls whose PMBC did not proliferate to fVIII had predominantly fVIII-induced CD4\(^+\) T blasts that produced TGF-\(\beta\). The presence in controls and patients without inhibitors of fVIII-induced IFN-\(\gamma\)-producing CD4\(^+\) T cells, but not IL-4-producing CD4\(^+\) T cells, which are abundant in inhibitor patients, suggests a role of Th1 cells in initiating the immune response to fVIII, and of Th2 cells in the development of strong inhibitor production. The polarized high ratios of Th3/Th1 and Th3/Th2 in controls and patients without inhibitors suggest that a preponderance of Th3 cells in the response to fVIII may help to maintain tolerance to fVIII.

Keywords

CD4\(^+\) T cells, cytokines, factor VIII, hemophilia A, inhibitors

Introduction

A proportion of patients with haemophilia A who undergo substitution therapy with human coagulation factor VIII (fVIII) products may develop antibodies (Ab) that block the pro-coagulant activity of fVIII (inhibitors) (1, 2). Inhibitor synthesis requires fVIII-specific CD4\(^+\) T cells, which are common in the blood of haemophilia A patients (3, 4), but also in healthy subjects (3, 5).

The common finding of fVIII-specific CD4\(^+\) T cells in healthy subjects is puzzling, because their presence does not correlate with synthesis of clinically significant anti-fVIII Ab: in fact, only 15% of healthy blood donors produce small amounts of anti-fVIII Ab, which may block fVIII in vitro but never cause coagulation deficits in vivo (6–8).

After antigen (Ag) activation, CD4\(^+\) T cells differentiate into different subsets, which carry out characteristic functions by virtue of the cytokines they produce (9, 10). The two subsets of Ag-primed CD4\(^+\) T cells, termed Th1 and Th2, have been well-characterized in both mice (11–14) and humans (15–17). Several types of T regulatory cells, including Th3, have also been identified in both mice (11–14) and humans in recent years (18–22).

Th1 cells secrete pro-inflammatory cytokines, such as interferon (IFN)-\(\gamma\), interleukin (IL)-2 and tumor necrosis factor (TNF)-\(\alpha\), which facilitate the development of immune responses by activating antigen-presenting cells (9). Th1 cytokines stimulate B cells to produce Ab that bind complement, such as IgG1 and IgG2 in humans (23). Th2 cells produce anti-inflammatory cytokines, such as IL-4 and IL-10, which reduce the intensity of immune responses by down-regulating activated antigen-pres-
entating cells and Th1 cells (9). However, IL-4 and IL-10 also induce growth and differentiation of B cells, and promote synthesis of Ab that do not fix complement, such as IgE and IgG4 in humans (23, 24). Th3 cells produce transforming growth factor (TGF)-β cytokines, which are potent down-regulators of both T cell and humoral Ab responses (13, 18–22).

Both Th1 and Th2 cells induce synthesis of anti-fVIII Ab and inhibitors in haemophilia A patients (17, 25, 26) and in the analogous mouse model (27–29). In haemophilia A patients, most inhibitors are Th2-driven IgG4, although some are Th1-driven IgG1 (17, 26); anti-fVIII Ab and inhibitors are mostly of the IgG4 subtype in patients with a strong anti-fVIII Ab response, whereas they are mostly IgG1 in patients with a modest Ab response (17, 25). Haemophilia A mice exposed to fVIII intra-venously (i.v.) synthesize Th1-driven anti-fVIII Ab at the beginning of their immune response to fVIII, whereas a Th2-driven Ab response becomes prominent when the anti-fVIII Ab response is robust (27, 29). However, to date, nothing is known about the presence of fVIII-specific Th3 cells, or about any modulatory effect on anti-fVIII CD4+ T cells in human or murine haemophil-
ia A.

Here, we have examined whether fVIII-specific CD4+ T cells of healthy subjects and haemophilia A patients synthesize different cytokines, by determining the synthesis of IFN-γ, IL-4 and TGF-β1 by CD4+ T cells isolated from the blood of healthy sub-
jects and haemophilia A patients with or without inhibitors, after stimulation in vitro by exposure to physiological concentrations of human fVIII.

### Materials and methods

#### Subjects

We studied 44 healthy blood donors (“controls”: 31 men and 13 women, 21–65 years old), recruited randomly at the American Red Cross Blood Donor Center (St. Paul, MN, USA), and 30 haemophilia A patients (Table 1), chosen among those followed at the Hemophilia and Thrombosis Center at the University of Minnesota. All subjects were HIV-negative. Nineteen haemophil-
ia A patients had no history of inhibitors and 11 had active in-
hibitors. For three patients with inhibitors we carried out two experiments, 12–18 months apart. The protocol was approved by the Institutional Review Boards of the University of Minnesota and the American Red Cross Donor Center. All donors gave informed consent.

#### Preparation of CD4+ PBMC and proliferation assay

We isolated PBMC (peripheral blood mononuclear cells) from heparinized venous blood, and eliminated the CD8+ T cells as previously described (5). The CD8+ depleted, CD4+ enriched PBMC (CD4+ PBMC) were evaluated in five-day proliferation assays (5), using recombinant human fVIII (1 U/ml; Baxter, Glendale, CA, USA) or tetanus toxoid (TT, 10 µg/ml; Connaught Laboratories, Swiftwater, PA, USA) as stimulants. CD4+ PBMC cultured without any stimulus served as background controls. We determined the extent of cell proliferation from the incorporation of 3H-thymidine (1 µCi/well; specific activity: 6.7 Ci/mm; Dupont-NEN, Boston, MA, USA), measured in a liquid scintillation counter (LS 6500, Beckman Coulter, Fullerton, CA, USA). A two-tailed Student’s t-test was used to determine the significance of an increase in 3H-thymidine incorporation in the presence of an Ag, as compared to the incorporation in unstimu-
lated CD4+ PBMC. When a significant (p<0.05) difference was noted, we calculated the stimulation index (SI: ratio between the

### Table 1: Characteristics of haemophilia A patients.

<table>
<thead>
<tr>
<th>Patients</th>
<th>With inhibitors</th>
<th>Without inhibitors</th>
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<tbody>
<tr>
<td>Total number</td>
<td>11</td>
<td>19</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (inter-quartile range)</td>
<td>24 (12, 45)</td>
<td>19 (8, 42)</td>
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<tr>
<td>Current treatment</td>
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<td></td>
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<tr>
<td>rhfVIII, on demand (n)</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>rhfVIII, prophylaxis (n)</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Inhibitor titer: Median (inter-quartile range)</td>
<td>5 (1, 17)</td>
<td></td>
</tr>
<tr>
<td>Historical peak</td>
<td>312 (24, 3600)</td>
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### Detection of CD3+CD4+ T blasts

We cultured CD4+ PBMC in 24-well plates [2 x 10^5 cells/well in 2 ml of RPMI 1640 with 5% heat-inactivated human AB serum (Bioreclamation Inc., Hicksville, NY, USA)], in the presence of fVIII (1 U/ml) or TT (10 µg/ml), or without any stimulant as background controls, for one to five days. Cells were harvested and stained with PerCp-labeled anti-human CD3 mAb (PharMingen, San Diego, CA, USA) and FITC- labeled anti-human CD4 mAb (PharMingen). We analyzed the cells by flow cyto-

### Analysis of intracellular cytokines in CD3+CD4+ T blasts

Most cytokines, including those we tested here, are also produced by cells other than CD4+ T cells (e.g. 31). To ensure that the fVIII-induced cytokine synthesis we tested was accounted for by CD4+ T cells, we used a multi-parameter flow cytometry assay that detects both intracellular cytokines and the CD3 and CD4 T cell markers at the single cell level (32).

We cultured CD4+ PBMC with fVIII or TT, or without any stimulus for five days, as described above. During the final six hours, we co-stimulated the cells with a CD28/CD49d mAb cocktail (1 µg/ml, Pharmingen) in the presence of GolgiStop (Pharmingen). GolgiStop contains monensin, an inhibitor of intracellular transport that prevents secretion of synthesized cyto-
kines, thereby facilitating their detection by intracellular staining. Cells were harvested and stained with PerCp-labeled anti-

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The above text is a summary of a scientific article discussing the synthesis of anti-fVIII antibodies and inhibitors in haemophilia A patients, focusing on the role of CD4+ Th3 cells in modulating immune responses to fVIII. The study includes methods for isolation and stimulation of PBMC, measurement of H-thymidine incorporation, and detection of intracellular cytokines. The table provides characteristics of haemophilia A patients, and the article concludes with discussions on the detection of CD3+CD4+ T cell blasts and analysis of intracellular cytokines.
Cells were fixed and permeabilized in Cytofix/Cytoperm solution (PharMingen). After washing, Perm/wash buffer containing 5% dry milk was added to block any non-specific binding. Cells were then stained intracellularly with PE-labeled anti-human IFN-γ (PharMingen) or anti-IL-4 (PharMingen) or anti-TGF-β1 (IQ Products, Rozenburglaan, The Netherlands) mAb. We used isotype-matched control mAbs (Pharmingen) to detect non-specific staining. Cell samples were analysed in a FACSCalibur flow cytometer (Becton Dickinson). Viable cells were characterized by SSC and FSC, and analysed for CD3, CD4 and cytokine staining.

Data were expressed as box and whisker plots (median, 25th and 75th percentile, minimum, and maximum). Mann-Whitney-U test was used for unpaired comparisons between different experimental groups, and Wilcoxon signed-rank test was used for paired comparisons within the same group. Simple linear regression and the Excel-ANOVA program (Microsoft Corp., Redmond, WA, USA) were used for correlation analysis. We considered a difference to be significant when p < 0.05.

Results

Proliferative responses to fVIII of CD4+ PBMC from controls

We tested the proliferative response to fVIII or TT of CD4+ PBMC from all controls. Twenty-three controls had significant proliferative responses to fVIII, which were usually modest (median: 1.93, inter-quartile range: 1.61–2.85). In contrast, the CD4+ PBMC of all controls responded to TT significantly and strongly (median: 11.30, inter-quartile range: 6.50–43.53) (data not shown). These results are consistent with our previous observation (5). Henceforth, we refer to controls whose CD4+ PBMC had significant proliferative responses to fVIII as “fVIII responders”, and those who did not as “fVIII non-responders”.

fVIII-induced blastic transformation of blood CD4+ T cells

We cultured CD4+ PBMC from five controls with TT for up to five days, to determine the time course of the Ag-induced blastic transformation of blood CD4+ T cells. The presence of TT increased the number of CD3+CD4+ T blasts as early as after two days of culture. The difference in the frequency of CD3+CD4+ T blasts in TT-stimulated and background control was significant only from day four onwards (data not shown). Based on these results, we measured the frequency of the CD3+CD4+ T blasts after five days of culture with fVIII. To compare the intensity of the responses obtained from different subjects and in different experiments, we divided the fVIII-induced intensity by the background control as the intensity of fVIII-induced specific response.

We used cultures of CD4+ PBMC from 11 fVIII responders and nine fVIII non-responders to determine whether the presence of 1 U/ml of fVIII induced an increase in CD3+CD4+ T blasts. fVIII exposure significantly increased the CD3+CD4+ T blasts in fVIII responders, but not in non-responders (Fig. 1, left plot). The intensity of fVIII-induced specific increase of CD3+CD4+ T blasts (the ratio between the frequencies of CD3+CD4+ T blasts in the presence of fVIII and the background controls) in the 11 fVIII responders correlated significantly with the intensities of their global CD4+ PBMC proliferative response to fVIII (Fig. 1, right plot).

fVIII-specific CD3+CD4+ T blasts of controls secrete IFN-γ; but not IL-4

We used CD4+ PBMC from the 11 fVIII responders and nine fVIII non-responders used in the experiments described above to examine whether the fVIII-induced CD3+CD4+ T blasts synthesized IFN-γ or IL-4. As a positive control, we examined the TT-induced IFN-γ or IL-4-producing CD3+CD4+ T blasts in three fVIII responders and four fVIII non-responders. fVIII exposure significantly increased the IFN-γ-producing CD3+CD4+ T blasts in the fVIII responder group (Fig. 2A), but not in non-responder group (Fig. 2B). fVIII exposure did not induce IL-4-producing CD3+CD4+ T blasts in either group. Among fVIII responders, the intensities of fVIII-induced specific increase of IFN-γ-producing CD3+CD4+ T blasts (the ratio between the frequencies in the presence of fVIII and the background controls) correlated significantly with the intensities of the proliferative response to fVIII of the donor’s CD4+ PBMC (Fig. 2C).

The presence of TT strongly increased the frequency of both IFN-γ and IL-4 synthesizing CD3+CD4+ T blasts (Fig. 2D).
**Figure 2: Cytokine-producing CD3+CD4+ T blasts in controls.** FVIII stimulation increased the IFγ-producing CD3+CD4+ T blasts in the FVIII responder group (A), but not in the non-responder group (B). The intensities of FVIII-induced specific increase of IFN-γ-producing CD3+CD4+ T blasts in responders correlated with the intensities of their proliferative response to FVIII (C). TT, used as a positive control antigen, induced a strong increase of IFN-γ or IL-4-producing CD3+CD4+ T blasts (D). The open circle marks a outlier; M, medium; **, p<0.01.

**FVIII-induced TGF-β1-producing CD3+CD4+ T blasts in controls**

To further determine whether Th3 cells are involved in modulating the immune response to FVIII, we used the multi-parameter flow cytometry assay described above to simultaneously detect the FVIII-specific CD3+CD4+ T blasts that secreted TGF-β1, or IFN-γ or IL-4. This approach yields a snapshot of the relative frequency of FVIII-sensitized CD4+ T cells that produce Th1 or Th2 or Th3 cytokines in an individual subject at any given time. We carried out these experiments using CD4+ PBMC from 12 FVIII responders and 12 non-responders. For four FVIII responders and six non-responders, we also set up CD4+ PBMC cultures stimulated with TT.

Culture with FVIII of CD4+ PBMC from the FVIII responders significantly increased IFN-γ and TGF-β1, but not IL-4, -producing CD3+CD4+ T blasts (Fig. 3A). In contrast, culture with FVIII of CD4+ PBMC from FVIII non-responders significantly increased only CD3+CD4+ T blasts that stained for TGF-β1 (Fig. 3A).

Among the FVIII responders, FVIII induced a specific increase of IFN-γ-producing CD3+CD4+ T blasts significantly higher than the increases in CD3+CD4+ T blasts producing IL-4 or TGF-β1 (data not shown). In contrast, among FVIII non-responders, FVIII induced a specific increase in TGF-β1-producing CD3+CD4+ T blasts significantly larger than the increases in IFN-γ or IL-4-producing T blasts (data not shown). The FVIII responders had significantly more FVIII-induced specific IFN-γ-producing CD3+CD4+ T blasts and significantly less FVIII-induced TGF-β1-producing CD3+CD4+ T blasts than the FVIII non-responders (data not shown).

**FVIII-induced Th1, Th2 and Th3 cells in haemophilia A patients**

We next investigated the intensity of FVIII-induced CD3+CD4+ T blasts that produced IFN-γ, IL-4, or TGF-β1 in CD4+ PBMC from haemophilia A patients with or without inhibitors. Figure 3A and B reports the results of those experiments. Among inhibitor patients, FVIII exposure strongly and significantly increased the frequency of IFN-γ and IL-4, but not TGF-β1, -producing CD3+CD4+ T blasts. In contrast, FVIII caused a modest yet significant increase in IFN-γ and TGF-β1, but not IL-4, -producing CD3+CD4+ T blasts in patients without inhibitors. When cultured with TT, CD4+ PBMC from both controls and haemophilia A patients showed strong and significant increases in the proportion of IFN-γ and IL-4-producing CD3+CD4+ T blasts (Fig. 3B).

Among inhibitor patients, the FVIII-induced specific increase of CD3+CD4+ T blasts that produced IFN-γ or IL-4 was significantly higher than the increase in those producing TGF-β1, whereas among the patients without inhibitors, the numbers of FVIII-induced specific T blasts of these three subsets were equal (data not shown). Patients with inhibitors had significantly more FVIII-induced specific IFN-γ and IL-4-producing CD3+CD4+ T blasts than patients without inhibitors (data not shown).

We calculated the ratios between the intensities of the FVIII-
induced specific cytokine-producing CD3⁺CD4⁺ T blasts in each individual, and grouped them in Figure 3C. Patients with inhibitors had significantly lower Th3/Th1 and Th3/Th2 ratios than any other groups. In contrast, fVIII non-responders had higher or equal Th3/Th1 and Th3/Th2 ratios compared to fVIII responders or patients without inhibitors (Fig. 3C).

**Discussion**

In previous studies we found that controls commonly have fVIII-sensitized CD4⁺ T cells: the intensity of their CD4⁺ T cell response to fVIII, although less intense than those observed in haemophilia patients, increases with age (3–5), suggesting that multiple episodes of activation of CD4⁺ T cells to fVIII occur over time, with accumulation of memory T cells. The CD4⁺ T cell responses that we detected in controls were short-lived, whereas in haemophilia patients with inhibitors they persist over time (3). These previous findings indicated that an immune response to fVIII is not unique to haemophilia A patients who lack tolerance to fVIII due to their congenital deficiency in fVIII: they suggested that immunomodulatory mechanisms curb the immune response to fVIII in controls and in patients without inhibitors. Those studies also found that Th1- and especially Th2-cells have a role in the anti-fVIII response that results in inhibitors, whereas in controls the fVIII-sensitized Th1 cells are much more com-
mon than Th2 cells (5).

The present results agree with those conclusions. They suggest that both therapeutic administration of FVIII or exposure to endogenous FVIII sensitizes Th1 cells first, and that sensitization of Th2 cells needs the pro-inflammatory action of Th1 cell-derived cytokines. This conclusion is supported by the present findings that patients without inhibitors and control FVIII responders had FVIII-induced CD4+ T cells that produce IFN-γ, but not IL-4 (Fig. 3A), suggesting that Th1 cells are primed more effectively than Th2 cells. The present finding that patients with inhibitors had more FVIII-induced Th1 cells than patients without inhibitors suggests that FVIII-sensitized Th1 cells continue to have an important role in the immune response to FVIII. However, patients with inhibitors also had abundant FVIII-induced Th2 cells, verifying the crucial role of FVIII-sensitized Th2 cells in inhibitor formation.

Moreover, these results suggest that FVIII-induced specific TGF-β1-producing CD4+ T cells may be involved in modulating the immune response to FVIII, and preventing its progression to a clinically significant Ab synthesis. The following lines of evidence in this study support the hypothesis. First, controls have FVIII-induced Th3 cells, particularly in FVIII non-responders, who had a predominantly FVIII-sensitized Th3 immune response (Fig. 3A). Second, in haemophilia A patients without inhibitors, FVIII stimulation sensitized the Th3 cells significantly. In contrast, in haemophilia A patients with inhibitors, the majority of the CD4+ T cells sensitized to FVIII are Ab-inducing Th2 and Th1 cells, but not Th3 cells (Fig. 3A). Finally, the cytokine expression pattern of CD4+ T cells may regulate the Ag-primed immune responses (33–35). Controls, especially FVIII non-responders, have polarized (higher) Th3/Th1 and Th3/Th2 ratios (Fig. 3C), indicating that FVIII has facilitated the sensitization of TGF-β1-producing CD4+ T cells in controls, although FVIII may also have the potential of sensitizing IFN-γ or IL-4-producing CD4+ T cells. In contrast, although FVIII has the potential to cause a weak or modest increase in TGF-β1-producing CD4+ T cells in inhibitor patients, who thus may have similar amount of FVIII-specific Th3 cells compared to controls or haemophilia A patients without inhibitors, FVIII is more likely to facilitate the sensitization of IFN-γ and IL-4-producing CD4+ T cells (Fig. 3A). This preponderance of Th1 and Th2 type response, which is essential for the development of inhibitors, caused the lower ratios of Th3/Th1 and Th3/Th2 in inhibitor patients (Fig. 3C).

In summary, these results indicate that endogenous or therapeutically administered FVIII may activate different CD4+ T cell subsets, in a sequence that might be dictated by the Ag presentation requirements for activation of the different CD4+ T cells (35, 36). They also suggest that Th1 cells are activated first: the pro-inflammatory Th1 cytokines may then facilitate the activation of Th2 cells, which are predominant when the anti-FVIII response is intense. Activation of potentially modulatory CD4+ T cells that produce TGF-β1 might occur only after prolonged exposure to FVIII, and it may not be favored in severe haemophilia A patients because of lack of sustained levels of FVIII: impaired activation of FVIII-specific Th3 cells would permit robust Ab synthesis, and inhibitor formation.

This study begins to identify functional properties of FVIII-primed CD4+ T cells in normal subjects and haemophilia A patients, and to define how they may correlate with inhibitor synthesis. An improved understanding of the immune mechanisms that trigger and maintain the synthesis of anti-FVIII Ab and inhibitors, and those which down-regulate the immune response to FVIII, may have implications for the design of safe and effective therapeutic protocols to curb inhibitor synthesis in haemophilia A patients in the future.

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References