Effect of *in vivo* induction of regulatory T cells during immune toleristaion on FVIII inhibitor anamnestic response.

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INTRODUCTION

The development of an alloantibody to infused factor VIII (FVIII) concentrate (FVIII inhibitor) is the most significant complication of replacement therapy in severe haemophilia A. Up to 30% of individuals with severe haemophilia A will develop a FVIII inhibitor, of which approximately half will be transient. The remaining FVIII inhibitors, especially if high titre (defined as >5 BU/mL), necessitate immune tolerisation induction (ITI) to eradicate the inhibitor. ITI involves the regular administration of FVIII concentrate (up to doses of 200 units/kg/day) and has a success rate of up to 80%. This leaves a proportion of individuals who fail first line ITI; these individuals commonly have high titre FVIII inhibitors and require by-passing agent therapy (e.g. FEIBA® or Novoseven®) to manage bleeding episodes and as prophylaxis for invasive procedures. Over time various strategies have been explored in an attempt to induce tolerisation in these 'refractory' cases. Such approaches include switching to plasma derived FVIII (pdFVIII) concentrates and the use of novel immunosuppressant agents such as Rituximab (ant-CD20 monoclonal antibody).

Regulatory T cells (Tregs) are a discreet subset of T cells that are important in tolerance to self-antigens, and the prevention of autoimmune disease and inappropriate immune responses in inflammatory/allergic conditions. Tregs are characterised by expression of surface markers (CD3, CD4, CD25Bright and CD127low). In addition, intranuclear expression of *FoxP3* is a requirement for Treg cells. In healthy donors Treg cells represent 5-10% of CD4+ T cells in PB or ~0.7 – 5.5% of mononuclear cells. Immunomodulation of Tregs either through induction of Tregs (e.g. with histone deacetylase inhibitors (HDACi)) or relative preservation of Tregs (e.g. with sirolimus) may have a role in therapy for autoimmune diseases and induction of tolerance.

We describe a case of a 15 year old boy with a refractory high titre FVIII inhibitor. He has suffered repeated life threatening intracranial haemorrhages (ICH), including two following recurrence of the FVIII inhibitor during ITI with pdFVIII (Biostate) and Rituximab.

CASE REPORT

The current course of ITI commenced when the patient was 15 years old. His severe haemophilia was confirmed after development of a cephalohaematoma at birth. He has an affected older sibling who has not developed a FVIII inhibitor, and F8 gene analysis has demonstrated a gross deletion of multiple Exons. The high titre FVIII inhibitor was first detected when he was 19 months old (initial titre 40 BU/mL), and management was with on demand activated Prothrombin Complex Concentrate (aPCC). The child had suffered two ICH by the age of 9 years. Following a third ICH when 11 years old, ITI was commenced with plasma derived FVIII and Rituximab (fig. 1 A). Commencement of ITI was associated with the typical anamnestic immune response with the FVIII inhibitor titre peaking at 1661 BU/mL during the 2nd month of ITI. The last dose of Rituximab was given 30 months after initiation of this 1st course of ITI. Seven months later he presented with a muscle bleed and the FVIII inhibitor titre was 23 BU/mL, having been <0.6 BU/mL two months earlier. During the following 8 months he suffered two further ICH, when the FVIII inhibitor titre was between 3 and 55 BU/mL.

Given the severity of the bleeding symptoms, despite by-passing agent prophylaxis, alternative strategies including haemopoietic stem cell transplant (HSCT) were sought for eradicating the FVIII inhibitor.

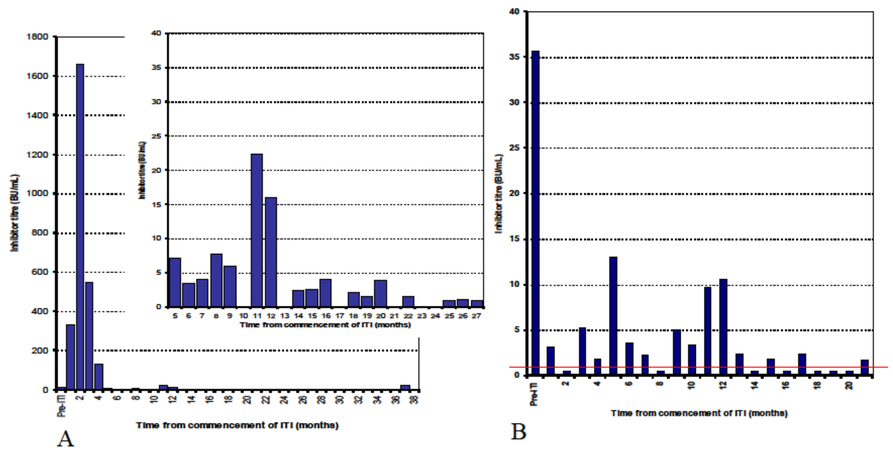

Figure 1. A) FVIII inhibitor titre response during previous ITI with pdFVIII (Biostate) & Rituximab. Insert shows FVIII inhibitor titre weeks 5 to27 in detail. B) FVIII inhibitor titre following commencement of current course of ITI. Red line denotes level of detection for Nijmegen assay (<0.6 BU/mL).

METHODS

ITI therapy

The protocol for ITI was of a 4 week pulse of oral HDACi and sirolimus with an initial 5 days IVIg at the start of the 4 weeks, together with high dose plasma derived FVIII (200 units/kg/day) and Rituximab. The timing of the Rituximab, and 4 week pulse of combine oral HDACi/sirolimus therapy and IVIg has evolved with time (fig. 2).

Measurement of Tregs (fig. 3)

Antibodies – Monoclonal antibodies (mAbs) specific for CD3 peridinin-chlorophyll-protein (PerCp)-conjugated, CD4 Fluorescein isothiocyanate (FITC)-conjugated, CD25 allophycocyanin-conjugated and CD45 were obtained from BD Biosciences (Sydney, NSW, Australia). Monoclonal antibodies (mAbs) specific for IgG2a (rat), FoxP3 (PCH101) and CD127 were obtained from eBioScience (Gladesville, NSW, Australia).

Cell preparation & labelling – Peripheral blood (EDTA), either lysed whole blood (ammonium chloride) or PBMC preparations were adjusted to a WCC of 1x107 cells/mL. Briefly, 1x106 cells were stained with mAb to identify CD3+/CD4+/CD25bright/CD127low population, combined with either isotype control IgG2b-PE or FoxP3-PE mAb. Intranuclear staining procedure was performed as per kit instructions using eBioscience FoxP3 staining kit. A healthy control sample was stained alongside the patient sample as adequate in-house reference ranges have yet to be determined.

Data acquisition & analysis – Data was acquired on a BD FACSCanto II flow cytometer. Standard daily FACS Canto II quality control procedures (CS&T beads, BD Biosciences, Australia) are required to be accepted before data acquisition. FCS files were analysed using Kaluza (1.2) analysis software (Beckman Coulter, Australia).

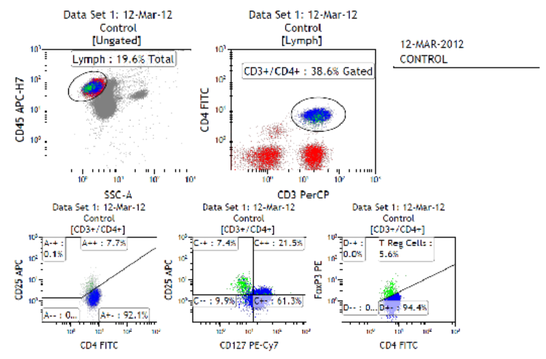

Figure 3 – Representative analysis of control sample for Tregs as outlined in method.

RESULTS

The response of the FVIII inhibitor titre to re-commencement of ITI is shown in figure 1B. There was no demonstrable anamnestic response. Since week 66 there has been measurable FVIII levels several hours after a dose of pd FVIII, and 12 out of 16 inhibitor titres are <0.6 BU/mL (4 titres range from 1.4 to 2.4 BU/mL). By-passing agent prophylaxis was ceased at week 86 and the dose of pdFVIII was reduced to 100 U/kg/day from week 89 (fig. 2). Measurement of Tregs pre- (day 1), during (day 15) and post- (day 31) the 4 week course of IVIg/HDACi/sirolimus demonstrates a relative increase in Tregs compared to the total CD3+/CD4+ T cell population (figs. 4 & 5).

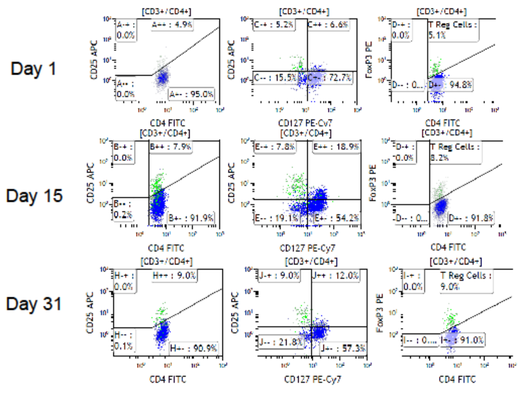

Figure 4 – Flow cytometry for Tregs in patient.
Treg (green) population of total T cells. Day 1 – sample pre-IVIg & oral HDACi/sirolimus; day 31 – 3 days after completing 4 week course of HDACi/sirolimus.

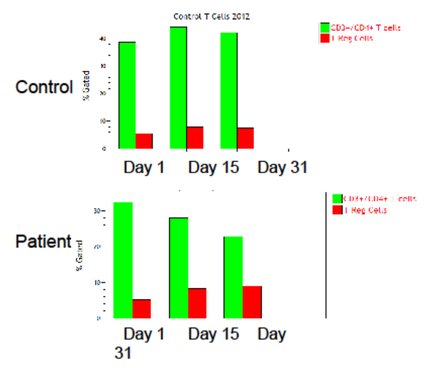

Figure 5 – Comparison of flow cytometry for CD3+/CD4+ T cells & Tregs in patient and control.
Blood samples collected and processed on the same days. For patient; day 1 – sample pre-IVIg & oral HDACi/sirolimus; day 31 – 3 days after completing 4 week course of HDACi/sirolimus.

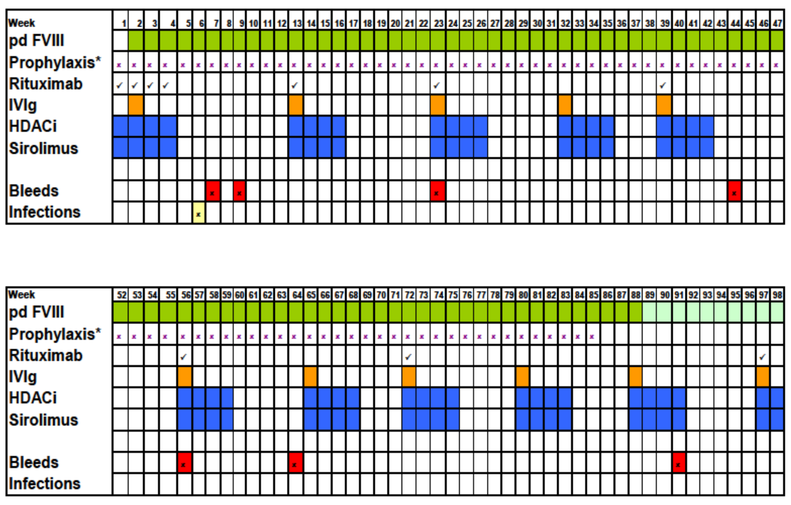

Figure 2 – Protocol for ITI by week.
pdFVIII – plasma derived factor VIII concentrate (Biostate) 200 units/kg body weight daily, until decreased to 100 units/kg/day at week 89. Prophylaxis = prophylaxis with by-passing agents (FEIBA® or Novosven®). Bleeds – bleeds requiring hospitalisation.

DISCUSSION

The approach to refractory FVIII inhibitors is complicated, with a lack of data to inform treatment decisions (Haemophilia 2007, 13 (suppl 1), 1-22; Eur J Haem 2012, 88, 371-9). A single case report of a sibling HSCT was associated with the recurrence of the FVIII inhibitor and death due to sepsis (Haemophilia 2010, 16, 143-7). In this context the options for patients with life threatening bleeds while on by-passing agent prophylaxis for refractory FVIII inhibitors is limited. In this case the potential induction and/or preservation of Tregs utilising a combination of agents (HDACi, sirolimus and IVIg) may have been associated with a loss of the anamnestic response at the time of commencement of ITI. It would be interesting to see if this abrogation of the anamnestic response could be replicated in other patients with refractory FVIII inhibitors. Whether this approach or a refinement of Treg induction/preservation can be utilised to induce tolerisation in salvage ITI is unknown.

DOI: 10.3252/pso.eu.WFH2012.2012