



UNIVERSITÀ DEL PIEMONTE ORIENTALE

Department of Health Science

School of Medicine

Master degree in Medical Biotechnologies

**In vitro and in vivo T-cell proliferative response
in a hemophilia A mouse model**

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SUMMARY

Rationale of the study: Haemophilia A (HA) is an X-linked bleeding disorder caused by congenital deficiency of factor VIII (FVIII). Besides its role in coagulation, previous work from our laboratory suggested that FVIII deficiency may alter T-cell responsiveness, with HA T cells showing enhanced responses to relatively low-intensity TCR stimulation. This study further investigated the kinetics of CD4⁺ and CD8⁺ T cell *in vitro* proliferation and early splenic T cell responses following immunization.

Planning of the study: Splenic leukocytes from HA and WT BALB/c mice were labelled with CellTrace Far Red and stimulated *in vitro* with α -CD3 (10 or 25 ng/mL) and α -CD28 (500 ng/mL). Proliferation was assessed by flow cytometry after 24, 48, and 72 hours. In parallel, HA and WT mice were immunized once with OVA/IFA and analysed 8 days later for splenic CD4⁺ and CD8⁺ T cells, memory-associated populations, and Tregs.

Results: Both CD4⁺ and CD8⁺ T cells showed increasing proliferation with culture time and higher α -CD3 stimulation. The difference between HA and WT was most evident at later time points under relatively low-intensity stimulation, while stronger stimulation reduced the difference between the two groups. At day 8 after OVA/IFA immunization, no significant differences were detected in the percentage and number of the major splenic T-cell populations analysed.

Conclusions: These findings suggest a subtle, context-dependent alteration in T cell responsiveness associated with FVIII deficiency rather than generalized T cell hyper-responsiveness. Further studies using purified T cell populations, complementary proliferation and viability assays, functional approaches, and antigen-specific stimulation are needed to define the underlying mechanism and its relevance to immune responses against therapeutic FVIII.

INTRODUCTION

Hemophilia A

Hemophilia A (HA) is a bleeding disorder due to a deficiency or dysfunction of a plasma glycoprotein called coagulation Factor VIII (FVIII), which acts as a co-factor in the intrinsic pathway of blood coagulation and is linked to the X chromosome. It is defined by a defect in thrombin generation that fails to produce fibrin clots, resulting in recurrent, often spontaneous, hemorrhagic episodes. The most frequent bleeds are into the joints (hemarthroses), muscle and soft tissues, and, when neglected, lead to continued joint damage, chronic pain, and considerable disability. Hemophilia A is a secondary hemostasis disorder, meaning that primary plug formation is normal. Still, the plug is not reinforced by a strong fibrin network because of the lack or abnormality of FVIII. (1)

Indeed, the coagulation process can be divided into two pathways: the extrinsic (tissue factor) pathway and the intrinsic (contact activation) pathway (Figure 1). Both pathways lead to a common pathway that includes the generation of thrombin and the polymerization of fibrin. Tissue factor (TF), which is exposed at the site of vascular injury, binds circulating activated Factor VII (FVIIa) to form the TF–FVIIa complex, which activates both Factor X (FX) and Factor IX (FIX) in the extrinsic pathway. The intrinsic pathway amplifies this response: when FIX is activated, it combines with its cofactor, FVIIIa, and on the activated platelet surface with calcium ions to form the intrinsic tenase complex. (2)

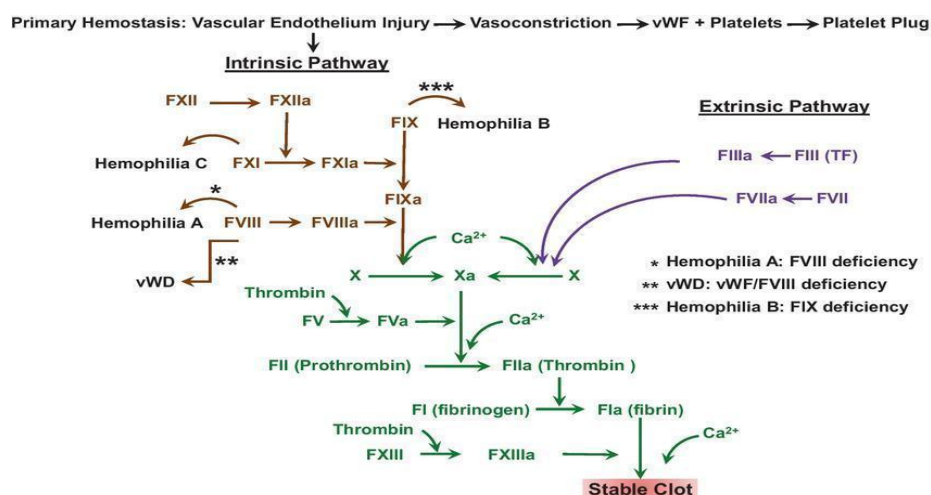


Figure 1. Overview of the coagulation cascade (3)

HA occurs in 1 in 5,000 male births around the world. The disease is transmitted as an X-linked recessive trait and so affects mainly males, as they have one copy of the X chromosome and no compensatory allele. Females with one FVIII gene mutation usually have no symptoms or symptoms that are mild. Females affected by Turner syndrome (45, X karyotype) may also suffer from HA, but with just one X chromosome. Nearly two-thirds of cases are familial (passed down through the mother), and one-third result from spontaneous de novo mutations in the FVIII gene, located on the long arm of the X chromosome at Xq28. (4)

The severity of HA is based on the level of residual FVIII activity in plasma and ranges from severe, moderate, and mild: severe is defined as FVIII activity that is below 1 international unit per deciliter (IU/dL) (1% of normal activity) and is seen in about 43% of cases. Patients have recurrent bleeds, especially into joints and muscles, usually from a young age; moderate is defined as having FVIII levels between 1 and 5 IU/dL, and bleeding events are often triggered by minor surgery/trauma, but spontaneous bleeding is still present, although less frequent. In mild HA (FVIII activity 6–40 IU/dL), the condition becomes clinically apparent only after a significant trauma. (5)

Beyond its clinical manifestations, HA is associated with musculoskeletal comorbidities, particularly hemophilia arthropathy resulting from repeated joint hemorrhages and reduced bone mineral density, which increases the risk of osteopenia and osteoporosis. Emerging evidence also suggests that FVIII deficiency may influence vascular and angiogenic processes beyond its established role in coagulation. (6)

Factor VIII Structure

FVIII is a large plasma glycoprotein that weighs around 330 kDa and is synthesized by liver sinusoidal endothelial cells, along with other extrahepatic organs such as the lung and kidney endothelium. The domain structure is organized as follows: A1-A2-B-A3-C1-C2. The large B domain is not known to have a catalytic role and is cleaved off when FVIII is activated. In the absence of activation, FVIII is present in plasma as non-covalently bound to the von Willebrand Factor (VWF), mostly through the C2 and A3 domains. This interaction is physiologically important because VWF is a protector and helps keep FVIII from being attacked by other molecules, like activated protein C and Factor Xa, and prevents its early binding to phospholipid membranes. Because of this, VWF increases the plasma half-life of FVIII from about 1-2 hours (free FVIII) to about 12-14 hours (VWF-bound FVIII). (7) Upon damage to the vascular wall, thrombin, generated by the extrinsic pathway, cleaves FVIII at specific sites in the A1, A2, and A3 domains, releasing FVIII from VWF and forming the heterotrimer FVIIIa, which comprises the A1, A2, and A3-C1-C2 subunits bound together by

metal-ion interactions. The intrinsic tenase complex is immediately formed as FVIIIa binds to FIXa on the surface of the phospholipid. Importantly, FVIIIa is intrinsically unstable and spontaneously inactivates through dissociation of the A2 subunit, which is a natural mechanism for stopping coagulation after haemostasis has been attained. This self-limitation of the activation of FVIII is an important characteristic of the hemostatic balance, which prevents pathological clot formation. (8)

Treatment of Hemophilia A

Over the last 60 years, HA treatment has come a long way from the application of fresh frozen plasma and cryoprecipitate in the 1960s to the use of highly purified plasma-derived and recombinant FVIII concentrates in modern times. Current treatment for severe HA is the use of prophylactic FVIII replacement therapy, where FVIII is given intravenously every two to three days (usually three times a week) to keep FVIII levels above 1 IU/dL and to reduce the risk of spontaneous bleeding. Early childhood prophylaxis has been found to maintain joint function and substantially reduce long-term disability in comparison to on-demand treatment, where treatment only occurs following bleeding episodes. (5)

Recombinant products of FVIII have provided a significant improvement in safety over plasma-derived products because of the absence of transmission of blood-borne pathogens. More recently, extended half-life (EHL) recombinant FVIII products have been developed to reduce the need for frequent injections and increase the duration of FVIII activity, increasing convenience and adherence. These are engineered by means of Fc fusion, PEGylation, or albumin fusion. Another major paradigm shift in the treatment of HA is the approval of the bispecific monoclonal antibody, emicizumab (Hemlibra®), a drug that is administered subcutaneously and acts as a cofactor replacement for FVIII, bypassing the need for FVIII. Emicizumab can be used in people who have inhibitors to FVIII. (2) More advanced stages of clinical development are gene therapy approaches with the goal of obtaining long-term endogenous FVIII expression after a single dose of an adeno-associated viral (AAV) vector, which could be a curative strategy. (9)

Despite these advances, the most serious and challenging complication of HA treatment is the development of neutralizing antibodies against FVIII, known as inhibitors. In people with severe HA, inhibitors develop in approximately 30% of previously untreated patients, with the majority occurring during the early exposure period. In people with mild or moderate HA, the cumulative incidence of inhibitor development is approximately 5–10%. (5) Coagulators such as recombinant activated rFVIIa or activated prothrombin complex concentrate (aPCC) are required to obtain haemostasis, as standard FVIII replacement is less effective and predictable in the presence of inhibitors. The

development of inhibitors significantly increases the risk of morbidity and mortality related to bleeding and severely restricts treatment choices, and dramatically increases healthcare costs. Induction of immune tolerance (ITI) involves giving high doses of FVIII many times over months to years to eliminate inhibitors and can induce immune tolerance in about 60-70% of patients, but is very difficult to use, costly, and not always successful. (10)

Immune system activation and the development of FVIII Inhibitors

The development of inhibitors against recombinant FVIII is an immune-mediated complication of replacement therapy in HA. Following the administration of the therapeutic FVIII, the protein can be recognized and processed by the immune system, leading to the generation of FVIII-specific neutralizing antibodies in patients. However, exposure to the therapeutic FVIII does not result in inhibitor formation in all patients, indicating that some outcome of FVIII exposure is determined by the interaction of genetic, cellular, and treatment-related factors. The immune response involves the coordinated interactions between the antigen-presenting cells (APCs), CD4⁺ T cells, B cells, and regulatory mechanisms that determine whether FVIII exposure results in an immunogenic or tolerogenic response (11,12). Following its administration, FVIII can be internalized and processed by professional APCs, particularly dendritic cells (DCs). FVIII peptides can subsequently be presented to major histocompatibility complex class II (MHC II) molecules expressed on CD4⁺ T cells. This process provides a mechanism by which FVIII-specific CD4⁺ T cells can be activated and contribute to the development of the humoral adaptive immune response. DCs have been shown to play an important role in the initial presentation of FVIII to T cells, while other APC populations, including macrophages and B cells, may contribute to the subsequent phase of the immune response. (13,14)

Activation of FVIII-specific T cells provides an important link between antigen recognition and the subsequent humoral response. Recognition of FVIII-derived peptide MHC II complexes by the T-cell receptor (TCR), with appropriate co-stimulatory signals, promotes T-cell activation and expansion. Activated CD4⁺ T cells can subsequently provide help to FVIII-specific B cells, promoting their proliferation, differentiation, and antibody production. The development of high-affinity anti-FVIII antibodies is dependent not only on B cell recognition of FVIII but also on effective T cell help. (15)

The balance between immune activation and tolerance is central to the development of inhibitors. Regulatory T cells (Tregs) and other mechanisms of peripheral immune regulation can limit the expansion of FVIII-reactive lymphocytes, while effective activation of helper T cells and B cells favors the generation of sustained antibody responses.

Recent studies have highlighted that the different populations of CD4⁺ T cells play different roles in the immunogenic and tolerogenic responses to FVIII. T Follicular helper (Tfh) cells promote humoral immune responses, providing critical help to B cells, especially in germinal centers, which is important for immunoglobulin class switching, affinity maturation, and establishment of long-lasting antibody responses. Regulatory T cells (Tregs), on the other hand, play an important role in immune tolerance by regulating excessive antigen-specific effector T cell responses and also dampening the development of antibody-mediated immune responses. The activity of Tfh cells is also regulated by another regulatory population, T follicular regulatory (Tfr) cells, which are localized in germinal centers and are also involved in the regulation of antibody production. Thus, the balance between Tfh-mediated immune activation and Treg/Tfr mediated regulation may determine whether exposure to therapeutic FVIII produces an immunogenic response and the development of inhibitors, or a more tolerogenic outcome. (11,16)

In addition to the cellular mechanisms involved in antigen recognition, the likelihood of the development of inhibitors is influenced by genetic characteristics of the patient. The underlying *F8* mutation is an important determinant because mutations associated with complete absence of endogenous FVIII are generally associated with a greater risk of inhibitor development rather than mutations that permit production of a partially functional FVIII protein. The HLA genotype also influences the repertoire of FVIII-derived peptides that can be presented to CD4⁺ T cells and may contribute to differences in inhibitor risk among individuals. The capacity to generate an effective FVIII-specific response depends on both the molecular characteristics of FVIII and the antigen presentation context in which FVIII-derived peptides are encountered. (17) Treatment-related and environmental factors can further influence the development of the inhibitors. The intensity and timing of FVIII exposure, age at the first treatment, treatment regimen, and inflammatory conditions surrounding the FVIII administration have been investigated as potential risk modifiers for the inhibitor. These factors may influence the inflammatory environment in which FVIII is presented to the immune system and affect whether antigen exposure elicits a more activating or tolerogenic response. (18)

The inhibitor development cannot be attributed to a single immune cell population and pathway. Rather, it represents the outcome of interactions between FVIII availability, antigen presentation, T cell activation, B cell responses, immune regulation, and patient-specific genetic and environmental factors. In this network, CD4⁺ T cells occupy a central position because they link antigen recognition to the development of a sustained antibody response. Understanding how T cell activation and

responsiveness are regulated in hemophilia A is important for elucidating not only the mechanisms underlying FVIII inhibitor formation but also the broader immunological consequences of FVIII deficiency. (11)

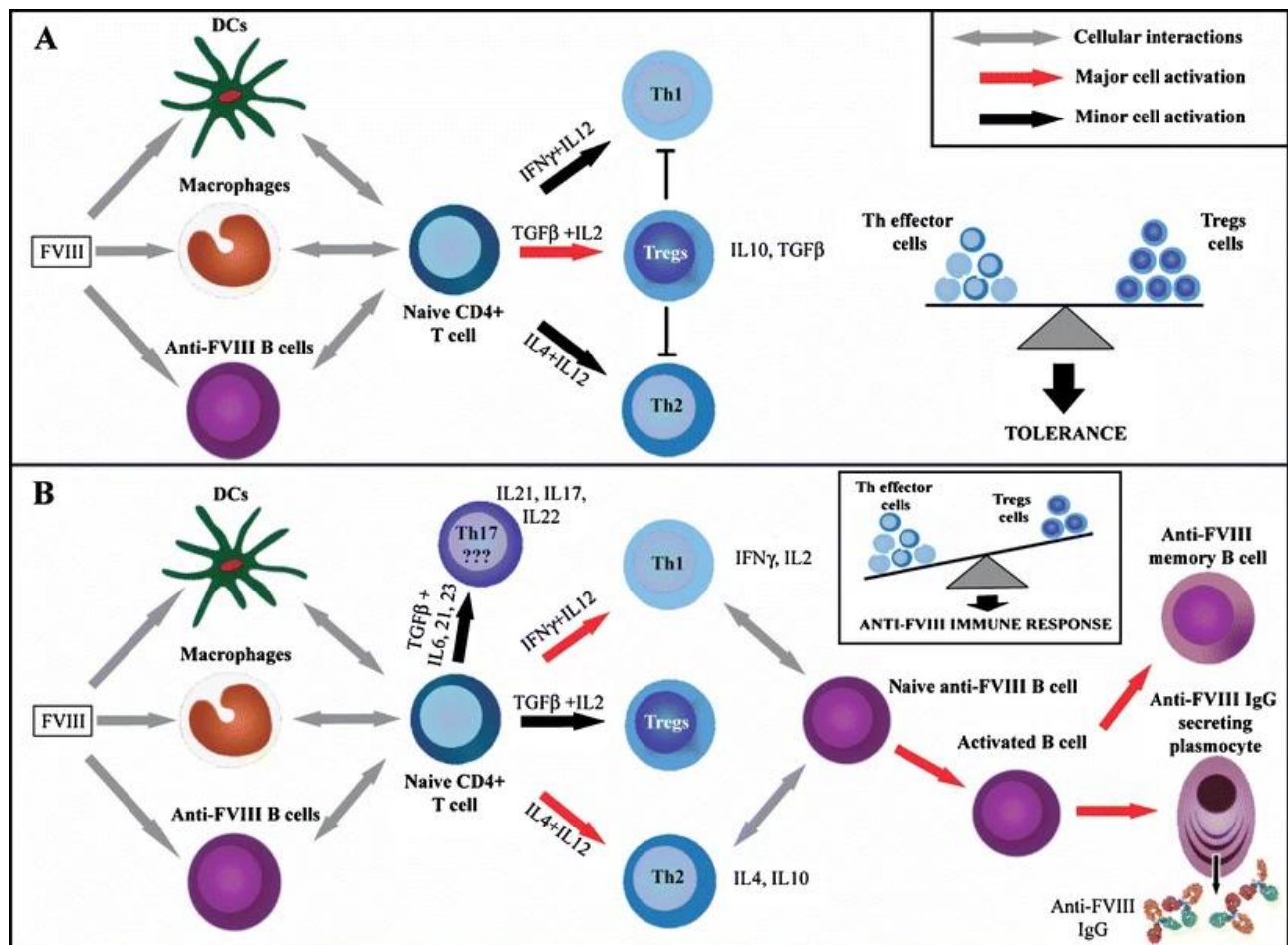


Figure 2. Mechanisms of FVIII tolerance (a) versus anti-FVIII immune response (b). (a) In healthy individuals and inhibitor-negative hemophilia A patients, FVIII is internalized by APCs (dendritic cells, macrophages, anti-FVIII B cells) and presented to naïve T cells, which become tolerized; Treg cells mediate this peripheral tolerance. (b) In inhibitor-positive patients, APC-presented FVIII activates FVIII-specific Th1, Th2, and possibly Th17 cells, which drive naïve B cells to proliferate into anti-FVIII antibody-secreting plasma cells and memory B cells. (19)

T cell Activation and Regulation

The T cell receptor (TCR) must recognize an antigen-MHC complex to initiate T cell activation. The TCR itself is not intrinsically capable of signaling, and is associated with the CD3 complex that has intracellular signaling motifs, which are transmitted when the receptor is engaged. The activation TCR/CD3 signal leads to the activation of the downstream pathway that controls the transcription of genes and consequently dictates the fate of the T cell: either proliferation, differentiation, cytokine

production, or being unresponsive. The strength and duration of TCR signaling are important determinants of these cellular outcomes. (20)

Conventional T cells need to receive both signals from the TCR and co-stimulatory signals: the most significant of these is provided by CD28. CD28 binds to CD80 and CD86 on the surface of APCs, which enhances signaling by the TCR. The coordinated TCR and CD28 signaling affects the capacity of T cells to expand clonally and to gain effector functions. The activation of T cells is not only dependent on the engagement of the TCR but also on whether the integrated TCR signaling input is of a sufficient magnitude to elicit a productive cellular response. (20)

The activation threshold is especially important in the context of differences in T cell responsiveness. TCR stimulation, co-stimulatory, and inhibitory signals are constantly combined by T cells to determine the strength, duration, and context of the TCR stimulation. The amplitude of proliferation and the functional responses of the target cells may be modified by changes in this signaling balance. Experimental strategies involving varying degrees of TCR activation therefore offer a way to explore how easily T cells respond to antigen receptor binding. (21)

Effects of FVIII Deficiency on T Cell Responses

Studies investigating the pathophysiology of FVIII inhibitor formation have traditionally focused on the immune responses to therapeutic FVIII; relatively few studies have examined the potential immunological changes in the absence of endogenous FVIII. This difference is significant, as T cells in HA mature and function without the presence of endogenous FVIII, before the replacement therapy is introduced. To investigate this in our laboratory, we have studied whether FVIII deficiency affects T-cell activity and responsiveness. It was previously demonstrated that FVIII and ovalbumin (OVA) result in an increased number of long-term memory T-cells in hemophilic mice as compared to wild-type (wt) mice. (22). In the same study, the hemophilia-derived T cells showed greater sensitivity to TCR-mediated stimulation in vitro. Hemophilia-derived CD4⁺ T cells demonstrated enhanced proliferative response after stimulation with a low concentration of anti-CD3, especially at the lower concentrations of 10ng and 25ng/ml. These results suggest that the threshold for the activation of HA CD4⁺ T cells might be lower, and they might respond to relatively weak signals from the TCR with a strong proliferative response. Similar, but more limited, effects were observed for CD8⁺ T cells. Splenic CD8⁺ T cells isolated from HA mice demonstrated increased in vitro proliferation at the lowest concentration of anti-CD3. In the same condition, fewer HA-derived CD8⁺ T cells remained non-dividing, whereas a greater proportion underwent multiple rounds of cell division compared to wt cells. The higher responsiveness of both CD4⁺ and CD8⁺ T cells suggest that FVIII deficiency

might affect the responsiveness of T cells to TCR-mediated stimulation, particularly when the strength of the activating signal is relatively low.

These observations precipitate an important question about the cellular mechanisms responsible for the enhanced proliferative phenotype of HA-derived T cells. The increased proliferation can result from a variety of factors, such as initiation of the cell cycle or the survival of activated cells. The relationship between the FVIII deficiency and these processes is not well understood. It remains unclear whether these changes in cell function have the same effect on CD4⁺ and CD8⁺ T cells.

The present study further extends the observations of previous studies by investigating the cellular responses underlying the altered T cell reactivity observed in FVIII-deficient mice. The proliferation of CD4⁺ and CD8⁺ T cells and the kinetics of this proliferative response were examined following *in vitro* α -CD3/CD28 stimulation. In addition, a short-term *in vivo* immunization experiment was performed to investigate early changes in splenic CD4⁺ and CD8⁺ T cell populations following antigenic stimulation. Together, these approaches were used to further characterize T cell responses associated with congenital FVIII deficiency.

OBJECTIVE OF THESIS

Based on our laboratory's previous findings demonstrating increased T cell reactivity and enhanced proliferative responses in HA mice, this master's thesis aims to further investigate the immunological consequences of congenital FVIII deficiency, specifically in CD4⁺ and CD8⁺ T cells. The study aims to determine whether the enhanced proliferative capacity observed in HA mice is associated with alterations in T cell activation and proliferation kinetics after both in vitro and in vivo challenge.

This was done by comparing CD4⁺ and CD8⁺ T cells after in vitro stimulation with anti-CD3/anti-CD28, isolated from hemophilic and WT mice. In parallel, a short-term in vivo immunization experiment was performed using OVA/IFA, with HA and WT mice analyzed on the 8th day after immunization to characterize CD4⁺ and CD8⁺ T-cell responses following antigenic stimulation.

MATERIALS AND METHODS

Mice. Hemophilic Balb/c (HA Balb/c) mice were generated in our laboratory by backcrossing the original FVIII knockout mouse (B6;129-*F8*^{tm1Kaz}/J, Jackson # 004424) (Bi et al., 1995) for 10 generations into the Balb/cJ strain (Jackson # 000651). We bred HA males with heterozygous females to obtain 50% HA and 50% wt males to match in the single experiments. All animals were kept under specific pathogen-free (SPF) conditions, and all procedures were reviewed and approved by the Animal Care and Use Committee of Università del Piemonte Orientale (Italian Health Ministry Authorization n.1169/2024-PR, protocol DB064.95).

Organ collection and cell isolation. At the time of euthanasia, spleens were collected from 8-week-old male Balb/c HA and WT mice in a 6-well plate containing 10 mL of sterile media (RPMI 1640 + 10% Fetal Bovine Serum (FBS) + 100U/ml penicillin + 100µg/ml streptomycin + 2mM L-glutamine 1% + 0,05 mM β- mercaptoethanol). Single-cell suspension was obtained by mechanical disaggregation of the spleen followed by filtration (40µm) and centrifugation at 1500rpm for 5 minutes at 4°C. Red blood cells were lysed by adding 5ml of red blood cell lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA) and incubated for 5 minutes at room temperature (RT). Supernatant was discarded; cells were resuspended in 10ml of media and counted in the Bürker chamber using trypan blue to exclude dead cells.

***In vitro* proliferation assay.** After isolation of the spleen leukocytes, 10⁷ cells were stained with CellTrace™ Far Red Cell stain (ThermoFisher) according to the manufacturer's instructions. Briefly, CellTrace™ was prepared at a concentration of 1 mM by adding 20 µl DMSO to one vial of CellTrace™ reagent. The final working solution concentration was 0,5 mM in PBS. The cells were incubated for 20 minutes at RT, protected from light. At least 2 times the original staining volume of the buffer was added to the cells and incubated for 5 minutes on ice to remove free dye. The cells were washed by centrifugation 2 times and resuspended in complete RPMI. The cells were washed by centrifugation 2 times and resuspended in complete RPMI. After staining with proliferation dye, cells were counted as mentioned above; 150000 (3.0x10⁶/ml) cells were plated in a 96-well plate and stimulated with two different α-CD3 concentrations (25 and 10ng/ml) and α-CD28 (500ng/ml). As control groups, T cells were cultured with medium only. In vitro cell proliferation was evaluated by FACS after 24, 48, and 72 hours. Once labelled cells divide, the cytoplasmic proliferation dye is divided equally between the two daughter cells, leading to a characteristic kinetic profile where the number of peaks of progressively halving proliferation dye fluorescence intensity are observed. Each sample was plated at least in duplicate.

Table 1. Summary of splenic leukocyte tested conditions

Leukocyte culture conditions:
1. 25 ng/ml α-CD3, α-CD28 500 ng/ml
2. 10 ng/ml α-CD3, α-CD28 500 ng/ml
3. Only Media

***In vivo* immunization.** For the *in vivo* immunization, 8-week-old male Balb/c HA and WT mice were immunized subcutaneously with 50mg recombinant ovalbumin (OVA). OVA was emulsified at a 1:1 ratio with incomplete Freund's adjuvant (IFA) before injection. Following the single subcutaneous immunization, mice were maintained for 8 days and subsequently euthanized for analysis. Spleens were collected in sterile complete RPMI medium and processed to obtain single-cell suspensions as described above. Splenic leukocytes were subsequently analyzed by flow cytometry to characterize CD4⁺ and CD8⁺ T-cell populations, including central memory (CM) and effector memory (EM) subsets, as well as regulatory T cells (Tregs)

Flow cytometry analysis. Samples were labelled for 15 minutes at 4°C with the anti-mouse fluorochrome-conjugate monoclonal antibodies listed in Table 2. For Treg detection, after surface staining, cells were incubated with fixation/permeabilization buffer at 4°C overnight and subsequently stained with the anti-FoxP3 antibody for 45 minutes at room temperature. Samples were acquired on the Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific), and analysis was performed using FlowJo Software. The number of cells and fluorescence intensity were recorded.

Table 2. Antibodies used for flow cytometry analysis

Antibody	Reactivity	Clone	Manufacturer	Format	Incubation condition
CD3	mouse	145-2C11	e-Bioscience	PE-Cy7	15 min at 4°C
CD4	mouse	RM4-5	e-Bioscience	FITC	15 min at 4°C
CD8	mouse	53-6.7	e-Bioscience	PE	15 min at 4°C
CD62L	mouse	MEL-14	e-Bioscience	Alexa Fluor780TM	15 min at 4°C
CD44	mouse	IM7	e-Bioscience	APC	15 min at 4°C
Fox P3	mouse	FJK-16s	e-Bioscience	PE	45 mins at RT

CD25	mouse	PC61.5	e-Bioscience	PE	15 min at 4°C
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Statistical analysis. Statistical analysis and comparisons were performed with GraphPad Prism 9.0 (GraphPad). All data are expressed as the mean $\pm$ standard deviation (SD). Mann-Whitney U test was used to compare differences between the two independent groups of WT and HA mice when $n \geq 3$. A p-value of less than 0.05 was considered statistically significant.

RESULTS

In vitro proliferation of CD4⁺ and CD8⁺ T cells in HA and WT mice

To investigate the altered proliferative T cell response of HA mice, leukocytes were isolated from the spleen of 8-week-old HA and WT BALB/c mice and were assessed using an *in vitro* proliferation assay (Figure 3A). Cells were labelled with CellTrace™ Far Red Cell stain and then cultured for 24, 48, or 72 hours in medium containing 10 or 25 ng/mL α -CD3, in the presence of a constant concentration of α -CD28; control samples were cultured only in the medium. CD4⁺ and CD8⁺ T-cell populations were detected by flow cytometry, and proliferation was measured by dilution of CFTR signal. Splenic leukocytes had a narrow and high-intensity fluorescence profile at day 0, which immediately follows the CFTR labelling, and which served as the reference distribution for assessing dye dilution in later measurements (Figure 3B). At day 0, the relative proportion of the two major T-cell subsets, CD4⁺ T cells and CD8⁺ T cells, was similar in HA and WT samples, suggesting that the absence of FVIII did not significantly affect the relative proportions of the two major T-cell subsets under the experimental conditions (Figure 3C).

After stimulation, progressive CFTR dilution was observed as a function of culture time, as successive rounds of cell division occurred (Figure 3D, E); notably, after 3 days, the peaks indicating the proliferating cells were higher for the CD8⁺ compared to the CD4⁺ T cells, confirming the greater proliferation capability of the former population (23). At day 1, few or no CD4⁺ or CD8⁺ T cells gained the fluorescence characteristic of proliferating cells even in the presence of α -CD3 (Figure 4A–D). Stimulation via TCR led to a gradual shift in the distribution of the fluorescence to lower intensity levels of CFTR; this effect was more pronounced at day 3 than day 2, due to the accumulation of the proliferating cells over time. This was seen in both subsets of T cells and was greater with stronger stimulation. In summary, both subsets of T cells could be stimulated by α -CD3/ α -CD28, and the amount of dye that was diluted depended on the intensity of the stimulation and the length of the culture.

The proportions of total live CD4⁺ and CD8⁺ T cells did not significantly differ throughout the culture period between HA and WT samples when cultured under medium-only conditions (Figure 5A and 5D). The CD4⁺ T population remained constantly higher than the CD8⁺ T cells even following stimulation with α -CD3 (Figure 5B,C and 5E,F) although after 72 hours the total CD8⁺ T cells increased their frequency, suggesting a faster proliferation (or better survival over time) than the CD4⁺ T cells.

As expected, the percentage of proliferating CD4⁺ and CD8⁺ T cells rose from both genotypes with

time for both α -CD3 concentrations (Figure 5H,I and 5K,L). Proliferation was greater in both genotypes at the higher concentration of α -CD3 (25 ng/mL) (Figure 5I,L). Compared to WT T cells, the proliferating fraction of both HA CD4⁺ and CD8⁺ T cells was higher at 72 hours, with the greater difference observed at 10 ng/mL α -CD3 (Figure 5H and K). Thus, the difference between HA and WT was more evident at the later time point during culture than at the earliest time point (in relatively low-intensity TCR stimulation).

Overall the comparison among the different stimulation strengths revealed that higher concentrations of α -CD3 (25 ng/mL) increased proliferation in HA and WT T cells, but decreased the difference between the two genotypes. The above observations suggest that there was not a uniform difference in proliferation between the HA and non-HA groups throughout the time course and/or stimulus.

The time-course experiment provides more information than the previous analysis, that was carried out only at 72 hours, on when the differences between HA and WT T cells become apparent. At the lower α -CD3 stimulation the superior HA proliferation profile remained evident while it dropped at the higher concentration. This behavior may reflect a lower threshold of activation of the HA T cells.

Short-term *in vivo* immune response following OVA/IFA immunization

Previous experiments performed in our laboratory showed differences in memory T cell populations in HA mice following immunization with FVIII and OVA, but these differences were observed at a later stage of the immune response (22). To investigate if the difference in T cell responsiveness was reflecting an early change in splenic T cell composition after antigen exposure, HA and WT mice were immunized a single time with OVA/IFA and analyzed 8 days after immunization (Figure 6A). We focused on the OVA since it represents a xenogeneic antigen to which HA and WT mice should respond equally and on day 8 since it is normally the time of the peak of *in vivo* response (22). The present experiment, therefore, tested whether late changes in splenic T cell populations could be related to an altered early immune response during the *in vivo* expansion phase or reflecting a later abnormal memory T cell formation and/or maintenance.

There was no significant difference in splenic leukocyte, total CD4⁺ and CD8⁺ T and Treg cell numbers after OVA/IFA immunization (Figure 6B-E). At day 8 post-immunization, therefore, no major change in splenic overall cellularity nor in absolute numbers of the major T cell populations evaluated was associated with FVIII deficiency.

CD4⁺ memory-associated populations were then analyzed based on the expression of CD44 and CD62L. A significant fraction of the CD4⁺ in HA and WT mice was CM (CD44^{hi}CD62L⁺) cells (Figure 6F). Similarly, EM (CD44^{hi}CD62L⁻) cells were also seen in both genotypes, but there was

no significant difference in the relative proportion of these cells in HA versus (vs) WT mice (Figure 6G). In line with this, the absolute levels of CD4⁺ CM and EM were similar between the two genotypes (Figure 6J and 6K). This same analysis was conducted for CD8⁺ T cells: CD8⁺ CM cells were the most abundant memory-associated subset in both HA and WT mice (Figure 6H), while the CD8⁺ EM cells were less abundant (Figure 6I). There was no significant difference in the relative distribution of these populations between HA and WT mice in both frequency (Figure 6H,I) and number (Figure 6L,M). Differently from the long-term analysis, the present day-8 analysis did not detect any difference in the total and memory T cell populations, suggesting that the event reflecting the greater memory T cell conversion in HA mice should take place later in the immune response.

FIGURES

FIGURE 3

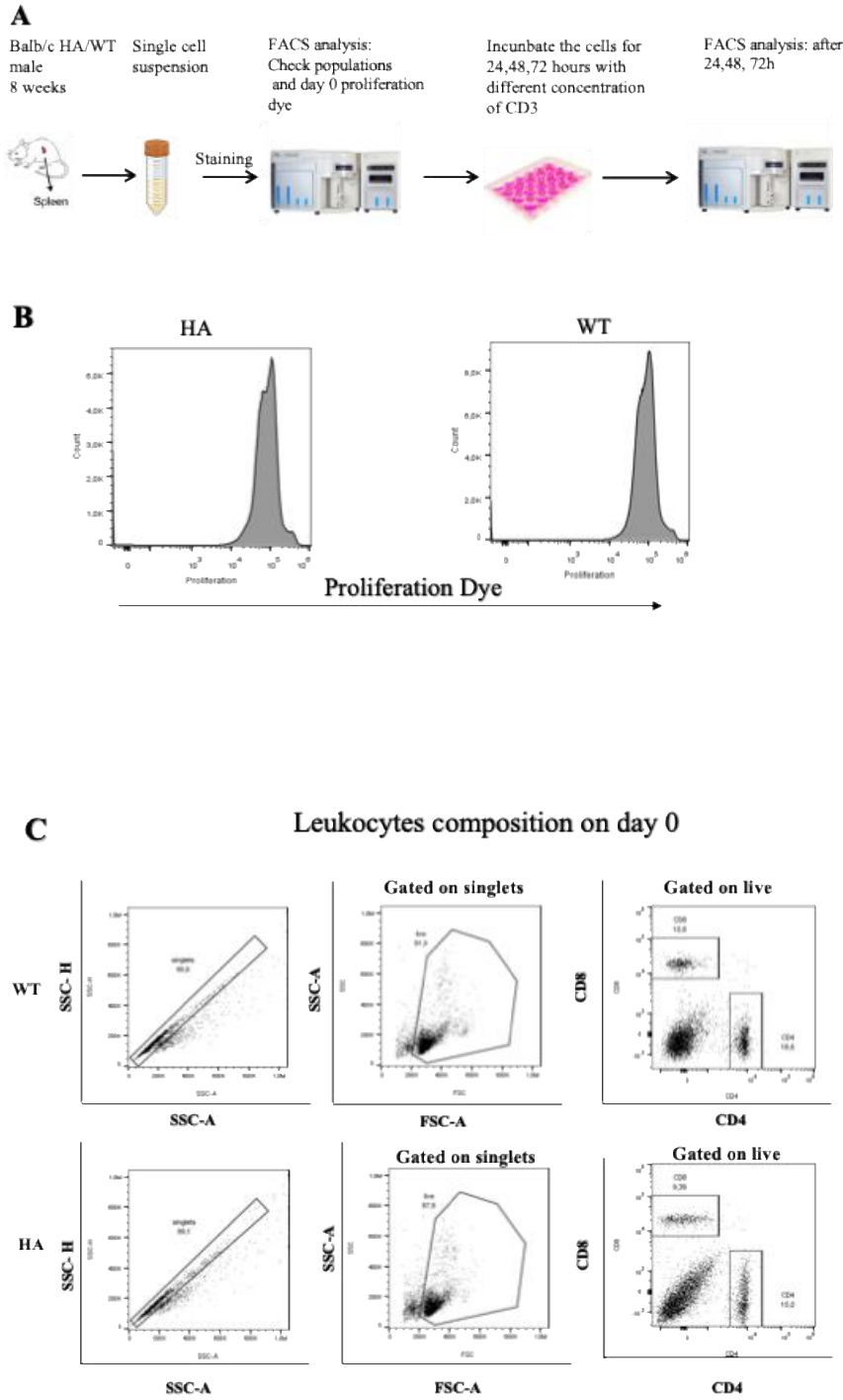
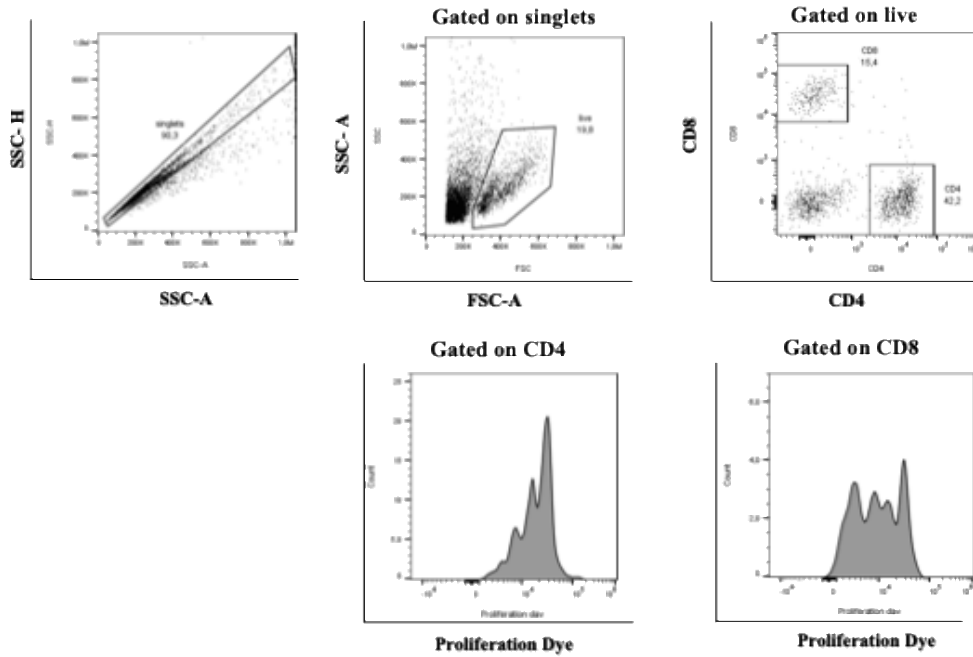
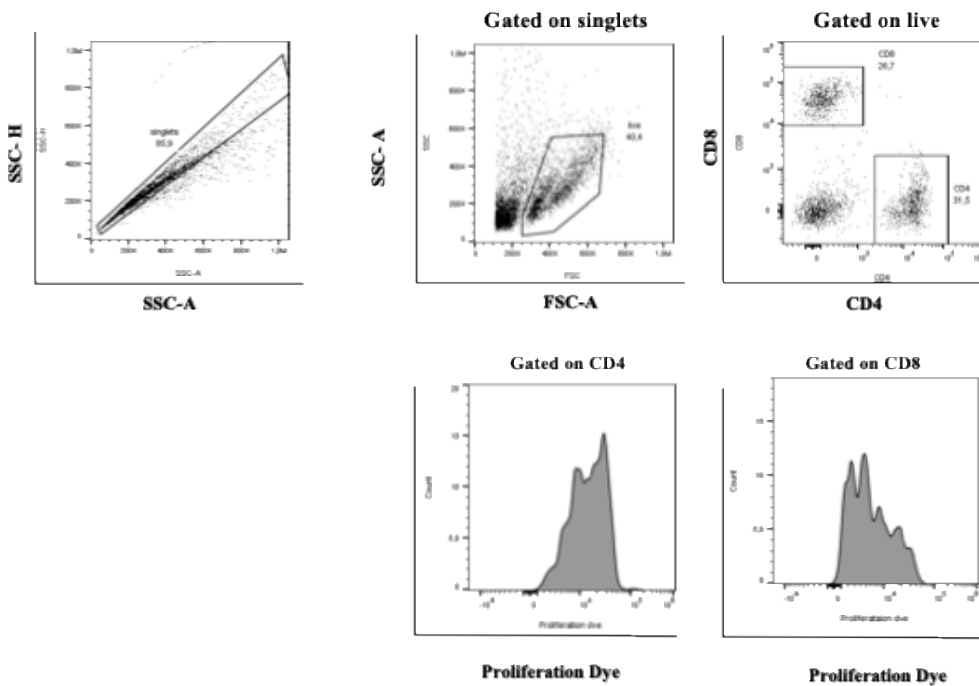


Figure3A–C. Experimental workflow and flow-cytometric gating strategy. (A) Experimental workflow for splenic leukocyte isolation, CellTrace Far Red labelling, culture, and flow-cytometric analysis. (B) Representative day 0 CellTrace Far Red profiles from HA and WT mice. (C) Gating strategy for identification of singlets, live cells, and CD4⁺ and CD8⁺ T cells.

D WT DAY 3 10ng CD3



E HA DAY 3 10ng CD3



Figures 3D–E. Proliferative profile and gating strategy of splenic T cells at Day 3. Representative flow-cytometry histograms and sequential gating strategies used to identify proliferating CD4⁺ and CD8⁺ T-cell populations in (D) WT and (E) HA mice following 3 days of culture with 10 ng/mL α -CD3 and 500 ng/mL α -CD28.

FIGURE 4

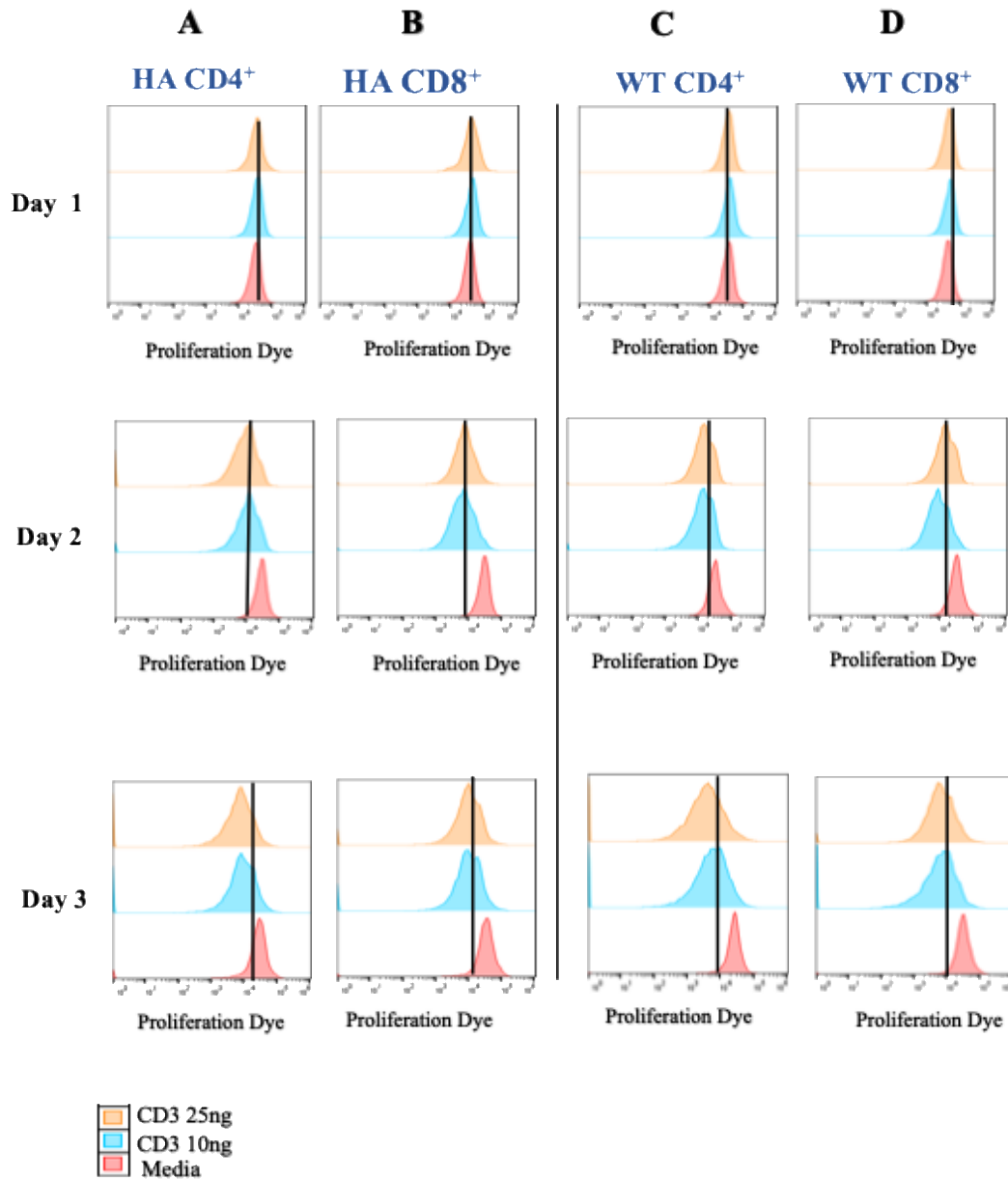


Figure 4. Representative CellTrace Far Red fluorescence profiles showing T-cell proliferation over time. Representative flow-cytometric proliferation profiles of CD4⁺ and CD8⁺ T cells from HA and WT mice following culture in medium alone or stimulation with α -CD3 at 10 or 25 ng/mL in combination with α -CD28 at 500 ng/mL. CellTrace Far Red fluorescence was analyzed after 1, 2, and 3 Days of culture. (A) HA CD4⁺ T cells, (B) HA CD8⁺ T cells, (C) WT CD4⁺ T cells, and (D) WT CD8⁺ T cells. The vertical black line indicates the baseline fluorescence peak of non-dividing cells. Progressive displacement of the fluorescence profile towards lower CellTrace intensity represents dye dilution associated with cell division

FIGURE 5

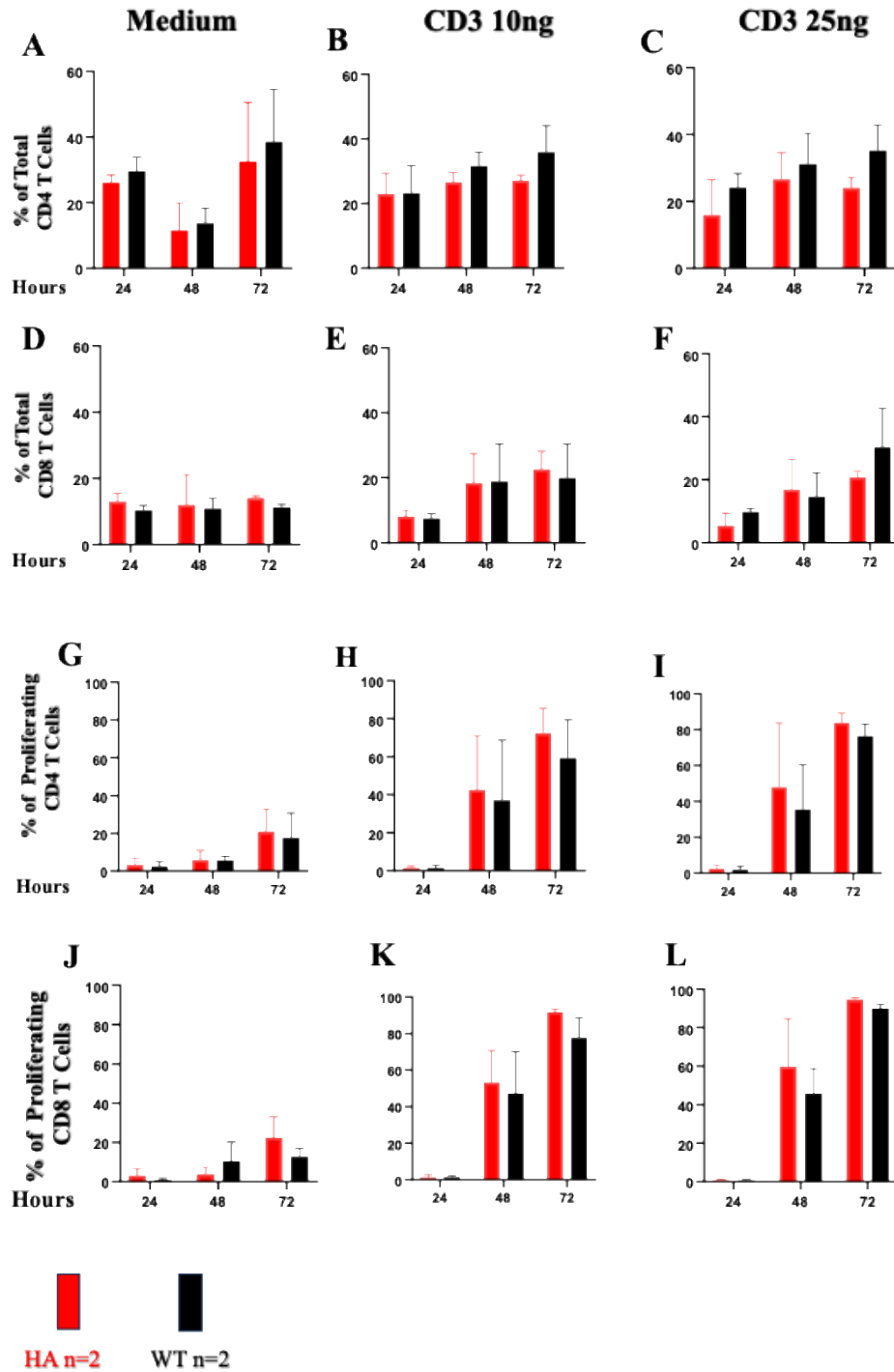


Figure 5. Quantification of CD4⁺ and CD8⁺ T-cell proportions and proliferation over time. Total percentages and proliferation of CD4⁺ and CD8⁺ T cells from HA (red) and WT (black) mice were evaluated at 24, 48, and 72 hours of culture in medium alone or after stimulation with α -CD3 (10 or 25 ng/mL) and α -CD28 (500 ng/mL). (A–C) Total CD4⁺ T cells, (D–F) total CD8⁺ T cells, (G–I) proliferating CD4⁺ T cells, and (J–L) proliferating CD8⁺ T cells. Total CD4⁺ and CD8⁺ T-cell percentages were calculated among live leukocytes; proliferation was determined from CellTrace Far Red dilution. Time of culture is indicated on the x-axis in hours. Data are presented as mean $\pm$ SD.

FIGURE 6

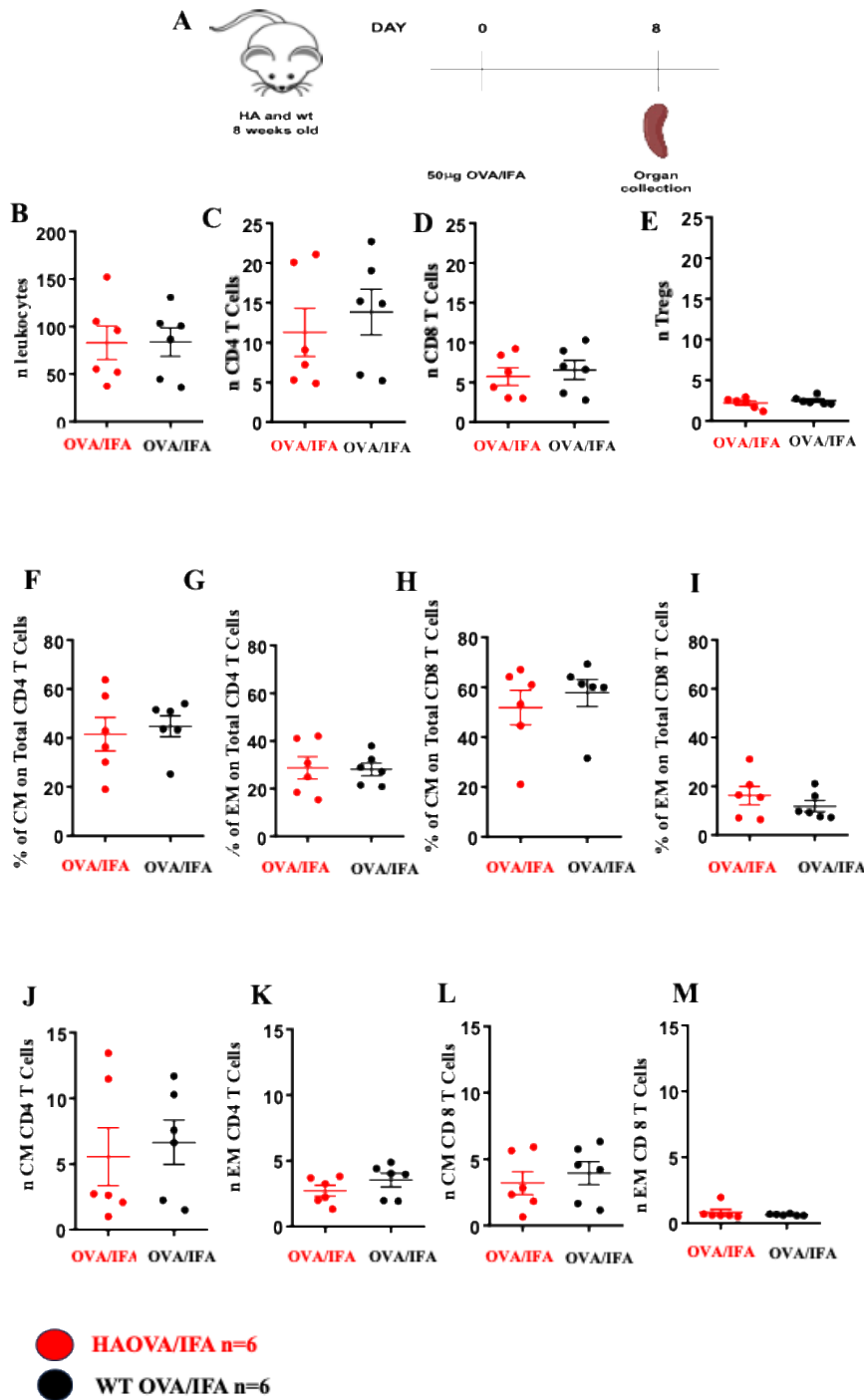


Figure 6. Short-term in vivo immune response following OVA/IFA immunization. HA and WT mice were immunized with OVA/IFA and analyzed 8 days later. (A) Experimental design. (B) Total splenic leukocytes; (C–D) CD4⁺ and CD8⁺ T cell numbers; (E) regulatory T cells; (F–G) CD4⁺ central memory (CM) and effector memory (EM) T cell frequencies; (H–I) CD8⁺ CM and EM T-cell frequencies; (J–M) absolute numbers of CD4⁺ and CD8⁺ CM and EM T cells. Data are shown as mean $\pm$ SD.

DISCUSSION

The primary clinical association of HA is the deficiency of coagulation FVIII. However, previous studies have suggested that FVIII deficiency may also be associated with alterations in immune regulation and T cell responses (24). Our previous work showed that HA T cells could respond more strongly than WT T cells to relatively low-intensity in vitro TCR stimulation (22). This study further extended these observations by studying the kinetics of the proliferation of CD4⁺ and CD8⁺ T cells in response to α -CD3/ α -CD28 stimulation and selected populations of splenic T cells during the early phase of response to in vivo immunization.

The progressive CellTrace Far Red dilution of both HA and WT T cells after α -CD3 stimulation demonstrated accumulation of cell divisions over the course of the culture. Concentration of α -CD3 and length of culture affected magnitude of the proliferative response. The higher concentration of α -CD3 increased proliferation in both genotypes, while the differences between the two were more evident in the lower stimulation condition. This time-course analysis also revealed that these differences were not stable over the culture period. At 24 hours no difference was detected, likely due to the low number of cells already in proliferation, in accordance with the known Lag phase for the T cells to enter in the cell cycle(26). From day 2 instead, the T cells enter in the exponential expansion phase in which they can divide very rapidly (every 4/6 hours); this explains the sudden rise of proliferating cells observed at 48 hours, followed by the further increase at 72 hours. Noteworthy the HA surpassed the WT samples already after 48 hours confirming the hypothesis that the HA T cells hold a faster cell cycle, especially at lower level of stimulation. A possible reason could be that the threshold for T cell activation may be altered by FVIII deficiency. The activation of T cells require the integration of TCR signaling with co-stimulatory signal(s) such as CD28 (25,. If the activation stimulus is relatively weak, a difference in the cellular responsiveness might lead to a measurable difference in the activation response that follows, the proliferation response. However, if stimulation is strong enough, both HA and WT T cells can be stimulated into a significant proliferative response, thus diminishing the apparent genotype differences.

The rates also varied between CD4⁺ and CD8⁺ T cells. The proliferation of both populations was related to the culture time, with both populations exhibiting increasing proliferation in response to culture time; however, the magnitude and time of the response were not the same. This reflects the fact that the effect seen in the present system might be dependent on the T cell subset and on the intensity and duration of the stimulation. The response also differed between CD4⁺ and CD8⁺ T cells, suggesting that the magnitude and timing of proliferation may depend on the activation threshold of

each T cell subset. This threshold can be influenced by intrinsic factors as well as by extrinsic factors such as costimulatory signals, cytokines, and interactions with other cells present in the splenic culture. The present experimental design does not allow these factors to be distinguished. Future experiments using purified CD4⁺ and CD8⁺ T cell populations, different concentrations of α -CD3 and α -CD28, or controlled addition of specific cytokines could help determine whether the genotype-dependent difference is related primarily to intrinsic T cell responsiveness or to signals provided by the surrounding cellular environment.

The in vitro proliferation data have some limitations: assays were done with splenic leukocytes instead of purified T cells. Thus, the response seen reflects the behavior of the T cells in a population of mixed splenic-cell populations. There are other immune cells that can modulate T cell activation by cell-cell contact or by soluble mediators. The present experiments cannot determine if the difference is due to an intrinsic property of HA T cells or if it is, at least in part, mediated by other cells that are present in the culture. There are also issues of interpreting the CellTrace assay itself. The more a cell is diluted, the more times it has been divided, but the degree of dilution cannot be used to distinguish the difference from increased entry into division, differences in the number of times the cell has divided, or differences in cell survival during culture. (26)

No significant genotype-dependent differences were observed in the absolute number of splenic leukocytes (CD4⁺ T, CD8⁺ T, and Tregs) 8 days after OVA/IFA immunization. Likewise, the CD4⁺ and CD8⁺ CM and EM populations that were analyzed showed no significant differences between HA and WT mice. These results reveal that significant changes in the proportion of analyzed T cell subsets did not occur at this early time point compared to the difference observed at later time points. Partially these results are not surprising since the memory populations should become evident 14 days after the immune challenge. Nevertheless, no change was detected in the number and frequency of total T cell subpopulations, even if day 8 corresponds to the usual peak of immune T cell expansion. Therefore, we can exclude that in this setting the proliferation of T cells was higher in the HA mice, differently from what observed in vitro. It is worthy to mention that the OVA dose used for the challenge and the presence of IFA gave a strong immune stimulation overcoming the threshold, while the higher proliferative in vitro response was seen at low α -CD3 concentration.

Indeed, the two experimental conditions are significantly different. In the in vitro assay, splenic leukocytes are stimulated with a well-defined mixture of α -CD3 and α -CD28 for a defined period of time in controlled culture conditions. The in vivo response is very complex and includes antigen presentation, co-stimulatory signals, cytokines, regulatory cells, and interactions with other

compartments of the immune system and tissue.(27) These other regulatory mechanisms might affect the size and makeup of the response and might obscure more subtle differences in T cell reactivity.

The results of the memory-cell studies must also be discussed in comparison with the experiments previously conducted in our laboratory. Differences in memory T-cell populations were previously observed at a later stage following immunization (22). The difference between the examined CM and EM populations was not significant in the present experiment that was analyzed on day 8 after the first immunization. The difference between the studies might be due to the different phases of the immune response analyzed. Memory-associated populations are still evolving even after exposure to an antigen, and thus it is possible that the difference detected at later time points may not have been observed in the early response. Further longitudinal study is needed to see if these differences in these populations appear at later ages as a function of genotype. Moreover other markers could be assessed for detecting possible alterations in the early immune reactivity of HA T cells, such as CD137 (28) and KLRG1 (29). CD137 (4-1BB) is an activation-associated co-stimulatory receptor induced following T cell activation and can provide information on the activation and persistence of responding T cells. KLRG1 is expressed on antigen-experienced T cells and is associated with T-cell differentiation and effector-cell characteristics, while also exerting an inhibitory function that can influence T cell responses. Therefore, analysis of these markers could provide complementary information on the activation and differentiation state of T cells that may not be evident from the frequency of memory T cell populations alone. Previous analyses performed in our laboratory also evaluated CD137 and KLRG1 expression and identified preliminary differences between HA and WT T cells. Although these findings were promising, they require confirmation in further experiments and could help determine whether the altered T cell responsiveness associated with FVIII deficiency is accompanied by changes in T cell activation or differentiation.

Similarly, the comparable number of Tregs between HA and WT mice does not establish that their functional activity is identical. Tregs are important regulators of immune responses to FVIII, and alterations in their frequency, activation state, or antigen specificity have been associated with differences in immune tolerance in HA mouse models (30). In particular, FVIII-responsive CD4⁺ CD25⁺ FoxP3⁺ Tregs have been shown to contribute to the suppression of FVIII-specific effector T-cell responses and the establishment or maintenance of tolerance. Therefore, differences in Treg-mediated regulation may not necessarily be reflected by changes in the overall abundance of Tregs. In the present analysis, Tregs were identified based on their phenotype and quantified, but their activation state or suppressive function was not measured directly. Functional assays, such as

evaluating their ability to suppress T cell proliferation or assessing additional markers associated with Treg activation and suppressive capacity, would be needed to determine whether Treg function differs between HA and WT mice..

Combined, the present results suggest that FVIII deficiency affects T cell responsiveness, but do not suggest a general up-regulation of T cell proliferation or a general change in the composition of splenic T cells during the early in vivo response. By contrast, the in vitro time-course experiment suggested that the differences between HA and WT T cells were related to the intensity of TCR stimulation and to the time point in the proliferative response. The fact that there was no difference in the splenic T cell composition at day 8 further underlines that the change in responsiveness upon controlled stimulation does not necessarily correspond to a quick alteration in the numbers of major splenic T cell populations during the early stages of the response in vivo. To elucidate the cellular mechanism underlying this phenotype and to assess its relevance to immune responses to therapeutic FVIII, additional purified T cell experiments, as well as functional assays, and antigen-specific stimulation will be necessary.

CONCLUSION

The present study evaluates the impact of congenital FVIII deficiency on T cell responses in an HA mouse model, including CD4⁺ and CD8⁺ T cell proliferation upon in vitro CD3/CD28 stimulation, and specific splenic T cell populations during initial immune responses after in vivo immunization.

The time-course analysis revealed that α -CD3/ α -CD28 stimulation of both HA and WT T cells resulted in proliferation, the level of which depended on the T cell subset, α -CD3 concentration, and culture time. The effect of HA vs WT differed from relatively low to relatively high intensity of stimulation, with the greatest effect at later time points and the least effect at higher intensities. Compared with the previous single time-point analysis, these results indicate that the HA-associated phenotype is not a constant increase in proliferative capacity but is dependent on the conditions and stage of T cell stimulation.

The in vivo findings provide important context for interpreting the context-dependent T-cell responsiveness observed in vitro. The absence of an early alteration in the major splenic T-cell populations suggests that the difference detected under controlled in vitro stimulation does not necessarily translate into an immediate change in overall T cell composition following in vivo antigenic stimulation. Instead, the altered responsiveness may represent a more subtle functional phenotype that becomes apparent only under specific conditions of TCR stimulation or at later stages

of the immune response. This distinction is relevant to understanding the immunological consequences of FVIII deficiency and highlights the importance of further studies using antigen-specific stimulation and functional assays to determine whether this altered T cell responsiveness contributes to immune responses against therapeutic FVIII.

In general, the results suggest that congenital FVIII deficiency could be related to a subtle context-dependent change in the responsiveness of T cells, but not to a generalized hyper-responsiveness of the T cells. The use of purified T cell subsets, complementary proliferation and viability assays, functional evaluation, and antigen-specific stimulation will play a crucial role in establishing whether this altered responsiveness is intrinsic to HA T cells and whether this plays a role in the immune response to therapeutic FVIII.

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