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Phenotypic characterization of immune cell populations in homeostatic and FVIII-challenged severe HA mice

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SUMMARY

Rationale of the study. Hemophilia A (HA) is a recessive X-linked bleeding disorder caused by the lack of coagulation factor VIII (FVIII). Current treatment of the disease consists of FVIII infusions, which leads to formation of neutralizing antibodies (inhibitors) in 20-30% of patients. Both the absence of central tolerance, due to the congenital absence of FVIII, and the presence of danger signal play a role in the immune system response. It has been shown that hematopoietic stem cells from FVIII deficient mice showed a reduced long-term repopulating capacity, suggesting a novel role for FVIII in hematopoiesis. Moreover, cytokine analysis of plasma from pediatric previous untreated patients showed increased level of inflammatory cytokines in comparison to healthy young boys. Inflammatory environment could alter the immune population balance and/or predispose HA patients to altered immune responses. For time being very little is known about any possible immunological alteration correlating with the absence of FVIII prior to treatments and no extensive analysis of hemato-lymphoid populations has been conducted in patients and even in the mouse model for HA. Moreover, there are limited studies done comparing the changes in immune populations after immune challenging between HA and wild-type (wt) mice, since there is no real wt control for the original B6/129 HA mice.
Planning of the study. Since there are limited studies done in humans for investigating the immune system of HA patients in homeostatic condition, we first decided to longitudinal characterize in different organs the main immune cell populations in a mouse model of severe HA. Moreover, to assess the role played by the endogenous environment established in untreated HA mice on the strength and specificity of the humoral and cellular immune response we then challenged the mice with either FVIII or ovalbumin (OVA) in presence or absence of a danger signal. For testing the intrinsic Tregs differentiation capability, we performed an in vitro Tregs differentiation protocol starting from naïve CD4 T cells isolated from both HA and wt mice.
Results. No differences were observed in the immune populations between wt and HA mice in homeostatic conditions. After immune challenging with FVIII/IFA or OVA/IFA both wt and HA mice showed similar antigen-specific humoral response. FVIII/IFA challenged HA mice exhibited higher number of CD4 T cells in comparison to wt mice, while OVA/IFA HA mice showed higher number of CD8 T cells compare to OVA/IFA wt mice. Moreover HA mice challenged with both FVIII/IFA and OVA/IFA showed higher conversion of naïve to memory T cells than wt mice, which indicates that HA T cells have lower activation threshold; these differences were only observed when using the adjuvant while it was absent in mice treated with FVIII without the addition of IFA, which suggests that the combination of exogenously given danger signal with endogenous inflammatory microenvironment could be involved in immune response of HA mice.
Conclusions. Our study established, that absence of FVIII did not affect the HA main immune populations number in homeostatic condition. Overall the results obtained here suggest that immunogenic versus tolerogenic response to FVIII in HA patients could depend on the reciprocal interactions of extrinsic and intrinsic factors among which the pro-inflammatory microenvironment determined by the recurrent micro-bleedings that should be taken in consideration.

Introduction

1. Hemophilia A

Hemophilia A(HA) is a recessive X-linked bleeding disorder which effects 1 in 5000 newborn males, caused by lack or reduced activity of coagulation factor VIII (FVIII). FVIII is a non-enzymatic cofactor in the coagulation cascade. While circulating in the bloodstream in its inactive form, it is bound to another molecule called von Willebrand factor (vWF). Despite the fact that most of the coagulation factors are produced by hepatocytes in the liver, it has been shown that FVIII is mainly produced by liver sinusoidal endothelial cells(LSEC)[1][2]. In response to injury, FVIII is activated by thrombin which is able to bind to factor IX (FIX) in a complex that is needed to activate factor X (FX), which converges in the common pathway of the coagulation cascade to convert fibrinogen into fibrin. Based on FVIII residual activity HA is divided in 3 forms: mild form where FVIII activity is from 5 to 40%, moderate 1 to 5%, and severe form where the FVIII activity is less than 1%[3]. HA can be caused by different mutations in the *F8* gene; more than 2000 mutations have been reported: missense, nonsense, frameshift, splice site change, synonymous and inside the promoter[4], but the most common is intron 22 inversion, followed by point mutations and rarely insertions and deletions. Clinical manifestations of HA vary based on the severity of the disease, which can range from bleedings following a trauma, rare spontaneous bleedings and hemarthrosis in milder form to spontaneous bleedings, with frequent hemarthrosis in the severe form. Recurrent joint bleeds in hemophilic patients may lead to the development of joint disease and in turn to the destruction of joints, ultimately leading also to chronic synovitis[5]. Diagnosis of HA is based on family history or following a continuous bleeding after a trauma. Main diagnostic technique is the evaluation of activated partial thromboplastin time (aPTT), which evaluated the ability of patient's plasma to shorten time needed for clot formation in a standard FVIII-free plasma. However, this method is less sensitive when it comes to mild form of hemophilia, which sometimes can provide false negative results; this provides the need of using more sensitive methods like, two-stage clotting, chromogenic assay and genetic investigation. For the time being, there is no definitive cure for HA while the standard treatment consists of continuous plasma derived (pd) or recombinant (r) FVIII infusion, which can be on demand, when the bleeding occurs, or prophylactic when FVIII is continuously infused to prevent the bleeding[6]. However, this treatment has some downsides: high cost of the treatment; short half-life of both pd and rFVIII (approximately 12 hours), which requires more frequent infusions and development of inhibitory antibodies against infused FVIII, which effects around 20-30% of the severe HA patients[7]. The development of inhibitors against infused FVIII makes the management of bleed more difficult and even more expensive[5]. The clinical protocols available for inhibitor eradication are immune tolerance induction (ITI) or administration of bypassing agents. ITI is based on high amount and frequent injections of FVIII over many months, which aims to establish antigen-specific tolerance. Due to its frequency, ITI treatment is highly expensive and invasive as the use of a central venous catheter is required. Since there is a need to overcome those problems and there are around 29% ITI refractory patients[8], in the last decades different approaches have emerged mainly focused on immunomodulation (e.g. oral

tolerance induction, T cell therapy, immunosuppression)[9], or on non-FVIII therapies which are acting by enhancing the coagulation (i.e. emicizumab) or by inhibition of anticoagulant pathways (i.e. fitusiran and concizumab)[10]. Lately there have been several improvements to the current treatment such as the development of new molecules with extended half-life (EHL): for instance FVIII is linked to human IgG1 domain which creates a fusion protein (rFVIIIFc), this allows the molecule to bind to the neonatal Fc receptor, whose expression is stable, and avoids protein degradation by lysosomes and enables recycling, which in turn increases its half-life 1,5- to 2 fold over rFVIII[11][12]. HA represents a good candidate for gene therapy, since restoring FVIII levels higher than 1% is enough to ameliorate the bleeding phenotype of patients and increase the quality of life. This approach has been used for hemophilia B patients for more than 20 years [13]. So far, recombinant adeno-associated viral vectors (rAAV) are the predominant in use for transgene delivery. rAAV are non-integrating into the host genome[14] but there has always been interest in utilizing also lentiviral vectors (LV), as an alternative approach for patients which have pre-existing neutralizing anti-AAV antibodies. LV integrating nature could facilitate the maintenance of the transgene in replicating cell types, for example in the growing livers of children, which would widen the potential benefit from this treatment[15]. A single administration of rAAV in patients with severe HA has provided evidence of no inhibitor formation and a stable dose-dependent increase in FVIII levels with a follow up of 5-years [16]. Even though gene therapy is pretty pervasive, there are still challenges to be solved, such as: ineffective vector delivery linked to the presence of pre-existing antibodies, which in some patients can be prevented by immunosuppression and possible rejection of AAV-transduced cells due to the expression of the "foreign" protein. The dose of the vector and the transgene expression levels have a significant effect on whether an immune response is induced against both AAV and/or transgene product[17].

1.1 Risk factors involved in inhibitor development

Inhibitor development is a very complex process, which involves not only genetic, FVIII mutation, single-nucleotide polymorphism in HLA locus, ethnicity, but also environmental factors such as manufacturing process of the concentrates, timing of the first exposure, dosage and the clinical conditions that could evolve in immunological danger signals[18]. Null mutations in the FVIII gene are associated with the highest rates of inhibitor formation[19], which is around 20-30%, while it is less common to see inhibitors in patients with mild or moderate HA (about 10%)[20]. This suggests that there is a lack of immunological central tolerance to FVIII in the patients which are affected by the severe form while in patients who do not have a null mutation, there can be still an expression of a truncated protein, which would be presented to the immune system. There have been many studies for investigating the role played by pdFVIII versus (vs) rFVIII[21][22][20], but still no consistent or clear evidence of a difference has been determined[23][24]. It could be possible that pdFVIII is less immunogenic due to the human origin of FVIII glycosylation and presence of FVIII-vWF complex, which confers longer half-life. Unlike second-generation rFVIII products, which are made by different cell lines such as Chinese hamster ovary (CHO) and baby hamster kidney (BHK), the fourth generation is made in the human embryonic kidney (HEK) cell line and therefore it shows human post-

translational modification, which helps to avoid high immunogenicity. Even though there have been significant improvements to the treatment of HA patients and on the biological knowledge of FVIII, there are still many unanswered questions and unsolved problems remaining, such as immunogenicity of FVIII and why only some patients develop inhibitors while others do not. Recent studies have also proposed a FVIII direct or indirect role outside the coagulation. For instance Aronovich et al.[25] showed a reduced *in vivo* long term repopulation capability of transplanted HA HSC in a competitive assay, raising the question if absence of FVIII could have a direct effect on the immune populations of HA patients. Moreover, a study conducted in pediatric previous untreated patients (PUPs)[26] has shown that PUPs have more inflammatory cytokines present in sera prior to FVIII treatment compare to healthy young boy, suggesting an alteration of the endogenous environment which could predispose patients to undesired immune responses.

2. Pathophysiology of inhibitor formation

FVIII is made up of 6 domains: A1; A2; B; A3; C1, C2[27]. Studies have shown that B domain seems to have no involvement in the coagulation activity[28] and it is removed after FVIII activation by thrombin. The liver is the major source of FVIII in the body and its main producers are LSECs[1]. Liver is considered to be a tolerogenic organ, because of its constant balancing of pro- and anti-inflammatory signals as a result of continuous exposure to blood borne antigens[29]. In normal plasma, FVIII is non-covalently bound to vWF which regulates its half-life in circulation and prevents its proteolysis by FXa and activated protein C[30]. After proteolytic activation of the coagulation factor by thrombin, FVIII dissociates from vWF and is able to bind platelets surface and interact with FIXa and FX, which leads to coagulation[31]. As mentioned above, treatment with FVIII products is complicated by the development of neutralizing antibodies. These makes factor replacement therapy ineffective and those who develop inhibitors worsen their morbidity. This occurs more commonly in severe HA patients, usually within 50 days from the first exposure. Among the patients who develop inhibitors, we can distinguish low-titer patients, in which the anti-FVIII antibodies can be transient and may disappear without treatment, and high-titer patients, which are found with Bethesda units (BU) higher of 5[32]. 1 BU refers to capacity of the patients' plasma to inhibit 50% of FVIII activity in a pooled normal plasma. It has been shown that the major component of anti-FVIII antibodies consists of IgG4, followed by IgG1[33][30]. High levels of IgG4 anti-FVIII antibodies were observed only in patients with high-titer of inhibitors [33]. It is still not clear why some patients develop anti-FVIII antibodies while others do not. Like mentioned before, mutation of *F8* gene plays a role in inhibitor developments: those who carry large deletions, non-sense mutation and intron 22 inversions have 7- to 10-fold higher probability of developing inhibitors compared to those who have small mutations[34]. Those who have small mutations still have small quantities of the non-functional coagulation factor produced and this is might be enough for tolerance induction[30]. Another risk factor can be represented by the age of first exposure[35] and by the type of replacement therapy. It has also been

shown that reduction of FVIII dosage and of infusion number causes inhibitors in less patients compared to the standard treatment[36], even though it was not conclusive if the results observed was due to low dose/less exposure or due to minimizing the injection during danger signals (bleeding, vaccination, surgery). First line treatment for patients who are inhibitor-positive is to attempt immune tolerance induction (ITI), ITI is the most widely used therapy to eradicate inhibitors by repeated administration of high dose of FVIII [30][37]. This technique has shown around 80% efficiency in eliminating inhibitors in HA patients and achieving tolerance in few months[38]. Even though ITI is highly expensive, it is able to reduce long-term costs associated with inhibitor therapy[39]. Studies have suggested that successful ITI can be promoted by B cell depletion, hence sometimes it is performed in combination with anti-CD20 Abs[40][41]. Even though ITI sounds compelling, around 29% of the patients fail to respond to the therapy, for which novel therapies of tolerance induction have been made. One of the treatments is based on bypassing agents use, such as recombinant activated factor VII (FVIIa) and activated prothrombin complex concentrates (aPCCs). These can help the progression of coagulation by bypassing the FVIII-dependent step and enhancing thrombin generation: they control at least 80% of the bleeding episodes associated with high-titer inhibitors[42]. However, their hemostatic efficacy is not easy to predict and it does not have the same success rates in patients without inhibitors[43]. Emicizumab, which is a humanized bispecific antibody, has gained a lot of attention in recent years and it can be used either as prophylaxis or after an unsuccessful ITI. It has the capacity to bridge FIXa and FX, which resembles FVIIIa action[44], leading to the production of thrombin in a dose-dependent manner.

3. Immune system

FVIII inhibitors development involves both innate and adaptive arms of the immune system[45]. The immune system is a collection of cells, molecules and processes that function to protect the organism from foreign antigens (e.g. microbes, viruses, cancer cells and toxins)[46]. The immune system comprises of two arms: innate and adaptive. The innate arm of the immune system represents the first line of defense to the intruding pathogens, it is antigen-independent, and therefore nonspecific. It is used by the host immediately or within hours from the encountering with antigen/s. Even though it is believed that innate immune response has no immunologic memory there have been studies suggesting otherwise[47][48][49]. The first evidence for NK cell-associated recall responses came from mice deficient in T cells and B cells developing antigen-specific immunological memory to haptens, small molecules that form immunogenic adducts with proteins. When mice lacking B and T cells were sensitized by applying the hapten to the skin, they still developed a measurable recall response within a few days. Re-challenge with the hapten that had been applied before induced ear swelling, while application of a different hapten to sensitized mice did not induce a contact hypersensitivity reaction, indicating antigen specificity of this phenomenon[48].

The innate immunity comprised four types of defensive barriers: anatomic, physiologic, endocytic/phagocytic and inflammatory. As mentioned before, innate immunity is antigen-independent

so it relies on pattern recognition receptors (PRRs), which allow a limited range of immune cells to detect and rapidly response to a wide range of pathogens that share common structures, known as pathogen associated molecular patters (PAMPs)[50]. One of the most important function of the innate immunity is the production of cytokines and chemokines (small proteins involved in cell-cell communication and recruitment) and the rapid recruitment of immune cells to the infection sites. Cytokine production during innate immunity is key to mobilizing many defensive mechanisms throughout the body and also to activate local cellular responses to infection or injury. Cytokines are also critical for initiating cell recruitment and the local inflammation which is essential for clearance of many pathogens. The phagocytic action of the innate immune response promotes clearance of antibody complexes or dead cells and removes foreign substances present in lymph, blood and other tissues. It participates also in the activation of the adaptive immune response through mobilization and activation of antigen-presenting cells (APCs)[51]. Innate immune cells are: macrophages, granulocytes, dendritic cells (DCs), mast cells, natural killer (NK) cells, and innate lymphoid cells (iLC). Macrophages are long-lived cells which not only play a role in phagocytosis, but also are involved in antigen presentation to T cells. DCs are professional APCs and fundamental in initiating the acquired immune response acting as an important link between innate and adaptive immunity[52]. NK cells are a prominent source of interferon-gamma (IFN-γ), which mobilizes APCs and promotes the development of effective antiviral immunity[53]. Moreover, they play a major role in tumor rejection and induction of apoptosis of cells infected by viruses. NK cells kill infected cells through release of perforin and granzymes (proteins that cause lysis of target cells) from their granules, which in turn induce the apoptosis.

Development of the adaptive immunity is crucial when the innate immunity is ineffective in eliminating infectious agents. Adaptive immune system includes two main cell populations: T cells, which are activated by the action of APCs, and B cells which can differentiate into antibody secreting cells (ASC) and plasma cells (PCs). B cells originate in the bone marrow (BM) from hematopoietic stem cells (HSCs) throughout multiple differentiation stages and once mature they leave BM expressing a unique antigen-binding receptor, the B cell receptor (BCR) on their membrane[54]. Unlike T cells, they are able to recognize antigens directly, without the help of APCs. The main function of B cells is to produce antibodies against foreign antigens, which requires their further differentiation[55] but they can also act as APCs under certain circumstances. When B cells are activated, they undergo proliferation and differentiation into ASC followed by formation of PCs or memory B cells. Memory B cells are long-lived, continue to express their BCR and they can be called to rapidly respond by production of antibodies upon re-exposure. PCs can be either short-lived, which stay locally where they form, or long-lived, which reside in BM where they produce large amounts of antibodies that enter into circulation providing effective protection against pathogens. T cells as well derive from HSCs but the common lymphoid progenitors (CLP)s has to migrate into thymus for completing the differentiation into naïve T cells. The main defining characteristic of T cells is the expression of the T cell receptor (TCR): each T cell expresses a single type of TCR[56]. As mentioned before, T cell antigen-specific recognition requires its presentation by APCs. APCs express the major histocompatibility complex

(MHC) molecules on their surface, which are either defined as class I (which in humans are HLA A, B and C), found on all nucleated cells, or class II (termed HLA DP, DQ and DR) localized on specific cells of the immune system: macrophages, DCs and B cells. Class I MHC molecules present endogenous (intracellular) peptides while class II present exogenous (extracellular) peptides. Each MHC protein can present short peptides, derived by the degradation of larger proteins when a cell is infected by an intracellular pathogen or phagocytes foreign organisms or proteins. During the development T cells undergo positive and negative selection in thymus in order to be selected based on the recognition of the own MHC molecules and to avoid autoimmune responses. The opportunity for the specific T cell to encounter an APC carrying the correct peptide on the MHC complex is increased by the circulation of T cells throughout the body and their accumulation in lymph nodes together with APCs. In response to TCR activation, T cells further control immune responses by secretion of cytokines. There are 3 main types of T cells: CD8 T cytotoxic cells, CD4 T helpers and T regulatory cells (Tregs). CD8 T cells are mainly involved in killing cells which are infected by foreign agents, like viruses, and killing tumor cells[57]. Their activation depends on the interaction of their TCR with peptide bound to MHC class I molecules. Upon activation they undergo clonal expansion and differentiation into effector cells, which release substances that induce target cell apoptosis. After resolution of infection, most of the effector cells undergo apoptosis and are cleared by phagocytes and the cells which remain are retained as memory cells, which can quickly differentiate into effector cells upon subsequent encounter with the same antigen. CD4 T cells play an important role in establishing and maximizing the immune response since they do not have cytotoxic or phagocytic activity and are unable to directly kill infected cells or clear pathogens but they are the mediators of the immune response by directing other cells and regulating the type of the immune response that would develop. Unlike CD8 T cells, they are activated through TCR recognition of antigen bound to MHC class II molecules, and once activated, they release cytokines which influence the activity of many cell types including, APCs which in turn interact with them. Like CD8 T cells, most CD4 T cells will also undergo apoptosis upon resolution of infection, with few remaining as memory T cells. Tregs play an important role in limiting and suppressing immune responses, thereby they function to control responses to self-antigens and prevent the development of autoimmune disease.

For preventing autoimmune responses there are two main mechanisms: central and peripheral tolerance[58]. Central tolerance mechanisms act on T and B cell development in thymus and BM respectively. Immature B cells that recognize self-antigen in the BM with high avidity undergo either receptor editing or cell death by apoptosis. Receptor editing involves reactivation of RAG genes and rearrangement of another Ig light chain gene to let the immature B cell express another BCR. If after receptor editing B cells still recognize self-antigen with high avidity they undergo apoptosis [56]. In thymus the double positive (DP) $CD4^+CD8^+$ population which do not bind self-peptide MHC complexes die by neglect, which is called a positive selection, in which self-antigens play an important role and the affinity of interaction is low. Furthermore, DP population that can recognize self-peptide MHC complexes with high affinity undergo cell death by apoptosis, which is known as negative selection, or develop into natural regulatory T cells. Only those which recognize self-antigens with low

affinity can further continue their development. Thymocytes that recognize MHC I develop into spCD8, whereas those that recognize MHC II develop into spCD4.

While central tolerance occurs in primary lymphoid organs, peripheral tolerance occurs in secondary lymphoid organs. Although central-tolerance is efficient, it cannot eliminate all self-reactive lymphocytes, partly because not all self-antigens are expressed at the primary site of lymphocyte development[59]. Therefore, mechanisms of peripheral tolerance are required to control lymphocytes that first encounter their cognate self-antigens outside of thymus, such as food antigens, developmental antigens and antigens displayed during chronic infection. Both anergy and deletion of self-reactive T cells can occur in the periphery. DCs are the inducers of immune response, but they are also crucial regulators of tolerance induction and maintenance. Tolerogenic DCs can also be induced and maintained by various anti-inflammatory and immunosuppressive agents *in vitro* and *in vivo*, including IL-10, TGF-β, corticosteroids and rapamycin[60]. Indoleamine 2,3-dioxygenase 1 (IDO1) is a key regulatory enzyme that supports Treg function and peripheral tolerance in adult life, which can also be found in APCs[61]. Study has shown[62] that IDO in APCs produce immunosuppressive metabolites through the breakdown of tryptophan, which has been linked to inhibitor development in humans. Tregs also play a major role in maintaining peripheral tolerance mechanisms as discussed below.

3.1 Immune cells in Hemophilia A

It is still not completely understood why and how FVIII elicits immune response, but it is believed that in patients completely lacking FVIII there is an absence of central tolerance. It has also been shown that some healthy patients have anti-FVIII antibodies, while simultaneously having specific anti-idiotypic antibodies, which prevents binding of anti-FVIII antibodies to FVIII[63]. There has also been evidence that healthy patients have existing FVIII reactive T cells[64], suggesting that immune response towards FVIII depends on both central and peripheral tolerance. Since in the late 90s it was observed that HIV infected HA patients had decreasing inhibitor titer in parallel to decreasing CD4 T cells[65], it is generally accepted that a classical T-cell dependent immune response is required for the generation of inhibitors. The first step for developing a CD4 T mediated immunity to infused FVIII requires uptake by APCs. Several studies have specifically implicated both macrophages and DCs in the spleen as the main APCs which bind and internalize FVIII[61]. *In vivo* studies in mice revealed that upon intravenous infusion, FVIII preferentially accumulates in the spleen, co-localizing with macrophages within the red pulp and to a lesser extent follicular DCs within the antigen-sampling boundary of the marginal zone (MZ)[66]. Innate-like MZ B cells have been shown to be critical in development of inhibitors, because of their enhanced ability to shuttle antigen into the follicle to promote formation of germinal-center and to work in concert with follicular DCs to potently activate follicular T cells[67]. Although it has been shown that antigen presentation by B cells is dispensable, S. Delignat et al.[68] showed that mice fed with selective inhibitor of Bruton tyrosine kinase to inhibit B-cell receptor signaling prior to challenge with FVIII did not prevent the development of inhibitory anti-FVIII IgG. Several *in vitro* and *in vivo* studies have identified an important role of the FVIII C1 domain

in mediating endocytosis. Steric hindrance of this domain using the monoclonal antibody KM33, or mutating the epitope at amino acids 2090, 2092 and 2093, inhibits FVIII uptake by human and mouse DCs, which subsequently reduces FVIII immune response in HA mice[69][70]. It has also been shown that the C2-specific antibody BO2C11, as well as mutation in the epitope for this antibody at amino acids 2215 and 2220, similarly inhibited FVIII uptake by DCs and decreased FVIII immune response in HA mice[71]. Naïve CD4 T cells can differentiate into 5 major subsets: Th1, Th2, Th17, T follicular helper (Tfh) cells and Tregs[72]. Th1 cells are defined by their ability of expressing interferon(IFN)-γ and the master transcription factor T-bet, and they mainly participate in type 1 immune responses to intracellular pathogens. Th2 cells instead express IL-4, IL-5, IL-13 and the master transcription factor GATA3, and they are mostly involved in type 2 immune response to larger extracellular pathogens. M. Qadura et al, showed that formation of inhibitors against infused FVIII involved both Th1 and Th2 subsets of CD4 T helper cells[73]. Tfh cells are characterized by expression of the B-cell follicle-homing CXCR5 and the programmed cell death(PD)-1 receptors; they secrete IL-21 and express Bcl6 and their main function is to help B cells to produce high affinity antibodies. Recently it has also been shown that FVIII inhibitor-producing mice had increased formation of germinal centers (GC) and increased Tfh cells in response to FVIII immunization compared to the mice which did not develop any inhibitors against FVIII[74].

Tregs are defined by the expression of IL-2 receptor α chain(CD25) and the master transcription factor Forkhead box P3(FoxP3)[75], and their function is to maintain the immune homeostasis. Tregs can develop in the thymus during negative selection, and these Tregs are called thymic-derived Treg(tTreg) cells[76]. By self-antigens recognition, they mainly control self-reactive effector T cells that escaped negative selection to maintain immune tolerance[76]. In contrast, Tregs differentiated from naïve CD4 T cells outside of the thymus are called peripheral Tregs (pTregs). Compared to tTregs, pTregs not only recognize self-antigens, but also recognize non-self antigens including food antigens and innocuous commensal microbiota derived antigens, therefore, they are important for the maintenance of mucosal tolerance[77]. Cytokines, especially IL-2 and TGF-β, play essential roles during the development and differentiation of Tregs. IL-2 and TGF-β promote FoxP3 expression by activating STAT5 and SMAD2/3, respectively[78][79][80]. There are multiple mechanisms through which Tregs can demonstrate their regulatory functions: by secreting regulatory cytokines such as IL-10, TGF-β and IL-35[81], by consuming IL-2, which is important for effective survival and proliferation of all CD4 T cells[82]; by expressing cell surface receptors that transmit negative signals such as CTLA-4, CD39 and CD73[83] and by inducing trogocytosis to strip off the antigen-MHCII complexes from APCs in an antigen-specific manner[84]. Mutations in the FoxP3 gene in mice or in humans can lead to a severe autoimmune diseases[85][86][87]. On the other hand they can also be involved in tumor development by suppressing the development of antitumor immunity[88]. IL2 is critical for Tregs development since in its absence, they fail to survive or expand in the periphery or even in thymus[89][90]. Studies showed that it is possible to selectively expand Tregs with IL-2 if it is bound to a specific anti-IL-2 antibody[91]. In fact Chao-Lien Liu et al. showed that treating HA mice with IL-2 complexed with an anti-IL-2 monoclonal antibody expanded T regs *in vivo* and when administered in combination with

FVIII gene therapy in mice, they did not develop inhibitors, while the control mice quickly lost the FVIII activity[92]. B. Waters et al. also showed[93] that low doses of anti-CD3 antibody prevented the development of FVIII inhibitors in mice due to the expansion of Tregs while destroying the effector T cells in HA mice. Our lab has also shown that Tregs depletion, after in vivo FVIII delivery by lentiviral transduction in HA mice, provoked FVIII activity reduction and inhibitor formation[94], Interestingly, this effect disappeared as soon as new Tregs were produced and detected in periphery. Altogether these observations are interesting and raises questions about the Tregs functionality in HA patients.

Aims of the thesis

1. In vivo hemophilic immune cells analysis in homeostatic condition. Since there are limited studies done in humans for investigating the immune system of HA patients in homeostatic condition, we first decided to longitudinal characterize in different organs the main immune cell populations in a mouse model of severe HA. Unlike HA patients, performing this kind of analysis on HA mice gave us some advantages, since mice can be maintained without FVIII treatment and compared with a full-matched group of wild-type (wt) mice, and allow the access to organs that are not easy to sample in patients, such as BM and thymus.

2. In vivo hemophilic humoral and cell immune response assessment upon specific antigen challenge. To assess the role played by the endogenous environment established in untreated HA mice on the strength and specificity of the humoral and cellular immune response we then challenged the mice with either FVIII or ovalbumin (OVA) in presence or absence of a danger signal. The experiments were planned for elucidating if differences would have been observed between wt and HA mice after FVIII immunization and if those disparities would have been linked exclusively to the FVIII or even to another given antigen.

3. In vitro Tregs differentiation evaluation of hemophilic CD4 T cells. In order to test the intrinsic Tregs differentiation capability, we performed an in vitro Tregs differentiation protocol starting from naïve CD4 T cells isolated from both HA and wt mice.

Materials and Methods

Mice

Hemophilic Balb/c (HA Balb/c) mice were generated in our laboratory by backcrossing the original FVIII knock out mouse (B6;129-*F8*^{tm1Kaz}/J, Jackson # 004424)[95] for 10 generations into the Balb/cJ strain(Jackson # 000651). We bred HA males with heterozygote females in order to obtain 50% of HA and 50% of wt males to match in the single experiments. All animals were kept under specific pathogen-free (SPF) conditions and all the procedures were reviewed and approved by the Animal Care and Use Committee of Università del Piemonte Orientale (Italian Health Ministry Authorization # 370/2019-PR, project DB064.45).

PCR Genotyping of mice

A small piece of tail was cut from mice with scissors after the mice reached 4 weeks, bleeding was stop by using a cauterize machine. Digestion of the tail snips and DNA isolation was performed by using DNA extraction kit (Promega) according to the manufacturer's protocol. DNA concentration was determined by Nano Drop TM 2000c spectrophotometer (Thermo Scientific) by measuring the absorbance of nucleic acid at 260nm ultraviolet light. PCRs were performed with GoTaqR Flexi DNA polymerase (Promega). PCR protocol was as follows: initial denaturation at 95°C for 3 minutes followed by 10 cycles of denaturation at 95°C for 30 seconds, annealing starting at 61°C for 40 seconds which was decreasing by 1°C after each cycle, extension at 72°C for 40 seconds; after 28 cycles of denaturation at 95°C for 30 seconds, annealing at 51°C for 40 seconds, extension at 72°C for 40 seconds and final extension at 72°C for 5 minutes.

Primers used: Sequence 5'--> 3': Mutant Forward – TGT GTC CCG CCC CTT CCT TT
Wild type Forward – TGC AAG GCC TGG GCT TAT TT
Common – GAG CAA ATT CCT GTA CTG AC

Organ collection and flow cytometry analysis of immune cells

At the time of euthanasia, the following organs were harvested from mice in cold media (RPMI 1640 + 5% Fetal bovine serum (FBS)): spleen, thymus, BM. Peripheral blood (PB) was collected in 1,5ml tube containing Sodium citrate (1:10 sodium citrate:PB). A single cell suspension was obtained by

mechanical disaggregation of spleen and thymus followed by filtration (40μm filter) and centrifugation at 1500rpm for 5 minutes at 4°C. Red blood cells were lysed by adding Red blood cell lysis buffer (155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA) and incubated for 5 minutes at RT. BM cells were flushed from tibia and femur by using 1ml syringe with 27G needle and passed through a 19G needle for facilitating the cell release. Live cells were counted for each organ by using Trypan Blue. Cell suspensions were resuspended in cold FACS buffer (PBS + 2%FBS + 2mM EDTA) for the staining. Master mix of antibodies was made for each staining and the cells were incubated for 15 minutes at 4°C. After incubation samples were washed and resuspended in FACS buffer for acquisition. For Tregs staining, the surface antigens were stained as mentioned above while the FoxP3 intracellular staining was performed with eBiosciense mouse Regulatory T cell staining kit accordingly to the manufacturer instruction. Briefly, the cells were kept overnight in Permabilization/Fixation buffer at 4°C overnight followed by a wash with the Permabilization buffer; samples were stained with α-FoxP3 antibody, for 45 minutes at RT. All the antibodies used are listed in Table 1, while the gating strategies are showed in Figure 1-4. Samples were acquired on the Attune NxT Acoustic Focusing Cytometer (ThermoFisher Scientific) and analysis was performed by FlowJov10 software (LLC).

Immunization with human FVIII or OVA

8 week old HA and wt Balb/c male mice were subcutaneously (sc) immunized 3 times(Day 0, 14, 35) with 2U of human B-domain deleted (BDD) recombinant FVIII (ReFacto AF, Pfizer) or with 50μg of ovalbumin (OVA, SERVA), in emulsion (1:1 ratio) with incomplete Freund's adjuvant (IFA). Control mice were injected with PBS/IFA emulsion. Blood was taken from mice at different timepoints (Day -3, 18, 45, 64, 77, 81 and 100) and serum stored at -80°C until ELISA was performed for detecting the formation of antibodies against immunized antigen. At day 100 tail bleeding assay was performed. Mice were killed and different organs (spleen, thymus, BM) were taken for flow cytometry analysis.

Treatment with human FVIII

8 weeks old HA and wt male mice were injected with 3U of human B-domain deleted recombinant FVIII (ReFacto), resuspended in PBS by intravenous (iv) injection(Day 0, 7, 14, 21 and 28). Control mice were injected with just PBS. Blood was taken from mice at different timepoints (Day -3, 18, 35, 50, 65, 77 and 88) and serum stored at -80°C until ELISA was run. At day 88 tail bleeding assay was

performed. Mice were killed and different organs different organs (spleen, thymus, BM) were taken for flow cytometry analysis.

Bleeding assay

At the end of the experiment the mice were anesthetized and the distal portion of the tail was cut at a diameter of 2 mm. Tails were placed in a 15 ml conical tube containing 14 ml of 37°C pre-warmed saline. Blood was collected for 10 min and, following centrifugation, resuspended in red blood lysis buffer. Blood loss was evaluated by estimating hemoglobin concentration by measuring the absorbance of serial sample dilutions at 575 nm on a Victor X4 (PerkinElmer) spectrophotometer.

Mouse specific IgG detection

Serum samples were collected regularly from the mice under isofluorane anesthesia and stored at -80°C. Specific α-FVIII or α-OVA IgG were measured by Enzyme-linked immunosorbent assay (ELISA). 96 well plates were coated overnight at 4°C with 4U/ml of ReFacto or 1μg/ml OVA. After plates were washed with 200μl of PBS + 0,05% Tween, 100μl of PBS containing 1% bovine serum albumin (BSA, Merck) was added for blocking and incubated for 1-2 hours at RT, meanwhile the serum samples were diluted in PBS +1% BSA. After incubation plates were washed and 50μl of diluted serum samples were added and the plates were incubated for 2 hours at RT. After plates were washed and 50ul of HRP conjugated α-mouse IgG diluted in PBS1x+1%BSA was added and plates were incubated for 1 hour at RT. After a final wash the enzyme activity was determined using the 100μl of TMB substrate (DiaSorin) solution followed by 100μl of stop solution (0.16M Sulfuric Acid, DiaSorin). The absorbance was measured at 450nm on a Victor X4 (PerkinElmer) spectrophotometer.

Bethesda assay

The presence of FVIII inhibitors in fresh plasma of injected mice was assessed by the Nijmegen modification of Bethesda assay as previously described[96] with slight modifications. Briefly, serum samples from immunized mice were diluted at different dilutions in pooled HA plasma, after which it was mixed 1:1 with HA plasma containing 1U/ml FVIII. Standards were made by mixing pooled HA plasma with HA plasma containing 1U/ml FVIII(100% FVIII activity) after which it was diluted in pooled HA plasma for getting 75%, 60%, 50%, 32,5%, 25% and 12,5% FVIII activity. Mixed samples

were incubated for 2 hours at 37°C, after which aPTT was performed. Briefly, 25µl of the incubated samples were resuspended in 25µl of aPTT reagent SynthASil(HemosIL) and incubated for 3 minutes at 37°C, after which 25µl of $CaCl_2$ was added and coagulation time was recorded.

Naïve CD4 T isolation and Treg differentiation

Spleen was harvested from 8 weeks old male Balb/c HA and wt mice in a complete media (RPMI 1640 + 10%FBS + 100U/ml penicillin + 100µg/ml streptomycin + 2mM L-glutamine + 0,05mM β-mercaptoethanol) and single cell suspension was obtained. Splenic naïve CD4 T cells were isolated by immunomagnetic negative selection: total splenocytes were incubated with Biotin-antibody cocktail(CD8a, CD11b, CD11c, CD19, CD25, CD45R, CD49b, CD105, Anti-MHC II, Ter-119 and TCRγ/δ) for 5 minutes at 4°C followed by incubation with Anti-Biotin MicroBeads and CD44 Microbeads for 10 minutes at 4°C and finally isolated by LS separation Columns (Miltenyi Biotec), according to the manufacturer's protocol. Flow cytometry analysis was done pre- and post-isolation for checking the purity of isolated naïve CD4 T cells (Figure 4A,B) and only samples with purity higher than 90% were used for the experiments. Isolated naïve CD4T cells were plated at the density of $2{,}5x10^5$ cells per well in a 96 well plate, all of the samples were plated in complete media in presence of 100ng/ml α-CD3, 500ng/ml α-CD28, 10ng/ml TGF-β and 50U/ml IL-2 and incubated at 37°C for 4 days. As controls naïve CD4 T cells were plated with complete media only or α-CD3/CD28. Each condition was plated in triplicate. After 4 days cells were washed and prepared for the Treg staining as mentioned above, after same volume was acquired on FACS machine for all the samples, cell counts were done by FlowJo analysis.

Statistical analysis

Statistical analysis and comparisons were performed with GraphPad Prism 9.0 (GraphPad). All data are expressed as average ±standard error of mean (SEM). One-way analysis of variance (ANOVA) with post-hoc Tukey test was performed to compare three/four groups. Two-way ANOVA was used for immuno-phenotypic characterization and to resolve overall effects on specific IgG levels between HA or wt groups over time. A p-value of less than 0.05 was considered to be statistically significant.

Table 1: 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	AF700	15 min at 4°C
CD19	mouse	1D3	e-Bioscience	APC	15 min at 4°C
CD25	mouse	PC61.5	e-Bioscience	PE	15 min at 4°C
CD27	mouse	LG.7F9	e-Bioscience	FITC	15 min at 4°C
CD44	mouse	IM7	e-Bioscience	APC	15 min at 4°C
CD45	mouse	30-F11	e-Bioscience	AF780	15 min at 4°C
CD62L	mouse	MEL-14	e-Bioscience	AF700	15 min at 4°C
B220	mouse	RA3-6B2	e-Bioscience	PE	15 min at 4°C
Gr1	mouse	RB6-8C5	eBioscience	PerCP5.5	15 min at 4°C
MHC II	mouse	M5/114.15.2	eBioscience	AF700	15 min at 4°C
FoxP3	mouse	FJK-16s	Invitrogen	APC	45 min RT
ckit	mouse	ACK2	eBioscience	APC	15 min at 4°C
Sca1	mouse	D7	eBioscience	PE-Cy7	15 min at 4°C

Results

1. In vivo hemophilic immune cells analysis in homeostatic condition.

Male Balb/c HA and wt mice were killed at different ages: 2, 8, 14, 24 and 52 week old. Total cell counts in Spleen, BM and thymus were not statistically different at all timepoints between the two groups (Figure 5A-C). Spleen and BM similarly increased the number of cells from 2 to 8 weeks and mostly remained stable without big changes overtime, while in kinetic graph of thymus we can see how total counts of thymus starts to diminish overtime, as expected by the aging. Overall the number of splenic T (Figure 5D-F) and B (Figure 5G) cells was not in the HA mice; by the different timepoint analysis we could appreciate the quick rise in T and partially B cell observed from week 2 to 8 corresponding to the full supply of the adaptive immune system cells from birth to adult life. No change in the thymic output of DP, SP CD4, SP CD8, $CD3^+$ T cells and tTregs (Figure 6A-E) was detected between HA and wt mice with both groups showing the abrupt decline already at week 8 with the exception of the SP CD8. Enumeration of main immune cells and hematopoietic stem and progenitor cells (HSPC, here defined as LSK, lineage [CD3, CD11b, Ter119, B220, Gr1] negative and $Sca1^+c\text{-}Kit^+$) in BM revealed stable B cells output (Figure 7A), quick increase of granulocytes at the beginning followed by unchanged level overtime (Figure 7B) and constant slow rise of LSK (Figure 5C,D), it is already known that overtime LSK numbers start to increase, while their functionality decreases[97]. Again, no alteration was seen between the two investigated groups. Splenic T cells memory compartment, defined by the $CD44^{hi}$ expression for both CD4 and CD8 (Figure 1A), similarly increased overtime in HA and wt mice (Figure 8A-D), which reflects the physiologic conversion of naïve toward memory phenotype related to the progressive encounter with self and environmental antigens even in mice kept in SPF condition[98].

2. In vivo hemophilic humoral and cell immune response assessment upon specific antigen challenge.

2.1 FVIII immunization elicited specific antibody formation in both HA and wt mice but only the former ones developed inhibitors

After observing no differences in homeostatic condition, we moved to the immune challenging experiments in which eight week old Balb/c HA and wt mice were sc injected 3 times with 2U of FVIII

in emulsion with IFA to provide a strong adjuvant signal (Figure 9A). HA and wt mice developed serum level of FVIII-specific IgG with the same increasing kinetic (Figure 9B), indicating that FVIII delivered in presence of IFA was immunogenic for both groups. Nevertheless, the tail bleeding assay, conducted 100 days after the first FVIII immunization, indicated an increase blood loss in HA FVIII/IFA compare to HA PBS/IFA mice (Figure 9C) while the blood loss was minimal in FVIII-immunized wt animals (Figure 9D). Presence of inhibitors was tested by performing the Bethesda assay: all FVIII-immunized HA mice showed more than 5BU (Figure 9E). Total splenic cell count showed a trend towards elevation in HA FVIII/IFA mice (Figure 10A), supported by the greater number of CD4 T cells ($28{,}03x10^6 \pm 2{,}82$) found in those mice compare to all other three groups (wt FVIII/IFA=$15{,}91x10^6 \pm 4{,}5$; wt PBS/IFA=$13{,}69x10^6 \pm 1{,}66$; HA PBS/IFA=$15{,}98x10^6 \pm 1{,}94$) (Figure 10B). Even splenic CD8 T cells were slightly increased in HA FVIII/IFA mice (Figure 10C) while Tregs were higher in HA FVIII-immunized versus (vs) HA PBS/IFA control mice ($3{,}62x10^6 \pm 0{,}35$ vs$1{,}97x10^6 \pm 0{,}27$) (Figure 10D). The increment in peripheral Tregs was not matched by a greater thymic output (Figure 10E) and overall the thymus analysis did not display any difference in the number of total and mature single positive SP CD4 and CD8 T cells among the different groups (data not shown). These observations suggest that the splenic changes in the number of T cells was mainly due to peripheral events rather than an augmented production from the thymus. Notably, the HA FVIII/IFA mice showed a number rise in the spleen for all the T cells compared to the average of HA control (no treatment) mice of similar age, depicted by the red dashed line in the graphs, confirming that the number alteration was related to the FVIII injection and not to IFA alone. Despite the similar humoral response to FVIII immunization in wt and HA mice, our data suggest that inhibitor formation was higher in HA mice accompanied by a long-term elevation of the splenic CD4 T cells number.

2.2 Humoral response and splenic T cell compartment after OVA immunization was similar in wt and HA mice

We sought to compare the humoral and cell response observed after the FVIII immunization with the ones elicited by an antigen, ovalbumin (OVA), which represents a full non-self protein for both HA and wt mice. OVA immunization protocol copied the FVIII one (Figure 11A). Again, both HA and wt mice developed similar OVA-specific IgG level (Figure 11B), but OVA immunization induced a stronger

humoral response compare to FVIII since the serum optimal dilution for detecting the respective specific IgG was 1:5000 for the former and 1:50 for the latter one (Figure 9B). Differently from HA immunization, OVA/IFA injections provoked a similar elevation in splenic immune cell count for both wt (84,33x10^6±10,22) and HA (94,04x10^6±9,02) mice above the average of control non-injected mice (wt=61,93x10^6±5,1; HA=57,84x10^6±4,06)(Figure 11C), mainly attributable to CD4 T cells for both groups (Figure 11D) and even to CD8 T cells for HA mice (Figure 11E). No differences were observed in number of Tregs in spleen (Figure 11F) or in the thymus (Figure 11G) between any of the groups. Overall, no main difference in thymocytes were detected among the groups, as observed in FVIII vaccinated mice (data not shown). These data suggest that both the humoral and T cell immune response to OVA challenge was similar between wt and HA mice.

2.3 FVIII immunization promoted conversion of CD4 and CD8 T cells to a memory phenotype in both wt and HA mice

The general splenic CD4 and CD8 T cell increase in immunized mice prompted us to investigate by flow cytometry their naïve vs memory ratio. Interestingly, the percentage of $CD44^{hi}$ memory CD4 T cells was higher in HA (OVA/IFA=38,37%±1,65; FVIII/IFA=41,68%±1,24) than in wt (OVA/IFA=28,6%±2,31; FVIII/IFA=37,6%±0,56) mice after the challenge with both OVA and FVIII (Figure 12A) while their amount was significantly greater in HA FVIII/IFA (11,61x10^6±0,99) than in wt OVA/IFA (4,8x10^6±0,43) mice (Figure 12B). Similarly, $CD44^{hi}$ memory CD8 T cells percentage (Figure 12C) and number (Figure 12D) were increased in the challenged HA group in comparison to wt mice. Interestingly, while conversion and/or maintenance of memory CD4 and CD8 T cells was similar between wt and HA mice immunized with FVIII, challenge with OVA promoted the acquisition of a T cell memory phenotype in HA but not in the wt mice, with the latter maintaining the same percentage and number of the untreated wt and HA controls (black and red dashed lines respectively in the graphs). Therefore, FVIII seems to be a strong immunogenic antigen even for the wt animals. On the other hand, the HA mice might be more prone to generate and maintain memory T cells once they are activated by an antigen, not limited to FVIII.

2.4 OVA immunization induced higher granulocytes and B cell increment than FVIII in BM of both wt and HA mice

In parallel to the analysis of the T cell compartment, we conducted an evaluation of other immune cell populations in spleen and BM. While in the spleen we did not detect any further significative difference, total BM cells were found higher after OVA/IFA (wt=41,6x10^6±1,6; HA=56,38x10^6±2,55) than after FVIII/IFA (wt=22,72x10^6±2; HA=27,26x10^6±3,02) injection in both wt and HA mice, with HA OVA/IFA mice showing the greatest count among the four groups (Figure 13A). Both B cells (Figure 11B) and granulocytes (Figure 13C) were responsible for this increment. Percentage of memory $CD27^+$ B cells were raised in HA mice after challenge with both OVA and FVIII (Figure 13D) although their number was high only after OVA injection (Figure 13E); instead no difference was detected in splenic memory $CD27^+$B cells among the different groups (Figure 13F,G).

2.5 FVIII treatment promoted stronger humoral response in HA compare to wt mice

All HA mice immunized with FVIII displayed specific IgG formation while in clinic only 30-40% of treated HA patients develop neutralizing antibodies against infused FVIII[7]. Therefore we decided to test in our mouse model a FVIII regimen (hereafter called FVIII treatment) resembling more closely the usual therapeutic approach, while in clinics for prophylactic regimen dosage is usually 25-40U/kg FVIII up to three time weekly, we injected mice weekly once 120U/kg FVIII : both wt and HA mice were weekly iv injected 5 times with 3U FVIII or PBS as control (Figure 14A). Serum FVIII-specific IgG were first detected in both wt and HA mice only after all five FVIII infusions, precisely 7 days after the last injection (Figure 14B). While initially the antibody level was equal between the two groups, starting from day 50 it significantly increased in HA mice remaining higher until the last checked timepoint (day 77), when it started to decrease in wt mice. Notably the maximum FVIII antibody concentration reached a much lower value (evaluated by the OD reading of the same serum dilution) than in FVIII immunized wt and HA mice (Figure 9B). Moreover, 5 out of 6 HA mice and 4 out of 5 wt mice injected with FVIII developed anti-FVIII antibodies (data not shown). The blood loss test performed 50 days after the last FVIII infusion resulted in no difference between treated and no-treated HA (Figure 14C) or wt (Figure 14D) animals. While 66% of FVIII-treated HA mice showed high titer of inhibitors (BU>5) no wt mice displayed their formation (Figure 14E). Overall splenic CD4

and CD8 T cell numbers did not differ among the groups after FVIII treatment (Figure 15B,C), with the FVIII HA mice not showing the same CD4 T cell increase detected after FVIII immunization (Figure 9B)(HA FVIII=19,51x10^6±1,35 vs HA FVIII/IFA=28,03x10^6±2,82; p=0,026*). Similarly, splenic (Figure 15D) and thymic (Figure 15E) Tregs number was comparable among the groups but again the FVIII treatment did not induce their raise in HA mice (2,37x10^6±0,16) as it happened in FVIII immunized mice (Figure 10D, 3,62x10^6±0,35). Despite the observed inhibitor formation in the majority of HA mice after repeated FVIII iv injections, the humoral and cellular response displayed different features than in FVIII-immunized mice suggesting the relevance of the danger signal in promoting a strong and long lasting response to the FVIII protein.

2.6 FVIII treatment stimulated a small conversion of CD4 and CD8 T cells to a memory phenotype

Naïve vs memory splenic T cells analysis highlighted the similar relative increase of $CD44^{hi}$ memory CD4 T cells in wt (34,74%±1,32) and HA (35,42%±1,61) mice after the FVIII treatment in comparison to their respective PBS injected counterparts (wt=27,62%±1,77; HA=27,33%±0,59) (Figure 16A). Instead $CD44^{hi}$ memory CD8 T percentage was higher only in FVIII-treated HA animals (38,62%±2,21) confronted with the HA PBS ones (28,87%±0,57) (Figure 16C). Counts of memory T cells were not found different among the 4 groups (Figure 16B,D). As done in BM and spleen of immunized mice, we assessed granulocytes (data not shown) and B cells of FVIII treated mice. While BM analysis did not reveal any significative difference (Figure 17A-D), the number of splenic memory $CD27^+$ B cells was increased after FVIII injections in HA (1,25x10^6±0,18) compare to wt group (0,82x10^6±0,05) (Figure 17F) with no change in their percentage (Figure 17E). In absence of a concomitant danger signal, FVIII injections elicited a limited memory CD4 T cell increase, still greater than in control PBS-injected mice, and an interesting raise of memory $CD27^+$ B cells in the spleen of HA vs wt mice.

3. In vitro Tregs differentiation evaluation of hemophilic CD4 T cells.

Naïve CD4 T cells yield after immunomagnetic isolation was $3,89x10^6 \pm 0,69$ and $3,51x10^6 \pm 0,34$ cells for wt and HA mice respectively, while purity was always similar between the two groups and greater of 90%. After 4 days of culture, plated HA naïve CD4 T cells showed higher survival than wt in all the tested conditions (media only; α-CD3/CD28 only or IL-2 + TGF-β stimulation) as shown by the ratio lower than one obtained by dividing the number of cells found in the wt by the ones found in the HA wells (Figure 18A-C). Similarly, Tregs number was lower in wt compare to HA wells when stimulated with both α-CD3/CD28 and IL-2 + TGF-β, with the exception of one experiment out of three (Figure 18D,E), indicating a biological variability among the same groups. Even though we recovered more Tregs in all HA samples, the percentages of Tregs obtained after the differentiation from the plated naïve CD4 T cells was similar if not higher in the wt wells in both α-CD3/CD28 (Figure 19A) and IL-2 + TGF-β (Figure 19B) condition. While the overall survival of HA CD4 T cells was greater, the Tregs differentiation efficacy in vitro seemed enhanced starting from wt CD4 T cells.

Discussion

The current therapy for HA patients is the administration of pdFVIII or rFVIII, but this treatment still has some complications. One of the major complications of FVIII infusion is the development of inhibitory antibodies against infused FVIII, which affects around 30-40% of the patients with severe form of HA, thus those patients undergo different replacement therapy, such as use of bypassing agents or ITI. It is still not fully understood why inhibitors develop against infused FVIII, one of the plausible explanations could be the high described immunogenicity of FVIII[99], but this does not explain why some patients develop inhibitors while others do not. Moreover, many groups have investigated its peculiar behavior and demonstrated that FVIII itself does not represent a danger signal activating pathogen-associated molecular patterns (PAMPs)[100], FVIII aggregates related to protein manufacturing do not induce immune responses[101], surgery associated DAMPs do not influence the strength of anti-FVIII immune response[102], while the involvement of the thrombin formation as costimulatory signal in the activation of immune cells has been controversial[103][104]. Skupsy et al.[105] also showed that injection of heat inactivated FVIII elicited significantly lower immune response in mice compared to the ones receiving normal FVIII, which suggests that immune response towards FVIII could be associated to its coagulation function. They also showed that OVA injection in combination with either FVIII or thrombin elicited significantly higher OVA-specific antibody level than OVA alone. Overall the coagulation cascade promoted by FVIII seems not to play a direct major role in delivering a danger signal but inflammation condition linked to the recurrent bleedings might favor the immune system activation since there is a well known crosslink between coagulation and inflammation[106] and FVIII has been recently proposed as a player in regulating immune biology[107]. The idea that FVIII could play a role in the hemato-immune system is also supported by Aronovich et al.[25] which showed a reduced *in vivo* long term repopulation capability of transplanted HA HSC in a competitive assay with wt HSC. There are still limited studies done for comparing immune populations of wt and HA mice under homeostatic condition, hence we decided to characterize immune populations of male Balb/c HA mice prior to any FVIII treatment and to compare them to their wt counterpart. This should facilitate the identification of possible difference in the immune cell populations of this two groups not attributable to FVIII administration. For all of our experiments we employed the common used FVIII exon 16-disrupted HA mouse model[95] backcrossed for 10 generation to the Balb/c background since this strain is known for its strong humoral response linked to a Th2 biased response[108][109][110]. Interestingly, Balb/c HA and wt mice did not display any significative difference in number of the main immune cells of BM, spleen and thymus from early (2

weeks) to late (52 weeks) timepoint, which suggest that absence of FVIII and/or the microenvironment alone in absence of external stimuli does not affect the immune populations homeostasis. Pinchuk et al. has performed a splenic immuno-phenotypic characterization of Balb/c and C57BL/6 mice mice[111] at 1, 3, 5, 10 and 18 month age, where they observed different percentage of memory T cells compared to us. In particular the memory CD4 T cells were 85% at 12 weeks while we observed 29%±0,56 at 14 weeks, and were 90% at 20 weeks vs 34%±0,66 at 24 weeks, similar differences were observed also in the memory CD8 T cells, where they observed 80% at 12 weeks while we observed 32%±0,66 at 14 weeks and at 85% at 20 weeks vs 35,5%±0,63 at 24 weeks. This discrepancy could be due to the different level of CD44 expression used for the definition of the memory phenotype: while we considered only $CD44^{hi}$ T cells they included the CD44 intermediate. Another factor could be played by the animal facility in which the mice were hosted: our mice were kept in a SPF room, which limits the amount of new antigens that T cells can encounter, while they did not mention their mouse facility condition. Aronovich et al.[25] showed that C57BL/6 HA mice had lower percentage of long-term HPSC in comparison to wt counterparts, but they did not observe any difference in total percentage or numbers of LSK, even though a direct comparison with our results is hard to make due to the different mouse strain, C57BL/6, used by them. Nevertheless, we also did not observe any difference in LSK population between HA and wt. The results of this first homeostatic analysis of the HA immune system set up a baseline for the second sets of experiments, focused on the immune challenge of the HA mice.

The HEMFIL study[26] showed that HA PUP patients had significantly increased pro-inflammatory cytokines compared to healthy young boys, suggesting that there could be an environment predisposing the HA patients to inhibitor formation. Unfortunately, a deep investigation of that pro-inflammatory milieu is not easy to conduct in HA patients due to their therapeutic requirements which could affect the naïve status, and the difficulty of sampling primary and secondary immune tissues (e.g. BM, spleen). Therefore, we decided to compare the specific humoral response and related immune cells changes in HA mice upon FVIII or OVA challenge for further discriminating the impact of the environment from the antigen itself in the immune responses towards the two proteins. One caveat of this comparison was the use of human recombinant FVIII as immunogen in the HA murine model. Therefore the human FVIII might be recognized as a foreign protein as OVA in both wt and HA animals, as demonstrated by higher percentage of HA mice developing anti-FVIII response against human FVIII than to mouse FVIII[112]. It is noteworthy to mention that we injected the BDD form of

human recombinant FVIII, while the main differences in the coding sequence between mouse and human FVIII are concentrated in the B domain, and the homology at the amino acid level is equal to 87% if that domain is excluded[113].

While most of the studies on FVIII immunogenicity were conducted exclusively on HA mice, we compared immune reaction to those proteins between wt and HA mice. Indeed, when challenged sc with either FVIII or OVA in emulsion with IFA both wt and HA mice developed equal high and stable titers of antigen-specific IgG even if α-OVA antibodies were much greater than α-FVIII ones as indicated by the 100 fold increase in the serum dilution used in the ELISA for their detection, which could be due to the amount of antigen given, since for FVIII we injected only 0,5μg while for OVA 50μg, this could have been enough to elicit stronger humoral response against OVA in both HA and wt mice compare to FVIII. Reipertet al.[114] described a similar α-FVIII IgG rate of development in both wt and HA C57BL/6 mice injected sc with FVIII highlighting the fundamental role played by a strong adjuvant in eliciting an immune response even toward a self-protein. Differently, FVIII was more immunogenic than OVA in terms of specific antibodies formation when they were intraperitoneally delivered in absence of adjuvant[105].

Despite the total FVIII-specific IgG levels were analogous, the bleeding assay performed many days after the last FVIII administration showed that HA FVIII/IFA mice lost more blood than the HA PBS/IFA, while the loss was minimal for the wt animals, regardless of the FVIII injection. Indeed, the HA FVIII/IFA group developed inhibitors as confirmed by the BU greater than 5 while the FVIII-specific IgG detected in the wt mice were probably not directed against functional epitopes of the coagulation factor as shown in wt C57BL6 mice[114]. The significative higher blood loss in HA FVIII/IFA compare to HA PBS/IFA is puzzling and it might be attributed to a cross-reactivity of some FVIII-specific IgG with factor V (FV). Indeed Arsiccio et al.[115] showed that isolated FVIII inhibitors from some HA patients could also inhibit FV activity. This could be explained by the fact that FV and FVIII share around 40% homology, have similar structure and function[116], and are thought to descend from a common ancestral A1-A2-A3-containing protein through a gene-duplication event[117]. After the acquisition of C-type and B-domain, a second gene-duplication ultimately separated ancestral genes for FV and FVIII.

Notably, when FVIII was delivered iv, as it happens in patient treatment, HA mice showed higher FVIII-specific IgG level than wt controls and the kinetic of their development was different compare to the sc FVIII-injected mice: they were detectable only after the fifth dose and reached a lower plateau, especially in the wt mice. Conversely, C57BL/6 wt and HA mice were reported to display similar kinetic of anti-FVIII antibody upon iv injections[114] suggesting a stronger overall humoral immunoreactivity towards FVIII in the C57BL/6 background, which has been described in other studies directly comparing the two murine strains[118]. Qadura et al[73] also showed that C57BL/6 developed stronger humoral response against infused FVIII than Balb/c mice, which could be explained by Balb/c being more prone to Th2 response, while in FVIII inhibitor formation both Th1 and Th2 subsets of CD4 T helper cells are involved. The FVIII treatment elicited inhibitor formation in more than 80% of our HA mice, differently from what commonly observed in severe HA patients[119], but this agrees with most of the studies conducted in the HA murine models[120], probably related to the usage of human FVIII as mentioned above. More intriguing was the formation of FVIII-specific antibodies in the FVIII-treated wt group, although none of those mice developed inhibitors. Again the xenogeneic origin of human FVIII might have played a role while the presence of anti-FVIII IgG, but not inhibitors, has been reported in healthy subjects[63][121].

Despite the similar kinetic in antigen-specific IgG development between HA and wt mice, only the HA FVIII/IFA group displayed a higher splenic CD4 T cell number, including Tregs, while HA OVA/IFA mice showed a slightly not significant increase. Considering that the cell enumeration was performed more than 9 weeks after the last antigen administration, that rise could be linked to a boosted initial conversion from naïve towards memory phenotype, a longest maintenance and/or a reduction in the contraction of memory T cells[122]. The memory vs naïve analysis based on the CD44 acquisition confirmed that both CD4 and CD8 T cells in the HA mice were in general more prone to acquire a memory phenotype, regardless of the antigen, FVIII or OVA, supplied. We did not establish how many of those memory T cells were antigen-specific but the pro-inflammatory environment reported in HA patients[26] might favor a more robust immune response, especially in presence of a concurrent danger signal. In fact, the FVIII treatment caused nor significative increase in splenic T cell number neither higher conversion in HA compared to wt mice, even if both groups receiving FVIII showed greater percentage of $CD44^{hi}$ CD4 T cells compared to their respective PBS control animals. Few studies have highlighted that splenic T cells isolated from iv FVIII-injected HA mice and restimulated *in vitro* with

FVIII made both Th1 (IFN-γ and IL2) and Th2 (IL4, IL10) cytokines[123][124] with the Balb/c strain producing higher levels than C57BL/6, especially of the Th2 type[73]. Those analysis were not in parallel conducted on wt mice and based on our results it would be important to perform a comparison of those specific cytokine responses for elucidating if HA patients carry a pro-inflammatory milieu that predispose them to produce and/or maintain easily memory T cells upon their presentation and recognition.

In accordance with the augmented FVIII-specific IgG secretion, HA FVIII mice displayed higher number of memory splenic $CD27^+$ B cells while the difference with the wt FVIII was not detected in the BM. Notably, the number of splenic memory B cells developing in FVIII-treated HA mice was similar to the ones found in FVIII-immunized HA mice suggesting that for the FVIII antigen the danger signal provided by IFA was not necessary for supporting the B cell switch toward the memory phenotype. Surprisingly, cell BM analysis highlighted an increased cellularity in OVA vaccinated HA mice compared to all other experimental groups and that was ascribable to both $Gr1^+$ granulocytes and $B220^+CD19^+$ B cell rise. That behavior was linked to the combination of the OVA antigen in the HA mice, since no rise was detected in BM among the FVIII-treated mice while IFA in combination with FVIII did not alter the cell counts. Moreover, no effect on number of mature granulocytes and B cells was observed in the spleen. The "emergency granulopoiesis" cascade represents a regulatory loop which consists of three phases: first, presence of a pathogen, which needs to be effectively and rapidly sensed by the immune and hematopoietic system of emergency state; second, neutrophil production in the BM needs to be enhanced through the emergency state translated into molecular events; and third, the state of "emergency granulopoiesis" needs to be restrained after the infection has been cleared, in order to enable the hematopoietic system to restore the homeostatic steady-state[125]. Increased B cell lymphopoiesis could be associated to an immunological challenge as well. Fulop et al. showed that mice injected with sheep red blood cells for four weeks increased the production of BM B cell lymphocytes, demonstrating that a chronic increase in exposure to extrinsic agents can produce a long-term elevation of the BM B lineage cells[126]. It is difficult to link our observation to any of those on demand mechanisms since our analysis was conducted more than 65 days after the last antigen challenge when the acute immune response should have been turned off. Here it might be that there was an extension of the acute inflammatory response with a delay in the restoration of the homeostatic steady-state condition, but more analysis is required for the determination of the possible causes.

Based on the described pro-inflammatory milieu described in HA PUP [26], we hypothesized that constant exposure to pro-inflammatory environment could affect the ability of naïve CD4 T cells to differentiate into Tregs. Therefore, we isolated splenic naïve CD4 T cells from both HA and wt Balb/c mice and tested *in vitro* their Tregs differentiation capability.

Interestingly our data suggest that HA isolated splenic naïve CD4 T cells survived more *in vitro* compare to wt cells, not only when cultured in media only but also when stimulated with α-CD3/CD28 only or IL-2 + TGF-β. Since the naïve CD4 T cells from HA mice could come from an already inflammatory microenvironment unlike wt, they could be more "resistant to apoptosis". Ponchel et al.[127] showed that naïve CD4 T cells isolated from rheumatoid arthritis (RA) patients blood, proliferated more than those from the healthy subjects under stimulation of PHA and α-CD3/CD28, similar to our in vitro results. Even though RA and HA are two different diseases it seems that both of them could share similar pro-inflammatory microenvironment[26][128], which could influence the T cells coming from these patients and make them hyper-responsive to activation and lower the threshold of their immune activation. Since our initial aim was not to see the proliferation capability of CD4 T cells, and we did not use proliferation die or check the apoptosis by staining, we cannot exclude survivability/resistance to apoptosis/proliferation for recovering higher number of CD4 T cells from HA mice samples than from wt. Unfortunately, there have been no other reporting of apoptosis resistant CD4 T cells and for better understanding we would have to look more into the molecular mechanisms and to pro- and anti-apoptotic pathways of T cells.

Even though we observed that more HA naïve CD4 T cells differentiated into Tregs than in wt samples, the percentage of Tregs on CD4 T cells was higher or similar in wt mice. However, since it was observed only in 2 experiments out of 3, we will have to conduct more experiments before drawing any definitive conclusion, if there is a difference between capability of HA and wt splenic naïve CD4 T cells differentiation into Tregs. For better understanding the Tregs functions it would be necessary to perform a classical Tregs suppression assay testing different ratio of Tregs with T effector cells. Like mentioned before[92], IL-2 therapy with a specific α-IL2 antibody could suppress the immune response to FVIII by specifically increasing the number of Tregs; it is important to note that the increased number of Tregs was mainly due tTregs, since most of the Tregs where expressing the Helios marker, which is known to be restricted to tTregs.

In conclusion our study shows that 1. Both HA and wt mice have similar number of the main immune cell populations in BM, spleen and thymus prior to FVIII treatment; 2. There is a role played not only by danger signal and the self/non-self discrimination but also by the intrinsic pro-inflammatory microenvironment in inhibitor formation; 3. There could be failure of immune suppression in HA Tregs and/or robust CD4 T cell proliferation due to hyper-responsiveness.

Mechanism of inhibitors formation is hard to predict, but our results suggest that potential limitation of external stimuli (danger signal), could reduce the formation of inhibitors. Moreover, our results suggest that HA T cells could have lower activation threshold, due to which it would be necessary to decrease the FVIII dose, which could potentially decrease the amount of inhibitor development in HA patients.

Finally, it would also be important to conduct similar studies on other mouse strains, for excluding the possibility of the results described being strain specific.

Figures

Figure 1: Representative gating strategies for detecting splenic T cells

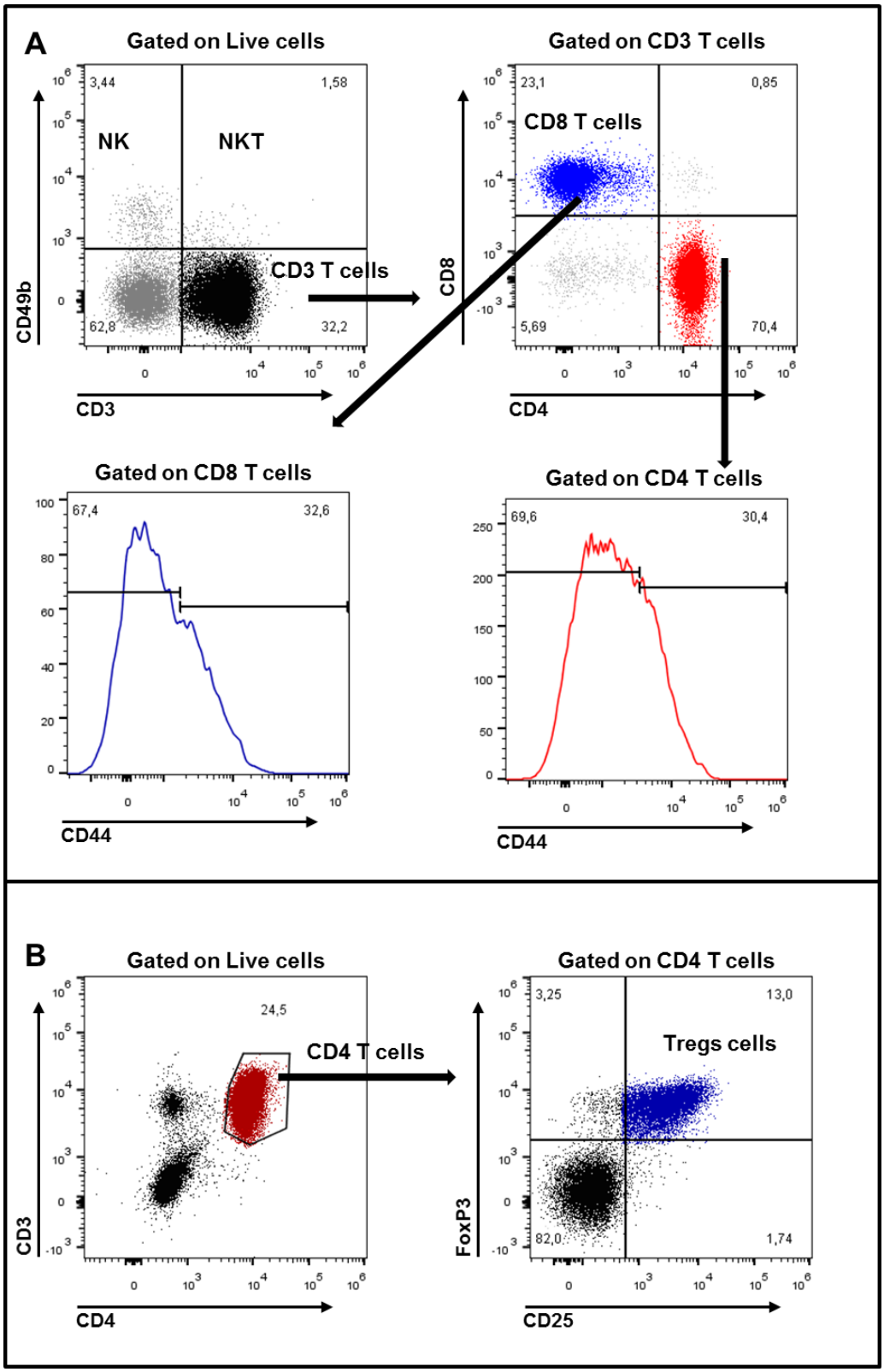

Figure 2: Representative gating strategies for detecting BM cells

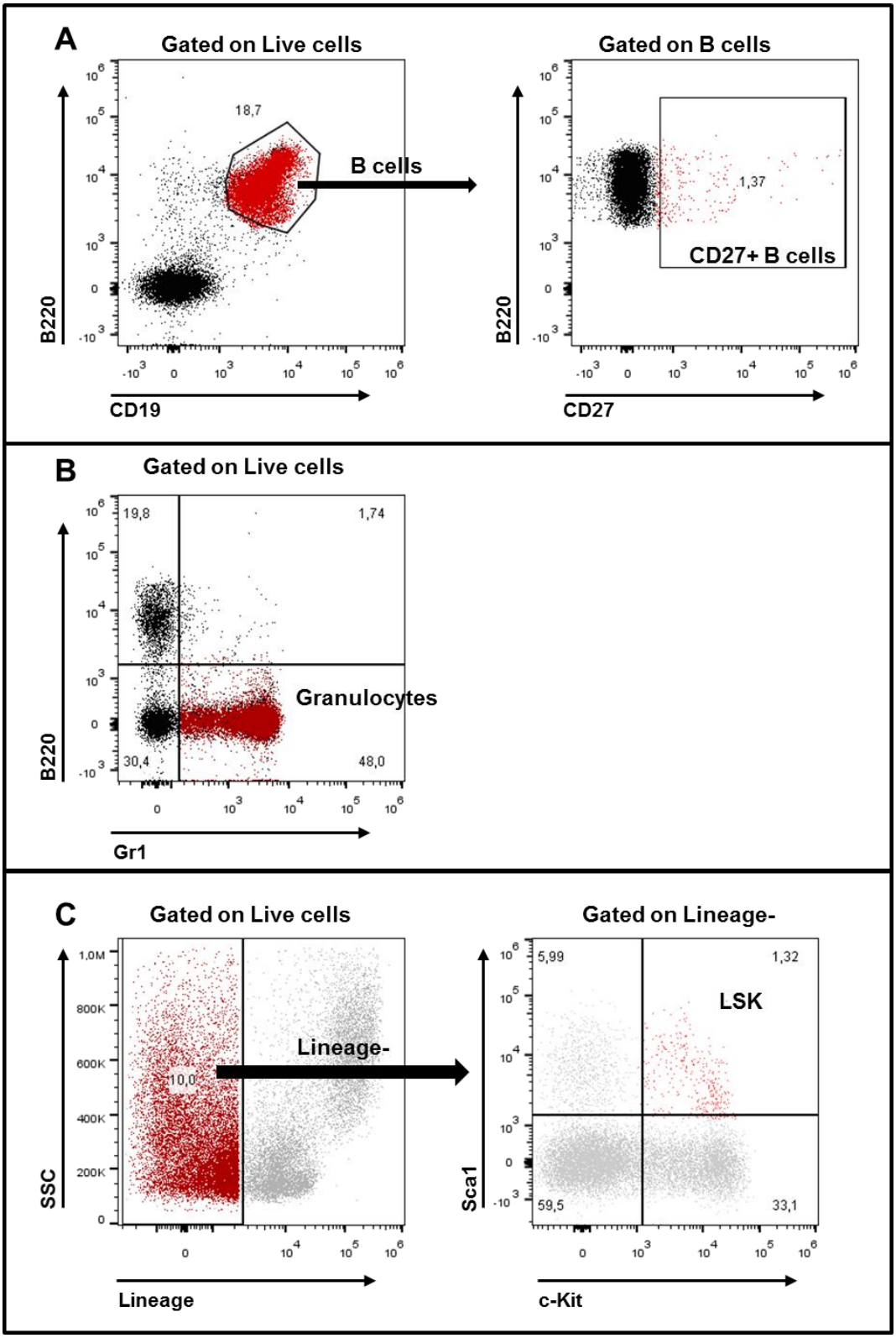

Figure 3: Representative gating strategies for detecting thymocytes

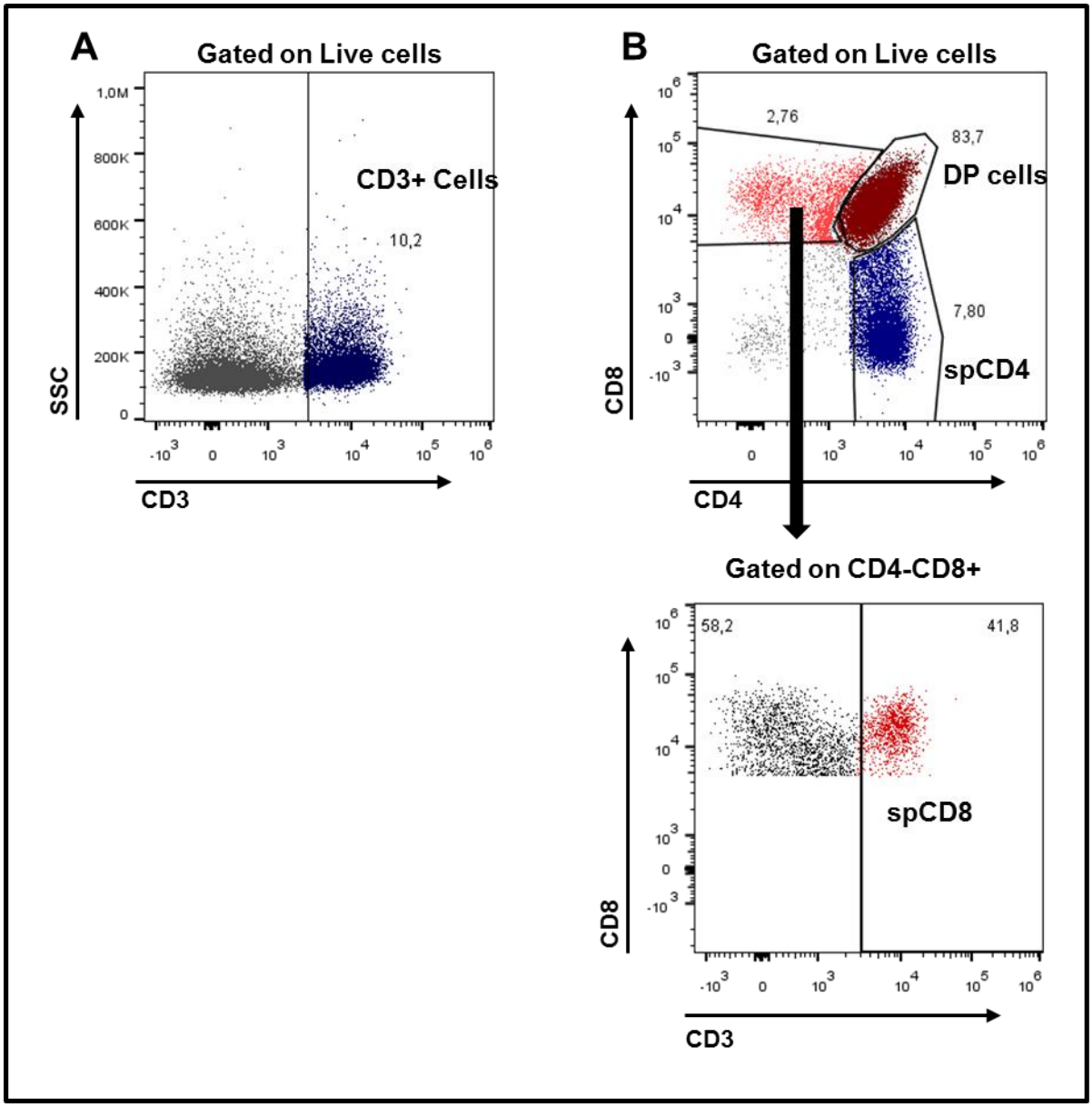

Figure 4: Isolated naïve CD4 T cells gating strategy and the difference between pre and post stimulation

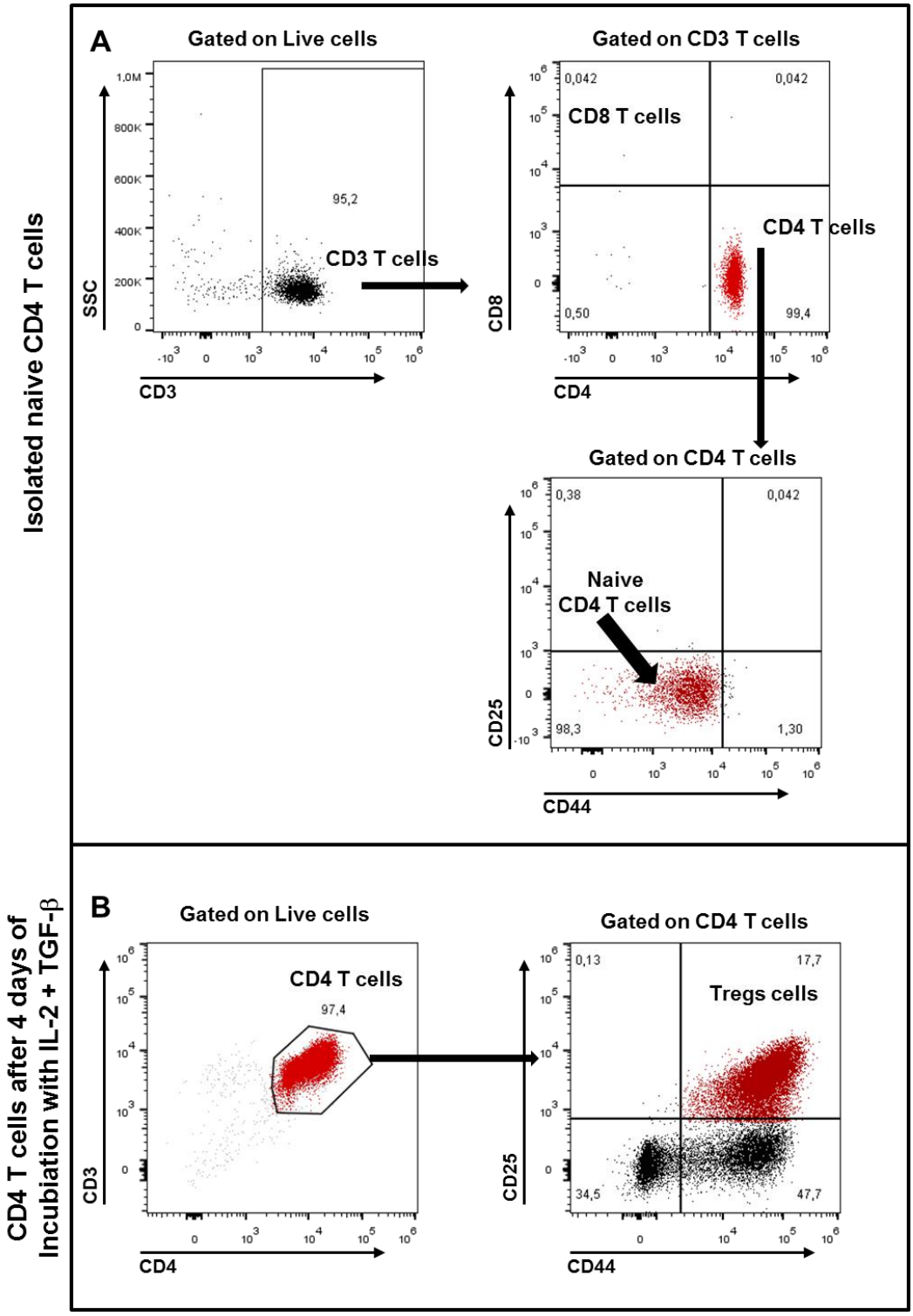

Figure 5: Immuno-phenotypic characterization of Balb/c HA and wt

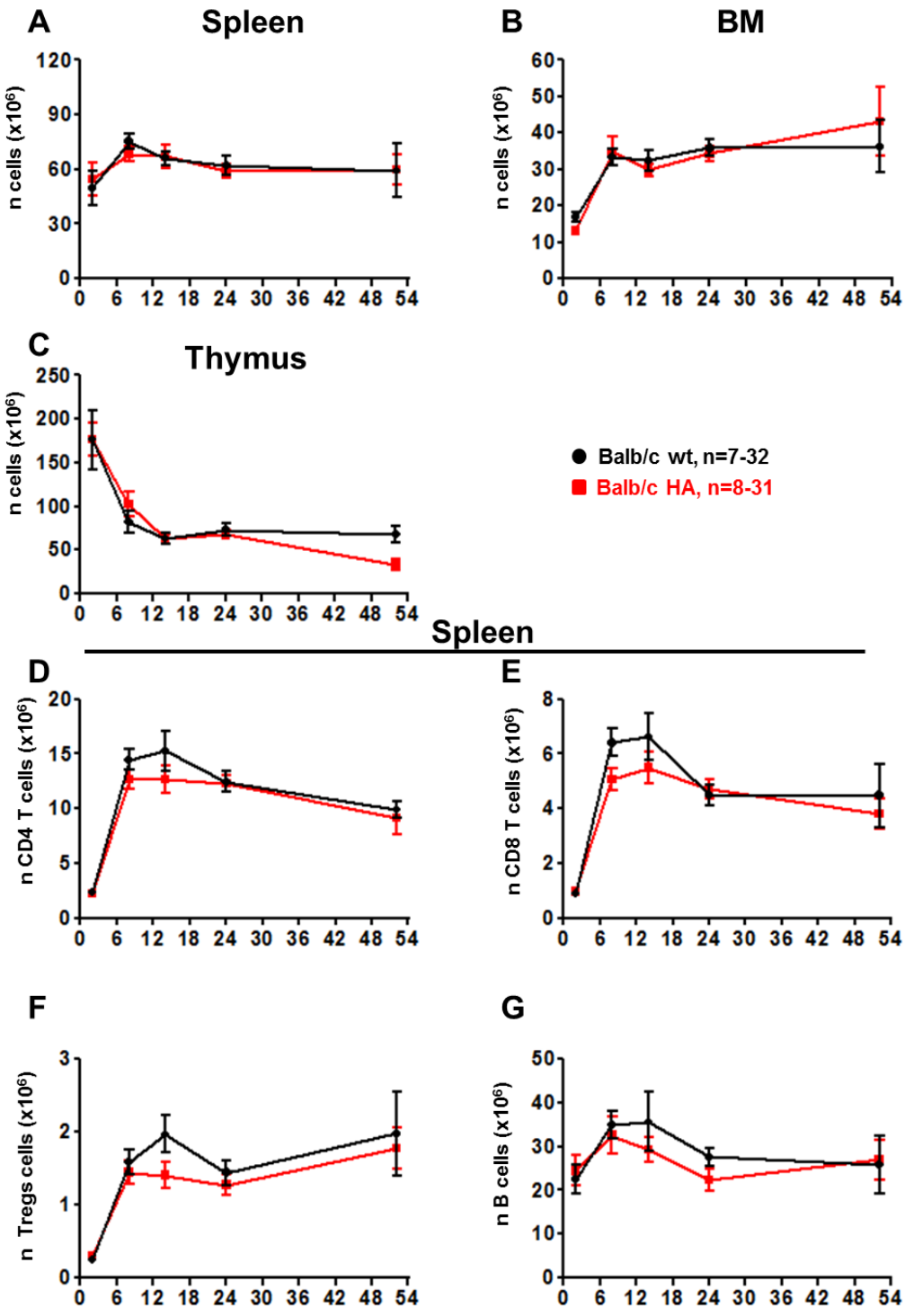

Figure 6: Immuno-phenotypic characterization of Balb/c HA and wt thymus

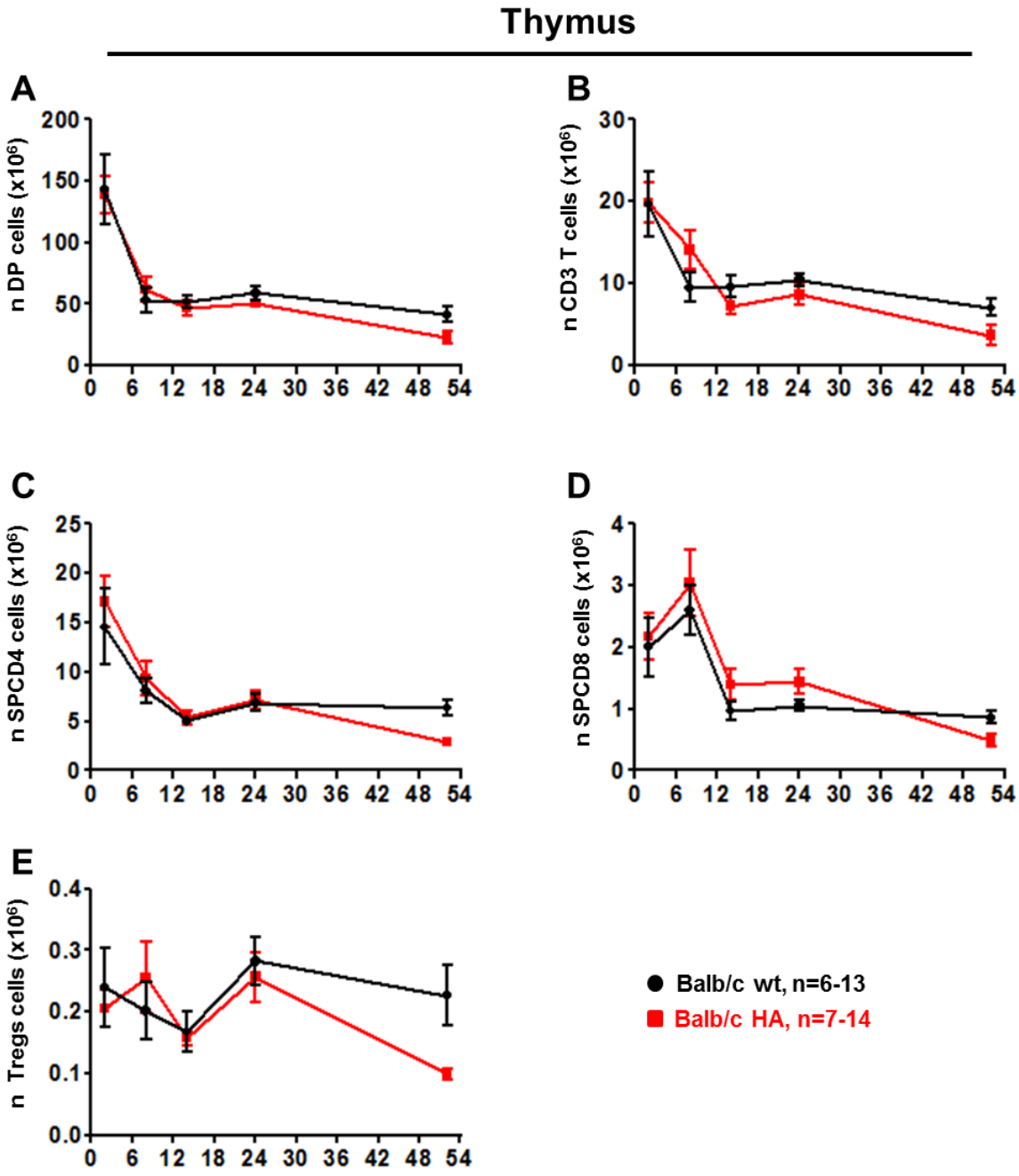

Figure 7: Immuno-phenotypic characterization of Balb/c HA and wt BM

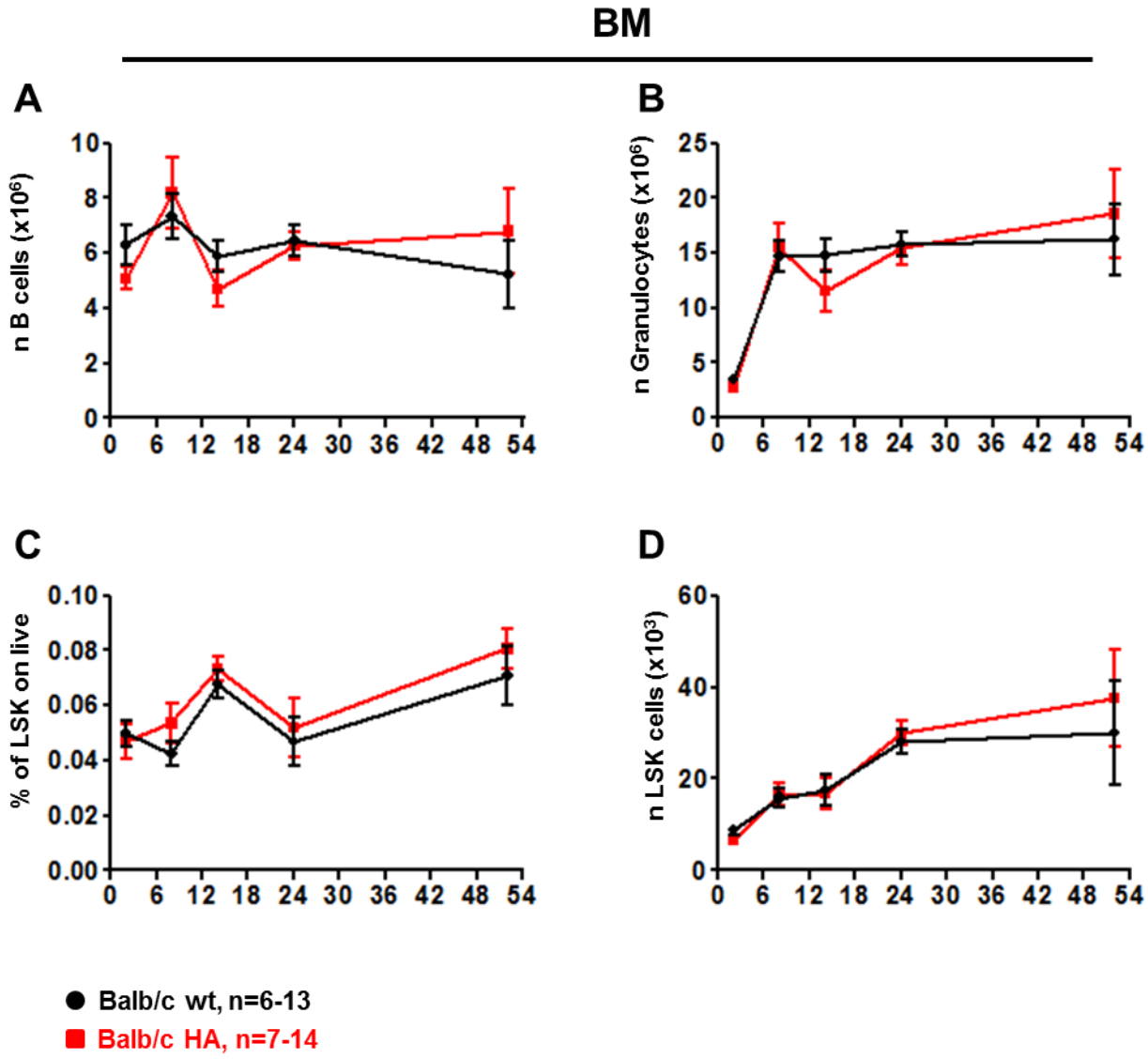

Figure 8: Immuno-phenotypic characterization of Balb/c HA and wt splenic Memory T cells

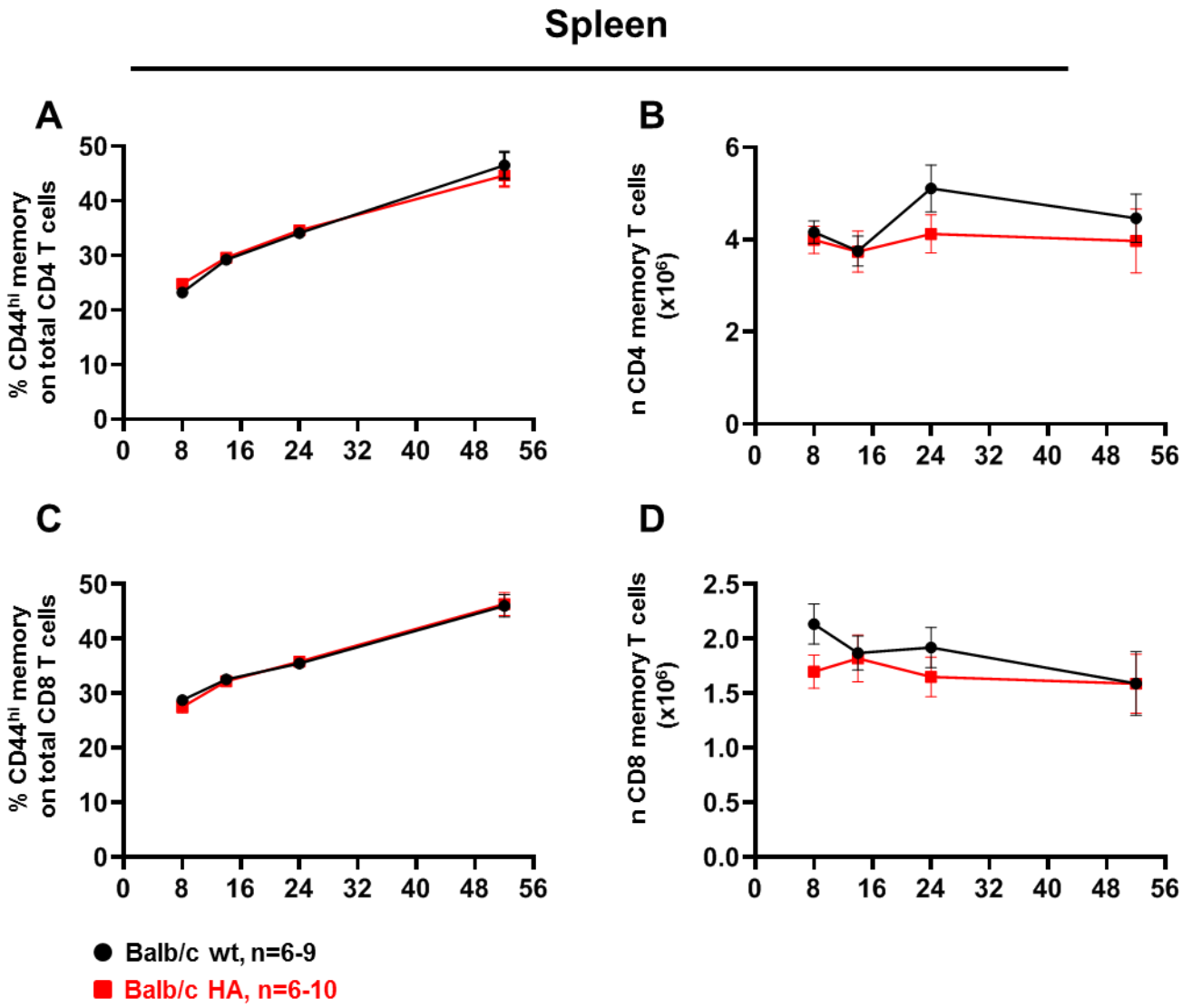

Figure 9: Similar humoral response against FVIII immunized wt and HA mice

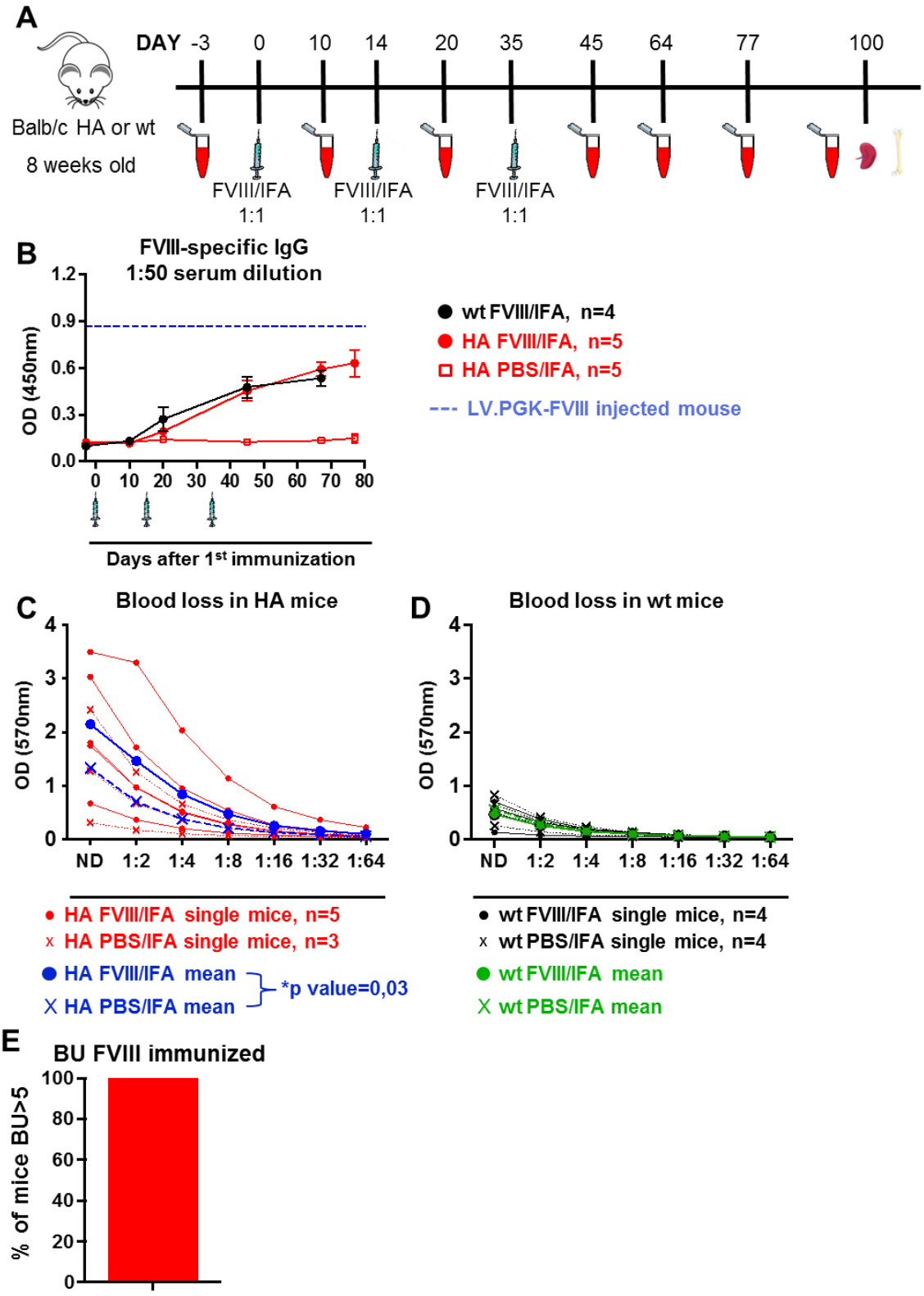

Figure 10: Higher CD4 T cells in FVIII immunized HA mice

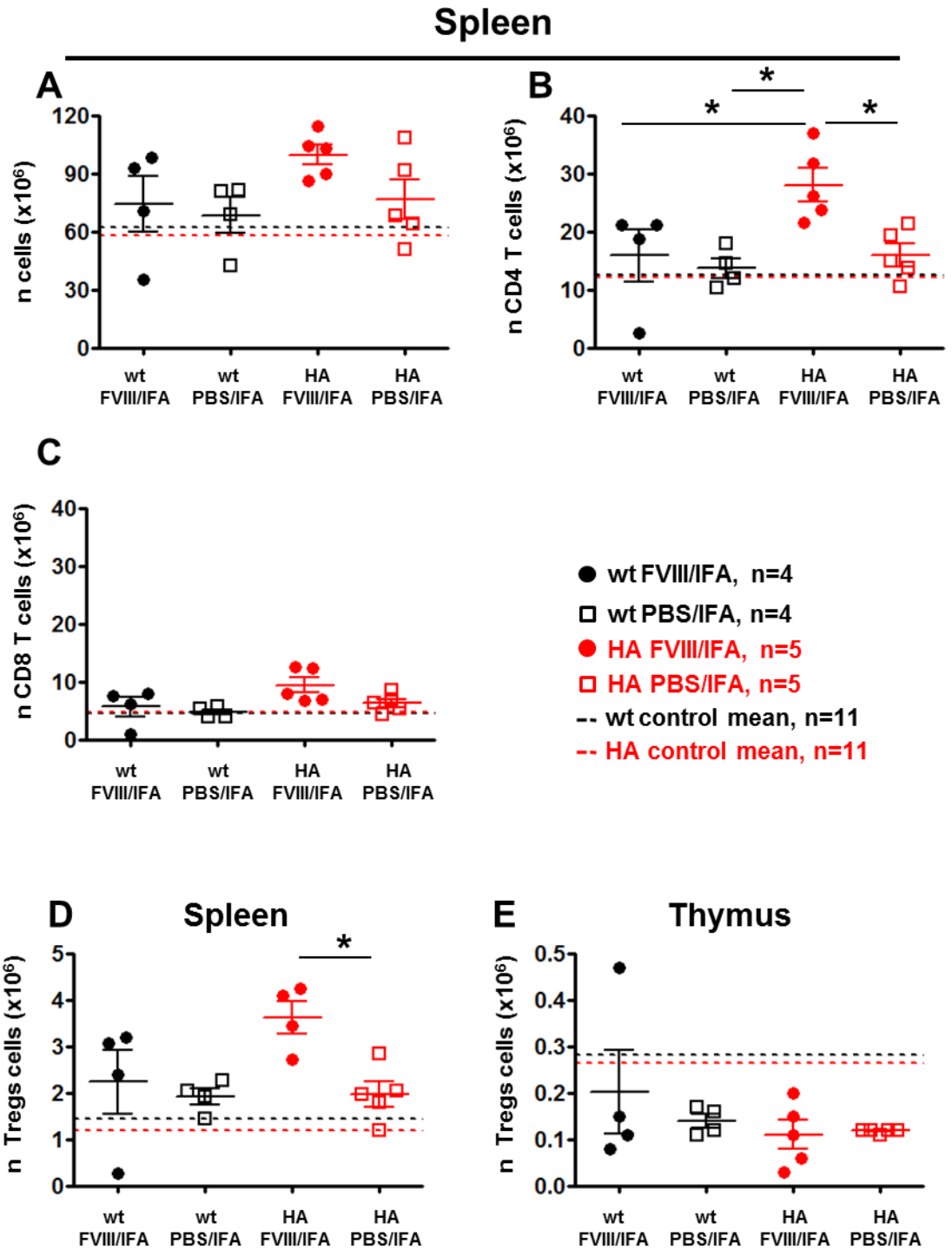

Figure 11: OVA-specific humoral response and T cell enumeration in HA and wt mice immunized with OVA

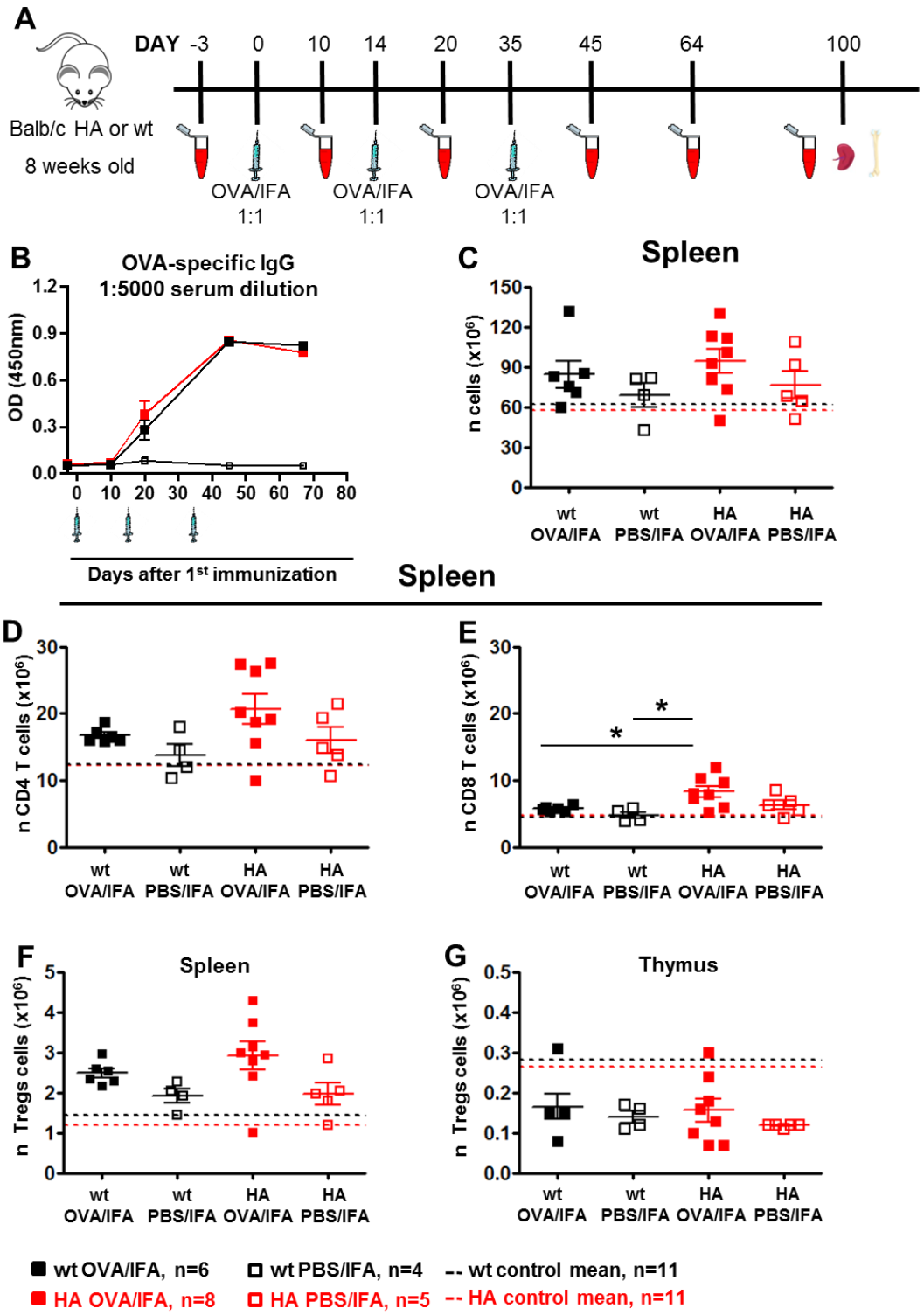

Figure 12: Increased naïve to memory T cell conversion in immunized HA mice

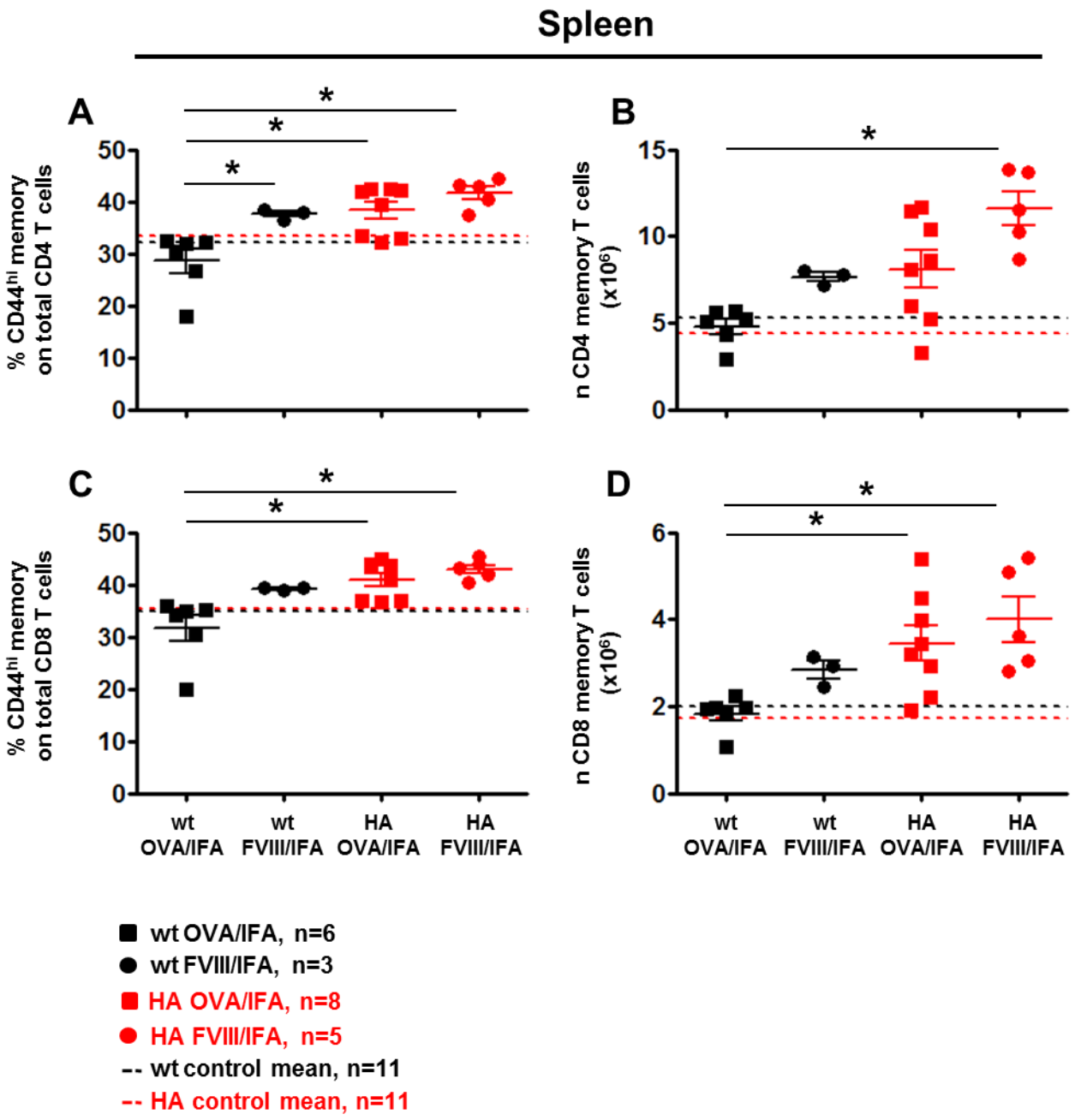

Figure 13: OVA immunization induced increase of BM B cells and Granulocytes

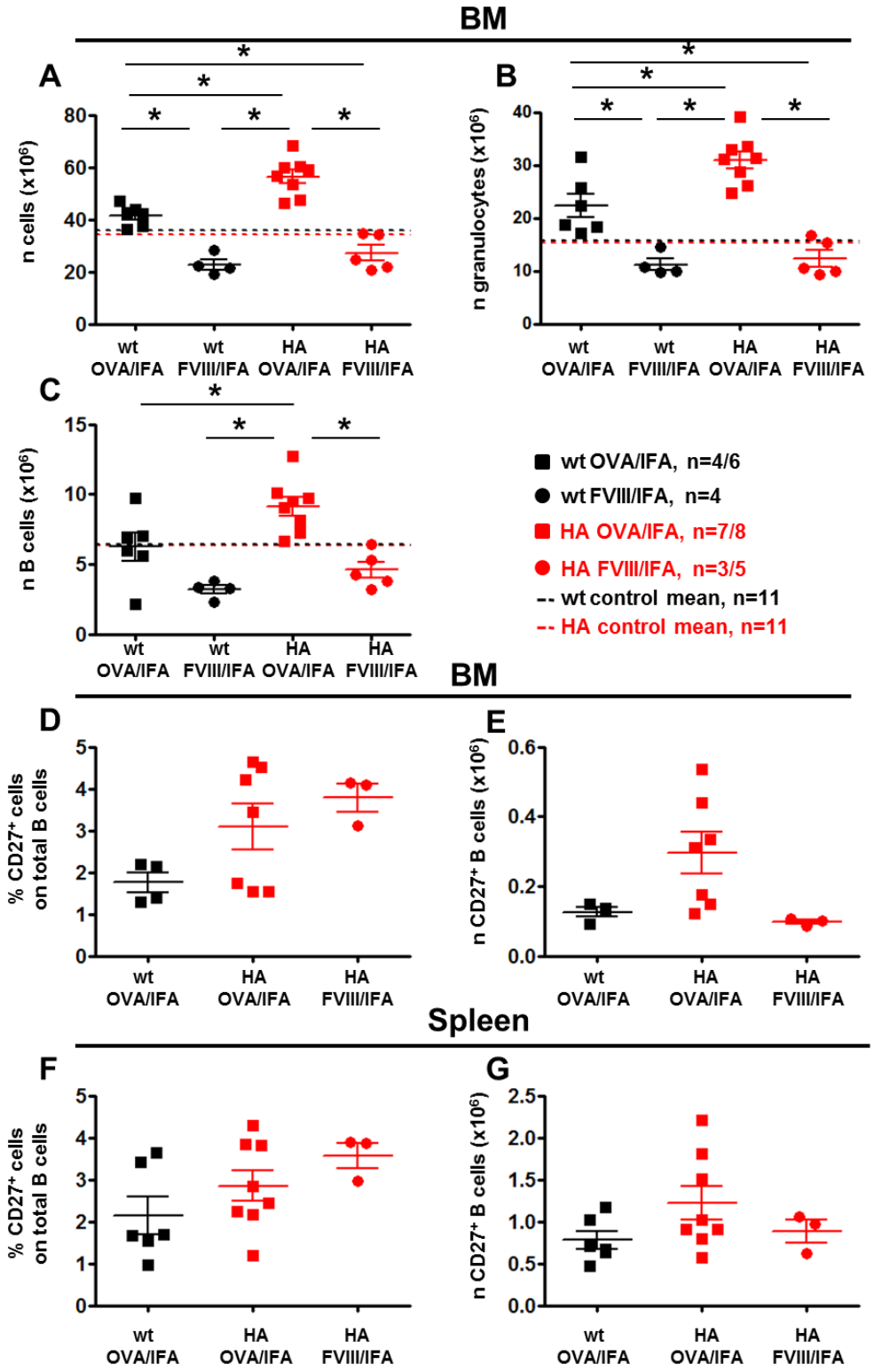

Figure 14: Stronger humoral response in FVIII treated HA mice

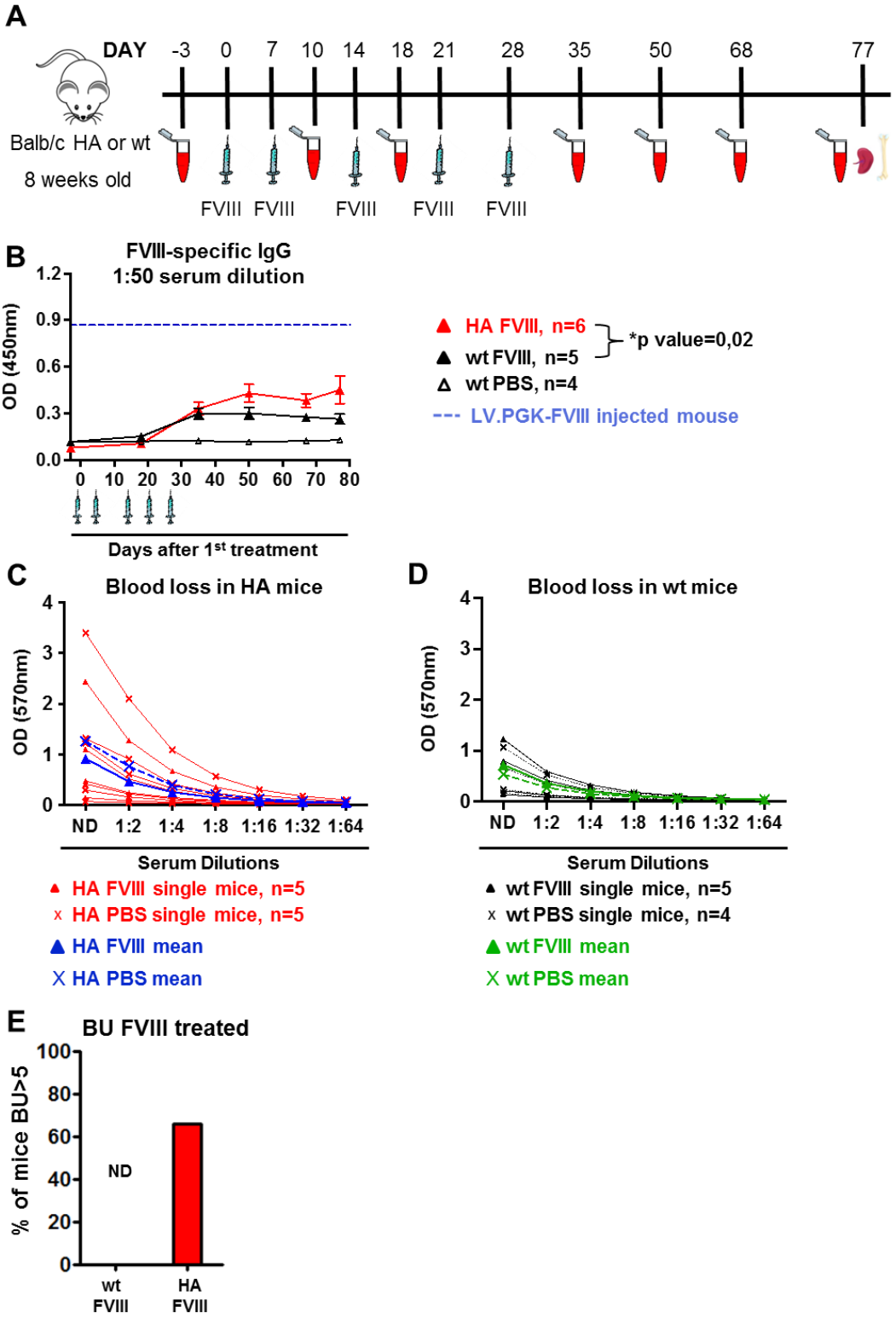

Figure 15: No Differences observed in immune populations between FVIII treated HA and wt mice

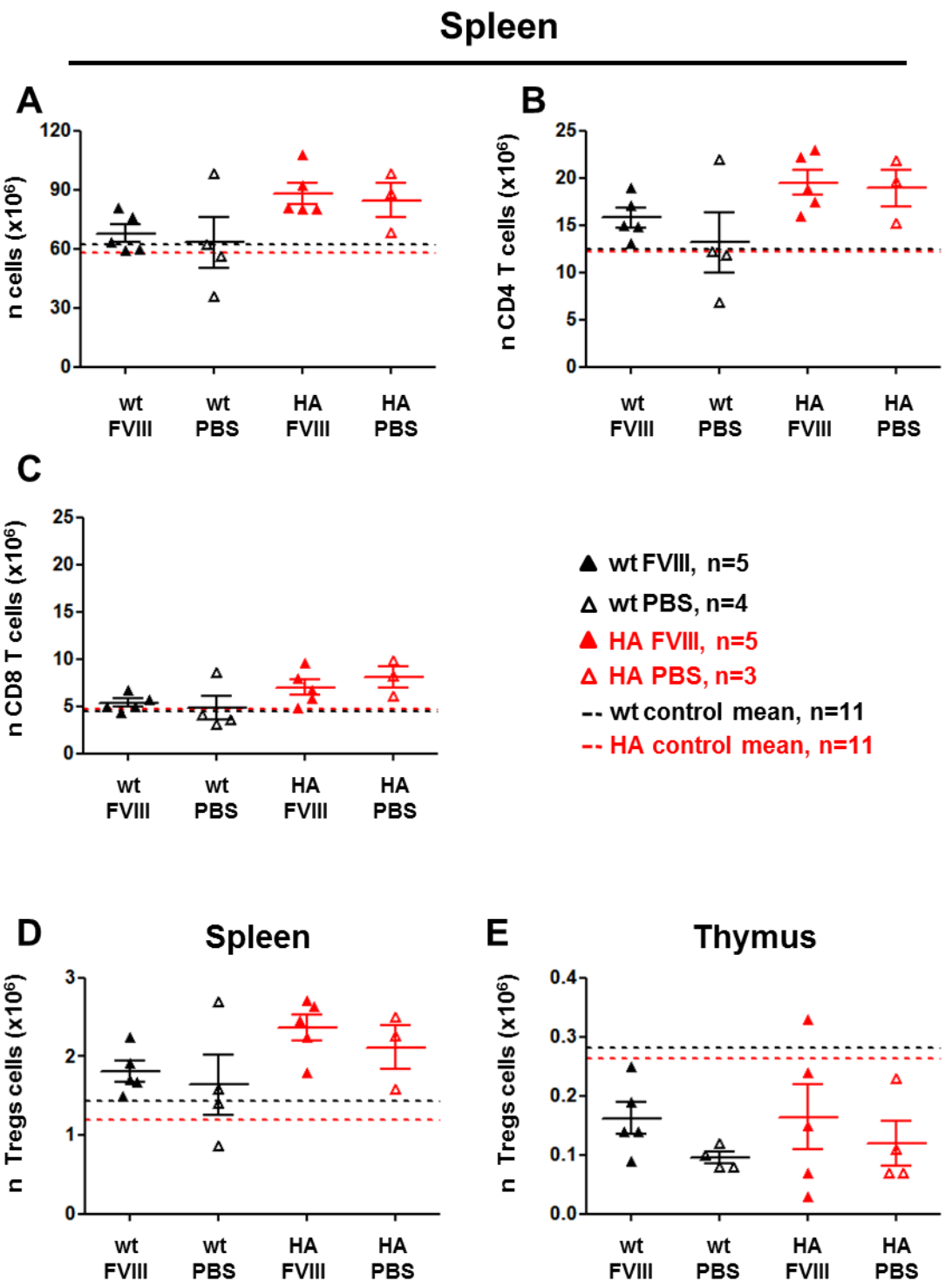

Figure 16: FVIII treatment increased naïve to memory T cells conversion in both groups

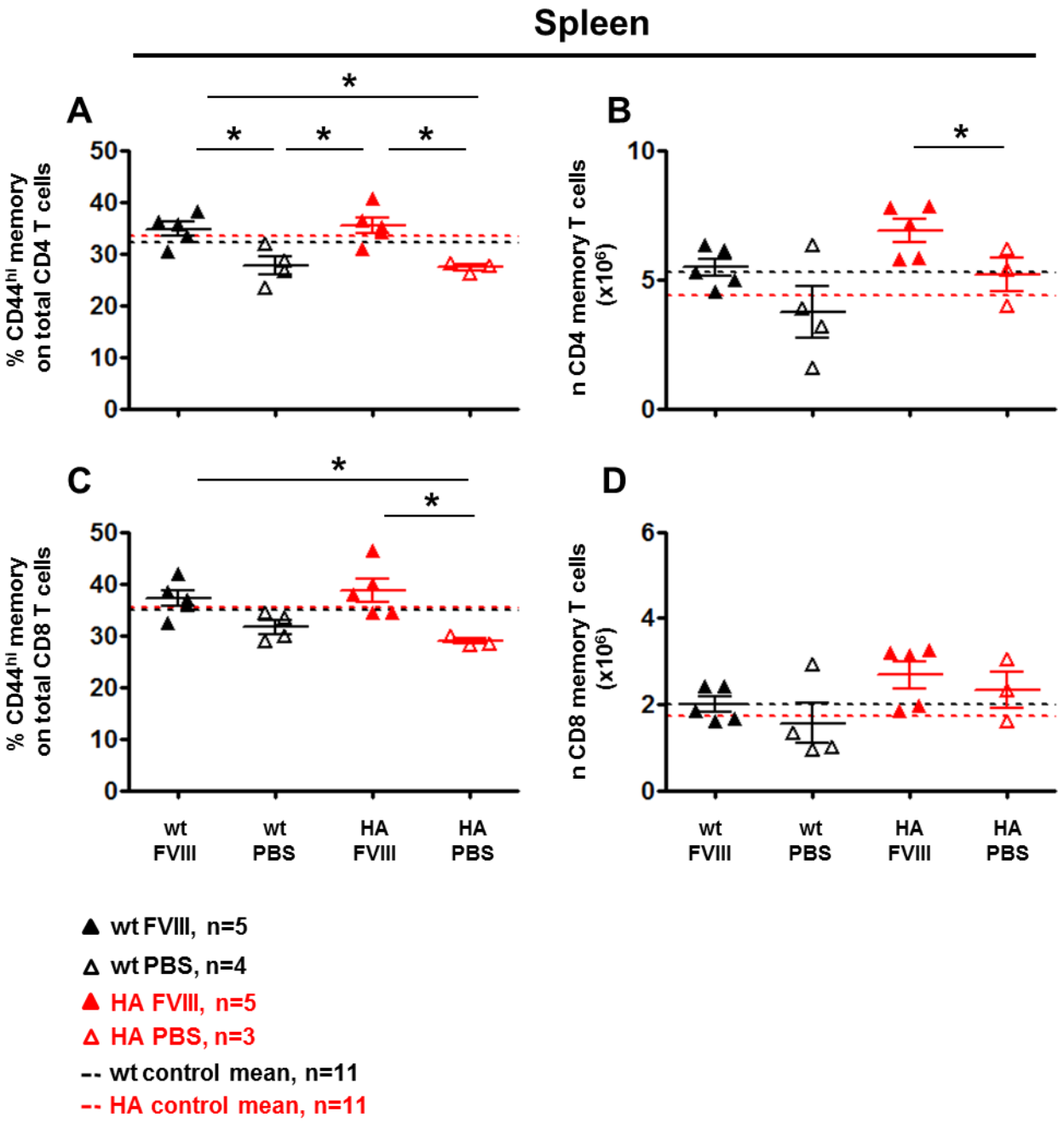

Figure 17: Memory B cells and B cells in BM and spleen

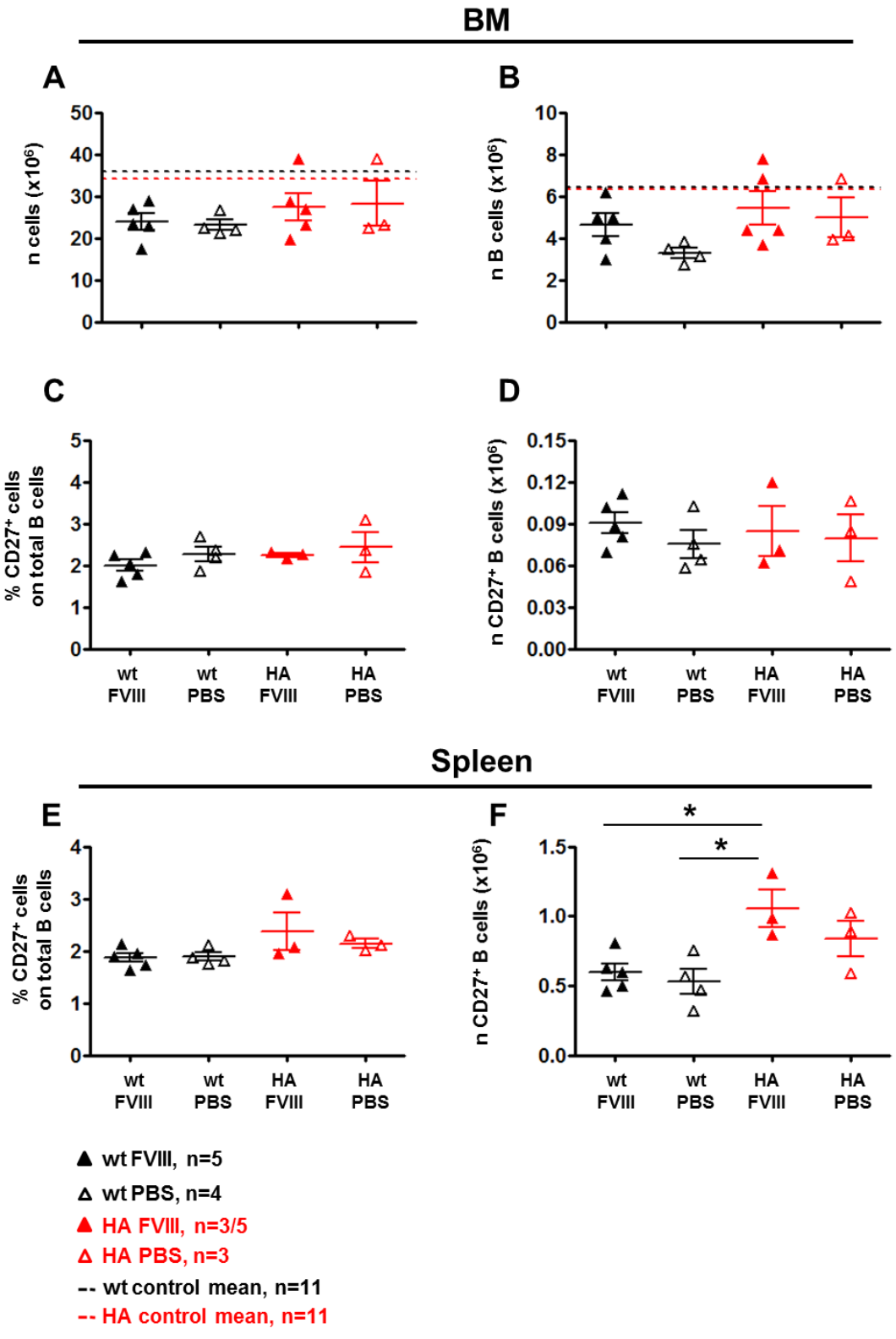

Figure 18: wt samples had lower number of CD4 T cells compared to HA

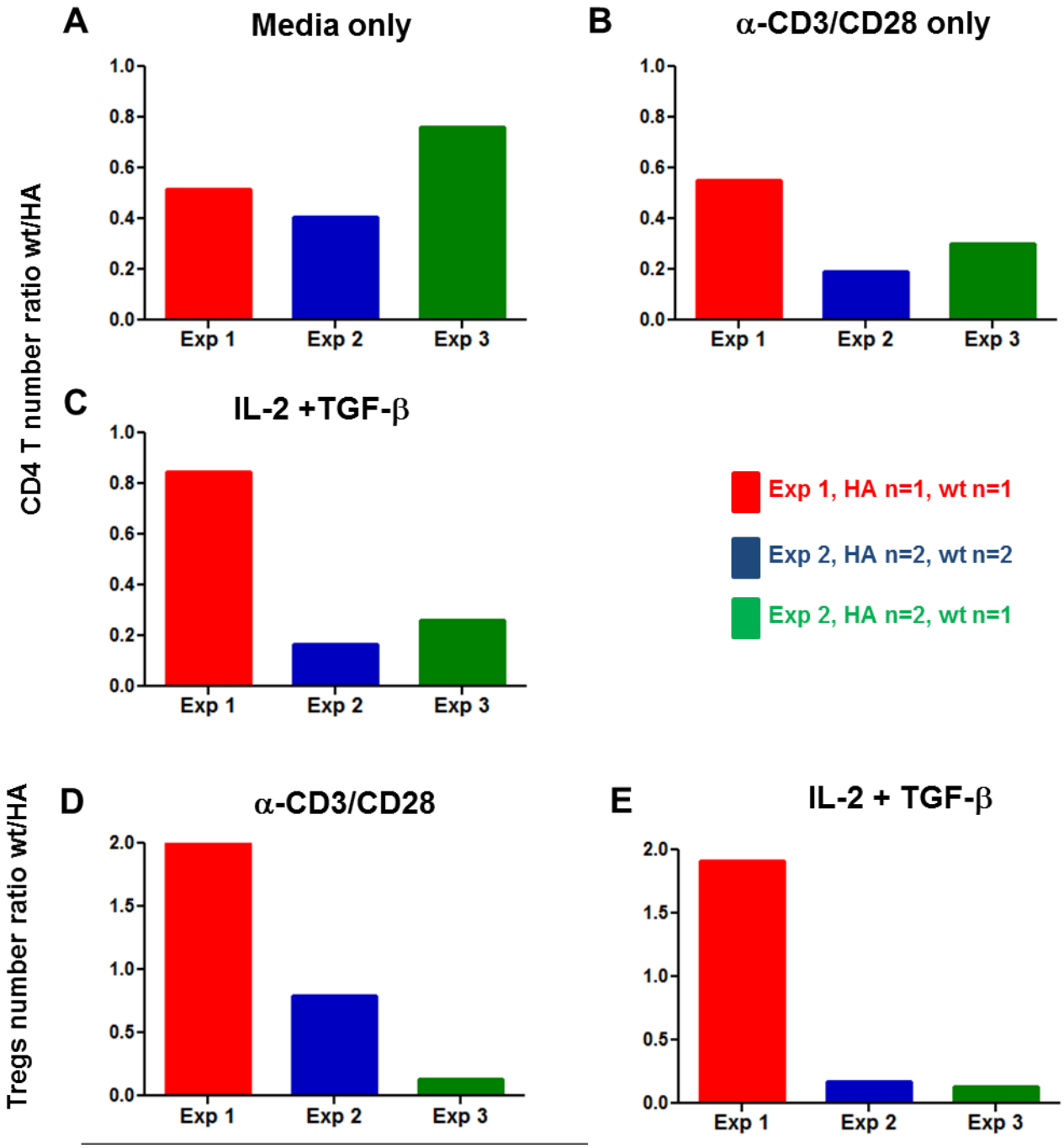

Figure 19: wt samples had higher % of Tregs under only α-CD3/CD28 stimulation

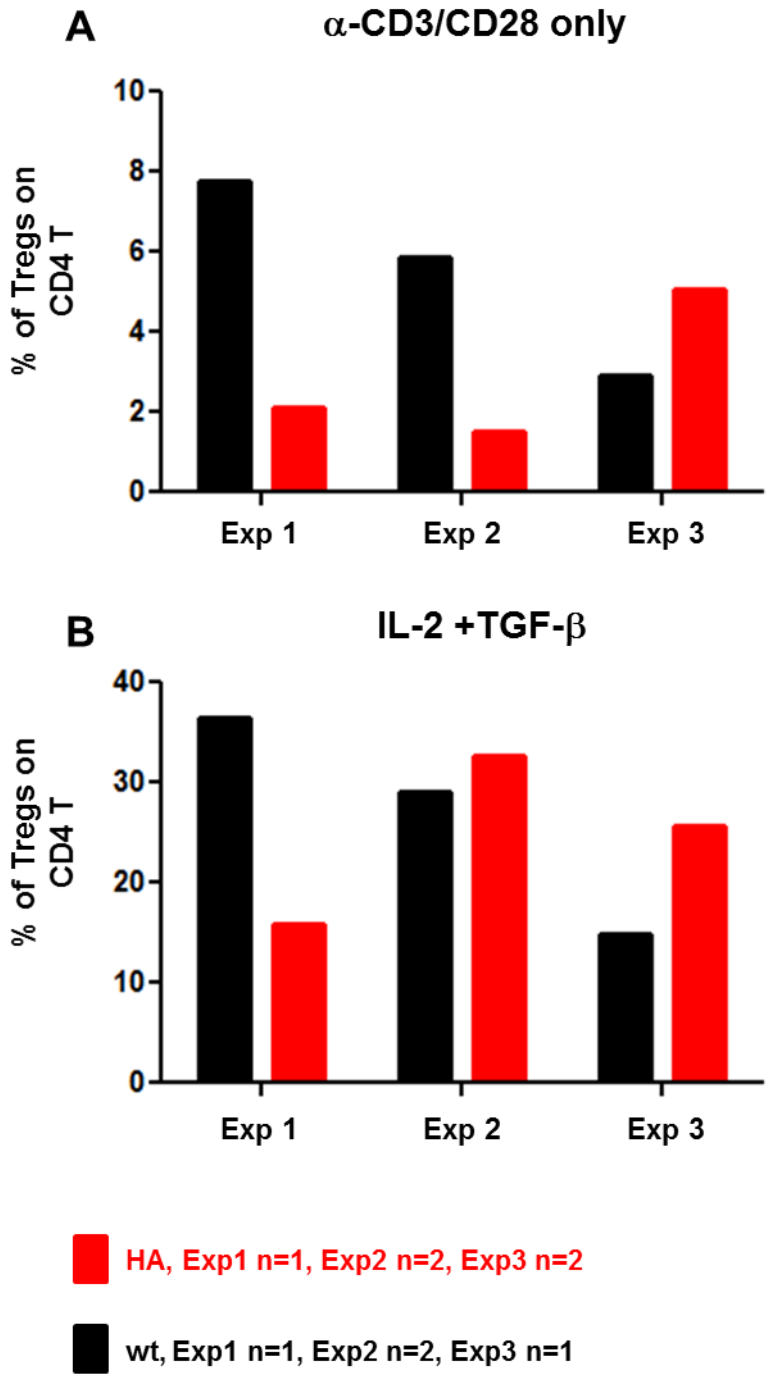

Figure legends

Figure 1. Representative gating strategies for detecting splenic T cells. **A.** CD4 and CD8 T cells were defined as CD49b-CD3+CD4+ and CD49b-CD3+CD8+ respectively while the memory was identified as CD49b-CD3+CD4+CD44^{hi} or CD49b-CD3+CD8+CD44^{hi}. **B.** T regulatory cells were defined as CD3+CD4+CD25+FoxP3.

Figure 2. Representative gating strategies for detecting BM cells. **A.** B cells were B220+CD19+ while memory B cells were B220+CD19+CD27+. **B.** Granulocytes were B220-Gr1+. **C.** LSK was defined as Lineage-(CD3, CD11b, Ter119, B220, Gr1) Sca1+c-Kit+.

Figure 3. Representative gating strategies for detecting thymic cells. **A.** CD3+ cells were gated on live cells. **B.** DP cells were CD4+CD8+, while spCD4 was CD4+CD8- and spCD8 cells were CD3+CD4-CD8+

Figure 4. Isolated naïve CD4 T cells gating strategy and the difference between pre and post stimulation. **A.** Representative dot plots of isolated splenic naïve CD4 T cells which are defined as CD3+CD4+CD25-CD44-. **B.** Representative dot plots of isolated splenic naïve CD4 T cells after 4 days of incubation with α-CD3/CD28, IL-2 + TGF-β.

Figure 5. A. Kinetic of total splenocyte count. **B.** Kinetic of total cell counts obtained from one femur + one tibia/mouse. **C.** Total cell numbers of splenic CD4 T cells. **D.** Total cell numbers of splenic CD8 T cells. **E.** Total cell numbers of splenic T regulatory cells (CD3+CD4+CD25+FoxP3+). **F.** Total cell numbers of thymic T regulatory cells (CD3+CD4+CD25+FoxP3+). Y axis represents number of cells(x10^6), X axis represents different timepoints (weeks). Red line with red squares represents HA (n=8-31) and the black line with black circles represents wt (n=7-32). Results were obtained from 32 independent experiments.

Figure 6. A. Total numbers of thymic DP cells. **B.** Total number of thymic CD3+ T cells. **C.** Total cell numbers of thymic spCD4 T cells. **D.** Total cell numbers of thymic spCD8 T cells. **E.** Total cell numbers of thymic T regulatory cells (CD3+CD4+CD25+FoxP3+). Y axis represents number of cells(x10^6), X axis represents different timepoints (weeks). Red line with red squares represents HA (n=7-14) and the black line with black circles represents wt (n=6-13). Results were obtained from 22 independent experiments.

Figure 7. A. Total numbers of BM B cells. **B.** Total number of BM granulocytes. **C.** % of BM LSK. **D.** Total cell numbers of BM LSK(x10^3). Y axis represents number of cells (x10^6 or x10^3 for LSK) or %, X axis

represents different timepoints (weeks). Red line with red squares represents HA (n=7-14) and the black line with black circles represents wt (n=6-13). Results were obtained from 22 independent experiments.

Figure 8. A. Percentage of splenic $CD44^{hi}$ Memory CD4 T cells. **B.** Total number of splenic $CD44^{hi}$ memory CD4 T cells. **C.** Percentage of splenic memory $CD44^{hi}$ CD8 T cells. **D.** Total number of splenic memory $CD44^{hi}$ CD8 T cells. Y axis represents either % or number of cells($x10^6$), X axis represents different timepoints (weeks). Red line with red squares represents HA and the Black line with black circles represents wt. Results were obtained from 22 independent experiments.

Figure 9. A. Scheme of immunization, representing days of injection of FVIII/IFA and days of blood collection. **B.** FVIII ELISA, average level of FVIII-specific IgG in HA and wt immunized mice, Y axis represents OD(450nm), while X axis represents days. Black circles represent FVIII immunized wt mice (n=4), red circles represent FVIII immunized HA mice (n=5), empty red square represents HA mice injected with PBS/IFA (n=3), dashed blue line represents serum from LV.PGK.FVIII injected mouse. **C,D.** Blood loss reading on spectrophotometer, Y axis represents OD(570nm), while X axis represents the blood dilutions. Black circles represent FVIII immunized wt mice (n=4), red circles represent FVIII immunized HA mice (n=5), black X represents wt mice injected with PBS/IFA (n=4), red X represent HA mice injected with PBS/IFA (n=5), green circle represents mean of FVIII immunized wt mice, green X represents mean of represents mean of PBS/IFA injected wt mice, blue circle represents mean of FVIII immunized HA mice, blue X represents mean of PBS/IFA injected HA mice. **E.** Bethesda assay, percentage of mice developing high titer of BU >5. Y axis represents % of mice with BU >5.

Figure 10. **A.** Total number of splenocytes. **B.** Total number of splenic CD4 T cells. **C**. Total number of splenic CD8T cells. **D.** Total number of splenic T regulatory cells. **E.** Total number of thymic T regulatory cells. Y axis represents number of cells($x10^6$), black circles represent FVIII immunized wt mice(n=4), black empty squares represent wt mice injected with PBS/IFA(n=4), red circles represent FVIII immunized HA mice(n=5), empty red squares represent HA mice injected with PBS/IFA(n=5), The dashed red and black line in each scatter plot shows the mean of control (not injected) Balb/c HA and wt mice respectively. means ± SEM are plotted together with the individual values.

Figure 11. A. Scheme of immunization, representing days of injection of OVA/IFA and days of blood collection. **B.** OVA ELISA, average level of OVA-specific IgG in HA and wt immunized mice, Y axis represents OD(450nm), while X axis represents days, red squares represent OVA immunized HA mice(n=8), empty red square represents HA mice injected with PBS/IFA(n=3), black squares represent OVA immunized wt mice(n=4) and empty black squares represent wt mice injected with PBS/IFA(n=2). **C.** Total number of splenocytes. **D.** Total number of splenic CD4 T cells. **E**. Total number of splenic CD8T cells. **F.** Total number of splenic T regulatory cells. **G.** Total number of thymic T regulatory cells. Y axis represents number of cells($x10^6$). Black squares represent OVA immunized wt mice(n=6), red squares represent OVA immunized HA

mice(n=8), empty black squares represent wt mice injected with PBS/IFA(n=4), empty red squares represent HA mice injected with PBS/IFA(n=5), The dashed red and black line in each scatter plot shows the mean of control (not injected) Balb/c HA and wt mice respectively. Means ± SEM are plotted together with the individual values.

Figure 12. A. Percentage of splenic $CD44^{hi}$ memory CD4 T cells. **B.** Total number of splenic $CD44^{hi}$ memory CD4 T cells. **C.** Percentage of splenic memory $CD44^{hi}$ CD8 T cells. **D.** Total number of splenic memory $CD44^{hi}$ CD8 T cells. Y axis represents either % or number of cells($x10^6$). Black squares represent OVA immunized wt mice(n=6), black circles represent FVIII immunized wt mice(n=3), red squares represent OVA immunized HA mice(n=8), red circles represent FVIII immunized HA mice(n=5). The dashed red and black line in each scatter plot shows the mean of control (not injected) Balb/c HA and wt mice respectively. means ± SEM are plotted together with the individual values.

Figure 13. A. Total BM cells. **B.** Total number of BM granulocytes. **C.** Total number of BM B cells. **D.** Percentage of CD27 on BM B cells. **E.** Total number of BM CD27+ B cells. **F.** Percentage of CD27 on splenic B cells. **G.** Total number of splenic CD27+ B cells. Y axis represents either % or number of cells($x10^6$). Black squares represent OVA immunized wt mice(n=4-6), black circles represent FVIII immunized wt mice(n=4), red squares represent OVA immunized HA mice(n=7-8), red circles represent FVIII immunized HA mice(n=3-5). The dashed red and black line in each scatter plot shows the mean of control (not injected) Balb/c HA and wt mice respectively. Means ± SEM are plotted together with the individual values.

Figure 14. A. Scheme of Treatment, representing days of injection of FVIII and days of blood collection. **B.** FVIII ELISA, average level of FVIII-specific IgG in HA and wt treated mice, Y axis represents OD(450nm), while X axis represents days, black triangles represents FVIII treated wt mice(n=5), empty black triangles represents PBS injected wt mice(n=4), red triangles represents FVIII treated HA mice(n=6), dashed blue line represents serum from LV.PGK.FVIII injected mouse. **C,D.** Blood loss reading on spectophotometer, Y axis represents OD(570nm), while X axis represents the dilutions, black triangles represent FVIII treated wt mice(n=5), red triangles represent FVIII treated HA mice(n=6), empty black triangles represent PBS injected wt mice(n=4), blue triangle represents mean of FVIII treated HA mice, blue X represents mean of PBS injected HA mice, green triangle represents mean of FVIII treated wt mice, green X represents mean of PBS injected wt mice. **E.** Bethesda assay, percentage of mice developing high titer of BU >5. Y axis represents % of mice with BU >5.

Figure 15. A. Total number of splenocytes. **B.** Total number of splenic CD4 T cells. **C**. Total number of splenic CD8T cells. **D.** Total number of splenic T regulatory cells. **E.** Total number of thymic T regulatory cells. Y axis represents number of cells($x10^6$). Black triangles represent FVIII treated wt mice(n=5), red triangles represent FVIII treated HA mice(n=5), empty black triangles represent PBS injected wt mice(n=4), empty red triangles represent HA mice injected with PBS(n=3). The dashed red and black line in each scatter plot shows the mean of

control (not injected) Balb/c HA and wt mice respectively. means ± SEM are plotted together with the individual values.

Figure 16. A. Percentage of splenic $CD44^{hi}$ memory CD4 T cells. **B.** Total number of splenic $CD44^{hi}$ memory CD4 T cells. **C.** Percentage of splenic memory $CD44^{hi}$ CD8 T cells. **D.** Total number of splenic memory $CD44^{hi}$ CD8 T cells. Y axis represents either % or number of cells($x10^6$). Black triangles represent FVIII treated wt mice(n=5), red triangles represent FVIII treated HA mice(n=5), empty black triangles represent PBS injected wt mice(n=4), empty red triangles represent HA mice injected with PBS(n=3). The dashed red and black line in each scatter plot shows the mean of control (not injected) Balb/c HA and wt mice respectively. Means ± SEM are plotted together with the individual values.

Figure 17. A. Total BM cells. **B.** Total number of BM B cells. **C.** Percentage of CD27 on BM B cells. **D.** Total number of BM CD27+ B cells. **E.** Percentage of CD27 on splenic B cells. **F.** Total number of splenic CD27+ B cells. Y axis represents either % or number of cells($x10^6$). Y axis represents either % or number of cells($x10^6$). Black triangles represent FVIII treated wt mice(n=5), empty black triangles represent PBS injected wt mice(n=4), red triangles represent FVIII treated HA mice(n=5), empty red triangles represent HA mice injected with PBS(n=3). The dashed red and black line in each scatter plot shows the mean of control (not injected) Balb/c HA and wt mice respectively. Means ± SEM are plotted together with the individual values.

Figure 18. A, B, C. Ratio between wt and HA (wt/HA) CD4 T cells recovered after 4 days of incubation in media only, α-CD3/CD28 or TGF-β + IL-2 stimulation respectively. **D, E.** Ratio between wt and HA (wt/HA) T regulatory cells after 4 days of incubation in α-CD3/CD28 or TGF-β + IL-2 stimulation respectively. Y axis represents CD4T cell number ratio wt/HA or Tregs number ratio wt/HA, while X axis represents the independent experiment.

Figure 19. A. Percentage of Tregs on CD4 T cells in α-CD3/CD28 only. **B.** Percentage of Tregs on CD4 T cells under IL-2 + TGF-β stimulation.

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