Supplemental Material

Factor VIII trafficking to $CD4^+$ T cells shapes its immunogenicity and requires several types of antigen presenting cells

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Supplemental Methods

Generation of FVIII-AF488

Recombinant FVIII-SQ concentration was determined at 280 nm absorbance based on an $E^{0.1\%}$ of 1.6 and M_r of 165,000. There are 3 described free cysteines in FVIII-SQ, at the C310 and C692 in the heavy chain and C2000 in the light chain (Figure S1A). To determine site specificity of labelling, rFVIII-SQ-A488 was loaded on an SDS-Page gel with equimolar amounts of control proteins including rFVIII-SQ and factor V-810-SYA-A488 (FV-810 with C585S, C1960Y, C2113A), a site-specifically labelled FV variant with a known 1:1 molar ratio of protein to probe generated as previously described. The majority of rFVIII-SQ-A488 was labelled in the A2 domain consistent with labelling at C692 and lesser labeling was observed in the light chain consistent with C2000. Densitometry studies, using FV-810-SYA-A488 as a control, demonstrated rFVIII-SQ-A488 had a ratio of 1M of protein to 1.5M of A488-maleimide. As expected, the cleavage fragments of rFVIII-SQ-488 in the presence of thrombin (IIa) were the same as the rFVIII-SQ control (Figure S1B). The specific activity determined by an activated partial thromboplastin time one-stage assay of rFVIII-SQ-A488 was decreased at 4,000 IU/mg versus standard rFVIII-SQ specific activity of 7,000-10,000 IU/mg (Table S1); we hypothesize this diminished specific activity is related to the minor Alexa488 labelling at C2000 and resultant diminished membrane binding.

Generation of FOVA

Recombinant FVIII-OVA concentration was determined at 280 nm absorbance based on an $E^{0.1\%}$ of 1.6 and M_r of 165,000. As expected, the cleavage fragments of FVIII-OVA in the presence of thrombin (IIa) were the same as the rFVIII-SQ control. The specific activity determined by an activated partial thromboplastin time one-stage assay demonstrated approximately normal FVIII specific activity analogous to rFVIII-SQ generated by the same methods or commercially available rFVIII-SQ (moroctocog alfa or “ReFacto”, Pfizer, New York, NY).

Plasma isolation and *in vivo* administration of reagents

For plasma samples, mice were anesthetized with isoflurane and blood was collected via the retro-orbital plexus. Sodium citrate (3.2%) was added to the samples at one-tenth total volume. Plasma was isolated by centrifugation.

In the APC uptake experiments, S129/C57BL/6 HA mice received 5-10 µg FVIII-AF488 or OVA-AF488 (Molecular Probes, Eugene, OR) IV into a tail vein. In the DC phenotyping and Tfh/GC/antibody development time-course experiments, S129/C57BL/6 HA mice received a single and repeated weekly injections of 1.5 IU FVIII (Xyntha, Pfizer, New York, NY) ± 50 µg ODN1826 (Invivogen, San Diego, CA) IV, respectively. For antigen presentation assays, animals received 5 µg FOVA and 5E6 CTV-stained OT-II $CD4^+$ cells IV in PBS on the following day. For MF inactivation, C57BL/6 HA mice received 250 µg $GdCl_3$ IP 1 day before and on the day of IV FOVA administration. For MZB cell depletion, C57BL/6 HA mice received 100 µg anti-CD11a and anti-CD49d or isotype control antibodies (clones 2A3 and LTF-2) (Bio X Cell, Lebanon, NH) IP 4 and 2 days before IV FOVA administration. For DC depletion, CD11c-DTR/GFP mice received 100 ng DT IP 1 day before and on the day of FOVA administration. For pDC depletion, BDCA2-DTR/GFP mice received 300 ng DT IP 1 day before, 1 day after and 3 days after FOVA administration. For depletion of cDC1s, XCR1-DTRvenus mice received 1 µg DT IP 1 day before and 2 days after IV FOVA administration. For intravital microscopy, CD11c-DTR/GFP mice received 5 µg FOVA ID in the groin area and 1E7 CTV-stained OT-II $CD4^+$ cells IV. Inflammatory signaling-deficient and control mice (C57BL/6) received 4 weekly IV injections of 2.5 IU FVIII. For immunogenicity comparison, S129/C57BL/6 HA mice received 4 weekly IV injections of 300 ng FVIII (1.5 IU), FIX (Benefix, Pfizer, New York, NY) or OVA (Invivogen, San Diego, CA).

Intravital microscopy

Male CD11c-DTR/GFP mice were anesthetized with isoflurane. Inguinal lymph nodes were exposed by surgical incision and securing the skin flap on a modified glass-bottom dish insert for imaging on an inverted microscope. During imaging, the core body temperature of the animals was maintained at 36–37 °C with a temperature controller consisting of a rectal probe and a heating pad. Images were collected using a Leica SP8 confocal/multiphoton microscope with a HCX IRAPO L 25x/0.95 water dipping objective. Excitation was delivered at $\lambda = 860$ nm for GFP, CTV and collagen with signals collected at $\lambda = 495–575$ nm, $\lambda = 450–490$ nm and $\lambda = 420–440$ nm, respectively. A conventional PMT was used for collagen, with HyD detectors used for GFP and CTV. After imaging, unconscious mice were euthanized by cervical dislocation.

Flow cytometry

Spleens were harvested and turned into single-cell suspensions using 70-µm cell strainer in cold PBS buffer. Surface staining was performed at 4°C for 30 min. Live cells were stained using Zombie Aqua (BioLegend, San Diego, CA). For intracellular Bcl-6 and IFN-β staining, cells were treated with Fixation/Permeabilization set (Thermo Fisher Scientific). Before IFN-β staining,

DCs were isolated using Pan Dendritic Cell Isolation Kit (Miltenyi Biotec, Auburn, CA) and treated with Cell Activation Cocktail with Brefeldin A (BioLegend, San Diego, CA). All antibodies are listed below in Table S2. All gating schemes are shown in Figure S9.

Supplemental Tables

Table S1. Specific activity of FVIII-AF488. Specific activity was determined by partial thromboplastin time one-stage clotting assay measurement relative to protein concentration. Data shown as mean ± standard error measurements from 3 independent experiments.

	Specific Activity (U/mg)
rFVIII-SQ	8000 ± 800
rFVIII-SQ-A488	4000 ± 600

Table S2. Antibodies used in the study.

Antibody/target antigen	Fluorochrome	Clone	Company
CXCR5	BV421	L138D7	BioLegend, San Diego, CA
CD95	BV605	SA367H8	
CD4	BV711	RM4-5	
GL7	AF488	GL7	
PD-1	PE-Cy7	29F.1A12	
PDCA-1	BV421	927	
CD11b	AF488, PE-Cy7	M1/70	
CD8α	BV711	53-6.7	
CD64	BV605	X54-5/7.1	
CD11c	PE	N418	
CD86	PE-Cy7	GL-1	
MAR-1	AF647	MAR-1	
B220	BV605	RA3-6B2	
F4/80	APC	BM8	
CD21	AF647	7E9	
CD23	PerCP-Cy5.5	B3B4	
IFN-beta	FITC	orb526032	Biorbyt, Cambridge, UK
TIM-4	PerCP-eFluor 710	54(RMT4-54)	eBioscience, Thermo Fisher Scientific, Waltham, MA
CD19	APC-eFluor 780	1D3	
Bcl-6	PE	K112-91	
MHC-II	APC-eFluor 780	M5/114.15.2	
FoxP3	eFluor660	FJK-16s	
CD11a	-	M17/4	Bio X Cell, Lebanon, NH
CD49d	-	PS/2	
TNP	-	2A3	
KLH	-	LTF-2	

Supplemental Figures

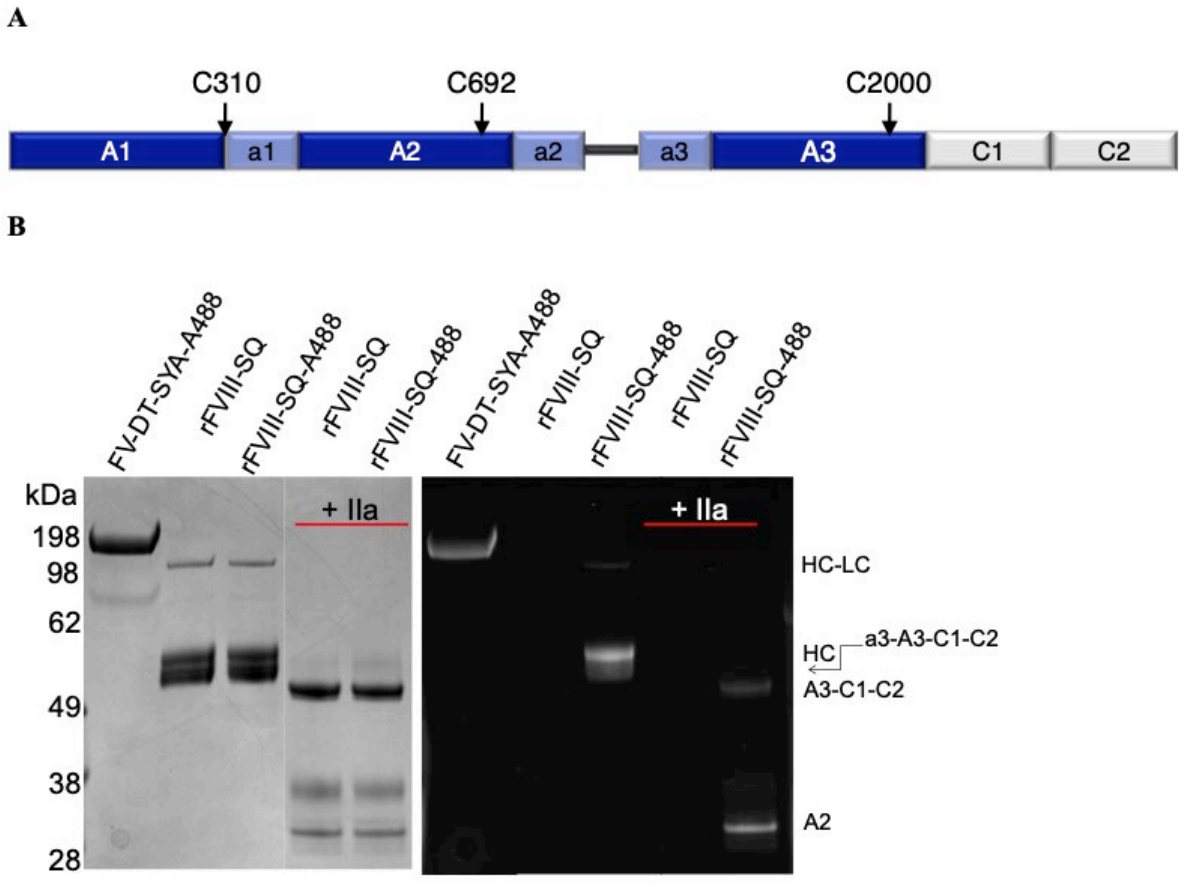
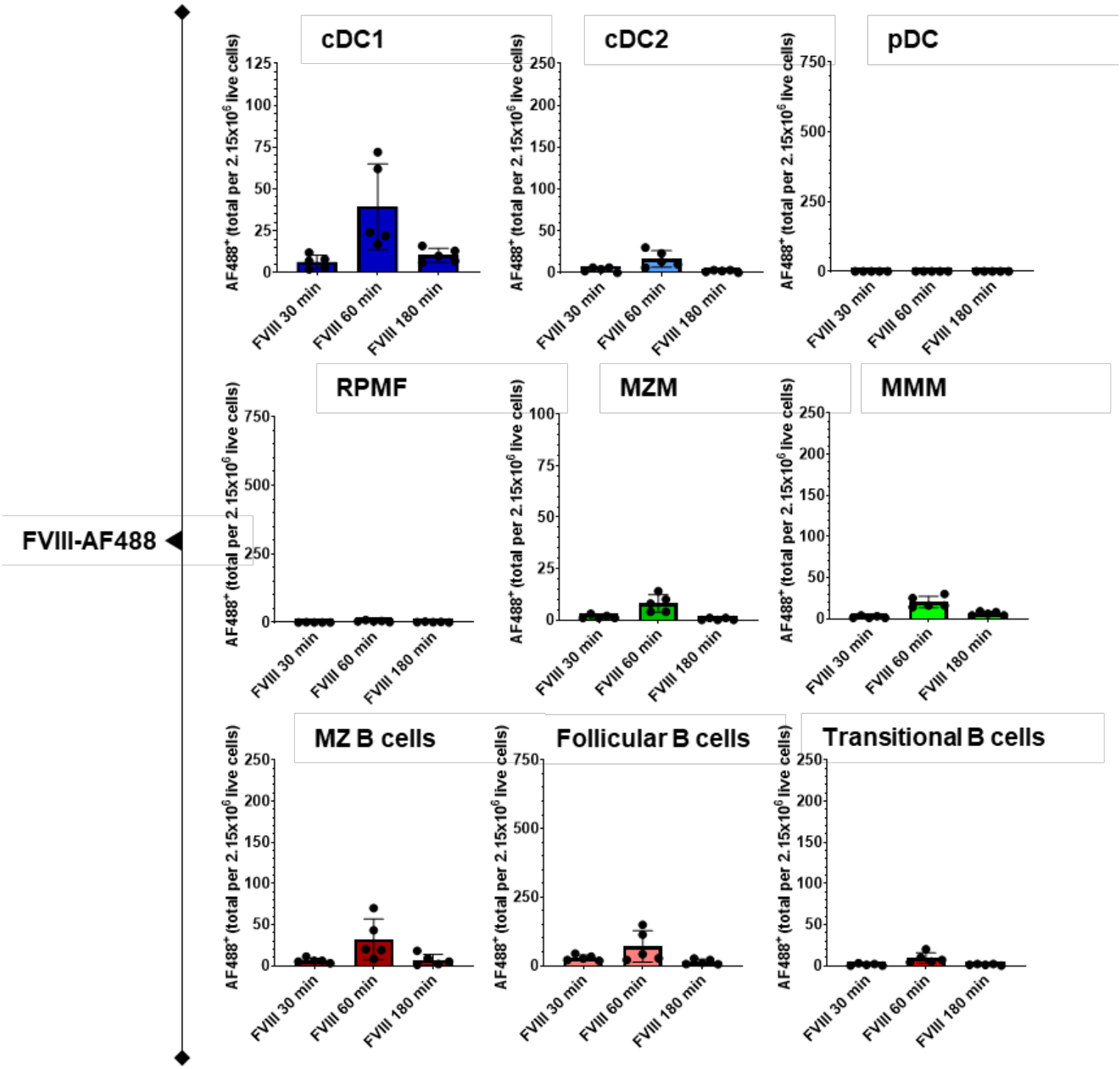


Figure S1. Characterization of rFVIII-SQ-A488: (A) FVIII domain structure with free cysteins noted. (B) SDS-PAGE analysis of 1.5 uM rFVIII-SQ and rFVIII-SQ-A488 in the presence or absence of 10nM thrombin. The gel was stained with Coomassie blue and fluorescent images are shown (right).


FVIII-AF488
cDC1
cDC2
pDC
RPMF
MZM
MMM
MZ B cells
Follicular B cells
Transitional B cells
AF488+ (total per 2.15x10^6 live cells)
FVIII 30 min
FVIII 60 min
FVIII 180 min

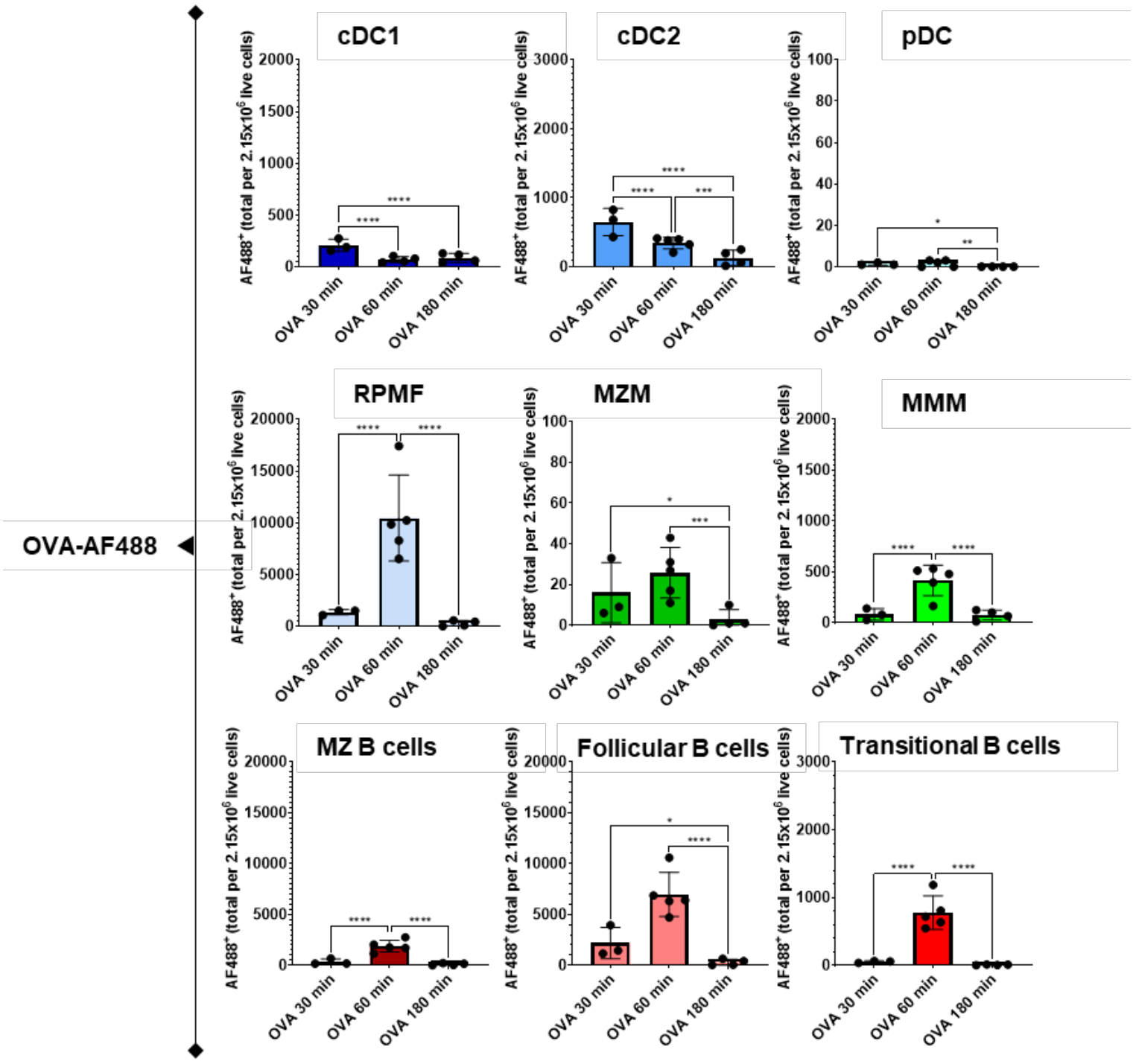
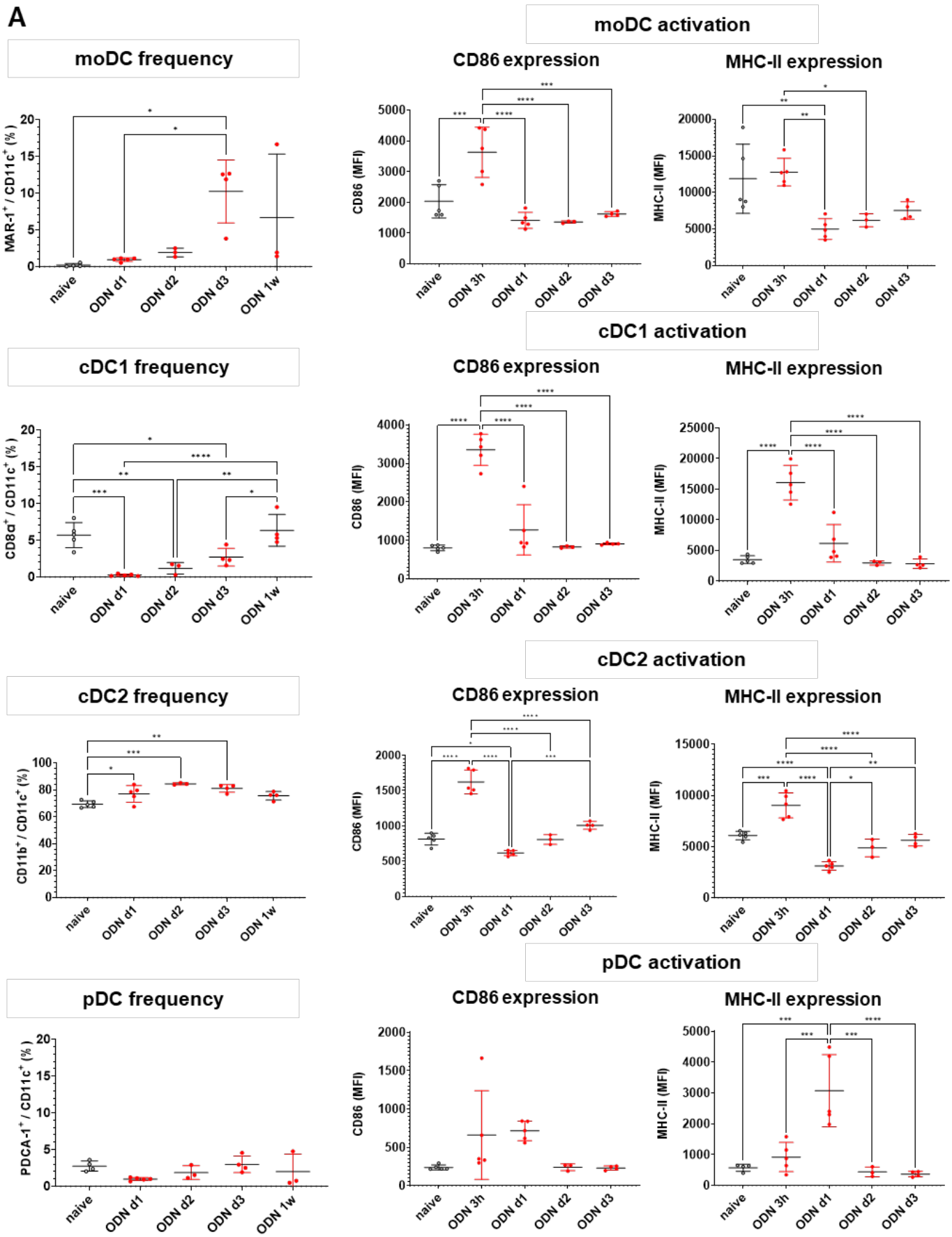


Fig. S2. FVIII and OVA are taken up by multiple types of DCs, MFs and B cells, and show different uptake kinetics. S129/C57BL/6 HA mice received 10µg FVIII-AF488 or OVA-488 IV (n=3-5/group). After 30-180 minutes, spleens were collected for flow cytometry. Total FVIII-AF488$^+$ or OVA-AF488$^+$ cell numbers were quantified in cDC1s (CD8α$^+$CD11b$^-$CD11c$^+$), cDC2s (CD8α$^-$CD11b$^+$CD11c$^+$), pDCs (PDCA-1$^+$CD11c$^+$), RPMFs (F4/80$^+$CD11b$^-$), MZMs (F4/80^{lo}CD11b$^+$TIM-4^{hi}), MMMs (F4/80^{lo}CD11b$^+$TIM-4^{lo}), MZB cells (B220$^+$CD21^{hi}CD23$^{lo/-}$),

transitional B cells ($B220^{+}CD21^{-}CD23^{lo/-}$), and follicular B cells ($B220^{+}CD21^{med}CD23^{lo/-}$). Shown are means±SD and P values from analysis of variance.


A
moDC frequency
MAR-1+ / CD11c+ (%)
naive
ODN d1
ODN d2
ODN d3
ODN 1w
moDC activation
CD86 expression
CD86 (MFI)
MHC-II expression
MHC-II (MFI)
ODN 3h
cDC1 frequency
CD8α+ / CD11c+ (%)
cDC1 activation
cDC2 frequency
CD11b+ / CD11c+ (%)
cDC2 activation
pDC frequency
PDCA-1+ / CD11c+ (%)
pDC activation

B

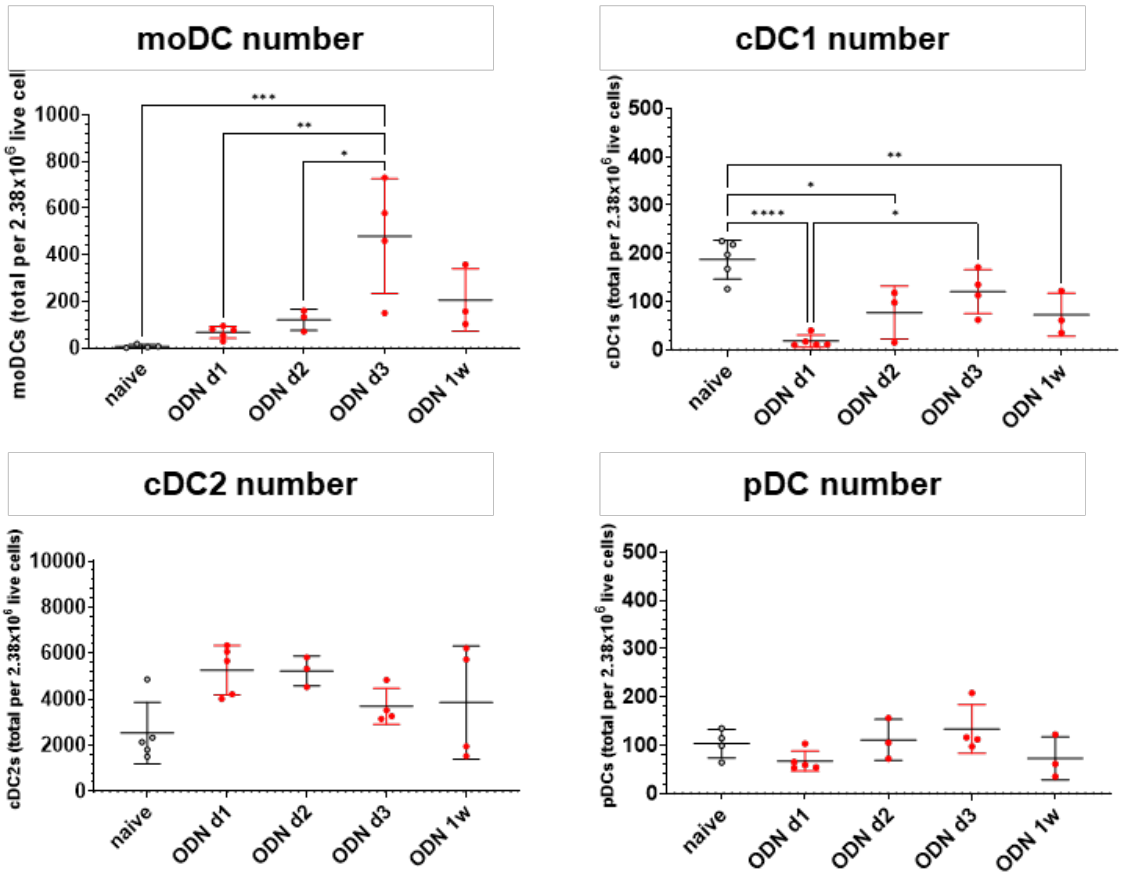


Fig. S3. Effects of TLR9 agonist administration on DC numbers in the spleen. S129/C57BL/6 HA mice received a single injection of ODN1826 (50µg) IV (n=3-5/group). After 3-154 hours (3 hours or 1, 2, 3 and 7 days) after administration, spleens were collected for flow cytometry analysis. (A) Flow cytometry analysis of moDC (MAR-1$^+$CD64$^+$CD11b$^+$CD11c$^+$), cDC1 (CD8α$^+$ CD11b$^-$CD11c$^+$), cDC2 (CD8α$^-$CD11b$^+$CD11c$^+$) and pDC (PDCA-1$^+$CD11c$^+$) frequencies and expression of activation markers CD86 and MHC-II on each subset (1, 2, 3 days and 1 week after administration). (B) Flow cytometry analysis of total moDC (MAR-1$^+$CD64$^+$CD11b$^+$CD11c$^+$), cDC1 (CD8α$^+$ CD11b$^-$CD11c$^+$), cDC2 (CD8α$^-$CD11b$^+$CD11c$^+$) and pDC (PDCA-1$^+$CD11c$^+$) numbers 1, 2, 3 days and 1 week after administration. All plots (A-B) show means±SD and P values by analysis of variance.

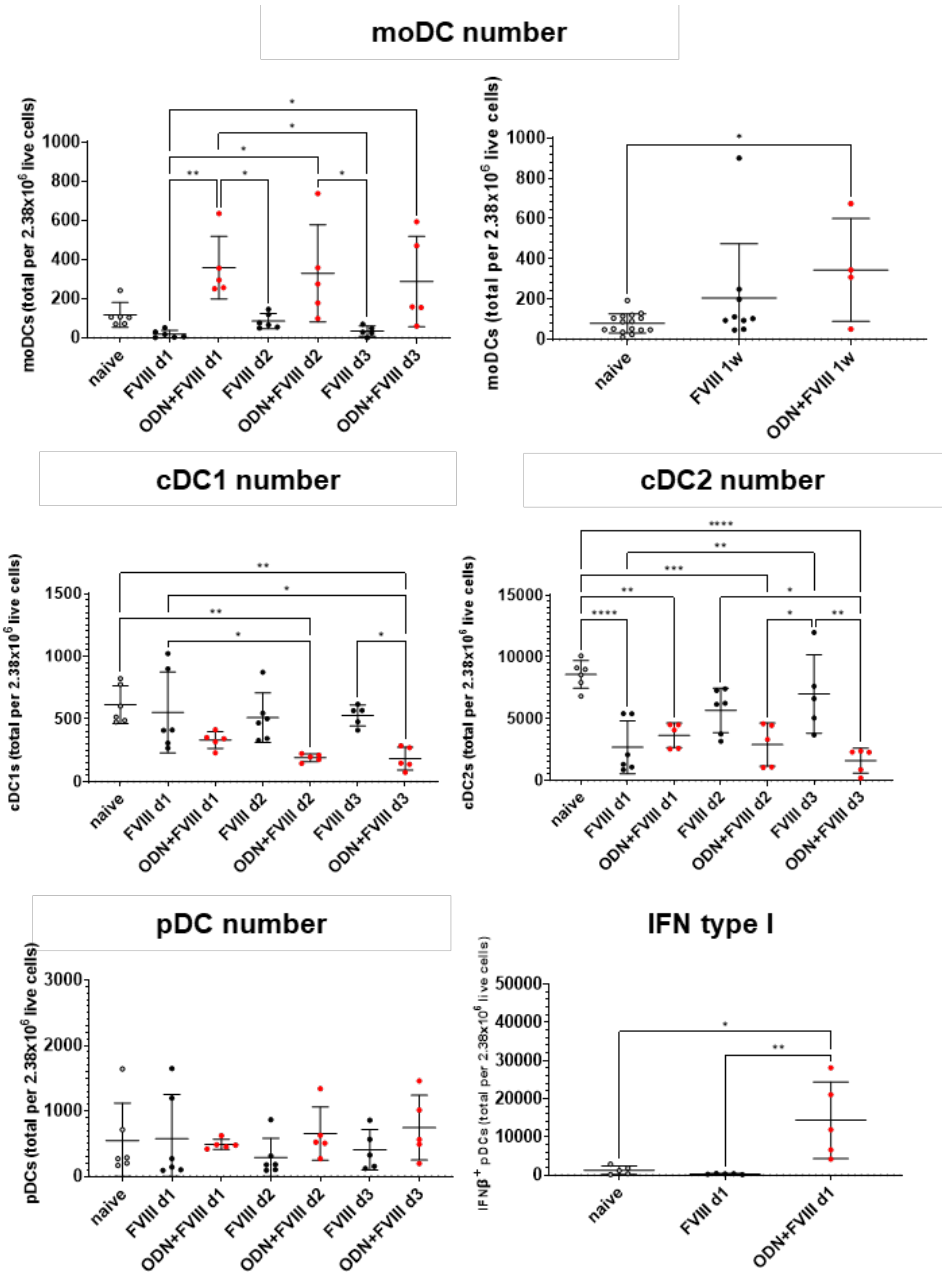


Fig. S4. Changes in total DC numbers in response to systemic administration of FVIII with or without TLR9 agonist. S129/C57BL/6 HA mice received a single injection of FVIII (1.5IU)±ODN1826 (50µg) IV (n=4-16/group). After 3-154 hours (3 hours or 1, 2, 3 and 7 days) after administration, spleens were collected for flow cytometry analysis. (B) Flow cytometry analysis of total moDC (MAR-1$^+$CD64$^+$CD11b$^+$CD11c$^+$) numbers (1, 2, 3 days and 1 week after administration), cDC1 (CD8α$^+$ CD11b$^-$CD11c$^+$) numbers (1,2 and 3 days after administration), cDC2 (CD8α$^-$CD11b$^+$CD11c$^+$) numbers (1, 2 and 3 days after administration), pDC (PDCA-1$^+$CD11c$^+$) numbers (1, 2 and 3 days after administration), and IFN-β$^+$ pDC numbers (1 day after administration). All plots (B-E) show means±SD and P values by analysis of variance.

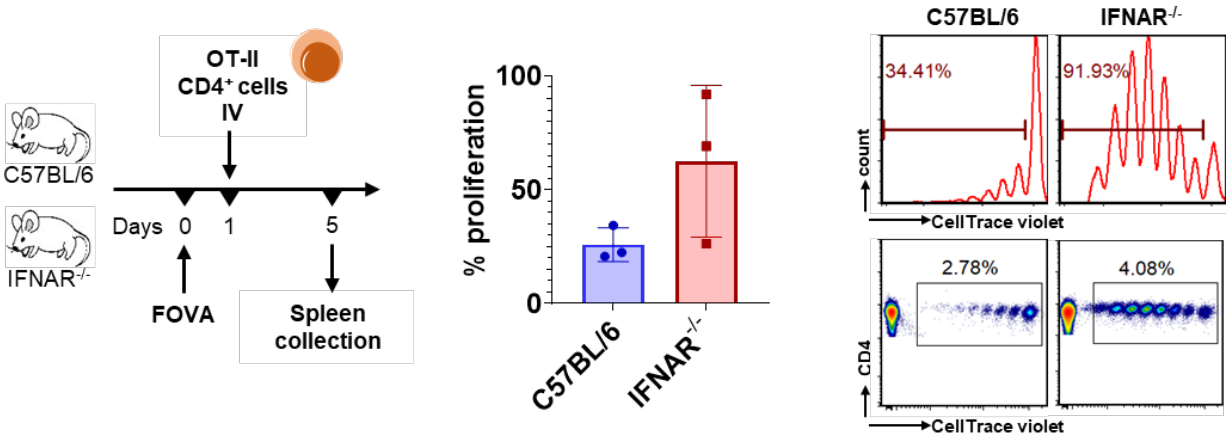


Fig. S5. IFNAR$^{-/-}$ mice exhibit elevated helper T cell proliferative responses to FVIII. *In vivo* antigen presentation assay in C57BL/6 IFNAR$^{-/-}$ mice. IFNAR$^{-/-}$ or wild-type C57BL/6 mice (n=3) received FOVA. One day later, CD4^{+} T cells were magnetically sorted from OT-II mice, labeled with CTV and adoptively transferred to the experimental mice. Four days later spleens were collected for flow cytometry analysis of proliferation. The plots show a summary of %proliferation (mean±SD), representative proliferation histograms and respective dot plots.

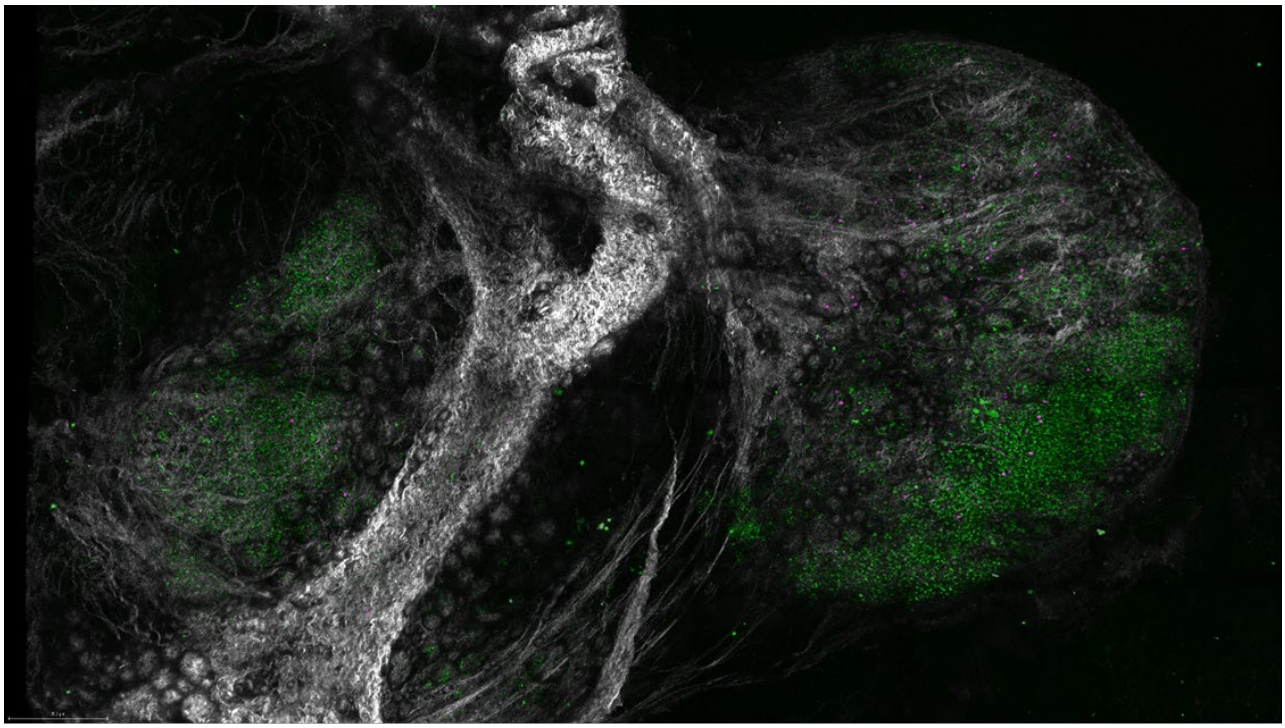


Fig. S6. Intravital microscopy of inguinal lymph nodes 20h after injection of an irrelevant antigen (FIX). Multiphoton intravital microscopy (IVM) in C57BL/6 CD11c-DTR/GFP mice (n=2). Twenty four hours before microscopy, $CD4^+$ T cells were magnetically sorted from OT-II mice, labeled with CTV and adoptively transferred to the experimental animals. FIX (5µg) was injected intradermally in the groin area 20 h before microscopy. LNs were surgically exposed in anesthetized mice and imaged *in vivo* on an inverted Leica SP8 confocal/multiphoton microscope with a HCX IRAPO L 25x/0.95 water dipping objective. During imaging, the core body temperature of the animals was maintained at 36–37 °C with a temperature controller consisting of a rectal probe and a heating pad. Imaging data were collected and processed using Leica LASX software. Sixty-minute time series were captured with Z-stacks collected once every minute to a depth of ~100µm. The full-field view shows a whole lymph node with observable $CD11c^+GFP^+$ cells (green), collagen (grey), and $CD4^+CTV^+$ cells (purple).

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Fig. S7. Mice receiving repeated injections of FIX or OVA do not produce IgG2c in the absence of IgG1. S129/C57BL/6 HA mice received 4 weekly injections of FVIII, FIX, or OVA IV (n=7-9/group). Blood samples were collected for ELISA and Bethesda assays 1 week after the last injection. FVIII-, FIX- or OVA-specific IgG2c titers were measured 1 week after the last injection. Shown are means±SD.

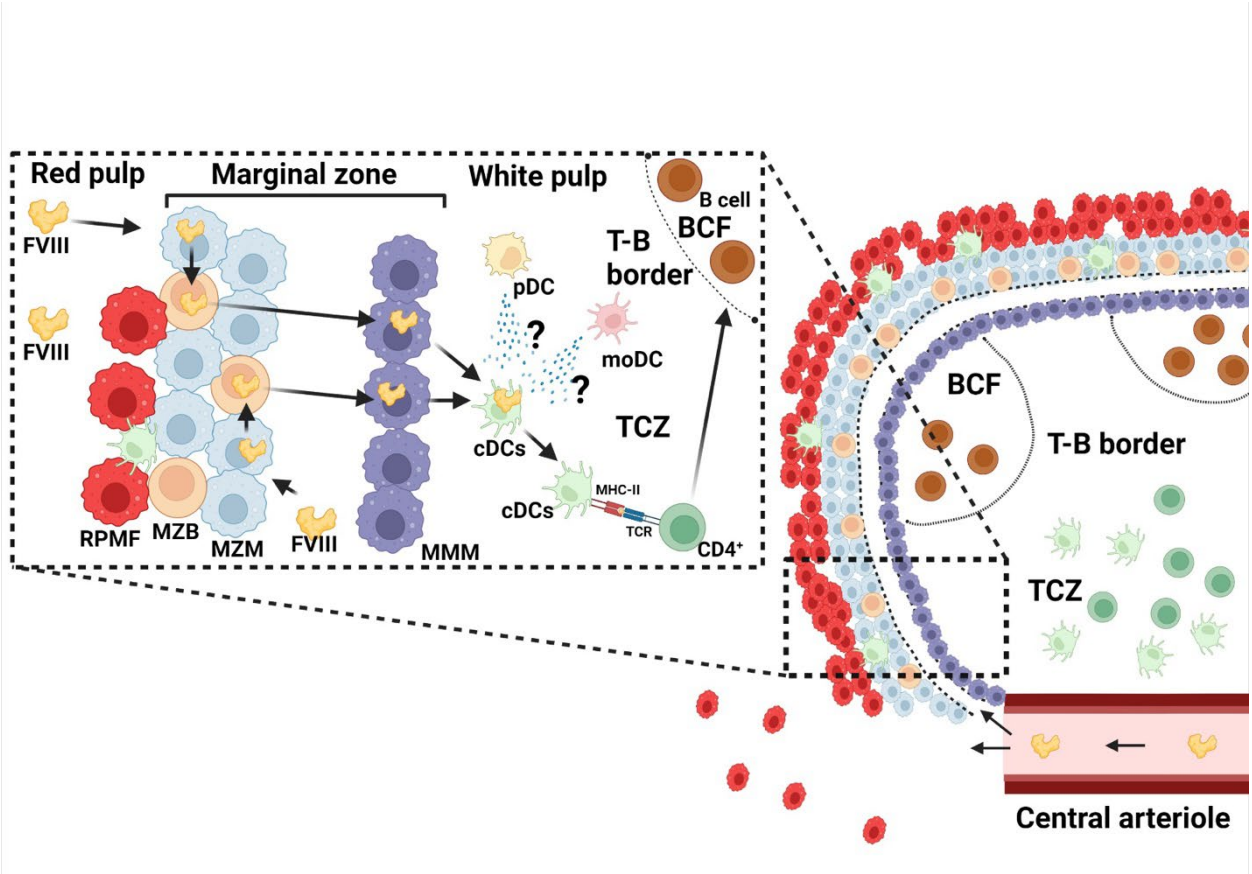
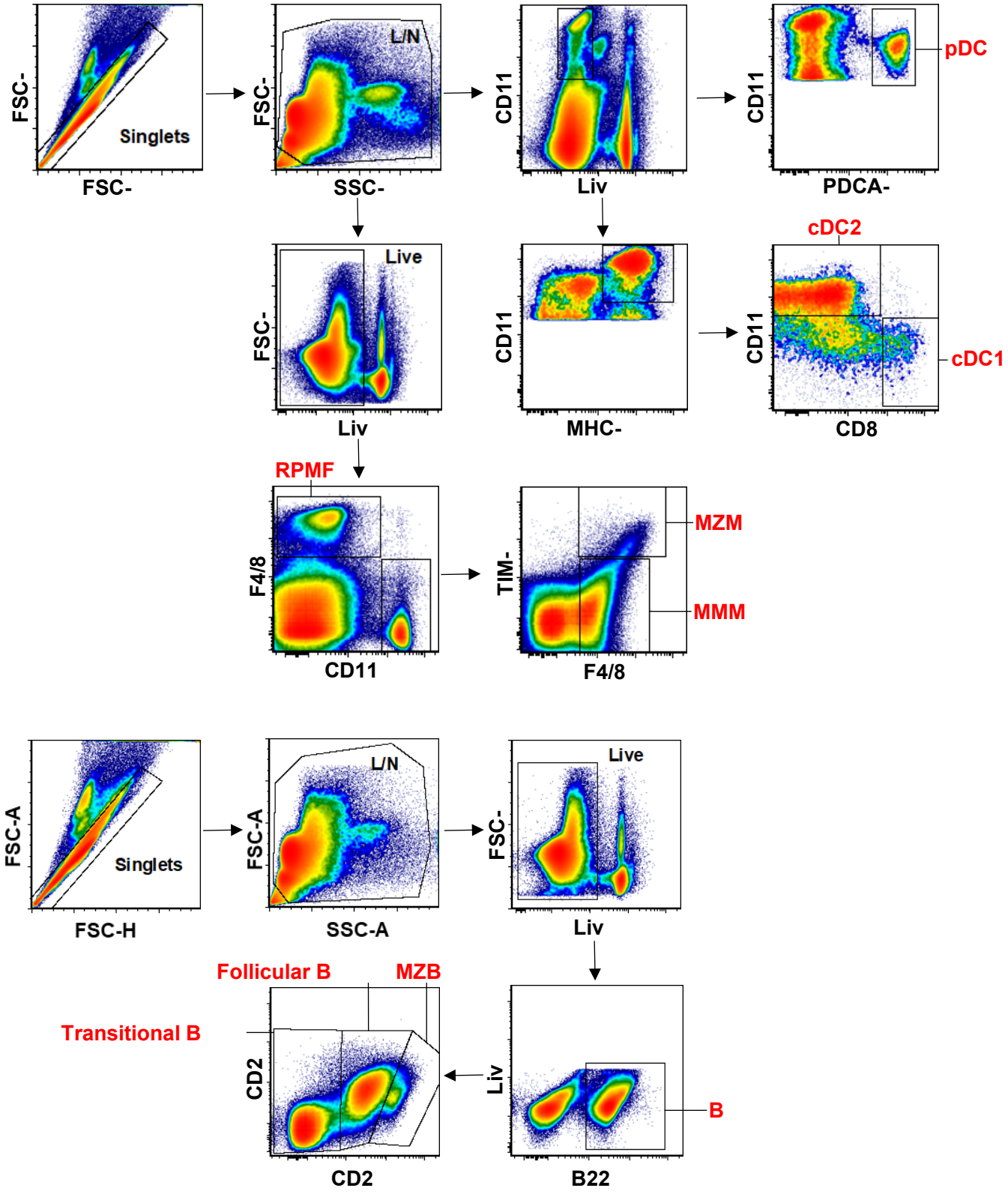


Fig. S8. A proposed model of FVIII immune response mechanism. Upon exiting central arterioles in the spleen and entering the marginal zone sinus and the red pulp, FVIII is likely captured by marginal zone macrophages (MZMs) which cooperate with marginal zone B (MZB) cells and marginal metalophilic macrophages (MMMs) to shuttle FVIII across the marginal zone to the white pulp, possibly in a bucket-relay like manner. Once in the white pulp, FVIII is presented by conventional dendritic cells (cDCs) to CD4+ T cells in the T cell zone (TCZ) with bystander involvement of plasmacytoid (pDCs) and monocyte-derived dendritic cells (moDCs). Then, primed $CD4^+$ T cells differentiate into follicular helper T (Tfh) cells and relocate to the T-B border to provide help to B cells in the B cell follicles (BCF). Created with BioRender.com

A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CD11
cDC1
CD8
RPMF
F4/8
CD11
TIM-
MZM
MMM
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CD2
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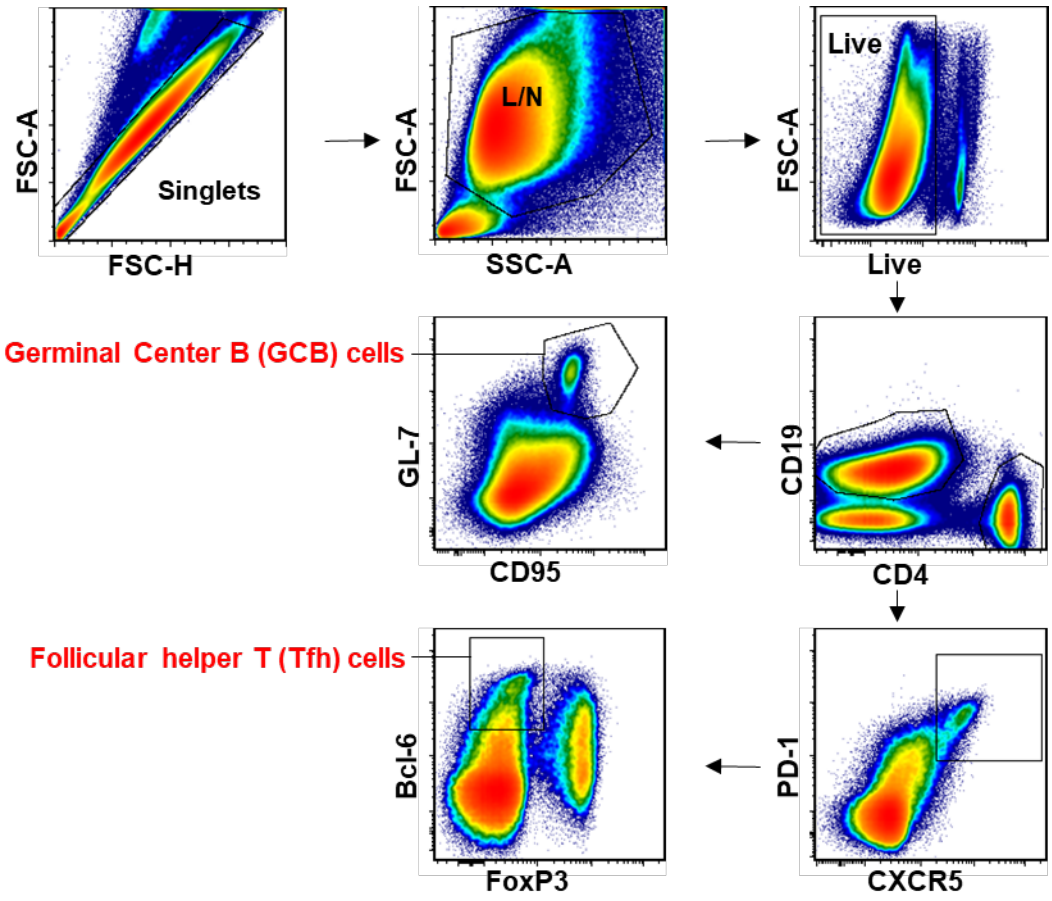
B22

B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Follicular helper T (Tfh) cells

C

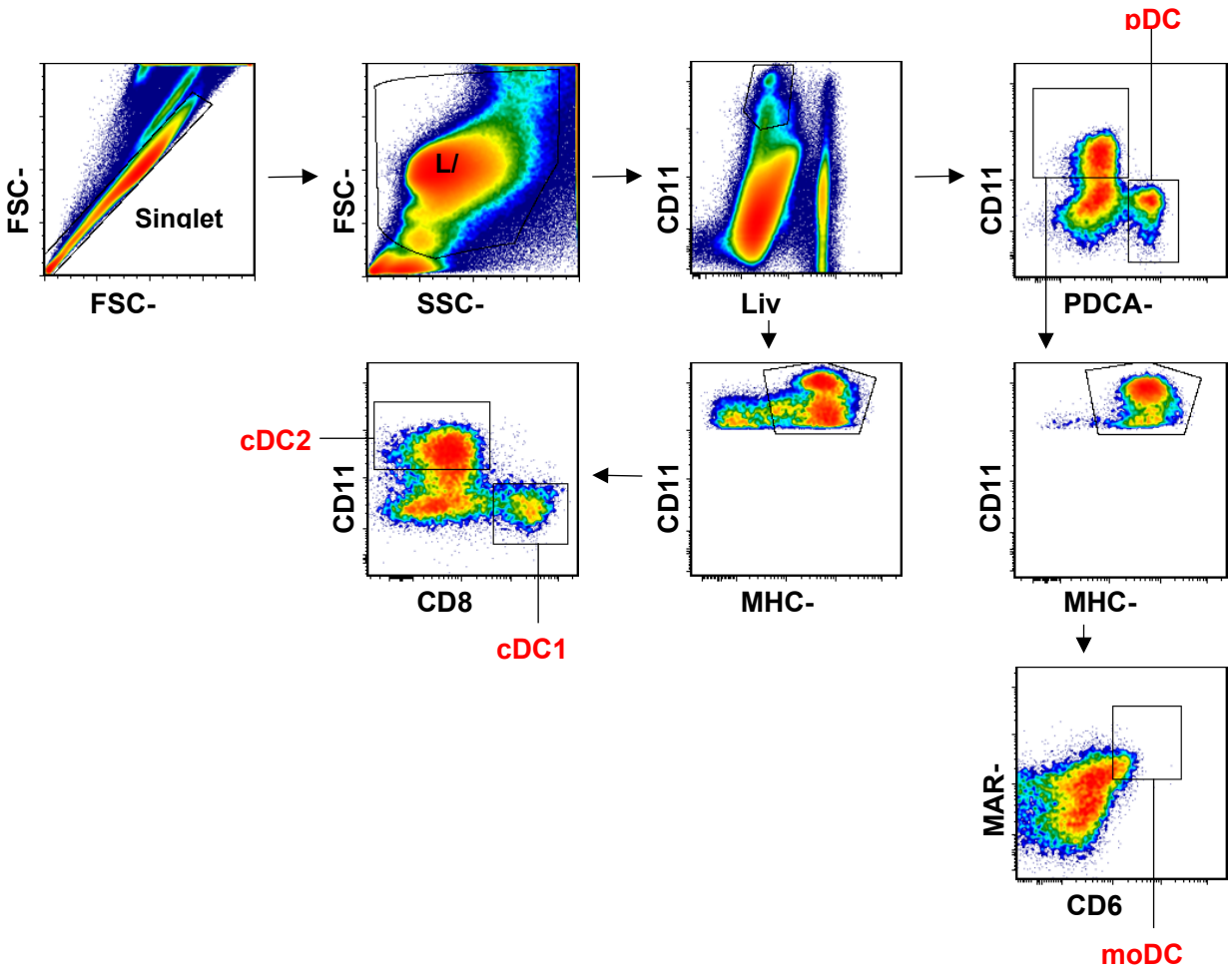

Fig. S9. Flow cytometry gating schemes. (A) Gating scheme for FVIII and OVA uptake analysis. Conventional DC1s were defined as CD11$^{med/hi}$MHC-II^{+}CD8α^{+}CD11b^{-}, cDC2s as CD11c$^{med/hi}$MHC-II^{+}CD8α^{-}CD11b^{+}, pDCs as CD11c$^{med/hi}$PDCA-1^{+}, RPMFs as F4/80^{hi}CD11b^{-}, MZMs as F4/80^{lo}CD11b^{+}TIM-4^{hi}, MMMs as F4/80^{lo}CD11b^{+}TIM-4^{lo}, B cells as B220^{+}, MZB cells as B220^{+}CD21^{hi}CD23$^{lo/-}$, follicular B cells as B220^{+}CD21^{med}CD23$^{lo/-}$, and transitional B cells as B220^{+}CD21^{-}CD23$^{lo/-}$. (B) Gating scheme for Tfh and GCB cell analysis. Tfh cells were defined as CD4^{+}CXCR5^{+}PD1^{+}Bcl6^{+}FoxP3^{-} and GCB cells as CD19^{+}GL7^{+}CD95^{+}. (C) Gating scheme for DC phenotyping. Conventional DC1s were defined as CD11$^{med/hi}$MHC-II^{+}CD8α^{+}CD11b^{-}, cDC2s as CD11c$^{med/hi}$MHC-II^{+}CD8α^{-}CD11b^{+}, pDCs as CD11c$^{med/hi}$PDCA-1^{+} and moDCs as CD11c$^{med/hi}$MHC-II^{+}CD11b^{+}CD64^{+}MAR-1^{+}).

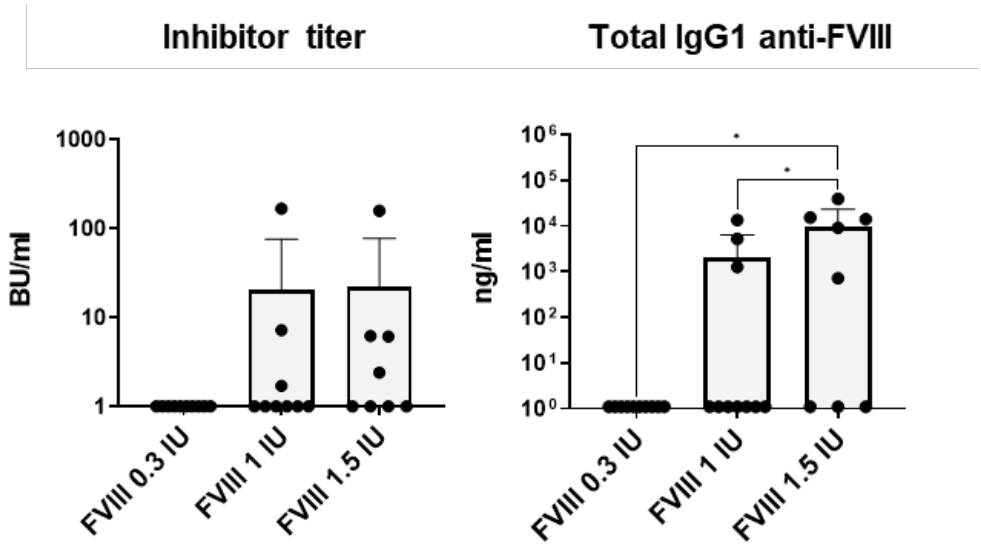

Fig. S10. FVIII dose-dependent inhibitor and total IgG1 anti-FVIII responses. S129/C57BL/6 HA mice (n=8-10/group) received 4 weekly injections of 0.3, 1.0 or 1.5 IU FVIII. Blood samples were collected one week after the last injection. Shown are means±SD and P values by analysis of variance.

Supplemental videos

Movie S1. Full-field view across an inguinal lymph node in a CD11c-DTR/GFP mouse 24h after adoptive transfer of CTV-stained CD4$^+$ T cells without FOVA administration. Intravital microscopy z-series showing CD11c$^+$GFP$^+$ cells (green), CTV$^+$CD4$^+$ cells (purple), and SHG signals from collagen (grey).

Movie S2. Motile CD4$^+$ T cells surveying the B cell follicle. Four-dimensional intravital imaging (Z-series projection over time) for 60 min, 5h after FOVA injection. Shown are CD11c$^+$GFP$^+$ (green) and CTV$^+$CD4$^+$ cells (purple).

Movie S3. CD4$^+$ T cells on the T-B border. Intravital microscopy z-series showing CD11c$^+$GFP$^+$ cells (green) and CTV$^+$CD4$^+$ cells (purple).