Supplemental Methods

ELISA and assessments of cytokine production

Mouse serum cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ and TNF-α) were measured by means using the bead-based immunofluorescence assay MilliplexKit (Millipore Corporation) using multiplex cytokine reagents. Measurements and data analysis were performed with xPonent software. TGF-β1 was measured by a specific ELISA kit (R&D Systems and Abnova Corporation) as described(24). Human cytokines (IFN-γ, IL-1β, IL-4, IL-6, IL-10, IL-12, IL-17, TGF-β1, TGF-β2, and TGF-β3) were detected in culture supernatants using a Bio-Plex human cytokine assay (Biorad), IP10 and IFNα were measured by specific ELISA kits (Abcam and PBL Assay Science). The lower and higher detection limits for each cytokine were (pg/ml)—IFN-γ, 1.14–23,200; IL-1β, 10–1,000; IL-4, 1.5–4,780; IL-6, 2.00–200; IL-10, 1.55–21,500; IL-12, 2.19–32,700; IL-17, 1.95–25,500; TGF-β1, 1.3–29,700; TGF-β2, 1.60–21,300; and TGF-β3, 1.10–15,600; IP10, 6.2–200; IFN-α, 6.1–500.Cell culture supernatants were analyzed for cytokine content at 24 (IL-1β, IL-4 and IL-6) or 48 (IL-10, IL-12, IL-17, IP-10, IFNα, TGF-β1, TGF-β2, TGF-β3, and IFN-γ) h. An ELISA-based TransAM Flexi NF-κB Family Kit (Active Motif) was used to monitor the activity of NF-κB family members, as described (33, 62).

L-kynurenine determination

Briefly, plasma samples and supernatants were collected and kept frozen at -20 °C until analysis. Tryptophan and kynurenine concentrations were measured by HPLC using 3-nitro-L-tyrosine as an internal standard. Frozen serum specimens were thawed at room temperature, 100 ml of serum was diluted with 100 µL of potassium phosphate buffer (0.05 mol/L, pH 6.0) containing the internal calibrator 3-nitro-L-tyrosine (100 µmol/L). Protein was precipitated with 50 µL of trichloroacetic acid (2 mol/L). The capped tubes with the precipitate were immediately vortex-mixed and centrifuged for 10 min at 13,000 × *g*. One hundred fifty microliters of the supernatants were transferred into microvials and placed into the autosampling device. The external calibrator was prepared from freshly thawed stock solutions of tryptophan and kynurenine (1 mmol/L in bidistilled water, stored at -20 °C) and albumin (70 g/L, which corresponds to the average physiological protein content in human serum). Fifty microliters of tryptophan, 10-µL kynurenine, and 940-µL albumin stock solution were mixed together. Aliquots of 200 µL calibrator preparations were then treated in the same way as the serum specimens. For separation, reversed-phase cartridges LiChroCART RP18 columns were used. Tryptophan was detected by a fluorescence detector at an excitation wavelength of 285 nm and an emission wavelength of 365 nm. A Shimadzu SPD-6A UV-detector in flow stream series connection was used for detection of both kynurenine and nitrotyrosine at a wavelength of 360 nm. The elution buffer was a degassed potassium phosphate solution (0.015 mol/L, pH 6.4) containing 27-mL/L

acetonitrile. Analyses were carried out at a flow rate of 0.9 ml/min and a temperature of 25 °C. To estimate IDO activity, the kynurenine-to-tryptophan ratio (kyn/trp) was calculated and expressed as µmol kynurenine/mmol tryptophan.

DNA isolation and SNP selection and genotyping

Genomic DNA from hemophilic patients and healthy controls was isolated from whole blood using the QIAamp DNA Blood Mini kit (Qiagen). Single-nucleotide polymorphisms (SNPs) in the human *TLR9* gene were selected, based on previously published evidence of functional consequences(M). No SNPs in *IDO1* are currently known as being associated with altered IDO1 immune regulatory function, and none was thus examined. Specifically, we analyzed rs187084, rs5743836 and rs352140 SNPs. Genotyping was performed using SNP-specific assays and KASPar chemistry (KBioscience) according to manufacturer's instructions. For internal quality control, about 5% of samples were randomly selected and included as duplicates. Concordance between the original and the duplicate samples for the three SNPs analyzed was ≥99%.

Real-Time RT-PCR

Real-Time RT-PCR (for mouse *Ido1*, *Bcl6, Prdm1, Pax5, Xbp1 a*nd *Gapdh* and human *IDO1*, *TLR9*, *TBX21*, *GATA3*, RORC, *FOXP3* and *ACTB*) analyses were carried out as described(24), using primers listed in **Supplementary Table 4**. RT-PCR data were calculated as the ratio of gene to *ACTB* (for human cells) or to *Gapdh* (for mouse cells) expressions, by the relative quantification method (ΔΔCT; means ± s.d. of triplicate determinations), and data are presented as normalized transcript expression in the samples relative to normalized transcript expression in control cultures (in which fold change = 1; dotted line).

1. Munde EO, et al. Polymorphisms in the Fc gamma receptor IIIA and Toll-like receptor 9 are associated with protection against severe malarial anemia and changes in circulating γ interferon levels. Infect Immun. 2012;80(12):4435–4443.

Supplementary Table 1

(%)	Inhibitor-free	Inhibitor-positive
Monocytes ($CD14^+$)	17.2± 6.7	18.1 ± 10.3
mDCs ($CD11c^+/BDCA1^+$, $CD11c^+/BDCA3^+$)	1.1 ± 0.4	1.3 ± 0.6
pDCs ($CD123^+/BDCA4^+$)	0.4 ± 0.1	0.3± 0.2
T cells ($CD3^+$)	62.6 ± 16.7	59.5 ± 14.6
B cells ($CD19^+$)	18.7 ± 9.4	20.8 ± 7.8

Percentages of different cells found in PMBCs from inhibitor-positive and-negative patients. Data are presented as means ± s.d.

Supplementary Table 2. IDO competence in hemophilia A patients

IDO competence	Inhibitor negative	Inhibitor positive	Total
Yes			
mRNA (+), Protein (+), Activity (+)	36	18	54
No			
total	14	32	46
mRNA (-), Protein (-), Activity (-)	13	27	40
mRNA (+), Protein (-), Activity (-)	1	3	4
mRNA (+), Protein (+), Activity (-)	0	2	2

+, presence; -, absence Pearson χ^2 = 17.4436 Pr = 0.001

Supplementary Table 3. *TLR9* SNPs in hemophilic patients according to IDO1 induction*

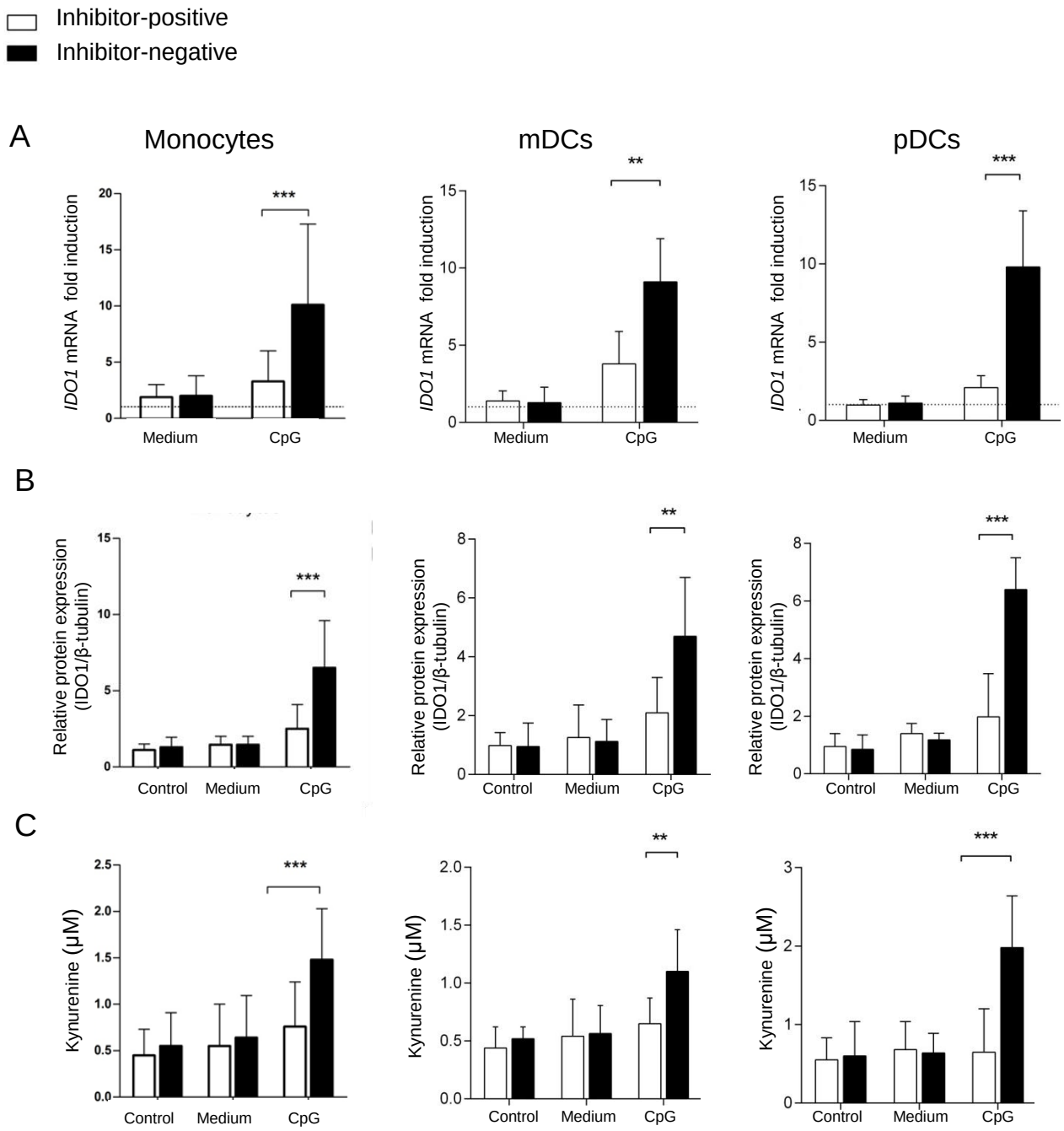
Gene	SNP rs# number	Genotype	Hemophilic patients	
			IDO^+ (N = 54)	IDO^- (N = 46)
TLR9	rs187084	T/T	15	20
		T/C	24	20
		C/C	4	5
	rs5743836	T/T	35	37
		T/C	7	7
		C/C	1	0
	rs352140	C/C	15	13
		C/T	17	23
		T/T	11	9

**TLR9,* Toll-Like Receptor 9-encoding gene

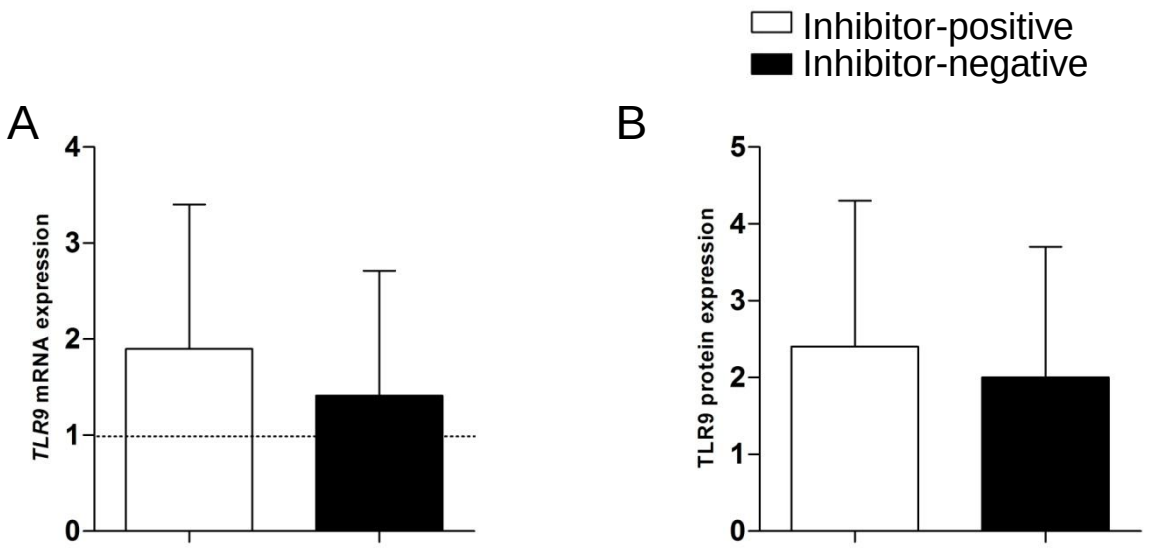
Supplementary Table 4. Primers used in Real-Time reverse transcriptase PCR

GENE	FORWARD SEQUENCE	REVERSE SEQUENCE
Gapdh	5' - CTG CCC AGA ACA TCA TCC CT -3'	5' - ACT TGG CAG GTT TCT CCA GG -3'
Ido1	5’ - GTG GGC TTT GCT CTA CCA CA -3’	5’ - AAG CTG CCC GTT CTC AAT CA -3’
Rorc	5’ - ACA ACA GCA GCA AGT GAT GG - 3’	5’ - CCT GGA TTT ATC CCT GCT GA - 3’
Foxp3	5’ - CCC AGG AAA GAC AGC AAC CTT TT -3’	5’ - TTC TCA CAA CCA GGC CAC TTG - 3’
Il17a	5’ – GACTACCTCAACCGTTCCAC -3’	5’ – CCTCCGCATTGACACAGC - 3’
Bcl6	5' - CGC GAA CCT TGA TCT CCA GT - 3'	5' - TGA CTC TCA CTG CTG CTT CG -3'
Prdm1	5' - AGG GTT TTA CTC AGC TCG CC - 3'	5' - GTA AAC TTG GCA GGG CAC AC -3'
Pax5	5' - CCT ATT GTC ACA GGC CGA GA -3'	5' - AAA TTC ACT CCC AGG CAC CA -3'
Xbp1	5' - AAT GGA CAC GCT GGA TCC TG -3'	5' - ACA TAG TCT GAG TGC TGC GG -3'

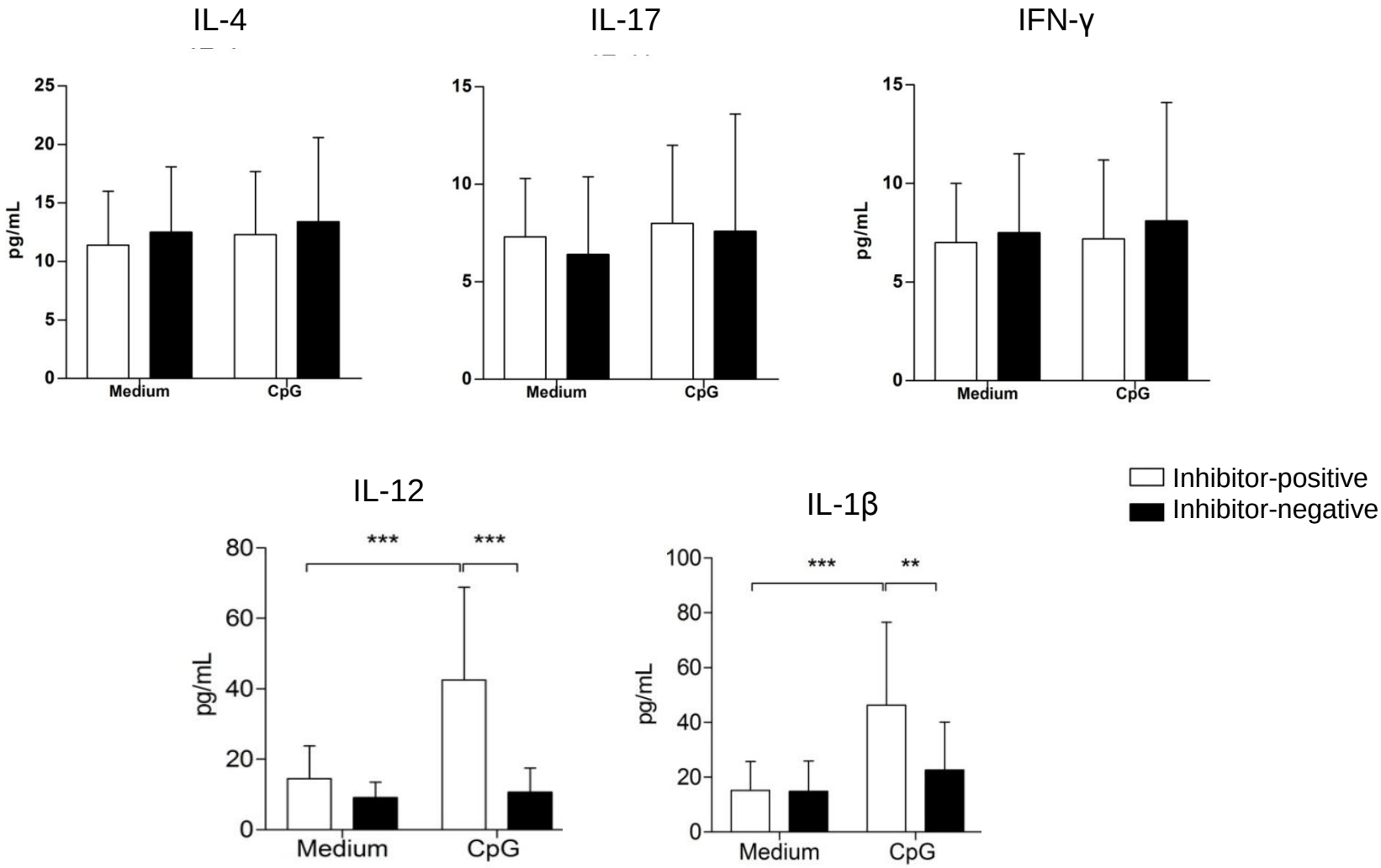
GENE	FORWARD SEQUENCE	REVERSE SEQUENCE
β-ACTIN	5’-CTCGTCGTCGACAACGGCT-3’	5’-TCAGGGTGAGGATGCCTCTC-3’
IDO1	5’-TCACAGACCACAAGTCACAG-3’	5’-GCAAGACCTTACGGACATCT-3’
TLR9	5’-TCAGCCATACCAACATCCT-3’	5’-GTAATAACAGTTGCCGTCCAT-3’
TBX21	5' - AAC CCA GTT CAT TGC CGT GA - 3'	5' - GGG ATG CTG GTG TCA ACA GA -3'
GATA3	5' - ACA GAA CCG GCC CCT CAT TA -3'	5' - CAG AGT GTG GTT GTG GTG GT - 3'
RORC	5' - AGG CCA TTC AGT ACG TGG TG -3'	5' - TGC CAC CGT ATT TGC CTT CA - 3'
FOXP3	5' - TGT GGG GTA GCC ATG GAA AC -3'	5' - TCA TTG AGT GTC CGC TGC TT -3'



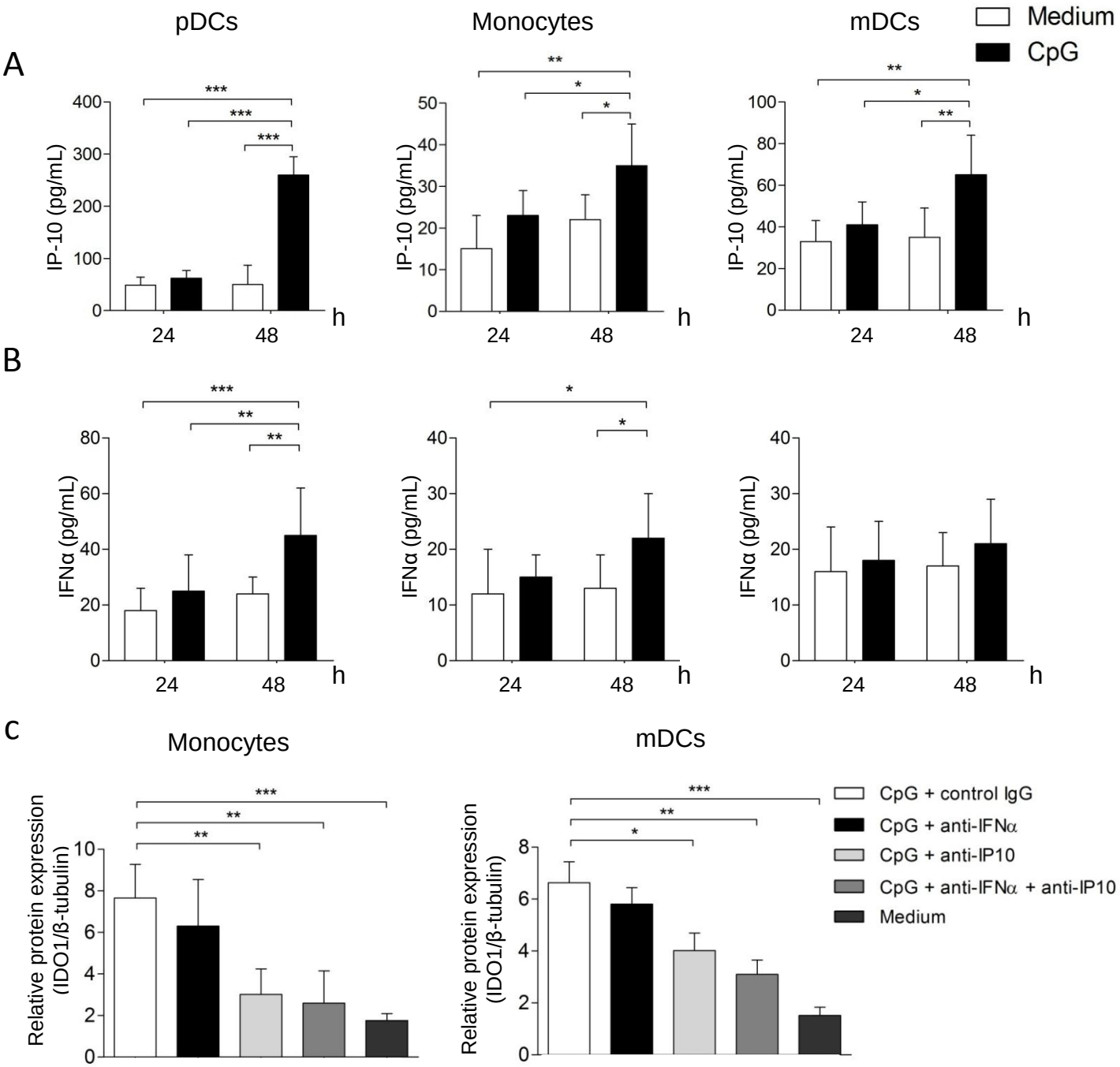
Supplementary Fig. 1. IDO1 induction by CpG-ODN in cell subsets from hemophilic patients with (N = 50) or without (N = 50) inhibitors. (**A**) pDCs, mDCs and monocytes were purified from PBMCs and cultured with CpG-ODN or medium alone. Expression of mRNA is presented relative to expression in the respective freshly harvested cell subsets (in which fold change = 1; dotted line). Data are means ± s.d., with ** $P < 0.01$, *** $P < 0.001$. (**B**) pDCs, mDCs and monocytes were purified from PBMCs and cultured as in **A**, and IDO1 protein expression was analyzed by immunoblotting. IDO1 protein expression was quantified by densitometry, as normalized to β-tubulin. Data are presented as means ± s.d., with **$P < 0.01$, ***$P < 0.001$. (**C**) pDCs, mDCs and monocytes were cultured with CpG-ODN or in medium alone. Culture supernatants were collected after 48 h and L-kynurenine was measured by HPLC. Results are presented as mean ± s.d., with **$P < 0.01$, ***$P < 0.001$ (ANOVA and Bonferroni multiple comparison test).



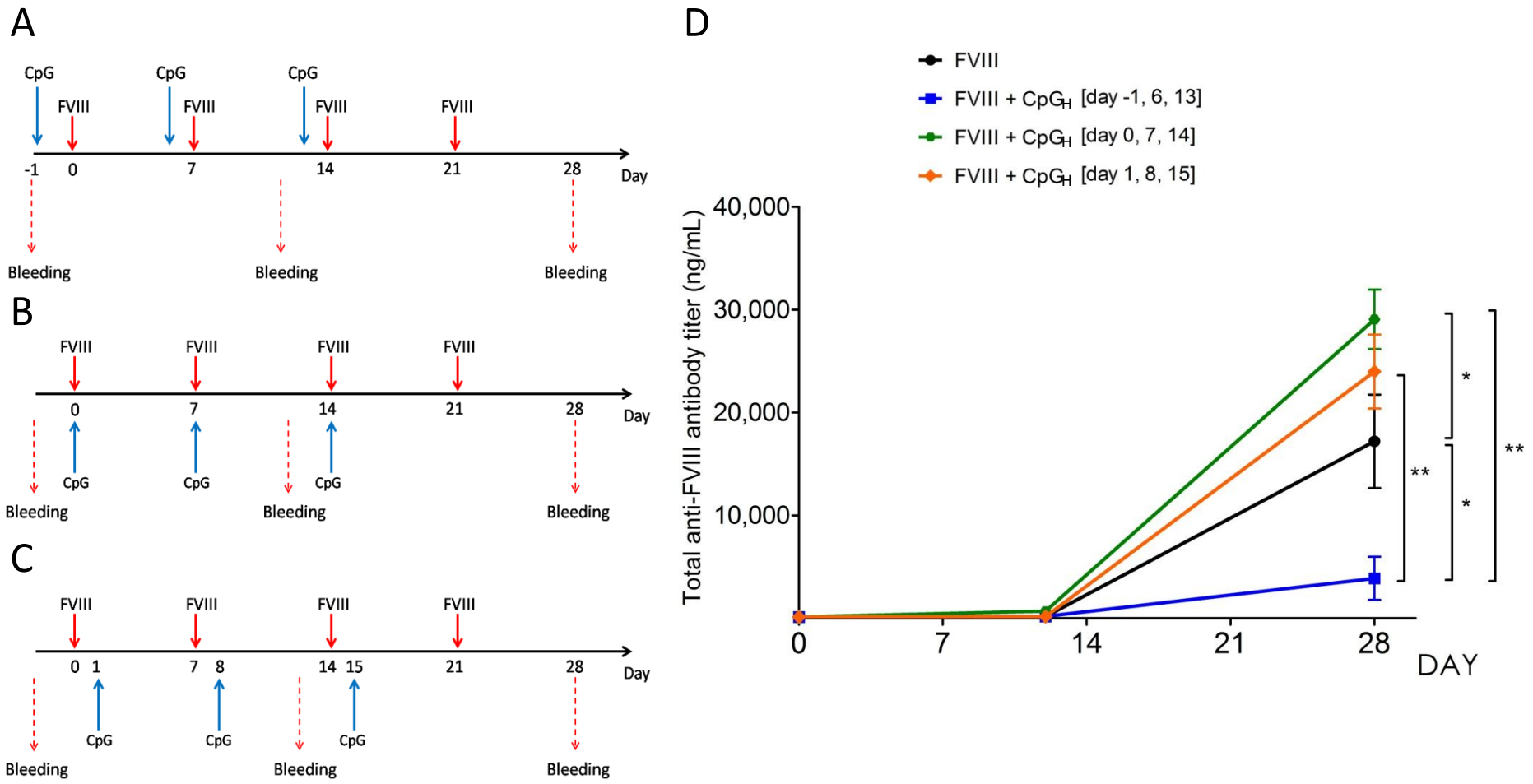
Supplementary Fig. 2. TLR9 expression in PBMCs from hemophilic patients with (N = 50) or without (N = 50) inhibitors. (**A**) PBMCs were purified and expression of *TLR9* mRNA was quantified. Data presented are relative to transcript expression in healthy controls (in which fold change = 1; dotted line); mean ± s.d. (**B**) TLR9 protein expression in PBMCs was quantified by densitometry, as normalized to β-tubulin, and data are presented as means ± s.d. Groups were compared with two-tailed Student's t-test.



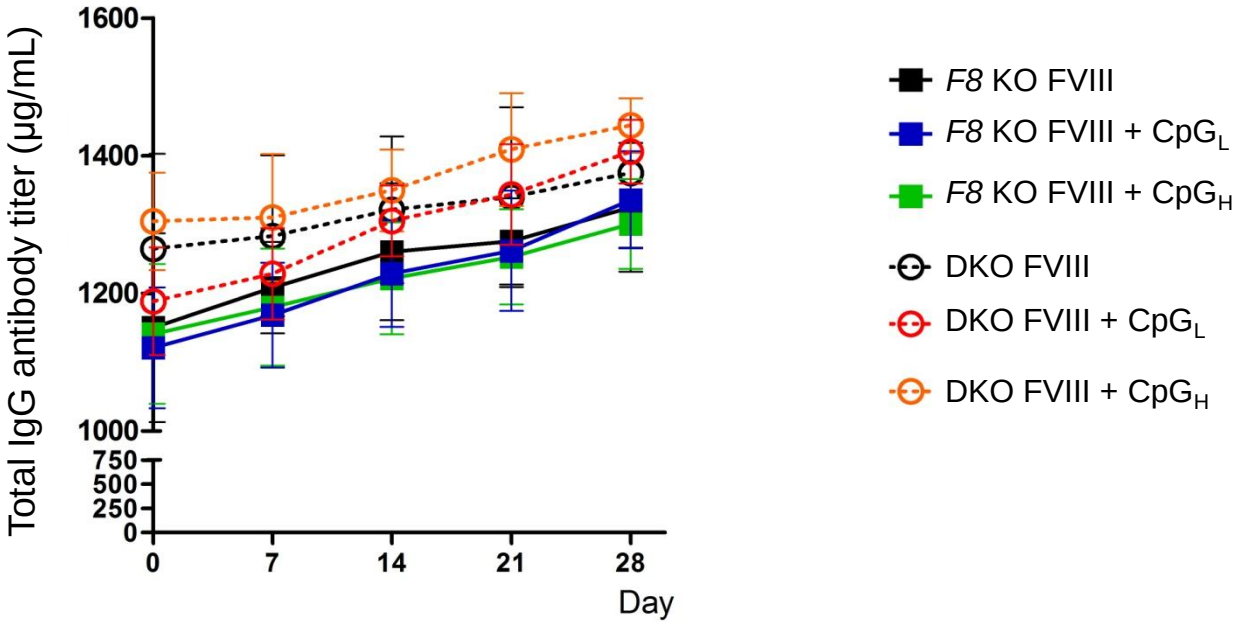
Supplementary Fig. 3. Cytokine production in response to CpG-ODN in hemophilic patients with (N = 50) or without (N = 50) inhibitors. PBMCs were cultured with CpG- ODN or medium alone. Supernatants were collected and analyzed for cytokines production after 24 (IL-4, IL-1β) or 48 h (IL-12, IL-17, IFN-γ). Data are represented as mean ± sd, with $^{**}P < 0.01$, $^{***}P < 0.001$ (ANOVA and Bonferroni multiple comparison test).



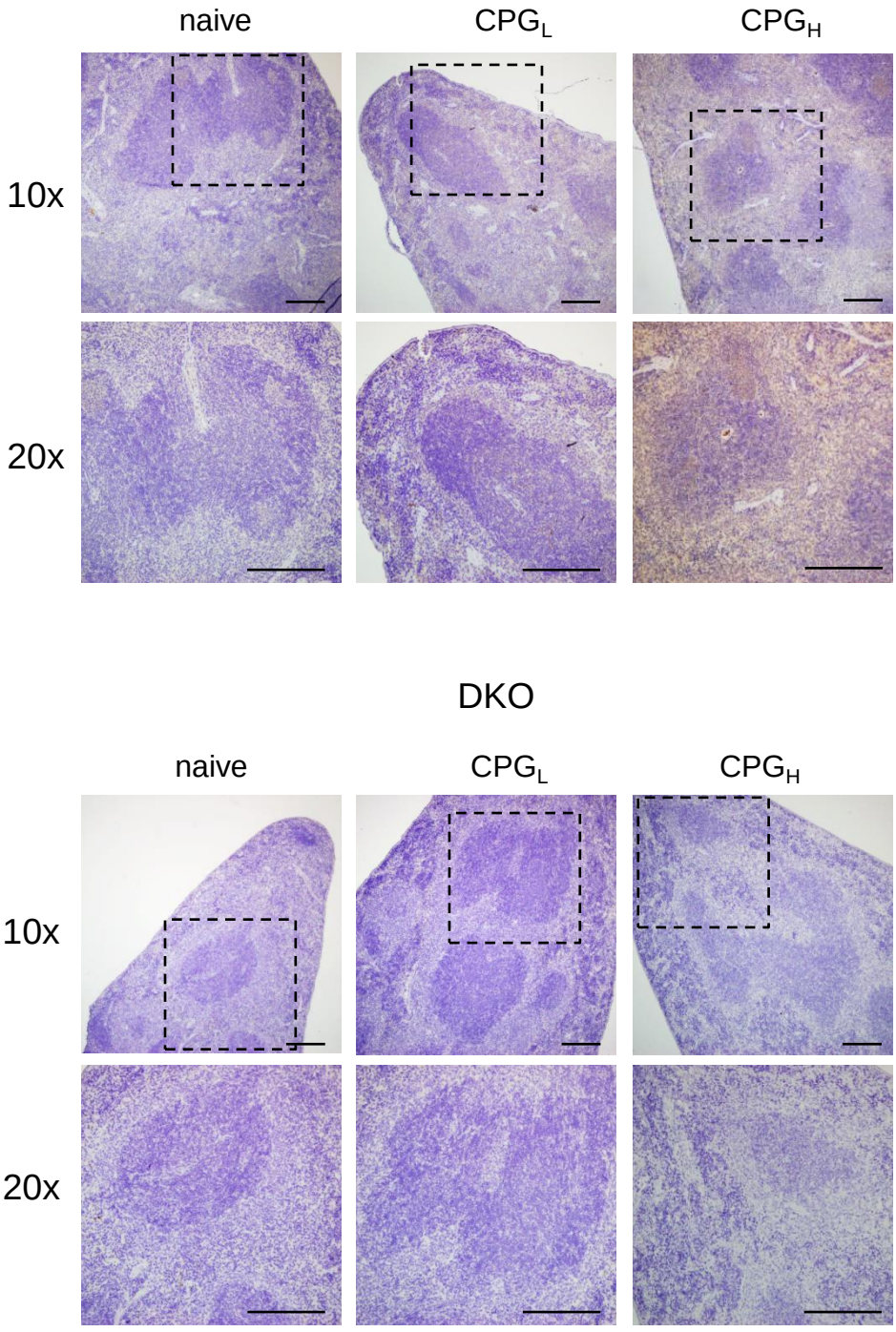
Supplementary Fig. 4. Cytokine production in response to CpG-ODN in Monocytes, pDCs and mDCs purified from PBMCs of hemophilic. Cells purified from patients without inhibitors (N = 30) were cultured with CpG- ODN or medium alone and supernatants were collected and analyzed for IFNα (**A**) and IP-10 (**B**). (**C**) Neutralization of both IFNα or IP-10 reverses IDO1 induction by CpG Monoytes and mDCs. Monocytes and mDCs, purified as in **A** were stimulated with CpG-ODN in the presence or absence of antibodies to IFNα or IP-10 or their combination, isotype-matched antibodies served as control treatments. IDO1 protein expression was analyzed by immunoblotting and quantified by densitometry as normalized to β-tubulin induction. Data are represented as mean ± sd., with **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (ANOVA and Bonferroni multiple comparison test).



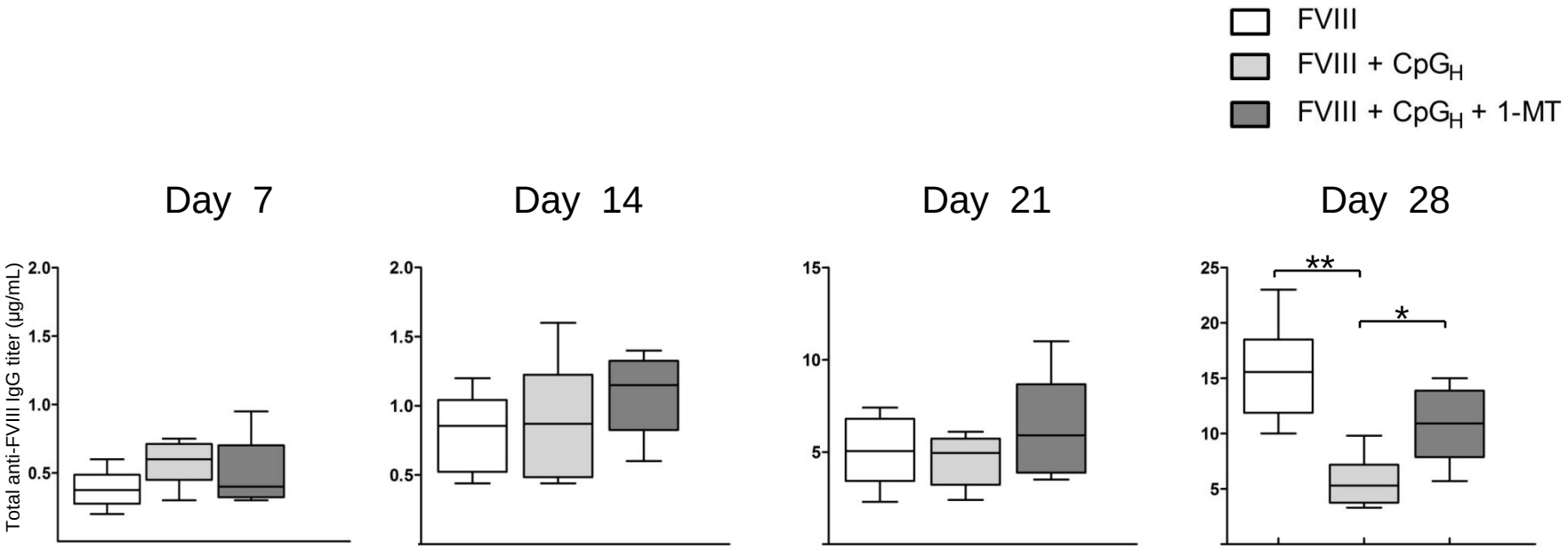
Supplementary Fig. 5. Treatment with CpG_H suppresses anti-FVIII antibody titers only when given before rhFVIII administration. *F8* KO mice (n = 6) received intravenous rhFVIII (200 ng; weekly). In the first three weeks, CpG_H was given 24 h before (**A**), at the same time (**B**) or 24 h after (**C**) rhFVIII administration. (**D**) The anti-FVIII antibody response was measured by ELISA at the indicated days. Data are represented as mean ± sd., with $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (ANOVA and Bonferroni multiple comparison test).



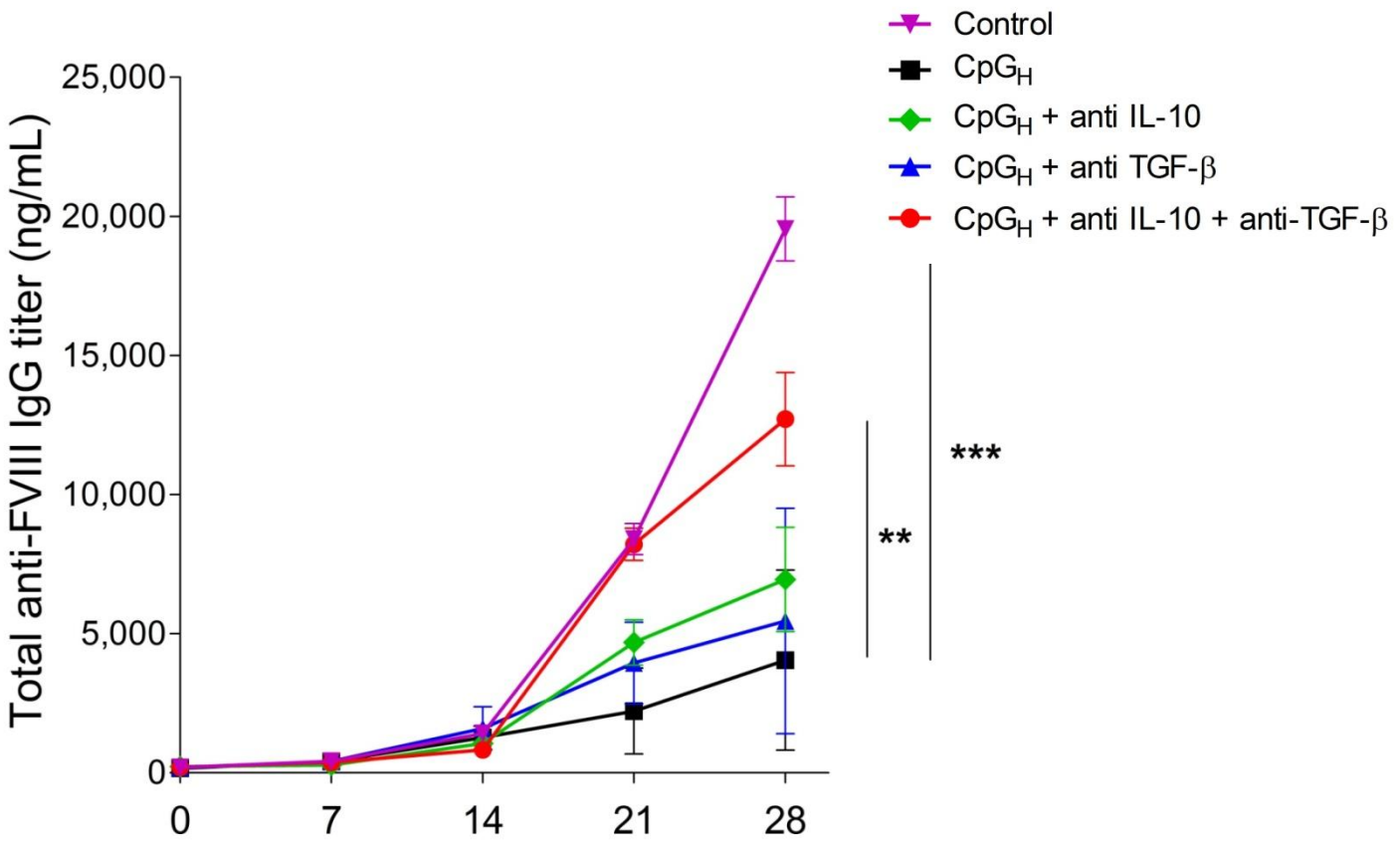
Supplementary Fig. 6. Total IgG titers in *F8* KO and DKO mice treated with CpG_L or CpG_H and rhFVIII. *F8* KO and DKO mice (n = 8) received intravenous rhFVIII (200 ng; weekly). In the first three weeks, CpG_H, CpG_L or vehicle (PBS) was given 24 h before FVIII administration. Serum total IgG levels were measured by ELISA. Data are presented as means ± s.d. from three experiments (ANOVA and Bonferroni multiple comparison test).



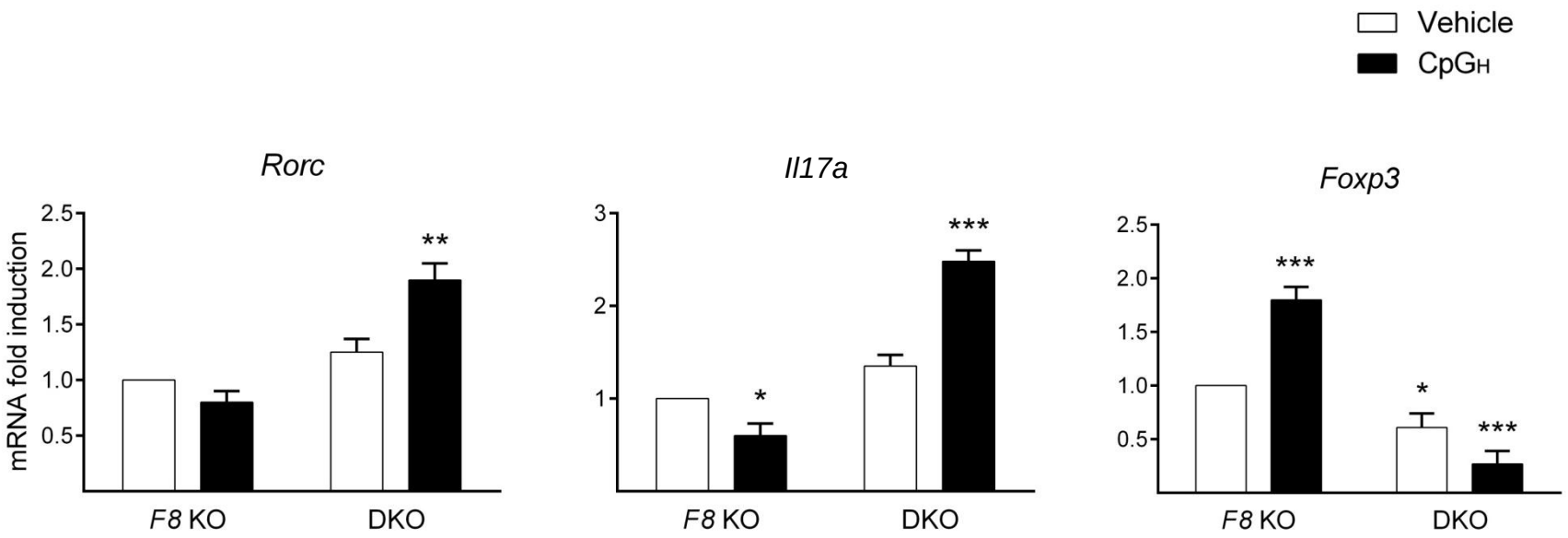
Supplementary Fig. 7. CpG$_H$ promotes IDO1 expression in specific areas of the spleen**.** Spleens from vehicle- or CpG$_H$-stimulated mice were analyzed by immunohistochemistry for IDO1 expression. Cell nuclei were counterstained with haematoxylin. Representative images of three different experiments, with three mice per group per experiment. Bars 200μm.



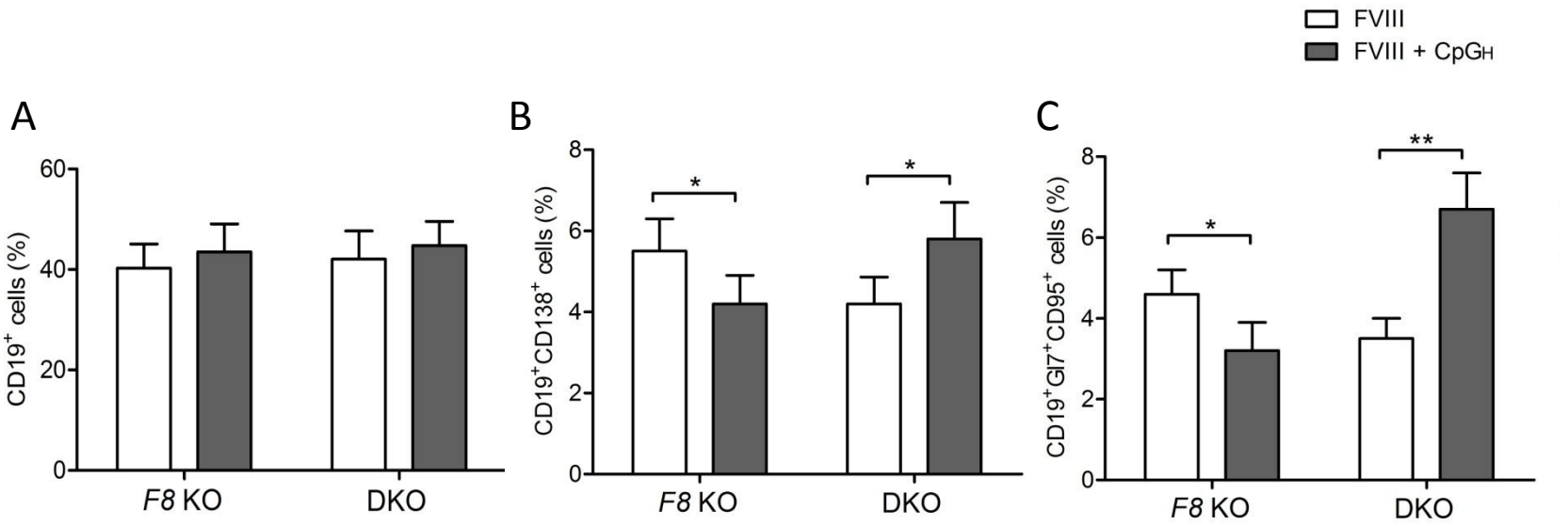
Supplementary Fig. 8. Administration of 1-methyl-tryptophan (1-MT) reverses the protective effect of CpG_H in hemophilic mice. *F8* KO (*n* = 8) received intravenous rhFVIII (200 ng; weekly). In the first three weeks, CpG_H alone or in combination with 1-MT was given 24 h before FVIII administration. The anti-FVIII antibody response was measured by ELISA on the indicated days. Vertical middle lines represent the first and the third quartiles, horizontal lines indicating the median values (with whiskers being minimum and maximum values); three independent experiments. **P* < 0.05, ***P* < 0.01. ANOVA followed by Bonferroni multiple comparison test.



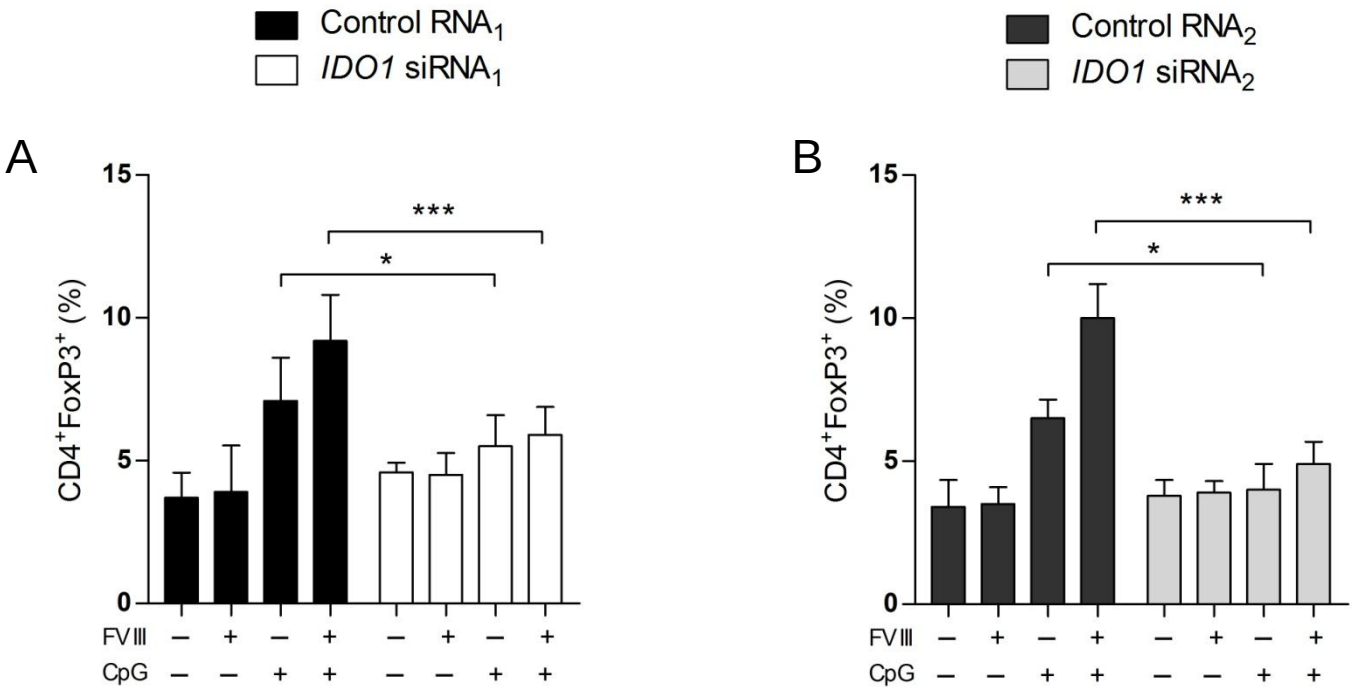
Supplementary Fig. 9. Neutralization of both TGF-β and IL-10 reverses the protective effect of CpG$_H$ in hemophilic mice. *F8* KO (*n* = 8) received intravenous rhFVIII as described in **Fig. 2A**. Neutralizing antibodies to IL-10 (200 µg/mouse) or TGF-β (100 µg/mouse), or their combination (250 ng /mouse) were given 24 h before FVIII administration, isotype-matched antibodies serving as control treatments. The anti-FVIII antibody response was measured by ELISA on the indicated days. Means s.d. of three independent experiments. ***P* < 0.01, ****P* < 0.001 by ANOVA followed by Bonferroni multiple comparison test.



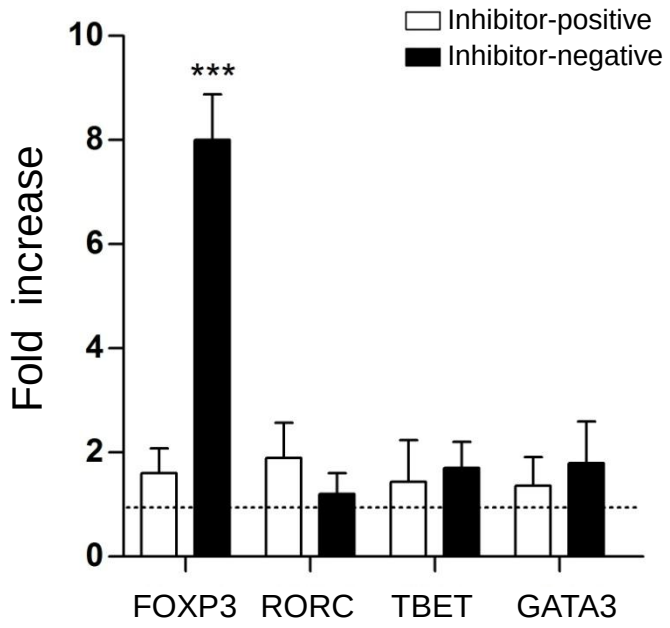
Supplementary Fig. 10. CpG_H administration induces Treg when IDO1 is active.
Mice were treated with vehicle or CpG_H. RT-PCR expression of *Rorc*, *Il17a* and *Foxp3* transcripts were evaluated in splenic $CD4^+$ cells from hemophilic mice 24 h after the second treatment with vehicle or CpG_H. Data presented are relative to transcript expression in $CD4^+$ T cells from *F8* KO mice and are reported as means ± s.d. (n = 3; **P* < 0.05, ***P* < 0.01, ****P* < 0.01; two-tailed Student's *t*-test).



Supplementary Fig. 11. Administration of CpG_H reduces activated B cells and plasma cells in *Ido1*-competent hemophilic mice. *F8* KO or DKO (n = 8) received intravenous rhFVIII (200 ng; weekly). In the first three weeks, CpG_H or vehicle was given 24 h before FVIII administration. The percent (%) of total (A), plasma cells (B) and activated B cells (C) were assessed by means of citofluorimetric analysis at day 20. Means s.d. of three independent experiments. *P < 0.05, **P < 0.01, two-tailed Student's t-test.



Supplementary Fig. 12. *IDO1* silencing in FL-DCs prevents CpG_H-dependent induction of FVIII-specific Treg cells in hemophilia A patients. $CD4^+$ $FOXP3^+$ cell frequency in $CD4^+$ T cells, co-cultured (for 5 days) with DCs (either untreated or treated with CpG) that had been transfected with $control_1$ or *IDO1*-specific $siRNA_1$ (**A**) or $control_2$ or *IDO1*-specific $siRNA_2$ (**B**) . FVIII was present in a portion of the co-cultures. $N = 5$. Data are presented as means ± s.d., with $^*P < 0.05$ and $^{***}P < 0.001$ (ANOVA followed by Bonferroni multiple comparison test) .



Supplementary Fig. 13. CpG_H-treated FL-DCs increase FOXP3 expression in CD4+ T cells from inhibitor-positive hemophilia A patients. T-helper cell transcription factors transcript levels in $CD4^+$ T cells cultured with FL-DCs from, either untreated or treated with CpG-ODN (3 µg/mL); *N* = 5. mRNA of each transcription factor is presented relative to expression in the respective, freshly isolated $CD4^+$ (in which fold change = 1; dotted line). Data are means ± s.d. of three experiments. ***P < 0.001; two-tailed Student's t-test.