JEM

Supplemental material

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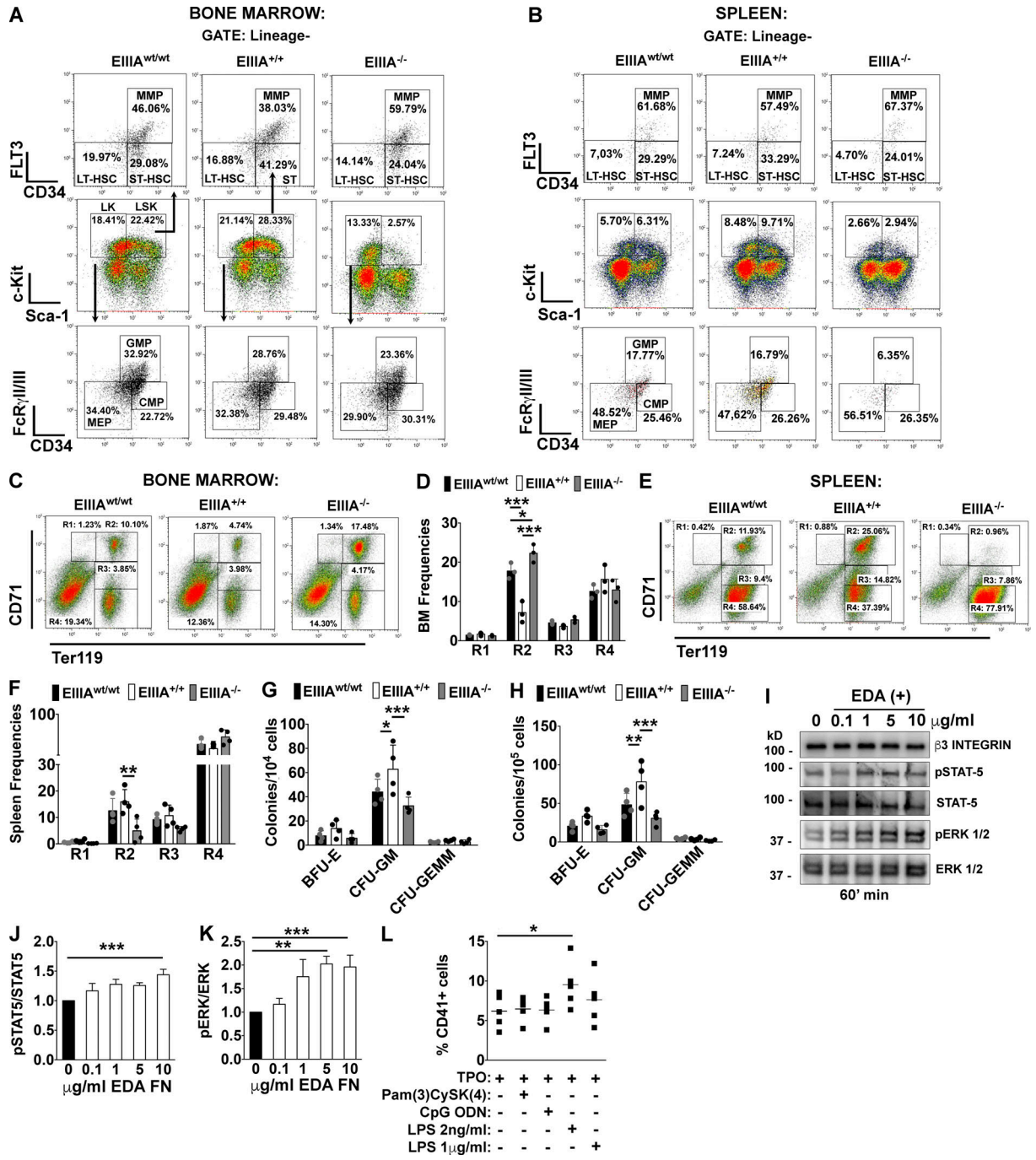

Figure S1. **Effects of EDA overexpression on hematopoietic progenitors in mice and concentration-dependent effects on Mk signaling pathway activation. (A and B)** Representative contour plots and gate strategy of flow cytometric analysis of LSK, LT-HSC ($Lin^-Sca\text{-}1^+c\text{-}kit^+CD34^-CD135^-$), ST-HSC ($Lin^-Sca\text{-}1^+c\text{-}kit^+CD34^+CD135^-$), MMP ($Lin^-Sca\text{-}1^+c\text{-}kit^+CD34^+CD135^+$), CMP ($Lin^-Sca\text{-}1^-c\text{-}kit^+CD34^+FcγRII/II^{low}$), GMP ($Lin^-Sca\text{-}1^-c\text{-}kit^+CD34^+FcγRII/II^{high}$), and MEP ($Lin^-Sca\text{-}1^-c\text{-}kit^+CD34^-FcγRII/III^-$) cells in the BM (A) and spleens (B) of $EIIIA^{wt/wt}$, $EIIIA^{+/+}$, and $EIIIA^{-/-}$ mice after experimental fibrosis. **(C and D)** Representative dot plots of flow cytometric analysis of erythroid precursors in the BM of $EIIIA^{wt/wt}$, $EIIIA^{+/+}$ and $EIIIA^{-/-}$ mice after experimental fibrosis using surface markers CD71 and Ter119 (C). Frequencies of erythroid precursor cells are shown in bar graphs as mean ± SD (n = 3; D). **(E and F)** Representative dot plots of flow cytometric analysis of erythroid precursors in the spleens of $EIIIA^{wt/wt}$, $EIIIA^{+/+}$, and $EIIIA^{-/-}$ mice after experimental fibrosis using surface markers CD71 and Ter119 (E). Frequencies of erythroid precursor cells are shown in bar graphs as mean ± SD (n = 3; F). **(G and H)** BM cells (10^4; G) and spleen cells (10^5; H) from $EIIIA^{wt/wt}$, $EIIIA^{+/+}$, and $EIIIA^{-/-}$ mice (n = 4) were plated in methylcellulose medium supplemented with cytokines. BFU-E, CFU-GM, and CFU-GEMM colonies were scored 7 d after plating. **(I)** Purified Mk's were stimulated for 1 h with increasing concentration of cFN and the phosphorylation of STAT-5 and ERK 1/2 evaluated by Western blotting. β3 integrin was revealed to ensure the same number of Mk used in the experiment, while total STAT-5 and ERK 1/2 were detected to ensure equal protein loading. **(J and K)** Histograms showing the ratio of phosphorylated and total STAT-5 (J) and ERK 1/2 (K) proteins in mature Mk's stimulated with increasing amounts of cFN for 1 h. At least three independent experiments were performed. **, $P < 0.01$; ***, $P < 0.001$. **(L)** Quantification of Mk output after 3 d of culture treated only with TPO, TPO plus 1 μg/ml of Pam3CsK4, TPO plus 3 μM CpG ODN, and TPO plus LPS (2 ng/ml and 1 μg/ml, respectively). Five independent experiments were performed. *, $P < 0.05$.

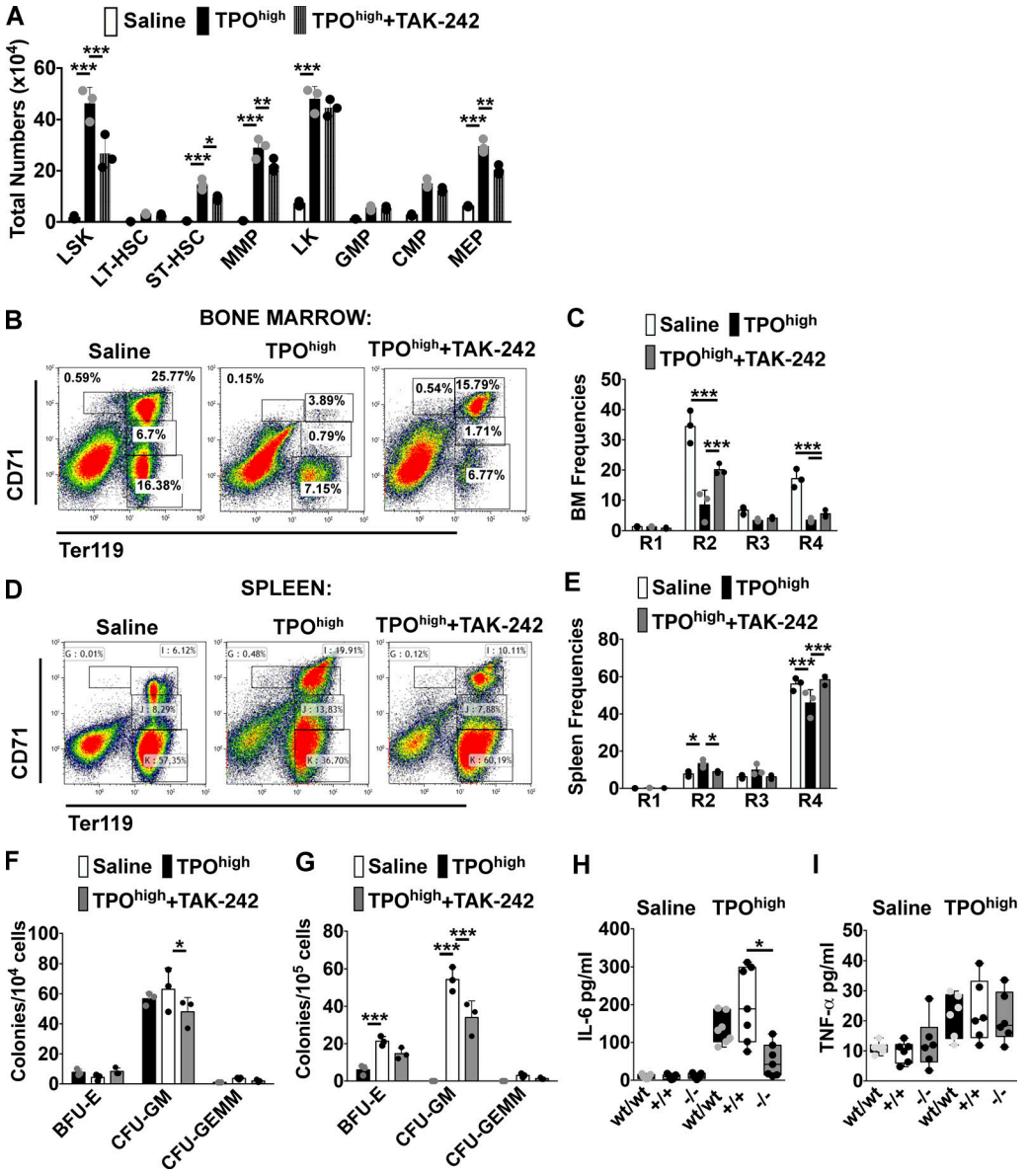

Figure S2. **Effects of TLR4 inhibition on hematopoietic progenitors in mice. (A)** Total numbers of LSK, LT-HSC, ST-HSC, MMP, CMP, GMP, and MEP cells in the spleens of saline-, TPO^{high}-, and TPO^{high} plus TAK-242–treated mice are shown in bar graphs as mean ± SD (n = 3). *, P < 0.05; **, P < 0.005; ***, P < 0.001. **(B and C)** Representative dot plots of flow cytometric analysis of erythroid precursors in the BM of saline-, TPO^{high}-, and TPO^{high} plus TAK-242–treated mice using surface markers CD71 and Ter119 (B). Frequencies of erythroid precursor cells are shown in bar graphs as mean ± SD (n = 3; C). **(D and E)** Representative dot plots of flow cytometric analysis of erythroid precursors in the BM of saline-, TPO^{high}-, and TPO^{high} plus TAK-242–treated mice using surface markers CD71 and Ter119 (D). Frequencies of erythroid precursor cells are shown in bar graphs as mean ± SD (n = 3; E). **(F and G)** BM cells (10^4; F) and spleen cells (10^5; G) of saline-, TPO^{high}-, and TPO^{high} plus TAK-242–treated mice (n = 3) were plated in methylcellulose medium supplemented with cytokines. BFU-E, CFU-GM, and CFU-GEMM colonies were scored 7 d after plating. **(H and I)** Quantification of TNF-α (H) and IL-6 (n = 7; I) by ELISA in BM cell-free supernatants of wild-type, EIIIA$^{+/+}$, and EIIIA$^{-/-}$ mice treated with saline or TPO. n = 7. *, P < 0.05.

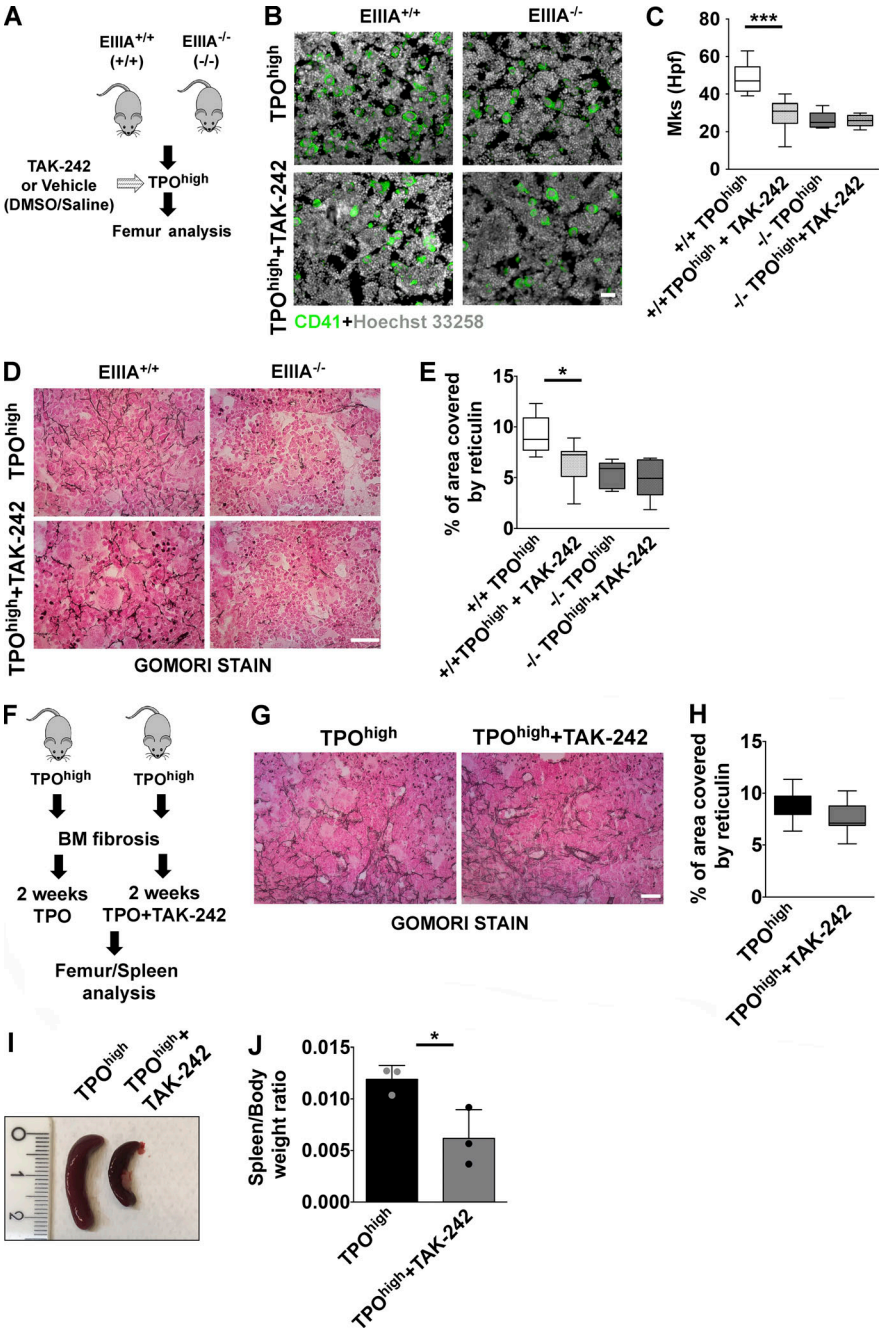

Figure S3. **TAK-242 treatment ameliorates Mk hyperplasia and BM fibrosis in EIIIA$^{+/+}$ mice while it has null effects in EIIIA$^{-/-}$ mice. (A)** Schematic representation of in vivo strategy for TLR4 inhibition in EIIIA$^{+/+}$ and EIIIA$^{-/-}$ mice during experimental fibrosis. **(B)** Representative immunofluorescence staining of CD41 Mk's in BM sections of EIIIA$^{+/+}$ and EIIIA$^{-/-}$ mice treated with TPO plus vehicle or TPO plus TAK-242. Nuclei were stained with Hoechst 33258. Bar, 50 µm. **(C)** Quantification of CD41^{+} Mk's per high power field (hpf) in BM sections of EIIIA$^{+/+}$ and EIIIA$^{-/-}$ mice treated with TPO plus vehicle or TPO plus TAK-242. ≥100 Mk's were randomly counted from five sections of three different mice per genotype in each experimental condition. ***, P < 0.001. **(D)** Representative Gomori staining in BM sections of EIIIA$^{+/+}$ and EIIIA$^{-/-}$ mice treated with TPO plus vehicle or TPO plus TAK-242. Bar, 50 µm. Objective, 40×. **(E)** Assessment of reticulin deposition in EIIIA$^{+/+}$ and EIIIA$^{-/-}$ mice treated with TPO plus vehicle or TPO plus TAK-242. ≥15 random images from BM diaphysis per mice, with three mice per treatment and genotype, were analyzed using ImageJ software. *, P < 0.05. **(F)** Schematic representation of in vivo strategy for TLR4 inhibition in TPO^{high} mice after the BM fibrosis is already established. **(G and H)** Representative Gomori staining (G) and assessment of reticulin deposition (H) in BM sections of TPO^{high}- and TPO^{high} plus TAK-242–treated mice. Bar, 50 µm. Objective, 40×. **(I and J)** Representative spleens (I) and spleen/body weight ratios (J) were calculated in TPO^{high}- and TPO^{high} plus TAK-242–treated mice.

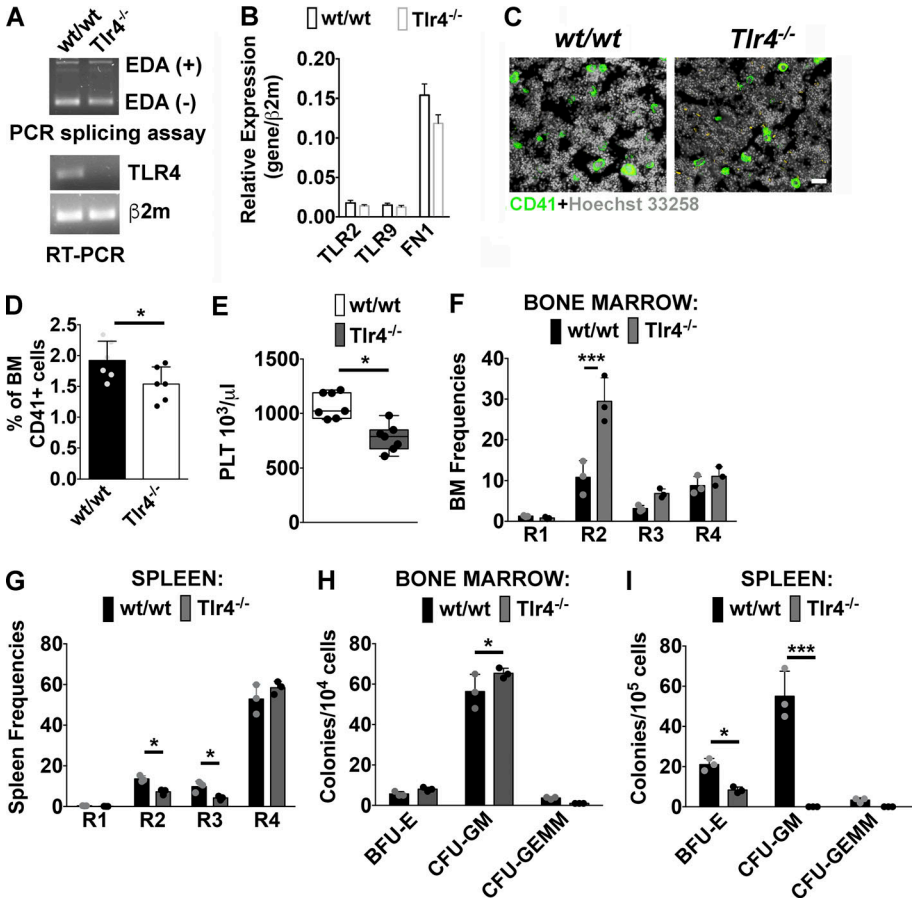

Figure S4. **TLR4 deletion in vivo induces a modest thrombocytopenia but restrains EDA FN effects on hematopoietic progenitors after experimental fibrosis. (A)** PCR splicing assay of EDA FN in BM cells recovered from untreated wild-type (*wt/wt*) and *Tlr4*$^{-/-}$ mice. Real-time PCR products of TLR4 expression confirmed the targeted deletion of TLR4 in the knockout mice. β2-microglobulin was amplified for sample normalization. **(B)** Real-time PCR analysis of FN, TLR2, and TLR9 expression relative to β2-microglobulin in BM cells of wild-type and *Tlr4*$^{-/-}$ mice. *n* = 3. **(C and D)** Immunofluorescence of CD41^{+} Mk's in BM sections (C) and relative quantification (D) in untreated wild-type and *Tlr4*$^{-/-}$ mice. Bar, 50 µm. *n* = 6. Nuclei were stained with Hoechst 33258. **(E)** Peripheral platelet (PLT) counts in untreated wild-type and *Tlr4*$^{-/-}$ mice. *n* = 7. **(F and G)** Frequencies of erythroid progenitors in BM (F) and spleens (G) of wild-type and *Tlr4*$^{-/-}$ mice after experimental fibrosis. *n* = 3. **(H and I)** BM cells (10^4; H) and spleen cells (10^5; I) of wild-type and *Tlr4*$^{-/-}$ mice after experimental fibrosis (*n* = 3) were plated in methylcellulose medium supplemented with cytokines. BFU-E, CFU-GM, and CFU-GEMM colonies were scored 7 d after plating. *, P < 0.05; ***, P < 0.001.

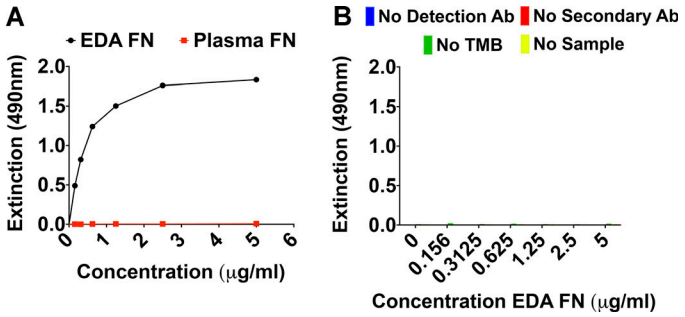

Figure S5. **Establishment of the specific ELISA for quantification of EDA FN in plasma samples. (A)** Evaluation of different EDA FN standard concentrations (black circles) and exclusion of cross-reactions to pFN (red squares). **(B)** Exclusion of cross-reactions to different components of the ELISA setup (no detection antibody (Ab), blue histograms; no secondary antibody, red histograms; no tetramethylbenzidine (TMB) ELISA substrate, green histograms; no sample, yellow histograms).