ARTICLE - Red Cell Biology & its Disorders

Splenic erythrophagocytosis is regulated by ALX/FPR2 signaling

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SUPPLEMENTARY MATERIALS

1. Materials and Methods

1.1 Animals and Reagents

C57BL/6J (#00664), *Spic*-EGFP (#025673), LysM-Cre (#004781) and *Alox15*$^{-/-}$ (#002778) mice were purchased from The Jackson Laboratories (Bar Harbor, ME) at 10 to 16 weeks of age and allowed to acclimate for at least 1 week prior to inclusion in experimental studies or used as breeders. *Fpr2*$^{-/-}$ and humanized *ALX/FPR2-GFP* floxed (h*FPR2*$^{fl/fl}$) mice were provided by Idorsia Pharmaceuticals and bred in-house. All mice were maintained on normal laboratory chow diet, housed in a temperature controlled, 12-hour light–dark cycling environment, and were randomized to experimental groups. All animal procedures were performed in accordance with ethical regulations and pre-approved by the University of Louisville Institutional Animal Care and Use Committee. LXA_4 was purchased from Cayman Chemical (Ann Arbor, MI). Antibodies used for flow cytometry were purchased from BioLegend (San Diego, CA) and included: Streptavidin-FITC, F4/80-APC (BM8), CD11b-Brilliant Violet 421 (M1/70), CD47-PE (miap301), Ter119-Brilliant Violet 421 (TER-119), Ter119-PE (TER-119), CD71-APC (RI7217), CD45-FITC (S18009F), CD11b-FITC (M1/70), Gr1-FITC (RB6-8C5), DRAQ5, CD44-Brilliant Violet 711 (IM7).

1.2 *In vivo* RBC turnover

For *in vivo* tracking of circulating RBC, EZ-Link Sulfo-NHS Biotin (Thermo Scientific) was administered in a two-step labelling protocol, essentially as described[1,2]. Briefly, biotin was dissolved in water and further diluted in sterile saline prior to i.v. retro-orbital injection

(100 µl). In the first step, daily 1 mg biotin doses were administered for three days. Five days after the third administration, a second labelling dose of 0.6 mg was injected. Serial blood samples were collected via tail bleed at the indicated days and biotin expression was determined by flow cytometry.

1.2 Bone marrow erythroid precursors

Bone marrow from femur and tibia of WT and *Fpr2*$^{-/-}$ mice was flushed in cell staining buffer (BioLegend) using a 25G needle. Single cell solutions were obtained using a 70 µm cell strainer and subjected to flow cytometry analysis. Erythroid precursors were identified as CD45^{-}CD11b^{-}Gr1^{-}Ter119^{+}DRAQ5^{+}. Subpopulations of erythroblasts and reticulocytes were differentiated based on CD44 expression and cell size.

1.3 Histology

Spleens were collected and fixed in 10% neutral buffered formalin prior to being embedded in paraffin wax. Tissue sections (5 µM) were deparaffinized and stained with Mayer's Hematoxylin (Electron Microscopy Sciences, PA, USA) and Eosin Y Solution (Sigma-Aldrich, MO, USA), or with Prussian blue (Abcam, UK) according to manufacturer's instructions. For immunofluorescence staining, deparaffinized sections were rehydrated prior to antigen retrieval using a citrate buffer and incubation with Anti-F4/80-APC rat monoclonal antibody (clone: BM8; BioLegend). Imaging and analysis were performed using a Keyence BZ-X810 fluorescence microscope (IL, USA).

1.4 Gene Expression Analysis

1.4.1 RNA-Sequencing

An antibody-based magnetic bead isolation kit (Stem Cell Technologies) was used according to manufacturer's instructions to obtain F4/80$^+$ cells from whole spleen. Total RNA was isolated from mouse spleen and F4/80$^+$ splenocytes using the RNeasy Plus Universal Mini Kit (Qiagen). RNA quality was evaluated using NanoDrop ONEC (Thermo Scientific) and then subjected to poly-A RNA sequencing. RNA integrity was assessed using the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). For library construction, the abclonal second strand synthesis module kit was utilized (Cat. # RK20346). Equimolar pooling of libraries was performed based on Q.C. values, and paired-end sequencing was performed on an Illumina NovaSeq X Plus (Illumina, California, USA) using the 25B Flow Cell. FASTQ files were generated utilizing BCL2fastq.

1.4.2 Bioinformatics analysis

Quality control (Q.C.) of the raw sequence data was performed using FastQC (version 0.10.0) for each sequencing sample. The sequences were aligned to the mm10 mouse reference genome using STAR version 2.6 [3]. Differential expression of ENSEMBL protein-coding transcripts was performed using DESeq2[4], and raw counts were obtained from the STAR-aligned bam format files using HTSeq version 0.10.0[5]. The raw counts were normalized using the Relative Log Expression (RLE) method and then filtered to exclude genes with fewer than ten counts across the samples. DESeq2 guidelines were used to identify differentially expressed genes, and all P values were adjusted for testing multiple genes (Benjamini–Hochberg procedure; $p \leq 0.05$). Functional

enrichment analysis was performed using the clusterProfiler R package to identify enriched Gene Ontology biological processes for each set of differentially expressed genes (DEGs)[6]. RNA-seq data have been deposited in the NCBI GEO database with the accession numbers GSE292685 and GSE292686.

1.4.3 qRT-PCR

RNA from flow cytometry-sorted or differentiated macrophages was isolated using RNeasy Plus Universal Mini Kit (Qiagen) according to manufacturer's instructions. cDNA was synthesized using Applied Biosystems High-Capacity cDNA Reverse Transcription Kit and qPCR was performed with Applied Biosystems PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) on an Applied Biosystems QuantStudio 5. RT^2 qPCR Primer Assays (Qiagen) were used for each target gene and relative expression was determined using the $2^{-\Delta\Delta Ct}$ method normalized to expression of *Hprt*.

1.5 *In vivo* splenic RBC uptake

Erythrophagocytosis *in vivo* was assessed essentially as described[7,8]. Blood was collected from a naïve donor mouse via cardiac puncture and mixed with 0.2 M EDTA. RBC were separated from plasma by centrifugation at 3,500 rpm for 10 minutes at 4°C and washed twice with PBS. Washed RBC were resuspended in PBS and either oxidized with copper(ii) sulfate ($CuSO_4$) and L-ascorbic acid or incubated with CD47 blocking antibody. For oxidation, RBC were incubated in 0.2 mM $CuSO_4$ and 5 mM L-ascorbic acid in PBS at 4% hematocrit for 30 minutes at 37°C, with gentle agitation every 10 minutes. After incubation, RBC were washed twice with PBS containing 5 mM EDTA followed by two washes with PBS alone. For CD47 antibody blockage, RBC were

incubated with 10 µg/mL CD47 antibody (Bio X Cell) or 10 µg/mL mouse IgG isotype control (Bio X Cell, BE0083) for 1 hour at 37°C. Treated RBC were labeled with carboxyfluorescein succinimidyl ester (CFSE; BioLegend, CA, USA) according to the manufacturer's instructions. Briefly, $1x10^8$ cells/mL were incubated with 5 µM/mL CFSE in PBS for 20 minutes at room temperature then washed with PBS. CFSE-labeled RBC ($2x10^8$ cells) were administered to mice via retro-orbital injection (200 µL sterile saline). After 1 hour, mice were euthanized and spleens collected for flow cytometry. A single cell suspension was generated using a 70 µM cell strainer with PBS using the plunger from a 5 mL syringe. The suspension was centrifuged and resuspended in Cell Staining Buffer (BioLegend). Cells were incubated in 2 µg Fc-block (BioLegend) for 5-10 minutes, stained with fluorescent antibodies for 15-20 minutes, and fixed in 1% paraformaldehyde. Data were acquired using a BD Fortessa X-20 Flow Cytometer and analyzed using FlowJo software.

1.6 Targeted lipidomics

1.6.1 Tissue preparation and solid phase extraction (SPE)

All reagents used in lipid mediator extractions and analyses were LC/MS grade, ensuring the highest level of purity. Spleens were removed from animals and placed directly in ice-cold methanol containing commercially available deuterium-labeled synthetic standards (PGE_2-d4, 15d-PGJ_2-d4, LTB_4-d4, LXA_4-d5, 11,12-EET-d11, 15-HETE-d8, 5-HETE-d8, RvE1-d4, RvD2-d5, RvD3-d5, MaR1-d5, and MaR2-d5; Cayman Chemical, Ann Arbor, MI, USA), then stored at -80°C. Prior to lipid extraction, tissue was minced with surgical

scissors on ice and then centrifuged (13,000 rpm; 10 min; 4°C). The supernatant was collected and subjected to solid phase extraction (SPE) essentially as described previously[9]. Briefly, samples were acidified to a pH of 3.5 and added to C18 SPE columns (Biotage, Uppsala, Sweden) that were conditioned with successive washes of methanol and water. Neutral lipids were removed from the column using *n*-hexanes while lipid mediators were eluted with methyl formate. Using N_2 gas, the solvent was evaporated and samples resuspended in methanol:water (50:50, v/v).

1.6.2 LC-MS/MS analysis

Extracted lipid mediator samples were analyzed using a Shimadzu (Kyoto, Japan) liquid chromatography system (LC 20-AD with an SIL-20AC autoinjector) coupled to a QTrap5500 mass spectrometer (AB Sciex, Framingham, MA, USA) operated in negative polarity mode. Samples were injected onto a Kinetex Polar C18 HPLC column (100 mm x 3 mm x 2.6 µm; Phenomenex, CA, USA) held at 59°C. A gradient elution of methanol:water:acetic acid beginning at 45:55:0.01 (v/v/v) and ending at 80:20:0.01 (v/v/v) was used at a flow rate of 0.5 ml/min. Data was acquired using Analyst software (v 1.7.1) and analyzed with Sciex OS-Q. Identified mediators had matching chromatographic peak retention time with that of synthetic standards run in parallel (+/- 0.1 min), signal:noise ratio >5, and an on-column calculated concentration above the lower limit of quantification for each mediator. Fragmentation spectra of synthetic standards compared with that of samples was also used. Absolute quantification was achieved by comparing samples to a 12-point standard curve of synthetic standards run

in parallel. SCIEX OS (v.2.0.1) software was used for peak identification and quantification.

1.6.3 Data analysis

Statistical analyses were performed using the freely available Metaboanalyst software platform (Metaboanalyst.ca). Analytes with missing data in more than 50% of samples in both experimental groups were excluded from analysis. Missing values were imputed with 1/5 the minimum value of that analyte. Data were then log transformed and autoscaled prior to downstream analyses (partial least squares discriminant, volcano plot, VIP).

1.7 iRPM differentiation

Differentiation of iRPM was performed essentially as described[10]. Briefly, murine bone marrow was isolated and plated in RPMI-1640 (Thermo Fisher) supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin, and 20 ng/mL M-CSF (StemCell Technologies). On day 4, media was supplemented with IL-33 (10 ng/mL; BioLegend) and hemin (20 μM; Sigma-Aldrich). After 5 additional days, cells were ready for experiments. For gene expression studies, BMDM were generated using M-CSF media without hemin and IL-33.

2. References

1. Khandelwal, S. & Saxena, R.K. Assessment of survival of aging erythrocyte in circulation and attendant changes in size and CD147 expression by a novel two step biotinylation method. Exp Gerontol. 2006;41(9):855-861.
2. Saxena, R.K., Bhardwaj, N., Sachar, S.*, et al.* A Double in vivo Biotinylation Technique for Objective Assessment of Aging and Clearance of Mouse Erythrocytes in Blood Circulation. Transfus Med Hemother. 2012;39(5):335-341.
3. Dobin, A., Davis, C.A., Schlesinger, F.*, et al.* STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29(1):15-21.
4. Love, M.I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550.
5. Anders, S., Pyl, P.T. & Huber, W. HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015;31(2):166-169.
6. Yu, G., Wang, L.G., Han, Y.*, et al.* clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS. 2012;16(5):284-287.
7. Sambrano, G.R., Parthasarathy, S. & Steinberg, D. Recognition of oxidatively damaged erythrocytes by a macrophage receptor with specificity for oxidized low density lipoprotein. Proc Natl Acad Sci U S A. 1994;91(8):3265-3269.
8. Turpin, C., Meilhac, O., Bourdon, E.*, et al.* Methodologies and tools to shed light on erythrophagocytosis. Biochimie. 2022;202(166-179.
9. Dalli, J., Colas, R.A., Walker, M.E.*, et al.* Lipid Mediator Metabolomics Via LC-MS/MS Profiling and Analysis. Methods Mol Biol. 2018;1730(59-72.
10. Lu, Y., Basatemur, G., Scott, I.C.*, et al.* Interleukin-33 Signaling Controls the Development of Iron-Recycling Macrophages. Immunity. 2020;52(5):782-793 e785.

3. Figure Legends

Supplementary Figure 1. Bone marrow erythropoiesis. Representative flow cytometry dot plots and quantification of erythroid precursors in the bone marrow of WT and *Fpr2*$^{-/-}$ mice. n = 6-7 per group. Data are mean ± SEM. *p<0.05 as determined by unpaired Student's t-test.

Supplementary Figure 2. Erythrocyte parameters of complete blood count analysis in WT and *Fpr2*$^{-/-}$ mice. Quantification is shown. Data are mean ± SEM. n = 34 per group. *RBC – red blood cell count, Hb – hemoglobin, HCT – hematocrit, MCV – mean corpuscular volume, MCH – mean corpuscular hemoglobin, MCHC – mean corpuscular hemoglobin concentration, RDW – red cell distribution width*

Supplementary Figure 3. Flow cytometry gating of splenic red pulp macrophages. Representative flow cytometry dot plots of spleens of WT and *Spic*-EGFP mice. GFP+ cells cluster into a population of CD11b^{dim}F4/80^{+} cells.

Supplementary Figure 4. Validation of myeloid-specific hALX/FPR2 knockout mice. (**A**) Humanized *FPR2-GFP* knockin floxed mice (h*FPR2*$^{fl/fl}$) were crossed with mice expressing Cre recombinase under the Lysozyme M promoter (LysM-Cre) to generate mice with myeloid-specific deletion of *FPR2* (h*FPR2*^{MKO}). (**B**) Representative PCR gel electrophoresis used for genotyping of h*FPR2*^{MKO} colony. (**C**) Expression of GFP and human ALX/FPR2 was determined in peripheral blood leukocytes by flow cytometry using Ly6C and Ly6G to identify monocytes and neutrophils, respectively.

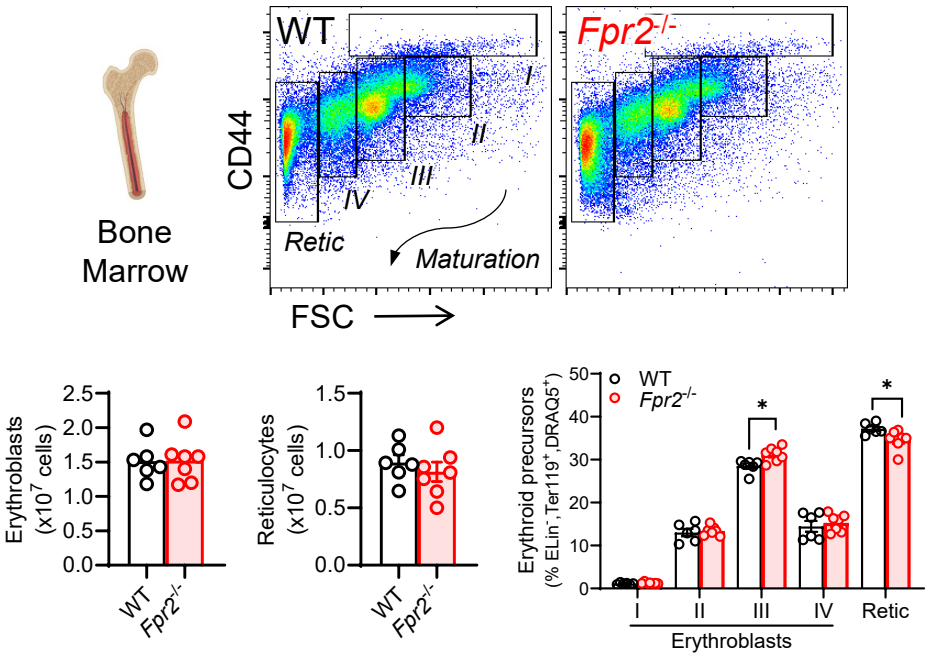
Supplementary Figure 5. Quantification of CFSE+ cells in spleens after adoptive transfer of oxidized (Ox) or CD47 blocking antibody (CD47 Ab) treated RBC. Data

are mean ± SEM. n = 4-6 per group. ns: $p>0.05$ as determined by unpaired Student's t-test.

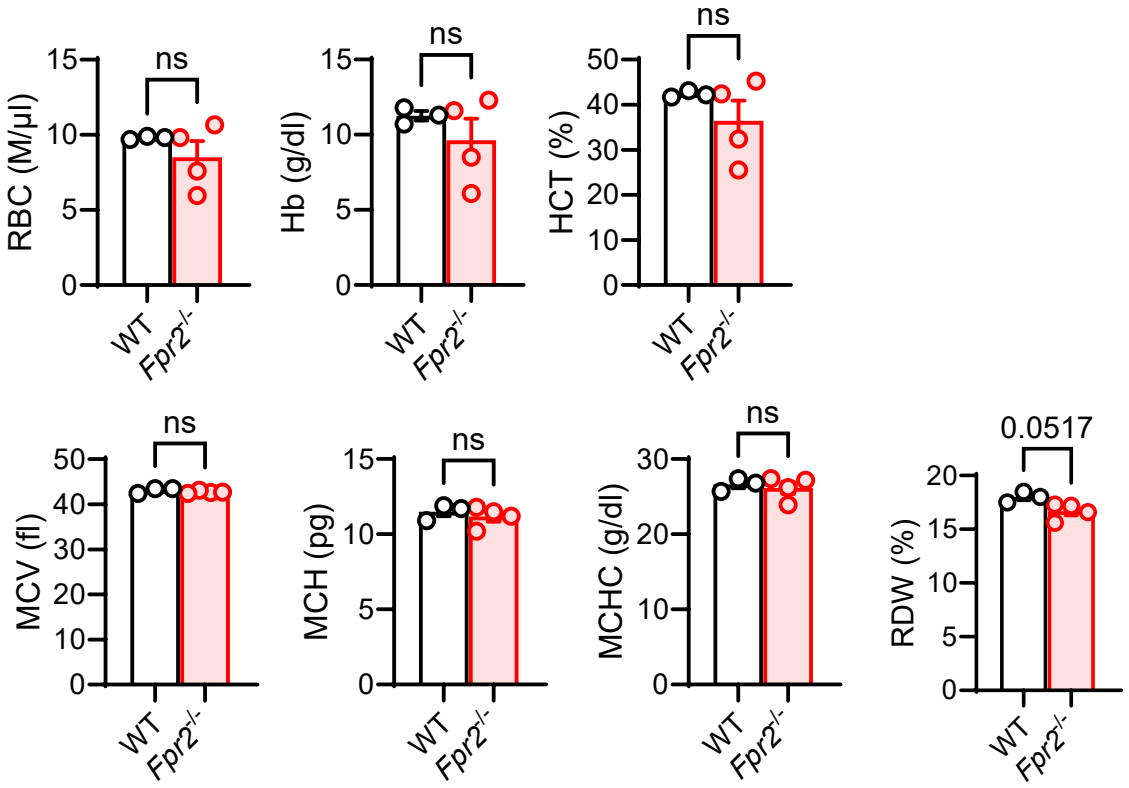
Supplementary Figure 6. Chromatograms from Arachidonic Acid Metabolome. Representative extracted ion mass chromatograms are shown for arachidonic acid-derived lipid mediators that were significantly changed in spleens after adoptive transfer of damaged red blood cells. A representative synthetic standard is shown in addition to a sample. Shaded area illustrates the peak area that was used for quantitation.

Supplementary Figure 7. Chromatograms from Docosahexaenoic Acid, Docosapentaenoic Acid, and Eicosapentaenoic Acid Metabolomes. Representative extracted ion mass chromatograms are shown for docosahexaenoic, docosapentaenoic, and eicosapentaenoic acid-derived lipid mediators that were significantly changed in spleens after adoptive transfer of damaged red blood cells. A representative synthetic standard is shown in addition to a sample. Shaded area illustrates the peak area that was used for quantitation.

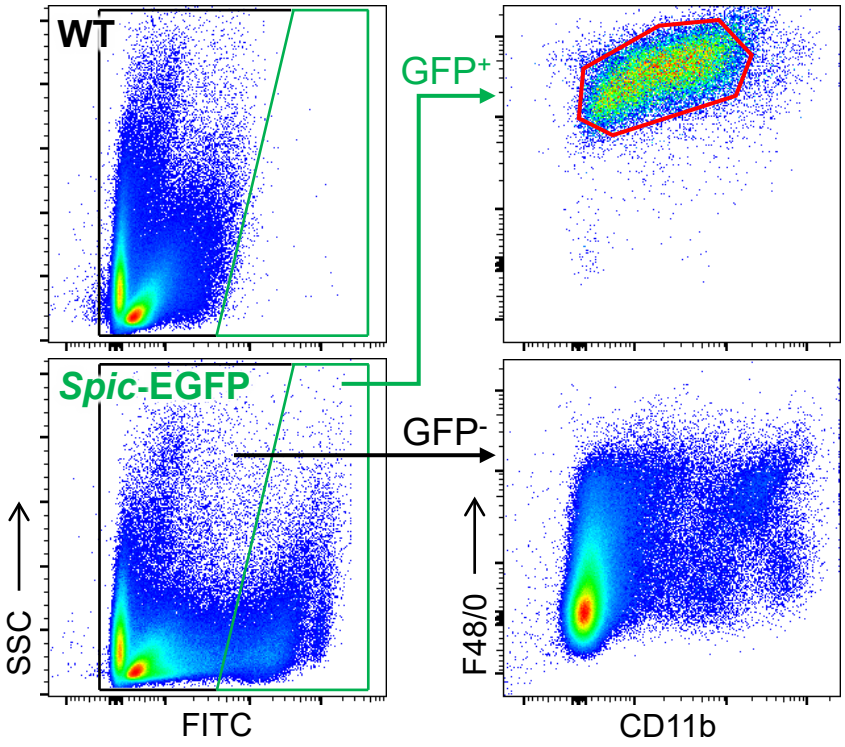
Supplementary Figure 1



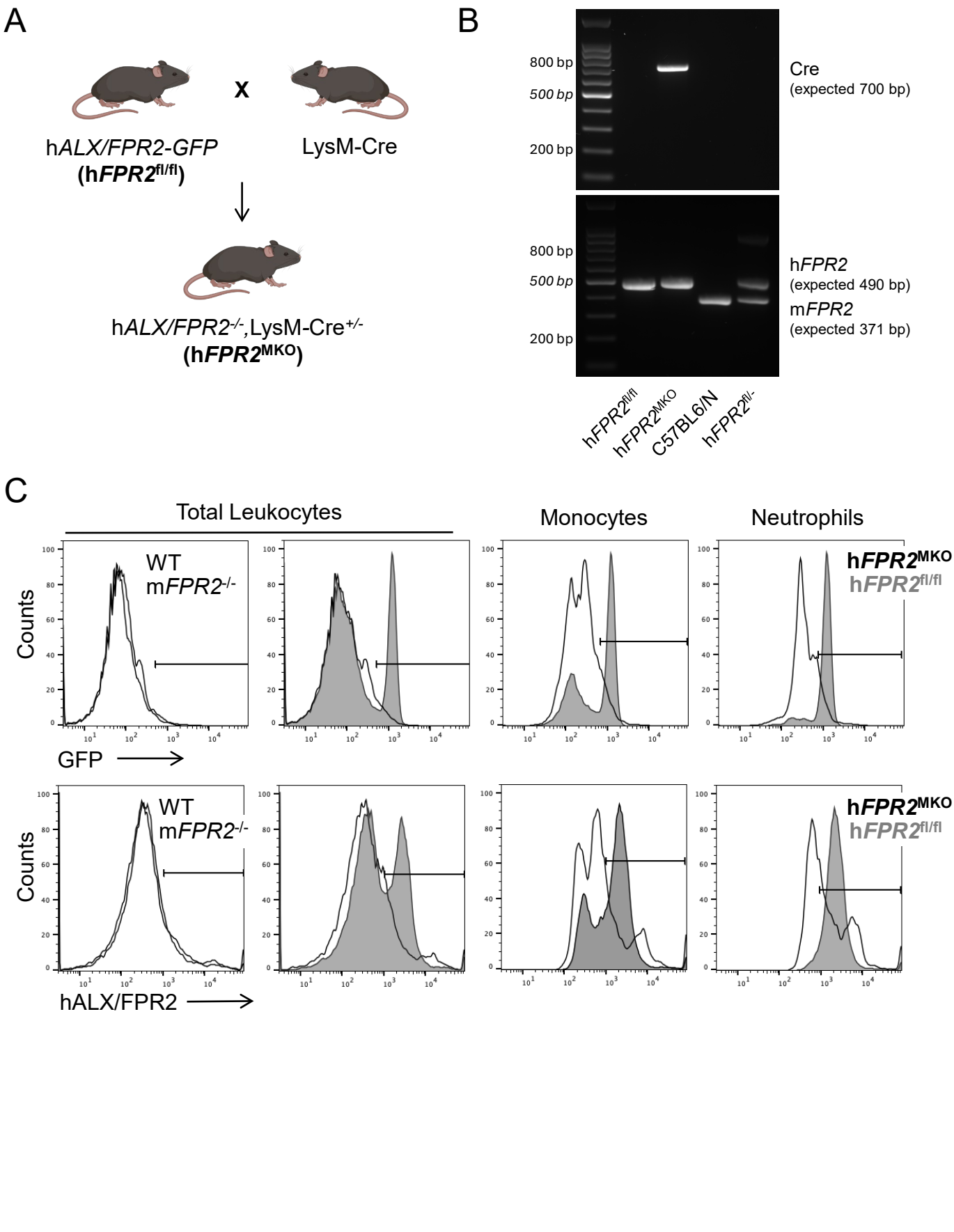
Supplementary Figure 2



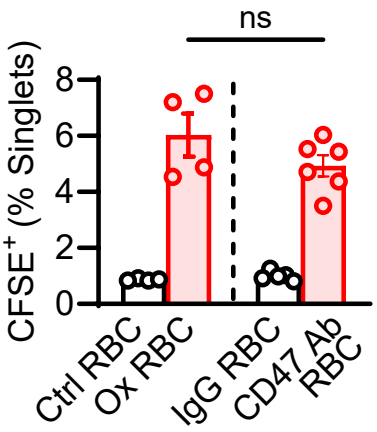
Supplementary Figure 3



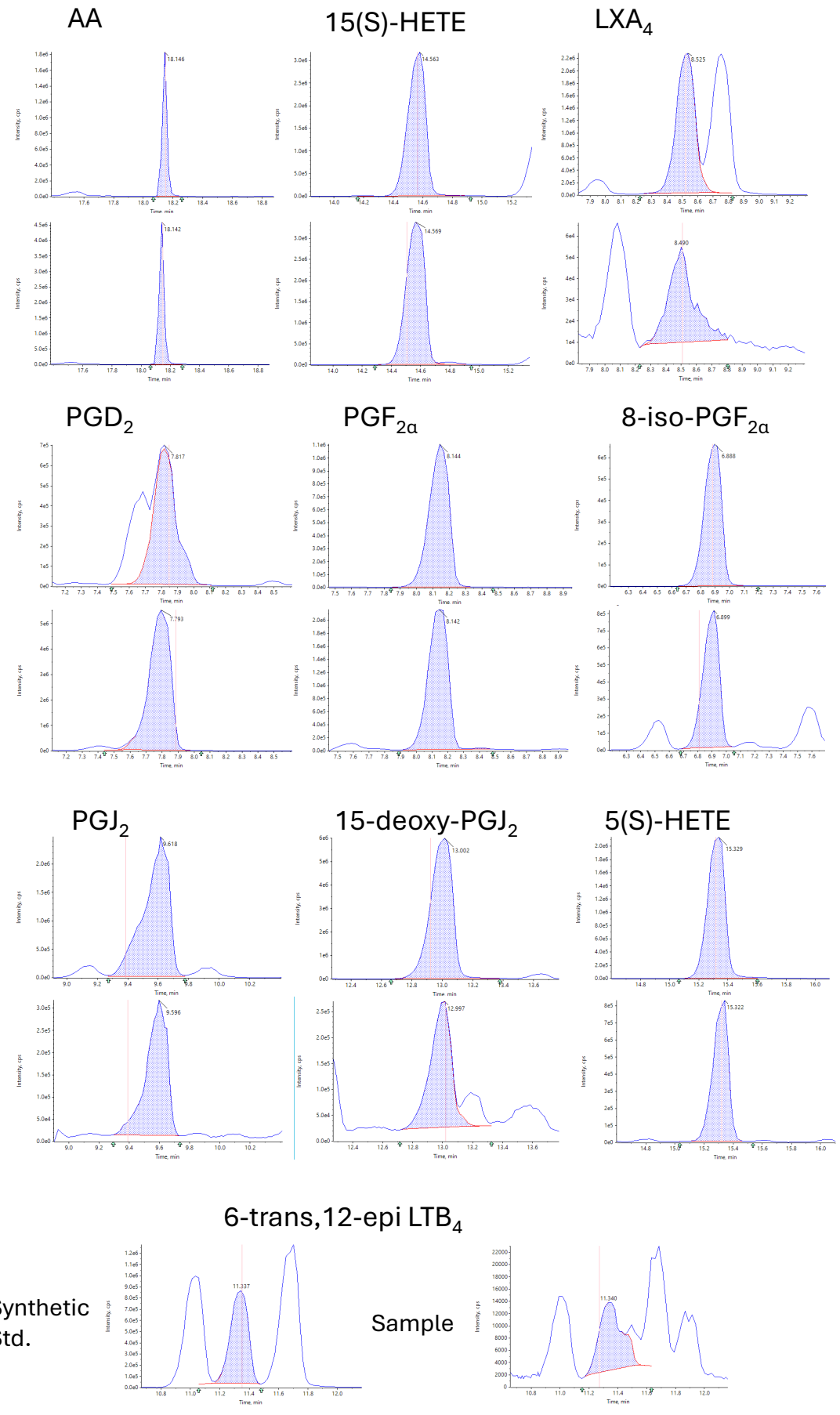
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7