Pregnenolone sulfate induces transcriptional and immunoregulatory effects on T cells

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Supplemental figures and legends

Figure S1

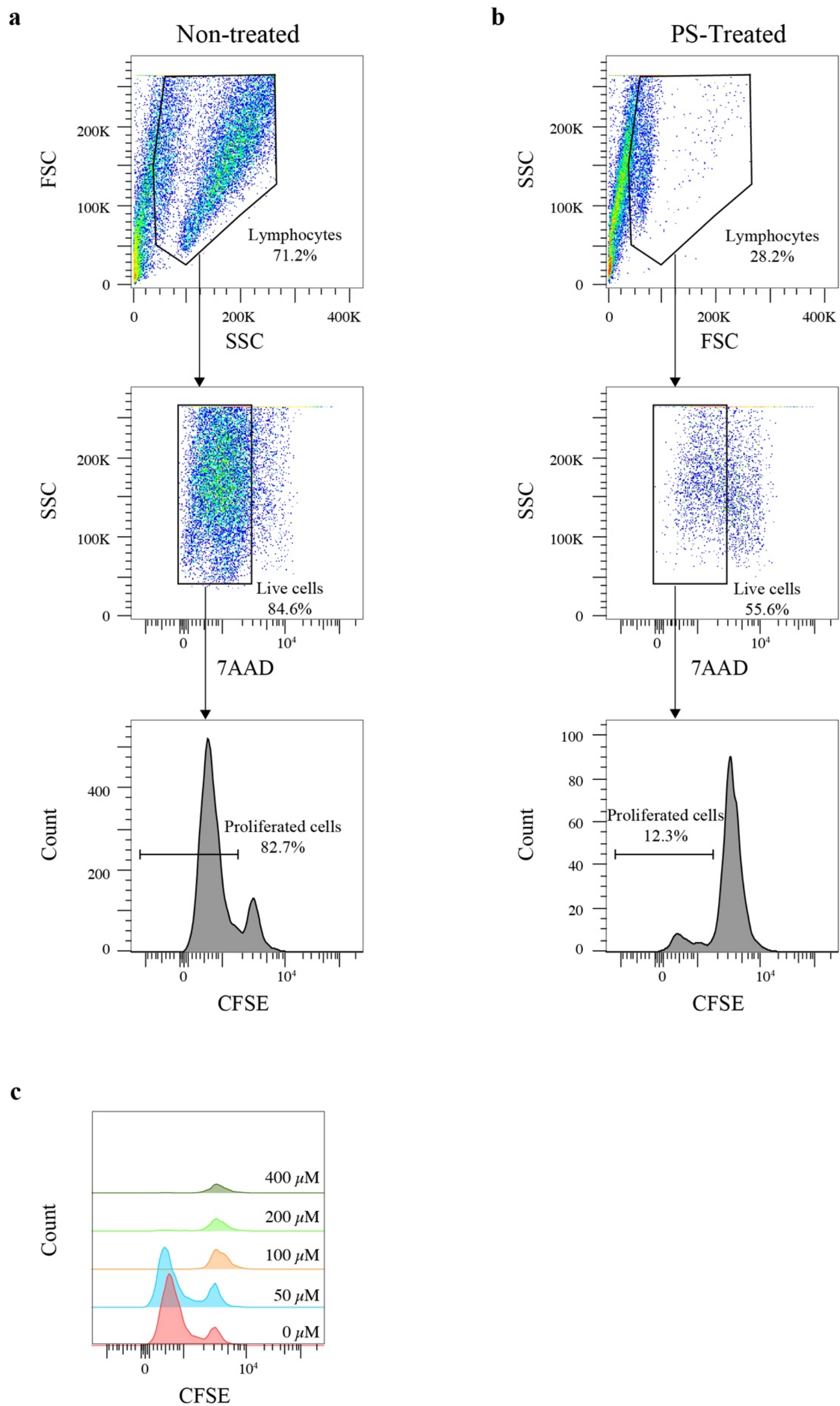

Figure S1. Gating strategy used in PBMC cell viability and proliferation assays. Flow cytometric plots showing the gating strategy used in the (**a**) non-treated and (**b**) 200 μM pregnenolone sulfate (PS)-treated PBMC cell viability and proliferation assays from one individual. Lymphocytes were gated according to FSC and SSC, and live cells were gated according to 7-AAD. CFSE-stained live cells were detected using CFSE FITC. (**c**) Flow cytometric plot of CFSE-stained live lymphocytes for the same individual under the indicated pregnenolone sulfate concentrations.

Figure S2

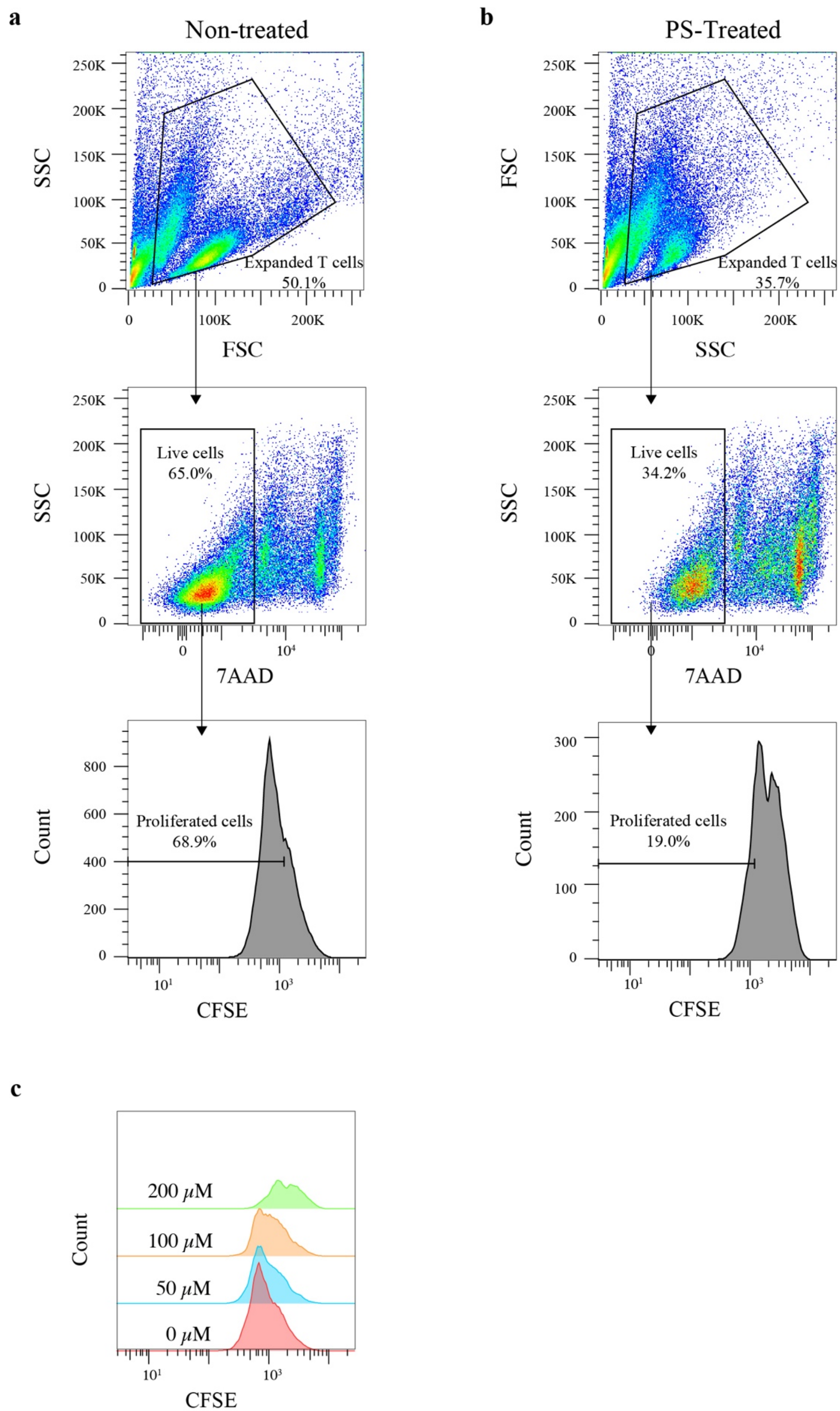

Figure S2. Gating strategy used in expanded T cells viability and proliferation assays. Flow cytometric plots showing the gating strategy used in the (**a**) non-treated and (**b**) 200 μM pregnenolone sulfate (PS)-treated expanded T cells viability and proliferation assays from one individual. Expanded T cells were gated according to FSC and SSC, and live cells were gated according to 7-AAD. CFSE-stained live cells were detected using CFSE FITC. (**c**) Flow cytometric plot of CFSE stained live expanded T cells for the same individual under the indicated pregnenolone sulfate concentrations.

Figure S3

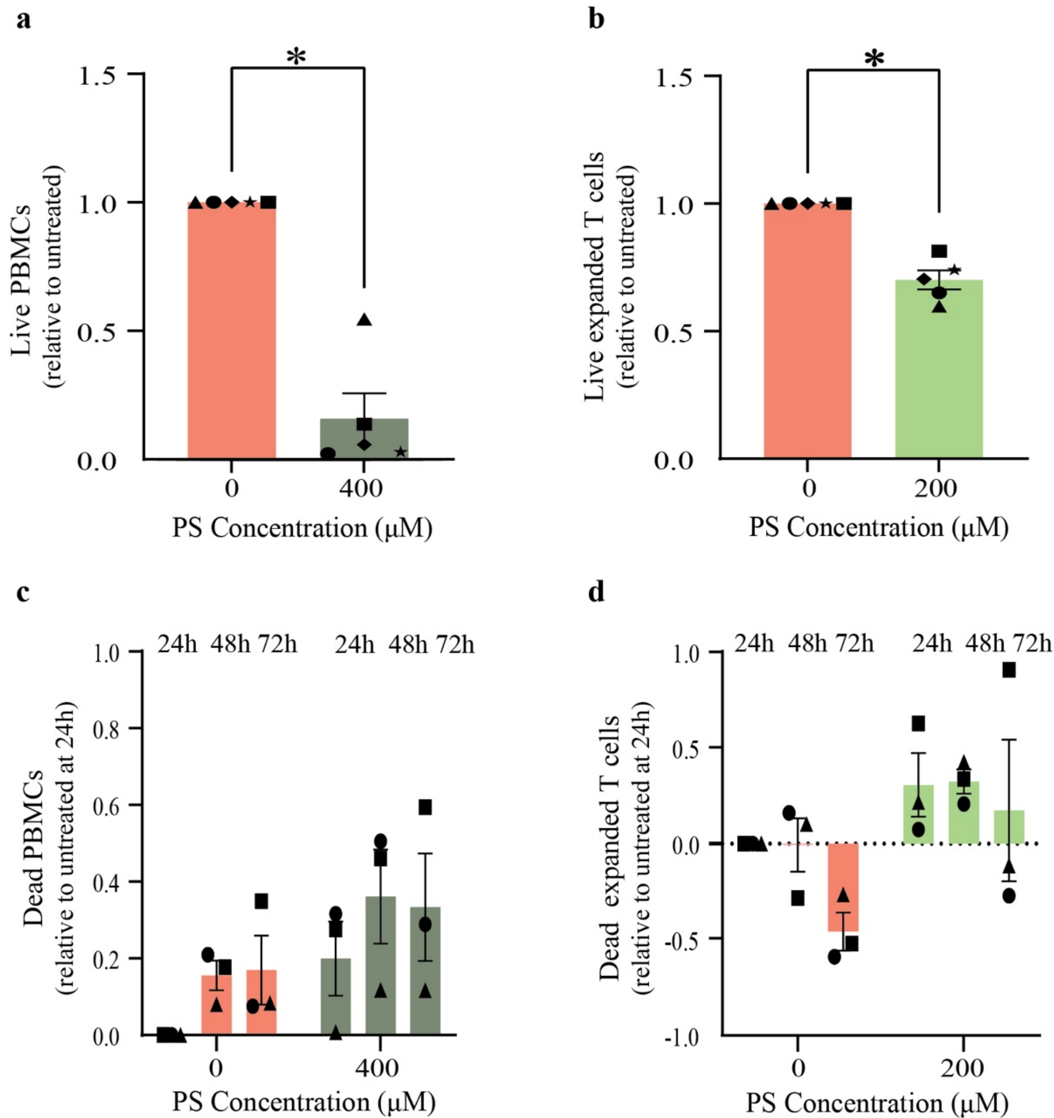

Figure S3. ***In vitro*** **inhibition of T cell viability and apoptosis post-pregnenolone sulfate treatment of non-activated T cells.** Normalized bar plots showing the viability of non-activated (**a**) PBMCs and (**b**) expanded T cells across pregnenolone sulfate (PS) treatment concentrations relative to the non-treated, non-activated corresponding samples. Normalized bar plots showing the apoptosis of non-activated (**c**) PBMCs and (**d**) expanded T cells across pregnenolone sulfate treatment concentrations relative to non-activated corresponding samples. Statistical analyses were performed using paired *t*-test for viability assays, and two-way ANOVA for apoptosis test. Experimental design of assays based on outline in Fig. 1a.

Figure S4

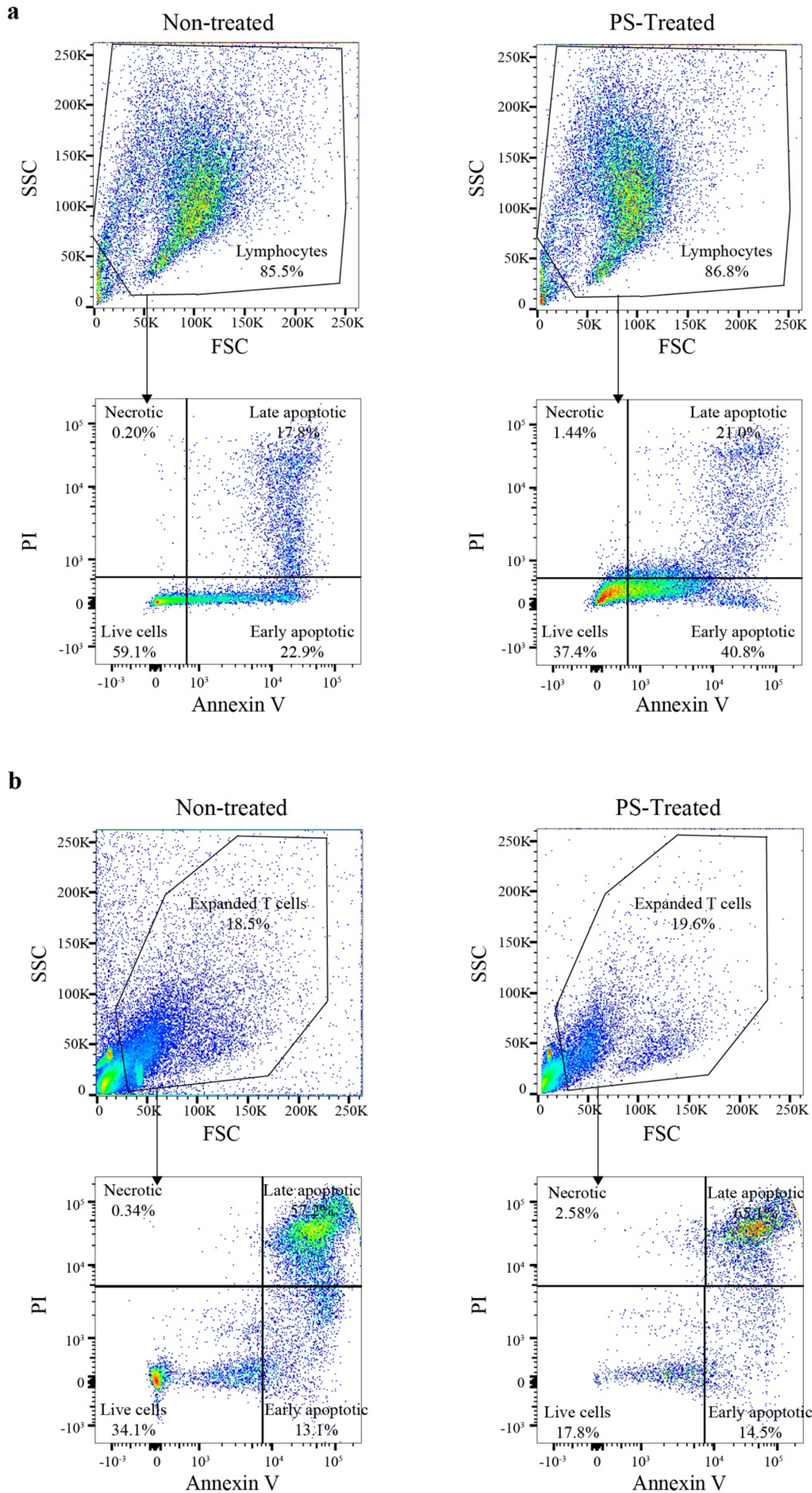

Figure S4. Gating strategy used in apoptosis assay. Flow cytometric plots showing the gating strategy used in the non-treated and pregnenolone sulfate (PS)-treated (**a**) PBMCs and (**b**) expanded T cells apoptosis assays from one individual. PBMCs and expanded T cells were gated according to FSC and SSC. Live (AnnexinV$^-$PI$^-$), early apoptotic (AnnexinV$^+$PI$^-$), late apoptotic (AnnexinV$^+$PI$^+$) and necrotic (AnnexinV$^-$PI$^+$) lymphocytes were gated according to PI and Annexin-V.

Figure S5

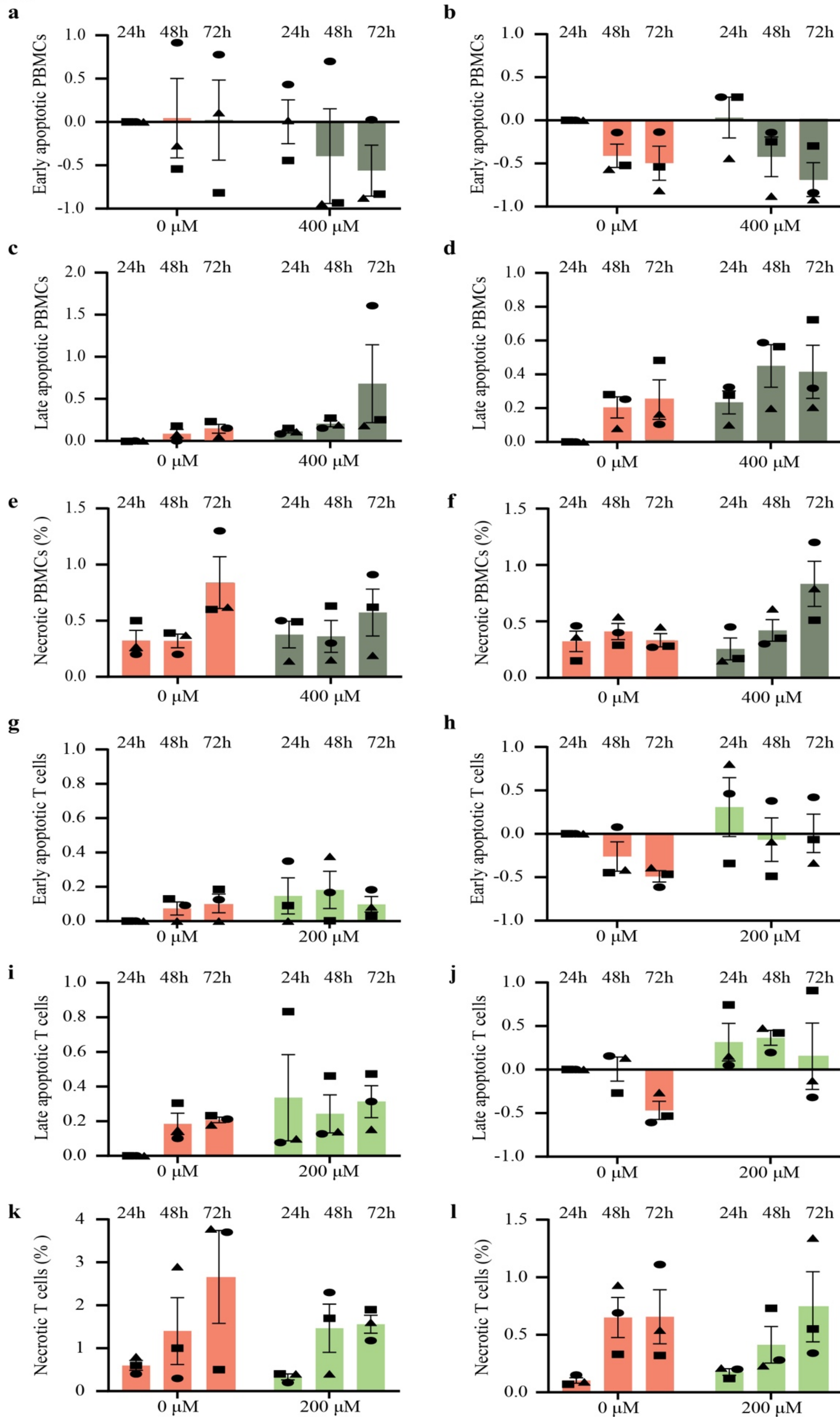

Figure S5. *In vitro* induction of apoptotic stages post-pregnenolone sulfate treatment of activated and non-activated PBMCs and expanded T cells. Normalized bar plots showing the early apoptosis of (**a**) activated and (**b**) non-activated PBMCs, and late apoptosis of (**c**) activated and (**d**) non-activated PBMCs, treated with 200 μM of pregnenolone sulfate relative to non-treated corresponding samples at the 24-hour time point. Percentage of necrotic (**e**) activated and (**f**) non-activated PBMCs are shown here due to their relatively lower number. Normalized bar plots showing the early apoptosis of (**g**) activated and (**h**) non-activated expanded T cells, and late apoptosis of (**i**) activated and (**j**) non-activated expanded T cells treated with 200 μM of pregnenolone sulfate relative to non-treated corresponding samples at the 24-hour time point. The percentage of necrotic (**k**) activated and (**l**) non-activated expanded T cells are shown here due to their relatively lower number. Experimental design of assays based on outline in Fig. 1a.

Figure S6

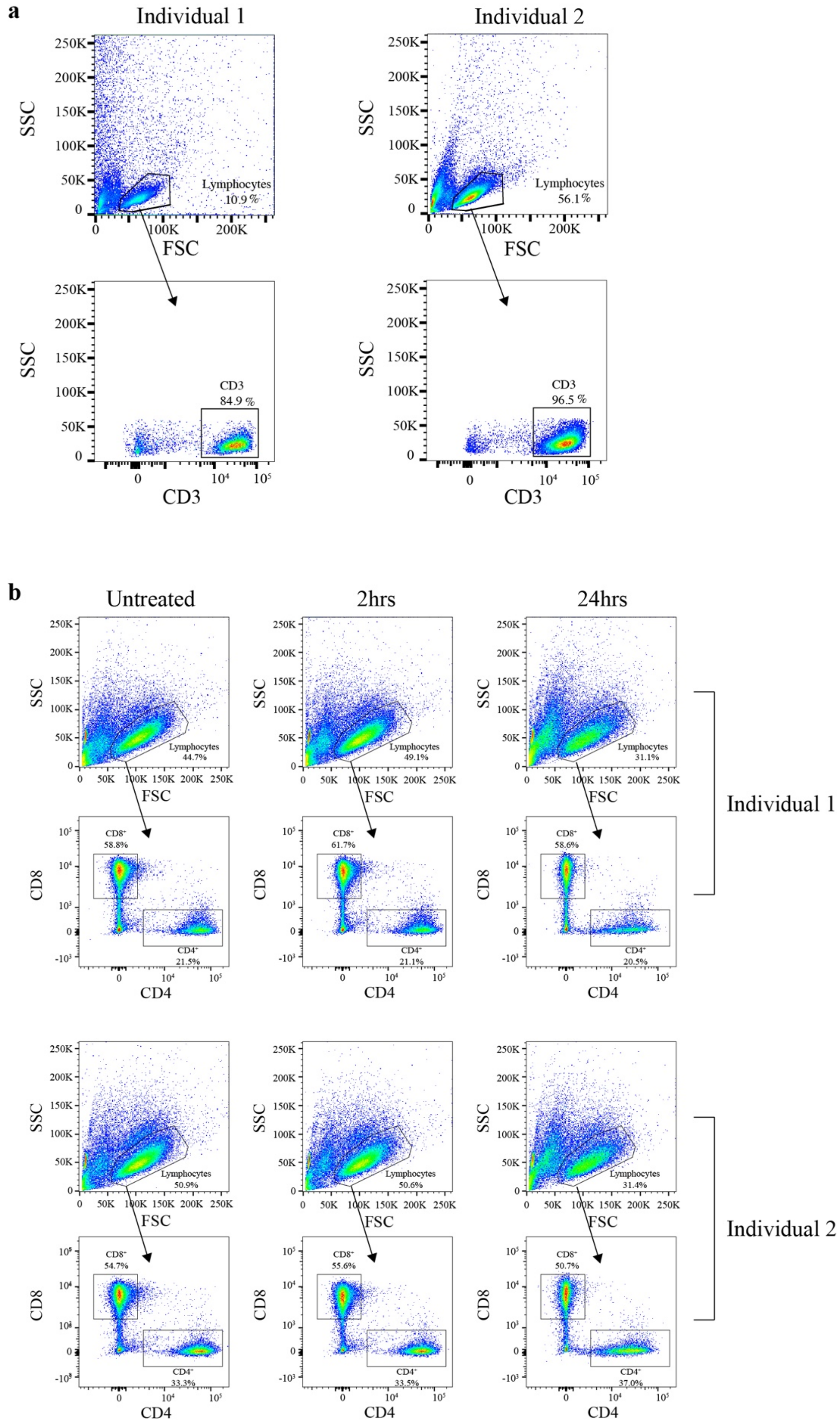

Figure S6. Immunophenotyping of T cells after expansion for RNA-sequencing experiments. (**a**) Flow cytometry plots showing T cells expanded from PBMCs of 2 donors stained with CD3-PE antibody. (**b**) Flow cytometry plots of expanded T cells from 2 donors that were non-treated, treated for 2 hours or 24 hours with 200 μM of pregnenolone sulfate and stained with CD4-PE and CD8-PB antibodies. Live T cells were primarily gated using FSC and SSC, followed by gating on (**a**) $CD3^+$ cells or (**b**) $CD4^+$/$CD8^+$ cells.

Figure S7

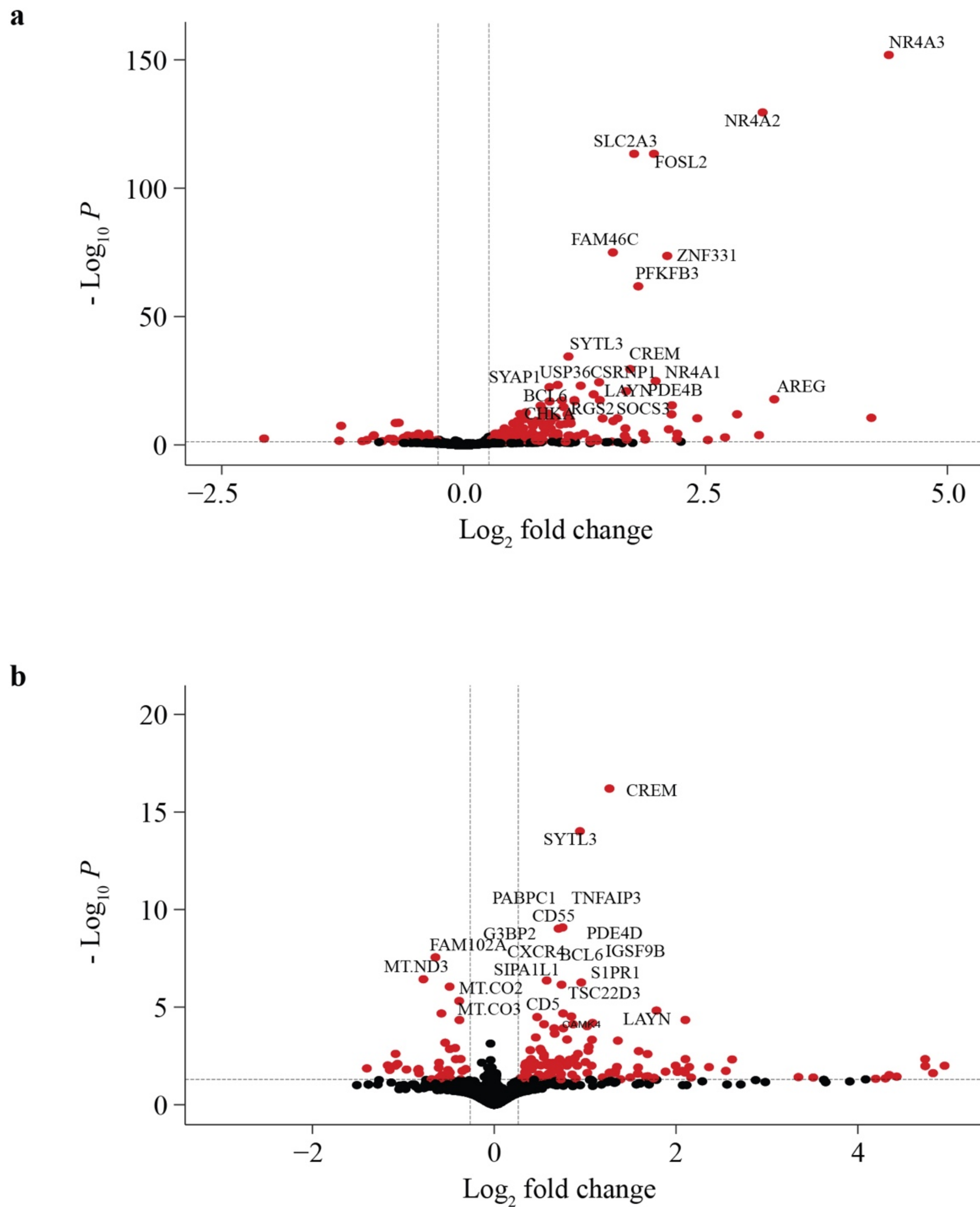

Figure S7. Differential gene expression of pregnenolone sulfate treated and non-treated samples in non-activated T cells. Volcano plots showing differential gene expression before and after pregnenolone sulfate-treatment at (**a**) 2 hours and (**b**) 24 hours post-treatment of the non-activated samples. Significant DEGs after treatment (|FC| ≥ 1.2, B-H FDR < 0.05) are shown in red and the top 20 DEGs are labeled.

Figure S8

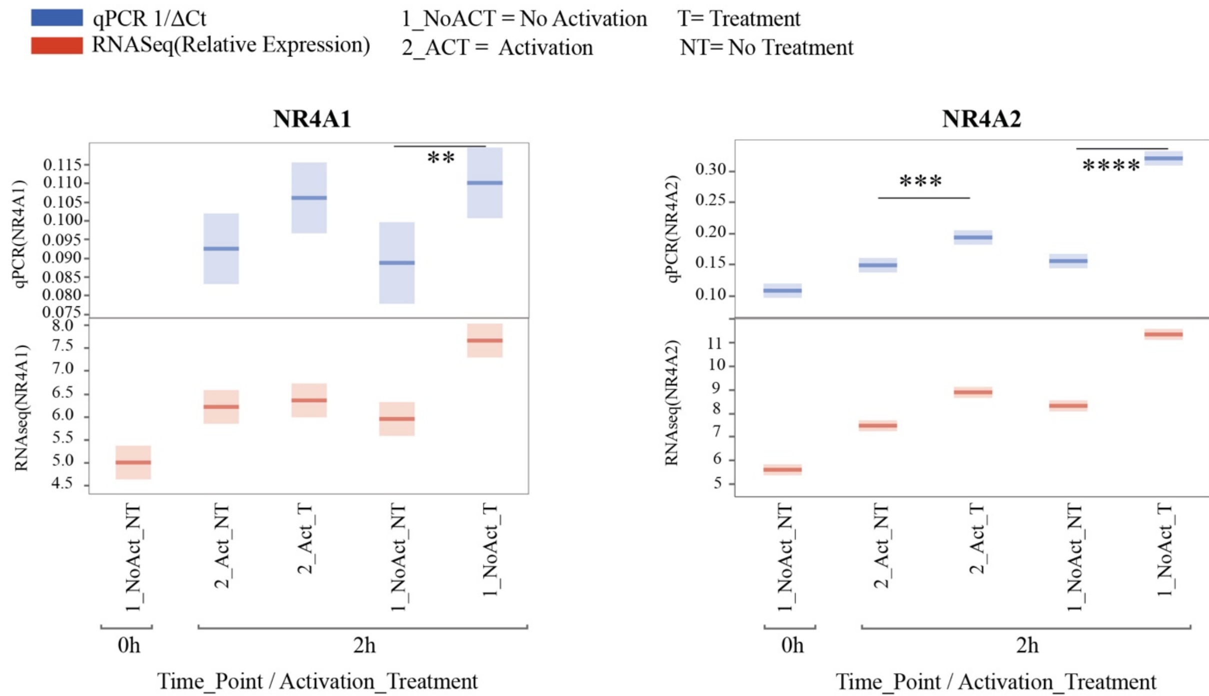

Figure S8. qPCR validation of *NRA41* and *NR4A2* expression. Box plots representing qPCR (1/ΔCt) and RNA-sequencing (VST-normalized expression) expression levels of *NR4A1* and *NR4A2* genes from total RNA of 20 samples (NoACT_NT_0hrs, n = 4; NoACT_NT_2hrs, n = 4; NoACT_T_2hrs, n = 4; ACT_NT_2hrs, n = 4; ACT_T_2hrs, n = 4). Unpaired *t*-test was performed and significance is indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Figure S9

qPCR 1/ΔCt | 1_NoACT = No Activation | T= Treatment

RNASeq(Relative Expression) | 2_ACT = Activation | NT= No Treatment

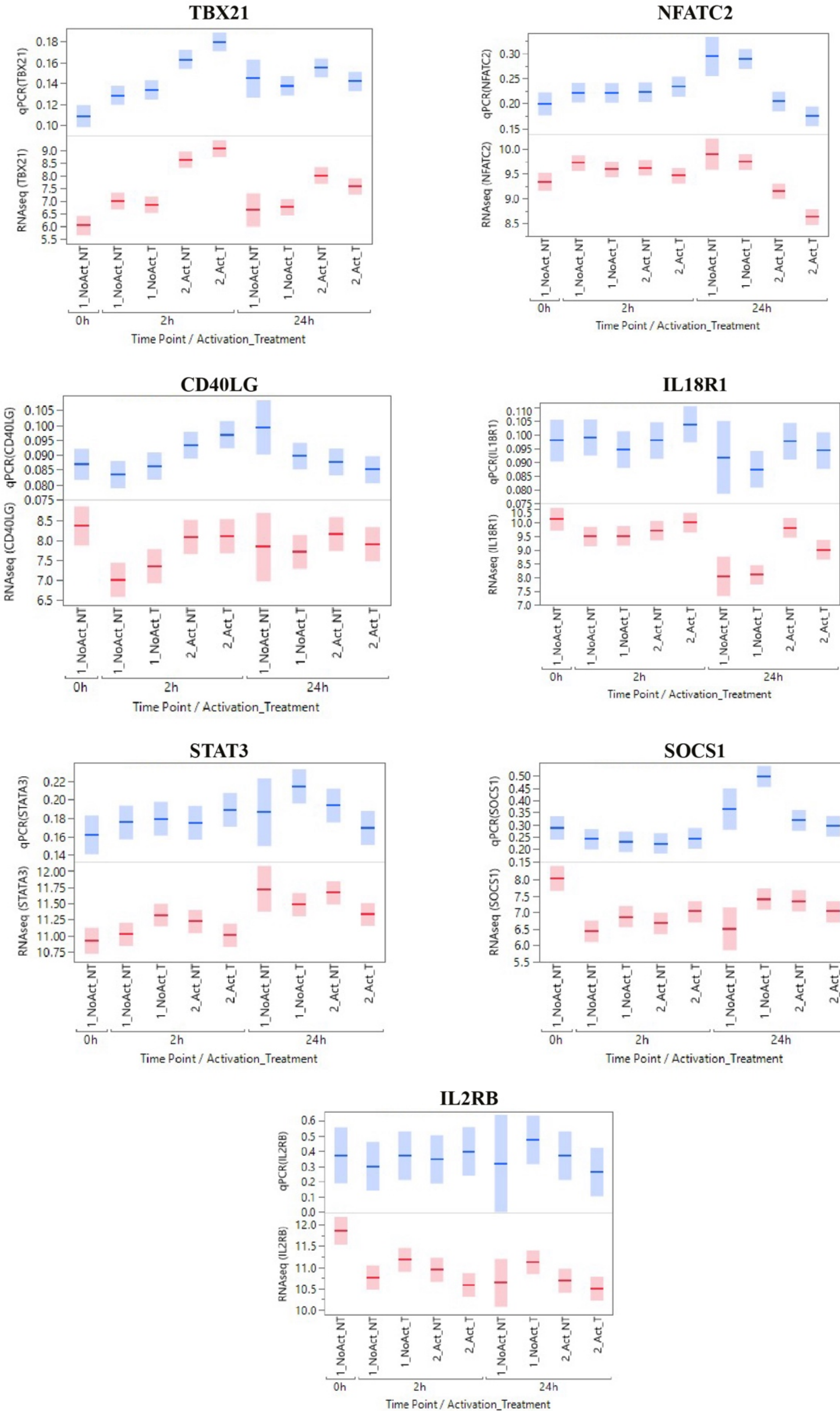

Figure S9. qPCR validation of select differentially expressed genes from RNA-seq. Box plots representing qPCR (1/ΔCt) and RNA-sequencing (VST-normalized expression) expression levels of *TBX1*, *NFATC1*, *CD40LG*, *IL18R1*, *STAT3*, *SOCS1* and *IL2RB* genes from total RNA of 32 samples (NoACT_NT_0hrs, n = 3; NoACT_NT_2hrs, n = 4; NoACT_NT_24hrs, n = 1; NoACT_T_2hrs, n = 4; NoACT_T_24hrs, n = 4; ACT_NT_2hrs, n = 4; ACT_NT_24hrs, n = 4; ACT_T_2hrs, n = 4; ACT_T_24hrs, n = 4).

Figure S10

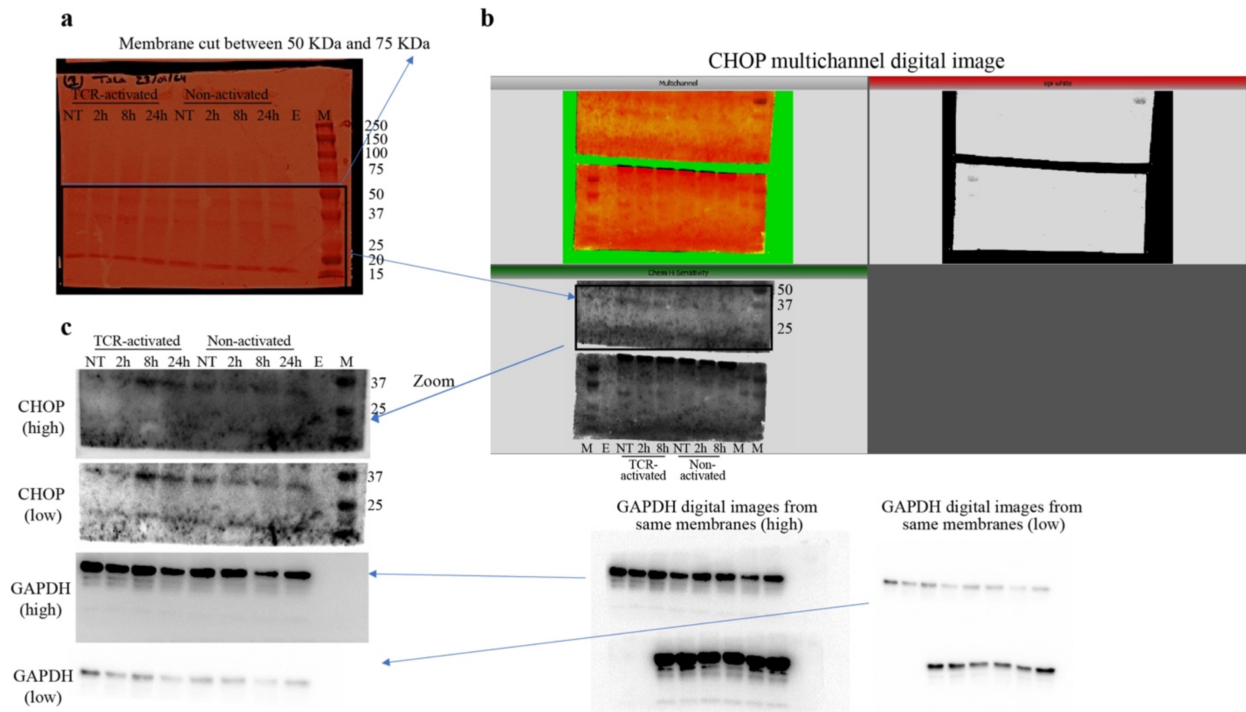

Figure S10. Original Western blots. (**a**) Ponceau stain image of the uncut nitrocellulose membrane used for blotting for CHOP and GAPDH shown in Figure 3b. The box shows where the membrane was cut (between 50 KDa and 75 KDa). The ladder and corresponding sizes in KDa are shown. Whole-cell lysates from expanded T cells of one donor that were TCR-activated (left) or non-activated (right), and either non-treated (NT), or treated with 200µM of pregnenolone sulfate (PS) for 2, 8, or 24 hours, run on a gel and blotted with CHOP rabbit monoclonal antibody, followed by GAPDH mouse monoclonal antibody. M: marker; NT: non-treated; E: empty. (**b**) (Top) Multichannel digital images captured after blotting for CHOP, with the top cut membrane being from (a) and used for Figure 3b, and the bottom cut membrane being a technical replicate (without the 24-hour timepoint). Membrane borders are clearly shown. The blots at the bottom of the panel show high and low exposures after blotting both membranes with GAPDH. (**c**) Images of the first top membrane with high and low exposures of CHOP and GAPDH are shown, which were cropped to show CHOP expression in activated T cells in Figure 3b.

Figure S11

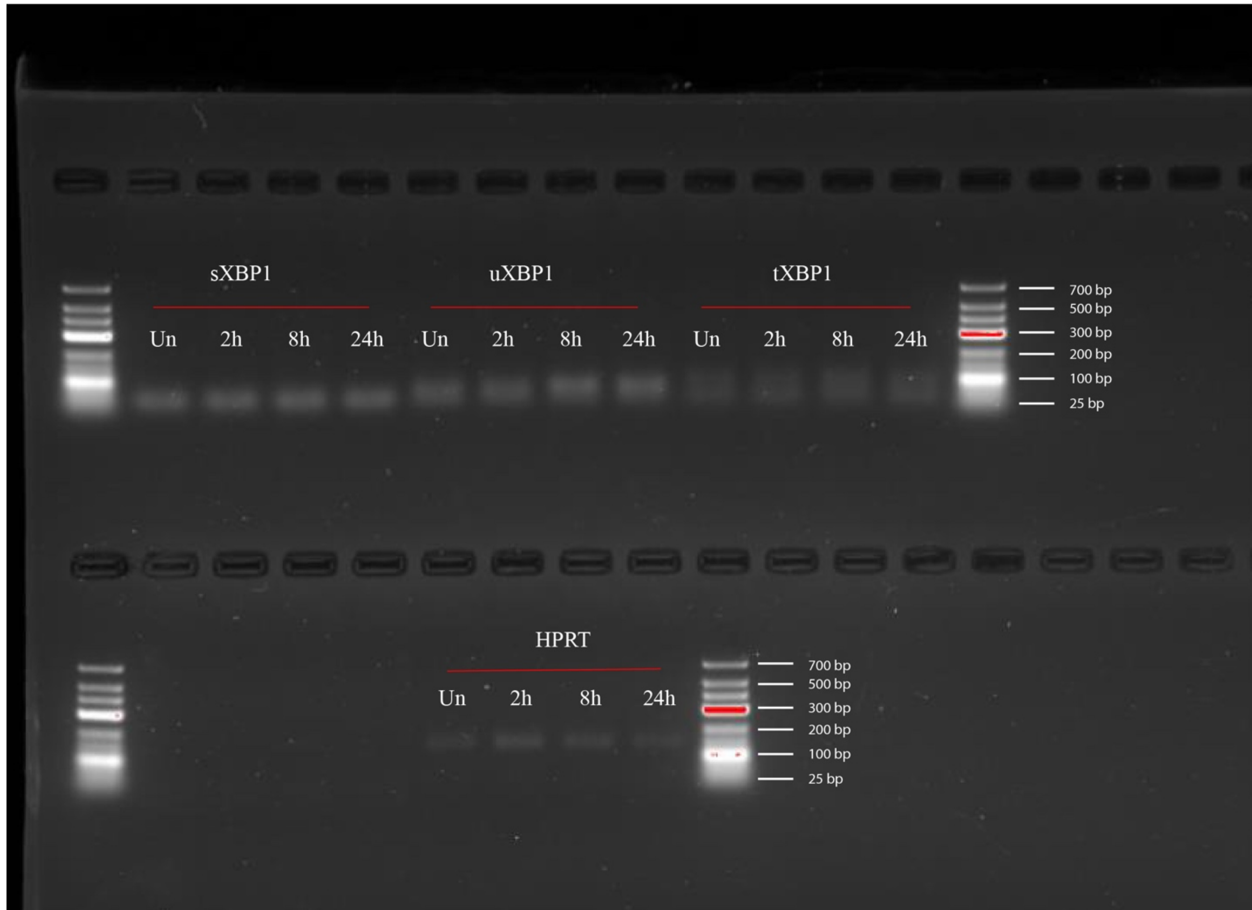

Figure S11. PCR gel for XBP1 splicing. Original uncropped gel image showing bands from PCR amplified cDNA of spliced XBP1 (sXBP1, 59 bp), unspliced XBP1(uXBP1, 76 bp), and total XBP1 (tXBP1, 71 and 97 bp) in the top gel, and HPRT (139 bp) in the bottom gel. PCR amplification was performed on cDNA synthesized mRNA extracted from expanded T cells of one donor that were TCR-activated for 24 hours, followed by no pregnenolone sulfate treatment (Un), or treatment for 2, 8 or 24 hours as indicated.

Figure S12

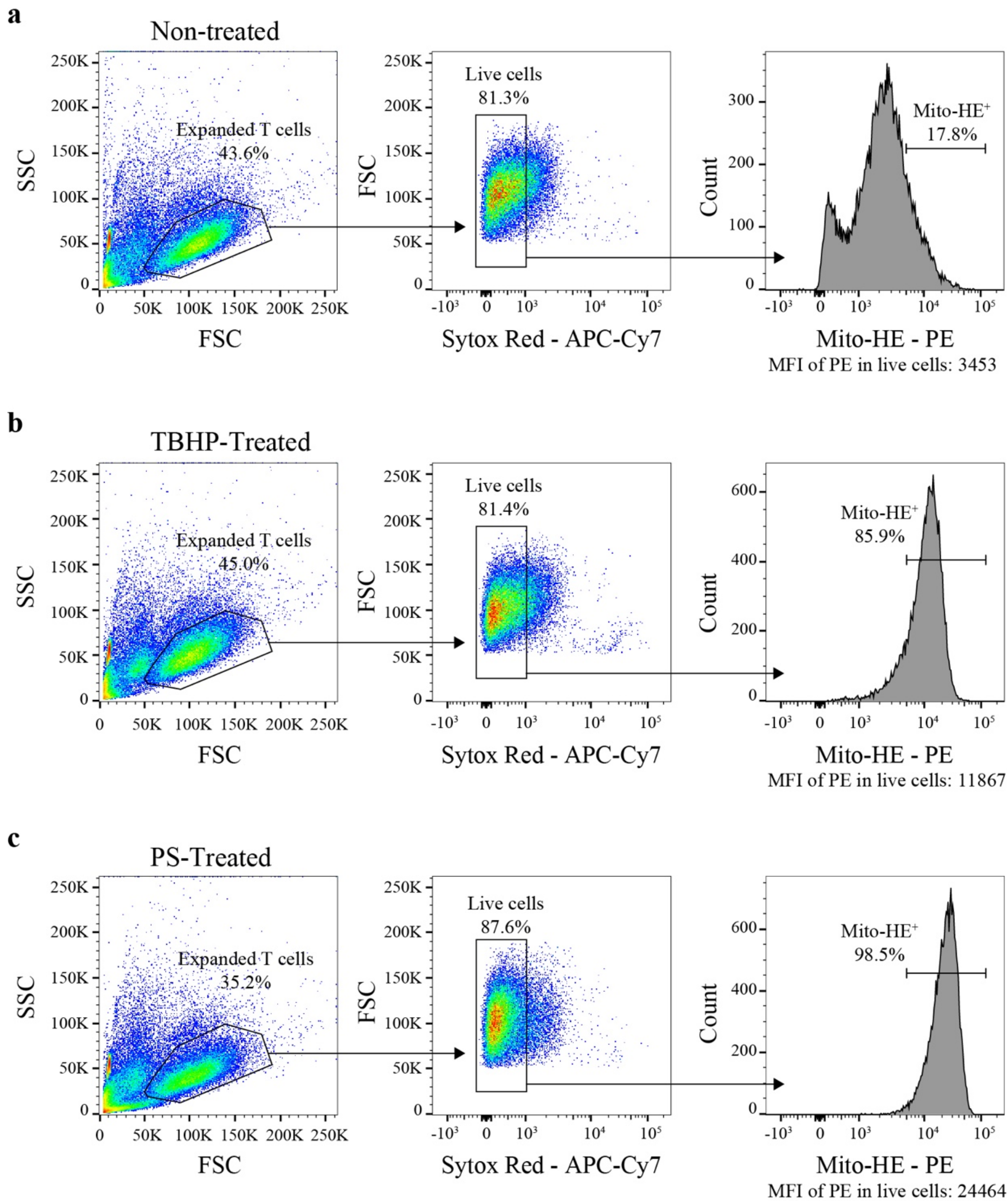

Figure S12. Gating strategy for Mitochondrial superoxide flow cytometric assay. Flow cytometric plots showing the gating strategy used in the (**a**) non-treated, (**b**) tert-Butyl hydroperoxide (TBHP, 250µM)-treated and (**c**) pregnenolone sulfate (PS, 200µM)-treated expanded T cells following mitochondrial superoxide assay from one individual. Expanded T cells were gated according to FSC and SSC, followed by live cell gating using the SYTOX™ Red. Live cells were then gated for Mito-HE and the mean fluorescence intensity (MFI) of the Mito-HE dye within the live cell gate was recorded by FlowJo™.