ARTICLE - Stem Cell Transplantation

# Targeting TFH cells is a novel approach for donor-specific antibody desensitization of allograft candidates: an *in vitro* and *in vivo* study

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## Supplementary methods

### Flow Cytometry

The samples were processed in batches to account for the batch effects. Briefly, 2–10 million PBMCs were thawed and incubated with a mixture of antibodies diluted in 75% PBS and 25% Brilliant Violet Buffer (BD Biosciences) for 30 minutes at 4°C. The cells were surface-stained with Fc receptor-blocking medium (10% FCS PBS). The PBMCs were then washed, fixed, and permeabilized with fixation/permeabilization buffer (eBioscience) for 40 minutes at 4°C. After this the cells were washed with permeabilization buffer (eBioscience), incubated in the dark for 30 minutes at 4°C with intracellular antibodies, and washed before data acquisition on an Aurora spectral flow cytometer (Cytek). PBMCs were stimulated with donor-cell lysate at 1:5 ratio for 6 hours in the presence of Golgi plug (BD Biosciences) and Monensin (eBioscience), at 37°C and 5% $CO_2$. Responder cells were washed, surface stained with CD3, CD8, and CXCR5 antibodies for 20 minutes at 4°C, after which they were washed and fixed with 1% paraformaldehyde (Sigma) for 40 minutes. Subsequently, the cells were permeabilized and incubated with mouse serum (Invitrogen) for 5 minutes at room temperature, followed by incubation with CD40L, and IL-21 antibodies for 30 minutes at room temperature. The cells were then washed and analyzed using a BD Biosciences cytometer (FACSCantoII).

### ELISA

Cytokine levels were measured in coculture supernatants or plasma using the Human CXCL13 Quantikine ELISA kit (R&D Systems), Human IL-21 Quantikine ELISA kit (R&D Systems), and human IL-6 Quantikine ELISA kit (R&D Systems) according to the manufacturer's protocol. Total IgG production was measured in coculture supernatants or plasma using the Human IgG Total ELISA kit (eBioscience), Human IgM Total ELISA kit (eBioscience), and Human IgA Total ELISA kit (eBioscience).

### RNA Sequencing

Total RNA was extracted from cTfh were sorted directly into a lysis buffer. RNA was isolated using the miRNeasy Mini Kit (Qiagen). Complementary DNA synthesis and amplification were performed using a SMARTer Stranded Total RNA-Seq Kit v2 Pico Input Mammalian (Takara). Libraries were sequenced on an Illumina NextSeq 500 platform using 75-bp paired-end reads. Paired-end reads were checked for quality and adapters using FastQC software (version 0.11.7). These quality-trimmed reads were later mapped against the Ensembl human reference genome (GRCh38 version 91) using HISAT2 mapper (version 2.1.0). Gene counts were generated on the mapped files using HT-Seq (version 0.11.2). EdgeR (version 3.24.1), a bioconductor R (version 3.8) package, was used to analyze differentially expressed genes. Differentially expressed genes (two-fold difference and false discovery rate, 0.05) were analyzed using Ingenuity Pathway Analysis (IPA) (Qiagen), with a focus on canonical pathways and upstream regulator analyses. Principal component analysis (PCA) plots were generated based on normalized and scaled fragments per kilobase of transcripts per million read counts, using the factoextra and ggplot2 packages in R.

### ATAC-Sequencing

ATAC sequencing was performed as described previously.[1] Briefly, the cTfh cells were isolated by flow cytometric sorting. Following this, $5\times10^4$ sorted cells were resuspended and lysed in 50 µl Lysis Buffer (10 mM NaCl; 10 mM Tris-HCl, pH 7.4; 3 mM $MgCl_2$; and 0.1% ICEPALEA-630) after this the cells were washed twice in PBS. DNA was fragmented using the TruePrep DNA Library Prep Kit V2 from Illumina (Vazyme, TD501-01) and purified using the MinElute PCR Purification Kit (QIAGEN, 28006). Purified DNA was barcoded using the TruePrep Index Kit V2 (Vazyme, TD202) and amplified by PCR using the TruePrep DNA Library Prep Kit V2 (Illumina). Clean VAHTS DNA beads (Vazyme, N411) were used to purify cDNA libraries between 100 and 1,000 bp. Paired-end sequencing was performed using a NovaSeq6000 (Illumina). Raw ATAC-seq FASTQ files from paired-end sequencing were processed as described previously. Clean fastq files were aligned to the hg38 reference genome using Bowtie2. SAMtools was used to remove unmapped and unpaired mitochondrial reads. PCR duplicates were removed using Picard software. Reads were shifted +4 bp and -5 bp for the positive and negative strand, respectively. Peak calling was performed using MACS2 with an FDR q value of 0.01. We combined the peaks of all samples to create a union peak list and merged the overlapping peaks with the BedTools merge command. The number of reads per peak was determined using BedTools. Differentially accessible regions were identified following DESeq2 normalization using an FDR cut-off q value < 0.05. Motif enrichment was calculated using HOMER (default parameters) on the peaks that were differentially accessible across the LV-PRDM1 and LV-control groups. Transcription binding site prediction analysis was performed using a known motif-discovery strategy.

**Statistical Analyses**

Mean, SD, and SEM values and frequencies were used to describe continuous and categorical variables, respectively. The means and proportions were compared using the t-test and chi-squared test (or the Mann–Whitney U test and Fisher's exact test wherever applicable). Multiple groups were analyzed using the Kruskal–Wallis test or one-way ANOVA with Tukey's post-hoc test for adjustment for multiple comparisons. Statistical significance was set at $P < 0.05$, and all tests were two-sided. Analyses were performed using GraphPad Prism version 8, Cytobank (http://www.cytobank.org/), Partek Flow (http://www.partek.com/partek-flow/), and R software (https://www.r-project.org/, R Development Core Team, Vienna, Austria).

**References**

1. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nat Methods. 2013;10(12):1213-1218.

## Supplementary Tables

### Table S1. List of flow cytometry antibodies

| Antibody | Fluorescent labeling | Clone | Manufacturer |
|---|---|---|---|
| CD196 | BB515 | 11A9 | BD Pharmingen |
| CD279 | PE | MIH4 | BD Pharmingen |
| 7-AAD | PerCP-Cy5.5 | - | BD Pharmingen |
| CD183 | PE-Cy7 | 1C6/CXCR3 | BD Pharmingen |
| CXCR5 | Alexa 647 | Rf8b2 | BD Pharmingen |
| CD3 | Alexa 700 | UCHT1 | BD Pharmingen |
| CD278 | BV421 | DX29 | BD Pharmingen |
| CCR7 | BV510 | 2-L1-A | BD Pharmingen |
| CD45RA | BV605 | HI100 | BD Pharmingen |
| CD4 | APC-H7 | SK3 | BD Pharmingen |
| CD28 | PE/Cy7 | CD28.2 | Biolegend |
| CD154 | PE | 24-31 | Biolegend |
| CD278 | BV510 | C398.4A | Biolegend |
| CD80 | BV650 | 2D10 | Biolegend |
| CD86 | BV421 | IT2.2 | Biolegend |
| CD54 | PE | HCD54 | Biolegend |
| CD40 | AF700 | 5C3 | Biolegend |
| LFA-1 | BV421 | M24 | Biolegend |
| CD3 | FITC | SK7 | Biolegend |
| CD20 | AF750 | 2H7 | Biolegend |
| CD38 | BV421 | HB-7 | Biolegend |
| CD19 | BV510 | HIB19 | Biolegend |
| CD27 | PE-Cy7 | M-T271 | Biolegend |
| CD27 | PerCP-Cy5.5 | M-T271 | Biolegend |
| CD275 | APC | 2D3 | Biolegend |
| IL-21 | PE | 3A3-N2 | Biolegend |

**Abbreviations:** CD= cluster of differentiation; 7-AAD= 7-aminoactinomycin D; CXCR5= C-X-C chemokine receptor type 5; LFA-1= lymphocyte function-associated antigen-1; IL-21= interleukin-21.

**Table S2. Characteristics of DSA positive patients treated with sirolimus and/or Rituximab**

| | Single drug group | | Combined group | *P* |
|---|---|---|---|---|
| | Sirolimus group | Rituximab group | | |
| number，n | 16 | 10 | 8 | - |
| Sex，male/female，n | 4/12 | 3/7 | 2/6 | 0.9577 |
| Median age（range） | 45.0 (32-63) | 47 (34-71) | 41 (10-62) | 0.324 |
| Diagnosis | | | | 0.421 |
| AML | 9 | 5 | 4 | |
| ALL | 2 | 2 | 1 | |
| MDS | 5 | 3 | 3 | |
| Median DSA MFI （range） | 13532.5 (3553-26693) | 9168.5 (5911-12473) | 15619 (9842-17687) | 0.054 |
| Number of courses | | | | |
| 1 | 2 | 0 | 0 | |
| 2 | 2 | 4 | 3 | 0.316 |
| >2 | 12 | 6 | 5 | |
| Pregnancy (female) | | | | |
| Yes | 12 | 7 | 5 | 0.1922 |
| No | 0 | 0 | 1 | |
| Times of transfusion | 6 (4-20) | 5（2-16） | 10（6-32） | 0.354 |
| Median course of disease (months) | 9（2-18） | 7（3-25） | 11（3-27） | 0.753 |

**Abbreviations:** AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; HLA, human leukocyte antigen; DSA, donor-specific anti-HLA antibody; MFI, mean fluorescence intensity

**Table S3 Statistical table of adverse drug reaction of patients**

| | S group | R group | S+R group |
|---|---|---|---|
| number | 16 | 10 | 8 |
| COVID-19 | 10 | 6 | 4 |
| Bacterial infection | 2 | 1 | 2 |
| Virus infection | 2 | 1 | 1 |
| Fungal infection | 0 | 1 | 0 |
| Abdominal pain | 4 | 2 | 3 |
| constipation | 1 | 1 | 2 |
| diarrhea | 2 | 2 | 1 |
| Tachycardia | 1 | 0 | 1 |
| Proteinuria | 0 | 0 | 1 |

**Abbreviations:** COVID-19, Corona Virus Disease 2019. S group, Sirolimus group. R group Rituximab group. S+R group, Combined group.

**Supplementary figure legends**

**Figure S1. DSA desensitization treatment flow chart**

DSA positive patients underwent desensitization treatment 40 days before transplantation for a duration off our 4 weeks. After one week of treatment, patients who met the DSA requirements (DSA MFI < 2,000) could enter the conditioning regimens and were prepared for hematopoietic stem cell transplantation. (A) Flow chart of the sirolimus desensitization treatment. (B) Flow chart of the rituximab desensitization treatment. (C) Flow chart of the rituximab and sirolimus desensitization treatment.

**Abbreviations:** DSA, donor specific anti-HLA antibodies; Ara-C, cytrarabine; Bu, busulfan; CTX, cyclophosphamide; M-CCNU, semustine; ATG, thymoglobulin; MTX, methotrexate; CSA, cyclosporine; MMF, mycophenolate mofetil; MFI, mean fluorescent intensity.

**Figure S2. Flow cytometry representation graphs of cTfh and its subsets.** (A) Nucleated cell gate strategy; (B) CD4 cell gate strategy ($CD3^{+}CD4^{+}$); (C) cTfh cell gate strategy ($CD3^{+}CD4^{+}CXCR5^{+}CD45RA^{-}$); (D-E) cTfhem cell gate strategy ($CD3^{+}CD4^{+}ICOS^{+}CCR7^{-}PD\text{-}1^{+}$); (F) cTfh1 cell ($CD3^{+}CD4^{+}CXCR3^{+}CCR6^{-}$), cTfh2 cell ($CD3^{+}CD4^{+}CXCR3^{-}CCR6^{-}$) and cTfh17 cell ($CD3^{+}CD4^{+}CXCR3^{-}CCR6^{+}$) gate strategy.

**Abbreviations:** NC, nucleated cell; cTfh, circulating T follicular helper cells.

**Figure S3. Analysis of the proportion and correlation of cTfh subgroups**

(A) The proportion of cTfh in T cells. (B) Correlation analysis of the cTfh ratio and absolute value with anti-HLA antibody MFI. (C) Correlation analysis of the cTfh ratio and absolute value with DSA-MFI.

**Abbreviations:** HD, healthy donor; HLA, human leukocyte antigen; cTfh, circulating T follicular helper cells; Bm, memoery B cells; DSA, donor-specific antibodies; Ab, antibody; MFI, mean fluorescent intensity.

**Figure S4. Flow cytometry representation graphs of memory B cells and purity identification by flow cytometry sorting.** (A) B cell gate strategy ($CD3^{-}CD19^{+}$), gated on nucleated cell; (B) Memory B cell gate strategy ($CD3^{-}CD19^{+}CD27^{+}$); (C) Plasmablast gate strategy ($CD20^{-}CD38^{+}$); (D) cTfh cell purity identification (>99%); (E) memory B cell purity identification (>97%).

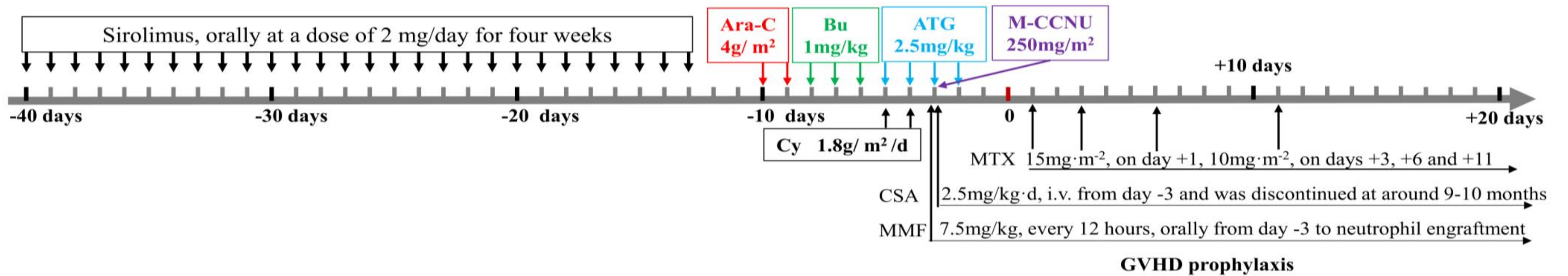
**Figure S5. Comparing anti-HLA antibodies in transplant candidates' sera (*in vivo*) and DSA positive patients' co-cultured supernatants (*in vitro*).** The patients' cTfh cells and memory B cells were co-cultured after sorting (n=5), and then the titer of anti-HLA antibody in the co-culture supernatant was determined. (A) Patient 1; (B–C) Patient 2; (D–E) Patient 3; (F) Patient 4; (G) Patient 5. The antibody test results *in vivo*

are displayed in red columns, and *in vitro* are displayed in black columns.

**Figure S6. Combined RNA-seq and ATAC-seq analysis suggests baiap3 as a potential ctfh function regulator.** The cTfh cells of healthy donors (n=2), patients with positive (n=3) and negative (n=3) were analyzed. (A) Heat map of RNA-seq gene differential expression heat map; (B) ATAC-seq chromatin accessible differential peak calorigram; (C) Enrichment analysis of differential gene pathways; (C) RNA-seq and ATAC-seq analysis of BAIAP3; Transcription binding site prediction analysis performed using known motif discovery strategy. EdgeR (version 3.24.1), a bioconductor R (version 3.8) package, was used to analyze differentially expressed genes. Principal component analysis (PCA) plots were generated on the basis of normalized and scaled fragments per kilobase of transcripts per million read counts using the factoextra and ggplot2 packages in R.

**Figure S7. Dynamic changes of DSA in sirolimus treatment cohort.** The changes of DSA in the first week (n=16), the second week (n=16), the third week (n=12) and the fourth week (n=12) were shown. Paired T test is used in A-E diagram.

Figure S1

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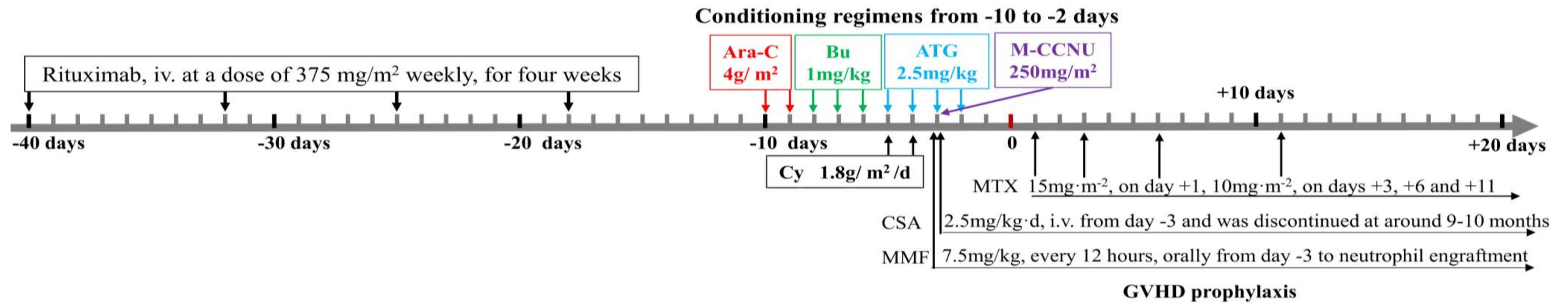


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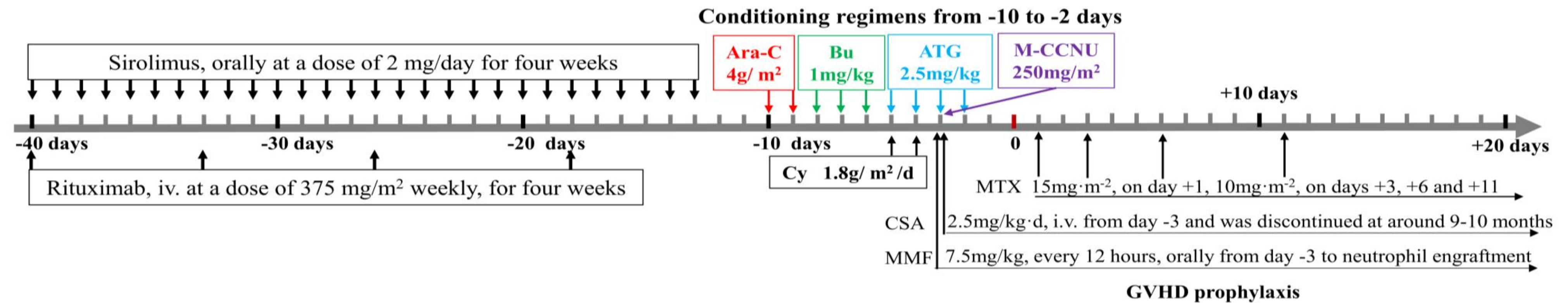


**Figure S2**

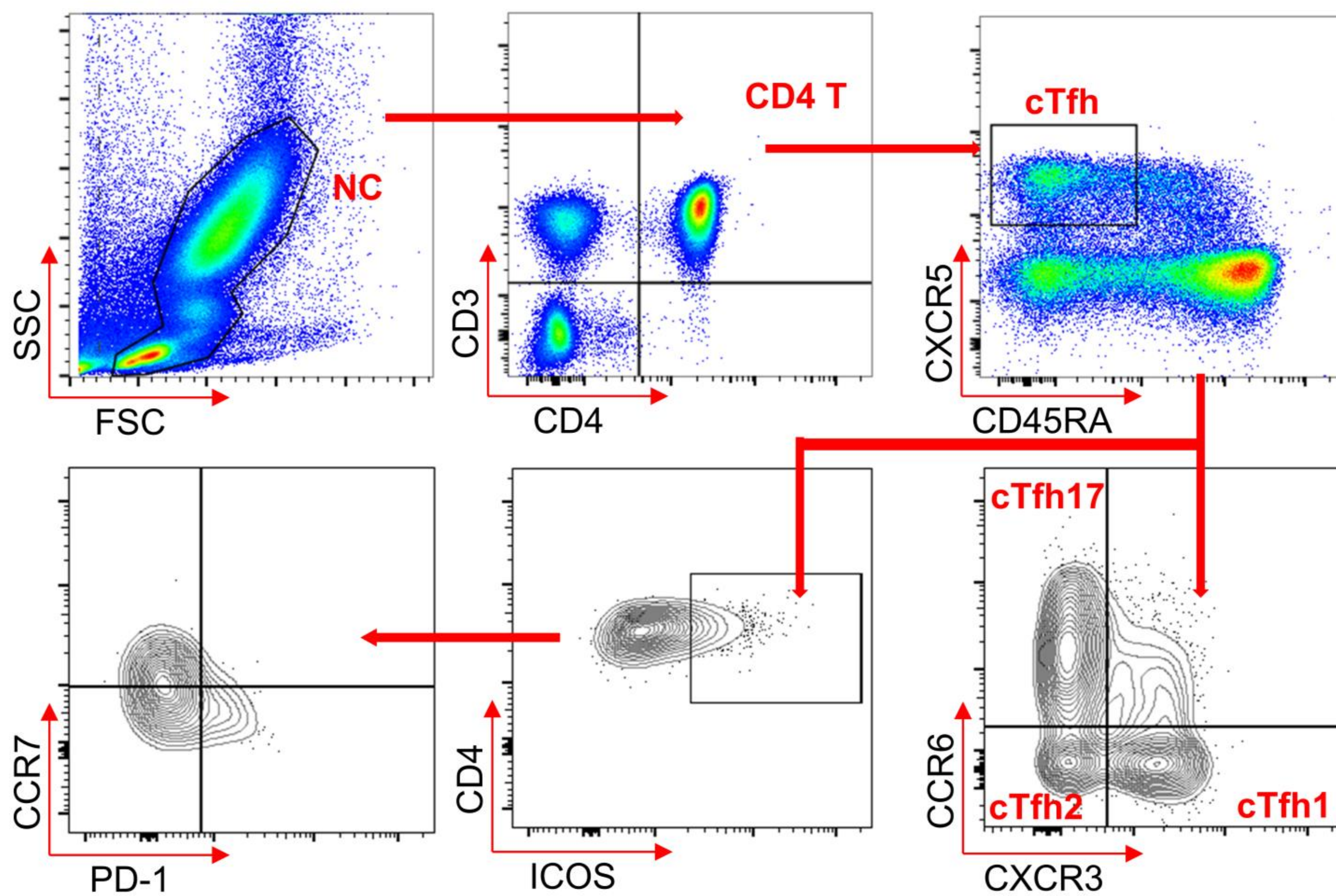
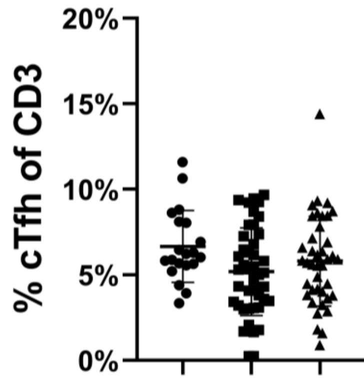


**Figure S3**

● HD (n=20)
■ Patients with negative anti-HLA antibody (n=43)
▲ Patients with positive anti-HLA antibody (n=40)

A

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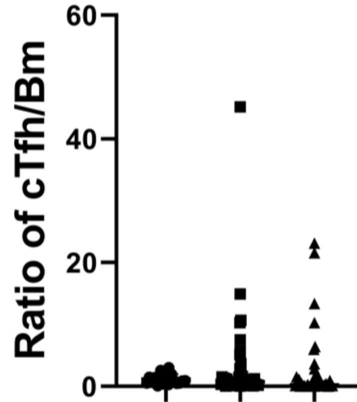


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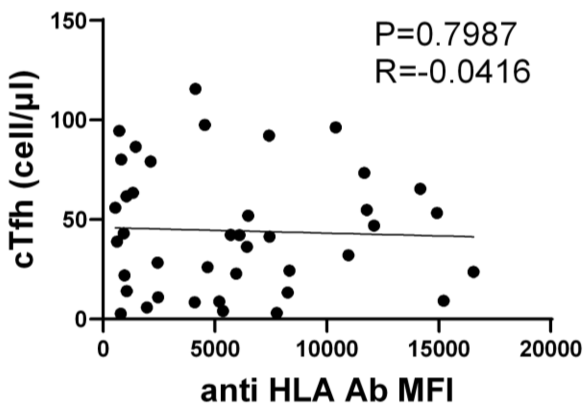
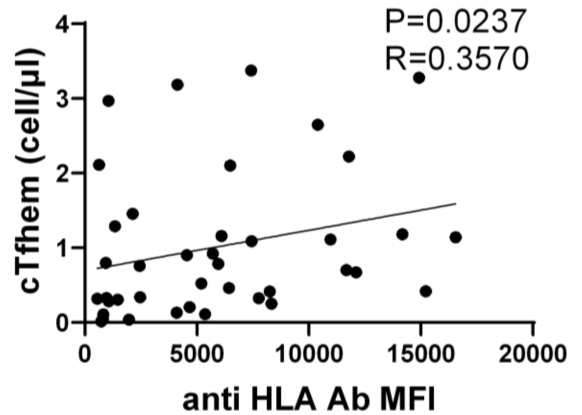


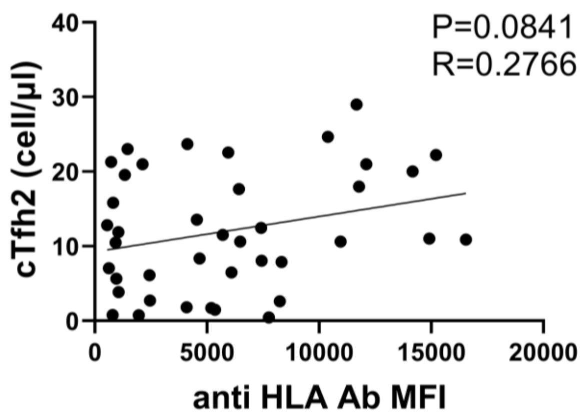
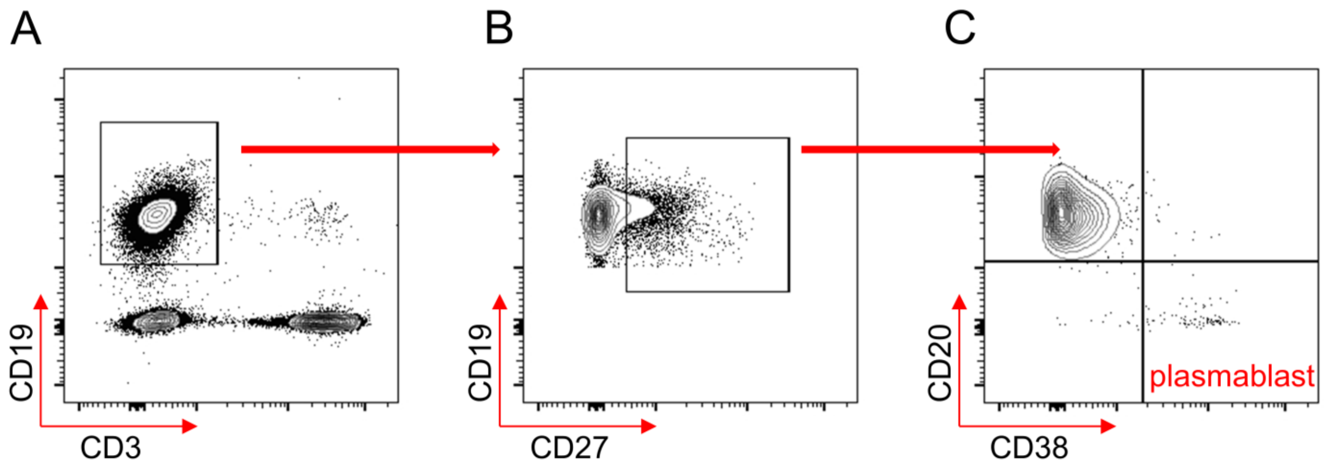
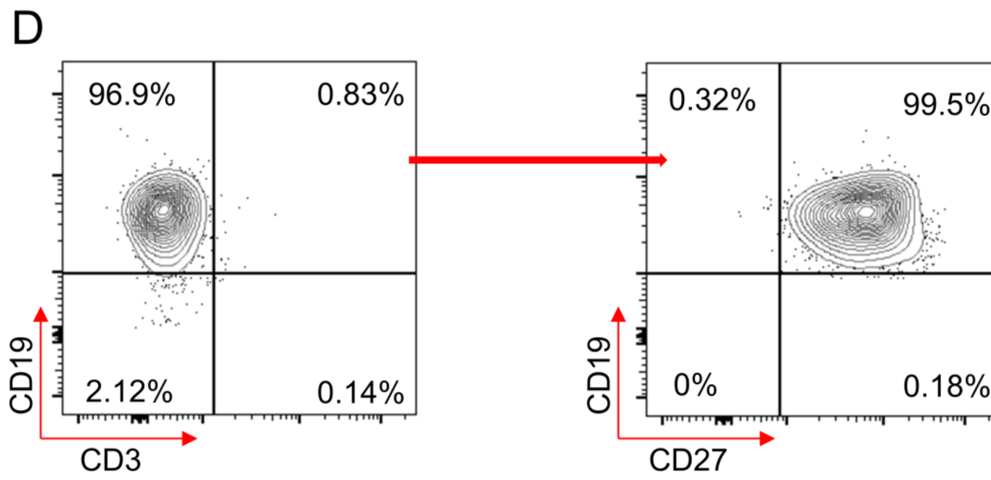



**Figure S4**



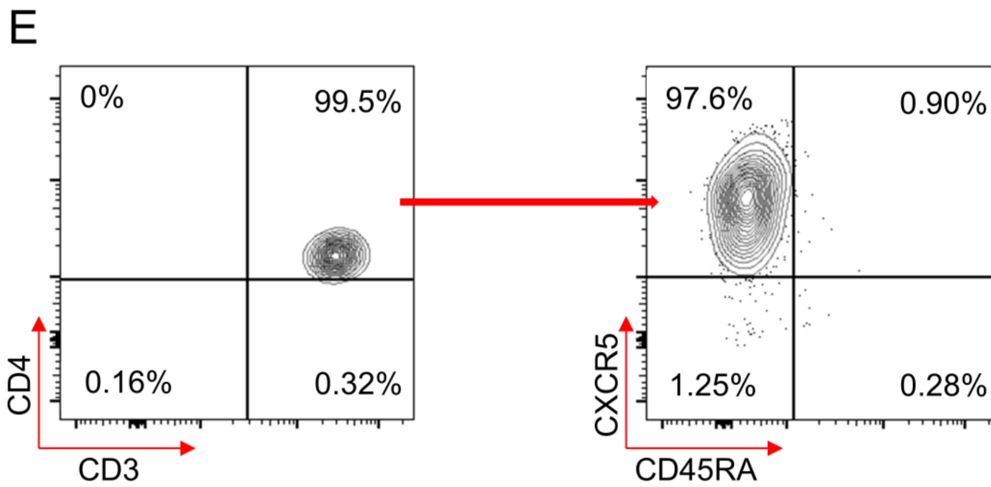



**Figure S5-1**

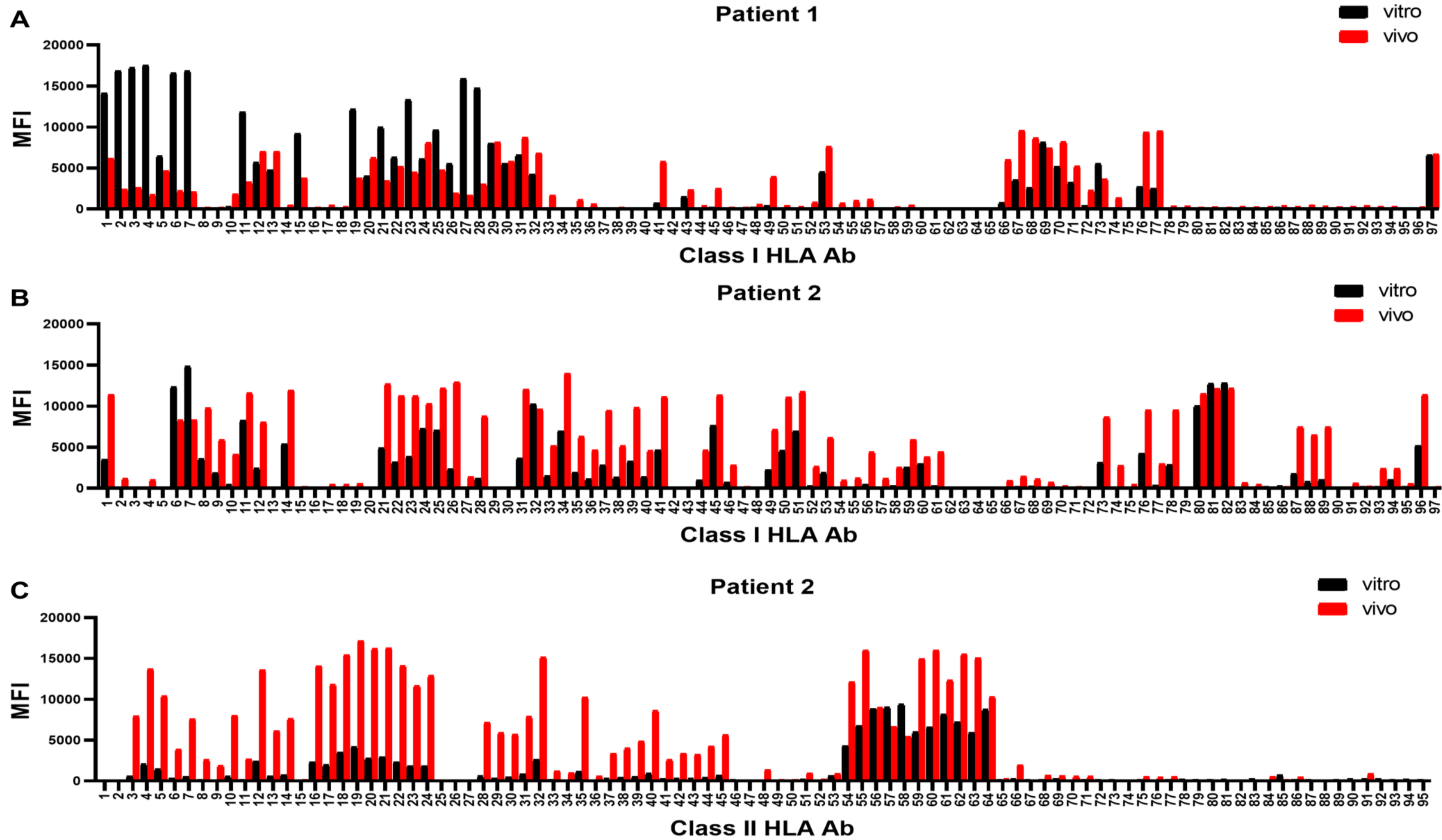
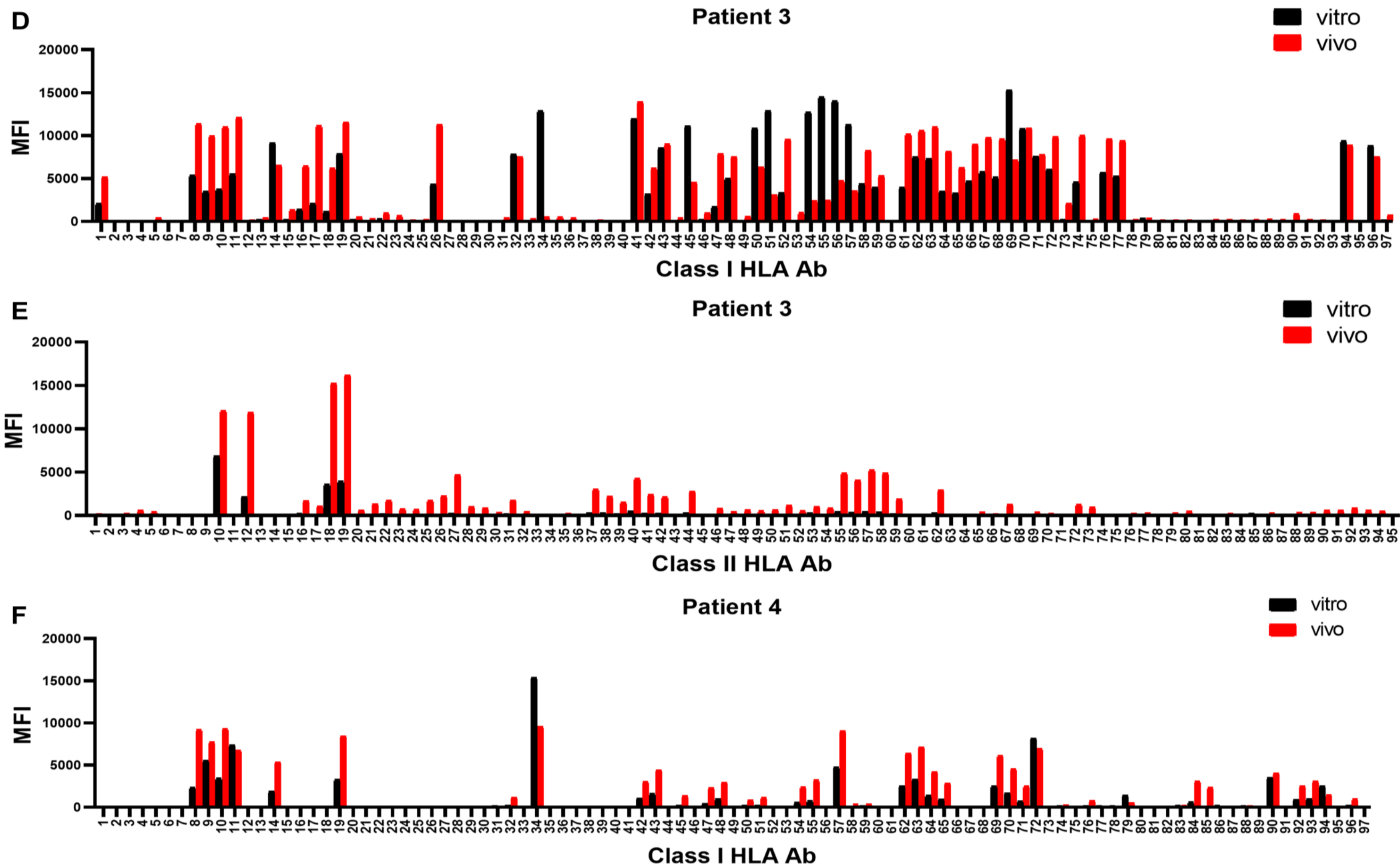


**Figure S5-2**



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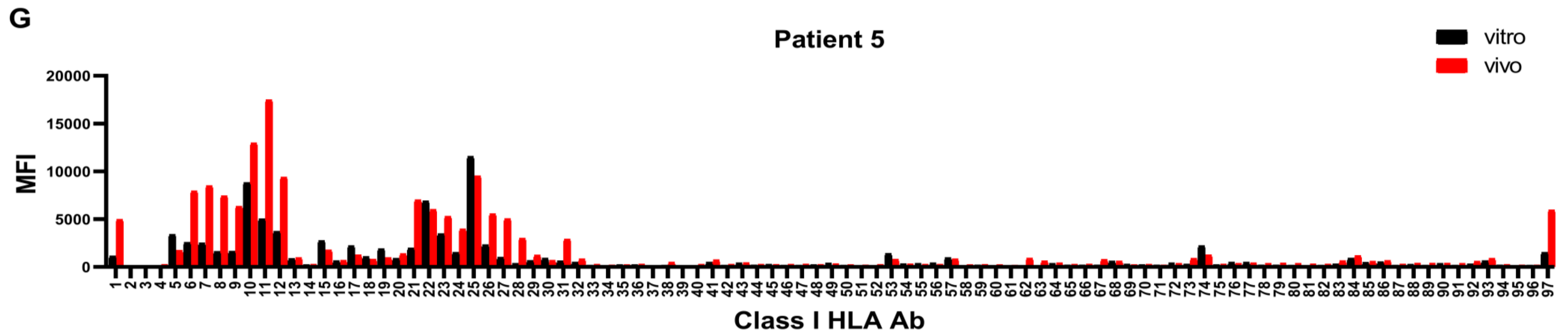

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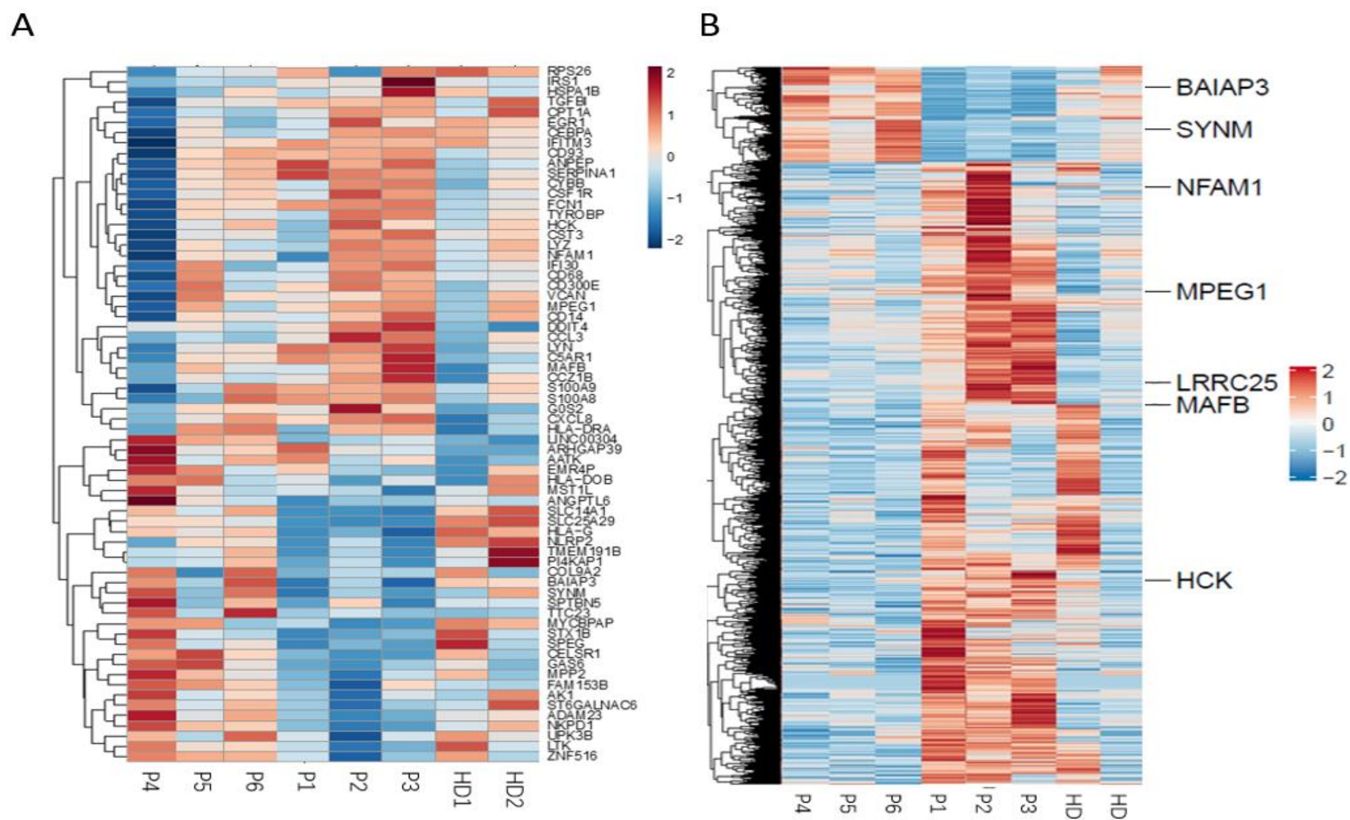


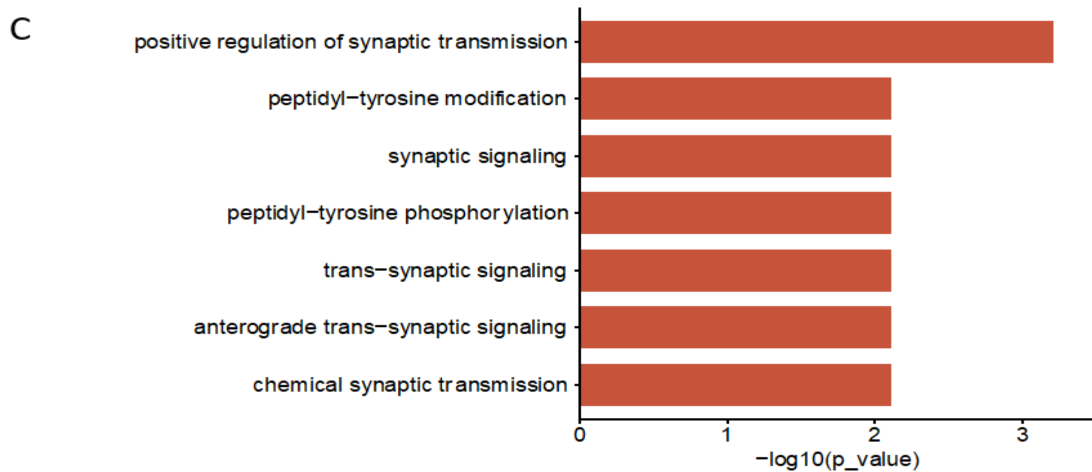


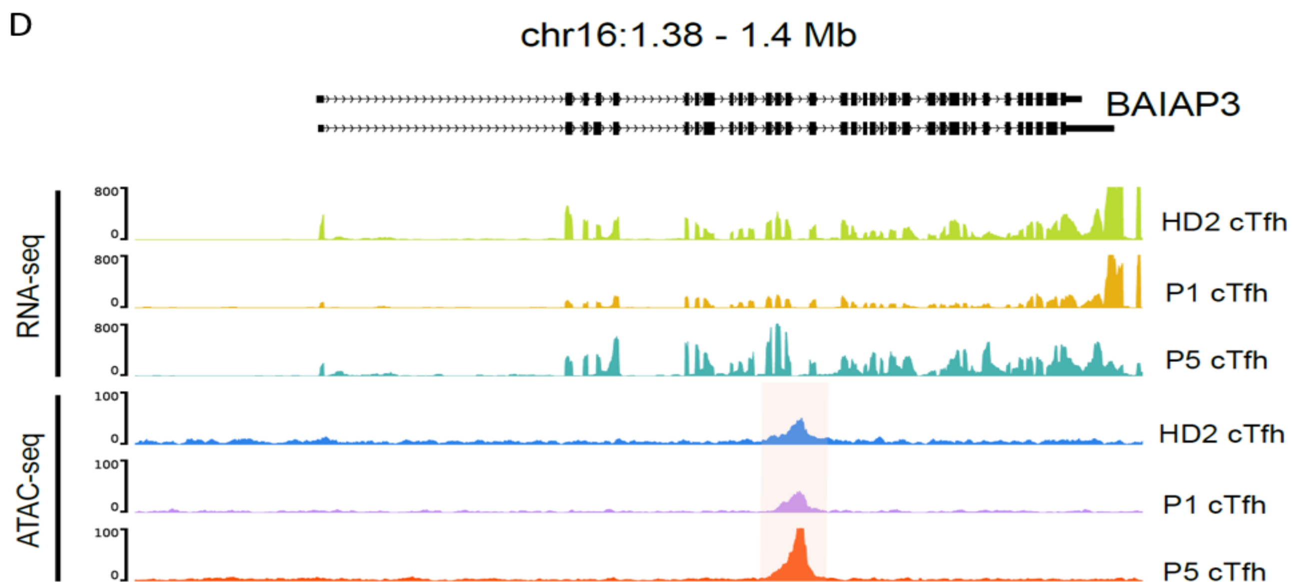

**Figure S7**