ISCI, Volume *23*

Supplemental Information

Human Plasma-like Medium Improves T Lymphocyte Activation

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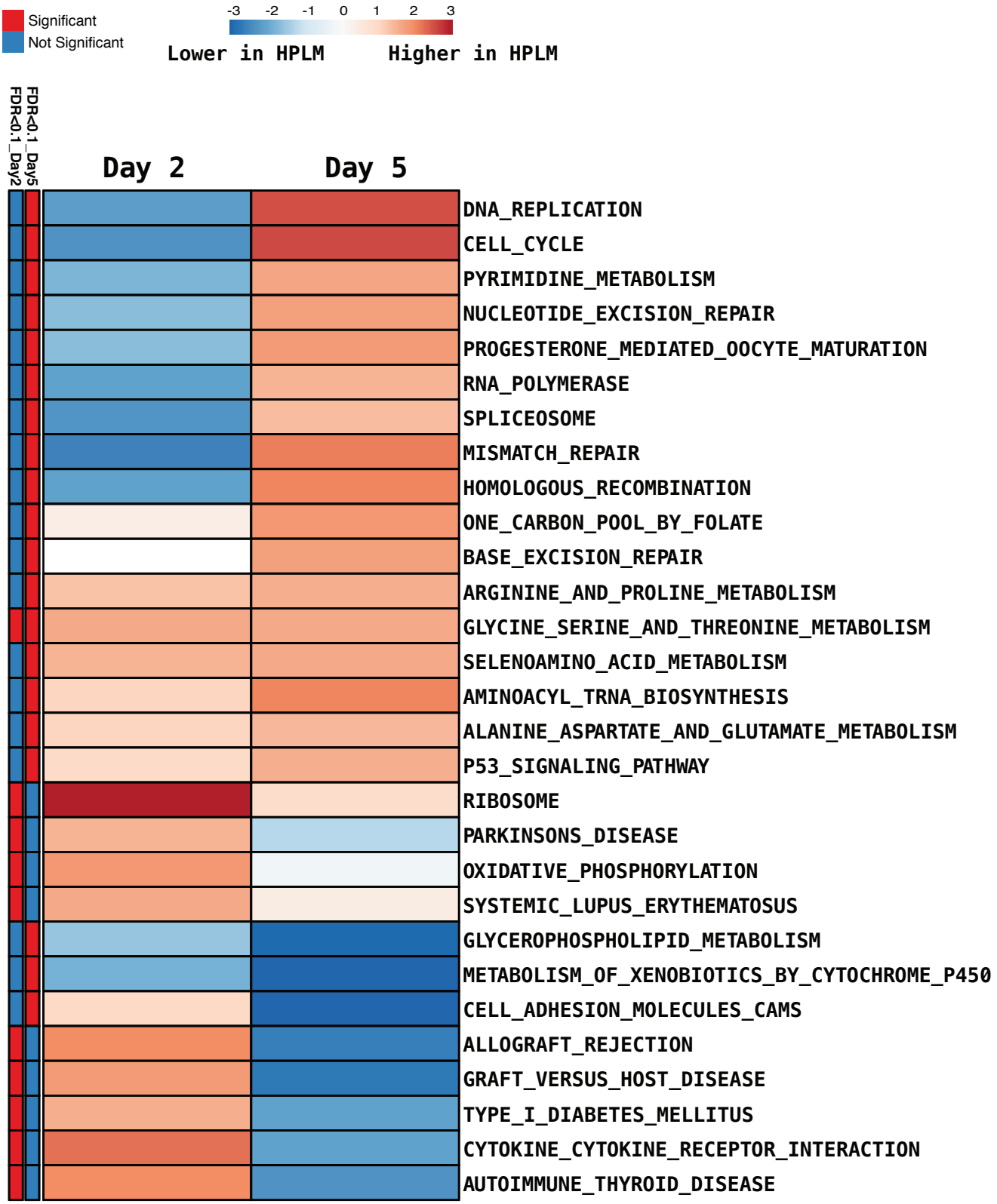
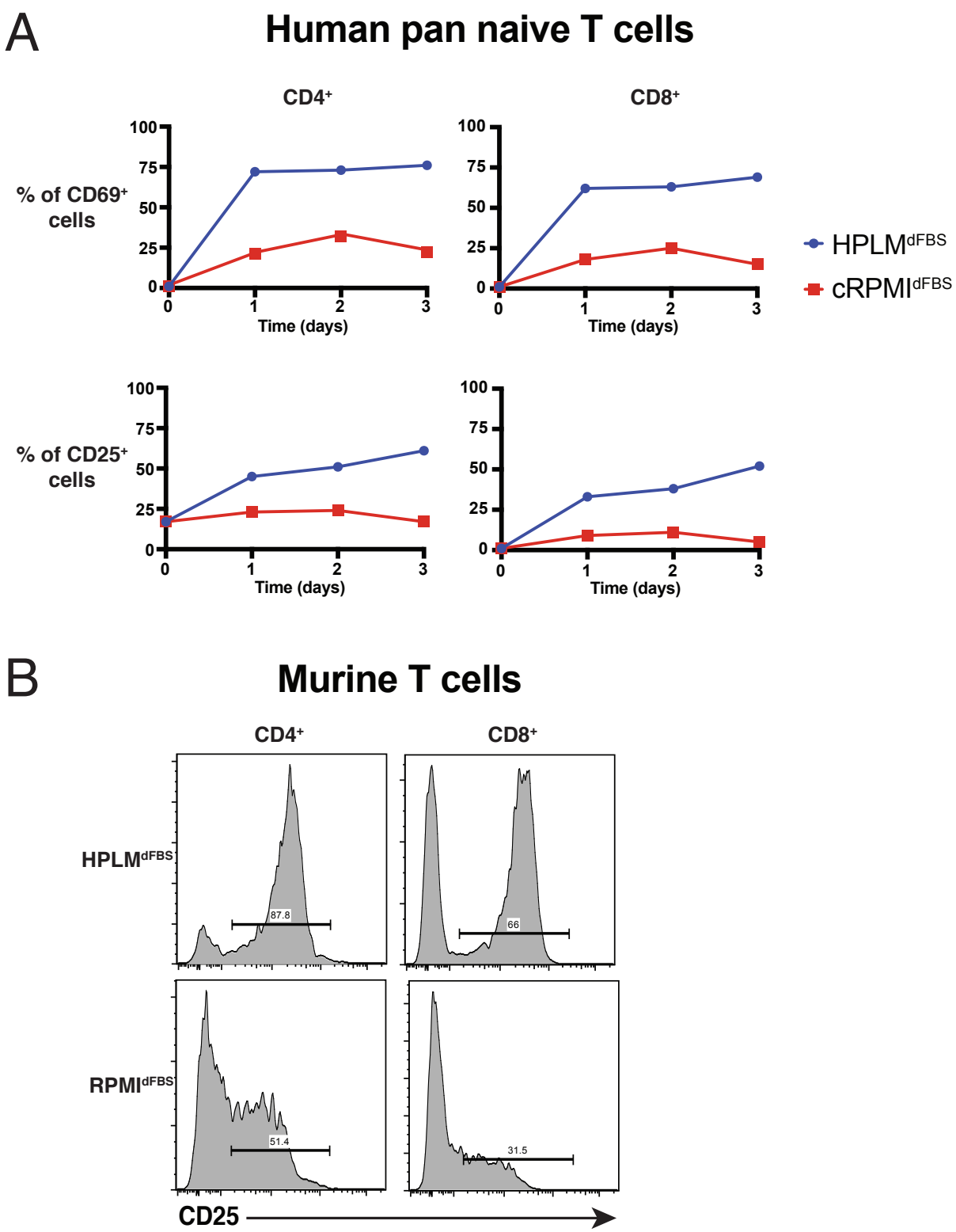


Supplemental Figure 1 – GSEA of purified naïve human T cells activated in either HPLM^{dFBS} or RPMI^{dfBS}, related to Figure 1C-D.

Plot showing KEGG pathways enriched at either 48 or 120 hours following stimulation in either HPLM^{dFBS} or RPMI^{dFBS}.



Supplemental Figure 2 – Activation kinetics of murine/human T cells cultured in HPLM^{dFBS} or RPMI^{dFBS}. Data related to Figure 2A.

(A) Daily quantification of CD25 and CD69 of pan human naïve T cells cultured in either HPLM^{dFBS} or RPMI^{dFBS}. Data shown are a single experiment representative of 4 repeats.

(B) Quantification of CD25 and CD69 24 hours post-activation of splenic murine T cells cultured in either HPLM^{dFBS} or RPMI^{dFBS}. Data is representative of two experiments.

Supplemental Table 1: Detailed Media Composition, Related to Figure 1

	Concentration (μM)		
	RPMI	**HPLM-MIN**	**HPLM**
Glucose	11111	5000	5000
Proteinogenic Amino acids			
Alanine	0	430	430
Arginine	1149	110	110
Asparagine	378	50	50
Aspartate	150	20	20
Cysteine	0	40	40
Cystine	208	100	100
Glutamate	136	80	80
Glutamine	2055	550	550
Glycine	133	300	300
Histidine	97	110	110
Hydroxyproline	153	0	0
Isoleucine	382	70	70
Leucine	382	160	160
Lysine	219	200	200
Methionine	101	30	30
Phenylalanine	91	80	80
Proline	174	200	200
Serine	286	150	150
Threonine	168	140	140
Tryptophan	25	60	60
Tyrosine	11	80	80
Valine	171	220	220
Ions			
Na^+	138525	132271	132271
K^+	5333	4142	4142
Ca^{2+}	424	2390	2390
Mg^{2+}	407	830	830
NH^{4+}	0	40	40
Cl^-	108781	116196	116196
HCO^{3-}	23809	24000	24000
PO_4^{3-}	5634	966	966
SO_4^{2-}	407	350	350
NO^{3-}	848	80	80

Additional Polar Metabolites			
2-hydroxybutyrate			50
3-hydroxybutyrate			50
4-hydroxyproline			20
Acetate			40
Acetone			60
Acetylcarnitine			5
Acetylglycine			90
α-aminobutyrate			20
α-ketoglutarate			5
Betaine			70
Carnitine			40
Citrate			130
Citrulline			40
Creatine			40
Creatinine			75
Formate			50
Fructose			40
Galactose			60
Glutathione			25
Glycerol			120
Hypoxanthine			10
Lactate			1600
Malate			5
Malonate			10
Ornithine			70
Pyruvate			50
Succinate			20
Taurine			90
Urea			5000
Uric Acid			350
Uridine			3

MATERIALS AND METHODS

Study Subjects

Anonymized blood samples were received from the NIH Department of Laboratory Medicine and processed in order to isolate PBMCs.

Cell Culture

In order to maintain consistency and reduce potential batch effects RPMI was prepared from powder (US Biologicals). Fresh L-glutamine (Gibco) and dialyzed FBS (Thermo Fisher Scientific) were added immediately prior to use at a final concentration of 2 mM and 10% by volume respectively. $HPLM^{dFBS}$ was prepared as previously described (Cantor *et al.*, 2017) along with four additional compounds: Uridine (3 μM), α-ketoglutarate (5 μM), acetylcarnitine (5 μM) and malate (5 μM). $HPLM^{dFBS}$-Min was prepared as $HPLM^{dFBS}$ but lacking all defined components other than glucose, proteinogenic amino acids, vitamins, salts, and dialyzed serum. (see Supplemental Table 1). RPMI was supplemented with 10% of either dialyzed ($RPMI^{dFBS}$) or unmodified FBS ($RPMI^{FBS}$). For long-term T cell proliferation assays, human recombinant IL-2 (Roche) was also added fresh immediately prior to use at a final concentration of 100 IU/ml. X-VIVO 15 media (Lonza) was used without additional serum. AIM-V media was a mixture of 50% RPMI and 50% AIM-V (Gibco) supplemented with 5% Normal Human Serum (Valley Biomedical). RPMI-Metabolites was prepared by supplementing RPMI with both 10% dialyzed FBS and 31 additional polar metabolites at concentrations defined in basal HPLM.

Flow Cytometry

For simple surface stains, cells were washed once in phosphate buffered saline (PBS) and stained with 50 μl of Zombie Aqua™ viability (Biolegend) dye for 20 minutes on ice. After washing once with FACS buffer (PBS containing 5% FBS and 0.1% NaN_3), cells were stained in 50 μl of FACS buffer containing diluted antibodies for 30 minutes on ice. Cells were then washed three times in FACS buffer and fixed before acquisition on either a LSRII or LSRFortessa (BD Biosciences). For intracellular cytokine staining following restimulation, cells were washed with PBS, stained in antibodies in 50 ul of FACS buffer for 30 minutes on ice. Cells were then washed three times in FACS buffer, then incubated for 20 minutes in 50 ul of Fixation/Permeabilization solution (BD Biolegend). Cells were washed three times in Perm/Wash buffer (BD Biolegend), then stained for 30 minutes on ice in antibodies diluted in

Perm/Wash buffer. Cells were washed three times, then resuspended in Perm/Wash buffer and acquired on an LSRII. Data was analyzed using FlowJo v. 9.9.5.

T cell Activation and expansion Analysis

Peripheral blood mononuclear cells were freshly isolated from blood from healthy human donors of both sexes ranging from 25-60 years old. Naïve T cells were enriched via a negative selection kit (Miltenyi Biotech, 130-097-095) and either used immediately or stored in liquid nitrogen in a 9:1 mixture of FBS: dimethyl sulfoxide. For short-term activation assays, 96 well Nunc Maxisorp plates were coated with 1 to 10 ug/ml of anti-CD3/anti-CD28 antibodies in PBS for 2 to 4 hours at 37°C. Plates were then washed twice with PBS and enriched naïve T cells were added in the indicated media formulations. Levels of activation markers were measured by flow cytometry 16 to 24 hours later. For proliferation assays, cells were stained with Celltrace Violet and then activated as above. After three days, cells were transferred to a new plate with fresh media and analyzed by flow cytometry after another two days. Alternatively, for murine T cells, crude splenic cells were isolated and cultured on plate-bound anti-CD3/CD28 antibodies (clones 2C11 and clone 37.51 respectively, Biolegend). One day later cells were analyzed by flow cytometry as described above to measure levels of activation markers.

Calcium flux measurements

Purified naïve human T cells were cultured in RPMI for 16 to 24 hours following isolation or thawing from liquid nitrogen storage. Cells were then loaded with Indo-1 calcium Indicator dye (Thermo Fisher Scientific) resuspended in PowerLoad™ (Thermo Fisher Scientific) at a concentration for 20 minutes at room temperature. and stained for surface markers. Anti-CD3 (Hit3α) was added at 10 ug/ml, samples were acquired for 20 seconds to determine background levels, then F(ab')$_2$ fragments (Jackson Immunoresearch) were added at 13 ug/ml in order to crosslink the TCR. The flux was then recorded for another 130-180 seconds and analyzed by measuring the ratio of fluorescence in the two channels.

T cell cytokine production assays

Human T lymphocytes were activated and expanded in IL-2-containing media as described above for 15 to 20 days. The night before the assay, cells were placed in fresh media (ether HPLM^{dFBS} or RPMI^{dFBS}) containing IL-2, and the following day were restimulated with either 1 μg/ml each of anti-CD3 (HIT3α, Biolegend) or PMA and ionomycin (Biolegend) for 6 or 5 hours respectively. Four hours prior to analysis, Brefeldin A was added (Biolegend). Following the

restimulation, cells were washed in PBS, placed on ice and stained according to the protocol described above.

Retroviral Transductions

CD19-CAR expressing retrovirus was produced as previously described (Kerkar *et al.*, 2011). 96-well plates were coated with Retronectin (Clontech) overnight at 4°C, washed and blocked with 2.5% bovine serum albumin (BSA), washed again and then bound to retrovirus by centrifuging the plates with viral supernatant for two hours at 37°C. The supernatants were then aspirated and human T lymphocytes that had been activated the previous day were added to the plate in the medium indicated. 48 hours following transduction, the cells were stained with Biotin-Protein L (Genscript) followed by fluorescently-conjugated streptavidin.

RNA-Sequencing

RNA was isolated from cells using trizol (Thermo Fisher). We then used 0.1 – 1 ug of total RNA as input for mRNA capture with oligo-dT coated magnetic beads using the Illumina TruSeq protocol. The mRNA was fragmented, and then a random-primed cDNA synthesis was performed. The resulting double-strand cDNA was used as the input for a standard Illumina library prep with end-repair, adapter ligation and PCR amplification and then quantitated by qPCR followed by cluster generation and sequencing. RNA-Seq processing was conducting using the Pipeliner RNA-Seq workflow for quality assessment. For gene expression analysis, reads were trimmed to remove adapters and low-quality regions using Trimmomatic v0.33 (Bolger, Lohse and Usadel, 2014). Trimmed reads were aligned to the human GRCh38 reference genome and Gencode release 28 annotation using STAR v2.5.3 run in 2-pass mode (Dobin *et al.*, 2013). MultiQC v1.1 was used to aggregate QC metrics from picard, FastQC v0.11.5, FastQ screen v0.9.3 and RseQC to assess read and alignment quality (Wang, Wang and Li, 2012; Ewels *et al.*, 2016; Brown, Pirrung and Mccue, 2017; Wingett and Andrews, 2018). RSEM v1.3.0 was used for gene-level quantification and the resultant raw counts were voom-quantile normalized and batch corrected using the R package limma v3.38.3. Only genes that passed a 1 CPM threshold across a minimum of 3 samples based on the size of the smallest library were carried forward for differential expression testing. Pre-ranked gene set enrichment analysis (GSEA) was carried out using Gene Set Enrichment Analysis tool (GSEA v3.0) and Kegg pathways from MSigDB (Liberzon *et al.*, 2011). Heatmap figures for visualization of gene

expression and normalized enrichment score (NES) were generated using the heatmap.2 function from the R package gplots v3.0.1.1 and Clustvis respectively (Metsalu and Vilo, 2015).

Quantification and Statistical Analyses

One-way ANOVA with Dunnett's/Tukey's multiple comparisons test and Student's t test were performed using Prism software (GraphPad). Descriptions of sample size and particular tests used can be found in the figure legends.

Supplemental References

Bolger, A. M., Lohse, M. and Usadel, B. (2014) 'Trimmomatic: A flexible trimmer for Illumina sequence data', *Bioinformatics*, 30(15), pp. 2114–2120. doi: 10.1093/bioinformatics/btu170.

Brown, J., Pirrung, M. and Mccue, L. A. (2017) 'FQC Dashboard: Integrates FastQC results into a web-based, interactive, and extensible FASTQ quality control tool', *Bioinformatics*, 33(19), pp. 3137–3139. doi: 10.1093/bioinformatics/btx373.

Cantor, J. R. *et al.* (2017) 'Physiologic Medium Rewires Cellular Metabolism and Reveals Uric Acid as an Endogenous Inhibitor of UMP Synthase', *Cell*. Elsevier, 169(2), pp. 258-272.e17. doi: 10.1016/j.cell.2017.03.023.

Dobin, A. *et al.* (2013) 'STAR: Ultrafast universal RNA-seq aligner', *Bioinformatics*, 29(1), pp. 15–21. doi: 10.1093/bioinformatics/bts635.

Ewels, P. *et al.* (2016) 'MultiQC: Summarize analysis results for multiple tools and samples in a single report', *Bioinformatics*, 32(19), pp. 3047–3048. doi: 10.1093/bioinformatics/btw354.

Kerkar, S. P. *et al.* (2011) 'Genetic engineering of murine CD8+ and CD4+ T cells for pre- clinical adoptive immunotherapy studies', *J. Immunother.*, 34(4), pp. 343–352. doi: 10.1097/CJI.0b013e3182187600.Genetic.

Liberzon, A. *et al.* (2011) 'Molecular signatures database (MSigDB) 3.0', *Bioinformatics*, 27(12), pp. 1739–1740. doi: 10.1093/bioinformatics/btr260.

Metsalu, T. and Vilo, J. (2015) 'ClustVis: A web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap', *Nucleic Acids Research*, 43(W1), pp. W566–W570. doi: 10.1093/nar/gkv468.

Wang, L., Wang, S. and Li, W. (2012) 'RSeQC: Quality control of RNA-seq experiments', *Bioinformatics*, 28(16), pp. 2184–2185. doi: 10.1093/bioinformatics/bts356.

Wingett, S. W. and Andrews, S. (2018) 'FastQ Screen: A tool for multi-genome mapping and quality control', *F1000Research*, 7(May), p. 1338. doi: 10.12688/f1000research.15931.2.