Progress report on the practical training at LIMCR research institute

Stephan Drothler[1]

[1] *LIMCR - SALK Salzburg*

18.01.2021 - 26.02.2021

In this report, an overview over the theoretical and practical work conducted during the lab internship is given. The theoretical work includes the study of papers and protocols, as well as the design and planning of performed experiments. Relevant data from these experiments will be thoroughly presented and discussed. The aim of the practical training was the preparation of genetic material (RNA/DNA) from blood and splenocytes of tumor engrafted mice for next-generation sequencing. The training was focused on methods relevant to the study of clonal evolution, T cell interaction and tumor heterogeneity in chronic lymphatic leukemia (CLL). During the practical training, we worked with immunodeficient NSG mice, which were transplanted with tumors from TCL-1 mice in October or December. Weekly tumor load checks and visual inspections by animal technicians were used as a basis for determining humane endpoints. After sacrificing the animals, we prepared isolated RNA and DNA from sorted T cells with (CD4+, CD8+ and CD4-/CD8-), sorted CLL and splenocytes, to identify the clonal tumor and T cell architecture by subsequent NGS analysis. During the research training, most of the work has gone in the preparation of organs, sorting of rare cell population and analysis of single cell suspensions via flow cytometry. Additionally, we started with the preparation of a TCR library from the venous blood samples of the recipient mice. We saw a significantly shorter survival of one mouse group transplanted with the TCL-1 tumor 703. We found differences in the reasons for sacrifice between the different transplantation groups. Generally, we saw higher levels of CD8 than CD4 cells after sorting the splenocytes of recipient mice, as well as in the final venous blood samples before euthanasia. The work of the past weeks was tailored towards sequencing analysis, which is critical to decipher the heterogeneity of this complex disease. To understand the disease progression of the individual mice, identification of pro or anti-tumor T cells and the mutational profile of malignant B cells is essential to improve future therapies.

Contents

1 Introduction

Chronic lymphatic leukemia (CLL) is a heterogenous malignant disease of the B-cell lineage, leading to severe immune defects or death. In the past few years, research has demonstrated the pivotal role of T cells in

the progression of the disease. Thereby, growth of specific tumor clones is either supported by cytokines and cell-cell interactions with T cells, or inhibited by tumor-specific cytotoxic T cells. The conflicting results from bulk T cell analyses have indicated the need for very precise characterization of the individual T cell repertoires, to identify tumor-interacting T cell clones. With the help of increasingly precise multiplexed techniques on a single cell level, a surprisingly large heterogeneity of T cells was discovered and linked to differences in responses to immunotherapies [1].

During the internship, I joined a project of Christian Scherhäufl, based on CLL engraftment in immunodeficient mice. For transplantation, splenocytes from four primary tumor lines were injected into four recipient mice (n = 16). The recipient, severely immunodeficient NSG mice lack endogenous T cells, therefore we hypothesize that tumor specific T cell clones would be easier to identify. The overall aim of the project is, to study the clonal distribution, evolution and transcriptome of T cells and to describe CLL clones based on their B cell receptor and exome sequences 1. In order to monitor disease progression after CLL transplantation in recipient mice, regular characterization and counting of malignant tumor cells is essential. The tumor load (TL) is calculated as the fraction of $CD5^+/CD19^+$ T cells of total lymphocytes (see. Eq. 1) measured by flow cytometry (see. 2.1). After the TL exceeds a defined threshold ($\geq$ 80 % CLL of lymphocytes) or the individual mice show symptoms of suffering, recipient mice are sacrificed. Organs (eg. bone marrow, lymph nodes, liver, spleen) are harvested and single cell suspensions are analyzed by flow cytometry, sorted or cryopreserved.

To study clonal evolution under different selective pressures (e.g. transplantation, immune cells, treatment studies), CLL and corresponding T cell receptor (BCR/TCR) diversity are of major interest. Consequently, we want to perform next-generation sequencing of TCR and BCR, to analyze the clonal composition of the respective tumor lines before and after transplantation in different recipient mice (e.g. NSG vs WT). For identification of cytotoxic T lymphocytes (CTL) and immunogenic peptides from tumor specific MHC-peptide (pMHC) complexes in the future, a relevant paper from Purcell et al. [2] was examined and summarized (see. 3.2). In the article, a method for mass spectrometry analysis of MHC-bound peptides is described, which could be adapted for future experiments. Different mouse strains were used as recipient mice to study the impact of different immune cells on clonal evolution of TCL-1 derived tumors. C57BL/6 mice are the dominant background of our murine models and declared as wild type (WT). The established, immuno-modulated strains are NSG, OT-I/II and TCL-1 (see. 3.4). To study the T cell interaction with CLL, OT and NSG mice are used as model environments with no tumor specific clones.

The aim of this practical training was the library preparation of RNA and DNA from CLL bearing NSG mice for next-generation sequencing. We isolated the genetic material for receptor analysis from single cell suspensions of splenocytes or venous blood, amplified the target sequences and indexed the samples for analysis. For transcriptome and whole exome analysis, we sorted T cells of interest based on expression of the TCR co-receptors CD4 and CD8 (CD4+, CD8+. CD4-/CD8-) and CLL cells based on CD5 and CD19 expression (CD5+/CD19+) (see. Fig. 1). In the following weeks, our samples will be sequenced and analyzed. Thereby, we hope to improve the development of future therapies by better understanding the complexity of the disease.

2 Methods

2.1 Tumor load screening

Briefly, 10 µl (+30 µl EDTA) of tail vein blood from recipient mice (Tab. 6) were added to 10 µl (+100 µl PBS) FACS beads in FACS tubes. Beforehand, one sample of FACS beads (Flow-count Fluorospheres, Beckman & Coulter) was counted with an *Eve* automatic cell analyzer (NanoEntek)[1] to account for dispersion irregularities in the stock. We measured 6000-10000 $\frac{\text{beads}}{10\mu L}$, which was used to calculate absolute cell counts in our samples for every flow cytometry run. All blood samples were stained with the premixed staining master mix T4 (see. Tab 4), additionally one blood sample) was used as isotype control (T4iso) (see. Tab 4). [2] Staining was executed at room temperature for 15 minutes in the dark, then erythrocytes were eliminated in 2 mL lysis-fixation buffer (FACS™ Lysing solution, BD) until the suspensions became clear ($\approx$ 1 minute). Cells were centrifuged at 350g for 5 minutes, then supernatants were discarded and tubes filled with 150 µL PBS. All samples were transferred ($\approx$ 200 µL) to a 96-deep well plate and analyzed on a Cytoflex Flow cytometer (Beckman & Coulter) [Volume to record: 160 µL, flow rate: 110 - 125 $\frac{\mu L}{min}$]

$$\text{TL [\%]} = \frac{CD5^+/CD19^+}{\text{live, singlet } CD45^{\text{high}}} \times 100 \qquad (1)$$

2.2 Organ and cell preparation

Tumor bearing mice with a high tumor load or signs of weakness, were sacrificed by CO_2 inhalation. Subsequently, $\approx$ 5 mL of DPBS flushed peritoneal cavity fluid, $\leq$ 1 mL heart-blood, bone marrow (femurs) and

[1] Optical counting method. Samples were diluted 1:2 with 0.4% tryphan blue (NanoEntek) and mixed by pipetting. Cell/bead suspensions were transferred into counting slide and analyzed after manually adjusting the focus.

[2] On 18.01.20 - the first three mice were also accidentally stained with T4iso instead of T4. This was documented in the corresponding data sheet.

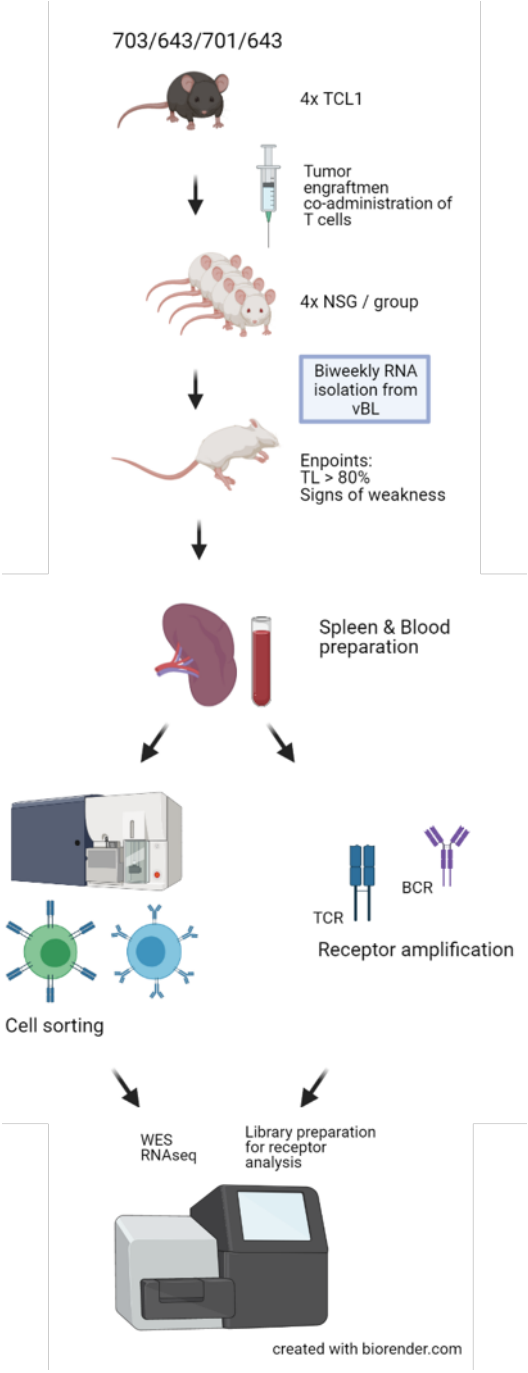

Figure 1: *Schematic workflow of the tumor transplantation (Tx) project. Splenocytes from four primary tumor lines were injected into four recipient mice (n = 16). Relative tumor loads (see. Eq. 1) from venous blood (vBL) samples were checked weekly while RNA from vBL was isolated every two weeks. Mice were euthanized, when they showed signs of weakness (see. Subsection 3.1) or the relative tumor load exceeded 80% of CD45+ lymphocytes (see. Fig. 6). Organs were prepared as described in Section 2.2. CLL/CD4+/CD8+/CD4-CD8- cells were sorted for whole exome sequencing (CLL) or RNAseq (T cells). B and T cell receptors (BCR/TCR) were amplified (see. 2.3) and indexed for NGS analysis.*

spleens were taken. Single cell suspensions were prepared from all organs, then cell counts and viabilities were measured. 1 x 10^6 cells from each organ, except the spleen, were used for flow cytometry analysis. Isotype controls with one million cells were prepared for splenocytes and blood circulating cells. Residual cells were diluted to $\approx$ 50 x 10^6 $\frac{cells}{mL}$ in Freezing solution (90% FBS + 10 % DMSO) and frozen at - 80 °C. In addition, we analyzed 10 µL of vBL stained with the T4 panel in the flow cytometer. Next, we sorted splenocytes, based on the cell counts, analyzed by flow cytometry. Thereby, we calculated the desired amount of cells for sufficient RNA and DNA isolation from the vBL cytometry analysis (Gating 6). Then, RNA was extracted from 20 µL vBL (15 min ACK lysis in 200 µL), sorted $CD4^+$, $CD8^+$, $CD4^-/CD8^-$ with the RNeasy micro kit (Qiagen) and sorted CLLs (1-2 x 10^6) with the RNeasy mini kit (Qiagen) or High Pure RNA Isolation Kit (Roche) according to the manufacturer's protocol.[3] For DNA isolation 5 x 10^6 sorted splenocytes or 1 -2 x 10^6 sorted CLL were prepared with a DNeasy Blood & Tissue Kits (Qiagen), whereby all non-specified centrifugation step were either carried out at max speed (2 x 10^4 g) or 1.4 x 10^4 g. All nucleic acid concentrations were estimated with a NanoDrop (Thermo). If sample volumes were higher than the maximal loading volume of the spin-columns, the initial binding steps were repeated until the whole samples were loaded ($\leq$ 3x).

2.3 T cell receptor library preparation

RNA samples from sorted CD4 and CD8 T cells from different transplantation experiments were pooled for NGS. In that, more sample heterogeneity is achieved, which in turn reduces the amount of PhiX control library. For library generation target sequences containing the variable regions of TCR α - and β -chains were amplified with the SMARTer Mouse TCR a/b profiling kit (Takara Bio, USA). Briefly, mouse RNA samples (Tab. 1) were transcribed into cDNA with SMART Overhangs. These overhangs are used for binding the forward primers in the variable regions, while reverse primers hybridized in the constant regions in the first PCR reaction. The second PCR was used to add adaptor and indexing sequences to for Illumina sequencing of the samples. After the second amplification round, cDNA samples of interest ($\approx$ 650 - 1150 bp) were purified with AMPure XP Beads and validated on a Agilent 4200 Tapestation with a DNA 1000 Kit.

2.4 Statistical Analysis

Statistical analysis was performed with either GraphPad Prism (V 8) or R (V 3.0.1) using the Rstudio (V

[3] All centrifugation steps were carried out for 30 instead of 15 seconds. This was due to technical limitations of our table top centrifuge, which only allows for 30 second interval alterations.

Table 1: *Overview of TCR library preparation. Samples are identified in the left column. The Volume (second column) was adjusted based on RNA (NanoDrop) concentrations. FWD and REV primers are used for identification of samples after Illumina sequencing. Abb. $_p$ = Primer, FWD = forward, REV = reverse*

Sample ID	RNA [µl]	FWDp	REVp(α +β)
656 d13	9,5	F5	R3
656 d27	9,5	F6	R3
656d 41	9,5	F7	R3
658 d13	9,5	F8	R3
658 d27	9,5	F9	R3
658d 41	7,3	F10	R3
Tumor 643/20	4	F11	R3
602 d13	9,5	F12	R3
602 d22	9,5	F5	R4
602 d27	9,5	F6	R4
602d 41	8,2	F7	R4
602d 55	9,5	F8	R4
602 d67	9,5	F9	R4
602 d81	9,5	F10	R4
602 d95	9,5	F11	R4
602 d 97 sac	9,5	F12	R4
Negative control	0	F8	R2

1.4.1) interface. Group differences were calculated using the nonparametric paired Wilcoxon test (GraphPad) or a parametric Tukey's adjusted 2-way Anova (GraphPad). Group-wise and global differences in survival were calculated with a log-rank test from the *survminer()* package (Rstudio).

3 Results

3.1 Tumor variants influence median survival

Four different Tcl1 tumors were transplanted in four NSG mice each. The 16 mice were subsequently analyzed for differences in T cell clonality. Each mouse was injected with 5-8 x 10^6 splenocytes from either 701, 703, 643 or 717 Tcl-1 mice (6). After the individual mice showed signs of weakness or had a high tumor load (> 80 %) mice were sacrificed and their organs harvested. One mouse in the 701 groups died in the cage (TL 23%), while three mice in the 717 group were sacrificed due to enlarged intestine ("belly") or signs of weakness with a TL < 70%. Generally, most mice were euthanized with a high tumor load (n = 8/16), while two mice from the 643 line were sacrificed with 70 % TL due to weakness (see. 2B). The "belly" phenotype is clearly attributable to an enlarged spleen. Weakness is used as a collective term for symptoms of suffering. Animal well-being was attested by experienced animal technicians based on multiple parameters, like fur quality, behavior and physical appearance. No statistical analysis was performed because of low sample sizes in they respective categories. Furthermore, NSG mice are prone to infections and might suffer from non-CLL induced malady. Direct comparisons of endpoints must therefore only be made with utmost caution.

In order to isolate cell populations of interest, positive selection cell sorting of splenocytes using a FACSAriaIII (BD) device was performed. For downstream analysis, we were interested in pure CD4, CD8 and CLL populations, to analyze expression profiles. As described in Methods (see. Section 2.2), we analyzed splenocytes with flow cytometry and calculated the total sorting volume accordingly. Generally, we aimed for $\geq$ 1-2 x 10^4 CD4 and CD8 cells respectively, to ensure suitable numbers for RNA isolation. As shown in Figure 2A, we obtained higher numbers of CD8 and CD4 cells (Paired Wilcoxon: Median of differences: 7965, $p < 0.04$). Tumor variants did not show significant differences in either CD8 or CD4 T cell yield (2 way Anova, Tukey's adjusted for multiple comparisons, Tab. 3). In the 701 recipient mice, CD4 cells were nearly absent, while in the 703 group - CD8 cells were highest.

Figure 2C visualizes the survival after tumor transplantation. Overall survival of the experiment groups differed significantly (log-rank $p < 0.0001$), multiple log-rank testing with the Bonferroni-Holm correction revealed a significantly lower survival in the 703 group than in the other groups ($p < 0.018$).

3.2 Immunopeptidomic analysis

For unbiased analysis of clinical samples, manipulation of cell lines or tissues for peptide isolation is undesired (eg acid stripping or transfection). The outlined purification approach uses mechanical cell lysis, antibody dependent MHC isolation and reversed-phase HPLC to minimize sample loss and possible contamination. Peptide analysis is subsequently performed with nUPLC–MS/MS. The authors state a low difficulty of the protocol on the biochemical side, but recommend an early consulting of a mass spectrometry expert. The authors indicate the importance of LS grade materials and provide a detailed list of their reagents - this list should be discussed with the MS-expert prior to sample preparation. The biochemical preparation involves the homogenization of sample (eg. tissue, cell culture), purification of pMHCs via immuno-precipitation and subsequent peptide isolation by RP-HPLC. Preparation of the immunoprecipitation column requires the appropriate antibodies against the respective mouse strain specific MHC epitopes. Anti $H2K^b$ and $H2D^b$ antibodies are readily available from different manufacturers (eg. Thermo [3][4]) for MHC capture. After baiting the pMHC complexes, the peptides are dissociated from their MHCs with 10% acetic acid. Subsequently, the peptides are enriched via fractionized collection after R-HPLC purification. Finally, the eluted peptides are analyzed via LC–MS/MS. A recommended count for sufficient yield is 5×10^7 - 1×10^9 cells per experiment.

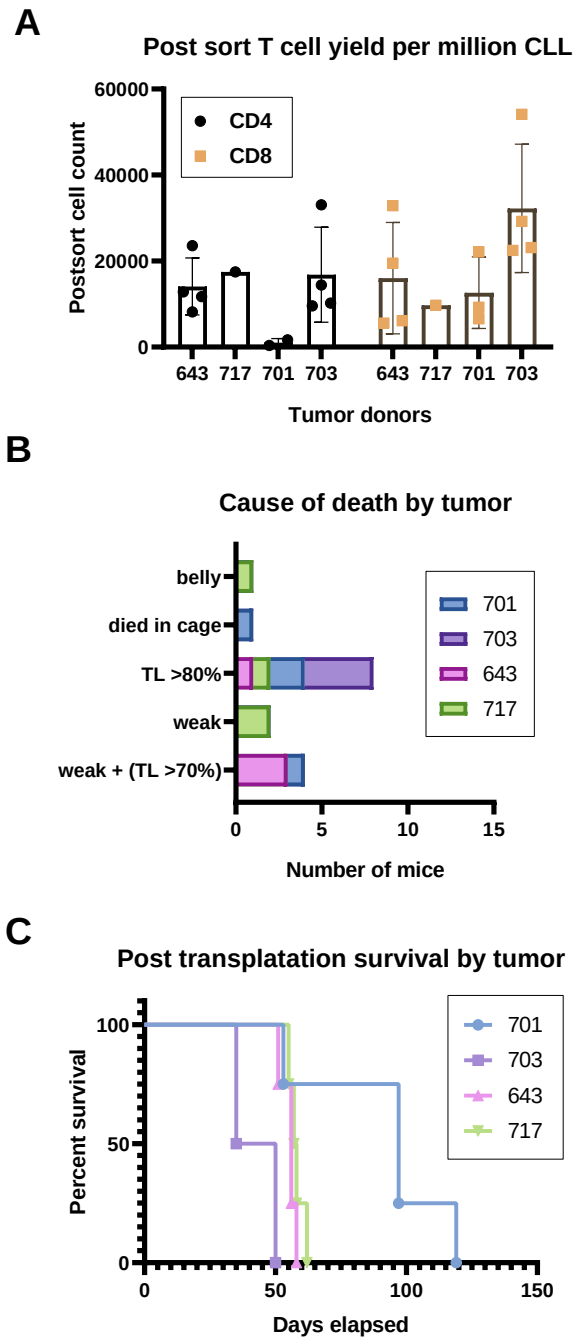

Figure 2: ***Transplantation experiment of four different CLL strains from 643, 701, 703 and 717 TCL-1 mice in NSG mice (n = 4/tumor).*** *The NSG mice were sacrificed at humane endpoints or when the relative tumor load was higher than 80 % (**B**). Kaplan-Meier survival curves were constructed for the different tumor groups (**C**), whereby the 703 group showed significantly lower survival than the other groups ($p < 0.018$). After the sacrifice, mice spleocytes were sorted for CD8 and CD4 cells and are shown here per million sorted CLL cells (**A**). No sorted cells were available for the mice which died in the cage (701 group). Additionally, for three of the four 717 transplanted mice, no sorted cell data is available yet, but splenocytes were frozen for analysis in the future. Data is shown in means + standard deviation.*

As for any new method it is critical to asses every step thoroughly to minimize error propagation. Equally vital is the initial execution with an adequate cell line, ideally one with a known peptide repertoire. Additionally, experimental costs for suitable chemicals were calculated. Table 7 lists MS grade chemicals, necessary for the experiment. Some reagents were assumed to be available, therefore the list must be validated based on the LIMCR inventory. Additionally, the authors use mechanical methods to lyse cells which is not routinely performed in our Lab - this is still subject to discussion.

3.3 Most blood samples are suitable for library preparation

Library preparation with the SMARTer Kit yielded suitable cDNA fragments in the desired size range (650 - 1150 bp) for all samples except 602 day 27 (see. 4/LaneB2). As expected, the tumor sample from the TCL1 mouse showed the highest DNA concentration (7.71 $\frac{ng}{\mu L}$), because it has endogenous T cells.[4] Upon analysis of the individual electropherograms (data not shown), we confirmed that most samples had a peak concentration around 600 - 700 bp, which is in line with the reports of the manufacturer.[5] During the last days of the practical training, additional blood samples from the mice (Tab 5) are prepared for a sequencing run.

3.4 Mouse models

In this section I aim to provide an overview of the established mouse models used for our CLL transplantation experiments. TCL1 mice are used as tumor donors, while backcrossed OT, WT and NSG mice are used as syngeneic recipients.

3.4.1 TCL1 - a model for aggressive human CLL

The TCL1 strain carries the eponymous transgene *TCL1*, which codes for a co-activator of AKT. This protein is a critical part of the PI3-K pathway directly involved in mTOR and NKκB activation. These mice are widely used as a CLL tumor model, because it mimics the complex heterogeneity of this disease very well. CLL is known to be dependent on multiple drivers from the environment or genetics of the malignant cells. Studies with TCL-1 mice have successfully led to many scientific discoveries concerning CLL. For example, TCL-1 mouse studies showed the mechanism of action for Ibrutinib, a novel drug targeting Brutons tyrosine Kinase [5].

[4] NSG mice only posses T cell which were co-administered during tumor engraftment. Thus we expect a higher cDNA yield from the TCL1 sample, since it possesses a regular T cell repertoire.

[5] They showed data from murine spleen samples (10 ng RNA) and obtained maximal peaks at the 700 to 800 bp range.

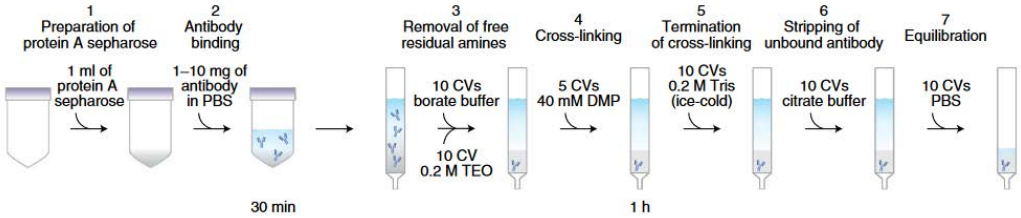

Fig. 3 | Generation of MHC immunoaffinity column. The first seven steps of the Procedure are shown. CV, column volume; DMP, dimethyl pimelimidate dihydrochloride; TEO, triethanolamine.

Figure 3: *Step by step visualization for generating a immuno-precipitation column. Taken from "Mass spectrometry–based identification of MHC-bound peptides for immunopeptidomics" (Nature Protocol)s [2].*

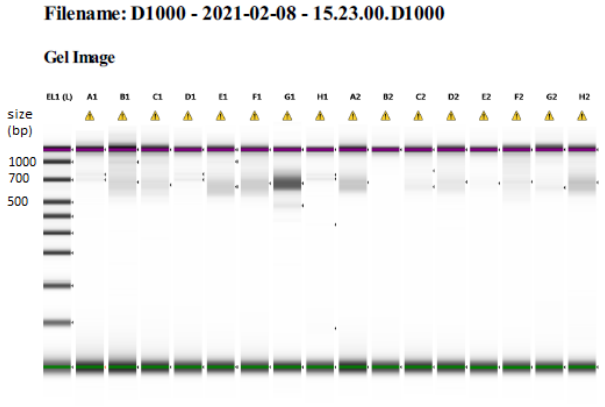

Figure 4: *D1000 Kit on the Tapestation4200 (Agilent). Electronic Marker for DNA1000 was used as a reference. All samples have their major peak concentration around 600 - 650 bp. The gel image was fitted to the upper and lower boundaries of the kit, therefore the DNA bands from the samples appear very dim. The different concentrations can be found in Table 2.*

Table 2: *Results from the Tapestation run with a DNA1000 Kit. Successful library preparation was expected to yield in a 650 - 1150 bp range (see. 4). All samples except the blood sample from the mouse 602 on day 27 yielded a suitable library for sequencing. The G1 sample was derived from the tumor donor mouse 643 pre-transplantation, thus no time-point was stated.*

Lane	Conc [ng/µl]	Mouse ID	Timepoint
A1	0.3	656/182	d13
B1	1.03	656/182	d27
C1	0.41	656/182	d41
D1	0.345	658/182	d13
E1	2.03	658/182	d27
F1	1.05	658/182	d41
G1	7.71	Tumor	643/20
H1	0.474	602/182	d13
A2	1.04	602/182	d22
B2		602/182	d27
C2	0.3809	602/182	d41
D2	0.558	602/182	d55
E2	0.117	602/182	d67
F2	0.399	602/187	d81
G2	0.248	602/182	d95
H2	2.39	602/182	d97

3.4.2 OT-I/OT-II - OVA specific T cells

Both OT mouse models carry individual transgenes which makes them express OVA-specific Vβand Vα chains. These mouse models are very popular and usually used to study immune responses to ovalbumin, the dominant protein fraction in egg white [6]. The large success of the mouse model can be attributed to Hogquist et al. and Barnden et al. - who generated $CD8^+$ OVA-specific (OT-I) and $CD4^+$ OVA-specific (OT-II) T cell clones, respectively [7], [8]. The use of a single transgene induced the expression of Vβ2 and Vβ5 regions of the TCR, recognizing $OVA_{257\text{-}264}$ (Seq: SIINFKEL) for OT-I and $OVA_{323\text{-}339}$ (Seq: ISQAVHAAHAEINEAGR) for OT-II. The transgenic mice have a narrow repertoire of T cells with a high frequency of the described clones and thereby enable uncluttered tracing of immune responses.

3.4.3 NSG - Immunodeficient mouse models

NOD-scid-γ (NSG) mice are almost completely immunodeficient and are derived from NOD x CD17-scid mice matings with $(IL\text{-}2R)\gamma^{-/-}$ mice. The initially crossed mice already had a compromised NK-cell activity and innate immune functionality, which enabled engraftments from human haematopoietic cells. The additional dysfunctionalization of the IL-2 receptor then resulted in complete absence of of NK-cell responses, minimal reactivity of the innate immune system and no adaptive immune responses [9]. We use these mice to study the reaction of co-transplanted T cells to the tumor proliferation and map differences in clonality to clinical parameters of disease progression.

4 Conclusion

During the practical research training I was able to gain valuable insight into the sample preparation to analyze T cell and B cell clonality by NGS. Flow cytometry and cell sorting are very versatile methods for a very large range of immunological research questions and thus indispensable further research in this field. Due to the high degree of heterogeneity in CLL, it is still unclear which exact cellular pathways or which T cell phenotypes support or prevent disease progression. The dramatically reduced cost of next generation sequencing made it an accessible technique for a growing number of laboratories all over the world. It allows for precise definition of T and B cell clones based on their uniquely recombined hypervariable receptor regions. With variations in murine xenograft models, the hunt for tumor specific clones, or the role of bystander T cells can be further described, possibly leading to better understanding of their impact and function in disease progression. Concretely, knowledge about tumor editing pathways with regards to T cell exhaustion, might help to design more robust tumor specific T cells for future immunotherapies. I was also able to gather experience in other projects which are not described in this report. Thereby I studied material for the design of *in situ* hybridizing RNA probes, joined meetings with application scientists or participated in device trainings.

I am very thankful for the opportunities I was given during the practical training. It was always a progressive atmosphere with active encouragement to ask questions. The cell sorting of rare populations was a time intensive manner and a valuable skill, demanding experience and careful execution. Furthermore, I learned to make plans and adjust them unexpectedly, as endpoints of mouse experiments are not under the full control of the experimenter. Hopefully, the data from the past weeks helps to shed more light on the T and B cell dynamics in CLL and supports the growing knowledge for the successful combat of the disease.

5 Appendix

5.1 Code for multiple log-rank test of survival

Listing 1: *Data import and log-rank test with the survminer package in Rstudio.*

```
#data import
survivalNSG <- suvivalNSG %>%
  mutate(tumor = as.factor(tumor),
         status = as.numeric(status))

pairwise_survdiff(Surv(survival,status) ~
    tumor,
                  data = survivalNSG,
                  p.adjust.method = "BH")
```

5.2 Two way ANOVA of CD4 and CD8 post sort yield

Table 3: *Analysis of post sort T cell yield (CD4 and CD8) between four transplanted tumor variants (643, 701, 703 and 717). Groups were tested Tukey's multiple comparisons test correction for 2way ANOVA was used in GraphPad Prism 8.*

Groups	Adj. P Value
CD4	
643 vs. 717	0,9918
643 vs. 701	0,3807
643 vs. 703	0,9831
717 vs. 701	0,5375
717 vs. 703	>0,999
701 vs. 703	0,2341
CD8	
643 vs. 717	0,9512
643 vs. 701	0,9752
643 vs. 703	0,1777
717 vs. 701	0,9952
717 vs. 703	0,2702
701 vs. 703	0,1148

Table 4: *T4 panel for staining cell suspensions from NSG mice during the transplatation experiment. For the isotype control master mix (T4iso) - CD69, 4-1BB and PD1 antibodies were substituted for an Isotype control (Orange).*

Channel	Antibody	Company	Clone	Cat / ref	Concentration	Isotype	µl/1 mio cells in 150 µl
FITC	anti-mouse CD62L	Invitrogen	MEL-14	11-0621-85	0.5 mg/ml	Rat / IgG2a, kappa	0.125µl
PE	anti-mouse CD69 PE	BioLegend	H1.2F3	104507	0.2 mg/ml	Armenian Hamster IgG	0.25µl
ECD							
PC5.5	anti-mouse CD44	BioLegend	IM7	103032	0.2 mg/ml	Rat / IgG2b, kappa	0.25µl
PC7	anti-mouse CD45	BioLegend	I3/2.3	147703	0.2 mg/ml	Rat/ IgG2b	0.25µl
APC	anti-mouse 4-1BB APC	BioLegend	17B5	106109	0.2 mg/ml	Syrian Hamster IgG	0.25µl
APC-A700	anti-mouse CD3 AF700	BioLegend	17A2	100216	0.5mg/ml	Rat / IgG2b, kappa	0.125µl
APC-A750	anti-mouse CD8 APC-H7	BD	53-6.7	560182			0.25µl
PB 450	anti-mouse CD5 BV421	BioLegend	53-7.3	100629	0.2mg/ml	Rat / IgG2a, kappa	0.25µl
KO525							
Violet 610	anti-mouse CD19 BV 605	BioLegend	6D5	115540	0.2 mg/ml	Rat / IgG2a, kappa	0.25µl
Violet 660	anti-mouse CD4 BV 650	BioLegend	RM4-5	100555	0.2 mg/ml	Rat / IgG2a, kappa	0.25µl
Violet 780	anti-mouse PD1 BV785	BioLegend	29F.1A12	135225	0.2 mg/ml	Rat / IgG2a, kappa	0.25µl

Table 5: *Overview of the post sort statistics of the NSG mice.*

Mouse ID	Tumor		sCD4+ cells	RNA CD4+ [ng]	sCD8+ cells	RNA CD8+ [ng]	sCLL cells	RNA CLL [ng]
694	643/20		8181	67,1	5556	18	1 Mio	380
695	643/20		12848	906,4	19511	117	1 Mio	200
696	643/20		23573	45,1	32877	19	1 Mio	540
697	643/20		11744	148,5	6128	216	1 Mio	70
681	717/20			0,0		0		0
682	717/20			0,0		0		0
683	717/20		17452	45,1	9748	131	1 Mio	670
641	717/20			0,0		0		0
602	701/20		2528	56,1	33197	133	1,5 Mio	54
722	701/20			0,0		0		0
723	701/20	CD4 not isolated	0	0,0	9267	75	1 Mio	835
724	701/20		751	61,6	13103	154	2 Mio	129
655	703/20		21611	51,7	81224	68	1,5 Mio	510
656	703/20		49614	14,3	34670	119	1,5 Mio	189
657	703/20		14357	121,0	43817	66	1,5 Mio	270
658	703/20		15300	8,8	33727	18	1,5 Mio	225
							B-cells	
747/20	WT SPL		20042	3,3	30317	40	250000	392
762/20	WT SPL		72195	63,8	71336	15	250000	278
796/20	WT SPL		29539	79,2	43075	405	250000	271

Table 6: *Overview of individual mice and the transplanted tumors. Survival in days for Kaplan Meier plots were calculated by substraction of Tx Date from Sac. Date (see. 2A). Reason for sacrifice were condensed into less categories for display on 2B.Abb: TX: transplantation, Sac: Sacrificed*

Mouse ID	Tumor	Birthday	TX Date	Sac. Date	Reason Sac.	Cells TX [Mio cells]
694	643/20	21.05.2020	15.12.2020	09.02.2021	weak	8
695	643/20	21.05.2020	15.12.2020	09.02.2021	weak, belly	8
696	643/20	21.05.2020	15.12.2020	11.02.2021	weak, TL >70%	8
697	643/20	21.05.2020	15.12.2020	04.02.2021	TL >80%	8
681	717/20	15.05.2020	15.12.2020	10.02.2021	belly; enlarged intestine	8
682	717/20	15.05.2020	15.12.2020	15.02.2021	weak	8
683	717/20	15.05.2020	15.12.2020	11.02.2021	TL >80%	8
641	717/20	07.04.2020	15.12.2020	08.02.2021	very weak	8
602	701/20	07.03.2020	22.10.2020	27.01.2021	TL>80%	6,6
722	701/20	28.05.2020	22.10.2020	14.12.2020	Died in cage	6,6
723	701/20	28.05.2020	22.10.2020	18.02.2021	TL>80%	6,6
724	701/20	28.05.2020	22.10.2020	27.01.2021	TL>70%	6,6
655	703/20	03.05.2020	22.10.2020	26.11.2020	TL >80%	5,07
656	703/20	03.05.2020	22.10.2020	11.12.2020	TL >80%	5,07
657	703/20	03.05.2020	22.10.2020	26.11.2020	TL >80%	5,07
658	703/20	03.05.2020	22.10.2020	11.12.2020	TL >80%	5,07

Table 7: *Estimated costs of LC-MS/MS grade chemicals for p(MHC) analysis described by Purcell et al. [2]*

Note	Recommended reagents	Cost (Eur)	Quantity
cat: 00940	Formic acid (FA, LC–MS Ultra, >98% purity; Sigma-Aldrich, cat. no. 14265-1ML)	80	50 mL
	Igepal CA-630 (Sigma-Aldrich, cat. no. I8896)	100	50 mL
	Triethanolamine (Thermo Fisher Scientific, cat. no. 787-500ml)	25	500 mL
	Pepstatin A (MP Biochemicals, cat. no. 219536810)	75	5 mg
	Dimethyl pimelimidate dihydrochloride (DMP-2HCl; Sigma, cat. no. D8388-250MG)	40	250 mg
Keep in glass	Complete protease inhibitor cocktail tablets, provided in glass vials (Roche, cat. no. 11836145001)	810	60 tabs
Keep in glass	Acetic acid (>99.8% purity, 1 liter in glass bottle; Sigma-Aldrich, cat. no. 33209-1L-GL)	50	1 L
	Water, Optima LC/MS-grade 4L (Thermo Fisher Scientific, cat. no. FSBW6-4)	45	1 L
	Acetonitrile, Optima LC/MS-grade (Thermo Fisher Scientific, cat. no. FSBA955-4)	515	4 L
Corrosive	Trifluoroacetic acid (TFA; sequanal-grade, 10 × 1 ml; Thermo Fisher Scientific, cat. no. PIE28904)	150	10x 1 mL
	Isopropanol (LC–MS Chromasolv; Sigma-Aldrich, cat. no. 34965-2.5L)	100	2.5 L
	Methanol for LC, LiChrosolv (Merck Millipore, cat. no. 1.06018.4000)	125	4 L
	[Glu1]-Fibrinopeptide B human (≥90% purity by HPLC, 0.1 mg; Sigma-Aldrich, cat. no. F3261)	60	0.1 mg
	Potassium chloride (KCl; Ajax Finechem, cat. no. 383)		
	Sodium dihydrogen orthophosphate (NaH2PO4; Ajax Finechem, cat. no. 3964)		
	Potassium dihydrogen phosphate (KH2PO4; Ajax Finechem, cat. no. 391)		
	Sodium hydroxide (NaOH; Merck Millipore, cat. no. 1.06498.0500)		
	Boric acid (Astral Scientific, cat. no. BIOBB0044)		
	Citric acid monohydrate (Merck Millipore, cat. no. 1.00244.0500)		
	Ethanol, absolute (Ajax Finechem, cat. no. AJA214)		
	Ethylenediaminetetraacetic acid disodium salt (EDTA; Chemsupply, cat. no. 9326410003617)		
	Dimethyl sulfoxide (DMSO; Sigma-Aldrich, cat. no. 472301-100ML)		
	SDS–PAGE gel (Mini-PROTEAN TGX 12% Precast Gel, Bio-Rad cat. no. 456-1046)		
	Coomassie blue		
	Sodium azide (NaN3)		
	Liquid nitrogen		
	Total:	**2175**	

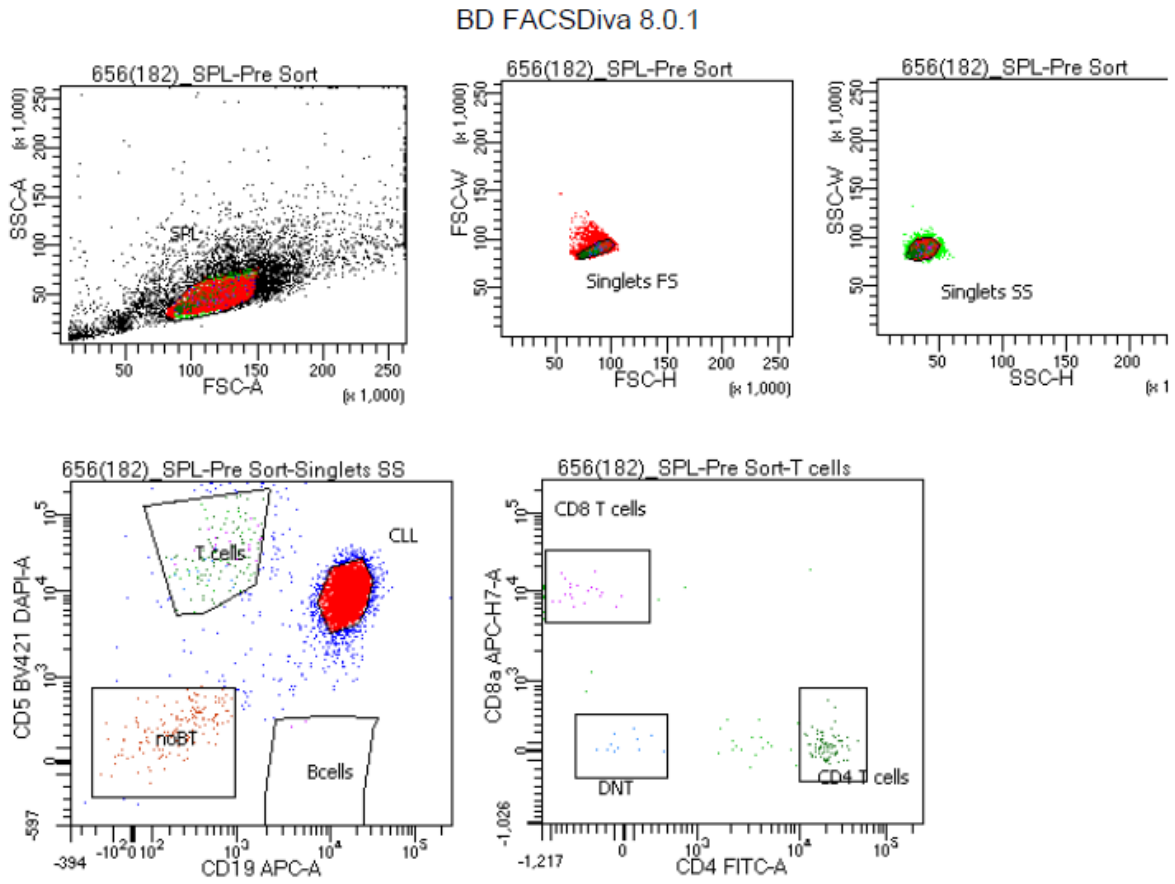

Figure 5: *Gating strategy for cell sorting of splenocytes. First Lymphocytes were gated based on forward and side scatter values, before two doublet discrimination steps were performed (FSC-H/FSC-W) and (SSC-H/SSC-W) (Three plots on the top, left to right). Next, doublet excluded lymphocytes were distinguished by expression of CD5 and CD19, where we gated CLL and T cells respectively. T cells were further discriminated based on CD4 and CD8 expression, double negative T cells (DNT) were also sorted (Two bottom plots, left to right). All sorts were performed with a 4-way purity mask.*

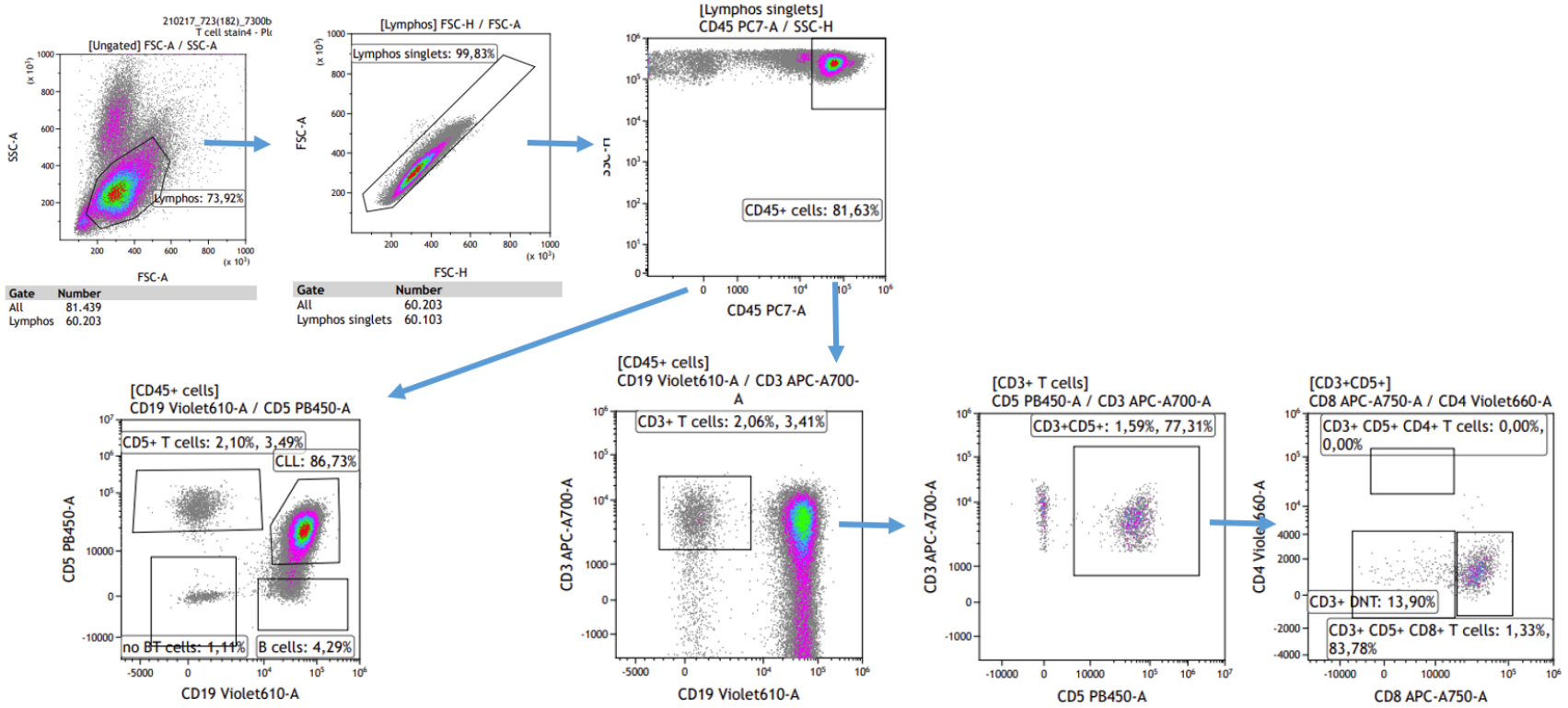

Figure 6: *Gating strategy for flow cytometry analysis of venous blood samples. After gating for Lymphocytes (Top, left) based on forward and side scatter, doublets were excluded by forward scatter height over area. Next, singlets were gated for high CD45 expression. CLL cells were identified by high expression of CD19 and CD5 (Bottom, left). Additionally, CD45+ singlets were gated on for CD3+ and CD19- expression, before excluding CD5+ cells. Finally, CD3+ CD5+ T cell singlets were distinguished based in CD8 and CD4 co-receptors.*

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