Chapter 7

Conclusions and future perspectives

7.1 Summary of findings

Cytotoxic T cells are a crucial component of the adaptive immune system. They are of great interest for medical therapies as they can kill infected and cancerous cells. They do so via direct contact to target cells and the focused secretion of lytic granules, also known as degranulation, which ensures that target cells are killed while leaving surrounding cells intact. The importance of cytotoxic T cells to maintain a healthy immune system is demonstrated by the CTL defects observed in patients with life-threatening immunodeficiency diseases (Clark and Griffiths, 2003; Feldmann et al., 2003; Stepp et al., 1999).

This study aimed to use the CRISPR-Cas9 gene editing technology in a screening setting to test for genes that regulate CTL killing. In chapter 3, I established the amount of CRISPR-Cas9-RNP reagents needed to efficiently edit primary mCTL by targeting the abundant cell surface protein Thy1. Conditions resulting in reproducible and efficient CRISPR-mediated KO in mCTL were successfully established, in some cases reducing protein expression by over 90% (Figure 3.6). Targeting *Rab27a*, a known mediator of cytotoxic T cell function, using CRISPR-Cas9-RNPs significantly reduced RAB27A protein levels and resulted in a degranulation and killing phenotype (Figure 3.7). CRISPR-mediated KO of other known regulators of CTL cytotoxicity, the lytic granule component perforin and MUNC13-4, a part of the lytic granule fusion machinery, was further used to set up an assay that measures degranulation and killing simultaneously (Figure 3.11). This flow cytometry-based assay allowed detection of regulators of T cell killing, while the degranulation readout gave immediate insight into the underlying molecular mechanism by which the killing is mediated.

The combined degranulation and killing assay was suitable for screening as it could be performed in a 96 well format. To identify targets for a screen I performed an RNA-seq study that compared the transcriptome of naive and effector CD8 T cells (chapter 4). Genes highly expressed in activated but not naive CD8 T cells are likely to be important for CTL effector functions, including killing. This gave a large set (1803 genes) of upregulated differentially expressed genes (Appendix B), which were predominantly associated with the cell cycle (Figure 4.3). 13 genes, chosen based on functional annotation analysis and literature research, were targeted by CRISPR and tested in the combined degranulation and killing assay. *Hif1*α and *Nfil3* were identified as interesting genetic targets where CRISPR-mediated KO decreased the killing but not the degranulation capability of effector CTL (Figures 4.5 and 4.6), a phenotype reminiscent of perforin CRISPR samples (Figure 3.8). This was in line with published studies, which identified that perforin protein expression was affected by the absence of members of the HIF complex and NFIL3 (Finlay et al., 2012; Rollings et al., 2018). However, the precise mechanism by which HIF-1α and NFIL3 affect perforin protein levels remains to be elucidated. Other studies have shown that these genes are important when lost in naive T cells or during T cell development in vivo (Doedens et al., 2013; Finlay et al., 2012; Rollings et al., 2018). The data generated in this thesis demonstrated a direct importance of NFIL3 and HIF-1α in effector CTL to enable killing. This is likely mediated through their effect on perforin. Furthermore, the RNA-seq dataset was used to identify genes that are differentially expressed between males and females. Interestingly this included some genes that are implicated in CTL killing function (such as *Gzma*, *Il2ra* and *Nfil3*) (Table 4.3). It might therefore be interesting to explore whether there are differences between the killing capabilities of males and females.

The screen performed in chapter 4 confirmed that the combined degranulation and killing assay could be used to identify regulators of CTL killing in mCTL. In chapter 5, I successfully tested this assay in hCTL using cells derived from FHL2 and FHL3 patients. The traditional degranulation assay is currently used for diagnostic purposes, but cannot detect defects resulting from perforin mutations (Bryceson et al., 2012). Diagnosis of FHL therefore requires an additional investigation of perforin protein expression (Bryceson et al., 2012). The combined degranulation and killing assay would therefore reduce the number of experiments that need to be performed, making the diagnostic process more efficient. This highlights the potential of the combined degranulation and killing assay as a diagnostic test.

The scalability of the screen was tested in hCTL using a library containing 64 compounds that targeted the NF-κB signalling pathway. In this screen for chemical mediators of CTL

killing, I identified that parthenolide (drug 19) affected CTL killing within 1 h of treatment (Figure 5.8). Parthenolide was found to affect NF-κB activity via the phosphorylation status of p65 at Ser536 (Figure 5.9). However, parthenolide has also been reported to affect HDAC1 and DNA methyltransferase 1 (Gopal et al., 2007; Liu et al., 2009), meaning that I could not conclude that the degranulation and killing defect observed upon CTL treatment with parthenolide was due to its inhibition of p65. KO of p65 using CRISPR could help to clarify the role of NF-κB in CTL killing. The drug screen therefore highlighted two important points. Firstly, that the combined degranulation and killing assay could be used to screen many dozens of compounds. Secondly, it emphasised the advantage of a CRISPR-based screen over a drug screen, as the genetic approach provides a cleaner system that is, at least in theory, not complicated by unanticipated off-target effects.

Finally, I successfully used the CRISPR gene editing technology using the RNP approach in hCTL (Figure 6.9). I also explored stable Cas9 expression through lentiviral transduction as an alternative approach to CRISPR in primary human T cells and related cell lines. This approach could provide an even cleaner system as stable incorporation of antibiotic resistance cassettes or fluorescent markers would allow selection of cells that contain all necessary CRISPR reagents. Additionally, this approach has the potential to allow much larger CRISPR-based screens, as discussed in section 7.4. The difficulties I experienced with lentiviral transduction of human T cells with Cas9 are in line with observations from other labs (Legut et al., 2018; Shifrut et al., 2018; Wang et al., 2014b). However, through careful optimisation of transduction methods I achieved a transduction efficiency of up to 17% (Figure 6.7). Transposon-based stable integration of Cas9 could be explored in parallel to single cell cloning of the Cas9-2A-mCherry transduced cells in future experiments, in order to achieve the aim of creating primary human T cells that stably express Cas9.

7.2 Comparison of findings to published studies using CRISPR in primary T cells

7.2.1 CRISPR in mouse T cells

As this PhD project was ongoing, several papers have been published that use the CRISPR-Cas9 technique in primary mouse T cells. These can broadly be divided into studies using primary cells derived from Cas9 expressing mice and studies that use Cas9-RNPs, two options that were also explored in this thesis. Meanwhile, several studies noted that all-in-one viral vectors encoding both Cas9 and sgRNAs resulted in low transduction efficiencies, likely

due to the large size of Cas9, and are not as efficient at generating KOs (Huang et al., 2019; Seki and Rutz, 2018).

Several Cas9 expressing mice have been generated (Chu et al., 2016b; Platt et al., 2014; Tzelepis et al., 2016). Chu et al. (2016a) used primary T cells derived from transgenic Cas9 mice and achieved KO of CD44 in around 50% of the cells four days after transduction with retroviral vectors encoding sgRNAs. Interestingly they showed that their Cas9 mice expressed more Cas9 in lymphocytes and generate KOs at higher efficiencies than the Cas9 mice generated by Platt et al. (2014).

A paper published after this thesis was submitted used the same transgenic Cas9 mice (generated by Tzelepis et al. (2016)) that I used for the experiments in Figure 3.4 for genome-wide CRISPR screens in primary CD4 T cells (Henriksson et al., 2019). They transduced the Cas9 expressing T cells with a genome-wide retroviral sgRNA library, where each virus encoded a single sgRNA together with BFP and puromycin resistance. This enabled selection of cells that stably expressed sgRNAs. In contrast, I transiently nucleofected synthetic crRNAs and tracrRNAs into primary T cells derived from the transgenic Cas9 mice, a process which does not allow stable expression or selection of cells that have taken up the reagents. Using this approach, I achieved an average protein loss of 41.6% in nucleofected cells when using 10 μg crRNA/tracrRNA (Figure 3.4). In the experimental setup of Henriksson et al. (2019) all cells should have been expressing a sgRNA and Cas9 after selection procedures. Therefore, their approach, in contrast to mine, was not limited by nucleofection efficiency or the stability of synthetic RNAs. Unfortunately, a direct comparison of CRISPR efficiency between my study and Henriksson et al. (2019) is not possible, because they do not show the percentage loss of protein expression they achieved in response to CRISPR. In their methods they state that they used a viral vector encoding BFP, GFP and a sgRNA against GFP to test Cas9 function. With this approach, Cas9 cutting efficiency can be validated and quantified by loss of GFP expression in the presence of the GFP-targeting sgRNA. As the results are not shown it is unclear how efficiently they lose protein expression in their experimental setup. However, it seems that their CRISPR KO worked to some extent as they observed signs of altered CD4 T cell differentiation in response to some sgRNAs (Henriksson et al., 2019).

I was able to improve on the CRISPR KO efficiency I achieved with cells derived from Cas9-expressing mice by using Cas9-RNPs in cells derived from WT mice (64.7% Thy1 KO achieved with Cas9-RNP (Figure 3.5) in contrast to 33.3% Thy1 KO achieved using the same concentration of synthetic crRNA/tracrRNA in cells from Cas9-expressing mice (Figure 3.4)).

Cas9-RNPs have the advantage of allowing gene-editing in primary cells regardless of their genetic background. Due to its higher efficiency, the Cas9-RNP approach was subsequently used throughout this thesis.

While Cas9-RNPs have been widely used in primary human T cells, as outlined in the next section, the first study that used this approach in primary mouse T cells was only published in 2018 (Seki and Rutz, 2018). Using one guide to target Thy1, Seki and Rutz (2018) reported 60% loss of Thy1 expression in primary mouse T cells using the Cas9-RNP approach, which corresponds well to the 64.7% loss of Thy1 expression I achieved with one guide (Figure 3.5). To further improve CRISPR efficiencies, Seki and Rutz (2018) carefully optimised a myriad of further experimental parameters. They tested a range of nucleofection conditions (optimising buffers and pulses), showed that using two or three sgRNAs per gene rather than one sgRNA per gene resulted in improved KO efficiency and even found that some commercially available Cas9 proteins lead to higher KO efficiencies than others (Seki and Rutz, 2018). Using their optimised conditions, Seki and Rutz (2018) achieved 85%-98% KO across a variety of targets in activated and nonactivated primary human and mouse CD4 and CD8 T cells. Similarly to Seki and Rutz (2018), I routinely used three guides per gene, resulting in comparable KO efficiencies across many target genes (*Rab27a* (Figure 3.7), *Perforin* (Figure 3.8), *Munc13-4* (Figure 3.9), *Nfil3* (Figure 4.5), *Hif1a* (Figure 4.6)). The study by Seki and Rutz (2018) indicates that there may be potential to improve the KO efficiency I achieved even further. This could be explore in future experiments, for example by testing a variety of commercially available Cas9 proteins.

7.2.2 CRISPR in human T cells

Several technical approaches have been pursued to achieve CRISPR-Cas9 mediated gene editing in primary human T cells. These include nucleofection of DNA plasmids, viral delivery of CRISPR components and nucleofection of crRNAs and tracrRNAs in combination with Cas9 protein or Cas9 mRNA.

The first published study that used CRISPR in primary human T cells nucleofected plasmids encoding Cas9 and sgRNAs (Mandal et al., 2014). Interestingly, they found that a sgRNA that led to loss of B2M expression in 48% of HEK293T cells only resulted in loss of B2M expression in less than 5% of primary T cells. Even after targeting the gene with two guides, they only achieved 18% loss of B2M expression (Mandal et al., 2014), indicating that their approach to gene-editing in primary T cells was inefficient.

Further technical difficulties were highlighted by several studies that attempted to use viral delivery of CRISPR components to primary human T cells. Wang et al. (2014b) tried to deliver Cas9 and sgRNAs using lentiviral vectors to primary human T cells, but while they were able to KO CCR5 in a human CD4 T cell line, they stated that attempts to disrupt CCR5 gene expression in primary human T cells using the same experimental approach were unsuccessful, although the data is not shown. Similarly, Griffin et al. (2015) tried to deliver Cas9 and sgRNAs using viral vectors and observed that editing efficiency was lower in primary CD4 T cells than in related cell lines. More recently, Legut et al. (2018) managed to transduce primary human T cells with a viral vector encoding Cas9, sgRNA and puromycin resistance. However, 90% of cells died following selection, indicating that only 10% of the cells were successfully transduced (Legut et al., 2018). These results correlate well with my findings in chapter 6, and demonstrate that low transduction efficiency is a key challenge to stable expression of Cas9 in primary T cells.

The most widely published approach to CRISPR-Cas9 gene editing in primary human T cells is the Cas9-RNP method. Schumann et al. (2015) achieved ∼40% loss of cell surface expression of CXCR4 in primary human T cells in response to nucleofection of in vitro transcribed crRNA and tracrRNA alongside Cas9 protein. Nucleofection of CRISPR reagents as RNAs and proteins was found to be less toxic and more efficient at generating KOs than nucleofection of plasmids (Hendel et al., 2015). Another study using Cas9-RNPs in primary human T cells achieved 86% loss of CD45 in response to nucleofecting CRISPR reagents using the neon transfection system (Gundry et al., 2016). These results are comparable to the efficiencies I obtained when targeting *CD2* by CRISPR in primary human T cells, where I achieved 75.8% KO on average at day 7 post nucleofection when using the neon transfection system (Figure 6.9). Further studies have successfully used the Cas9-RNP approach to create more potent CAR T cells, for example by disrupting PD-1 to enhance CAR T cell mediated killing (Rupp et al., 2017) and by disrupting CD7 to avoid fratricide of CD7 specific CAR T cells (Gomes-Silva et al., 2017). While not the focus of this thesis, it is also noteworthy that knock-ins have been achieved using the Cas9-RNP approach (Roth et al., 2018; Schumann et al., 2015).

Similarly to my approach in chapter 4, where I performed an arrayed screen using Cas9-RNPs in primary mouse T cells, the Cas9-RNP approach has also been used for an arrayed screen in primary human T cells. While I targeted 13 genes, Hultquist et al. (2016) targeted 45 genes in order to identify genes that control HIV infection of primary human T cells. Hultquist et al. (2016) were able to perform an arrayed screen at a larger scale because they

had access to a 96-well electroporation device. Together, both my study and the paper by Hultquist et al. (2016) demonstrate the value of the Cas9-RNP technology for the purpose of small to medium sized arrayed CRISPR screens.

Further to the technical approaches outlined above, nucleofection of Cas9 mRNA, rather than Cas9 protein, alongside in vitro transcribed RNA has also been used for successful gene editing in primary human T cells (Ren et al., 2017a). Additionally, the lentiviral delivery of sgRNAs followed by nucleofection of Cas9 mRNA was shown to result in loss of CD3 expression in 90% of cells, in comparison to 82% KOs achieved with synthetic sgRNA and Cas9 protein co-nucleofection and 76% KOs achieved with co-nucleofection of synthetic sgRNAs and Cas9 mRNA (Ren et al., 2017b). Similarly, Shifrut et al. (2018) achieved loss of CD8 expression in over 80% of cells when using lentiviral delivery of sgRNAs followed by nucleofection of Cas9 protein. While the KO efficiencies achieved are convincing across several different technical approaches, the viral delivery of sgRNAs has the additional benefit of enabling delivery of genome-wide sgRNA libraries, allowing large-scale unbiased screens, as recently performed by Shifrut et al. (2018).

In summary, the toolbox for disrupting gene expression using CRISPR-Cas9 in primary human and mouse T cells has been expanding rapidly over the last 5 years as this PhD project was ongoing. The findings in this thesis correlate well with published findings, and contribute to knowledge of how to efficiently and robustly disrupt gene expression in primary T cells. KO efficiencies over 80% have been achieved across many genetic targets, both in this thesis and in the published literature. Cas9-RNPs or viral expression of sgRNAs in combination with Cas9 protein/mRNA nucleofection or cells derived from Cas9-expressing mice have emerged as the most convincing approaches to generate KOs in primary T cells at high efficiencies.

7.3 Evaluation of the Cas9-RNP approach to CRISPR gene editing

The results obtained in this thesis indicate that Cas9-RNPs can generate true genetic KOs in a substantial subset of target cells as measured by WB and flow cytometry (Figures 3.4 - 3.9). Cas9-RNPs generated permanent genetic modifications, which were shown to be stably maintained, at least until 1 month after nucleofection (Figure 6.9). While some cells treated with Cas9-RNP appeared to have lost protein expression completely, other cells had only

partially lost protein expression or not lost it at all. This heterogeneity can be seen clearly in flow cytometry plots where *Thy1* was targeted in mCTL (Figure 3.5) and *CD2* or *B2M* were targeted in Jurkat cells or hCTL (Figures 6.9, 6.10 and 6.11). The variability of the fluorescent signal likely correlated to homozygous KOs, heterozygous KOs and unedited cells. This heterogeneous mix of cells is likely the result of limited transfection efficiency and variability in DNA repair outcomes. This heterogeneity may create noise in a phenotypic readout that can conceal the phenotype of genes where KO only has a moderate effect. One way to limit this noise is to derive clones from the mixed population in order to be able to identify homozygously edited clones and the precise genetic alteration that occurred.

The benefit of analysing clones was also emphasised by a recently published study, that showed that CRISPR can result in complex gene rearrangements, such as deletions of many kilobases, around the cut site (Kosicki et al., 2018). The authors highlighted the need for a comprehensive analysis of CRISPR edited cells before drawing functional conclusion, let alone administering the edited cells to patients. Such comprehensive analysis would best be achieved by cloning cells, followed by performing long-range PCR or sequencing to identify all genetic changes caused by gene editing (Kosicki et al., 2018).

7.4 The potential to perform large scale screens in primary human T cells

Secretion of lytic granules is a crucial part of the CTL killing response. Degranulation is measured by the exposure of LAMP1 on the extracellular surface of CTL. While degranulation was found to correlate with killing (Betts et al., 2003), it only gives an indirect killing readout. The combined degranulation and killing assay presented in this thesis measures target cell death directly, while still including the LAMP1 readout. This allowed me to directly correlate the target cell lysis and degranulation readouts and gave immediate limited mechanistic insight. The new assay is therefore an improvement over the traditional degranulation assay.

I showed that this assay can be used to detect genetic defects and compounds that reduce degranulation and killing. For example, CRISPR-mediated KO of *Rab27a* (Figure 3.7), *Munc13-4* (Figure 3.9) and parthenolide treatment (Figure 5.7) decreased degranulation and killing. I also showed that the assay can detect genetic defects that cause an increase in degranulation while decreasing CTL killing, such as CRISPR-mediated KO of *Prf1* (Figure 3.8) and *Nfil3* (Figure 4.5). This demonstrated that CTL degranulation does not always

correlate to cytolytic activity. No genetic targets or chemical compounds tested in this thesis increased CTL killing, however, the assay should in theory also be able to detect this phenotype, as long as the percentage of target cells killed throughout the assay duration is kept below 100% in the control samples.

It would be interesting to scale this assay up to a 384 well format, where less cells and reagents would be needed per sample. Fixation could be explored in order to stably maintain the end point of the assay across the plate. Furthermore, the assay could be expanded to include a cytokine readout, enabling comprehensive analysis of CTL function at scale. Such an assay would be useful for large-scale arrayed screening approaches of compounds and genetic targets.

While arrayed screens are potentially powerful, they are limited by the quality of available datasets and the literature to inform the choice of genetic targets. In order to scale up the screen even further it would have to be switched from an arrayed to a pooled format. The pooled format is of particular interest as it would allow a genome-wide screen using a lentiviral sgRNA library. This would provide an unbiased approach and maximise the potential of identifying novel regulators of CTL function.

There are three main challenges that I anticipate for a genome-wide screen in primary hCTL. The first challenge would be to produce primary CTL stably expressing Cas9. As demonstrated in chapter 6, and acknowledged in the literature, stable expression of Cas9 in primary human T cells through lentiviral transduction is inefficient (Seki and Rutz, 2018; Shifrut et al., 2018). A recent study transduced primary human T cells with a genome-wide sgRNA library and subsequently introduced Cas9 protein through nucleofection (Shifrut et al., 2018). This approach, however, is limited by nucleofection efficiency, which may not equal the level of Cas9 expression that would be achieved after stable transduction and selection procedures. Given that I was able to produce up to 17% Cas9-2A-mCherry positive hCTL through transduction (Figure 6.7), I would be keen to repeat these experiments and attempt to clone out individual Cas9-expressing cells.

The second potential hurdle after Cas9 expression will be to select for cells that have incorporated an sgRNA after transduction with the lentiviral library. This is usually achieved through antibiotic selection if the sgRNA library contains an antibiotic resistance cassette. While I had difficulty to select Cas9-expressing cells with blasticidin after transduction with the Cas9-Blast lentivirus in chapter 6, I would expect that the transduction efficiency with

the lentiviral sgRNA library would be higher, as the sgRNA construct is smaller than the Cas9 construct. Higher transduction efficiency would result in better survival upon antibiotic selection.

Thirdly, pooled screens are dependent on an appropriate flow cytometry based assay to sort cells with the phenotype of interest. After sorting, samples are sequenced to identify which sgRNAs were present in the cells with the desired phenotype (Joung et al., 2017). LAMP1 expression in the degranulation assay could be used as the sorting phenotype. Similarly to Shang et al. (2018), who sorted Jurkat cells according to increased or decreased expression of CD69, I could sort cells with low or high LAMP1 signal in response to stimulation. However, in the degranulation assay I often observed a proportion of cells that, despite being stimulated, overlapped with the non-degranulating control in terms of the LAMP1 signal (e.g. see Figure 3.7). These cells likely did not degranulate as they did not encounter a target cell throughout the assay. This indicated that further optimisation of the E:T ratio and assay duration would be necessary to minimise the background noise in the degranulation assay.

Additional technical difficulties can be anticipated. Genome-wide screens require sorting of tens of millions of cells, which takes many hours (Doench, 2017). The effect of this sorting time on cell viability and αLAMP1-PE antibody signal needs to be taken into account. How will cell viability be affected if they are kept at room temperature for a prolonged period of time during sorting? Would the αLAMP1-PE antibody still be bound, and how would its brightness be affected over time? These factors have the potential to create further noise in the degranulation assay. Splitting the assay over several days, as done in chapter 5 for the drug screen, would address some of these concerns.

In addition to the technical difficulties identified, the biological caveat that a killing phenotype does not always overlap with a degranulation phenotype, as outlined in section 7.1, needs to be taken into account. In summary, the degranulation assay would be an immensely interesting readout for a genome-wide screen, but requires careful planning and further optimisation.

7.5 Conclusions

In conclusion, the techniques established and optimised in this thesis will be of value to research into the fundamental biology underlying the CTL killing mechanism. Detailed

analysis of individual gene targets as well as larger screens are possible. Additionally, the combined degranulation and killing assay could potentially be valuable as a diagnostic tool to identify immunodeficiency disorders. Efficient CRISPR-Cas9 gene editing in primary human CTL is furthermore of great interest for medical purposes, including cell-based immunotherapies. While better techniques are required to modify T cells, we also need to improve the understanding of fundamental T cell biology to inform future therapies. The methods developed here can be used to discover, study the mechanism of and validate genetic targets for T cell therapies.