

# Identifying regulators of cytotoxic T cell function through molecular and genetic screening



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For Mama, Papa, Isabell, Charlotte and Annette



## **Declaration**

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared here and specified in the text. The RNA-sequencing study described in chapter 4 was analysed in collaboration with Martin Del Castillo Velasco-Herrera.

This thesis is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution.

This dissertation contains less than 60,000 words as prescribed by the Degree Committee for the Faculty of Biology.

Katharina Strege  
December 2018



# Abstract

## Identifying regulators of cytotoxic T cell function through molecular and genetic screening

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Cytotoxic T lymphocytes (CTL) are crucial components of the adaptive immune system that kill infected and tumourigenic cells. CTL killing requires focused secretion of cytotoxic compounds from lytic granules. This process is known as degranulation. In this study, I aimed to establish the CRISPR-Cas9 gene editing technology in primary T cells and to optimise screening approaches to identify regulators of CTL killing.

The first half of the thesis focuses on primary mouse CTL. The CRISPR technology was successfully optimised in CTL using Cas9-ribonucleoprotein complexes resulting in efficient CRISPR-mediated loss of target proteins. Genes encoding known mediators of CTL cytotoxicity, *Rab27a*, *Munc13-4* and *Prf1*, were targeted using CRISPR. The resulting samples were used to establish a flow cytometry-based assay that simultaneously measures CTL degranulation and target cell death.

This assay enabled me to screen for mediators of CTL killing, while providing mechanistic insight by detecting degranulation. The screen was informed by a transcriptomic study that compared naive and effector CD8 T cells. 1803 significantly upregulated differentially expressed genes [ $\log_2(\text{fold change}) > 2$ ] were identified. Functional annotation analysis and literature research were used to select genes for the targeted CRISPR screen, which highlighted the importance of HIF-1 $\alpha$  and NFIL3 in CTL killing.

The second half of the thesis focuses on primary human CTL. The combined degranulation and killing assay was further validated using patient-derived CTL, indicating its potential as a diagnostic test. I showed that the assay is suitable for mid-sized screens using a library of 64 compounds targeting the NF- $\kappa$ B signalling pathway. Further opportunities for increasing the scale of this screening technique are discussed.

Finally, I successfully tested CRISPR using Cas9-ribonucleoprotein complexes in the human system. Additionally, stable Cas9 expression through lentiviral transduction was explored in primary CTL and related cell lines. This has the potential to allow selection of cells expressing the CRISPR machinery, providing a cleaner experimental system and the possibility of large-scale screening approaches.

In summary, the techniques established in this thesis will be valuable for studying the genetics underlying CTL killing and the combined degranulation and killing assay furthermore shows great potential for diagnostic purposes.



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into perspective. His support has meant so much to me, especially over the last few months.

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# Abbreviations

|        |   |
|--------|---|
| AF488  | - Alexa Fluor 488   |
| APC    | - Allophycocyanin   |
| APCs   | - Antigen presenting cells                                  |
| B2M    | - $\beta$ 2 microglobulin                                   |
| BFP    | - Blue fluorescent protein                                  |
| BGP    | - $\beta$ -glycerol phosphate                               |
| bp     | - Base pairs  |
| BV421  | - Brilliant Violet 421                                      |
| BV711  | - Brilliant Violet 711                                      |
| CARs   | - Chimeric antigen receptors                                |
| Cas    | - CRISPR-associated proteins                                |
| CCR5   | - C-C motif chemokine receptor 5                            |
| CD     | - Cluster of differentiation                                |
| CER1   | - Cytoplasmic extraction reagent 1                          |
| CER2   | - Cytoplasmic extraction reagent 2                          |
| CHS    | - Chediak-Higashi Syndrome                                  |
| CRISPR | - Clustered regularly interspaced short palindromic repeats |
| crRNA  | - CRISPR RNA  |

---

|        |   |
|--------|---|
| cSMAC  | - Central supramolecular activation complex                       |
| CTL    | - Cytotoxic T lymphocytes   |
| CTLA-4 | - Cytotoxic T-lymphocyte-associated antigen 4                     |
| DAG    | - Diacylglycerol  |
| DAPI   | - 4',6-diamidino-2-phenylindole                                   |
| DAVID  | - Database for Annotation, Visualization and Integrated Discovery |
| DMEM   | - Dulbecco's Modified Eagle Medium                                |
| DMSO   | - Dimethyl sulfoxide  |
| DPBS   | - Dulbecco's Phosphate-Buffered Saline                            |
| dSMAC  | - Distal supramolecular activation complex                        |
| DTT    | - DL-Dithiotheitol  |
| E:T    | - Effector-to-target  |
| EDTA   | - Ethylene-Diamine Tetraacetic acid                               |
| ERK    | - Extracellular signal-regulated kinase                           |
| FACS   | - Fluorescence-activated cell sorting                             |
| FasL   | - Fas ligand  |
| FBS    | - Fetal bovine serum  |
| FDR    | - False discovery rate  |
| FHL    | - Familial hemophagocytic lymphohistiocytosis                     |
| FIDEA  | - Functional Interpretation of Differential Expression Analysis   |
| FITC   | - Fluorescein isothiocyanate                                      |
| FPKM   | - Fragments per kilobase per million                              |
| FSC    | - Forward scatter   |
| GAPs   | - GTPase-activation proteins                                      |

---

|              |                                       |
|--------------|---------------------------------------|
| GDP          | - Guanosine diphosphate               |
| GEFs         | - Guanine nucleotide exchange factors |
| GO           | - Gene ontology                       |
| GS           | - Griscelli syndrome                  |
| GTP          | - Guanosine triphosphate              |
| h            | - Hours                               |
| hCTL         | - Human CTL                           |
| HDR          | - Homology-directed repair            |
| HDs          | - Healthy donors                      |
| HEK          | - Human embryonic kidney              |
| het          | - Heterozygous                        |
| HIF          | - Hypoxia-inducible factor            |
| HIV          | - Human immunodeficiency virus        |
| HLH          | - Hemophagocytic lymphohistiocytosis  |
| HNTC         | - Highest non-toxic concentrations    |
| hom          | - Homozygous                          |
| HPS          | - Hermansky-Pudlak Syndrome           |
| HRP          | - Horseradish peroxidase              |
| hTCM         | - Human T cell media                  |
| I $\kappa$ B | - Inhibitor of $\kappa$ B             |
| IFNs         | - Interferons                         |
| IKK          | - I $\kappa$ B kinase                 |
| IL           | - Interleukin                         |
| indels       | - Insertions or deletions             |

---

|                   |   |
|-------------------|---|
| IP <sub>3</sub>   | - Inositol-(1,4,5)-triphosphate                   |
| IS                | - Immunological synapse                           |
| ITAM              | - Immunoreceptor tyrosine-based activation motifs |
| ITK               | - Interleukin-2-inducible T cell kinase           |
| kDa               | - Kilodalton                                      |
| KEGG              | - Kyoto Encyclopaedia of Genes and Genomes        |
| KO                | - Knockout  |
| LAG-3             | - Lymphocyte activation gene 3                    |
| LAMP1             | - Lysosomal-associated membrane protein 1         |
| LAT               | - Linker for activation of T cells                |
| LB                | - Luria-Bertani                                   |
| Lck               | - Lymphocyte-specific protein tyrosine kinase     |
| LDH               | - Lactate dehydrogenase                           |
| MAPK              | - Mitogen-activated protein kinase                |
| mCTL              | - Mouse CTL                                       |
| MgCl <sub>2</sub> | - Magnesium chloride                              |
| MHC               | - Major histocompatibility complex                |
| min               | - Minutes   |
| MOI               | - Multiplicity of infection                       |
| mRNA              | - Messenger RNA                                   |
| mTCM              | - Mouse T cell media                              |
| MW                | - Molecular weight                                |
| NaCl              | - Sodium chloride                                 |
| NaF               | - Sodium fluoride                                 |

---

|                       |  |
|-----------------------|--|
| NaOV                  | - Sodium orthovanadate                             |
| NaPPi                 | - Sodium pyrophosphate                             |
| NER                   | - Nuclear extraction reagent                       |
| NF- $\kappa$ B        | - Nuclear factor- $\kappa$ B                       |
| NFAT                  | - Nuclear factor of activated T cells              |
| NFIL3                 | - Nuclear factor, interleukin 3 regulated          |
| NHEJ                  | - Non-homologous end-joining                       |
| NK                    | - Natural killer                                   |
| NP40                  | - Nonidet P40                                      |
| NT                    | - Non-targeting                                    |
| OVA                   | - Ovalbumin  |
| padj                  | - Adjusted p-value                                 |
| PAM                   | - Protospacer adjacent motif                       |
| PBMCs                 | - Peripheral blood mononuclear cells               |
| PCA                   | - Principal component analysis                     |
| PD-1                  | - Programmed cell death 1                          |
| PE                    | - Phycoerythrin                                    |
| Pen                   | - Penicillin                                       |
| PHA                   | - Phytohaemagglutinin                              |
| pHRSIN-mCh            | - pHRSIN-mCherry                                   |
| PI(4,5)P <sub>2</sub> | - Phosphatidylinositol-(4,5)-biphosphate           |
| PIP5K                 | - Phosphatidylinositol 4-phosphate 5-kinase type I |
| PKC                   | - Protein kinase C                                 |
| PKD                   | - Protein kinase D                                 |

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|                |   |
|----------------|---|
| PLC $\gamma$ 1 | - Phospholipase C $\gamma$ 1  |
| PMA            | - Phorbol 12-myristate 13-acetate                                   |
| PMSF           | - Phenylmethylsulfonyl fluoride                                     |
| pSMAC          | - Peripheral supramolecular activation complex                      |
| RAS-GRP1       | - Guanine nucleotide exchange factor RAS guanyl-releasing protein 1 |
| RE             | - Restriction enzyme  |
| RIN            | - RNA integrity number  |
| RISC           | - RNA-induced silencing complex                                     |
| RNA-seq        | - RNA-sequencing  |
| RNPs           | - Ribonucleoprotein complexes                                       |
| RPMI           | - Roswell Park Memorial Institute                                   |
| RT             | - Room temperature  |
| SD             | - Standard deviation  |
| SDS            | - Sodium dodecyl sulfate  |
| sec            | - Seconds   |
| sgRNA          | - Single guide RNA  |
| siRNA          | - Small interfering RNA   |
| SLACs          | - Synaptotagmin-like protein lacking C2 domains                     |
| SLP76          | - Src homology 2 domain-containing leukocyte protein of 76 kDa      |
| SLPs           | - Synaptotagmin-like proteins                                       |
| SNAP           | - Synaptosome associated protein                                    |
| SNARE          | - Soluble NSF attachment protein receptor                           |
| SOC            | - Super optimal broth with catabolite repression                    |
| SSC            | - Side scatter  |



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|          |  |
|----------|--|
| Strep    | - Streptomycin                                       |
| STX11    | - Syntaxin-11  |
| TAE      | - Tris-acetate-EDTA                                  |
| TALENs   | - Transcription activator-like effector nucleases    |
| TBS-T    | - Tris buffered saline-Tween                         |
| TCR      | - T cell receptor                                    |
| TIGIT    | - T Cell ITIM Domain                                 |
| TNF      | - Tumour necrosis factor                             |
| tracrRNA | - Trans-activating crRNA                             |
| Tris-HCl | - Tris(hydroxymethyl)aminomethane hydrochloride      |
| TU       | - Transducing units                                  |
| VAMP     | - Vesicle-associated membrane protein                |
| VAV1     | - Vav Guanine Nucleotide Exchange Factor 1           |
| WB       | - Western blotting                                   |
| WT       | - Wild-type  |
| Zap70    | - $\zeta$ -chain-associated protein kinase of 70 kDa |
| ZFNs     | - Zinc finger nucleases                              |

