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.

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Abstract

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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 [log2(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-1α 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-κB 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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Abbreviations

AF488	- Alexa Fluor 488
APC	- Allophycocyanin
APCs	- Antigen presenting cells
B2M	- $\beta 2$ microglobulin
BFP	- Blue fluorescent protein
BGP	- β-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

IκB - Inhibitor of κB

IFNs - Interferons

IKK - IκB kinase

IL - Interleukin

indels - Insertions or deletions

IP_3 - 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

$MgCl_2$ - 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-κB - Nuclear factor-κB

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_2 - Phosphatidylinositol-(4,5)-biphosphate

PIP5K - Phosphatidylinositol 4-phosphate 5-kinase type I

PKC - Protein kinase C

PKD - Protein kinase D

PLCγ1 - Phospholipase Cγ1

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

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	- ζ-chain-associated protein kinase of 70 kDa
ZFNs	- Zinc finger nucleases