Chapter 2

Materials and Methods

2.1 Cell culture

2.1.1 Cell culture conditions and media

The media compositions for all cell types used in this study are listed in Table 2.1. Fetal bovine serum (FBS) (Labtech) was heat inactivated at 56 °C for 30 min before addition to Roswell Park Memorial Institute (RPMI, Gibco, #21875-034 or Sigma, #R8758) or Dulbecco's Modified Eagle Medium (DMEM, Gibco, #41966-029) media. All media contained antibiotics (penicillin (pen) , streptomycin (strep)) (Sigma, #P0781) and was filter sterilised through a 0.22 μm filter. Mouse and human T cell media will subsequently be referred to by their abbreviations: human T cell media (hTCM) , mouse T cell media (mTCM).

All centrifugation steps were performed at 200xg for 5-10 min at room temperature (RT) unless otherwise indicated. Cells were cultured at 37 °C and 10% CO_2 in a humidified atmosphere.

2.1.2 Primary mouse T cell culture

Mice

Mice were bred under specific pathogen-free conditions in accordance with UK home office guidelines. T cells from OT-I mice (Hogquist et al., 1994) (genotype OT-I Rag1^{tm1Bal} on a C57BL/6 background) express a TCR that specifically recognises amino acids 257-264 (peptide sequence: SIINFEKL) of the chicken OVA protein presented in the context of H-2K^{b} MHC class I molecules. This peptide is referred to as 'OVA peptide' or simply 'OVA'

Table 2.1 ***Cell culture media.***

Name	Composition	Source
Human T cell media (hTCM)	RPMI 1640	Gibco, #21875-034, Sigma, #R8758
	2% (v/v) recombinant IL-2 protein	Produced in-house (Ruprecht et al., 2005)
	5% (v/v) human serum	Sigma, #H6914
	2 mM (1% (v/v)) L-glutamine	Sigma, #G7513
	1 mM (1% (v/v)) sodium pyruvate	Gibco, #11360-039
	50 μM β-mercaptoethanol	Gibco, #31350-010
	100 U/ml penicillin, 0.1 mg/ml streptomycin	Sigma, #P0781
Mouse T cell media (mTCM)	RPMI 1640	Gibco, #21875-034, Sigma, #R8758
	20 ng/ml recombinant murine IL-2	Peprotech, #212-12
	10 % (v/v) heat-inactivated FBS	Labtech
	2 mM (1% (v/v)) L-glutamine	Sigma, #G7513
	1 mM (1% (v/v)) sodium pyruvate	Gibco, #11360-039
	50 μM β-mercaptoethanol	Gibco, #31350-010
	100 U/ml penicillin, 0.1 mg/ml streptomycin	Sigma, #P0781
Target cell media	DMEM	Gibco, #41966-029
	10 % (v/v) heat-inactivated FBS	Labtech
	100 U/ml penicillin, 0.1 mg/ml streptomycin	Sigma, #P0781
HEK media	DMEM	Gibco, #41966-029
	10 % (v/v) heat-inactivated FBS	Labtech
YT media	RPMI 1640	Gibco, #21875-034, Sigma, #R8758
	2% (v/v) recombinant IL-2 protein	Produced in-house (Ruprecht et al., 2005)
	10 % (v/v) heat-inactivated FBS	Labtech
	2 mM (1% (v/v)) L-glutamine	Sigma, #G7513
	1 mM (1% (v/v)) sodium pyruvate	Gibco, #11360-039
	50 μM β-mercaptoethanol	Gibco, #31350-010
	100 U/ml penicillin, 0.1 mg/ml streptomycin	Sigma, #P0781
Jurkat media	RPMI 1640	Gibco, #21875-034, Sigma, #R8758
	10 % (v/v) heat-inactivated FBS	Labtech
	100 U/ml penicillin, 0.1 mg/ml streptomycin	Sigma, #P0781

throughout this thesis. Due to breeding on a *Rag1* -/- background, all OT-I T cells express the same TCR from the double positive stage in T cell development and are CD8-positive. This system allows uniform stimulation upon addition of OVA peptide in vitro, resulting in CTL that can kill EL4 target cells loaded with OVA.

C57BL/6N WT mice were obtained through the 3i Consortium (Wellcome Trust Sanger Institute). Spleens from C57BL/6 Cas9 mice, where Cas9 under the control of the human *EF1*α promoter was inserted into the *Rosa26* locus (Tzelepis et al., 2016), were kindly provided by Dr Gabriel Balmus and Dr Kosuke Yusa (Wellcome Trust Sanger Institute). Spleens from *ashen* mice (Wilson et al., 2000) were kindly provided by Dr Melina Schuh (MRC Laboratory for Molecular Biology).

Preparation and stimulation of murine splenocytes

Spleens were manually disrupted using a sterile syringe plunger and resulting splenocytes were filtered through a 70 μm cell strainer before centrifugation at 200xg for 10 min. For OT-I splenocytes, each pellet was resuspended in 100 ml mTCM (Table 2.1) containing 10 nM OVA peptide (Anaspec, #AS-60193-5) and cultured at 37 °C for 3 days. From day 3 onwards, cells were washed daily and resuspended at roughly 1x10^6 cells/ml. OT-I cells were used in functional assays from day 6 - 9 post in vitro stimulation.

To stimulate splenocytes derived from WT, Cas9 or *ashen* mice, 6 well plates were coated with 0.5 µg/ml αCD3ε (clone Bio500A2, eBioscience, #16-0033-86) and 1 µg/ml αCD28 (clone 37.51, eBioscience, #16-0281-86) overnight at 4 °C. 13.6x10^6 splenocytes were seeded per well in 8 ml mTCM on the pre-coated plates. Cells were cultured for 48 h at 37 °C and 10% CO_2. The resulting stimulated T cells were washed, resuspended at 1x10^6 cells/ml in fresh mTCM and transferred to uncoated plates. Subsequently, mCTL were split every 1-2 days and functional assays were performed from day 6 - 9 post in vitro stimulation. CD8 T cells usually grew out to >80% during expansion, but were purified using the mouse CD8a T cell isolation kit (Miltenyi Biotec, #130-104-075) in experiments where a pure CD8 population was required.

2.1.3 Primary human T cell culture

Phytohaemagglutinin (PHA)-blasts from FHL patient samples (Table 2.2) were kindly provided by Professor Stephan Ehl (Medical center, University of Freiburg). T cells derived from healthy donors (HDs) were obtained from buffycoat (NHS Blood and Transplant Centre). Peripheral blood mononuclear cells (PBMCs) were isolated from buffycoats by Ficoll-Paque (GE healthcare, #17-1440-02) gradient centrifugation and washed 5x in RPMI containing 1% FBS.

Human T cells were stimulated with 1 µg/ml PHA (Sigma, #L1668) and allogeneic PBMCs derived from buffycoat residue (NHS Blood and Transplant Centre). Allogeneic PBMCs were isolated as described for HDs, followed by irradiation at 3000 radiation absorbed dose (rad) of gamma rays prior to addition to human T cells. Cells were cultured in hTCM (Table 2.1) at 37 °C and 10% CO_2. Cells were expanded when confluent, roughly

Table 2.2 ***Details of mutations in FHL patients.*** *Cells were derived from patients that presented with FHL2, caused by mutations in PRF1, or FHL3, caused by mutations in MUNC13-4. Patient mutations are described according to published guidelines (Ogino et al., 2007). Mutations are indicated as homozygous (hom) if the same mutation is present on both alleles, heterozygous (het) if only one allele is affected by the mutation and compound het if both alleles are affected, but the mutations differ on each allele. The abbreviation 'c.' stands for coding DNA and is followed by the exact nucleotide mutation at the DNA level (del = deletion). Intronic locations are numbered with reference to exonic sequences (+1 indicates that the mutation affects an intron). The abbreviation 'p.' stands for the protein amino acid sequence and is followed by the change in amino acids (three letter code) caused by the mutation (fs = frameshift, X = early stop codon). The affected exons are given in square brackets.*

Patient	FHL subtype	Mutation
6981	FHL2	hom c.1288G>C; p.Asp430His [Exon 3]
3026	FHL2	compound het c.272C>T; p.Ala91Val [Exon 2] and c.1213C>T; p.Gln405X [Exon 3]
392	FHL2	hom c.1349C>T; p.Thr450Met [Exon 3]
4166	FHL2	c.112G>A; p.Val38Met and c.272C>T; p.Ala91Val [Exon 2 and 3] (het/hom information not provided)
3778	FHL3	hom c.1208C>T; p.Leu403Pro [Exon 14]
1310	FHL3	het c.753(+1) splice site [Exon 9] and c.2346_49del; p.Arg782fsX12 [Exon 24]
4413	FHL3	het c.1389(+1) G>A splice donor site [Exon15] and c.2346_2349del; p.Arg782Ser fsX12 [Exon24]

every 1-3 days until day 14 post in vitro stimulation, at which time the cells usually started to proliferate less. hCTL were used for functional assays from day 12 - 15 post stimulation and were re-stimulated as described above roughly every 21 days. The percentage of CD8 and CD4 positive cells was checked on a regular basis by flow cytometry. If necessary, CD8 T cells were purified using the human CD8 T cell isolation kit (Miltenyi Biotec, #130-045-201). The human CD4 T cell isolation kit (Miltenyi Biotec, #130-045-101) was used to exclude CD4 cells. For blasticidin concentration response and selection experiments human T cells were treated with blasticidin (Sigma, #15205) at the concentrations indicated in figure legends.

2.1.4 Cell line culture

P815 (mouse mastocytoma) cells were used as target cells for human CTL and WT mouse CTL. P815s express Fc receptors on their cell surface that bind to the constant region of antibodies. P815s loaded with αCD3ε can trigger activation of T cells. EL4 (mouse lymphoma) cells were used as target cells for OT-I CTL as they can present the OVA peptide

in the context of H-2K^b MHC class I molecules. Blue fluorescent protein (BFP) P815s and EL4s, stably expressing Farnesyl-5-TagBFP2 through retroviral transduction were established in the Griffiths lab (Ritter et al. (2015) and Alex Ritter's PhD thesis). These target cells are subsequently referred to as blue EL4 or blue P815s for simplicity. I established red P815s and EL4s using lentiviral transduction (see section 2.10). All P815 and EL4 cell lines were cultured in target cell media (Table 2.1) and split 1 in 10 every 2-3 days. For red P815s and EL4s, the target cell media was supplemented with 1 µg/ml puromycin (Thermo Fisher Scientific, #A1113803).

Human embryonic kidney (HEK) 293T cells were maintained in target cell media without antibiotics and split when reaching roughly 80% confluency using trypsin (Sigma, #T3924) for detachment. The human NK cell line YT was maintained in YT media (Table 2.1) and the human Jurkat T cell line was maintained in Jurkat media (Table 2.1). YT cells and Jurkat cells were split 1 in 5 every 2-3 days.

2.2 Nucleofection

2.2.1 Preparation of synthetic RNAs

Synthetic RNAs were obtained as dried pellets from Dharmacon, reconstituted in Tris-EDTA pH 7.4 (Sigma, #93302) to give stock solutions between 1-10 μg/μl and mixed on an orbital shaker at 700 rpm for 30 min at RT. Aliquots were stored at $-20\,^{\circ}$C. Details of all synthetic RNA reagents used are outlined in Table 2.3. siRNA and crRNA sequences were predesigned by Dharmacon/Horizon Discovery. Only crRNAs targeting exons were chosen, with preference given to crRNAs targeting different exons in the same gene in order to increase the possibility of cutting out a large section of the gene.

2.2.2 Nucleofection of primary mouse T cells

mCTLs were nucleofected at day 4-7 post in vitro stimulation. Nucleofections were performed with the Lonza mouse T cell nucleofection kit (Lonza, #VPA-1006) using the 2b nucleofection machine (Lonza) or the P3 primary cell kit (Lonza, #V4XP-3024) using the 4D nucleofector (Lonza). Mouse T cell nucleofection media (Lonza, #VZB-1001) was supplemented with 1% (v/v) medium component A (Lonza, #VZB-1001), 2 mM L-glutamine (Sigma, #G7513) and 5% (v/v) FBS (Labtech). Before use, 1% (v/v) component B (Lonza, #VZB-1001) was added and nucleofection media was pre-warmed at 37 °C for at least 30 min. Subsequently, 5x10^6 cells per condition were centrifuged at 200xg for 5 min. All supernatant

was removed carefully and pellets were resuspended in 100 μl nucleofection solution (Lonza) containing the desired reagents. For siRNA experiments, 5x10^6 T cells were nucleofected with 3 μg scramble or Rab27a siRNA (Table 2.3). For CRISPR experiments, 5x10^6 T cells were nucleofected with 5 μg per crRNA (3 crRNAs per gene means 15 μg crRNAs in total), 15 μg tracrRNA and 10 μg Cas9 protein (Takara, #632640), unless otherwise indicated in figure legends. Nucleofection controls and non-targeting (NT) CRISPR controls were included in every experiment. Cells resuspended in nucleofection solution containing all necessary reagents were transferred to nucleofection cuvettes and nucleofected using program X-001 (2b machine) or program DN-100 (4D machine). Next, each nucleofected sample, containing 5x10^6 mCTL, was transferred to 1 ml prewarmed nucleofection media in 1 well of a 12 well plate. Cells were left to recover in the incubator for 3-4 h before splitting across 3 wells in a 12 well plate and topping up each well with 3 ml mTCM. The following day, cells were pelleted and resuspended in fresh mTCM at roughly 1x10^6 cells/ml.

2.2.3 Nucleofection of primary human T cells

Human T cells were stimulated with 5 μg/ml PHA for 3 days prior to nucleofection. The P3 primary cell kit (Lonza, #V4XP-3024) was used for nucleofection with the 4D nucleofector (Lonza). Prior to nucleofection, hTCM was pre-warmed at 37 °C for at least 30 min. 1-1.5x10^6 cells were pelleted and resuspended in nucleofection solution containing 2 μg lifeact-EGFP plasmid or CRISPR reagents. As only 1-1.5x10^6 cells were nucleofected per condition, the amount of CRISPR reagents used was scaled down accordingly (e.g. as I used 5 μg per crRNA for 5x10^6 mouse T cells, I only used 1 μg per crRNA when nucleofecting 1x10^6 human T cells). Human T cells were nucleofected using program E0-115 on the 4D nucleofector.

For nucleofection with the Neon transfection system (Thermo Fisher Scientific), human T cells were washed once in Dulbecco's Phosphate-Buffered Saline (DPBS) (Gibco, #14190094) and then resuspended in 110 μl buffer T (Neon transfection system kit, Thermo Fisher Scientific, #MPK10025) containing the appropriate CRISPR reagents. The reaction conditions were otherwise identical to the Lonza 4D samples (same number of cells and same amount of reagent). Cells were transfected using the following settings: 1600V, 10 ms, 3 pulses.

Upon nucleofection with neon or 4D nucleofectors, 1-1.5 million human T cells were transferred into 500 μl pre-warmed hTCM in one well of a 24 well plate. Cells were left to recover in the incubator for 2 h before topping up with an additional 500 μl pre-warmed

hTCM. The next day, cells were pelleted at 200xg for 10 min and resuspended in fresh hTCM.

2.2.4 Nucleofection of Jurkat cells

Jurkat cells were nucleofected using the SE Cell line nucleofection kit (Lonza, #V4XC-1012). For nucleofections using cuvettes, 1x10^6 Jurkat cells were pelleted and resuspended in 100 μl nucleofection solution containing CRISPR reagents. For nucleofections using 16-well strips, 0.2x10^6 Jurkat cells were pelleted and resuspended in 20 μl nucleofection solution containing CRISPR reagents. In both experimental conditions, Jurkat cells were nucleofected with 2 μg Cas9 protein, 3 μg tracrRNA, and 1 μg per crRNA (3 crRNAs per gene) targeting *CD2* or the gene encoding β2 microglobulin (*B2M*) using program CK-116 on the 4D nucleofector. Cells were transferred into pre-warmed Jurkat media in 24 well plates (1x10^6 cells samples) or 96 well plates (0.2x10^6 cells samples) and left to recover for 2 h before topping up with pre-warmed Jurkat media.

Table 2.3 ***Overview of synthetic RNAs (siRNAs, crRNAs and tracrRNA) used in this study.*** *All reagents were predesigned and acquired from Dharmacon/Horizon Discovery.*

Reagent	**Catalogue number**
scramble siRNA	D-001810-10-20
Rab27a siRNA	L-060970-01
tracrRNA	U-002005-50
Non-targeting crRNA	U-007501-20
mouse *Thy1* crRNA	CR-041986-01-0005
mouse *Rab27a* crRNA1	CR-060970-01-0005
mouse *Rab27a* crRNA2	CR-060970-04-0005
mouse *Rab27a* crRNA3	CR-060970-05-0005
mouse *Munc13-4* crRNA1	CM-064384-01-0002
mouse *Munc13-4* crRNA2	CM-064384-02-0002
mouse *Munc13-4* crRNA3	CM-064384-03-0002
mouse *Perforin* crRNA1	CM-064701-01-0002
mouse *Perforin* crRNA2	CM-064701-02-0002
mouse *Perforin* crRNA3	CM-064701-03-0002
mouse *Cav2* crRNA1	CM-063000-01-0002
mouse *Cav2* crRNA2	CM-063000-02-0002

Table 2.3 continued from previous page

Reagent	Catalogue number
mouse *Cav2* crRNA3	CM-063000-03-0002
mouse *Anxa1* crRNA1	CM-040923-01-0002
mouse *Anxa1* crRNA2	CM-040923-02-0002
mouse *Anxa1* crRNA3	CM-040923-03-0002
mouse *Anxa2* crRNA3	CM-061993-01-0002
mouse *Anxa2* crRNA3	CM-061993-04-0002
mouse *Anxa2* crRNA3	CM-061993-05-0002
mouse *Anxa3* crRNA1	CM-046409-01-0002
mouse *Anxa3* crRNA2	CM-046409-02-0002
mouse *Anxa3* crRNA3	CM-046409-05-0002
mouse *Anxa4* crRNA1	CM-057375-02-0002
mouse *Anxa4* crRNA2	CM-057375-04-0002
mouse *Anxa4* crRNA3	CM-057375-05-0002
mouse *Nfil3* crRNA1	CM-063246-01-0002
mouse *Nfil3* crRNA2	CM-063246-02-0002
mouse *Nfil3* crRNA3	CM-063246-03-0002
mouse *Slc7a5* crRNA1	CM-041166-01-0002
mouse *Slc7a5* crRNA2	CM-041166-02-0002
mouse *Slc7a5* crRNA3	CM-041166-03-0002
mouse *Hif1α* crRNA1	CM-040638-01-0002
mouse *Hif1α* crRNA2	CM-040638-03-0002
mouse *Hif1α* crRNA3	CM-040638-04-0002
mouse *Dysf* crRNA1	CM-040311-02-0002
mouse *Dysf* crRNA2	CM-040311-03-0002
mouse *Dysf* crRNA3	CM-040311-04-0002
mouse *Cpne5* crRNA1	CM-053834-01-0002
mouse *Cpne5* crRNA2	CM-053834-02-0002
mouse *Cpne5* crRNA3	CM-053834-03-0002
mouse *Ppfia3* crRNA1	CM-066090-01-0002
mouse *Ppfia3* crRNA2	CM-066090-02-0002
mouse *Ppfia3* crRNA3	CM-066090-05-0002
mouse *Tns2* crRNA1	CM-040926-01-0002
mouse *Tns2* crRNA2	CM-040926-02-0002

Table 2.3 continued from previous page

Reagent	Catalogue number
mouse *Tns2* crRNA3	CM-040926-03-0002
human *CD2* crRNA1	CM-017854-01-0002
human *CD2* crRNA2	CM-017854-02-0002
human *CD2* crRNA3	CM-017854-05-0002
human *B2M* crRNA1	CM-004366-01-0002
human *B2M* crRNA2	CM-004366-02-0002
human *B2M* crRNA3	CM-004366-03-0002

2.3 Western blotting

2.3.1 Lysate preparation

Cells were counted and pelleted at 200xg for 5 min, resuspended in 1 ml ice-cold DPBS and pelleted again at 200xg for 5 min at 4 °C. Pellets were dried by removing the supernatant, frozen on dry ice before storage at −80 °C or lysed directly at 2-5x10^7 cells/ml for Western blotting (WB).

Cytoplasmic lysate preparation

Dried pellets were resuspended in lysis buffer [50 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) pH 8, 150 mM sodium chloride (NaCl) , 1 mM magnesium chloride ($MgCl_2$) (all obtained from CIMR media kitchen), 2% Triton X-100 (Sigma, #T8787) in ultrapure H_2O (CIMR media kitchen)]. 1X protease inhibitor cocktail (Roche, #04693132001) was added just before use. Additionally, 1X PhosSTOP (Roche, #4906845001) was included in the lysis buffer for lysates used to blot for phosphorylated proteins. After incubation at 4 °C for 30 min, lysates were centrifuged at 18,800 xg for 25 min at 4 °C. The supernatant was transferred into a fresh tube and sample buffer added as outlined in section 2.3.2.

Nuclear lysate preparation

For the detection of the protein Nuclear factor, interleukin 3 regulated (NFIL3), nuclear proteins were extracted from cell pellets using the NE-PER Nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, #78835). This kit uses 3 buffers, cytoplasmic

extraction reagent 1 (CER1), cytoplasmic extraction reagent 2 (CER2) and nuclear extraction reagent (NER). CER1, CER2 and NER were added at a ratio of 200:11:100, as detailed in the manufacturer's instructions, to give a final lysate concentration of $5x10^7$ cells/ml. Protease inhibitors (Roche, #04693132001) were added to CER1 and NER buffers immediately before use. Cell pellets were resuspended in ice-cold CER1, vortexed for 15 seconds (sec) and incubated on ice for 10 min. Next, ice-cold CER2 reagent was added, samples were vortexed for 5 sec and incubated on ice for 1 min prior to centrifugation at 18,800 xg for 5 min at 4 °C. The supernatant, containing cytoplasmic proteins, was transferred to a clean tube. The pellet, containing nuclear proteins, was resuspended in ice-cold NER, vortexed for 15 sec every 10 min for a total of 40 min. Samples were pelleted at 18,800 xg for 5 min at 4 °C and the supernatant, containing nuclear proteins, was transferred to a clean tube and sample buffer added as outlined in section 2.3.2.

Whole cell lysate preparation

The following method was used to enable detection of Hypoxia-inducible factor (HIF)-1α by WB. Four days after nucleofection, control (nuc control and NT CRISPR) mCTL and mCTL nucleofected with *Hif1*α CRISPR reagents were transferred to plates coated with 1 µg/ml αCD3ε (clone Bio500A2, eBioscience, #16-0033-86). $5x10^6$ mCTL were seeded per well and the plate was transferred to a hypoxia chamber (Whitley H35 hypoxystation) kept at 1% O_2 and 37 °C. After 4 h, samples were collected on ice, centrifuged briefly for 1 min at 200xg and 4 °C, washed 1x with ice-cold DPBS containing 1x protease inhibitor (Roche, #04693132001), and pelleted again. After removing the supernantant, pellets were resupsended at $5x10^7$ cells/ml in the following lysis buffer, kindly provided by Natalie Burrows (Patrick Maxwell's lab, Cambridge Institute for Medical Research): H_2O containing 50 mM Tris-HCl pH 7.4, 120 mM NaCl, 5 mM ethylene-Diamine Tetraacetic acid (EDTA), 0.5% nonidet P40 (NP40), 1mM DL-Dithiotheitol (DTT), 1 mM Phenylmethylsulfonyl fluoride (PMSF), 2 mM sodium orthovanadate (NaOV), 2 mM sodium fluoride (NaF), 20 mM β-glycerol phosphate (BGP), 5 mM sodium pyrophosphate (NaPPi) and 1x protease inhibitor cocktail (Roche, #04693132001). Each sample was sonicated twice for 2 sec at an amplitude of 8 using a soniprep 150 plus sonicator (MSE). After 10 min incubation on ice samples were centrifuged at 16,200xg for 10 min at 4 °C. The resulting supernatant was transferred to a clean tube and sample buffer added as outlined in section 2.3.2.

Table 2.4 ***Primary antibodies used for WB.*** *Abbreviations: Rb = Rabbit, Ms = mouse, Rt = rat, Gt = goat.*

Target	Species reactivity	Clonality	Dilution	Source
Ms α-Rab27a	mouse	monoclonal	1 in 100	Abcam, #ab55667
Rb α-Munc13-4	mouse	monoclonal clone 1223B	1 in 200	R&D Systems, #MAB89662
Gt α-Munc13-4	human	polyclonal	1 in 300	Eversest Biotech, #EB06383
Rt α-Perforin	mouse	monoclonal clone CB5.4	1 in 500	Enzo life sciences, #ALX-804-057-C100
Ms α-Perforin	human	monoclonal (supernatant) clone 2D4	1 in 50	(Baetz et al., 1995)
Ms α-Cas9	not applicable	monoclonal clone 7A9	1 in 100	Epigentek, #A9000-100
Rb α-Calnexin	mouse/human	polyclonal	1 in 2000	Sigma, #C4731
Ms α-βactin	mouse/human	monoclonal clone AC-15	1 in 5000	Sigma, #CA5441
Rb α-NFIL3	mouse	monoclonal clone DK580	1 in 500	Cell Signaling Technology, #14312S
Ms α-Hif1α	mouse	monoclonal clone 241809	1 in 500	R&D Systems, #MAB1536
Rb α-Lamin B1	mouse/human	polyclonal	1 in 5000	Abcam, #ab16048
Rb α-p65 (S536)	human	monoclonal clone 93H1	1 in 500	Cell Signaling Technology, #3033S
Ms α-IkBα	human	monoclonal clone L35A5	1 in 500	Cell Signaling Technology, #4814S
Rb α-p105/p50	human	monoclonal clone D4P4D	1 in 100	Cell Signaling Technology, #13586S

2.3.2 Protein electrophoresis and transfer

Nupage LDS sample buffer (Invitrogen, #NP0007) containing 10% NuPAGE Sample reducing reagent (Invitrogen, #NP0009) was added to WB lysates at a final concentration of 1X. Samples were boiled at 95 °C for 5 min prior to loading onto pre-cast NuPAGE 4-12% Bis-Tris gels (Invitrogen, #NP0335) alongside 5 μl of Precision Plus Protein Kaleidoscope MW marker (BioRad, #161-0375). Gels were run at 110-130 V for 1-2 h in MES (life technologies, #NP0002) or MOPS (for high MW proteins, life technologies, #NP0001) sodium dodecyl sulfate (SDS) running buffer diluted to 1X in distilled H_2O. Proteins were transferred to nitrocellulose membranes (GE Healthcare life sciences, #10600003) in NuPAGE transfer buffer (life technologies, #NP0006) diluted to 1X in distilled H_2O and 10% (v/v) methanol at RT for 95-100 min at 100 V. Successful transfer was confirmed by staining with ponceau red (0.1% (w/v) ponceau, 5% (v/v) acetic acid in H_2O) for 5 min at RT while shaking, followed by several washes in H_2O to remove the red stain.

2.3.3 Incubation with antibodies and protein detection

Blocking was performed in 1X Tris buffered saline-Tween (TBS-T) (1XTBS + 0.05% (v/v) Tween-20) containing 5% (w/v) skimmed milk. When blotting for phosphorylated proteins 1X TBS-T (0.05% Tween-20) containing 5% (w/v) BSA was used instead. Blots were incubated with primary antibodies (Table 2.4) diluted into blocking buffer while rotating overnight at 4 °C. The following day, blots were washed four times for a total of 20 min in 1x TBS-T solution before addition of horseradish peroxidase (HRP) conjugated secondary antibodies (Table 2.5) diluted into blocking buffer for 1 h at RT. Following four more

Table 2.5 ***Secondary antibodies used for WB.***

Target	Conjugation	Dilution	Source
Goat α-mouse	Peroxidase	1 in 3000	Thermo Fisher Scientific, #32430
Goat α-rabbit	Peroxidase	1 in 3000	Thermo Fisher Scientific, #32460
Goat α-rat	Peroxidase	1 in 10000	SouthernBiotech, #3030-05
Donkey α-goat	Peroxidase	1 in 10000	Jackson ImmunoResearch, #705-035-147

washes, proteins were detected using ECL prime western blotting reagent (GE Healthcare, #RPN2232) and images were acquired using the ChemiDoc MP imaging system (Bio-Rad). Band intensities were quantified using the ImageLab4.1 software (Bio-Rad) and samples were normalised to loading controls and expressed relative to an appropriate experimental control, as detailed in figure legends.

2.4 Flow cytometry

2.4.1 Cell surface staining

Cells were washed in fluorescence-activated cell sorting (FACS) buffer (DPBS + 1% (v/v) FBS) and stained with live dead markers (Table 2.6) and fluorescently-conjugated antibodies (Table 2.7) diluted into FACS buffer for 10-15 min at RT or at 4 °C for 30 min. Subsequently, cells were washed to remove excess antibody and resuspended in FACS buffer for sample acquisition on LSRFortessa (BD biosciences), Accuri C6 (BD biosciences) or Attune NxT (Thermo Fisher Scientific) flow cytometers. Data was analysed with FlowJo version 10.4.2 (FlowJo, LLC).

Table 2.6 ***Live/dead stains used in flow cytometry experiments.***

Reagent	Dilution	Source
Fixable yellow	1 in 1000	Thermo Fisher Scientific, #L34959
Zombie Red	1 in 500	Biolegend, #423110
DAPI	1 in 5000 (1 μg/ml)	Thermo Fisher Scientific, #D3571

Table 2.7 ***Directly conjugated antibodies used in flow cytometry experiments.*** *Abbreviations: FITC = Fluorescein isothiocyanate, PE = Phycoerythrin, APC = Allophycocyanin, BV421 = Brilliant Violet 421, BV711 = Brilliant Violet 711, AF488 = Alexa Fluor 488.*

Antibody specificity and conjugation	Clone	Isotype	Species specificity	Dilution	Source
B2M-FITC	2M2	mouse IgG1 κ	human	1 in 400	Biolegend, #316304
CD107a(LAMP1)-PE	1D4B	rat IgG2a κ	mouse	1 in 100	eBioscience, #12-1071-83
CD107a(LAMP1)-PE	H4A3	mouse IgG1 κ	human	1 in 200	eBioscience, #12-1079-42
CD2-FITC	RPA-2.10	mouse IgG1 κ	human	1 in 200	Biolegend, #300206
CD2-PE	RPA-2.10	mouse IgG1 κ	human	1 in 200	Biolegend, #300208
CD4-PE	RM4-5	rat IgG2a κ	mouse	1 in 200	Biolegend, #100511
CD4-PE	A161A1	rat IgG2b κ	human	1 in 200	Biolegend, #357404
CD8-APC	53-6.7	rat IgG2a κ	mouse	1 in 200	Biolegend, #100712
CD8-BV421	RPA-T8	mouse IgG1 κ	human	1 in 400	Biolegend, #301036
CD8-BV711	53-6.7	rat IgG2a κ	mouse	1 in 200	Biolegend, #100747
CD8-BV711	RPA-T8	mouse IgG1 κ	human	1 in 200	Biolegend, #301044
Thy1.2-APC	53-2.1	rat IgG2a κ	mouse	1 in 200	BD Biosciences, #561974
Thy1.2-AF488	30-H12	rat IgG2b κ	mouse	1 in 200	Biolegend, #105316
TNF-PE	MAb11	mouse IgG1 κ	human	1 in 100	BD Biosciences, #554513

2.4.2 Intracellular staining for TNFα detection

Flat bottom 96 well plates were coated with 1 μg/ml αCD3ε (clone OKT3, eBioscience, #14-0037-82) overnight at 4 °C. hCTL were treated with drug 19/parthenolide (Medchem express, #HY-N0141) or dimethyl sulfoxide (DMSO) (Sigma, #D2650) vehicle control overnight at 8.3 μM. The following day, coated wells were washed with hTCM before addition of 200,000 hCTL treated with drug 19 or DMSO per well. Golgi stop (BD biosciences, #554715), containing the protein transport inhibitor monensin, was added at 0.67 μl/ml to inhibit protein secretion, including the secretion of TNFα. Cells were incubated with and without the αCD3ε stimulus at 37 °C. After 4 h, cells were washed in ice-cold DPBS and stained with αhuman-CD8-BV711 (Table 2.7) and fixable yellow live dead dye (Table 2.6) for 30 min at 4 °C. After washing with FACS buffer, cells were fixed and permeabilised using 100 μl of fixation/permeabilisation solution (BD biosciences, #554715) per well for 20 min at 4 °C. After 2 washes with 1x permeabilisation/wash buffer (BD biosciences, #554715), samples were incubated with human Fc-block (Miltenyi, #130-059-901) at 4 μl/1x10^6 cells for 15 min at RT. Next, mouse αhuman-TNFα-PE (Table 2.7) or mouse IgG1-PE isotype control (Abcam, #ab81200) were added and incubated for a further 30 min at 4 °C. Following 2 washes in 1x permeabilisation/wash buffer, samples were resuspended in FACS buffer for analysis.

2.4.3 Cell sorting

Samples were washed with sterile FACS buffer containing 100 U/ml pen, 0.1 mg/ml strep, and stained with 4',6-diamidino-2-phenylindole (DAPI) (Table 2.6) to distinguish live cells from dying cells. P815 and EL4 positive for nuclight red or hCTL positive for Cas9-2A-mCh were sorted into appropriate media containing 100 U/ml pen, 0.1 mg/ml strep using an Influx cell sorter (Becton Dickinson). After sorting, cells were pelleted, resuspended in fresh pen/strep-containing media and transferred to the incubator.

2.5 Assays to measure CTL effector function

In this thesis I used degranulation and killing assays to measure CTL effector function. The conditions used to trigger an effector response differed according to whether CTLs were derived from OT-I mice, WT mice or human PBMCs. To measure degranulation and killing capabilities of OT-I CTL, EL4 target cells were used, which present the OVA peptide in the context of their H-2K^b MHC class I molecule. EL4s were pulsed with 1 μM OVA peptide for 1 h at 37 °C. Subsequently, cells were washed three times into mTCM prior to addition to OT-I CTL. Unpulsed EL4 that were incubated and washed in parallel served as a negative control.

To trigger a degranulation and killing response in WT mCTL or hCTL I used P815 target cells, which express Fc receptors on their cell surface that are able to bind to the constant region of antibodies. P815s were loaded with αCD3ε (for hCTL: clone UCHT1, BDPharmingen, #555330; for WT mCTL: clone 145-2C11, eBioscience, #16-0031-86) for 20 min at RT, resulting in a final αCD3ε concentration of 0.5 μg/ml for mCTL and 0.25 μg/ml for hCTL upon addition of target cells to CTL.

The conditions outlined above were used throughout. For simplicity, I will refer to hCTL and mCTL as 'CTL', to P815 and EL4s as 'targets' and to OVA peptide and αCD3ε as 'stimulus' for the remainder of section 2.5. In general, two - four technical replicates were included per condition in every experiment.

2.5.1 Degranulation assay

Both CTL and targets with stimulus were resuspended at 2x10^6 cells/ml and 200,000 cells were seeded per well in a round bottom 96 well plate to give an effector-to-target (E:T) ratio of 1, unless otherwise stated in figure legends. PE-conjugated αCD107a (αLAMP1) antibody (Table 2.7) was added for the duration of the assay in all wells except staining

controls. Unstimulated control wells were included for all conditions, which contained CTL and target cells without stimulus or no target cells at all.

Following incubation at 37 °C for 3 h, or the time periods indicated in figure legends, samples were pelleted at 4 °C and washed in ice-cold DPBS. Next, samples were incubated with fluorescently-conjugated antibodies to stain CD8 and Thy1 cell surface molecules (Table 2.7) and with live/dead markers (Table 2.6) for 30 min on ice. After washing in FACS buffer, samples were resuspended in FACS buffer before acquisition on Accuri C6 (BD biosciences), Attune NxT (Thermo Fisher Scientific) or LSRFortessa (BD biosciences) flow cytometers. Results were analysed with FlowJo version 10.4.2 (FlowJo, LLC). The gating strategy used for analysis with FlowJo is explained in Figure 3.1. A forward scatter (FSC) and side scatter (SSC) gate was used to separate the cell population from debris, a second gate was used to isolate single cells from doublets, a third gate was used to isolate live cells from dead cells, a fourth gate was used to isolate CD8 cells and a final gate was used to isolate LAMP1-PE positive cells. Gates were set according to fluorescence minus one controls and experimental controls (unstimulated CTL).

2.5.2 Combined degranulation and killing assay

For the combined degranulation and killing assay, it was important to use stained target cells in order to be able to clearly distinguish CTL and targets during analysis steps. Initially, CFSE-stained target cells were used in this assay. Later on, targets that were permanently stained blue, as described under section 2.1.4, were used instead. For CFSE staining, target cells were incubated with 0.25 μM CFSE (Thermo Fisher Scientific, #C34570) for 10 min at RT in the dark. Following addition of FBS-containing media to quench the dye for 5 min at RT in the dark cells were washed twice before addition to T cells.

CTL and targets with stimulus were co-cultured at an E:T of 2.5:1 in 96 well round bottom plates, with 125,000 CTL and 50,000 target cells seeded per well. Controls that contained CTL and targets without stimulus, CTL on their own or targets on their own were included in every experiment. After incubation at 37 °C for 3 h, samples were pelleted and washed in ice-cold DPBS. Cells were stained with fluorescently-conjugated αCD8 antibodies (Table 2.7) and a live dead stain (Table 2.6) for 30 min at 4 °C. After washing with FACS buffer, and just before analysis on the Attune NxT flow cytometer (Thermo Fisher Scientific), 10 μl 123count ebeads (Invitrogen, #01-1234-42) were added per well. The contents of each well were mixed thoroughly with a mutlichannel pipette.

The analysis process for the beads killing assay readout is explained in detail in Figure 3.11 . Gates were set around the beads population and the stained target cell population in FlowJo version 10.4.2 (FlowJo, LLC). This enabled to count the number of target cells and beads in each well, and allowed to derive a targets:beads ratio. This ratio was multiplied by the number of beads known to be present in the 10 μl added to each sample (information supplied by the manufacturer) in order to determine the total number of target cells remaining in each well. The number of target cells in wells containing CTL + targets + stimulus, was compared to the number of target cells in wells containing CTL + targets - stimulus. This allowed to calculate the percentage of live and dead target cells in each stimulus-containing well. The degranulation assay readout was analysed as explained in section 2.5.1, with the exception that beads were gated out in the first gate before FSC/SSC and subsequent gating.

2.5.3 Incucyte killing assay

CTLs were co-cultured with targets stably expressing a red nuclear marker (Essen Bioscience, #4625, see section 2.10) in ultralow attachment round-bottomed microplates (VWR, #7007). Cells were mixed at E:T 10:1 or E:T 2.5:1, as indicated in the figure legends. 2000-4000 targets with and without stimulus were plated per well and pelleted at 200xg for 2 min. The appropriate number of CTL, to give the desired E:T, were carefully added on top in order to leave the target cell pellet intact. Plates were briefly centrifuged at 200xg for 1 min, then transferred to an Incucyte S3 live cell analysis system (Essen Bioscience) maintained at 37 °C and 5% CO_2. Samples were imaged once an hour using the 4x objective. Target cell death was detected as loss of red fluorescence and was quantitated using the spheroid quantification tool (Incucyte S3 software). Results were exported and the percentage of target cell lysis over time in each well was calculated relative to the first time point measured.

2.5.4 LDH release killing assay

The CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, #G1780) was used to quantitate the enzyme lactate dehydrogenase (LDH), which is released from the cytoplasm of cells upon cell death. The released LDH participates in an enzymatic reaction where a tetrazolium salt is converted into a red formazan product. The amount of cell death is proportional to the amount of red product formed.

The LDH killing assay was performed in a 96 well round bottom plate in killing assay media (RPMI - phenol red + 2%FBS + 100 U/ml pen, 0.1 mg/ml Strep). 100,000 CTLs were seeded per well in one row and serially diluted two fold down the plate. Next, drug

19/parthenolide (Medchem express, #HY-N0141), or equivalent amount of DMSO vehicle control, was added at a final concentration of 8.3 μM. Subsequently, 10,000 targets with stimulus or without stimulus (controls) were added per well, resulting in different E:T ratios ranging from 10:1 to 1.25:1. Samples were mixed by pipetting, centrifuged at 200xg for 1 min, then incubated for 3 h at 37 °C. Lysis buffer (Promega, #G1780) was added to positive control wells to lyse all cells 45 min before the end of the incubation period. After 3 h, samples were pelleted at 200 xg for 2 min and 50 μl supernatant was transferred into a 96 well flat bottom plate and mixed with 50 μl substrate mix (Promega, #G1780). After incubation for 30 min in the dark, absorbance at 490 nm was measured using a spectramax plate reader (Molecular devices) and Softmax software (Molecular devices). The cell death readout was determined by subtracting the absorbance of unstimulated (CTL + target) wells from their stimulated (CTL + targets + stimulus) equivalent, and dividing this number by the absorbance recorded in positive control wells containing lysis buffer. The percentage lysis was calculated by multiplying the resulting number by 100.

2.6 RNA preparation and sequencing

10 spleens were collected from 16 week old WT mice (4 males, 6 females) on two different spleen collection dates. Splenocytes were isolated as described under section 2.1.2. The mouse CD8a T cell isolation kit (Miltenyi Biotec, #130-104-075) was used to purify CD8 cells. 6x10^6 purified CD8 cells were pelleted, washed 2x in ice-cold DPBS and frozen at -80 °C (day 0 samples). An aliquot of each sample was used to determine the CD8 and CD4 expression profile by flow cytometric analysis. The remainder of the cells were transferred onto plates coated with 0.5 µg/ml αCD3ε (clone Bio500A2, eBioscience, #16-0033-86) and 1 µg/ml αCD28 (clone 37.51, eBioscience, #16-0281-86) and cultured as described under section 2.1.2. On day 7, the CD8 purity of each sample was again confirmed by flow cytometry. 6x10^6 cells per sample were pelleted, washed 2x in ice-cold DPBS and frozen at -80 °C (day 7 samples). This resulted in 10 biological replicates for both day 0 and day 7 time points.

RNA was extracted from the cell pellets following the manufacturer's protocol of the RNeasy mini kit (Qiagen, #74104). The concentration and quality of the extracted RNA were determined using a Qubit fluorometer (Thermo Fisher Scientific) and a bioanalyser (Agilent), respectively. A total of 1 μg of RNA per sample was submitted to the Illumina bespoke team at the Wellcome Trust Sanger Institute. The Illumina bespoke team prepared stranded 75

base pairs (bp) paired-end barcoded libraries with oligodT pulldown. For sequencing, all libraries were pooled across 3 different lanes on an llumina Hiseq platform.

2.7 RNA-seq analysis

The following bioinformatic analyses were performed with Martin Del Castillo Velasco-Herrera, following a published workflow (Anders et al., 2013): read alignment, fragment counting, quality control and differential expression analysis of day 7 vs day 0. Reads were aligned to the mouse reference genome (GRCm38) using STAR, a splice-aware mapper (Dobin et al., 2013), guided by the ENSEMBL mouse annotation v84. Htseq count (Anders et al., 2015) was used to count the number of total mapped and uniquely mapped reads. From this, the percent of uniquely mapped reads was determined. The raw counts were normalised by calculating the fragments (read pairs) per kilobase per million (FPKM) reads mapped. Quality control examinations were performed following the DESeq analysis vignette. This included unsupervised hierarchical clustering of samples and principal component analysis (PCA).

The DESeq2 Bioconductor package (Anders and Huber, 2010) was used to identify differentially expressed genes between day 7 and day 0 groups and between males and females within the day 7 group. The spleen collection date was included as a covariate in the analysis. P-values were corrected for multiple testing using the Benjamini-Hochberg method, giving an adjusted p-value (padj). Genes were considered differentially expressed with a padj <0.01 and a log2(fold change) <-2 or >2.

2.8 Toxicity testing and screening of the NF-κB compound library

The NF-κB signalling compound library was obtained from MedChem Express (#HY-L014). Compound toxicity was tested over a range of concentrations in hCTL. First, compounds were added to 96 well plates at 50 μM in duplicates or triplicates and serial threefold dilutions were performed down the plate. The appropriate amount of DMSO (Sigma, #D2650) vehicle control was included on every plate. An equal volume of media containing 125,000 hCTL was added to every well, which halved the concentration of drug. The final concentration range was between 25 μM and 0.1 μM. hCTLs were left to incubate with drugs or DMSO overnight.

The following day the Celltitre96 AQueous One Solution Cell Proliferation Assay (Promega, #G3580) was used to determine drug toxicity after overnight treatment. This kit contains an electron coupling reagent (phenazine ethosulfate, PES) and a tetrazolium compound, which is reduced into a coloured formazan product in metabolically active cells, meaning the quantity of coloured product detected at 490 nm is proportional to the number of live cells. 20 µl reagent were added per well and mixed thoroughly. The plates were incubated for 4 h at 37 °C in the dark, then absorbance at 490 nm was recorded using a spectramax plate reader (Molecular devices) and Softmax software (Molecular devices). hCTL were treated with compounds for 24 h in total. Concentration response curves were plotted using Prism software (Graphpad). For every drug, the highest concentration that did not have toxic effects on hCTLs over a 24 h period was chosen for screening using the combined degranulation and killing assay (section 2.5.2). Assays were performed in hCTL at day 13-15 post in vitro stimulation. Treatments with compounds was performed overnight. In every assay, target cells alone were treated with the compounds in order to test for any effects of the drugs on target cells.

For follow up of drug 19/parthenolide (Medchem express, #HY-N0141), hCTLs were treated with 2.075 - 8.3 µM drug 19/parthenolide for time periods indicated in figure legends followed by analysis using the Incucyte killing assay (section 2.5.3). hCTLs treated with 8.3 µM drug 19/parthenolide were also analysed using the LDH release killing assay (section 2.5.4) and the TNF-expression assay (section 2.4.2).

Additionally, the effect of 8.3 µM drug 19/parthenolide on LAMP1-exposure triggered by treating hCTLs with 4 µM phorbol 12-myristate 13-acetate (PMA) (Sigma, #P8139) and 1 µg/ml Ionomycin (Sigma, #IP657) was tested. PMA and Ionomycin were both prepared in DMSO and controls containing the appropriate amount of DMSO were included in every experiment. After treatment for 3 h in the presence of αhuman-LAMP1-PE (Table 2.7), cells were stained with αhuman-CD8-BV711 (Table 2.7) and the live/dead marker Zombie yellow (Table 2.6) for 30 min on ice. After washing in FACS buffer, samples were resuspended in FACS buffer before acquisition on Attune NxT (Thermo Fisher Scientific) or LSRFortessa (BD biosciences) flow cytometers. Results were analysed with FlowJo version 10.4.2 (FlowJo, LLC).

To investigate the effect of drug 19/parthenolide on p65 protein levels, hCTLs were treated with 8.3 µM drug 19/parthenolide, 4 µM PMA and 1 µg/ml Ionomycin or appropriate DMSO control for 3 h followed by WB lysate preparation (section 2.3.1).

2.9 Molecular cloning

The *Cas9* gene was amplified from the lenti Cas9-Blast vector (Addgene, #52962, see chapter 6 Figure 6.3 for plasmid map) using a forward primer containing upstream homology sequences to pHRSIN-mCherry (pHRSIN-mCh) and a XhoI restriction enzyme (RE) site and one of two different reverse primers. Reverse primer 1 contained a downstream BamHI RE site and homology region to pHRSIN-mCh. Reverse primer 2 contained a downstream P2A sequence, as well as the BamHI RE site and homology region to pHRSIN-mCh. The primer sequences are given in Table 2.8. The PCR mastermix used is given in Table 2.9 and the PCR conditions in Table 2.10.

Table 2.8 ***Overview of primers used in this thesis.*** *Primers were acquired from Sigma and resuspended in nuclease free water to give 100 μM stock solutions.*

Purpose	**Primer**	**Sequence 5' to 3'**
Cloning Cas9 into pHRSIN	Cas9-Fwd	GGGGATCTGGAGCTCTCGAGAAATGGACAAGAAGTACAGCATCGGCCTG
	Cas9-mCh-Rev	ATGGTGGCGACCGGTGGATCCCCCTTATCGTCATCGTCTTTGTAATCTTTCTTCTTCTTAG
	Cas9-2A-mCh-Rev	ATGGTGGCGACCGGTGGATCCCCCGGTCCAGGATTCTCTTCGACATCTCCGGCTTGTTT CAGCAGAGAGAAGTTTGTTGCCTTATCGTCATCGTCTTTGTAATCTTTCTTCTTCTTAG
Sequencing Cas9-Blast	Cas9-Blast Fwd	CCAAAGAGGTGCTGGACG
	Cas9-Blast Rev	GCTCTTTCAATGAGGGTGGA
Sequencing pHRSIN	pHRSIN_SFFV_Fwd	TGCTTCTCGCTTCTGTTCG
	pHRSIN_WPRE_Rev	CCACATAGCGTAAAAGGAGC
Sequence Cas9 fully	Cas9_1_Fwd	CTTCGGCAACATCGTGGA
	Cas9_1_Rev	GGTTCTTCTTGATGCTGTGC
	Cas9_2_Fwd	ACGACCTGGACAACCTGCT
	Cas9_2_Rev	CCGTCTGCTCTTGCTCAGTC
	Cas9_3_Fwd	GAAGATTTTTACCCATTCCTGAAG
	Cas9_3_Rev	TTGCTCTGGTCGAAGAAAATC
	Cas9_4_Fwd	AAATCTCCGGCGTGGAAG
	Cas9_4_Rev	CGCTCGATGAAGCTCTGG
	Cas9_5_Fwd	AGCCCCGCCATTAAGAAG
	Cas9_5_Rev	GCTGCTTCATCACTTTGTCGT
	Cas9_6_Fwd	GAGGTCGTGAAGAAGATGAAGAA
	Cas9_6_Rev	CGGGGTGTTCTTTCAGGAT
	Cas9_7_Fwd	GCAAGGCTACCGCCAAGT
	Cas9_7_Rev	TCAGCTTGTCATTCTCGTCGT
	Cas9_8_Fwd	GGCTACAAAGAAGTGAAAAAGGA
	Cas9_8_Rev	GTCTGCACCTCGGTCTTTTTC
	Cas9_9_Fwd	GTACACCAGCACCAAAGAGGT
	Cas9_9_Rev	AGCTGTTTCTGCTCATTATCCTC
	Cas9_10_Fwd	AAGGCTGGACAGGCTAAGAA
	Cas9_10_Rev	CGACATCTCCGGCTTGTTT

Table 2.9 ***PCR mastermix composition for Cas9 amplification from the Cas9-Blast plasmid.***

Reagents	**μl/reaction**
10X Pfx reaction mix	5 μl
10 μM Fwd primer	1.5 μl
10 μM Rev primer	1.5 μl
10 ng/μl template DNA	1 μl
Accuprime Pfx DNA polymerase	0.4 μl
$MgSO_4$	1 μl
Nuclease free water	39.6 μl

Agarose gels were prepared by adding the desired amount of agarose (Sigma, #A9539) to Tris-acetate-EDTA (TAE) buffer (CIMR media kitchen). This mixture was heated in the microwave to mix and allowed to cool before addition of gel red (Biotium, #41003, final dilution 1:10,000). PCR products were mixed with Orange loading dye (Thermo Fisher Scientific, #SM1173) and run alongside O'gene ruler DNA ladder mix (Thermo Fisher Scientific, #SM1173) on a 0.7% (w/v) agarose gel in 1x TAE buffer (CIMR media kitchen). 5 μg of pHRSIN-mCh plasmid was digested with 1x fast digest green buffer (Thermo Fisher Scientific, #B72), 1 μl alkaline phosphatase (Sigma, #11097075001), 1 μl XhoI (Thermo Fisher Scientific, #FD0694), 1 μl BamHI (Thermo Fisher Scientific, #FD0055) and topped up to 50 μl with nuclease free H_2O. The digest was performed at 37 °C overnight and products were separated on a 0.7% agarose gel. The expected size of the PCR products were 4220 bp for the Cas9 construct and 4278 bp for the Cas9+P2A construct. The expected size of pHRSIN digested with XhoI and BamHI was 9665 bp.

Table 2.10 ***PCR conditions for amplification of Cas9 from the Cas9-Blast plasmid.*** *Cas9 was amplified from the Cas9-Blast plasmid and XhoI and BamHI restriction enzyme sites as well as homology regions to the pHRSIN plasmid were added.*

	Step	Temperature and time
Cycle 5 times	denaturation	95 °C for 15 sec
	annealing	59 °C for 30 sec
	elongation	68 °C for 4 min 20 sec
Cycle 35 times	denaturation	95 °C for 15 sec
	annealing and elongation	68 °C for 4 min 20 sec

Both PCR products and the digested plasmid were extracted from gels using the QIAquick gel extraction kit (Qiagen, #28704). 100 ng recipient vector and 2x excess insert were mixed with the Gibson Assembly mastermix (New England Biolabs, #E2611S) and topped up with nuclease free H_2O to 20 μl per reaction. This mix was kept for 1 h at 50 °C on a heatblock, then transferred to ice for 3 min before addition to NEB 10-beta *E.coli* (New England Biolabs, #C3019H).

2 μl of the gibson product were added to NEB 10-beta *E.coli*, mixed by flicking 5 times and allowed to recover for 30 min on ice. Subsequently, *E.coli* were heatshocked for 30 sec at 42 °C, then placed on ice for 5 min before addition of 950 μl super optimal broth with catabolite repression (SOC) media. After rotating at 250 rpm for 60 min at 37 °C, 100-150 μl of bacteria were streaked out onto Luria-Bertani (LB) agar plates containing 100 µg/ml ampicillin (CIMR media kitchen), and left to grow at 37 °C overnight. 8 individual colonies were picked per plate (plate 1 = XhoI-Cas9-BamHI plasmid, plate 2 = XhoI-Cas9-2A-BamHI plasmid) and expanded while shaking at 230 rpm and 37 °C for two days in 4 ml ampicillin containing media (LB media + 100 µg/ml ampicilin). Plasmids were purified using the QIAprep Spin Miniprep kit (Qiagen, #27106).

A restriction digest was performed with 1x fast digest green buffer (Thermo Fisher Scientific, #B72), 1 μl XhoI (Thermo Fisher Scientific, #FD0694), 1 μl BamHI (Thermo Fisher Scientific, #FD0055) and 5 μl of DNA from the miniprep, topped up to 25 μl with nuclease free H_2O. Resulting products were separated on a 0.7% (w/v) agarose gel for size confirmation. This allowed isolation of colonies that contained intact XhoI and BamHI RE sites and an insert corresponding to the *Cas9* gene in size. Sequencing by Source BioScience confirmed that *Cas9* was correctly inserted. These cultures were expanded to 250 ml, and plasmids were purfied using Qiafilter plasmid maxiprep kit (Qiagen, #12263). DNA concentration and purity was determined using a DS-11 spectrophotometer (DeNovix). Primers were designed roughly every 400 bp along the *Cas9* gene to be able to sequence it fully (Table 2.8) and the correct sequence was confirmed by sanger sequencing carried out by Source BioScience. Plasmid maps of the resulting pHRSIN-Cas9-mCh and pHRSIN-Cas9-2A-mCh were prepared using snapgene (GSL Biotech).

2.10 Nuclight red lentiviral transduction

The nuclight red lentivirus (Essen bioscience, #4625, #lot611110) encodes a nuclear-restricted red fluorescent protein (mKate2) and a puromycin resistance gene. P815 and

blue EL4s were transduced with nuclight red lentivirus at a low multiplicity of infection (MOI) of 0.07 in the presence of 6 µg/ml protamine sulfate. Cells expressing mKate2 were isolated by FACS, as explained in section 2.4.3, and subsequently cells were cultured in the presence of 1 µg/ml puromycin (see section 2.1.4).

2.11 pHRSIN lentiviral production and transduction

The following conditions were used to produce lentivirus with pHRSIN-GFP (for plasmid map see Appendix A, Figure A.1), pHRSIN-mCh (for plasmid map see chapter 6, Figure 6.3), pHRSIN-Cas9-mCh (for plasmid map see chapter 6, Figure 6.4) and pHRSIN-Cas9-2A-mCh (for plasmid map see chapter 6, Figure 6.4) plasmids. The pHRSIN-GFP plasmid was a gift from Adrian Thrasher (Institute of Child Health). The pHRSIN-mCh plasmid (James and Vale, 2012) was a gift from John James (MRC Laboratory for Molecular Biology). pCMVδ8.91 (packaging plasmid, for plasmid map see Appendix A, Figure A.2) and pMD-G (encoding the viral envelope protein VSV-G, for plasmid map see Appendix A, Figure A.3) plasmids were a gift from Paul Lehner's lab (Cambridge Institute for Medical Research).

HEK293Ts were seeded in T75 flasks so they reached 70% confluency overnight. The next day, the following 2 tubes were prepared for each T75: Tube 1 contained 1.5 ml Opti-MEM (Gibco, #31985-047), 30 μl 1M HEPES (Gibco, #15630-080) and 42 μl TransIT 293 reagent (Mirus, #MIR2700). Reagents were mixed and incubated at RT for 5 min. Tube 2 contained 4 μg pCMVδ8.91, 4 μg pMD-G and 6 μg of the desired pHRSIN plasmid. The contents of tube 1 were added dropwise to tube 2 and mixed gently by bubbling air through a pipette boy held under the liquid for 1 min. After incubation for 15 min at RT, 12 ml pre-warmed HEK media were added slowly. This mix was added dropwise to HEK293Ts and cells were transferred to a 37 °C incubator. Viral supernatant was collected after 48 h and 72 h. The supernatant was centrifuged at 500xg for 10 min and filtered through a 0.45 μM pvdf filter to remove cell debris. Next, the viral supernatant was concentrated by incubation with lenti-X (Takara Clontech, #631232) for 1 h at 4 °C at a lenti-X-to-viral supernatant ratio of 1-to-3 . After centrifugation at 1500xg for 45 min at 4 °C, the viral pellet from one T75 was resuspended in 100 μl ice-cold hTCM. This effectively resulted in a 100-120X more concentrated lentivirus. Concentrated lentivirus was aliquoted and stored at -80 °C.

The following conditions were used to transduce cell lines with pHRSIN-Cas9 lentiviruses: For transduction of P815s, 2x10^6 P815s were resuspended in target cell media containing 6 µg/ml protamine sulfate (Sigma, #P4020) and 100 μl concentrated pHRSIN-Cas9-2A-mCh

or pHRSIN-Cas9-mCh lentivirus. For transduction of YTs, 1x10^6 cells were resuspended in YT media containing 6 µg/ml protamine sulfate and 55 μl concentrated pHRSIN-Cas9-2A-mCh lentivirus. For transduction of Jurkats, 1x10^6 cells were resuspended in Jurkat media containing 6 µg/ml protamine sulfate and 45 μl concentrated pHRSIN-Cas9-2A-mCh lentivirus.

For transduction of primary human T cells with pHRSIN-GFP or pHRSIN-mCh lentiviruses, 1x10^6 T cells were resuspended in 300 μl hTCM containing lentivirus and 6 µg/ml protamine sulfate or lentiboost (1 in 100 dilution, meaning 8 µg/ml reagent A and 1 mg/ml reagent B, Sirion Biotech, #SB-P-LV-101-01), as indicated in figure legends. Cells were incubated in this small volume in one well of a 24 well plate for 4 h at 37 °C. After 4 h, wells were topped up to 2 ml with hTCM containing 1x10^6 irradiated allogeneic PBMCs and 1 μg/ml PHA.

Before transduction of primary human T cells with pHRSIN-Cas9-2A-mCh lentivirus the viral titre was determined by transducing Jurkat cells. 0.2x10^6 Jurkats were infected with varying volumes of concentrated lentivirus (between 50 μl and 0.05 μl) in a total volume of 2.5 ml Jurkat media containing 6 µg/ml protamine sulfate. The lentiviral titre was determined as outlined in Figure 6.7. The number of transduced cells was multiplied by the percentage of fluorescent cells and the dilution factor. The resulting number was divided by the transduction volume to give the number of transducing units (TU) per ml. For transductions, primary human T cells were activated on plates coated with 1 µg/ml αCD3ε (clone OKT3, eBioscience, #14-0037-82) and 2 µg/ml αCD28 (clone CD28.2, BD Biosciences, #555726). The next day, T cells were transduced with pHRSIN-Cas9-2A-mCh lentivirus at an MOI of 1.17 in the presence of 6 µg/ml protamine sulfate or lentiboost (1 in 100 dilution, Sirion Biotech, #SB-P-LV-101-01), as an alternative transduction enhancer.

2.12 Cas9-Blast lentiviral production and transduction

The virapower packaging mix (Thermo Fisher Scientific, #K497500) was used for lentiviral production with the lenti Cas9-Blast plasmid (Addgene, #52962), a gift from Professor Feng Zhang (Massachusetts Institute of Technology). HEK293T were seeded in T75 flasks so they reached 70% confluency overnight. The next day, the following 2 tubes were prepared for each T75: Tube 1 contained 1.5 ml Opti-MEM, 9 μg virapower, 3 μg lenti Cas9-Blast and 12 μl plus reagent (Thermo Fisher Scientific, #15338100). Tube 2 contained 1.5 ml Opti-MEM containing 36 μl lipofectamine LTX (Thermo Fisher Scientific, #15338100). Both tubes were incubated at RT for 5 min, then mixed together and incubated for a further 30 min at

RT. HEK293T media was removed and replaced with 5 ml Opti-MEM. Subsequently, the DNA-lipofectamine mix was added to the cells and left overnight. The following day, the cell culture supernatant was removed and replaced with HEK media. Viral supernatant was collected after 48 h and 72 h and concentrated using ultracentrifugation (SW-28 swinging-bucket rotor, Beckmann) at 25,000 rpm for 90 min at 4 °C. The resulting viral pellets were resuspended in 200 μl ice-cold DPBS + 1% FBS per flask, aliquoted and stored at −80 °C.

For transduction of primary T cells, 3x10^6 cells were resuspended in concentrated lentivirus from one T75 containing 6 µg/ml protamine sulfate. Samples were centrifuged at 754xg for 15 min at 32 °C. 1x10^6 cells were aliquoted per well in a 24 well plate and topped up to 2 ml with hTCM containing 1x10^6 irradiated allogeneic PBMC and 1 μg/ml PHA. Selection with 5 µg/ml Blasticidin was started on day 6 post transduction.

2.13 PCR for Cas9-Blast

DNA was extracted from frozen pellets of HEK293T cells transduced with Cas9-Blast lentivirus (10,000 cells) or treated with protamine sulfate alone (0.25x10^6 cells) and from frozen pellets of hCTL transduced with Cas9-Blast lentivirus (0.45x10^6 cells) or treated with protamine sulfate alone (1.3x10^6 cells). Each pellet was resuspended in 200 μl of DNA extraction buffer. 1 ml DNA extraction buffer contained 975 μl 10% (w/v) Chelex 100 chelating resin (Bio-Rad, #1421253) diluted into nuclease-free H_2O, 10 μl 10% (v/v) Tween 20 (Sigma, #P1379) and 15 μl proteinase K (Sigma, #P8044). The resulting samples were incubated at 50 °C for 90 min, followed by heating at 95 °C for 25 min and then cooled to RT.

PCR was used to test for the presence of Cas9-Blast in the extracted DNA. PCR was performed with primers targeting a region between the 3' end of the *Cas9* gene and the blasticidin resistance gene (Table 2.8), producing a product of 274 bp. PCR was performed using the mastermix outlined in Table 2.11 and the conditions outlined in Table 2.12. Negative controls including nuclease-free water instead of DNA were included in the PCR reaction.

Table 2.11 ***PCR mastermix for Cas9-Blast PCR.*** *PCR mastermix for amplifying a stretch of DNA between Cas9 and the Blasticidin resistance gene in the Cas9-Blast plasmid.*

Reagents	**μl/reaction**
10X PCR buffer	2.5 μl
50 nM $MgCl_2$	0.75 μl
10 mM dNTP mix	0.5 μl
Fwd primer	0.5 μl
Rev primer	0.5 μl
Platinum taq DNA polymerase	0.1 μl
Template DNA	1 μl
Nuclease free water	18.75 μl

Table 2.12 ***PCR conditions for Cas9-Blast PCR.*** *PCR conditions to amplify a stretch of DNA between Cas9 and the Blasticidin resistance gene in the Cas9-Blast plasmid.*

	Step	**Temperature and time**
Cycle 35 times	initial denaturation	94 °C for 2 min
	denaturation	94 °C for 30 sec
	annealing	58 °C for 30 sec
	elongation	72 °C for 30 sec

The resulting PCR products were separated on a 1.5% (w/v) agarose gel, prepared as explained in section 2.9. Gel extraction was performed with the QIAquick gel extraction kit (Qiagen, #28704) and DNA concentration was determined using a DS-11 spectrophotometer (DeNovix). Sanger sequencing to confirm the sequence of the PCR product was performed by Source BioSciences.

2.14 Statistical analysis

Graphs were created and statistical analyses were performed using Prism 7 (GraphPad Software). The statistical tests used are stated in every figure legend and where relevant in the text. Statistical analysis was only performed on data obtained from three or more independent experiments. Paired t-tests were applied for statistical analysis of degranulation and killing assay results. Samples were paired according to the days that the experiments

were performed, in order to account for any day-to-day variations. Statistical analysis of WB results was performed using one sample t-tests. For all other comparisons, unpaired t-tests with Welch's correction, which does not assume equal standard deviations (SD), were performed. For all statistical analyses, differences between samples were considered statistically significant if $p<0.05$.