Cellular and molecular events controlling acquisition of cytotoxic activity by melanoma-reactive CD4^{+} T cells *in vivo*

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I, Katharina Franziska Bergerhoff, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

While there is an abundance of studies on cytotoxic $CD8^+$ T cells in cancer immunotherapy, $CD4^+$ T cells with cytotoxic potential are receiving increasing attention from the scientific community. Previously, our lab has underscored the significance of tumour reactive $CD4^+$ T cells which acquire cytotoxic activity during immunotherapy of malignant melanoma. This study aims to analyse and characterise the molecular and cellular mechanisms underlying the function of these cytotoxic $CD4^+$ T cells. On protein and transcript level, cytotoxic tumour infiltrating $CD4^+$Trp1 T cells exhibited a highly plastic phenotype: Th1 and Th2 specific transcription factors Gata3 and T-bet were co-expressed and inflammatory cytokines IFNγ, TNF-α and IL-2 were secreted. Additionally, $CD8^+$ lineage specific transcription factor Runx3 expression was elevated and correlated highly with GzmB expression. However, and in contrast to classical $CD8^+$ CTLs, cytotoxic $CD4^+$Trp1 T cells lacked expression of $CD8^+$ transcription factor Eomes. In depth microarray analysis via Canonical Correspondence Analysis (CCAM) revealed a high correlation of tumour infiltrating $CD4^+$Trp1 cells with a full effector $CD8^+$ T cell gene signature rather than a $CD4^+$ or $CD8^+$ memory phenotype. The strong correspondence with differentiated $CD8^+$ effector T cells prompted the investigation of the role of mTOR signalling in $CD4^+$ cytotoxicity as mTOR activity is crucial for $CD8^+$ effector differentiation. Inhibition of mTORC1 activity by administration of rapamycin and genetic engineering of $CD4^+$Trp1 cells was evaluated. Disruption of mTORC1 signalling counteracted the acquisition and/or maintenance of a cytotoxic phenotype whilst preserving the capacity to produce inflammatory cytokines. This study illustrates the complexity of this highly plastic, cytotoxic $CD4^+$ T cell subset and highlights the importance of mTORC1 signalling for the cytotoxic activity of tumour specific $CD4^+$Trp1 T cells.

This thesis is dedicated to my family and especially my mother.

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1 List of abbreviations and genes

4EBP1	4E binding protein 1
5'TOP	5' terminal oligopyrimide
αCD3/4/…	anti-CD3/4/… antibody
ACT	adoptive cellular transfer
AHR	aryl hydrocarbon receptor
Ahr	gene encoding AHR
ANOVA	analysis of variance
AP-1	activator protein 1
APC	antigen presenting cell
APC (flow cytometry)	allophycocyanin
Areg	gene encoding Amphiregulin
Bach2	BTB and CNC homolog 2
Bach2	gene encoding Bach
BATF	B-cell activating transcription factor
Batf	gene encoding BATF
BCL-6	B cell leukemia/lymphoma 6
Bcl6	gene encoding BCL-6
Bhlhe41	gene encoding Dec2
Blimp-1	B lymphocyte induced maturation protein 1
BTLA	B- and T-lymphocyte attenuator
Btla	gene encoding BTLA
c-Maf	avian musculoaponeurotic fibrosarcoma protooncogene
CAR	chimeric antigen receptor
CCA	Canonical correspondence Analysis
CCAM	Canonical Correspondence Analysis of Microarray data
CCL17/20/…	chemokine (C-C motif) ligand 17/20/…
Ccl17/20/…	gene encoding CCL17/20/…
CCR3/4/…	C-C chemokine receptor type 3/4/…
Ccr3/4/…	gene encoding CCR3/4/…
$CD4^+$Trp1	$CD4^+$ T cell isolated from Trp1 mice
CFSE	carboxyfluorescein succinimidyl ester
ChIP	chromatin immunoprecipitation
CMV	cytomegalovirus
Csf2	gene encoding GM-CSF

CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte associated antigen 4
CXCR4/5/…	C-X-C chemokine receptor type 4/5/…
Cxcr4/*5*/…	gene encoding CXCR4/5/…
Cy	cyanine
DC	dendritc cell
DOX	doxycycline
DT	diphtheria toxin
Elf4	gene encoding Mef
eIF-4E	eukaryotic initiation factor 4 E
eIF4F	eukaryotic translation initiation factor 4 F protein complex
Entpd1	gene encoding CD39
ERM	Ets-related molecule
Eomes	Eomesodermin
Eomes	gene encoding Eomes
Epas1	gene encoding Hif-2α
Ermn	gene encoding ERM
FACS	fluorescence-activated cell sorting
FasL	Fas Ligand
Fasl	gene encoding FasL
FcR	Fc receptor
FITC	fluorescein isothiocyanate
FKBP12	12-kDa FK506-binding protein
Flt3L	Fms-like tyrosine kinase 3 ligand
FMD-2A	foot-and-mouth disease 2A sequence
Foxp3	forkhead box P3
Foxp3	gene encoding Foxp3
GADPH	glyceraldehyde 3-phosphate dehydrogenase
Gata3	GATA Binding Protein 3
Gata3	gene encoding Gata3
GEO	Gene Expression Omnibus
Gfi1	growth factor independent 1
Gfi1	gene encoding Gfi1
GFP	green fluorescent protein
GITR	glucocorticoid-induced tumor necrosis factor receptor
Glut1	glucose transporter 1

Glut3	glucose transporter 3
GM-CSF	granulocyte-macrophage colony-stimulating factor
GVAX	GM-CSF secreting B16/BL6 cell-based vaccine
Gy	Gray (unit)
GzmA/B/…	Granzyme A/B/…
Gzma/b/…	gene encoding GzmA/B/…
Havcr1	gene encoding TIM1
Havcr2	gene encoding TIM3
Hif-1α/2α	hypoxia inducible factor 1α/2α
HIV	human immunodeficiency virus
HLX	H2.0-Like Homeobox
Hlx	gene encoding HLX
hPGK	human phosphoglycerate kinase
ICOS	inducible T-cell co-stimulator, also: CD278
Icos	gene encoding ICOS
IELs	intraepithelial lymphocytes
IFNγ	interferon γ
Ifng	gene encoding IFNγ
IFNγ R2	IFNγ receptor 2
Ifngr2	gene encoding IFNγ R2
IL-2/3/…	interleukin 2/3/…
Il2/3/…	gene encoding IL-2/3/…
Il2ra	gene encoding CD25 (IL-2 receptor α)
Il2rb	gene encoding CD122
IL-27 R-α/WSX-1	IL-27 receptor α
Il27ra	IL-27 R-α/WSX-1
Il1rl1	gene encoding ST2 (Interleukin 1 receptor like 1)
i.d.	intradermal
i.p.	intraperitoneal
IRES	internal ribosome entry site
IRF4	Interferon regulatory factor 4
Irf4	gene encoding IRF4
Itgae	gene encoding CD103
Itgal	gene encoding LFA-1 (CD11a)
Itgam	gene encoding CD11b
Itgax	gene encoding CD11c

i.v.	intravenous
JunB	Jun B proto-oncogene
Junb	gene encoding JunB
KLF2	Krüppel like factor 2
Klrb1c	gene encoding CD161
KLRG1	killer cell lectin-like receptor G1
Klrg1	gene encoding KLRG1
LFA-1	leukocyte function-associated molecule 1 (CD11a)
LN	lymph nodes
Lta	gene encoding TNF-β
LTR	long terminal repeat
LUC	firefly luciferase
mAb	monoclonal antibody
Maf	gene encoding c-MAF
mCAT	murine cationic amino acid transporter
MEF	myeloid Elf-1-like factor
MHC I/II	major histocompatibility complex class I/II
Mki67	gene encoding Ki67
mLST8	mammalian lethal with SEC13 protein 8
MoMLV	moloney murine leukemia virus
Ms4a4b	gene encoding Chandra
mTOR	mammalian target of rapamycin
mTORC1/2	mTOR complex 1/2
NK	natural killer cell
ns	not significant
Ova / $Ova_{257-264}$	SIINFEKL peptide derived from ovalbumin
P2A	picornavirus 2A sequence
p.s.	per sample
PBS	phosphate buffered saline
PCA	Principal Component Analysis
PD-1	programmed death receptor 1
Pdcd1	gene encoding PD-1
PD-L1	programmed death ligand 1
PE	phycoerythrin
PFA	paraformaldehyde
Ph-Eco	Phoenix Eco cell line

PHD	prolyl-4-hydroxylase
PRAS40	proline-rich Akt substrate of 40 kDa
Pras40	gene encoding PRAS40
Prf1	gene encoding Perforin
Prdm1	gene encoding Blimp-1
pS6	phospho-S6 ribosomal protein (phosphorylation at $Ser_{235/236}$)
pVHL	von Hippel-Lindau protein
Q8	truncated human CD34 (QBend10 epitope) linked to a CD8α stalk
RAG1	recombination activating gene 1
RAPA	rapamycin
Raptor	regulatory-associated protein of mTOR
Rheb	Ras homolog enriched in brain
RORα	retinoid-acid receptor-related orphan receptor α
Rora	gene encoding RORα
Rorc	gene encoding RORγt
RORγt	retinoid-acid receptor-related orphan receptor gamma t
RT	radiation therapy
rtA2-M2	optimised reverse tetracycline-controlled transactivator
Runx3	Runt-related transcription factor 3
Runx3	gene encoding Runx3
S6K1/2	S6 kinase 1/2
SD	standard deviation
Sell	gene encoding CD62L
SJL	Swiss/Jackson Laboratory, mouse strain
Slc2a1/3	solute carrier family 2, facilitated glucose transporter member 1/3, encodes Glut1/3
Spi1	gene encoding PU.1
SRT	SJL $RAG^{-/-}$ Tan
STAT3/4/…	signal transducer and activator of transcription 3/4/…
Stat3/*4*/…	gene encoding STAT3/4/…
T-bet	T-box expressed in T cells
Tbx21	gene encoding T-bet
TC	tissue culture
TCF-1	T-Cell-specific transcription factor 1

Tcf7	gene encoding TCF-1
TCR	T cell receptor
T_{eff}	$CD4^+Foxp3^-$ effector T cells
TetO	tetracycline operator
Tfh	follicular helper T cell
TGF-β	Transforming growth factor beta
Tgfb1	gene encoding TGF-β
Tfrc	gene encoding CD71
Tg	transgenic
Th1/2/…	type 1/2/… $CD4^+$ helper T cell
ThPok	Th inducing POZ-Krüppel Factor
TIM1/2/…	T-cell immunoglobulin domain and mucin domain 1/2/…
Timd2	gene encoding TIM2
TIL	tumour infiltrating lymphocytes
Tnf	gene encoding TNF-α
TNF-α	tumour necrosis factor α
TNF-β	tumour necrosis factor β
Tnfrsf4	gene encoding OX-40/CD134
Tnfsf10	gene encoding TRAIL
Tnfrsf18	gene encoding GITR
TRAF-3	TNF receptor-associated factor 3
Traf3	gene encoding TRAF-3
TRAIL	TNF related apoptosis inducing ligand
Treg	$CD4^+Foxp3^+$ regulatory T cell
Trp1	tyrosinase-related protein 1 (gp75)
u/ml	units/ml
VSV	Vesicular Stomatitis Virus
VSV-ova	Vesicular Stomatitis Virus encoding Ova
VZV	Varicella-zoster virus
W-PRE	Woodchuck hepatitis post-transcriptional regulatory element
WT	wild type
Zbtb7b	gene encoding ThPok
-/-	knock out

2 Introduction

2.1 Immune cells and specific subset characteristics

Supplying essential stimulatory and inhibitory signals to $CD8^+$ T cells and B cells, $CD4^+$ T cells are considered the orchestrators of the adaptive immune response. $CD8^+$ and $CD4^+$ T cells display distinct differences in function, antigen recognition, effector molecules and transcription factor signature.

The separation of the two T cell lineages takes place in the thymus when $CD4^+CD8^+$ double positive thymocytes mature and start to express one of the transcription factors Runx3 and ThPok. Runt-related transcription factor 3 (Runx3) is the CD8 lineage specific transcription factor and is associated with the repression of ThPok transcription and ablation of CD4 expression; hence, the $CD8^+$ T cell lineage is finalised. For the development of single positive $CD4^+$ T cells, however, Th inducing POZ-Kruppel Factor (ThPok) is expressed and in turn assists to repress Runx3 transcription to stabilise CD4 T cell fate (Kappes et al. 2006; Egawa & Littman 2008; Setoguchi et al. 2008).

Upon antigen encounter and specific stimuli by the microenviroment, the naïve $CD4^+$ T cell compartment divides into several functionally diverse subsets (Fig. 1). The cells of each subset express key transcription factors, cytokines and surface markers which correlate with their specific functionality.

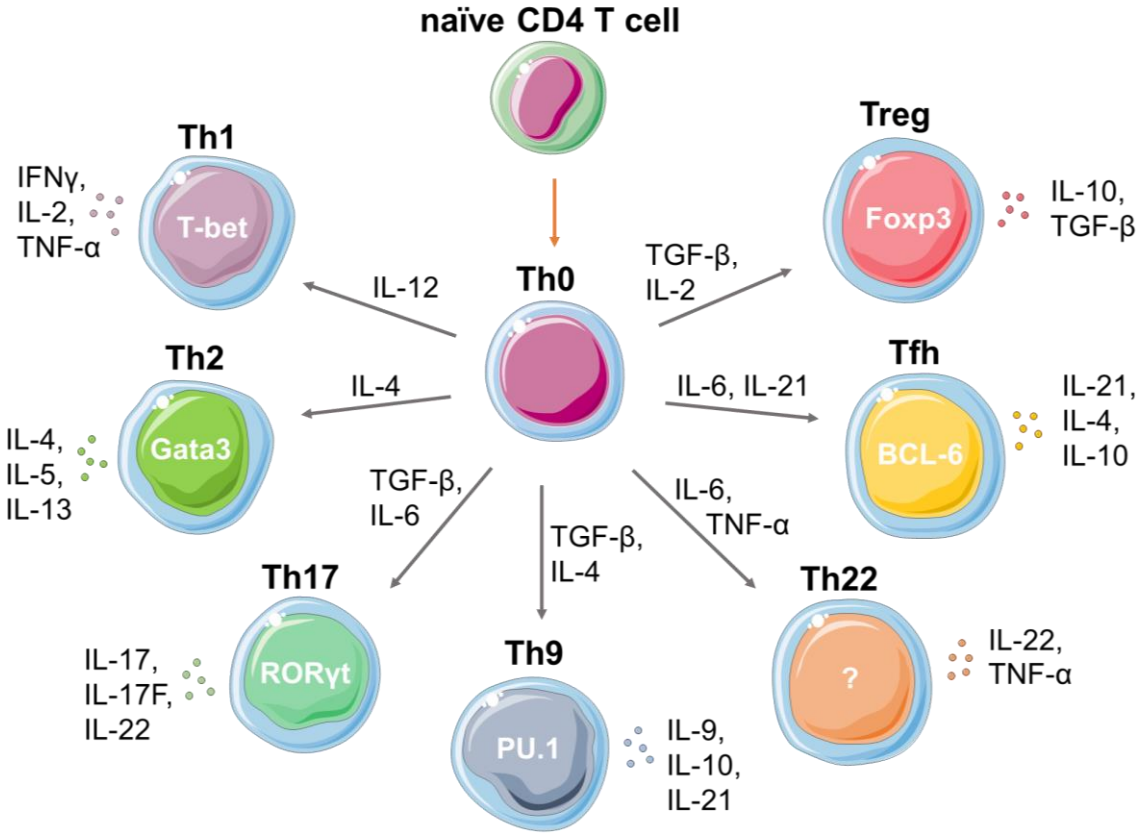

Figure 1 | Schematic of the differentiation of naïve CD4⁺ T cells into different T helper subsets. Shown are transcription factors (white) and key molecules which promote the differentiation of Th1, Th2, Th17, Th9, Th22, follicular T helper (Tfh) and regulatory (Treg) T cells after activation of naïve CD4⁺ T cells into Th0 cells (orange arrow). Further depicted are characteristic cytokines of each subset which correlate with their specific functionality. The figure was generated using Servier Medical Art (Servier 2015).

2.1.1 Type 1 T helper cells (Th1)

Type 1 T helper cells (Th1) are crucial for the adaptive immune responses against intracellular pathogens and viruses (Romagnani 1999). The T box transcription factor T-bet and the cytokine Interleukin 12 (IL-12) present the key regulators of Th1 lineage commitment (Fig. 1) (Szabo et al. 2000; Athie-Morales et al. 2003; Kanhere et al. 2012). The transcription factors HLX, ERM, IRF4 and STAT4 further also play a role in Th1 differentiation and/or maintenance: HLX physically interacts with T-bet and enhances Th1 cytokine expression (Mullen et al. 2002; Zheng et al. 2003; Martins et al. 2005) while ERM is induced by IL-12 signalling during Th1 development (Ouyang et al. 1999). STAT4

and IRF4 were both shown to significantly contribute to Th1 differentiation and STAT4 to additionally play an essential part in Th1 proliferation (Kaplan et al. 1996; Nishikomori et al. 2002; Veldhoen 2010; Yang et al. 2015).

Typical cytokines produced by Th1 cells are IFNγ, IL-2 and TNF-α (Fig. 1) but can also include IL-10, IL-15, IL-3, TNF-β and GM-CSF (Mosmann et al. 1986). Other molecules such as the chemokine receptors CCR5 and CXCR3 (Loetscher et al. 1998) and the co-stimulatory receptor CD225 (DNAM-1) which regulates Th1 expansion and effector functions (Dardalhon et al. 2005), are highly expressed on Th1 T cells. The co-inhibitory molecule TIM-3 is also characteristically expressed on Th1 cells and was shown to be regulated cell intrinsically by T-bet (Zhu et al. 2005; Hastings et al. 2009; Anderson et al. 2010).

Th1 cells further express the CD20 homologue Chandra which is absent on Th2 T cells (Venkataraman et al. 2000; Xu et al. 2006). The second chain of IFNγ receptor (IFNγ R2) is specifically lost in Th1 cells (Bach et al. 1995; Pernis et al. 1995) while IL-12 receptor (IL-12R) and its homologue IL-27 R-α (WSX-1) are highly expressed. IL-12R expression is lost on Th2 cells but persists in Th1 cells post stimulation of the TCR (Szabo et al. 1997; Usui et al. 2006; Becskei & Grusby 2007); IL-27 R-α was found to be crucial for the early stages of Th1 differentiation and induction of cytokine production (Yoshida et al. 2001; Takeda et al. 2003).

2.1.2 Type 2 T helper cells (Th2)

Type 2 helper $CD4^+$ T cells (Th2) play an essential role in allergic diseases such as asthma (Lloyd & Hessel 2010) and in the immune response against extracellular bacteria and helminth parasites (Romagnani 1999; Pearce et al. 2004; Taylor et al. 2008). Th2 immunity is elicited through specific cytokine secretion and often through activation of eosinophils (Spencer & Weller 2010) and IgE producing B cells (Maggi 1998). It has further been suggested that Th2 cells can be recruited to a tumour site and facilitate tumour growth by dysregulated cytokine expression (De Monte et al. 2011).

The transcription factor Gata3 and the cytokine IL-4 are indispensable for Th2 differentiation and Th2 cytokine expression (Fig. 1) (Paul & Seder 1994; Zheng & Flavell 1997; D. H. Zhang et al. 1998; Kishikawa et al. 2001; Kanhere et al. 2012). Also Gfi1, a transcriptional repressor which stabilises Gata3 expression and promotes proliferation (Zhu et al. 2002; Pargmann et al. 2007; Shinnakasu et al. 2008), Dec2 (Liu et al. 2009) and STAT5 and STAT6 are important for Th2 differentiation (Zhu et al. 2003; Sahoo et al. 2011).

Upon activation, the Th2 subset produces the characteristic inflammatory cytokines IL-4, IL-5 (Mosmann et al. 1986; Cherwinski et al. 1987; Romagnani 1991) and IL-13 (Brown et al. 1989; de Waal Malefyt et al. 1995) (Fig. 1) but can further secret IL-3, IL-6 (Zubiaga et al. 1990), IL-9 (Gessner et al. 1993; Schmitt et al. 1994) as well as IL-24 (Sahoo et al. 2011), IL-25 (Fort et al. 2001; Mearns et al. 2014), IL-31 (Dillon et al. 2004; Castellani et al. 2010) and the epidermal growth factor family member Amphiregulin (Zaiss et al. 2006). The transcription factors IRF4 (Rengarajan et al. 2002; Hu et al. 2002; Lohoff et al. 2002), JunB and c-Maf play important roles in the regulation of Th2 cytokine expression, for instance IL-4 and IL-5 (Ho et al. 1996; Kim et al. 1999; Li et al. 1999; Hartenstein et al. 2002; Voice et al. 2004).

Th2 cells express the Toll-like receptor superfamily member ST2 (also called IL1RL1) (Löhning et al. 1998) and a specific set of chemokine receptors: CCR3, CCR4 (Yamamoto et al. 2000), CCR8 (Zingoni et al. 1998; Soler et al. 2006) and CXCR4 (Jagodziński & Trzeciak 2000; Piao et al. 2012). Furthermore, immune checkpoint molecules TIM1, TIM2 and ICOS (inducible T-cell co-stimulator, also: CD278) are highly expressed; TIM1 and ICOS stimulate and enhance Th2 function while TIM2 inhibits Th2 activation (Tesciuba et al. 2001; Kuchroo et al. 2003; Khademi et al. 2004; Meyers et al. 2005; Shilling et al. 2005).

2.1.3 Type 0 T helper cells (Th0)

Activated but non-polarised $CD4^+$ T cells are termed Th0 and displays characteristics of both Th1 and Th2 T cells and produce for instance GM-CSF, IFNγ, IL-2, IL-3, IL-4, IL-5, IL-6, IL-13, TNF-α and –β (Gajewski et al. 1994).

2.1.4 T helper 17 cells (Th17)

Th17 cells play an important role in autoimmunity, especially tissue and organ-specific autoimmune inflammation, and contribute strongly to the immune response against extra- and intracellular pathogens such as fungi and bacteria which evade Th1 and Th2 responses (Korn et al. 2007; Ouyang et al. 2008). The orphan nuclear receptor RORγt is the key transcription factor of this $CD4^+$ T cells subset (Harrington et al. 2005; Ivanov et al. 2006) and the cytokines transforming growth factor β (TGF-β) and IL-6 are required for induction of this T cell subset from naïve $CD4^+$ T cells (Fig. 1) (Veldhoen et al. 2006; Mangan et al. 2006). Also transcription factors RORα, IRF4, AHR, TCF-1, c-Maf, BATF and STAT3 were shown to be important for Th17 differentiation and development

although their expression is not limited to only the Th17 subset (Brüstle et al. 2007; Harris et al. 2007; Mathur et al. 2007; Yang et al. 2007; Quintana et al. 2008; Yang et al. 2008; Bauquet et al. 2009; Schraml et al. 2009; Betz et al. 2010; Muranski et al. 2011).
Th17 cells highly express IL-17, IL-17F and IL-22 (Fig. 1) (Harrington et al. 2005; Ivanov et al. 2006) but also produce increased levels of IL-21, IL-6 and TNF-α (Langrish et al. 2005; Wei et al. 2007). Furthermore, chemokine CCL20 and its respective receptor CCR6 are highly expressed (Hirota et al. 2007; Eyerich et al. 2009), as well as CD161 which is also found on NK and NKT cells (Cosmi et al. 2008; Kleinschek et al. 2009; Ramirez et al. 2010).

2.1.5 T helper 9 cells (Th9)

In 2008, another T helper subset was identified in mouse and human and was termed Th9 cells based on their high expression of IL-9. Th9 cells are further characterised by their production of IL-21 and IL-10 (Veldhoen et al. 2008; Dardalhon et al. 2008; Tan et al. 2010; Végran et al. 2014) and their key transcription factor PU.1 (Fig. 1). Polarisation of $CD4^+$ T cells into Th9 cells requires a combination of T cell activation and the cytokines IL-4 and TGF-β (Fig. 1) (Veldhoen et al. 2008; Dardalhon et al. 2008; Elyaman et al. 2009). PU.1 and also Interferon-regulatory factor 4 (IRF4) are instrumental for the differentiation of Th9 cells but are also found to be involved in Th2 and Th17 cell development (Rengarajan et al. 2002; Jäger et al. 2009; Chang et al. 2010; Staudt et al. 2010). Additionally, transcription factors IRF1 and STAT6 were also shown to be involved in Th9 development and are directly connected to their pro-inflammatory effector function (Goswami et al. 2012; Végran et al. 2014). Th9 cells further express chemokines CCL17 and CCL22 (Chang et al. 2010) and chemokine receptors CCR3, CCR6 and CXCR3 (Kara et al. 2013; Végran et al. 2014).
Th9 cells were shown to promote tissue inflammation and considerably contribute to the pathogenesis of asthma, allergic diseases and autoimmune responses, for instance CNS autoimmune disorders (Veldhoen et al. 2008; Jäger et al. 2009; Staudt et al. 2010; Chang et al. 2010). Recently, Th9 cells were also reported to have anti-cancer efficacy *in vivo* by ameliorating the immune response against tumours via intra-tumoural secretion of IL-9 and IL-21 leading to the activation of $CD8^+$ T cells and potentially DCs, mast cells and natural killer cells (Purwar et al. 2012; Végran et al. 2014; Végran et al. 2015). Interestingly, Purwar and colleagues also reported TCR-specific cytotoxic activity of differentiated Th9 OT-II cells against B16-ova *in vitro* (Purwar et al. 2012).

2.1.6 T helper 22 cells (Th22)

The Th22 subset partially resembles Th1, Th2 and Th17 T cells but emerged in human and mice as a new and distinct T cell subset in epidermal immunity and were found enriched in inflammatory skin disorders (Duhen et al. 2009; Trifari et al. 2009; Eyerich et al. 2009; Basu et al. 2012). Th22 cells produce high levels of IL-22, TNF-α and IL-13 (Fig. 1) but lack the expression of Th1/Th17 cytokines IFNγ, IL-4 and IL-17 and the Th17 specific chemokine CCL20 (Eyerich et al. 2009; Duhen et al. 2009). So far, no single key transcription factor could be identified to be exclusively required for Th22 differentiation but cytokines IL-6 and TNF-α were shown indispensable for Th22 induction (Yan Zheng et al. 2007; Duhen et al. 2009; Basu et al. 2012). Th22 cells only display very low levels of Th1/Th2/Th17 specific transcription factors T-bet, Gata3 and RORγt. However, Th22 cells highly express chemokine receptors CCR10, CCR4 and CCR6 and transcription factor aryl hydrocarbon receptor (AHR), which plays a crucial role in both Th17 and Th22 differentiation and IL-22 production (Duhen et al. 2009; Ramirez et al. 2010).

2.1.7 Follicular T helper cells (Tfh)

Follicular T helper $CD4^+$ T cells (Tfh) are crucial for humoral immunity as this $CD4^+$ subset promotes the antigen-specific B cell immune response: Tfh activity is required for germinal centre formation (Johnston et al. 2009; Nurieva et al. 2009; Yu et al. 2009; Nurieva et al. 2008), B cell differentiation into plasma cells (Bryant et al. 2007; Ozaki et al. 2004; Ettinger et al. 2005) and orchestration of isotype switching and antibody production in B cells (Pene et al. 2004). Tfh lineage specific transcription factor BCL-6 (B cell leukemia/lymphoma 6) and cytokines IL-6 and IL-21 are required for Tfh formation (Fig. 1) (Nurieva et al. 2008; Nurieva et al. 2009; Yu et al. 2009; Eto et al. 2011). Also the transcription factors BATF and c-Maf were shown to be involved in Tfh development and expansion but are not exclusive for this T cell subset (Bauquet et al. 2009; Betz et al. 2010; Ise et al. 2011).

The chemokine receptor CXCR5 and the co-stimulatory molecule ICOS are characteristic markers for Tfh cells (Rasheed et al. 2006) and necessary for homing to B cell follicles and exerting Tfh function (Breitfeld et al. 2000; Schaerli et al. 2000; Akiba et al. 2005; Bossaller et al. 2006; Odegard et al. 2008)**.** Chemokine receptor CCR7 is a dynamically modulated marker of Tfh which can be highly up- or downregulated depending on the microenvironment (Breitfeld et al. 2000; Haynes et al. 2007).

IL-21 is the main Tfh effector cytokine (Chtanova et al. 2004; Bryant et al. 2007) but also IL-10 (Kim et al. 2001) and IL-4 (Kim et al. 2001) are produced and contribute to Tfh

functionality (King & Mohrs 2009). Besides BCL-6, transcription factors IRF4 and STAT3 were shown to cooperate in gene regulation (Kwon et al. 2009) and play an important part in Tfh development, effector function and expression of key molecules, for instance ICOS (Eddahri et al. 2009; Zheng et al. 2009; Bollig et al. 2012; Wu et al. 2015). Tfh cells are also characterized by a high expression of further immune checkpoint molecules, for instance CD200, Programmed cell death protein 1 (PD-1) and B and T lymphocyte attenuator (BTLA) (Chtanova et al. 2004; Rasheed et al. 2006; Haynes et al. 2007; Nurieva et al. 2008; M'Hidi et al. 2009; Yusuf et al. 2010).

2.1.8 Regulatory T cells (Treg)

Regulatory $CD4^+$ T cells (Tregs) are essential for regulating effector T cell responses and maintaining immunological self-tolerance to prevent autoimmunity. However, Tregs also crucially dampen and suppress T cell responses against tumours thereby limiting the efficacy of cancer therapy (Zou 2006).

Characteristically, Tregs express the forkhead transcription factor Foxp3 and produce the cytokines IL-10 and TGF-β (Fig.1) (Asseman et al. 1999; Nakamura et al. 2001; Fontenot et al. 2003; Hori et al. 2003; Eller et al. 2011). Two different populations of regulatory T cells have been described: natural Tregs (nTreg) which are formed in the thymus and induced Treg (iTreg) which are induced in the periphery (Itoh et al. 1999; Zhang et al. 2001; Fantini et al. 2004). While thymic nTregs are generated solely by Foxp3 expression, iTreg differentiation is induced by TGF-β and IL-2 (Chen et al. 2003; Fantini et al. 2004; S. G. Zheng et al. 2007; Davidson et al. 2007) which induce and maintain Foxp3 expression in in $Foxp3^-$ $CD25^-$ $CD4^+$ T cells (Fantini et al. 2004; Marie 2005). Additionally, AHR and STAT5 were shown to have a crucial role in early Treg differentiation as both transcription factors bind the Foxp3 promoter can regulate its expression (Burchill et al. 2006; Quintana et al. 2008) while Bach2 was shown to stabilise Foxp3 expression (Roychoudhuri et al. 2013). Tregs display an array of specific surface markers such as CD25 (IL-2R) (Shevach 2002; Fontenot et al. 2003), CD39 (Deaglio et al. 2007; Borsellino et al. 2007; Mandapathil et al. 2009) and chemokine receptors CCR4 and CCR6 (Yamazaki et al. 2008; Sugiyama et al. 2013). Also LFA-1 (Wohler et al. 2009), CD103 (Anz et al. 2011) and transferrin receptor (CD71) were found on highly activated Tregs (Sagoo et al. 2011; Zeng et al. 2013). Furthermore, a high level of co-inhibitory molecule CTLA-4 (Takahashi et al. 2000) and co-stimulatory molecules OX40 (McHugh et al. 2002b; Takeda et al. 2004), ICOS (Vocanson et al. 2010; Kornete et al. 2012; Redpath et al. 2013) and GITR (Glucocorticoid-induced tumour-necrosis-factor-

receptor-related protein) (Shimizu et al. 2002; McHugh et al. 2002a) are detected on Tregs.

2.1.9 $CD8^+$ T cells

$CD8^+$ T cells represent the traditional effector arm of the adaptive immune system. Upon antigen recognition on MHC class I, $CD8^+$ T cells differentiate into cytotoxic effector cells which are able to kill target cells, for instance virus infected or tumour cells (Shiku 1975). These cytotoxic T lymphocytes (CTL) kill target cells by either the secretion of the cytotoxic molecules perforin and a family of serine proteases, Granzymes, e.g. GzmB, which induce apoptosis in the targeted cell, or by engaging the death receptor FAS on the target cell with its ligand FASL on the T cell membrane causing caspase-dependent apoptosis (Smyth & Trapani 1995; Trapani et al. 1998). $CD8^+$ T cells also express TRAF3 (Xie et al. 2011) and produce Th1 cytokine IFNγ (Ghanekar et al. 2001; Glimcher et al. 2004). The $CD8^+$ lineage specific transcription factor Runx3 collaborates with the transcription factors T-bet (Cruz-Guilloty et al. 2009), Mef (Koizumi et al. 1993; Youn et al. 1996; Lacorazza et al. 2002), IRF4 and BATF (Grusdat et al. 2014; Kurachi et al. 2014) to differentiate naïve $CD8^+$ T cells into effector cells and induce the cytotoxic phenotype.

2.1.10 Memory and effector T cells

After clonal expansion of short-lived effector T cells and clearance of, for instance, the infection, the $CD8^+$ T cell compartment contracts and only a population of long-lived, antigen-specific memory T cells remains (Golstein et al. 1972; Callan et al. 1996; Butz & Bevan 1998). $CD8^+$ effector and memory T cells express different key transcription factors: While T-bet is a Th1 lineage-specific transcription factor, it also has great importance for the development and maintenance of effector CD8 T cells. In addition, the transcription factor Eomesodermin (Eomes) is expressed to a low degree along T-bet in cytotoxic $CD8^+$ T cells but increases drastically in memory $CD8^+$ T cells in which T-bet expression is, in turn, strongly decreased.

BCL-6 and Blimp-1 (B lymphocyte induced maturation protein 1) are two other important effector/memory differentiation controlling transcription factors which are not only found in $CD8^+$ but also $CD4^+$ T cells: effector $CD8^+$ and $CD4^+$ T cells have high expression of Blimp-1 but display no or only low Bcl-6 expression while both, $CD8^+$ and $CD4^+$ memory cells, upregulate Bcl-6 and downregulate Blimp-1 (Kallies et al. 2009; Crotty et al. 2010).

Highly activated $CD8^+$ effector T cells characteristically express CD11b, CD11c (Huleatt & Lefrançois 1995; Christensen et al. 2001; Cooney et al. 2013) and CXCR3 (Hu et al. 2011; Kurachi et al. 2011) as well as LFA-1 (CD11a) (Anikeeva et al. 2005) and CD69 (Simms & Ellis 1996). Futhermore, the transcription factor Id2 (Cannarile et al. 2006; Masson et al. 2013) is an important modulator of $CD8^+$ immunity modulator and is often co-expressed with other effector markers such as KLRG1 (Simms & Ellis 1996; Knell et al. 2013). In both $CD4^+$ and $CD8^+$ T cells, loss of the expression of co-stimulatory receptor CD27 marks an antigen-experienced effector phenotype (De Jong et al. 1992; Hamann et al. 1997).
The nuclear protein Ki67 is often used to investigate proliferative potential of lymphocytes as it is abundantly expressed in dividing cells and lost only in quiescent cells and during DNA repair. Highly proliferative effector T cells therefore express Ki67 while memory T cells are increasingly proliferative senescent and express less Ki67 (Gerdes et al. 1984; Soares et al. 2010).

The traditional memory markers CD44 and CD62L display a characteristic expression pattern: CD44 itself is known to be expressed at intermediate to high levels on memory $CD8^+$ T cells (Sprent 1993; Xiaohong Zhang et al. 1998; Mbitikon-Kobo et al. 2009) while CD62L expression in antigen-experienced, effector memory T cells is low (Sprent & Surh 2002; Seder & Ahmed 2003).
Further memory T cell markers on the other hand include CD122 (Xiaohong Zhang et al. 1998; Goldrath et al. 2000; Cho et al. 2000; Mbitikon-Kobo et al. 2009), CXCR5 (Quigley et al. 2007) and the transcription factors Klf2 (Schober et al. 1999; Grayson et al. 2001) and Id3 (Ji et al. 2011; Hu & Chen 2013) which are essential for memory T cell differentiation.
Chemokine receptor CXCR5 is expressed in a subset of central memory $CD4^+$ T cells which bear strong similarities to Tfh cells (Chevalier et al. 2011). Another, more Th17-like, antigen-experienced memory $CD4^+$ T cell subset expresses CCR6 (Liao et al. 1999; Kleinewietfeld et al. 2005). CCR7, however, is not a definite memory marker: depending on the expression of other markers, for instance CD62L and CXCR5, certain $CD4^+$ and $CD8^+$ memory subsets lack or express the chemokine receptor (Sallusto et al. 1999; Seder & Ahmed 2003) (Quigley et al. 2007).
Interestingly, the expression of the α-chain (CD103) of the integrin $\alpha_E\beta_7$ together with activation marker CD69 on $CD8^+$ T cells marks 'tissue-resident memory T cells' in the skin (Gebhardt et al. 2009; Sheridan & Lefrançois 2011; Mackay et al. 2013).

2.2 A novel CD4$^+$ T cell subset: cytotoxic CD4$^+$ T cells

Whereas there is a multitude of research studies on cytotoxic activity of CD8$^+$ T cells and Natural killer cells (NK), cytotoxic CD4$^+$ T cells have only started to be characterised over the last decade (Appay 2004). Originally, the contribution of CD4+ T cells in the immune response against cancer was thought to only be a passive, exclusively helper function (Pardoll & Topalian 1998; Antony et al. 2005); for instance, intra-tumoural secretion of IFNγ (Qin & Blankenstein 2000), direct priming of CD8$^+$ CTL priming via IL-2 production (Ossendorp et al. 1998) or indirect priming of CTLs via CD40L stimulation of dendritic cells (DCs) (French et al. 1999; Sotomayor et al. 1999).

In the past 5-10 years, however, an increasing amount of reports describing cytolytic molecule expression on CD4$^+$ T cells and directed target cell killing (Quezada et al. 2010; Xie et al. 2010; Muranski et al. 2008). This unconventional CD4$^+$ subset was first detected in human inflammatory pathologies such as human immunodeficiency virus (HIV), Varicella-zoster virus (VZV) and cytomegalovirus (CMV) infections (Huang et al. 1992; Norris et al. 2001; Casazza et al. 2006). Several recent studies, however, illustrated the significance of tumour reactive CD4$^+$ T cells during tumour progression and immunotherapy of cancer (Hunder et al. 2008).

In a model of B16 melanoma, tumour antigen-specific CD4$^+$T cells displayed cytotoxicity and maintained the ability to differentiate into Th0, Th1 and Th17 helper cells *in vitro* (Muranski et al. 2008). In the same model, CD4$^+$T cells developed cytotoxicity upon ligation of the co-stimulatory receptor OX40 in lymphopenic recipients. The CD4$^+$T cells eradicated established tumours and displayed potential to produce Th1 and Th2 cytokines (Hirschhorn-Cymerman et al. 2012). This publication as well as a study from Weiskopf and colleagues which characterised cytotoxic Dengue virus-specific CD4$^+$ T cells, suggested an important role of Eomesodermin for the cytotoxic activity of the cells (Weiskopf et al. 2015).

Mucida and colleagues further reported a downregulation of CD4$^+$ lineage transcription factor ThPok in cytotoxic CD4$^+$ T cells and hypothesise that this event is directly correlated with increased cytotoxic potential (Mucida et al. 2013).

A recent report from Hildemann and colleagues illustrated the generation of antigen-specific CD4$^+$ cells with cytotoxic potential against viral and bacterial antigens *in vitro*. Interestingly, the authors compare the killing efficiency of CD4$^+$ and CD8$^+$ T cells and found the MHC II restricted cytotoxicity of CD4$^+$ T cells to be almost identical with CD8$^+$ cytotoxic potential (Hildemann et al. 2013).

This research project, however, focuses on tumour reactive $CD4^+$ T cells which exhibit cytotoxic activity and the ability to eradicate large tumour lesions in a model of adoptive therapy of B16/BL6 melanoma (Quezada et al. 2010). Over the last 25 years, adoptive cellular transfer (ACT) based immunotherapy has consistently advanced and proved to be very effective, for example in metastatic melanoma (Rosenberg et al. 1988; Dudley et al. 2002; Rosenberg 2011). ACT emerged to be especially efficacious in combination with lymphopenia and $CD8^+$ T cells carrying a transgenic T cell receptor (TCR) engineered to recognise a tumour antigen (Dudley et al. 2005; Morgan et al. 2006). Quezada et al. adapted these conditions and demonstrated $CD4^+$ cytotoxicity in lymphopenic, tumour bearing mice after adoptive transfer of antigen-specific $CD4^+$T cells. The tumour reactive $CD4^+$ T cells in this work originate from the TCR transgenic Trp1 model. Via their transgenic MHC class II-restricted T cell receptor (TCR), these $CD4^+$ T cells recognize a peptide derived from the melanocyte differentiation and melanoma antigen tyrosinase-related protein 1 (TRP-1 or gp75) presented on IA^b (Houghton 1994). The Trp1 model is based on the 'cappucino' white-based brown mutant B^w mouse which does not express TRP1 due to an irradiation induced gene inversion. After identification of a TRP1-reactive TCR from TRP1-immunised B^w mice, the mice were crossed into black $RAG1^{-/-}$ mice and subsequently into B^w $RAG1^{-/-}$ to prevent negative selection of the T cells expressing the highly avid TRP1-reactive TCR, due to the lack of TRP1 expression (Muranski et al. 2008).
As additional part of the therapy, the immunomodulatory antibody αCTLA-4 was used and transpired to be necessary for long term survival (Quezada et al. 2010). The blockade of immune checkpoints, i.e. skewing the balance between co-stimulatory and inhibitory signals T cells receive, through blocking the co-inhibitory molecule CTLA-4 was previously demonstrated to induce significant anti-tumour immunity in immunogenic tumours (Leach et al. 1996) as a monotherapy and against less immunogenic tumours when combined with a Granulocyte-macrophage colony-stimulating factor (GM-CSF) secreting tumour cell-based vaccine (GVAX) (van Elsas et al. 1999).

This study aims to analyse the cellular and molecular mechanisms which direct the acquisition and maintenance of cytotoxicity in antigen-specific $CD4^+$ T cells. In order to characterise and dissect the cytotoxic activity, we established a novel *in vivo* differentiation protocol which allows the generation and analysis of cytotoxic $CD4^+$Trp1 T cells in juxtaposition to tolerant and helper $CD4^+$ T cells with the same specificity but different phenotypes. Particular focus hereby lies on the expression of $CD4^+$ and $CD8^+$ lineage specific differentiation markers and transcription factors such as Runx3 and

ThPok, Gata3 and T-bet. Special attention is paid to the expression pattern of these transcription factors and the effector molecule GzmB as well as inflammatory cytokines. Furthermore, microarray analysis of tolerant, helper and killer $CD4^+$Trp1 T cells is performed to find potential correlations with T helper subsets and effector/memory CD8 T cells. Due to the similarity of cytotoxic $CD4^+$Trp1 T cells to $CD8^+$ CTLs and the importance of mTOR signalling for CTL differentiation, mTORC1 inhibition studies will be performed to determine the role of mTORC1 in $CD4^+$ cytotoxicity.
These experiments aim to elucidate the cellular and molecular events which *control in vivo* differentiated, cytotoxic $CD4^+$ T cells.

2.3 Tumour immunology and immunosurveillance in cancer

Tumourigenisis, i.e. the transformation of healthy cells into a malignant neoplasm, and understanding the complex mechanisms underpinning tumour evolution have been a widely analysed and discussed topics in biomedical research for decades. Hanahan and Weinberg famously first coined 6 hallmarks of cancer in 2000 depicting the essential changes in cell physiology underlying carcinogenesis: 1) self-sufficiency in growth signals, 2) insensitivity to growth-inhibitory signals, 3) evasion of apoptosis, 4) limitless replicative potential, 5) sustained angiogenesis and 6) tissue invasion and metastasis (Hanahan & Weinberg 2000). Following a decade of considerable advances in cancer research and immunology, however, the dogma was augmented and extended in 2011 with four more hallmarks: 7) evasion of immune destruction, 8) promotion of tumour growth via chronic inflammation, 9) reprogramming of energy metabolism and 10) sustained genome instability and mutation (Hanahan & Weinberg 2011).
With the thus increasing attention on the effect of the immune system on tumour development and progression, the concept of immunosurveillance emerged as a crucial element in tumour immunology: Tumour immunosurveillance describes the ability of the immune system to regulate tumour development by detecting cancerous cells and controlling or eliminating them.
Along with a multitude of studies using mouse models of cancer and human cancers demonstrating a functional immune system with the potential for tumour surveillance (Burnet 1970; Thomas 1982; Street et al. 2001; Shankaran et al. 2001; Street et al. 2002; Pagès et al. 2005; Galon et al. 2006) came the realisation that tumours can acquire the ability to escape the immune response, a process termed immunoediting (Dunn et al. 2004). Schreiber and colleagues revolutionised the field of immuno-oncology when they presented their research outlining the three phases of cancer immunoediting, the ‘Three

Es': elimination, equilibrium, and escape. The elimination phase represents the original concept of cancer immunosurveillance and sees malignant cells being destroyed by the immune system. However, if the elimination is incomplete, a temporary state of equilibrium can be reached: although the progress of the developing tumour is halted and contained, the immune system fails to fully extinguishing all transformed cells. During the equilibrium phase tumour cells can further evolve, acquiring additional genetic and epigenetic changes which allow them to eventually evade the immune response and outgrow. In this last phase, the escape, selected tumour cell variants which survived both previous phases of elimination and equilibrium can now overcome, resist or suppress anti-tumour immune responses and expand unimpededly.

There are a variety of mechanisms how tumour cells can effectively evade the immune response: As the detection of tumours by immune cells is based on the recognition of tumour antigens such as viral proteins or peptides originating from oncogenes, mutated proteins or the overexpression of non-mutated proteins, antigen loss and the impairment of antigen presentation are potent escape strategies (Restifo et al. 1996; Benitez et al. 1998; Khong & Restifo 2002; Matsushita et al. 2012). Furthermore, tumours can create a highly immunosuppressive microenvironment (Rabinovich et al. 2007; Vesely et al. 2011), for instance by secreting the suppressive cytokines TGF-β (transforming growth factor β) and IL-10 (Aruga et al. 1997; Flavell et al. 2010; Zhang et al. 2013) or express co-inhibitory receptors such as for instance PD-L1 (programmed death ligand 1) (Blank et al. 2005). Further immune evasion mechanisms include also tumours induced tolerance of T cells against the antigen they normally recognise (Willimsky & Blankenstein 2005) and recruitment of regulatory T cells which further induce immune-inhibition (Onizuka et al. 1999; Woo et al. 2002; Liyanage et al. 2002; Golgher et al. 2002; Terabe & Berzofsky 2004).

2.4 Immune checkpoint blockade in cancer

One of the strategies to counteract immunoediting and thus overcome the immune resistance of tumours is the blockade of inhibitory immune checkpoints. These molecules are inhibitory co-receptors expressed on T cells which negatively regulate T cell function. Indispensable for peripheral self-tolerance they also promote T cell exhaustion and can be directly manipulated by tumours which often express their specific ligands and thereby suppress anti-tumour immunity. Immune checkpoint therapy aims to interrupt the interaction of the inhibitory co-receptor with their ligands using monoclonal antibodies to allow an effective anti-tumour response (Śledzińska et al. 2015).

There are a number of inhibitory receptors which are preferentially expressed on different tumour infiltrating T cell subsets and are currently analysed on their potential as target for immunomodulatory cancer therapy in pre-clinical models and clinical trials, for instance PD-1, TIGIT, CD200, TIM-1/2/3 and CTLA-4 (cytotoxic T-lymphocyte associated antigen 4) (Śledzińska et al. 2015). Especially PD-1 and CTLA-4 have proven very successful targets and antibodies developed to block binding of their ligands have shown promising clinical results in a variety of cancers for instance metastatic melanoma, renal carcinoma and non-small-cell lung cancer (Hodi et al. 2010; Topalian et al. 2012).

2.4.1 CTLA-4

CTLA-4 (also: CD152) is a type 1 transmembrane glycoprotein of the immunoglobulin superfamily and a CD28 homologue (Dariavach et al. 1988). The crucial immunomodulatory molecule is found on all T cell subsets after activation and is constitutively expressed at high frequency on regulatory $CD4^+$ T cells (Walunas et al. 1994; Sansom & Walker 2006). Its importance in immune regulation was demonstrated by studies showing that CTLA-4 deficiency in mice led to fatal lymphoproliferative disease with multi-organ lymphocytic infiltration and organ destruction resulting in premature death by 3-4 weeks of age (Tivol et al. 1995; Waterhouse et al. 1995; Chambers et al. 1997). CTLA-4 interacts with the CD28 ligands CD80 (B7-1) and CD86 (B7-2) but binds them with a 20-100 fold higher affinity and avidity than CD28 (Greene et al. 1996; Collins et al. 2002).

Numerous studies demonstrate the negative regulatory effect of CTLA-4, however the mechanism of action has been largely disputed. CTLA-4 has been shown to affect the lymphocyte expressing the molecule and thus acts in a cell-intrinsic manner, but also to exert its inhibitory function through other cells, in a cell-extrinsic manner. Cell-extrinsic functions of CTLA-4 result in an impairment of CD28 co-stimulation and thereby inhibition of T cell activation. This effect is caused by the disruption of CD28 recruitment to the immunological synapse by direct competition for its ligands CD80 and CD86 and/or by induction of trans-endocytosis of both CD80 and CD86 from antigen presenting cells. Interestingly, this phenomenon was found to not be limited to regulatory T cells but was also shown on Foxp3- effector $CD4^+$ T cells (Greene et al. 1996; Qureshi et al. 2011). Cell-intrinsic negative signalling was demonstrated to be induced by agonistic antibody engagement and crosslinking of CTLA-4 (Walunas et al. 1994; Krummel 1995; Krummel 1996). The molecular events underlying the cell-intrinsic effect of CTLA-4, however, are still unclear and are often presented by conflicting results in the scientific literature (Walker & Sansom 2015): for instance, while some studies suggest that CTLA-4 disrupts

phosphorylation of CD3ζ chains and/or the formation of ZAP-70 microclusters, both indispensable molecular events for T cell activation (Lee et al. 1998; Schneider, Smith, et al. 2008), other reports claim to find no such effects (Schneider et al. 2001; Calvo et al. 1997). Similarly, interaction of CTLA-4 with important regulatory enzymes PI3K and SHP-2 and increase of AKT signalling following CTLA-4 engagement were reported (Marengère et al. 1996; Hu et al. 2001; Schneider, Valk, et al. 2008) and subsequently disputed (Stein et al. 1994; Parry et al. 2005; Yokosuka et al. 2010). A more recent study suggests a new molecular pathway to be involved in CTLA-4 mediated T cell suppression: Kong and colleagues demonstrated a direct interaction of the CTLA-4 cytoplasmic tail with protein kinase C-η (PKC-η) and emphasised its importance in T cell inhibition by showing an impaired contact-dependent inhibitory activity of PKC-η -deficient regulatory T cells (Kong et al. 2014).

Employing the immunomodulatory nature of anti-CTLA-4 antibodies in cancer therapy has seen increasing success over the past 20 years: Blocking CTLA-4 with therapeutic antibodies in several mouse models of cancer, for instance colon, prostate and renal carcinoma, lymphoma and melanoma, increased anti-tumour immunity and induced control and/or rejection of the tumour lesions (Leach et al. 1996; Kwon et al. 1997; van Elsas et al. 1999). More recently, two fully human monoclonal anti-CTLA-4 antibodies were developed by Medarex (Ipilimumab, MDX-010) and Pfizer (Tremelimumab) and entered 42 clinical studies for metastatic/stage IV melanoma as single agent or combined therapy with vaccination or chemotherapeutic agents such as dacarbazine. αCTLA-4 treatment successfully increased the median overall survival in most of the clinical studies and had an overall response rate of up to 15 %. However, due to the crucial function of CTLA-4 in immune regulation, up 60 % of patients displayed immune-related adverse effects which could be reversed by treatment with steroids or other immune-suppressive drugs (Hodi et al. 2010; Robert et al. 2011; Margolin et al. 2012; Ribas et al. 2013; http://clinicltrials.gov 2016; Śledzińska et al. 2015).

One of the major obstacles in immunotherapy of cancer is the potent immunosuppression performed by tumour infiltrating Tregs (Terabe & Berzofsky 2004). Interestingly, we recently demonstrated the mechanism of action of anti-CTLA-4 antibodies such as Ipilimumab to be the depletion of intra-tumoural Tregs by macrophages via Fcγ-RIV (Simpson et al. 2013). The use of anti-CTLA-4 (αCTLA-4) therefore removes the element of immunosuppression and peripheral tolerance and increases the balance of effector to regulatory T cells in the tumour. The ratio between these two subsets has been shown to be a critical indicator for tumour eradication and survival (Quezada et al. 2011).

2.5 The BL6/B16 mouse model of melanoma

The availability of syngeneic mouse models of human disease such as melanoma is indispensable for translational immunological research. The murine melanoma cell line B16 which is used in the study presented here has emerged as one of the most frequently used melanoma models and has been widely demonstrated to be notoriously difficult to treat. It derived from a spontaneously formed melanoma lesion from a C57BL/6 mouse and was originally established in 1973 as a tumour cell line with potential to metastasise to the lung (Fidler 1973). Different varieties of B16 melanoma have since been generated and exhibit different growth rates and metastatic behaviour *in vivo* (Fidler 1975; Briles & Kornfeld 1978). The TRP-1$^+$ B16 melanoma was further found to be poorly immunogenic, i.e. fails to illicit a strong immune response and only displays very low immune infiltration (Celik et al. 1983). It was therefore chosen as xenograft in this study to present a particular challenge for the TRP-1-specific CD4$^+$ T cells.

2.6 mTOR signalling and its role in T cells

T cell differentiation requires specific signals and cues in form of extracellular cytokines and T cell receptor stimulation. Both of these stimuli induce signalling through a central and important serine/threonine kinase, mTOR. Mammalian target of rapamycin (mTOR) is highly conserved and part of a signalling network which regulates cell growth, proliferation and survival. It was first identified in yeast as the target of the macrolide rapamycin of the bacterium *Streptomyces hygroscopicus* (Heitman et al. 1991; Keith & Schreiber 1995). mTOR is the catalytic subunit of two protein complexes, mTORC1 and mTORC2, which each feature different substrate specificities. mTORC1 consists of mTOR, Raptor and mLST8 (Hara et al. 2002; Kim et al. 2002; Loewith et al. 2002) while mTORC2 contains mTOR, Rictor, mLST8 and SIN1 (Loewith et al. 2002; Jacinto et al. 2004; Sarbassov et al. 2004). This study, however, is focused on mTORC1 signalling and its role in CD4$^+$T cell cytotoxicity in particular.

mTORC1 signalling is induced by a large variety of signals, for instance amino acids or insulin (Long et al. 2005; Sancak et al. 2007), oxygen (Sofer et al. 2005) or cellular energy levels and glucose (Inoki et al. 2003) but also growth factors, cytokines (Ballif et al. 2005) and TCR engagement (Gorentla et al. 2011). mTORC1 controls crucial cellular events such as cell cycle progression, translation and survival by phosphorylating its downstream targets S6K1 and S6K2 (p70S6K and p70S6Kβ) and the eukaryotic

initiation factor 4 E binding protein (4E-BP1) (Fig. 2). Through the kinases S6K1 and S6K2, mTORC1 regulates cell homeostasis and growth by inactivating pro-apoptotic BAD (Harada et al. 2001) but the main function of S6K1/2 is the regulation of protein synthesis. The p70 kinases are regulators of protein synthesis as they phosphorylate 40S ribosomal protein S6 which is responsible for the translation of 5'TOP

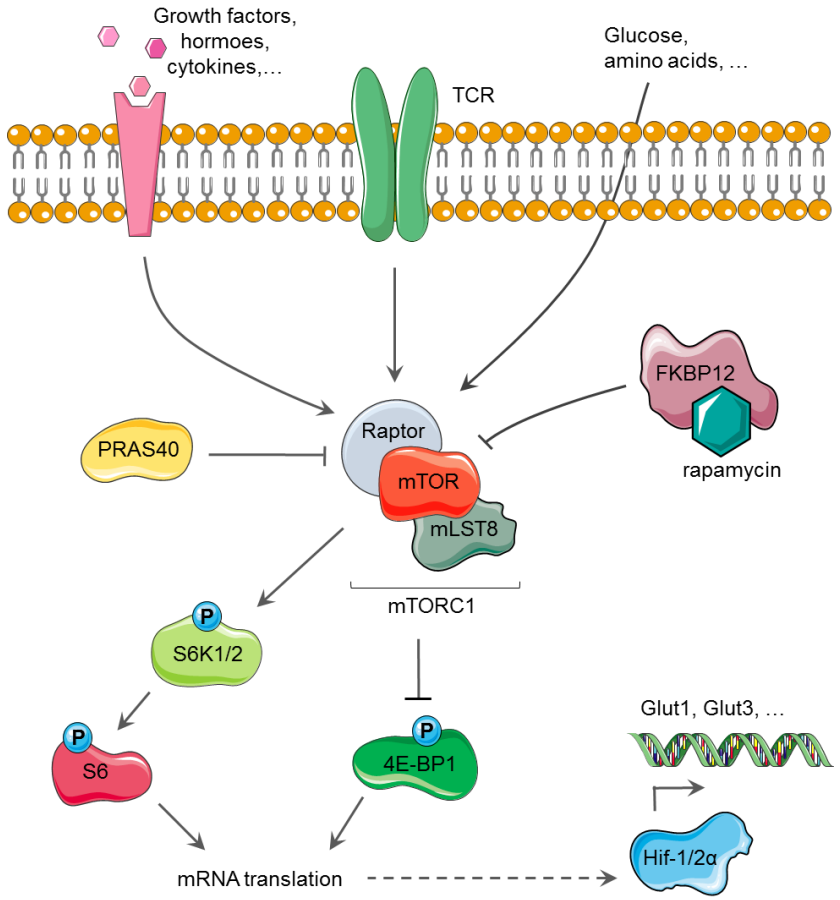

Figure 2 | Schematic of the mTORC1 pathway. Engagement of the T cell receptor (TCR), signalling induced by growth factors, hormones, cytokines and nutrients eventually induce activity of mTORC1 (mammalian target of rapamycin complex 1). The heterotrimeric protein complex consists of mTOR, mLST8 and Raptor and can be inhibited by PRAS40 or rapamycin bound to FKBP12. When activated, mTORC1 phosphorylates S6K1 and S6K2 which in turn phosphorylates ribosomal protein S6 and results in increased mRNA translation. mTORC1 phosphorylation of 4E-BP1 inhibits its function in eIF-4F complex and thereby enables initiation of mRNA translation. Hypoxia inducible factor 1α and 2α (Hif-1α/-2α) expression is increased by mTORC1 signalling and induces expression of target genes such as Glut1 and Glut3 upon hypoxia. The figure was generated using Servier Medical Art (Servier 2015).

(5' terminal oligopyrimide) mRNA transcripts. 5'TOP mRNA encode important components of translation such as ribosomal proteins, elongation factors and translational regulators (Jefferies et al. 1997; Loreni et al. 2000).

4E-BP1, on the other hand, is a translational repressor which, unphosphorylated, inhibits translation by binding the eukaryotic initiation factor 4 E (eIF-4E) of the multi-enzyme complex eIF4F and prevents the formation of the complex. The eIF4F complex has a crucial role in the translation initiation machinery as it binds mRNA, transports it to the 40 S ribosomal subunit and unwinds its secondary structure to facilitate ribosome binding (Gingras et al. 1999). Phosphorylation of 4E-BP1 by mTORC1 causes dissociation of the protein from the eIF-4F complex and thereby enables translation initiation (Hay & Sonenberg 2004; Thoreen et al. 2009).

The enhancement of mRNA translation plays a crucial role in various processes such as cell growth, division, proliferation and metabolism but mTORC1 also was shown to specifically induce expression of Hypoxia inducible factor 1α (Hif-1α) and its target genes Glut1 and Glut3 through 4E-BP1, S6K1 and STAT3 pathways (Iyer et al. 1998) (Fig. 2).

mTORC1 activity can be disrupted by the bacterical molecule rapamycin. It associates with the immunophilin FKBP12 (Schreiber 1991) which in turn binds mTORC1 and prevents phosphorylation of its substrates (Fig. 2). mTORC2, however, is rapamycin insensitive as the FKBP12-rapamycin complex only binds and inhibits mTORC1 (Jacinto et al. 2004). In the clinic, rapamycin is used as immunosuppressant to inhibit mTOR activity or as anti-fungal or even anti-cancer agent (Sehgal 2003). mTORC1 activity can further be inhibited by PRAS40 (proline-rich Akt substrate of 40 kDa) as its binding to mTORC1 complex component Raptor disrupts the catalytic activity of mTORC1 (Wang et al. 2007; Wang et al. 2012) (Fig. 2).

Interestingly, Araki and Rao and colleagues recently demonstrated the importance of mTOR signalling in T cell fate and cytotoxic activity in $CD8^+$ CTLs (Rao et al. 2010; Araki et al. 2009). Specifically, inhibition of mTORC1 via rapamycin administration had two main effects: 1) it disrupted migration of $CD8^+$ CTLs into the tumour and tumour draining lymph nodes and diminished their cytotoxic potential (Chaoul et al. 2015) and 2) it increased the number of memory $CD8^+$ T cells in anti-viral and anti-tumour immune responses (Araki et al. 2009; Turner et al. 2011; Veliça et al. 2015).

3 Materials and methods

3.1 Mice

6–8 week old male C57BL/6 were supplied from Charles River Laboratories. The Trp1 SRT (SJL RAG$^{-/-}$ Tan) Tg mouse is based on a *Rag1^{tm1Mom}* background and also carries the white-based brown radiation-induced mutation of TRP1, *Tyrp1^{B-w}* (Muranski et al. 2008; Quezada et al. 2010). Additionally, the Trp1 SRT mouse carries transgenic TCR α and β transgenes which encode an MHC class II–restricted (I-A^{b}) TCR recognizing the endogenous melanocyte differentiation antigen–minimal TRP-1 epitope corresponding to amino acids 113–127. The Trp1 SRT mouse is furthermore homozygous for CD45.1, which allows the tracking of the CD4^{+}Trp1 T cells in the CD45.2^{+} C57BL/6 recipient after the adoptive cellular transfer. Furthermore, Trp1 FoxGFP, Trp1 FoxDTR and Trp1 LUC mice were used; these lines are based on the Trp1 SRT background but were crossed to FoxGFP, Foxp3-DTR and B6-LUC mice, respectively, to introduce additional transgenes. Trp1 FoxGFP mice express the regulatory T cell specific transcription factor Foxp3 only as the chimeric GFP-Foxp3 fusion protein Foxp3^{gfp} due to the knock in of GFP in the first coding exon of the *Foxp3* gene (Fontenot et al. 2005).

Trp1 FoxDTR mice carry cDNA encoding the human diphtheria toxin receptor (DTR) in the 3' untranslated region of the Foxp3. Thereby, Foxp3^{+} (regulatory) T cells are rendered sensitive to diphtheria toxin (DT) which allows the specific elimination of regulatory T cells via administration of DT (Kim et al. 2007; Goding et al. 2013). The B6-LUC mice, which were used to generate the Trp1 LUC strain, carry the firefly luciferase transgene (LUC) driven by the c-FOS promoter. The c-FOS promoter causes constitutive expression of the luciferase reporter gene in all tissues of these mice (Geusz et al. 1997). Animals were maintained in specific pathogen-free conditions in individually ventilated cages in accordance with institutional guidelines. All animal experiments were performed according to protocol 4 (Generation of tumours by transplant of malignant cells) covered by the project licence 70/7301 (Study and manipulation of immune-regulatory pathways controlling anti-tumour immunity: Applications to immunotherapy of cancer) and were approved by the Home Office and UCL Biological Services.

3.2 Cell lines

The poorly immunogenic B16/BL6 cell line was originally obtained from I.J. Fidler (M.D. Anderson Cancer Center, Houston, TX). The cellular vaccine GVAX, a stable B16/Bl6 melanoma line which constitutively expresses and secrets Granulocyte-macrophage colony-stimulating factor (GM-CSF), was established in our laboratory and described in previous studies (Dranoff et al. 1993; van Elsas et al. 1999).

To produce ecotropic MoMLV retrovirus the packaging cell line Phoenix Eco (Ph-Eco) was used. Ph-Eco is based on the highly transfectable human embryonic kidney HEK-293T/17 cell line which stably expresses the viral proteins *gag-pol* and the ecotropic virus envelope *env* (Pear et al. 1993; Swift et al. 2001; He et al. 1998). The cells were kindly supplied by Pedro Veliça (Royal Free Hospital, London, UK) and Eva Kokalaki (UCL Cancer Institute, London, UK).

The Flt3L-secreting B16/BL6 cell line was created in our laboratory and was kindly supplied by Fred Arce (UCL Cancer Institute, London, UK).

3.3 Antibodies

The therapeutic antibody αCTLA-4 (clone 9H10) and the blocking anti-FcR (Fc receptor, rat anti-mouse CD16/32, clone 2.4G2) were purchased from BioXCell. The clones, fluorochromes, concentrations and suppliers of antibodies used for flow cytometry are outlined in table 1.

Table 1 | Conjugated antibodies used for flow cytometry.

Antibody specificity	Clone	Conjugate	Manufacturer	Dilution
BCL-6	K112-91	V450	BD Biosciences	2 µl p.s.
Blimp-1	5E7	Alexa Fluor® 647	BD Biosciences	1:50
CD3	17A2	PerCP/Cy5.5	Biolegend	1:300
CD3	17A2	Alexa Fluor® 700	eBioscience	1:200
CD4	RM4-5	Brilliant Violet 785™	Biolegend	1:300
CD4	RM4-5	V500	BD Biosciences	1:200
CD4	RM4-5	V450	BD Biosciences	1:200
CD8	53-6.7	eFluor® 450	eBioscience	1:200
CD8	53-6.7	eFluor® 650 NC	eBioscience	1:200

CD8	53-6.7	Brilliant Violet 785™	Biolegend	1:200
CD8	53-6.7	Brilliant Violet 650™	Biolegend	1:200
CD34	QBEND/10	Biotin	AbD Serotec	1.5 µl p.s.
CD44	IM7	Brilliant Violet 785™	Biolegend	1:100
CD45.1	A20	PE-Cy7	eBioscience	1:200
CD45.1	A20	APC-eFluor® 780	eBioscience	1:200
CD62L	MEL-14	eFluor® 450	eBioscience	1:100
Eomes	Dan11mag	PE	eBioscience	1:100
Eomes	Dan11mag	PerCP-eFluor® 710	eBioscience	1:50
Foxp3	FJK-16s	eFluor® 450	eBioscience	1:200
Foxp3	FJK-16s	Alexa Fluor® 700	eBioscience	1:100
Gata3	L50-823	Alexa Fluor® 647	BD Biosciences	1:100
Gata3	TWAJ	PerCP-eFluor® 710	eBioscience	1:100
GzmB	GB12	PE	Invitrogen	1:100
GzmB	GB11	APC	Invitrogen	1:100
Glut3	*polyclonal*	FITC	Abcam	1:100
IFNγ	XMG1.2	eFluor® 450	eBioscience	1:100
IFNγ	XMG1.2	Alexa Fluor® 488	eBioscience	1:100
IL-2	JES6-5H4	PE-Cy7	eBioscience	1:100
IL-2	JES6-5H4	PerCP/Cy5.5	Biolegend	1:100
Ki67	SolA15	FITC	eBioscience	1:400
Ki67	SolA15	PerCP-eFluor® 710	eBioscience	1:400
Ki67	B56	FITC	BD Biosciences	5 µl p.s.
KLRG1	2F1	PerCP-eFluor® 710	eBioscience	1:100
pS6	D57.2.2E	PE	Cell Signaling Technology	1:50
pS6	D57.2.2E	Alexa Fluor® 647	Cell Signaling Technology	1:50
T-bet	4B10	Brilliant Violet 711™	Biolegend	1:50
T-bet	4B10	PE	Biolegend	1:100
ThPok	T43-94	Alexa Fluor® 647	BD Biosciences	5 µl p.s.
TNF-α	MP6-XT22	PE	BD Biosciences	1:100
TNF-α	MP6-XT22	PE/Cy7	Biolegend	1:100
Runx3	527327	APC	R&D Systems	1:100
Vβ14	14-2	FITC	BD Biosciences	1:200

For the detection of biotinylated antibodies, Streptavidin Brilliant Violet 711™ (Biolegend) was used at a concentration of 1:200. To distinguish viable from dead cells and debris, Fixable Viability Dye (eBioscience) in eFluor® 450 or eFluor® 780 was applied along with the extracellular staining before the permeabilisation. Antibody targets for extracellular staining were CD3, CD8, CD45.1, CD4, CD34, CD44, CD62L, Glut3, KLRG1 and Vβ14 while Ki67, IFNγ, TNF-α, IL-2, T-bet, ThPok, Gata3, Runx3, Eomes, FoxP3, p-S6, Blimp-1, Bcl-6 and GzmB were stained for intracellularly, after the cell permeabilisation.

3.4 *In vivo* studies

3.4.1 Tolerant, helper and killer treatment regimens

C57BL/6 mice were anaesthetised with inhaled isoflurane and injected with $2.5x10^5$ B16/BL6 tumor cells intradermally (i.d.) on one flank on day 0. For the *in vivo* differentiation of cytotoxic $CD4^+$Trp1 T cells ('killer'), the mice were lymphodepleted with either 5 Gy of total body irradiation (radiation therapy; RT) on day 8. To promote the differentiation into a helper phenotype ('helper'), the tumour bearing mice were injected i.d. in the opposite flank with 150 Gy irradiated 10^6 GVAX on day 8. Both experimental groups received $6x10^4$ purified $CD4^+$ cells from Trp1 SRT, Trp1 LUC or Trp1 FoxDTR mice intravenously (i.v.) and 200 µg αCTLA-4 (clone 9H10) intraperitoneally (i.p.); two additional injections of 100 µg αCTLA-4 were given on days 11 and 14. For the untreated, 'tolerant' phenotype, the mice only received the adoptive cellular transfer (ACT) of $6x10^4$ purified $CD4^+$Trp1 cells.

3.4.2 mTOR inhibition studies

For the analysis of the impact of mTORC1 inhibition on $CD4^+$Trp1 cytotoxicity, C57BL/6 mice received the same B16/BL6 tumour challenge and 'killer' treatment as described in *3.4.1 Tolerant, helper and killer treatment regimens* but received additional daily i.p. injections of 375 µg/kg rapamycin (RAPA) (Insight Biotechnology). Rapamycin was dissolved at high concentration (25 mg/ml) in DMSO (Sigma) subsequently diluted further in sterile Hank's Balanced Salt Solution (HBSS; PAA) or PBS (Invitrogen) to 375 µg/kg in 200 µl for injection. To avoid freeze-thaw cycles, aliquots of the final concentrations were frozen and thawed each day prior to injection.

Equally, for mTORC1 inhibition studies with iPRAS40 transduced CD4$^+$Trp1 T cells C57BL/6 mice received the B16/BL6 tumour challenge and 'killer' treatment as described in *3.4.1 Tolerant, helper and killer treatment regimens* but instead of naïve T cells the mice received 3x10^5 iPRAS40 or iGFP (mock) transduced CD4$^+$Trp1 T cells (see *3.6.4 Transduction of CD4$^+$Trp1 T cells* and *3.4.3 Lymphocyte isolation and adoptive cellular transfer*) on day 8 and 2 mg/ml doxycycline (DOX) (Alfa Aesar, MP Biomedicals Europe) with 2 % sucrose (Sigma) in the drinking water from day 12 on until the end of the experiment. The DOX drinking water was kept in darkened bottles to protect the photosensitive DOX and was renewed every 3 days.

3.4.3 Lymphocyte isolation and adoptive cellular transfer

For the tolerant, helper and killer regimens, the axillary, brachial, inguinal, superficial cervical, lumbar aortic and mesentery lymph nodes and spleen were dissected from donor Trp1 SRT, Trp1 LUC or Trp1 FoxDTR mice. On average, for adoptive transfer experiments, one transgenic donor per three C57BL/6 recipient mice was required. The lymph nodes were kept on ice in supplemented RPMI (Sigma): 10 % FCS (Sigma), 100 units/100 µg Penicillin/Streptomycin (Sigma) per ml, 2 mM L-Glutamine (Sigma), 50 uM 2-mercaptoethanol (Invitrogen). In order to obtain a single cell suspension, the tissues were passed through 70 µm filters in cold medium and the lymphocytes were purified by density gradient centrifugation with Histopaque 1119™ (Sigma-Aldrich). For the CD4$^+$ T cells purification, CD4 (L3T4) MicroBeads and LS columns (both Miltenyi) on a MACS separator were used. Cells were constantly kept on ice in MACS buffer (0.5 % BSA (Sigma-Aldrich), 2 mM EDTA (Sigma) in HBSS). The purity of the TRP1 TCR carrying CD4$^+$ cells was examined by CD4 and Vβ14 staining via flow cytometry. After warming the recipient mice in an atmosphere of 40 °C to increase the blood flow to superficial veins, 6x10^4 purified CD4$^+$Trp1 cells were injected in 200 µl i.v. into the tail vein.

For mTORC1 inhibition studies with iPRAS40 and iGFP transduced CD4$^+$Trp1 T cells, the transduced cells were selected for the transduction marker CD34 after 65 to 72 hours incubation with IL-2 (see *3.6.4 Transduction of CD4$^+$Trp1 T cells*) using human CD34 MicroBeads and LS columns (both Miltenyi). The manufacturer's protocol was slightly changed to compensate for the high quantity of cells expressing the target molecule: prior to incubation with the beads, the transduced cells were incubated with 50 µg/ml anti-FcR for 30 minutes on ice to block Fc receptors and were washed subsequently. To label the transduced cells, 150 µl of CD34 MicroBeads were added to each iGFP and

iPRAS40 transduced cell suspension in a total volume of 300 µl each and incubated for 30 minutes at 4 °C. After assessing the purity of transduced cells by flow cytometry, $3x10^5$ purified $CD34^+$ $CD4^+$Trp1 cells were injected in 200 µl i.v. into the tail vein of warmed recipient mice.

3.4.4 Isolation and *ex vivo* re-stimulation of T cells

Tumour bearing mice were sacrificed on day 17-19 after tumour implantation and peripheral (axillary, brachial, inguinal) lymph nodes and the tumour were dissected. The lymph nodes were constantly kept on ice in supplemented RMPI, the tumours, however, were placed in RPMI without supplements or HBSS/PBS. The lymph nodes of each mouse were pooled, passed through a 70 µm cell strainer and the cells were counted on the Muse™ cell analyser (Millipore). For the purification of tumour infiltrating lymphocytes, the tumour was dissociated by cutting with fine scissors or using the Miltenyi gentleMACS™ Dissociator with the respective C Tubes (Miltenyi) and the program *tumour_2*. The tissue was further digested with a mixture of 0.2 mg/ml DNase (Roche) and 0.33 mg/ml Liberase TL or Liberase DL (Roche, Sigma) in supplement and serum-free RPMI at 37 °C for 30 minutes. After passing the tumour suspension through a 70 µm filter, the lymphocytes were separated from the tumour cells, dead cells and debris by density gradient centrifugation with Histopaque 1119™. For all functional experiments except for the rapamycin treatment of tumour bearing mice, the tumour and lymph node samples were evenly distributed between the different FACS staining panels. The samples were split to allow the staining for a high number of different proteins of interest per sample (tumour/lymph nodes). However, due to the low number of tumour infiltrating T cells in the rapamycin treated mice, the tumour samples from these experiments were not split between different staining panels.
For re-stimulation of the lymphocytes and cytokine analysis, 5×10^4 dendritic cells (DCs) pulsed with B16/BL6 tumour lysate and 2 µM TRP1 peptide (Pepceuticals) were added to each sample from lymph nodes or tumours of each mouse and incubated at 37 °C. After 1 hour, 2 µg/ml GolgiPlug™ (BD) was added and the cell suspensions were incubated for further 3 hours at 37 °C.

3.4.5 Quantification of tumour infiltrating lymphocytes

For quantification of the tumour infiltrating lymphocytes flow cytometry reference beads (PeakFlow™ blue or Cell Sorting Set-up Beads (for UV lasers), both Molecular Probes®,

Life Technologies) were added to the samples prior to analysis to normalize for the volume of the sample acquired. The absolute number of the respective lymphocyte population (e.g. $CD4^+$Trp1 effectors [$CD4^+$ $CD45.1^+$ $Foxp3^-$]) per gram of tumour was calculated using the following formula:

$$\text{absolute cell number of } CD4^+\text{Trp1 cells/mg tumour} = \left(\frac{\text{acquired number of } CD4^+\text{Trp1 cells}}{\text{acquired number of beads}} \cdot \text{number of beads added to sample}\right) / \text{tumour weight}$$

3.4.6 Antibody staining for flow cytometry

Immediately after tissue dissociation or re-stimulation, respectively, cells were stained with appropriate antibodies against extracellular molecules in a blocking solution (5 % mouse serum (AbD Serotec), 5 % rat serum (AbD Serotec), 2 % fetal calf serum (FCS, Gibco®, Life Technologies), 2 % anti-FcR (rat anti-mouse CD16/32, 2.4G2), 0.1 % sodium azide (Sigma-Aldrich) in HBSS). The cells were then permeabilised and fixed with the Foxp3 staining kit (eBioscience) according to the manufacturer's instructions, and stained with the antibodies for intracellular targets in the Foxp3 perm buffer solution + 10 % of blocking solution. When appropriate, streptavidin conjugated with a fluorochrome was used to detect biotinylated antibodies. If the biotinylated antibody detected an extracellular epitope the streptavidin was applied separately for 10 minutes in blocking solution before the permeabilisation, if the epitope was intracellular, streptavidin was applied in Foxp3 perm buffer + 10 % of blocking solution after the intracellular antibody staining. After the staining, both *ex vivo* and re-stimulated samples were fixed with 1-2 % paraformaldehyde (PFA; Fisher Scientific, VWR).
The cytometer used was a BD LSRFortessa™ cell analyser configured with configured with a 405 nm octagon, 488 nm octagon, 561 nm octagon and 640 nm trigon laser excitation lines and filters.

3.4.7 Tumour protection experiments

C57BL/6 mice received the same B16/BL6 tumour challenge and tolerant, helper and killer treatments as described in *3.4.1 Tolerant, helper and killer treatment regimens* and *3.4.2 mTOR inhibition studies* and adoptive transfer of naïve and/or transduced $CD4^+$Trp1 T cells as outlined in *3.4.3 Lymphocyte isolation and adoptive cellular transfer*. Additionally, tumour size and/or body weight of individual animals were recorded every

2-3 days throughout the experiment, up to 100 days in total. Importantly, the animals were closely monitored and pain or distress levels were assessed according to UKCCCR guidelines. Mice were killed by a schedule 1 method if one of the following end points were reached (as outlined in PIL *70/7301)*: a) The tumour reached a diameter of 12 mm for untreated and 15 mm for therapeutic studies, b) The tumour limits the animal's normal behavioural repertoire or causes undue distress as reflected by: dehydration, dyspnoea, anorexia, lethargy, ruffled fur, piloerection, hunched posture, difficulty in moving, cachexia, signs of neurological impairment such as difficulty in moving or partial paralysis, c) The animal loses > 10% of its body weight, d) Severe ulceration of the tumour occurs.

3.5 Microarray set up

The GeneChip® Mouse Genome 430 2.0 Array (Affymetrix) was used to analyse the transcriptome of tolerant, helper and killer $CD4^+$Trp1 cells from both lymph nodes and tumours as well as $CD4^+$ cells from naïve Trp1 FoxGFP mice. Dr. Sergio Quezada (UCL Cancer Institute, London, UK) performed the experimental procedures to set up the microarray while I participated in the analysis and guided the direction of the bioinformatic investigation of the microarray data.

C57BL/6 mice were injected with $2x10^5$ B16/BL6 tumor cells i.d. on the flank on day 0. For the generation of cytotoxic $CD4^+$Trp1 T cells ('killer'), the mice were lymphodepleted on day 9-10 with 5 Gy total body irradiation while for the generation of helper phenotype, tumour bearing mice were injected subcutaneous (s.c.) on the opposite flank with previously irradiated (150 Gy) 10^6 GVAX. On the same day, both groups received $5x10^4$ - $8x10^4$ purified $CD4^+$Trp1 FoxGFP cells i.v. and were injected i.p. with 200 µg αCTLA-4 (9H10) on days 10, 13 and 16. As additional control, the untreated ('tolerant' phenotype) group only received the adoptive cellular transfer of $5x10^4$ - $8x10^4$ purified $CD4^+$Trp1 FoxGFP cells. Mice were killed 8 days after the adoptive transfer and the lymphocytes from tumour and peripheral (axillary, brachial, inguinal) lymph nodes were extracted respectively (see *4.3.3*). In addition, the lymphocytes from the lymph nodes, and the tumours, respectively, were pooled within each of the three groups. Effector ($Foxp3^-$) $CD4^+$Trp1 cells from the tumours or the lymph nodes were sorted on a $CD4^+GFP^-$ gate to exclude the Tregs ($CD4^+GFP^+$ cells) and $CD4^+CD45.1^+$ gate to exclude the endogenous $CD4^+$ T cells originating from the recipient C57BL/6. To obtain naïve $CD4^+$Trp1 cells, lymphocytes from the lymph nodes of Trp1 FoxGFP mice were purified and sorted for $CD25^-$ GFP^- cells. RNA was isolated from the purified $CD4^+$Trp1 cells using TRIzol® (Invitrogen) and glycogen according to the manufacturer's guidelines.

In order to reach the required amount of RNA for the gene array, there were 8-10 mice per group in each experiment and the experiments were performed in triplicates.

3.6 Cell culture

3.6.1 Preparation of cellular components for *ex vivo* and *in vitro* assays

To prepare B16/BL6 lysate for pulsing DCs, B16/BL6 cells were cultured for 2-3 days and irradiated with a target dose of 150 Gy in supplemented RPMI medium. The irradiated cells were incubated 3-4 hours at 37 °C and subsequently lysed by three freeze/thaw cycles, by alternately submerging the cells in liquid nitrogen and the 37 °C water bath.

To produce a high number of dendritic cells for the *ex vivo* re-stimulation assays, C57BL/6 mice simulated with Flt3L either by s.c. injection of 2-3x10^6 Flt3L-secreting B16 cells in the scruff of the neck or by injection of 10 µg/day recombinant Flt3L i.p. for 10 days. 10 days of Flt3L stimulation, spleens were harvested and injected lengthwise with a mixture of 0.2 mg/ml DNase and 0.33 mg/ml Liberase TL in serum free RPMI (1 ml per spleen). The spleens were cut and incubated in the Liberase/DNAse solution for 30 minutes at 37 °C. Afterwards, the tissue was passed through a 70 µm cell strainer, washed with an HBSS or PBS based 5 mM EDTA buffer and kept below 4 °C at all times. The DCs were purified according to manufacturer's guidelines with CD11c microbeads and LS columns on the MACS separator (all three Miltenyi) in MACS buffer. To generate unloaded control DCs, the purified cells were incubated overnight at a density of 5x10^7 DCs per 15 cm plate in 20 ml total volume in supplemented RPMI with 5 ml of filtered GMCSF supernatant obtained from a confluent 15 cm plate of GVAX at 37 °C and 5 % CO_2. To load DCs with B16/BL6 lysate, the tumour lysate was added in a ratio of 1:1 to 1:4 (DCs to tumour cells) to the purified cells and incubated overnight as described for the unloaded control DCs. The next day, the B16 pulsed DCs were purified by density gradient centrifugation with Histopaque 1119™ and frozen in 1 ml of freezing medium (80 % FCS, 10 % RPMI, 10 % DMSO) at a concentration of 2x10^6 cells/ml.

3.6.2 *In vitro* activation assays and CFSE staining

Spleen and peripheral (axillary, brachial, inguinal) lymph nodes from Trp1 SRT, Trp1 LUC or Trp1 FoxDTR mice were dissected and kept on ice in supplemented RPMI. The

tissues were passed through a 70 µm filter and the viable lymphocytes were purified by a density gradient centrifugation with Histopaque 1119™.

To analyse the proliferation of activated CD4$^+$Trp1 T cells, the cells were labelled with CFSE (carboxyfluorescein diacetate succinimidyl ester) or CellTrace™ violet (both Molecular Probes®, Life Technologies). CFSE and CellTrace violet are succinimidyl ester dyes which diffuse through the cell membrane into the cytoplasm where esterases cleave their acetyl groups. The resulting fluorescent dyes bind to amino groups on intracellular proteins and are thereby maintained during cell division and passed on equally to daughter cells. As the fluorescent signal decreases with each division by approximately half, several successive cell generations can be detected individually by flow cytometry. CFSE has an excitation peak of 492 nm and emission peak of 517 nm, the excitation peak of CellTrace violet is at 405 nm and the emission peak at 450 nm (Lyons & Parish 1994; Graziano et al. 1998; Lyons 2000; Quah & Parish 2012).

The purified naïve cells were resuspended up to 2x10^7 cells/ml in 1 ml of 37 °C warm HBSS + 0.1 % BSA. CFSE or CellTrace violet was added to the cells to a manufacturer recommended final concentration of 5 µM. After incubation for 10 minutes at 37 °C, the reaction was quenched by adding cold supplemented RPMI and incubating the cell suspension on ice for 5 minutes. The cells were washed three times with cold HBSS or PBS to remove excess CFSE/CellTrace violet and 4-9x10^4 cells were plated out in duplicates or triplicates in a round bottom 96-well plate. The CD4$^+$Trp1 were activated with 2 µM TRP1 peptide and exogenous, B16 pulsed DCs (see *3.6.1 Preparation of cellular components for ex vivo and in vitro assays*) in a ratio of 1 : 2.5-4 (lymphocytes to DCs) and incubated for 72 hours at 37 °C.

For the analysis of the impact of mTOR inhibition on CD4$^+$Trp1 proliferation and cytotoxicity, 0.5 µM RAPA was added to the CFSE/ CellTrace violet labelled cells, DCs and TRP1 peptide and before incubating the cells at 37 °C for 72 hours.

For the short *in vitro* activation of iPRAS40 and iGFP transduced CD4$^+$Trp1 T cells, the cells were removed from the IL-2 rich medium after transduction and 72 hours *in vitro* incubation (see *3.6.4 Transduction of CD4$^+$Trp1 T cells*) and were rested overnight in supplemented RPMI at 37 °C without IL-2. CD4$^+$Trp1 T cells were then either left untreated or re-activated with exogenous DCs (1:1 ratio) and 2 µM TRP1 peptide. Additionally, the cells were treated or not with 0.5 µM RAPA and/or 1 µg/ml DOX and were incubated for 24 hours at 37 °C.

3.6.3 Retrovirus production and concentration

The retrovirus chosen for the transduction of murine $CD4^+$Trp1 T cells was ecotropic Moloney murine leukemia virus (MoMLV) as murine cells express the respective ecotropic receptor mCAT (murine cationic amino acid transporter) which allows their infection by MoMLV particles (Chattopadhyay et al. 1981; Albritton et al. 1989; Wang et al. 1991).

The inducible retroviral expression vectors iPRAS40 and iGFP were designed, validated and kindly provided by Pedro Veliça (Royal Free Hospital, London, UK). Both inducible expression plasmids were used *in vivo* for selective mTORC1 inhibition in murine tumour specific $CD8^+$ T cells in a recent publication (Veliça et al. 2015).

The *Pras40* encoding vector iPRAS40 is based on a pSERS backbone which contains a Tet-ON "All-In-One" inducible system allowing regulated expression of *Pras40* by administration of tetracycline or doxycycline. The tetracycline inducible expression cassette consists of the optimised reverse tetracycline-controlled transactivator protein (rtTA-M2) which is expressed under the constitutively active hPGK (human phosphoglycerate kinase) promoter, and the Tet operon (TetO, tetracycline operator) which controls the expression of PRAS40 through a minimal promoter (Fig. 3). Only in the presence of tetracycline or doxycycline the transactivator rtTA2-M2 can bind the TetO and induce the transcription of *Pras40* (Heinz et al. 2011; Veliça et al. 2015).

In order to easily assess the induction of PRAS40 expression, Green fluorescent protein (GFP) was added downstream of *Pras40* and linked with a P2A (picornavirus 2A sequence) resulting in nearly equimolar production of the P2A connected genes (induction marker). Additionally, the transactivator rtTA-M2 was linked through a FMD-2A (foot-and-mouth disease 2A) to a Q8 tag which allows the detection of successfully transduced cells (transduction marker) (Fig. 3). The Q8 tag is a fusion protein originally designed by Philip et al. which consists of a 42-amino-acid-long human CD8α stalk and transmembrane domain (‘8’) and a 16-amino-acid-long truncated human CD34 extracellular tail which presents the minimised epitope for the QBEnd10 anti-CD34 antibody (‘Q’) (Philip et al. 2014; Veliça et al. 2015).

To increase the expression of the transgenes, the cis-acting post-translational enhancer W-PRE (Woodchuck hepatitis post-transcriptional regulatory element) was added downstream of the tetracycline inducible cassette (Donello et al. 1998; Lee et al. 2005).

The control vector iGFP is identical with iPRAS40 but lacks the *Pras40* gene and the P2A sequence downstream of TetO (Fig. 3) (Veliça et al. 2015).

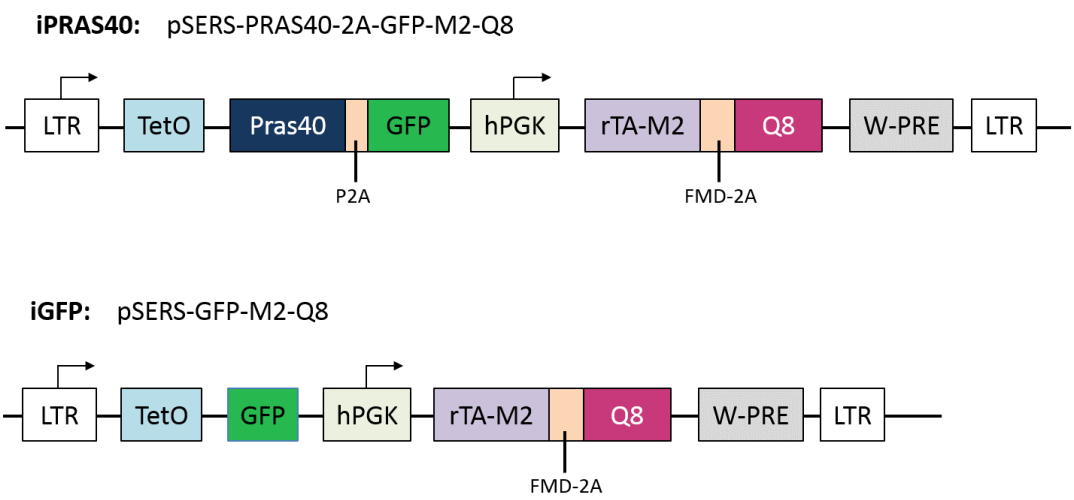

Figure 3 | Maps of doxycycline-inducible retroviral vectors iPRAS40 and iGFP. The pSERS based iPRAS40 vector encodes *Pras40* connected with a P2A sequence to GFP and contains the following further essential components: LTR: long terminal repeat, TetO: tetracycline operator, hPGK: human phosphoglycerate kinase, rtA2-M2: optimised reverse tetracycline-controlled transactivator, FMD-2A: foot-and-mouth disease 2A sequence; P2A: picornavirus 2A sequence, Q8: truncated human CD34 (QBend10 epitope) linked to a CD8α stalk, W-PRE: Woodchuck hepatitis post-transcriptional regulatory element. The control vector iGFP is identical with iPRAS40 but lacks the *Pras40* and P2A sequences upstream of GFP. The Q8 tag is used to determine transduction efficiency while GFP is the marker for induction.

For the generation of retrovirus the following plasmids were produced in *Escherichia coli* DH5a: the iPRAS40 and iGFP expression vectors which were kindly supplied by Pedro Veliça (Royal Free Hospital, London, UK), the PeqPam plasmid which contains Gag and Pol structural genes and the pMono-Eco plasmid which encodes the ecotropic viral envelope gene, both kindly supplied from Fred Arce and Claire Roddie (UCL Cancer Institute, London, UK).

To produce iGFP and iPRAS40 retroviral particles, $9x10^6$ Ph-Eco cells were plated in 14 cm dish in 20 ml IMDM (Sigma) supplemented with 10 % FCS, 100 units/100 µg Penicillin/Streptomycin per ml and 2 mM L-Glutamine and incubated at 37 °C (day 1). After 24 hours the cells were ready to be transfected if they had reached ~70-80% confluency, were fully attached and had formed visible protrusions. Per 14 cm plate, 8.69 µg expression plasmid (iGFP or iPRAS40), 8.69 µg PeqPam plasmid and 4.34 µg pMono-Eco plasmid were mixed with 702 µl TE buffer (10 mM Tris-HCl (Sigma), 1 mM EDTA (Sigma) in dH_2O, pH adjusted to 8.0 with HCl) in one tube while in a second tube 65 µl Fugene (Promega) was carefully added to 724 µl of Optimem (Gibco). The DNA

mixture was added to the Fugene and Optimem solution and left to incubate for 15 min at room temperature. The transfection mix was then added dropwise to the Ph-Eco cells and returned to incubate at 37 °C (day 2). After 16 hours, the transfection medium was carefully removed and replaced with 20 ml supplemented IMDM (day 3). After 24 hours, the transfected cells were transferred into a 32 °C incubator and left to produce virus for another 24 hours (day 4). On day 5, the retrovirus-containing supernatant were harvested and pooled if several 14 cm plates were used.

To concentrate the retrovirus, the supernatants were filtered through a 0.45 µm polyethersulfone (PES) filter (Sartorius) and mixed and processed with Retro-X™ Concentrator (Clontech) according to the manufacturer's guidelines. After incubation overnight at 4 °C and centrifugation at 1,500 x g for 45 minutes at 4 °C, the retrovirus-containing pellet was carefully resuspended in 1/10 or 1/50 of the original volume using fresh supplemented RPMI (day 6). Immediately afterwards, the retroviral solutions were quickly transferred to storage at -80 °C in appropriate, single-use aliquots (1.5 ml – 5 ml).

3.6.4 Transduction of CD4$^+$Trp1 T cells

For the transduction of primary CD4$^+$Trp1 T cells, non-tissue culture (TC)-coated 24 well plates were coated with RetroNectin (Takara): the 1 mg/ml RetroNectin stock solution was aliquoted in small volumes and kept for single-use at -20°C. The RetroNectin was thawed and diluted in sterile dH_2O to a working concentration of 25 µg/ml. To coat the wells, 0.5 ml of the 25 µg/ml RetroNectin solution were added per well of the sterile, non-TC-coated plate. After sealing the plate with parafilm, it was either left overnight at 4°C or for a minimum of 3 hours at room temperature until immediate use.

Lymphocytes were isolated from Trp1 SRT, TRP1 LUC or TRP1 FoxDTR mice according to *3.4.3 Lymphocyte isolation and adoptive cellular transfer* (day 1). After the CD4$^+$ T cell selection, the T cells were stimulated with exogenous DCs (see *3.6.1 Preparation of cellular components for ex vivo and in vitro assays*), 2 µM TRP1 peptide and 10 u/ml human recombinant IL-2 (Peprotech). The cells were mixed at a ratio of 2-3 CD4$^+$ T cell to 1 DC then plated out at $2x10^6$ cells per well (1 ml/well) in a TC treated 24 well plate or at $29.8x10^6$ cells/ml in 5 ml supplemented RPMI in an 6 cm plate and incubated for 24 hours at 37°C. On day 2, the RetroNectin solution was transferred into the same number of wells of a new non-TC-treated 24 well plate which was then sealed with parafilm and kept at 4°C for future use within 30 days. This recycling could be performed twice, yielding a total of 3 uses from the same RetroNectin solution. Immediately after removing the RetroNectin solution from the now-coated plate, the wells were blocked with a sterile-

filtered BSA blocking solution (2 % BSA (Sigma) in PBS) for 30 minutes at room temperature. The wells were washed twice with PBS and left covered in PBS at room temperature until the plate was used. Throughout this process, treat care was taken to avoid letting the RetroNectin coated wells stand dry. The activated cells were carefully removed from the TC plates and resuspended in fresh supplemented RPMI with 100 u/ml IL-2 (day 2) at a concentration of $4x10^6$ cells/ml. An appropriate amount of retrovirus solution was quickly thawed and kept on ice for immediate use. After removing the PBS from the RetroNectin covered wells, 10 µl (50x concentrated virus) or 50 µl (10x concentrated virus) retrovirus solution were placed directly onto the surface of the well, quickly followed by 500 µl of the cell suspension ($2x10^6$ cells). The plate was sealed with Parafilm and transferred into a 32°C preheated centrifuge to spin at 800 x g for 90 minutes without break. After the centrifugation, 500 µl of supplemented RPMI with 100 u/ml IL-2 were added to each well and the cells were incubated at 37°C for 3 days. Throughout this period the cells were checked regularly and when the medium turned yellow the wells were topped up to 2 ml with fresh supplemented RPMI with 100 u/ml IL-2. As soon as the medium changed colour after that, 1 ml was carefully removed from the surface of the wells and replaced with 1 ml of fresh supplemented RPMI with 100 u/ml IL-2. This process was repeated as often as necessary throughout the 3 days to keep the proliferating T cells supplied with nutrients and IL-2.

3.7 Statistical analyses

Data were analysed using Excel (Microsoft Office) and Prism 5.0 (GraphPad Software, Inc.). The statistical significance was determined by a Student's *t* test (between two groups or conditions) or one-way analysis of variance (ANOVA) with a Bonferroni post-hoc test (three or more groups or conditions). The data from survival experiments were analysed with the Kaplan-Meier method and a log-rank (Mantel-Cox) test was performed to calculate statistical differences between survival curves. P values below 0.05 were considered statistically significant with significance increasing accordingly: * : $P \leq 0.05$, ** : $P \leq 0.01$, *** : $P \leq 0.001$, **** : $P \leq 0.0001$.

3.8 Gene array analysis

3.8.1 General set up, principle component analysis and heat map generation

The labels of the experimental groups were set as follows: Killer.TIL and Killer.LN represent the gene expression data of killer (RT + $CD4^+$Trp1 + αCTLA-4) $CD4^+$Trp1 T cells isolated from either tumour (TIL) or axillary, brachial and inguinal lymph nodes (LN). In the same manner, helper and tolerant $CD4^+$Trp1 T cell gene expression sets from the tumour or the lymph nodes were labelled Helper.TIL, Helper.LN and Tolerant.LN. The gene array data set originating from naïve $CD4^+$ T cells was called naïve.TRP1.

The microarray data from the GeneChip® Mouse Genome 430 2.0 Array was analysed by Masahiro Ono, John Ambrose and myself using the statistical programming language R (R Core Team 2013) with the Bioconductor packages *limma* (Linear Models for Microarray Data), *affy*, *mouse4302.db* and a*ffyQCReport* as well as the CRAN (The Comprehensive R Archive Network) packages *gplots* and *ade4*.

To normalise the expression data and correct background noise the *RMA* (robust multichip averaging) algorithm of a*ffy* was used (Gautier et al. 2004). The package *mouse4302.db* (Carlson 2011) was utilised to annotate the expression data and quality control was performed using a*ffyQCReport* (Parman et al. 2011). For statistical weighting and analysis of the microarray data, thus to assess the differential expression of genes between the different experimental groups, the functions *scalewt* of *ade4* (Chessel & Dufour 2004; Dray & Dufour 2007; Dray et al. 2007) and *lmFit*, *topTable* and *eBayes* of *limma* (Smyth 2004; Ritchie et al. 2015) were employed. This computation yielded a total of 7228 significant genes from the following comparison sets: KillerVsHelper.TIL, KillerVsHelper.LN and KillerVsTolerant.LN. The set KillerVsHelper.TIL, for instance, includes all genes whose expression values were significantly up- or downregulated in the Killer.TIL data set in comparison to the Helper.TIL data set. The other two comparison sets (KillerVsHelper.LN and KillerVsTolerant.LN) were established in an analogous manner.

For the computation of a Principle Component Analysis (PCA) Masahiro Ono used the function *dudi.pca* of *ade4*. To create the heat map analyses John Ambrose used the functions *heatmap.2* and *breaks* from the CRAN package *gplots* (Warnes et al. 2012). The data was first normalised by subtracting the mean expression of each probe set (gene) from the expression value of each individual sample. The expression values were

then scaled by dividing the standardised values by the standard deviation across all samples. In the heat maps, the scaled expression value (Row Z-Score) is illustrated in a red and green colour scale, red denoting high and green low expression of the respective gene. If there were multiple probes for one gene on the microarray, the probe set with the largest signal variance was used to analyse and display the respective gene.
Despite the normalisation of expression values a few outliers, i.e. random aberrant very high or very low expression values, persisted. As these deviating values would determine the limits for the colour scale, the outliers would cause the colour spectrum to stretch and the majority of the data to appear faint on the heat map and conceal changes in gene expression. Therefore the limits of the Row Z-Score (colour scale) were customised to include 95 % of the data and set a cut off for the 5 % which of aberrantly high/low expression values. Extreme values are displayed in the colour of the highest or lowest expression value of the 95 %. For instance, an aberrant expression value of 5 in an analysis in which 95 % of the data falls in the Row Z-Score range of -3 to 3 will be displayed in the same colour as an expression value of 3. This colour scale customisation was performed using the *breaks* function.

3.8.2 Canonical Correspondence Analysis of Microarray data (CCAM)

Masahiro Ono created several Canonical Correspondence Analyses on Microarray data (CCAM). CCAM is an adaptation of CCAM which allows simultaneous analysis of two microarray data sets and was performed as previously described (Ono et al. 2013). For the computation of the different CCAMs, several microarray data sets from the ImmGen database were used (Heng et al. 2008). As some of the ImmGen datasets originated from a different platform, normalisation across two platforms by the function *virtualArrayExpressionSets* of the Bioconductor package *virtualArray* was required to merge the data sets into a virtual array to be able to compute CCAM (Heider & Alt 2013).

For the CCAM of 'CD4$^+$ and CD8$^+$ likeness' the 7228 differentially expressed genes were analysed in context with two ImmGen gene expression data sets from subcutaneous B16 tumour infiltrating CD8$^+$ (CD4$^-$ TCRβ$^+$ CD45$^+$, T.8.TI.B16) and CD4$^+$ T cells (CD8$^-$ TCRβ$^+$ CD45$^+$, T.4.TI.B16) from 6 week old C57BL/6 mice; for this CCAM T.8.TI.B16 - T.4.TI.B16 was used as explanatory variable.

For the CCAM of tolerant, helper and killer CD4$^+$Trp1 in context with CD8$^+$ differentiation phenotypes (effector, memory), several ImmGen datasets encompassing the genetic signature of OT-I CD8$^+$ T cells from different time points of the response against the

$Ova_{257-264}$ peptide SIINFEKL from ovalbumin encoded by Vesicular Stomatitis Virus (VSV-ova) were used. For the generation of these datasets, adoptively transferred OT-I $CD8^+$ T cells were isolated from the spleen of 6-week old C57BL/6J mice 5 days (T.8Eff.Sp.OT1.d5.VSVOva, GEO accession no. GSM538388, GSM538389, GSM605897), 6 days (T.8Eff.Sp.OT1.d6.VSVOva, GEO accession no. GSM538389, GSM538390, GSM538391), 8 days (T.8Eff.Sp.OT1.d8.VSVOva, GEO accession no. GSM538392, GSM538393, GSM538394) or 15 days (T.8Eff.Sp.OT1.d15.VSVOva, GEO accession no. GSM538385, GSM538386) after virus inoculation.

4 Results

4.1 Establishment of a model to study the function and plasticity of tumour reactive $CD4^+$Trp1 T cells *in vivo*

Our lab previously demonstrated that a triple therapy with lymphopenia, adoptive cellular transfer of $CD4^+$Trp1 cells and αCTLA-4 treatment results in complete eradication of established B16/BL6 melanoma lesions and long term protection (Quezada et al. 2010). In order to characterise and dissect the cytotoxic activity of these $CD4^+$ T cells, a novel *in vivo* model was established which allows the analysis of the cytotoxic $CD4^+$Trp1 T cells in juxtaposition to tolerant and helper $CD4^+$ T cell phenotypes.

Three different treatments were applied to B16/BL6 melanoma bearing mice to promote different functionalities of tumour reactive $CD4^+$ cells.

To generate a hyporesponsive (tolerant) $CD4^+$ phenotype, we chose to perform adoptive transfer of $CD4^+$Trp1 T cells into tumour bearing animals without further treatment as preliminary data from our laboratory suggested that transferred $CD4^+$Trp1 T cells in this experimental set up expanded but then rapidly contracted and did not display any anti-tumour activity. We hypothesised that this effect is due to anergy or peripheral tolerance and considered this $CD4^+$ T cell population a highly contrasting subset to compare with the particularly active cytotoxic $CD4^+$ phenotype.

To compare the killer $CD4^+$ T cells with a 'conventional' helper $CD4^+$ phenotype, a combination of the tumour cell based vaccine GVAX and αCTLA-4 was used. GVAX consists of B16/BL6 cells which are genetically engineered to secret Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Dranoff et al. 1993; Huang et al. 1994). The cells are lethally irradiated (150 Gy) prior to injection intradermally where the residual GM-CSF secretion recruits antigen presenting cells (APCs) such as granulocytes,

macrophages, and dendritic cells to the vaccination site (Dranoff et al. 1993; Mach et al. 2000). The APCs are promoted to take up irradiated tumour cells and cell debris for antigen presentation in MHC II and cross-presentation in MHC I, leading to increased priming of B16 melanoma reactive $CD4^+$ T cells and $CD8^+$ respectively (Huang et al. 1994; Quezada 2006). GVAX therapy was shown to successfully promote prophylactic immunity to B16 melanoma and combination with αCTLA-4 results in tumour rejection; this strong anti-tumour effect, however, was only reported when αCTLA-4 and GVAX were given earlier (day 3) after a much lower tumour challenge ($1.2x10^4$) than in this study (Dranoff et al. 1993; Quezada 2006).

The therapy of adoptive transfer of $CD4^+$Trp1 T cells together with GVAX and αCTLA-4 was chosen to generate a comparable 'traditional' $CD4^+$ helper T cells as preliminary data from our laboratory and a recently published report suggests that this therapy induces $CD4^+$ helper activity, but not cytotoxicity, and contributes to tumour protection (Simpson et al. 2013).

To generate the phenotypically diverse $CD4^+$ T cells 6-8 week old, male C57BL/6 mice were challenged with $2.5x10^5$ B16/BL6 melanoma cells intradermally (i.d.) on one flank on day 0; by day 8 the tumours were established lesions of 3-6 mm in diameter.

For a hyporesponsive (tolerant) $CD4^+$ T cell phenotype the tumour bearing mice received $6x10^4$ naïve $CD4^+$Trp1 T cells purified from Trp1 mice intravenously (i.v.) on day 8 and no further additional treatment.

To promote a helper phenotype, the mice received $1x10^6$ cells of the previously irradiated cellular vaccine GVAX i.d. and 200 µg αCTLA-4 (9H10) intraperitoneally (i.p.) following the same $CD4^+$Trp1 transfer on day 8. Additionally, 100 µg αCTLA-4 were injected on day 11 and 14.

For the generation of cytotoxic ('killer') $CD4^+$Trp1 cells, tumour bearing mice were treated according to the original report by Quezada et al: animals were lymphodepleted prior to adoptive transfer of $CD4^+$Trp1 with 5 Gy full-body irradiation (radiation therapy, RT) and received 200 µg of αCTLA-4 i.p. after the injection of T cells on day 8. Furthermore, mice were also injected twice with 100 µg αCTLA-4 on day 11 and 14 (Quezada et al. 2010). The three treatments are summarised in figure 4.

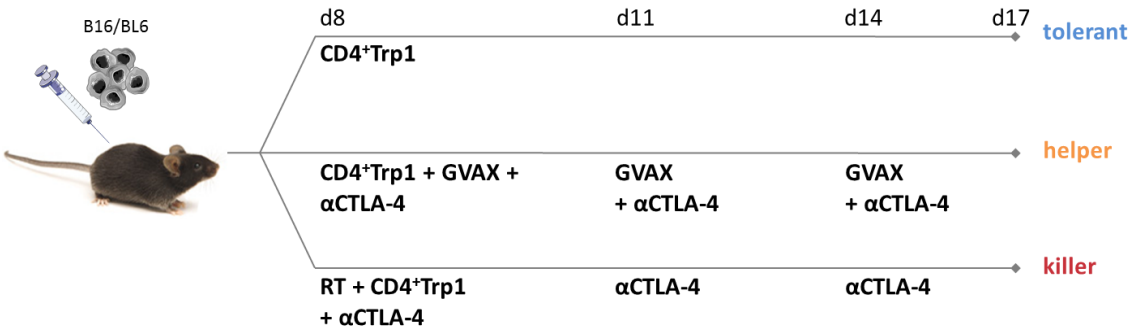

Figure 4 | Experimental set up for the in vivo generation of the three different CD4$^+$ phenotypes – tolerant, helper and killer CD4$^+$ T Cells. The different treatments of the B16/BL6 tumour bearing C57BL/6 mouse give rise to three disparate phenotypes: hyporesponsive (tolerant), helper and cytotoxic (killer) CD4$^+$ T cells. C57BL/6 mice were challenged with 2.5x10^5 B16/BL6 tumor cells intradermally (i.d.) on the flank on day 0. For the generation of the killer CD4$^+$ T cells, the mice were lymphodepleted on day 8 with 5 Gy total body irradiation (radiation therapy, RT). On the same day, killer and helper groups received 6x10^4 purified CD4$^+$Trp1 T cells intravenously and were injected intraperitoneally (i.p.) with 200 µg αCTLA-4 (9H10) and received further injections of 100 µg αCTLA-4 on days 11 and 14. For the helper phenotype, mice were additionally injected i.d. on the opposite flank to the tumour with 150 Gy irradiated 10^6 GVAX, a cellular vaccine based on GM-CFS secreting B16/BL6 cells on day 8, 11 and 14. The 'tolerant' phenotype group received only the adoptive cellular transfer of 6x10^4 purified CD4$^+$Trp1 cells. Mice were sacrificed on day 17 and the lymphocytes were extracted from tumour and peripheral (axillary, brachial, inguinal) lymph nodes for functional analysis.

4.1.1 Tolerant, helper and killer tumour reactive CD4$^+$Trp1 T cells generated *in vivo* exhibit different phenotypic properties

To study the phenotypic differences between tolerant, helper and killer CD4$^+$Trp1 T cells, tumour bearing mice treated with the three different therapies (Fig. 4) were sacrificed 9 days after treatment. T cells were isolated separately from tumours and axillary, brachial and inguinal lymph nodes and were stained with fluorescently labelled monoclonal antibodies and analysed by flow cytometry either immediately or after a 4 hour restimulation with exogenous DCs and TRP1 peptide. Additionally, tumour growth, survival and infiltration of T cells into the tumours were measured.

To simplify and for the purpose of clarity and consistency, CD4$^+$Trp1 T cells isolated from lymph nodes and tumours from mice receiving the 'killer' therapy, i.e. 5 Gy radiation (RT),

$6x10^4$ naïve $CD4^+$Trp1 T cells and αCTLA-4 injection, will henceforth be referred to as cytotoxic/killer $CD4^+$Trp1 T cells. This designation does not imply that 100 % of these cells show expression of Granzyme B (GzmB) but relates to the ability of this particular treatment to induce upregulation of GzmB and cytotoxic potential in these $CD4^+$ T cells leading to the eradication of established melanoma in mice (Quezada et al. 2010). Correspondingly, $CD4^+$Trp1 T cells isolated from mice receiving the 'helper' treatment ($CD4^+$Trp1 T cells, GVAX and αCTLA-4) will be referred to as helper $CD4^+$Trp1 T cells, although not 100 % of the isolated lymphocytes display a specific Th1 FACS profile; and $CD4^+$Trp1 T cells from mice receiving only $CD4^+$Trp1 adoptive transfer will be called tolerant $CD4^+$Trp1 T cells.

In keeping with previous published data (Quezada et al. 2010), the treatment consisting of lymphodepletion, $CD4^+$Trp1 T cell adoptive transfer and αCTLA-4 promoted long-term survival without relapse and eradication of the B16/BL6 tumour within 15 days of treatment (Fig. 5 A, B). Treatment of the tumour bearing mice with $CD4^+$Trp1 T cell transfer alone ('tolerant') or T cell transfer, GVAX and αCTLA-4 ('helper) failed to control tumour growth and induce rejection, resulting in poor survival (Fig. 5 A, B).

Historically, the poorly immunogenic B16 melanoma fails to be controlled and cleared by the endogenous immune compartments alone (Leveson et al. 1979) and also this study showed only low infiltration by endogenous effector lymphocytes with all three treatments (Fig. 5 E). The exceptionally low intratumoural $CD8^+$ and $CD4^+$ effector and regulatory lymphocyte count in the killer condition (RT + Trp1 + αCTLA-4) is most likely, at least in part, due to the lymphodepletion 9 days prior to analysis of the tissue. Mice which did not receive radiation therapy but instead were treated with either $CD4^+$Trp1 T cell transfer alone (tolerant) or T cell transfer, GVAX and αCTLA-4 (helper) displayed similar numbers of endogenous $Foxp3^-$ $CD4^+$ effector and $CD8^+$ T cells (Fig. 5 E). However, a lower $Foxp3^+$ $CD4^+$ regulatory T cell number was found in mice receiving the helper treatment (Fig 5 E); this was expected as we recently published that αCTLA-4 mediates selective depletion of regulatory T cells by macrophages in the tumour (Simpson et al. 2013). As shown in (Quezada et al. 2010), this depletion of Tregs is also observed in the killer condition and contributes, alongside the lymphodepletion, to the low Trp1 regulatory T cell number in the tumour (Fig. 5 E).

$CD4^+$Trp1 effector T cells successfully infiltrate the tumour when transferred into lymphodepleted, tumour bearing animal along with αCTLA-4 (Fig. 5 C). In contrast, the adoptive transfer of $CD4^+$Trp1 T cells alone (tolerant) or alongside GVAX and αCTLA-4 (helper) failed to induce potent infiltration and/or accumulation of the melanoma specific $CD4^+$ effector T cells in the tumour (Fig. 5 C). The low number of endogenous and Trp1

Tregs and accumulation of CD4$^+$Trp1 effector T cells in the tumour treated with the 'killer' therapy result in a high CD4$^+$Trp1 eff/total Treg (endogenous and Trp1) ratio (Fig. 5 D)

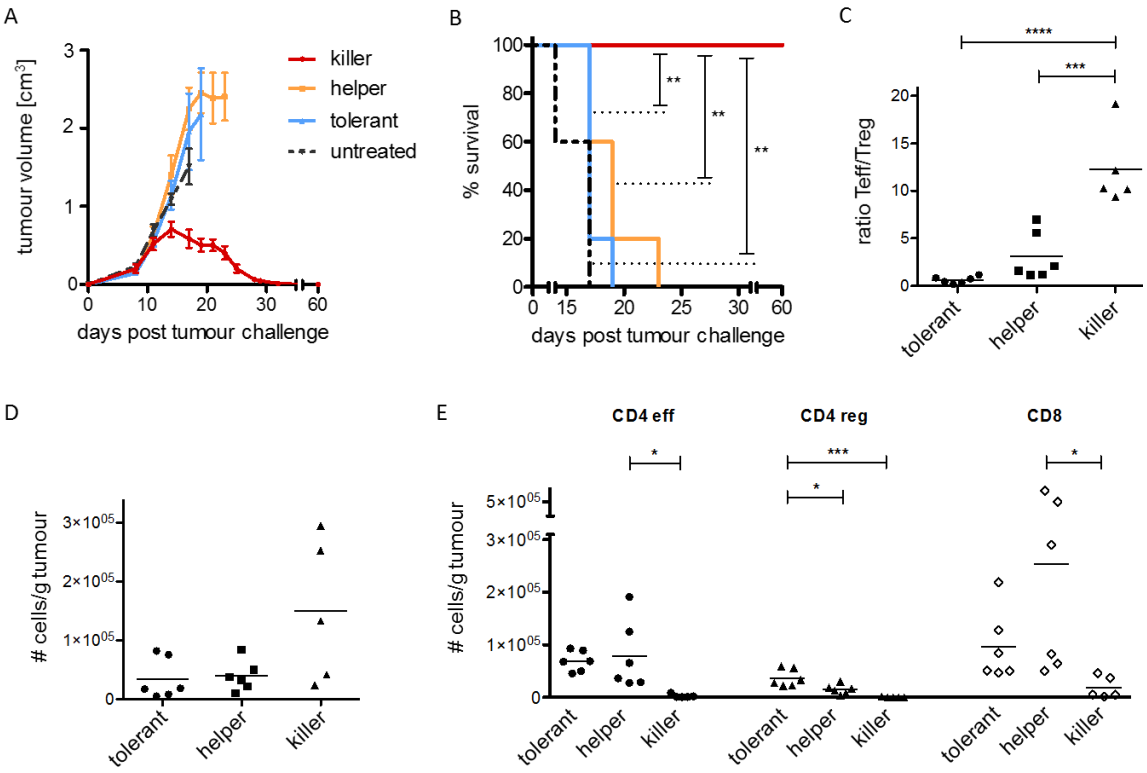

Figure 5 | Killer, but not tolerant and helper CD4$^+$Trp1 T cells promote survival and decrease B16/BL6 melanoma burden by infiltration of high numbers of CD4$^+$Trp1 effector and low numbers of regulatory T cells. The three adoptive cellular therapy treatments (tolerant: CD4$^+$Trp1 transfer, helper: CD4$^+$Trp1 transfer + GVAX + αCTLA-4 and killer: RT + CD4$^+$Trp1 transfer + αCTLA-4) have different impacts on the survival, tumour growth and CD4$^+$ effector and regulatory T cell infiltration. Treatments as described in Fig. 4. **A:** mean tumour growth of mice receiving the three different T cell treatments. **B:** survival of B16/BL6 tumour bearing C57BL/6 mice following different treatments. **C:** Quantification of the absolute number of Foxp3$^-$ CD4$^+$Trp1 effector cells per gram tumour. **D:** ratio of intratumoural Foxp3$^-$ CD4$^+$Trp1 effector cells over the number of total Foxp3$^+$ Tregs (Trp1 and endogenous). **E:** Quantification of the absolute number of endogenous CD8, Foxp3$^-$ CD4$^+$ effector and Foxp3$^+$ CD4$^+$ regulatory T cells per gram tumour. Presented is representative data from one of three independent experiments, n = 3-6 mice per group. Numbers of tumour infiltrating T cells were calculated as described in Materials and methods. A one way ANOVA with a Bonferroni post-test was performed to calculate statistical differences between groups, * : P ≤ 0.05, ** : P ≤ 0.01, *** : P ≤ 0.001.

which has previously been shown to be favourable for overall survival (Quezada & Peggs 2011).
Correspondingly, the higher number of endogenous and $CD4^+$Trp1 regulatory T cells and low $CD4^+$Trp1 effector accumulation observed in tumours treated with 'tolerant' or 'helper' treatment regime lead to an unfavourable, significantly lower ratio of Teff/Treg (Fig. 5 C).

To characterise and assess the functional phenotype of the cytotoxic $CD4^+$Trp1 T cells in comparison to their helper and tolerant counterparts, T cells isolated from tumours and lymph nodes were stained for multi-colour flow cytometry analysis. Granzyme B (GzmB) expression was analysed as well as Th1 and Th2 specific transcription factors T-bet and Gata3, Ki67 as a proliferation marker and inflammatory cytokines restimulation of the $CD4^+$Trp1 T cells with their cognate antigen.
In keeping with published data (Quezada et al. 2010), a high proportion (67.6 %) of killer $CD4^+$Trp1 effector T cells expressed GzmB in the tumour while only 33 % of tumour infiltrating helper $CD4^+$Trp1 expressed the cytotoxicity marker (Fig. 6 A). Cytotoxic activity of killer $CD4^+$Trp1 effectors was further verified by assessment of the expression of degranulation markers CD107a/b (LAMP-1/2) after re-stimulation: ~91 % of tumour infiltrating killer T cells expressed both degranulation markers while only up to 37 % of peripheral $CD4^+$Trp1 effectors from the lymph nodes displayed degranulation (Fig. 6 B). Tolerant $CD4^+$Trp1 isolated from the tumour showed low cytotoxic activity based on GzmB expression *ex vivo* (up to 16.5 %) (Fig. 6 A) and did not secrete the Th1 cytokines IFNγ, IL-2 and TNF-α after restimulation with their cognate antigen (Fig. 6 E-G). However, varying proportions of tumour infiltrating killer and helper $CD4^+$Trp1 effector cells expressed all three effector cytokines (Fig. 6 E-G). As published, killer $CD4^+$Trp1 cells displayed high levels of Th1 cytokine secretion (Quezada et al. 2010), while the frequency of helper $CD4^+$Trp1 T cells expressing IFNγ, IL-2 and/or TNFα was significantly lower (Fig. 6 E-G).
The three different treatment regimens also influenced the *in vivo* proliferative status of the $CD4^+$Trp1 effector T cells in the tumour: based on Ki67 expression, hyporesponsive ('tolerant') $CD4^+$Trp1 cells displayed only a low proliferation rate whereas 90-95 % of both helper and killer $CD4^+$Trp1 T cells were found to be highly proliferative (Fig. 6 C).
Further analysis of the three subsets revealed differences in the expression of Th1 and Th2 lineage specific transcription factors T-bet and Gata3 but none of the groups displayed a specific Th1 or Th2 commitment. The tolerant $CD4^+$Trp1 in the tumour were a mixed population with up to 13 % of the cells being either T-bet^+ or $Gata3^+$ (single positive, dark and light grey) and 26 % T-bet^+ $Gata3^+$ (double positive, white, dotted) cells (Fig 6 D). Interestingly, there was less variability in the killer and helper $CD4^+$Trp1

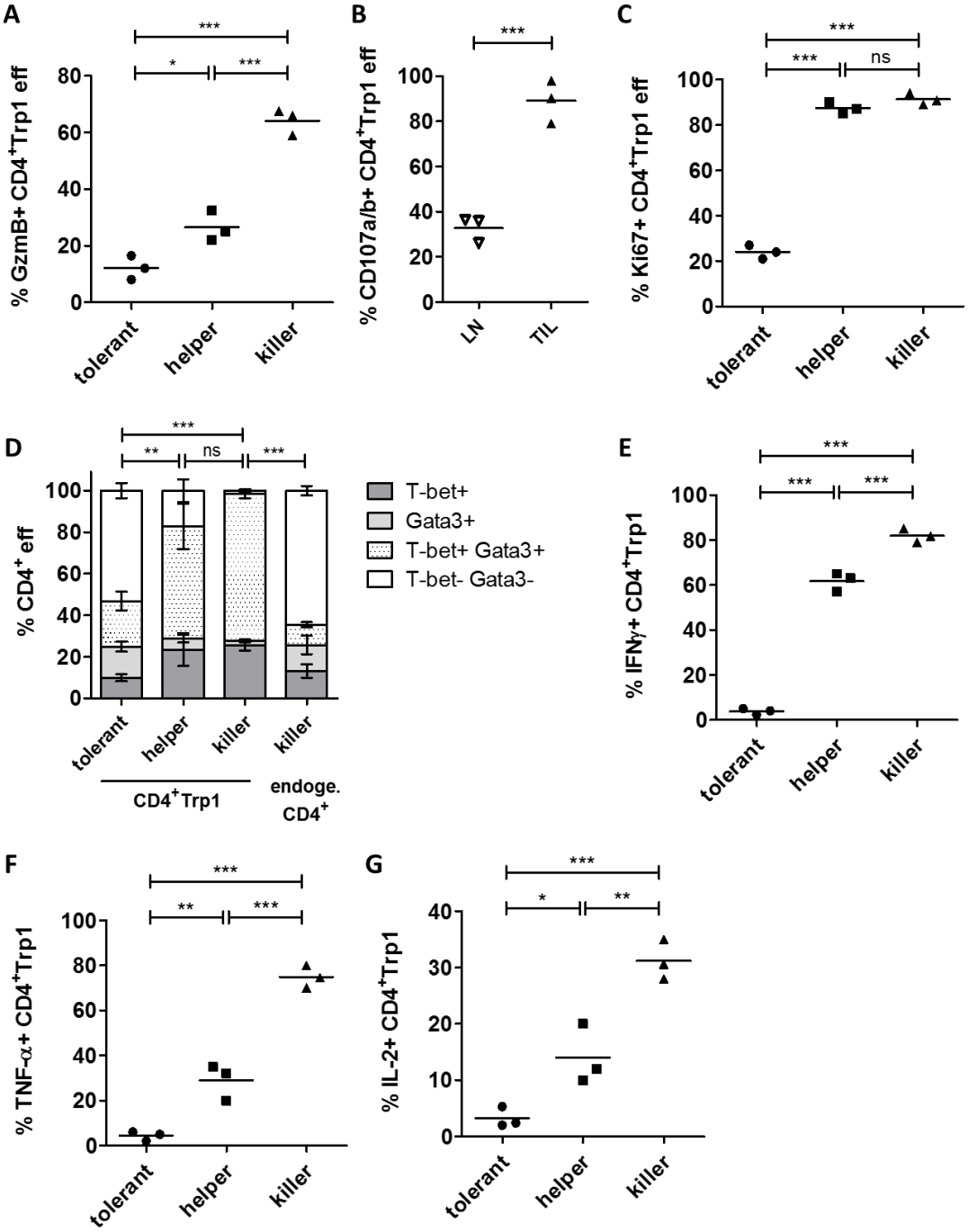

Figure 6 | Tumour infiltrating tolerant, helper and killer CD4⁺Trp1 display phenotypic differences by proliferation, transcription factor and effector molecule expression. Foxp3⁻ CD4⁺Trp1 effector T cells isolated from B16/BL6 tumours treated with either the tolerant, helper or killer therapy (treatments as described in Fig. 4) differ in GzmB expression, proliferation, cytokine secretion after re-stimulation and Th1/Th2 specific transcription factor expression. **A:** GzmB expression of Foxp3⁻ CD4⁺Trp1 effector T cells after isolation from B16/BL6 tumours. **B:** Expression of degranulation markers CD107a and CD107b on CD4⁺Trp1 effectors from the periphery (LN) and

tumour (TIL) after re-stimulation ex vivo for 4 hours. **C:** Expression of proliferation marker Ki67 on Foxp3$^-$ CD4$^+$Trp1 effector T cells after isolation from B16/BL6 tumours. **D:** Expression pattern of Th1 and Th2 specific transcription factors T-bet and Gata3 on Foxp3$^-$ CD4$^+$Trp1 and endogenous CD4$^+$ effector T cells after isolation from B16/BL6 tumours. T-bet+ and Gata3+ (light and dark grey) represent cells expressing only one *or* the other transcription factor. **E-G:** Expression of IFNγ, IL-2 and TNF-α of tumour infiltrating Foxp3$^-$ CD4$^+$Trp1 effector T cells after re-stimulation ex vivo for 4 hours. Presented is representative data from one of three independent experiments, n = 3-6 mice per group. A one way ANOVA with a Bonferroni post-test was performed to calculate statistical differences between groups, * : $P \leq 0.05$, ** : $P \leq 0.01$, *** : $P \leq 0.001$.

T cells with the vast majority of the tumour infiltrating cells co-expressing both Th1 and Th2 transcription factors (Fig 6 D). Lower numbers of helper CD4$^+$Trp1 T cells were single positive for only one of the transcription factors (up to 12 % T-bet$^+$, 7% Gata3$^+$) whereas 95 % of killer CD4$^+$Trp1 T cells expressed T-bet and 75 % of these co-expressed Gata3 (Fig 6 D). The endogenous effector CD4$^+$ compartment only co-expressed Gata3 and T-bet in up to 12 % of tumour infiltrating cells while the majority (up to 65 %) did not express either of the transcription factors (Fig. 6 D).

Despite of the CD4$^+$ T cells having an identical specificity through carrying the same T cell receptor (TCR), this study demonstrates that different therapeutic interventions can have a tremendous impact on the differentiation of the CD4$^+$Trp1 T cells *in vivo*. The three types of melanoma specific T cells presented in this work do not only display differences in phenotype and effector function but also their ability to proliferate and be recruited or infiltrate the tumour site efficiently and eradicate the melanoma lesion.

Collectively, this data supports this model of generating tolerant and helper CD4$^+$ T cell phenotypes in juxtaposition to the described cytotoxic CD4$^+$Trp1 T cells. The availability of this practical model will prove advantageous for further analysis of the cytotoxic CD4$^+$ phenotype and understanding of the mechanisms underpinning the *in vivo* differentiation of tumour reactive T cells into different effector subsets

4.2 Gene expression profiling of tumour reactive killer T cells reveals significant differences to naïve, tolerant and helper CD4$^+$Trp1 T cells

In order to characterise of the molecular components such as transcription factors, important signalling pathway enzymes or effector molecules that contribute and control

the potent cytotoxic phenotype of the CD4$^+$Trp1 T cells, a microarray was performed. The analysis of the transcriptomes of CD4$^+$ cells from naïve Trp1 mice and CD4$^+$Trp1 cells from tumour and lymph nodes of the tolerant, helper and killer condition treated mice allows a comparison between the subsets but also between the two sites, which correlates with different degrees of activation, within a subset (e.g. CD4$^+$Trp1 killer T cells in the lymph node vs CD4$^+$Trp1 killer T cells in the tumour).
Having several phenotypically diverse subsets as comparison to the killer CD4$^+$ T cells allows a broader and more realistic analysis of the subsets and the genes involved in the differentiation of the cytotoxic CD4$^+$Trp1 T cells than a simple comparison between highly activated killer and antigen-inexperienced naïve T cells.

The microarray of choice was the GeneChip® Mouse Genome 430 2.0 Array which allows the simultaneous analysis of over 39,000 transcripts (Affymetrix® 2004). C57BL/6 mice were injected with $2x10^5$ B16/BL6 cells followed by $5x10^4$ - $8x10^4$ purified naïve CD4$^+$Trp1 FoxGFP cells 9 to 10 after tumour challenge. As previously described, the mice of the tolerant group were left untreated while the mice were irradiated prior to T cell transfer to promote the killer phenotype or vaccinated with GVAX for the helper phenotype (see chapter *4.1*). Additionally, the helper and killer groups received 200 µg αCTLA-4 (9H10) on days 10, 13 and 16. The lymph nodes and tumours were dissected 8 days after therapy and the T cells were sorted for CD4$^+$Trp1 effectors (CD4$^+$GFP$^-$ CD45.1$^+$). Naïve CD25$^-$ GFP$^-$ CD4$^+$Trp1 cells were isolated from lymph nodes of Trp1 FoxGFP mice and RNA for the array was isolated from the purified CD4$^+$Trp1 cells of the different conditions.
The different CD4$^+$ samples in the following analyses are termed based on the treatment cohort and site of origin: CD4$^+$ T cell subset (tolerant, helpers, killers, naïve) from tumour (TIL) or lymph nodes (LN).

As the first analysis, principal component analysis (PCA) was performed using the unfiltered expression values of all subsets. For the computation of PCA the function *dudi.pca* of CRAN package *ade4* was used. PCA showed a favourable separation of the T cell subsets and hence indicated a good quality gene profiling with low gene expression variability amongst triplicates (Fig. 7). Furthermore, PCA demonstrated qualitatively that killer CD4$^+$Trp1 T cells isolated from the tumour or lymph nodes were highly distinct from naïve, tolerant and helper CD4$^+$Trp1 T cells.

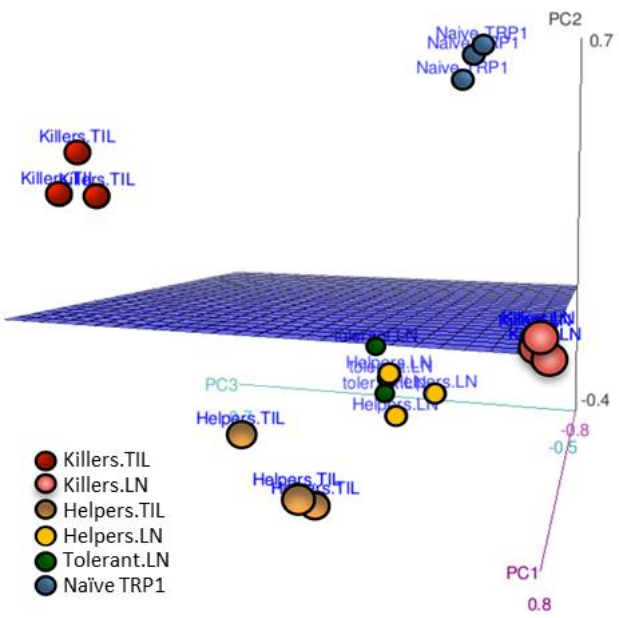

Figure 7 | Tumour infiltrating CD4⁺Trp1 effector T cells from the killer condition are transcriptionally distinct from CD4⁺Trp1 cells from naïve TRP1 mice and the tolerant and helper condition. 3D PCA plot of the different phenotypically diverse CD4 subsets (tolerant, helpers, killers, naïve) from different sites (tumour (TIL) and lymph nodes (LN)) using the gene expression values from the GeneChip® Mouse Genome 430 2.0 Array. C57BL/6 mice were challenged with B16/BL6 and treated with killer, helper and tolerant regimens as described in chapter *4.1*. Mice were sacrificed 8 days after adoptive transfer and RNA was isolated from GFP⁻ CD4⁺Trp1 effector cells and used for the GeneChip® Mouse Genome 430 2.0 Array. Naïve GFP⁻ CD4⁺Trp1 cells were isolated from lymph nodes of Trp1 FoxGFP mice. n=8-10 mice per group. Experiments were performed in triplicates.

4.2.1 Transcriptome analysis of killer CD4⁺Trp1 TILs reveals a mixed Th and CD8⁺ CTL phenotype

In order to investigate the phenotype of the killer CD4⁺Trp1 further and examine potential similarities to CD4⁺ helper subsets and CD8⁺ T cells, the expression values of key transcription factors, effector molecules and cell-type specific markers of Th0, Th1, Th2, Th9, Th17, Th22, follicular helper (Tfh) and regulatory CD4⁺ T cells (Treg) as well as cytotoxic CD8⁺ T cells were analysed.

To provide a comprehensive overview, the different T cell subsets are presented as heat map analyses, each depicting a panel of genes encoding characteristic markers for each subset. For this analysis the functions *heatmap.2* and *breaks* of the CRAN package *gplots* were used in R. In the heat maps, the scaled expression value (Row Z-Score) is illustrated in a red and green colour scale, red denoting high and green low expression of the respective gene.

It is important to note that as the expression values were standardised per individual probe set (gene), the expression values of a gene can be compared across samples but the expression levels cannot be directly compared between genes.
In the heat maps each column represents a sample, for instance 'Tolerant.LN1' denotes the microarray data from $CD4^+$ T cells which were isolated from lymph nodes (LN) from mice of the tolerant cohort from experiment 1 out of 3, and each row represents a probe set corresponding to a specific gene.

4.2.1.1 Examination of Th0, Th1 and Th2 marker expression

Killer $CD4^+$Trp1 in the tumour (Killers.TIL1/2/3) and in the periphery (Killers.LN1/2/3) show higher expression of *Zbtb7b*, which encodes $CD4^+$ lineage transcription factor ThPok, than tolerant, helper and even naïve $CD4^+$Trp1 T cells (Fig. 8). Between the two killer groups (LN and tumour), *Zbtb7b* expression was higher in the tumour infiltrating T cells than in the periphery. This suggests that the acquired cytotoxic phenotype was not caused by the loss of the $CD4^+$ lineage specific transcription factor.

Emphasising the highly plastic phenotype of the cytotoxic $CD4^+$Trp1 T cells, Th0 related cytokines such as TNF-α (encoded by *Tnf*) and –β (*Lta*), IFNγ, IL-13, IL-2, IL-3, IL-4, IL-5, IL-6 and GM-CSF (*Csf2*) were highly expressed in the tumour infiltrating killer $CD4^+$Trp1 (Fig. 8). But not only cytotoxic $CD4^+$Trp1 T cells but also helper $CD4^+$Trp1 TILs (Helpers.TIL1/2/3) showed upregulation and comparable expression of GM-CSF, IFNγ, IL-2, IL-3, IL-5 and IL-13 to the $CD4^+$ killer T cells in the tumour which points to a possible involvement of the tumour microenvironment on cytokine expression of $CD4^+$Trp1. IL-4 and IL-6 expression, however, was significantly increased on killer TILs while TNF-β expression was highest in helper TILs.

When examining the expression of characteristic Th1 makers, killer $CD4^+$Trp1 T cells in the tumour display a striking Th1-like expression pattern (Fig. 8): Th1 lineage transcription factor T-bet (encoded by *Tbx21*) expression was elevated at a similar level across killer $CD4^+$Trp1 T cells from lymph nodes and tumour and helper TILs and was only slightly reduced in tolerant and helper $CD4^+$Trp1 T cells in the periphery (LN). Similarly, IRF4 expression was increased in killer TILs and the other differentiated T cell

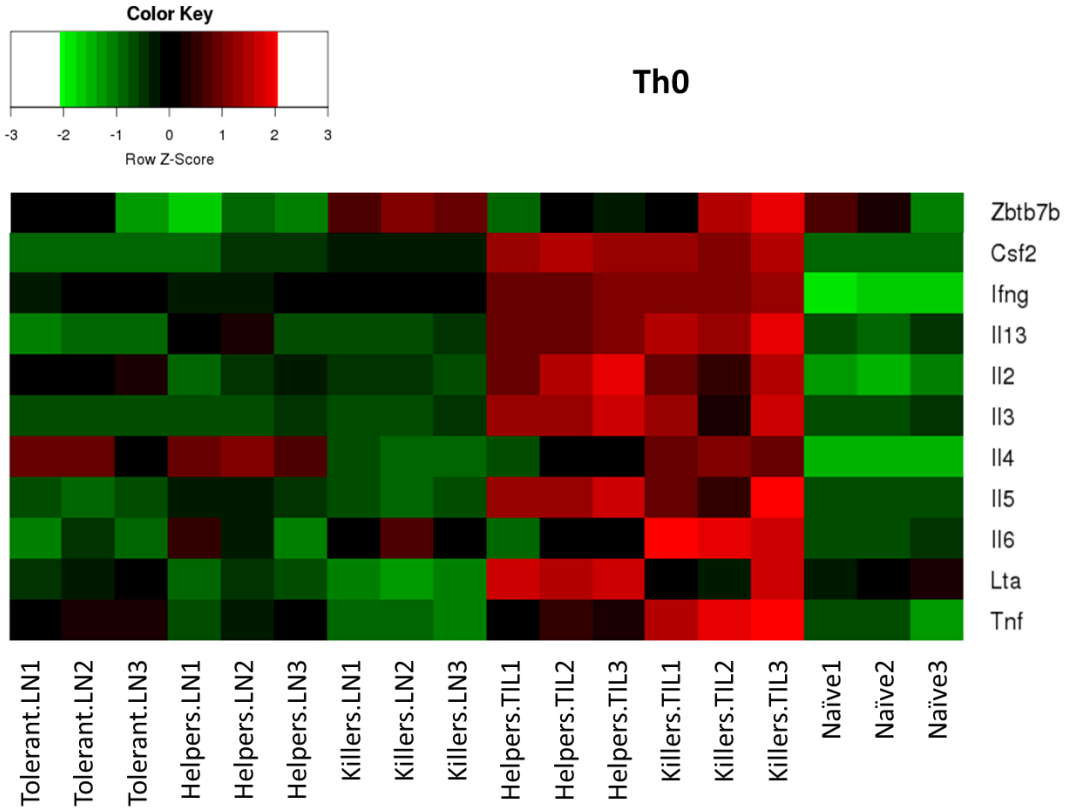

Figure 8 | Heat map analysis of microarray data using a panel of genes encoding Th0 markers and CD4⁺ T cell lineage marker ThPok. Displayed are genes encoding ThPok (*Zbtb7b*), GM-CSF (*Csf2*), IFNγ (*Ifng*), IL-13 (*Il13*), IL-2 (*Il2*), IL-3 (*Il3*), IL-4 (*Il4*), IL-5 (*Il5*), IL-6 (*Il6*), TNF-β (*Lta*) and TNF-α (*Tnf*). Colour coding is used to display high (red) and low (green) expression of a genes across the experimental groups: tolerant, helper, killer and naïve CD4⁺Trp1 effector T cells extracted from tumour (TIL) or lymph nodes (LN) from treated mice (treatment regimens as described in chapter *4.1*). Mice were sacrificed 8 days after adoptive transfer and RNA was isolated from GFP⁻ CD4⁺Trp1 effector cells and used for the GeneChip® Mouse Genome 430 2.0 Array. Naïve GFP⁻ CD4⁺Trp1 cells were isolated from lymph nodes of Trp1 FoxGFP mice. n=8-10 mice per group. Experiments were performed in triplicates, each indicated by 1/2/3.

subsets (tolerant, helper) except for naïve CD4⁺Trp1 T cells. Ets transcription factor ERM (encoded by *Ermn*) expression was upregulated in killer CD4⁺Trp1 in the tumour and LN and slightly lower in helper TILs. Also STAT4 expression was upregulated on killer and helper TILs but also in tolerant CD4⁺Trp1 T cells. Transcription factor HLX was significantly upregulated in killer TILs in comparison to helper TILs and killer CD4⁺Trp1 T cells from the LN (Fig. 9). The specific high expression of these transcription factors on tumour infiltrating CD4⁺Trp1 T cells suggests a potential importance for these proteins for the development and/or maintenance of the cytotoxic CD4⁺ phenotype.

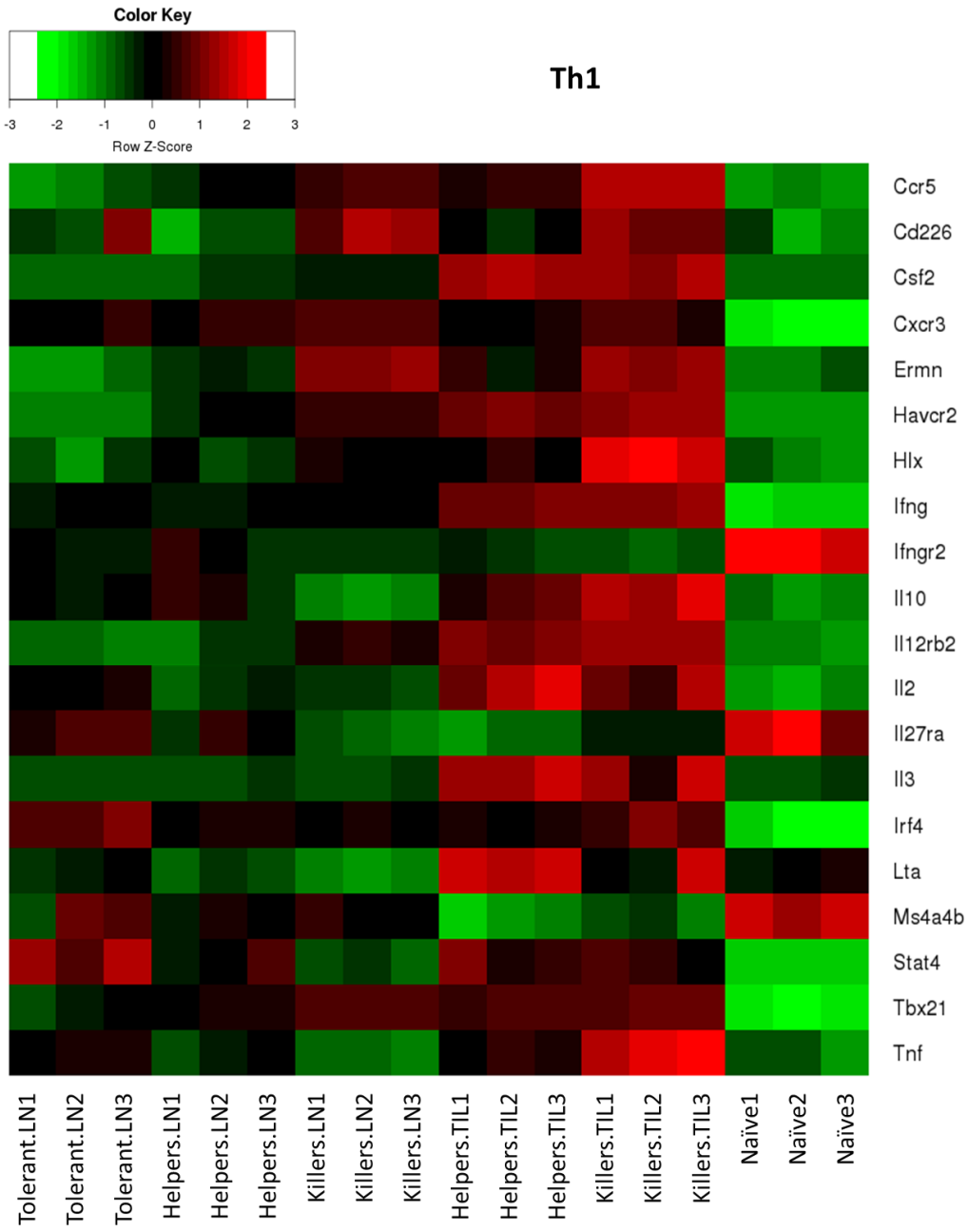

Figure 9 | Heat map analysis of microarray data using a panel of genes encoding Th1 markers. Displayed are genes encoding GM-CSF (*Csf2*), chemokine receptors CCR5 (*Ccr5*) and CXCR3 (*Cxcr3*), CD226 (*Cd226*), ERM (*Ermn*), TIM3 (*Havcr2*), HLX (*Hlx*), IFNγ (*Ifng*), IFNγ R2 (*Ifngr2*), IL-10 (*Il10*), IL-12R (*Il12rb2*), IL-2 (*Il2*), IL-27 R-α/WSX-1 (*Il27ra*), IL-3 (*Il3*), IRF4 (*Irf4*), TNF-β (*Lta*), Chandra (*Ms4a4b*), STAT4 (*Stat4*), T-bet (*Tbx21*) and TNF-α (*Tnf*). Colour coding is used to display high (red) and low (green) expression of a genes across the experimental groups: tolerant, helper, killer and naïve $CD4^{+}$Trp1 effector T cells extracted from tumour (TIL) or lymph nodes (LN) from treated mice (treatment regimens as described in chapter *4.1*). Mice were sacrificed 8 days after adoptive transfer and RNA was isolated from GFP^{-} $CD4^{+}$Trp1 effector cells

and used for the GeneChip® Mouse Genome 430 2.0 Array. Naïve GFP$^-$ CD4$^+$Trp1 cells were isolated from lymph nodes of Trp1 FoxGFP mice. n=8-10 mice per group. Experiments were performed in triplicates, each indicated by 1/2/3.

Besides transcription factors, also Th1 cytokines GM-CSF (*Csf2*), IFNγ, IL-2, TNF-α (*Tnf*), IL-10 and IL-3 were all highly expressed in tumour infiltrating killer CD4$^+$Trp1 T cells. *Tnf* and *Il10* expression was significantly increased in killer TILs in comparison with helper TILs while *Lta* (encoding TNF-β) expression levels were significantly higher in the helper T cells (Fig. 9).
Also expression of the co-inhibitory molecule TIM-3 (encoded by *Havcr2*) and chemokine receptors CCR5 and CXCR3 was increased on killer TILs. *Ccr5* upregulation was statistically significantly in comparison with all experimental group on the array. The co-stimulatory molecule CD226 showed significantly higher expression in killer CD4$^+$Trp1 T cells from the tumour and the periphery (LN) than in any other group (Fig. 9).
Interestingly, as typically seen in Th1, killer TILs show a distinct downregulation of the second chain of IFNγ receptor (IFNγ R2), encoded by *Ifngr2*, while the IL-12 receptor (IL-12R, *Il12rb2*) whose expression is specific for Th1 and is lost in Th2 T cells (Szabo et al. 1997; Usui et al. 2006) was highly expressed in helper TILs and killer CD4$^+$ T cells from the tumour and lymph nodes (Fig. 9).
However, CD4$^+$Trp1 killer TILs only displayed low expression of the IL-12 receptor homologue IL-27 R-α (WSX-1), encoded by *Il27ra*, which is normally found highly expressed on Th1 T cells. Similarly, the expression of the CD20 homologue Chandra (encoded by *Ms4a4b*) which has been shown to be exclusively expressed on Th1 but not Th2 T cells (Venkataraman et al. 2000; Xu et al. 2006), however, was low on CD4$^+$Trp1 killers in the tumour and only slightly elevated in the periphery (LN) (Fig. 9) and therefore might not be necessary for the killer CD4$^+$ functionality.

Examination of Th2 markers revealed a partially Th2 skewed phenotype: cytotoxic CD4$^+$Trp1 TILs did not display high levels of Th2 specific transcription factor *Gata3* mRNA but other Th2 associated transcription factors such as Amphiregulin (encoded by *Areg*), Gfi1, IRF4, JunB, c-Maf (*Maf*), STAT5 and STAT6 were moderately to highly expressed (Fig. 10) and might thus be important for the differentiation of the CD4$^+$ cytotoxic phenotype.
Also important type 2 cytokines IL-3, IL-4, IL-5, IL-6, IL-13 and IL-25 were highly abundant in killer CD4$^+$Trp1 TILs; the expression of IL-4, IL-6 and IL-25 transcripts was even significantly increased in comparison with helper CD4$^+$Trp1 TILs. On the other

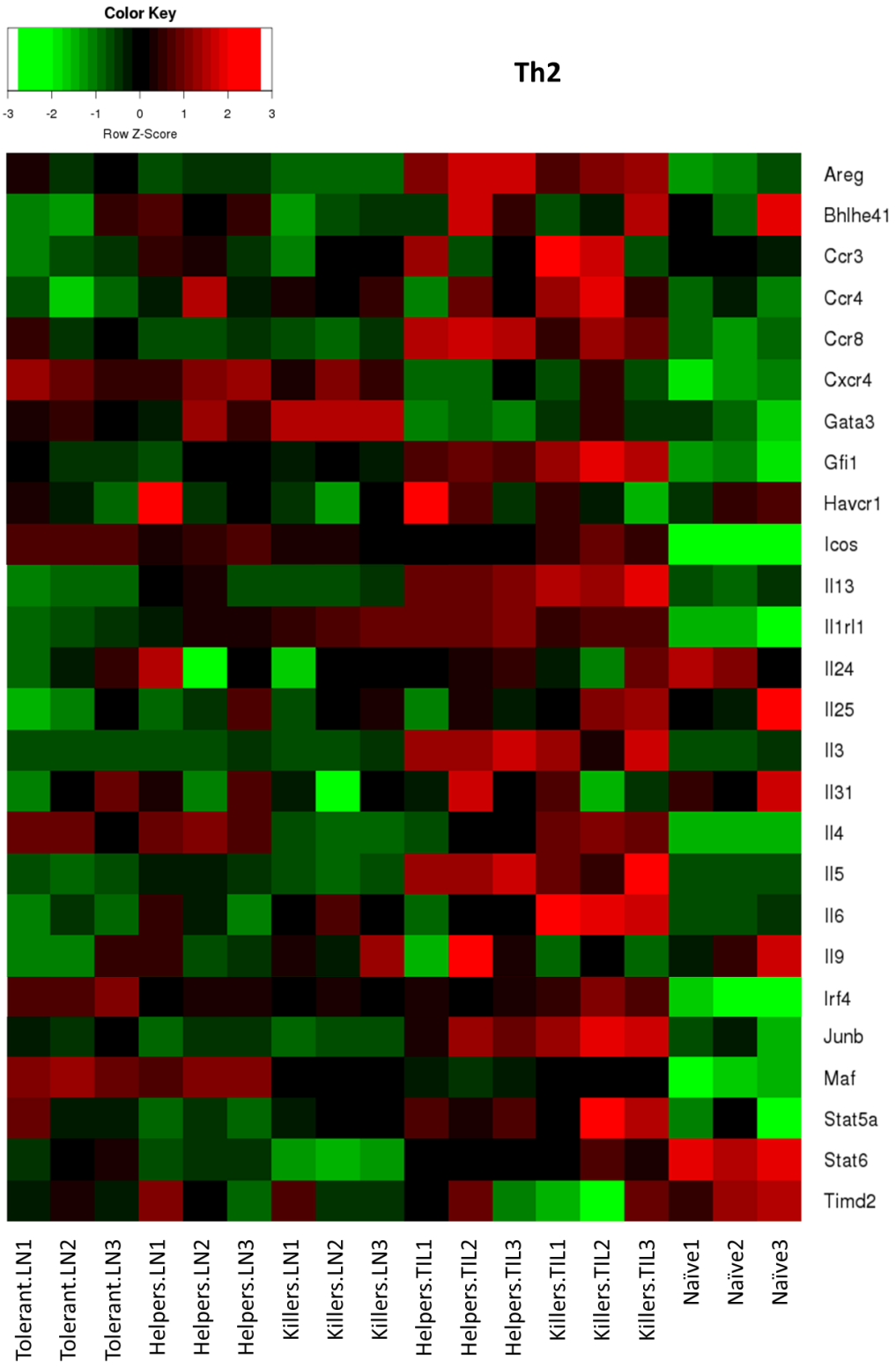

Figure 10 | Heat map analysis of microarray data using a panel of genes encoding Th2 markers. Displayed are genes encoding Amphiregulin (*Areg*), Dec2 (*Bhlhe41*), chemokine receptors CCR3 (*Ccr3*), CCR4 (*Ccr4*), CCR8 (*Ccr8*) and CXCR4 (*Cxcr4*), Gata3 (*Gata3*), Gfi1 (*Gfi1*), TIM1 (*Havcr1*), ICOS (*Icos*), IL-13 (*Il13*), ST2 (*Il1rl1*), IL-24 (*Il24*), IL-25 (*Il25*), IL-3 (*Il3*), IL-31 (*Il31*), IL-4 (*Il4*), IL-5 (*Il5*), IL-6 (*Il6*), IL- 9 (*Il9*), IRF4

(*Irf4*), JunB (*Junb*), c-Maf (*Maf*), STAT5 (*Stat5*), STAT6 (*Stat6*) and TIM2 (*Timd2*). Colour coding is used to display high (red) and low (green) expression of a genes across the experimental groups: tolerant, helper, killer and naïve CD4$^+$Trp1 effector T cells extracted from tumour (TIL) or lymph nodes (LN) from treated mice (treatment regimens as described in chapter *4.1*). Mice were sacrificed 8 days after adoptive transfer and RNA was isolated from GFP$^-$ CD4$^+$Trp1 effector cells and used for the GeneChip® Mouse Genome 430 2.0 Array. Naïve GFP$^-$ CD4$^+$Trp1 cells were isolated from lymph nodes of Trp1 FoxGFP mice. n=8-10 mice per group. Experiments were performed in triplicates, each indicated by 1/2/3.

hand, Th2 associated cytokines IL-31, IL- 9 and IL-24 showed only low levels of expression on killer CD4$^+$ TILs (Fig. 10).
Also the characteristic Th2 chemokine receptors CCR3, CCR4 and CCR8 and the Toll-like receptor superfamily member ST2 (*Il1rl1*) were highly expressed on tumour infiltrating killer CD4$^+$Trp1 cells, only CXCR4 mRNA levels were found to be low.
Despite the highly activated state of CD4$^+$Trp1 TILs, the immune checkpoint and Th2 associated molecules TIM1 (encoded by Havcr1) and TIM2 (Timd2) displayed only moderate and low expression in comparison to helper CD4$^+$Trp1 TILs, respectively, and ICOS expression was only slightly increased (Fig. 10).

4.2.1.2 Examination of Th17, Th22 and Th9 marker expression

Killer CD4$^+$Trp1 TILs do not display a Th17 phenotype on transcript level as only few Th17 markers were found to be expressed: cytokines IL-17 (encoded by *Il17a*) and IL-21 and chemokine CCL20 were not upregulated in comparison to helper TILs but IL-6 and TNF-α (*Tnf*) were significantly increased (Fig. 11).
Th17 specific transcription factor RORγt (*Rorc*) was slightly downregulated on the killers in the tumour and TCF-1 (*Tcf7*) expression was the lowest in comparison to all experimental groups; high expression was only observed in naïve and tolerant and helper LN. This suggests that RORγt does not play an important part in the development and/or maintenance of the cytotoxic CD4$^+$ phenotype and fits with published research which shows that TCF-1 expression is rapidly downregulated in effector T cells (Willinger et al. 2006).
Other transcription factors such as BATF, AHR, IRF4, RORα and STAT3 which are not exclusive to Th17 cells displayed very similarly high expression values in the both tumour infiltrating T cell datasets (helper and killer). The Th17 and NK receptor CD161 (*Klrb1c*),

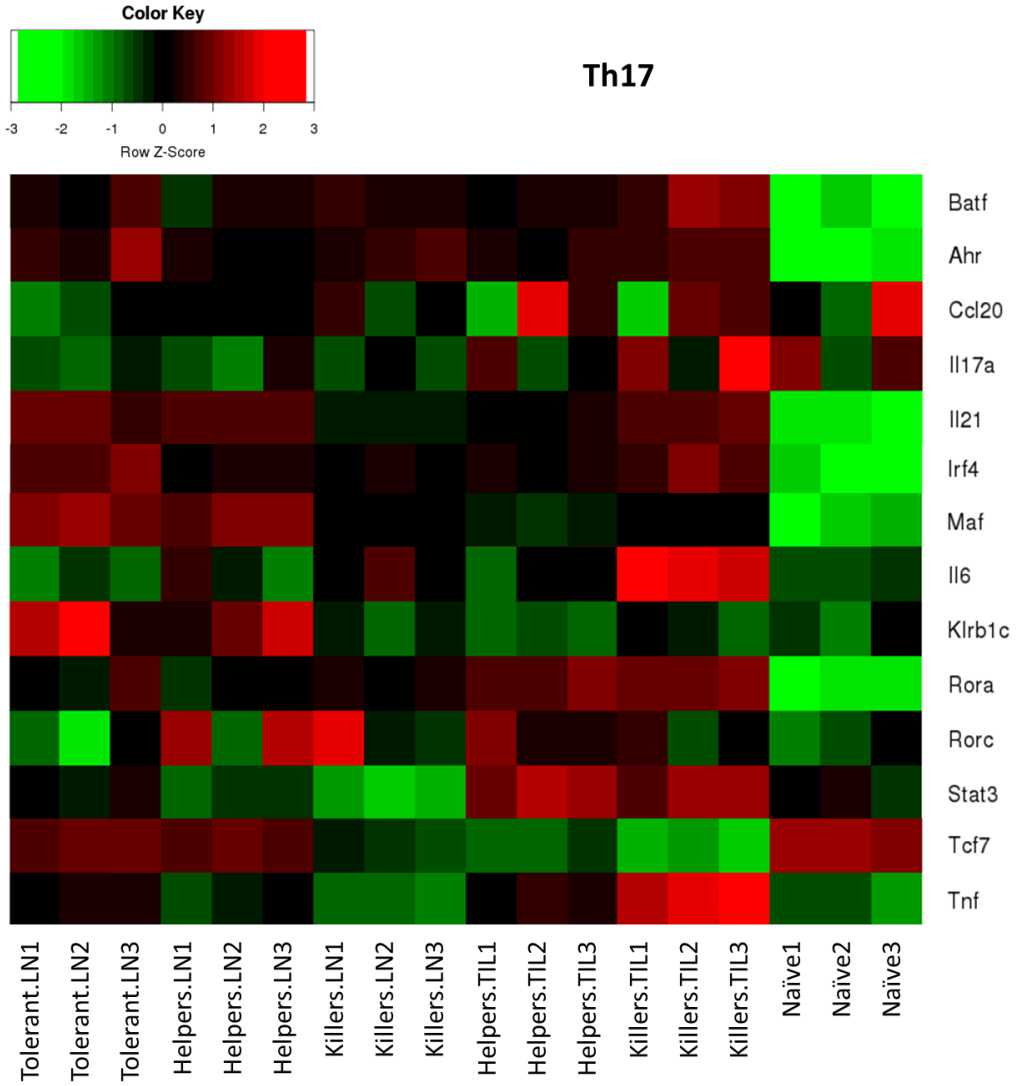

Figure 11 | Heat map analysis of microarray data using a panel of genes encoding Th17 markers. Displayed are genes encoding BATF, (*Batf*), AHR (Ahr), CCL20 (*Ccl20*), IL-17 (*Il17a*), IL-21 (*Il21*), IL-6 (*Il6*), IRF4 (*Irf4*), c-Maf (*Maf*), CD161 (*Klrb1c*), RORα (*Rora*), RORγt (*Rorc*), STAT3 (*Stat3*), TCF-1 (*Tcf7*) and TNF-α (*Tnf*). Colour coding is used to display high (red) and low (green) expression of a genes across the experimental groups: tolerant, helper, killer and naïve $CD4^{+}$Trp1 effector T cells extracted from tumour (TIL) or lymph nodes (LN) from treated mice (treatment regimens as described in chapter *4.1*). Mice were sacrificed 8 days after adoptive transfer and RNA was isolated from GFP^{-} $CD4^{+}$Trp1 effector cells and used for the GeneChip® Mouse Genome 430 2.0 Array. Naïve GFP^{-} $CD4^{+}$Trp1 cells were isolated from lymph nodes of Trp1 FoxGFP mice. n=8-10 mice per group. Experiments were performed in triplicates, each indicated by 1/2/3.

however, was only weakly expressed on killer CD4$^+$Trp1 TILs (Fig. 11) and emphasises the notion that Th17 signature is not part of the highly plastic killer CD4$^+$Trp1 phenotype. CD4$^+$Trp1 killers in the tumour also showed only little resemblance to Th22 cells: expression of the transcription factor *Ahr* which was found important for Th22 differentiation and maintenance, was moderately high but not increased in comparison to any differentiated subset (Fig. 12).

Of the Th22 characteristic chemokine receptor combination of CCR10, CCR4 and CCR6 (Duhen et al. 2009; Ramirez et al. 2010) only *Ccr4* expression was increased on killer CD4$^+$Trp1 TILs while *Ccr6* was significantly downregulated and *Ccr10* only slightly increased in comparison with helper TIL (Fig. 12).

Genes encoding Th22 cytokines TNF-α and IL-13 (Eyerich et al. 2009; Duhen et al. 2009), however, were significantly upregulated in killer CD4$^+$T cells (Fig. 12).

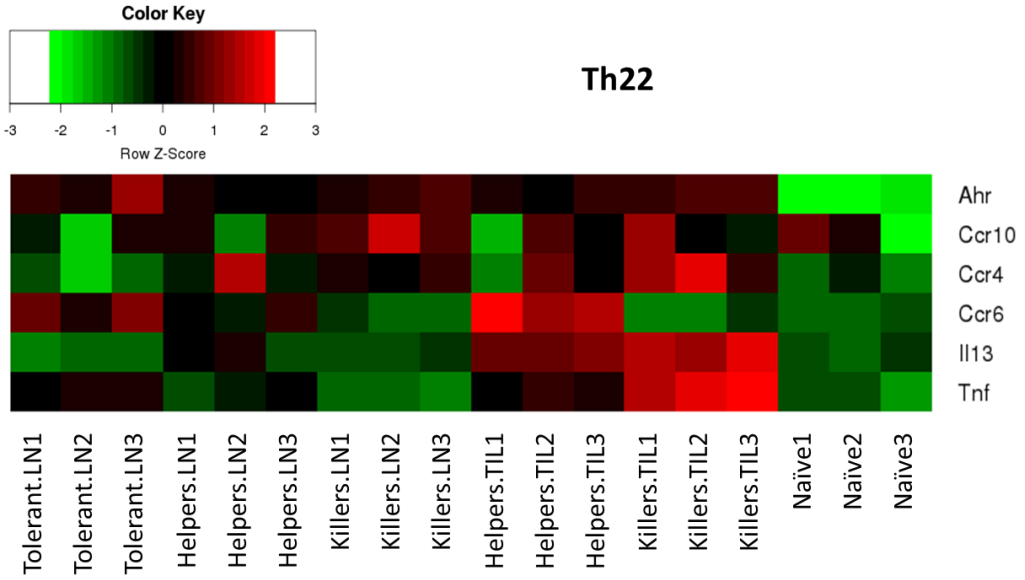

Figure 12 | Heat map analysis of microarray data using a panel of genes encoding Th22 markers. Displayed are genes encoding aryl hydrocarbon receptor AHR (*Ahr*), chemokine receptors CCR10 (*Ccr10*), CCR4 (*Ccr4*) and CCR6 (*Ccr6*), IL-13 (*Il13*) and TNF-α (*Tnf*). Colour coding is used to display high (red) and low (green) expression of a genes across the experimental groups: tolerant, helper, killer and naïve CD4$^+$Trp1 effector T cells extracted from tumour (TIL) or lymph nodes (LN) from treated mice (treatment regimens as described in chapter *4.1*). Mice were sacrificed 8 days after adoptive transfer and RNA was isolated from GFP$^-$ CD4$^+$Trp1 effector cells and used for the GeneChip® Mouse Genome 430 2.0 Array. Naïve GFP$^-$ CD4$^+$Trp1 cells were isolated from lymph nodes of Trp1 FoxGFP mice. n=8-10 mice per group. Experiments were performed in triplicates, each indicated by 1/2/3.

Unfortunately, it was not possible to analyse IL-22 expression as the *Il22* probe was not included on the gene array.

Furthermore, killer CD4$^+$Trp1 TILs only showed little resemblance to Th9 cells based on their expression of Th9 markers (Fig. 13): the expression values of the characteristic Th9 cytokine *Il9*, chemokines *Ccl17* and *Ccl22* as well as chemokine receptor *Ccr6* and suggested Th9 specific transcription factor PU.1 (endocded by *Spi1*) (Chang et al. 2010) were significantly decreased in comparison to helper CD4$^+$Trp1 TILs while the expression values of *Il21*, *Cxcr3* and transcription factors *Irf4* and *Stat6* were increased

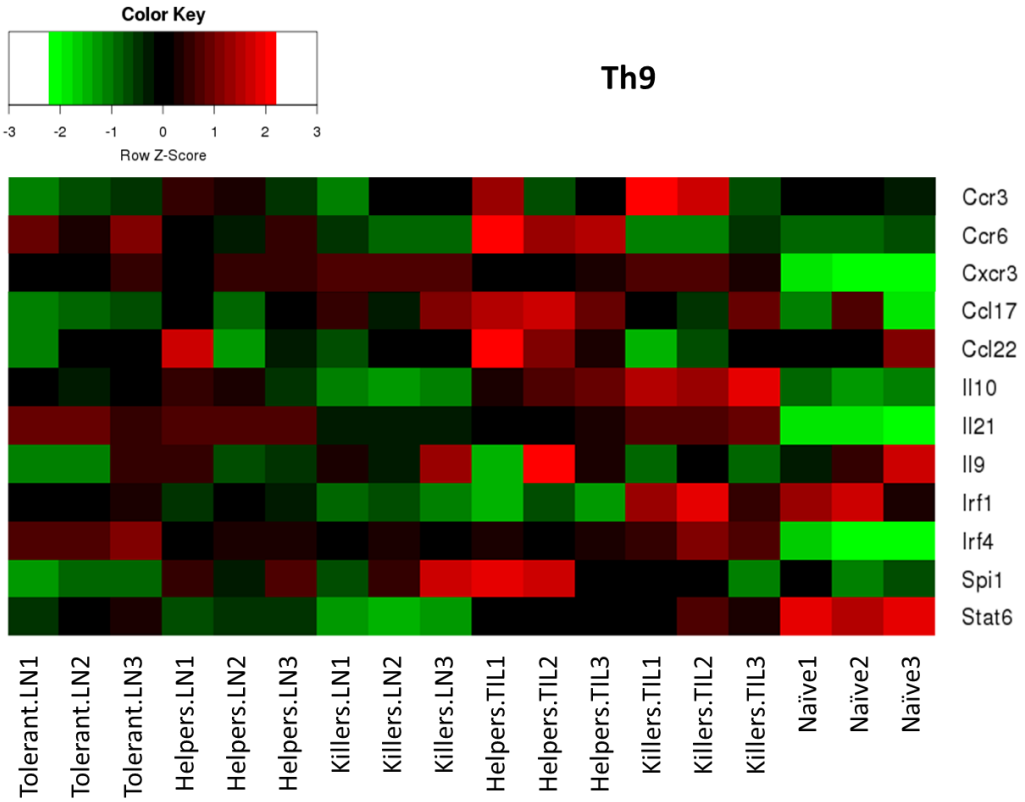

Figure 13 | Heat map analysis of microarray data using a panel of genes encoding Th9 markers. Displayed are genes encoding CCR3 (*Ccr3*), CCR6 (*Ccr6*), CXCR3 (*Cxcr3*), CCL17 (*Ccl17*), CCL22 (*Ccl22*), IL-10 (*Il10*), IL-21 (*Il21*), IL-9 (*Il9*), IRF1 (*Irf1*), IRF4 (*Irf4*), PU.1 (*Spi1*) and STAT6 (*Stat6*). Colour coding is used to display high (red) and low (green) expression of a genes across the experimental groups: tolerant, helper, killer and naïve CD4$^+$Trp1 effector T cells extracted from tumour (TIL) or lymph nodes (LN) from treated mice (treatment regimens as described in chapter *4.1*). Mice were sacrificed 8 days after adoptive transfer and RNA was isolated from GFP$^-$ CD4$^+$Trp1 effector cells and used for the GeneChip® Mouse Genome 430 2.0 Array. Naïve GFP$^-$ CD4$^+$Trp1 cells were isolated from lymph nodes of Trp1 FoxGFP mice. n=8-10 mice per group. Experiments were performed in triplicates, each indicated by 1/2/3.

but not statistically different. *Il10*, *Ccr3* and *Irf1* expression, however, was high on tumour infiltrating killer CD4$^+$Trp1 T cells and significantly increased in comparison with helper TILs (Fig. 13).

4.2.1.3 Examination of Tfh and Treg marker expression

When analysing the microarray data in respect to follicular helper T cell (Tfh) markers, it became clear that killer CD4$^+$Trp1 TILs also did not resemble Tfh cells based on their gene signature as their expression levels of crucial Tfh markers BCL-6 and CXCR5 (Breitfeld et al. 2000; Schaerli et al. 2000; Johnston et al. 2009; Nurieva et al. 2009; Yu et al. 2009) as well as Tfh associated molecules CD200 and BTLA were very low in the tumour and LN (Fig. 14). Also the expression of CCR7, a molecule which can be expressed highly on Tfh depending on the microenvironment (Breitfeld et al. 2000), was low on killer CD4$^+$Trp1 T cells.

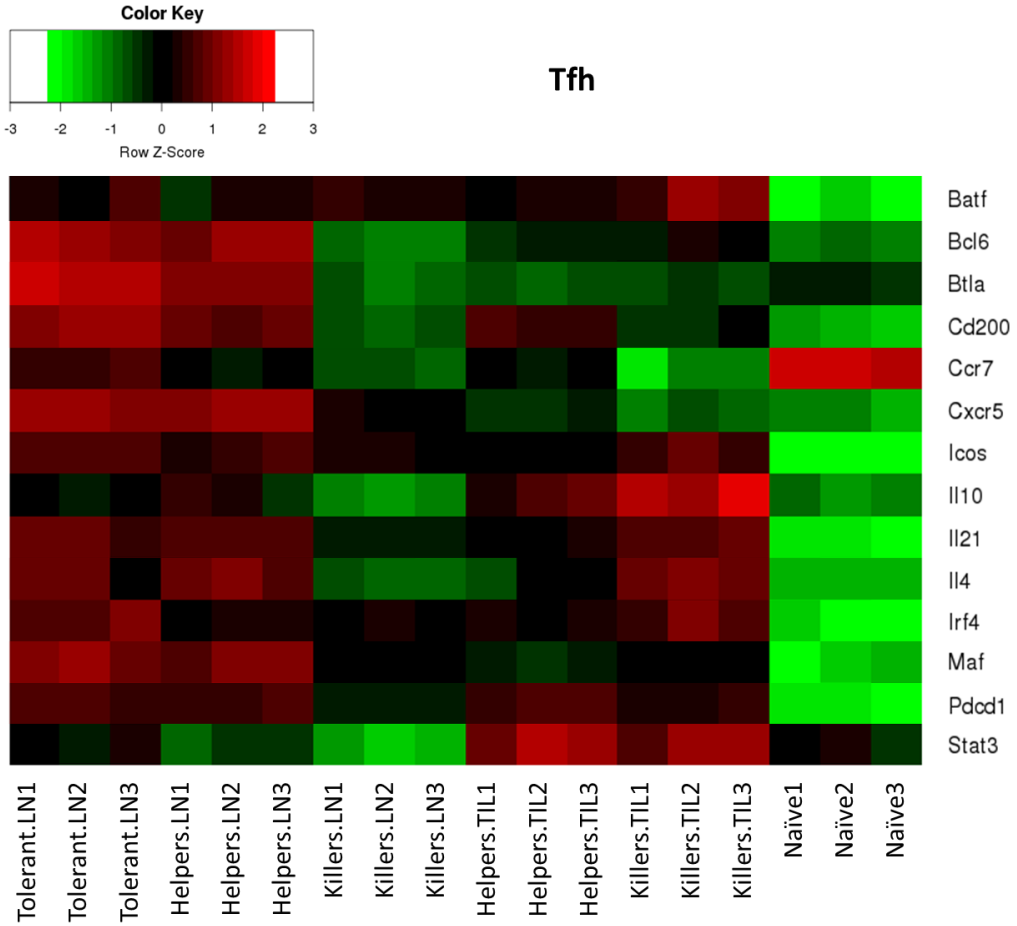

Figure 14 | Heat map analysis of microarray data using a panel of genes encoding follicular B helper T cell (Tfh) markers. Displayed are genes encoding BATF (*Batf*),

BCL-6 (*Bcl6*), BTLA (*Btla*), CD200 (*Cd200*), CCR7 (*Ccr7*), CXCR5 (*Cxcr5*), ICOS (*Icos*), IL-10 (*Il10*), IL-21 (*Il21*), IL-4 (*Il4*), IRF4 (*Irf4*), c-Maf (*Maf*), PD-1 (*Pdcd1*) and STAT3 (*Stat3*). Colour coding is used to display high (red) and low (green) expression of a genes across the experimental groups: tolerant, helper, killer and naïve $CD4^+$Trp1 effector T cells extracted from tumour (TIL) or lymph nodes (LN) from treated mice (treatment regimens as described in chapter *4.1*). Mice were sacrificed 8 days after adoptive transfer and RNA was isolated from GFP^- $CD4^+$Trp1 effector cells and used for the GeneChip® Mouse Genome 430 2.0 Array. Naïve GFP^- $CD4^+$Trp1 cells were isolated from lymph nodes of Trp1 FoxGFP mice. n=8-10 mice per group. Experiments were performed in triplicates, each indicated by 1/2/3.

Other Tfh markers such as the co-stimulatory and co-inhibitory receptors ICOS and PD-1, inflammatory cytokines IL-10, IL-21, IL-4 and transcription factors IRF4, STAT3, BATF and MEF were highly expressed in tumour infiltrating killer TILs and all showed lower levels in the periphery (LN) except for IRF4, BATF and c-Maf. However, out of these markers, only IL-10 was expressed significantly higher in killer TILs in comparison with helper TILs (Fig. 14).

When analysing regulatory T cell markers, it was expected that due to the electronic sorting for GFP- $CD4^+$ effector T cells ($Foxp3^+$ cells are GFP^+) prior to mRNA collection, that there was only very little expression of Treg specific molecules. This was validated as tumour infiltrating killer $CD4^+$Trp1 T cells displayed only very low *Foxp3* expression level and are therefore not regulatory T cells (Fig. 15). Also the expression level of other Treg markers such as CCR6, IL-9 and CD103 (encoded by *Itgae*) was very low in the killer subset. Interestingly and in keeping with recent literature (Roychoudhuri et al. 2013), Bach2 expression was particularly low in killer $CD4^+$ T cells in the lymph node (LN) and tumour which further emphasises their effector and not regulatory function.
However, killer $CD4^+$Trp1 TILs showed high expression of markers which are characteristically found on Tregs but are not exclusive to this subset: immune checkpoint suppressors/activators CTLA-4, ICOS, GITR (*Tnfrsf18*) and OX40 (*Tnfrsf4*) as well as the cytokines IL-10 and TGF-β (*Tgfb1*) were all expressed in killer $CD4^+$ T cells in the tumours (Fig. 15). Also the expression of effector T cell markers such as CD25 (*Il2ra*) and CD39 (*Entpd1*), CD71 (*Tfrc*) and transcription factors AHR and STAT5 and chemokine receptor CCR4 was high but LFA-1 (*Itgal*) was significantly upregulated in killer TILs in comparison to helper TILs (Fig. 15) and therefore might play a role in the novel killer $CD4^+$ T cell phenotype.

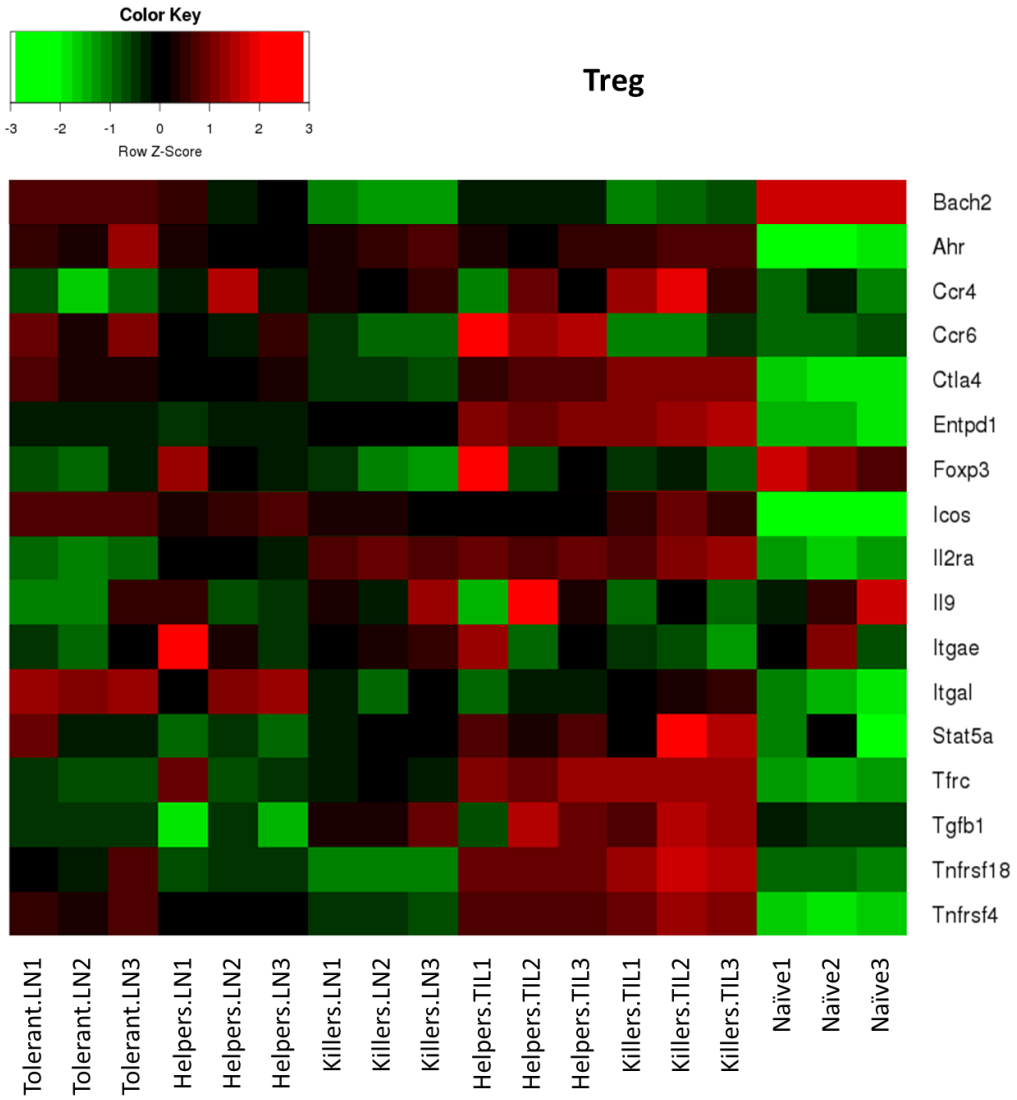

Figure 15 | Heat map analysis of microarray data using a panel of genes encoding regulatory T cell (Tregs) markers. Displayed are genes encoding Bach2 (*Bach2*), AHR (*Ahr*), CCR4 (*Ccr4*), CCR6 (*Ccr6*), CTLA-4 (*Ctla4*), CD39 (*Entpd1*), Foxp3 (*Foxp3*), ICOS (*Icos*), IL-10 (*Il10*), CD25 (*Il2ra*), IL-9 (*Il9*), CD103 (*Itgae*), LFA-1 (*Itgal*), STAT5 (*Stat5*), CD71 (*Tfrc*), TGF-β (*Tgfb1*), GITR (*Tnfrsf18*) and OX40 (*Tnfrsf4*). Colour coding is used to display high (red) and low (green) expression of a genes across the experimental groups: tolerant, helper, killer and naïve $CD4^{+}$Trp1 effector T cells extracted from tumour (TIL) or lymph nodes (LN) from treated mice (treatment regimens as described in chapter *4.1*). Mice were sacrificed 8 days after adoptive transfer and RNA was isolated from GFP^{-} $CD4^{+}$Trp1 effector cells and used for the GeneChip® Mouse Genome 430 2.0 Array. Naïve GFP^{-} $CD4^{+}$Trp1 cells were isolated from lymph nodes of Trp1 FoxGFP mice. n=8-10 mice per group. Experiments were performed in triplicates, each indicated by 1/2/3.

4.2.1.4 Examination of CD8⁺ marker and effector molecule expression

Because of their cytotoxic potential *in vivo*, it was critical to analyse CD4⁺Trp1 T cells in regards to their expression of CD8⁺ T cell markers.

CD8⁺ lineage transcription factor *Runx3* was highly expressed on tumour infiltrating killer CD4⁺Trp1 cells (Killers.TIL) but was also detected in only slightly lower levels on the helper TILs and killer CD4⁺ T cells in the periphery (Helpers.TIL, Killers.LN, Fig. 16). This data emphasises the likely importance of the characteristic CD8⁺ transcription factor for the killer CD4⁺Trp1 phenotype and correlates with previous studies which showed its direct link with GzmB expression and overall cytotoxic potential of T cells (Wang et al. 2008; Cruz-Guilloty et al. 2009).

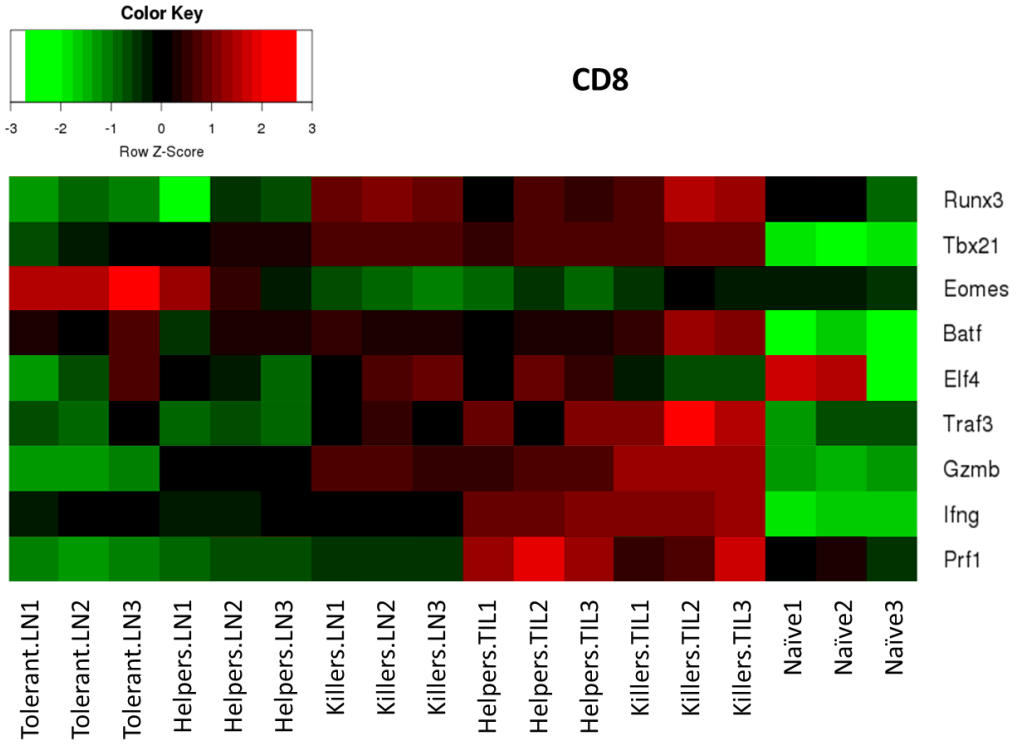

Figure 16 | Heat map analysis of microarray data using a panel of genes encoding CD8⁺ T cell markers. Displayed are genes encoding Runx3 (*Runx3*), T-bet (*Tbx21*), Eomesodermin (*Eomes*), BATF (*Batf*), MEF (*Elf4*), IFNγ (*Ifng*), TRAF3 (*Traf3*), GzmB (*Gzmb*) and Perforin (*Prf1*). Colour coding is used to display high (red) and low (green) expression of a genes across the experimental groups: tolerant, helper, killer and naïve CD4⁺Trp1 effector T cells extracted from tumour (TIL) or lymph nodes (LN) from treated mice (treatment regimens as described in chapter *4.1*). Mice were sacrificed 8 days after adoptive transfer and RNA was isolated from GFP⁻ CD4⁺Trp1 effector cells and used for the GeneChip® Mouse Genome 430 2.0 Array. Naïve GFP⁻ CD4⁺Trp1 cells were isolated from lymph nodes of Trp1 FoxGFP mice. n=8-10 mice per group. Experiments were performed in triplicates, each indicated by 1/2/3.

Also two other important and often co-expressed $CD8^+$ transcription factors, T-bet and Eomes, displayed a unique expression pattern: as reported earlier, *Tbx21* (encoding for T-bet), which is not only important for Th1 differentiation but also instrumental for effector $CD8^+$ T cell development (Cruz-Guilloty et al. 2009), was highly expressed while transcription factor *Eomes* (Eomesodermin) expression was very low in killer $CD4^+$Trp1 TILs (Fig. 16). AP-1 transcription factor *Batf*, was expressed at moderately high level in killer TILs and displayed an expression range across all samples which was very similar to *Tbx21*. This suggests that, together with Runx3, T-bet is important but Eomes dispensable for the cytotoxic $CD4^+$ phenotype.
Furthermore, expression of *Elf4* (encoding transcription factor MEF) was low and significantly decreased in killer $CD4^+$Trp1 TILs in comparison with helper TILs (Fig. 16). Killer $CD4^+$Trp1 TILs also highly expressed TNF receptor-associated factor 3 (*Traf3*) mRNA, which was found to be essential for optimal T cell immunity (Xie et al. 2011).
High levels of $CD8^+$ CTL effector molecules *Gzmb*, *Prf1* (encoding Perforin) and *Ifng* transcripts were found in the tumour infiltrating killer and helper $CD4^+$Trp1 subsets. *Gzmb* was also expressed to a lesser extent on killer $CD4^+$ T cells in the lymph nodes (Fig. 16).

To have a further look into the expression of cytotoxic molecules expressed by killer $CD4^+$Trp1 TILs, a new heat map was created. The analysis indicated that killer $CD4^+$ TILs displayed particularly high levels of mRNA from all cytotoxic molecules we analysed: FasL, GzmA-K, Perforin (encoded by *Prf1*) and TRAIL (*Tnfsf10*) (Fig. 17). However, it is important to note that most of these genes, with the exception of *Gzma* and *Gzmk*, were also highly expressed on helper TILs. Killer $CD4^+$Trp1 T cells from the lymph nodes were devoid of cytotoxic molecule expression except for *Gzma*, *Gzmb* and *Gzmk*. Remarkably, *Gzma* and *Gzmk* are the only two genes which were significantly higher expressed in killer $CD4^+$Trp1 T cells from the tumour and lymph node in comparison to helper TILs (Fig. 17).

Taken together, the microarray analysis demonstrated that tumour infiltrating killer $CD4^+$Trp1 T cells display a high degree of activation by expressing a multitude of inflammatory cytokines along with the cytolytic molecules GzmA – K, Perforin and even FasL and TRAIL, although the latter two were previously shown to be dispensable for $CD4^+$Trp1 effector function (Quezada et al. 2010). Interestingly, killer $CD4^+$ T cells in the lymph node, however, lacked transcripts of all cytokines and Perforin and only expressed a minority of Granzymes (Fig. 17); this underscores the importance of the tumour micro-

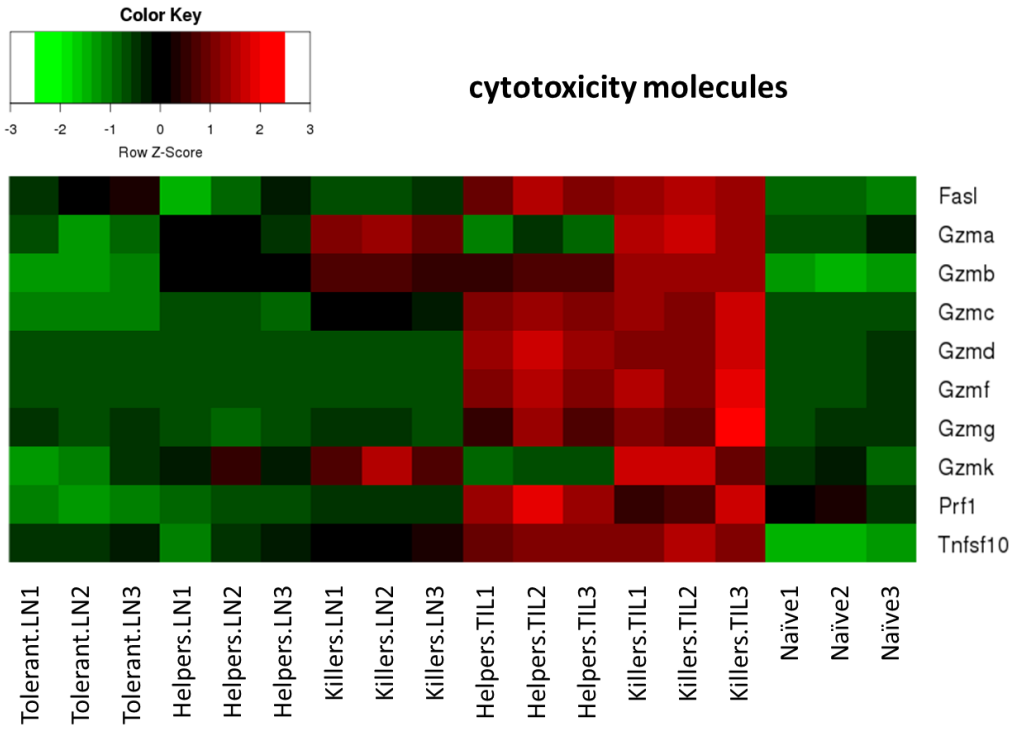

Figure 17 | Heat map analysis of microarray data using a panel of genes encoding CD8⁺ cytotoxic effector molecules. Displayed are genes encoding FasL (*Fasl*), GzmA - K (*Gzma – Gzmk*), Perforin (*Prf1*), TRAIL (*Tnfsf10*). Colour coding is used to display high (red) and low (green) expression of a genes across the experimental groups: tolerant, helper, killer and naïve CD4⁺Trp1 effector T cells extracted from tumour (TIL) or lymph nodes (LN) from treated mice (treatment regimens as described in chapter *4.1*). Mice were sacrificed 8 days after adoptive transfer and RNA was isolated from GFP⁻ CD4⁺Trp1 effector cells and used for the GeneChip® Mouse Genome 430 2.0 Array. Naïve GFP⁻ CD4⁺Trp1 cells were isolated from lymph nodes of Trp1 FoxGFP mice. n=8-10 mice per group. Experiments were performed in triplicates, each indicated by 1/2/3.

environment on the differentiation of the CD4⁺Trp1 T cells into a cytotoxic phenotype.

In conclusion, tumour infiltrating, cytotoxic CD4⁺Trp1 T cells expressed Th1, Th2 and CD8⁺ CTL specific transcription factors and effector molecules on transcript level. These results indicate that cytotoxic CD4⁺Trp1 T cells do correlate with one specific T cell subset alone but exhibit a complex multifunctional phenotype.

4.2.1.5 Unbiased analysis of gene array data

Beyond the assessment of expression of a hand-curated selection of T cell subset markers, the microarray was further analysed in an unbiased approach. After

normalisation, gene expression values of tumour infiltrating CD4$^+$Trp1 T cells from mice receiving the killer treatment (Killers.TIL1-3) and mice receiving the helper therapy (Helpers.TIL1-3) were compared and genes with a significant change in expression value (p value < 0.05) between the two cohorts were selected. The 75 most differentially expressed (up- or downregulated) genes on killer TILs in the context and direct comparison with helper TILs are presented in figure 18.

Interestingly, the gene with the highest increase in expression in comparison to helper CD4$^+$Trp1 T cells was the cytotoxic molecule encoding gene *Gzma* (Fig. 18). Additionally to GzmA, the unbiased analysis revealed two more members of the Granzyme family, GzmK and GzmB in the top 75 highest expressed genes on killer TILs. This further confirms the crucial difference in cytotoxic potential of killer CD4$^+$ T cells in comparison with helper CD4$^+$ effectors.

Furthermore, chemokine receptors CCR2 and CCR5, which have been found connected with an active Th1 phenotype, were highly expressed on killer CD4$^+$ effectors but not on helper TILs (red, Fig. 18). In addition, genes associated with metabolism such as the thiamine transporter TC1 (encoded by *Slc19a2*), Glut3 (*Slc2a3*) and Hif-2α (*Epas1*) were also found significantly upregulated in killer TILs. This could be due to the induced lymphopenia of the host prior to T cell transfer which might induce homeostatic proliferation and thus increased metabolic rate of the T cells.

On the opposite end of the scale, for instance *Ccr6* and *Id3* stand out: Th9/22 and Treg chemokine receptor CCR6 and memory marker Id3 (Ji et al. 2011; Hu & Chen 2013) show especially low expression on killer TILs in comparison with helper TILs. This might suggest that these molecules are not involved/not important for the cytotoxic CD4$^+$ phenotype and further emphasises the previous analysis showing a Th1/Th2 rather than Th9/22 or Treg skewing of the killer CD4$^+$Trp1 T cells.

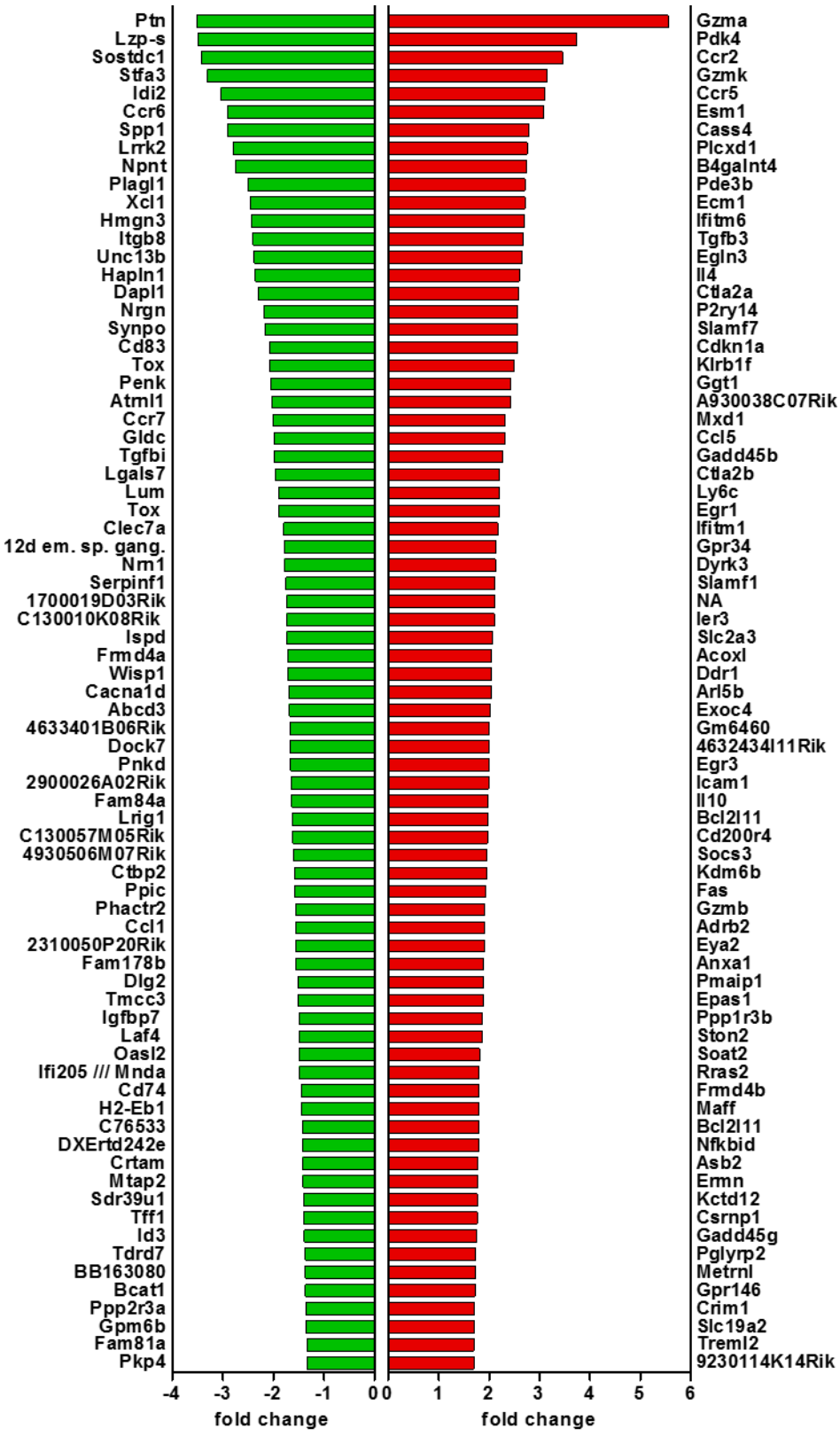

Figure 18 | Unbiased analysis of the most differentially expressed genes in tumour infiltrating killer CD4$^+$Trp1 T cells in comparison with helper TILs. Displayed are the top 75 most up- (red) or downregulated (green) genes, respectively, of killer CD4$^+$Trp1 effector T cells extracted from tumours from treated mice in the context of the gene

expression in helper CD4$^+$Trp1 TILs (treatment regimens as described in chapter 4.1). Genes are displayed in ascending order of fold change between expression values of killer TILs vs helper TILs. All depicted genes display statistically significant differences (p value below 0.05) in expression values between triplicates of the groups between helper and killer TILs. Mice were sacrificed 8 days after adoptive transfer and RNA was isolated from GFP$^-$ CD4$^+$Trp1 effector cells and used for the GeneChip® Mouse Genome 430 2.0 Array. n=8-10 mice per group. Experiments were performed in triplicates.

4.2.2 Killer CD4$^+$Trp1 T cells display a transcriptional signature resembling CD8$^+$ rather than CD4$^+$ T cells

Based on the directed and unbiased analyses killer CD4$^+$Trp1 T cells display a mixed phenotype which is not skewed towards one particular CD4$^+$ subset but rather illustrates a multi-functional phenotype. Their cytotoxic potential and resemblance with CD8$^+$ T cells were further striking features of this killer CD4$^+$ T cell population. Therefore, we chose to investigate the similarities to CD4$^+$ and CD8$^+$ T cells more extensively.

For further investigation of gene signature Canonical Correspondence Analysis (CCA), a multidimensional method which allows the analysis of interrelationships between several sets of gene array data, was chosen. The adaptation of CCA for microarray analysis, CCA on Microarray data (CCAM), has previously proven effective when it was used to reproduce and visualise known relationships between lymphocyte differentiation, leukaemia subtypes and specific genes (Ono et al. 2013).

In order to determine the 'CD4$^+$- or CD8$^+$-likeness' of the cytotoxic CD4$^+$Trp1 T cells by CCAM, the transcriptomic expression profiles of the tolerant, helper and killer CD4$^+$Trp1 cells from both tumour and lymph nodes were compared with specific microarray data subsets from the ImmGen database (Heng et al. 2008). The naïve CD4$^+$Trp1 T cell samples were removed from the analysis at this point because they were too phenotypically diverse in comparison to the other antigen-experienced subsets.
To stay as close to the original experimental set up as possible, the two data sets used for comparison were T.8.TI.B16 and T.4.TI.B16 which originate from tumour infiltrating CD8$^+$ (CD4$^-$ TCRβ$^+$ CD45$^+$) and CD4$^+$ (CD8$^-$ TCRβ$^+$ CD45$^+$) T cells. Likewise, the implanted tumour was B16/BL6 melanoma and grew as subcutaneous implant on 6 week old C57BL/6 mice (Heng et al. 2008).

Masahiro Ono performed the CCAM according to his established method in (Ono et al. 2013; Ono et al. 2014).

Before the CCAM, the microarray data was first normalised and background noise was removed (see chapter *3.8*) before differentially expressed genes were extracted from the context of two experimental groups, for example Killer.TIL (killer $CD4^+$Trp1 cells isolated from the tumour) and Helper.TIL (helper $CD4^+$Trp1 cells isolated from the tumour). A total of 7228 significant genes were filtered from the following comparison sets: KillerVsHelper.TIL, KillerVsHelper.LN and KillerVsTolerant.LN. Furthermore, the two ImmGen datasets (T.8.TI.B16 and T.4.TI.B16) were treated in the same fashion to obtain a reference data set (CD8vsCD4.TIL).

A CCAM is performed with a minimum of two sets of data, one microarray of interest (the 'response variable') and another transcriptomic dataset which is used to define the context of the analysis (the 'explanatory variable') (Ono et al. 2013). In this case, the microarray dataset for tolerant, helper and killer $CD4^+$ T cells represents the response variable and CD8vsCD4.TIL (T.8.TI.B16 - T.4.TI.B16) was used as explanatory variable for CCAM to investigate the relationships between the 7228 differentially expressed genes from the microarray and the CD4/CD8 transcriptomic ImmGen data.

The CCAM revealed that tumour infiltrating killer $CD4^+$Trp1 cells (Killers.TIL) showed more resemblance to tumour infiltrating $CD8^+$ T cells than to $CD4^+$ T cells. This is illustrated in figure 19 by the position of the data points: the higher the score on the CD8vsCD4.TIL axis, the more correlation exists between the particular subset and the tumour infiltrating $CD8^+$ phenotype. Interestingly, the CCAM also revealed a higher correlation of genes expressed in $CD4^+$Trp1 killer cells in the lymph node (Killers.LN) and helper $CD4^+$Trp1 TILs (Helpers.LN) with the $CD8^+$ than with the $CD4^+$ ImmGen dataset (Fig. 19). Based on this analysis, only the expression profiles of tolerant and helper $CD4^+$Trp1 from the lymph nodes resembled the gene set of the B16/BL6 infiltrating $CD4^+$ cells.

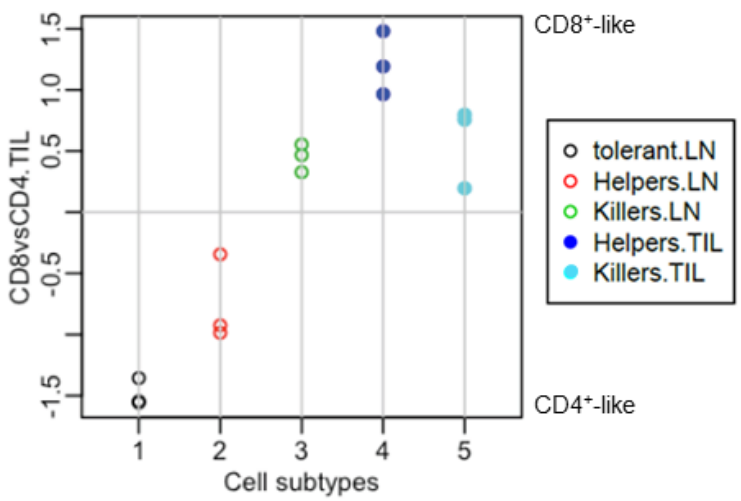

Figure 19 | CCAM result comparing killer, helper and tolerant CD4⁺Trp1 with CD8⁺ and CD4⁺ T cells. The 1D plot illustrates CCAM of the 7228 differentially expressed genes from the microarray in context with two ImmGen data sets from BL6/B16 tumour infiltrating CD8⁺ (T.8.TI.B16) and CD4⁺ T cells (T.4.TI.B16); T.8.TI.B16 - T.4.TI.B16 (CD8vsCD4.TIL) was used as explanatory variable. RNA was isolated from GFP⁻ CD4⁺Trp1 tolerant, helper and killer CD4⁺Trp1 effector T cells extracted from tumour (TIL) or lymph nodes (LN) from treated mice (treatment regimens as described in chapter *4.1*). Mice were sacrificed 8 days after adoptive transfer and RNA used for the GeneChip® Mouse Genome 430 2.0 Array. n=8-10 mice per group. Experiments were performed in triplicates.

Although the tumour infiltrating CD8⁺ dataset from the ImmGen database was useful for the CD4/CD8 comparison, it is unclear which level of activation and which effector phenotype the CD8⁺ T cells possessed. In order to better understand which CD8⁺ phenotype (effector / memory) the killer CD4⁺Trp1 T cells share the most phenotypical and genetic properties with, further investigation was required.

4.2.3 The genetic signature of cytotoxic CD4⁺Trp1 T cells correlates with an effector, not a memory or less mature CD8⁺ T cell phenotype

To determine the transcriptional similarities of tumour infiltrating killer CD4⁺Trp1 cells with effector and memory CD8⁺ phenotypes, an array of ImmGen data sets were used. These data sets encompass the genetic signature of antigen-specific OT-I CD8⁺ T cells from different time points of a CD8⁺ mediated response against Ova expressing recombinant Vesicular Stomatitis Virus (VSV-ova). CD8⁺ T cells from the TCR transgenic OT-I model are MHC Class I restricted T cells that express a TCR reactive to the $Ova_{257-264}$ peptide SIINFEKL derived from ovalbumin. The ImmGen data sets present OT-I CD8⁺ T cells (CD4⁻ CD45.1⁺) from the spleen of VSV-ova infected 6 week old C57BL/6 mice. As the recombinant Vesicular Stomatitis Virus (VSV-ova) is engineered to encode and

induce the expression of $Ova_{257-264}$, adoptively transferred OT-I $CD8^+$ T cells mount an immune response against VSV-ova infected cells which present $Ova_{257-264}$ peptide in MHC I (Kim et al. 1998; Clarke et al. 2000).
At the time point of analysis no tumour-specific $CD8^+$ data sets were readily available from the ImmGen database, therefore this VSV-ova and OT-I data set was chosen to model the immune response of antigen-specific $CD8^+$ T cells.

Again, the 7228 differentially expressed genes from the microarray were used for this second CCAM to compare the gene signatures of tolerant, helper and killer $CD4^+$Trp1 T cells with four time points of the course of the antigen-specific OT-I $CD8^+$ response: day 5 (d5.1 and d5.2, duplicates from the same day), day 6, day 8 and day 15 after infection/OT-I $CD8^+$ transfer (T.8Eff.Sp.OT1.d5.VSVOva, T.8Eff.Sp.OT1.d6.VSVOva, T.8Eff.Sp.OT1.d8.VSVOva, T.8Eff.Sp.OT1.d15.VSVOva) (Heng et al. 2008). These fours data sets include $CD8^+$ effectors at different stages of their activity: the d5 data sets are expected to exhibit the gene expression pattern of an early effector $CD8^+$ while the data sets from d6 and 8 should portray the fully differentiated effector phenotype; the d15 data set correlates with a clear development from effector to memory cell fate (Turner et al. 2006).

The result of the multiple dimension CCAM is displayed as a two-dimensional triplot: the map illustrates the relationships between genes, $CD4^+$Trp1 subsets and OT-I $CD8^+$ phenotypes based on the day after adoptive transfer. The OT-I $CD8^+$ effector/memory phenotypes present the explanatory variables in this analysis which are depicted as vectors in the triplot.

In order to draw a conclusion from the map, it is important to note the proximity of a gene or a $CD4^+$Trp1 subset to a vector and its direction. Furthermore, the higher the distance of the genes/subsets from the cluster in the midpoint of the graph, the higher the correlation with the respective vectors (ImmGen data set). Taken together this means, the closer two components are on the triplot, the more correlated they are. For instance, the CCA illustrates that the expression of *Egr2*, a tolerance gene which is expressed on $CD44^{high}$ effector and memory $CD8^+$ T cells (Zhu et al. 2008), (Fig. 20, orange dotted square) is associated with the ImmGen data set from d8 of the VSV-ova infection as it situated very close to the d8 vector and almost lies in its linear extrapolation. On the other hand, *Egr2* does not show a correlation to the data sets from d5.1 and d5.2 as these vectors point in the opposite direction. However, rather than correlating specific genes with a phenotype, this analysis is aimed at revealing qualitative relationships be-

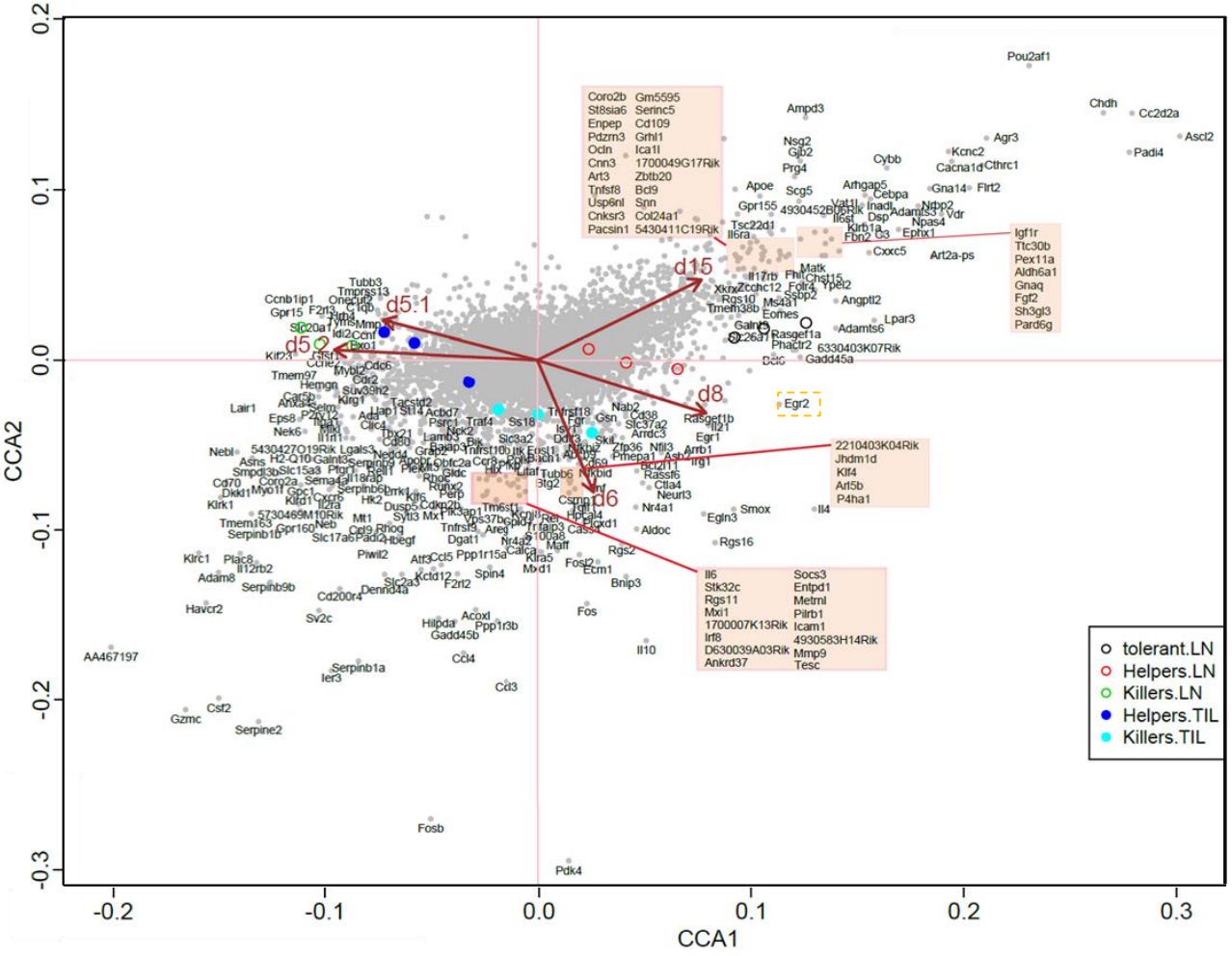

Figure 20 | CCAM triplot illustrating relationships between tolerant, helper and killer CD4⁺Trp1 T cells with different CD8⁺ phenotypes. The 2D plot presents the CCAM of the 7228 differentially expressed genes from the microarray in context with ImmGen data sets taken from different time points of the OT-I CD8⁺ response against VSV-ova: day 5 (d5.1, d5.2), day 6 (d6), day 8 (d8) and day 15 (d15) after adoptive transfer of OT-1 T cells. These datasets were used as explanatory variables. RNA was isolated from GFP⁻ CD4⁺Trp1 tolerant, helper and killer CD4⁺Trp1 effector T cells extracted from tumour (TIL) or lymph nodes (LN) from treated mice (for treatment regimens see chapter *4.1*). Mice were sacrificed 8 days after adoptive transfer and RNA used for the GeneChip® Mouse Genome 430 2.0 Array. n=8-10 mice per group. Experiments were performed in triplicates.

tween the OT-I $CD8^+$ T cell signatures and the tolerant, helper and killer $CD4^+$Trp1 subsets.

Remarkably, the different explanatory variables, i.e. the vectors depicting OT-I $CD8^+$ gene signatures of day 5, 6, 8 and 15 after adoptive transfer, are well separated on the CCAM triplot (Fig. 2): while the two d5 vectors had a very similar directed and were both located in the second quadrant of the plot (positive values on the CCA2 axis and negative values on the CCA1 axis), d6 and d8 vectors were contained in the direct opposite fourth quadrant of the triplot (positive CCA1 and negative CCA2 values). The direction of the d15 vector was separate from all others and situated in the first quadrant (positive CCA1 and CCA2 values).

The CCAM map illustrates that helper $CD4^+$Trp1 T cells from the tumour (Helpers.TIL) and killer T cells from the lymph node (Killers.LN) were mostly situated in the second quadrant and close to the d5 vectors (Fig. 20). This suggests a higher correlation of those two sets with the genetic footprint of an early effector $CD8^+$ rather than a fully differentiated effector.
The tumour infiltrating killer $CD4^+$Trp1 cells (Killers.TIL), however, were located closer to the origin of the CCA1 and 2 axes and the d6 and d8 vectors than any other vector (Fig. 20). This emphasises an association with the gene signature of d6 and slightly d8, the proposed peak of the OT-I $CD8^+$ response.
The tolerant and helper $CD4^+$Trp1 from the lymph nodes (Helpers.LN and tolerant.LN) were mostly situated in quadrant 1, between the d8 and d15 vectors; tolerant $CD4^+$ T cells were located closest to the d15 vector. This points towards higher correlation with a more resting late effector/early memory $CD8^+$ phenotype. However, it is important to note that these two data sets were previously found to be most correlated with a $CD4^+$ rather than $CD8^+$ T cell signature (Fig. 19); it is therefore difficult to draw specific conclusions about these subsets.

The expression profiling by CCAM thereby confirmed that the overall transcriptomic signature of the cytotoxic $CD4^+$Trp1 cells in the tumour is positively correlated to an effector CTL rather than an early effector or memory $CD8^+$ signature.

A more detailed analysis of the expression values of effector and memory fate markers overall confirm the effector phenotype of killer $CD4^+$Trp1 TILs: activation and effector markers such as T-bet (encoded by *Tbx21*), Id2, CD25 (*Il2ra*), LFA-1 (*Itgal*), CD11b (*Itgam*), CD11c (*Itgax*), KLRG1, CD69, Blimp-1 (*Prdm1*) and proliferation marker Ki67 (*Mki67*) were all moderately to highly expressed. Of these effector molecules, LFA-1, CD11c, KLRG1 and CD69 were significantly upregulated in killer TILs in comparison to

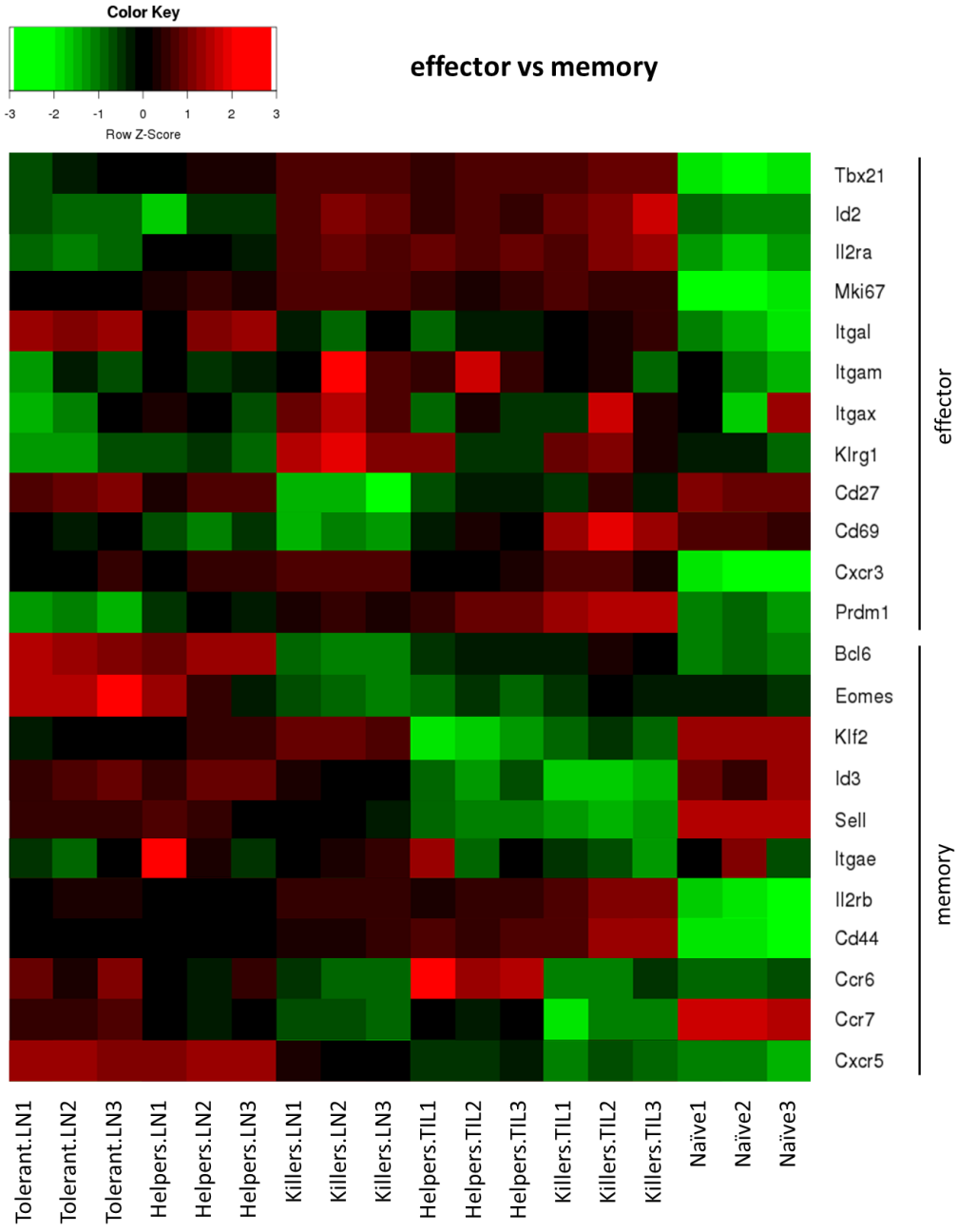

Figure 21 | Heat map analysis of microarray data using a panel of genes encoding effector and memory T cell markers. Displayed are genes encoding effector markers: T-bet (*Tbx21*), Id2 (*Id2*), CD25 (*Il2ra*), Ki67 (*Mki67*), LFA-1 (*Itgal*), CD11b (*Itgam*), CD11c (*Itgax*), KLRG1 (*Klrg1*), CD27 (*Cd27*), CD69 (*Cd69*), CXCR3 (*Cxcr3*) and Blimp-1 (*Prdm1*) and memory markers: BCL-6 (*Bcl6*), Eomesodermin (*Eomes*), Klf2 (*Klf2*), Id3 (*Id3*), CD62L (*Sell*), CD103 (*Itgae*), CD122 (*Il2rb*), CD44 (*Cd44*), CCR6 (*Ccr6*), CCR7 (*Ccr7*), and CXCR5 (*Cxcr5*). Colour coding is used to display high (red) and low (green) expression of a genes across the experimental groups: tolerant, helper, killer and naïve

$CD4^+$Trp1 effector T cells extracted from tumour (TIL) or lymph nodes (LN) from treated mice (treatment regimens as described in chapter *4.1*). Mice were sacrificed 8 days after adoptive transfer and RNA was isolated from GFP^- $CD4^+$Trp1 effector cells and used for the GeneChip® Mouse Genome 430 2.0 Array. Naïve GFP^- $CD4^+$Trp1 cells were isolated from lymph nodes of Trp1 FoxGFP mice. n=8-10 mice per group. Experiments were performed in triplicates, each indicated by 1/2/3.

helper TILs (Fig. 21). Interestingly, both CD69 and KLRG1 are not only associated with a $CD4^+$ and $CD8^+$ effector phenotype (Simms & Ellis 1996) but also acts as stimulatory receptor and activation marker for another subset of cytotoxic cells: Natural Killer (NK) cells (Blaser et al. 1998; Borrego et al. 1999).

On the other hand, the expression levels of memory markers such as BCL-6, Eomes, Klf2, Id3, CCR6, CCR7 and CXCR5 were particularly low on tumour infiltrating killer $CD4^+$Trp1 T cells, but only CCR6 was significantly decreased in context with helper TILs (Fig. 21).

A direct comparison of the expression of the $CD8^+$ T cell effector and memory differentiation regulators T-bet and Eomes (Pipkin & Rao 2009; Ansel et al. 2006) and the reciprocally antagonistic Blimp-1 and BCL-6 (Johnston et al. 2009) reveals a very clear $CD8^+$ effector-like phenotype on $CD4^+$Trp1 killer TILs: high expression of T-bet and Blimp-1 and simultaneous low expression of Eomes and BCL-6 (Fig. 21).

Other memory markers (CD122 (*Il2rb*) and CD44), however, displayed increased expression on killer $CD4^+$ TILs (Fig. 21). Interestingly, CD122 and CD44 were moderately to highly expressed on all antigen-experienced subsets originating from tumour and lymph nodes, only naïve $CD4^+$Trp1 did not show any expression. This suggests that these markers are inherently upregulated in adoptively transferred $CD4^+$Trp1 T cells – irrespective of their phenotype.

Alongside the high expression of CD44, low expression of CD62L (encoded by *Sell*) was detected which suggests an effector memory phenotype (Seder & Ahmed 2003). Also the low expression of CD103 (encoded by *Itgae*) points towards a $CD8^+$ memory phenotype but CD103 has been shown to have different expression pattern and associated functionality in $CD4^+$ T cells, for instance in naïve Tregs (Lehmann et al. 2002).

Taken together, the CCAM and the individual analysis of effector and memory markers illustrate that cytotoxic $CD4^+$Trp1 cells in the tumour represent a new $CD4^+$ subset which closely correlates with the gene signature of a $CD8^+$ effector CTL rather than memory phenotype.

4.2.4 Protein expression of selected microarray target genes correlates with their observed transcript level

Analysis of gene expression is a powerful tool to qualitatively and quantitatively investigate expression patterns on transcript level, however, observed mRNA levels do not always directly correlate with the level of expression and activity of the corresponding protein. Transcript quantities often do not directly translate into the same protein abundance due to regulation of protein expression through post-transcriptional and post-translational modifications as well as protein degradation (de Sousa Abreu et al. 2009; Vogel & Marcotte 2012). These potential discrepancies between transcript and protein level make it critical to assess and compare the expression of selected genes on protein level.

To assess whether the up- or downregulation of important $CD4^+$ and $CD8^+$ T cell, effector and memory marker genes on the microarray correlated with expression of the corresponding protein, tumour infiltrating $CD4^+$Trp1 T cells from the killer cohort were analysed via multicolour flow cytometry. According to chapter *4.1*, B16/BL6 tumour bearing C57BL/6 mice received 5 Gy RT, $6x10^4$ $CD4^+$Trp1 T cells and αCTLA-4. After the mice were sacrificed on day 17 or 18, the tumours and lymph nodes were dissected and processed as outlined in chapter *3.4.3*.
$CD4^+$Trp1 TILs were gated on viable $CD45.1^+$ $Foxp3^-$ $CD4^+$ singlets to exclude endogenous $CD4^+$, $CD8^+$ and regulatory T cells as well as tumour cells and debris. The expression of a protein on $CD4^+$Trp1 effector T cells was compared with the expression of the target in an appropriate reference T cell population. The reference subset varied depending on the specific protein in accordance with its established expression pattern: For ThPok and Runx3, for instance, tumour infiltrating endogenous $CD8^+$ T cells from the tolerant cohort were chosen as the reference subset because peripheral, antigen-experienced $CD8^+$ T cells have a high abundance of $CD8^+$ lineage transcription factor

Runx3 while lacking expression of $CD4^+$ lineage transcription factor ThPok (Wang et al. 2008; Cruz-Guilloty et al. 2009).

Overall, the protein expression of selected $CD4^+$, $CD8^+$, effector and memory markers on tumour infiltrating $CD4^+$Trp1 T cells correlated with their transcript level in the microarray in the vast majority of cases. The high abundance of $CD4^+$ and $CD8^+$ lineage transcription factors mRNA encoding ThPok and Runx3 was also detected protein level with up to 98 % $CD4^+$Trp1 TILs expressing ThPok and 79 % expressing Runx3 (Fig. 22). This level of Runx3 expression was similar to the expression in the endogenous $CD8^+$ T cell and significantly higher than in the endogenous $CD4^+$ compartment (Fig. 23 B).
Up to 86 % of killer $CD4^+$Trp1 TILs expressed Th1 transcription factor T-bet and, surprisingly, it was found that the majority of the T-bet^+ $CD4^+$ T cells co-expressed the Th2 specific transcription factor Gata3 (Fig. 23 C). 78 % (± 8.3 % SD) of $CD4^+$Trp1 T cells co-expressed Gata3 and T-bet while the endogenous $CD4^+$ T cells only showed T-bet and Gata3 co-expression in up to 10 % of the cells (Fig. 23 C).
As seen on the microarray, $CD4^+$Trp1 TILs did not express Eomes (Fig. 22). It was further evident that GzmB protein levels correlated with Runx3 and T-bet: 91 % of the $Runx3^+$ $CD4^+$Trp1 T cells also expressed $GzmB^+$ and 89 % of the T-bet expressing $CD4^+$Trp1 effectors co-expressed GzmB (Fig. 23 A).
The specific effector T cell expression pattern of memory and effector associated transcription factors Blimp-1 and BCL-6 was also validated by flow cytometry: no BCL-6 protein was detected while Blimp-1 was expressed in up to 70 % of tumour infiltrating $CD4^+$Trp1 T cells (Fig. 22) and highly co-expressed with GzmB (Fig. 23 A).
Also the expression of another activation marker, KLRG1, showed high correlation with the microarray: up to 56 % of Killer TILs expressed KLRG1 and 73 % of $KLRG1^+$ $CD4^+$Trp1 T cells also expressed T-bet. It was previously demonstrated that high levels of T-bet expression in $CD8^+$ T cells induced a short lived, $KLRG1^{high}$ effector cell state with increased expression of KLRG1 and effector rather than memory potential (Joshi et al. 2007). This strongly correlates with the described gene and protein expression pattern observed in cytotoxic $CD4^+$Trp1 T cells.
Examining two more effector/memory markers, FACS analysis showed that, as on the gene array, CD27 expression was very low and only expressed in up to 15 % of $CD4^+$Trp1 TILs. *Cd2*5 expression, however, was high on transcript level but translated poorly into CD25 protein expression resulting in only up to 7 % $CD25^+$ $CD4^+$Trp1 TILs (Fig. 22).

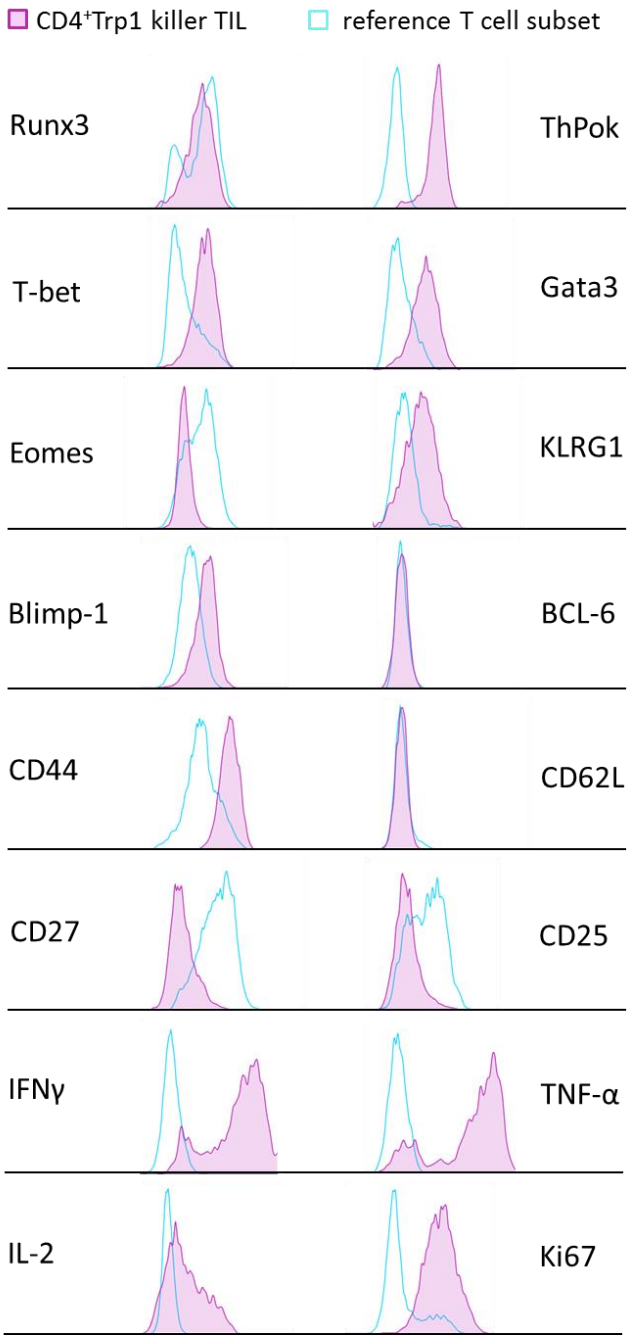

Figure 22 | Protein expression of selected CD4⁺, CD8⁺, effector and memory markers on tumour infiltrating CD4⁺Trp1 T cells correlates with their transcript level on the microarray in most cases. C57BL/6 mice were challenged with 2.5x10^5 B16/BL6 cells and received treatment to differentiate CD4⁺Trp1 T cells into tolerant, helper or killer T cells (see chapter *4.1*). Mice were sacrificed on day 17 or 18. CD4⁺Trp1 T cells isolated from the tumours of mice receiving the killer treatment were gated on CD45.1⁺ Foxp3⁻ (purple). The reference T cell population (blue) for ThPok, Runx3, Eomes, Bcl-6, KLRG1, CD62L, CD44 and Ki67 were endogenous CD8⁺ TILs from the tolerant cohort; the reference set for T-bet, Gata3, Blimp-1, IFNγ, TNF-α and IL-2 were endogenous CD4⁺ effector TILs from the tolerant cohort; the reference T cell population for CD25 were endogenous Tregs from the LN of killer treated mice and the CD27 reference set were endogenous CD8⁺ T cells from the LN of the killer cohort. Presented is representative data from four independent experiments which were each repeated two or three times, n=3 mice per group; with the exception of ThPok: the staining was only performed in one experiment.

In regards to Th1 cytokines, the results from the gene expression profiling were also reproduced on protein level: up to 92 % of tumour infiltrating killer CD4⁺Trp1 T cells ex-

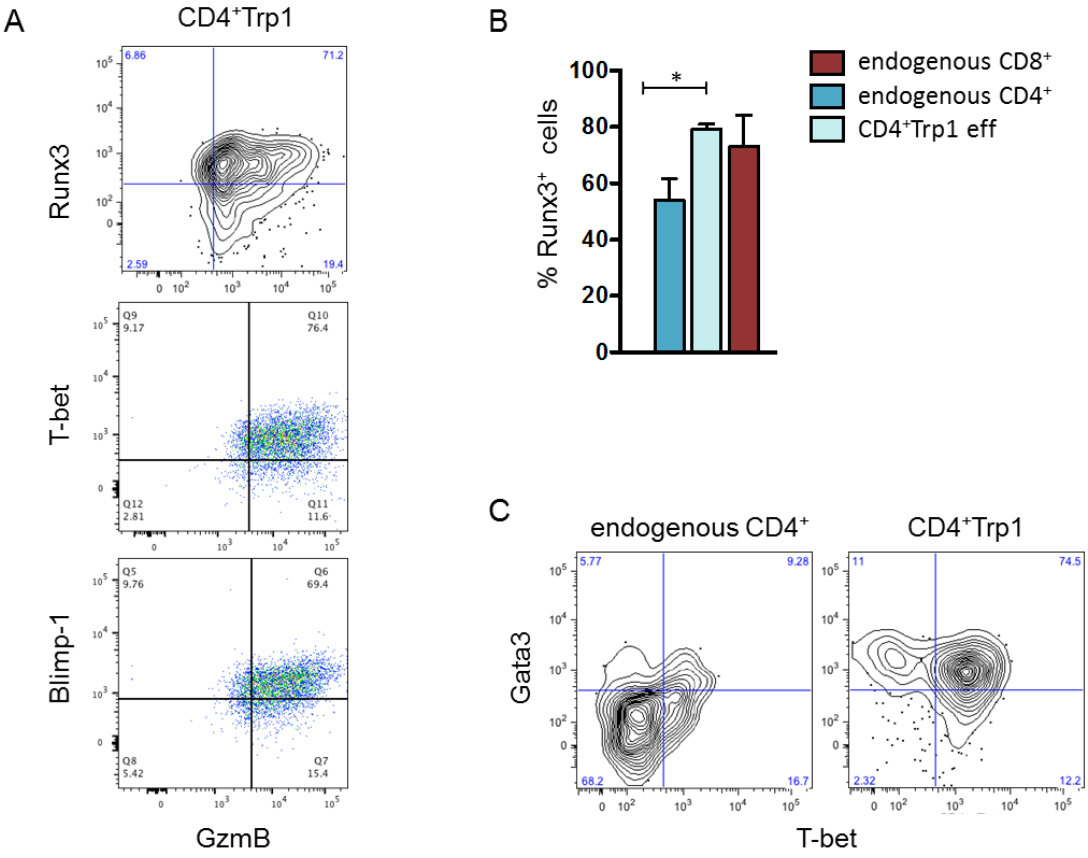

Figure 23 | Protein expression of CD8⁺ lineage and effector transcription factors Runx3 and Blimp-1 and Th1 and Th2 specific transcription factors T-bet and Gata3 on tumour infiltrating killer CD4⁺Trp1 T cells. C57BL/6 mice were challenged with 2.5x10^5 B16/BL6 cells and received treatment to differentiate CD4⁺Trp1 T cells into killer T cells (see chapter *4.1*). Mice were sacrificed on day 17 or 18. CD4⁺Trp1 T cells were isolated from the tumours and were gated on CD45.1⁺ Foxp3⁻ to exclude Tregs. **A:** Representative contour and pseudo colour plots depicting the co-expression of GzmB and transcription factors Runx3, T-bet and Blimp-1 on CD4⁺Trp1 TILs. **B:** Percentage of Runx3 expressing tumour infiltrating CD4⁺Trp1 effector T cells. **C:** Representative contour plot detailing T-bet and Gata3 expression on endogenous and CD4⁺Trp1 effector T cells isolated from the tumour. Presented is representative data from four independent experiments which were each repeated two or three times, n=3 mice per group. To calculate statistical differences a one way ANOVA with Bonferroni post-test was performed: * : P ≤ 0.05.

pressed IFNγ and TNF-α after re-stimulation. IL-2 expression was slightly lower with up to 40 % of killer CD4⁺ TILs producing the cytokine (Fig. 22).

The high expression of *Mki67* (encoding Ki67) mRNA on the microarray could be validated on protein level as well: the vast majority of killer CD4$^+$Trp1 TILs (up to 95 %) expressed the proliferation marker (Fig. 22).

To summarize, in keeping with the transcriptome analysis, the high expression on protein level of inflammatory Th1 cytokines, GzmB and transcription factors T-bet, Runx3 and Blimp-1 in tumour infiltrating killer CD4$^+$Trp1 T cells combined with the lack BCL-6 expression points towards a CD8$^+$ effector rather than a traditional CD4$^+$ Th1- or Th2-like phenotype. Importantly, and contrary to previous reports (Qui et al. 2011; Hirschhorn-Cymerman et al. 2012; Curran et al. 2013), the absence of Eomes emphasises that cytotoxic CD4$^+$Trp1 TILs present a new, multi-functional subset which, despite strong similarities, is distinct from CD8$^+$ CTLs.

4.3 The role of mTORC1 in the acquisition and maintenance of CD4$^+$Trp1 cytotoxicity

Previously, an association of the serine/threonine kinase mammalian target of rapamycin (mTOR) with T cell differentiation was illustrated (Delgoffe et al. 2009; Araki et al. 2009) and recent reports correlate mTORC1 inhibition with a decrease in GzmB and T-bet expression in CD8$^+$ CTLs (Rao et al. 2010). As these findings support a role for mTORC1 signalling in the differentiation of cytotoxic CD8$^+$ T cells, they also point to a potential role of this pathway in the acquisition of killer activity by CD4$^+$Trp1 T cells due to their high similarity to CD8$^+$ CTLs (see chapter *4.2.3* and *4.2.4*).

4.3.1 Effects of rapamycin induced mTORC1 inhibition on CD4$^+$Trp1 T cells

In order to address the role of mTOR activity in the cytotoxicity and proliferative potential of CD4$^+$Trp1 cells, naïve CD4$^+$Trp1 T cells were stimulated with their cognate antigen in an *in vitro* activation assay and treated with Rapamycin (RAPA). RAPA inhibits mTOR complex 1 (mTORC1) signalling by binding to the immunophilin FKBP12 (Schreiber 1991) which in turn binds mTORC1 and prevents phosphorylation of mTORC1

substrates. mTOR complex 2 (mTORC2) signalling, however, is insensitive to inhibition by RAPA as the FKBP12-rapamycin complex only binds to mTORC1 (Jacinto et al. 2004).

4.3.1.1 Rapamycin induced mTORC1 inhibition diminishes GzmB expression *in vitro*

For the *in vitro* activation assay, naïve $CD4^+$Trp cells were isolated from spleen and peripheral lymph nodes from TRP1 SRT, TRP1 LUC or TRP1 DTR mice and labelled with CFSE (carboxyfluorescein succinimidyl ester) or CellTrace violet to allow precise T cell proliferation analysis. The fluorescent succinimidyl ester dyes CFSE and CellTrace violet diffuse through the cell membrane, bind to intracellular proteins and thereby mark live cells. As the labelled cells divide, the fluorescence signal is diluted with each daughter cell receiving approximately half the fluorescence of the parental cell. The resulting generations can be detected by individual sharp peaks via flow cytometry (Lyons & Parish 1994; Graziano et al. 1998; Lyons 2000; Quah & Parish 2012).

The CFSE labelled $CD4^+$Trp cells were specifically stimulated by adding exogenous dendritic cells (DCs), irradiated, IFNγ-treated B16 cells and 2 µM TRP1 peptide. To inhibit mTORC1 signalling, 0.5 µM was added to the cell suspension; GzmB expression and proliferation were examined after 3 days of incubation.

The specific activation of the $CD4^+$Trp1 cells with their cognate antigen presented by DCs induced a high proliferation rate of up to 7 divisions over 72 hours ($CD4^+$ act, red, Fig. 24 A). 87 % of the $CD4^+$Trp1 T cells showed a fluorescence intensity correlating to 6 and 7 cell divisions and almost no cells were detected with a higher fluorescence signal corresponding to cells with less than 5 cell divisions (red, Fig. 24 A).

Treatment with RAPA slightly reduced the proliferative capacity of the $CD4^+$Trp1 cells as the T cells only reached a maximum of 5 divisions and peaked at the 4th cell division ($CD4^+$ act + RAPA, blue, Fig. 24 A). Additionally, evidenced by a peak of high fluorescence signal, there was a small population (9 %) of T cells in the RAPA treated sample which did not divide over the 72 hour incubation period. Non-stimulated T cells did not proliferate, as indicated by a singular peak of high fluorescence intensity ($CD4^+$, orange, fig. 24 A).

Activated $CD4^+$Trp1 T cells produced high levels of GzmB with up to 90 % of the cells expressing the cytolytic molecule ($CD4^+$ act, red, Fig. 24 B). Inhibition of mTORC1 in ac-

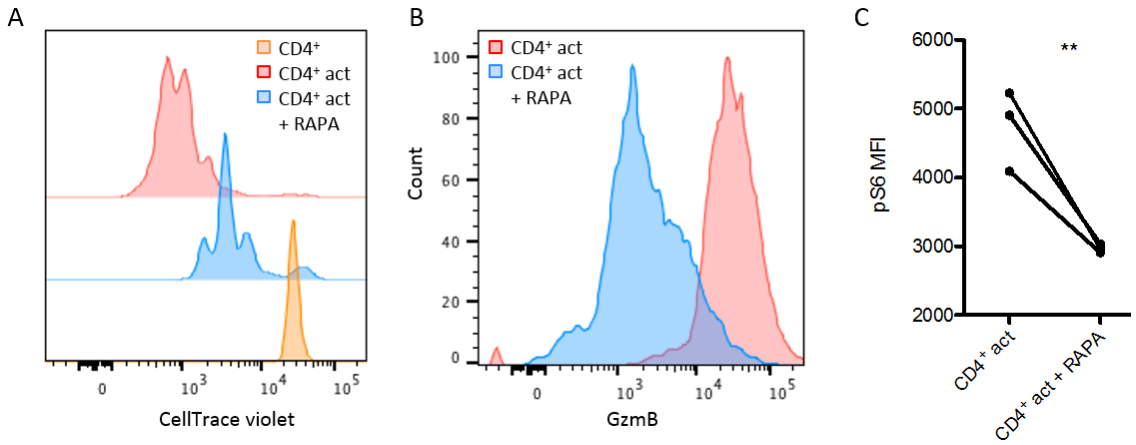

Figure 24 | Inhibition of mTORC1 in activated CD4⁺Trp1 cells slightly reduces proliferation and diminishes GzmB expression. Purified naïve CD4⁺Trp1 cells from lymph nodes and spleen of Trp1 SRT/LUC/FoxDTR mice were labelled with CellTrace violet, left unstimulated (CD4⁺, orange) or were activated for 72 hours with B16 pulsed DCs and 2 µM TRP1 peptide (CD4⁺ act, red) and treated with 0.5 µM rapamycin (CD4⁺ act + RAPA, blue). **A:** Depiction of cell divisions by decreasing fluorescence of CellTrace violet with each division. **B:** GzmB expression in activated CD4⁺Trp1 T cells without (CD4⁺ act, red) and with RAPA treatment (CD4⁺ act + RAPA, blue). **C:** Geometric mean fluorescence intensity (MFI) of Ser235/236 S6 phosphorylation (pS6) in activated CD4⁺Trp1 T cells without (CD4⁺ act) and with RAPA treatment (CD4⁺ act + RAPA). Shown are representative graphs of one out of three independent experiments, all conditions were performed in duplicates or triplicates. A Student's t test was used to determine statistical significance: * : P ≤ 0.05, ** : P ≤ 0.01.

tivated CD4⁺Trp1 T cells by RAPA treatment caused a distinct decrease of GzmB⁺ T cells (CD4⁺ act + RAPA, blue, Fig. 24 B).

Inhibition of mTORC1 signalling was verified by staining for the presence of the Ser235/236 phosphorylation of S6 (pS6), the target of the mTORC1 substrate S6K. To measure the S6 phosphorylation per cell, the pS6 geometric mean fluorescence intensity (MFI) was calculated. In comparison with the activated control sample (CD4+ act), RAPA treatment inhibited mTORC1 signalling as evidenced by a mean reduction in pS6 MFI of 63.3 % (± 5.6 %) in CD4⁺Trp1 T cells (Fig. 24 C).

4.3.1.2 mTORC1 inhibition decreases GzmB expression *in vivo* whilst preserving the production of effector cytokines

To assess whether the decrease of GzmB caused by mTORC1 inhibition observed *in vitro* also translates into a functional difference in cytotoxic activity *in vivo*, RAPA was administered to mice receiving the 'killer' treatment. As outlined in chapter *4.1*, B16/BL6 tumour bearing C57BL/6 mice received 5 Gy RT, $6x10^4$ $CD4^+$Trp1 T cells and αCTLA-4 and received additional daily i.p. injections of 375 µg/kg RAPA from day 12 on. The mice were sacrificed on day 17 or 18 and the tumours and lymph nodes were dissected and processed according to chapter *3.4.4*.

The concentration of 375 µg/kg/d rapamycin was chosen based on multiple previous studies of mTORC1 inhibition in T cells *in vivo*; the concentration of i.p. RAPA treatment typically ranged from 75 or 750 µg/kg/day (Araki et al. 2009; Li et al. 2011; Li et al. 2012; Keating et al. 2013; Chaoul et al. 2015; Pollizzi et al. 2015). Preliminary experiments with the highest concentration 750 µg/kg/d showed that this concentration caused such a drastic decrease in the number of tumour infiltrating $CD4^+$Trp1 T cells that there were too few cells to analyse. Therefore, the intermediate concentration of 375 µg/kg/d was used for further experiments.

The RAPA treatment significantly decreased the infiltration of $CD4^+$Trp1 effector T cells into the tumour (Fig 23 A) in comparison to the non-RAPA treated cohort.
In keeping with previous reports on $CD8^+$ CTL (Rao et al. 2010), RAPA treatment during the immune response significantly reduced GzmB expression on both endogenous $CD8^+$ as well as $CD4^+$Trp1 T cells in the tumour (Fig. 25 B). A reduction of up to 56.0 % of GzmB expression was observed, from 67.6 % (± 10.8 % SD) of $CD4^+$Trp1 effector T cells expressing the cytolytic molecule down to 29.7 % (± 13.7 % SD) (Fig. 25 B, C).
Despite the lower infiltration rate, $CD4^+$Trp1 T cell proliferation, as measured by Ki67 expression, was only slightly reduced in the tumours of the RAPA treated mice and statistically not significant (Fig. 25 D, E).
The impact of RAPA treatment on T-bet expression of $CD4^+$Trp1 effector T cells, however, did not correlate with the published $CD8^+$ T cell data from Rao et al. which showed significant decrease of T-bet expression upon RAPA treatment (Rao et al. 2010): There was no significant change in T-bet expression in $CD4^+$Trp1 killer T cells in the RAPA treated cohort (Fig. 25 F).

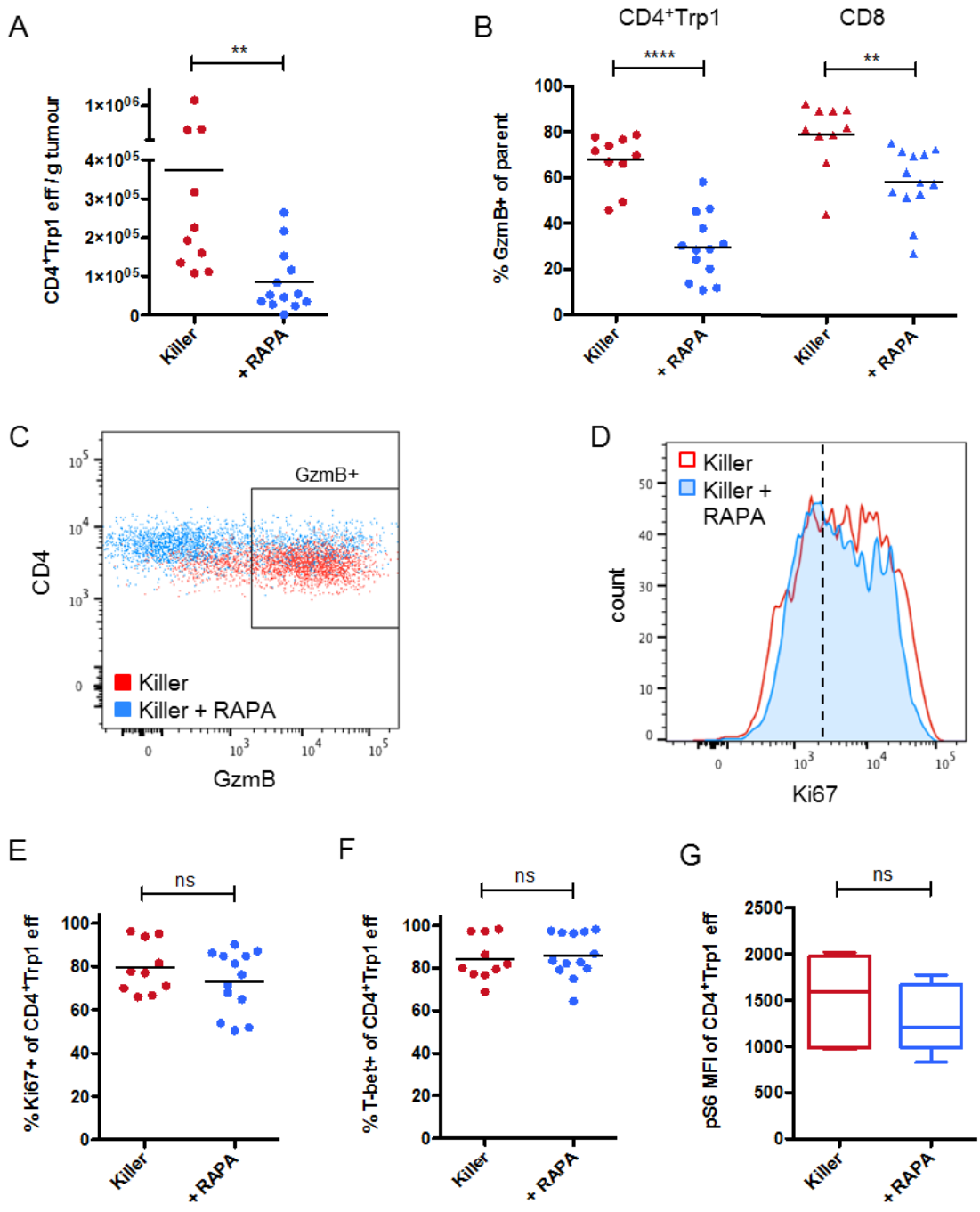

Figure 25 | Inhibition of mTORC1 activity by rapamycin treatment causes reduction of CD4⁺Trp1 infiltration into the tumour and GzmB expression on tumour infiltrating lymphocytes but does not affect their proliferation. C57BL/6 mice were challenged with 2.5x10^5 B16/BL6 tumour cells and received only the killer (RT + CD4⁺Trp1 transfer + αCTLA-4, chapter *4.1*) treatment (Killer, red) or the killer treatment with additional daily i.p. injections of 375 µg/kg rapamycin (+RAPA, blue) from day 12 until the end of the experiment. Mice were sacrificed on day 17 or 18. **A:** Quantification

of the absolute number of tumour infiltrating Foxp3$^-$ CD4$^+$Trp1 effector cells per gram tumour. **B:** Percentage of GzmB expressing CD4$^+$Trp1 effector and endogenous CD8$^+$ T cells in the tumour. **C:** Dot plot detailing GzmB expression of CD4$^+$Trp1 effector T cells from tumours treated with (blue) and without RAPA (red). **D:** Histogram depicting Ki67 expression on CD4$^+$Trp1 effector T cells from tumours treated with (blue) and without RAPA (red). **E:** Percentage of Ki67 expressing tumour infiltrating CD4$^+$Trp1 effector T cells. **F:** Percentage of T-bet expressing CD4$^+$Trp1 effector T cells in the tumour. **G:** MFI of pS6 in tumour infiltrating CD4$^+$Trp1 T cells. Presented is pooled (A, B, E-G) or representative (C, D) data from one of three independent experiments, n = 3-5 mice per group. Numbers of tumour infiltrating T cells were calculated as described in Materials and methods. A one way ANOVA with a Bonferroni post-test was performed to calculate statistical differences between groups: * : P ≤ 0.05, ** : P ≤ 0.01, *** : P ≤ 0.001, **** : P ≤ 0.0001, ns: not significant.

Unfortunately, it was not possible to verify the actual inhibition of mTORC1 activity in CD4$^+$Trp1 T cells by RAPA treatment. The direct *ex vivo* staining of mTORC1 downstream target pS6 after the processing of the tissues was poor and yielded only a slight and statistically not significant decrease in pS6 MFI of CD4$^+$Trp1 T cells from RAPA treated mice (Fig. 25 G). This result could suggest that the administered RAPA concentration of 375 µg/kg/d was not sufficient to downregulate mTORC1 activity. However, it is also possible that the overall decreased staining of pS6 was caused by the lengthy process of extracting the lymphocytes from the tumours, which included 30 minutes incubation of the tumours at 37 °C and gradient centrifugation at RT. The processing and change of temperatures might have allowed intracellular phosphatases to remove the phosphorylation of S6 before the FACS staining.

In order to analyse the cytokine expression, CD4$^+$Trp1 T cells from tumour and lymph node samples were re-stimulated with exogenous DCs and TRP1 peptide for 4 hours without adding any additional RAPA.

Due to the lower number of infiltrating CD4$^+$Trp1 effector T cells it was not possible to split the tumour and perform two different FACS staining panels (direct *ex vivo* staining and after re-stimulation) from the same sample (tumour). This is important to note as it means that these re-stimulation results, unlike the other re-stimulation data presented in this study, do not show cells from the same population (tumour) as in the direct *ex vivo* analysis.

The direct stimulation through the TCR by DCs presenting the cognate antigen in in MHC II results in mTORC1 activity (Chi 2012), this is illustrated in the untreated killer CD4$^+$Trp1 T cells and, interestingly, the re-stimulated CD4$^+$Trp1 T cells from RAPA treated mice showed a distinct and significant downregulation of pS6: tumour infiltrating CD4$^+$Trp1 cells from the RAPA treated cohort showed a mean reduction of pS6 MFI of 33.5 % (± 20.5 % SD) in comparison to the non-treated killer cohort (Fig. 26 B). This illustrates that enough RAPA was retained in cells throughout the lymphocyte isolation process to inhibit mTORC1 activity when the cells were re-activated. This furthermore strongly suggests that although there was no difference in pS6 MFI detectable in the direct *ex vivo* analysis of the CD4$^+$Trp1 T cells (Fig. 25 G), the RAPA treatment was probably sufficient to induce mTORC1 inhibition in T cells *in vivo*. The observed loss of S6 phosphorylation conceivably was not due to a lack of mTORC1 inhibition but to the lengthy tissue processing before the FACS staining.

After re-stimulation, the tumour infiltrating CD4$^+$Trp1 T cells from the RAPA treated cohort exhibited a mean reduction of GzmB expression of 52,4 % (± 20.3 % SD) in comparison with the non-treated killer cohort (Fig. 26 A). This reduction in GzmB expression was almost identical to the observed decrease in the directly *ex vivo* stained tumour samples (Fig. 25 B). This combined data supports and emphasizes the negative impact of mTORC1 inhibition on the cytotoxicity of CD4$^+$Trp1 T cells *in vivo*.

However, despite the persistent effect of mTORC1 inhibition on GzmB expression, the production of inflammatory cytokines IFNγ, IL-2 and TNF-α after re-stimulation was not significantly reduced (Fig. 26 C).

It is important to note, however, that the pleiotropic nature of mTOR and the systemic administration of RAPA in this experiment make it difficult to conclude with certainty that the GzmB down-regulation was exclusively due to mTORC1 inhibition in the transferred CD4$^+$Trp1 T cells. Furthermore, a negative effect of RAPA on B16 melanoma proliferation *in vitro* and growth and metastasis *in vivo* which was demonstrated in previous studies (Eng et al. 1984; Buscà et al. 1996; Guba et al. 2002; Yang et al. 2010). The observed effect could therefore also be caused or increased by an interplay of different intercellular factors caused by the ubiquitous mTORC inhibition in other cells, including for instance endogenous lymphocytes, myeloid and tumour cells.

Because of this lack of specificity of mTORC1 inhibition, I chose to genetically engineer CD4$^+$Trp1 T cells to decrease mTORC1 activity in these cells exclusively.

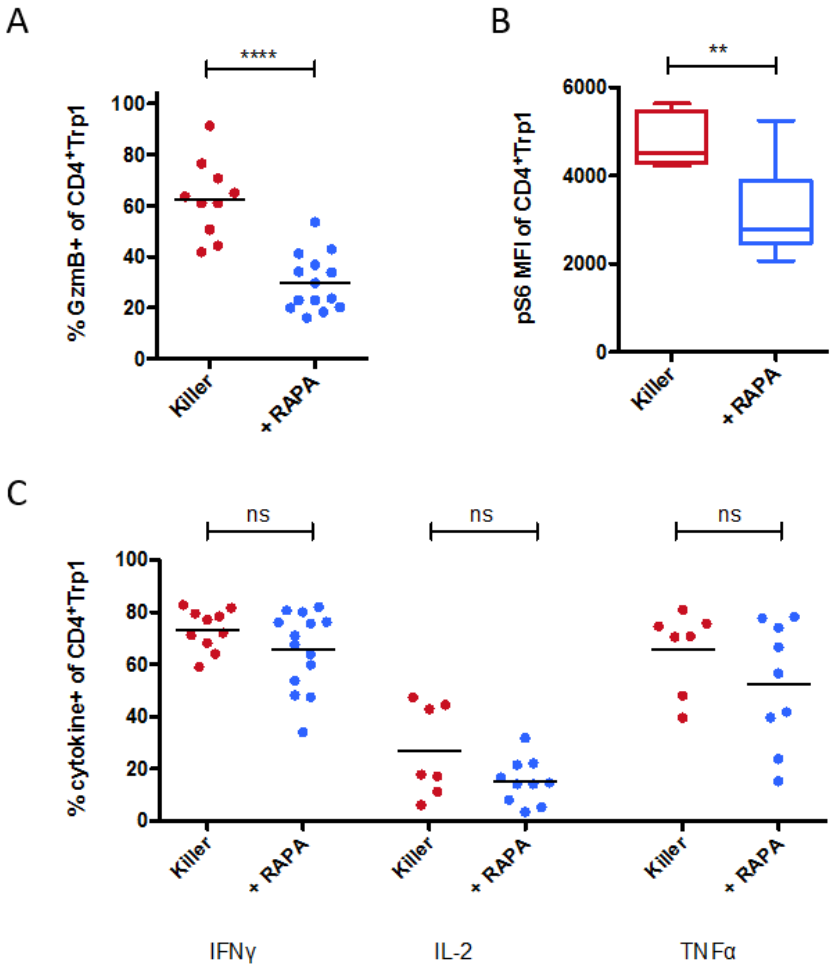

Figure 26 | Re-stimulated CD4⁺Trp1 T cells from RAPA treated tumours display decreased ability to express GzmB whilst maintaining the capacity for cytokine production. C57BL/6 mice were challenged with $2.5x10^5$ B16/BL6 tumour cells and received only the killer (RT + CD4⁺Trp1 transfer + αCTLA-4, see chapter *4.1*) treatment (Killer, red) or the killer treatment with additional daily i.p. injections of 375 µg/kg rapamycin (+RAPA, blue) from day 12 until the end of the experiment. Mice were sacrificed on day 17 or 18 and tumour infiltrating lymphocytes were re-stimulated for 4 hours with exogenous DCs and 2 µM TRP1 peptide. **A:** Percentage of GzmB expressing tumour infiltrating CD4⁺Trp1 T cells after re-stimulation. **B:** MFI of pS6 in tumour infiltrating CD4⁺Trp1 T cells after re-stimulation. **C:** Percentage of tumour infiltrating CD4⁺Trp1 T cells expressing IFNγ, IL-2 or TNF-α after re-stimulation. Presented is pooled data from two independent experiments, n = 3-5 mice per group. A one way ANOVA with a Bonferroni post-test was performed to calculate statistical differences between groups: * : P ≤ 0.05, ** : P ≤ 0.01, *** : P ≤ 0.001, **** : P ≤ 0.0001, ns: not significant.

4.3.1.3 Genetic approach of CD4$^+$Trp1 T cell-selective mTORC1 inhibition

To assess the impact of mTORC1 inhibition on the acquisition and maintenance of the cytotoxic phenotype more selectively and avoid systemic mTORC1 dysregulation, the CD4$^+$Trp1 T cells were genetically engineered prior to the adoptive transfer. The transgenic CD4$^+$ T cells were transduced with the inducible MoMLV retroviral plasmid iPRAS40 to overexpress proline-rich Akt substrate of 40 kDa (PRAS40), a negative regulator of mTORC1 activity (Wang et al. 2012). The expression vectors iPRAS40 and the respective control vector iGFP which lacks the *Pras40* transgene were designed, validated and kindly provided by Pedro Veliça (Royal Free Hospital, London, UK) and were used *in vivo* for selective mTORC1 inhibition in murine tumour specific CD8$^+$ T cells in a recent publication (Veliça et al. 2015). Both vectors iPRAS40 and iGFP encode a Q8 tag, a fusion protein of human CD8α and CD34, as transduction marker and GFP as induction marker which is only expressed alone (iGFP) or along with the *Pras40* transgene (iPRAS40) upon treatment with doxycycline (DOX) (see chapter *4.1*) (Philip et al. 2014; Veliça et al. 2015).

To test the effect of inducible PRAS40 expression on mTORC1 signalling in CD4$^+$Trp1 T cells, CD4$^+$ T cells were purified from naïve Trp1 SRT/LUC or DTR mice and were transduced with the retroviral plasmids iGFP and iPRAS40 after 24 hour specific stimulation with exogenous DCs and TRP1 peptide (see chapter *3.4.4*). The cells were expanded for 72 hours supplemented with 100 u/ml IL-2 *in vitro* and subsequently rested overnight without IL-2. The cells were then left untreated (CD4$^+$) or re-stimulated with exogenous DCs and 2 µM TRP1 peptide (CD4$^+$ act) and received treatment or not with 0.5 µM RAPA (+ RAPA) and/or 1 µg/ml doxycycline (+ DOX) for 24 hours. 1 µg/ml doxycycline was previously validated to be the ideal DOX concentration for maximum induction *in vitro* with these vectors (Veliça et al. 2015).

The transduction efficiency in CD4$^+$Trp1 T cells was typically 65-90% for iGFP and 60-80 % for iPRAS40 vectors. In the absence of DOX, no GFP expression and no effect on pS6, i.e. mTORC1 activity, was observed in iPRAS40 and iGFP transduced cells (Fig. 27 A, B, grey). Upon treatment with DOX, GFP expression was induced in both cell types but only iPRAS40 transduced cells showed a decrease in pS6 expression cells (Fig. 27 A, B dark red/blue). Treatment with RAPA robustly reduced mTORC1 activity and thus

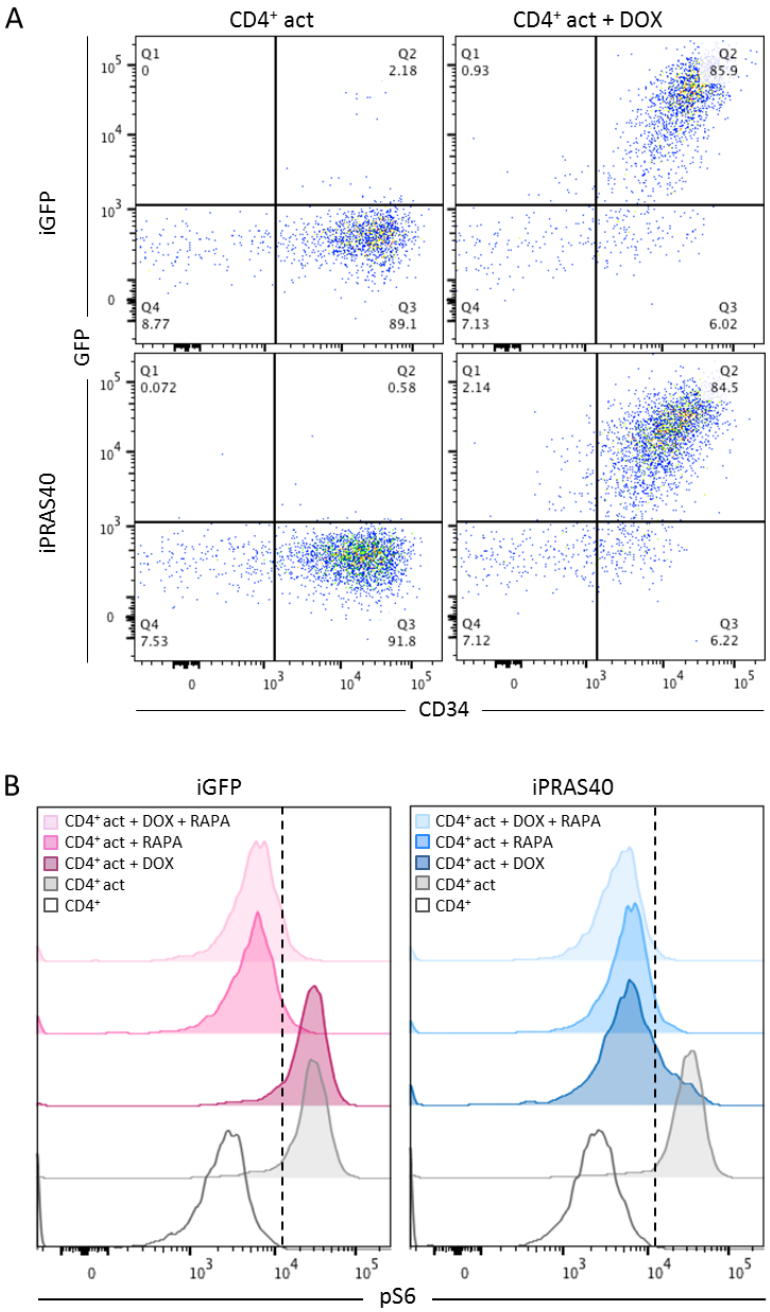

Figure 27 | After successful transduction and induction, CD4⁺Trp1 T cells transduced with the iPRAS40 construct show inhibition of mTORC1 activity. CD4⁺Trp1 T cells were transduced with retroviral plasmids iGFP and iPRAS40 (see chapter *3.6.4*) and were kept for 72 hours supplemented with 100 u/ml IL-2 *in vitro*. After resting overnight without IL-2, CD4⁺Trp1 T cells were left untreated (CD4⁺) or re-stimulated with exogenous DCs and 2 µM TRP1 peptide (CD4⁺ act) and received treatment or not with 0.5 µM RAPA (+ RAPA) and/or 1 µg/ml doxycycline (+ DOX) for 24 hours. **A:** Dot plots detailing the expression of transduction marker CD34 and induction marker GFP on re-stimulated CD4⁺Trp1 T cells when treated with DOX (CD4⁺ act + DOX) or not (CD4⁺ act). **B:** Histograms depicting the phosphorylation of S6 (pS6) on CD4⁺Trp1 T cells after re-stimulation and treatment with DOX and/or RAPA. Shown are representative graphs of one out of two independent experiments, all conditions were performed in duplicates or triplicates.

phosphorylation of S6 (pS6) in both iGFP and iPRAS40 transduced cells (Fig. 27 B, CD4⁺ act + RAPA).

Addition of RAPA to the induction with DOX did not decrease pS6 significantly more than RAPA treatment alone (Fig. 27 B, $CD4^+$ act + DOX + RAPA).
In comparison with RAPA treated T cells, mTORC1 inhibition by inducible PRAS40 expression pS6 was not completely downregulated (Fig. 27 B, $CD4^+$ act + DOX, blue). This suggests that iPRAS40 transduced $CD4^+$Trp1 T cells retain residual, but still highly reduced mTORC1 activity in comparison to non-induced iPRAS40 transduced T cells and induced iGFP $CD4^+$Trp1 T cells (Fig. 27 B, $CD4^+$ act, grey and $CD4^+$ act + DOX, dark red).

As inducible PRAS40 expression resulted in a successful reduction in pS6 and hence mTORC1 inhibition of up to 80 %, the iPRAS40 transduced $CD4^+$Trp1 T cells were considered effective enough to use in functional *in vivo* experiments.

4.3.1.4 iPRAS40 $CD4^+$Trp1 T cells display reduced GzmB expression *in vivo* but maintain ability to produce IFNγ

In order to test the effect of $CD4^+$Trp1 T cell specific mTORC1 inhibition on the development and maintenance of the cytotoxic phenotype *in vivo*, iPRAS40 transduced $CD4^+$Trp1 T cells were injected instead of naïve T cells in the 'killer' treatment. As described in chapter *4.1*, B16/BL6 tumour bearing C57BL/6 mice received 5 Gy RT and αCTLA-4 i.p. and received adoptive transfer of either $6x10^4$ naïve $CD4^+$Trp1 T cells as reference cohort ('naïve killer'), $3x10^5$ iGFP transduced $CD4^+$Trp1 T cells as control cohort ('iGFP killer') or $3x10^5$ iPRAS40 transduced $CD4^+$Trp1 T cells ('iPRAS40 killer') for the experimental mTORC1 inhibition cohort. Additionally, all groups received 2 mg/ml DOX in the drinking water from day 12 on until the end of the experiment to induce PRAS40 expression in iPRAS40 transduced $CD4^+$Trp1 T cells. The mice were sacrificed on day 18 or 19 and the tumours and lymph nodes were dissected and processed according to chapter *3.4.4*.
After transduction and subsequent 72 hour *in vitro* culture of $CD4^+$Trp1, the T cells were purified based on the transduction marker CD34. The $CD4^+$ and $CD34^+$ purity of iPRAS40 and iGFP transduced T cells that were then adoptively transferred typically reached 90 to 100 %.

In order to achieve a similar number of transduced tumour infiltrating $CD4^+$Trp1 T cells as in the naïve killer cohort, the number of adoptively transferred T cells was increased

from $6x10^4$ to $3x10^5$ as preliminary experiments showed a decreased infiltration and engraftment of transduced cells at lower numbers of adoptively transferred transduced $CD4^+$Trp1 T cells.

Analysis of the lymph nodes and tumours demonstrated an efficient engraftment of transduced T cells in both tissues and a successful induction of transgene expression as measured by the transduction marker Q8 (CD34) and induction marker GFP (Fig. 28 A, mid blue). A large proportion of engrafted tumour infiltrating $CD4^+$Trp1 T cells (85 % of iGFP and 62 % of iPRAS40) was $CD34^+$ GFP^+ (Fig. 28 A); only this $CD45.1^+$ $CD4^+$ $CD34^+$ GFP^+ population (mid blue) was used for subsequent analysis of marker expression. The engraftment of T cells was reduced in comparison with the naïve killer (red) in the iPRAS40 transduced cohort (blue) while the iGFP transduced group (green) showed no significant change in tumour infiltrating T cell numbers (Fig. 28 B).

Direct *ex vivo* staining of GzmB of tumour infiltrating lymphocytes showed that significantly less iPRAS40 transduced and induced ($CD34^+$ GFP^+) $CD4^+$Trp1 T cells expressed the cytotoxic molecule than the mock control iGFP and the reference naïve killer cohort (Fig. 28 C). The percentage of GzmB expression $CD4^+$Trp1 T cells reduced from 51.4 % (± 11.9 % SD) in the iGFP cohort to 35.3 % (± 8.9 % SD) in the iPRAS40 group. This significant decrease in GzmB expression upon mTORC1 inhibition is in agreement with the rapamycin induced mTORC1 inhibition data and further supports the hypothesis that mTORC1 activity is instrumental for the acquisition or maintenance of the cytotoxic phenotype.

The proliferative status of the transferred cells, as evidenced by Ki67 expression, differed significantly between the naïve and transduced $CD4^+$Trp1 T cells (Fig. 28 D). Although iPRAS40 transduced cells showed a further decrease of proliferation in comparison to the iGFP control group but an average of 76 % iPRAS40 $CD4^+$Trp1 effectors still displayed high expression of Ki67 (Fig. 28 D). This is an important piece of data as it demonstrates that despite the mTORC1 inhibition the tumour reactive cells maintain an overall high proliferative status.

Interestingly, the significant reduction in GzmB expression between the naïve killer reference group and the iGFP mock control cohort illustrates that the transduction process and/or *in vitro* culture prior to the adoptive transfer already compromises $CD4^+$Trp1 T cell cytotoxic to a certain extent (Fig. 28 C).

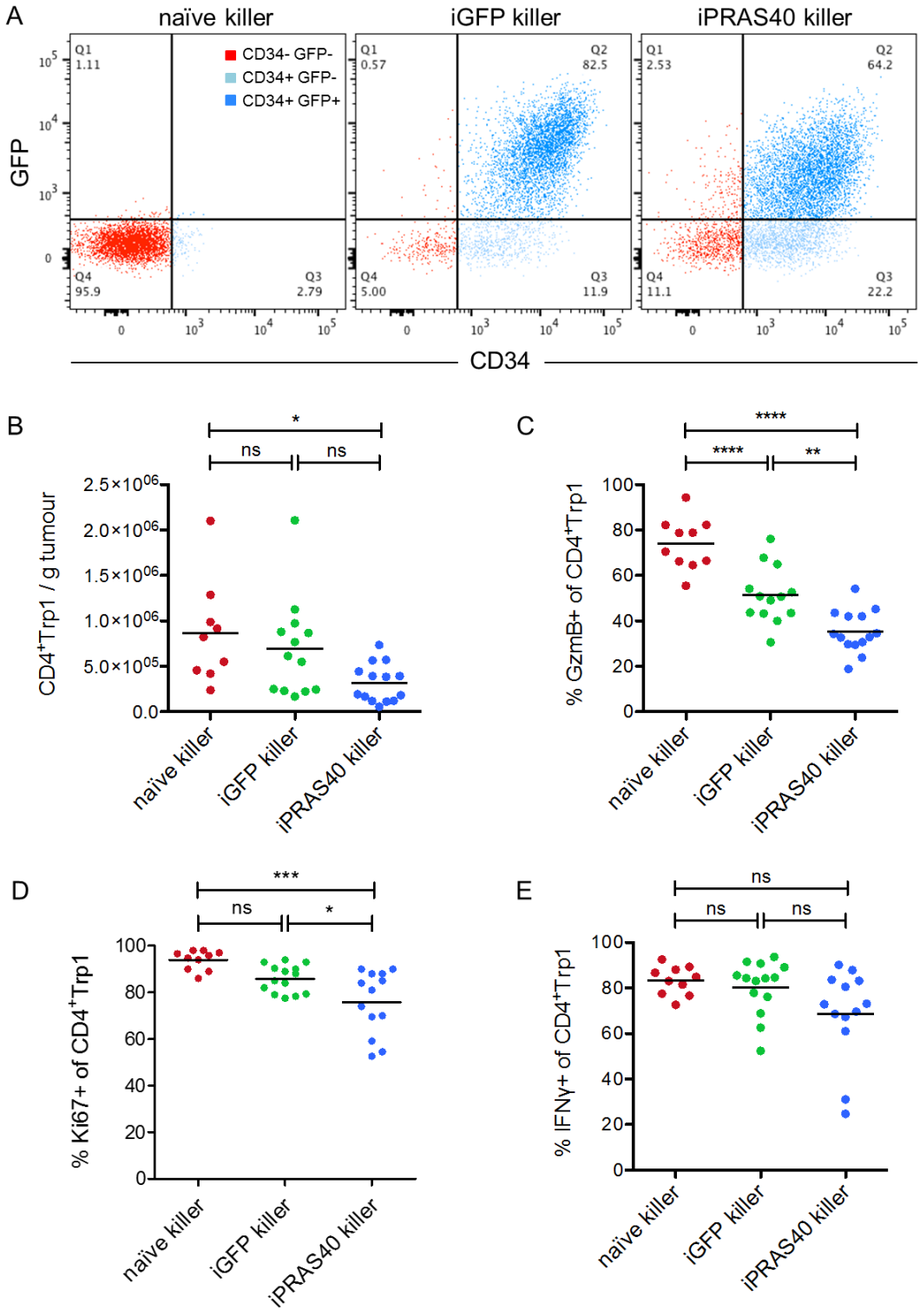

Figure 28 | mTORC1 inhibition via overexpression of PRAS40 induces a reduction of GzmB+ CD4⁺Trp1 T cells whilst maintaining the production of IFNγ after re-stimulation. CD4⁺Trp1 T cells were transduced with retroviral plasmid iPRAS40 and the control plasmid iGFP (see chapter *3.6.4*) and were kept for 72 hours supplemented with

100 u/ml IL-2 *in vitro*. C57BL/6 mice were challenged with B16/BL6 tumour cells and received the killer treatment (RT + $CD4^+$Trp1 transfer + αCTLA-4, chapter *4.1*) with $6x10^4$ naïve $CD4^+$Trp1 T cells (naïve killer, red) or $3x10^5$ iGFP/ iPRAS40 transduced $CD4^+$Trp1 T cells (iGFP killer/iPRAS40 killer). Additionally, all groups received doxycycline (DOX) from day 12 until the end of the experiment to induce the expression of the PRAS40 transgene. Mice were sacrificed on day 18 or 19 and to measure cytokine expression, tumour infiltrating lymphocytes were re-stimulated for 4 hours with exogenous DCs and 2 µM TRP1 peptide. **A:** Representative depiction of the expression of transduction marker CD34 and induction marker GFP on iGFP/iRPAS40 transduced and induced tumour infiltrating $CD4^+$Trp1 T cells. **B:** Quantification of the absolute number of tumour infiltrating $CD4^+$Trp1 cells per gram tumour. **C:** Percentage of GzmB expressing tumour infiltrating $CD4^+$Trp1 T cells. **D:** Percentage of Ki67 expressing tumour infiltrating $CD4^+$Trp1 T cells. **E:** Percentage of tumour infiltrating $CD4^+$Trp1 T cells expressing IFNγ after re-stimulation. Presented is representative or pooled data from three independent experiments, n = 3-6 mice per group. A one way ANOVA with a Bonferroni post-test was performed to calculate statistical differences between groups: * : $P \leq 0.05$, ** : $P \leq 0.01$, *** : $P \leq 0.001$, **** : $P \leq 0.0001$, ns: not significant.

To re-stimulate and analyse inflammatory cytokine expression of tumour infiltrating $CD4^+$Trp1 T cells, half of the tumour samples were incubated with exogenous DCs and 2 µM TRP1 peptide for 4 hours without adding any additional DOX. As also observed when inhibiting mTORC1 by administration of RAPA, also the $CD4^+$Trp1 T cell specific mTORC1 inhibition on iPRAS40 $CD4^+$Trp1 T cells showed no impact on IFNγ expression in comparison to the naïve killer reference and iGFP mock control (Fig. 28 E).

4.3.1.5 Genetic engineering of $CD4^+$Trp1 T cells causes reduced *in vivo* anti-tumour activity and loss of long-term protection

To assess the impact of mTORC1 inhibition on the *in vivo* anti-tumour activity of CD4Trp1 and their ability to reject established tumours, we tracked tumour growth and overall survival after therapy over a period of 100 days. Due to the observed significant difference in GzmB expression upon mTORC1 inhibition (Fig. 28 C) the protection experiments aimed to determine whether the observed reduction of cytotoxicity marker GzmB translated into a decreased potential in tumour eradication and recurrence. This

was an essential question as GzmB expression was not completely ablated in the iPRAS40 transduced CD4$^+$Trp1 T cells, but only reduced; it was therefore possible that the residual GzmB expression, along with potential compensation mechanisms, could still reduce or control tumour growth. Furthermore, it was critical to determine if the transduction process and the decreased GzmB expression in iGFP CD4$^+$Trp1 killer T cells in comparison with the naïve CD4$^+$Trp1 killer T cells (Fig. 28 C) impaired the *in vivo* functionality of the transduced T cells.

The experimental set up of the tumour survival experiments was identical with the previous functional experiment: B16/BL6 tumour bearing C57BL/6 mice received the killer treatment (5 Gy RT + αCTLA-4 + CD4$^+$Trp1 T cell transfer) with either 6x10^4 naïve CD4$^+$Trp1 T cells as reference cohort ('naïve killer + DOX'), 3x10^5 iGFP transduced CD4$^+$Trp1 T cells as control cohort ('iGFP killer + DOX') or 3x10^5 iPRAS40 transduced CD4$^+$Trp1 T cells ('iPRAS40 killer (+ DOX)'). All groups except for a subgroup of the iRPAS40 cohort ('iPRAS40 killer') received 2 mg/ml DOX in the drinking water from day 12 on until the end of the experiment to induce PRAS40 expression in iPRAS40 transduced CD4$^+$Trp1 T cells. The mice were closely monitored and tumour size of individual animals was recorded every 2-3 days throughout the 100 days of the experiment.

In keeping with previous work (Quezada et al. 2010), the reference cohort (naïve killer + DOX) rejected the implanted tumours efficiently. One out 10 mice in this group had to be euthanised on day 30 due to poor wellbeing and despite having a regressing tumour (red, Fig. 29 A, B). The control group iGFP + DOX showed a heterogeneous response with 3 out of 12 mice completely rejecting their tumours whist the rest showed only temporary control of tumour growth and subsequent outgrowth (green, Fig. 29 A). Overall this resulted in long-term survival of 25 % of the iGFP + DOX cohort (green, Fig. 29 B). The additional control group which only received iPRAS40 transduced cells but no induction of PRAS40 expression through DOX (iPRAS40 killer) yielded similar results: 7 out of 10 mice eventually succumbed to the outgrowth of transiently controlled tumours but 30 % displayed tumour eradication and long-term survival (purple, Fig. 29 A, B). The experimental cohort which received iPRAS40 transduced cells and DOX (iPRAS40 killer + DOX) did not differ significantly from the control iGFP killer + DOX and the iPRAS40 killer groups: the majority (8 out of 12 mice) displayed temporary tumour control followed by consequent outgrowth while 33.3 % saw tumour rejection and survival for 100 days (blue, Fig. 29 A, B).

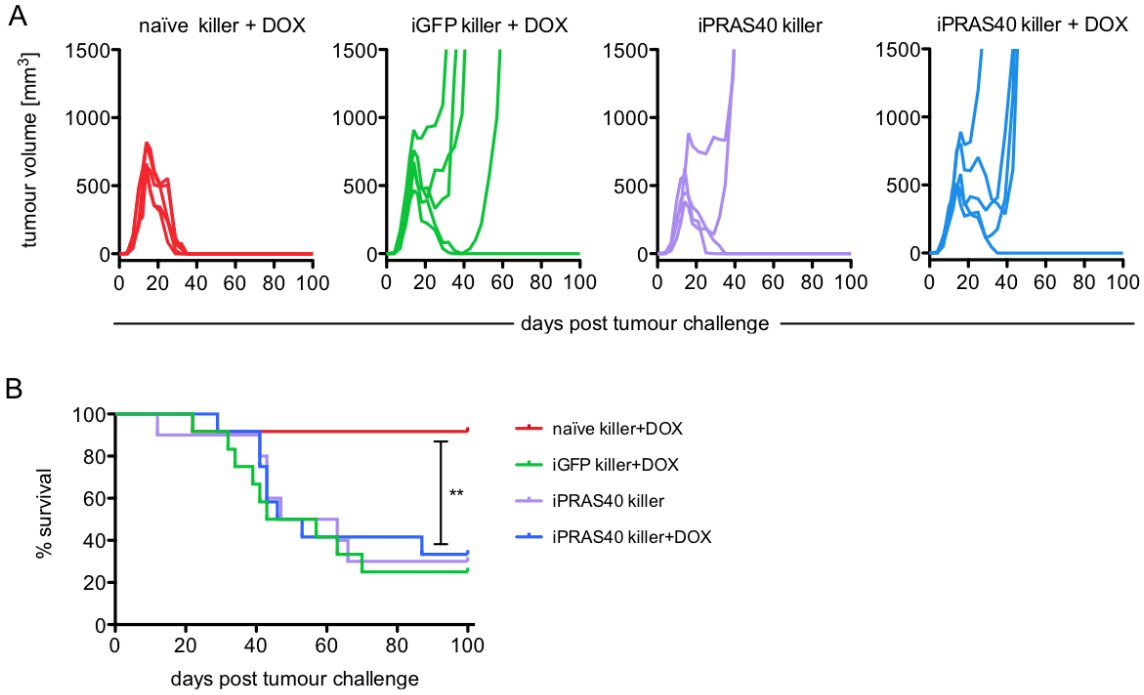

Figure 29 | Genetic engineering of CD4$^+$Trp1 T cells results in loss of complete tumour rejection and long-term protection. CD4$^+$Trp1 T cells were transduced with retroviral plasmid iPRAS40 and the control plasmid iGFP (see chapter *3.6.4*) and were expanded for 72 hours with IL-2 *in vitro*. C57BL/6 mice were challenged with B16/BL6 tumour cells and received the killer treatment (RT + CD4$^+$Trp1 transfer + αCTLA-4 chapter *4.1*) with 6x10^4 naïve CD4$^+$Trp1 T cells (naïve killer) or 3x10^5 iGFP/ iPRAS40 transduced CD4$^+$Trp1 T cells (iGFP killer/iPRAS40 killer) and DOX or not from day 12 until the end of the experiment to induce the expression of the PRAS40 transgene. **A:** tumour growth (volume) over time in individual mice from one representative experiment. **B:** Cumulative survival of mice from two independent experiments. Presented is representative or pooled data from two independent experiments, n = 5-6 mice per group. Tumour survival data was analysed with the Kaplan-Meier method and a log-rank (Mantel-Cox) test was performed to calculate statistical differences between survival curves, * : P ≤ 0.05, ** : P ≤ 0.01.

These results demonstrate that in this model, mTORC1 inhibition did not have a statistically significant impact on tumour eradication and long-term survival when compared to the two control groups (iPRAS40 killer + DOX vs iPRAS40 killer or iGFP killer + DOX). However, it is important to note that there was a drastic loss of tumour

protection and survival advantage in the control groups iPRAS40 killer and iGFP killer + DOX in comparison to the naïve killer + DOX reference cohort (Fig. 29 B).
The loss of the characteristic potent anti-tumour activity of killer CD4$^+$Trp1 T cells in all the cohorts receiving transduced T cells (irrespective of mTORC1 inhibition) strongly suggests that the *in vitro* transduction process (either the expansion, the viral transduction or both) reduced the transduced T cells' ability to fully eradicate the established tumours. A potential explanation would be the exhaustion of the transduced T cells due to long-term *in vitro* expansion in high IL-2.

It is important to note that the end point of the functional experiment was on day 18/19 after tumour inoculation, a time point at which all experimental groups display a decrease in tumour size (Fig. 29 A). This tumour protection and survival experiment illustrates that the percentage of GzmB producing transduced CD4$^+$Trp1 T cells at this time point (d18/19) does not directly correlate with the T cells' capacity to control, reduce or eradicate the tumours. Despite a low variance in GzmB expression at d18/19 (Fig. 28 C), each experimental cohort which received transduced T cells displayed very heterogeneous results with some mice successfully rejecting the tumour while others within the same group displayed only a temporary decrease and control of tumour size before succumbing to unregulated tumour growth (Fig. 29 A).

Taken together, these data demonstrate that mTORC1 inhibition by inducible PRAS40 expression does not have a negative effect on tumour eradication and survival in comparison to the transduced controls. However, this study further illustrates that the process of transduction and *in vitro* expansion of CD4$^+$Trp1 T cells prior to adoptive transfer impairs their functionality *in vivo* and results in a critical loss of tumour rejection and long-term survival.

5 Discussion

Harnessing cytotoxic, tumour-specific CD4$^+$ T cells to treat cancer has great potential to improve current T cell therapies which are largely based on the transfer of CD8$^+$ T cells. In this PhD project, I endeavoured to characterise and define cellular and molecular mechanisms underlying the development of CD4$^+$ cytotoxic T cells.

Here we demonstrated that that tumour reactive killer CD4$^+$Trp1 cells induced by lymphopenia and treatment with αCTLA-4 exhibit a multifunctional effector phenotype *in vivo* which is distinct from helper and hyporesponsive CD4$^+$ phenotypes.

Killer, and not helper or tolerant, CD4$^+$Trp1 cells are able to eradicate established B16/BL6 melanoma lesions by infiltrating the tumour in high numbers and acquiring cytotoxic activity in form of GzmB expression (Fig. 5, 6).

Both transcriptome and protein analysis of tumour infiltrating cytotoxic CD4$^+$Trp1 T cells showed mixed phenotypical characteristics: while expressing the CD8$^+$ and CD4$^+$ lineage specific transcription factors Runx3 and ThPok, respectively, also Th1 and Th2 key transcription factors T-bet and Gata3 were co-expressed in up to 85 % of cells (Fig. 6). Furthermore, high GzmB expression at transcriptional and protein level did not correlate with Eomes expression as is found on CD8$^+$ CTLs; CD4$^+$Trp1 cells lacked expression of Eomes entirely (Fig. 6).

Tumour infiltrating killer CD4$^+$Trp1 T cells further expressed transcripts of a large variety of inflammatory cytokines correlating with different T helper subsets: GM-CSF, IFNγ, TNF-α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-21, IL-25, IL-17, IL-13 and IL-10 (Fig. 8-15). However, only a high expression of IFNγ, TNF-α and IL-2 was verified so far also on protein level (Fig. 22). Furthermore, killer CD4$^+$Trp1 TILs did not express transcripts of Th17, Th9, Tfh and Treg specific transcription factors RORγt, PU.1, BCL-6 and Foxp3 or Th22 exclusive chemokine receptor pattern of CCR10, CCR4 and CCR6 (Fig. 12). Together with the high cytotoxic molecule (e.g. GzmB) expression, these results emphasise the multi-functional highly plastic, Th1/Th2 phenotype of cytotoxic CD4$^+$Trp1 T cells.

Canonical Correspondence Analysis of Microarray data (CCAM) demonstrated a higher correlation of tumour infiltrating CD4$^+$Trp1 CTLs with the gene signature of CD8$^+$ rather than CD4$^+$ T cells (Fig. 19). Another CCAM further illustrated that cytotoxic CD4$^+$Trp1 TILs highly correspond with full effector, antigen-specific CD8$^+$ T cell transcriptional signature and not a memory phenotype (Fig. 20).

Due to the crucial role of mTOR signalling in CD8$^+$ effector differentiation, the importance of mTOR signalling for cytotoxic CD4$^+$Trp1 T cells was assessed. Disruption of mTORC1 activity via treatment with rapamycin or genetic engineering of CD4$^+$Trp1 cells to overexpress mTORC1 inhibiting PRAS40 caused a down-regulation of GzmB whilst the production of inflammatory cytokines IFNγ, TNF-α and IL-2 was maintained *in vitro* and *in vivo* (Fig. 26, 28). The impact of the inhibition of mTOR signalling on GzmB expression

suggests a potential involvement of the mTOR pathway in the acquisition or maintenance of cytotoxic activity on the $CD4^+$ T cells.
This study underscores the complexity of the unconventional Th1/Th2/CTL phenotype of tumour reactive cytotoxic $CD4^+$Trp1 T cells but also highlight the benefit of their multi-functionality in immunotherapy of melanoma.

A recent publication criticised that although cytotoxic $CD4^+$ T cells were relevant in human disease, they might not be clinically translatable as this subset would not be able to perform a 'traditional helper function' (Kline et al. 2012). However, we demonstrate that cytotoxic $CD4^+$Trp1 T cells not only express cytolytic molecules but also a large variety of cytokines which we hypothesise enables this $CD4^+$ subset to provide 'classical' help to other lymphocytes.

Cancer immunotherapy with cytotoxic $CD4^+$ T cells could have several advantages over $CD8^+$ T cells therapies: Rosenberg and colleagues illustrated that tumour protection was often incomplete in patients with melanoma, even when transferring tumour antigen-specific and not polyclonal $CD8^+$ T cells (Rosenberg et al. 2005). Furthermore, the generation of a large number of autologous tumour-specific $CD8^+$ T cells for adoptive transfer is a lengthy and complicated *ex vivo* process as this tumour reactive subset is present at very low frequencies in peripheral blood. Most methods are too elaborate and time-consuming to be employed in the clinic or even induce activation induced apoptosis of the expanded $CD8^+$ T cells (Cebecauer et al. 2005; Vinay et al. 2015).
As previously published and based on the results of the current study, tumour reactive cytotoxic $CD4^+$ T cells are not only able to eradicate large established, poorly immunogenic tumours and promote long-term protection (Fig. 5 A) (Quezada et al. 2010), they are also producing inflammatory cytokines (Fig. 6 E-G) and have a transcriptional T helper profile (Fig. 8-15) which could enable them to simultaneously orchestrate the function of other immune cell compartments (CD8, NK cells, B cells) in the immune response to cancer. Furthermore, the $CD4^+$Trp1 T cell induced tumour rejection is independent from $CD8^+$ T cells, only requires a low number of T cells and does not involve extensive *in vitro* expansion of manipulation of differentiation prior to T cell transfer (Quezada et al. 2010).

In addition, $CD4^+$ T cells benefit from their proficiency to recognise antigens presented on MHC class II molecules instead of class I as $CD8^+$ T cells. Antigen recognition in MHC I / II constitutes a critical difference as $CD8^+$ T cell cytotoxicity depends predominantly on the presentation of intracellular, endogenous antigens in MHC Class I molecules on the surface of target cells which, e.g. in the case of cancerous cells, display high genetic instability. Due to uncontrolled mutagenisis in the tumour cells, defects in antigen processing and presentation can occur; for instance affecting antigen processing key proteins β_2-microglobulin, TAP1, and TAP2 activity or even causing the loss of MHC I chains (Korkolopoulou et al. 1996; Cabrera et al. 2003). Collectively, these attributes can cause tumour cells to be unreliable targets for $CD8^+$ CTLs (Restifo et al. 1996; Muranski & Restifo 2009; Iwai et al. 2002).

$CD4^+$ T cells on the other hand, recognise antigens presented in MHC II on professional antigen presenting cells (APCs), for instance dendritic cells, which capture antigen by taking up exogenous, tumour derived fragments by phagocytosis and present them in MHC II (Savina & Amigorena 2007). Furthermore, it was demonstrated that tumour cells are also able to present endogenous peptides in MHC II by taking up fragments of apoptotic tumour cells via autophagy (Nuchtern et al. 1990). Although $CD4^+$ cells are then likewise affected by the unreliable expression of MHC II and presentation of antigens on cancer cells, it has been shown that, even though most solid cancers do not express MHC II constitutively, the expression of the molecule is upregulated in many tissues and tumours when exposed to IFNγ (Propper et al. 2003; Boehm et al. 1997; Boss 1997). As shown also by Quezada and colleagues, presence of IFNγ was indispensable for MHC Class II up-regulation on B16/BL6 melanoma cells (Quezada et al. 2010).

This further emphasises the importance of the mixed phenotype of the tumour reactive $CD4^+$Trp1 T cells presented in this study: the cytotoxic $CD4^+$Trp1 cells not only express GzmB but simultaneously high levels of IFNγ rendering the tumour cells susceptible to $CD4^+$ T cell cytotoxic activity by upregulating MHC II (Fig. 5 A, 6 E-G). Even if the tumour fails to express MHC II, the tumour stroma and accessory macrophages will cross-present tumour antigens. Importantly, destruction of the tumour stroma by cytotoxic lymphocytes was shown to also lead to the elimination of tumour cells as bystanders (Spiotto & Schreiber 2005).

Collectively, this suggests that cancer therapy with cytotoxic $CD4^+$ T cells can overcome traditional $CD8^+$ CTL complications and offer an effective way to treat even poorly immunogenic tumours such as B16/BL6 melanoma.

5.1 The tolerant, helper and killer $CD4^+$ phenotype model

The three different $CD4^+$ T cell subsets, tolerant, helper and killer, were generated by adoptive transfer of naïve $CD4^+$Trp1 T cells in three different treatment cohorts. As expected, tolerant $CD4^+$ T cells presented a hyporesponsive phenotype with very low tumour infiltration and hardly any effector molecule production while helper $CD4^+$Trp1 T cells displayed moderate expression of inflammatory Th1 cytokines (Fig. 5 A, 6 E-G).
The treatments used to give rise to the three phenotypically diverse subsets were very different: only adoptive transfer of $CD4^+$Trp1 T cells for the tolerant, adoptive transfer in combination with GVAX and αCTLA-4 for the helper and radiation therapy (RT), adoptive transfer and αCTLA-4 for the killer (chapter *4.1*). It is important to acknowledge that in this model, there were no control groups receiving only RT, only αCTLA-4, only GVAX or RT + GVAX in addition to the adoptive transfer of $CD4^+$Trp1 T cells which would allow to precisely compare tolerant, helper and killer cohorts with each other. However, the conclusions drawn from this model were not directed towards the effect of a specific treatment as anti-tumour therapy but rather used to compare two phenotypically different T cell subsets (tolerant and helper) with the published killer $CD4^+$Trp1 T cell phenotype.

A crucially different part of the killer $CD4^+$T cell inducing treatment in comparison with the helper and tolerant therapy is the irradiation (RT) prior to adoptive transfer. As previously reported, cytotoxicity, tumour eradication and substantial proliferation was also induced in $CD4^+$Trp1 T cells when transferred into tumour bearing $RAG^{-/-}$ mice instead of irradiated C57BL/6. As the only difference between $RAG^{-/-}$ animals and C57BL/6 is the lack B and T cells (Mombaerts et al. 1992), it is suggested that irradiation is mostly needed to deplete the endogenous B and T cell compartment of the recipient (Quezada et al. 2010). The lymphodepletion caused by RT is thought to induce homeostatic proliferation of the transferred naïve T cells (Goldrath & Bevan 1999; Marrack et al. 2000). This characteristic rapid expansion of T cells in lymphopenic recipients has been correlated with differentiation of naïve lymphocytes into effector T cells (Murali-Krishna & Ahmed 2000; Gudmundsdottir & Turka 2001; Min et al. 2005) and

reversal of tumour-induced anergy of antigen-specific T cells (Brown et al. 2006; Kline et al. 2008). Interestingly, the avidity of the TCR is an important determinant of the degree of homeostasis induced proliferation; the higher the avidity, the more extensive the expansion of T cells (Ge et al. 2001; Kassiotis et al. 2003).
In light of these reports, it is proposed that naïve $CD4^+$Trp1 T cells, which carry the highly avid Trp1 TCR, undergo rapid homeostatic expansion due to the lymphodepletion and presentation of their cognate antigen in the tumour bearing recipients. It is further suggested that this homeostatic expansion might have an important role in the development of the highly plastic, multi-functional $CD4^+$ killer phenotype.

A distinct feature of cytotoxic $CD4^+$Trp1 T cells is the co-expression of Th1 and Th2 transcription factors T-bet and Gata3 (Fig. 23 C). As Kanhere et al. recently elaborately demonstrated, a multitude of key immune regulatory genes such as transcription factors (Blimp-1, BATF, IRF1, BCL-6, STAT4, STAT5, …), cytokines (IL-4, IL-5, IL-13), and cytokine and chemokine receptors (IL1RL2, CCR2, CCR5, …) display T-bet and Gata3 binding domains and thereby display the possibility of regulation through the Th1 and Th2 specific transcription factors (Kanhere et al. 2012). Cytotoxic $CD4^+$ T cells thus have potential to highly benefit from the expression of both transcription factors to gain Th1/Th2 dual functionality.
Furthermore, Jenner et al. previously elucidated a set of shared target genes between Gata3 and T-bet and emphasised the importance of T-bet and Gata3 expression, exclusively or simultaneously to control the development of T cell fates. The authors verified previous studies (Cousins et al. 2002; Messi et al. 2003; De Fanis et al. 2007) showing Gata3 expression in both human Th1 and Th2 cells but added that nearly all Gata3 expression in Th1 cells coincides with T-bet expression (Jenner et al. 2009). Interestingly, a similar co-expression pattern of the previously considered ‘exclusive’ Th17 and Treg specific transcription factors RORγt and Foxp3 emerged to be essential for the functionality of a subset of regulatory T cells (Zhou et al. 2008). This suggests that T helper phenotype commitment is not a static, single transcription factor induced event but is caused by a more complex interplay between transcription factors, such as Gata3 and T-bet, and their exclusive and shared target genes. These studies emphasise the great extent in which the simultaneous expression of Gata3 and T-bet might precipitate or contribute to the highly plastic, multifunctional killer $CD4^+$Trp1 phenotype.

5.2 The microarray analysis

The microarray analysis revealed a highly plastic, multi-functional helper and CTL phenotype of tumour infiltrating killer $CD4^+$Trp1 T cells and a high correlation of cytotoxic $CD4^+$ T cells with the genetic signature of effector $CD8^+$ T cells.

The tolerant and helper subsets were suitable and crucial for the comparison with the killer $CD4^+$Trp1 T cells, however, the tumour infiltrating helper $CD4^+$Trp1 T cells appear to not completely fit one type of 'traditional' $CD4^+$ helper T cells: Although helper $CD4^+$Trp1 T cells did not develop anti-tumour activity or promoted tumour protection, they appeared to be in a strongly activated state and up to 32 % of TILs expressed GzmB (Fig. 6 A). It is overall difficult to define a subset as 'general' helper population as there are distinct phenotypic differences between CD4 helper subsets Th1, Th2, Th17, Tfh, Th9, Th22 and regulatory T cells. Based on the transcriptional expression of T helper subset markers, helper TILs most resembled Th1 T cells by expressing Th1 transcription factor T-bet as well as most Th1 but also a variety of Th2 cytokines (Fig. 9, 10).

However, it was surprising to find the helper $CD4^+$Trp1 TIL gene signature correlated so highly with $CD8^+$ related genetic patterns in the CCAM. A potential explanation for this discrepancy lies in the nature of the comparison with the ImmGen dataset T.8.TI.B16: it is unknown which phenotype the $CD8^+$ T cells had which were used to generate this dataset – they could be effector, memory or exhausted CD8+ T cells or even constitute of a mix of the three. Furthermore, this result could be due to the particularly high transcript expression of cytotoxic molecules on helper TILs and the similarities in $CD8^+$ T cell marker expression pattern between helper and killer TILs (Fig. 16, 17).
In the second CCAM, the helpers were shown to correlate with an early effector $CD8^+$ phenotype (Fig. 20). This activated cell state might be due to the very avid transgenic Trp1 TCR which causes chronic stimulation of helper $CD4^+$Trp1 T cells in the tumour.

Among the large number of T helper and CD8 T cell markers expressed on cytotoxic $CD4^+$Trp1 T cells, there are several which stand out due to their exclusive expression of killer (and not helper) TILs or their connections with (cytotoxic) effector T cells:
Interestingly, $CD4^+$Trp1 TILs highly express the $CD8^+$ lineage specific transcription factor Runx3 on transcript and protein level. Previous reports show that Runx3 was imperative for GzmB expression (Wang et al. 2008) but also that Runx3 expression alone is not

sufficient to drive cytotoxic activity of $CD8^+$ effectors (Kohu et al. 2005; Grueter et al. 2005; Taniuchi 2009). In this study, expression of Runx3 coincided with GzmB expression on up to 91 % of cytotoxic $CD4^+$Trp1 effectors in the tumour while T-bet was co-expressed with GzmB in up to 89 % of $CD4^+$Trp1 TILs (Fig. 23). When simultaneously expressed as observed on killer $CD4^+$Trp1 TILs, Runx3 and T-bet were shown to cooperate to induce CTL differentiation by induction of IFNγ, GzmB and Perforin expression (Cruz-Guilloty et al. 2009) This strongly suggests an association of Runx3 and T-bet with the cytotoxic $CD4^+$ T cell fate. However, the importance of the transcription factors for the acquisition of cytotoxic activity remains to be investigated for instance by knock down of one, two or all three (Runx3, T-bet, Gata3) of the transcription factors via shRNA in $CD4^+$Trp1 T cells prior to adoptive transfer.

The microarray analysis revealed an up-regulation of Gfi1 (growth factor independent-1) on killer and helper $CD4^+$ T cells in the tumour (Fig. 10). Gfi1 is a transcriptional repressor which is induced by IL-4/STAT6 and was shown to promote expansion of Gata3 expressing cells and Th2 responses (Zhu et al. 2006). A recent study demonstrated that TCR-mediated upregulation of Gfi1 supports Th2 cell differentiation by enhancing Gata3 stability (Shinnakasu et al. 2008). This points to a potential role of Gfi1 expression in driving or supporting the Th2 branch of the mixed Th1/Th2/CTL $CD4^+$ phenotype by stabilising Gata3 whilst T-bet is expressed. Whether Gfi1 expression contributes to the acquisition of the cytotoxic activity, however, is not clear and still needs to be examined.

Contrary to other reports on cytotoxic $CD4^+$ cells (Qui et al. 2011; Hirschhorn-Cymerman et al. 2012; Curran et al. 2013; Weiskopf et al. 2015), Eomes was not expressed on the killer $CD4^+$Trp1 TILs. This lack of Eomes expression suggests that the transcription factor is not necessary for the cytotoxic effector function in this model. In fact, Eomes has not been portrayed as an exclusive effector marker at all: In several studies of lymphocytic choriomeningitis virus (LCMV) infection, Eomes and T-bet were both found to be expressed in effector and circulating memory $CD8^+$ T cells. However, Eomes expression was higher in memory cells while T-bet transcripts were increased in effector $CD8^+$ cells (Intlekofer et al. 2005; Joshi et al. 2011).
Furthermore, Pipkin et al. observed higher T-bet, but not Eomes, expression upon TCR stimulation while only several days after TCR engagement Eomes was upregulated (Pipkin et al. 2010). Eomes deficient memory $CD8^+$ cells were also shown to have several functional memory defects such as reduced long-term persistence and

expansion rate after antigen-reencounter and less capacity to localize or populate the memory T cell niche in the bone marrow (Banerjee et al. 2010). Based on these reports, an inverse correlation of T-bet and Eomes in effector and memory $CD8^+$ T cells emerges: T-bet expression is high and Eomes expression low (not necessarily absent) in effector $CD8^+$ T cells and conversely, Eomes expression increases and T-bet decreases as the cell fate changes to a memory phenotype. Thus, it is clear that high expression of Eomes is not a hallmark of effector but memory $CD8^+$ T cells, although Eomes is most likely not a master T cell memory regulator such as BCL-6. The strong evidence of Eomes involvement in memory, not effector, functionality combined with the results from this immunotherapy study suggest that tumour reactive $CD4^+$ T cells do not depend on Eomes expression to develop cytotoxic activity. However, as $CD4^+$Trp1 T cells were only analysed at a time point when cytotoxic activity is already acquired, one cannot rule out the possible involvement of Eomes in the induction and development of the cytotoxic phenotype at an earlier time point without further experiments such as knock down via shRNA.

The expression of $CD4^+$ lineage specific transcription factor ThPok was high on killer $CD4^+$Trp1 T cells in the tumour and the periphery, likewise, on transcript and protein level (Fig. 8, 22). This data stands in contrast with previous publications which emphasised the important role of ThPok in suppressing $CD8^+$ CTL fate in $CD4^+$ T cells in the periphery by blocking Perforin and GzmB expression (Wang et al. 2008; Sun et al. 2005; He et al. 2005). However, these studies also often report high expression of Eomes on cytotoxic $CD4^+$ T cells which killer $CD4^+$Trp1 characteristically lack in this presented work (Fig. 22) and depict Runx3 as orchestrator of gene expression of cytotoxic molecules such as GzmB in ThPok-deficient $CD4^+$ T cells (Wang et al. 2008).
The co-expression of ThPok and Runx3 was recently reported in $CD4^+$ T cells found in the intestine (IELs, intraepithelial lymphocytes) (Reis et al. 2013). Killer $CD4^+$Trp1 TILs partially resemble IELs as both T cell populations exhibit high expression of CD69, GzmB, Runx3, T-bet and Thpok; however, $CD4^+$Trp1 TILs lack IELs characteristic CD103 expression (Reis et al. 2014) (Fig. 15).
Interestingly, Mucida and colleagues further illustrated that activated IELs lose expression of ThPok, and thereby caused de-repression of the $CD8^+$ CTL lineage. The emerging cytotoxic potential in IELs was a result of potent or repetitive antigen specific activation of the $CD4^+$ effector T cells. However, cytotoxic IELs lacking ThPok displayed

decreased expression of T helper (Th1, Th2, Th17 and Treg) gene signatures (Mucida et al. 2013).
In light of these results and the data presented in this study, ThPok expression appears to not be required for $CD4^+$ cytotoxicity but drives or contributes to the Th component of the highly plastic killer $CD4^+$Trp1 phenotype.

Interestingly, transcript expression of IL-12R β2 is high on killer.TIL, killer.LN and helper.TIL. Loss of the beta subunit of IL-12R is a characteristic Th2 phenomenon which is highly connected with the cytokine milieu in the microenvironment: IL-4 inhibits while IFNγ helps to maintain IL-12R β2 expression during early Th2 development (Szabo et al. 1997). The high expression of IL-12R β2 on killer $CD4^+$Trp1 TILs was expected as its expression is specifically induced by TCR stimulation (Usui et al. 2006) and $CD4^+$Trp1 killer TILs express a large variety of cytokines but particularly high levels of IFNγ which stimulates its expression. However, IL-12R β2 expression is inhibited by Gata3 and stimulated by STAT4 (Usui et al. 2006) – two molecules which were shown to be highly expressed on the killer $CD4^+$Trp1 TILs. This suggests a phenotype which is in a delicate balance between positive and negative regulation of gene expression due to simultaneous expression of T-bet, Gata3, Runx3 and ThPok.

Recent studies emphasized the importance of chemokine receptor CCR5 on $CD4^+$ T cells expressing GzmB, Perforin and the degranulation marker LAMP-1: Zaunders and colleagues detected this phenotype in human HIV-1 and CMV-specific $CD4^+$T cells while Naito et al. demonstrated that CCR5 expression was instrumental for $CD4^+$ T cell recruitment to the tumour site and intratumoural migration mediated by CCL3 (Zaunders et al. 2004; Naito et al. 2015). Interestingly, not only do killer $CD4^+$Trp1 TILs express high levels of CCR5 transcript but also of CCL3 (Fig. 9). This data suggests that CCR5 could also be involved in $CD4^+$Trp1 T cell migration but also proposes the possibility of $CD4^+$Trp1 TILs recruiting more killer $CD4^+$ T cells to the tumour site by production of CCL3. To validate this hypothesis, CCL3 and CCR5 expression should be analysed via flow cytometry and, if found upregulated, knocked down/out to investigate their importance for the $CD4^+$Trp1 cytotoxic phenotype.

Another molecule which is highly expressed only on killer $CD4^+$Trp1 T cells in the tumour and periphery is CD226 (Fig. 9). CD226 is a co-stimulatory molecule expressed on Th1 $CD4^+$, $CD8^+$ T cells and NK cells and shares the ligand CD155 (PVR) with co-inhibitory

receptor TIGIT (Gilfillan et al. 2008). In fact, similar to the CTLA-4 and CD28 axis, the key mechanism of action of TIGIT is the interference with CD226 homodimerisation required for co-stimulation (Johnston et al. 2014). Gilfillan and colleagues further demonstrated CD226 co-stimulation to be instrumental for directing $CD8^+$ effector functions against non-professional APCs and NK targets which evade immunosurveillance (Gilfillan et al. 2008). The very selective expression of CD226 on only killer $CD4^+$ T cells suggests a potential involvement of CD226 in the development and/or maintenance of the highly plastic phenotype of cytotoxic $CD4^+$Trp1 T cells.
Also the CD226 associated integrin LFA-1 (CD11a) (Shibuya et al. 2003) was significantly higher expressed on transcript level in killer $CD4^+$Trp1 TILs in comparison to helper TILs (Fig. 15, 21). LFA-1 was found to be crucial for mediating the release of cytolytic granules in the immune synapse between $CD8^+$ CTLs and their target cell (Anikeeva et al. 2005). The function of LFA-1 could contribute of the highly cytotoxic activity of $CD4^+$Trp1 T cells. Both CD226 and LFA-1 expression still remain to be verified on protein level.

Furthermore, $CD4^+$Trp1 TILs lack CD62L expression on protein and transcript level. This phenotype on $CD4^+$ T cells was reported to be accompanied with a high expression of effector molecule expression, for instance IFNγ, in comparison with $CD62L^+$ cells (Hengel et al. 2003) which aligns with the high IFNγ expression of killer $CD4^+$Trp1 T cells. Yang et al. further illustrated that CD62L is cleaved from the cell surface tumour specific, lytic $CD8^+$ T cells after antigen encounter and that the shedding was directly correlating with the expression of degranulation marker LAMP-1 (CD107a) (Yang et al. 2011). It is unclear whether CD62L shedding also occurs on killer $CD4^+$Trp1 T cells as CD62L expression was also very low on transcript level. However, without further experiments such as investigating the presence of CD62L in the supernatant of restimulated cells by ELISA, the possibility of shedding cannot be excluded. Moreover, LAMP-1 (CD107a) expression was low on transcript level but was detected when stained together with LAMP-2 (CD107b) after restimulation of $CD4^+$Trp1 T cells *ex vivo* (Fig. 6 B).

Expression of another memory marker, CCR7, was particularly low on killer TILs on transcript level (Fig. 21). This data not only suggests an effector phenotype but more specifically correlates with a report by Harari et al. demonstrating that the vast majority of antigen specific $CD4^+$ T cells which highly express IL-2 and IFNγ are $CCR7^-$ *in vitro* and *in vivo* (Harari et al. 2004).

Furthermore, killer CD4$^+$Trp1 TILs expressed significantly higher levels of the effector markers KLRG1 and CD11c on transcript level than helper TILs (Fig. 21). The two molecules were previously shown to be co-expressed and highly upregulated on CD8 T cells. Increased expression of T-bet in CD8$^+$ T cells was further demonstrated to induce a short lived, KLRG1^{HIGH} effector cell state (Huleatt & Lefrançois 1995; Joshi et al. 2007). These data strongly correlates with the described gene and protein expression pattern observed in cytotoxic CD4$^+$Trp1 T cells and suggests a potential role of KLRG1 and CD11c in the acquisition and/or maintenance of the cytotoxic phenotype.

Besides specific markers, the microarray also illustrated the importance of the TRP1 antigen-rich microenvironment of the tumour as crucial component for the development of CD4$^+$Trp1 cytotoxicity: killer CD4$^+$Trp1 cells in the lymph node lacked transcripts or expressed significantly lower levels of most cytokines and cytotoxic molecules, with only few exceptions, in comparison to killer TILs (Fig. 8-12, 21). Collectively, this emphasises the importance of the tumour microenvironment on the cytotoxic activity of CD4$^+$Trp1 T cells.

5.3 The mTORC1 inhibition experiments

As it was recently demonstrated that mTOR signalling is associated with T cell differentiation (Delgoffe et al. 2009; Araki et al. 2009) and GzmB and T-bet expression in CD8$^+$ CTLs (Rao et al. 2010), we inhibited mTORC1 by treating mice killer condition (RT + CD4$^+$Trp1 + αCTLA-4) with rapamycin to investigate the potential role of mTORC1 signalling in the differentiation of the cytotoxic CD4$^+$Trp1 T cells.

Inhibition of mTORC1 *in vivo* by administration of rapamycin ablated GzmB expression without impacting the effector-typical high T-bet expression or cytokine secretion (Fig. 25, 26). However, it is not possible to distinguish if the observed effect of GzmB down-regulation were due to mTORC1 inhibition or an interplay of different factors caused by ubiquitous mTORC inhibition. Nonetheless, the reduction of GzmB was confirmed *in vitro* when CD4$^+$Trp1 T cells were activated and cultured with rapamycin (Fig. 24). The finding that inhibition of mTORC1 ablates GzmB expression therefore suggests that mTORC1 activity might be important for the acquisition or maintenance of cytotoxicity.

Interestingly, and in contrast to (Rao et al. 2010), there was only a mild change in the expression of the T-bet with rapamycin treatment (Fig. 25 B). Conversely, prolonged T-bet expression could not override the effect of mTORC1 inhibition and induce GzmB expression. This suggests that in killer CD4$^+$Trp1 T cells either T-bet controls GzmB expression via mTORC1 signalling or that T-bet expression is independent from GzmB and vice versa.

Genetically engineering of CD4$^+$Trp1 T cells to overexpress PRAS40, a negative regulator of mTORC1 activity, instead of treatment with rapamycin limited the mTORC1 inhibitory effect to only transduced CD4$^+$Trp1 T cells and avoided systemic mTORC1 dysregulation.

Forced PRAS40 expression in killer CD4+Trp1 T cells (iPRAS40 killer) produced correlating results with the preceding rapamycin experiments: iPRAS40 killer CD4$^+$Trp1 T cells displayed significantly decreased GzmB expression *in vivo* whilst IFNγ expression was maintained (Fig. 28 C, D).

iPRAS40 transduced CD4$^+$Trp1 T cells were then used in a long-term survival experiment to investigate the effects of mTORC1 inhibition on tumour eradication and protection. It was demonstrated that mTORC1 inhibition did not have a statistically significant impact on tumour eradication and long-term survival when compared to the two control groups (iPRAS40 killer + DOX vs iPRAS40 killer or iGFP killer + DOX). Surprisingly, however, there was a drastic overall loss of tumour protection and survival advantage in all the groups which received transduced T cells, irrespective of their transgene or DOX treatment (Fig. 29 B).

This loss of the characterised killer functionality of the CD4$^+$Trp1 T cells in all the cohorts which received transduced T cells, irrespective of mTORC1 inhibition, demonstrates that the genetic engineering had severe side effects which impaired the transduced T cells' ability to eradicate the tumour. This could be due to exhaustion of the transduced T cells caused by receiving the first TCR specific stimulation *in vitro*, expanding for several days with high survival stimulus (IL-2) *in vitro* and finally undergoing homeostatic expansion and receiving chronic stimulation with their cognate antigen *in vivo*.

Gattinoni and colleagues have shown that tumour specific Pmel CD8$^+$ T cells which are activated and kill target cells efficiently *in vitro*, are not as effective in tumour eradication *in vivo* (Gattinoni et al. 2005). High expression of Granzymes and IFNγ in particular was correlated with decreased anti-tumour activity *in vivo*. The authors suggest this effect is

due to either an inability to produce IL-2 and respond to homeostatic cytokines such as IL-7 and IL-15, a downregulation of co-stimulatory and lymphoid-homing molecules or differentiation into an exhausted, pro-apoptotic state with impaired proliferation.
As it was necessary to transfer 5x more transduced cells to reach a comparable CD4$^+$Trp1 T cell number to the naïve killer in tumour and lymph node on day 18/19 of the experiment, it is possible that transduced CD4$^+$Trp1 T cells indeed possess a decreased proliferative potential early after the transfer. After 10 days *in vivo* (day 18/19), however, proliferative capacity of iGFP and naïve CD4$^+$Trp1 T cells was identical as evidenced by high Ki67 expression in both cohorts (up to 95 % Ki67$^+$, Fig. 28 D). Interestingly, the authors suggest that the use of IL-15 instead of IL-2 for the *in vitro* T cell culture equally expanded the lymphocytes and delayed the differentiation into an effector state (Gattinoni et al. 2005). The exchange of IL-2 with IL-15 in the transduction protocol could be tested to investigate if it can restore CD4$^+$ T cell effector function *in vivo*.

It is notable, however, that 30 % of the mTORC1 inhibited cohort (iPRAS40 killer + DOX) did eradicate the tumours in a timely manner equivalent to not only the transduced control groups but even to the reference ‘naïve killer’ subset (Fig. 29). This could be due to the residual GzmB expression (35.3 % ± 8.9 % SD) and high cytokine expression which was detected on d18/19 (Fig. 28). High IFNγ expression in itself can contribute to tumour eradication by other immune cell subsets (Mumberg et al. 1999; Dighe et al. 1994; Coughlin et al. 1998; Qin et al. 2002; Zhang et al. 2008; Kline et al. 2012) and could play an important part in this scenario. Furthermore, although GzmB is used as a marker for cytotoxicity in this study, there are a number of different Granzyme (A-K) molecules which were found upregulated on transcript level in killer CD4$^+$Trp1 T cells. So although mTORC1 inhibition has an effect on GzmB expression, we did not study any of the other Granzyme molecules in this context. There could be an occurrence of compensation mechanisms such as an increased expression of other Granzymes, Perforin, or even TRAIL or FasL. Although the latter two were previously described to be dispensable for cytotoxic CD4$^+$Trp1 mediated tumour eradication (Quezada et al. 2010), TRAIL and FasL could still have a role as compensatory anti-tumour mechanism in this setting.

As mTOR is widely associated with metabolic changes in T cells and homeostatic proliferation potentially plays a part in the development of the cytotoxic phenotype, this data connects $CD4^+$ killer activity with cellular metabolism.

Interestingly, T cells change their metabolism upon activation: Following antigen encounter, $CD8^+$ and $CD4^+$ T cells change from naïve, quiescent cells to effector cells with various new functions. Effector T cell characteristics such as enhanced proliferation, directed migration, cytotoxic molecule and cytokine production and secretion require more energy. In order to keep up with the metabolic demand, effector lymphocytes switch their ATP synthesis from the universal mitochondrial oxidative phosphorylation to aerobic glycolysis (Cham et al. 2008; Finlay & Cantrell 2011). This enables activated T cells to meet their energy requirements even in hypoxic environments as aerobic glycolysis allows ATP production from glucose regardless of oxygen availability (Vander Heiden et al. 2009).

It has not been clear why T cells switch to the much less efficient glycolysis even during abundant oxygen supply when they require higher ATP levels. Recently, however, Chang et al. illustrated the significance of the glycolysis enzyme GADPH (Glyceraldehyde 3-phosphate dehydrogenase) on inflammatory cytokine production. When GAPDH is not engaged in aerobic glycolysis, thus in naïve, unactivated T cells, it binds AU-rich elements within the 3' UTR of *Ifng* and *Il2* mRNA and thereby inhibits their translation. It was demonstrated that only during occupation of GAPDH in the aerobic glycolysis pathway, IFNγ and IL-2 production is permitted. Therefore, aerobic glycolysis appears to be a metabolic regulator for effector T cell function (Chang et al. 2013; Nagy & Rigby 1995).

These findings indicate the change to aerobic glycolysis serves as a switch to an effector function gene/protein set and highlights the potential role of GAPDH or other multi-functional glycolysis enzymes in the acquisition of cytotoxicity on $CD4^+$ T cells via post-transcriptional gene silencing.

In keeping with these results, transcriptional analysis of *in vitro* stimulated $CD8^+$ T cells demonstrated a direct dependence of GzmB and perforin with active glycolysis: glucose deprivation during stimulation caused a significant down-regulation of the cytolytic molecules (Cham et al. 2008).

Interestingly, the microarray analysis in the current study did not only reveal high expression of the mTORC1 downstream proteins Hypoxia inducible factors 1α (Hif-1α)

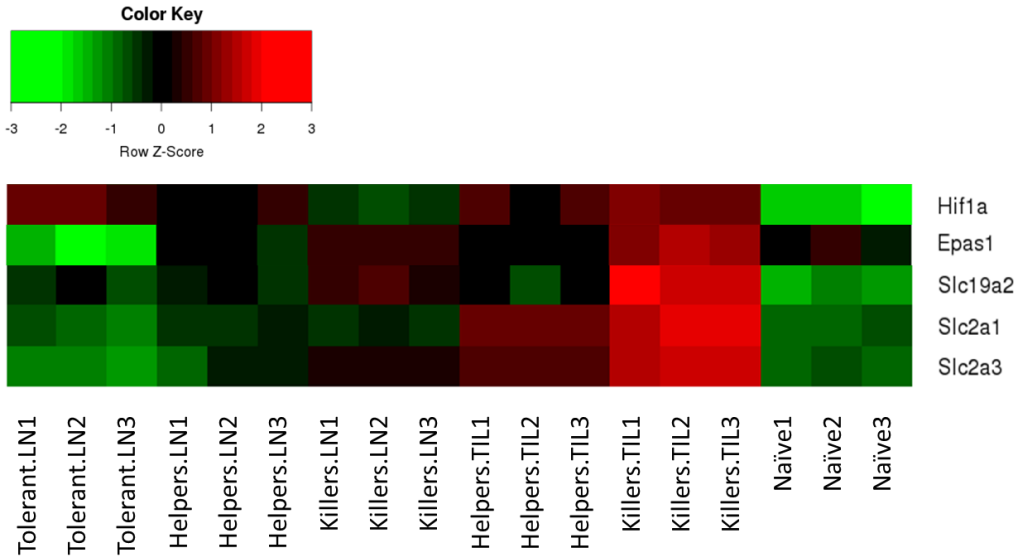

Figure 30 | Tumour infiltrating killer CD4$^+$Trp1 cells highly express mTORC1 downstream proteins involved in metabolism. Displayed are genes encoding HIF-1α (*Hif1a*), HIF-2α (*Epas1*), thiamine transporter TC1 (*Slc19a2*), Glut1 (*Slc2a1*) and Glut3 (*Slc2a3*). Colour coding is used to display high (red) and low (green) expression of a genes across the experimental groups: tolerant, helper, killer and naïve CD4$^+$Trp1 effector T cells extracted from tumour (TIL) or lymph nodes (LN) from treated mice (treatment regimens as described in chapter *4.1*). Mice were sacrificed 8 days after adoptive transfer and RNA was isolated from GFP$^-$ CD4$^+$Trp1 effector cells and used for the GeneChip® Mouse Genome 430 2.0 Array. Naïve GFP$^-$ CD4$^+$Trp1 cells were isolated from lymph nodes of Trp1 FoxGFP mice. n=8-10 mice per group. Experiments were performed in triplicates, each indicated by 1/2/3.

and 2α (Hif-2α, encoded by *Epas1*) on tumour infiltrating killer CD4$^+$Trp1 T cells but also of its metabolic target genes glucose transporter type 1 (Glut1 encoded by *Slc2a1*) and type 3 (Glut3 encoded by *Slc2a3*) (Fig. 30) (Iyer et al. 1998; Dodd et al. 2015). Up-regulation of Glut1 was previously shown to be an essential process in CD8$^+$ T cell activation (Jacobs et al. 2008). Additionally to Glut1 and Glut3, thiamine transporter 1 (TC1, encoded by *Slc19a2*), another metabolically important transporter, was also highly abundant (Fig. 30). All three genes *Slc2a1*, *Slc2a3* and *Slc19a* were differentially expressed on the killer CD4$^+$Trp1 cells in comparison with the tolerant and helper subsets in the lymph node or tumour and the killer T cells in the lymph node (Fig. 30). In fact, *Slc2a3* and *Slc19a2* were found in the top 75 genes which were significantly higher expressed on killer CD4$^+$Trp1 TILs in comparison to helper TILs (Fig 18).

The killer $CD4^+$ TIL restricted expression pattern was also detected for the transcripts of Hif-1α and Hif-2α (Fig. 30). Hif-1α and -2α represents the α-subunits of an oxygen-responsive, heterodimeric transcription factors complex which either factor forms with the constitutively expressed β subunit ARNT. The complex binds hypoxia response elements (HREs) on the DNA to induce expression of specific genes involved in erythropoiesis, apoptosis, glycolysis and proliferation. This can only occur under hypoxic conditions as Hif-1α and Hif-2α proteins are rapidly degraded in the presence of oxygen. This mechanism is based on the hydroxylation on three specific proline residues of both Hif-1α and Hif-2α by prolyl-4-hydroxylase (PHD) which requires molecular oxygen (Ivan et al. 2001). The pVHL (von Hippel-Lindau protein) then binds the hydroxylated sites and induces polyubiquitination of Hif-1α and Hif-2α by E3 ubiquitin ligases, marking the proteins for rapid proteosomal degradation (Salceda & Caro 1997; Maxwell et al. 1999). Hif-1α and Hif-2α each target some unique and some overlapping genes, Hif-1α, for instance, induces glycolytic gene expression while Hif-2α does not (Maltepe et al. 1997; Semenza & Wang 1992; Iyer et al. 1998; Hu et al. 2003; Semenza 2000).

In this study, the *Hif1a* and *Epas1* gene up-regulation on killer $CD4^+$Trp1 TILs is probably only partly due to the potentially hypoxic environment in the tumour because the helper TILs display significantly lower expression of the oxygen-responsive transcription factors; especially Hif-2α expression is significantly ($P < 0.05$) increased (Fig. 18, 30). However, because regulation of Hif-1α/2α is mainly post-translational (Iyer et al. 1998), it is vital to analyse the protein expression and activity of Hif-1α and Hif-2α.

Hence, whilst killer and helper $CD4^+$Trp1 TILs display a similar gene profile for some effector cytokines, Hif-1α, Hif-2α and the metabolically essential glucose/thiamine transporters Glut1, Glut3 and TC1 seem to be preferentially upregulated in the killer setting. Based on these preliminary data Hif-1α, Hif-2α, Glut1, Glut3 and TC1 are promising new targets for the characterisation of the molecular mechanisms underlying the $CD4^+$ cytotoxic phenotype.

Interestingly, recent findings illustrate novel functions of HIFs in lymphocytes: Hif-1α and -2α protein expression is induced by TCR stimulation in a mTORC1-dependent manner under hypoxia in peripheral T cells *in vitro* and *in vivo* (Lukashev et al. 2001; Nakamura et al. 2005; Lukashev et al. 2006). Shi and colleagues further demonstrated that the Hif-1α induced glycolytic pathway was crucial for the differentiation of Th17 and regulatory T cells (Shi et al. 2011). Hif-1α also plays an important role in T cell survival by inhibiting

activation induced cell death (Makino et al. 2003). In fact, CTLs which differentiate in a hypoxic environment and display high levels of Hif-1α were shown to have increased cytotoxic capacity (Caldwell et al. 2001).
This data correlates with recent reports that Nur77, an orphan nuclear hormone receptor which is rapidly expressed after TCR stimulus (Osborne et al. 1994; Au-Yeung et al. 2014), binds pVHL and thereby inhibits ubiquitination and subsequent degradation of Hif1-1α. The stabilisation of Hif-1α by Nur77 allows for activity of the transcription factor resulting in increased expression of Hif-1α targets such as Glut1 (Kim et al. 2008). Furthermore, Nur77 expression was found to directly correlate with the strength of TCR stimulation (Moran et al. 2011). In light of these combined results, it seems possible that the high Hif-1α and Glut1 expression on killer $CD4^+$Trp1 TILs (Fig. 30) is the result of strong stimulation of the Tg Trp1 TCR causing increased expression of Nur77 which in turn inhibits Hif-1α degradation and allows elevated expression of Glut1.

Preliminary *in vitro* experiments with the Hif-1α and Hif-2α small molecule inhibitor Chetomin (Kung et al. 2004), however, did not result in a differential expression of GzmB on activated $CD4^+$Trp1 T cells upon treatment.

5.4 Outlook

Although this present study has revealed specific characteristics of the highly plastic, cytotoxic $CD4^+$Trp1 phenotype and underscored the importance of mTORC1 signalling in the acquisition and maintenance of cytotoxic potential, there are further experiments to be done to further elucidate the mechanisms underlying this $CD4^+$ effector subset.

The high expression of GzmB and other Granzyme proteins is a crucial component of the cytotoxic potential of $CD4^+$Trp1 T cells. Therefore it would be interesting to analyse transcription factor binding sites on the Granzyme A-K gene promoter sequences *in silico*, as for instance outlined by Whitfield and Rao and colleagues (Rao et al. 2008; Whitfield et al. 2012), to find specific transcription factors involved in the expression of the cytolytic molecules. In addition, this analysis would greatly benefit by performing a DNA pull-down (Drewett 2001; Deng et al. 2003) or chromatin immunoprecipitation (ChIP) assay (Kuo & Allis 1999; Wells & Farnham 2002; Dryer & Covey 2006) followed

by mass spectrometry on either *in vitro* activated or *in vivo* differentiated killer CD4$^+$Trp1 T cells to identify in detail the transcription factors bound to the GzmA-K promoters.

Due to the importance of mTORC1 signalling and homeostatic expansion for the cytotoxic phenotype, further studies into downstream proteins of mTORC1 could reveal more of the molecular mechanisms connected with metabolism underlying CD4$^+$ cytotoxicity. It would be interesting to investigate Glut1, Glut3 and TC1 expression on killer CD4$^+$Trp1 T cells to verify the transcriptional data on protein level (Fig. 30) and assess potential co-expression of these proteins with transcription factors Runx3, T-bet and Gata3 and effector molecules such as GzmB, IFNγ, IL-2 and TNF-α.
Although preliminary experiments with HIF-1α and HIF-2α inhibitor Chetomin did not show any inhibitory effect on GzmB expression of CD4$^+$Trp1 T cells *in vitro*, another inhibitor or genetically ablating/knocking down HIF-1α and/or HIF-2α via TALEN or CRISPR technology or shRNA knock down should be attempted. Chetomin was highly toxic and problematic to use on the T cells so another inhibitor such as PX-478 or 103D5R (Tan et al. 2005; Koh et al. 2008) could be used instead, although a knock down/out of the gene by shRNA or TALEN/CRISPR, respectively, would be a more specific option which could be carried further into functional *in vivo* experiments.
Likewise, it would be interesting to investigate the impact on cytotoxic potential of overexpression of HIF-1α, HIF-2α or also other molecules which were exclusively expressed on killer CD4$^+$Trp1 TILs such as CCR5, CD226, LFA-1, KLRG1 or CD11c in either a) helper or tolerant CD4$^+$Trp1 cells or b) polyclonal naïve or B16/BL6 tumour sensitised CD4$^+$ cells from C57BL/6 mice in the killer setting (RT + CD4$^+$ T cells + αCTLA-4). Hu and colleagues, for instance, created plasmids carrying oxygen independent mutant HIF-1α or HIF-2α which could be used for these experiments (Hu et al. 2007). These experiments could pinpoint the potential involvement of said molecules in the acquisition and maintenance of cytotoxic activity on the CD4$^+$Trp1 T cells and investigate their importance for the development of the effector phenotype.

However, the negative impact of the transduction procedure on CD4$^+$ T cell anti-tumour activity observed in the mTORC1 inhibition studies via PRAS40 expression poses a problem for future clinical applications. We hypothesise that the highly avid Trp1 TCR is a key component for the acquisition of the cytotoxic phenotype as it allows directed killing activity of tumour cells in an MHC II dependent manner, enhances homeostatic expansion and increases mTORC1 signalling. Therefore, it will be necessary to

transduce a polyclonal population with a tumour-reactive TCR prior to T cell transfer into patients to elicit $CD4^{+}$Trp1-comparable responses. This emphasises the need to further improve the transduction protocol but also test to transduce polyclonal with different TCRs reactive to known tumour associated proteins such as NY-ESO1 or even neo-antigens (Hunder et al. 2008; Linnemann et al. 2015) or chimeric antigen receptors (CARs) (Kalos 2012).

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