

THE MOLECULAR REGULATION OF CD40L IN CD8⁺ T CELLS

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ABSTRACT

The T cell compartment consists of two major subsets with diverse assignments. CD4⁺ T cells express CD40L upon activation, a central co-stimulatory receptor to induce B cell mediated humoral immunity, activate APCs and prime efficient effector CD8⁺ T cell development (“helper function”). In contrast, cytotoxic CD8⁺ T cells are predetermined to kill infected or malignant cells directly. However, a fraction of CD8⁺ T cells expressing CD40L upon activation was identified. So far, it is not understood in CD8⁺ T cells a) how CD40L expression is regulated, b) when and how the ability of CD40L expression is implemented and c) what are the implications for the immune system.

In this thesis, we found that CD40L expression is regulated by DNA-methylation of regulatory regions of the *CD40LG* locus in CD4⁺ as well as CD8⁺ T cells. The de-methylation of central elements is implemented in the thymus and increases with T cell maturation reflected by enhanced stability of CD40L expression. Elevated CD5 and NUR77 expression of CD40L⁺ CD8⁺ SP thymocytes suggests that high affine detection of self-peptides during positive selection in the thymus implements CD40L expression ability and predetermines the fate of the CD40L imprinted CD8⁺ T cells. CD40L⁺ naïve CD8⁺ T cells differ in their TCR repertoire from their CD40L⁻ counterparts and preferentially mature into memory cell subsets with cytokine and chemokine receptor profiles of Tc2, Tc17 and Tc22 cells. With their non-cytotoxic phenotype and gene expression signatures, the CD40L⁺ memory CD8⁺ T cell subsets Tc2, Tc17 and Tc22 widely resemble helper CD4⁺ T cells and can be distinguished from classical cytotoxic Tc1 and Tc17+1 cells by their IL-6R and absent SLAMF7 expression and their skin migratory phenotype.

Altogether, we demonstrate that from the earliest developmental stages in thymus onwards naïve CD8⁺ T cells are not homogenous and the abilities to provide “CD40L based help” or “cytotoxicity mediated killing” are independent of the CD4⁺ or CD8⁺ T cell status. Cells with helper-type CD8⁺ T cell cytokine and gene-expression signatures were found at barrier sites (skin, lung) by us and others where they contribute to multiple autoinflammatory diseases. Therefore, this work insinuates the need to revisit CD8⁺ T cell capabilities and function in immune responses.

ZUSAMMENFASSUNG

T Zellen können in zwei Hauptpopulationen mit unterschiedlichen Aufgaben unterschieden werden. CD4⁺ T Zellen exprimieren im Zuge ihrer Aktivierung CD40L, welches ein zentraler kostimulatorischer Rezeptor zur Induktion von B-Zell basierter humoraler Immunität, APC Aktivierung und einer effizienten Effektor CD8⁺ T Zell Entwicklung ist („Helfer-Funktion“). Im Gegensatz dazu sind die zytotoxischen CD8⁺ T Zellen dazu vorbestimmt, infizierte oder maligne Zellen direkt abzutöten. Jedoch wurde eine Fraktion von CD8⁺ T Zellen identifiziert, die nach Aktivierung CD40L exprimiert. Bisher ist nicht verstanden, wie in solchen CD8⁺ T Zellen a) die CD40L Expression reguliert ist, b) wann und wie die Fähigkeit CD40L zu exprimieren implementiert wird und c) was die Folgen für das Immunsystem sind.

In dieser Arbeit konnten wir zeigen, dass sowohl in CD4⁺ als auch in CD8⁺ T Zellen die CD40L Expression durch DNA-Methylierung regulatorischer Regionen des CD40LG Locus reguliert wird. Die Demethylierung zentraler Elemente wird im Thymus implementiert, manifestiert sich mit der T-Zell Reifung und geht mit einer zunehmenden Stabilität der CD40L Expression einher. Erhöhte Expression von CD5 und NUR77 in CD40L⁺ CD8⁺ SP Thymozyten weisen auf eine positive Selektion mit hoher Affinität gegen Selbst-peptide während der Reifung im Thymus hin, welche das weitere Schicksal der CD40L exprimierenden CD8⁺ T Zellen beeinflusst. Naive CD40L⁺ CD8⁺ T Zellen besitzen ein anderes TCR Repertoire als CD40L⁻ CD8⁺ T Zellen und reifen im Zuge ihrer Aktivierung bevorzugt zu Gedächtniszellen mit Zytokin- und Chemokinrezeptorprofilen von Tc2, Tc17 und Tc22 Zellen heran. Mit ihrem nicht-zytotoxischen Phänotyp und ihrer Genexpressionsnatur ähneln diese Zellen stark Helfer-CD4⁺ T Zellen und können von den klassisch zytotoxischen Tc1 und Tc17+1 Zellen durch ihre IL-6R und fehlende SLAMF7 Expression sowie der Expression von Markern die auf eine Fähigkeit in die Haut zu wandern schließen lassen, unterschieden werden.

Zusammenfassend zeigen wir hier, dass naive CD8⁺ T Zellen von den frühesten Entwicklungsstadien im Thymus an nicht homogen sind und die Fähigkeiten über CD40L Expression eine Helferefunktion auszuüben beziehungsweise über die Sekretion zytolytischer Moleküle Zielzellen abzutöten unabhängig vom CD4⁺ or CD8⁺ T-Zell Status sind. Zellen mit Zytokin- und Genexpressionssignaturen, die

mit denen der CD8⁺ Helfer-T Zellen übereinstimmen, wurden von uns und anderen in Geweben (Haut, Lunge) identifiziert und tragen zu den verschiedensten autoinflammatorischen Erkrankungen bei. Diese Arbeit insinuiert daher die Notwendigkeit einer grundlegenden Neubewertung der CD8⁺ T Zell Fähigkeiten und Funktionen in Immunantworten.

ABBREVIATIONS

APC	Antigen Presenting Cell
BrefA	BrefeldinA
BSA	Bovine Serum Albumin
CaN	Calcineurin
CCR	C-C motive Chemokine Receptor
CM	Central Memory
CsA	Cyclosporine A
CXCR	C-X-C motive Chemokine Receptor
DAPI	4',6-Diamidin-2-Phenylindol
DC	Dendritic Cell
DN	Double Negative (CD4 ⁻ CD8 ⁻) T cell
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
DP	Double Positive (CD4 ⁺ CD8 ⁺) T cell
E	Effector
EM	Effector Memory
ER	Endoplasmatic Reticulum
FACS	Fluorescence Activated Cell Sorting
IFN	Interferon
IL	Interleukin
Iono	Ionomycin
KO	Knockout
Kb	Kilobase
MACS	Magnetic Cell Separation
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MΦ	Macrophage
N	Naïve
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
O/N	Over Night

PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
PMA	Phorbol 12-myristat-13-acetate
PRR	Pattern Recognition Receptors
qPCR	Quantitative Real Time PCR
RNA	Ribonucleic Acid
RT	Room Temperature
SEB	Staphylococcus Aureus Enterotoxin B
Seq	Sequencing
SP	Single Positive (CD4 ⁺ CD8 ⁻ or CD4 ⁻ CD8 ⁺) T cell
TCR	T Cell Receptor
Tc	T Cytotoxic
TF	Transcription Factor
T _{FH}	T Follicular Helper Cell
Th	T Helper
T _{MNP}	Memory T Cells with Naïve Phenotype
TREC	T Cell Receptor Excision Circles
Treg	T Regulatory Cell
T _{RM}	Tissue Resident Memory T Cell
T _{SCM}	T Memory Stem Cell
TSST1	Toxic Shock Syndrome Toxin 1
WT	Wild Type

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1. INTRODUCTION

1.1 The immune system

The evolution not only led to the development of highly evolved multicellular organisms but also gave rise to organisms with a parasitic and/or symbiotic lifestyle. The existence of these potential harmful threats forced the development of protective mechanisms. Prokaryotes possess a rudimentary defense system based on DNA cleaving that recently became well known as genome editing tool CRISPR (Marraffini, 2015). Among eukaryotes, invertebrates express germline-encoded pattern recognition receptors (PRRs) of the innate immune system that detect conserved structures among pathogens and provoke according immune responses whereas an adaptive immunity developed later with the rise of the vertebrates (Kawai and Akira, 2011). The adaptive immune system is characterized by a sheer unlimited ability of pathogen sensing provided by a flexible arrangement of antigen recognizing receptors of the two pillars of adaptive immunity: B cells and T cells. B cells mediate humoral immunity that protects the liquid systems by antibody secretion whereas T cells are capable to exert cell-mediated immunity (Iwasaki and Medzhitov, 2015).

1.2 T cells

1.2.1 The T cell receptor

Some lymphoid progenitors mature in the bone marrow to B cells whereas others migrate into the thymic cortex where they develop into T cells. T cells are characterized by a highly diverse T cell receptor (TCR) that is responsible for the detection of potential threats. In most T cells, the TCR consist of α - and β -chain that are formed by random joining of multiple regions of exonic DNA. The region for the first rearranged β -chain consists of 42 $V\beta$ (variable), 2 $D\beta$ (diversity), 13 $J\beta$ (joining) and 2 $C\beta$ (constant) segments. RAG enzymes splice one segment of each region together to form a functional V-D-J variable region that is linked to a constant segment to form a complete β -chain. Successful arrangement leads to a proliferation burst and the recombination of the α -chain locus consisting of 43 $V\alpha$ and 58 $J\alpha$ segments of which one each is spliced together with one $C\alpha$

segment into a V-J-C α -chain resulting in several cells presenting a complete α/β TCR at the cell surface with the same β - but various α -chains (Kreslavsky et al., 2012; Laydon et al., 2015). Off note, a small fraction of lymphoid progenitors in the thymus instead rearrange γ - and δ -locus and become γ/δ T cells that do not express CD4 or CD8 coreceptor and possess distinct properties (Vantourout and Hayday, 2013).

The variable regions of the α - and the β - chain consisting of the variously recombined V, D and J segments define the antigen detection properties of the TCR. The TCR detects antigens presented on major histocompatibility complex (MHC) by an antigen presenting cell (APC). This interaction mostly occurs at specific regions called complementary-determining regions (CDRs). While CDR1 and CDR2 are involved in MHC binding, CDR3 is critical for the antigen recognition (Kranz, 2005) (Figure 1). This variation leads to 3×10^{11} possible TCR recombinations of which around 2.5×10^7 can be found among naïve T cells in human. As each TCR is capable of detecting up to 10^6 peptide:MHC complexes, T cells can provide immunity against nearly every threat that is encountered during lifetime (Wooldridge et al., 2012).

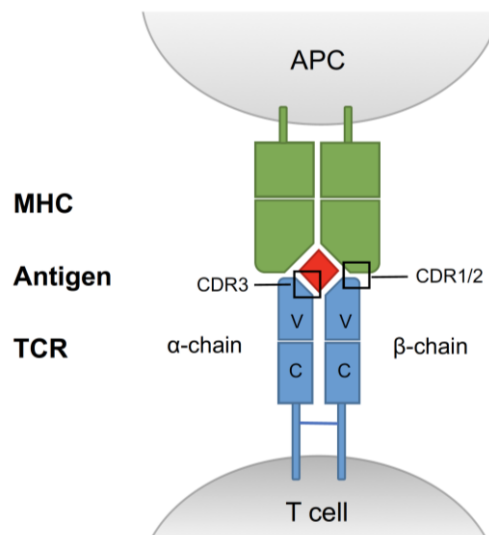


Figure 1: Antigen/MHC complex detection by the TCR.

Antigen presenting cells (APC) present the antigen to the TCR on the T cell which consists of an α - and a β - chain. Both chains have a constant (C) region and a variable (V) region that interacts with the antigen in the complementary-determining region 3 (CDR3) and with the MHC in the CDR1 and CDR2 regions.

1.2.2 T cell development

Maturation of T cells in the thymus progresses over several consecutive steps from CD4⁻CD8⁻ double negative stage 1 (DN1) via DN2 to DN3 stage while they rearrange their β -chain of their TCR and present it with a non-rearranged α -chain as preliminary T cell receptor. Successful presentation leads to a rearrangement of the α -chain in DN4 stage and the presentation of a fully rearranged TCR together with CD4 and CD8 co-receptors in double positive (DP) stage (Germain, 2002) (Figure 2).

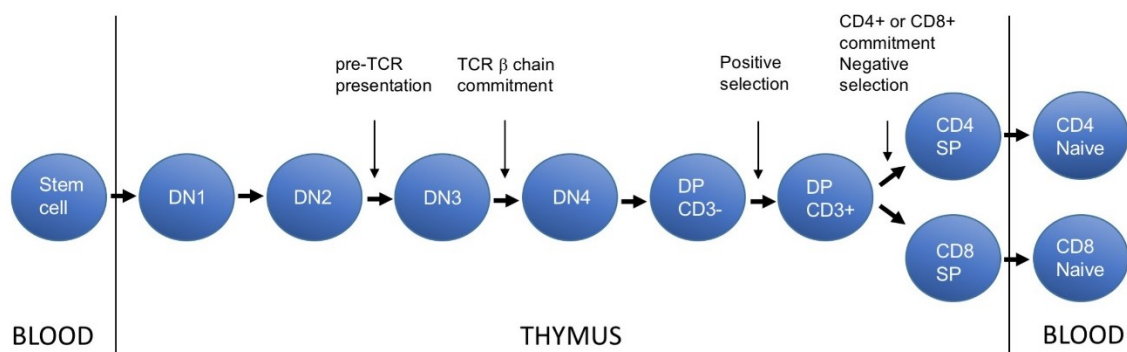


Figure 2: Stages of T cell development. Adapted from (Weerkamp et al., 2006).

Stem cell like cells migrate from the bone marrow via blood into the thymus where they enter the CD4⁻CD8⁻ double negative (DN) stage 1. TCR recombination events during the maturation steps DN2, DN3 and DN4 lead to co-expression of CD4 and CD8 in the double positive (DP) stage. Successful selection is accompanied by upregulation of CD3 and a CD4⁺CD8⁻ or CD4⁻CD8⁺ single positive (SP) lineage commitment. Mature cells egress from thymus and circulate as naïve cells through blood and lymph until they encounter their cognate antigen.

The responsiveness of the newly arranged TCR is tested by a highly specialized variant of APCs, thymic epithelial cells (TECs) that present autoantigens on major histocompatibility complex (MHC) type I or type II. Too weak or absent MHC detection by the TCR leads to cell death by neglect (positive selection) in the cortex whereas too strong self affine clones are eliminated in the medulla by negative selection to avoid autoantigen targeting T cells in the periphery (Klein et al., 2014; Takaba and Takayanagi, 2017) (Figure 3).

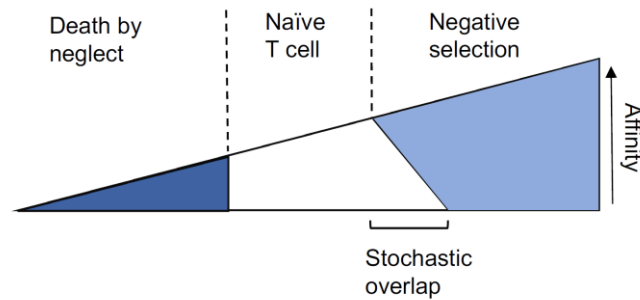


Figure 3: TCR affinity during T cell selection decides T cell fate. Adapted from (Klein et al., 2014)

Lack of TCR stimuli leads to death by neglect. Weak affinity TCR signaling induces positive selection and give rise to naïve T cells. Increasing affinity of the TCR induces the differentiation of Tregs from CD4⁺ T cells or elimination by negative selection.

1.2.3 The thymic cortex: positive selection and CD4⁺ vs. CD8⁺ T cell fate decision

The cTECs of the thymic cortex mediate positive selection by presenting a unique MHC ligandome. This ligandome consists of “private peptides” provided by the thymoproteasome subunit $\beta 5t$, which possesses a unique substrate preference responsible for MHC I specific peptide processing and loading (Murata et al., 2007). The cTECs furthermore exclusively utilize cathepsin L and TSSP1 combined with strong autophagy for the processing of MHC II specific peptides. It is speculated that these highly specialized mechanisms for antigen presentation are evolved in order to increase the overall TCR repertoire by enhancing the numbers of clones with a low affinity passing the positive selection (Gommeaux et al., 2009; Hsieh et al., 2002; Xing et al., 2013). Furthermore, it creates a peptide pool different from constitutive proteasomes acting during negative selection in order to avoid elimination of all positively selected T cells (peptide-switch model) (Kincaid et al., 2016).

CD4⁺CD8⁺ DP T cells wander through the cortex and scan the cTECS for their TCR specific peptide presented either on MHC type I or MHC type II. Upon binding to peptide-MHC complexes, independent of the MHC class, the CD8 co-receptor is downregulated (Brugnera et al., 2000). Since the CD8 co-receptor specifically binds to MHC I whereas CD4 co-receptor attaches to MHC II, downregulation of CD8 destabilizes MHC I:TCR interactions compared to

MHCII:TCR complexes. Therefore, detection of an MHCII:peptide complex leads to a comparatively long and strong TCR signaling activating a CD4 lineage manifesting transcription factor network (Liu and Bosselut, 2004). In contrast, MHC I specific cells loose contact to the cTEC after short MHC signaling due to the downregulated CD8 co-receptor and become susceptible to intrathymic IL-7 signaling (Brugnera et al., 2000; Park et al., 2010). This combination activates CD8-associated transcription factors that not only promote the development of CD8⁺ T cells but also repress CD4⁺ T cell related genes and therefore determine a CD8⁺ T cell fate (Cruz-Guilloty et al., 2009; Setoguchi et al., 2008).

The avidity of the TCR for the MHC presented peptide during positive selection influences the functional attributes of the T cell in periphery in terms of responsiveness, survival and development (Persaud et al., 2014). Avidity composes of the sum of multiple interactions with specific affinities that result from the binding strength of the TCR to the MHC presented peptide. High avidity goes along with prolonged TCR signaling and an increase of the TCR signaling repressors CD5 and NUR77 that are important for the fine tuning of TCR signaling response (Azzam et al., 1998; Moran et al., 2011) (Figure 4).

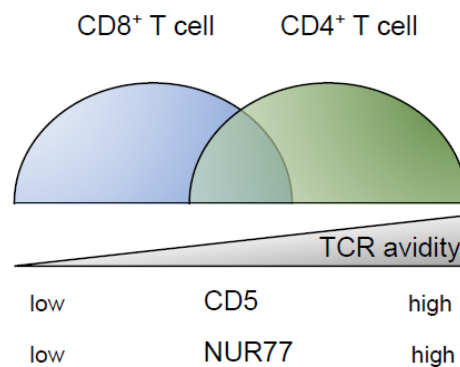


Figure 4: TCR avidity during positive selection decides CD8⁺ versus CD4⁺ T cell fate and is reflected by CD5 and NUR77 expression levels. Adapted from (Hogquist and Jameson, 2014).

1.2.4 The T cell lineage transcription factors: ThPOK and Runx3

The CD4 versus CD8 lineage decision is mainly regulated by the transcription factors ThPOK and Runx3 accompanied by concerted changes in the epigenetic landscape. Long and strong TCR signaling activates the CD4 lineage transcription factor ThPOK, which represses CD8 lineage associated genes,

opposes the Runx3 dependent activation of the CD4 silencer and imprints CD4 lineage fate by TET mediated DNA demethylation of the CD4 locus (Egawa and Littman, 2008; Muroi et al., 2008; Wang et al., 2008). Efficient CD4 imprinting further requires concerted action of several other transcription factors, which are also activated by strong TCR stimuli. A potent activation of ThPOK is dependent on GATA3 induction by c-MYB (Maurice et al., 2007). GATA3 not only promote ThPOK expression itself but also represses CD8 lineage decision by inhibiting Runx3 expression (Wang et al., 2008; Xiong et al., 2013). Besides, the transcription factors TCF-1 and LEF-1 are indispensable to reach sufficient ThPOK levels for CD4⁺ T cell differentiation as well as TOX for complete CD4⁺ T cell maturation (Aliahmad et al., 2011; Steinke et al., 2014) (Figure 5).

In contrast, short TCR signaling leads to Runx3 activation by IL-7 and IL-15 signaling in a STAT5 dependent manner and results in the re-expression of the CD8 co-receptor (Park et al., 2010) (Figure 5). Besides IL-6, IFN- γ , TGF β and TSLP can induce Runx3 and drive CD8⁺ lineage development (Etzensperger et al., 2017). The CD8⁺ T cell fate is stabilized by the ThPOK silencer, which mediates a strong epigenetic silencing of the *ThPOK* locus in the absence of strong TCR stimuli as well as by histone deacetylase mediated silencing of the CD4 locus (Boucheron et al., 2014; Tanaka et al., 2013).

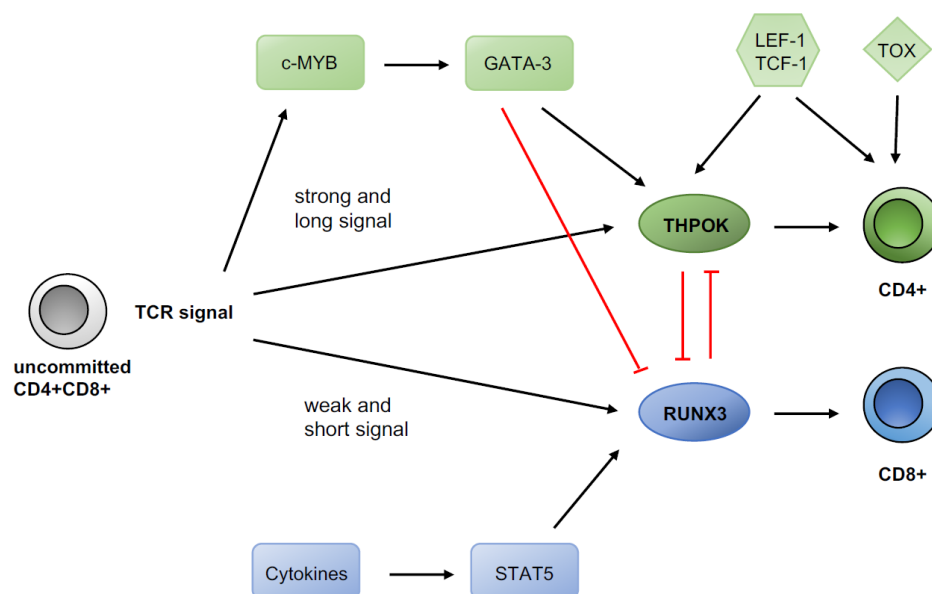


Figure 5: Lineage transcription factors ThPOK and Runx3 regulate CD4⁺ versus CD8⁺ T cell fate. Adapted from (Mookerjee-Basu and Kappes, 2014).

1.2.5 The thymic medulla: negative selection and tolerance induction

After positive selection and CD4 versus CD8 fate decision, induced CCR7 expression mediates the migration of developing T cells into the thymic medulla that consists of a network of mTECs, dendritic cells (DCs) and B cells (Kurobe et al., 2006). Medullary TECs present high levels of self-peptides controlled by the AIRE gene (Derbinski et al., 2001; Heino et al., 1999). AIRE orchestrates the activation of silent gene loci and initiates promiscuous gene expression (Anderson et al., 2002; Meredith et al., 2015). T cells wander through the network and scan the APC for cognate antigens. Persistent TCR signaling in response to autoantigens leads to the induction of apoptosis and therefore elimination of the T cells from the repertoire by negative selection (Strasser et al., 2007). From CD4⁺ T cells, a stochastic overlap between survival and elimination is known (see Figure 3). A strong but transient interaction of high self-affine CD4⁺ T cells with medullary APC can result into the implementation of a natural regulatory T cell (nTreg) fate by FoxP3 induction - a process called agonistic selection as it bases on self-antigen MHC complex detection (Hsieh et al., 2012; Li and Rudensky, 2016; Takaba and Takayanagi, 2017). Finally, the cells that succeeded to pass selection undergo several rounds of proliferation while they upregulate S1P1 receptor and egress from the thymus into the periphery as naïve T cells (Carlson et al., 2006; Pénit and Vasseur, 1997).

1.2.6 T cells in immune response

In the periphery, mature T cells migrate through blood and secondary lymphatic organs where they scan APCs for their cognate antigen (Masopust and Schenkel, 2013). The specificity of T cells for a particular antigen lies at around 1 in 100.000. For proper immune defense, a clonal burst of short-lived effector cells occurs following antigen recognition, increasing the cell numbers up to 10⁷ progenies while migrating to the site of infection (Blattman et al., 2002). After the infection is cleared, 90-95% of the effector cells die in the contraction phase leaving behind a small memory fraction characterized by a strong, cytokine independent response upon secondary pathogen exposure (Williams and Bevan, 2007) (Figure 6).

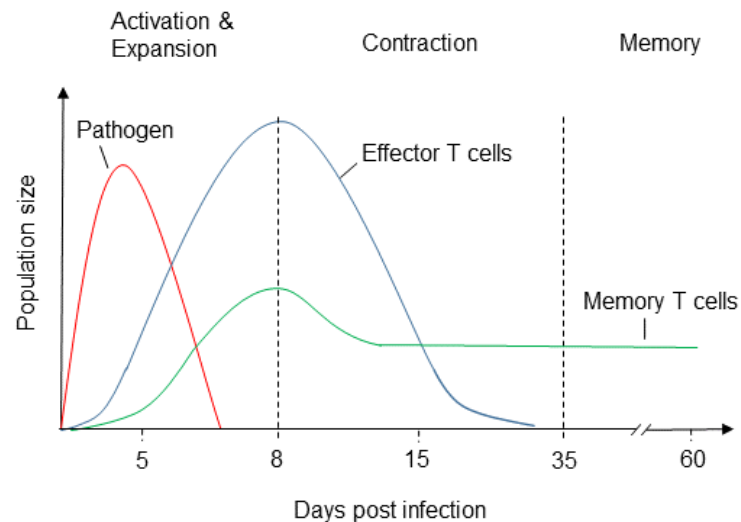


Figure 6: Kinetic of CD8⁺ T cell response. Adapted from (Kaech and Cui, 2012).

This memory compartment – the hallmark of adaptive immunity – can be roughly subdivided according to their steady state migration potential and tissue surveillance capacity into CD45RA⁻CD45RO⁺CCR7⁺ central memory (CM) and CCR7⁻ effector memory (EM) cells (Sallusto et al., 1999). CCR7 enables CM to migrate through areas of secondary lymphoid organs. They produce high levels of IL-2 and efficiently differentiate into effector cells after re-activation. In contrast, EM home into inflamed tissue and can provide rapid effector function by secretion of cytokines and cytolytic molecules. Some effector cells instead re-express CD45RA and become terminally differentiated effectors (EMRA) (Sallusto et al., 2004). Upon activation and inflammation T cells also can migrate into tissue where they upregulate CD103 and/or CD69 and become tissue resident memory (T_{RM}) cells (Jameson and Masopust, 2018).

There are different models how the differentiation into short-lived effector and different memory populations is regulated (Figure 7). The **separate-precursor model** suggests a fate imprinting in the thymus whereas the **decreasing-potential model** implies an effect of repeated stimuli on differentiation. According to the **signal strength model**, the strength of TCR stimulus combined with co-stimulus and cytokine signal decide the fate. A weak stimulus gives rise to CM followed EM and effector cells. Finally, the **asymmetric cell fate model** implies that the daughter cell, which is connected to the APC by

the immunological synapse, receives a stronger stimulation leading to effector cell differentiation, while the other cell experiences a weaker stimulus and differentiates into a memory cell (Kaech and Cui, 2012). Extensive analyses on single cell levels in murine models revealed that memory formation is only predictable on population but not on single cell level (probably due to local differences in antigen presentation or cytokines) with an overall tendency to progressively differentiate from N to CM to EM to EMRA (Aalderen et al., 2017; Buchholz et al., 2013; Gerlach et al., 2013). Global TCR sequencing of T cells derived from diverse human organs suggest that effector and EMRA cells develop separately from a common precursor that can give rise to CM, EM and T_{RM} as postulated in the separate precursor model (D. Farber, unpublished).

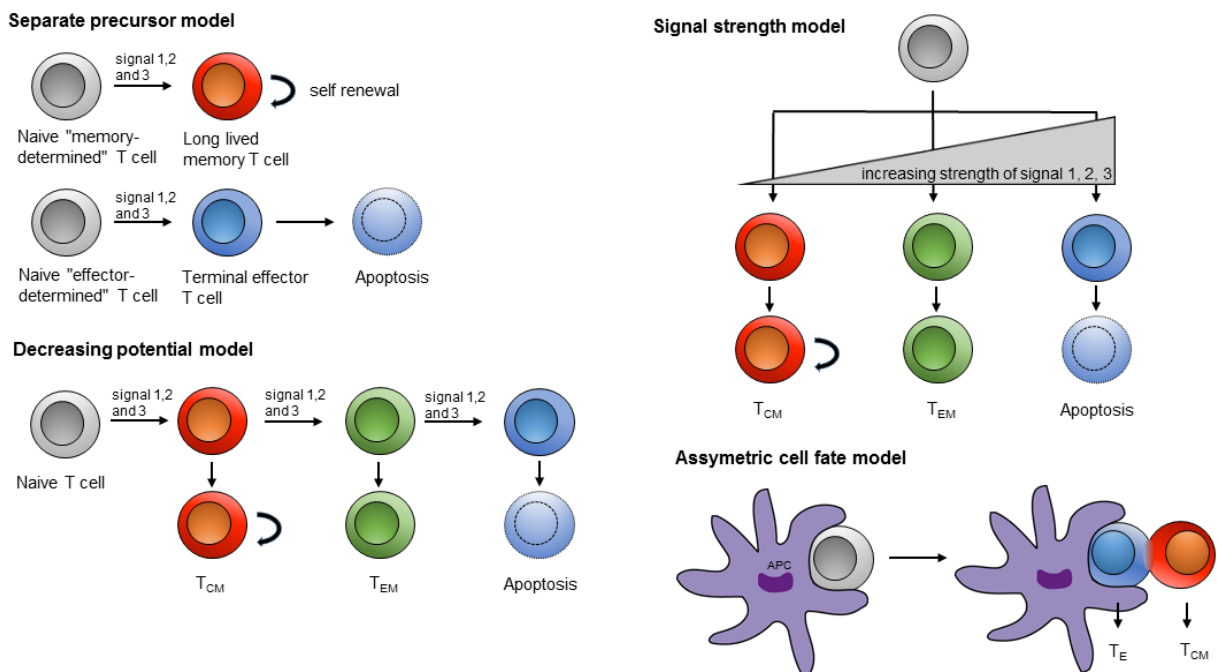


Figure 7: Different models of T cell differentiation. Adapted from (Kaech and Cui, 2012).

1.2.7 $CD4^+$ T cells

$CD4^+$ T cells are specialized to detect antigens bound to MHCII receptors that are expressed on professional APCs such as dendritic cells (DC), macrophages ($M\Phi$), B cells as well as on endothelial and epithelial cells. The antigen presenting cells sample potential threats from extracellular fluids, internalize them and load them onto MHCII in the endolysosomal compartment before transporting to the surface (Blum et al., 2013; Neefjes et al., 2011; Vyas et al., 2008). Danger signals

by pattern recognition receptors that detect conserved pathogen structures activate the APCs and they wander into draining lymph nodes while differentiating into mature APCs with increasing expression of MHCII and costimulatory molecules such as CD80 and CD86 (Tan and O'Neill, 2005). Naïve CD4⁺ T cells become activated upon recognition of the subsequent antigen presented on the APC leading to the differentiation into highly specialized subsets dependent on the local cytokine milieu (Sallusto, 2016). The first described subsets Th1 and Th2 are induced by the cytokines IL-12/ IFN- γ and IL-4 respectively that activate the lineage specific transcription factors (TF) T-bet (Th1) and GATA3 (Th2) (Constant and Bottomly, 1997). While IFN- γ secreting Th1 are important for inflammatory responses, IL-4, IL-5 and IL-13 secreting Th2 can activate mast cells and eosinophils. Both promote humoral immune response by activating B cells in a CD40L dependent manner and inducing hyperproliferation as well as class-switching into diverse immunoglobulins (Smith et al., 2000). Since, several further CD4⁺ T cell subsets were identified as summarized in Table 1.

Subset	Transcription-factors	Cytokines	Surface-markers	Function
Th1	T-bet	IFN- γ	CCR6 ⁻ CXCR3 ⁺ CCR4 ⁻	Intracellular bacteria, viruses, autoimmunity
Th2	GATA3	IL-4 IL-5 IL-13	CCR6 ⁻ CXCR3 ⁻ CCR4 ⁺	B cell help, extracellular parasites, allergy
Th17	ROR γ t	IL-17	CCR6 ⁺ CXCR3 ⁻ CCR4 ⁺ CCR10 ⁻	Bacteria, fungi, autoimmunity
Th22	AHR	IL-22	CCR6 ⁺ CXCR3 ⁻ CCR4 ⁺ CCR10 ⁺	Skin inflammation
Th17+1	T-bet ROR γ t	IFN- γ IL-17	CCR6 ⁺ CXCR3 ⁺ CCR4 ⁻	Autoimmunity
Th9	PU.1 IRF4	IL-9	CCR6 ⁺ CXCR3 ⁺ CCR3 ⁺	Allergy

Treg	FoxP3	IL-10 TGF- β	CD25 ⁺	Immune regulation
T _{FH}	Bcl6	diverse	CXCR5 ⁺ CCR7 ⁺	Germinal center help
T _{RM}	?	diverse	CD103 ⁺ CD62L ⁻ CCR7 ⁻ CD69 ⁺	Barrier defense
Cytotoxic CD4	Eomes? T-bet?	IFN- γ	CD27 ⁻ CD28 ⁻ CD57 ⁺ CRTAM ⁺	Intracellular bacteria, viruses

Table 1: The diversity of CD4⁺ T cell subsets. Adapted and modified from (Sallusto, 2016).

Within memory cells, subsets with specific cytokine secretion profiles can be distinguished by the expression of specialized homing receptors that regulate the migration to tissue and sites of inflammation. Among these the chemokine receptor CCR6 separates the non-IL-17 producing cells from IL-17 producers. Within the CCR6 negative fraction, CXCR3 and CCR4 mark Th1 and Th2 cells respectively. In contrast, the CCR6⁺ cells can be separated into a CCR4⁺ Th17 subset and a CXCR3⁺, IFN- γ co-expressing Th17+1 population (Acosta-Rodriguez et al., 2007). Th17 differentiate upon ROR γ t activation in the presence of IL-6 and TGF β and provide immunity against bacteria and fungi in barriers such as mucosa and skin while some further develop to IFN- γ co-expressing Th17+1 cells in the presence of IL-12 (Lexberg et al., 2010; Yang et al., 2014). Furthermore, CCR10 separates an AHR expressing and IL-22 secreting Th22 fraction from the Th17 population, which are central players in skin protection (Fujita, 2013; Trifari et al., 2009; Zheng et al., 2007). These CD4⁺ T helper subsets are complemented by IL-9 secreting, CCR3 expressing Th9 cells that are located in mucosa and lung and associated with allergy (Danilova et al., 2015; Kaplan, 2013). CD4⁺ T cells can differentiate into tissue specific populations such as T follicular helper cells (T_{FH}) that express CXCR5 and exclusively reside in germinal centers to provide B cell help as well as CD103 and/or CD69 expressing tissue resident memory cells (T_{RM}), which provide barrier defense (Crotty, 2014; Mueller and Mackay, 2016). Natural regulatory T cells (nTregs) derive from the thymus or arise in the periphery (“induced” iTregs) by the induction of FoxP3

transcription factor. They inherit the ability to suppress unwanted immune responses and therefore protect the immune system from autoreactivity (Vignali et al., 2008). Some CD4⁺ T cells exhibit CD8⁺ associated features such as secretion of lytic enzymes (Granzymes, Perforin) together with IFN- γ . Those cytotoxic CD4⁺ T cells kill the target cells in a MHCII dependent manner and contribute to the defense against intracellular pathogens (Takeuchi and Saito, 2017). Off note, those classifications are not always strict. For example, some Tregs can gain tissue residency by expressing CD103 and/or CD69 (Schenkel and Masopust, 2014). Moreover, Tregs as well as T_{RM} were described to secrete distinct cytokine profiles that match those of Th1, Th2 or Th17 cells (Hondowicz et al., 2016; Wilk et al., 2017).

1.2.8 CD8⁺ T cells

In contrast to CD4⁺ T cells, CD8⁺ T cells detect antigens presented by MHC type I receptors expressed on all nucleated cells. MHCI is presenting cytosolic molecules such as virus particles that were degraded by the proteasome, loaded onto the MHC in the endoplasmic reticulum (ER) and transported to the surface. CD8⁺ T cells kill infected target or malignant cells either by secretion of cytolytic molecules such as Perforin and Granzymes or by Fas mediated apoptosis (Kägi et al., 1994; Lowin et al., 1994; Metkar et al., 2002). The CD8⁺ T cell compartment was diversified into different memory subsets based on the CX3CR1 expression as a marker of CD8⁺ T cell cytotoxicity (Böttcher et al., 2015; Gerlach et al., 2016). Alternatively, the markers CXCR3/CCR5 and CCR4/CRTH2/CCR8 known from CD4⁺ T cell subset diversification were used to identify Tc1 (T cytotoxic type 1) and Tc2 (T cytotoxic type 2) CD8⁺ T cells respectively to distinguish IFN- γ from IL-4 producers (Cosmi et al., 2000; D'Ambrosio et al., 1998). Beyond, the usage of further receptor combinations remained controversial. IL-17 producing CCR5⁺CCR6⁺ and a CCR4⁺CCR5⁻ subset lacking cytolytic molecules and secreting IL-4, IFN- γ and IL-2 instead were described (Kondo and Takiguchi, 2009; Kondo et al., 2009). Moreover, functional analyses of CD8⁺ T cell subsets indicate the existence of IL-22 producing Tc22 and IL-17/ IFN- γ coproducing Tc17+1 cells (Liu et al., 2011; Yen et al., 2009). Just recently, the complexity of

CD8⁺ T cell compartment was elevated by the introduction and further subdivision of skin CD8⁺ T_{RM} into a CD103⁺CD49a⁺ cytotoxic and a CD103⁺CD49a⁻ non-cytotoxic subset (Cheuk et al., 2017; Gebhardt et al., 2009; Jiang et al., 2012).

1.2.9 T cell activation

Proper T cell mediated adaptive immunity requires orchestrated coincidence of three independent signals: TCR signaling, costimulatory signal and cytokine milieu. The first signal is provided by TCR activation by an MHC presented peptide. Additional costimulation, mostly mediated by CD28 which can partially be replaced or complemented by CD27, 4-1BB or OX-40, is required to prevent anergy and avoid unspecific T cell activation (Chen and Flies, 2013). The tertiary signal is provided by the cytokine milieu during activation and guides T cell differentiation into a specialized subset to handle the diverse threats. These cytokines are usually provided by PRR activated APC but can also derive from other lymphoid cells (Curtsinger et al., 1999).

Optimal CD8⁺ T cell activation, memory formation and secondary expansion is thought to depend on CD4⁺ T cell help (Bennett et al., 1998; Janssen et al., 2003; Schoenberger et al., 1998; Shedlock and Shen, 2003). CD4⁺ T cell help is provided by CD40L dependent DC “licensing” leading to increased antigen presentation and expression of costimulatory signals (Frentsch et al., 2005; Kawabe et al., 1994). Additionally, activated CD4⁺ T cells provide IL-2 required for optimal clonal expansion and effector function of CD8⁺ T cells (Lai et al., 2009). There are two models how help is provided: the three-cell model that requires simultaneous binding of CD4⁺ and CD8⁺ T cell to the same APC and the sequential two-cell model/kinetic model, that allows a APC licensing by CD4⁺ T cells followed by a later CD8⁺ T cell activation through the activated APC (Ridge et al., 1998). Besides, a variant of “self”-help was also reported for CD8⁺ T cells. Sufficient numbers of activated, IFN- γ secreting CD8⁺ T cells activates DC detectable by CD80, CD83 and IL-12 secretion (Mailliard et al., 2002, 2013; Nakamura et al., 2007; Stark et al., 2013; Wang et al., 2001). Some CD8⁺ T cell clones furthermore produce IL-2 instead of IFN- γ similar to CD4⁺ helper cells,

which is utilized in an autocrine manner for robust secondary expansion (Feau et al., 2011; Mailliard et al., 2013; Pira et al., 2007).

1.3. CD40L

CD40L (gp39, CD154, TRAP) is a transmembrane protein of the TNF superfamily originally identified in mouse (Armitage et al., 1992). CD40L is expressed on activated CD4⁺ T cells, B cells, monocytes, macrophages, granulocytes but also on endothelial cells, epithelial cells, fibroblasts, keratinocytes and platelets (Chatzigeorgiou et al., 2009; Grewal and Flavell, 1998). The CD40L protein comprises of 32-39 kDa dependent on posttranslational modifications is presented as trimeric complex on cell surface or released as soluble form (Graf et al., 1995; Mazzei et al., 1995). CD40L is a central player of humoral immunity. Activated CD4⁺ T cells transiently express CD40L and migrate to the boarder of the B cell follicles in the lymph nodes. Upon TCR:MHCII interaction they can provide CD40L-CD40 mediated co-stimulation and induce germinal center formation, somatic hypermutations and class switch in the B cell (Eertwegh et al., 1993; Foy et al., 1993). Besides, CD40L signaling is a central player of cell-mediated immunity. CD40L dependent activation can enable B cells, DCs and monocytes to mature to highly efficient APCs (Ahmadi et al., 2008; Caux et al., 1994; Kiener et al., 1995). This CD40L mediated DC “licensing” describes the induction of APC maturation processes including the upregulation of MHC and costimulatory molecules as well as the secretion of the pro-inflammatory cytokines IL-1, IL-6, IL-8 and TNF- α . CD40L-CD40 interaction with DCs moreover is necessary to promote IL-12 secretion by the DCs, a cytokine critical for the induction of proper cytolytic responses of CD8⁺ T cells (Fujii et al., 2004).

1.3.1 Structure

The *CD40L* gene stretches over 12kb of the X-chromosome and consists of 5 exons. Gene expression is induced upon TCR stimulation dependent translocation of activated transcription factors into the nucleus and binding to regulatory elements of the *CD40L* gene. NFAT was identified as central TF, which binds at two sites of the proximal and one site of the distal promotor and is supported by CD28 dependent binding of EGR1 (Cron et al., 2006; Lindgren et

al., 2001; Lobo et al., 2000; Schubert et al., 1995). The promoter further contain a NF- κ B (p65 homodimer) binding site, TFE3/TFEB binding sites required for maximal activity in mouse, an AKNA binding element and a CD28 response element bound by NF- κ B and AP-1 components (Huan et al., 2006; Parra et al., 2001; Siddiqa et al., 2001; Srahna et al., 2001) (Figure 8). CD40L expression is further supported by one upstream enhancer with NFAT and GATA3 binding sites and a downstream enhancer with a NF- κ B (p50 homodimer) binding site (Brunner et al., 2008; Schubert et al., 2002).

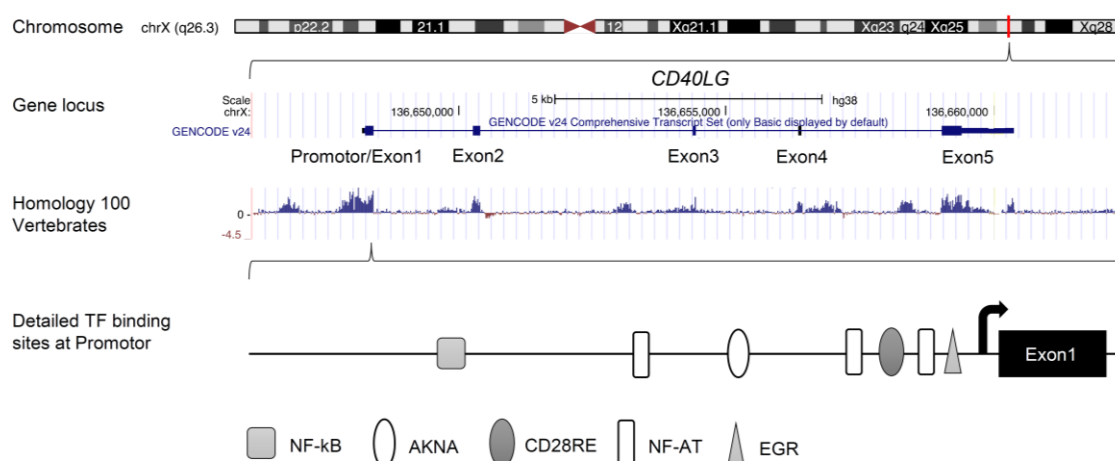


Figure 8: Location and organization of the *CD40LG* gene. Adapted from (Steiper et al., 2008).

CD40LG is located on the X-chromosome, stretches 12kb and consists of 5 exons that are highly conserved among vertebrates. The *CD40L* promoter inherits binding sites for several transcription factors such as NFAT, NF- κ B, EGR as well as a CD28 responsive element (RE) which are responsible for induction and tuning of *CD40L* expression.

1.3.2 Regulation

De novo *CD40L* expression in $CD4^+$ T cells can be detected as early as 2 hours following TCR stimulation, peaks at around 6 hours and is almost undetectable after 24 hours (Quezada et al., 2004). Effector and memory $CD4^+$ T cells can store *CD40L* in secretory lysosomes and release as early as 15min following activation (Koguchi et al., 2007). In the presence of CD28 and IL-2 signaling, a second peak occurs at 48 hours after T cell activation (McDyer et al., 2002; Snyder et al., 2007). Prolonged TCR signaling exceeding 24 hours enhances the *CD40L* stability by post-transcriptional mechanisms that influence RNA and protein stability and location in human and mouse (Matus-Nicodemos et al., 2011; Vavassori et al., 2009). Additionally, IL-15 prolongs *CD40L* expression in $CD4^+$ T

cells in a STAT5 dependent manner (Lowe et al., 2014). However, CD40L surface expression is tightly regulated. Interaction with its receptor CD40 leads to immediate internalization of CD40L (Kooten and Banchereau, 2000).

1.3.3 Interaction partners

CD40L has highest affinity to the CD40 receptor, which is expressed constitutively or upon activation by most cells of the immune system and the vasculature (Schönbeck and Libby, 2001). However, CD40L can also bind α IIb β 3, α 5 β 1 and Mac-1 (α M β 2, CD11b) (Alturaihi et al., 2015; L veill  et al., 2007; Zirlik et al., 2007). The three integrins were shown to be bound by the soluble form of CD40L. While α IIb β 3 is abundantly expressed on platelets, required for aggregation and adhesion was shown to regulate thrombus formation stabilization upon CD40L binding, the physiological role of fibronectin receptor α 5 β 1 is still unknown (Andr  et al., 2002). In contrast, Mac-1 binding of CD40L regulates the migration of leukocytes to sites of inflammation (Wolf et al., 2011; Zirlik et al., 2007).

1.3.4 Diseases

The importance of a tightly controlled CD40L expression is shown by aberrant CD40L expression. CD40L deficiency caused by gene mutations in the CD40L locus leads to X-linked hyper IgM syndrome characterized by absent class switching and consequent lack of IgG, IgA and IgE antibodies but also impaired T cell function as an indirect result of absent CD40 mediated activation of DCs (Allen et al., 1993; DiSanto et al., 1993). On the other hand, deregulated expression levels caused by CD40LG demethylation on the silenced X-Chromosome was observed in women with systemic sclerosis, systemic lupus erythematosus and rheumatoid arthritis leading to increased levels of autoantibodies (Lian et al., 2012; Liao et al., 2012; Lu et al., 2007). Increased RNA and serum levels of CD40L were detected in multiple sclerosis patients and CD40L blockage in murine EAE could prevent disease onset (Gerritse et al., 1996; Howard et al., 1999; Huang et al., 2000; Zhong et al., 2016). Besides, in

several CD40 expressing cancer cell types, dependent on the signaling properties, CD40L mediated signaling can result into opposing effects including the upregulation of anti-apoptotic factors leading to an increased proliferative capacity and survival of the tumor cells but also the induction of apoptosis (Elgueta et al., 2009; Frentsch et al., submitted).

1.3.5 CD40L on CD8⁺ T cells

A fraction of CD8⁺ T cell was reported to express the CD4⁺ helper T cell molecule CD40L by us and others (Cronin et al., 1995; Durlanik et al., 2016; Frentsch et al., 2013; Hermann et al., 1995; Stark et al., 2013). While around 10% of naïve (CD45RA⁺CCR7⁺) cells express CD40L, the frequency increases to around 30% in the CD45RA⁻ memory compartment (Frentsch et al., 2013). In contrast, none of the effector/EMRA (CD45RA⁺CCR7⁻) CD8⁺ T cells can express CD40L. CD8⁺ T cells do not possess prestored CD40L but expression is rapidly activated upon polyclonal stimulation and detectable within 2hrs comparable to CD4⁺ T cells. After 3-4hrs the CD40L protein is detectable at the surface, reaching a peak between 6-8hrs and slowly declines after 12hrs. CD40L expressing CD8⁺ T cells are capable of DC licensing and B cell activation similar to CD4⁺ helper cells (Frentsch et al., 2013).

The CD40L⁺ CD8⁺ T cell fraction can be subdivided into two populations of which the first display weak CD40L expression and classical cytotoxic CD8⁺ T cell properties including the expression of IFN- γ , Granzymes and Perforin. They can be generated *in vitro* from naïve CD8⁺ T cells upon stimulation in the presence of IL-12 and were found to participate to *in vivo* immune responses against viruses (human and murine Cytomegalovirus, Epstein-Barr virus, Influenza, Yellow Fever) and bacteria (*Listeria monocytogenes*) (Durlanik et al., 2016; Frentsch et al., 2013; Stark et al., 2013; AR. Schulz, R.Stark, A.Hartung unpublished). CD40L expressed by CD8⁺ T cells promotes CD4⁺ memory T cell expansion and induces secondary expansion of CD8⁺ memory T cells themselves when inflammation is limited. Recently, Tay et al. showed that the observed positive effects on proliferation and secondary expansion of CD8⁺ T cells in defined bacterial and viral infections are mediated by a cell extrinsic

positive feedback loop. Activated DC provide IL-12 that induce CD40L in CD8⁺ T cells, which in turn receive proliferation supportive signals via CD40L:CD40 interaction with the DC (Tay et al., 2017). Besides, CD40L can induce apoptosis of CD40 expressing solid tumor cells in a caspase 8 pathway dependent manner (Frentsch, Japp et al.; submitted).

The second fraction of CD40L⁺ CD8⁺ T cells is characterized by strong CD40L expression (high MFI), lack of cytotoxic molecules and high levels of IL-2 (S. Warth, unpublished; Frentsch et al., 2013). This CD40L bright population resembles CD4⁺ T cells in their gene expression profile and was shown to be able to activate dendritic cells and B cells similar to those classical CD40L⁺ CD4⁺ helper T cells (Frentsch et al., 2013; Hernandez et al., 2007). However, nor their specific role in the immune system neither an induction mechanism could be identified for the CD40L bright CD8⁺ T cells so far.

1.4 Aim of this thesis

CD40L is a central molecule of activated CD4⁺ T cell to provide help to B cells and induce maturation of APCs whereas CD8⁺ T cells classically directly kill infected or abnormal cells in the body. Previous work could show that CD40L is also expressed by some activated CD8⁺ T cells (Cronin et al., 1995; Durlanik et al., 2016; Frentsch et al., 2013; Hermann et al., 1995; Stark et al., 2013). CD40L expressing CD8⁺ T cells have been shown to be involved in the defense against various forms of pathogens including viruses, bacteria, parasites and cancer cells, of which the vast majority belongs to classical IFN- γ co-expressing, cytotoxic CD8⁺ T cells that arise upon proliferation in the presence of IL-12 (Durlanik et al., 2016; Frentsch et al., 2013; Stark et al., 2013; AR. Schulz, A.Hartung, unpublished). Those IFN- γ ⁺ CD8⁺ T cells differ from a second fraction that express high levels of CD40L together with IL-2 and exhibit CD4⁺ T helper like gene expression pattern (Frentsch et al., 2013). However, it is not understood how CD40L is induced in those IFN- γ CD40L⁺CD8⁺ T cells and how they contribute to the immune defense. Besides, the general mechanism of CD40L gene regulation in CD8⁺ T cell is unknown. Hence, this work aims to investigate 1) how CD40L expression is regulated in CD8⁺ T cells on gene level, 2) when and where is the CD40L expression induced, 3) what is the impact on immune

response. In a first step *CD40LG* gene locus accessibility, transcription factor usage and induction mechanism following TCR activation of CD40L⁺ CD8⁺ T cells will be compared to common knowledge gathered from CD4⁺ T cells. Next, this work should assess when and in response to what the ability to express CD40L is imprinted (or lost) in CD8⁺ T cells and the resulting implications in the overall T cell fate. Finally, the collected knowledge should be placed into the context of immune defense and provide a better understanding of the role of CD40L⁺ CD8⁺ T cells in the periphery. Together, the gathered results should broaden the knowledge about the flexibility of the immune system and give useful information about the function of CD40L expressing CD8⁺ T cells resulting into potential therapeutic applications of CD40L⁺ CD8⁺ T cells.

2. MATERIALS AND METHODS

2.1 General material and equipment

2.1.1 Cell culture

Complete medium for human T cell culture (AB)

RPMI 1640 – GlutaMAX™	Gibco
+ Penicillin (100U/ml final)/ Streptomycin (100µg/ml final)	Biochrom
+ 10% human AB serum, heat inactivated (56°C, 30min)	Pan Biotech

Complete medium for mouse T cell culture

RPMI 1640 – GlutaMAX™	Gibco
+ 10% fetal calf serum (FCS), heat inactivated (56°C, 30min)	Lonza
+ Penicillin (100U/ml final)/ Streptomycin (100µg/ml final)	Biochrom
+ 50µM β-Mercaptoethanol	Gibco

ACK lysing buffer

Gibco

Trypsin/EDTA solution (0.05%/0.02%)

Biochrom

2.1.2 Buffers

dH₂O (desalted)

House made

PBS (phosphate buffered saline)

Gibco

PBS

+ BSA (bovine serum albumin, 0.2% final)

PAA

TAE buffer: dH₂O

+ 40mM Tris (pH 7.6)

Roth

+ 20mM acetic acid

Sigma

+ 1mM EDTA

AppliChem

2.1.3 Chemicals

Isopropanol

Merck

Ethanol

Roth

Methanol	Merck
Sodium hydroxide (NaOH)	Roth
Hydrochloric acid (HCl)	Roth

2.1.4 Material

Conical tubes (15ml and 50ml)	Corning
Cell strainer (40µm and 70µm)	BD Bioscience
CellTris filter (30µm)	Partec
Cell culture plates (96/48/24/6 well)	Greiner Bio-one
Cell culture dish	Greiner Bio-one
Cell culture flasks (T25 and T75)	Corning
Syringes	BD Bioscience
Vacutainer blood collection tubes (Lithium Heparin)	BD Bioscience

2.1.5 Equipment

Allegra X-15R Centrifuge	Beckmann Coulter
Allegra X-22 Centrifuge	Beckmann Coulter
Centrifuge 5810R	Eppendorf
Microfuge16	Beckmann Coulter
Microfuge22R	Beckmann Coulter
Vacuum pump (BVC21)	Vacuubrand
Vortexer (Genie2)	Scientific Industries
CASY Cell counter	Innovartis
CO ₂ Incubator	New Brunswick
Water bath	GFL
Pipettes	Eppendorf
MasterCycler personal	Eppendorf
Thermomixer comfort	Eppendorf
Mini Shaker	VWR
Innova44	New Brunswick
ND-1000	NanoDrop Tech.

2.2 Cell culture methods

2.2.1 Preparation of human peripheral blood cells (PBMCs)

All experiments involving human material were approved by the Ethics committee of the Charité.

Tested buffy coats were obtained from DRK-Blutspendedienst Nord-Ost. Venous blood was collected in Vacutainer Lithium Heparin blood collection tubes (BD Bioscience) from healthy volunteers or SLE patients after informed consent.

If HLA information was required, 50µl of whole blood was diluted 1:1 with PBS/BSA and stained with HLA-A2 antibody for 15min at RT prior to erythrocyte lysis with 1ml Buffer EL (Quiagen) for 15min on ice. After washing with PBS/BSA, HLA status was assessed by flow cytometry.

For PBMC isolation, whole blood was diluted 1:1 with PBS/BSA and overlaid to 1/5th volume Ficoll (Biocoll separating solution, Biochrom). After centrifugation (20min, 800g, acceleration: 7, deceleration: 1) the white interphase was collected and washed with PBS/BSA (10min, 490g). After resuspending in PBS/BSA, cells were counted (CASY, Innovatis) and centrifuged (10min, 300g) before further processing.

Human cells were cultivated in RPMI (Gibco) supplemented with 10% AB Serum (Pan Biotec) and 1% Penicillin (100U/ml final)/Streptomycin (100µg/ml final, both Biochrom).

2.2.2 Preparation of human thymus

Human thymi were provided from Deutsches Herzzentrum Berlin (DHZB), Berlin after surgical removal during heart operation of children <12 months of age. Thymi were sliced into smaller pieces and reduced to single cell suspension by passing through a 70µm cell strainer using the plunger of a syringe while washing with PBS/BSA. After centrifugation and resuspension, the cells were passed through a 30µm filter before cultivation in complete human T cell medium or flow cytometric staining and analysis.

2.2.3 Isolation of murine cells

C57BL/6, MHCI KO and MHCII KO mice (on C57/BL6 background) were obtained from the Jackson Laboratory. OT-I mice were kindly provided by Thomas Blankenstein (MDC) and CD8⁺ specific H-Y (Mata Hari) mice from Il-Kang Na (MDC/Charité).

All mice were bred, housed and used at the Charité in accordance with the German law for animal protection with permission from the local veterinary offices and the LAGeSo (Landesamt für Gesundheit und Soziales), Berlin. Genotypes of transgenic animals were analyzed routinely by PCR or flow cytometry before use.

Mice were anesthetized with Isoflurane (AbbVie) prior to sacrificing by cervical dislocation. The fur was disinfected with ethanol followed by removal of the thymus and spleen and transfer into PBS/BSA. The organs were passed through a 70µm cell strainer using the plunger of a syringe to gain single cell suspensions. After centrifugation (5min, 350g) the supernatant was discarded and splenocytes were resuspended in 1ml ACK buffer (Gibco) for 3min at RT for the lysis of erythrocytes. Lysis was stopped with PBS/BSA followed by centrifugation (5min, 350g). For all further steps cells were passed through a 30µm filter and kept in PBS/BSA or cultivated in mouse medium containing RPMI 1640 (Gibco) supplemented with 10% fetal calf serum (FCS, heat inactivated 56°C, 30min, Lonza), Penicillin (100U/ml final)/ Streptomycin (100µg/ml final, both Biochrom) and 50µM β-Mercaptoethanol (Gibco).

2.2.4 Cell counting

Cells were counted by mixing a defined volume of cell suspension with appropriate electrolytic buffer (CASYton, Roche) and measurement at CASY (Roche/Innovartis). CASY works with a low voltage field applied to a pore that is filled with the cell suspension. Particles in the electrolyte solution cause a measurable increase in resistance dependent on their diameter used to distinguish cell numbers and debris. From given cell numbers, total cell counts were calculated.

2.2.5 Magnetic cell enrichment

For enrichment of defined populations prior to flow cytometric sorting or other applications supramagnetic nanoparticles bound to antibodies were applied (MACS, Miltenyi Biotech). Cells were labelled with appropriate MicroBead-conjugated antibodies 15min at RT. After washing with PBS/BSA (5min, 350g) cells were applied through a 30µm filter to a MACS column (Miltenyi Biotec) bound to a magnetic field. The ferromagnetic matrix of the columns binds MicroBead labeled cells while all unbound cells were washed away with PBS/BSA. After washing, the column was removed from the magnetic field and cells were eluted from the column with 5ml PBS/BSA. For up to 1×10^7 magnetic labelled or up to 2×10^8 total cells, MS columns were used. For larger quantities, LS columns were applied. MACS technology was utilized for the enrichment of CD4⁺ and CD8⁺ T cells respectively.

2.2.6 Cell stimulation

Characterization of cytokine profiles or CD40L expression as well as cell expansion required activation of the T cell cells. For maximal cytokine/CD40L readout, polyclonal stimulation was applied using chemical compounds phorbol 12-myristate 13-acetate (PMA, Sigma) and Ionomycin (Iono, Sigma) that mimic TCR- plus co-stimulation. Both activate the protein kinase C, a central player of intracellular TCR signaling. Furthermore, Ionomycin induces activation of NFAT by promoting influx of calcium into the cytosol. For stimulation, $1-2 \times 10^6$ cells/ml in medium were supplemented with 10ng/ml PMA and 1µg Iono for 6hrs at 37°C in the incubator (5% CO₂, >90% relative humidity).

More physiological stimulations as well as long term cultures were achieved with αCD3/αCD28 stimulations. 1µg/ml αCD3ε antibody (UCHT1, BD) and 3µg/ml αCD28 antibody (CD28.2, BD) (if not stated otherwise) were loaded onto 96 well plates (PS U plate, Greiner Bio-one) in PBS and incubated for 2hrs at 37°C or O/N at 4°C. Unbound antibodies were washed away with PBS. 1×10^5 cells in 100µl AB medium were stimulated per well for 6 hours (short term stimulation).

Human thymocytes were stimulated with a combination of superantigens Staphylococcus aureus enterotoxin B (SEB) and Toxic shock syndrome toxin 1 (TSST1). Superantigens bridge the binding of TCR and MHC in a peptide independent manner. Thymocytes were stimulated at 2×10^6 cells/ml with 1.5 $\mu\text{g/ml}$ SEB (Sigma-Aldrich) and 1 $\mu\text{g/ml}$ TSST1 (Sigma-Aldrich) for 6hrs at 37°C in the incubator (5% CO_2 , >90% relative humidity).

2.2.7 Cell cultivation (long term)

For CD40L stability experiments and T cell priming, long term culture was applied. Sorted human naïve/memory CD8^+ T cells or SP CD8^+ thymocytes were loaded at 5×10^4 - 1×10^5 cells/well in human complete culture medium supplemented with various cytokines (10ng/ml, see Table 2) into $\alpha\text{CD3}/\alpha\text{CD28}$ coated 96 well plates. After 2 days incubation at 37°C in the incubator, the cells were transferred into an uncoated 96 well plate and incubated for further 5 days with additional cytokine supplemented medium. If needed, cells were transferred into larger wells with higher volumina of medium. At day 7 cells were removed from the plates, counted and re-stimulated with PMA/Iono for 6hrs for CD40L/cytokine readout.

Cytokine	Manufacturer
IL-2 IS	Miltenyi
IL-7	Miltenyi
IL-12	Miltenyi
IL-15	Miltenyi
IL-18	MBL

Table 2: Cytokines and blocking antibodies used in long term cultivation

2.3 Flow cytometry and fluorescence-activated cell sorting (FACS)

Fluorescence based flow cytometry allows high throughput characterization of cells on single cell basis by fluorophores bound to cells. Those fluorophores can be coupled to specific antibodies against defined proteins, intercalate with DNA (DAPI/Propidium iodide) or bind to amine groups of cells (CFSE). An accordingly stained cell suspension is diluted with sheath fluid to single cell level while running through a laser beam. The forward scatter measures the distraction of the laser

beam and correlates with cell diameter while the side scatter detects cell granularity. The laser beam furthermore excites fluorophores bound to the cell. The emitted fluorescence signal is amplified and run through different dichroic filters to separate detectors allowing the measurement of several fluorophores at the same time.

The cells were measured with FACSCantoll (BD Bioscience) or LSRII (BD Bioscience) and analyzed with FlowJo software (Treestar). For cell sorting, a FACSriaII Machine (BD Bioscience) was used by the Flow Cytometry core facility of the BCRT.

Instrument	Laser	Detectors BP filters
FACSCantoll	405nm	450/50; 510/50
	488nm	488/10; 530/30; 585/42; 670LP; 780/60
	633nm	660/20; 780/60
LSRII	405nm	450/50; 525/50; 610/20; 710/50; 780/60
	488nm	488/10; 525/50; 685/35
	561nm	582/15; 610/20; 670/14; 710/50; 780/60
	640nm	660/13; 720/30; 780/60

Table 3: Configurations of the flow cytometers

2.4 Immunofluorescence staining

2.4.1 Surface staining

Cells were stained in 100ul PBS/BSA with fluorophore coupled antibodies plus Fc receptor antibodies to prevent unspecific binding for 10min at RT in the dark. For human cells, Beriglobin (1mg/ml, CSL Behring) was used as Fc block; mouse cells were supplemented with FcR antibody (2ug/ml, DRFZ). All antibodies were titrated to determine their optimal concentration prior to use. After staining, cells were washed with 4ml PBS/BSA to reduce background and spun down 350g, 5min. Prior to flow cytometric measurement, DAPI (0.4µM) or PI (0.4mg/ml) were added to detect dead cells.

For the surface detection of CD40L the stimulation was performed in the presence of antibodies against CD40 to prevent internalization of CD40L (Yellin et al., 1994).

Antibody	Clone	Final conc.	Manufacturer
α CD40 (human)	G28-5	3 μ g/ml	DRFZ
α CD40 (mouse)	FGK-45	3 μ g/ml	DRFZ

Table 4: Antibodies for surface detection of CD40L

2.4.2 Intracellular staining

For detection of secretory proteins such as cytokines Brefeldin A (10 μ g/ml final, Sigma) was added during stimulation to prevent a release from the Golgi complex. Detection of intracellular proteins requires fixation and permeabilization prior to staining. This was accomplished by washing with PBS after the stimulation followed by LiveDead™ staining (1 μ l/ml final, ThermoFisher) for 20min. During the last 10min, surface markers were added. After washing with PBS/BSA, 5min, 350g cells were fixated with 1ml 1:10 diluted in H₂O BD Lysing solution (BD Bioscience) for 10min at RT followed by centrifugation 5min, 350g. The supernatant was discarded and cells were resuspended in 500ul BD Permeabilization solution 2 (diluted 1:10 in H₂O, BD Bioscience) for 10min, RT. After washing with PBS/BSA, cells were stained intracellularly with appropriate antibodies for 30min at RT in the dark, washed and measured on a flow cytometer.

2.4.3 Intracellular staining of phosphorylated transcription factors

Cells were stimulated 13min with PMA/Ionomycin, washed at 350g, 5min and fixated with 16% PFA (paraformaldehyde, ThermoFischer) for 10min. After a second washing step, surface staining was applied for 30min prior to permeabilization with 600 μ l Methanol (Sigma) over night at -80°C. Staining of phosphorylated transcription factors was applied for 30min at RT prior to flow cytometric measurement.

2.4.4 Intranuclear staining

Detection of transcription factors was done separately with specific nuclear factor fixation and permeabilization solutions. Cells were stained with LiveDead™ and surface markers as indicated above followed by fixation with 1ml Nuclear Factor Fixation buffer (diluted 1:4 in PBS, Biolegend) for 20min at RT. Following washing with 1ml Nuclear Factor Permeabilization buffer (diluted 1:10 in PBS, Biolegend) the cells were incubated in 1ml Permeabilization buffer for 20min. After centrifugation and discarding the supernatant, the cells were stained for intranuclear factors 30min at RT in the dark prior to measurement.

Intranuclear FoxP3 stainings were conducted for 30min in 100µl FoxP3 Perm solution after treatment of the cells with 1ml 1x Biolegend FoxP3 Fix/Perm solution (diluted in PBS) for 20min, followed by a washing step with 1ml 1x FoxP3 Perm buffer (diluted in PBS, Biolegend) and further 15min incubation in 1ml FoxP3 Perm buffer.

2.4.6 Antibodies

Following antibodies were titrated for their optimal concentrations and used for cell labelling in human and mouse:

Human

Antigen	Clone	Manufacturer
CD3	OKT3/UCHT-1	Biolegend/House made
CD4	RPA-T4	Biolegend
CD8	RPA-T8	BD
CCR7	G43H7	Biolegend
CD45RA	HI-100	Biolegend
CD45RO	UCHL1	Biolegend
CCR4	L291H4	Biolegend
CCR6	G034E3	Biolegend
CCR10	6588-5	Biolegend
CXCR3	G025H7	Biolegend
CD40L	34-31	Biolegend
IFN- γ	B27	Biolegend
IL-2	MQ1-17H12	Biolegend
IL-4	MP4-25D2	Biolegend
IL-22	22URT1	eBioscience
IL-17A	BL168	Biolegend

SLAMF7	162.1	Biologend
Granzyme B	GB11	BD
Perforin	B-D48	abcam
CD1a	HI149	BD
Runx3	527327	R&D
ThPOK	11H11A14	Biologend
AHR	FF3399	eBioscience
GATA3	REA174	Miltenyi
NFκB p65	K10-895.12.50	BD
HLA-A2	BB7.2	Biologend
CD5	UCHT2	Biologend
CD69	FN50	Biologend
CD56	HCD56	Biologend
CD25	M-T271	BD
FoxP3	259D/C7	BD
CD11a	HI11	Biologend
CD95	DX2	Biologend
CD28	CD28.2	Biologend
CD103	Ber-ACT8	Miltenyi
CD57	HCD57	Biologend
CLA	HECA-452	Miltenyi

Table 5: Human antibodies

Mouse

Antigen	Clone	Manufacturer
CD3	145-2C11	Miltenyi
CD4	GK1.5	Miltenyi
CD8	53-6.7	Biologend
CD24	M1/69	Biologend
CD25	PC62	Biologend
CD44	IM7	Biologend
CD5	53-7.3	Biologend
CD40L	MR1	Miltenyi

Table 6: Mouse antibodies

2.5 Multiplex ELISA

For quantification of secreted cytokines, sorted CD8⁺ and CD4⁺ T cell subsets were cultivated at 1x10⁴ cells/100μl AB medium in αCD3/αCD28 coated 96 well plates for 24hrs or 72hrs. Pooled supernatants were snap frozen in liquid nitrogen (N₂) and stored at -80°C until further use. Multiplex ELISA (Q-Plex™ Array, Quansys) was performed with 50μl supernatant according to the manufacturer's protocol. Infrared emission was analyzed with Odyssey (LiCor) at various

intensities. Data analysis was performed with Q-view software (version 3.09, Quansys).

2.6 Keratinocyte Activation Assay

Cytokine supernatants of sorted T cells as described in Multiplex ELISA or medium alone were applied at 2% final concentration to primary human keratinocytes (CellSystems) cultured in KGM medium (Lonza) and incubated for further 24hrs. Cells were harvested, RNA isolated and activation assessed by RT-qPCR conducted by K. Wolk, Charité. Reverse transcription of mRNA was performed after preincubation of 25 µg/µl total RNA of each sample with 16 ng/µl oligo d(T)18 primer (ThermoFisher Scientific) at 75 °C for 5 min followed by cooling in an ice bath. 5U/µl M-MLV reverse transcriptase, first strand buffer, 10 mM DTT (all from ThermoFisher Scientific), 2U/µl of RNasin ribonuclease inhibitor (Promega) and 250 mM of ultrapure dNTP Set (Merck) were added and the reaction conducted at RT for 10min followed by 42°C step for 60min and a reaction stop at 95°C, 5min. Triplicates of each sample were analyzed by quantitative real-time PCR (StepOne plus, ThermoFisher scientific) using Maxima Probe/ROX qPCR Master Mix, ready-to-use detection assays for CCL26, CXCL11 and the house keeping gene HPRT containing double-labeled probes (all ThermoFisher Scientific). Gene expression was calculated relative to HPRT expression.

2.7 Quantitative real time PCR

Cells of interest were resuspended in 350µl lysis buffer RA1 (Macherey&Nagel) containing 1% β-Mercaptoethanol (Sigma), snap frozen in liquid N₂ and stored at -80°C until further use. RNA was isolated with NucleoSpin RNA Isolation kit (Macherey & Nagel) according to the manufacturers' protocol. The quality of total RNA isolates was quantified using a NanoDrop ND-100 spectrophotometer and normalized with qPCR grade H₂O. Reverse transcription of RNA to cDNA was performed with TaqMan reverse transcription kit (Applied Biosystems). 7.7µl RNA was mixed with 2µl TaqMan reaction buffer (10x), 4.4µl MgCl₂ (25mM), 4µl dNTPs, 0.5µl random hexamers, 0.5µl oligo (dT), 0.4µl RNase Inhibitor and 0.5

μ l reverse transcriptase to 20 μ l total volume. Reverse transcription was performed at 10min 25°C, 40min 48°C, 5min 95°C followed by cooling to 4°C.

For quantitative analysis of RNA expression levels TaqMan qPCR was performed with cDNA. TaqMan technology bases on probes that bind the cDNA between the primer binding sites. Probes are small stretches of complementary sequences with a fluorophore and a quencher bound to its opposite ends. The quencher suppresses fluorescence by FRET (Forester Resonance Energy Transfer) until amplification of the cDNA leads to dissociation and cleavage of the probe accompanied by a local separation of fluorophore and quencher resulting in detectable fluorescence. The additional need for binding to complementary sequences by the probe reduces possible off target effects compared to quantification with classical DNA intercalating fluorescence reagents such as SYBR Green. All primers were designed intron spanning to avoid amplification of genomic DNA and obtained from TIB MolBiol. Probes with FAM fluorophore and Dark quencher were obtained from Roche. Optimal concentrations of primers and probe were assessed prior to use. 5 μ l of cDNA was added to a total volume of 15 μ l qPCR mix containing 2 μ l 10x concentrated primer/probe mixture, 3 μ l qPCR grade H₂O (Roche) and 10 μ l LightCycler MasterMix (2x, Roche) into a 384 well plate (Roche). qPCR was run with a pre-incubation stage 5min, 95°C; 45 cycles of amplification with 10sec 95°C, 30sec 60°C, 1sec 72°C and a cooling phase for 10sec 40°C using a LightCycler® 480 II (Roche). All samples were run in duplicate and normalized to the housekeeping gene ACTB. Data analysis was performed with LightCycler® 480 Software (Version 1.5, Roche).

Primer	Sequence 5' -> 3'	Probe number (Roche)
ACTB fwd	ATTGGCAATGAGCGGTTC	11
ACTB rev	CGTGGATGCCACAGGACT	11
CD40L fwd	TCATGAAAACGATACAGAGATGC	2
CD40L rev	CTTCGTCTCCTCTTTGTTTAAACATT	2

Table 7: Primers and Probes used for quantitative real time PCR

2.8 RNA-sequencing

RNA was isolated from sorted cells with NucleoSpin RNA Isolation kit (Macherey & Nagel) according to the manufacturers' protocol. The quality of total RNA isolates was quantified using a NanoDrop ND-100 spectrophotometer and

integrity further analyzed using the Agilent RNA 6000 Nano kit with Agilent 2100 Bioanalyzer. Following RNA-sequencing was conducted by the BCRT Next Generation Sequencing Core Facility. In detail, Poly-(A)-selection was performed utilizing the NEBNext Poly(A)mRNA Magnetic Isolation Module (NEB) according to the manufacturers requirements. mRNA libraries were prepared with the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB). All libraries were analyzed with the Agilent DNA 1000 Kit and quantified using the Qubit® dsDNA BR Assay Kit (ThermoFisher). After equimolar pooling all samples were sequenced using an Illumina HiSeq 1500 system with High Output chemistry v4 (50 cycles, single-read). The RNA-sequencing data is deposited and accessible at NCBI GEO Expression Omnibus under: GSE115103.

Raw data were quality controlled by fastQC and reads were aligned to the GRCh37 (Ensembl) human genome using bowtie2 (Langmead and Salzberg, 2012). Reads were summarized per gene using the featureCount algorithm implemented in the R-package "Rsubread" and the further processing and analysis of the sequencing data were done in R (version 3.3.3). Raw counts of protein coding genes were normalized and transformed. The combat algorithm (package "sva") was used to remove variances in gene expression which were associated with the CD4-CD8 contrast only and donor specific differences (Leek et al., 2012). Therefore, CD4 or CD8 samples were combined with the donor id and used as batch definition. Top 1000 most variable expressed genes across all samples of either non-compensated or compensated data were used to generate heatmaps of unsupervised hierarchical clustering (euclidean distances of scaled data and complete linkage).

Principal component analysis (PCA): Normalized and compensated data for all genes were scaled and subjected to a principle component analysis using singular value decomposition. The first two principle components were shown in the plot. Signature genes distinguishing the cell subsets were identified by fitting generalized linear multinomial models to the normalized data via penalized maximum likelihood (package "glmnet"). RNA-seq data for dermal and epidermal T cell subsets were obtained from GEO database (GSE83637). Raw counts were combined with raw expression data from this study, normalized and variance stabilized transformed (package "DESeq2"). In order to remove technical differences between the two studies, a batch compensation using the donor ids

as batch definition was done (package "sva"). Top 1000 most variable expressed genes across all samples from this set were used in a principle component analysis. PC1 and PC2 were displayed in the plot. Overrepresentation of genes related to specific GO terms were analyzed using the topGO package with all protein-coding genes as background.

2.9 TCR-sequencing

Analyses of TCR repertoires was performed by next generation sequencing of CDR3 beta chain as described previously (Bacher et al., 2016; Dziubianau et al., 2013). Genomic DNA was isolated from FACS sorted Tc subsets using AllPrep DNA Micro Kit (Qiagen) followed by recombined TCR- β locus amplification with Multiplex PCR Kit (Quiagen) and sequencing with the Illumina MiSeq System. The primary analysis of raw sequencing data including subsequent clone grouping and clonotype generation was performed as previously described using the free open-source clonotyping IMSEQ analytic platform (Kuchenbecker et al., 2015). Reads with an average quality score below 30 were excluded from the analysis.

Shared clonotypes and shared reads were calculated as in (Becattini et al., 2015). Shared clonotypes were assessed using the Jaccard index defined as: $(\text{shared clonotypes of 1 and 2}) / (\text{total clonotypes 1} + \text{total clonotypes 2} - \text{shared clonotypes of 1 and 2})$. To calculate shared reads, the frequency of shared clonotypes within total reads was assessed from population 1 with population 2 and other way round. Mean values were plotted.

2.10 TREC quantification

TREC numbers were quantified by C.Schmidt, Universität Greifswald using a TaqMan qPCR with an ABI PRISM 7700 Sequence Detector Taqman (Thermo Fisher) as described in (Kimmig et al., 2002). In brief, qPCR was performed with 100ng genomic DNA, using a serial dilution of an RAG2 and δ Rec- ϕ J α signal joint (TREC) expressing vector as standard curve. DNA, 10nM dNTP (PerkinElmer), PCR buffer including 4.5mM MgCl₂ (Life Technologies), 1.25 U PlatinumTaq polymerase (Life Technologies), 25 pmol of primers and 5pmol 6FAM-TAMRA probe were used in 50 μ l total volume for amplification. After initial denaturation

step at 95°C for 5min, 45 cycles of 95°C, 30sec and 66°C, 30sec were performed. For TREC analysis the primers ϕ J α (-258): AAC AGC CTT TGG GAC ACT ATC G, δ Recsj (+104): AAC AGC CTT TGG GAC ACT ATC G and the probe: 6FAM-CCA CAT CCC TTT CAA CCA TGC TGA TGA CAC CTC T-TAMRA were used. For RAG2 analysis the primers RAG2 (2160) GCA ACA TGG GAA ATG GAA CTG, RAG2 (2404) GGT GTC AAA TTC ATC ATC ACC ATC and the probe 6FAM-CCC CTG GAT CTT CTG TTG ATG TTT GAC TGT TTG TGA-TAMRA were utilized.

2.11 DNA Methylation analysis

Genomic DNA was isolated with the DNeasy tissue kit (Quiagen) according to the manufacturers protocol and applicated to bisulfite treatment by Epiontis as described in (Olek et al., 1996). Sodium bisulphite treatment leads to conversion of unmethylated cytosines into uracil that is detected as thymine in following PCR whereas methylated cytosines remain unchanged. The PCR products were purified with ExoSAP-IT (USB Corp.) and sequenced using the ABI Big Dye Terminator v 1.1 chemistry (Applied Biosystems) followed by capillary electrophoresis on an ABI 3100 genetic analyzer. The data was interpreted with ESME (Lewin et al., 2004).

2.12 Statistics

Statistical analysis was conducted with Prism (Version 7, GraphPad Software) as specified in text and figure legends. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3. RESULTS

3.1 TCR dependent induction of CD40L gene expression

3.1.1 NFAT but not NF- κ B is essential for CD40L expression

It is not understood why all CD4⁺ but only some CD8⁺ T cells are capable to express CD40L upon T cell activation. One possibility is a difference in CD40L gene expression induction between CD40L⁻ and CD40L⁺ T cells. Gene expression is regulated by a vast interplay of transcription factors that bind to regulatory elements within and around gene loci and control induction as well as repression of the gene. CD40L expression in CD4⁺ T cells is induced by TCR dependent T cell activation, which has been shown to be critically dependent on the transcription factors NFAT and NF- κ B (Brunner et al., 2008; Lobo et al., 2000; Schubert et al., 1995). TCR ligation activates an intracellular signaling cascade resulting into a calcium (Ca²⁺) flux into the cell (Figure 9).

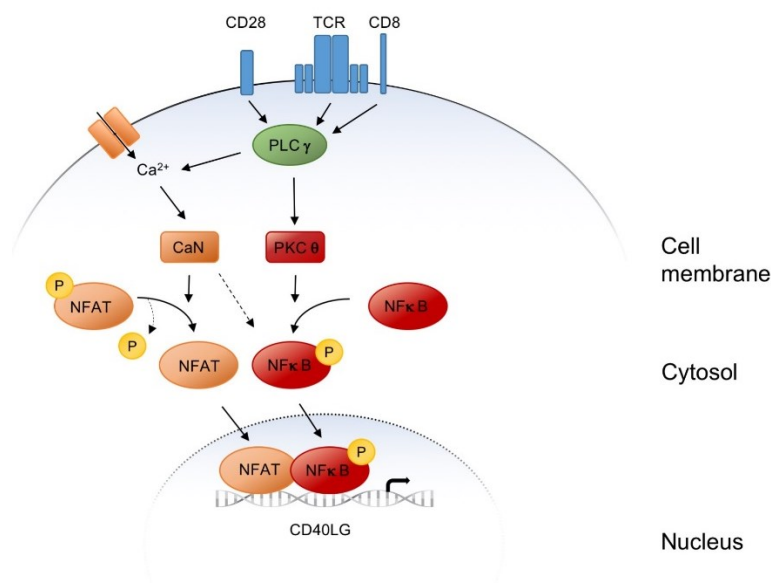


Figure 9: T cell activation is translated into de-phosphorylation (P) of NFAT and phosphorylation of NF- κ B by intracellular signaling cascades allowing the translocation into the nucleus and induction of CD40L expression.

This cascade can be supported by a parallel activation of the T cell co-receptor CD28 (Andres et al., 2004). Intracellular calcium forms a complex with calmodulin which in turn activates calcineurin (CaN). The active form of calcineurin dephosphorylates cytosolic NFAT leading to the translocation of NFAT into the

nucleus and subsequent binding to *CD40LG* locus (Brunner et al., 2008; Lobo et al., 2000; Macian, 2005; Schubert et al., 1995).

In a first step, we assessed whether CD8⁺ T cells utilize the same mechanisms of CD40L gene expression induction as known from CD4⁺ T cells. We analyzed the role of NFAT for CD40L induction in CD8⁺ T cells by an inhibition of the calcineurin phosphatase activity with Cyclosporine A (CsA) (Clipstone and Crabtree, 1992; Mattila et al., 1990). CsA was shown to be an efficient inhibitor of CD40L in CD3⁺ T cells (Fuleihan et al., 1994). By subdividing the CD3⁺ fraction, we could show that CsA efficiently abolishes CD40L expression in polyclonally activated CD8⁺ T cells at concentrations of 12.5 nM comparable to CD4⁺ T cells (Figure 10A). Strong inhibitory effects of CsA were also observed for IFN- γ and IL-2 production.

NF- κ B, the second central TF in the induction of CD40L expression is also activated by TCR signaling and binds to the *CD40LG* promotor and the downstream enhancer as p65 homo- or p50/p65 heterodimer (Schubert et al., 2002; Srahna et al., 2001). In resting cells, bound I κ B kinase keeps NF- κ B dimers in the cytosol. Following TCR activation, phosphorylation of the I κ B kinase releases NF- κ B to translocate into the nucleus (Karin, 1999). As CsA is shown to affect not only NFAT but also NF- κ B, we used ML120B to assess the effect of NF- κ B specific inhibition on CD40L expression (Nishiyama et al., 2005; Srahna et al., 2001). ML120B is a potent and highly selective I κ B Kinase β -inhibitor that exhibit an IC₅₀ (half maximal inhibitory concentration) value of 3.4 μ M assessed by IL-2 inhibition in activated human PBMCs (Nagashima et al., 2006; Wen et al., 2006). Despite we observe reduced levels of IL-2 and efficient block of p65 phosphorylation, ML120B treatment did not affected CD40L expression in activated CD4⁺ or CD8⁺ T cells (Figure 10B, C). In order to exclude possible non-physiological effects of the strong, chemical stimulus with PMA and Ionomycin, the inhibition experiments were repeated with plate bound α CD3/ α CD28, resulting in the same result (not shown).

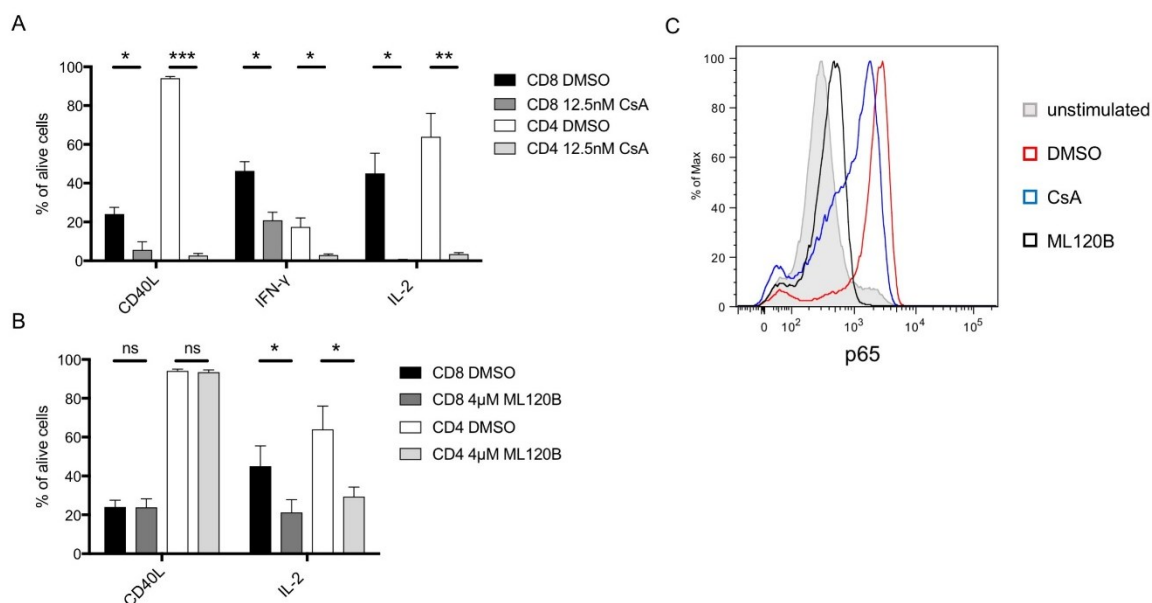


Figure 10: Role of NFAT and NF- κ B in the induction of CD40L expression in CD4⁺ and CD8⁺ T cells.

CD4⁺ and CD8⁺ T cells were enriched from human PBMCs by MACS. Cells were treated with 12.5nM CsA (**A**), 4 μ M ML120B (**B**) or according concentrations of DMSO for 45min prior to polyclonal stimulation with PMA/Iono for 6hrs in the presence of BrefA. Cytokines were stained intracellularly and measured on a flow cytometer. Data is summarized from three independent experiments. Mean \pm SEM, Student's t-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. (**C**) Enriched CD8⁺ T cells were stimulated with PMA/Iono for 13 min prior to fixation followed intranuclear staining for phosphorylated p65 NF- κ B subunit and measurement on a flow cytometer.

In summary, the induction of CD40L by TCR stimulation via the activation and subsequent binding of NFAT to the *CD40LG* locus is equally important in CD4⁺ as well as CD8⁺ T cells, whereas the contribution of NF- κ B is neglectable in both cell types.

3.1.2 Identification of potential regulatory elements at the *CD40LG* locus

While NFAT is indispensable for CD40L induction in CD4⁺ as well as CD8⁺ T cells, it remains unclear why the majority of CD8⁺ T cells is incapable to express CD40L. Another mechanism of gene expression regulation are epigenetic modulations including DNA methylation and histone modifications. Interestingly, aberrant DNA demethylation of the *CD40LG* promotor accompanied by deregulated CD40L expression was observed in several autoimmune disorders (Belkhir et al., 2014; Lian et al., 2012; Liao et al., 2012; Lleo et al., 2012; Lu et

al., 2007). Hence, we hypothesized that DNA methylation might be a central regulator of CD40L expression in T cells. DNA methylation describes the active addition of a methyl group to a cytosine within a CpG element by DNA methyltransferases (DNMTs). Cytosine methylation affects the binding sites of transcription factors and generally results in a blockage of gene expression (Zhu et al., 2016).

So far, the DNA methylation analyses of the *CD40LG* locus were limited to the promotor of CD4⁺ T cells and from transcription factor analyses two enhancers were described as additional regulatory elements of CD40L expression. However, all CD4⁺ T cells are homogenously capable of expressing CD40L in contrast to CD8⁺ T cells. Therefore, we cannot exclude the possibility that CD8⁺ T cells use other/additional regulatory elements. In order to identify possible further regulatory elements, we performed an extensive *in silico* comparative sequence analysis stretching +/- 100kb around the *CD40LG* gene, which would include possible long distance enhancers (Figure 11) (Chepelev et al., 2012). Orthologous sequences of human and mouse were aligned and coding as well as non-coding sequences with a homology >75% identified in mVISTA (Mayor et al., 2000). Next, we scanned the identified homologous sequences for conserved transcription factor binding sites with a focus on those transcription factors known to regulate CD40L expression (see 1.3.1) in rVISTA (Loots et al., 2002). As sites of gene expression and gene regulation furthermore exhibit an uncoiled, open chromatin formation, they are more susceptible to DNase I dependent digestion (Crawford et al., 2006). We utilized published data of DNase I hypersensitive sites gained from CD40L expressing cells such as Th1 CD4⁺ cells (GEO: GSM1008604), EBV transformed lymphoblastoid cell line (Cell line GM12878, GEO: GSM816665) and Jurkat cells (Clone E61, GEO: GSM736501) and overlaid them with the TF binding sites (Boyle et al., 2008; Imadome et al., 2003).

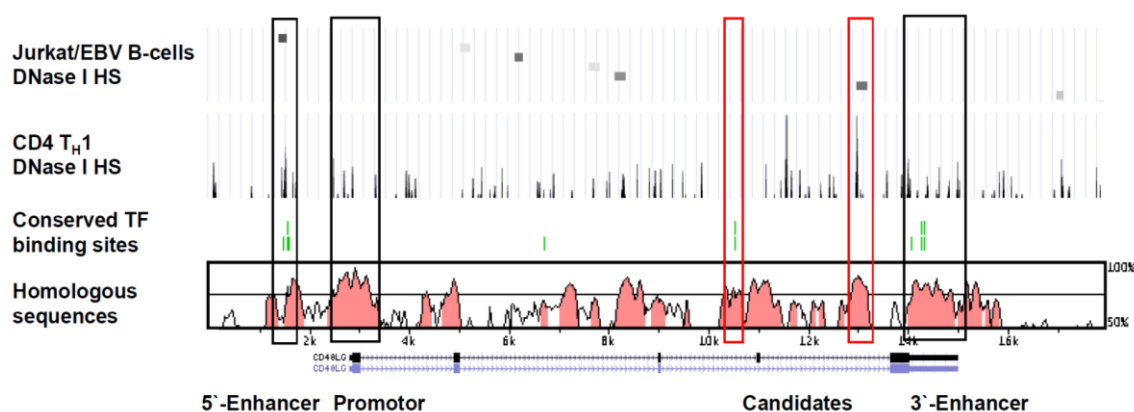


Figure 11: Identification strategy of potential regulatory elements within and around the *CD40LG* locus.

Potential regulatory elements of the *CD40LG* locus were identified by an overlay of high homologous sequences between human and mouse (mVISTA) with transcription factor binding sites (rVISTA) and DNase I hypersensitive sites from CD40L expressing CD4 T_H1, Jurkat and EBV transformed B-cells. Besides the known regulatory elements promotor, 3'-enhancer and 5'-enhancer (black boxes), two intragenic candidates at -8kb and -10kb (red boxes) are shown.

The combination of these approaches identified two intragenic, intronic (at -8kb and -10kb) (Figure 11) and four extragenic sequences (at +35kb, +17kb, -20kb and -27kb) (not shown) as potential regulatory elements beyond the published promotor and two enhancers. These regions were finally assessed for the presence of CpG sequences before they were included in the DNA methylation analysis.

3.1.3 CD40L expression correlates with DNA demethylation

DNA methylation and demethylation are transient processes in T cell maturation, differentiation and division (Berkley et al., 2013; Thomas et al., 2012; Tsagaratou et al., 2017). Therefore, we separated polyclonally activated CD40L⁺ or CD40L⁻ peripheral CD3⁺CD8⁺ T cells into the developmental stages naïve (N, CD45RA⁺CCR7⁺), effector (E, CD45RA⁺CCR7⁻), effector memory (EM, CD45RA⁻CCR7⁻) and central memory (CM, CD45RA⁻CCR7⁺) prior to bisulphite sequencing. CD3⁺CD4⁺ T cells and CD3⁻CD19⁺ B cells were included as respective CD40L⁺ or CD40L⁻ control. We observed demethylation at the promotor, both enhancers and the -8kb intragenic region in CD40L⁺CD4⁺ T cells, while all those regions except of the 5'-enhancer were methylated in CD40L⁻ B cells. In CD8⁺ T cells, independent of their maturation level (N, EM, CM, E), the

methylation data exhibit a clear correlation between methylation status and CD40L expression ability at the promotor. In line with the observations from CD4⁺ T cells and B cells, the 5'-enhancer is also demethylated in a CD40L independent manner in CD8⁺ T cells (Figure 12). In contrast, the 3'-enhancer is only demethylated in CD4⁺ but not CD8⁺ T cells. One intragenic region at -8kb exhibit a diverse methylation status in CD40L⁺ versus CD40L⁻ CD8⁺ N, EM and CM T cells similar to the promotor but however also CD40L⁻ effector CD8⁺ T cells are demethylated in this area. The further candidates, the 4 extragenic regulatory elements and one intragenic candidate did not show a correlation with CD40L expression in the initial experiment and were excluded from further analyses. Altogether, DNA-methylation seem to regulate CD40L expression ability by different methylation solely at the promotor whereas the 5'- enhancer is continuously accessible and the 3'-enhancer is CD4⁺ but not CD8⁺ T cell specific. For the novel identified -8kb intragenic region an impact on CD40L expression ability is possible but would require another mechanism to silence CD40L in effector compared to CD40L⁻ N, EM and CM CD8⁺ T cells.

	CD4 ⁺ N CD40L ⁺	CD8 ⁺ N CD40L ⁻	CD8 ⁺ N CD40L ⁺	CD8 ⁺ CM CD40L ⁻	CD8 ⁺ CM CD40L ⁺	CD8 ⁺ EM CD40L ⁻	CD8 ⁺ EM CD40L ⁺	CD8 ⁺ E CD40L ⁻	B cells CD40L ⁻
PROMOTOR	1	56	5,5	94,5	1	84,5	0,25	92	58,5
	5,5	60	4	78,5	0	81	0	83,5	59,75
	11,5	91,25	20	99	0	90,25	9,75	100	100
	0	96,75	82,25	100	0	89,75	0	100	87
	0	100	87,25	99,25	0	90,25	0	100	100
	1	100	95	97	0	90,5	0	100	100
5'-ENHANCER	0,5	0,5	0,5	26	2	23	3,5	45	0
	0,25	7,5	0	15,5	1,75	23,5	2	33	69,5
INTRAGENIC REGION	13	100		89	4	95	23	0	100
	57	100	0	100	12	100	63	0	100
	58		0	88	8		51	0	
3'-ENHANCER	38	100	100	96	82	83,5	68,75	94,75	100
	24	96,5	98	95,75	96	95,25	74,5	93,5	91
	32,5	96,75	92,5	94,75	93,5	95	64,5	84,25	91
	9,75	91,25	94	95	90	95,5	54,75	90,75	94,25
	4,75	89	91,5	96,25	81,5	96	50	93,25	92,5
	15,25	100	96,25	98,5	97	97,5	75,5	100	96,75
	17	93	94,5	91	82,25	92,25	69,25	90,5	94,5
	3,5	92,25	98	94	91	96,25	70,25	96,75	95,25
	10,5	89,5	88	91,75	73,5	97	43,75	93,25	94,25
	13,5	100	100	99	85	93,5	56,5	99	97,5
18,5	98	100	100	100	84	100	52	97	87

Figure 12: DNA methylation profile of peripheral cells.

Naïve (CD45RA⁺CCR7⁺), effector (CD45RA⁺CCR7⁻), effector memory (CD45RA⁻CCR7⁻) and central memory (CD45RA⁻CCR7⁺) CD3⁺CD8⁺ T cells as well as CD3⁺CD4⁺ T cells and CD3⁻CD19⁺ B cells were sorted from human PBMCs. After 6hrs polyclonal stimulation with PMA/Iono in the presence of αCD40, CD8⁺ T cells were separated into CD40L⁺ and CD40L⁻ subsets. Following DNA isolation, bisulphite sequencing was performed. Methylation level shown as heat map with blue = full methylation, yellow = no methylation, white = no data available. n=2.

3.1.4 DNA demethylation of the CD40L promotor is activation independent

Polyclonal activation that was required to identify the CD40L expressing T cells among N, CM, EM and E CD8⁺ T cells can alter DNA methylation pattern as rapid as 5 hours following stimulation (Kersh et al., 2006). In order to exclude that stimulation dependent changes caused the differences in the methylation pattern, we screened the CD8⁺ T cell compartment for markers that identifies CD40L expressing cells *ex vivo*.

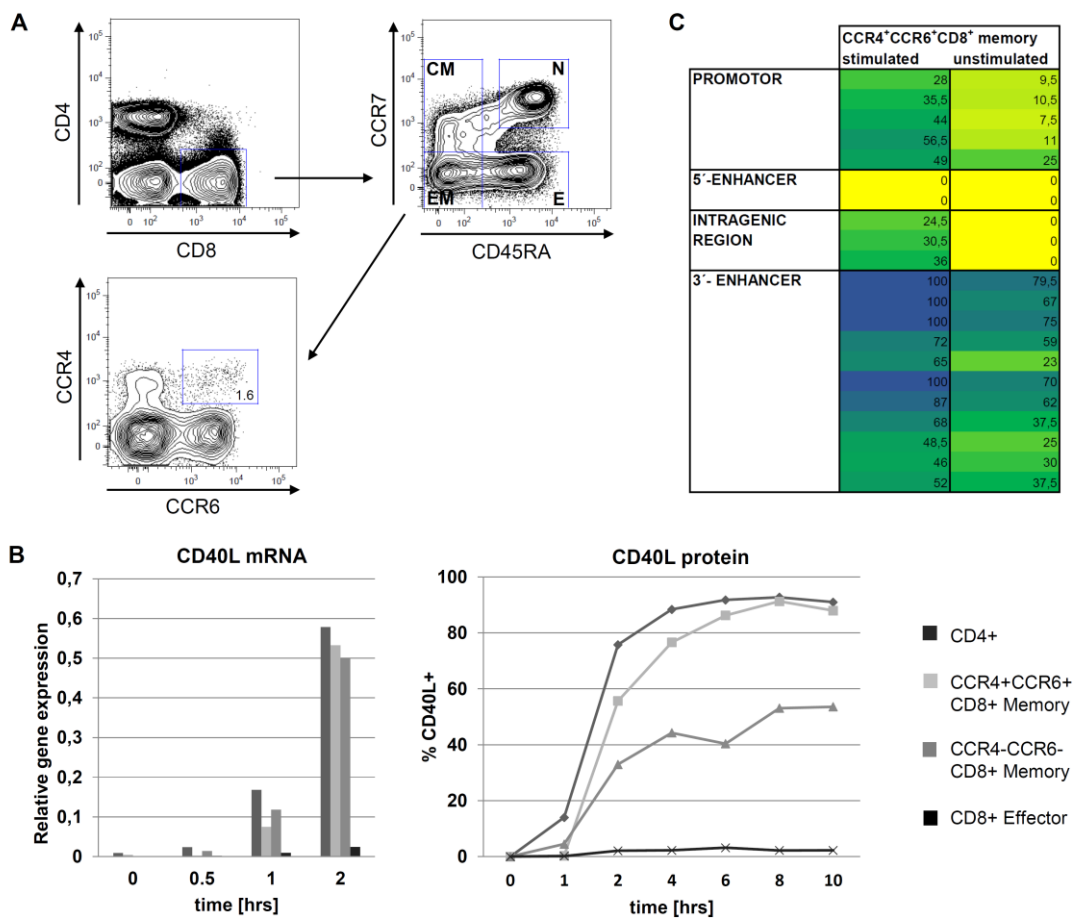


Figure 13: DNA methylation imprinting is stimulation independent.

(A) Example gating strategy: CD8⁺ T cells were distinguished into CCR7⁺CD45RA⁺ naïve (N), CCR7⁻CD45RA⁺ effector (E) and CD45RA⁻ memory cells consisting of CCR7⁺ central memory (CM) and CCR7⁻ effector memory cells (EM). Within total CD45RA⁻ memory cells, 1-2% coexpress CCR6⁺ CCR4⁺. (B,C) CD40L expression of CCR6⁺CCR4⁺ memory CD8⁺ T cells compared to CCR6⁻CCR4⁻ memory CD8⁺ T cells, CD8⁺ effector T cells and CD4⁺ T cells after polyclonal stimulation with PMA/Iono for indicated time. (B) CD40L mRNA analyzed by qPCR, (C) CD40L surface staining in the presence of αCD40 analyzed on a flow cytometer. (D) CD8⁺ T cells were enriched from human PBMCs by MACS and CD45RA⁻CCR6⁺CCR4⁺ CD8⁺ T cells sorted. One half was stimulated polyclonally with PMA/Iono for 6hrs and used with the unstimulated control for bisulphite sequencing.

We found a CD45RA⁻ memory CD8⁺ T cell fraction expressing the chemokine receptors CCR6 and CCR4 that homogenously expressed high levels of CD40L mRNA and protein upon stimulation (Figure 13A, B). The DNA methylation pattern of the regulatory regions of this subset was compared prior and after polyclonal activation. We observed no stimulation dependent demethylation but instead rather a slight increase in methylation for the promotor, the -8kb intragenic region and the 3'-enhancer while the 5'-enhancer retained its demethylated conformation. Therefore, we conclude that DNA demethylation of regulatory elements is an intrinsic and critical regulator of CD40L expression in CD8⁺ T cells, which is implemented in a stimulation independent manner (Figure 13C).

3.2 The CD40L expression ability of T cells is implemented in the thymus

3.2.1 Naïve CD40L expressing CD8⁺ T cells are true naïve cells

The DNA methylation pattern can be altered by the inhibition of the *de novo* methylation after cell division or in an active process by TET enzymes (Smith and Meissner, 2013; Wu and Zhang, 2014). The clear difference between the CD40L⁺ and CD40L⁻ naïve CD8⁺ T cells in DNA methylation pattern therefore raises the question whether the CD40L⁺ T cells are truly naïve cells or antigen experienced cells with a naïve phenotype. A fraction of memory cells, T memory stem cells (T_{SCM}) and memory T cells with naïve phenotype (T_{MNP}), can express the naïve cell markers CD45RA and CCR7 we used for our experiments (Gattinoni et al., 2011; Pulko et al., 2016). Therefore, we wanted to exclude the possibility that naïve CD40L⁺ CD8⁺ T cells from our DNA methylation experiment had already encountered their cognate antigen. First, we controlled the gating strategy by co-staining of CD45RA and CCR7 with CD95 and CD11a, markers that are shared by T_{SCM} and T_{MNP} cells. Only 2-3% of the CD8⁺ T cells in the CD45RA⁺CCR7⁺ compartment co-expressed CD95 and CD11a and therefore were lower in their frequency than CD40L⁺ CD8⁺ T cells in this compartment that account for around 10% (Figure 14A). Furthermore, we assessed the number of T cell receptor excision circles (TRECs) in sorted CD45RA⁺CCR7⁺ naïve CD8⁺ T cells that were either CD40L⁺ or CD40L⁻ and compared it to respective CD45RA⁻ memory CD8⁺

T cells. T cell receptor excision circles are formed during TCR rearrangement of T cells in the thymus but are not duplicated during cell division and therefore dilute out with increasing cell division. As the TREC number was comparable among the two naïve subsets and hardly detectable in the memory cells (Figure 14B), we conclude that CD40L⁺ naïve CD8⁺ T cells are not antigen experienced cells that differentiated into the CD40L⁺ phenotype upon antigen encounter. Instead, CD40L⁺ naïve CD8⁺ T cells must have their DNA methylation pattern and CD40L expression ability imprinted at earlier stages of development. Moreover, the slightly reduced number of TRECs in naïve CD40L⁻ cells might be caused by a relative higher frequency of T_{SCM} or T_{MNP} cells among them.

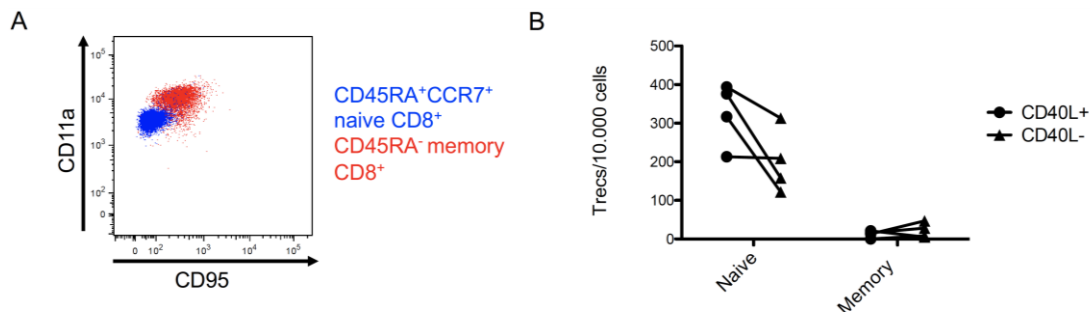


Figure 14: Naïve CD40L⁺ CD8⁺ T cells are not antigen experienced.

(A) CD8⁺ T cells were enriched from human PBMC by MACS and CD95 and CD11a expression on naïve CD45RA⁺CCR7⁺ versus memory CD45RA⁻ CD8⁺ T cells assessed by flow cytometry. **(B)** Following stimulation with PMA/Iono in the presence of α CD40, CD40L⁺ and CD40L⁻ naïve (CD45RA⁺CCR7⁺) and memory (CD45RA⁻) CD8⁺ T cells were sorted. DNA was isolated and TRECs quantified by qPCR. n = 4

3.2.2 CD40L expression ability of CD8⁺ T cells is implemented in the thymus

As already naïve CD8⁺ T cells could be distinguished into CD40L⁺ and CD40L⁻ fractions by their DNA methylation pattern, the DNA demethylation imprinting probably takes place at an earlier time point in T cell development. Therefore, we assessed whether precursors of naïve cells in thymus are already able to express CD40L. T cell precursors wander from the bone marrow into the thymus. Here they undergo several developmental stages from DN1-4 over DP while rearranging their TCR. After positive selection validated TCR functionality and negative selection depleted strong autoreactive cells, the remaining cells mature into CD4⁺ or CD8⁺ SP cells that egress into the blood as naïve T cell (see 1.2.2).

Those developmental stages can be dissected by the expression of CD1a and CD3 in human thymocytes. CD1a is expressed from DN1 stage until successful TCR rearrangement occurred, positive and negative selection was passed and CD3 expression is induced at DP stage (Figure 15A) (Weerkamp et al., 2006). We observed that CD40L expression accompanies CD3 induction at DP stage and continues in CD4⁺ and CD8⁺ SP stage (Figure 15B). While almost all CD4⁺ SP thymocytes express CD40L upon stimulation, an average of 5% of CD8⁺ SP thymocytes are CD40L⁺ (Figure 15C, D).

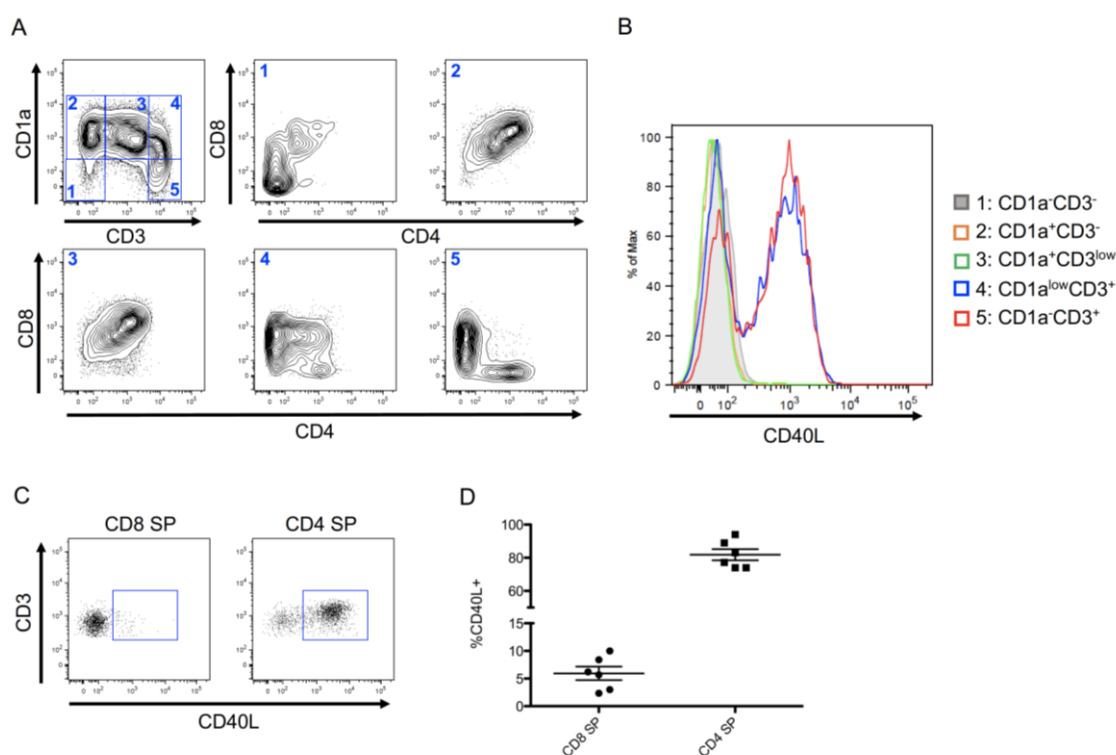


Figure 15: CD40L expression among human thymocytes.

(A) Developmental stages in thymus from CD4⁻CD8⁻CD1a⁻CD3⁻DN (1) CD4⁻CD8⁻CD1a⁺CD3⁻ early DP (2), CD4⁺CD8⁺CD1a^{+/low}CD3^{low/+} DP (3 and 4) to CD4⁺ or CD8⁺ CD1a⁻CD3⁺ SP (5) according to *ex vivo* CD1a and CD3 expression. **(B)** CD40L expression in different thymic developmental stages as shown in (A) stimulated 6hrs with SEB/TSST-1 in the presence of α CD40. **(C)** Example dot plot of CD40L frequencies in CD8⁺ and CD4⁺ SP thymocytes stimulated 6hrs with SEB/TSST-1 in the presence of α CD40. **(D)** Summarized frequencies of CD40L⁺ T cells among indicated CD1a⁻ CD3⁺ CD4⁺ or CD8⁺ SP cells. Mean \pm SEM.

In murine thymus, T cell development stages can be dissected by the expression of CD3, CD24 and CD25. While CD25 is exclusively expressed in DN stage, CD24 expression declines with proceeding maturation of the T cells (Baldwin et al., 2005). Positive selection induces expression of CD3 in DP cells that now further mature to CD4⁺ or CD8⁺ SP cells. Co-staining of CD40L revealed

induction of CD40L expression at the DP to SP transition accompanied by CD3 expression in line with our observations in human thymus (Figure 16A). At CD3⁺ SP stage, around 3-4% of murine CD8⁺ thymocytes were expressing CD40L (Figure 16B, C). Taken together in human and mouse the ability to express CD40L is implemented during DP stage in CD4⁺ and some CD8⁺ T cells.

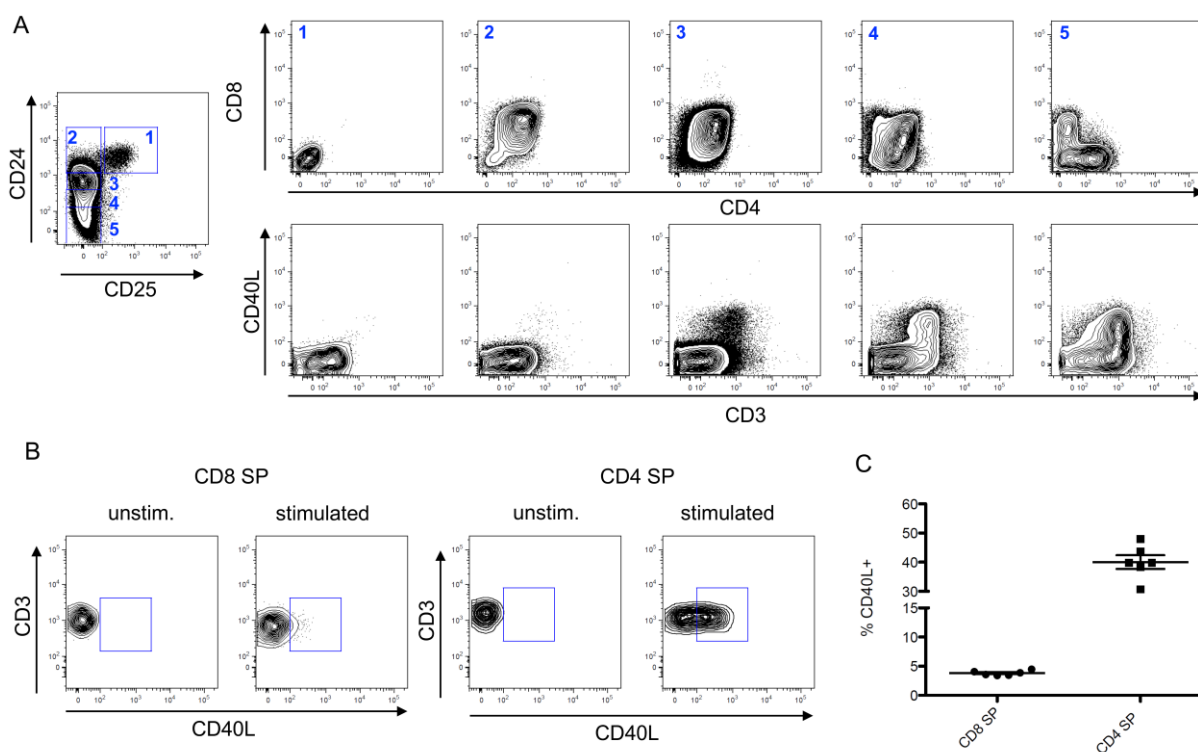


Figure 16: CD40L expression in murine thymocytes.

(A) T cell developmental stages in murine thymus from CD25⁺CD24⁺⁺ DN (1), CD25⁺CD24⁺⁺ early DP (2), CD25⁺CD24⁺ DP (3), CD25⁺CD24^{low} DP to SP transition to CD25⁻CD24⁻ SP stage. (B) Expression of CD40L in CD3⁺CD24⁻ CD8⁺ SP or CD4⁺ SP thymocytes after stimulation with PMA/Iono for 6hrs in the presence of BrefA. (C) Summarized frequencies of CD40L⁺ cells among CD4⁺ or CD8⁺ SP cells. Mean \pm SEM. n=6.

3.2.3 The DNA methylation pattern of the *CD40LG* locus is imprinted in the thymus

In order to understand whether CD40L expression ability from DP stage onwards is reflected by subsequent changes in the DNA methylation, we assessed *CD40LG* locus methylation in the different stages of T cell development. DN, DP, CD4⁺ SP and CD8⁺ SP were separated into CD40L⁻ or CD40L⁺ fractions after polyclonal stimulation and analyzed by bisulphite sequencing. Methylation analysis revealed that the detection of CD40L protein on the surface is accompanied by a subsequent demethylation of the *CD40LG* promoter beginning

with DP and established at SP stage (Figure 17). Moreover, the 5'-enhancer and the intragenic region became demethylated during thymic T cell maturation. The 3'-enhancer displayed a beginning demethylation only in CD4⁺ SP T cells in line with data gathered from circulating T cells where demethylation was CD4⁺ T cell exclusive and complete (see Figure 12). Therefore, the ability to express CD40L by some CD8⁺ (and all CD4⁺) T cells is regulated by DNA methylation status of the *CD40LG* promotor which is implemented in the thymus.

	DN CD40L-	DP CD40L-	DP CD40L+	CD8 ⁺ SP CD40L-	CD8 ⁺ SP CD40L+	CD4 ⁺ SP CD40L-	CD4 ⁺ SP CD40L+
PROMOTOR	50,75	81	14,5	10,5	3,25	0,5	0
	46,5	58,75	39,5	17	1,5	24,75	4,75
	77,5	97	63,25	45,5	4,5	42,75	4,5
	77,5	94	88,5	87	52	39,75	4
	82,25	98,75	89,25	95	67,5	56	0
		98,5	94	100	100	97	0
5'-ENHANCER	75	85,5	28,5	11,5	4,5	47	0
	73,75	84	65	7,75	2,5	44,25	0
INTRAGENIC REGION	100	100	70	52	0	75	0
	84	77	77,5	54,5	14,5	72,5	0
	84	79	86,5	53	22,5	57	0
3'-ENHANCER	97	93	88	97	75	90	44
	95,75	96,5	97,25	97,5	93	95,25	69,25
	96	94,5	89,5	92	94,75	93,75	94
	84,25	96	96,5	92,25	95	94	39
	91,25	93,5	92	85,75	89,75	79,5	23,75
	100	100	100	100	98	94,25	53,75
	93,75	94,5	94,5	91,75	92,25	87,25	44,5
	82	96,5	96,25	92,5	88	75,75	39,25
	82,5	90,5	95,25	75	77,5	78	25,25
	83,5	99,5	86	77	59,25	92	38
	90,5	100	97	90	88,5	100	81,5

Figure 17: Demethylation of the *CD40LG* locus is imprinted in the thymus.

Thymocytes were sorted into DN (CD1a⁺CD3⁻CD4⁻CD8⁻), DP (CD3^{int}CD4⁺CD8⁺) and SP (CD3⁺CD4⁺ or CD3⁺CD8⁺) subsets and stimulated polyclonally with SEB/TSST-1 for 6hrs in the presence of αCD40. After separation of CD40L⁺ from CD40L⁻ cells by sort, bisulphite sequencing was conducted. n=2.

3.3 The CD40L expression stability

3.3.1 Stability of CD40L expression correlates with the DNA methylation status

Our data suggest an imprinting of CD40L expression ability during thymic development by DNA demethylation of regulatory elements of the *CD40LG* locus. However, previous work also revealed an induction of CD40L expression in naïve cells upon stimulation and cultivation in the presence of IL-12 resulting in CD40L⁺ CD8⁺ T cells with cytotoxic phenotype including co-expression of IFN-γ as well as lytic molecules (Stark et al., 2013). In order to understand the interplay of these

two mechanisms, we analyzed the stability of CD40L expression at different stages of T cell development in the presence and absence of cytokines, especially IL-12. To this purpose, isolated CD8⁺ SP thymocytes as well as naïve and memory CD8⁺ T cells were stimulated polyclonally to separate CD40L⁺ from CD40L⁻ cells and cultivated in the presence of cytokines. In all three cell types, only a minority of sorted CD40L⁻ cells expressed CD40L after cultivation, which most likely results from incomplete sort due to a delayed CD40L expression kinetic during activation. Among the sorted CD40L⁺ cells from CD8⁺ SP thymocytes as well as naïve CD8⁺ T cells, CD40L expression turned out to be unstable. In line with data gathered from naïve cells (Stark et al., 2013), IL-2 stabilizes the ability to express CD40L also in CD8⁺ SP thymocytes (Figure 18A). Furthermore, thymocyte survival was dependent on the presence of either IL-2 or IL-7. However, IL-12 did not contribute to CD40L stability or resulted in increased CD40L⁺ frequencies in thymocytes. In naïve cells, IL-12 but neither IL-7/IL-15 nor IL-18 as control were capable to induce CD40L expression in a mechanism that is independent of the thymic imprint but required proliferation as the effect is observable earliest from day 2 on (Figure 18B). In contrast, memory CD8⁺ T cells have stable CD40L expression that is unaffected by the presence of cytokines (Figure 18C). This increasing stability of CD40L expression in CD8⁺ T cells from thymic SP over naïve to memory stages parallels the progressing DNA demethylation at regulatory elements of the *CD40LG* locus during T cell maturation (see Figures 12, 17). Altogether, thymic implementation and IL-12 dependent imprint in proliferating cells compose two separate processes of CD40L induction in CD8⁺ T cells.

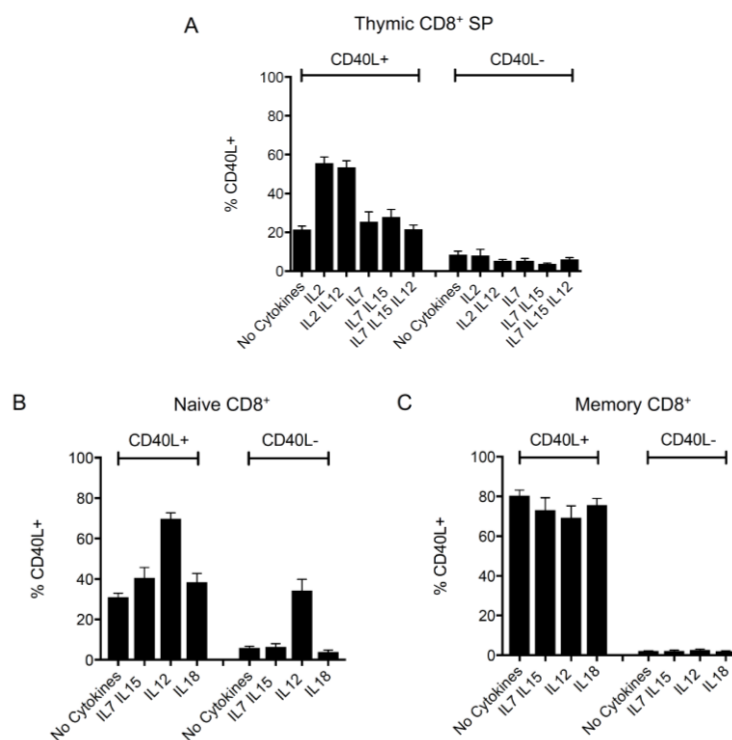


Figure 18: Stability of CD40L expression in different developmental stages of CD8⁺ T cells.

Sorted CD3⁺CD8⁺ SP thymocytes (**A**) as well as CCR7⁺CD45RA⁺ naïve (**B**) and CD45RA⁻memory (**C**) PBMC derived CD8⁺ T cells were stimulated with plate bound α CD3 and α CD28 for 6hrs in the presence of α CD40 and sorted into CD40L⁺ and CD40L⁻ cells. Separated cells were further stimulated with α CD3/ α CD28 for 40hrs and cultivated in the presence of indicated cytokines for 7 days in total. Frequencies of CD40L expressing cells were assessed by restimulation with PMA/Iono for 6hrs in the presence of α CD40 and subsequent flow cytometric analysis. $n = 3$. Mean \pm SEM.

3.3.2 Stability of CD40L expression is regulated by CD28 mediated co-stimulation

As stability of CD40L was reported to be dependent on the presence and duration of CD28 signaling and strong TCR signaling during activation of naïve cells increased the frequency of CD40L⁺ CD8⁺ T cells (McDyer et al., 2002; Snyder et al., 2007; Stark et al., 2013), we assessed the effect of different concentrations of α CD28 and α CD3 in the initial T cell activation on CD40L stability in 7 day cultures (Figure 19).

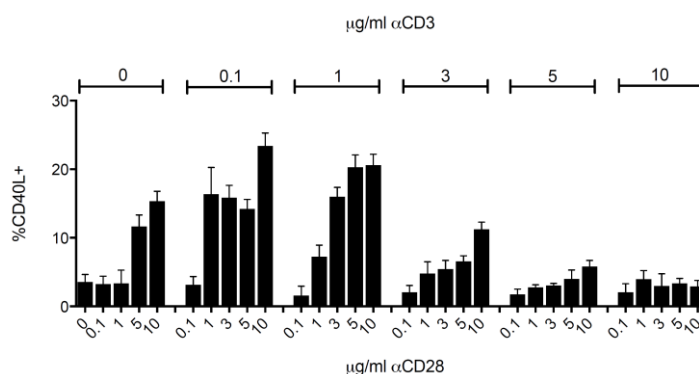


Figure 19: High levels of α CD28 increase while high levels of α CD3 impair the stability of CD40L expression.

Sorted naïve CCR7⁺CD45RA⁺ CD8⁺ T cells were stimulated 2 days with plate bound α CD3 and α CD28 in indicated concentrations. After 5 more days of cultivation, cells were restimulated with PMA/Iono for 6hrs in the presence of α CD40 and CD40L expression assessed on a flow cytometer. n = 2. Mean \pm SEM.

While stimulation with high concentrations of CD28 significantly increased the stability of CD40L expression, CD3 contributed to this effect only in concentrations between 0.1-1 μ g/ml. Higher concentrations exhibited a negative effect on CD40L expression. Altogether, CD40L can be imprinted in the thymus or recruited from naïve CD8⁺ T cells in the presence of IL-12. The stability of CD40L expression in imprinted cells depends on the strength of CD28 co-stimulation during T cell activation and the presence of IL-2 during proliferation (also see Stark et al., 2013).

3.4 CD40L imprinting in the thymus is accompanied by higher TCR avidity during positive selection

3.4.1 Thymic CD40L⁺ CD8⁺ T cells are selected in a MHCI dependent manner

Next, we aimed to assess which mechanism is responsible for the implementation of CD40L expression in some but not all CD8⁺ T cells in the thymus. As CD40L expression is accompanied by CD3 expression - a marker of successful positive and negative selection in the thymus - positive T cell selection might play a role in CD40L imprinting. CD4⁺ T cells that are all capable of expressing CD40L are selected by MHCI presented peptides. Therefore, we first wanted to assess whether CD40L imprint in CD8⁺ T cells occurs by classic MHCI dependent

peptide recognition or instead by a MHCII dependent mechanism. Therefore, we analyzed the CD40L expression in CD8⁺ SP thymocytes of MHC I and MHCII knockout (KO) mice. MHC I knockout led to an impaired maturation of CD8⁺ SP cells accompanied with increased frequencies of CD4⁺ SP T cells while MHCII KO caused the opposite effect. However, in MHCII KO mice CD40L expression on CD8⁺ SP T cells was comparable to WT mice, excluding the possibility of a MHCII dependent selection of those cells (Figure 20A). The absence of CD40L expression on the few CD8⁺ T cells that were detectable in the spleen of MHC I KO further support the concept of a MHC I dependent selection mechanism (Figure 20B). However, it cannot be completely ruled out that this effect is caused by the impaired CD8⁺ SP T cell development in MHC I knockout.

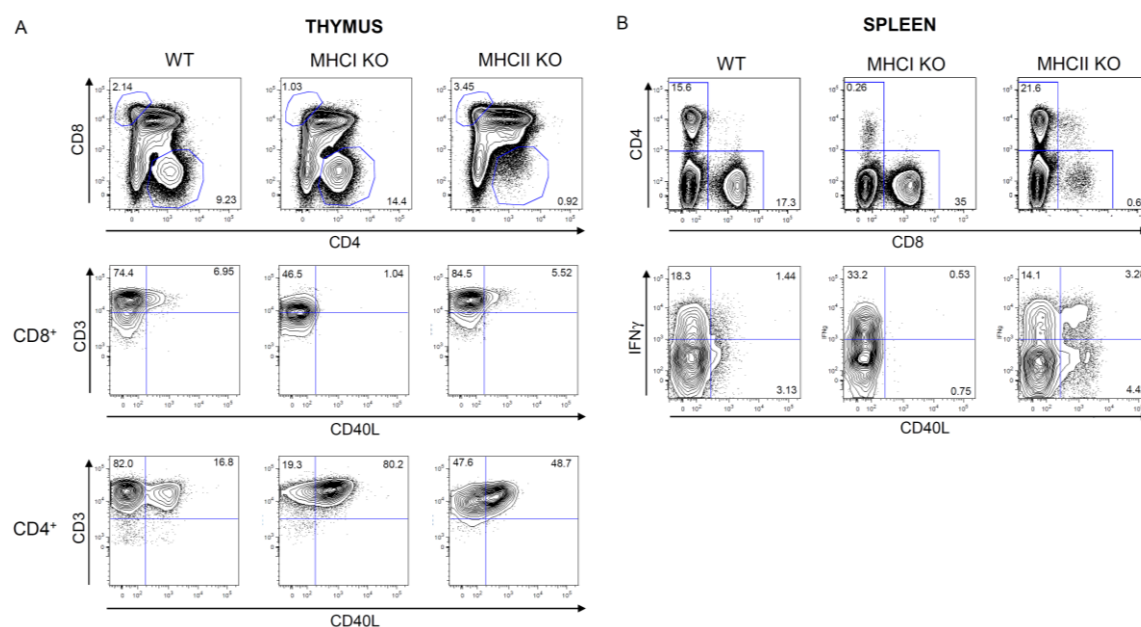


Figure 20: CD40L⁺ CD8⁺ T cells are selected in a MHCII independent but MHC I dependent manner.

(A) Murine thymocytes or (B) splenocytes from WT, MHC I KO and MHCII KO mice were stimulated polyclonally with PMA/Iono for 6hrs in the presence of BrefA. Intracellular CD40L and IFN- γ expression was analyzed by flow cytometry.

3.4.2 CD40L⁺ CD8⁺ T cells express higher levels of the TCR avidity markers

The mechanism of positive selection of CD4⁺ and CD8⁺ T cells in thymus not only differs in the utilized MHC, these cells are selected to, but also in the signaling intensity that they experience during selection (Liu and Bosselut, 2004) (see 1.2.3). Detection of MHCII presented peptides results into a longer binding of the

DP cell to the APC and subsequent stronger TCR signaling than detection of MHC I presented peptides. This long, strong signaling during MHC II dependent selection implements CD4⁺ T cell fate which is paralleled by the ability to express CD40L. We therefore hypothesized that CD40L imprinting in CD8⁺ T cells might be caused by a stronger TCR signaling during selection compared to those CD8⁺ T cells that are unable to express CD40L. CD5 is a marker that is expressed relative to the TCR avidity the cell is selected to (Azzam et al., 1998; Moran et al., 2011). Therefore, we assessed CD5 expression of polyclonally activated murine CD8⁺ SP thymocytes (Figure 21). CD40L⁺ CD8⁺ SP thymocytes expressed higher CD5 levels compared to CD40L⁻ CD8⁺ SP cells suggesting a stronger TCR signal in CD40L⁺ T cells during selection.

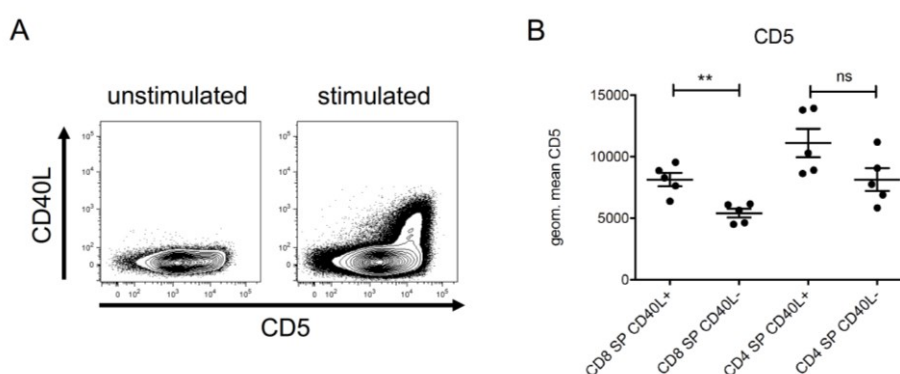


Figure 21: Murine CD40L⁺ CD8⁺ SP T cells express higher levels of the TCR avidity marker CD5. (A, B) Thymocytes from B6 mice were stimulated 6h with PMA/Iono, stained intracellularly and subsequently analyzed on a flow cytometer. (A) Exemplary CD40L and CD5 staining of unstimulated and stimulated total alive thymocytes. (B) Geometric mean of CD5 in CD40L⁺ or CD40L⁻ T cells pre-gated to CD3⁺CD8⁺ or CD3⁺CD4⁺ SP thymocytes respectively. One representative experiment out of 2 shown. $n_{\text{total}} = 10$. Mean \pm SEM. Student's t-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

However, CD4⁺ SP T cells also differ in CD5 MFI among CD40L⁺ and CD40L⁻ cells. Therefore, we utilized different murine models with transgenic MHC class I restricted TCR to exclude a possible bias in CD5 readout caused by different responsiveness/viability during polyclonal activation. H-Y and OT-I mouse strains differ in their CD5 expression and reactivity (Fulton et al., 2015; Kieper et al., 2004). H-Y mice express a TCR against a Y chromosome encoded male antigen resulting in T cells that have imprinted low CD5 levels from positive selection and are depleted during negative selection in male but not female mice. In contrast, OT-I cells express an ovalbumin specific TCR and high levels of CD5 indicating they were selected with strong avidity to some unknown self-peptide (Clarke et

al., 2000). We first validated the CD5 expression levels in female H-Y and OT-I mice that were bred on RAG2^{KO} background to exclude endogenous TCR and compared to WT mice (Figure 22A). Subsequent polyclonal stimulation of splenocytes revealed large differences in CD40L expression with almost absent CD40L expression in H-Y mice followed by 5-10% in WT mice and frequencies close to 60% in OT-I mice (Figure 22B). These results suggest, that the TCR affinity during MHC class I dependent T cell selection in the thymus regulates CD40L expression.

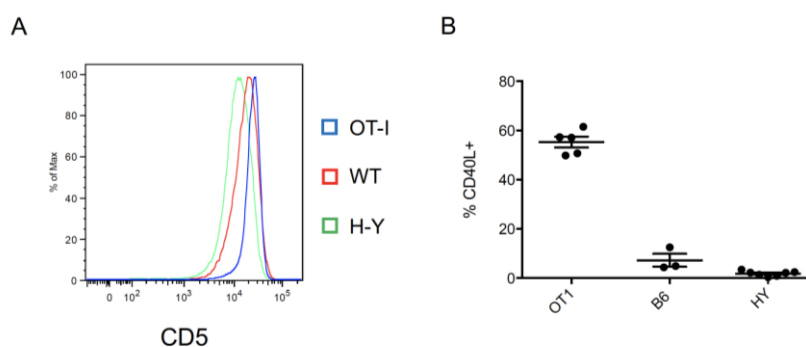


Figure 22: High TCR affinity promotes maturation of CD40L⁺ CD8⁺ T cells.

(A) CD5 expression of CD3⁺CD8⁺ splenocytes of WT, female H-Y RAG2^{KO} and OT-I RAG2^{KO} mice was measured *ex vivo* on a flow cytometer. **(B)** Splenocytes were stimulated polyclonally with PMA/Iono in the presence of BrefA. Intracellular CD40L expression of CD3⁺CD8⁺ T cells was measured. Mean \pm SEM. Data of 2 independent experiments.

In order to validate whether also human CD40L⁺ CD8⁺ T cells were selected with high affinity to self-peptides during positive selection, we assessed the gene expression of human CD40L⁺ versus CD40L⁻ CD8⁺ SP thymocytes. In line with the previous mouse data, gene expression of CD5 and a second TCR avidity marker NUR77 was higher in CD40L⁺ cells compared to CD40L⁻ CD8⁺ SP cells (Figure 23A). Furthermore, sort of the 6-7% of naïve CD8⁺ T cells with lowest and highest CD5 MFI respectively resulted in twice as much CD40L⁺ frequencies within the CD5^{high} fraction after polyclonal activation (Figure 23B, C). Altogether, the ability to express CD40L seem to be imprinted by strong detection of self-peptides during maturation in the thymus in human and mouse.

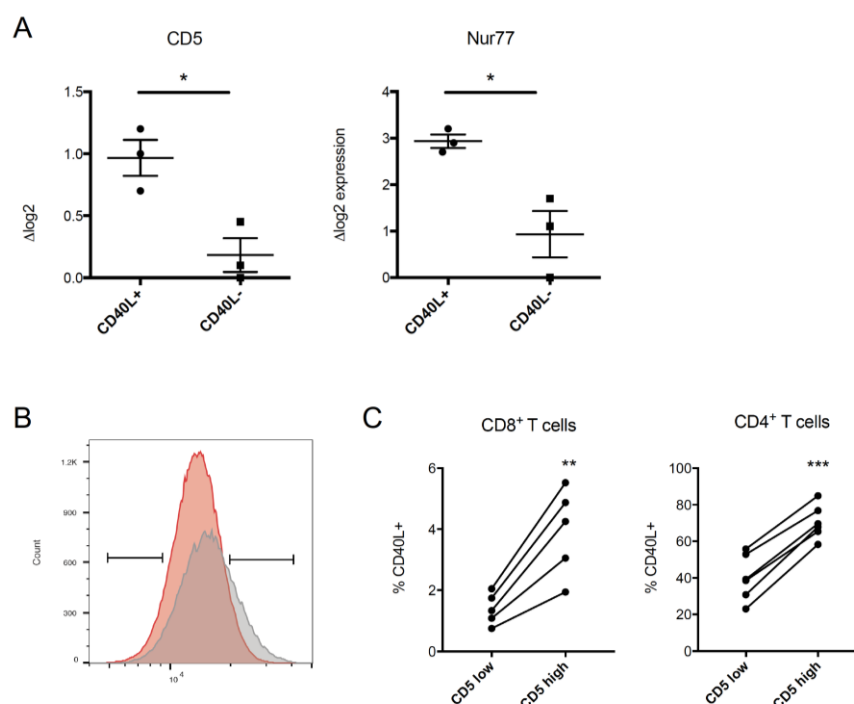


Figure 23: Human CD40L⁺ CD8⁺ T cells express higher levels of CD5 and NUR77.

(A) Human thymocytes were stimulated polyclonally with SEB/TSST-1 in the presence of αCD40 and CD40L for 6hrs. CD1a⁻ CD3⁺ CD4⁻ CD8⁺ SP cells were sorted into CD40L⁺ and CD40L⁻ fraction and used for RNA-sequencing. Mean ± SEM. **(B)** Representative histogram of CD5^{low} and CD5^{high} gating among naïve CD45RA⁺CCR7⁺CD11a⁻CD95⁻ CD8⁺ T cells. **(C)** CD5^{low} and CD5^{high} cells were sorted from naïve CD8⁺ and CD4⁺ T cells, stimulated with plate bound αCD3/αCD28 in the presence of αCD40 and CD40L antibody for 12hrs and CD40L frequencies assessed by flow cytometry. Data represents 2 independent experiments. Student's t-test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

3.4.3 CD40L expression is implemented during positive and not negative selection

We observed CD40L implementation in T cells from DP stage on beginning with the co-expression of CD3 which is upregulated upon positive and negative selection. Among CD4⁺ T cells that egress from positive selection, those CD5^{high} cells that strongly bind to AIRE dependent autoantigens presented in the thymic medulla undergo agonistic selection and become FoxP3⁺ regulatory T cells (Ono and Tanaka, 2016). In order to distinguish whether implementation of CD40L in CD8⁺ T cells already occurs during positive- or similar to Tregs during negative selection, we utilized the markers CD69 and CCR7 (Figure 24A). The early T cell stages in the thymic cortex lack CD69 and CCR7 expression. TCR signaling during positive selection results in CD69 upregulation followed by CCR7 expression. CCR7 is crucial for migration from the cortex into the medulla where

the cells undergo negative selection as $CD69^+CCR7^{high}$ cells. Mature cells downregulate CD69 and egress from thymus into blood (Takada et al., 2015; Van Laethem et al., 2013). All $CD1a-CD3^+CD40L^+$ cells were $CD69^+$ but just partially $CCR7^+$ suggesting an implementation during positive selection in the cortex. However, this approach is challenged by the necessity of a stimulation for CD40L detection that also induces CD69 upregulation. Therefore, we additionally compared the expression of CCR7 and CD25 of $CD40L^+$ T cells with $FoxP3^+$ Tregs as those must have passed negative selection in the medulla for successful $FoxP3$ implementation (Figure 24B). As a large fraction of $CD40L^+$ T cells are $CCR7^-CD25^-$ in contrast to $FoxP3^+$ Tregs, induction of CD40L took place prior to migration into the medulla. In summary, we conclude that CD40L expression is implemented during positive but not negative selection.

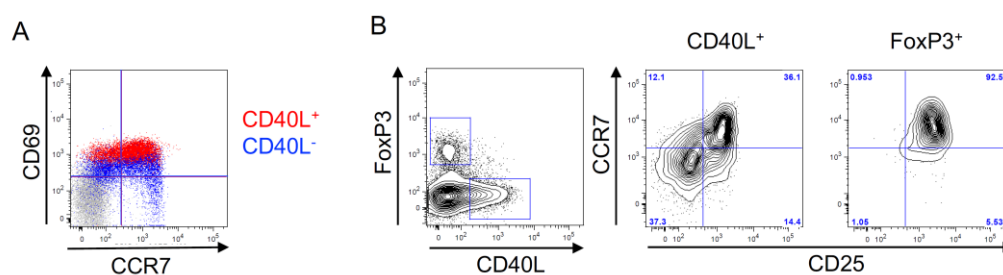


Figure 24: CD40L expression begins with CD69 upregulation in thymic cortex

Human thymocytes were stimulated polyclonally with SEB/TSST-1 in the presence of aCD40 and CD40L for 6hrs. **(A)** CD69 and CCR7 expression in total thymocytes (grey) compared to $CD1a-CD3^+CD40L^-$ (blue) or $CD40L^+$ (red) T cells. **(B)** $FoxP3$ and CD40L expression in polyclonally activated $CD3^+$ thymocytes (left). CCR7 and CD25 in $CD40L^+$ or $FoxP3^+$ cells (right).

3.5 $CD40L^+CD8^+$ T cells develop into unique memory T cells

3.5.1 The $CD8^+$ memory T cell compartment consists of different Tc subsets

We demonstrated that the ability to express CD40L in $CD8^+$ T cells is implemented during high affine detection of self-antigens during positive selection. Next, we wanted to assess whether this affects the further fate of the cells. The cytokine milieu during the activation of naïve T cells and the subsequent proliferation regulates the induction of diverse transcription factors resulting in the formation of distinct memory subsets with unique functions and cytokine profiles (Sallusto, 2016). Peripheral $CD4^+$ memory T cells can be distinguished *ex vivo* into Th1, Th2, Th17, Th17+1 and Th22 subsets based on

the expression patterns of the chemokine receptors CCR4, CCR6, CCR10 and CXCR3 (Acosta-Rodriguez et al., 2007; Bonecchi et al., 1998; Sallusto et al., 1998; Trifari et al., 2009). We saw before that the chemokine receptors CCR4 and CCR6 identify a CD40L expressing memory CD8⁺ T cell fraction (see Figure 13). This raised the question, whether the concept of chemokine receptor expression can be transferred to CD8⁺ memory T cells and how CD40L expression is distributed within these subsets. We could show that the CD8⁺ memory T cell compartment contains subsets with CD4⁺ T cell matching chemokine receptor expression pattern: Tc1 (CCR6⁻CXCR3⁺CCR4⁻), Tc2 (CCR6⁻CXCR3⁻CCR4⁺), Tc17+1 (CCR6⁺CXCR3⁺CCR4⁻), Tc17 (CCR6⁺CXCR3⁻CCR4⁺CCR10⁻) and Tc22 (CCR6⁺CXCR3⁻CCR4⁺CCR10⁺) (S. Warth, PhD thesis; Loyal et al., submitted). While the different CD4⁺ T cell subsets account relatively equally for around 10-20% of memory CD4⁺ T cells respectively, the Tc1 fraction dominates in CD8⁺ memory with 60% followed by Tc17+1 and Tc2 with around 10% and Tc17 and Tc22 with around 1% (S. Warth, PhD thesis; Loyal et al. submitted). Interestingly, all cells assigned to the subsets Tc2, Tc17, and Tc22 express CD40L (Figure 25). In contrast, only a minor fraction of Tc1 and Tc17+1 cells co-expressed CD40L together with IFN- γ suggesting an IL-12 dependent imprint (S. Warth, PhD Thesis; Stark et al., 2013). This data suggests, naïve CD40L⁺ CD8⁺ T cells preferentially differentiate into cells with chemokine receptor expression patterns that would assign them to Tc2, Tc17 and Tc22 cell subsets.

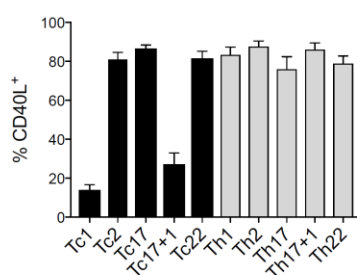


Figure 25: Tc2, Tc17 and Tc22 memory CD8⁺ T cells express CD40L similar to CD4⁺ T cells.

Proportion of CD40L expression among sorted and polyclonally activated Tc and Th cell subsets (n=6). Data adapted and extended from S.Warth. Mean \pm SEM.

However, we first wanted to assess in depth the reliability of the CD8⁺ memory compartment separation by their chemokine receptor expression pattern. We validated whether these subsets secrete according cytokines as known from the different CD4⁺ Th subsets by performing a multiplex cytokine array from the supernatants of sorted and polyclonally stimulated memory CD8⁺ and CD4⁺ T cell subsets. In line with published data (Maggi et al., 1994; Salgame et al., 1991), only Tc/Th1 and Tc/Th17+1 subsets produced IFN- γ and the Tc/Th2 subsets were the main IL-4 and IL-13 producers (Figure 26A). However, Tc17 and Tc22 cells also responded with rapid IL-13 secretion within the first 24hrs and even produced IL-4 after 72hrs of stimulation. The Tc/Th17 and Tc/Th17+1 cells secreted IL-17 that is also produced by Tc/h22 cells at the later timepoint (Figure 26A). IL-22 and the anti-inflammatory cytokine IL-10 were only detected after prolonged stimulation in Tc/h17 and Tc/h22 cells (Figure 26B). TNF- α was produced relatively equally by all cell types and GM-CSF dominantly by Tc2 and Th17+1 cells. None of the cell subsets produced IL-1b, IL-8 or IL-12p70 (not shown).

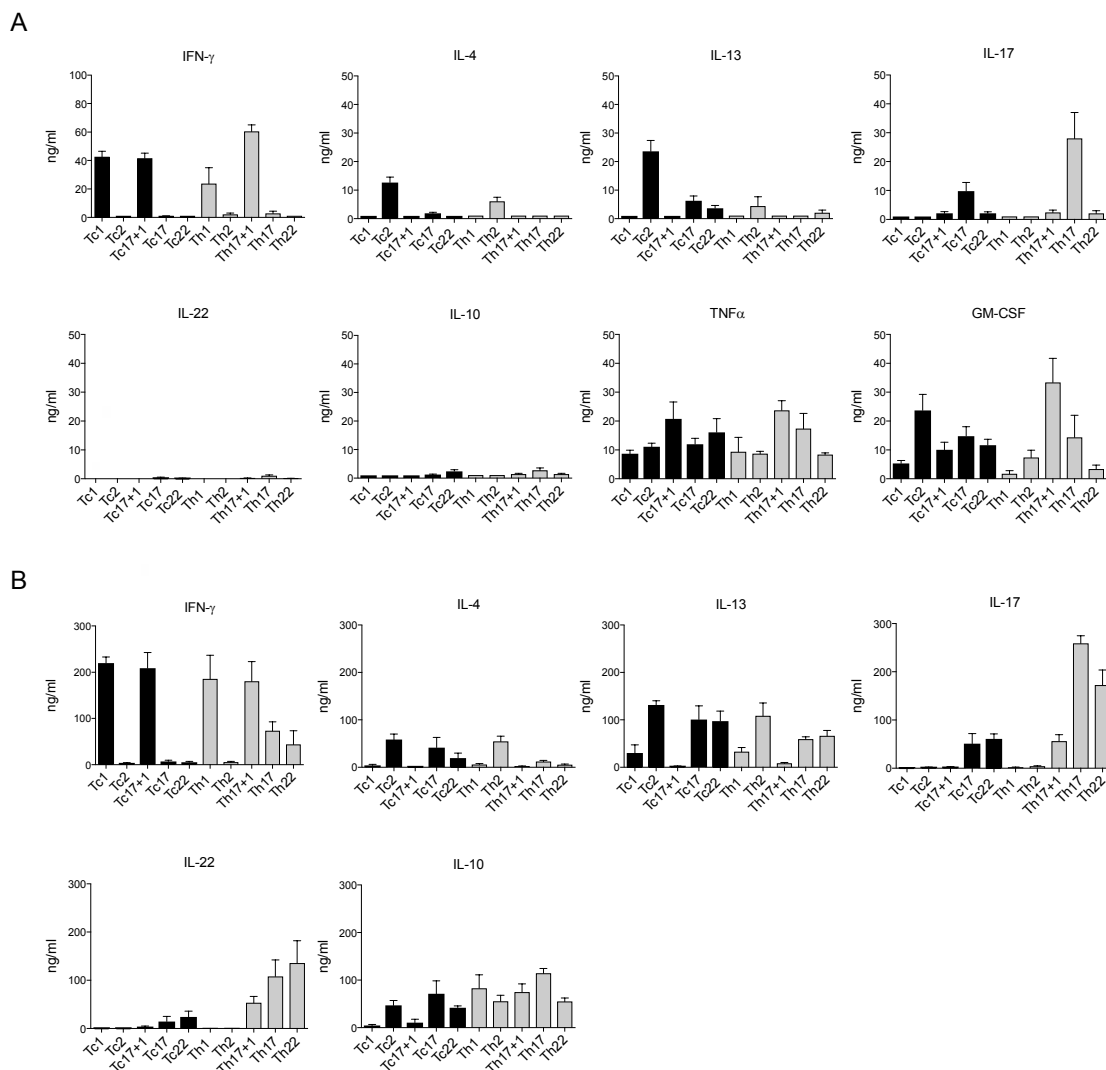


Figure 26: Cytokine secretion profile of the memory CD8⁺ T cell subsets Tc1, Tc2, Tc17+1, Tc 17 and Tc22 compared to their respective CD4⁺ T cell subsets.

CD45RA⁻ CD8⁺ (black) or CD4⁺ (grey) memory T cell subsets Tc1/Th1 (CCR6⁻CXCR3⁺CCR4⁻), Tc2/Th2 (CCR6⁻CXCR3⁻CCR4⁺), Tc17+1/Th17+1 (CCR6⁺CXCR3⁺CCR4⁻), Tc17/Th17 (CCR6⁺CXCR3⁻CCR4⁺CCR10⁻) and Tc22/Th22 (CCR6⁺CXCR3⁻CCR4⁺CCR10⁺) were sorted and stimulated at 1x10⁴ Cells/100ul for 24hrs (A) or 72hrs (B) with αCD3/αCD28. The supernatant was utilized for multiplex cytokine ELISA. n = 10/3 (Tc/Th, 24hrs), n = 4/4 (Tc/Th, 72hrs). Mean ± SEM.

3.5.2 RNA-Sequencing revealed similarities in the gene expression of CD4⁺ and CD8⁺ memory T cell subsets

In order to understand whether there is a common gene expression signature in the CD40L expressing memory CD8⁺ T cell subsets Tc2, Tc17 and Tc22 and how these subsets are related to the CD40L⁺ CD4⁺ T cell subsets, we performed RNA-sequencing of highly purified CD4⁺ and analogous CD8⁺ memory T cell subsets.

Strikingly, unsupervised clustering of normalized data separated the CD8⁺ subsets Tc1 and Tc17+1 from Tc2, Tc17 and Tc22 and placed the latter ones in the proximity of the CD4⁺ T cell subsets (Figure 27).

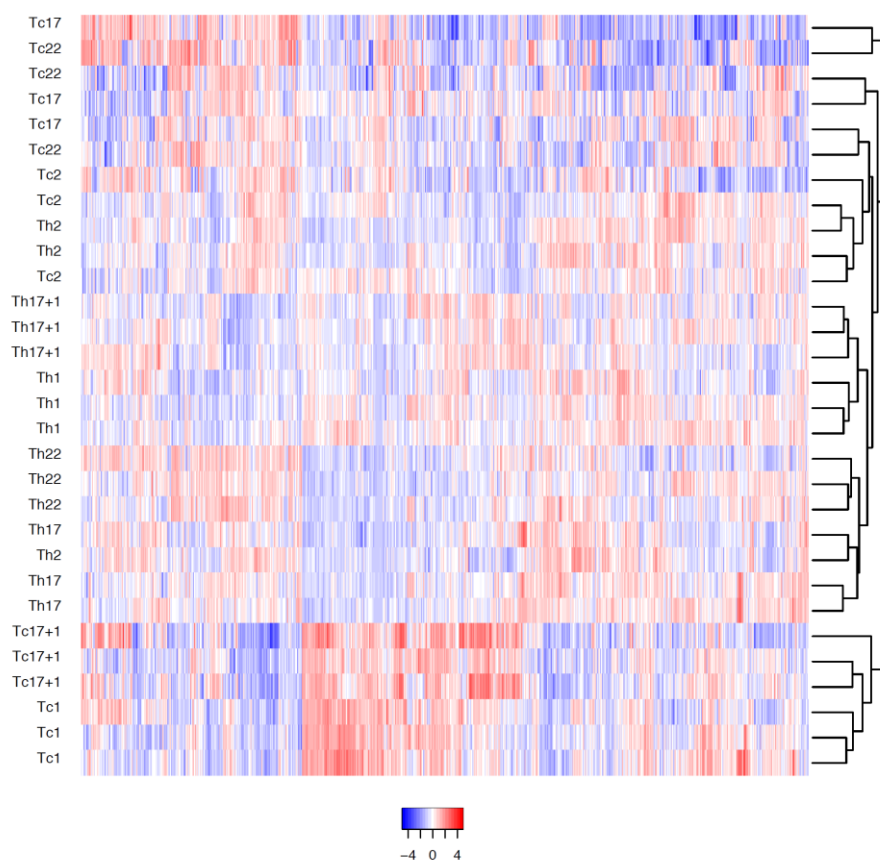


Figure 27: Top 1000 differentially expressed genes among CD8⁺ and CD4⁺ memory T cell subsets.

RNA was isolated from sorted memory CD45RO⁺CD8⁺ or CD4⁺ T cell subsets Tc1/Th1, Tc2/Th2, Tc17+1/Th17+1, Tc17/Th17 and Tc22/Th22 and used for RNA-sequencing. Heatmap displays top 1000 most variable expressed genes. n = 3.

The high analogy of distinct CD4⁺ and corresponding CD8⁺ T cell subsets with respect to their chemokine receptor profiles and cytokine secretion patterns moreover suggested that similar molecular differentiation mechanisms might act for both CD4⁺ and CD8⁺ T cell subtypes. In order to avoid general differences between CD4⁺ and CD8⁺ T cells as well as between donors cover common signatures among the Tc and Th subsets, we performed a batch compensation. This resulted in clusters of distinct CD8⁺ T cell subsets with their corresponding CD4⁺ T cell counterpart. The clustering pattern furthermore revealed a similarity between Tc1/Th1 and Tc1/Th17+1 cell subsets, while Tc2/Th2, Tc17/Th17 and Tc22/Th22 cell subsets established a separate cluster (Figure 28).

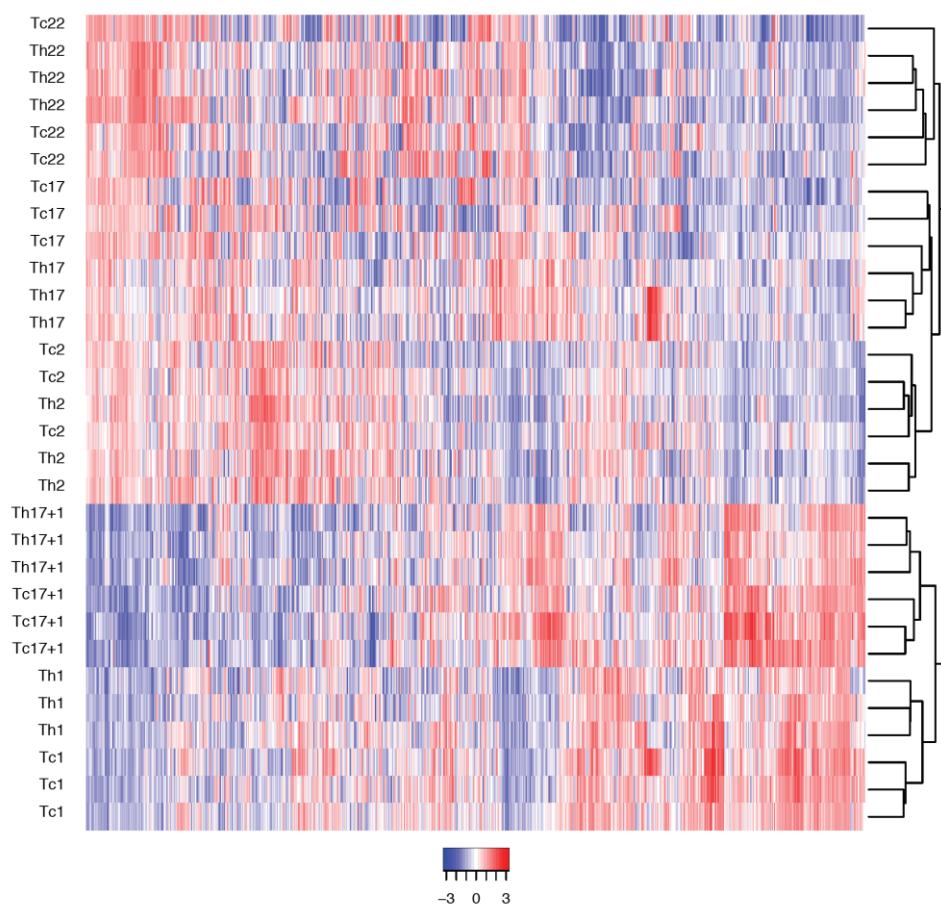


Figure 28: Top 1000 differentially expressed genes among CD8⁺ and CD4⁺ memory T cell subsets after batch compensation.

Heatmap of top 1000 most variable expressed genes after normalization and batch compensation of RNA-seq analyses. $n = 3$.

A principal component analysis (PCA) of all differentially expressed genes of the compensated data set confirmed a strong correlation of the respective Tc and Th cell subsets across all donors with a particular similarity of Tc17/Th17 and Tc22/Th22 cells (Figure 29A). The similarity between distinct CD4⁺ T cell subsets and corresponding CD8⁺ T cell subsets was further supported by the analysis of key transcription factors controlling differentiation and maintenance of distinct functional memory T cells (Coghill et al., 2011). While the expression of *TBX21* and *RORC* was restricted to IFN- γ - and IL-17A-secreting subsets in both CD4⁺ and CD8⁺ T-cell subsets, *GATA3* gene expression and protein was detectable in Th2, Tc2 but also in Tc17 and Tc22 cell subsets (Figure 29B, C). In contrast, all CD4⁺ Th subsets as well as Tc2, Tc17 and Tc22 CD8⁺ T cells express *AHR* that is strongest in Th/c17, and Th/c22 subsets respectively (Figure 29B, C). We

therefore conclude that similar molecular mechanisms are responsible for memory T-cell subset differentiation and/or maintenance in both CD4⁺ and CD8⁺ T-cell subsets, respectively. Additionally, Tc2, Tc17 and Tc22 cells also display strong overall similarities with CD4⁺ T cells in their gene expression including the expression of the classical helper molecule CD40L suggesting they might represent a subset of CD8⁺ T cells with helper-type features.

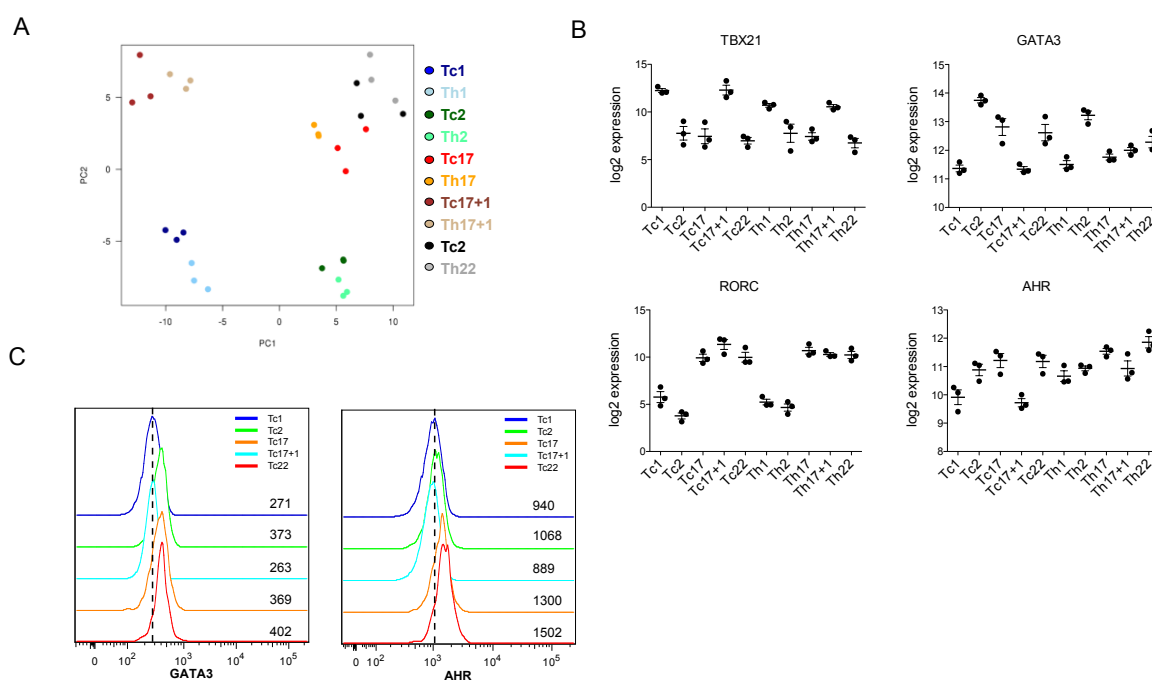


Figure 29: CD8⁺ and CD4⁺ memory T cell subsets resemble in TF gene expression.

(A) Principal component analysis (PCA) of all protein coding expressed genes **(B)** Normalized, log₂ transformed expression of indicated transcription factors among Th and Tc subsets. Mean ± SEM. **(C)** Intracellular staining for TF of indicated Tc subsets.

3.5.3 CD8⁺ memory T cells subdivide into cytotoxic and non-cytotoxic subsets

In order to assess in depth what differs the single subsets from each other we performed a gene ontology overrepresentation analysis (ORA) of the differentially expressed genes, which revealed an enrichment of signatures associated with the gene clusters “immune response”, “cell activation”, “communication” and “trafficking”. These mainly comprised of immunomodulatory and cytotoxicity markers as well as cytokine and chemokine receptors. For example, the chemokine receptor *CCR5* was expressed by Tc1 and Tc17+1 CD8⁺ cells together with the cytotoxicity markers *Granzymes A, B, H, K, M, Granulysin*,

Perforin as well as other markers such as *LAG3*, *CRTAM* and *NKG7* (Figure 29) (Boles et al., 2005; Hidalgo et al., 2008; Kohlmeier et al., 2008). However, Tc1 and Tc17+1 cells could be clearly distinguished from each other by cytokine receptors as *IL-17RE*, *IL-18R* and *IL-23R* as well as Tc1 markers *ITGAM*, *CD38* and *TIGIT*.

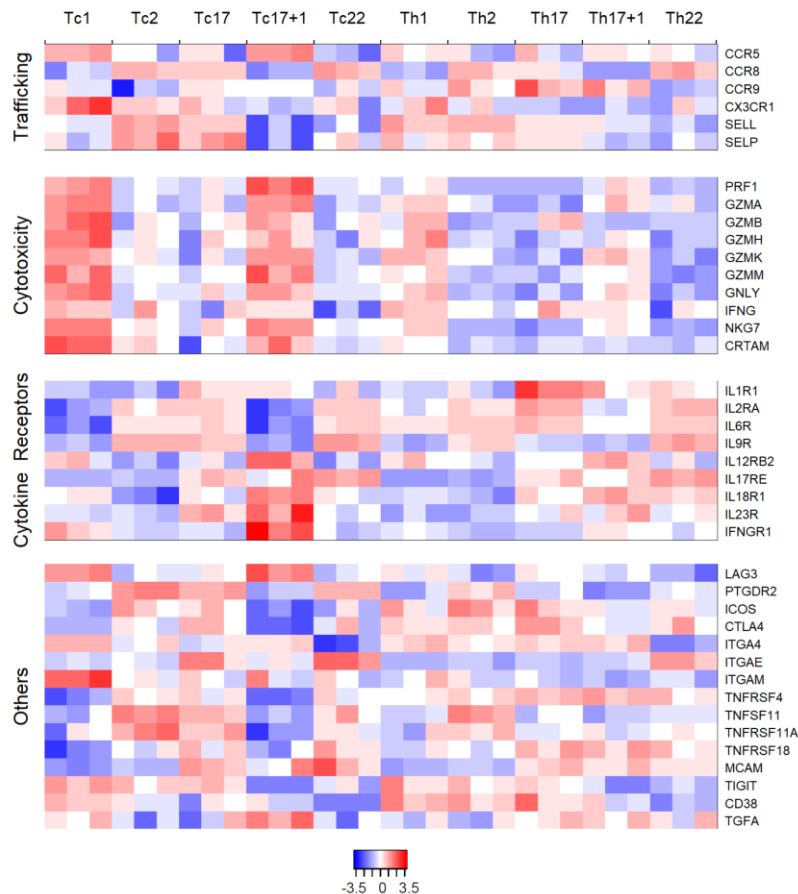


Fig.30: Tc1 and Tc17+1 differ from Tc2, Tc17 and Tc22 in the expression of genes associated with cytotoxicity, immunomodulation, activation and migration.

Heatmap representation of normalized, log₂ transformed RNA-seq expression values of selected genes enriched in gene ontology ORA analysis.

In contrast, Tc2, Tc17 and Tc22 lacked expression of cytotoxicity-associated genes. Instead, they expressed the Th2 CD4⁺ T cell related gene *PTGDR2* (*CRTM2*) (Figure 30) (Fanis et al., 2007; Nagata et al., 1999). Together with *CCR8*, the receptors used for cell subset identification (*CCR4*, *CCR10*) and the cytokine receptors (*IL-6R*, *IL-9R*), Tc2, Tc17 and Tc22 cell subsets displayed many characteristics of skin migratory T cells, where non-cytotoxic subsets of CD8⁺ T cells have been demonstrated to exert a barrier protection function (Fu

et al., 2016; Huber et al., 2009; Schaerli et al., 2004; Tubo et al., 2011). Notably Tc2, Tc17 and Tc22 subsets also expressed more genes associated with immunomodulatory function of the B7-CD28 and TNFRSF superfamilies than the cytotoxic subsets Tc1 and Tc17+1 (Chen and Flies, 2013).

Expression of cytotoxicity related genes was one of the most prominent signatures that distinguished Tc1 and Tc17+1 from Tc2, Tc17 and Tc22 cell subsets. The mRNA of Perforin and Granzyme B was shown to be pre-stored in cytotoxic cells but only translated into protein upon T cell activation (Pipkin et al., 2010). Therefore, we assessed the protein levels of the lytic molecules Perforin and Granzyme B in the different T cell subsets *ex vivo*. Among Tc1 cells, 40% co-expressed Perforin and Granzyme B protein but almost none of the Tc17+1 cells (Figure 31A). Furthermore, we found a small fraction Th1 CD4⁺ T cells expressed cytolytic molecules which are the characteristic markers for cytotoxic CD4⁺ T cells (Mattoo et al., 2016). Next, we aimed to analyze whether there is a common mechanism that regulates the helper (CD40L expression) versus cytotoxic (expression of lytic molecules) phenotype in both CD4⁺ and CD8⁺ T cells and whether this mechanism might affect the ability to express CD40L, too. We could show that compared to non-cytotoxic, classical helper CD4⁺ T cells cytotoxic CD4⁺ T cells completely lack CD40L expression (Figure 31B). Cytotoxic CD4⁺ T cells acquire their cytotoxic phenotype in a Runx3-dependent manner and Runx3 overexpression was shown to impair CD40L expression (Mucida et al., 2013). Therefore, we assessed Runx3 levels in sorted CD4⁺ and CD8⁺ T-cell subsets. Cytotoxic CD8⁺ memory (Tc1/Tc1+1), cytotoxic CD4⁺ and effector CD8⁺ T cells expressed comparable levels of Runx3, whereas helper-type memory CD8⁺ T cells (Tc2, Tc17, Tc22) possessed as little Runx3 as helper CD4⁺ memory T cells (Figure 31C). However, naïve CD40L⁺ and CD40L⁻ CD8⁺ T cells did not differ in their expression of Runx3 or the Runx3 repressor ThPOK (Figure 31D). Therefore, among memory T cells both CD4⁺ and CD8⁺ fractions can be subdivided into different Th/Tc subsets according their cytokine profile as well as into cytotoxic and non-cytotoxic, helper-type subsets that differ in their CD40L and Runx3 expression.

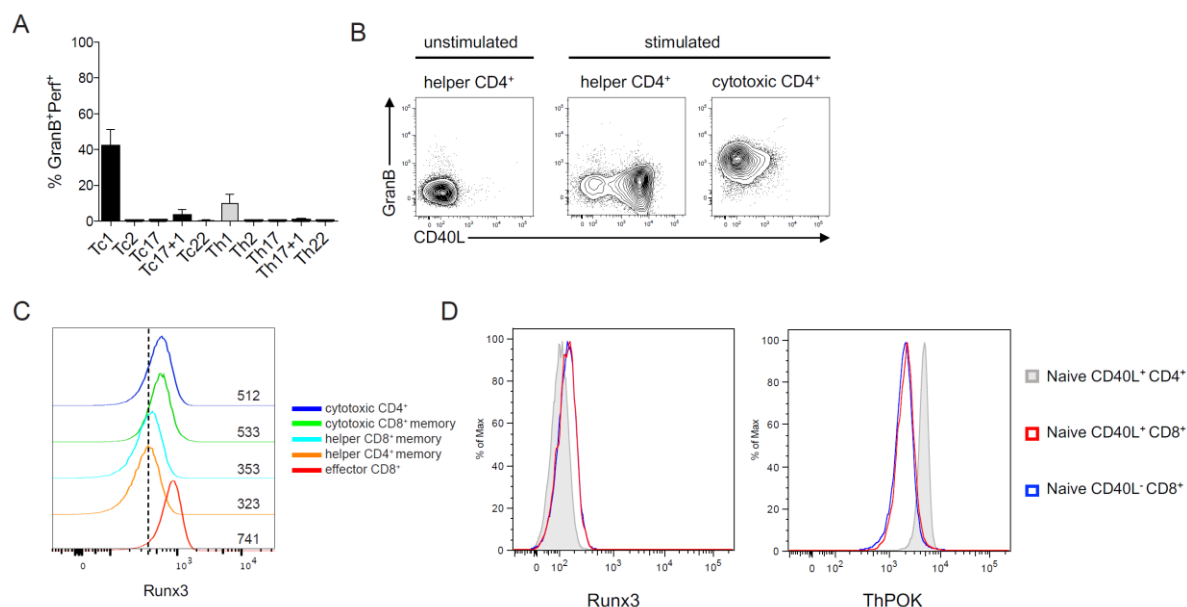


Figure 31: Cytotoxic CD4⁺ and CD8⁺ memory T cells do not express CD40L but Runx3.

(A) PBMCs were stained *ex vivo* for Granzyme B and Perforin and measured by flow cytometry. Gated on respective Tc/Th subset according to their chemokine receptor pattern. **(B)** Granzyme B and CD40L co-expression of sorted, polyclonally activated helper memory CD4⁺ (CD45RA⁺CD28⁺CD57⁻) and cytotoxic memory CD4⁺ (CD45RA⁺CD28⁻CD57⁺) T cells compared to unstimulated control. **(C)** Effector CD8⁺ (CD45RA⁺CCR7⁻), cytotoxic CD8⁺ memory (CD45RA⁺CXCR3⁺CCR4⁻), cytotoxic CD4⁺ (CD45RA⁺CD28⁻CD57⁺), helper CD8⁺ memory (CD45RA⁺CXCR3⁺CCR4⁻) and helper CD4⁺ (CD45RA⁺CD28⁺CD57⁻) T cells were sorted and intracellular Runx3 expression assessed. Numbers in plot indicate the MFI. **(D)** MACS enriched CD4⁺ and CD8⁺ T cells were polyclonally activated and sorted into CD40L⁻ and CD40L⁺ naive subsets. Runx3 and ThPOK were assessed intracellularly.

3.5.4 SLAMF7 and IL-6R distinguish cytotoxic from non-cytotoxic T cells

In order to identify further markers that can distinguish cytotoxic from helper-type T-cell subsets we screened for genes that were differentially expressed in the cytotoxic T-cell populations (Tc1, Tc17+1) compared to all other non-cytotoxic Tc and Th subsets. We could identify 28 genes that were more weakly and 22 genes that were more strongly expressed among the cytotoxic T cell subsets (Figure 32A). Strikingly, the two cell surface molecules SLAMF7 and IL-6R displayed a converse expression pattern among all lymphocytes (Figure 32B). In concordance, all CD4⁺ as well as CD8⁺ Tc2, Tc17 and Tc22 helper T-cell subsets but not the cytotoxic Tc1 and Tc17+1 cell subsets expressed high levels of IL-6R (Figure 32C). Opposing expression patterns were observed for SLAMF7 that was only expressed by the cytotoxic Tc1 and Tc1+17 CD8⁺ memory T-cell subsets and all Granzyme B and/or Perforin expressing CD8⁺ T cells (Figure 32 D). Naive

CD8⁺ and CD4⁺ T cells lacked SLAMF7 but expressed IL-6R, whereas cytotoxic CD8⁺ effector T cells expressed the highest levels of SLAMF7 (Figure 32E). SLAMF7 was co-expressed on all CD8⁺ T cells expressing Granzyme B, Perforin and/or IFN- γ after in vitro activation (Figure 32F). Moreover, SLAMF7 is detectable among Granzyme B⁺ Perforin⁺ cytotoxic CD4⁺ T cells (Figure 32F). Since all further cytotoxic subsets among lymphocytes, the CD56 expressing NK, NKT and ILC1 cells were SLAMF7⁺IL-6R⁻ (Figure 32G), SLAMF7 represents a highly suitable marker to assess the cytotoxic potential of lymphocytes while IL-6R represents the non-cytotoxic fraction.

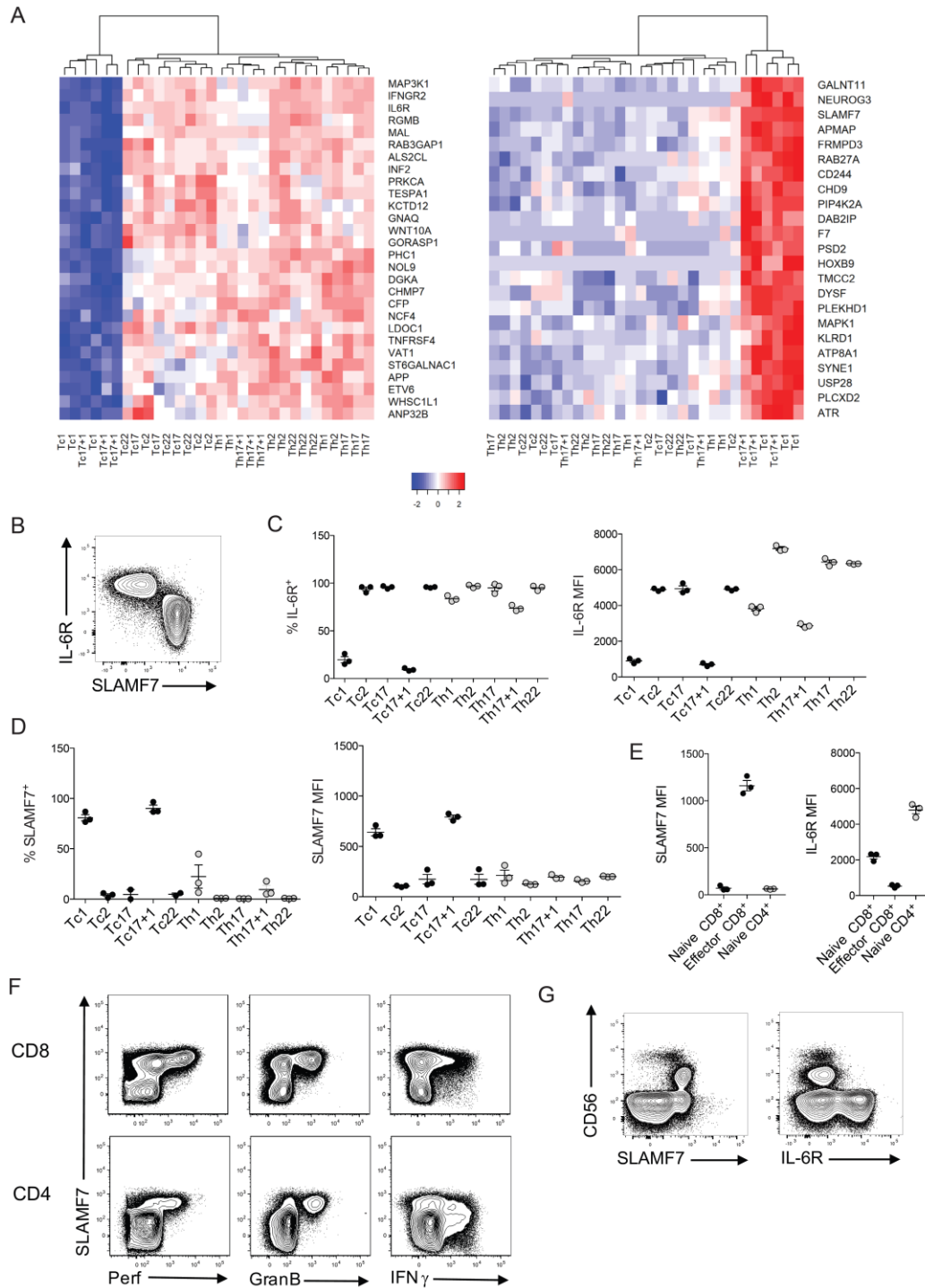


Figure 32: SLAMF7 and IL-6R expression distinguishes cytotoxic memory CD8⁺ and CD4⁺ T cells from non-cytotoxic cells.

(A) Heatmaps of normalized and scaled genes distinguishing cytotoxic (Tc1 and Tc17+1) and non-cytotoxic CD8⁺ and CD4⁺ T cell subsets (Tc2, Tc17, Tc22, Th1, Th2, Th17, Th17+1, Th22). **(B)** Representative dot plot of IL-6R and SLAMF7 expression among lymphocytes. **(C, D)** Flow cytometric stainings indicating frequency and MFI of of IL-6R **(C)** and SLAMF7 **(D)** among CD45RA⁻CD4⁺ and CD8⁺ T cell subsets. **(E)** MFI of of IL-6R and SLAMF7 of CD45RA⁺CCR7⁺ naïve CD4⁺ and CD8⁺ T cells and CD45RA⁺CCR7⁻ effector CD8⁺ T cells **(F)** Flow cytometric co-stainings of SLAMF7 with Granzyme B, Perforin and IFN-γ after 6hrs of polyclonal activation in the presence of BrefA, gated on CD8⁺ T cells or CD4⁺ T cells. **(G)** Representative dot plot of SLAMF7 and IL-6R coexpression with CD56 among lymphocytes.

3.5.5 CD40L⁺ memory CD8⁺ T cell subsets possess a unique TCR repertoire

Previous studies have suggested a relative plasticity in cytokine production and lineage transcription factor expression among differentiated CD4⁺ memory T-cell subsets (Kunicki et al., 2017; O'Shea and Paul, 2010). Becattini et al. furthermore demonstrated heterogeneous differentiation of pathogen-specific CD4⁺ T cells into distinct CD4⁺ helper T cell sister clones (Becattini et al., 2015). However our CD40L expression pattern suggests a preferential differentiation of CD40L⁺ naive CD8⁺ T cells into Tc2, Tc17 and Tc22 memory CD8⁺ T cells. To assess a potential corporate contribution of the different CD8⁺ T-cell subsets to specific immune responses, we performed TCR β -chain deep sequencing of isolated Tc subsets.

The relative numbers of clonotypes were smaller in Tc17 and Tc22 cells compared to Tc1, Tc2 and Tc17+1 with a few clones dominating their repertoire (Figure 33A, B). While less than 0.6% of the clones are present in frequencies above 1%, they accounted for 60% (Tc17) and 90% (Tc22) of the reads (Figure 33C, D). The oligoclonal repertoire in Tc17 and Tc22 cells with a few dominating clones suggests a highly specialized response in contrast to the rather polyclonal Tc1, Tc17+1 and Tc2 cells.

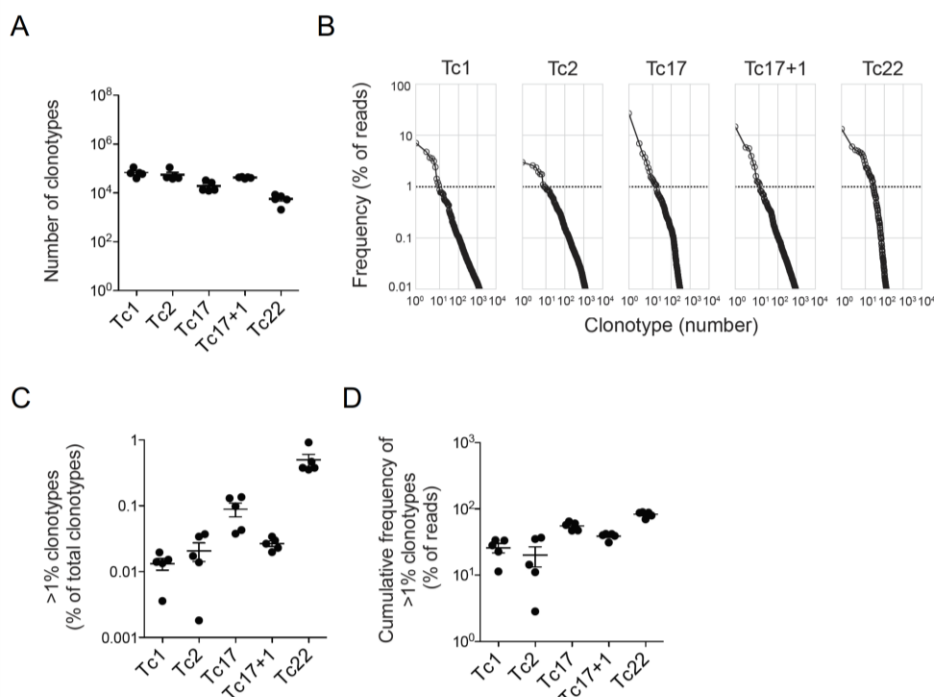


Figure 33: Some clones are enriched among Tc17 and especially Tc22 cells.

Tc1, Tc2, Tc17, Tc17+1 and Tc22 were sorted from human PBMC and analyzed by TCR-sequencing. **(A)** Number of clones found in different subsets. Mean \pm SEM. **(B)** Number of clonotypes and their frequency of reads of one representative donor. **(C)** Number of clonotypes present at frequencies >1% in the total clonotype pool. **(D)** Cumulative frequency of all clonotypes present at frequencies >1%.

Next, we asked whether the oligoclonality of Tc17 and Tc22 cells is caused by a limited selection of TCRs, which would suggest a detection of invariant MHCs (MHC-Ib). We therefore assessed the TCR-V β usage of the different donors among naïve and the different Tc subsets (Figure 34). The V β families varied among donors as well as among Tc17 and Tc22 indicating they detect diverse antigen:MHCs combinations. However, Tc17+1 cells displayed a strong enrichment for TCR-V β chains of the V6 family and therefore might recognize antigens presented in a MHC-Ib dependent manner.

reads were shared by the cytotoxic and by the helper-type subsets, respectively (Figure 35B, C). Given the small overlap between those two groups in their shared clonotypes and shared reads, cytotoxic and helper-type CD8⁺ T cells detect distinct epitopes and were most likely involved in diverse antigenic responses. However, within such response heterogeneous differentiation or plasticity allows the differentiation of either Tc1 or Tc17+1 cells and accordingly either Tc2, Tc17 or Tc22 cells.

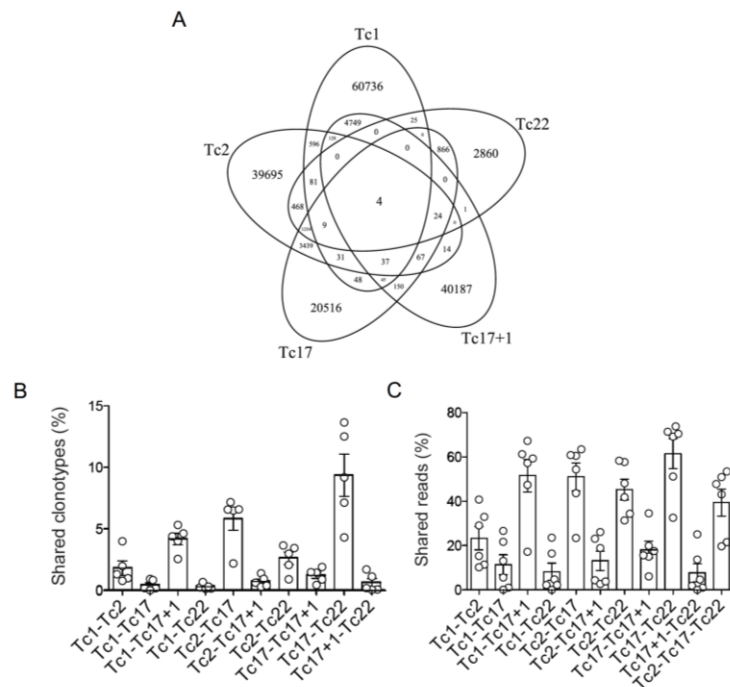


Figure 35: The clone repertoire overlaps among the cytotoxic and among the non-cytotoxic CD8⁺ memory T cell subsets.

(A) Venn Diagram of an example donor visualizes number clonotypes shared among different Tc subsets. **(B)** Summary of clonotype numbers shared among different Tc subsets from all donors. **(C)** Summary of reads shared among different Tc subsets from all donors.

3.5.6 Naïve CD40L⁺ and CD40L⁻ CD8⁺ T cells have distinct TCR repertoires

The implementation of CD40L expression in some CD8⁺ T cells in the thymus and the striking different clone repertoire of memory CD40L⁺ CD8⁺ T cells suggest that the imprinting in the thymus dictates the fate of the CD8⁺ T cell in the periphery. Given this assumption, thymic imprinted CD40L⁺ CD8⁺ SP T cells shape the naïve CD40L⁺ repertoire, which preferentially differentiate into Tc2, Tc17 or Tc22 T cell upon activation. First, we therefore compared the clone

repertoire of naïve CD40L⁺ and naïve CD40L⁻ CD8⁺ T cells. After thymic egress, estimated 50-100 sister clones circulate in the blood (Gonçalves et al., 2017). As 10% of the naïve cells in blood express CD40L, either 1 clone out of 10 sister clones randomly gain CD40L expression ability (same clone, different fates) or every 10th clone experiences a CD40L imprinting signal and shares the CD40L expression ability with its sister clones (one clone, one fate) (Figure 36).

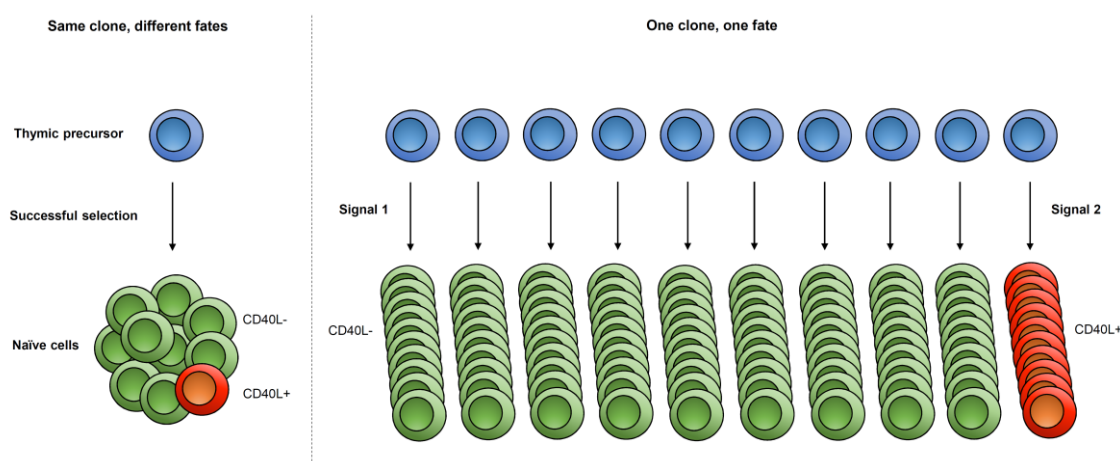


Fig. 36: Hypotheses for CD40L⁺ CD8⁺ T cells development.

Same clone, different fates: during positive selection and proliferation in the thymus a small fraction out of a population of sister clones gains the ability to express CD40L. One clone, one fate: some clones receive a different signal during selection and differentiate into CD40L⁺ cells.

Due to physiological and technical limitations, approximately 1/20th of the total naïve CD8⁺ T cell TCR repertoire can be assessed reducing the possibility of overlaps in the repertoire of two naïve populations. To understand the significance of the overlap among CD40L⁺ and CD40L⁻ naïve CD8⁺ T cells, we calculated the frequency of clones that were found in numbers higher than one clone in the complete repertoire consisting of CD40L⁺ and CD40L⁻ cells. In average 37% of the clones were found multiple times in the total pool as well as among CD40L⁺ or CD40L⁻ naïve CD8⁺ T cells alone (Figure 37A). In a next step, we assessed the overlap of CD40L⁺ and CD40L⁻ clones in the overall pool and a singleton free pool resulting in maximum frequencies around 0.1-1% (Figure 37B). The difference between the two values suggests diverse clone repertoires of CD40L⁺ and CD40L⁻ naïve CD8⁺ T cells with the small overlap rather derived from incomplete stimulation of some CD40L⁺ clones that therefore falsely were assigned as CD40L⁻ clones. As 99% of the TCR β chains were shown to be unique

(Gonçalves et al., 2017), the overlap could also be generated by random overlaps in TCR β sequences that in the end belong to different TCRs with diverse α -chains. Therefore, TCR sequencing demonstrated that naïve CD40L⁺ and CD40L⁻ CD8⁺ T cells are two subsets with diverse TCR repertoires.

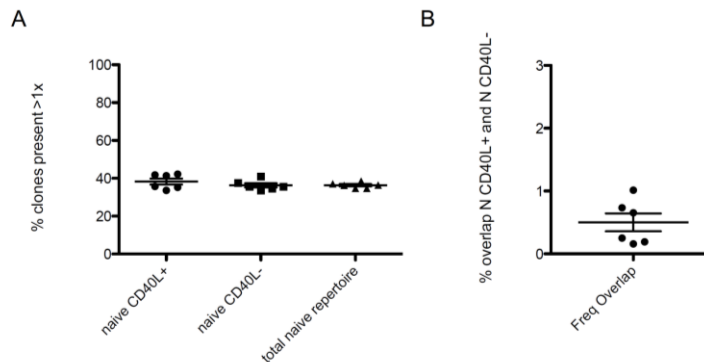


Figure 37: Overlaps in the clonotypes of naïve CD40L⁺ and CD40L⁻ CD8⁺ T cells.

Naïve CD45RA⁺CCR7⁺ CD8⁺ T cells were sorted and stimulated polyclonally with PMA/Iono for 6hrs in presence of α CD40. CD40L⁺ and CD40L⁻ cells were sorted and subsequently used for TCR-sequencing. **(A)** Frequencies of clones present more than once in the clone repertoire. **(B)** Overlap of clonotypes present >1x among CD40L⁺ and CD40L⁻ naïve CD8⁺ T cells. Mean \pm SEM.

3.5.7 CD40L expressing memory T cells are recruited from CD40L⁺ naïve T cells

When a naïve clone encounters its antigen, the cell undergoes massive proliferation and contributes to the immune response. After pathogen/antigen clearance some of the initial clones remain as memory cells. In murine models, not all naïve sister clones that were released from thymus contribute to a specific immune response, therefore a clonal overlap between the naïve and the memory pool should be detectable by TCR sequencing (Quinn et al., 2016). In order to proof whether the clones in the human memory Tc subsets are recruited from the naïve CD40L⁺ or CD40L⁻ pool we compared the clonal overlap of the Tc subsets with naïve CD40L⁺ and CD40L⁻ cells. However, the clone overlap ranged from 0.01 to maximum 2%, suggesting that in humans either the majority of naïve sister clones is recruited to the immune response and/or the part of cells from the whole repertoire in human body we analyzed here was too limited for a reliable readout. However, the non-significant tendencies suggested that Tc1 cells are rather recruited from the CD40L⁻ whereas Tc2, Tc17 and Tc22 cells are rather

derived from the CD40L⁺ naïve CD8⁺ T cell repertoire (Figure 38). For Tc17+1 cells the picture remained inconclusive.

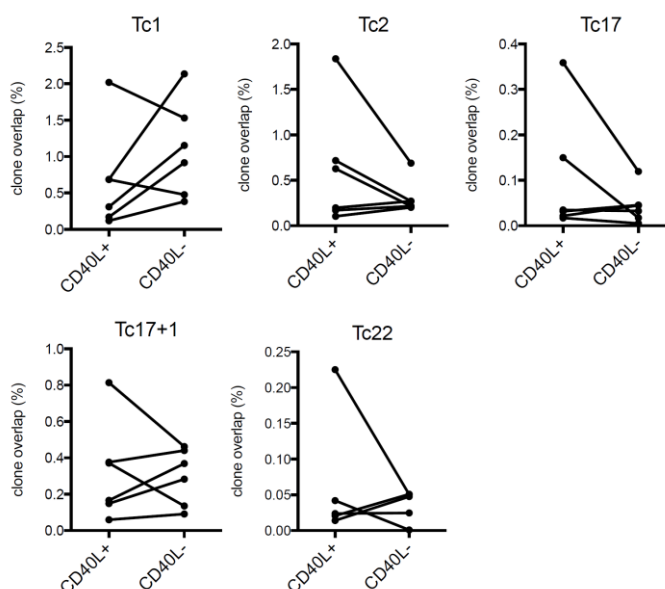


Figure 38: The overlap in clone repertoire among naïve CD40L⁺ and CD40L⁻ cells with memory Tc1, Tc2, Tc17, Tc17+1 and Tc22 cells.

Numbers of clonotypes from the respective naïve and Tc subset were summarized and the numbers of clones found in naïve as well as Tc subset plotted as frequency of total clone numbers.

3.6 Role of CD40L⁺ CD8⁺ T cells in immunity

3.6.1 CD40L expression in systemic lupus erythematosus

We demonstrated that naïve CD40L⁺ and CD40L⁻ CD8⁺ T cells give rise to distinct sets of memory T cell subsets and the high affinity of CD40L⁺ CD8⁺ T cells against self during positive selection suggests a potential role of those memory Tc2, Tc17 and Tc22 cells in the development of autoimmunity. In psoriasis, a chronic inflammatory skin disease, CD8⁺ T cells infiltrating the dermis and epidermis and characterized by IL-17A and IL-22 production have been implicated in the pathogenesis (Hijnen et al., 2013; Res et al., 2010; Zheng et al., 2007). Moreover, the frequencies of IL-22 producing, peripheral CD8⁺ T cells were increased in psoriasis patients (Luan et al., 2014). In line with that, we observed an increase of circulatory non-cytotoxic Tc2, Tc17 and Tc22 CD8⁺ T cells accompanied by

respective changes in cytokine secretion patterns in psoriasis but not atopic dermatitis (S. Warth, PhD Thesis; Loyal et al., submitted).

As increased CD40L levels have been reported in SLE patients (Desai-Mehta et al., 1996; Mak and Kow, 2014), we also analyzed the circulatory T cell compartment from SLE patients in comparison to healthy controls. As reported, we found deregulated cytokine production and increased levels of CD40L not only in CD8⁺ and CD4⁺ memory cells (Figure 39) but also in naïve and CD4⁻CD8⁻ T cell subsets (not shown) (Ohl and Tenbrock, 2011). However, the changes in cytokine levels were not significant and did not correlate with the disease severity measured by SLEDAI score.

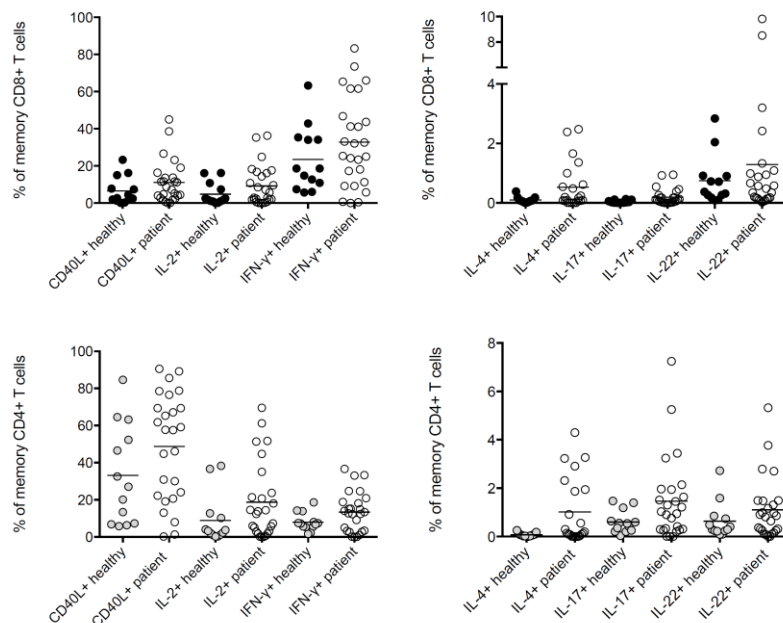


Figure 39: Cytokine secretion of CD4⁺ and CD8⁺ memory T cells in SLE patients.

Human PBMC from healthy donors or SLE patients were stimulated 6hrs with PMA/Iono in the presence of BrefA and cytokine as well as CD40L expression was assessed intracellularly. Pregated on CD3⁺CD45RA⁻ memory CD8⁺ or CD4⁺ T cells. Mean. $n_{\text{healthy}} = 13$, $n_{\text{patients}} = 26$.

Furthermore, the changes in cytokine expression were not reflected by changes in the Tc and Th subset composition. While the frequencies of Tc1 cells remained unaltered, Tc17+1 cells were decreased in all SLE patients. In contrast, frequencies of Tc17 and Tc22 cells tendentially increased in patients with a SLEDAI score >8, whereas Th subsets did not exhibit any significant difference (Figure 40). However, SLE is also associated with aberrant DNA methylation

patterns, which are already imprinted in naïve stage (Absher et al., 2013). Therefore, our data suggests, that the observed changes in cytokine secretion are not caused by deregulated T cell differentiation but rather by deregulated DNA methylation.

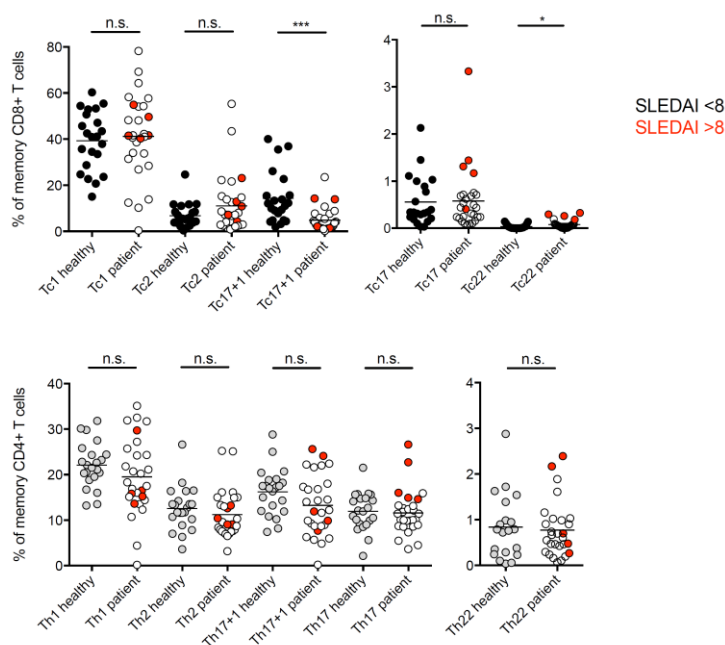


Figure 40: Frequencies of Tc and Th subsets in SLE patients.

Frequencies of Tc/Th subsets in healthy and SLE patients were assessed *ex vivo* on a flow cytometer. Mean. $n_{\text{healthy}} = 22$, $n_{\text{patients (SLEDAI<8)}} = 23$, $n_{\text{patients (SLEDAI>8)}} = 5$. Mann-Whitney t-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.6.2 Role of CD40L⁺CD8⁺ T cell subsets in barriers

Non-lymphoid tissues such as skin, lung and gut possess specialized, resident form of memory T cells that express CD69 and/or CD103 (Schenkel and Masopust, 2014). They can be formed upon priming of T cells in skin accompanied by the induction of a circulatory fraction providing immunity at adjacent barriers or alternatively recruited from the circulatory T cell pool upon inflammation (Gaide et al., 2015; Jameson and Masopust, 2018; Jiang et al., 2012). Among those CD8⁺ T cells located in dermal and epidermal layers of skin a large fraction exhibits a non-cytotoxic phenotype and characteristically lack CD49a expression (Cheuk et al., 2017; Purwar et al., 2011). Since helper-type CD8⁺ T cells lack cytotoxicity, express various markers of skin migration (see Figure 30) and we observed increased frequencies of circulatory Tc2, Tc17 and

Tc22 cells in patients with active psoriasis (S. Warth, PhD Thesis; Loyal et al., submitted), the non-cytotoxic CD8⁺ T cell subsets Tc2, Tc17 and Tc22 might be circulatory variants of skin resident cells (McCully et al., 2012, 2018). Comparison of the Tc-subset gene signatures with published signatures of skin derived dermal CD103⁺ and CD103⁻, epidermal CD103⁺CD49a⁺ and CD103⁺CD49a⁻ T_{RM} and as well as blood derived CLA⁺ and CLA⁻ T_{EM} cells as controls (Cheuk et al., 2017) placed helper-type CD8⁺ T cells in the proximity of the dermal cells (Figure 41A). Tc1 and Tc17+1 cells are heterogenous regarding their CLA expression and additionally contain not only T_{EM} but also T_{CM} cells (Figure 41B) resulting into adjacent but separate clusters of blood derived CLA⁺/CLA⁻ and Tc1/Tc17+1 cell subsets that were both distinct from skin derived cell populations.

The Tc2, Tc17, Tc22 and Th2 subsets that were enriched in psoriasis patients share the production of IL-13 (see Figure 26). IL-13 activates CCL26 secretion by keratinocytes (Kagami et al., 2005), which results in a positive feedback loop of CCR4 expressing T cells and in parallel antagonizes the migration of cytotoxic CXCR3⁺ cells (Nakayama et al., 2010; Purwar et al., 2006). We found high levels of CCL26 in keratinocytes treated with supernatants of stimulated Tc2, Tc17, Tc22 but not of stimulated Tc1 or Tc17+1 subsets (Figure 41C). In contrast, helper-type CD8⁺ T cells did not affect the levels of CXCL11 expression, which is a ligand for CXCR3 expressed by cytotoxic memory CD8⁺ T cell subsets. These results support the hypothesis that circulatory human non-cytotoxic CD8⁺ T cell subsets might migrate into the skin and may play a so far unrecognized role in the amplification of skin immunity.

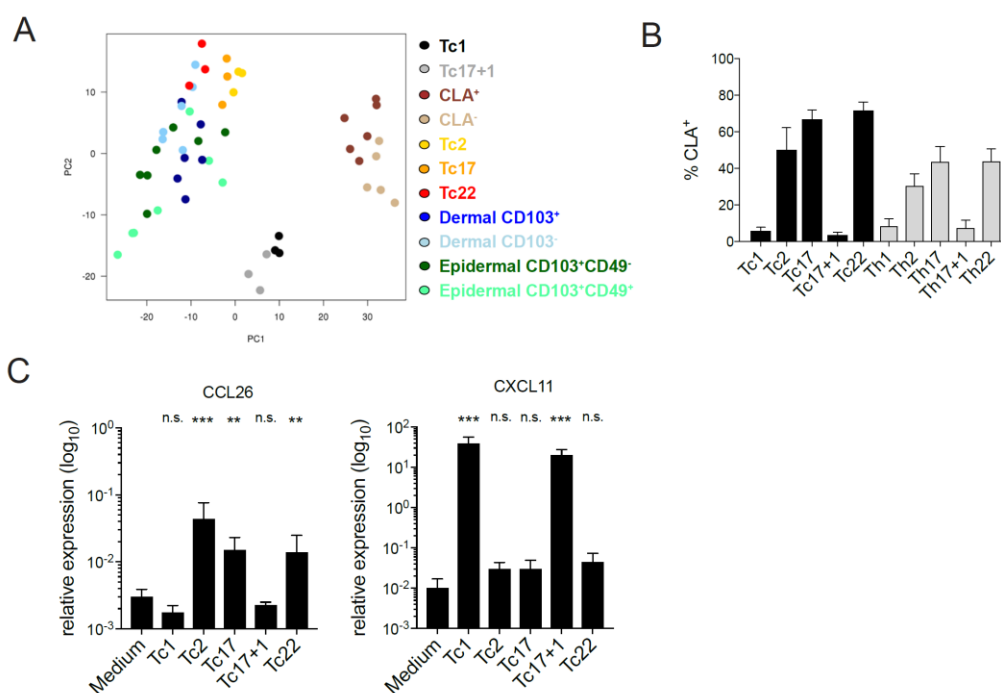


Figure 41: Skin associated signature of non-cytotoxic CD8⁺ T cell subsets.

(A) PCA of CD8⁺ memory T cell subsets and CLA⁺/CLA⁻ blood derived CD8⁺ T_{EM}, CD103⁺/CD103⁻ dermis derived, CD49a⁺/CD49a⁻ epidermis derived CD8⁺ T cells as described in (Cheuk et al., 2017). (B) CLA expression among Tc and Th subsets (n = 4). (C) Relative expression of CCL26 and CXCL11 by keratinocytes stimulated with supernatants derived from sorted and activated Tc cell subsets. Mean ± SEM. Student's t-test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 relative to medium as control.

4. DISCUSSION

CD40L, a central costimulatory receptor to provide help, is transiently expressed upon activation in all CD4⁺ and some CD8⁺ T cells. Previous work showed that around 10% of naïve CD8⁺ T cells are capable to express CD40L whereas the frequency increases to around 30% in the memory compartment (Frentsch et al., 2013). This increase suggests an implementation of CD40L expression during differentiation, which could be partially linked to IL-12 dependent induction in the proliferation phase upon antigen encounter (Stark et al., 2013). The origin of CD40L expressing CD8⁺ T cells with “naïve” phenotype however remained unclear. We demonstrated that CD40L expression requires absent DNA methylation at the *CD40LG* promotor in CD4⁺ and CD8⁺ T cells, which is implemented in the thymus. The ability of CD8⁺ T cells to express CD40L is induced by high affinity detection of self-peptidess during positive selection in human and mouse. While subdivision of thymic CD4⁺ T cells into classical CD4⁺ T cells and natural regulatory T cells is widely accepted, our data proves for the first time that thymic CD8⁺ T cells are also heterogeneous. Moreover, thymus derived CD40L⁺ CD8⁺ T cells differ from CD40L⁻ CD8⁺ T cells in their further development. They give rise to non-cytotoxic memory cells with defined cytokine secretion profiles of Tc2, Tc17 or Tc22 T cells while CD40L⁻ CD8⁺ T cells differentiate into classical IFN- γ ⁺ cells with a cytotoxic capacity. The CD40L⁺ “helper-type” CD8⁺ memory T cell subsets Tc2, Tc17 and Tc22 resemble CD4⁺ T cells in their gene expression and display a skin migratory phenotype. CD8⁺ T cells with helper-type cytokine profiles have been reported in allergy and various autoinflammatory diseases. Therefore, thymus derived helper-type CD8⁺ T cells might represent a unique force in cellular immunity requiring a re-evaluation of CD8⁺ T cell capabilities and functions in human health and disease.

4.1 TCR dependent induction of CD40L expression

Reports based on CD4⁺ T cell analyses indicate that TCR dependent CD40L induction is dependent on NFAT and NF- κ B signaling. Utilizing highly specific chemical blockage during *ex vivo* stimulation of enriched T cells we observed that the induction of CD40L expression is comparably dependent on NFAT but not

NF- κ B signaling in CD4⁺ and CD8⁺ T cells (Figure 10). However, the observation of NF- κ B dependent activation of CD40L based on a proteasome inhibitor mediated blockage of I κ B degradation, which also affects NFAT (Srahna et al., 2001) and binding of NF- κ B to the CD40L locus was shown in cell lines but not for primary cells (Schubert et al., 2002). Therefore, we conclude that NFAT is the central regulator of CD40L expression induction in both CD4⁺ and CD8⁺ T cells, while NF- κ B is abdicable in situations with sufficient TCR stimuli.

4.2 Regulation of CD40L gene accessibility

Another regulatory mechanism of gene expression is to modify the usage and the accessibility of gene regulatory elements. We first assessed whether there are other regulatory elements in CD8⁺ T cells than those described for CD4⁺ T cells by an extensive *in silico* analysis including evolutionary conservation (human-mouse homology), TF binding sites, DNase I hypersensitive sites and CpG sequences within a stretch of +/- 100kb from *CD40LG* gene (Figure 11). Since in diseases with deregulated CD40L expression in CD4⁺ T cells were related to changes in DNA methylation (Lian et al., 2012; Liao et al., 2012; Lu et al., 2007), we assessed the DNA methylation pattern of the known and novel identified candidates to understand a) the location of potential regulatory elements and b) the role of DNA methylation in CD40L expression. DNA methylation analysis of naïve, effector and memory CD8⁺ T cells revealed a strict correlation between absent DNA methylation and CD40L expression ability at the CD40L promotor region but not at other regions (Figure 12). At the 5'-enhancer the methylation pattern displayed an open DNA formation independent of CD40L expression ability. CD4⁺ T cells possess 2 DNase I hypersensitive sites bound by NFAT and GATA3 in this upstream enhancer and these sites were not found in CD8⁺ T cells (Brunner et al., 2008). Therefore, it is possible that these sites contribute to the differences in CD40L expression of CD4⁺ and CD8⁺ T cells. Moreover, the 3'-enhancer, containing two NF- κ B binding sites, was exclusively demethylated in CD4⁺ but not CD8⁺ T cells. Since we demonstrated, that NF- κ B is negligible for CD40L induction upon proper TCR stimulation, this enhancer might rather contribute to CD40L expression/stability in CD4⁺ T cells under suboptimal conditions. Altogether, the ability to express CD40L is highly dependent on the

DNA methylation status at the *CD40LG* promotor in CD4⁺ and CD8⁺ T cells. While the 5'-enhancer might contribute to the CD40L expression in both cell types, the 3'-enhancer is not accessible in CD8⁺ T cells.

4.3 Mechanism of CD40L implementation in the thymus

The absent DNA methylation at the *CD40LG* promotor of naïve CD40L⁺ but not CD40L⁻ CD8⁺ T cells might result from a falsely assignment of the naïve status to cells that have encountered their cognate antigen yet or from demethylation earlier in T cell development. By the exclusion of memory T cells expressing the naïve cell markers CCR7 and CD45RA (T_{SCM}/T_{MNP}) as well as TREC analysis, we demonstrated the true naïve status of naïve CD40L expressing CD8⁺ T cells (Figure 14). Therefore, we assessed CD40L expression in the earlier developmental stages in the thymus. CD40L staining of activated thymocytes revealed CD40L gene expression from DP stage accompanied by CD3 co-expression – a marker of sufficient TCR dependent T cell selection. In SP stage, around 3-5% of thymic CD8⁺ T cells expressed CD40L in human and mouse (Figures 15, 16). Correspondingly, bisulphite sequencing demonstrated absent DNA methylation at *CD40LG* promotor in DP as well as CD4⁺ and CD8⁺ SP CD40L⁺ thymocytes (Figure 17). Based on these results we conclude that CD40L expression is implemented in all CD4⁺ and some CD8⁺ T cells during thymic development at the transition to CD3⁺DP T cells.

Dissection of the developmental stages in human and mouse thymus narrowed down CD40L expression implementation to the CD3⁺CD4⁺CD8^{low} DP stage, a stage at which positive and negative selection as well as CD4 versus CD8 lineage decision has just taken place (Figures 15, 16) (Brugnera et al., 2000; Liu and Bosselut, 2004; Park et al., 2010). The major regulator of CD4⁺ versus CD8⁺ T cell decision is the length of the TCR signal during positive selection, which is dependent on the MHC and the TCR avidity to the presented peptide. While MHC I specific CD8⁺ T cells are selected with a weaker and shorter stimulus, CD4⁺ T cells, that are all expressing CD40L, are selected to a stronger stimulus in a MHC II dependent manner (Liu and Bosselut, 2004). With murine MHC I and MHC II knockout models, we could determine that positive selection of CD40L⁺ CD8⁺ T cells in the thymus is not dependent on the presence of MHC II

but requires the presence of MHCI (Figure 20). Accordingly, we found higher levels of CD5 and NUR77, markers of TCR stimulation strength during positive selection, in CD4⁺ T cells than CD8⁺ T cells. However, CD40L⁺ CD8⁺ T cells displayed higher levels of CD5 and NUR77 than CD40L⁻ CD8⁺ T cells in human and mouse (Figures 21, 23). Correspondingly, transgenic mouse models with different CD5 expressions, expressed according levels of CD40L (Fulton et al., 2015; Kieper et al., 2004). H-Y mice on RAG2^{-/-} background (to exclude any endogenous TCR expression) had low CD5 expression and almost absent CD40L whereas OT-1 mice on RAG2^{-/-} expressed high levels of CD5 and the majority of CD8⁺ T cells were CD40L⁺ (Figure 22).

Among CD4⁺ T cells, the CD5^{high} fraction develop into Tregs during negative selection in the medulla or become depleted (Ono and Tanaka, 2016). We utilized CCR7, a receptor necessary for migration from the cortex to the medulla, to identify whether positive selection in the cortex or negative selection in the medulla implements CD40L expression in the CD5^{high} CD8⁺ T cells (Takada et al., 2015; Van Laethem et al., 2013). As CD40L is detectable prior to CCR7 upregulation, T cells must have gained their ability to express CD40L during positive selection (Figure 24). Interestingly, the frequencies of thymus derived natural CD25⁺FoxP3⁺CD4⁺Tregs among CD4⁺ T cells and CD40L⁺CD8⁺ among CD8⁺ T cells are comparable with around 3-5% in thymic SP stage and around 10% among naïve PBMC derived T cells and therefore might reflect the thresholds of high affinity selected T cells generated by T cell maturation and selection in the thymus (Moran et al., 2011).

The intensity of TCR stimulus during positive selection not only regulates CD5 and NUR77 expression intensity but also influences the susceptibility of the pre-mature T cell to cytokine mediated signaling. A strong stimulus activates a CD4 lineage transcription factor ThPOK whereas weak signaling allows the induction of CD8 lineage transcription factor Runx3 by cytokines (Egawa and Littman, 2008; Muroi et al., 2008; Park et al., 2010; Wang et al., 2008). These two transcription factors ThPOK and Runx3 were associated with CD40L expression in several experimental setups. Lack of Runx3 expression or overexpression of ThPOK led to a moderate CD40L expression in cytotoxic CD8⁺ T cells (Shan et al., 2017; Tsuchiya et al., 2016). Furthermore, binding of Runx3 to the *CD40LG* locus adjacent to the downstream enhancer was observed in

mouse (GSE50130, Lotem et al., 2013), suggesting a possible direct regulation of CD40L expression by Runx3. High affinity selection of CD8⁺ T cells therefore might open a window in the lineage transcription factor network that induces a CD4⁺ cell like DNA demethylation of CD40L locus together with CD8⁺ lineage decision. Despite CD40L⁺ memory CD8⁺ T cells expressed reduced levels of Runx3, no clear difference was observed in naïve and thymic SP CD8⁺ T cell stages (Figure 31). However, repression of CD40L in the majority of CD8⁺ T cells not only rely on DNA but also histone methylation, which can be repressed by ThPOK utilizing CXXC5 (Tsuchiya et al., 2016). Therefore, further epigenetic analyses are needed to possibly link high affinity selection with DNA demethylation at the CD40L locus in CD8⁺ T cells.

4.4 Stability of CD40L expression

When we separated CD40L⁺ and CD40L⁻ CD8⁺ T cells *ex vivo* and cultivated them with or without cytokines, we could observe that CD40L⁻ cells stably lack CD40L expression in thymic SP CD8⁺, naïve CD8⁺ as well as memory CD8⁺ T cells suggesting that the central CD40L implementation mechanism is the DNA demethylation during positive selection in the thymus (Figure 18). One exception is the cytokine IL-12 which is capable to implement CD40L expression in proliferating CD40L⁻ naive cells resulting into cytotoxic, IFN- γ producing CD40L⁺ CD8⁺ T cells. We could previously show that the IL-12 dependent induction of CD40L occurs in a STAT4 dependent manner, which binds to the CD40L promotor (Stark et al., 2013). As the DNA methylation pattern in T_{EM} and T_{CM} cells (see Figure 12) does not distinguish between the thymus imprinted and the IL-12 imprinted CD40L⁺ cells, and the frequencies of IL-12 imprinted cells in peripheral blood are higher than those of the thymus imprinted cells, we conclude that IL-12 treatment induces CD40L gene expression by mediating *CD40LG* locus demethylation similar as during positive selection. This is in line with the observation, that the mechanism is strictly proliferation dependent suggesting STAT4 inhibits DNA remethylation after cell division by inducing DNA hyperacetylation of the loci it is bound to (Yu et al., 2007).

While CD40L expression in CD4⁺ T cells is stable beginning with the implementation in the thymus, CD40L⁺ CD8⁺ T cells exhibit unstable CD40L

expression in thymocytes with increasing stability over naïve to memory cells. This difference in stability is reflected by the progress of DNA demethylation in CD8⁺ T cells (Figures 12, 17). Besides, CD4⁺ but not CD8⁺ T cells have a demethylated downstream enhancer containing NF-κB binding sites (Schubert et al., 2002). By transfection of primary CD4⁺ T cells with luciferase reporter gene constructs containing either the promoter alone or together with the enhancer, Schubert et al. measured higher luciferase activity upon polyclonal activation when the enhancer was present. In striking contrast, we demonstrated that NF-κB blockage did not affect CD40L expression levels neither in CD4⁺ nor in CD8⁺ T cells. However, transfected constructs are susceptible to unwanted side effects. Therefore, genomic manipulation would be necessary to verify the role of this enhancer. As CD4⁺ T cells exhibit an overall higher stability of CD40L expression from the earliest developmental stages on, this enhancer might contribute rather to CD40L expression stability than its overall expression levels. Moreover, the upstream enhancer contains a GATA3 element, which is demethylated beginning with thymic SP T cell stage in all T cells and remains demethylated until memory stage where thymic imprinted CD40L⁺ CD8⁺ T cells (Tc2, Tc17, Tc22) begin to express GATA3 (Figures 12, 17) (Brunner et al., 2008). Since CD4⁺ T cells express GATA3 from thymic SP stage on, GATA3 binding to the enhancer might also support CD40L expression stability. Finally, we observed that in congruence with previous reports from CD4⁺ T cells, strong CD28 signals during naïve cell activation have a supportive effect onto CD40L stability of CD8⁺ T cells, too (Figure 19) (Johnson-Léger et al., 1998). This effect is probably regulated by the CD28 responsive element in the CD40L locus, which is bound by CD28 induced NFAT and NF-κB (Parra et al., 2001).

4.5 The fate of thymic imprinted CD40L expressing CD8⁺ T cells

We could show that the CD45RA⁻ CD8⁺ memory compartment can be dissected homologous to CD4⁺ T helper cells into the subsets Tc1, Tc2, Tc17, Tc17+1 and Tc22 according to their expression of the chemokine receptors CCR6, CCR4, CCR10 and CXCR3 with unique cytokine expression profiles (Figure 26) (S. Warth, PhD Thesis; Acosta-Rodriguez et al., 2007; Bonecchi et al., 1998; Sallusto et al., 1998; Trifari et al., 2009). Tc1 and Tc17+1 possess a cytotoxic phenotype

and contain some CD40L⁺ CD8⁺ T cells that are all IFN- γ co-producing and therefore most likely generated by IL-12 dependent priming upon naïve T cell activation. In contrast, Tc2, Tc17 and Tc22 are all expressing CD40L but no cytotoxic effector molecules such as Perforin, Granzymes or IFN- γ (Figures 25, 31). This raised the question whether thymic imprinted CD40L⁺ cells not only give rise to the naïve CD40L⁺ pool but also strongly affect the memory T cell fate. TCR sequencing showed that among naïve CD40L⁺ and CD40L⁻ cells around 40% of the clones are present more than one time in the repertoire – however only 0.5% of the total clones overlap between these two groups. Consequently, CD40L⁺ naïve cells seem to represent a separate cell type that recognizes different epitopes than CD40L⁻ cells (Figure 37). Strikingly, a similar separation could be observed with the TCR sequences of Tc1/Tc17+1 on one side and Tc2/Tc17/Tc22 on the other side (Figure 35). While the TCR sequences displayed a strong overlap among the Tc2, Tc17 and Tc22 and among the Tc1 and Tc17+1 subsets, there was little overlap between these two groups. We compared the TCR overlap between the naïve and the memory subsets but found too little overall overlap for a statistically significant correlation whether CD40L⁺ memory cells (Tc2/17/22) derived from the CD40L⁺ and Tc1/Tc17 from CD40L⁻ naïve pool respectively (Figure 38). Importantly, the readout from this strategy is limited by the potential different migration behavior of the Tc memory subsets as for example Tc2, Tc17 and Tc22 cells express markers pointing to a migration into skin and therefore large parts of their TCR repertoire would be missed by the analysis of blood derived, circulatory cells. Since it is additionally unknown how many of the naïve sister clones present at a defined time in the body are recruited into the memory response in humans and sequencing the whole TCR repertoire of a subject is practically not possible, we tried a bioinformatical approach. We utilized the GLIPH algorithm to assess the overlap of the TCR β CDR3 sequences – the areas of epitope recognition which exhibit highest inter-TCR variation – with a hamming distance of ≤ 1 (Glanville et al., 2017). Unfortunately, in large datasets this approach results in clusters above 50% of the CDR3 sequences and therefore requires additional TCR α and HLA information for a feasible output.

4.6 Phenotype of the CD40L expressing memory CD8⁺ T cells

Cytokine secretion analysis demonstrated that the separation of CD8⁺ memory T cells into the subsets Tc1, Tc2, Tc17, Tc17+1 and Tc22 directly *ex vivo* utilizing chemokine receptors adopted from CD4⁺ T cells is highly reliable. These subsets secrete the expected pattern of the cytokines IFN- γ , IL-4, IL-17 and IL-22 upon polyclonal activation (Figure 26). Furthermore, removal of donor and CD4⁺ versus CD8⁺ related differences in the RNA-seq data by batch algorithmus led to clustering of each CD8⁺ Tc subset with its respective CD4⁺ Th subset (Figure 27). This extreme similarity underlines the findings of publications claiming that both cell types utilize the same differentiation mechanisms based on the transcription factors T-bet, GATA3, ROR γ t and AHR (Figure 29) (Sallusto, 2016). Unsupervised clustering of the genes furthermore revealed a cluster consisting of the CD8⁺ T cell subsets Tc1 and Tc17+1 separated from the subsets Tc2, Tc17 and Tc22. Instead, the latter ones clustered with the CD4⁺ T cell subsets Th1, Th2, Th17, Th17+1 and Th22. This difference is strongly based on the cytotoxic signature of Tc1/Tc17+1 including cytolytic molecules (*Granzymes, Perforin*) and marker associated with a cytotoxic phenotype such as *CRTAM* and *LAG3* (Figure 30). However, in contrast to Tc1 we did not observed any Granzyme B and Perforin protein in Tc17+1 *ex vivo* (Figure 31). While mRNA of lytic enzymes is prestored, translation requires cell activation (Pipkin et al., 2010). Therefore, circulating Tc17+1 cells either did not encounter their cognate antigen recently or their ROR γ t expression actively repress the production of cytolytic molecules in a STAT3 dependent manner (Ciucci et al., 2017; Curtis et al., 2009).

Based on the observation that cytotoxic signatures are implemented and maintained by Runx3 in CD8⁺ as well as in cytotoxic CD4⁺ T cells and the non-cytotoxic Tc2, Tc17 and Tc22 cells CD8⁺ T cells express GATA3 instead, which counteracts cytolytic signatures (Figure 29) (Mucida et al., 2013; Pipkin et al., 2010; Woolf et al., 2003; Xiong et al., 2013), we assessed Runx3 levels in multiple cytotoxic and non-cytotoxic subsets. We could demonstrate that all cytotoxic subsets express Runx3 while Tc2, Tc17, Tc22 CD8⁺ and helper CD4⁺ T cells lacked Runx3 expression. Moreover, cytotoxic CD4⁺ T cells express Runx3 and at the same time are incapable to express CD40L, which indicates that Runx3

might be a central regulator of cytotoxic signature versus CD40L expression. As Runx3 and ThPOK control CD4⁺ versus CD8⁺ T cell fate in the thymus, reduced Runx3 levels might have been implemented in the thymus and underlie the CD40L expression ability of the thymic imprinted CD40L⁺ CD8⁺ T cells. However, we did not observe differences between Runx3 and ThPOK expression in thymic or naïve CD40L⁺ and CD40L⁻ CD8⁺ T cells (Figure 31). In concordance, it was just shown that Runx3 expression levels of memory cells are implemented upon TCR activation of naïve cells by remodeled genome access prior to the first cell division (Wang et al., 2018). Cytotoxic CD4⁺ T cells moreover have lost their ThPOK expression (Mucida et al., 2013). It has been shown that ThPOK induces CXXC5, which in turn prevents histone methylation and associated repression of the *CD40LG* locus. Therefore, the lack of CD40L expression in cytotoxic CD4⁺ T cells might be ThPOK dependent (Tsuchiya et al., 2016). However, CD40L expressing CD8⁺ T cells displayed repressed ThPOK in all developmental stages comparable to CD40L⁻ CD8⁺ T cells (Figure 31). Additionally, a CD4-Cre ThPOK^{fl/fl} mouse model that lacks ThPOK expression from DP stage on displayed a defective development of mature CD4⁺ T cells but still gave rise to CD40L⁺ CD8⁺ T cells (not shown). Nevertheless, it was shown that Runx3 knockdown as well as ThPOK overexpression are capable of inducing CD40L expression in CD8⁺ T cells (Shan et al., 2017; Tsuchiya et al., 2016). Therefore, Runx3 seems to be critical for the implementation of the cytotoxic phenotype in memory CD8⁺ T cell subsets but whether and how Runx3 and ThPOK contribute to the regulation of CD40L expression remains elusive.

In order to further assess how cytotoxic versus non-cytotoxic/helper-type CD4⁺ as well as CD8⁺ T cells differ from each other, we analyzed the most differentially expressed genes among the cytotoxic Tc1 and Tc17+1 compared to the non-cytotoxic Tc2/Tc17/Tc22 and CD4⁺ Th subsets. We could identify SLAMF7 and IL-6R as opposed expressed indicators of cytotoxic versus helper phenotype of T cells. SLAMF7 is an already well-known marker of NK and NKT cells (Boles and Mathew, 2001; Bouchon et al., 2001). However, together with IL-6R it distinguishes not only the non-cytotoxic naïve and Tc2/17/22 populations from the classical cytotoxic Tc1/17+1 and effector CD8⁺ T cells but also cytotoxic CD4⁺ from their normal helper counterparts (Figure 32). SLAMF7 is co-expressed on all Granzyme B⁺ and Perforin⁺ cells as well as on all CD56⁺ cells that includes

NK and NKT but also cytotoxic type1 innate lymphoid cells (ILC1) (Spits et al., 2016). Comte et al. just recently demonstrated that ligation of SLAMF7 increases cytotoxic degranulation and IFN- γ secretion capacity in NK but also CD8⁺ T cells (Comte et al., 2017). In contrast, classical IL-6R mediated IL-6 signaling is associated with anti-inflammatory processes (Schaper and Rose-John, 2015). It increases the expansion and maintenance of CD4⁺ T cells (Rochman et al., 2005) and prevents their conversion into FoxP3⁺ Tregs (Korn et al., 2008). Therefore, IL-6R expression on CD8⁺ T cells might counteract the implementation of a cytotoxic phenotype and contribute to cell survival instead. Altogether, our data demonstrates that SLAMF7 provides a novel marker of overall cytotoxicity among cells of the lymphoid lineage whereas IL-6R identifies non-cytotoxic cells among lymphocytes.

The CD40L⁺ memory CD8⁺ T cell subsets Tc2, Tc17 and Tc22 share their non-cytotoxic phenotype and a CD4⁺ helper cell related gene expression signature but they differ in their cytokine secretion profile. The strong overlap between CD4⁺ and CD8⁺ memory T cell subsets in the usage of the central differentiation regulating transcription factors T-bet, GATA3, ROR γ t and AHR suggests that similar stimulation/cytokine milieus underlie the priming. However, priming of naïve CD8⁺ T cells with CD4⁺ T cell based protocols worked very inefficiently with maximum 0.1% (Tc17), 0.7% (Tc22) and 10% (Tc2) of primed CD8⁺ expressing the respective dominant cytokine IL-17, IL-22 and IL-4 (Kondo et al., 2009; Liu et al., 2011; Vukmanovic-Stejic et al., 2000). Interestingly, these frequencies resemble the total frequencies of the subsets Tc17, Tc22 and Tc2 found *ex vivo* in blood (S. Warth, PhD Thesis). Together with the almost exclusive TCR repertoire, this might provide a hint that naïve CD8⁺ CD40L⁺ and CD40L⁻ cells have an intrinsic fate implemented. Alternatively, the used priming conditions might not reflect physiological conditions properly. CD4⁺ T cell priming has been shown to be dependent not only on the cytokine milieu but also on the strength and duration of the TCR stimulus, the stimulation dependent induction of cytokine receptor expression as well as the antigen dose and the type of APC that mediates the T cell activation (van Panhuys, 2016; Rothoefl et al., 2003). In CD8⁺ T cells such factors were hypothesized to affect the T_{CM} versus T_{EM} as well as T_{RM} differentiation but not taken into account for Tc subset differentiation so far (Kaech and Cui, 2012; Maru et al., 2017). The expression of different homing

receptors among CD8⁺ Tc subsets e.g. the skin homing receptors CCR4, CCR8, CCR10 among the non-cytotoxic subsets furthermore support the assumption that diverse priming conditions and associated APCs/sites of activation underlie their emergence. It has already been demonstrated that skin related APCs differ from classical dendritic cells, which dominate the systemic cytotoxic responses to viruses and bacteria in blood, an aspect that is further discussed below (Kashem et al., 2017).

The TCR repertoire of the memory CD8⁺ T cells inherited a striking observation. For CD4⁺ memory T cells it has been shown that the naïve cell preferentially differentiates into one specific Th subset based on the pathogen that is faced but can give rise to a small number of other Th subsets at the same time (Acosta-Rodriguez et al., 2007; Arlehamn et al., 2013). Such asymmetric cell division was also described for the Effector/T_{EM}/T_{CM} differentiation of CD8⁺ T cells (Buchholz et al., 2013; Chang et al., 2007; Gerlach et al., 2013; Plumlee et al., 2013; Stemberger et al., 2007). Our TCR sequencing data revealed that also among T_{EM} and T_{CM} CD8⁺ T cells the flexibility is restricted. A naïve T cells can develop into a cytotoxic subset with a certain flexibility whether it gains a Tc1 or a Tc17+1 phenotype or alternatively into a non-cytotoxic subset with flexibility limited to Tc2, Tc17 or Tc22 phenotypes (Figure 35). The little overlap among the cytotoxic and the non-cytotoxic, helper-like compartment in the TCR and in the priming strongly indicates different combinations of antigen epitopes/stimuli underlying their differentiation. These combinations at the same time have to meet the conditions to give rise to T_{CM} as well as T_{EM} phenotype since those were found among all Tc subsets with a tendency to T_{CM} for non-cytotoxic subset (S. Warth, PhD Thesis).

4.7 The role of CD40L expressing CD8⁺ T cells in immunity

We found IL-12 induced, IFN- γ co-expressing CD40L⁺ CD8⁺ T cells in large frequencies in several models of viral and bacterial immune responses (Durlanik et al., 2016; Schulz et al., 2015). These cells are capable of activating APC in a CD40L dependent manner similar to CD4⁺ T cells (Frentsch et al., 2013). Just recently, Tay et al. could show that this activation of DC by CD40L⁺ CD8⁺ T cells

provides a positive feedback loop that boost their expansion in inflammatory responses (Tay et al., 2017).

But what role do thymic imprinted CD40L⁺ CD8⁺ T cells play in the immune system? We demonstrated that these cells are selected with high affinity against self during positive selection, differ from their CD40L⁻ counterparts in naïve state and almost exclusively develop into type Tc2, Tc17 and Tc22 memory T cells, which exhibit a non-cytotoxic, helper-like phenotype and a TCR repertoire diverse from the one of the cytotoxic subsets. There are a couple of explanatory models that might unravel the role of thymic imprinted CD40L⁺ CD8⁺ T cells: their responsiveness & survival, migration & localization as well as their function.

4.7.1 Responsiveness and survival

The high affinity selection in thymus and higher CD5 expression of CD40L⁺ CD8⁺ T cells can have multiple consequences on their functionality in the periphery. The TCR sensitivity of T cells to self-peptides in the thymus influences the naïve phenotype of the cells. In murine models, CD5^{high} cells were more susceptible and consequently proliferated better in the presence of the cytokines IL-2, IL-7 and IL-15 (Cho et al., 2010; Fulton et al., 2015; Palmer et al., 2011). Higher affinity to self furthermore correlated with a stronger binding to foreign antigens accompanied by enhanced reactivity of CD4⁺ and CD8⁺ T cells (Fulton et al., 2015; Mandl et al., 2013). For CD4⁺ T cells it was shown that the higher responsiveness of CD5^{high} cells is not TCR dependent but an intrinsic feature (Persaud et al., 2014). Accordingly, those TCR with highest affinity among CD8⁺ T cells could be activated directly by peptides without CD8 co-receptor binding to the TCR:MHCI complex, which is usually required for efficient antigen recognition and CTL priming (Holler and Kranz, 2003; Wooldridge et al., 2007).

However, higher affinity to self is accompanied by a higher risk for unspecific and uncontrolled activation and consequent development of autoimmunity. So why and how does the immune system tolerate CD8⁺ T cells with high specificity to self in the periphery? There are several mechanisms of tolerance beyond the selection in thymus: induction of anergy, exclusion from areas of antigen presence/formation of immune privileged sites and repression

by Tregs. We could not find increased expression of anergy/senescence markers such as LAG-3, CD160, PD-1, TIM-3, KLRG-1 and CD57 in CD40L⁺ CD8⁺ T cells in thymus or periphery (not shown). Furthermore, type Tc2, Tc17 and Tc22 were reported to be located at almost every part of the body: skin, gut, lung and CNS (Cheuk et al., 2014; Song et al., 2018; Tang et al., 2012; Tzartos et al., 2008). Whether regulatory T cells prevent autoantigen specific responses of CD5^{high} CD8⁺ T cells in the periphery needs to be assessed. However, CD40L⁺ CD8⁺ memory T cells - similar to iTregs - are capable of producing the anti-inflammatory cytokine IL-10 and therefore might limit the extent of their own responses (Figure 26). Their lack of cytolytic effector functions moreover might provide a form of intrinsic protection mechanism that prevents aberrant cell destruction. Additionally, CD5 itself is capable of lowering the TCR activation threshold in periphery and even can keep cells unresponsive (Dalloul, 2009; Hawiger et al., 2004). Off note, T cell survival and maintenance of responsiveness in the periphery requires tonic TCR signaling by continuous TCR-self-peptide-MHC interaction (Takada and Jameson, 2009), which further modulate the CD5 levels (Smith et al., 2001). Therefore, high CD5 levels in thymic emigrants may be not fixed throughout naïve and memory stages of T cells and consequently in post-thymic stages other mechanisms are required to ensure that CD8⁺ T cells with a high affinity to self-peptides are only activated in controlled environments.

Lastly, there is inconsistent knowledge about how the mechanisms of the positive and negative selection is shaping the TCR affinity repertoire. Klein et al. proposed that the majority of T cells are selected to private peptides generated by the thymusproteasome in a way they are only bound with low affinity by the TCRs resulting into an increase of T cells passing positive and negative selection. However, some T cells instead detect public peptides generated by the immuneproteasome that are also presented and bind them with a comparably higher affinity resulting in re-encounter of these public peptides during AIRE mediated negative selection and (if they are not eliminated) into clones with a better reactivability in periphery (Klein et al., 2014). This share of labor between the two types of proteasomes increases the total TCR pool of T cells egressing into circulation. However, absence of thymusproteasomes (all variants) – allowing only the processing and presentation of public peptides by immunoproteasomes – resulted in the absence of CD5^{high} clones and altered the

TCR pool but did not diminish the overall diversity (Kincaid et al., 2016). Therefore, we would hypothesize that CD5 is implemented upon recognition of private peptides and those cells binding too strong to public peptides are depleted during AIRE dependent negative selection. Consequently, CD5^{high} CD40L⁺ CD8⁺ T cells would only bind those peptides with high affinity that by chance strongly resemble those designed by the thymus proteasome and usually are not present in the periphery as other proteasome variants shape the peptide repertoire in peripheral APCs. Since the CD5^{high} CD40L⁺ CD8⁺ T cell phenotype is already observable in the cortex, we could exclude the possibility, that similar to Tregs, CD8⁺ T cells with high TCR affinity to peripheral autoantigens were purposefully disarmed during negative selection by implementation of a phenotype that leads to CD40L expression and a selective development into non-cytotoxic memory T cells (Figure 24).

4.7.2 Migration and localization

With CCR4, CCR8, CCR10 and CLA, the CD40L⁺ memory CD8⁺ T cell subsets express multiple markers indicative of a skin homing capacity (Figure 30). While CCR8 expression is instructed in naïve cells by keratinocytes (Gaide et al., 2015; McCully et al., 2012), CCR4, CCR10 and CLA are induced upon antigen encounter presented by DC in skin draining lymph nodes (Campbell and Butcher, 2002; Campbell et al., 1999, 2007). Interestingly, similar to the circulatory cytotoxic versus non-cytotoxic memory CD8⁺ T cell subsets, CCR8⁻ and CCR8⁺ tissue resident memory CD8⁺ T cells from skin differ in their TCR repertoire (McCully et al., 2018). We could demonstrate that the gene expression signature of the circulatory, non-cytotoxic, CD40L⁺ memory cells strongly resembles that of CD8⁺ T cells isolated from skin (Figure 41). Together with the increased frequencies of circulating Tc2, Tc17 and Tc22 cells in psoriasis patients, the data gathered in this thesis indicate a role of CD40L⁺ helper-type memory CD8⁺ T cells in skin immunity. In clinically healed psoriasis patients enriched frequencies of IL-17 producing CD103⁺ T_{RM} were found in skin (Cheuk et al., 2014). Therefore, circulatory helper-type CD8⁺ T cells might be derived from skin based immune responses and able to (re)populate remote areas of skin. We showed that helper-type CD8⁺ T cell derived IL-13 induced CCL26 secretion by

keratinocytes, which might result in a positive feedback loop (Figure 41) (Kagami et al., 2005). CCL26 was demonstrated to recruit CCR4 expressing T cells and in parallel antagonizes the migration of cytotoxic memory and effector CD8⁺ T cells (Nakayama et al., 2010; Purwar et al., 2006). Moreover, the chemokine CCL20 is secreted by keratinocytes in healthy skin to promote wound healing (Kennedy-Crispin et al., 2012). However, it is also a central player in psoriasis as it recruits pathogenesis driving DC and T cells that express the CCL20 receptor CCR6 into lesions (Getschman et al., 2017; Kim et al., 2014). CCL20 is strongly enriched in psoriatic lesions and correlates with the CCR4 expression (Loyal et al., submitted). Therefore, CCR4⁺ helper-type CD8⁺ T cells with their CCL20 induction capacity may directly contribute to the pathogenesis of psoriasis.

The activation and priming conditions of helper-type CD8⁺ T cells in skin however are still a challenge to answer. While keratinocytes function as barrier and are capable of recruiting cells into the epidermis, they are no efficient antigen presenters. Instead, the human skin possesses 3 major DC subsets: Langerhans cells (LC), CD14⁺ DC and CD1a⁺ DC. Langerhans cells are located in the epidermis, can efficiently cross-presentate (uptake, process and present extracellular) antigens and strongly induce IFN- γ secretion in CD8⁺ T cells. They are competent CTL primers, possibly mediated by their ability to secrete the cytokine IL-15, which evokes cytotoxicity of CD49a⁺ epidermal CD8⁺ T cells (Cheuk et al., 2017; Oh et al., 2004). In contrast, CD14⁺ and CD1c⁺ DCs are located in the dermis. CD1c⁺ DC possess Th1 and Th2 priming capability and readily migrate into lymphatic vessels, carrying antigens to lymph nodes (Angel et al., 2006). CD14⁺ DC polarize naïve CD4⁺ T cells into follicular helper T cells and induce the rise of low cytotoxic, IL-13 secreting CD8⁺ T cells (Klechevsky et al., 2008). Since non-cytotoxic memory CD8⁺ T cells share the expression of IL-13 (Figure 26), CD14⁺ DC might preferentially interact with and prime CD40L⁺ CD8⁺ T cells. Interestingly, Langerhans cell-mediated priming in the presence of anti-CD8 blocking antibody give rise to IL-13⁺ CD8⁺ T cell at frequencies comparable to CD14⁺DC mediated priming. CD14⁺ DC express ILT2 and ILT4 receptors that bind classical (HLA-A and HLA-B) and non-classical (HLA-G1, -E, and -F) MHC and antagonistically compete with the CD8 coreceptor for its MHC binding (Banchereau et al., 2012; Endo et al., 2008; Shiroishi et al., 2003). CD8 coreceptor binding to MHCI is usually required to stabilize the MHC binding to

the TCR and augments the TCR sensitivity (Artyomov et al., 2010). This leaves two options: a) CD40L⁺ but not the CD40L⁻ CD8⁺ T cells were activated even in the absence of CD8 binding due to their high affinity or bridged by the additional signal derived from CD40L:CD40 interaction or b) specialized APCs such as CD14⁺ DC that block CD8 binding to the TCR:MHC complex inherit features that allow them to mediate a potent activation of CD40L⁺ CD8⁺ T cells. As CD40L⁺ naïve cells possess a TCR repertoire diverse from CD40L⁻ cells, it is possible that CD14⁺ DC present a unique peptide repertoire that is for example designed by a specialized proteasome and modified in a way that only TCR of CD5^{high} cells can bind strong enough and consequently become properly activated. CD14⁺ DC moreover were shown to secrete the cytokines IL-10 and TGF- β (Klechevsky et al., 2008). The presence of IL-10 enables also LC to prime non-cytotoxic CD8⁺ T cells whereas block of IL-10 in CD14⁺DC does not prevent it. As a prominent fraction of skin CD8⁺ T cells possess a non-cytotoxic phenotype (Cheuk et al., 2017; McCully et al., 2018) they might play a protective role in skin e.g. in wound healing or tolerance of commensal bacteria. It was recently demonstrated that murine non-cytotoxic skin Tc17 cells were primed in response to commensal bacteria derived peptides (Linehan et al., 2018). Those display an immunomodulatory signature that overlaps with the gene expression in our human circulatory non-cytotoxic CD8⁺ T cells including the upregulation of *TNFRSF4*, *TNFRSF11a*, *TNFRSF18*, *TNFSF11*, *ICOS* and *CTLA4* (Figure 30). The murine skin resident Tc17 cells detect peptides presented by the invariant MHCIIb molecule H2M3 and positively affect wound healing processes. H2M3 presents peptides that contain an N-formyl-Methionine (fMet) sequence, which is a protein translation initiation signal in bacteria and of a small group of mitochondrial proteins (Fischer Lindahl et al., 1997). The distinct TCR repertoire of helper-type cells compared to cytotoxic cells may therefore result from antigen recognition presented by a so far unidentified human homolog of murine H2M3 MHCIIb. As the V β -sequences of the TCR are also responsible for the binding of invariant types of MHCs display a high variance in helper-type CD8⁺ memory T cells, the searched invariant MHC has to display a broad V β -usage similar to the Qa-1 homolog HLA-E (Figure 34). Moreover, our data generated from the MHCII KO model verified that the priming of CD40L⁺ CD8⁺ T cells is dependent on the

presence of β -microglobulin which is a component of MHC I but also of invariant MHCs (Figure 20).

4.7.3 Function

a) Providing help when $CD4^+$ T cells are limited/ MHC I dependent manner:

Another hint regarding the role of $CD40L^+$ $CD8^+$ T cells in immunity comes from CD40L itself. Proper B cell activation and respective humoral immunity is dependent on T cell mediated help. The actual concept relies on MHC II presentation of antigens by B cells that are detected by $CD4^+$ T cells, leading to T cell activation and CD40L upregulation. Interaction of CD40L with CD40 on the B cell provides the necessary “help” signal to induce B cell maturation, class switching and somatic hypermutation, which can be further supported by T cell secreted cytokines (IL-4, IFN- γ). Helper-type Tc2, Tc17 and Tc22 $CD8^+$ T cells express CD40L with an intensity comparable to $CD4^+$ T cells. $CD40L^{high}$ IL-2 co-secreting clones were shown to be superior to $CD40L^{int}$ clones (IFN- γ co-producers) in B cell activation and induction of Ig secretion (Hermann et al., 1995). Furthermore, in HIV patients with reduced $CD4^+$ T cell levels, the majority of $CD8^+$ T cells exhibit an IL-4 secreting Tc2 phenotype and is capable of providing B cell help (Maggi et al., 1994, 1997). The increased frequency of $CD40L^+$ $CD8^+$ T cells when $CD4^+$ T cell-mediated help is limited such as in ThPOK^{-/-} and MHCII^{-/-} mice models and $CD4^+$ depletion experiments supports the ability of helper-type $CD8^+$ T cells to provide MHC I dependent B cell help. As humoral immunity is ineffective against intracellular infections that are classically presented on MHC I, this $CD8^+$ T cell mediated help might rather play a role in the response to cross-presented antigens.

Help was furthermore shown to be an important aspect of cell mediated immune responses (Bennett et al., 1998; Janssen et al., 2003; Schoenberger et al., 1998; Shedlock and Shen, 2003). $CD4^+$ T cells activate and license DC by CD40L-CD40 interaction, which leads to MHC upregulation and enables the DC to provide sufficient secondary (co-stimulatory receptors) and tertiary (cytokines) signals for optimal $CD8^+$ T cell activation and induction of an effector response. Huber and Lohoff showed that a reverse variant of mediating help also exists.

Non-cytotoxic, IL-17 producing CD8⁺ T cells were necessary for the accumulation of strongly pathogenic CD4⁺ T cells in the CNS of murine EAE model (Huber and Lohoff, 2015; Huber et al., 2009, 2013). Accordingly, IL-17 producing CD8⁺ T cells were identified in active lesions of MS patients (Tzartos et al., 2008). Therefore, CD40L⁺ CD8⁺ T cells might not only contribute to efficient effector responses of CD8⁺ T cells but also the recruitment and activation of antigen specific CD4⁺ T cells.

b) Cytokine secretion:

Helper-type CD8⁺ T cells secrete a specific repertoire of cytokines (IL-4, IL-13, IL-17, IL-22, IL-10) that differ from classical CD8⁺ T cell mediated IFN- γ secretion (Figure 26). The functions of these cytokines were mostly identified by analysis of CD4⁺ T cells. However, the effect of the cytokines on their target cell is independent of their origin and therefore should also be induced by CD8⁺ T cell derived cytokines. IL-4, IL-5 and IL-13 secretion by Th2 cells is critical for the recruitment of eosinophils, which mediate the expulsion of parasites (Sallusto, 2016). CD4⁺ T cell derived IL-4 and IL-5 moreover support B cell maturation and class switch in the presence CD40L-CD40 interaction. Th17 derived IL-17 in turn supports the clearance of extracellular bacteria and fungi by the mobilization of neutrophils (McDermott and Klein, 2018). And IL-22 produced by Th22 cells is assumed to play a role in skin regeneration as well as skin protection by inducing the production of antimicrobial peptides (Duhon et al., 2009; Sigmundsdottir et al., 2007). As helper-type CD8⁺ T cells are also capable to express CD40L in combination with IL-4 and IL-5, they can mediate according B cell class switching in a MHCII dependent manner (Hermann et al., 1995). IL-13, a feature of not only Th2 cells but also of all helper-type memory CD8⁺ T cells Tc2, Tc17 and Tc22 has a protective function in skin regulating basal cell activity and subsequently it contributes to wound healing (Dalessandri et al., 2016). The different cytokines also can induce the release of chemokines by other cells, which modulate the recruitment of further cells. Accordingly, helper-type CD8⁺ T cells are capable of inducing CCL26 in keratinocytes, which in turn mediate the recruitment of CCR4⁺ cells whereas cytotoxic CD8⁺ T cells were demonstrated to recruit further CXCR3⁺ cells by the induction of CXCL11, instead (Figure 41).

There are multiple conditions where Tc2, Tc17 and Tc22 cells were detected. Tumors at barrier sites such as skin or lung (melanoma, lung cancer) often contain Tc2 cells (Minkis et al., 2008; Roberts et al., 2009; Sheu et al., 2001). IL-13 producing Tc2 (most likely together with Tc17, Tc22) cells furthermore are widely associated with several autoimmune diseases in tissues. They are enriched in patients with systemic sclerosis (Cascio et al., 2017), alopecia areata (Czarnowicki et al., 2018), rheumatoid arthritis (Cho et al., 2012) and best described in asthma. Despite the vast majority of lung CD8⁺ T cells exhibit a cytotoxic memory (CXCR3⁺CCR5⁺) phenotype in patients with mild asthma only the small fraction expressing CCR4⁺ and producing IL-13 has been associated with disease progress (Campbell et al., 2001). Adoptive transfer of Tc2 cells that favored low antigen concentrations during priming but not Tc1 primed OT-1 cells led to lung eosinophilia and bronchial hyperresponsiveness, classical characteristics of asthma (Sawicka et al., 2004). Additionally, increased frequencies of IL-13 producing CD8⁺ T cells were found among bronchoalveolar lavage cells of asthmatic patients compared to healthy (Dakhama et al., 2013) and IFN- γ knockout led to a Tc2/Tc17 biased phenotype of lung CD8⁺ T cells with reduced cytotoxicity, paralleled by an aggravated disease (Tang et al., 2012). Hondowicz and his team showed that in mice sensitized with house dust CD4⁺ T_{RM} in lung arise together with circulatory T_{CM} in secondary lymphoid organs, a mechanism we propose to underlie the appearance of helper-type CD8⁺ T cell in the circulation (Hondowicz et al., 2016). A severe form of asthma is associated with high levels of Ig-E, which can mediate eosinophil and neutrophil activation. Secretion of Ig-E requires CD40L dependent activation of B cells in the presence of IL-4 and Tc2 cells could provide both as potential contributor to the worsening of asthma.

But how are these helper-type cells activated in autoimmune diseases? IL-17 and IL-22 producing CD8⁺ T cells in the epidermis were identified as major driver of psoriasis induction (Di Meglio et al., 2016). Triggers such as tissue traumata can result into a complex formation of keratinocyte derived antimicrobial peptide LL37 with self-DNA/RNA, which activates dendritic cells in skin to secrete IL-23. Those DC activate autoreactive CD8⁺ T cells which in turn release IL-17 and contribute to skin lesions – a process also known as Koebner phenomenon (Lande et al., 2014). Similarly, Mrp8 and Mrp14 can induce autoimmunity. They

belong to the group of DAMPS (damage-associated molecular pattern molecules) that are constitutively expressed endogenous proteins and released upon tissue damage. The Mrp8 and Mrp14 protein load is increased in several autoimmune disorders such as multiple sclerosis, rheumatoid arthritis and psoriasis. When presented on DC paralleled by CD40-CD40L interaction with CD8⁺ T cells Mrp8 and Mrp14 might mediate TLR4 ligation and IL-17 induction in the CD8⁺ T cells (Loser et al., 2010). Therefore, atypical structures that become accessible by cell disruption and/or complex formation possibly mimic high affine peptides for CD40L⁺ CD8⁺ T cells and activate them in a peptide independent mechanisms. While CD40L expressing CD8⁺ T cells might usually contribute to tissue maintenance and/or tolerance of commensal bacteria, their CD40L expression is a disadvantage when untypical structures become accessible for example by tissue disruption.

4.8 Conclusions

In summary, this work demonstrated that the naïve CD8⁺ T cell repertoire is not homogeneous but composed of cells expressing CD40L implemented during positive selection in the thymus by recognizing self-peptide:MHCI complexes with high avidity (Figure 42). These cells possess a special TCR repertoire, inherit different priming abilities and differentiate into unique memory CD8⁺ T cell subsets with a Tc2, Tc17 or Tc22 phenotype and non-cytotoxic properties. We could demonstrate that these non-cytotoxic, helper CD8⁺ T cells resemble helper CD4⁺ T cells in their transcriptome and phenotype. Consequently, the features “cytotoxicity” versus “helper-phenotype” are not CD8⁺ versus CD4⁺ T cell specific but valid for the whole T cell compartment instead, distinguishable by the expression of SLAMF7 and IL-6R.

Therefore, this work is not only challenging the unbiased flexibility of naïve CD8⁺ T cells but also the classical division of labor between MHC-II dependent, B-cell helping CD4⁺ T cells and cytotoxic CD8⁺ T cells killing infected/malignant cells in an MHC-I restricted manner. The CD4⁺ T cell compartment has already vastly diversified into different helper subsets, cytotoxic CD4⁺, T_{FH}, T_{RM} and Treg cells in recent years while actual reports indicate a similar plasticity of the CD8⁺ T compartment. We could demonstrate here that thymic imprinted, non-cytotoxic CD40L⁺ CD8⁺ T cells provides a unique CD8⁺ T cell type that has to be considered

when analyzing the contribution of CD8⁺ T cells to allergy, autoimmunity, inflammation and antitumorigenic responses.

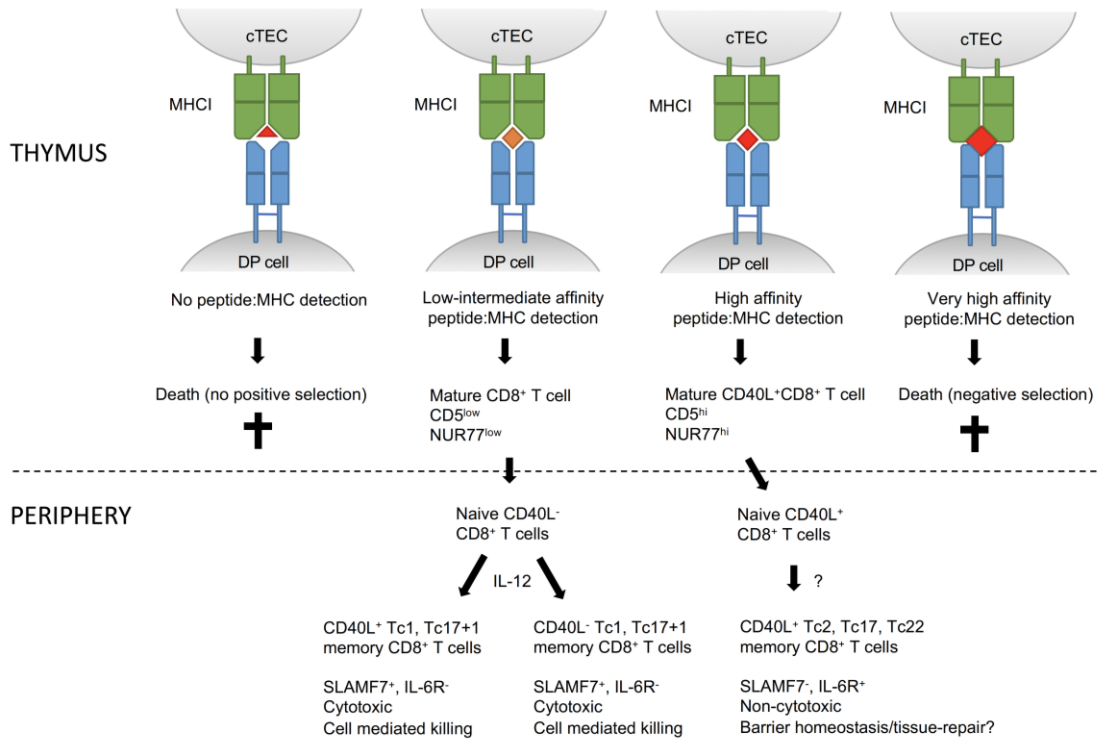


Figure 42: Concept of the role and fate of CD40L expressing CD8⁺ T cells in the immune system. During positive selection by cTECs in the thymic cortex a fraction of CD8⁺ T cells detects thymus proteasome processed private peptides with high affinity leading to the implementation of CD40L and high CD5 and NUR77 expression. These cells form a circulatory naïve T cell population with a unique TCR pool. Upon activation, they specifically differentiate into memory cells with skin migratory phenotype as well as type 2, 17 or 22 cytokine secretion profiles. In contrast, CD40L⁻ naïve cells can become CD40L⁺IFN- γ ⁺ upon differentiation in the presence of IL-12 and gain either a type 1 or a type 17+1 memory phenotype with cytotoxic properties. These cytotoxic and non-cytotoxic memory CD8⁺ T cells are distinguishable by their opposite expression of SLAMF7 and IL-6R. While the cytotoxic cells contribute to control and elimination of intracellular infections, non-cytotoxic Tc2, Tc17 and Tc22 memory cells might play an important role in skin barrier maintenance or commensal bacteria tolerance.

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6. EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Arbeit: „The molecular regulation of CD40L in CD8⁺ T cells“ selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Ich versichere, dass diese Arbeit in dieser oder anderer Form noch keiner anderen Prüfungsbehörde vorgelegt wurde. Der Inhalt der Promotionsordnung der Lebenswissenschaftlichen Fakultät der Humboldt-Universität zu Berlin vom 05.03.2015 ist mir bekannt. Die Grundsätze der Humboldt-Universität zu Berlin zur Sicherung guter wissenschaftlicher Praxis wurden eingehalten.

Berlin, den 03. Dezember 2018

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(Lucie Gloria Loyal)