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Differential antigen dependency of $CD4^+$ and $CD8^+$ T cells

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Abbreviations

aAPC	artificial antigen presenting cell
Ag	antigen
APC	antigen presenting cell
B10.BR	B10.BR/SgSnJ
B6	C57BL/6
bio	biotinylated
BMC	bone marrow chimera
bp	base pair
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
Cables1	Cdk5 and Abl enzyme substrate 1
CD	cluster determinant
CDK5	cycline dependent kinase 5
CFSE	carboxyfluorescein succinimidyl ester
CTLA-4	cytotoxic T-lymphocyte antigen 4
ctrl	control
CV	coefficient of variance
DAPI	4´,6-diaminidin-2-phenylindol
DC	dendritic cell
DMEM	Dulbeco's modified Eagle's medium
dNTP	deoxynucleioside triphosphate
dtg	double transgenic
ECAR	extracellular acidification rate
EDTA	ethylenediaminetetraacetic acid
Erk	extracellular signal-regulated kinase
ETS	electron transport chain
FACS	fluorescence-activated cell sorting
Fc	fragment crystalizing
FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FCS	fetal calf serum
FMO	fluorescence minus one (control staining)
FR4	folate receptor 4
FSC-A	forward scatter area
FSC-H	forward scatter height
GFP	green fluorescent protein
gMFI	geometric mean fluorescence intensity
i.p.	intra peritoneal
IC	isotype control
ICOS	inducible T cell co-stimulator
IFNγ	Interferon gamma
Ig	immunoglobulin
Ii	invariant chain of MHC II
IL	interleukin
Immgen	The Immunological Genome Project
IRF4	interferon regulatory factor 4
K14	human keratin 14
KRAB	Krüppel-associated box
L.	Listeria
LAT	linker of activated T cells
LFA-1	lymphocyte function-associated antigen 1
Lck	lymphocyte-specific protein tyrosine kinase
LCMV	lymphocytic choriomeningitis virus
mAb	monoclonal antibody
MACS	magnetic-activated cell sorting
MAP	mitogen-activated protein
MCC	moth cytochrome *c*
MCMV	mouse cytomegalovirus
MEM	minimal essential medium
MFI	mean fluorescence intensity
MHC	Major Histocompatibility Complex
miRNA	micro RNA
N	average division number
ns	not significant
OCR	oxygen consumption rate
OVA	chicken ovalbumin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed cell death 1
PD-L1	programmed cell death ligand 1
pfu	plaque forming units
PKC	protein kinase C
PLC-γ	phospholipase C-gamma
PMA	phorbol 12-myristate 13-acetate
pMHC	peptide-MHC-complex
RBC	red blood cell
RE	Rested Effector
RFU	relative fluorescent units
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute Medium
RTK	receptor tyrosine kinase
Runx2	runt-related transcription factor 2
SA	streptavidin
SRC	spare respiratory capacity
SSC	side scatter
TAP	transporter associated with antigen processing
T-bet	T cell-specific T-box transcription factor
TCR	T cell receptor
tg	transgenic
TGFβ	tumor growth factor beta
Th	T-helper
TIM	tetracycline inducible invariant chain with MCC_{93-103}
TNFα	tumor necrosis factor alpha
TSO	tetracycline regulated signal sequence with $OVA_{257-264}$
wt	wild type
α	anti
β_2m	beta-2 microglobulin

1 Abstract

Clonal expansion of antigen specific T cells is a major prerequisite for adaptive immune responses. Even though hints of differential requirements in the duration of antigen stimulation for $CD4^+$ and $CD8^+$ T cell proliferation are scattered through the literature, a careful side-by-side analysis of both subsets has only rarely been done.

A previous study showed that following a strong in vitro activation step, $CD8^+$ but not $CD4^+$ T cells proliferate in the absence of further antigen stimulation. The same experimental setup, consisting of an in vitro priming phase followed by adoptive transfer of TCR transgenic $CD4^+$ and $CD8^+$ T cells into mice expressing or not expressing the cognate antigen, was utilized for a deeper analysis of this phenomenon in the present study. The key finding was reproduced and potential methodological caveats such as cell number transferred or high O_2 concentration in the in vitro priming phase were excluded as causes. The differential proliferation patterns described previously were correlated to effector cell differentiation.

Several approaches were used to elucidate how this phenomenon might be regulated. Antigen-independent proliferation of $CD8^+$ T cells was found to be independent of the formation of homotypic T cell clusters and of triggering with self-peptide-MHC complexes. Antigen dependency of $CD4^+$ T cell proliferation on the other hand was unchanged in an inflammatory environment or following blockage of coinhibitory signaling pathways and was observed in memory and in Th1/Th2 differentiated $CD4^+$ T cells. Therefore, differential antigen dependency of $CD4^+$ and $CD8^+$ T cells seems to be T cell intrinsic.

The analysis of the proliferation kinetics of both subsets showed that the antigen-independent proliferation phase of $CD8^+$ T cells is time-limited. Additionally, $CD8^+$ T cells stayed in active stages of the cell cycle for longer periods of time if the stimulation persisted, pointing towards intrinsically distinct proliferative capacities of both subsets. Gene expression profiles of in vitro-stimulated T cells of both subsets were very similar. This indicates that the regulatory mechanisms causing the differential proliferation patterns and also the different functional properties of the subsets are not exclusively located at the transcription level, but may include the activity of micro RNAs, posttranslational protein modifications and epigenetic processes. Interestingly, $CD8^+$ T cells displayed a higher spare respiratory capacity (SRC) compared with $CD4^+$ T cells following in vitro stimulation. This parameter was suggested to correlate with the high proliferative potential of memory T cells and this observation might thus be correlated with the enhanced proliferative capacity of $CD8^+$ T cells.

Finally, differential T cell proliferation patterns seemed to reflect the biology of MHC I and MHC II molecules, as MHC II but not MHC I antigen presentation was found to be maintained on the surface of activated dendritic cells for prolonged periods of time in vivo.

Taken together, the data presented here suggest that $CD4^+$ T cells rely on continuous antigen presentation to expand and develop effector function, whereas their $CD8^+$ counterparts can be programmed to expand and differentiate in response to a transient antigen stimulus.

2 Zusammenfassung

Die Expansion antigen-spezifischer T-Zellen ist eine wesentliche Voraussetzung für eine effektive Immunantwort. Obwohl es Hinweise auf eine differentielle Antigenabhängigkeit von CD4- und CD8-T-Zellen in der Literatur gibt, wurden die Proliferationsmuster beider T-Zell-Populationen bisher nur selten direkt miteinander verglichen. In einer vorangegangenen Studie wurde eine differenzielle Antigenabhängigkeit von CD4- und CD8-T-Zell- in vivo beobachtet. Eine antigenunabhängige Proliferationsphase konnte für CD8-, aber nicht für CD4-T-Zellen beobachtet werden, wenn in vitro-aktivierte T Zellen in Mäuse transferiert wurden.

Unter Verwendung desselben experimentellen Systems wurde diese unterschiedliche Abhängigkeit beider T-Zell-Populationen von der Dauer der Antigenpräsentation in der vorliegenden Arbeit reproduziert. Zusätzlich konnte bestätigt werden, dass die antigenunabhängige Proliferation von CD8-T-Zellen mit der Entwicklung ihrer Effektorfunktionen einhergeht.

Daraufhin wurde die Robustheit des beobachteten Phänomens in verschiedenen experimentellen Ansätzen geprüft. Die antigenunabhängige Proliferation von CD8-T-Zellen beruhte nicht auf unspezifischer Stimulation durch Selbstpeptide im Kontext von MHC I oder der Bildung homotypischer T-Zell-Cluster während der in vitro-Stimulation. Weiterhin konnte eine antigenunabhängige Proliferation von CD4-T-Zellen weder im Kontext einer Entzündung noch unter Blockade costimulatorischer Signalwege beobachtet werden und blieb auch in CD4-Gedächtnis-T-Zellen und Th1- und Th2-Zellen aus. Die differentielle Antigenabhängigkeit von CD4- und CD8-T-Zellen scheint daher zellintrinsisch zu sein.

Die Analyse der Proliferationskinetik beider Subpopulationen offenbarte eine zeitliche Begrenzung der antigenunabhängigen Teilungsphase von CD8-T-Zellen. Zudem fanden sich unter persistenter Stimulation CD8-T-Zellen über einen längeren Zeitraum in aktiven Phasen des Zellzyklus, ein Hinweis auf grundsätzlich verschiedene proliferative Kapazitäten beider T-Zell-Populationen. Die Genexpressionsprofile von in vitro-stimulierten CD4- und CD8-T-Zellen unterschieden sich nur marginal. Dieser Umstand macht eine ausschließliche Regulation dieses Phänomens auf der Transkriptionsebene unwahrscheinlich. Ergänzend könnten posttranslationale Proteinmodifikationen, micro RNAs oder epigenetisch Prozesse an der Regulation der differenziellen Antigenabhängigkeit beteiligt sein. In diesem Zusammenhang erscheint es interessant, dass CD8-T-Zellen im Vergleich zu CD4-T-Zellen

eine höhere *spare respiratory capacity* (SRC) aufwiesen. Dieser Parameter wird unter anderem in Zusammenhang mit der schnellen Expansion von Gedächtnis-T-Zellen gebracht und könnte daher auch mit der erhöhten proliferativen Kapazität von CD8-T-Zellen korreliert sein.

Schließlich schienen die unterschiedlichen Proliferationsmuster von CD4- und CD8-T-Zellen die Biologie der entsprechenden MHC Moleküle widerzuspiegeln, da MHC II-Peptidkomplexe in vivo für längere Zeiträume auf der Oberfläche aktivierter dendritischer Zellen präsentiert wurden als MHC I-Peptidkomplexe.

Zusammenfassend legen die hier gezeigten Daten nahe, dass CD4-T-Zellen stärker von kontinuierlicher Antigenpräsentation abhängig sind als CD8-T-Zellen, deren Proliferation und Differenzierung durch einen starken Antigenstimulus programmiert werden kann.

3 Introduction

3.1 The role of T cells in the adaptive immune system

The synergy of antigen receptor rearrangement, clonal selection and long term maintenance of antigen-experienced memory cells enables the adaptive immune system to effectively eliminate pathogens as well as infected cells and to establish protection against reinfection and cancer. In contrast to the antigen receptors of the innate immune system, which recognize conserved structures associated with infections (pathogen associated molecular patterns, PAMPS) or general "danger signals", the antigen receptors of B cells and T cells can potentially recognize an enormous variety of molecular structures. Therefore, both B and T cells have to be selected against self-reactivity in the organs where their antigen receptors are generated, i.e. the bone marrow for B cells, and the thymus for T cells (central tolerance induction) and their reactivity must further be monitored by peripheral tolerance mechanisms after they left these organs. Upon infection with a pathogen, T cells bearing a cognate T cell receptor (TCR) will be activated, proliferate extensively, develop effector functions and eventually differentiate into long-lived memory cells.

Defined by the expression of the TCR coreceptors CD4 or CD8, $CD8^+$ Cytotoxic T cells (CTLs) and $CD4^+$ T-helper T cells (Th) are distinguished. $CD8^+$ T cells specialize in killing pathogen-infected or neoplastic cells. $CD4^+$ T cells are able to promote B cell responses by providing them with stimulatory signals and thereby allowing and directing antibody class switch in a process known as T cell help. T cell help is also necessary to optimize the functions of macrophages and enables them to kill phagocytosed pathogens efficiently. Additionally, $CD4^+$ T cells have the capacity to shape immune responses by a broad range of regulatory functions mainly mediated by the secretion of cytokines, which act on components of both the innate and adaptive immune system.

Both subsets of T cells and their sophisticated interactions with each other and with components of the adaptive and innate immune system are of paramount importance for the maintenance of immune integrity.

3.2 The context of T cell antigen recognition

The TCR recognizes protein-derived peptide antigens presented in the context of Major Histocompatibility Complex (MHC) molecules on the surface of antigen presenting cells

(APCs). $CD4^+$ and $CD8^+$ T cells recognize peptides in the context of different classes of MHC molecules. The CD4 coreceptor limits peptide recognition to the context of MHC class II molecules (MHC II), whereas T cells bearing the CD8 coreceptor recognize peptides in the context of MHC class I (MHC I). MHCs are found in all vertebrates and comprise a multigenic region in which not only MHC I and MHC II molecules but also genes associated with molecular assembly and peptide loading of these molecules are located. In mice, the MHC locus is called H-2 and contains two to three MHC I genes (H-2D, H-2K and in some strains H-2L) and two MHC II genes (H-2A, H-2E). MHC molecules are highly polymorphic and a given inbred mouse strain is therefore characterized by its MHC haplotype. The sequence variation is mostly concentrated in the regions that interact with the presented peptides.

MHC I and MHC II molecules display structural differences. MHC I consists of an α-chain containing one membrane-proximal domain anchoring the complex in the cell membrane and two membrane-distal domains that form the peptide-binding grove. Additionally, a β-chain (β_2-microglobulin, β_2m) is essential for the stability of the heterodimer but not involved in peptide binding or membrane anchoring. MHC II consists of one α- and one β-chain, each comprising a membrane-distal domain and a membrane-proximal-domain. Both domains are involved in peptide-binding.

Peptides presented by MHC I and MHC II derive from different subcellular compartments. MHC I molecules are loaded with peptides originating from cytosolic proteins that have been degraded by the proteasome. The resulting peptides are transported into the endoplasmic reticulum, where the loading takes place, in a TAP (transporter associated with antigen processing) dependent manner. Thus, MHC I peptides mirror the repertoire of cytoplasmic proteins and, in the case of a viral infection, also viral proteins. MHC II loading on the other hand occurs in late endosomes. Extracellular proteins are taken up by several pathways including endocytosis, pinocytosis and phagocytosis. Subsequently, they are degraded by lysosomal enzymes in the respective membrane compartments, which eventually fuse with endosomes. Thus, MHC II molecules are loaded with peptides derived from extracellular proteins and phagocytosed material.

There are exceptions to the general rules described above. MHC II molecules can be loaded with intracellular proteins if these were subjected to lysosomal degradation. On the other hand, extracellular protein-derived peptides can be presented on MHC I via a pathway termed cross-presentation. Specific subsets of dendritic cells (DCs) display a pronounced

ability to crosspresent antigen and are thus able to initiate $CD8^+$ T cell responses in situations where antigenic proteins are not directly targeted to APCs, i.e. some viral infections or tumors (Blum et al., 2013).

Both MHC classes are expressed differentially on APCs. MHC I molecules are expressed on all nucleated cells, MHC II expression on the other hand is limited to professional APCs, which are comprised of B cells, macrophages and DCs.

DCs, whose name derives from their morphology (Steinman and Cohn, 1973), are of critical importance for the induction and direction of both adaptive immune responses and tolerance mechanisms. They are an important interface between the adaptive and the innate immune systems. Being located in peripheral tissues, they constantly take up extracellular proteins and present them on MHC molecules. If an infection occurs, DCs are activated via Toll-like receptors (TLR), C-type lectin receptors (CLRs) and chemokine receptors following ligation with their respective ligands. Subsequently, DCs mature and migrate to the T cell areas of draining lymph nodes. In contrast to the immature state, mature DCs drastically reduce phagocytosis and in turn upregulate cell surface expression of peptide-loaded MHC II and MHC I molecules. Additionally, mature DCs express the costimulatory molecules CD80 and CD86 that deliver a second signal to T cells, which is necessary for their productive activation. Furthermore, DCs translate stimuli perceived during their activation into the expression of cytokines, which tailor the subsequently induced T cell responses (Mellman and Steinman, 2001; Steinman et al., 2003).

3.3 T cell development: the generation of a functional T cell repertoire

The development of T cells takes place in the thymus. Bone morrow derived early lymphoid progenitors enter the thymus and are committed to the T cell linage following Notch-signaling (Robey and Bluestone, 2004). During T cell development, each T cell precursor has to generate functional α- and β-chains of the TCR by genetic rearrangement of gene segments. This process is dependent on recombination-activating genes 1 and 2 (RAG-1 and RAG-2).

After entry into the thymus and commitment to the T cell lineage, all T cell precursors are negative for CD4 and CD8 and thus called double negative (DN) thymocytes. Upon generation of a functional TCR β-chain (tested by its pairing with pre-TCR α-chain), thymocytes express both CD4 and CD8 (double positive (DP) thymocytes). Subsequently, the

TCR α-chain is rearranged and thymocytes become either $CD4^+$ or $CD8^+$ (single positive, SP) and will leave the thymus after several days. Importantly, thymocytes expressing a TCR with a high affinity for self-peptides presented on MHC molecules are deleted by a process called negative selection. If a TCR is unable to interact with peptide-MHC (pMHC) complexes at all, the T cell dies by neglect (Starr et al., 2003).

During the process of TCR rearrangement, signaling via the pre-TCR made up of a functionally rearranged TCR β-chain and the pre-TCR α-chain commits thymocytes to the αβ T cell lineage. Alternatively, productive rearrangements of γδ TCR chains can occur and subsequently direct thymocytes to the γδ T cell lineage. γδ T cells are thought to provide immediate protection for a variety of tissues from pathogen invasion by recognition of microbial and damage-induced molecules (Jameson et al., 2002). The present study is focused on αβ T cells.

3.4 The physiology of T cell responses

The dynamics of cell populations are a key feature of the immune system. The rearrangement of TCRs during T cell development provides the adaptive immune system with an enormous spectrum of different TCRs. On the other hand, this process results in very low precursor frequencies of antigen-specific T cells (sometimes less than ten T cells per mouse, (Moon et al., 2007; Tubo et al., 2013)). Therefore, a large expansion of these rare cells following their activation is essential to the development of an effective immune response. However, as the body can only sustain the survival of a limited number of lymphocytes, the huge populations of effector cells generated during an immune response need to contract following clearance of the infection.

Accordingly, T cell responses are characterized by proliferation and contraction. By recognition of their cognate antigen on an APC, T cells become activated (priming phase), proliferate extensively and develop their effector functions (expansion and effector phase). After pathogen clearance, most antigen-specific T cells will die (contraction phase), leaving only a small population of memory T cells behind to provide immediate protection in the case of reinfection (memory phase).

3.4.1 T cell priming

Mature T cells circulate through blood and lymphoid tissues, scanning APCs for the presence of cognate pMHC complexes. If such a complex ligates their TCR, T cells will stop migrating and form stable contacts with the respective APC, which last a couple of hours. Subsequently, T cells will resume motility and begin to proliferate and develop effector functions (Bousso and Robey, 2003; Mempel et al., 2004). The successful activation of T cells requires signaling via the TCR and costimulatory receptors.

3.4.1.1 TCR ligation

T cells recognize peptides presented by MHC molecules via the TCR, a heterodimeric molecule embedded into a complex of signaling molecules. TCR α- and TCR β-chains interact with peptides that are non-covalently bound to the peptide-binding groove of MHC molecules. Both chains possess only very short intracellular domains without signaling capacity. The associated CD3 complex that consists of one δ-chain, one γ-chain and two ε-chains mediates signaling together with two ζ-chains.

These signaling molecules contain a total of ten immunoreceptor tyrosine based activating motifs (ITAMs), which are recognized and phosphorylated by the lymphocyte-specific protein tyrosine kinase (Lck) that is brought into close vicinity of the ITAMs following TCR ligation. Subsequently, a zeta chain associated protein of 70 kDa (Zap70) is recruited to the TCR complex. This starts a complex downstream signaling sequence including the formation of adaptor protein nucleated multimolecular signaling complexes and regulatory feedforward and feedback loops. These events finally result in the activation of the transcription factors activator protein 1 (AP-1), nuclear factor of activated T cells (NFAT) and nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB) and induce T cell proliferation and differentiation (Lin and Weiss, 2001).

The concrete mechanism by which TCR ligation and the subsequent formation of the immune synapse initiate signaling events in the TCR complex remains under discussion (Smith-Garvin et al., 2009), even though the model of “kinetic segregation” is supported by a recent publication (James and Vale, 2012). In this model, the ligation of the TCR leads to its partitioning into plasma membrane domains which contain Lck but not the phosphatase CD45 that prevents phosphorylation of ITAMs in the steady state.

3.4.1.2 Costimulation and coinhibition

Besides TCR triggering, the ligation of costimulatory receptors is a necessary prerequisite for productive T cell activation. The lack of such signaling results in unresponsiveness of T cells to further stimulation (anergy). APCs upregulate costimulatory molecules if they are activated in the presence of infection or danger-associated signals. If sensed by T cells via dedicated receptors, costimulatory signaling results in the amplification of the TCR signal.

Most prominently, the ligation of CD28 (expressed by T cells) by CD80 and CD86 (expressed on activated DCs) results in the recruitment and enhanced activation of phosphoinositide-3 kinase (PI3K). This signal is integrated together with the TCR signal and will finally result in increased engagement of the NFκB pathway. Additional costimulatory receptors such as CD40L, CD134 or inducible T cell co-stimulator (ICOS) are upregulated following CD28 ligation and further support T cell activation if triggered by their respective ligands (Acuto and Michel, 2003).

Cytotoxic T-lymphocyte antigen 4 (CTLA-4), an inhibitory receptor of CD80/CD86, competes with CD28. Its ligation results in T cell tolerance, inhibition of interleukin-2 (IL-2) production and cell cycle arrest. Further coinhibitory signals are mediated by the ligation of programmed cell death 1 (PD-1) and its ligands PD-L1 or PD-L2 and this is thought to participate in the maintenance of self-tolerance, as illustrated by the severe autoimmune phenotype of *Pd1*$^{-/-}$ mice (Acuto and Michel, 2003; Sharpe and Freeman, 2002). A delicate balance of costimulatory and coinhibitory signaling is essential to mediate immunity and protection from autoimmunity.

3.4.2 T cell effector functions

Following successful activation, T cells will proliferate extensively and develop their effector functions. As outlined above, $CD4^+$ and $CD8^+$ T cells possess fundamentally different effector functions.

3.4.2.1 $CD4^+$ T cell effector functions

Based on the expression of key transcription factors and cytokines, $CD4^+$ T cells can be further divided into subpopulations. $CD4^+$ T cell responses are polarized by the cytokines present during their activation. These cytokines are produced by APCs or by other cells in the priming microenvironment.

The first subsets defined by their distinct cytokine profiles were the Th1 and Th2 T cells (Mosmann et al., 1986). Interferon gamma (IFNγ) polarizes $CD4^+$ T cell responses towards the Th1 phenotype and is the key cytokine produced by these cells. IL-4 is of analogous importance for the differentiation and effector function of Th2 cells. The activity of key transcription factors (T cell-specific T-box transcription factor (T-bet) for Th1 and GATA binding protein 3 (Gata3) for Th2 T cells) is essential for the polarization of $CD4^+$ T cells. Subsequent epigenetic modifications result in the distinct effector functions of Th1 and Th2 cells (Grogan et al., 2001; Pipkin and Rao, 2009).

Th1-derived IFNγ has pleiotrophic effects, which synergize to induce the effective destruction of microbial pathogens by phagocytic cells and include the increase of NK cell cytotoxicity, the enhancement of phagocytosis and microbicidal activity of macrophages and the production of opsonizing immunoglobulin (Ig) G (Schroder et al., 2004).

Th2 cells on the other hand coordinate immune responses against parasites at mucosal and epithelial barriers. IL-4 recruits mast cells, eosinophils and basophils and activates mast cells, epithelial cells and smooth muscle cells in order to support parasite removal. Furthermore, IL-4 induces antibody class switch to IgE. These functions are mediated in concert with IL-13, IL-5 and several other cytokines produced by Th2 cells (Nair et al., 2006).

The subset of regulatory $CD4^+$ T cells (Treg) is recognized to be of paramount importance for the induction and maintenance of tolerance to self. The production of IL-10 and tumor growth factor beta (TGFβ) is essential for this function but the mechanistic details remain incompletely understood (von Boehmer, 2005). The expression of the transcription factor forkhead box p3 (Foxp3) is a hallmark of Tregs. Their development is dependent on TGFβ and IL-2 and their regulatory potential is impressively illustrated by the strong autoimmune phenotype of *Il-2*$^{-/-}$ mice (Sadlack et al., 1993).

A subset of IL-17A and IL-17E producing $CD4^+$ T cells (Th17) has been described to be induced by TGFβ and IL-6 and to be dependent on the transcription factor RAR-related orphan receptor gamma 2 (RoRγt). Th17 cells have been reported to orchestrate the clearance of extracellular bacterial and fungal infections. This effect is mediated by IL-17A and IL-17E. The secretion of these cytokines results in the recruitment and activation of neutrophils. Additionally, Th17 cells are potent inducers of autoimmune disease (Basu et al., 2013; Korn et al., 2007).

More recently, follicular helper T cells (Tfh) were identified due to their localization in B cell follicles. They express the surface markers chemokine (CXC-motif) receptor 5 (CXCR5)

and ICOS, the transcription factor B cell leukemia/lymphoma 6 (BCL6) and produce IL-21. These cells are specialized in providing B cell help. It remains unclear if they originate from $CD4^+$ T cells polarized into one of the above subsets and subsequently acquire the Tfh phenotype, or if they represent an autonomous subset. Tfh development requires contact with cognate antigen presenting B cells and the presence of IL-6 and IL-21 (Ma et al., 2012a).

It is currently thought that $CD4^+$ T cell subsets do not represent strictly separate lineages but rather display a certain plasticity so that the differentiation into one subset is not necessarily terminal (O'Shea and Paul, 2010). This phenomenon might be particularly pronounced for the Th17 and Tfh subsets (Basu et al., 2013).

3.4.2.2 $CD8^+$ T cell effector functions

In contrast to the many potential outcomes of $CD4^+$ T cell activation described above, the $CD8^+$ T cell effector functions are much less diverse. The predominant function is the direct killing of host cells infected with viruses or bacteria invading the cytoplasm (such as *Listeria (L.) monocytogenes* and *Salmonella spec.*) and neoplastic cells.

The secretion of IFNγ and tumor necrosis factor alpha (TNFα) induces anti-microbial responses mediated by cells of the innate and the adaptive immune system but also by epithelial cells. Furthermore, target cell apoptosis is induced by the delivery of granules containing perforin and granzymes. Following TCR ligation with cognate pMHC I complexes on the surface of a target cell, primed $CD8^+$ T cells release perforin and granzymes into the immune synapse formed between the cells. The change in pH induces perforin to polymerize and form pores in the cell membrane of the target cells. Through these pores, granzymes enter the cell and rapidly induce apoptosis via both caspase-dependent and -independent pathways. Additionally, $CD8^+$ T cells deliver death-signals by ligation of Fas (Trambas and Griffiths, 2003; Wong and Pamer, 2003a).

Besides cytotoxic $CD8^+$ T cells, a subset of $CD8^+$ T cells displaying regulatory functions may play a role in immune homeostasis, maintenance of immune privileged sites (Niederkorn, 2008; Vinay and Kwon, 2010) and the regulation of germinal center reactions (Ramiscal and Vinuesa, 2013). The importance of this subset is by far not as well understood as that of regulatory $CD4^+$ T cells.

3.4.3 Generation and maintenance of memory T cells

As a pathogen is cleared and both antigen presentation and the supply of T cell growth factors such as IL-7 ceases, up to 95% of effector T cells die. The causative relationships between these observations are difficult to assess and the effects of antigen persistence and cytokine presence on cell survival vary depending on the microenvironment and the type of infection. A metabolic switch is required for the successful transition from effector to memory cell status (Marrack et al., 2010).

The requirements for memory formation and maintenance of $CD4^+$ and $CD8^+$ T memory cells are distinct. For $CD4^+$ T cells, sufficient costimulation and IL-2 signaling during the initiation of the primary response is one prerequisite for effector to memory transition but the extent of this effect varies between $CD4^+$ T cell subsets. The time point of recruitment into the immune response and the associated changes in the microenvironment of priming might be critical, as $CD4^+$ T cell effector functions differ between cells that were engaged early or late during the course of an infection. Nevertheless, a specific effector differentiation or a minimal number of divisions is not essential for the transit to the memory stage. The long-term survival of $CD4^+$ T cells is dependent on IL-7 but not on TCR stimulation, as memory cells are maintained in the absence of MHC II (McKinstry et al., 2010).

A subset of $CD8^+$ effector T cells, characterized by high expression of the IL-7 receptor and low expression of KLRG1 (Killer cell lectin-like receptor subfamily G member 1), is is especially prone to transit to the memory stage. These cells are thus called memory precursor effector cells (MPEC). For long-term maintenance, $CD8^+$ T cells rely on the presence of both IL-7 and IL-15 (Kaech and Cui, 2012).

In general, memory T cells of both subsets are characterized by rapid unfolding of effector functions following restimulation. For both $CD4^+$ and $CD8^+$ T cells, at least two subtypes of memory cells have been described: central memory cells (Tcm), that express both L-selectin (CD62L) and C-C chemokine receptor type 7 (CCR7) at high levels, and effector memory cells (Tem), which display low expression of CCR7 and CD62L. Whereas Tcm cells are located in lymphoid organs, possess a high proliferative capacity and display effector functions only following stimulation, Tem cells are found in tissues, proliferate less but express effector molecules constitutively. Differential localization and functional properties of memory T cell subsets are thought to enhance the efficiency of memory T cell responses (Lanzavecchia and Sallusto, 2005; Sallusto et al., 2004).

3.4.4 T cell exhaustion

In many chronic viral infections and during cancer, a gradual loss of effector T cell functions is observed that finally leads to T cell deletion. This state of T cell dysfunction is called exhaustion and is described for both $CD4^+$ and $CD8^+$ T cells. The effect of persistent antigen presentation on the more diverse functions of $CD4^+$ T cell subsets are not as extensively studied as phenotypic changes associated with $CD8^+$ T cell exhaustion. The severity of T cell exhaustion is strongly correlated with the strength of T cell stimulation and the duration of the infection. Both cell-intrinsic (e.g. PD-1 signaling) and extrinsic negative regulatory pathways (e.g. cytokine mediated signals) are known to be involved in T cell exhaustion. In contrast to T cell anergy, a dysfunctional state induced during the initial antigen contact, exhausted T cells have experienced productive priming and effector differentiation before loosing their functionality. Both conditions are at least partially distinct at the gene expression level (Wherry, 2011).

3.5 The influence of antigen stimulus duration on T cell responses

In order to maintain immunity and prevent autoimmunity, T cell responses have to be both autonomous and controllable. This apparent contradiction is resolved in a concept where T cell functions are partially programmed during lineage commitment or priming and additionally tunable by cell extrinsic regulatory pathways. Besides costimulatory signals, whose impact on the subsequent T cell response is quite extensively studied, the influence of the duration of antigen stimulus on the fate of T cells is only incompletely understood.

The influence of TCR stimulus duration on the proliferation and differentiation of murine $CD4^+$ and $CD8^+$ T cells has been studied in several experimental models but only rarely in a comparative manner. A major experimental caveat is controlling the termination of antigen presentation. If T cell proliferation was analyzed in vitro, T cells were physically separated from the antigen. In vivo, treatment with antibiotics was used to limit antigen expression during bacterial infections. Alternatively, T cells stimulated in vitro or isolated from infected mice were adoptively transferred into uninfected mice. Furthermore, TCR interaction with cognate pMHC complexes was terminated by the administration of blocking monoclonal antibodies (mAbs) directed against these pMHC complexes.

3.5.1 The importance of antigen persistence for $CD4^+$ T cell responses

Several studies observed the proliferation of $CD4^+$ T cells in vitro to be antigen-dependent. Iezzi at al. showed that the proliferation and survival of naïve $CD4^+$ T cells depends on at least 15-20 h of antigen presentation (Iezzi et al., 1998). Stimulating TCR transgenic (tg) $CD4^+$ T cells with peptide-loaded macrophages, Schrum et al. observed that their proliferation is dependent on prolonged antigen stimulation, as the extent of CFSE dilution increased with prolongation of the stimulation period. In this study, the antigen presentation was interrupted by the removal of the T cells from the APCs in combination with the addition of a MHC II blocking mAb (Schrum and Turka, 2002). In a setup where the stimulation of TCR tg $CD4^+$ T cells was terminated with a mAb blocking the interaction of the TCR with its cognate pMHC complex presented by a B cell line, Huppa et al. demonstrated that $CD4^+$ T cell proliferation was gradually diminished with shortened stimulation periods (Huppa et al., 2003).

In contrast, other in vitro studies observed $CD4^+$ T cell proliferation to be rather independent of prolonged antigen presentation. Two phases of $CD4^+$ T cell proliferation were found by Jelley-Gibbs et al. in a study where the proliferation and effector functions of TCR tg $CD4^+$ T cells were assessed following stimulation with antigen expressing fibroblasts: an initial, antigen-dependent phase and a subsequent, cytokine-dependent but antigen-independent phase (Jelley-Gibbs et al., 2000). Lee at al. observed antigen-independent proliferation of TCR tg $CD4^+$ T cells following 60 h of antigen stimulation and further coculture with unpulsed APCs (Lee et al., 2002). Also Bajénoff et al. al found that $CD4^+$ T cells were able to proliferate without further antigen stimulation following 48 h in vitro priming with peptide-pulsed irradiated splenocytes (Bajénoff et al., 2002).

Taken together, these in vitro studies provide contradictory data. Some studies found that $CD4^+$ T cells are dependent on prolonged antigen presence, whereas others observed that $CD4^+$ T cells are able to proliferate even upon discontinued antigen presentation.

The assessment of $CD4^+$ T cell proliferation in vivo presents a more homogenous picture. The proliferation of TCR tg $CD4^+$ T cells was observed to depend on prolonged antigen presentation in an in vivo model of doxycycline-dependent antigen expression (Obst et al., 2005) that will be used in the present study. The repeated immunization with antigen presenting DCs enhanced the expression of the high affinity IL-2 receptor (CD25) and IFNγ on TCR tg $CD4^+$ T cells, but did not increase the number of cell divisions at an early time

point (38 h after the initial immunization) (Celli et al., 2005). Injection of peptide-immunized mice with a mAb blocking the TCR-pMHC interactions showed that the proliferation of TCR tg $CD4^+$ T cells decreased gradually with shortening of the in vivo stimulation period (Celli et al., 2007). In a similar approach, Yarke et al. stimulated TCR tg $CD4^+$ T cells in vitro, transferred them into mice immunized with the cognate antigen and terminated TCR stimulation by injection of a mAb blocking TCR-pMHC interactions. Here, $CD4^+$ T cells did not divide upon transfer into naïve mice and the extent of proliferation was dependent on the length of antigen stimulation allowed in vivo (Yarke et al., 2008). Additionally, Jusforgues-Saklani et al. observed that prolonged periods of antigen presentation to $CD4^+$ T cells were necessary for the induction of DC-crosspresentation. Here, splenocytes from male mice were used to immunize female mice. The NK cell-mediated rapid depletion of these cells hindered efficient cross presentation, an effect that could be abrogated by depletion of NK cells in the recipient females (Jusforgues-Saklani et al., 2008). Baumgartner et al. showed that the expansion of TCR tg $CD4^+$ T cells is directly correlated to the dose of cognate peptide immunization. This effect was more pronounced for a peptide that forms low-stability complexes with MHC II, indicating that $CD4^+$ T cells rely on repeated contacts with pMHC for their expansion (Baumgartner et al., 2010).

Taken together, all in vivo studies discussed above showed that the proliferation of $CD4^+$ T cells is dependent on the persistence of antigen.

3.5.2 The importance of antigen persistence for $CD8^+$ T cell responses

In several studies, an antigen-independent phase of $CD8^+$ T cell expansion has been observed. The termination of *L. monocytogenes* infection by ampicillin treatment did not result in a diminished expansion and differentiation of $CD8^+$ T effector cells if this treatment was started 24 h post infection (Mercado et al., 2000). If TCR tg $CD8^+$ T cells were stimulated in vitro with a fibroblast cell line expressing cognate antigen and costimulatory molecules, 2 h of stimulation were sufficient to induce proliferation and effector differentiation (van Stipdonk et al., 2001). In the same experimental setup, 20 h of in vitro stimulation were sufficient to allow the expansion and the development of effector functions in wild type (wt) mice (van Stipdonk et al., 2003). In another in vitro system, the number of peptide-pulsed APCs cocultured with TCR tg $CD8^+$ T cells correlated with the percentage of activated T cells, whereas the extent of proliferation and the acquisition of effector functions were independent of APC numbers (Kaech and Ahmed, 2001). In agreement with these

findings, the percentage of TCR tg $CD8^+$ T cells activated in a *L. monocytogenes* infection model was dependent on the infectious dose, but proliferation and effector functions of the recruited $CD8^+$ T cells were uniform (Kaech and Ahmed, 2001). If $CD8^+$ T cells isolated from *L. monocytogenes* infected mice were cultured in the absence of antigen stimulation, proliferation continued. Similarly, in vitro stimulated $CD8^+$ T cells kept on dividing if they were removed from the source of antigen (Wong and Pamer, 2001). In a different approach, Prlic et al. limited antigen presentation in vivo by diphtheria toxin mediated depletion of peptide-pulsed DCs. In the context of a *L. monocytogenes* infection, the depletion of DCs 1 h after transfer of TCR tg $CD8^+$ T cells resulted in 7 h of effective antigen presentation but still allowed complete proliferation and effector differentiation of $CD8^+$ T cells. Even though CFSE dilution and IFNγ production were not affected by shortened antigen presentation, the total expansion of $CD8^+$ T cells was reduced, indicating that antigen persistence supports the survival of differentiated $CD8^+$ T cells (Prlic et al., 2006). Following 20 h of in vitro stimulation with cognate peptide-loaded APCs and transfer into naïve mice, TCR tg $CD8^+$ T cells were able to proliferate extensively (van Stipdonk et al., 2008). Kalia et al. showed that virus-specific $CD8^+$ T cells isolated from lymphocytic choriomeningitisvirus (LCMV) infected mice expand similarly in secondary hosts infected with or immune to this virus (Kalia et al., 2010). Finally, in a viral meningitis model of intra cerebral LCMV infection, Kang et al. observed actively dividing $CD8^+$ T cells not only at the site of infection and antigen presentation but also in the blood, where antigen presentation is assumed not to take place. Furthermore, deficiency of MHC I did not abrogate but only reduced cell cycle activity of $CD8^+$ T cells in the infected brain (Kang et al., 2011). The increasing number of studies observing an antigen-independent proliferation phase for $CD8^+$ T cells led to the hypothesis that $CD8^+$ T cells proliferate "on autopilot" (Bevan and Fink, 2001).

In contrast, a similar number of publications observed that the proliferation of $CD8^+$ T cells is at least partially dependent on prolonged antigen presentation. In an in vitro setup where latex-microspheres coated with pMHC complexes and costimulatory ligands were used to stimulate $CD8^+$ T cells, Curtsinger et al. observed that the expansion of TCR tg $CD8^+$ T cells depends on the inflammatory cytokine IL-12. This dependency on IL-12 could be overcome by higher antigen doses in the culture, but the development of effector functions remained IL-12-dependent even following high-dose stimulation. In an in vivo immunization protocol, $CD8^+$ T cell expansion but not effector function became independent of IL-12 by increasing the antigen dose (Curtsinger et al., 2003). Storni et al. utilized replication deficient

virus-like particles (VLPs) to deliver antigen in a duration-limited manner in vivo. If mice received VLPs 1-3 d before transfer of cognate TCR tg $CD8^+$ T cells, the production of IFNγ was gradually reduced, accompanied by a slight impairment of proliferation (Storni et al., 2003). Shaulov et al. transferred in vitro-stimulated OVA-specific TCR tg $CD8^+$ T cells into naïve, LCMV infected or LCMV infected and OVA-immunized mice. In naïve and LCMV infected recipients, the proliferation but not the effector functions of $CD8^+$ T cells were reduced compared with mice that were immunized with OVA in the context of LCMV infection (Shaulov and Murali-Krishna, 2008). This finding points towards a differential antigen dependency of proliferation and effector differentiation of $CD8^+$ T cells. Tseng et al. assessed $CD8^+$ T cell proliferation in mice infected with *L. monocytogenes* and treated with ampicillin to terminate antigen presentation. In contrast to the studies discussed above, abrogation of infection 24 h after its onset reduced the expansion and the percentage of IFNγ positive endogenous listeria-specific $CD8^+$ T cells (Tseng et al., 2009). Aiming to optimize an immunization protocol, Johansen et al. observed that $CD8^+$ T cell responses to peptide immunization were maximal if repeated injections with increasing vaccine doses were applied. In comparison, a single bolus injection of peptide and adjuvant resulted in decreased IFNγ production and proliferation of specific $CD8^+$ T cells (Johansen et al., 2008). Abrogation of TCR stimulation can also be achieved by controlling TCR signaling pathways. Using a transgenic mouse where the expression of the TCR proximal kinase Lck can be regulated in a doxycycline-dependent manner, Tewari et al. observed that $CD8^+$ T cell expansion is dependent on prolonged Lck expression following viral infection (Tewari et al., 2006).

In conclusion, the literature on $CD8^+$ T cell antigen dependency is more heterogeneous than that for $CD4^+$ T cells. Between studies data are not always consistent and in some cases the use of similar experimental systems leads to different conclusions. An antigen-independent proliferation phase of $CD8^+$ T cells is supported by a similar number of studies as the antigen dependency of $CD8^+$ T cell expansion.

3.5.3 Comparative studies on $CD4^+$ and $CD8^+$ T cell antigen dependency

Even though many studies have analyzed the proliferative requirements of $CD4^+$ and $CD8^+$ T cells separately, a direct comparison has only rarely been done. Gett et al. transferred in vitro-stimulated $CD4^+$ and $CD8^+$ T cells into naïve mice and observed enhanced proliferation of $CD8^+$ T cells following 24 h of in vitro stimulation (Gett et al., 2003). Using *L. monocytogenes* infection, Corbin and Harty observed that the expansion and IFNγ production

of both subsets was only slightly affected if the infection was terminated by ampicillin treatment 24 h after its onset. Nevertheless, ampicillin application at the time point of infection led to a weaker expansion of $CD4^+$ compared to $CD8^+$ T cells (Corbin and Harty, 2004). Using the same experimental system, Williams and Bevan reported contradictory data. They showed that the magnitude of $CD4^+$ but not $CD8^+$ T cell responses is reduced following ampicillin treatment 24 h after the infection of the mice (Williams and Bevan, 2004). In contrast, Tseng et al. reported that the expansion of both $CD4^+$ and $CD8^+$ T cells was diminished upon ampicillin treatment of *L. monocytogenes* infected mice at the same time point (Tseng et al., 2009). Alternatively, Blair et al. used blocking mAbs against pMHC complexes to limit TCR triggering during a viral infection in vivo. In this system, the expansion of $CD4^+$ and $CD8^+$ T cells was observed to depend on prolonged antigen stimulation in vivo (Blair et al., 2011). Furthermore, a doxycycline regulated system of in vivo antigen expression to cognate TCR tg $CD4^+$ and $CD8^+$ T cells demonstrated antigen dependency of $CD4^+$ but not $CD8^+$ T cell proliferation during the expansion phase (Rabenstein, 2012). As this system is used in the present study, it will be described in detail in the results section (see section 5.1).

Thus, a small number of studies directly comparing the antigen dependency of $CD4^+$ and $CD8^+$ T cell expansion provide contradictory data.

3.5.4 The influence of antigen persistence on the formation of T cell memory

Many of the studies described above assessed the influence of restricted antigen presentation on secondary T cell responses. In some studies, the expansion and functionality of $CD8^+$ memory responses was unaffected by premature termination of antigen presentation during the primary response (Mercado et al., 2000; Tewari et al., 2006; van Stipdonk et al., 2008), whereas others reported impaired memory responses (Johansen et al., 2008; Kaech and Ahmed, 2001; Shaulov and Murali-Krishna, 2008; Storni et al., 2003; Tseng et al., 2009). Alternatively, prolonged antigen presentation to $CD8^+$ T cells during the primary response was reported to increase the magnitude but not the functionality of $CD8^+$ memory responses (Prlic et al., 2006). Recently, Henrickson et al. showed that the immunization with low doses of peptides impairs the $CD8^+$ memory response (Henrickson et al., 2013). Kim et al. provide data that indicate the requirement of sustained TCR-pMHC interactions for the transition of effector $CD4^+$ T cells into the memory pool (Kim et al., 2013).

The comparative analysis of $CD4^+$ and $CD8^+$ T cell memory generation also revealed a heterogeneous picture. Corbin et al. and Williams et al., even though using the same

experimental setup, reported contradictory observations of unaffected (Corbin and Harty, 2004) or impaired formation of both memory subsets (Williams and Bevan, 2004) following premature termination of *L. monocytogenes* infection during the primary response. Blair et al. observed that the secondary responses of both subsets were reduced if antigen presentation was curtailed during the primary response (Blair et al., 2011).

Thus, it remains incompletely understood how the duration of the antigen stimulus affects primary and secondary responses of $CD4^+$ and $CD8^+$ T cells. In vivo-studies on $CD4^+$ T cells do consistently report the enhancement of their proliferation if antigen persists. Studies on $CD8^+$ T cells come to more heterogeneous conclusions, possibly indicating that the proliferative capacity of this subset is more dependent on the experimental context or that different proliferative programs might be induced in the respective settings. Comparative studies present contradictory data, which cannot be explained by differences in experimental settings.

3.6 Aim of this thesis

A doxycycline-regulated system of in vivo antigen expression has recently been used to assess the requirement for continued antigen presentation during the expansion of $CD4^+$ and $CD8^+$ T cells (Rabenstein, 2012). In this system, $CD4^+$ T cells were found to depend on prolonged antigen presentation for strong proliferation in three independent experimental approaches. $CD8^+$ T cells on the other hand were able to proliferate extensively after a strong in vitro stimulation and transfer into antigen-free mice. The mechanisms responsible for this differential antigen dependency of $CD4^+$ and $CD8^+$ T cells remained to be analyzed in detail.

Therefore, potential causes of the differential proliferative patterns will be assessed in the present study. Given the broad expression of MHC I molecules and the relatively restricted expression of MHC II molecules in the body, antigen-independent proliferation of $CD8^+$ T cell could be due to self-peptide triggering of the TCR. $CD4^+$ T cell proliferation on the other hand might be dependent on proinflammatory cytokines, caused by coinhibitory signaling or might be related to a particular differentiation state of $CD4^+$ T cells. Furthermore, gene expression, proliferation kinetics and the metabolic capacities of both subsets will be studied in order to approach an understanding of the mechanisms responsible for differential antigen dependency of $CD4^+$ and $CD8^+$ T cells.

4 Material and Methods

4.1 Material

4.1.1 Chemicals and solutions

2-Mercaptoethanol	Carl Roth, Arlesheim, Switzerland
Agarose	Peqlab, Erlangen, Germany
Antibiotic/antimycotic	GE Healthcare, Chalfont St Giles, UK
Brefeldin A	Sigma-Aldrich, St. Louis, MO, USA
CFDA-SE Cell Tracer Kit	Invitrogen, Carlsbad, CA, USA
Chloroform	Carl Roth, Arlesheim, Switzerland
DAPI	Invitrogen, Carlsbad, CA, USA
DMEM (powder)	AppliChem, Darmstadt, Germany
DMEM, unbuffered	Seahorse Bioscience, North Billerica, MA, USA
DMSO	Sigma-Aldrich, St. Louis, MO, USA
dNTPs	Peqlab, Erlangen, Germany
Doxycycline	AppliChem, Darmstadt, Germany
EDTA (Gibco)	Life Technologies, Carlsbad, CA, USA
Ethanol	Diagonal, Münster, Germany
Ethidium bromide (1% solution)	AppliChem, Darmstadt, Germany
FCS (Gibco)	Life Technologies, Carlsbad, CA, USA
Ficoll (PAA)	GE Healthcare, Chalfont St Giles, UK
FVD (eFlour450 and eFlour660)	eBioscience, San Diego, CA, USA
Gelatin	Sigma-Aldrich, St. Louis, MO, USA
Gene ruler (Fermentas)	Thermo Fisher Scientific, Waltham, MA, USA
Glycerin	AppliChem, Darmstadt, Germany
HEPES (PAA)	GE Healthcare, Chalfont St Giles, UK
IL-2 (recombinant murine)	Immunotools, Friesoythe, Germany
IL-12 (recombinant murine)	Immunotools, Friesoythe, Germany
IL-4 (recombinant murine)	Immunotools, Friesoythe, Germany
IL-7 (recombinant murine)	Immunotools, Friesoythe, Germany

Ionomycin	Diagonal, Münster, Germany
Isopropanol	Diagonal, Münster, Germany
L-Glutamine (PAA)	GE Healthcare, Chalfont St Giles, UK
MEM non-essential amino acids (PAA)	GE Healthcare, Chalfont St Giles, UK
Neomycin	AppliChem, Darmstadt, Germany
$OVA_{257-264}$	Peptides & Elephants, Potsdam, Germany
Orange G	Sigma-Aldrich, St. Louis, MO, USA
PCR Buffer	Peqlab, Erlangen, Germany
PCR Enhancer	Peqlab, Erlangen, Germany
PCR Water	AppliChem, Darmstadt, Germany
PMA	Diagonal, Münster, Germany
Polymycin B	AppliChem, Darmstadt, Germany
Proteinase K	Diagonal, Münster, Germany
RCB Lysis Buffer	Biolegend, San Diego, CA, USA
Streptavidin (APC-Cy7, PE)	Biolegend, San Diego, CA, USA
Taq polymerase	Peqlab, Erlangen, Germany
TRIS	AppliChem, Darmstadt, Germany
Triton X-100 (Fluka)	Sigma-Aldrich, St. Louis, MO, USA
Trypan Blue	Carl Roth, Arlesheim, Switzerland
XF Cell Mito Stress Kit	Seahorse Bioscience, North Billerica, MA, USA

4.1.2 Consumables

Cell culture plate, 6-well	Sarstedt, Nümbrecht, Germany
Cell culture plate, 96-well round bottom	Sarstedt, Nümbrecht, Germany
Cell strainer (100 µm, sterile)	BD, Franklin Lakes, NJ, USA
Cover slides (glass)	Diagonal, Münster, Germany
FACS tubes	Sarstedt, Nümbrecht, Germany
Luminometer-plates, 96-well	Nunc, Penfield, NY, USA
Microfine Syringes for i.p and i.v. injections	BD, Franklin Lakes, NJ, USA
PCR-plates, 96-well	Diagonal, Münster, Germany
Polyamide-mesh, pore size 150 µm	RCT, Heidelberg, Germany
Polyamide-mesh, pore size 80 µm	RCT, Heidelberg, Germany
Reaction tubes 1.5 ml	Sarstedt, Nümbrecht, Germany

Round-bottom tubes 4 ml and 14 ml	BD, Franklin Lakes, NJ, USA
Serological pipettes (5 ml, 10 ml, 25 ml)	Sarstedt, Nümbrecht, Germany
XF96 Analyser Cell Culture Microplates and Sensor Cartridges	Seahorse Bioscience, North Billerica, MA, USA

4.1.3 Oligonucleotides

All oligonucleotides were derived from Eurofins MWG Operon, Ebersberg, Germany.

Target gene/construct	Primer name	Sequence (5'-3')
li-rTA	RO83	CTGGGAGTTGAGCAGCCTAC
	RO84	CTCCTGTTCCTCCAATACGC
li-rTA	RO281	GTCTCAGAAGTGGGGGCATA
	RO282	GGACAGGCATCATACCCACT
TIM	RO235	CTCATCTCAAACAAGAGCCA
	RO236	CACTGCTTACTTCCTGTACC
TIM	RO298	AGAGAGCCAGAAAGGTGCAG
	RO299	AGCAGATGCATCACATGGTC
TSO	RO267	TGTAAGCTCTTGGGAATGG
	RO268	TGGAGGGTGTCGGAATAGAC
TSO	RO267	TGTAAGCTCTTGGGAATGG
	RO288	GCATCCACTCACGGATTTCT
Act-mOVA	RO264	TTATTCGTTCAGCCTTGCCAGTAG
	RO265	GCTCCAGGATCTTCATTTTCTCAG
Cd274^{tmLpc}	RO368	AGAACGGGAGCTGGACCTGCTTGCGTTAG
	RO369	ATTGACTTTCAGCGTGATTCGCTTGTAG
	RO370	TTCTATCGCCTTCTTGACGAGTTCTTCTG
KRAB	RO405	GAGTGGAAGCTGCTGGACAC
	RO406	CAGGATGGGTCTCTTGGTGA

4.1.4 Antibodies for flow cytometry and cell sorting

Specificity	Clone	Conjugate(s)	Source
CD11b	M1/70	bio	Biolegend, San Diego, CA, USA

CD11c	N418	bio	Biolegend, San Diego, CA, USA
CD16/32	93	unconjugated	eBioscience, San Diego, CA, USA
CD25	PC61	PE	Biolegend, San Diego, CA, USA
CD4	RM4-5	bio, AL647, PerCP, Brilliant Violett 510	Biolegend, San Diego, CA, USA
CD4	Gk1.5	Al647	eBioscience, San Diego, CA, USA
CD4	Gk1.5	PerCP/Cy5.5	Biolegend, San Diego, CA, USA
CD44	IM7	FITC	Biolegend, San Diego, CA, USA
CD45.1	A20	Al647, PerCP	Biolegend, San Diego, CA, USA
CD45.2	104	Al647	Biolegend, San Diego, CA, USA
CD49b	DX5	bio	Biolegend, San Diego, CA, USA
CD5	53-7.3	bio	Biolegend, San Diego, CA, USA
CD62L	MEL-14	PE	Biolegend, San Diego, CA, USA
CD69	H1.2F3	PE	Biolegend, San Diego, CA, USA
CD71	RI7217	PE	Biolegend, San Diego, CA, USA
CD8α	53-6.7	Al647, bio, PerCP	Biolegend, San Diego, CA, USA
CD90.1	OX7	Al647, PerCP	Biolegend, San Diego, CA, USA
Eomes	DAN11MAG	Al647	Biolegend, San Diego, CA, USA
FC-receptor	2.4G2	unconjugated	BioXcell, West Lebanon, NH, USA
IFNγ	XMG1.2	PE	Biolegend, San Diego, CA, USA
IL-2	JES6-5A4	PE	eBioscience, San Diego, CA, USA
Isotype control (CD5)	MOPC-173	Al488	Biolegend, San Diego, CA, USA
Isotype control (Ki67)	RTK2758	bio	Biolegend, San Diego, CA, USA
Ki67	B56	Al488	BD, Franklin Lakes, NJ, USA
T-bet	eBio4B10	Al647	eBioscience, San Diego, CA, USA
TCR β-chain	H57-597	AL488	Biolegend, San Diego, CA, USA

Ter119	TER-119	bio	Biolegend, San Diego, CA, USA
TNFα	MP6-XT22	PE	Biolegend, San Diego, CA, USA
Vα2	CL7213F	FITC	Cedarlane, Burlington, Canada
Vβ3	Kj25	FITC	Kindly provided by N. Asinovski, C. Benoist and D. Mathis (Harvard Medical School, Boston, MA, USA)

4.1.5 Antibodies used in vitro or in vivo

All antibodies used in vitro or in vivo were obtained from BioXcell, West Lebanon, NH, USA.

Specificity	Clone
LFA-1	M17/4
Isotype control (LFA-1)	2A3
CD3	145-2C11
CD28	37.51
IFNγ	XMG1.2
IL-4	11B11
PD-1	J43
PD-L1	10F.9G2
CTLA-4	UC10-4F10-11
CD40	FGK45.5

4.1.6 Buffers and media

CFSE Medium	PBS
	0.1% BSA (w/v)
DMEM	DMEM
	10 mM HEPES
FACS Medium	DMEM
	10 mM HEPES
	1% BSA (w/v)

Gene ruler	100 µl Marker
	700 µl TAE
	200 µl Loading Buffer
Gitocher Buffer (10x)	H_2O
	670 mM TRIS, pH8.8
	166 M $(NH_4)_2SO_4$
	65 mM $MgCl_2$
	0.1% Gelatin
Loading Buffer (6x)	250 mg Orange G
(Gel electrophoresis)	30 ml Glycerin 30%
	70 ml H_2O
MACS Buffer	PBS
	0.5% BSA (w/v)
	1 mM EDTA
PBS EDTA	PBS
	5 mM EDTA
Seahorse Assay Medium	DMEM (unbuffered)
	25 mM Glucose
	1 mM Pyruvate
	2 mM L-Glutamine
T cell Medium	RMPI 1680
	10 % FCS (v/v)
	2 mM Glutamine
	MEM non-essential amino acids
	5 µM 2-Mercaptoethanol
TAE	242 g TRIS
	57.1 ml acetic acid 99%
	100 ml EDTA 0.5 M, pH8
	add up to 1 l with H_2O
TE	1 M TRIS, pH7.6
	0.5 M EDTA

Tissue digestion buffer	PCR H_2O
	1x Gitocher buffer
	0.3 mg/ml Proteinase K
	0.5% Triton X-100
Trypan Blue Solution (10x)	PBS
	0.05% Trypan Blue (w/v)

4.1.7 Laboratory equipment

BD FACSCalibur	BD, Franklin Lakes, NJ
BD FACSCanto II	BD, Franklin Lakes, NJ
Camera Olympus E-330 (for Labovert FS)	Olympus, Hamburg, Germany
Centrifuge 5417R	Eppendorf, Hamburg, Germany
Centrifuge Rotanta 46 RS	Hettich, Tuttlingen, Germany
Electrophoresis Power supply (EPS200)	Pharmacia Biotech, Upsalla, Sweden
Gamma-cell 40	AECL, Chalk River Laboratories, Canada
Gel-documentation system	Intas, Göttingen, Germany
Incubator 5% CO_2, 3% O2	Heraeus, Hanau, Germany
Incubator 5 % CO_2	Heraeus, Hanau, Germany
Laminar airflow cabinet HeraSafe	Heraeus, Hanau, Germany
Luminometer	Berthold Technologies, Bad Wildbad, Germany
Microscope Labovert FS	Leitz, Wetzlar, Germany
Multichannel pipette	Brandt, Wertheim, Germany
Pipettes	Gilson, Middleton, WI, USA
Seahorse XF96 Extracellular Flux Analyzer and Prep Station	Seahorse Bioscience, North Billerica, MA, USA
Thermocyler T1	Biometra, Göttingen, Germany
Water bath	Lauda, Lauda-Königshofen, Germany

4.1.8 Software

BD cell quest	BD, Franklin Lakes, NJ, USA
FACSDiva (Canto II)	BD, Franklin Lakes, NJ, USA

FACSDiva (Aria II)	BD, Franklin Lakes, NJ, USA
Flowjo 8.8.7 for Mac	Treestar Ashland, OR, USA
GenePattern 3.7.0	Broad Institute, Cambridge, MA, USA (Reich et al., 2006)
Prism 5.0c for Mac	GraphPad, La Jolla, CA, USA
Seahorse XF 96 Software	Seahorse Bioscience, North Billerica, MA, USA
Gel Documentation System	Intas, Göttingen, Germany

4.1.9 Statistic analysis

Statistic analysis was performed using the Prism 5.0c software. If not indicated otherwise, means, standard deviation and p values from unpaired two-tailed Student's *t*-test are shown.

4.2 Mice and treatments of mice

All mice were bred and maintained at the animal facility of the Institute for Immunology, Ludwig-Maximilians-University of Munich, Munich, Germany. All experiments were performed in compliance with German federal guidelines.

4.2.1 Wild type mice and congenic markers

Mice of B10.BR/SgSnJ (B10.BR, originally received from The Jackson Laboratory) C57BL/6 (B6) and BALB/c backgrounds were used. The congenic markers CD45.1 and CD90.1 used for the identification of adoptively transferred cells were originally derived from B6.SJL-Ptprca Pepcb/BoyJ and B6.PL-Thy1a/CyJ mice received from The Jackson Laboratory. BALB/c mice were obtained from Prof. Ludger Klein and Ksenija Jovanovic (Ludwig-Maximilians-University Munich, Munich, Germany).

4.2.2 T cell receptor transgenic mice

AND T cell receptor (TCR) transgenic (tg) animals (Tg(TcrAND)53Hed) display predominant expression of a MHC II-restricted TCR consisting of a Vα11 and a Vβ3 chain, recognizing a peptide derived from moth cytochrome *c* (MCC_{93-103}) in the context of H-$2E^k$ (Kaye et al., 1989). The Vα11 (derived from the $CD4^+$ T cell clone AN6.2) and Vβ3 constructs (derived from the $CD4^+$ T cell clone 5C.C7) containing endogenous promotor and

enhancer elements were coinjected to generate these mice. AND mice were received from Diane Mathis and Christophe Benoist (Harvard Medical School, Boston, MA, USA) and were maintained on the B10.BR background.

For some experiments, AND mice crossed to mice expressing the IL-4 reporter *Il4*^{tm1Lky} (4get, (Mohrs et al., 2001)), received from David Vöhringer, Friedrich-Alexander University Erlangen-Nürnberg, Erlangen, Germany) were used. The 4get transgene contains the murine IL-4 locus with an IRES (internal ribosomal entry site)-EGPF (enhanced GFP) construct with the polyadenylation signal from bovine growth hormone inserted just downstream of the translational stop and upstream of the polyadenylation site of intron 4. Thus, IL-4 is co-expressed with GFP in AND;4get mice.

T cells of OT1 TCR tg animals (Tg(TcraTcrb)1100Mjb) predominantly express a TCR that is specific for SIINFEKL peptide of chicken ovalbumin ($OVA_{257-264}$) in the context of H-2K^b and consists of a Vα2 and a Vβ5 chain (Hogquist et al., 1994). The TCR chains were derived from an OVA-specific CD8$^+$ T cell clone (149.42). OT1 mice were generated by coinjection of constructs coding for the α- and β-chains. OT1 mice were received from Thomas Brocker (Ludwig-Maximilians-University Munich, Munich, Germany) and were maintained on the B6 background.

TCR tg animals were crossed with mice expressing congenic markers (CD45.1 or CD90.1) in order to allow their tracing following adoptive transfer.

4.2.3 Double transgenic mice allowing doxycycline-dependent antigen expression

Double transgenic (dtg) dtg-M mice were obtained by crossing Ii-rTA tg mice (Tg(Cd74-rtTA)#Doi) (Obst et al., 2005) with mice carrying the TIM transgene (tetracycline inducible invariant chain with $MCC_{93-103,}$ Tg(tetO-Cd74/MCC)#Doi)(van Santen et al., 2004), resulting in doxycycline-dependent expression of MCC_{93-103} in the context of H-2E^k in MHC II positive cells and therefore cognate antigen presentation to AND TCR tg CD4$^+$ T cells (Obst et al., 2005). Dtg-M mice were received from Diane Mathis and Christophe Benoist (Harvard Medical School, Boston, MA, USA) and were maintained on the B10.BR background. For detailed descriptions of the Ii-rTA and TIM constructs and their functional properties refer to section 5.1.

In some experiments, dtg-M mice crossed to PD-L1-deficient *Cd274*^{tm1Lpc} mice were used (dtg-M;PD-L1$^{o/o}$, received from David Vöhringer, Friedrich-Alexander University Erlangen-Nürnberg, Erlangen, Germany). In these knock out mice, the signal peptide with the ATG

start codon and a large proportion of exon two of the CD274 gene was deleted by homologous recombination with a construct containing a neomycin cassette (Dong et al., 2004).

In Dtg-O mice, the Ii-rTA transgene is combined with the TSO transgene (tetracycline regulated signal sequence with $OVA_{257-264}$), resulting in doxycycline-dependent expression of $OVA_{257-264}$ peptide in MHC II positive cells and therefore cognate antigen presentation to OT1 TCR tg $CD8^+$ T cells (Rabenstein, 2012). Dtg-O mice were maintained on the B6 background. For detailed descriptions of the TSO construct and its functional properties refer to section 5.1.

In order to reduce the background expression of the TSO transgene in the absence of doxycycline, dtg-O mice were crossed to mice expressing the KRAB transgene (HPGKtTR-KRAB) coding for a tetracycline-dependent transrepressor under the control of the promotor of human phosphoglycerate kinase (hPGK) (Barde et al., 2009). For detailed description of the transgene and its supposed effect on $OVA_{257-264}$ expression in dtg-O;KRAB mice, refer to results section 5.7. KRAB mice were received from Andreas Trumpp (German Cancer Research Center, Heidelberg, Germany).

4.2.4 Mice expressing antigen constitutively

Ii-MCC mice (tg(H2-Ea-Cd74/MCC)37GNnak) constitutively express the Ii-MCC transgene, a fusion protein of invariant chain (Ii) of MHC II and the MCC_{88-103} peptide under the control of H-2Eα promotor elements, thus providing cognate antigen expression for AND TCR tg $CD4^+$ T cells on all MHC II expressing cells (Yamashiro et al., 2002). Ii-MCC mice were received from Diane Mathis and Christophe Benoist (Harvard Medical School, Boston, MA, USA) and were maintained on the B10.BR background.

Act-mOVA (Act-OVA, Tg(CAG-OVA)916Jen) mice constitutively express membrane-bound chicken ovalbumin under the control of β-actin promotor on all body cells (Ehst et al., 2003). In these transgenic mice, the leader sequence from $H\text{-}2K^b$ gene, containing a short extracellular spacer and the transmembrane region, is linked to the coding sequence of OVA under the control of CMV-immediate-early(IE)-enhancer and chicken-β-actin promotor, followed by a rabbit polyA sequence. Act-OVA mice were received from The Jackson Laboratory and were maintained on the B6 background.

4.2.5 MHCI$^{-/-}$ and MHCI$^{-/-}$DC-MHCI mice

In *B2m*^{tm1Jae} mice (Zijlstra et al., 1989), the gene coding for the beta-2 microglobulin (β_2m) is replaced with a construct containing a neomycin cassette by homologous recombination. Therefore, these mice are deficient for the β-chain of MHC I and thus lack MHC I on the surface of all body cells. Crossing them to mice that express β_2m under the control of the human keratin 14 (K14) promotor (K14-β_2m, (Capone et al., 2001)) allows for the surface-expression of MHC I in the thymus and thus undisturbed development of the endogenous CD8$^+$ T cell compartment. The K14-β_2m construct consists of the K14 promotor, β-globulin intron, the murine β_2m cDNA and the K14 polyA sequence. *B2m*^{tm1Jae};K14-β_2m mice are called MHCI$^{-/-}$ mice in this study. The combination with the CD11c-β_2m transgene (Kurts et al., 2001) allows for additional expression of MHC I on DCs in *B2m*^{tm1Jae};K14-β_2m;CD11c-β_2m (MCHI$^{-/-}$DC-MHCI) mice (Gruber et al., 2010). The CD11c-β_2m transgene consist of the β_2m cDNA ligated with the CD11c promotor region (Brocker et al., 1997).

Thomas Brocker and Caroline Bernhard (Ludwig-Maximilians-University Munich, Munich, Germany) kindly provided genotyped mice of both lines.

4.2.6 Genotyping of mice

Except for TCR tg animals, all mice were typed by polymerase chain reaction (PCR) using the primers and protocols described in sections 4.1.3 and 4.3.1.2. Ii-MCC mice were typed with the primers also used for identification of the TIM transgene. AND TCR tg mice were typed by surface staining of peripheral blood for the β-chain of the AND TCR (Vβ3) and flow cytometric analysis as described in section 4.3.3.1. Surface staining of the α-chain (Vα2) of OT1 TCR was utilized for the genotyping of OT1 TCR tg animals. In addition to the PCR, surface staining for PD-L1 was used for typing of PD-L1$^{o/o}$ mice. Congenic marker expression (CD45.1, CD90.1) was also assessed flow cytometrically.

For flow-cytometric typing, 2-3 drops of blood were collected from the tail vain and processes as described in section 4.3.2.1 and 4.3.3.1.

4.2.7 Mouse cytomegalovirus infection

If indicated, mice were infected with 2 x 10^6 plaque forming units (pfu) mouse cytomegalovirus (MCMV, obtained from Thomas Brocker, Ludwig-Maximilians-University Munich, Munich, Germany) diluted in PBS by intra peritoneal (i.p.) injection.

4.2.8 Intra peritoneal application of monoclonal antibodies

Sterile dilutions of monoclonal antibodies were prepared in PBS and a maximum of 200 µl per mouse injected i.p..

4.2.9 Doxycycline treatment

If indicated, mice were provided with 100 µg/ml doxycycline diluted in water low in divalent cations (Volvic, Danone Waters, Frankfurt, Germany) ad libitum.

4.2.10 Generation of bone marrow chimeras

Recipient mice were irradiated (5 Gy) twice at least 6 h apart and received 5 x 10^6 bone marrow cells from congenically distinct donors the same day. Recipient mice were supplied with 2 mg/ml neomycin and 0.1 mg/ml polymyxin B in their drinking water for the following 6 weeks. The chimerism was analyzed flow cytometrically when performing the final analysis of the experiments, using antibodies for the congenic markers of the recipients and the bone marrow donors and was higher than 97% in the B cell compartment ($CD45R^+$) in all experiments.

4.3 Methods

4.3.1 Molecular biology

4.3.1.1 Tissue digestion

For genotyping of mice, DNA from tail tissue (2-3 mm length) of mice was used. The tissue was digested in 100 µl tissue digestion buffer for 6 h at 56°C, followed by 10 min at 90°C for protein denaturation. For PCR, the lysate was diluted 1:10 in TE.

4.3.1.2 Polymerase chain reaction (PCR)

For PCR typing of transgenic mice, 24 µl of the master mix described below and 1 µl of diluted tissue lysate were used. Following initial DNA denaturation (5 min at 95 °C), 35 cycles of denaturation (30 sec at 95 °C), primer annealing (45 sec at 55 °C) and DNA

elongation (45 sec at 72 °C) were performed, followed by a final elongation period (5 min at 72 °C) and a cooling period (10 sec at 20 °C).

PCR master mix	
PCR H_2O	
PCR Buffer	1x
PCR Enhancer	0.5x
Oligonucleotide 1	0.5 µM
Oligonucleotide 2	0.5 µM
dNTPs	0.2 mM
Taq polymerase	0.026 U/µl

4.3.1.3 Gel electrophoresis

Agarose gels (TAE, 1.5% agarose, 0.005% ethidiumbromide) were used for size separation of PCR products, with a DNA marker (100 bp ladder) allowing for the determination of the PCR product size. The PCR products were mixed with loading buffer in a 1:5 ratio, loaded to gels and electrophoresis performed at 120 V in a horizontal gel chamber. The results were visualized using a UV light source and a gel documentation system.

4.3.1.4 RNA isolation

7-9 x 10^4 $CD4^+$ or $CD8^+$ T cells were directly FACS-sorted into 1 ml TRIzol (Invitrogen, Carlsbad, CA, USA) per sample and stored at −80 °C until further processing. Samples were thawed, 200 µl chloroform added and mixed for 15 sec, followed by 2 min incubation at room temperature. Samples were centrifuged at 12,000 g for 15 min at 4 °C. The RNA containing upper phase was transferred to a new tube, 500 µl isopropanol were added and incubated for 10 min at room temperature, followed by another 10 min centrifugation step. The supernatant was discarded and the pellet washed with 70% ethanol. The RNA was air-dried, resolved in 20 µl PCR H_2O and stored at −80 °C. RNA isolation was carried out by Simone Pentz (Ludwig-Maximilians-University Munich, Munich, Germany).

4.3.1.5 Gene expression analysis

The purity of the RNA isolated from sorted T cells was controlled with a 2100 Bioanalyzer (Agilent, Böblingen, Germany) and only samples with an RNA integrity number (RIN) > 7 used. The RNA was amplified using the two-cycle MessageAmp II aRNA Amplification Kit and further processed using the Message Amp II-Biotin Enhanced Kit according to manufacturer's instructions (both Ambion, Life technologies, Carlsbad, CA, USA). Samples from three independent experiments were hybridized on Affymetrix Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA, USA), using a GeneChip Hybrization oven 645. Microarrays were washed and stained using the GeneChip Fluidics Station 450 and scanned with the GeneChip Scanner 3000 (all from Affymetrics, Santa Clara, CA, USA). The above steps were carried out by Marion Horsch and Johannes Beckers (Helmholtz Center Munich, Munich, Germany).

The data were analyzed using the GenePattern platform (Reich et al., 2006). The Expression File Creator module was used to normalize .cel files, using Robust Multiarray Average background correction method (RMA, (Irizarry et al., 2003)). Subsequently, redundant probe sets were collapsed using the CollapseDataset module. The Multiplot module was used to visualize the data. A set of 128 genes specific for B cells (Painter et al., 2011) or encoded on the X- and Y-chromosome were excluded from visualization.

4.3.1.6 ATP Assay

The ATP Bioluminescence Assay Kit (Roche, Basel, Switzerland) was used to measure the ATP content of T cells in a 96-well format. According to manufacturers instructions, cells were lysed and the equivalent of 2.5 x 10^4 cells per well analyzed in triplicates for the ATP mediated bioluminescence of luciferase in a luminometer.

4.3.1.7 Seahorse XF96 Extracellular Flux Analyzer

The Seahorse XF96 Extracellular Flux Analyzer allows the measurement of changes in dissolved O_2 (O_2 consumption rate; OCR) and pH (extracellular acidification rate; ECAR) in the culture medium of live cells in vitro (Wu et al., 2007). In dedicated 96-well plates, a transient micro-chamber of 2.6 µl volume is generated above a monolayer of cells of interest in each well and changes in dissolved O_2 and pH are determined using optical fluorescent

biosensors during measuring cycles typically lasting 5-8 min. Between measuring cycles, mixing periods allow for the normalization of analytes in the medium. As cells stay alive and undisrupted, they can be maintained for hours in the analyzer (at 37 °C), and OCR and ECAR can be measured repeatedly. Four injection ports allow the application of drugs or inhibitors during the course of an experiment.

The Seahorse XF96 analyzer was used to perform a Mitochondrial Stress Test on stimulated T cells. As the XF96 analyzer relies on a monolayer of adherent cells for analysis, stimulated T cells (see section 4.3.2.3) were immobilized on the surface of XF96 Analyzer Cell Culture Microplates pretreated with 3.5 µg/cm^2 BD Cell Taq (BD, Franklin Lakes, NJ, USA) according to manufacturers instructions. To achieve monolayers, 0.8-1.6 x 10^6 unstimulated and 2-8 x 10^5 stimulated T cells were seeded in 80 µl Seahorse Assay Medium per well, centrifugated for 1 sec at 40 g and after reversion of plate orientation in the plate carrier for 1 sec at 80 g (both without brake) and incubated for 25 min at 37°C without CO_2 in the XF Prep Station. 120 µl of prewarmed Seahorse Assay Medium were added carefully and cells were incubated for further 15 min at 37 °C without CO_2 in the Seahorse XF Prep Station before the measurement was started. Three min mixing periods were alternated with 5 min measurement periods. After 5 measuring cycles for baseline acquisition, the H^+ pump inhibitor oligomycin was injected at a final concentration of 1 µM. With a delay of three measuring cycles each, FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, a decoupling agent, 1.5 µM final) and rotenone together with antimycin A (inhibitors of electron transport chain, both 1 µM final) were injected. The non-mitochondrial respiration was determined as the minimum of three rates measured following injection of antimycin A and rotenone and subsequently subtracted from all rates used for calculation of further parameters. Basal OCR and ECAR were calculated as the maximum of the 3 rates acquired before injection of oligomycin, OCR/ECAR as the rate of both independently calculated parameters. The ATP production was calculated as the minimum of three rates measured following oligomycin injection, the maximal respiration as the maximum of three rates measured following FCCP injection. The spare respiratory capacity (SRC) was calculated as the deviation of maximal respiration and basal OCR.

4.3.2 Cellular methods

4.3.2.1 Organ removal and generation of single cell suspensions

Mice were sacrificed in accordance with the German Protection of Animals Act by CO_2 fumigation. Lymph nodes (axillary, inguinal, brachial from recipients of T cell transfer, axillary, inguinal, brachial and cervical from T cell donors) and spleens or hind limb bones (femur and tibia, for generation of bone marrow chimeras) were removed under unsterile conditions after surficial disinfection of mice with ethanol. Lymph nodes and spleens were mechanically disrupted using sterile cell strainers if intended for adoptive T cell transfer or cell culture. If intended for flow cytometric analysis, organs were disrupted using glass cover slides and filtered trough a mesh (pore size of 150 µm). Splenocyte suspensions were centrifuged through a Ficoll cushion (10 min 2,000 rpm/984 g, brake grade 5 of 9) and the interphase harvested. Hind limb bones were mechanically disrupted in a sterile mortar, filtered trough a cell strainer and subjected to red blood cell lysis using RBC lysis buffer (Biolegend, San Diego, CA, USA) according to manufacturer's instructions.

Blood samples were collected directly into PBS/EDTA and erythrocytes removed using RBC Lysis Buffer according to manufacturer's instructions.

Single cell suspensions were pelleted (1,500 rpm/554 g, 4 min) and resuspended in a defined volume for cell counting using a Neubauer chamber and Trypan Blue solution. For organ collection and the generation of single cell suspensions, FACS medium or DMEM (if cells were intended for adoptive transfer without further processing) were used.

4.3.2.2 Magnetic-activated Cell Sorting (MACS)

Magnetic-activated Cell Sorting (MACS) was used to purify AND $CD4^+$ and OT1 $CD8^+$ T cells or polyclonal $CD4^+$ and $CD8^+$ T cells. This technic uses paramagnetic α-biotin-beads to separate cells labeled with biotinylated antibodies from unlabeled cells by passage over a column placed in a strong magnetic field. The cells of interest were negatively selected. Single cell suspensions from lymph nodes and spleens were incubated with a mix of biotinylated mAbs as indicated below. Incubation was carried out in a total of 200 µl FACS medium per donor mouse for 15 min on ice. Cells were washed twice with FACS medium and incubated with 10 µl α-biotin-beads (Milteny, Bergisch-Gladbach, Germany) + 190 µl FACS medium per donor mouse for 20 min at 4 °C (in the fridge). Cells were washed twice

with MACS buffer, resuspended in 0.7-2 ml MACS buffer per donor mouse and transferred onto equilibrated MACS LS-Columns (Milteny, Bergisch-Gladbach, Germany) at 2 ml (cells of 1-3 donor mice) per column. Columns were subsequently washed twice with 3 ml MACS buffer and the flow-through was collected. The obtained cells were washed once and counted. For quality control, an aliquot of purified cells was stained with α-CD4-PerCP, α-CD8-Al647 and SA-PE and analyzed by flow cytometry as described in section 4.3.3.1. A purity of 95% was routinely achieved.

Biotinylated mAb specificity	Target cells	µl/donor mouse
GR-1	Granulocytes	5 µl
Ter119	Erythrocytes	5 µl
CD49b	Natural killer (NK) cells	5 µl
CD11b	Macrophages	5 µl
CD11c	Dendritic cells	5 µl
CD45R	B cells	8 µl
CD4 or CD8 respectively	$CD4^+$ or $CD8^+$ T cells respectively	8 µl

4.3.2.3 T cell stimulation with plate-bound α-CD3 and α-CD28 mAbs

96-well round bottom plates were incubated with 70 µl/well PBS containing 10 µg/ml α-CD3 and α-CD28 mAbs each for at least 90 min at 37 °C and 5% CO_2. The plates were washed twice with cold PBS. T cells were cultured in T cell medium with 5 ng/ml IL-7 added to increase survival of unstimulated cells during the 2 d culture period. T cells were seeded at 10^5 cells and 200 µl per well and cultured at 37 °C and 5% CO_2 unless otherwise indicated.

4.3.2.4 T cell stimulation with antigen presenting cells

For the restimulation of T cell primed in vitro with plate-bound α-CD3 and α-CD28, a coculture with antigen presenting cells (APCs) was used. Splenocytes from wt or antigen-expressing mice (Ii-MCC for AND T cells, Act-OVA for OT1 T cells) were irradiated (10 Gy). Stimulated T cells were mixed with a known number of congenically distinct naïve wt splenocytes before CFSE labeling to provide undivided control cells. After labeling, the

equivalent of 10^4 stimulated T cells and 10^5 wt or antigen-expressing irradiated splenocytes per well were cocultured in 96-well round bottom plates. Culture was performed in T cell medium without additional IL-7 at 37 °C and 5% CO_2. 3 d later, the cultures were stained for respective congenic markers as well as CD4 and CD8 for flow cytometric analysis.

4.3.2.5 T cell restimulation for cytokine staining

For the quantification of cytokine production on the single cell level, it is necessary to block the secretion of cytokines to make them available for intracellular staining with mAbs. Therefore, splenocytes of T cell recipients or in vitro-stimulated T cells were cultured with 20 ng/ml PMA (a protein kinase C (PKC) stimulating agent) and 1 µg Ionomycin (a Ca^{2+}-ionophore) for 4 h at 5% CO_2 and 37 °C, causing very strong and TCR-independent (re)activation of T cells. BrefeldinA (an inhibitor of secretory vesicle formation at the Golgi apparatus) was added during the last 2 h of culture, leading to accumulation of produced cytokines in the T cells. The restimulation culture was performed in 6-well plates with 2-3 x 10^7 splenocytes in 3 ml T cell medium per well.

4.3.2.6 Th1/Th2 differentiation culture

To achieve differentiation of AND $CD4^+$ T cells towards the Th1 or Th2 phenotypes, cells were MACS-purified and stimulated with α-CD3 and α-CD28 as in section 4.3.2.3, but without IL-7. Additionally, 5 ng/ml IL-12 and 20 µg/ml α-IL-4 for Th1 or 50 ng/ml IL-4 and 50 µg/ml α-IFNγ for Th2 polarization were added as described in (Grogan et al., 2001). Control cells were stimulated as described in section 4.3.2.3.

4.3.2.7 Generation of Rested Effector $CD4^+$ T cells

Rested Effector (RE) T cells generated in vitro are very similar to in vivo generated memory cells (McKinstry et al., 2007). RE T cells were generated by culturing lymph node suspensions of AND T cells with irradiated (33 Gy) splenocytes from mice expressing $MCC_{88\text{-}103}$ constitutively (Ii-MCC) for 4 d, followed by a resting period of 3 d. Coculture was performed in 96-well round bottom plates with the equivalent of 0.25 x 10^5 AND T cells and 10^5 irradiated Ii-MCC splenocytes per well in T cell medium substituted with 80 U/ml IL-2. The cell suspensions were centrifuged over a Ficoll cushion as described in section 4.3.2.1 to

remove dead cells. As APCs had been irradiated before initial culture, they were removed by this procedure. The cells were counted and cultured at 10^5 cells per well in 96-well round bottom plates using T cell medium for 3 d to acquire the RE phenotype. The phenotypic similarity of in vivo generated AND memory cells and AND RE cells was described before (Tussing, 2008).

4.3.2.8 CFSE labeling

Carboxyfluorescein succinimidyl diacetate ester (CFDA-SE) is the highly cell permeable precursor of a fluorescence dye. Following entry into the cytoplasm, cellular esterases remove the acetate residues, generating fluorescent CFSE, which is subsequently covalently coupled to amino groups of cytoplasmic proteins. CFSE conjugated to cytoplasmic proteins is stable over days to months and in case of cell division symmetrically distributed between daughter cells. Therefore, the analysis of the CFSE dilution allows the monitoring of cell proliferation (Quah et al., 2007). For the labeling of cells with CFSE, single cell suspensions of 2 x 10^7 cells/ml were prepared in prewarmed CFSE medium and incubated with 10 µM/ml (lymphocyte or splenocyte preparations) or 5 µm/ml (cultured cells) CFDA-SA for 10 min at 37 °C. Cell suspensions were underlayed with 1 ml FCS and pelleted, followed by two washing steps with DMEM.

4.3.2.9 Adoptive T cell transfer

Adoptive transfer of T cells or bone marrow cells was performed via the tail vein. Mice were placed under a red light source for about 20 sec to allow widening of the tail veins. Cells were injected in a maximal volume of 200 µl in DMEM. Before transfer, cells were washed twice with DMEM if they had been maintained in medium containing BSA or FCS beforehand to remove these potential immunogenic proteins. If not indicated otherwise, 2 x 10^6 T cells (or the equivalent of 2 x 10^6 tg T cells, if unprocessed lymphocyte preparations were used) were transferred.

4.3.2.10 In vivo killing assay

To assess the capability of OT1 T cell to kill target cells presenting their cognate peptide ($OVA_{257-264}$) in vivo, we transferred congenically marked and peptide pulsed splenocytes

from wt mice into recipients of OT1 T cell transfer. Wt splenocytes were pulsed with 1 µg/ml $OVA_{257-264}$ in T cell medium for 4 h at 37 °C and 5% CO_2 or cultured without peptide (unpulsed). Splenocytes were subsequently labeled with 5 µM (pulsed) or 5 nM CFSE (unpulsed) as described in section 4.3.2.8, to allow discrimination of the two populations ex vivo. Pulsed and unpulsed cells were mixed in a 1:1 ratio and the equivalent of 2.5 x 10^6 cells per population transferred into mice that received OT1 T cell transfer 3 d earlier. 16 h later, mice were sacrificed and spleens analyzed by flow cytometry for frequencies of pulsed and unpulsed target cells.

4.3.3 Flow cytometry

The BD FACSCalibur and FACSCanto II flow cytometers were used for data acquisition. All stainings were performed in 96-well round bottom plates. All antibody dilutions were centrifuged at 21,000 g for 3 min at 4 °C prior to usage in order to remove protein aggregates. All incubations were carried out in the dark. Directly before analysis, cell suspensions were filtered through a mesh (pore size of 80 µm).

4.3.3.1 Staining of surface molecules

FACS medium was used for all washing steps, mAb dilution and sample acquisition. Cells were washed, pelleted, resuspended in 50 µl mAb solution and incubated for 20 min on ice. For staining with biotinylated mAbs, two washing steps and incubation with fluorochrome coupled SA-dilution for 10 min was carried out subsequently. All antibodies were diluted 1:400 and SA at 1:2,000. DAPI (4,6-diaminidin-2-phenylindol), used for the discrimination of dead cells, was applied at 1 µg/ml together with mAbs. Cells were washed before analysis.

4.3.3.2 Staining of intracellular markers and cytokines

The FoxP3 staining kit (eBiocience, San Diego, CA, USA) was used according to manufacturer's instructions for intracellular staining of transcription factors, cytokines, DAPI and the cell cycle activity marker Ki67. Beforehand, cells were stained with fixable viability dye (FVD) eFlour450 or eFlour660 diluted 1:1.000 in PBS for at least 20 min on ice. Cells were washed and stained for surface molecules as described above. Subsequently, cells were

fixed and permeabilized in 100 µl Fix/Perm per well for at least 30 min or up to 20 h at 4°C. Cells were washed twice with 150 µl Perm/Wash per well. Cells were incubated with an α-CD16/CD32 or an α-FC-receptor mAb for 5 min on ice to prevent the unspecific binding of mAbs later on. Cells were washed and stained intracellulary under the conditions described below. Before analysis, cells were washed with PermWash and FACS medium and resuspended in FACS medium. Stainings with isotype control antibodies (IC) or FMOs (fluorescence minus one; staining for all markers except the one to be analyzed) were used as controls.

Specificity/Dye	Dilution	Staining condition
T-bet	1:100	1 h, room temperature
IFNγ, TNFα, IL-2	1:200	20 min, room temperature
Ki67	1:10	1 h, room temperature
DAPI	1 µg/ml	10 min, 4 °C

4.3.3.3 Fluorescence-activated Cell Sorting (FACS)

To remove dead and apoptotic cells and achieve a purity of approximately 100% essential for gene expression analysis, AND and OT1 T cells stimulated as described in section 4.3.2.3 were stained with α-Ter119, α-GR1, α-CD49b, α-CD11c, α-CD11b, α-CD45R and α-CD4 (OT1 T cells) or α-CD8 (AND T cells) (all biotinylated) and α-CD4-Al647 (AND) or α-CD8-Al647 (OT1) and SA-PE as described in section 4.3.3.1. 1 µg/ml DAPI was added directly before FACS sorting performed at a MoFlo sorter (Beckham Coulter, Indianapolis, IN, USA) operated by Joachim Ellwart (Institute for Molecular Immunology, Helmholtz Center Munich, Munich, Germany). Single $DAPI^{-}PE^{-}Al647^{+}$ cells were directly sorted into TRIzol (Invitrogen, Carlsbad, CA, USA). One sorting procedure was sufficient to obtain ~100% purity of $CD4^{+}$ and $CD8^{+}$ T cells.

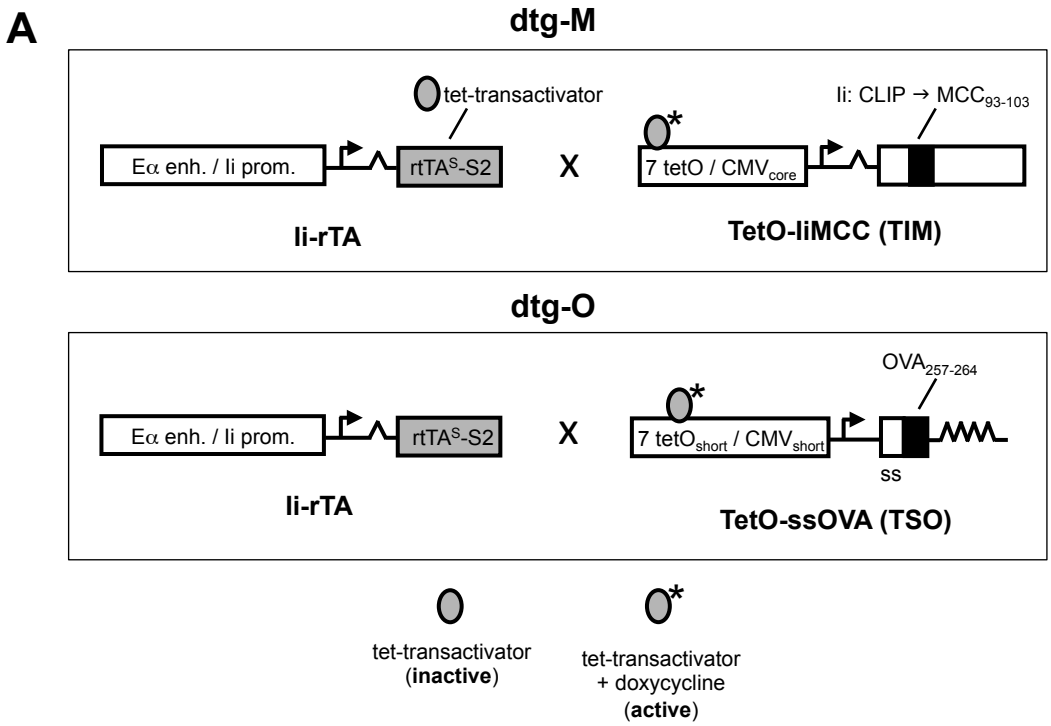
5 Results

5.1 Doxycycline-regulated antigen expression in vivo

In order to analyze how the duration of antigen presentation affects adaptive immune responses, a strict control of antigen expression is an inevitable prerequisite. In previous studies on T cell antigen dependency, termination of antigen expression in vivo has been achieved in systems of bacterial infection by antibiotic treatment. In this system, the antigenic stimulus cannot necessarily be separated from general inflammation and killed bacteria might persist for days (Corbin and Harty, 2004; Williams and Bevan, 2004). In addition, replicating pathogens add a further layer of complexity, as their antigens are presented by directly infected target cells and following phagocytic uptake in professional antigen presenting cells (APCs). The involvement of different cell types and antigen processing pathways hampers the predictability and also the analytic assessment of antigen presentation kinetics. Therefore, a doxycycline-dependent antigen expression system was used in this study to allow antigen expression to be tuned at will within a few professional APCs, creating conditions that allow the separate in vivo dissection of antigenic stimulation and inflammation.

To work with T cells of defined specificity, $CD4^+$ and $CD8^+$ T cell receptor (TCR) transgenic (tg) T cells were studied: AND TCR tg $CD4^+$ T cells, specific for amino acids 93-103 of moth cytochrome *c* ($MCC_{93\text{-}103}$) and OT1 TCR tg $CD8^+$ T cells, specific for amino acids 257-264 of chicken ovalbumin ($OVA_{257\text{-}264}$). Both TCRs display high affinities for their respective antigens (Alam et al., 1996; O'Donoghue et al., 2013) and are not self-reactive (Aichinger et al., 2013; Hogquist et al., 1994). As TCR tg T cells can never completely represent the respective polyclonal subsets, the transferability of findings generated with AND and OT1 T cells to polyclonal T cells will be assessed later. Both TCR tg strains carry congenic markers (CD45.1 or CD90.1) used for the identification of the cells in recipients of T cell transfer.

Two double-transgenic (dtg) mouse lines were used for the expression of cognate antigens of AND TCR tg $CD4^+$ T cells or OT1 TCR tg $CD8^+$ T cells respectively (Fig. 1A). Both dtg mice carry a transgene coding for the improved S2 mutant of the reverse tet-transactivator ($rtTA^S$-S2), which consists of a tetracycline binding domain and a transactivator domain (Urlinger Hillen 2000). The tet-transactivator is under the control of the invariant chain (Ii) promoter and H-2Eα enhancer elements derived from the pDOI-6 transgene expres-



B

	TCR tg T cells	cognate peptide	MHC restriction	Ag expression in vivo	background
$CD4^+$	AND	MCC_{93-103}	$H\text{-}2E^k$	dtg-M	B10.BR
$CD8^+$	OT1	$OVA_{257-264}$	$H\text{-}2K^b$	dtg-O	B6

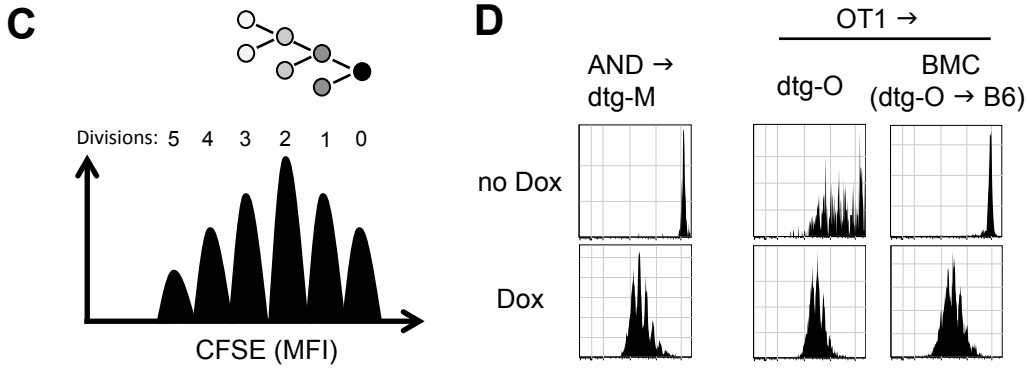


Fig. 1: Description of double-transgenic (dtg) mice expressing cognate antigen for AND (dtg-M) or OT1 T cells (dtg-O) in a doxycycline-dependent fashion. (A) Transgenes of dtg-M and dtg-O mice. In both lines, the reverse tetracycline-dependent transactivator ($rtTA^S$-S2) is expressed constitutively under the control of Eα enhancer and invariant chain (Ii) promotor elements. In dtg-M mice, this transgene is combined with the TIM transgene (tetracycline inducible invariant chain with MCC_{93-103}), where the CLIP region of Ii is replaced with the MCC_{93-103} peptide and expressed under the control of tetracycline operator (TetO) and CMV_{core} promotor sequences. In dtg-O mice, the TSO transgene (tetracycline regulated signal sequence with $OVA_{257-264}$) contains an improved tetracycline response element (pTRE-tight) consisting of $tetO_{short}$ and CMV_{short} promotor that regulates the expression of the ssOVA minigene consisting of the $H\text{-}2K^b$ signal sequence (ss) and $OVA_{257-264}$ followed by the human growth hormone splice substrate. In both dtg-M and dtg-O mice, the tet-transactivator

binds to the tetO sequences in the presence of doxycycline and induces the expression of the TIM and TSO transgenes. **(B)** Overview of experimental systems of cognate antigen expression. **(C)** The fluorescent dye CFSE quantifies proliferation in vivo (schematic). During each division, CFSE is equally distributed between daughter cells, resulting in distinct fluorescence peaks representing respective cell generations. **(D)** AND and OT1 T cells proliferate following adoptive transfer into dtg-M or dtg-O mice treated with doxycycline. Naïve CFSE labeled AND and OT1 T cells were adoptively transferred as indicated and the CFSE dilution analyzed 3 d later in the spleens by flow cytometry. As indicated, dtg mice received 100 µg/ml doxycycline in their drinking water during the experiment, starting 1 d before T cell transfer. Data shown have been gated on single live cells positive for congenic markers and CD4 or CD8, respectively. BMC: bone marrow chimera, lethally irradiated B6 mouse receiving dtg-O bone marrow transfer.

sion vector (van Santen et al., 2000) and thus is called Ii-rTA (Obst et al., 2005). The Ii-rTA construct contains intron one of Ii downstream of the transcription start, as introns are required for efficient expression of transgenes (Brinster et al., 1988), and the rabbit β-globulin polyA sequence.

In dtg-M mice, the second transgene TIM (tetracycline inducible invariant chain with $MCC_{93\text{-}103}$) consists of a modified Ii with the CLIP region replaced by $MCC_{93\text{-}103}$ under the control of the CMV_{core} promoter and seven tetO sequences (van Santen et al., 2004). An intron from rabbit β-globulin is inserted downstream of the transcription start site to allow efficient transgene expression. Only in the presence of a tetracycline such as doxycycline can the tet-transactivator bind to the tetracycline operator sequence (tetO). Together with factors bound to the adjacent minimal CMV promotor, expression of downstream genes is induced. Therefore, the cognate peptide of AND T cells is directly delivered to MHC II during its assembly in the endoplasmic reticulum in cells expressing this transgene. Since the tet-transactivator is under the control of Ii promoter elements, one would expect expression in cells positive for MHC II, i.e., predominantly in B cells and dendritic cells. The doxycycline-inducible gene expression system used here has been shown to lead to poor expression in B cells but efficient expression in dendritic cells (DCs) (Obst et al., 2005). However, for unclear reasons tetracycline dependent gene expression could not be observed in mature B cells in some studies (Hess et al., 2001; Witherden et al., 2000), whereas others were able to show such expression (Geraldes et al., 2007; Refaeli et al., 2005).

In dtg-O mice, the TSO (tetracycline regulated signal sequence with $OVA_{257\text{-}264}$) transgene encodes a minigene combining the $H\text{-}2K^b$ signal sequence (mediating translation into the endoplasmic reticulum) and $OVA_{257\text{-}264}$ (Rabenstein, 2012) followed by two stop codons and the human growth hormone splice substrate (Chaffin et al., 1990) required for

efficient expression. The cognate peptide of OT1 T cells is thus expressed without proteasomal processing and TAP-transport. Furthermore, crosspresentation of protein transferred to APCs besides the ones carrying the TSO transgene is avoided.

TSO is expressed under the control of an improved tetracycline response element (pTRE-tight, Clontech) in which an inadvertently included interferon response element (Rang and Will, 2000) is removed from the spacers separating the seven tetO sequences. The spacers themselves are shortened so that the tetO sequences are now separated by 3.5 instead of 4 helical turns ($tetO_{short}$), causing the transactivator molecules to bind on opposite sides of the DNA in an alternating fashion. Additionally, an enhancer element has been removed from the minimal CMV_{core} promotor, resulting in a shorter version (CMV_{short}). These changes have been shown to decrease background expression and increase inducibility of the tetracycline response element up to 1,000-fold (Agha-Mohammadi et al., 2004; Pluta et al., 2005).

In dtg mice, administration of doxycycline via the drinking water converts the tet-transactivator from an inactive into an active state, inducing the expression of the TIM or TSO transgenes, respectively. As AND TCR tg T cells recognize their cognate antigen in the context of H-$2E^k$, dtg-M mice were maintained on the B10.BR background, whereas the presentation of $OVA_{257-264}$ in the context of H-$2K^b$ required the dtg-O mice to be bred on the C57BL/6 (B6) background. For an overview of the two systems of TCR tg T cells and cognate antigen expressing mice see Fig. 1B.

To confirm the expression of cognate antigens for AND and OT1 T cells in dtg-M and dtg-O mice, respectively, the fluorescent dye CFSE was utilized to measure T cell proliferation in vivo following adoptive transfer. CFSE is derived from a colorless, highly cell permeable precursor substance by covalent coupling to cytoplasmic proteins during an in vitro labeling procedure. The covalently coupled CFSE is highly fluorescent and stable for weeks in vivo. During cell division, the dye is distributed equally between daughter cells, resulting in the loss of fluorescent intensity in each generation of the daughter cells that is measureable by flow cytometry (Quah et al., 2007). A schematic histogram of the CFSE mean fluorescent intensity (MFI) of a divided cell population is shown in Fig. 1C. The assumption of the CFSE fluorescence being equally distributed to daughter cells allows the assignation of each peak to one cell generation.

The adoptive transfer of naïve CFSE labeled AND T cells into dtg-M mice treated with 100 µg/ml doxycycline in the drinking water resulted in profound proliferation of these cells over 3 d, whereas cells transferred into untreated mice did not dilute CFSE (Fig. 1D, left).

This demonstrates the lack of unspecific expression of the TIM transgene in the absence of doxycycline, as far as AND T cells can detect it, and the inducible expression of MCC_{93-103} following treatment with doxycycline, as shown previously (Obst Benoist 2005, Obst Mathis 2007, Han Obst 2010, Rabenstein 2012).

Naïve OT1 T cells diluted CFSE extensively if transferred into dtg-O mice treated with doxycycline but in contrast divided also in the spleens of untreated dtg-O mice (Fig. 1D, middle). This leakiness of $OVA_{257-264}$ expression in untreated mice was abolished in bone marrow chimeras (BMCs), generated by reconstitution of lethally irradiated B6 mice with bone marrow from dtg-O mice, where expression of the TSO transgene is restricted to bone marrow derived cells. In these BMCs, proliferation of OT1 T cells is strictly dependent on doxycycline treatment (Fig. 1D, right), confirming previous data (Rabenstein, 2012).

Antigen expression in dtg mice cannot only be induced but also be switched off again. It has been shown previously that TIM mRNA disappears following doxycycline removal with a half-life of 2.8 h from the lymph nodes of dtg-M mice, following the kinetics of doxycycline in mouse serum (Böcker and Estler, 1981; Obst et al., 2005).

It has recently been proposed that CD5, a monomeric cell surface glycoprotein of T and B cells with an inhibitory influence on antigen receptor signaling, might be differentially expressed not only on $CD4^+$ and $CD8^+$ T cells but also dependent on the H-2 haplotype (Mandl et al., 2013). CD5 expression levels have been correlated with TCR affinity (Azzam et al., 2001), pointing towards the involvement of CD5 in TCR signal tuning. The hypothesis that higher CD5 levels reflect stronger tonic TCR signaling in $CD4^+$ compared with $CD8^+$ T cells is supported by higher expression of *Nr4a1* (Nur77), a nuclear receptor immediately upregulated by TCR stimulation, in this subset (Moran et al., 2011). The higher affinity of Lck for the cytoplasmic tail of CD4 than CD8 could be involved in generating these distinct prestimulation levels of both subsets (Itano et al., 1996; Wiest et al., 1993)

As our experimental systems are set up on two different genetic backgrounds displaying different H-2 haplotypes (see Fig. 1B), it had to be excluded that CD5 level differences on TCR tg AND and OT1 T cells would hamper the interpretation of experiments aimed to compare antigen dependency of both subsets. Therefore, the expression levels of CD5 on naïve polyclonal $CD4^+$ and $CD8^+$ T cells from B6 ($H\text{-}2^b$), B10.BR ($H\text{-}2^k$), BALB/c ($H\text{-}2^d$), AND TCR tg and OT1 TCR tg mice were analyzed.

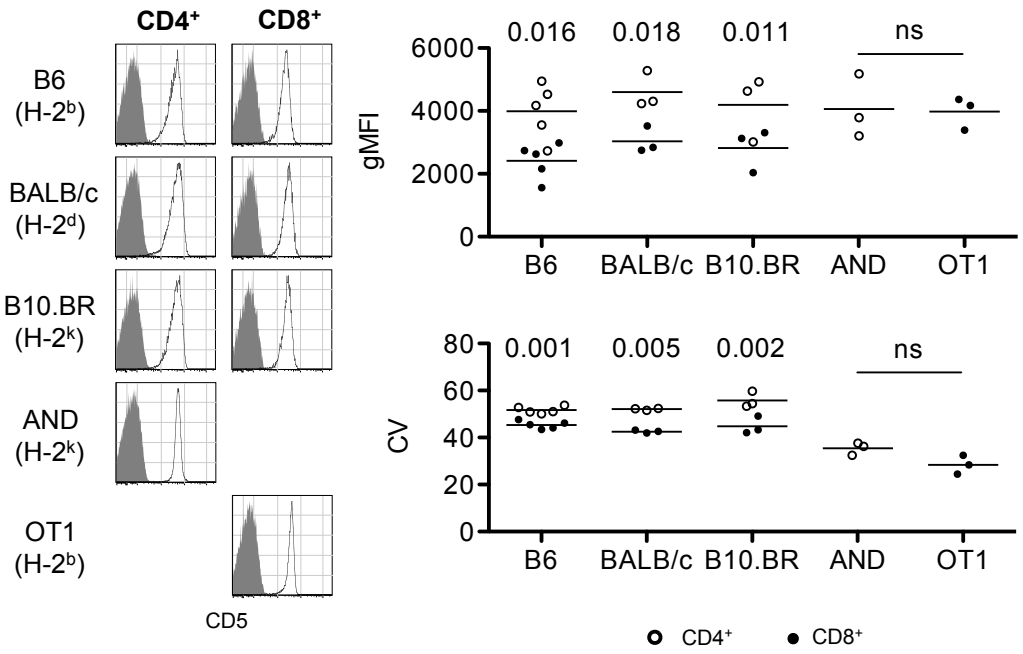


Fig. 2: **CD5 expression in mice of different genetic backgrounds and TCR tg T cells.** Splenocytes of naïve mice of B6 (H-2^b), BALB/c (H-2^d) and B10.BR (H-2^k) background as well as TCR tg AND and OT1 lines were stained for CD5. Isotype controls are shown in grey. Geometrical mean fluorescence intensities (gMFIs) and coefficient of variation (CV) of CD5 expression with means are shown in the graphs. P values were determined by unpaired Student's *t*-test. Cells shown have been gated on single live cells positive for CD4 or CD8, respectively. One of two similar, independent experiments with 3 to 5 mice per group is shown.

In agreement with previous data (Cho et al., 2010; Mandl et al., 2013), the gMFIs and coefficients of variance (CVs) of CD5 were significantly higher on CD4$^+$ compared to CD8$^+$ T cells from all tested strains. This observation might point towards a differential TCR affinity tuning in CD4$^+$ and CD8$^+$ T cells, with CD4$^+$ T cells adjusting the strength of TCR signaling via the expression level of CD5, whereas CD8$^+$ T cells regulate this by modulation of CD8 expression levels (Mandl et al., 2013; Park et al., 2007b).

No differences between geometrical mean fluorescence intensities (gMFIs) were found between CD4$^+$ and CD8$^+$ T cell populations from the tested backgrounds using one-way ANOVA (Fig. 2). The data shown in Fig. 2 indicate that the differential CD5 levels observed by Mandl et al. do most likely result from independent staining procedures.

On naïve AND and OT1 T cells, the expression levels (gMFI) and the CV of CD5 were very similar, indicating similar prestimulation of both subsets by self-pMHC complexes. CD5 expression on AND T cells is equal to that of polyclonal CD4$^+$ T cells, whereas OT1 T cells present higher CD5 levels than the average polyclonal CD8$^+$ T cell. Consistent with previous

observations, CVs of CD5 on both TCR tg T cell subsets are reduced compared to polyclonal subsets (Mandl et al., 2013).

In conclusion, it could be assumed that no intrinsically divergent inhibitory signal delivered by CD5 would hamper the direct comparison of AND and OT1 T cell proliferation.

5.2 Design and validation of the experimental setup

As the aim of this study was to compare the dependence of $CD4^+$ and $CD8^+$ T cell proliferation on the duration of antigen presentation, a similar activation of both subsets had to be achieved beforehand.

5.2.1 Transient and persistent TCR stimulation of AND and OT1 T cells

In order to compare the antigen dependency of $CD4^+$ and $CD8^+$ T cells as represented by AND and OT1 TCR tg T cells, it was important to design an experimental setting that allows very similar priming of both subsets. The physiological priming conditions of $CD4^+$ and $CD8^+$ T cells differ in the MHC class presenting the cognate peptides, the APC type, the density of pMHC molecules on the cellular and the systemic level and the TCR affinity. Even though dtg-M and dtg-O mice were generated to express antigen on the same APC population, they do not guarantee similar priming conditions due to the above reasons.

To circumvent these difficulties, AND and OT1 T cells were primed in vitro with a TCR independent stimulus (Fig. 3A). $CD4^+$ AND TCR tg or $CD8^+$ OT1 TCR tg T cells were isolated by Magnetic-activated Cell Sorting (MACS) from lymph nodes and spleens of TCR tg mice carrying a congenic marker (CD45.1 or CD90.1). Negative selection of $CD4^+$ and $CD8^+$ T cells routinely yielded cell preparations of more than 95% purity. This was necessary to exclude T cell help to $CD8^+$ T cells in the following culture. T cells were stimulated with plate-bound α-CD3 and α-CD28 mAbs. This stimulation delivers strong activating signals to both $CD4^+$ and $CD8^+$ T cells regardless of their TCR specificity and restriction. To increase T cell survival during the stimulation, 5 ng/ml interleukin-7 (IL-7) were added. Cells cultured with IL-7 alone were used as negative controls to account for unspecific stimuli in the cell culture system. After 2 d, cells were labeled with CFSE and 2 x 10^6 cells transferred into the respective wt (AND into B10.BR, OT1 into B6) mice, representing transient TCR stimulation or dtg mice (AND into dtg-M, OT1 into dtg-O) representing persistent TCR stimulation. All recipient mice received 100 µg/ml doxycycline in the drinking water during the experiment,

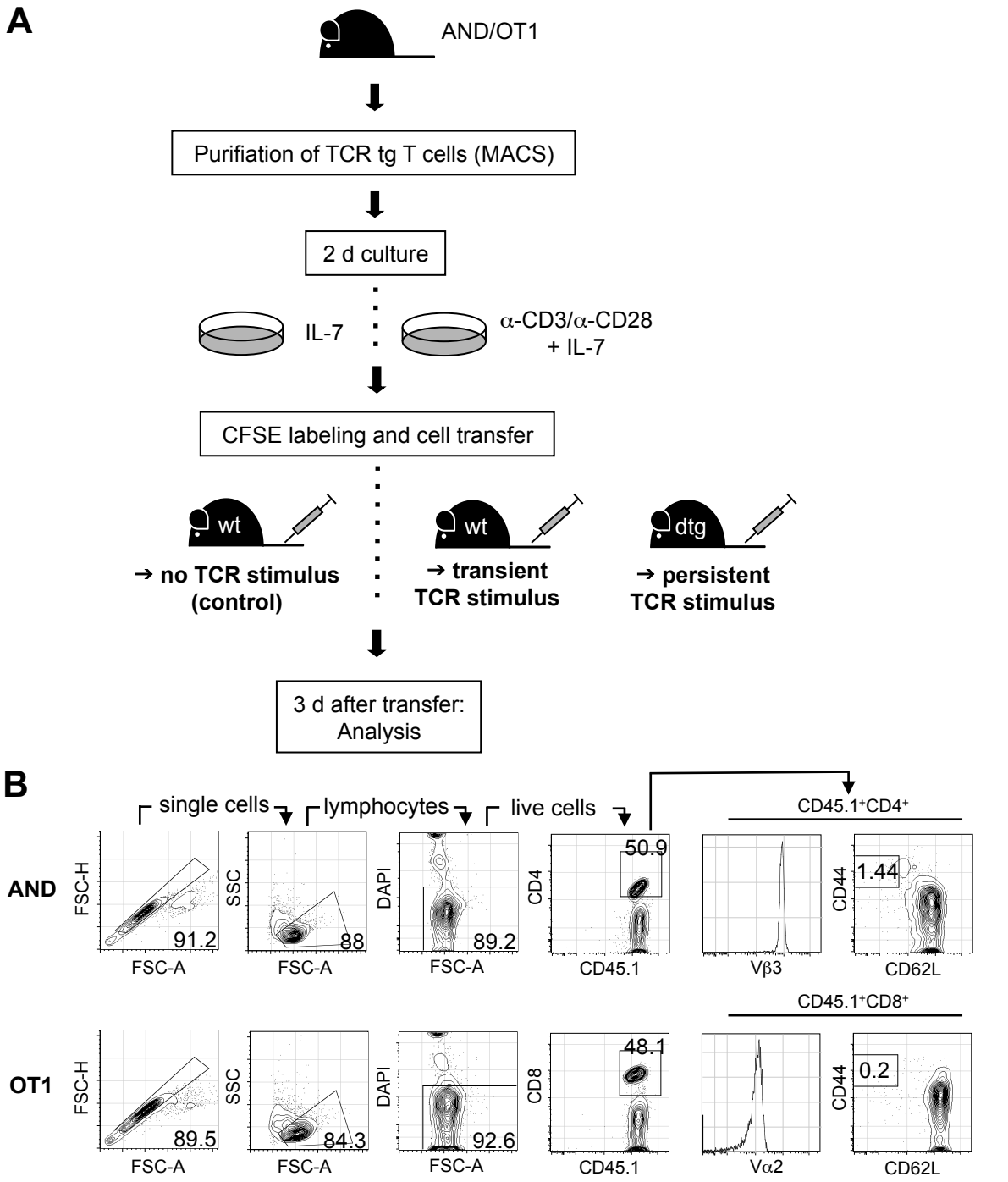


Fig. 3: Experimental setup used to assess the antigen dependency of T cells. (A) Congenically marked and MACS-purified AND ($CD4^+$) or OT1 ($CD8^+$) T cells were cultured with plate-bound α-CD3 and α-CD28 mAbs in the presence of 5 ng/ml IL-7 for 2 d. Cells cultured with IL-7 alone served as controls. T cells were CFSE labeled and 2 x 10^6 cells adoptively transferred into respective wt (transient TCR stimulus) or dtg mice (persistent TCR stimulus). Cells cultured with IL-7 alone and transferred into respective wt mice served as controls. 3 d after transfer, the CFSE dilution was analyzed in spleen and lymph nodes by flow cytometry. **(B)** Analysis of naïve AND and OT1 T cells prepared from lymph nodes of TCR tg animals. Cells were stained with the indicated cell surface markers and DAPI for discrimination of dead cells and analyzed by flow cytometry. For default analysis, the data were gated as indicated on single, live lymphocytes positive for the

congenic marker and CD4 (AND T cells) or CD8 (OT1 T cells). Staining for Vβ3 or Vα2 was used to confirm the transgenic identity of the T cells. Staining for CD44 and CD62L indicates the percentage of activated ($CD44^{high}CD62L^{low}$) T cells that was routinely found to be below 5%.

starting 1 d before T cell transfer. 3 d after transfer, the CFSE dilution of the transferred AND or OT1 T cells was analyzed in lymph nodes and spleen. This experimental setup quantifies T cell proliferation in the expansion phase in vivo and compares the antigen requirements of both subsets directly.

All mice used as T cell donors in this study were checked for transgene expression and phenotype, as depicted in Fig. 3B. An aliquot of single cell suspensions from lymph nodes or spleen was stained for the congenic marker (CD45.1 in Fig. 3B), activation markers CD44 and CD62L and tg TCR chains (Vβ3 for AND, Vα2 for OT1 T cells).

The gating strategy depicted is representative for all analyses of flow cytometric data in this study. A first gate in the forward scatter area (FSC-A) versus forward scatter height (FCS-H) plot was set to select single cells and exclude doublets, a procedure especially important if the CFSE dilution is analyzed. Next, a lymphocyte gate was set and the DAPI (4,6-diaminidin-2-phenylindol)-negative population (live cells) selected. If cells were fixed and permeabilized, a fixable viability dye (FVD) was used instead (see section 4.3.3.2). Subsequently, cells double positive for the congenic marker (CD45.1 in this case) and CD4 or CD8, respectively, were gated.

Expression of the tg TCR chains Vβ3 and Vα2 was used to confirm transgene expression of donor mice, the expression level of CD44 and CD62L were used to identify potentially pre-activated T cells ($CD44^{high}$, $CD62L^{low}$). The plots in Fig. 3B demonstrate that more than 98% of the T cells display a naïve $CD44^{low}CD62L^{high}$ phenotype.

5.2.2 AND and OT1 T cells are equally activated following in vitro stimulation

To confirm that the selected stimulation conditions result in similar activation of AND and OT1 T cells, both subsets were analyzed for activation marker expression after 2 d of culture. Representative histograms and summarizing statistics in Fig. 4A show very similar expression of the activation markers CD69, CD44, CD25 (all low on unstimulated and high on stimulated cells) and CD62L (high on unstimulated, low on stimulated cells) and metabolic activation markers CD71 (transferrin receptor) and CD98 (large neutral amino acid transporter, LAT1) on the T cell subsets. Both AND and OT1 T cells increased their cell size

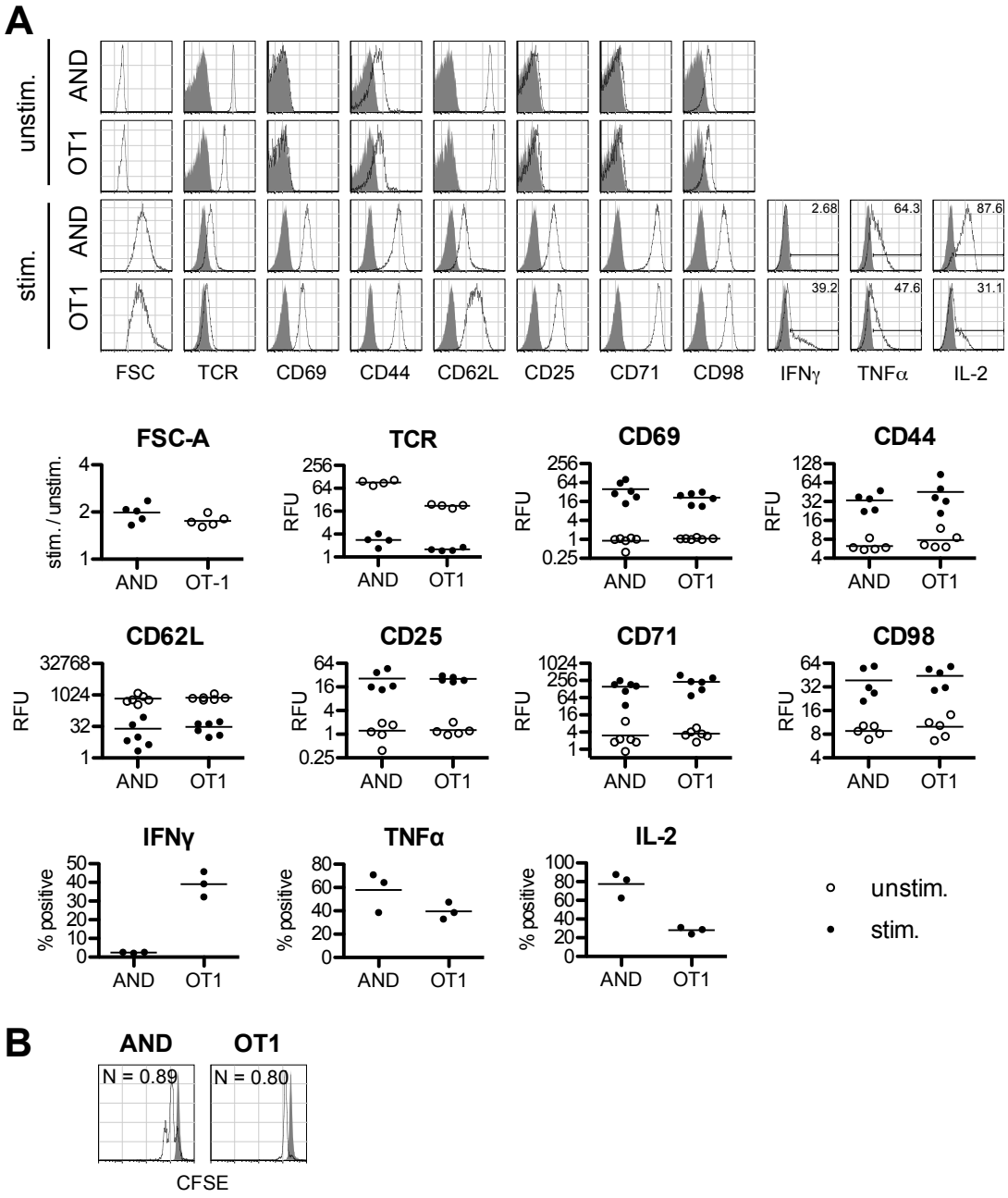


Fig. 4: Activation status of AND and OT1 T cells following 2 d of stimulation. AND or OT1 T cells were purified by MACS and cultured for 2 d in plates coated with α-CD3 and α-CD28 mAbs in the presence of 5 ng/ml IL-7. Cells cultured with IL-7 alone served as unstimulated controls (unstim.). **(A)** Expression of activation markers and production of cytokines on d 2. Before intracellular cytokine staining, cells were cultured for 4 h in the presence of PMA and Ionomycin, with Brefeldin A added for the last 2 h. A Fixable Viability Dye (FVD) was used to allow discrimination of cells that were dead before fixation. Data have been gated on single live $CD4^+$ or $CD8^+$ T cells respectively. FMO controls are shown in grey. The graphs show the mean values of data from 3-6 independent experiments. RFU (relative fluorescent units) were calculated as $gMFI_{sample}/gMFI_{FMO}$. **(B)** Proliferation of stimulated AND and OT1 T cells. MACS-purified AND and OT1 T cells were labeled with CFSE before culture. CFSE dilution was analyzed on d 2. Unstimulated cells are shown in grey. Average division numbers (N) were calculated from MFIs as $N = \log_2(MFI_{ctrl}/MFI_{sample})$ and are

indicated in the histograms. Shown are live cells. Data represent one of two independent experiments.

during stimulation, as reflected by the nearly doubled FCS-A (means and SD: 1.99 ± 0.27 fold for AND and 1.76 ± 0.15 fold for OT1). Significant differences between AND and OT1 T cells were only obvious for TCR-β expression levels, with AND T cells showing higher expression with stimulation (gMFIs of 91.2 ± 12.9 and 22.2 ± 2.3, *t*-test $p < 0.0001$) and without stimulation (gMFIs of 2.8 ± 1 and 1.6 ± 0.2, *t*-test $p = 0.045$). This has previously also been shown for polyclonal $CD4^+$ and $CD8^+$ T cells (Rabenstein, 2012).

Additionally, intracellular cytokine staining was performed following in vitro restimulation with PMA and ionomycin. Both AND and OT1 T cells showed a high percentage of cells positive for tumor necrosis factor alpha (TNFα) following in vitro restimulation (58 ± 17.1% and 39.7 ± 7.3%). Interferon gamma (IFNγ) expression was found in OT1 but not AND T cells at this time point (39.2 ± 6.8 % in OT1, 2.4 ± 0.3% in AND T cells, *t*-test $p = 0.0007$), even though the expression levels were rather low compared to later time points of the experiment (see section 5.3.2). IL-2 expression was obvious for both subsets but in a significantly higher proportion of AND than OT1 T cells (77.4 ± 13.1% and 28 ± 3.5%, *t*-test $p = 0.003$). During the 2 d culture period, AND and OT1 T cells underwent a similar numbers of divisions ($N = 0.89$ and $N = 0.8$) as determined by CFSE dilution (Fig. 4B).

This set of data confirms the equivalent activation of AND and OT1 T cells during in vitro stimulation with α-CD3 and α-CD28. Classical T cell activation markers as well as metabolic activation markers were equally induced or decreased following culture in the presence of α-CD3 and α-CD28 and no difference in the number of divisions during stimulation was obvious. Higher TCR expression in the AND T cells following stimulation would potentially allow them to react to the presentation of cognate peptide with a higher sensibility. Whereas the proportion of TNFα positive AND and OT1 T cells was similar following stimulation, the higher percentage of OT1 T cells producing relatively low levels of IFNγ most likely illustrated early effector differentiation. IFNγ-positivity of AND T cells indicative of Th1 differentiation was not obvious at this time point. As AND T cells expressed IFNγ at later time points (see section 5.3.2), this observation seems to be correlated to differential differentiation kinetics of the subsets. Finally, a higher proportion of AND than OT1 T cells produced IL-2.

Signaling via the IL-2 receptor has been repeatedly postulated to enhance primary T cell responses and $CD8^+$ T cell cytotoxicity in studies using mice or T cells deficient in components of this signaling pathway. However, primary T cell responses seem to be broadly independent of IL-2 (Malek, 2008). By cotransfer of IL-2 receptor alpha (IL-2Rα) -deficient and wt TCR tg $CD8^+$ T cells in a lymphocytic choriomeningitis virus (LCMV) infection model, Williams et al. showed that primary T cell expansion and effector function are unaffected by the lack of IL-2 signaling. Nevertheless, the lack of IL-2 signaling during the primary response strongly affected the magnitude of secondary expansion, suggesting that IL-2 is dispensable for T cell proliferation during primary but not recall responses (Williams et al., 2006). Thus, it seems unlikely that the higher IL-2 expression of AND T cells provides them with a proliferative advantage during the primary expansion phase.

5.3 Differential antigen dependency during the expansion phase

The experimental setup described in the previous section allows for a very similar stimulation of AND and OT1 T cells. Subsequently, the proliferation patterns of both subsets were compared following transient and persistent TCR stimulation in the expansion phase in vivo.

5.3.1 OT1 but not AND T cells continue proliferation if TCR stimulation ceases

In order to compare the antigen dependency of AND and OT1 T cell proliferation in the expansion phase in vivo, in vitro primed AND and OT1 T cells were transferred into wt or antigen expressing dtg mice as described in Fig 3A, subjecting them to transient (no antigen present in vivo) or persistent TCR stimulation (antigen presentation in vivo). T cells cultured without α-CD3 and α-CD28 were transferred into wt mice and served as unstimulated controls (ctrl). 3 d after transfer, the CFSE dilution was analyzed by flow cytometry, applying the gating strategy depicted in Fig. 3B.

The histograms in Fig. 5A show the CFSE dilution of AND and OT1 T cells subjected to the indicated experimental conditions. As T cells stimulated in vitro are heterogeneous is size, they cannot be homogenously labeled with CFSE and therefore the CFSE dilution does not result in distinguishable peaks. This circumstance makes software-assisted analysis of proliferation indices impossible. Thus, we calculated an average number of divisions (N) from CFSE MFIs as follows: $N = \log_2(\text{CFSE MFI}_{ctrl}/\text{CFSE MFI}_{sample})$. This parameter is indicative

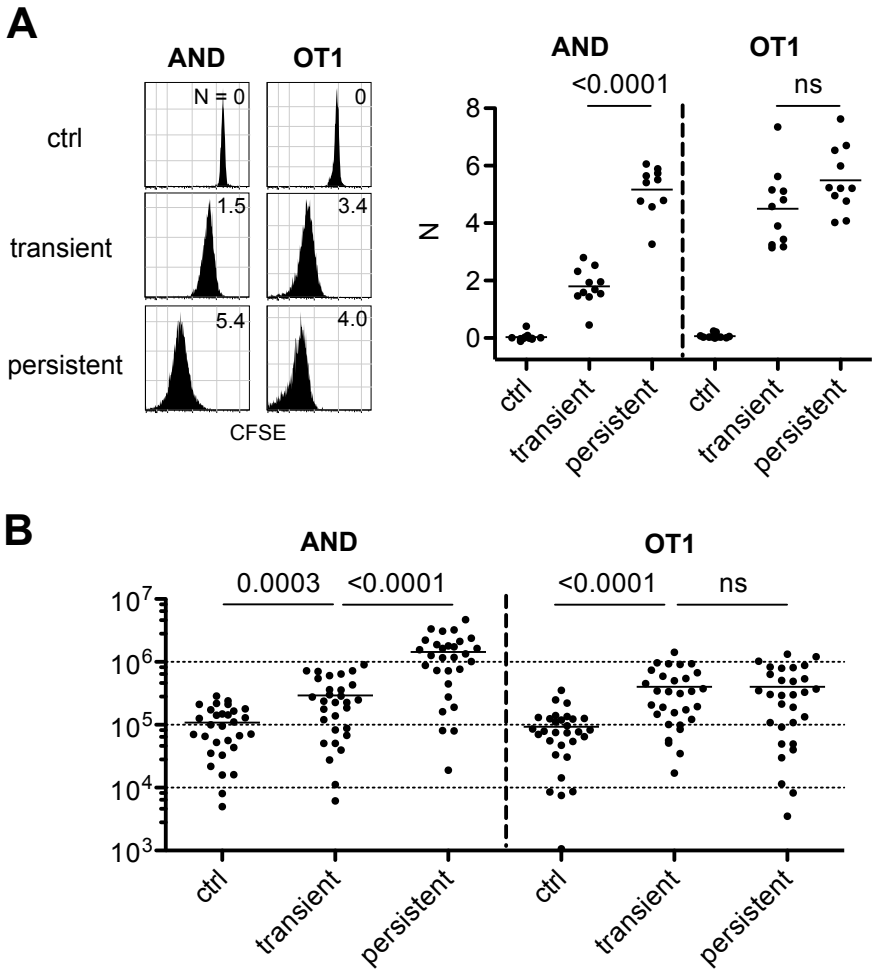


Fig. 5: OT1 but not AND T cells proliferate independently of persistent TCR stimulation during the expansion phase. Congenically marked AND or OT1 T cells were MACS-purified and stimulated with α-CD3 and α-CD28 mAbs in the presence of 5 ng/ml IL-7 for 2 d. Cells were labeled with CFSE and transferred into wt mice (transient condition) or antigen-expressing dtg mice (persistent condition, AND → dtg-M, OT1 → dtg-O). T cells cultured with IL-7 alone and transferred into wt mice served as controls (ctrl). All recipients received 100 µg/ml doxycycline in their drinking water during the experiment, starting 1 d before T cell transfer. **(A)** 3 d after T cell transfer, the CFSE dilution was analyzed in the spleen (depicted) and lymph nodes. The CFSE dilution in lymph nodes was very similar and is therefore not depicted. Shown are single live cells positive for the congenic marker and CD4 or CD8, respectively. Average division numbers were calculated from MFIs as $N = \log_2(MFI_{ctrl}/MFI_{sample})$ and are indicated in the histograms. **(B)** Absolute numbers of transferred tg T cells in the spleens of experimental mice. Data from 10 (A) or 30 (B) independent experiments with one mouse per condition are shown. Means are indicated by lines, p values were determined by unpaired Student's *t*-test. Absolute cell numbers presented in (B) include data from experiments shown in Fig. 6, 10, 14 and 15.

of the average number of divisions undergone by the whole population analyzed and is depicted in the histograms in Fig. 5A. AND T cells undergo only 1.5 divisions under the transient conditions while they proliferate extensively (5.4 divisions) following persistent

TCR triggering. In contrast, OT1 T cells divide to a similar extent in both the transient and the persistent conditions (N = 3.4 and 4, respectively). This holds true in the statistic evaluation, as average division numbers N of AND T cells are significantly reduced in the transient versus the persistent situation (N = 1.8 ± 0.6 and N = 5.2 ± 0.8), while there is no significant difference in OT1 T cell proliferation under both regimens (N = 4.1 ± 1.3 and N = 5.5 ± 1.1). These data depict the analyses of splenocytes but AND and OT1 T cell proliferation in lymph nodes was found to be very similar and is therefore not shown.

These findings are reflected in the absolute numbers of transferred AND and OT1 T cells in the spleens. Fig. 5B illustrates the enhanced accumulation of AND T cells in the persistent condition, whereas absolute OT1 T cells numbers are not significantly different in the transient versus persistent condition. It was noticed that AND T cells accumulated to a greater extent than OT1 T cells following persistent TCR stimulation. $CD4^+$ T cells can provide help to $CD8^+$ T cells and thus augment their primary responses (Castellino and Germain, 2006). However, as the strict separation of both subsets was at the center of interest in this study, the question of whether OT1 T cell expansion is impaired by a lack of T cell help was not further investigated.

Here, OT1 T cells were observed to proliferate independently of antigen presentation in the expansion phase in vivo following a strong in vitro priming stimulus. In contrast, AND T cell proliferation ceased in the absence of antigen in vivo, indicating differential antigen dependency of the subsets. The assessment of activation status following in vitro stimulation (Fig. 4) did not reveal a potential proliferative disadvantage of AND T cells. Instead, stimulated AND T cells displayed higher levels of TCR on the cell surface and an increased proportion of IL-2 positive cells compared to OT1 T cells. Thus, if anything, AND T cells could have gained a minor proliferative advantage during the stimulation period. This circumstance does not relativize but rather increases the significance of the finding of differential antigen dependency.

5.3.2 The effector differentiation of OT1 T cells is antigen-independent

It was unclear if the proliferation of OT1 T cells in the absence of antigen presentation in vivo would lead to the differentiation of functional cytotoxic T cells (CTLs) or if differentiation would be aberrant. Therefore, the correlation of T cell proliferation with T cell

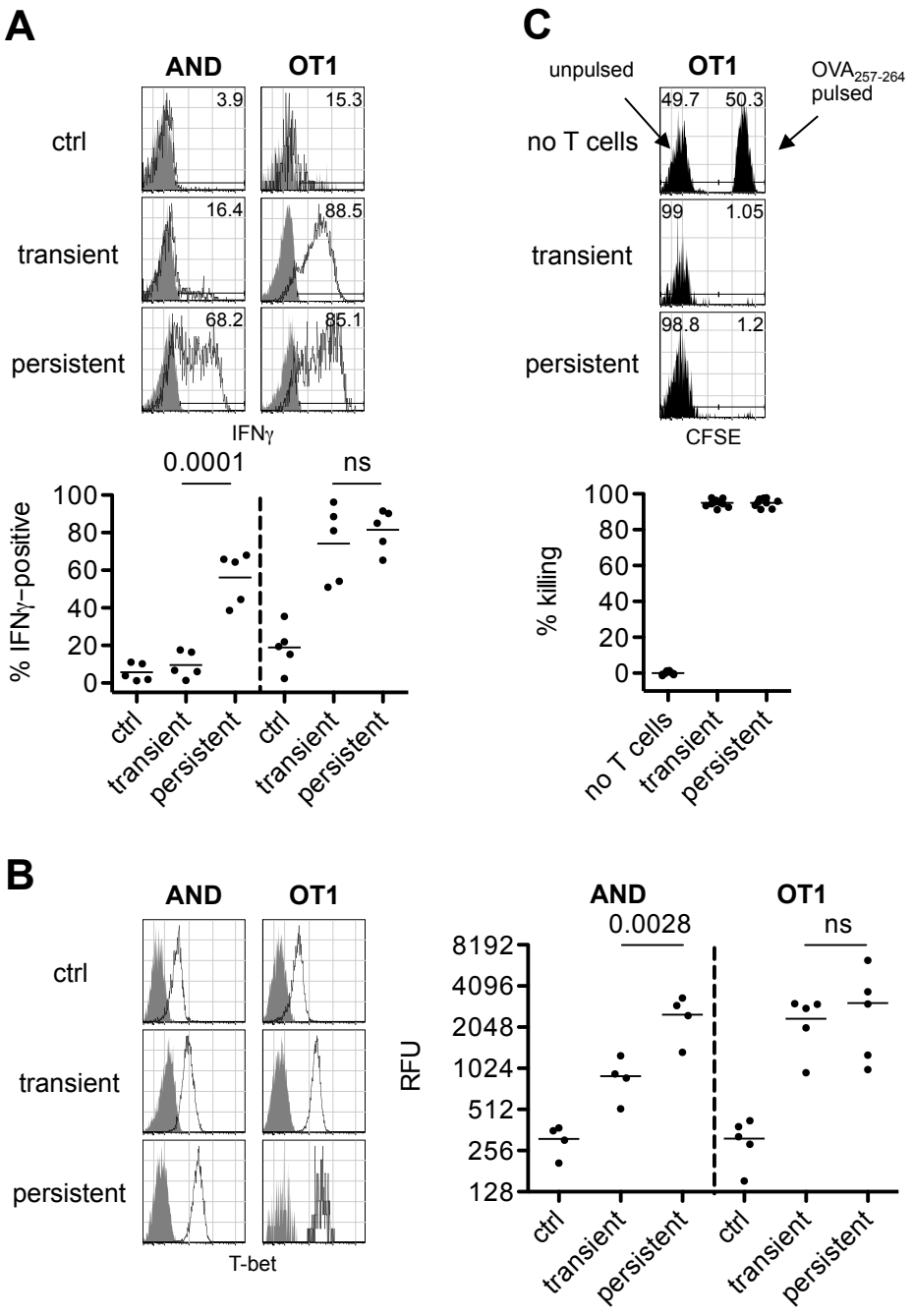


Fig. 6: Effector functions of stimulated AND and OT1 T cells following transient and persistent TCR stimulation. Congenically marked AND or OT1 T cells were MACS-purified and stimulated with α-CD3 and α-CD28 mAbs in the presence of 5 ng/ml IL-7 for 2 d. Stimulated AND or OT1 T cells were transferred into wt (transient) or antigen-expressing dtg mice (persistent). Cells left unstimulated during the culture and transferred into wt mice served as controls (ctrl). All recipients received 100 µg/ml doxycycline in their drinking water during the experiment, starting 1 d before T cell transfer. **(A)** On d 3 after T cell transfer, splenocytes were restimulated in vitro with PMA and Ionomycin. Cells were fixed, permeabilized and stained intracellulary for IFNγ. The percentage of IFNγ-positive cells is indicated in the histograms. The graph shows the means and Student's *t*-test p values of 5 experiments with one mouse per condition. **(B)** 3 d after transfer, lymph node suspensions were fixed and permeabilized for intracellular staining of the transcription factor T-bet. RFUs

(relative fluorescent units) were calculated as $gMFI_{sample}/gMFI_{FMO}$. Data from 4 (AND) or 5 (OT1) independent experiments with one mouse per condition and means are shown in the graph. **(C)** In vivo killing capability of in vitro-stimulated OT1 T cells following transfer into wt (transient) or antigen-expressing mice (persistent). 3 d after T cell transfer, mice received 2.5 x 10^6 $OVA_{257-264}$ pulsed, $CFSE^{high}$ splenocytes and 2.5 x 10^6 unpulsed, $CFSE^{low}$ splenocytes from congenically marked wt mice. 16 h later spleens were analyzed for frequencies of pulsed and unpulsed target cells by flow cytometry (shown in histograms). Mice that did not receive T cells served as controls. Percent killing was calculated as the reduction of target cell percentage relative to the mean percentage of pulsed target cells in control mice. The statistics panel shows data and means from 3 independent experiments with 2-5 mice per condition. All data shown have been gated on single live cells positive for the congenic marker defining target cells and CD4 or CD8, respectively. FMO controls are shown in grey. P values were determined by unpaired Student's *t*-test.

differentiation had to be assessed. To address this question, we stained transferred AND and OT1 T cells for IFNγ following in vitro restimulation.

Under conditions of transient as well as persistent TCR stimulation, OT1 T cells displayed a high percentage of IFNγ-positivity with no significant difference between transient and persistent TCR stimulation conditions (74.2 ± 20.5% versus 81.5 ± 11%, Fig. 6A). AND T cells on the other hand only acquired the ability to produce IFNγ following persistent TCR stimulation (56.3 ± 13.7%), with a significantly smaller proportion (9.7 ± 7.1%) stainable for IFNγ following transient TCR stimulation.

Additionally, levels of the IFNγ-expression inducing T cell-specific T-box transcription factor (T-bet) were examined by flow cytometry and found to be consistent with the IFNγ staining. High levels of T-bet were present in OT1 T cells under condition of both transient and persistent TCR stimulation, whereas AND T cells displayed lower levels of T-bet protein in the transient compared with the persistent situation (Fig. 6B).

Furthermore, the in vivo killing capacity of transiently and persistently stimulated OT1 T cells was analyzed. Wt or dtg-O mice receiving stimulated OT1 T cells 3 d before were injected with congenically marked wt splenocytes pulsed with $OVA_{257-264}$ in vitro and unpulsed control cells at a 1:1 ratio. To allow identification of target cells, the pulsed population was labeled with a high dose of CFSE before transfer and the unpulsed population with a low dose of CFSE. 16 h after target cell transfer, mice were sacrificed and the percentage of $OVA_{257-264}$ pulsed target cells and unpulsed cells analyzed (Fig. 6C). The percentage of killing was calculated as the reduction of target cell percentage relative to the mean percentage of pulsed target cells in control mice that did not receive OT1 T cells.

Following both transient and persistent TCR stimulation, OT1 T cells acquired the ability to kill target cells very efficiently (94.9 ± 2.4% and 95 ± 2.4% killing).

In conclusion, antigen-independent proliferation of OT1 T cells in the expansion phase was associated with differentiation into functional effector cells expressing IFNγ and T-bet. AND T cells were positive for the Th1 cytokine IFNγ and expressed high levels of T-bet only following persistent TCR stimulation. Differential antigen dependency of AND and OT1 T cells was therefore not restricted to proliferation as measured by CFSE dilution but extended to the differentiation of these cells.

5.3.3 Transiently stimulated polyclonal $CD8^+$ but not $CD4^+$ T cells divide extensively

To test if the above-described findings apply to polyclonal T cells, the experiment shown in Fig. 3 was carried out with polyclonal T cells from different genetic backgrounds. Here, a persistent TCR stimulation could not be performed but nevertheless proliferation of polyclonal T cells following transient TCR stimulation could be assessed.

Congenically marked polyclonal $CD4^+$ and $CD8^+$ T cell were MACS-sorted from wt mice of different genetic backgrounds (B6, BALB/c, B10.BR) and stimulated with α-CD3 and α-CD28 mAbs in vitro for 2 d. The T cells were CFSE labeled and transferred into wt mice and cultured unstimulated aliquots were transferred into wt mice as controls (ctrl). 3 d after transfer, the CFSE dilution was analyzed in the recipients' lymph nodes and spleen. In the experiments on B10.BR and BALB/c mice, congenically distinct naïve wt splenocytes were added at a 1:1 ratio to cultured T cells before the CFSE labeling procedure and thus served as an improved negative control during the CFSE dilution analysis. These CFSE spiked populations are shown in grey in the histograms in Fig. 7 and indicate the precise level of fluorescence of undivided cells.

For all genetic backgrounds, $CD8^+$ T cells were found to undergo more cell divisions than $CD4^+$ T cells following transfer into wt mice (Fig. 7). The extent of $CD8^+$ T cell proliferation showed small variations between the different genetic backgrounds, which did not reach significance (N = 4.7 ± 1.5 in B6, 3.3 ± 0.8 in B10.BR, 4 ± 0.6 in BALB/c). These data indicate that the proliferation patterns of TCR tg cells used here are representative for polyclonal T cell repertoires.

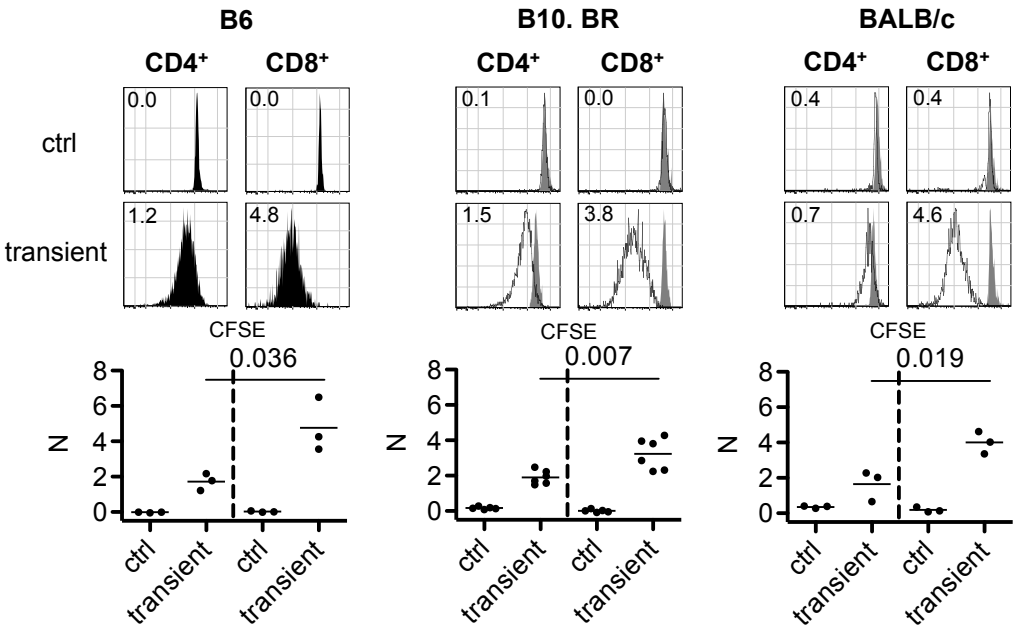


Fig. 7: Antigen-independent proliferation of polyclonal CD8$^+$ but not CD4$^+$ T cells from different genetic backgrounds. MACS-purified polyclonal CD4$^+$ or CD8$^+$ T cells from B10.BR, BALB/c and B6 mice were stimulated in vitro with plate-bound α-CD3 and α-CD28 mAbs in the presence of 5 ng/ml IL-7 for 2 d. Cells were labeled with CFSE and transferred into congenically distinct wt recipients of the same strain (transient condition). Cells left unstimulated in the culture and transferred into wt mice served as controls (ctrl). For experiments on B10.BR and BALB/c mice, stimulated cells were mixed 1:1 with congenically distinct naïve splenocytes of the same background before CFSE labeling to provide undivided control cells (shown in grey in the histograms). All mice received 100 µg/ml doxycycline in their drinking water during the experiment, starting 1 d before T cell transfer. 3 d after transfer, spleens were analyzed for the CFSE dilution of transferred cells by flow cytometry. Data shown have been gated on single live cells positive for the congenic marker and CD4 or CD8, respectively. CFSE dilution in lymph nodes was very similar and is therefore not shown. Average division numbers were calculated from MFIs as $N = \log_2(MFI_{ctrl}/MFI_{sample})$ and are depicted in the histograms. Means from 6 (B10.BR) or 3 (BALB/c, B6) independent experiments with one mouse per condition are shown in the graphs. P values were determined by unpaired Student's *t*-test. Please note that the data shown for B6 mice are also presented as control (ctrl) and transient condition in Fig. 12.

5.3.4 Antigen-independent proliferation of OT1 T cells does not occur in vitro

In order to dissect the factors mediating antigen-independent proliferation of OT1 T cells in the expansion phase, the in vitro reproducibility of this phenomenon was assessed. Even though less physiologic, an in vitro model of antigen-independent proliferation of OT1 T cells would allow direct interference with compounds manipulating potential causative processes (e.g. chromatin modification, signaling pathways or metabolic processes).

AND and OT1 T cells were MACS-sorted and stimulated with α-CD3 and α-CD28 for 2 d in vitro as before. Cells were CFSE labeled and subjected to a second culture period analo-

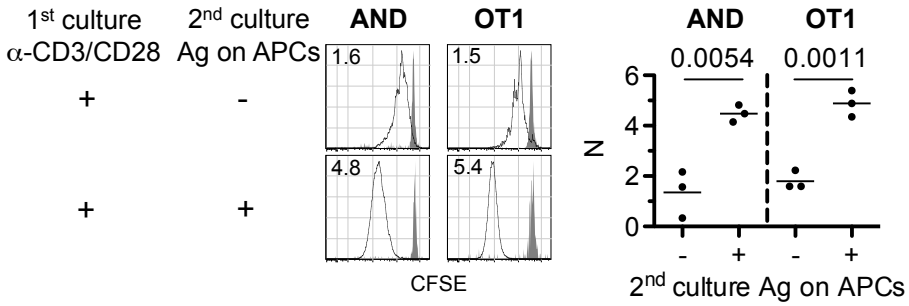


Fig. 8: Antigen-independent proliferation of OT1 T cells does not occur in vitro. AND or OT1 T cells were stimulated in vitro with plate-bound α-CD3 and α-CD28 mAbs in the presence of 5 ng/ml IL-7 for 2 d. T cells were CFSE labeled and cultured with irradiated splenocytes from respective wt or constitutively antigen-expressing mice (Ii-MCC for AND, Act-OVA for OT1) for further 3 d. Before CFSE labeling, a known number of congenically marked naïve wt splenocytes were added to serve as undivided controls for CFSE dilution analysis (shown in grey in the histograms). Data shown have been gated on single live cells positive for the congenic marker and CD4 or CD8, respectively. Average division numbers were calculated from MFIs as $N = \log_2(MFI_{undivided}/MFI_{sample})$ and are depicted in the histograms. The graph shows the mean values from 3 independent experiments with Student's *t*-test p values.

gous to transient or persistent TCR stimulation as in Fig. 3: Stimulated AND or OT1 T cells were cocultured with irradiated splenocytes from either wt (B10.BR or B6, respectively) or constitutively antigen expressing mice (Ii-MCC mice expressing $MCC_{88\text{-}103}$ under the control of invariant chain promotor elements to stimulate AND T cells, Act-OVA mice expressing OVA protein under the control of the actin promotor to stimulate OT1 T cells). Before the CFSE labeling procedure, a known number of congenically distinct naïve wt splenocytes was added to the T cells to indicate the level of fluorescence of undivided cells during the CFSE dilution analysis. After 3 d of coculture, the CFSE dilution of AND and OT1 T cells was analyzed by flow cytometry and the average number of cell divisions calculated.

Neither OT1 nor AND T cells were able to proliferate extensively in the absence of antigen during the second culture period (Fig. 8). Both subsets underwent significantly fewer divisions if cultured with wt splenocytes compared to antigen expressing splenocytes. The lack of antigen-independent proliferation of OT1 T cells in vitro was nevertheless informative, as it could be due to factors inhibiting OT1 T cell proliferation following stimulus removal in vitro or factors present in vivo but not in vitro, that are essential for antigen-independent proliferation of OT1 T cells. Potential candidates for both groups could be cytokines, oxygen concentration, flow conditions or a combination of several of these factors.

5.3.5 Proliferation patterns are unchanged if T cells are cultured at 3% O_2

The O_2 concentration in standard culture systems equals atmospheric conditions (21% O_2) whereas oxygen concentrations in lymphoid tissues of mice range from 0.5 to 4.5% (Caldwell et al., 2001). As reactive oxygen species (ROS), which are generated under oxidative stress, have recently been shown to interfere with the outcome of TCR signaling (Sena et al., 2013), the influence of the O_2 concentration during in vitro stimulation on the proliferation of AND and OT1 T cells was analyzed.

The experiment depicted in Fig. 3 was therefore repeated with T cells stimulated at 3% O_2 and 5% CO_2 at 37°C. Expression of activation markers CD44, CD62L, CD69, CD25 and TCR-β of cultured AND and OT1 T cells on d 2 did not differ from that of cells cultured at 21% O_2 (determined for one out of three experiments, data not shown, see Fig. 4). T cells were CFSE labeled and transferred into wt mice (transient TCR stimulation) or dtg mice (persistent TCR stimulation) and the CFSE dilution accessed by flow cytometry 3 d after transfer. Before CFSE labeling, congenically marked naïve wt splenocytes were added at a 1:1 ratio to cultured T cells to indicate the level of fluorescence of undivided cells during the CFSE dilution analysis. In analogy to T cells cultured at 21% O_2, OT1 but not AND T cells divided extensively under conditions of both transient and persistent TCR stimulation (Fig. 9A).

Furthermore, the antigen dependency of AND and OT1 T cell proliferation during a second culture period was tested in analogy to the experiment depicted in Fig. 8. AND and OT1 T cells stimulated at 3% O_2 were CFSE labeled and cultured with irradiated splenocytes of wt or constitutively antigen expressing mice (Ii-MCC to stimulate AND T cells, Act-OVA to stimulate OT1 T cells). Congenically marked naïve wt splenocytes were added at a 1:1 ratio to cultured T cells before the CFSE labeling procedure as controls. After 3 d of coculture, the CFSE dilution was analyzed by flow cytometry.

As observed before at 21% O_2, neither OT1 nor AND T cells proliferate extensively if antigen was not presented by APCs in the second culture period (Fig. 9B). However, OT1 T cell proliferation following persistent TCR stimulation was reduced compared to the second culture performed at 21% O_2 (N = 4.8 ± 0.5 at 21% O_2 versus N = 3.3 ± 0.2 at 3% O_2). Additionally, AND T cells showed only very limited proliferation following antigenic stimulation in the second culture period at 3% O_2 compared to 21% O_2 (means of N = 1.3 ± 0.7 at 3% O_2versus N = 4.5 ± 0.3 at 21% O_2). Therefore, in vitro but not in vivo proliferation of both subsets but especially AND T cells seemed to be supported by high O_2 concentrations.

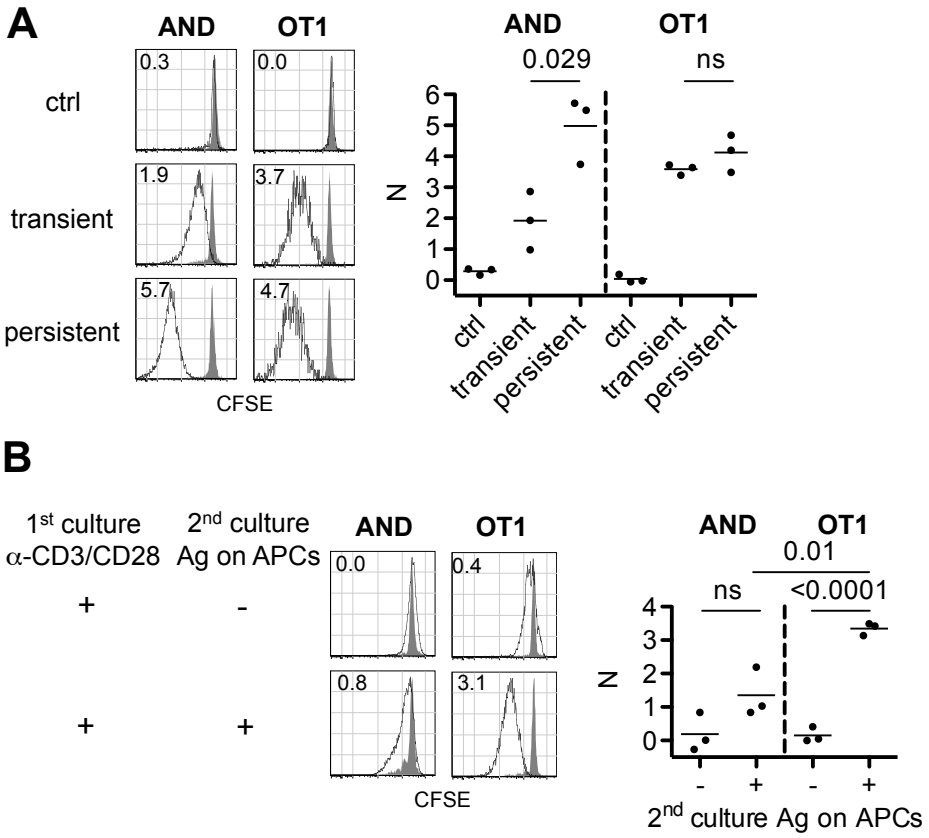


Fig. 9: In vitro stimulation at physiological O_2 concentration does not change the proliferation patterns of AND and OT1 T cells. Congenically marked AND or OT1 T cells were stimulated with plate-bound α-CD3 and α-CD28 mAbs in the presence of 5 ng/ml IL-7 for 2 d at 3% O_2, 5% CO_2 and 37 °C. Cells were mixed at a known ratio with congenically distinct naïve wt splenocytes before CFSE labeling to provide undivided controls (shown in grey in the histograms). **(A)** Stimulated T cells were transferred into wt (transient) or antigen-expressing dtg mice (persistent). Cells left unstimulated in the culture and transferred into wt mice served as controls (ctrl). All recipients received 100 µg/ml doxycycline in their drinking water during the experiment, starting 1 d before T cell transfer. 3 d after transfer, splenocytes were analyzed for the CFSE dilution of transferred T cells. The CFSE dilution in lymph nodes was very similar and is therefore not depicted. **(B)** Stimulated T cells were cultured for additional 3 d in the presence of irradiated APCs from wt or constitutively antigen-expressing mice (Ii-MCC for AND, Act-OVA for OT1). Again, cultures were performed at 3% O_2, 5% CO_2 and 37 °C. Data shown have been gated on single live cells positive for the congenic marker and CD4 or CD8, respectively. Undivided control populations are shown in grey. Average division numbers were calculated from MFIs as $N = \log_2(MFI_{undivided}/MFI_{sample})$ and are depicted in the histograms. Graphs show means and Student's *t*-test p values from 3 independent experiments.

This phenomenon has been previously described and was correlated with higher intracellular NO levels in cells cultured at O_2 concentrations close to those found in vivo (Atkuri et al., 2007).

In conclusion, the reduction of O_2 concentration in vitro did not influence differential antigen dependency of AND and OT1 T cells in the expansion phase in vivo or in vitro. Thus, the antigen-independent proliferation of OT1 T cells observed in vivo is not caused by high O_2 concentrations during the culture period.

5.4 Proliferative patterns of AND and OT1 T cells are cell intrinsic

Differential antigen dependency of AND and OT1 as well as polyclonal $CD4^+$ and $CD8^+$ T cells has been shown here. To elucidate how this phenomenon might be regulated, the modifiability of proliferation patterns of AND and OT1 T cells was assessed. The influence of MHC abundance, homotypic clustering behavior, bystander inflammation, coinhibitory signaling and $CD4^+$ T cell differentiation on the phenomenon described above was addressed. Additionally, the number of transferred T cells was excluded from impairing T cell proliferation.

5.4.1 OT1 T cell proliferation is not dependent on unspecific TCR triggering

The expression patterns of MHC I and MHC II molecules are broadly different. MHC II is only expressed by professional APCs and MHC I is expressed on all nucleated cells. Unspecific, low affinity triggering of TCR with self-peptides has been associated with homeostatic proliferation and T cell survival (Beutner and MacDonald, 1998; Brocker et al., 1997; Goldrath and Bevan, 1999; Kirberg et al., 1997; Takeda et al., 1996). Therefore, the question arose whether differential antigen dependency of $CD4^+$ and $CD8^+$ T cells is caused by the higher frequency of MHC I than MHC II molecules. Assuming that unspecific TCR triggering could maintain ongoing T cell proliferation, the higher number of triggering events could be responsible for antigen-independent proliferation of OT1 T cells.

To test this hypothesis, transgenic mice deficient for beta-2 microglobulin (β_2m), without which MHC I molecules cannot be assembled, were used. As $\beta_2m^{-/-}$ mice are partially lymphopenic due to the abrogated positive selection of $CD8^+$ T cells in the thymus, the β_2m gene was additionally expressed under the control of the human keratin 14 (K14) promotor, allowing its expression in the thymus and thus normal peripheral T cell compartments in mice that are for brevity referred to as $MHC^{-/-}$ mice. Crossing $MHC^{-/-}$ animals to mice carrying a transgene coding for β_2m under the control of the CD11c promotor resulted in $MHC^{-/-}$ DC-MHC I mice expressing MHC I in the thymus and on DCs. These two tg mice allowed analy-

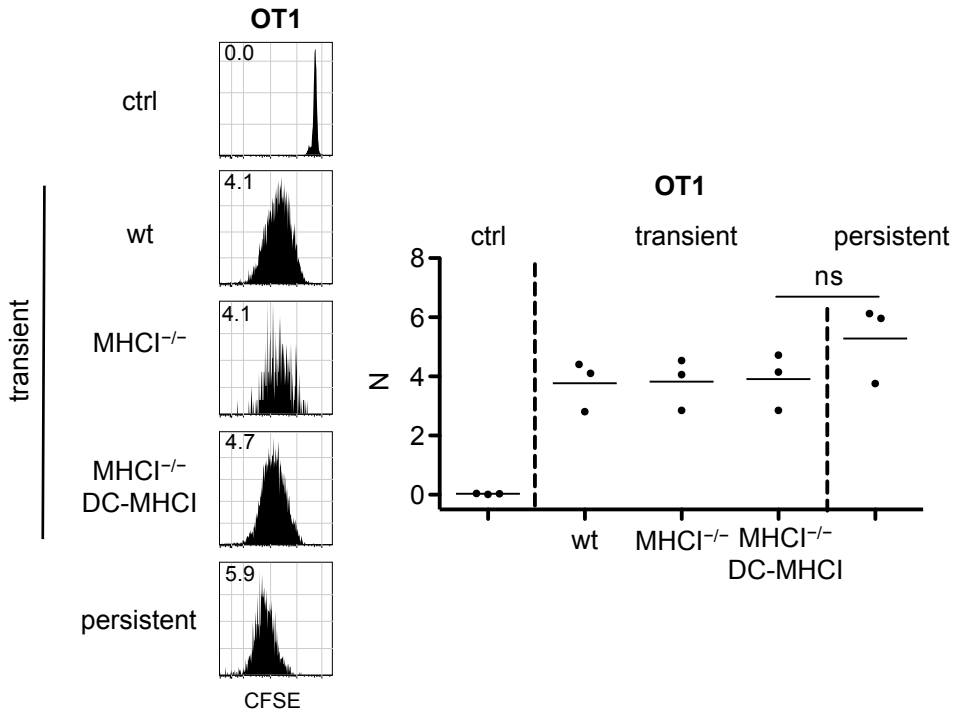


Fig. 10: Antigen independency of OT1 T cells does not rely on unspecific TCR triggering by self-peptide-MHC complexes. Congenically marked OT1 T cells were stimulated in vitro with plate-bound α-CD3 and α-CD28 mAbs in the presence of 5 ng/ml IL-7 for 2 d. Cells were CFSE labeled and transferred into wt mice, $MHCI^{-/-}$ mice, $MHCI^{-/-}$ DC-MHCI mice (all transient) or antigen-expressing dtg-O mice (persistent). Cells left unstimulated in the culture and transferred into wt mice served as controls (ctrl). All recipients received 100 µg/ml doxycycline in their drinking water during the experiment, starting 1 d before T cell transfer. 3 d after transfer, the CFSE dilution was analyzed in the spleen. The CFSE dilution in lymph nodes was very similar and is therefore not depicted. Data shown have been gated on single live cells positive for the congenic marker and CD8. Average division numbers were calculated from MFIs as $N = \log_2(MFI_{ctrl}/MFI_{sample})$ and are depicted in the histograms. Mean values from 3 independent experiments are shown. P values were calculated by unpaired Student's *t*-test.

sis of OT1 T cell proliferation in mice devoid of MHC I in the periphery ($MHCI^{-/-}$) and in mice expressing MHC I on DCs only ($MHC^{-/-}$DC-MHCI), creating a situation very much alike that encountered by $CD4^+$ T cells, whose cognate MHC molecules are only present on APCs.

In vitro-stimulated OT1 T cells were CFSE labeled and transferred into wt, $MHCI^{-/-}$, $MHCI^{-/-}$DC-MHCI mice (all representing transient TCR stimulation) or antigen expressing dtg-O mice (representing persistent TCR stimulation). 3 d after transfer, the CFSE dilution was analyzed in lymph nodes and spleen. Irrespective of MHC I expression, OT1 T cells had divided equally in all mice representing the transient condition, with average division numbers being similar compared to persistently stimulated OT1 T cells (Fig. 10).

Hence, antigen-independent proliferation of OT1 T cells is not due to unspecific TCR triggering mediated by self-peptides presented on MHC I. The differential antigen dependency of AND and OT1 T cells is thus not due to differential expression patterns of cognate MHC molecules.

5.4.2 The expansion of OT1 T cells is not dependent on homotypic T cell clusters

The formation of homotypic T cell clusters has been observed following T cell activation in vitro and in vivo for both $CD4^+$ (Hommel and Kyewski, 2003; Ingulli et al., 1997; Sabatos et al., 2008) and $CD8^+$ T cells (Bousso and Robey, 2003; Hommel and Kyewski, 2003). Recently, homotypic clustering dependent on the integrin LFA-1 (Lymphocyte function-associated antigen 1) has been suggested to enhance activation as well as effector cell differentiation and memory formation of $CD8^+$ T cells (Bose et al., 2013; Gérard et al., 2013), whereas others reported that T-T cell clusters attenuate $CD8^+$ T cell responses (Cox et al., 2013; Zumwalde et al., 2013).

Here, OT1 but not AND T cells were observed to form clusters during the second day of in vitro stimulation with α-CD3 and α-CD28 mAbs. This disparity could not be explained by differential expression of LFA-1 or ICAM-1 (intercellular adhesion molecule 1) on stimulated T cells (see section 5.5.1.2, Tables 1 and 2). The formation of these clusters was abrogated by a mAb blocking LFA-1 (Fig. 11A). Activation marker expression (CD44, CD62L, CD69, TCR-β) of OT1 T cells cultured in the presence of the blocking mAb was not altered compared to cells treated with isotype control (IC) mAb (data not shown) and comparable with data shown in Fig. 4.

To elucidate whether homotypic clusters were essential for antigen-independent proliferation, OT1 T cells stimulated in the presence of α-LFA-1 were CFSE labeled and transferred into wt (transient TCR stimulation) or antigen expressing dtg-O mice (persistent TCR stimulation). Mice received α-LFA-1 blocking mAb i.p. for continued blockage of T-T cell interactions. The CFSE dilution of transferred OT1 T cells was analyzed 3 d after transfer in lymph nodes and spleens of recipient animals.

The average number of divisions undergone by OT1 T cells under both transient and persistent TCR stimulation was unaffected by the blockage of LFA-1 (Fig. 11B) and very similar to that observed for OT1 T cell stimulated in the absence of α-LFA-1 (see Fig. 5; Means of $N = 4.5 \pm 1.3$ for transient condition, $N = 5.5 \pm 1.1$ for persistent stimulation). Thus,

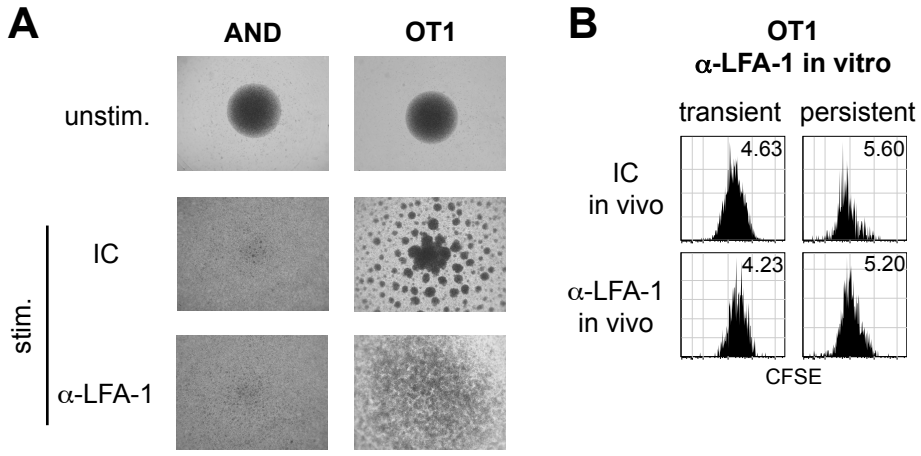


Fig. 11: LFA-1 dependent T-T clusters are not required for antigen-independent proliferation of OT1 T cells. Congenically marked AND or OT1 T cells were stimulated with α-CD3 and α-CD28 in round-bottom wells in the presence of 5 ng/ml IL-7 and 10 µg/ml α-LFA-1 or isotype control (IC) mAbs at 0.5 x 10^5 cells per well. After 24 h, 100 µl medium was replaced to maintain levels of mAbs. **(A)** Appearance of cultures on d 2 at 10x magnification. **(B)** OT1 T cells treated with α-LFA-1 during in vitro stimulation were transferred into wt (transient) and antigen-expressing dtg mice (persistent). Mice were injected with 200 µg α-LFA-1 i.p. 3 x every 12 h beginning 4 h before T cell transfer. All recipients received 100 µg/ml doxycycline in their drinking water during the experiment, starting 1 d before T cell transfer. 3 d after transfer, the CFSE dilution of transferred OT1 T cells was analyzed in the spleen. The CFSE dilution in lymph nodes was very similar and is therefore not depicted. Data shown have been gated on single live cells positive for the congenic marker and CD8. Average division numbers were calculated from MFIs as $N = \log_2(MFI_{ctrl}/MFI_{sample})$ and are depicted in the histograms. This experiment was performed once.

the formation of LFA-1 dependent homotypic T cell clusters was not responsible for antigen-independent proliferation of OT1 T cells in the experimental setup used here.

5.4.3 The proliferation of $CD4^+$ T cells is not dependent on inflammatory cytokines

The antigen-independent proliferation of OT1 T cells described above occurs in a sterile environment. Thus, differential antigen dependency of AND and OT1 T cells could be related to a differential dependency on inflammatory cytokines or costimulatory signals not provided in the experimental system used here.

To investigate this issue, wt mice were infected with mouse cytomegalovirus (MCMV), a pathogen known to cause massive cytokine release in infected animals. In order to circumvent potential different crossreactivity of AND and OT1 TCRs towards MCMV antigens, this experiment was performed with polyclonal $CD4^+$ and $CD8^+$ T cells from B6 mice. $CD4^+$ and $CD8^+$ T cells were stimulated with plate-bound α-CD3 and α-CD28 mAbs for 2 d in vitro.

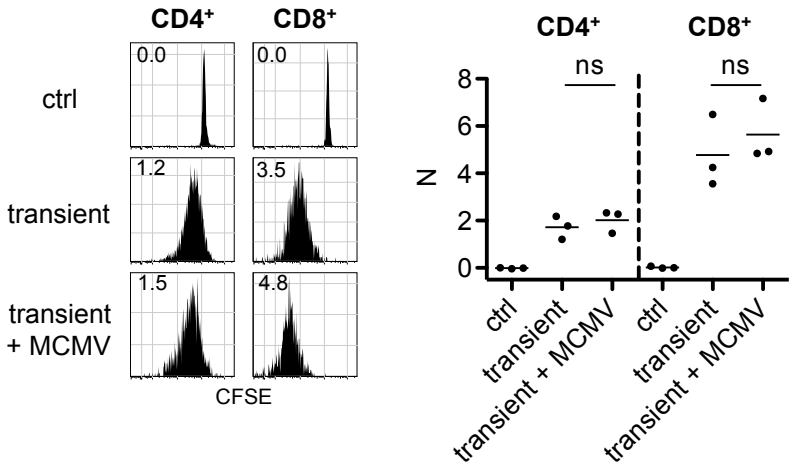


Fig. 12: Antigen-dependent proliferation of CD4$^+$ T cells is not released by bystander inflammation. Polyclonal CD4$^+$ or CD8$^+$ T cells from congenically marked B6 mice were stimulated with plate-bound α-CD3 and α-CD28 mAbs in the presence of 5 ng/ml IL-7 for 2 d. Cells were CFSE labeled and transferred into B6 mice that, if indicated, had been infected with 2 x 10^6 pfu MCMV 2 d earlier. 3 d after transfer, spleens were analyzed for the CFSE dilution of the transferred T cells. The CFSE dilution in lymph nodes was very similar and is therefore not depicted. Data shown have been gated on single live cells positive for the congenic marker and CD4 or CD8, respectively. Average division numbers were calculated from MFIs as N = $\log_2(MFI_{ctrl}/MFI_{sample})$ and are depicted in the histograms. The graph show means and unpaired Student's *t*-test results from 3 independent experiments with one mouse per condition. Please note that the data shown for the control (ctrl) and the transient condition (without MCMV) are also presented in Fig. 7.

The expression of activation markers (CD44, CD62L, CD25, CD71, CD98, TCR-β) was analyzed after the stimulation for two out of three experiments and found to be very similar to that of AND and OT1 T cells (data not shown, see Fig. 4). T cells were CFSE labeled and transferred into wt mice (representing transient TCR stimulation) infected with MCMV 2 d earlier. This time point of MCMV infection was selected for its documented high serum levels of the inflammatory cytokines IFNγ, IL-12, TNFα and IL-6 (Krug et al., 2004; Mitrović et al., 2012). T cells left unstimulated during culture and transferred into wt mice served as controls (ctrl). The CFSE dilution of transferred CD4$^+$ and CD8$^+$ T cells was analyzed in lymph nodes and spleens 3 d later.

MCMV infection did not enhance the proliferation of CD4$^+$ T cells in wt mice, as illustrated by very similar average division numbers (Fig. 12). If anything, the proliferation of CD8$^+$ T cells in MCMV infected mice was increased slightly but insignificantly. The lack of inflammatory cytokines in the experimental system used here was thus not responsible for the reduced proliferation of CD4$^+$ compared with CD8$^+$ T cells following transient TCR stimulation.

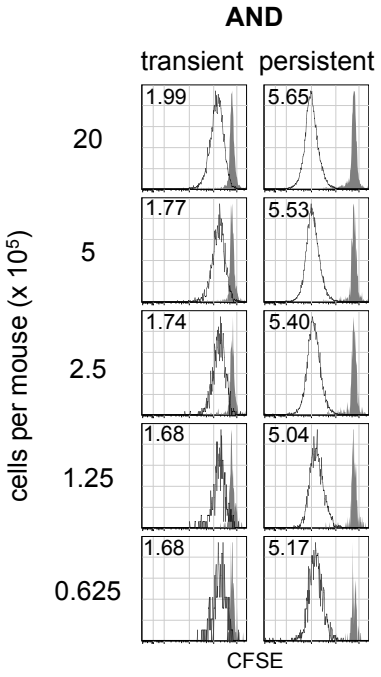


Fig. 13: The number of cells transferred does not affect the antigen dependency of AND T cell proliferation. Congenically marked AND T cells were stimulated with plate-bound α-CD3 and α-CD28 mAbs in the presence of 5 ng/ml IL-7 for 2 d. Cells were mixed at a 1:1 ratio with congenically distinct naïve wt splenocytes before CFSE labeling to provide undivided controls for CFSE dilution analysis (shown in grey in the plots). The indicated numbers of AND $CD4^+$ T cells were transferred into wt mice (transient) or antigen-expressing dtg-M mice (persistent). All recipients received 100 µg/ml doxycycline in their drinking water during the experiment, starting 1 d before T cell transfer. 3 d after transfer, the CFSE dilution was analyzed in the spleen. CFSE dilution in lymph nodes was very similar and is therefore not depicted. Data shown have been gated on single live cells positive for the congenic marker and CD4. Average division numbers depicted in the histograms were calculated from MFIs as $N = \log_2(MFI_{ctrl}/MFI_{sample})$.

5.4.4 Proliferation of AND T cells is not limited by the number of cells transferred

Clonal competition induced by transfer of high numbers of TCR tg T cells has been demonstrated to hamper proliferation and functionality of $CD4^+$ T cells (Badovinac et al., 2007; Quiel et al., 2011; Yarke et al., 2008). Hence it was necessary to investigate the influence of the number of cells transferred here (2×10^6 T cells per mouse) on AND T cell proliferation.

In vitro-stimulated AND T cells were CFSE labeled and 2×10^6 to 6.25×10^4 cells transferred into wt or antigen expressing dtg-M mice. The CFSE dilution was analyzed 3 d later in lymph nodes and spleen.

The number of AND T cells transferred did not have an obvious effect on the extent of proliferation, as average division numbers were found to vary only in the range of 0.5 divisions in animals receiving titrated numbers of AND T cells (Fig. 13). The number of cells transferred is thus not responsible for the antigen-dependency of AND T cell proliferation.

5.4.5 Blockage of coinhibitory signaling does not enhance AND T cell proliferation

Coinhibitory signals delivered via cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death 1 (PD-1) are able to modulate the outcome of TCR ligation. They are thought to reverse the TCR mediated cessation of T cell migration (Fife et al., 2009; Schneider et al., 2006) and therefore shorten the T cell-DC interaction in vivo. The prolifera-

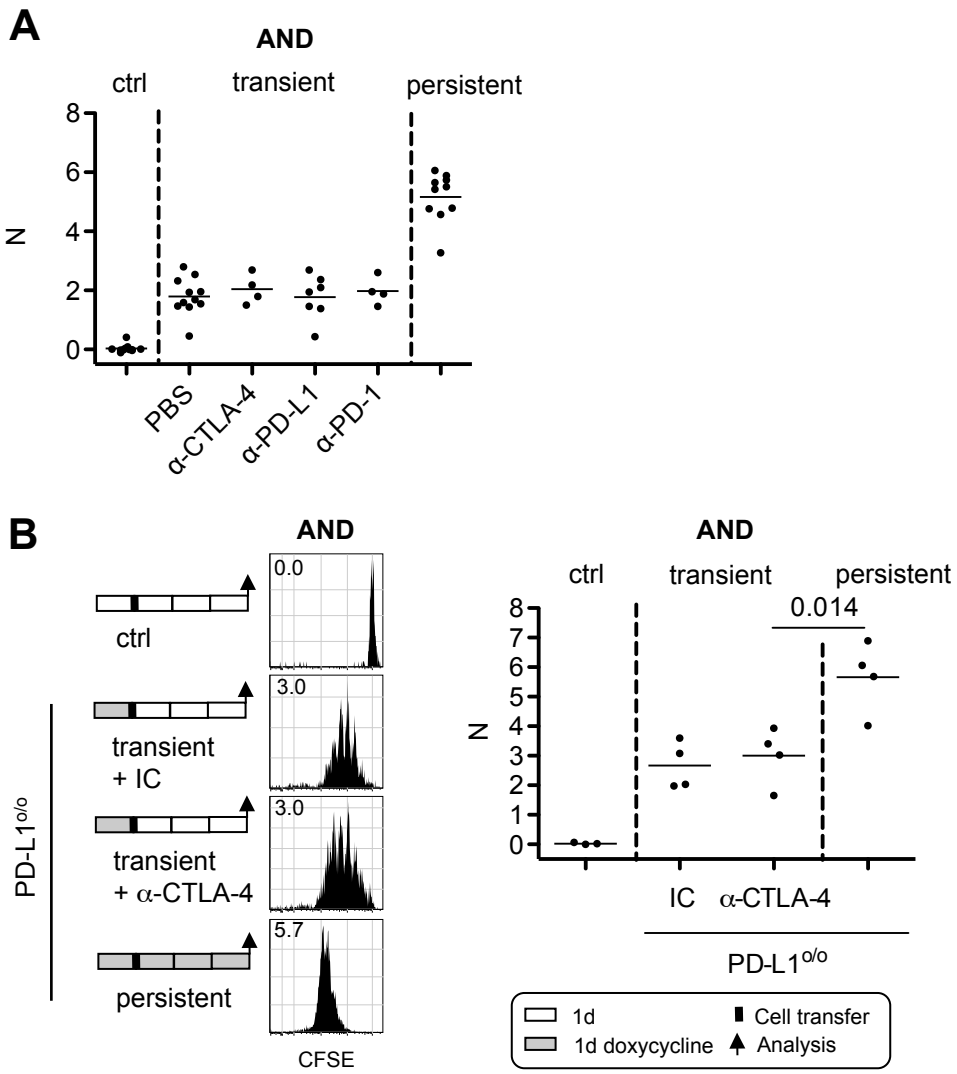


Fig. 14: Coinhibitory signaling does not cause the antigen dependency of AND T cell proliferation. (A) Congenically marked AND T cells were stimulated in vitro with plate-bound α-CD3 and α-CD28 mAbs in the presence of 5 ng/ml IL-7 for 2 d. Cells were CFSE labeled and transferred into wt mice (transient), which received 200 µg mAbs i.p. at the time point of T cell transfer as indicated, or antigen-expressing dtg-M mice (persistent). Cells left unstimulated during the culture and transferred into wt mice served as controls (ctrl). All mice received 100 µg/ml doxycycline in their drinking water during the experiment, starting 1 d before T cell transfer. 3 d after transfer, the CFSE dilution was analyzed in the spleen. The CFSE dilution in lymph nodes was very similar and is therefore not depicted. **(B)** Congenically marked naïve AND T cells were transferred into antigen-expressing dtg-M mice (PD-L1$^{o/o}$) treated with doxycycline and injected with 200 µg mAbs i.p. as indicated. 3 d after transfer, the CFSE dilution of transferred AND T cells was analyzed in spleens. The CFSE dilution in lymph nodes was very similar and is therefore not depicted. Average division numbers depicted in the histograms were calculated from MFIs as $N = \log_2(MFI_{ctrl}/MFI_{sample})$. Data shown have been gated on single live cells positive for the congenic marker and CD4. The mean values from 4-7 (A) or 4 (B) independent experiments with one mouse per condition are depicted. P values were calculated by unpaired Student's *t*-test.

tive blockage of AND T cells following transient TCR stimulation had therefore to be considered as being induced by coinhibitory signaling via CTLA-4 and/or PD-1 pathways.

As the in vitro stimulation used here does not contain any APCs that could potentially mediate coinhibitory signals, the importance of the CTLA-4 and PD-1 mediated signals was first explored in the expansion phase in vivo. AND T cells were stimulated in vitro for 2 d, CFSE labeled and transferred into wt (transient TCR stimulation) or antigen expressing dtg-M mice (persistent TCR stimulation). AND T cell left unstimulated during the culture and transferred into wt mice served as controls (ctrl). At the time point of T cell transfer, recipient mice were treated with blocking mAbs against CTLA-4, PD-1 or programmed cell death ligand 1 (PD-L1). The CFSE dilution of transferred AND T cells was analyzed in lymph nodes and spleens 3 d later.

The average number of divisions undergone by AND T cells in mice treated with blocking α-CTLA-4, α-PD-1 or α-PD-L1 mAbs were unchanged compared with PBS injected control mice (Fig. 14A) and remained significantly reduced in comparison to persistently stimulated T cells. Thus, proliferation of AND T cells during the expansion phase was unaffected by the coinhibitory signaling pathways targeted here.

To approach the principal possibility of coinhibitory signals mediating their effects during the priming phase, advantage was taken of the possibility to switch off antigen expression in dtg-M mice. Dtg-M mice provided with doxycycline in the drinking water for 1 d will display a fade out of antigen expression during the next 4 d (Obst et al., 2005). The transfer of naïve AND T cells at the time point of doxycycline removal thus creates a condition of transient TCR stimulation that includes the priming phase. To exclude coinhibitory signaling delivered by the PD-1 pathway, PD-L1 deficient (PD-L1$^{o/o}$) dtg-M mice were used as T cell recipients. Additionally, dtg-M;PD-L1$^{o/o}$ mice received a blocking α-CTLA-4 mAb at the time point of transfer, allowing the simultaneous blockage of both coinhibitory signaling pathways. Naïve AND T cells were CFSE labeled and transferred into dtg-M;PD-L1$^{o/o}$ mice treated with doxycycline and α-CTLA-4 or isotype control mAbs as indicated. Three days after transfer, the CFSE dilution was analyzed in lymph nodes and spleen.

Even during the double blockage of CTLA-4 and PD-1 signals, the difference in average division numbers between transient and persistent TCR stimulation was conserved (Fig. 14B). Together, these findings exclude that coinhibitory signals mediated by the PD-1 and CTLA-4 pathways are responsible for the antigen dependency of AND T cell proliferation.

5.4.6 Antigen dependency is conserved in Th1 or Th2 polarized AND T cells

Whereas $CD8^+$ T cell differentiation is limited to effector and memory subpopulations, $CD4^+$ effector T cells are known to fall into a variety of subpopulations with distinct cytokine profiles and functions. In the experimental system used here, AND T cells are not purposely conditioned towards Th1 or Th2 phenotype during in vitro stimulation. They express the transcription factor T-bet and produce IFNγ following persistent stimulation (see Fig. 6), indicative of preferential Th1 differentiation. When the expression of the Th2 cytokine IL-4 was analyzed using AND T cells expressing the IL-4 reporter construct 4get, no IL-4 gene translation as reported by green fluorescent protein (GFP) expression could be observed following transient or persistent TCR stimulation (Fig. 15A). It remained unclear why AND T cells show a preferential differentiation to the Th1 phenotype. Following *Leishmania* infection, mice from different backgrounds tend to generate Th1 or Th2-biased T cell responses (Locksley et al., 1999), indicating the involvement of genetic factors. Furthermore, the polarization of $CD4^+$ T cells might be influenced by gut microbiota (Chappert et al., 2013). As the present study was focused on the comparison of $CD4^+$ and $CD8^+$ T cells, this question was not further investigated.

Partially different proliferative capacities of Th1 and Th2 cells have been reported before (Grogan et al., 2001). To assess these potentially divergent proliferation patterns, Th1 or Th2 differentiating conditions were applied to the in vitro stimulation. Partial GPF expression of AND;4get T cells on d 2 of stimulation under Th2 differentiating conditions was observed (Fig. 15B). Nearly all AND;4get T cells expressed the IL-4 reporter GFP if a second culture period with APCs expressing $MCC_{88\text{-}103}$ (from Ii-MCC mice) was carried out under Th2 polarizing conditions (Fig. 15B), illustrating the susceptibility of AND cells to skew their differentiation towards the Th2 phenotype.

To assess the proliferation patterns of Th1 and Th2 cells, AND T cells were stimulated in vitro for 2 d under Th1 or Th2 polarizing conditions, CFSE labeled and transferred into wt (transient TCR stimulation) or antigen expressing dtg-M mice (persistent TCR stimulation). AND T cells stimulated in the presence of 5 ng/ml IL-7 served as unpolarized controls (unpol.) and AND cells left unstimulated during the culture and transferred into wt mice as unstimulated controls (not depicted). Three days after transfer, the CFSE dilution was analyzed in lymph nodes and spleens of recipient mice.

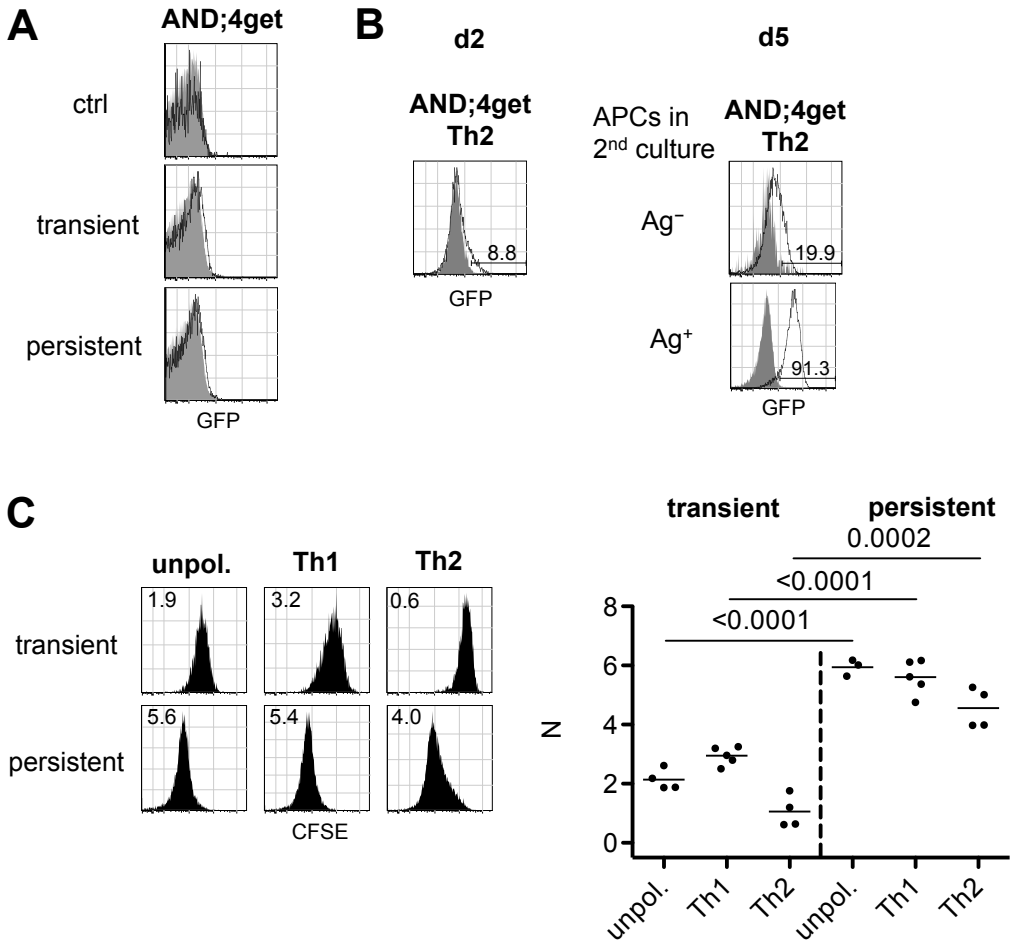


Fig. 15: Antigen dependency is conserved in Th1 or Th2 polarized AND T cells. (A) AND T cells do not express IL-4 following transient or persistent stimulation in vivo. Congenically marked AND;4get T cells were stimulated with α-CD3 and α-CD28 in the presence of 5 ng/ml IL-7 in vitro for 2 d and transferred into wt (transient) or antigen-expressing dtg-M mice (persistent). 3 d after transfer, splenocytes were analyzed for GFP expression. Endogenous $CD4^+$ T cells are shown in grey. **(B)** Congenically marked AND;4get T cells were stimulated with α-CD3 and α-CD28 under Th2 polarizing conditions for 2 d. Cells were cultured for further 3 d in the presence of APCs from wt or antigen-expressing APCs (from Ii-MCC mice) under Th2 polarizing conditions. On d 2 and d 5, GFP expression of AND;4get T cells was analyzed by flow cytometry. Cells cultured under non-polarizing conditions are shown in grey. The percentage of GFP^+ cells is indicated in the histograms. **(C)** Congenically marked AND T cells were stimulated with α-CD3 and α-CD28 in vitro under Th1 or Th2 polarizing conditions for 2 d. Cells were CFSE labeled and transferred into wt (transient) or antigen-expressing dtg-M mice (persistent). Cells left unstimulated during the culture and transferred into a wt mouse served as controls (histograms not shown). All recipients received 100 µg/ml doxycycline in their drinking water during the experiment, starting 1 d before T cell transfer. 3 d after transfer, spleens were analyzed for the CFSE dilution of transferred AND T cells. The CFSE dilution in lymph nodes was very similar and is therefore not depicted. Average division numbers were calculated from MFIs as $N = \log_2(MFI_{ctrl}/MFI_{sample})$ and are depicted in the histograms. Data shown have been gated on single live cells positive for the congenic marker and CD4. The Graph shows means from 3-5 independent experiments with one mouse per condition and p values calculated by unpaired Student's *t*-test.

Th1 or Th2 polarized AND T cells did not display changed proliferative patterns in comparison to the unpolarized control (Fig. 15C). Following all culture conditions, the extent of proliferation was significantly lower following transient TCR stimulation as compared with persistent TCR stimulation. Nevertheless, Th2 polarization did result in fewer divisions following transient TCR stimulation (N = 1.1 ± 0.5) compared to unpolarized AND T cells (N = 2.1 ± 0.4). In contrast, Th1 polarization seemed to enhance proliferation (N = 2.9 ± 0.3). Following persistent TCR stimulation, the degree of proliferation was reduced in Th2 compared to unpolarized AND T cells (N = 4.6 ± 0.7 versus 5.9 ± 0.3).

Taken together, these findings indicate a slightly reduced proliferative potential of Th2 polarized compared with Th1 or unpolarized AND T cells. This disagreement with the study of Grogan et al. (Grogan et al., 2001) will be discussed later (see section 6.2). Antigen-independent proliferation of AND T cells was not observed following any culture condition. Therefore, antigen-dependent proliferation was conserved across $CD4^+$ T cell subpopulations.

5.4.7 Antigen dependency of AND T cell proliferation is unchanged in memory cells

Memory T cells are thought to expand following shorter simulation periods than naïve T cells. It was therefore possible that the proliferation of AND memory T cells would not depend on prolonged TCR stimulation.

To address this question, memory-like Rested Effector (RE) $CD4^+$ T cells, whose phenotype and gene expression profile have been demonstrated to resemble that of in vivo differentiated memory cells (McKinstry et al., 2007; Strutt et al., 2012), were generated in vitro. Naïve AND T cells were stimulated with irradiated splenocytes from mice expressing $MCC_{88\text{-}103}$ (Ii-MCC) for 4 d in the presence of 80 U/ml IL-2, followed by a 3 d resting period without APCs or IL-2. Activation marker expression of RE AND T cells has been shown previously to resemble that of in vivo generated memory cells (Tussing, 2008). RE AND T cells were CFSE labeled and their proliferative capacity compared with naïve AND T cells in dtg-M mice treated with doxycycline 1 d before transfer (transient antigen presentation) or continuously during the experiment (persistent antigen stimulation). Naïve or RE AND T cells transferred into dtg-M mice not receiving doxycycline served as controls (ctrl). The CFSE dilution was analyzed 3 d after transfer in lymph nodes and spleen.

Both naïve and RE AND T cells displayed significantly diminished proliferation following transient as compared to persistent antigen presentation in vivo (Fig. 16). An antigen-independent proliferation phase could thus not be observed in memory-like RE AND

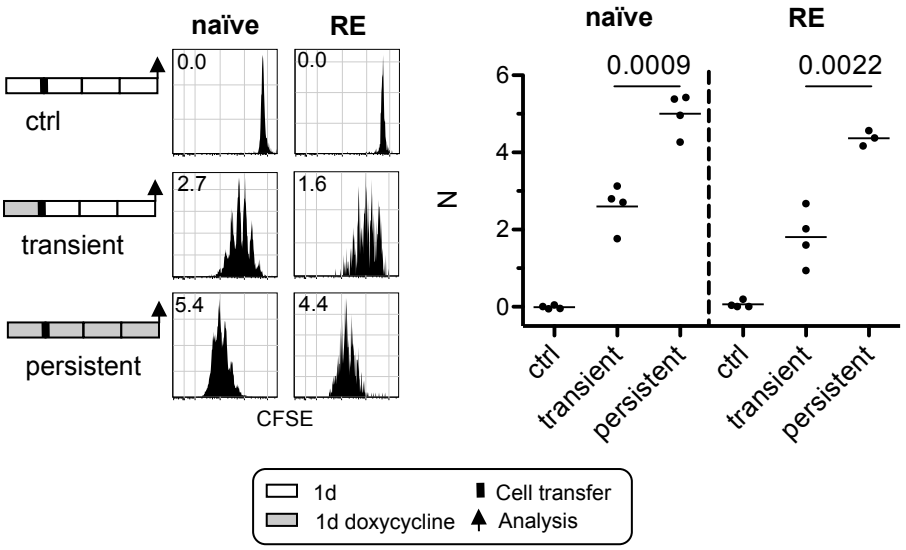


Fig. 16: The proliferation of Rested Effector (RE) AND T cells is antigen-dependent. Memory-like AND RE T cells were generated by culturing AND T cells in the presence of irradiated splenocytes from mice expressing $MCC_{88\text{-}103}$ constitutively (Ii-MCC) for 4 d with 80 U/ml IL-2, followed by a 3 d resting period of culture without APCs and IL-2. Before the second culture, APCs were removed by centrifugation trough a Ficoll cushion. Congenically marked naïve or RE AND T cells were CFSE labeled and transferred into dtg-M mice treated with doxycycline as indicated. Cells transferred into mice receiving no doxycycline served as controls (ctrl). 3 d after transfer, the CFSE dilution was analyzed in the spleen. The CFSE dilution in lymph nodes was very similar and is therefore not depicted. Data shown have been gated on single live cells positive for the congenic marker and CD4. Average division numbers depicted in the histograms were calculated from MFIs as $N = \log_2(MFI_{ctrl}/MFI_{sample})$. The mean values from 4-5 independent experiments with one mouse per condition are depicted. P values were determined by unpaired Student's *t*-test.

T cells, again showing the conservation of this phenomenon across the differentiation states of $CD4^+$ T cells.

In conclusion, the experiments presented in this section show that the differential antigen dependency of AND and OT1 T cells is resistant to all manipulations applied here. Thus this phenomenon seems to be intrinsic to AND and OT1 or $CD4^+$ and $CD8^+$ T cells, respectively.

5.5 The Mechanism of differential antigen dependency of AND and OT1 T cells

The data presented here point towards a cell intrinsic regulation of the differential antigen dependency of AND and OT1 T cells. Three strategies were used to approach the mechanism that is responsible for the differential antigen dependency of these subsets: gene expression

analysis, the assessment of proliferation kinetics and metabolic characterization of AND and OT1 T cells.

5.5.1 Gene expression analysis of AND and OT1 T cells

5.5.1.1 Gene expression of transiently and persistently stimulated AND and OT1 T cells

To find out whether the different proliferation patterns of $CD4^+$ and $CD8^+$ T cells are reflected at the transcriptional level, gene expression analysis of transiently and persistently stimulated AND and OT1 T cells has been carried out previously (Rabenstein, 2012). In that study, AND and OT1 T cells subjected to transient or persistent TCR stimulation as described in Fig. 3 were sorted from recipient mice 3 d after transfer and RNA isolated for microarray analysis. Here, the obtained data were reanalyzed (with the kind permission of H. Rabenstein).

In order to compare the gene expression of both subsets, the fold change (FC) of mean expression values of transiently stimulated versus control T cells was plotted against the FC of persistently stimulated versus control T cells (Fig. 17). In FC/FC plots, genes regulated similarly under both experimental conditions are located close to the diagonal (X = Y) of the plot, whereas genes whose expression is changed in one but not the other condition compared to the control condition will cluster around the FC = 1 lines.

Fold change (FC/FC) plots revealed broadly different gene expression patterns for AND and OT1 T cells under conditions of transient compared with persistent TCR stimulation. The overall appearance of the FC/FC plot displaying AND T cell gene expression resembled a cloud of dots clustered around the horizontal line. A group of genes was only upregulated following persistent TCR stimulation and few genes were strongly upregulated under both conditions. Gene expression of OT1 T cells on the other hand seemed to be more similar under both conditions, with the scatter-plot showing the tendency to locate around the X = Y line of the plot and a rather small group of genes upregulated only following persistent TCR stimulation (Fig 17). Linear regression analysis (performed by Cheng Guo) was used to assess the mathematic significance of this impression. The regression line for the FC/FC plot for AND T cells has a ~2-fold smaller slope than the FC/FC plot for OT1 T cells (P = 0.27 for AND and P = 0.46 for OT1 T cells). Furthermore, the correlation coefficient of the scatter-plot representing the gene expression of AND T cells is smaller than that of OT1 T cells (r^2 = 0.14 for AND, r^2 = 0.44 for OT1 T cells), indicating that the scatter-plot of OT1 T cell fits better to the regression line than that of AND T cells.

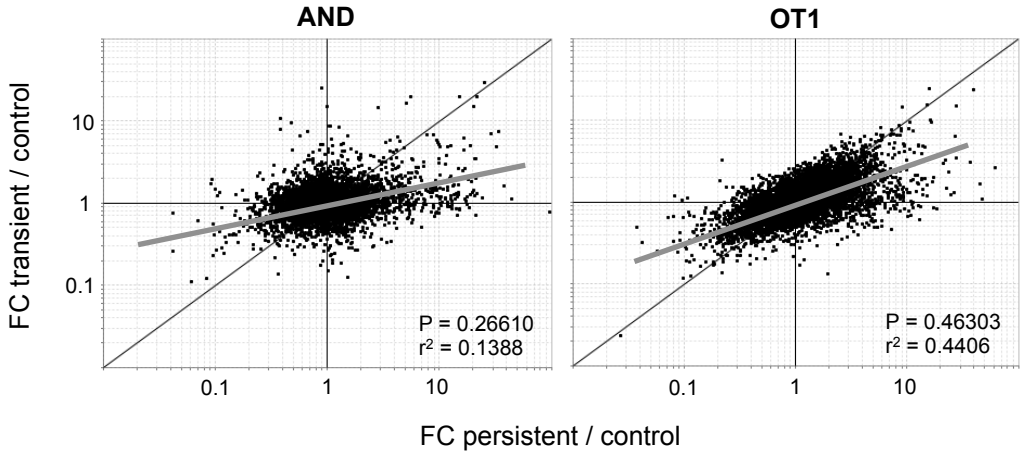


Fig. 17: Gene expression analysis of transiently and persistently stimulated AND and OT1 T cells. Congenically marked AND or OT1 T cells were purified by MACS and stimulated with α-CD3 and α-CD28 mAbs and 5 ng/ml IL-7 for 2 d, or cultured with IL-7 alone as controls. 4 x 10^6 Cells were transferred into wt mice (transient condition) or antigen-expressing dtg mice (persistent condition, AND →dtg-M, OT1 → dtg-O). Unstimulated T cells were transferred into wt mice and served as controls (ctrl). Dtg mice received 100 µg/ml doxycycline in their drinking water during the experiment, starting 1 d before T cell transfer. 3 d after T cell transfer, lymph nodes and spleens were pooled for FACS sorting. Cells were sorted twice for the presence of the congenic marker and CD4 or CD8, respectively, to achieve approximately 100% purity with the second sort being directly targeted into reaction tubes containing TRIzol. RNA was isolated, cDNA synthetized and gene expression of independent triplicates analyzed by hybridization to the Affymetrics Mouse Genome 430 2.0 microarray. These experiments were performed by H. Rabenstein (Rabenstein, 2012). Here, data were reanalyzed using the Genepattern software. Cheng Guo calculated regression lines using the Matlab software.

A detailed analysis of these datasets identified a small group of genes that were upregulated in persistently stimulated AND and OT1 T cells as well as in transiently stimulated OT1 but not AND T cells (Rabenstein 2012). Among them was the transcription factor T-bet, whose protein levels were demonstrated here to follow the same pattern (see Fig. 6). This group did not contain genes with a known role in the regulation of T cell proliferation and thus no promising candidates for master regulators of the antigen-independent proliferation of $CD8^+$ T cells.

In conclusion, the gene expression analysis of transiently and persistently stimulated AND and OT1 T cells revealed broad differences in gene expression patterns of both subsets under these conditions.

5.5.1.2 Gene expression of in vitro-stimulated AND and OT1 T cells

Even though activation marker expression of AND and OT1 T cells was very similar following the in vitro stimulation step, it remained possible that the differential proliferation and gene expression patterns observed on d 5 were already programmed during the priming phase. To address this question, gene expression analysis was performed on d 2 of in vitro stimulation, the point where T cell treatments diverged, in the present study. AND and OT1 T cells were stimulated with α-CD3 and α-CD28 for 2 d in vitro and FACS sorted to achieve approximately 100% purity and to eliminate dead or apoptotic cells. The RNA was isolated and independent triplicates used for the gene expression analysis on an Affymetrix Mouse genome 420 2.0 microarray. Data were analyzed using GenePattern open source software (for details refer to sections 4.3.1.4 and 4.3.1.5).

No genes are regulated in an opposing manner in AND and OT1 T cells

In order to find genes that could be responsible for the differential proliferation patterns of $CD4^+$ and $CD8^+$ T cells, a first analysis was dedicated to the identification of genes that are upregulated in one and downregulated in the other subset or vice versa. To visualize the changes in gene expression of AND and OT1 T cells following in vitro stimulation, the FC of mean expression values in the stimulated versus the unstimulated condition were plotted against their p value in so-called volcano plots, that allow for the simultaneous judgment of the extent of up- or downregulation of genes and their significance. These plots showed more genes to be significantly (*t*-test $p < 0.05$) upregulated more than 3-fold following stimulation in AND T cells (1,528, highlighted in blue) than in OT1 T cells (809, highlighted in green) (Fig. 18, upper panels). If genes upregulated in stimulated AND T cells were projected onto the data set of OT1 T cells and vice versa, it was obvious that the majority of genes are regulated similarly in the opposing T cell subset (Fig. 18, lower panel). Of the 1,528 genes significantly upregulated in AND T cells, only 19 (1.2%) were downregulated in OT1 T cells, with none of them reaching significance. Vice versa, of 809 genes significantly upregulated in OT1 T cells only 15 (1.9%) were downregulated in AND T cells (all with $p > 0.05$). Thus, no genes with opposing expression patterns in the subsets were identified.

Critical assessment of gene expression data

In order to judge the extent of variance between gene expression patterns of AND and OT1 T cells, these results were compared with gene expression data generated in an indepen-

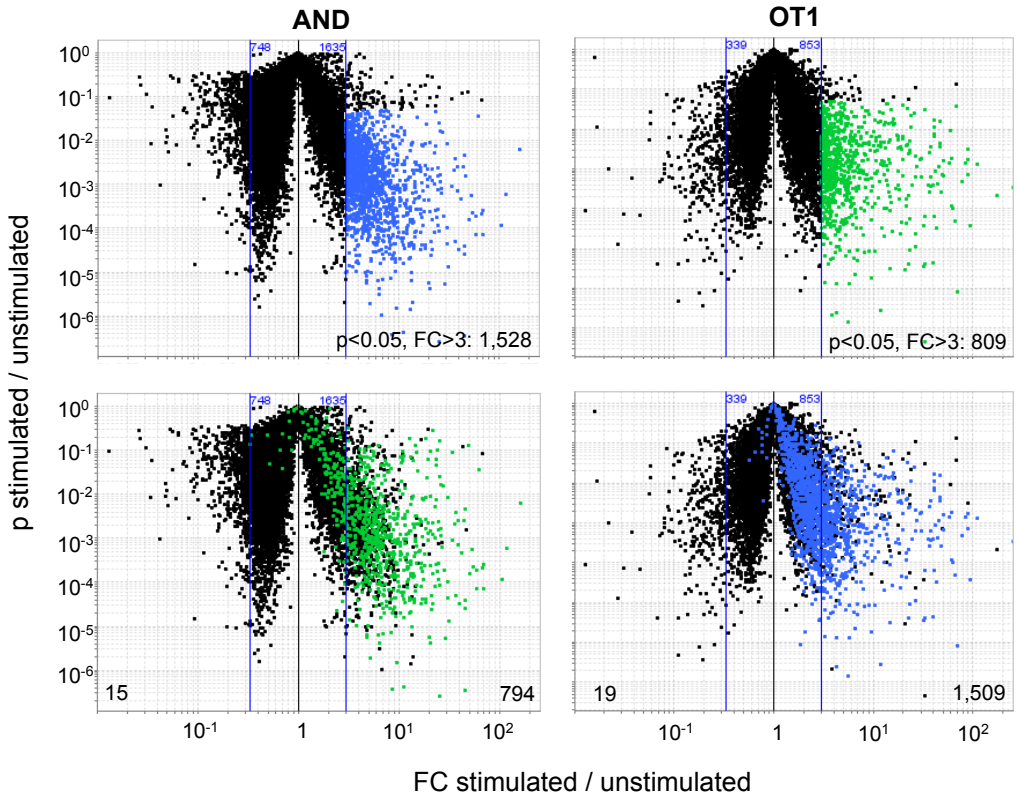


Fig. 18: **Gene expression of AND and OT1 T cells following 2 d of in vitro-stimulation.** AND or OT1 T cells were MACS-purified and stimulated with α-CD3 and α-CD28 mAbs and 5 ng/ml IL-7 for 2 d, or cultured with IL-7 alone as controls. Cells were FACS sorted (directly into TRIzol) to remove dead cells and achieve approximately 100% purity. The RNA was isolated and the gene expression in samples derived from 3 independent experiments analyzed with the Affymetrics Mouse genome 430 2.0 array. Data were processed using the Genepattern software. The blue lines mark a fold change (FC) of 3.

dent study. Gene expression data of $CD4^+$ and $CD8^+$ T cells stimulated with α-CD3 and α-CD28 for 24 h in vitro were recently published (Mingueneau et al., 2013) and would have been best suited for this purpose but were not available (as of February 24th, 2013). Instead, gene expression data derived from OT1 T cells stimulated with artificial APCs (aAPCs) in vitro (Agarwal et al., 2009) were used. In that study, aAPCs were generated by coating latex microspheres with the $H\text{-}2K^b$:Ig fusion protein and a CD80/Fc chimeric protein and loaded with $OVA_{257\text{-}264}$. OT1 T cells were cultured in the presence of aAPCs and 2.5 U/ml IL-2 for 2 d. Gene expression was analyzed on an Affymetrix Mouse Genome U74Av2 microarray. Naïve OT1 T cells freshly isolated from TCR tg animals were used as controls.

Here, microarray data from that study were reanalyzed in analogy to that of AND and OT1 T cells depicted in Fig. 18 to compare the gene expression of OT1 T cells stimulated under the two different conditions. As the data were generated using different Affymetrix microarrays, they could not be depicted in one single plot. In addition to different T cell stim-

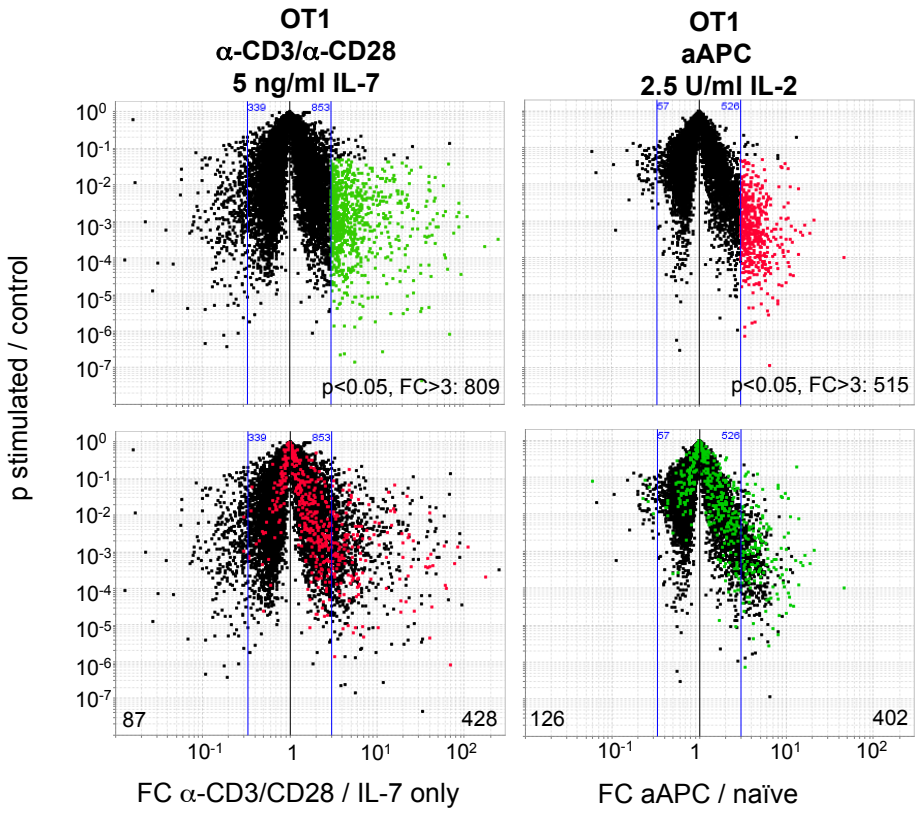


Fig. 19: Comparative analysis of gene expression data derived from OT1 T cells stimulated with two different protocols. Gene expression patterns of OT1 T cells stimulated with α-CD3 and α-CD28 in this study and OT1 T cells stimulated with artificial APCs (aAPCS; latex microspheres coated with H-2K^b and B7, loaded with $OVA_{257-264}$) by Agarwal et al. (Agarwal et al., 2009) were analyzed using the Genepattern software. The blue lines mark a fold change (FC) of 3.

ulation protocols, the control cells (IL-7 cultured versus naïve OT1 T cells) had to be considered as a possible cause of discrepancy. However, volcano plots revealed a similar overall impression of OT1 gene expression under both stimulation conditions. In general, OT1 T cells stimulated with aAPCs displayed a weaker up- and downregulation of most genes illustrated by a narrower distribution of the scatter-plot (Fig. 19, upper panel) that might be due to the use of different control cells in both studies. In these cells, 515 genes were significantly (*t*-test $p < 0.05$) upregulated more than 3-fold, compared to 809 genes in α-CD3/α-CD28 stimulated OT1 T cells.

If genes upregulated by one stimulation protocol were projected onto the data set of T cells stimulated with the other protocol, a picture similar to that presented for AND and OT1 T cells (Fig. 18) was observed (Fig 19, lower panel). Only 528 of the 809 genes significantly upregulated in α-CD3/α-CD28 stimulated OT1 T cell were also noted in the microarray used by Agarwal et al., but most of them (402, 76.2%) were also upregulated in aAPC-stimulated

cells. A total of 126 (23.8%) genes were downregulated but only two of them to a FC > 3 and reaching significance. The other way around, all 515 genes significantly upregulated in aAPC stimulated OT1 T cells could be plotted on the data set of α-CD3/α-CD28 stimulated OT1 T cells. Of these genes, 428 (83.1%) were upregulated and 87 (17%) were downregulated but only two reached a FC of 3 and significance. Only 149 genes were upregulated significantly at least 3-fold in both stimulated OT1 populations. This number is most likely underestimated by the fact that different microarrays were compared and different control cells used.

In the comparison of α-CD3/α-CD28-stimulated and aAPC-stimulated OT1 T cells, more genes tended to be regulated in an opposing manner (17% and 23%) than between α-CD3/α-CD28-stimulated AND and OT1 T cells (1.2% and 1.9%). In conclusion, gene expression variances between α-CD3/α-CD28 stimulated AND and OT1 T cells are lower than variances between OT1 T cells stimulated using two different protocols, indicating high reproducibility of the T cell stimulation used here on both subsets.

Few genes are differentially regulated in in vitro-stimulated AND and OT1 T cells

To compare the gene expression of in vitro-stimulated AND and OT1 T cells directly, mean expression values derived from stimulated AND and OT1 T cells were plotted against each other (Fig 20). In this plot, only differential gene expression of AND and OT1 T cells was visualized without reference to unstimulated cells set in volcano plots.

The majority of genes were regulated similarly in AND and OT1 T cells following in vitro stimulation. A total of only 58 genes are expressed at higher levels (FC > 3) in AND T cells, opposed by 71 genes upregulated specifically in OT1 T cells. These numbers were further reduced to 27 genes (AND) and 41 genes (OT1) if a *t*-test p value smaller than 0.05 was required, together representing only 0.5% of the genes depicted (Fig. 20, depicted in blue (AND) or green (OT1) and Tables 1, 2). Out of 41 genes differentially expressed in OT1 T cells, 19 were also upregulated in OT1 T cells sorted from mice infected with OVA-expressing *Listeria monocytogenes* 48 after the infection (as retrieved from The Immunological Genome Project (Immgen) database (Heng et al., 2008) on January 10th, 2014). These genes are marked with an asterisk in Table 2. As the Immgen database did not provide data on activated CD4^{+} T cells at that time point, this validation could not be carried out for the genes expressed at higher levels in AND T cells.

In addition to *Cd4* and *Cd8*, some genes that are associated with the specific effector functions of the subsets were differentially expressed. In AND T cells, the genes coding for

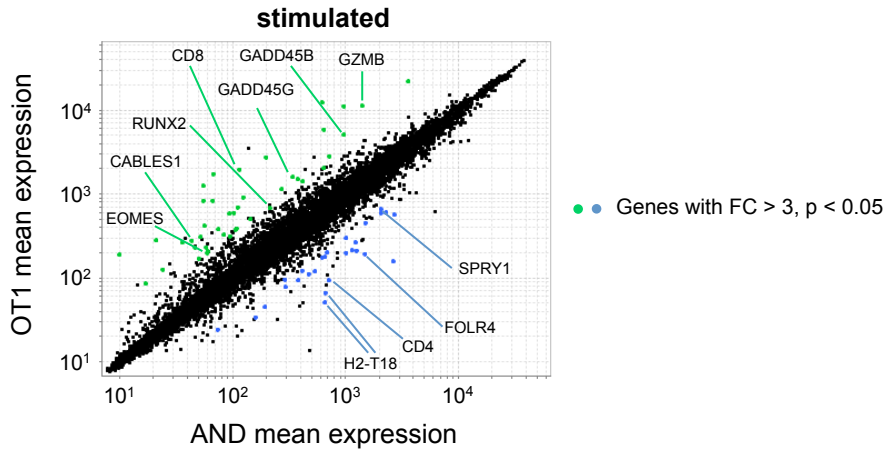


Fig. 20: Gene expression of AND and OT1 T cells following in vitro stimulation. AND or OT1 T cells were MACS-purified and stimulated with α-CD3 and α-CD28 mAbs in the presence of 5 ng/ml IL-7 for 2 d. Cells were FACS sorted (directly into TRIzol) to remove dead cells and achieve approximately 100% purity. The RNA was isolated and the gene expression in samples derived from 3 independent experiments analyzed with the Affymetrics Mouse genome 430 2.0 array. Data were processed using the Genepattern software.

the $CD4^+$ T cell cytokines IL-4 and IL-21 were expressed at higher levels than in OT1 T cells (FC of 34.35 and 5.53, respectively) but this did not reach significance. In OT1 T cells, the effector molecule granyzme B (*Gzmb*) and the transcription factor Eomes are significantly upregulated. Also the expression of the genes encoding H2-T18 and folate receptor 4 (*Folr4,* FR4) was upregulated in AND compared to OT1 T cells. H2-T18 (a variant allele of thymic leukemia Ag, TL) defines the genetic background of AND T cells (B10.BR). FR4 has been associated with the regulatory functions of $CD4^+$ T cells (Liang et al., 2013; Yamaguchi et al., 2007) and recently also with $CD4^+$ T cell anergy (Martinez et al., 2012). In agreement with its differential expression in $CD4^+$ T cells observed here, higher levels of FR4 on the surface of stimulated $CD4^+$ compared to $CD8^+$ T cells were reported previously (Yamaguchi et al., 2007).

Even though IFNγ production was shown to be limited to OT1 T cells following 2 d stimulation (Fig. 3), its transcription was not observed to be restricted to OT1 T cells. Besides Eomes, no transcription factors with a known role in T cells were found to be differentially regulated between AND and OT T cells. The expression of runt-related transcription factor 2 (*Runx2*), a transcription factor previously described for its importance in bone development (Ducy et al., 1997), was significantly upregulated in OT1 T cells in agreement with data available at the Immgen database (as retrieved on January 10th, 2014).

Probe name/ Gene symbol	Description	FC AND/OT1	p value
A630038E17RIK	RIKEN cDNA A630038E17 gene	4.63	0.007084
ADH1	alcohol dehydrogenase 1A (class I), alpha polypeptide	16.82	0.049588
AK3L1	adenylate kinase 3 alpha-like 1	4.07	0.049588
AKR1C12	aldo-keto reductase family 1, member C12	4.27	0.014625
AKR1C13	aldo-keto reductase family 1, member C13	3.42	0.011582
ASS1	argininosuccinate synthetase 1	4.48	0.006840
BC021614	cDNA sequence BC021614	3.39	0.035136
C80638	expressed sequence C80638	3.57	0.019331
CAP1	CAP, adenylate cyclase-associated protein 1 (yeast)	4.75	0.006840
CCR8	chemokine (C-C motif) receptor 8	3.06	0.035136
CD4	CD4 antigen	7.65	0.019331
CD9	CD9 antigen	3.69	0.000017
CHST2	carbohydrate sulfotransferase 2	4.73	0.001610
CKB	creatine kinase, brain	5.97	0.000002
FOLR4	folate receptor 4 (delta)	7.76	0.003587
H2-T18 /// H2-T3 /// H2-TW3 /// H2-T3-LIKE /// LOC633417 /// LOC674370	histocompatibility 2, T region locus 18 /// histocompatibility 2, T region locus 3 /// similar to histocompatibility 2, T region locus 3 /// MHC class I antigen /// similar to histocompatibility 2, T region locus 3 /// similar to histocompatibility 2, T region locus 3	9.99	0.003587
H2-T18 /// LOC633417 /// LOC674370	histocompatibility 2, T region locus 18 /// similar to histocompatibility 2, T region locus 3 /// similar to histocompatibility 2, T region locus 3	12.86	0.000013
HYI	hydroxypyruvate isomerase homolog (E. coli)	3.05	0.000373
IL12RB1	interleukin 12 receptor, beta 1	3.36	0.000608
LOC633417	similar to histocompatibility 2, T region locus 3	3.43	0.012419
MAPRE2	microtubule-associated protein, RP/EB family, member 2	3.78	0.000768
PTGIR	prostaglandin I receptor (IP)	4.30	0.001598
PTPRF	receptor type protein tyrosine phosphatase F	5.17	0.012419
SPRY1	sprouty homolog 1 (Drosophila)	3.49	0.020067
SYNPO	synaptopodin	3.78	0.004646
TESC	tescalcin	3.15	0.003043
UBXD5	UBX domain containing 5	5.35	0.000024

Table 1: Genes differentially expressed in in vitro-stimulated AND compared with OT1 T cells (highlighted in blue in Fig. 20). All genes with a FC (AND versus OT1) > 3 and Student's *t*-test p value < 0.05 are depicted. For experimental details refer to Fig. 20. P values were generated using the Multiplot module of the Genepattern software.

Recently, the expression of Runx2 in $CD8^+$ memory T cells was reported (Hu and Chen, 2013).

Even if no cyclins were differentially regulated in stimulated AND and OT1 T cells, three genes whose products could be involved in the regulation of cell cycle progression were found to be expressed at higher levels in OT1 T cells: growth arrest and DNA-damage-inducible 45 beta (*Gadd45b*), growth arrest and DNA-damage-inducible 45 gamma (*Gadd45g*) and Cdk5 and Abl enzyme substrate 1 (*Cables1*). AND T cells displayed significantly higher expression of sprout homology 1 (*Spry1*), a gene whose product is discussed as a regulator of T cell effector functions (Collins et al., 2012). While some data on the role of these genes in T cells are available (see section 6.3.1), their importance for the proliferation of T cells is still undefined.

Probe name/ Gene symbol	Description	FC OT1/AND	p value
0610037M15RIK	H2-Q6, histocompatobility 2, Q region locus 6	13.87	0.01905
2810439F02RIK*	TTC39C, tetratricopeptide repeat domain 39C	5.78	0.00623
5730557B15RIK	ANKRD33B, ankyrin repeat domain 33B	7.34	0.00002
ADM*	adrenomedullin	4.86	0.00248
AVPI1*	arginine vasopressin-induced 1	3.38	0.00622
BASP1*	brain abundant, membrane attached signal protein 1	4.19	0.00337
BC016495*	cDNA sequence BC016495	7.42	0.00162
C920025E04RIK	RIKEN cDNA C920025E04 gene	14.94	0.00108
CA2	carbonic anhydrase II	4.89	0.00166
CABLES1 /// LOC635753*	Cdk5 and Abl enzyme substrate 1 /// similar to Cdk5 and Abl enzyme substrate 1	6.36	0.00950
CD8A /// LOC636147 /// LOC669166	CD8 antigen, alpha chain /// similar to T-cell surface glycoprotein CD8 alpha chain precursor (T-cell surface glycoprotein Lyt-2) /// similar to T-cell surface glycoprotein CD8 alpha chain precursor (T-cell surface glycoprotein Lyt-2) (CD8a antigen)	25.53	0.01488
CRIM1	cysteine rich transmembrane BMP regulator 1 (chordin like)	3.95	0.00166
CRTAM*	cytotoxic and regulatory T cell molecule	3.58	0.00950
CXCL10*	chemokine (C-X-C motif) ligand 10	5.92	0.00119
EOMES*	eomesodermin homolog (Xenopus laevis)	3.43	0.01488
GADD45B*	growth arrest and DNA-damage-inducible 45 beta	5.28	0.01133
GADD45G*	growth arrest and DNA-damage-inducible 45 gamma	3.98	0.00016
GKAP1*	G kinase anchoring protein 1	3.88	0.01132
GZMB*	granzyme B	8.02	0.00035
H2-D1	histocompatibility 2, D region locus 1	19.83	0.00119
H2-D1 /// H2-L /// LOC547343 /// LOC636948	histocompatibility 2, D region locus 1 /// histocompatibility 2, D region /// similar to H-2 class I histocompatibility antigen, L-D alpha chain precursor /// similar to H-2 class I histocompatibility antigen, D-B alpha chain precursor (H-2D(B))	11.56	0.00735
H2-K1	Histocompatibility 2, K1, K region	6.10	0.01272
H2-L	histocompatibility 2, D region	9.03	0.00025
HIST1H4I*	histone 1, H4i	7.30	0.02895
HOD	homeobox only domain	3.91	0.03291
IL6	interleukin 6	5.04	0.04391
KLRC1 /// KLRC2*	killer cell lectin-like receptor subfamily C, member 1 /// killer cell lectin-like receptor subfamily C, member 2	19.04	0.02895
KLRD1	killer cell lectin-like receptor, subfamily D, member 1	22.83	0.00803
LOC547343	similar to H-2 class I histocompatibility antigen, L-D alpha chain precursor	17.07	0.01364
MALAT1	metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	3.30	0.00822
METRNL	meteorin, glial cell differentiation regulator-like	12.41	0.00453
OSTF1	osteoclast stimulating factor 1	3.18	0.00920
PERP*	PERP, TP53 apoptosis effector	4.68	0.00803
PKIB	protein kinase inhibitor beta, cAMP dependent, testis specific	13.24	0.01490
PLA1A	phospholipase A1 member A	3.52	0.02441
PLAGL1	pleiomorphic adenoma gene-like 1	6.20	0.00822
RAB34*	RAB34, member of RAS oncogene family	3.29	0.00368
RCN3	reticulocalbin 3, EF-hand calcium binding domain	3.35	0.00568
RUNX2*	runt related transcription factor 2	3.17	0.00568
SGIP1	SH3-domain GRB2-like (endophilin) interacting protein 1	5.22	0.01491
UPB1*	ureidopropionase, beta	3.48	0.01037

Table 2: Genes differentially expressed in in vitro-stimulated OT1 compared with AND T cells (highlighted in green in Fig. 20). All genes with a FC (OT1 versus AND) > 3 and Student's *t*-test p value < 0.05 are depicted. P values were generated using the Multiplot module of the Genepattern software. For experimental details refer to Fig. 20. Genes that are upregulated in OT1 T cells isolated from mice infected with OVA-expressing *Listeria monocytogenes* 48 h post infection (as retrieved from the Immgen database on January 10th, 2014) are marked with an asterisk.

Taken together, gene expression analyses confirmed the similar activation of AND and OT1 T cells following in vitro stimulation. Only a few genes were differentially expressed between the subsets.

In conclusion, a gene possibly responsible for the differential antigen dependency of AND and OT1 T cells was not identified by gene expression analysis, even though *Spry1* and the cell cycle regulatory genes *Gadd45b*, *Gadd45g* and *Cables1* could be involved in this process. Therefore, differential antigen dependency of both subsets is unlikely to be exclusively caused by a single gene product at the transcriptional level but might rather be elicited by a complex network of regulatory factors spanning transcriptional, translational, post-translational, epigenetic and potentially even metabolic processes.

5.5.2 Proliferation kinetics of AND and OT1 T cells differ

So far, the proliferation of AND and OT1 T cells has only been assessed at one time point in the expansion phase (d 5 of the experiment). As the CFSE dilution analysis only provided information on how often AND and OT1 T cells divided during the 3 d in vivo portion of the experiment (see Fig. 3 and Fig. 5), a strategy for deeper analysis of this issue was sought.

To access the cell cycle activity of T cells directly at the time point of analysis, the DNA stain DAPI was used. If stained on fixed and permeabilzed cells and acquired on a linear scale, DAPI gMFI is indicative of cellular DNA content, and therefore enables discrimination of cells with a single set of chromosomes (2n, G_1-phase of cell cycle) from those currently synthesizing DNA (> 2n and < 4n, S-phase) or being about to divide (4n, G_2-phase and early M-phase). The percentage of cells in these active stages of the cell cycle (% > 2n) is indicative of the division pace and cell cycle activity on the population level.

Furthermore, Ki67, a protein broadly used to identify cells in all active stages of cell cycle, was stained with a mAb. DNA content illustrated by DAPI staining and Ki67 protein expression was assessed simultaneously by flow cytometry.

To reveal potential differences in cell cycle activities of AND and OT1 T cells, T cells were analyzed directly after the second day of in vitro stimulation, the point of treatment divergence. Additionally, cell cycle activity was determined 3 d after transfer (d 5 of experiment) or 5 d after transfer (d 7 of experiment) into wt or antigen expressing dtg mice. T cells left unstimulated during the cultures and transferred into wt mice were used as controls (ctrl). The gate on actively dividing cells was set in a DAPI versus FSC-A plot where the increase of cell size during cell cycle progression facilitated the discrimination of this population. The quantification of Ki67 was complicated by the fact that following in vitro stimulation, T cells were falling into clearly distinct $Ki67^+$ and $Ki67^-$ populations, whereas

transferred T cells analyzed ex vivo appeared as uniform populations differing in their Ki67 gMFIs. Therefore, the percentage of positive cells or the relative fluorescent units (RFUs, calculated as $gMFI_{Ki67}/gMFI_{IC}$) was used to analyze Ki67 expression in these two situations as appropriate. The use of RFUs allowed the comparison of data from independent experiments and analysis time points.

Following in vitro stimulation, AND and OT1 T cells display equal percentages (42.2 ± 6.7% versus 36.5 ± 8.2%) of cells in S-, G_2- and M-phase of cell cycle (Fig. 21A left). The percentage of AND and OT1 T cells expressing Ki67 protein was much higher (94.8 ± 3.8% versus 81.9 ± 7.6%, Fig. 21B left). This finding had been anticipated as Ki67 protein levels were expected to be maintained during the lag phase between two divisions (G1) after a first cell cycle was completed. Significantly less AND than OT1 T cells expressed Ki67, most likely reflecting the fact that AND T cell proliferation during 2 d stimulation was less homogenous with some cells not yet in division, whereas almost all OT1 T cells had divided once (Fig. 4B). Thus, while the proliferative activity of AND and OT1 T cells was equal on the population level, Ki67 expression seemed to indicate a greater number of AND T cells not yet in division compared to OT1 T cells. This finding indicates that AND T cell proliferation following in vitro stimulation is induced with a slightly prolonged lag-time.

3 d after transfer (on d 5 of the experiment) of stimulated T cells, both AND and OT1 T cells displayed an equal percentage of cells with more than two chromosome sets following persistent TCR stimulation (11.2 ± 6.1% and 9.2 ± 6.5%, Fig. 21A middle). Following transient TCR stimulation, both subsets display a significantly smaller proportion of dividing cells (1.8 ± 0.8% of AND and 1.8 ± 1% of OT1 T cells). This finding shows that cell cycle activity of AND and OT1 T cells at this time point was similar. Thus, antigen-independent proliferation of OT1 T cells occurring after transfer into wt mice has ceased 3 d after transfer.

In contrast, Ki67 staining revealed no differences corresponding to those detected by DAPI staining on d 5 of the experiment. For both AND and OT1 T cells, the RFUs of persistently stimulated cells were not significantly elevated compared to transiently stimulated cells. RFUs of persistently stimulated AND and OT1 T cells were not significantly different, as well as RFUs of transiently stimulated AND and OT1 T cells (Fig. 21B middle).

5 d after transfer (on d 7 of the experiment) into wt or antigen expressing dtg mice, DAPI staining still showed a significantly higher proportion of AND and OT1 T cells actively dividing under persistent than under transient TCR stimulation (Fig. 21A, right). Additionally, significantly more OT1 than AND T cells showed active proliferation at that time point if

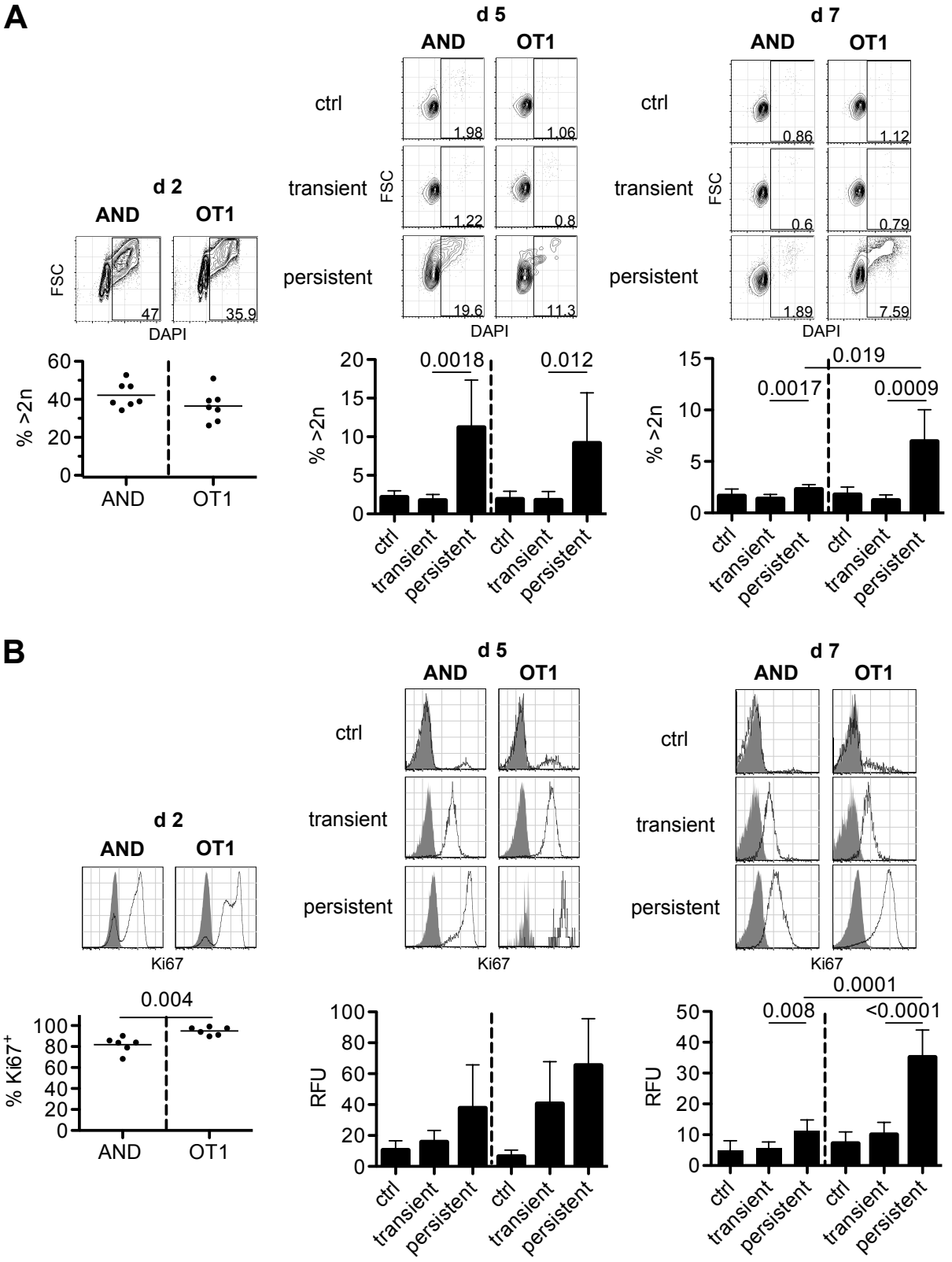


Fig. 21: Proliferation kinetics of AND and OT1 T cells following transient and persistent TCR stimulation. Congenically marked AND or OT1 T cells were stimulated with α-CD3 and α-CD28 in the presence of 5 ng/ml IL-7 in vitro for 2 d. An aliquot was fixed and permeabilized for staining with DAPI **(A)** and for Ki67 **(B)**. Cells were transferred into wt (transient TCR stimulation) or antigen-expressing dtg mice

(persistent TCR stimulation) for analysis of DNA content **(A)** and Ki67 **(B)** on d 3 after transfer (d 5 of the experiment) or d 5 after transfer (d 7 of the experiment) in lymph nodes. Cells left unstimulated and transferred into wt mice served as controls (ctrl). All recipients received 100 µg/ml doxycycline in their drinking water during the experiment, starting 1 d before T cell transfer. Data shown have been gated on single live cells positive for the congenic marker and CD4 or CD8, respectively. IC stainings are shown in grey. RFUs were calculated as $gMFI_{Ki67}/gMFI_{IC}$. The mean values from 6-7 independent experiments are shown in the graphs. P values were determined by unpaired Student's *t*-test.

subjected to persistent TCR stimulation (7 ± 3% of OT1 versus 2.3 ± 0.4% of AND T cells). This finding suggests that OT1 T cells are susceptible to TCR triggered T cell proliferation for longer periods of time than are AND T cells.

Ki67 staining at d 5 after transfer reveals a very similar picture: RFUs of persistently stimulated AND and OT1 T cells are significantly increased compared to transiently stimulated cells and OT1 T cells display significantly higher RFUs under persistent TCR stimulation than AND T cells (35.2 ± 8.8 versus 11.2 ± 3.6, Fig. 21B, right).

Taken together, the DAPI data demonstrate again similar activation of AND and OT1 T cells following 2 d in vitro culture. Cell cycle activity on d 5 revealed the antigen-independent proliferation of OT1 T cells to be time-limited and lasting for less than 3 d. Additionally, OT1 T cells seemed to be responsive to TCR stimulation for longer periods of time, indicating general differences in proliferation kinetics and capacities of the subsets.

Considering the kinetics of Ki67 positivity in direct comparison to cell cycle activity measured by DAPI staining, Ki67 seemed to be rather slowly fading following cessation of proliferation, resulting in disparate findings for both cell cycle activity markers on d 5 and accordance on d 7 of the experiment. Despite the fact that Ki67 protein expression has been described to be closely linked to cell cycle activity in a tumor cell line (Bruno and Darzynkiewicz, 1992) and that it is broadly used for the identification of actively proliferating cells, in this study and elsewhere Ki67 protein has been observed to be stable for prolonged periods of time following cessation of proliferation (Hettinger et al., 2013; Hogan et al., 2013).

5.5.3 The metabolic capacities of AND and OT1 T cells are partially distinct

The interest in the correlation of immune cell function and their metabolism is growing. Metabolic processes have been reported to influence the translation of TCR signaling into gene expression (Sena et al., 2013) and memory T cell formation has been correlated with metabolic changes in T cells (Gubser et al., 2013; van der Windt et al., 2013). As differentia-

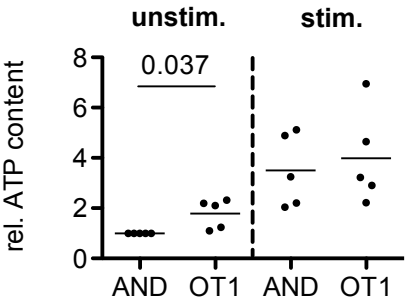


Fig. 22: ATP content of in vitro-stimulated AND and OT1 T cells. MACS-purified AND or OT1 T cells were stimulated with α-CD3 and α-CD28 in the presence of 5 ng/ml IL-7 in vitro for 2 d. The ATP content was measured using the ATP Bioluminescence Assay Kit (Roche). To allow inter-experimental comparison, the data were normalized to the samples with the lowest ATP content in each experiment (in all experiments: unstimulated AND T cells). The graph shows means and p values (paired Student's *t*-test) of data from 5 independent experiments.

tion and proliferation of T cells relies on and might be limited by their metabolic capacity, the differential antigen dependency of AND and OT1 T cells could be correlated with differential metabolic capacities.

To address this possibility, AND and OT1 T cells were analyzed for the amount of ATP per cell following 2 d in vitro stimulation, as the ATP content allows for a first estimate of metabolic activity of the subsets. As inter-experimental variance of this biochemical assay was high, data were related to the lowest value in each experiment (in all independent experiments unstimulated AND T cells) to allow statistical analysis of the results. The ATP content of in vitro-stimulated AND and OT1 T cells did not differ (Fig. 22). Nevertheless, unstimulated OT1 T cells contained significantly more ATP than unstimulated AND T cells, a hint of different metabolic states of both subsets following culture in the presence of IL-7.

In order to assess multiple metabolic parameters of live cells simultaneously, the Seahorse XF96 Extracellular Flux Analyzer was utilized. This machine determines dissolved O_2 and pH in the culture supernatant of live cells in vitro and allows calculation of the O_2 consumption rate (OCR) and the extracellular acidification rate (ECAR) (Wu et al., 2007). In a 96-well-format, optical fluorescent biosensors determine both analytes in a transient micro-chamber generated by lowering the sensor unit close to the bottom of the wells. In between measurement periods, the culture supernatant is mixed to allow equation of analyte concentrations. The measured OCR (Moles/min) is directly correlated to mitochondrial oxidative phosphorylation, whereas the ECAR (pH/min) is correlated to glycolysis-derived lactate responsible for the acidification of the culture medium. Additionally, four drug injection ports allow the application of components during the measurement period and changes in OCR and ECAR in response to the respective substances can be analyzed.

To assay the respiratory capacity of mitochondria, the OCR is measured following application of substances modifying the oxidative phosphorylation (Fig. 23A). After recor-

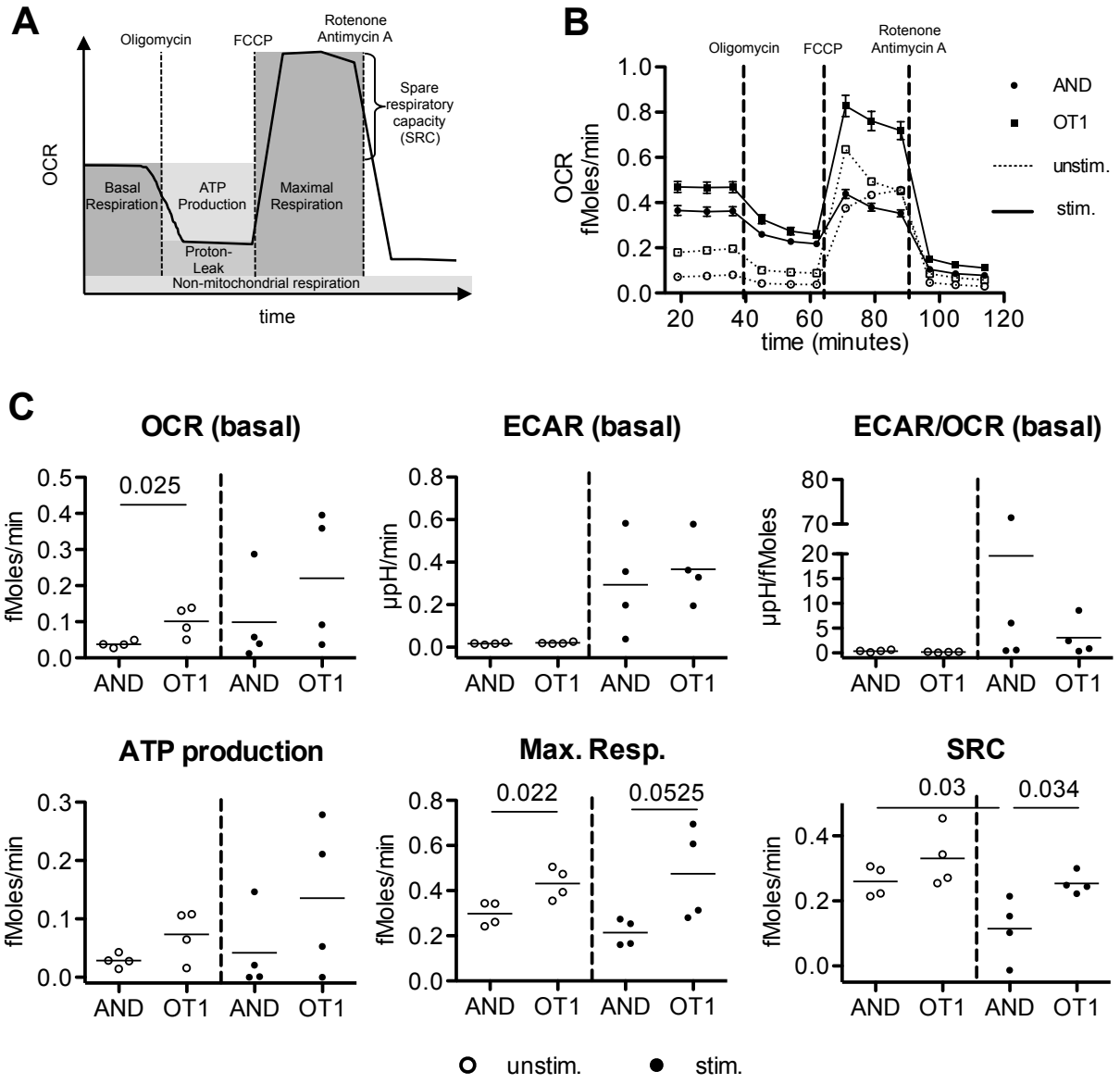


Fig. 23: Mitochondrial Stress Test using the Seahorse XF96 Extracellular Flux Analyzer. (A) Schematic: parameters of mitochondrial respiration accessible by the Mitochondrial Stress Test. **(B)**, **(C)** Purified AND or OT1 T cells were stimulated in vitro with α-CD3 and α-CD28 in the presence of 5 ng/ml IL-7 for 2 d. Cells cultured with IL-7 alone served as unstimulated controls. 0.8-1.6 x 10^6 unstimulated and 2-8 x 10^5 stimulated T cells per well were immobilized to the surface of Seahorse XF96 Cell Culture Microplates using BD Cell Taq. Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) were measured in the basal state and after separate injection of oligomycin, FCCP and rotenone/antimycin A. Data were normalized to the cell numbers in the wells. **(B)** Kinetic display of OCR data from one of 4 independent experiments. Mean and SEM from 5-8 replicates per condition are shown. **(C)** Statistical evaluation of 4 independent experiments with 5-8 technical replicates per condition. SRC: spare respiratory capacity. For details on parameter calculation see section 4.3.1.7. The mean values are shown in the graphs. P values were determined by Student's *t*-test.

ding the basal respiration, the ATP synthetase inhibitor oligomycin is injected, preventing phosphorylating respiration and therefore reducing OCR for the amount used for ATP

synthesis in the basal state. OCR is not completely reduced by this inhibitor, as protons can diffuse into the mitochondrial matrix to a certain extent (proton leak). The addition of the uncoupling agent FCCP leads to an increase of OCR to maximal levels, representing the maximal respiration that the cells are able to perform. The difference between basal respiration and maximal respiration is called spare respiratory capacity (SRC). Rotenone and antimycin A are inhibitors of electron transport chain (ETC) complexes I and III and their application reduces OCR to the base line representing non-mitochondrial respiration. Changes in the ratio of basal ECAR and basal OCR (ECAR/OCR) between experimental conditions are indicative of the enhanced use of glycolysis or oxidative phosphorylation in the cells analyzed.

The Mitochondrial Stress Test described above and depicted in Fig. 23A was performed on AND and OT1 T cells stimulated for 2 d in vitro with α-CD3 and α-CD28. T cells left unstimulated served as controls. Five initial measurement cycles (5 min measurement, 3 min mixing) were carried out before injection of oligomycin, with the last three being used to calculate basal OCR. After the injection of each drug, three measurement cycles were performed. During data analysis, OCR and ECAR were normalized to cell numbers. For details on parameter calculation refer to section 4.3.1.7.

Fig. 23B displays OCR kinetics of one out of four independent experiments. Statistical analysis revealed the basal OCR of unstimulated OT1 T cells to be significantly higher than that of unstimulated AND T cells (0.03 ± 0.01 fMoles/min and 0.1 ± 0.04 fMoles/min, Fig. 23C). A similar tendency was obvious for stimulated T cells but significance was not reached. Basal ECAR and the ECAR/OCR ratio were higher in stimulated AND and OT T cells than in unstimulated T cells, indicating increased glycolysis, but no differences between the subsets were detected. The ATP production of OT1 but not AND T cells was slightly but insignificantly increased in stimulated cells. The apparent contrast to the amount of ATP depicted in Fig. 22 can be explained by the use of two different experimental approaches; a correlation between the amount of O_2 used to produce ATP and the total amount of ATP per cell is not mandatory. Significantly higher maximal respiration was observed for unstimulated OT1 compared to AND T cells (0.29 ± 0.05 fMoles/min and 0.43 ± 0.07 fMoles/min). For stimulated AND and OT1 T cells, a *t*-test p value of 0.0525 indicated a strong tendency towards statistical significance. The SRC was similar in unstimulated AND and OT1 T cells but higher in stimulated OT1 than AND T cells (0.11 ± 0.1 fMoles/min and 0.25 ± 0.03

fMoles/min). Furthermore, stimulated AND T cells displayed a reduced SRC compared to unstimulated cells (0.11 ± 0.1 fMoles/min and 0.26 ± 0.05 fMoles/min).

For all parameters assessed, inter-experimental variances were relatively high. Thus, these results will require further validation by the future analysis of naïve T cells from both TCR tg and wt mice as well as transiently and persistently stimulated AND and OT1 T cells isolated from recipient mice (see Fig. 3).

In conclusion, unstimulated OT1 T cells displayed a higher basal and maximal respiration than unstimulated AND T cells. Additionally, the SRC is reduced in stimulated compared to unstimulated AND but not OT1 T cells and stimulated OT1 T cells display a higher SRC than stimulated AND T cells. This may provide OT1 T cells with a proliferative advantage contributing to antigen-independent proliferation of these cells.

Taken together, a mechanism of differential antigen dependency of $CD4^+$ and $CD8^+$ T cells could not be identified here. Gene expression analysis did not reveal potential candidates for the transcriptional regulation of this phenomenon. Instead, the gene expression patterns of AND and OT1 T cells were found to be very similar following two days of in vitro stimulation, indicating that the described phenomenon might in addition be regulated on the level of protein modifications, by miRNAs or by epigenetic processes. Partially divergent proliferation patterns and metabolic capacities could be involved in the differential antigen dependency of the subsets, but the causative relationships remain unclear.

5.6 T cell proliferation patterns reflect MHC biology

Considering the physiological context of T cell proliferation, the differential antigen dependency of AND and OT1 T cells might be related to the biology of MHC II and MHC I molecules. The stabilization of MHC II but not MHC I on the surface of activated human DCs has been reported in vitro (Cella et al., 1997). Subsequently, the reduced turnover of MHC II following DC maturation was revealed to be dependent on polyubiquitinylation of MHC II (Shin et al., 2006; van Niel et al., 2006) mediated by E3 ubiquitinase March 1 (De Gassart et al., 2008; Walseng et al., 2010). Differential stabilization of MHC molecules has so far not been demonstrated in vivo.

To address this question, DCs of dtg-M and dtg-O mice were activated in vivo by injection of α-CD40 mAb, a strong DC activating reagent (Hawiger Nussenzweig 2001).

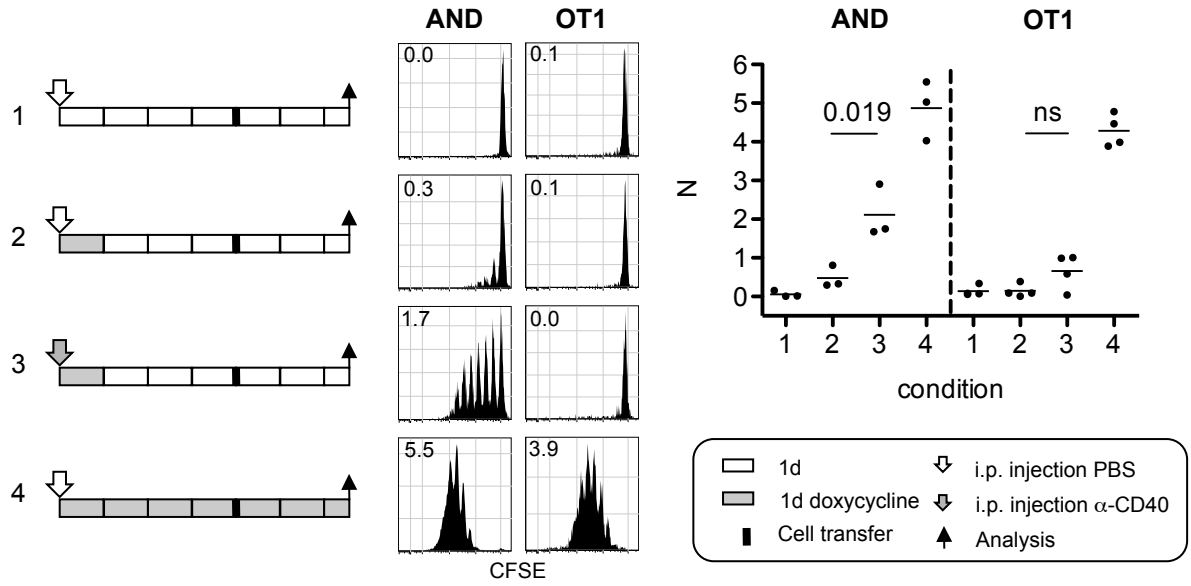


Fig. 24: The presentation of pMHC II but not pMHC I is prolonged on activated DCs in vivo. Mice (dtg-M, BMC: dtg-O → B6) were treated with doxycycline and injected with 50 µg α-CD40 mAb per mouse as indicated. Naïve CFSE labeled AND or OT1 T cells were transferred as sensors of respective pMHC complexes on d 4 of the experiment. The CFSE dilution of the transferred T cells was analyzed in lymph nodes and spleen 3 d after transfer. Data shown have been gated on single live cells positive for the congenic marker and CD4 or CD8, respectively. The CFSE dilution in lymph nodes was very similar and is therefore not depicted. Average division numbers depicted in the histograms were calculated from MFIs as $N = \log_2(MFI_{ctrl}/MFI_{sample})$. The means from 3 (AND) or 4 (OT1) independent experiments are depicted. Student's *t*-test was used to determine p values.

Starting at the time point of α-CD40 injection, mice were given doxycycline in their drinking water for 1 d. As a readout for the presence of pMHC I and pMHC II complexes on DCs, CFSE labeled AND or OT1 T cells were injected as sensors of cognate pMHC complexes. T cells were transferred 3 d after doxycycline removal, a time point where antigen expression has faded in dtg-M mice (Obst et al., 2005). This setup allows the assessment of pMHC I and pMHC II complex presence on activated versus immature DCs following transient antigen expression (Fig. 24, condition 2 and 3). The background expression of TSO transgene in dtg-O mice required the use of BMCs (dtg-O → B6) in this experiment. As BMCs on the B10.BR background did not display a chimerism of more than 70% (Rabenstein, 2012) and TIM expression in dtg-M mice is strictly doxycycline-dependent and antigen turn-off possible (see Fig. 1D), these mice where directly used in this experiment.

CFSE dilution analysis on d 3 after T cell transfer shows no proliferation of AND or OT1 T cells in negative control animals (condition 1) and strong proliferation in positive control animals (condition 4). Transient antigen expression terminated 3 d before T cell transfer alone

did not result in the presence of enough H-2E^k/MCC$_{92-103}$ or H-2K^b/OVA$_{257-264}$ complexes to induce the proliferation of transferred T cells (Fig. 24). If DCs had been activated at the time point of antigen turn-on (condition 3), the amount of presented H-2E^k/MCC$_{92-103}$ complexes were sufficient to induce significant proliferation of AND T cells compared with control mice receiving PBS (condition 2, N = 2.1 ± 0.7 and N = 0.5 ± 0.3). This was not the case for H-2K^b/OVA$_{257-264}$ complexes, as OT1 T cells transferred into mice of condition 3 did not show enhanced proliferation compared to cells transferred into mice of condition 2 (N = 0.7 ± 0.4 and N = 0.1 ± 0.2). These results show the prolonged presentation of pMHC II but not pMHC I complexes on activated DCs.

Therefore, T cell proliferation patterns might be adapted to differential stability of pMHC I and pMHC II complexes on DCs in an inflammatory environment. In vivo, CD4$^+$ T cell proliferation would subsequently be dependent to the presence of ongoing inflammation. In contrast, CD8$^+$ T cell proliferation may be independent of inflammation once it is initiated, resulting in a tighter regulation of CD4$^+$ T cell expansion.

5.7 Outlook: improving the tools for the analysis of T cell antigen dependency

Antigen dependency of CD4$^+$ and CD8$^+$ T cells is insufficiently compared also with respect to memory T cell generation and tolerance induction. In principle, doxycycline inducible neoself-Ag expression in dtg-M and dtg-O mice would allow the direct comparison of dose- and duration-requirements of both processes even in the endogenous polyclonal CD4$^+$ and CD8$^+$ T cell compartments. The leakiness of TSO expression in dtg-O mice (see Fig. 1D) and the difficulty to generate dtg-M → B10.BR BMCs with a homogenously high chimerism (Rabenstein 2012) impaired the design of a truly comparable experimental setup to address these questions.

To allow such experiments in the future, dtg-O mice were crossed to transgenic mice expressing the tetracycline-controllable transrepressor (tet-transrepressor) tTRKRAB, a fusion protein of the DNA binding domain of the tetracycline repressor (TetR) from *E. coli* and the Krüppel-associated box (KRAB) domain of the human Kox1 zinc finger protein (Deuschle et al., 1995), under the control of the human phosphoglycerate kinase (hPGK) promotor (Barde et al., 2009) as depicted in Fig. 25 (middle).

KRAB domains are found in many zinc finger proteins in mice and humans, where they mediate transcriptional repression of target genes by recruitment of histone deacetylases and

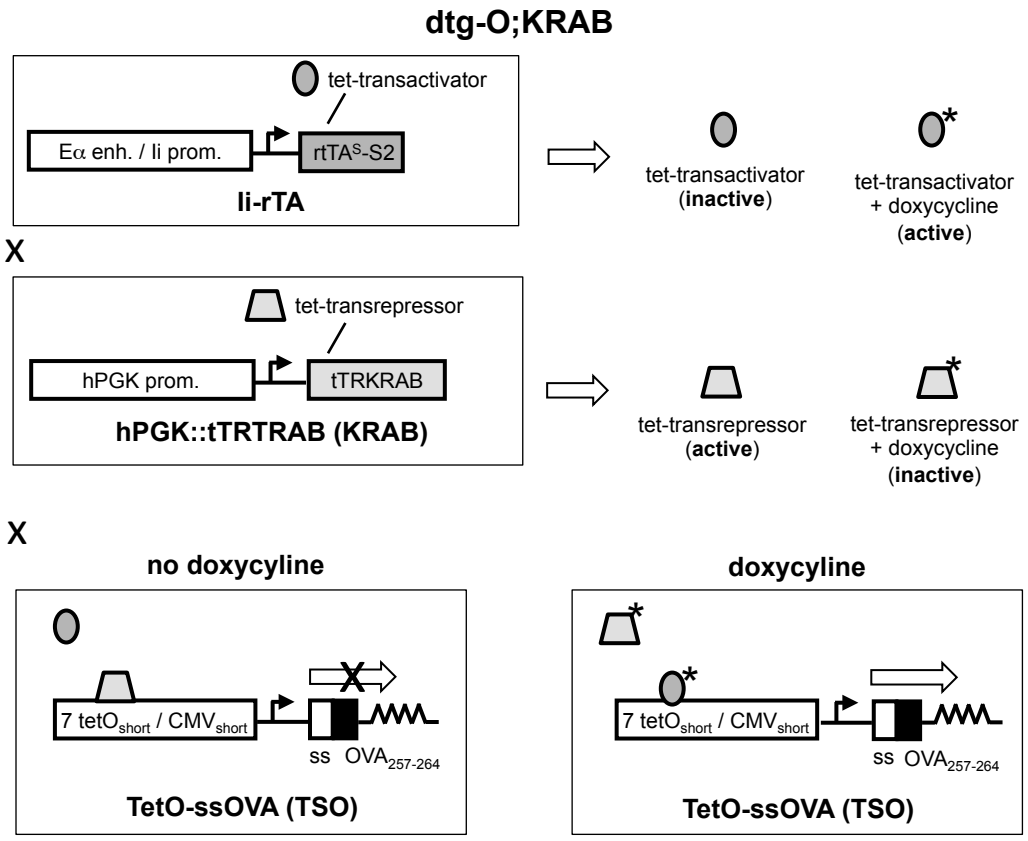


Fig. 25: Function of the KRAB repressor in dtg-O;KRAB mice. The KRAB transgene encodes the tetracycline-controllable transrepressor tTRKRAB, a fusion protein of the DNA binding domain of tetracycline repressor (tetR) from *E. coli* with the KRAB (Krüppel-associated box) domain of the human Kox1 protein under the control of human phosphoglycerate kinase (hPGK) promotor, allowing ubiquitous constitutive expression. In dtg-O;KRAB mice, the tet-transrepresor binds to tetO sequences in the absence of doxycycline, while the tet-transactivator cannot bind. In the presence of doxycycline, the tet-transrepressor is inactivated and the tet-transactivator is activated, leading to TSO-expression. For details on Ii-rTA^S and TSO transgenes refer to Fig. 1 and text section 5.1.

histone methyltransferases and subsequent heterochromatin spreading (Groner et al., 2010; Margolin et al., 1994). They can therefore mediate long-term but reversible transcriptional repression of target genes in mice (Groner et al., 2012). The tet-transrepressor protein is constitutively active in the absence of doxycycline, the presence of doxycycline leads to its deactivation. Therefore, in dtg-O;KRAB mice, the tet-transrepressor will be actively repressing TSO transgene expression in the absence of doxycycline. If mice are treated with doxycycline, the tet-transrepressor will be inactivated and the tet-transactivator will be activated, leading to expression of the TSO transgene (Fig. 25, bottom). This double control of TSO expression may eliminate the background expression of TSO observed in dtg-O mice.

The tight expression of a target gene in a similar triple transgenic mouse model has been reported before (Jellison et al., 2012).

If antigen expression kinetics in dtg-O;KRAB mice turn out to be similar to that of dtg-M mice, these two strains will provide a mouse model where the influence of TCR stimulus duration on tolerance induction and memory generation of $CD4^+$ and $CD8^+$ T cells can be studied. Furthermore, as antigen dose can be modulated in dtg-M mice (Obst et al., 2005) and potentially also in dtg-O mice, multiple aspects of antigen dependency of $CD4^+$ and $CD8^+$ T cells will be assessable.

5.8 Summary of results

In this study, differential antigen dependency of AND and OT1 T cells has been observed in the expansion phase on the proliferation and differentiation levels (Fig. 5, Fig. 6) and has been shown to be applicable to polyclonal $CD4^+$ and $CD8^+$ T cells (Fig. 7). This differential antigen dependence is not reproducible in vitro (Fig. 8) and is not induced by high O_2 concentrations during the in vitro stimulation period (Fig. 9), clonal competition (Fig. 13) or homotypic clustering of $CD8^+$ T cells (Fig. 11). Several T cell extrinsic factors including MHC class expression patterns (Fig. 10), inflammatory cytokines (Fig. 12), coinhibitory signaling (Fig. 14) and AND T cell differentiation (Fig. 15, Fig 16) have furthermore been shown not to modify the proliferative patterns of AND and OT1 T cells. Gene expression patterns reflect the differential antigen dependency of the subsets (Fig. 17) and demonstrated little differences between stimulated AND and OT1 T cells following 2 d in vitro stimulation (Fig. 18-20, Tables 1 and 2). The analysis of proliferation kinetics showed that the antigen-independent proliferation phase of OT1 T cells is rather short lasting and that OT1 T cells are responsive to TCR triggering for longer periods of time than are AND T cells (Fig. 21). On the metabolic level, Mitochondrial Stress Test showed that OT1 T cells possess a higher spare respiratory capacity (SRC) following in vitro stimulation (Fig. 23). Finally, T cell proliferation patterns have been demonstrated to reflect the biology of MHC I and MHC II, as the presentation of pMHC II but not pMHC I complexes was prolonged by the activation of DCs in vivo (Fig. 24). In the future, dtg-O;KRAB and dtg-M mice may allow for the comparison of the roles of antigen dose and the duration of antigen stimulation on $CD4^+$ and $CD8^+$ T cell tolerance induction and memory generation (Fig. 25).

6 Discussion

In this study, differential antigen dependency of $CD4^+$ and $CD8^+$ T cells during the expansion phase was shown in an experimental setup where both subsets were equally activated during the priming phase and the TCR stimulus duration was strictly controlled. This phenomenon seemed to be cell intrinsic and was correlated with partially divergent proliferation kinetics and metabolic capacities of the subsets. The transcriptional profiles of in vitro-stimulated AND and OT1 T cells were very similar. The differential proliferation patterns of both subsets seemed to reflect the biology of the respective MHC molecules, as pMHC II but not pMHC I complexes were presented for prolonged periods of time on activated DCs in vivo.

6.1 Differential antigen dependency of $CD4^+$ and $CD8^+$ T cells

In the experimental setup used here, the proliferation of $CD4^+$ and $CD8^+$ T cells was directly compared. As described in the introduction, such a direct comparison has rarely been done before but many studies have provided data on antigen dependency of only one of the two subsets. So far, only five studies provided comparative data on this issue.

Gett et al. stimulated polyclonal $CD4^+$ and $CD8^+$ T cells in vitro with α-CD3 and α-CD28 mAbs for 24 h and transferred them into naïve B6 mice. They observed slightly increased proliferation of $CD8^+$ compared with $CD4^+$ T cells during the following 3 d in vivo (Gett et al., 2003).

Williams et al. used a *Listeria (L.) monocytogenes* infection model and abrogated bacterial replication with ampicillin 24 h after infection to terminate antigen presentation in vivo (using BALB/c mice). In agreement with the data presented here, they observed $CD8^+$ T effector cell expansion to be unchanged if the bacteria were cleared early after infection, whereas $CD4^+$ T cell expansion was strongly reduced (Williams and Bevan, 2004). Even though *L. monocytogenes* infection represents a physiologic mode of TCR stimulation and ampicillin treatment was shown to reduce antigen presentation to a level insufficient to prime naïve $CD8^+$ T cells, the kinetics of the associated inflammation and its potential to influence T cell proliferation was not assessed. In this setup, $CD8^+$ T cell responses could thus not be shown to be independent of inflammation, i.e. costimulation and inflammatory cytokines.

Corbin et al. performed similar experiments in B6 mice. Antigen presentation was shown to persist until d 3 after infection if mice were treated with ampicillin 24 h after *L. monocytogenes* infection and was maintained for 7 d in untreated mice as determined by an ex vivo antigen detection assay (Corbin and Harty, 2004). In this study, the primary expansion of both $CD4^+$ and $CD8^+$ T cells was slightly reduced and the peak of T cell expansion reached one day earlier if ampicillin was administered 24 h after the infection. In another series of experiments, the extensive expansion of $CD4^+$ T cells required 48 h of unimpaired infection whereas $CD8^+$ T cells were able to expand as much as in control animals if the infection was cleared 24 h after the infection. Thus, even though the expansion of both subsets was slightly affected by a shortened antigen presentation period, $CD4^+$ T cells were shown to require longer antigen presentation for their maximal expansion than $CD8^+$ T cells.

In the same experimental setup, Tseng et al. observed the expansion of endogenous $CD4^+$ and $CD8^+$ effector T cell populations in CB1 mice (the offspring of a B6 x BALB/c mating) to be strongly reduced following termination of *L. monocytogenes* infection 24 h after its onset (Tseng et al., 2009).

Blair et al. used a pMHC-specific mAb to block the interaction of TCR and pMHC complexes in vivo and thereby terminated TCR triggering in the context of a vesicular stomatitis virus (VSV) infection. The magnitudes of $CD4^+$ and $CD8^+$ T cell effector responses were reduced by the application of a pMHC-specific mAb (Blair et al., 2011). In this setting, a differential effect of the viral infection on T cells of both subsets could not be excluded. Even though the blocking mAbs were shown not to deplete APCs, it was not ruled out that they possess different affinities for their targets and therefore block pMHC complexes with different efficiency and kinetics in vivo. The antibody used for the blockage of H-$2K^b$/$OVA_{257-264}$ complexes has a rather low affinity (Porgador et al., 1997). Furthermore, the responses of endogenous (polyclonal) $CD8^+$ and adoptively transferred TCR tg $CD4^+$ T cells were compared in this study. These experimental caveats limit the explanatory power of this study.

In all named studies, $CD4^+$ T cell responses were found to be impaired if the antigen presentation was terminated prematurely. The data on $CD8^+$ T cells are contradictory. $CD8^+$ T cells were unaffected by a shortened *L. monocytogenes* infection (Williams and Bevan, 2004) or did at least require shorter infection periods to expand as much as in control mice (Corbin and Harty, 2004; Gett et al., 2003). In contrast, $CD8^+$ T cell responses were observed to be

dependent on prolonged antigen presentation in two studies (Blair et al., 2011; Tseng et al., 2009).

The methodological caveats of the above studies are circumvented in the experimental setup used here. TCR stimulation is strictly separated from inflammation and terminated in a way that efficiently avoids residual stimulation. The stimulation of both subsets is carried out independently of their TCR specificity, affinity and the APC type. Thus, distinct antigen presentation- and costimulatory patterns, which might occur in infection models and may result in differential TCR triggering strength, are circumvented.

With the exception of (Gett et al., 2003), the above studies based their conclusions solely on the determination of $CD4^+$ and $CD8^+$ T cell expansion. Besides the extent of proliferation, this parameter may also be influenced by divergent survival rates, precursor frequencies and precursor commitment. In the present study, the extent of proliferation was quantified using CFSE dilution analysis. The additional assessment of cell cycle activity allowed for a detailed analysis of the proliferative patterns of $CD4^+$ and $CD8^+$ T cells.

The differential antigen dependency of $CD4^+$ and $CD8^+$ cells observed here was not restricted to TCR tg T cells, as the findings were reproduced with polyclonal T cells. The number of T cells transferred, previously observed to hamper T cell expansion (Badovinac et al., 2007; Quiel et al., 2011; Yarke et al., 2008) was ruled out to impair $CD4^+$ T cell proliferation here. T cell help was suggested to enhance the proliferation of $CD8^+$ T cells by supporting the antigen presentation to $CD8^+$ T cells (Jusforgues-Saklani et al., 2008) or by $CD4^+$ T cell derived IL-21 (Yi et al., 2009). The use of TCR tg T cells and the separation of $CD4^+$ and $CD8^+$ T cells excluded T cell help as a factor enhancing the proliferation of $CD8^+$ T cells in the present study. The cytokine IL-7 used here to enhance the survival of unstimulated T cells during the in vitro-priming-phase was chosen for its exclusive importance for homeostatic survival in vivo (Vivien et al., 2001). IL-7 was observed not to superimpose with TCR stimulation effects on T cell survival (Koenen et al., 2013). The O_2 concentration in the culture system used for T cell priming was, in contrast to previous in vitro-studies (Atkuri et al., 2007; Loisel-Meyer et al., 2012), shown not to affect the in vivo-proliferation of T cells.

Therefore, in the experimental setting used here differential antigen dependency of $CD4^+$ and $CD8^+$ T cells during the expansion phase in vivo was shown for the first time in a truly comparable manner (Fig. 5-9, (Rabenstein, 2012)), confirming the concept of $CD8^+$ but not $CD4^+$ T cell proliferation and differentiation as being set to “autopilot” following successful

priming (Bevan and Fink, 2001). This observation is in agreement with studies on the antigen dependency of $CD4^+$ T cell expansion (see also section 3.5.1). With respect to the substantial number of studies demonstrating antigen-dependent expansion of $CD8^+$ T cells, the data presented here indicate that $CD8^+$ T cells possess the potential to be programmed to expand and differentiate in an antigen-independent manner (see also section 3.5.2).

6.2 The differential antigen dependency of T cells is cell intrinsic

The experimental setup used here allowed for the further dissection of the mechanism causing the differential antigen dependency of $CD4^+$ and $CD8^+$ T cells, which pointed towards a cell intrinsic regulation of this phenomenon.

Homotypic clustering of $CD8^+$ T cells has been observed to regulate their proliferation and differentiation in recent studies. Independent studies showed that the abrogation of cluster formation reduces the accumulation of $CD8^+$ effector cells, their functionality and the protection from secondary infection (Bose et al., 2013; Gérard et al., 2013). In contrast, ICAM1-deficient and therefore non-clustering $CD8^+$ T cells showed enhanced effector cell accumulation and effector cell function in vivo, rather pointing towards an attenuating role of homotypic clusters (Cox et al., 2013; Zumwalde et al., 2013). While the mechanistic understanding of the regulatory role of homotypic T cell clusters is still incomplete, they were dispensable for antigen-independent proliferation of $CD8^+$ T cells here.

Tonic TCR triggering has been suggested to be essential for the homeostatic proliferation and the survival of naïve peripheral T cells (Beutner and MacDonald, 1998; Brocker, 1997; Goldrath and Bevan, 1999; Kirberg et al., 1997; Takeda et al., 1996) and was later shown to regulate the responsiveness of $CD8^+$ T cells to antigenic stimulation (Ebert et al., 2009; Lo et al., 2009; Santori et al., 2001). Antigen-independent proliferation of $CD8^+$ T cells has been observed here even in the absence of peripheral MHC I, segregating this phenomenon from homeostatic proliferation and excluding tonic TCR triggering as a cause of this behavior.

Even though proinflammatory cytokines were repeatedly shown to be required for $CD8^+$ T cell expansion (Curtsinger et al., 2003; Curtsinger et al., 1999; Kolumam et al., 2005; Lai et al., 2009; Starbeck-Miller et al., 2014), antigen-independent proliferation of $CD8^+$ T cells was observed here in a sterile environment. Furthermore, the lack of proinflammatory cytokines was not responsible for antigen dependency of $CD4^+$ T cells, as their proliferative block was not released in the presence of a bystander infection, contrasting with the dependency of $CD4^+$ T cell expansion on cytokines reported previously in an in vitro-study (Jelley-Gibbs et

al., 2000). Thus, the differential antigen dependency of T cell subsets seemed to be independent of a "third signal" meditated by inflammatory cytokines.

In opposition to costimulatory signals, coinhibitory signals delivered via CTLA-4 or PD-1 were observed to regulate T cell responses. The blockage of CTLA-4 signaling augmented T cell proliferation in vitro (Krummel and Allison, 1995; Walunas et al., 1994). The ligation of CTLA-4 reduced the motility of T cells and increased the duration of their contacts with DCs in vitro (Schneider et al., 2006). It was proposed that CTLA-4 signaling limits the in vitro-proliferation of $CD4^+$ T cells (Doyle et al., 2001). Signaling via PD-1 has been reported to be essential for tolerance maintenance by using knock out mice and blocking mAbs against PD-1 and its ligands PD-L1 and PD-L2 (Ansari et al., 2003; Fife et al., 2009; Nishimura et al., 1999; Salama et al., 2003). In mice deficient for PD-L1 (*Cd274*$^{-/-}$), $CD4^+$ and $CD8^+$ T cell responses were markedly enhanced (Latchman et al., 2004). Even if this body of literature may suggest that the antigen dependency of $CD4^+$ T cells is at least partially dependent on these coinhibitory pathways, the blockage of CTLA-4 and PD-1 signaling did not extend the proliferation of $CD4^+$ T cells following transient TCR stimulation in the study presented here. As a strong TCR stimulus was used here, this finding is in agreement with studies that observed the inhibition of T cell proliferation by PD-1 signaling if low antigen- or costimulation doses were used (Freeman et al., 2000; Latchman et al., 2001).

Antigen dependency was observed for both Th1 and Th2 cells in this study. These $CD4^+$ T cell subpopulations are distinguished on the functional level, based on the detection of distinct cytokine profiles and transcription factor expression (see also section 3.4.2.1). Compared to Th1 cells, Th2 polarized cells displayed a slightly reduced proliferative capacity in vivo in the present study. This was unexpected, as IFNγ has been suggested to limit the proliferation of T cells (Badovinac et al., 2004; Feuerer et al., 2006) and Grogan et al. reported more extensive proliferation of Th2 polarized $CD4^+$ T cells compared with Th1 polarized cells (Grogan et al., 2001). Grogan et al. used T cells from BALB/c mice, which are known to have a propensity towards Th2 responses (Locksley et al., 1999) and assessed the proliferation of Th1 and Th2 cells in vitro following a 7 d stimulation period. In the present study, the proliferation of the subsets was analyzed after a polarizing period of only 2 d and subsequent adoptive transfer into mice expressing or not expressing cognate antigen. The lack of further data on this issue and the differences in protocols and origin of Th cells make further studies necessary to elucidate the cause of these contradictory observations.

The proliferative capacity of memory T cells on the other hand has been broadly studied and the response to low doses of antigen, rapid proliferation and immediate cytokine production are thought to be hallmarks of memory T cell responses. Whereas these paradigms are supported by some studies (Rogers et al., 2000; Veiga-Fernandes et al., 2000), the details of memory T cell proliferation kinetics are still not fully understood. Macleod at al. observed that memory $CD4^+$ T cells expanded less than naïve ones, leaving the higher precursor frequencies of $CD4^+$ memory T cells as the cause of their enhanced accumulation (MacLeod et al., 2008). Whitmire et al. showed that memory T cell responses are not instantaneous. They observed both memory and naïve T cells to proliferate after a lag time of 2-3 d in vivo (Whitmire et al., 2008). Thus, in contrast to the paradigm of stronger, faster and higher memory responses, the antigen-requirements of memory T cells seem to be similar to that of naïve T cells in the described experimental settings. In the experimental system used here, antigen dependency of $CD4^+$ T cells was conserved in the memory compartment represented by in vitro-generated memory-like Rested Effector cells. These data support the idea that besides antigen responsiveness and proliferation rate, further factors, e.g. cytokine responsiveness or acquired survival advantages (even on the metabolic level, see section 6.3.3) may support the secondary responses of $CD4^+$ T cells. Methodological progress that facilitates the analysis of very small cell populations (Moon et al., 2007) will foster studies on the physiology of memory responses and will allow further elucidation this issue.

6.3 The mechanism of differential antigen dependency

6.3.1 On the transcriptional level, $CD4^+$ and $CD8^+$ T cells are very similar

In agreement with a recent publication (Mingueneau et al., 2013), the gene expression analysis of $CD4^+$ and $CD8^+$ T cells following in vitro stimulation (at the point of treatment divergence), revealed a high similarity of both subsets with only 0.5% of genes being differentially regulated. Even though the source of T cells and the stimulus duration limit the comparability of the datasets generated by Mingueneau et al. and presented here, the similarity of $CD4^+$ and $CD8^+$ T cells is a common feature of both analyses.

Except for Eomes, no further T cell specific transcription factors were differentially expressed in the subsets. Runt-related transcription factor 2 (*Runx2*), a transcription factor initially recognized for its importance in bone development (Ducy et al., 1997; Komori et al., 1997), was differentially upregulated in $CD8^+$ T cells in agreement with data available from

the Immunological Genome Project (Immgen) database (Heng et al., 2008), as retrieved on February 24th, 2014). Recently, a role for Runx2 in $CD8^+$ memory T cells has been discussed (Hu and Chen, 2013). In that study, the overexpression of *Runx2* in in vitro-generated $CD8^+$ memory T cells resulted in decreased expansion of these cells in vivo. Thus, Runx2 seems to be involved in T cell activation and perhaps also in their proliferation, but the details of its function in T cells are still unclear.

Among the genes differentially upregulated in $CD4^+$ T cell was *Sprouty1* (*Spry1*), a homolog of a gene first described to code for an inhibitor of fibroblast growth factor (FGF) signaling in *Drosophila melanogaster*, where it was suggested to be part of a negative feedback mechanism in receptor tyrosine kinase (RTK) signaling (Hacohen et al., 1998). Spry1 is one of four sprout-homologs defined in mammals and the only one known to be specifically upregulated in T cells following their activation (Choi et al., 2006). Whereas *Spry1*$^{-/-}$ $CD4^+$ T cells display enhanced IL-2 expression, IFNγ production is increased in *Spry1*$^{-/-}$ $CD8^+$ T cells, indicating an inhibitory influence of *Spry1* on T cell effector functions in both subsets. Under physiological conditions, Spry1 is recruited to the immune synapse following TCR ligation and its absences results in enhanced phosphorylation of LAT, PLC-γ and Erk (Collins et al., 2012; Lee et al., 2009). The role of Spry1 during later phases of the T cell response and its influence on T cell proliferation is only poorly studied, but tumor-specific $CD8^+$ T cells deficient for Spry1 display enhanced expansion compared to wt cells in a tumor model (Collins et al., 2012). From the data available to date, it cannot be excluded that *Spry1* regulates the proliferation of T cells. Despite the lack of data on its differential expression in $CD4^+$ and $CD8^+$ T cells in the above studies, it is therefore possible that the enhanced expression in $CD4^+$ T cells observed here contributes to the differential antigen dependency of $CD4^+$ and $CD8^+$ T cells.

Two members of the growth arrest and DNA-damage-inducible 45 (*Gadd45)* gene family, *Gadd45b* and *Gadd45g* were differentially upregulated in stimulated $CD8^+$ T cells in the study presented here. Gadd45 family members are small proteins (18-20 kDa) without enzymatic activity that are involved in the regulation of cell cycle arrest, DNA repair, cell survival and apoptosis following genotoxic stress in malignant cells (Liebermann et al., 2011). They are expressed in many cell types of the innate and the adaptive immune system. The expression of *Gadd45b* and *Gadd45g* in T cells can be induced by T cell activation and proinflammatory cytokines (Schmitz, 2013) or by genotoxic stress (Flint et al., 2005). Both molecules were reported to promote the expression of IFNγ in $CD4^+$ and $CD8^+$ T cells (Lu et

al., 2004; Lu et al., 2001; Yang et al., 2001). Lu et al. showed that Gadd45β is also important for the IL-2 production by $CD4^+$ T cells (Lu et al., 2004). Gadd45γ was observed to be dispensable for normal T cell development and proliferation (Hoffmeyer et al., 2001). As most of these studies were performed in vitro, the importance of Gadd45 proteins for T cell responses in vivo is still unclear. The stimulatory effect that Gadd45β and Gadd45γ have on cytokine expression is mediated by the activation of the MAP-Kinase pathway and the mitogen-activated protein kinase p38 (Lu et al., 2004; Lu et al., 2001; Yang et al., 2001). Here, a higher expression of *Gadd45b* and *Gadd45g* in $CD8^+$ compared to $CD4^+$ T cells was observed. In agreement with the above-described studies, this differential expression was correlated with the secretion of IFNγ in $CD8^+$ but not $CD4^+$ T cells at the time point of gene expression analysis. Thus, it seems likely that *Gadd45b* and *Gadd45g* expression is correlated with the differentiation status of both subsets.

Another gene differentially upregulated in $CD8^+$ T cells was Cdk5 and Abl enzyme substrate 1 (*Cables1*), a protein found to be interacting with cycline dependent kinases (CDKs) and expressed in all tissues but especially in neurons, where its knock-down inhibited cell growth (Matsuoka et al., 2000; Zukerberg et al., 2000). Human ovarian and colon cancers frequently present a loss of *Cables1* expression (Dong et al., 2003; Kirley et al., 2005; Park et al., 2007a; Zukerberg et al., 2004). Cables1 seems to be necessary for apoptosis induced by genotoxic stress (Wang et al., 2010) and *Cables1*-deficient mice present higher numbers of oocytes with reduced quality compared to wt mice (Lee et al., 2007). It can only by hypothesized that the differential upregulation of *Cables1* in stimulated $CD8^+$ T occurs in response to proliferation-associated stress and may support enhanced DNA-quality control during proliferation. The high expression of this gene may provide a proliferative advantage to $CD8^+$ T cells during the expansion phase.

Taken together, gene expression data illustrated the equality of $CD4^+$ and $CD8^+$ T cell stimulation reached here and revealed only few genes to be regulated differentially between the subsets. The differential expression of genes associated with the genotoxic stress response and *Spry1* could be correlated with the phenomenon of differential antigen dependency. The importance of these genes for the antigen dependency of T cell proliferation might be tested in the future with the help of T cells deficient in these genes or on T cells subjected to retroviral overexpression- or knock-down of the respective genes.

With respective to the differential proliferation patterns and the broadly different gene expression patterns observed following transient and persistent TCR stimulation (Rabenstein,

2012), the very similar gene expression of in vitro-stimulated T cells indicates that the differential proliferative response are not sole regulated at the transcriptional level. Additionally, potential regulatory mechanisms may include posttranslational protein modifications, miRNAs and epigenetic processes.

Micro RNAs (miRNAs) are emerging as important regulators of $CD4^+$ T cell differentiation and function (Baumjohann and Ansel, 2013) and were observed to regulate the effector functions of $CD8^+$ T cells (Gracias et al., 2013). However, they might not be essential for T cell survival (Jeker and Bluestone, 2013), possibly due to the fact that mRNAs of proliferating T cells possess fewer miRNA target sites (Sandberg et al., 2008). Nevertheless, miRNAs may be involved in the regulation of T cell proliferation and differentiation under conditions of limited antigen access and their role for the differential antigen dependency of $CD4^+$ and $CD8^+$ T cells could be addressed in future studies.

Epigenetic pathways are involved in the differentiation process of Th1 and Th2 cells by activating or silencing respective effector genes (Avni et al., 2002; Wei et al., 2009). Recently, the histone methylase SUV39H1 has been reported to be necessary for the silencing of Th1 genes and thus stabilizing Th2 differentiation (Allan et al., 2012). Thus, a specific histone-modifying enzyme could be required for the commitment towards $CD4^+$ and $CD8^+$ T cell phenotype and the programming of proliferative patterns. Mice deficient for chromatin-modifying enzymes like SUV39H1 can be used to elucidate this question in the future.

6.3.2 Differential proliferation kinetics of $CD4^+$ and $CD8^+$ T cells

In many viral infection models and following infection with *L. monocytogenes*, the magnitude of the $CD8^+$ responses exceeds that of $CD4^+$ T cell responses (Cauley et al., 2002; Foulds et al., 2002; Harrington et al., 2002; Homann et al., 2001). On the base of anecdotal data, $CD4^+$ and $CD8^+$ T cells are perceived to divide at different paces (Seder and Ahmed, 2003). In different experimental systems, $CD4^+$ T cells have been found to divide every 7-12 h (Gudmundsdottir et al., 1999; Jelley-Gibbs et al., 2000; Lee et al., 2002), whereas $CD8^+$ T cells divided every 2-6 h (Wong and Pamer, 2001; Yoon et al., 2010). However, the division pace of T cells might vary considerably between clones or depending on the experimental context (Hogan et al., 2013; Rai et al., 2009; Yoon et al., 2010), complicating a comparative analysis on this issue. The assessment of T cell proliferation kinetics in infection models revealed that TCR tg $CD4^+$ T cells expand slower than TCR tg $CD8^+$ T cells (Foulds et al., 2002), but polyclonal T cell populations displayed no differential kinetics of T cell expansion

(Schlub et al., 2011) as would have been suggested by the former study. Thus, the inflammatory environment during an infection further limits the explanatory power of these types of experiments, as $CD4^+$ and $CD8^+$ T cell might react differentially to the associated stimuli. As evidenced by the very similar activation and the extent of proliferation shown by both subsets following persistent TCR stimulation, these caveats were circumvented here. The combination of the CFSE dilution and cell cycle activity assessment using DAPI and Ki67 was used for a deeper analysis of proliferation kinetics of both subsets.

Here, $CD8^+$ T cells were observed to divide faster than $CD4^+$ T cells during the expansion phase following transient TCR stimulation, but this antigen-independent proliferation phase was time-limited. Additionally, $CD8^+$ T cells remained in active stages of the cell cycle for prolonged periods of time following persistent TCR stimulation, pointing towards a prolonged sensitivity to TCR triggering-induced proliferation. These findings suggest differences in the proliferation kinetics of $CD4^+$ and $CD8^+$ T cells that are more complex than previously assumed by Seder et al. (Seder and Ahmed, 2003).

In the future, the extension of the cell cycle analysis to further time points and the use of additional cell cycle activity probes could further complement these findings. If mice are treated with the thymidine analogue BrdU for a given time, the proliferation of a cell population during this period can be assessed by flow cytometry. Simultaneously, the percentage of cells in S- or G_2-phase (identified using a DNA dye) in a population that had divided during the previous BrdU-feeding period can be determined. The combination of both probes would allow for the direct comparison of G_1-phase duration of $CD4^+$ and $CD8^+$ T cells (Yoon et al., 2010). Contrary to the use of CFSE as a proliferation probe, the treatment with BrdU might be started not only at the time point of adoptive transfer but also at several time points through the experiment. By using this type of analysis to characterize proliferation velocities of T cells during the expansion phase, detailed knowledge of the proliferation kinetics of $CD4^+$ and $CD8^+$ T cells could be obtained.

From the data presented here, it can be hypothesized that $CD8^+$ T cells might divide faster during the whole expansion phase. For both subsets, the division pace might be highest at the starting point of expansion, with $CD4^+$ T cells slowing down earlier than $CD8^+$ T cells irrespective of continued or transient antigen presentation. A delayed responsiveness of $CD8^+$ compared to $CD4^+$ T cells to the termination of antigen presentation and an overall enhanced proliferative capacity of the former subset would be in agreement with both the phenomenon

of differential antigen dependency and the prolonged proliferative response of $CD8^+$ T cells to antigen.

6.3.3 T cell differentiation and proliferation are correlated to metabolic processes

T cell activation, differentiation, proliferation and the mediation of T cell effector functions are metabolically demanding processes. The importance of metabolic adaptations for T cell differentiation is receiving increasing interest. Fostered by methodological progress (Wu et al., 2007), T cell effector function, differentiation and memory formation have been revealed to depend on and be regulated by metabolic processes (Chang et al., 2013; Doedens et al., 2013; Man et al., 2013; Powell et al., 2013; van der Windt et al., 2012; Zeng et al., 2013), adding a new layer of complexity to the regulation of immune responses. Metabolic pathways are no longer perceived to be exclusively regulated by allosteric mechanisms but to be influenced by other regulatory networks and vice versa (Pearce et al., 2013).

For rapid proliferation during the expansion phase, T cells rely mainly on aerobic glycolysis (Frauwirth et al., 2002; Gerriets and Rathmell, 2012), a process known as the Warburg effect and recognized to provide proliferating cells not only with energy but also with metabolic precursors for catabolic building blocks. During resting or quiescent differentiation phases but also immediately following T cell activation, oxidative phosphorylation may be of paramount importance (Chang et al., 2013). Reactive oxygen species (ROS) that arise from the premature and incomplete reduction of oxygen during this process have been observed to act as secondary messengers. Complex II of the electron transport chain has been shown to be an important source of mitochondrial ROS (Tormos et al., 2011; Weinberg et al., 2010). T cells with a deficiency for a subunit of this complex needed for ROS production showed impaired proliferation and IL-2 production in response to in vitro activation and displayed a reduced in vivo-expansion following immunization (Sena et al., 2013). Thus, mitochondrial ROS might allow for the integration of metabolic status information with T cell activating signals and adapt T cell responses to potential metabolic limitations.

A rapid switch to aerobic glycolysis has repeatedly been shown to be important for memory T cells, enabling them to mediate effector functions almost immediately following restimulation (Fraser et al., 2013; Gubser et al., 2013; van der Windt et al., 2012; van der Windt et al., 2013). In these studies, the mitochondrial spare respiratory capacity (SRC), a parameter correlated with cell survival and stress resistance (Yadava and Nicholls, 2007), was reported to be a key metabolic feature of memory T cells. A high SRC is thought to provide

them with enhanced resistance to metabolic stress and allow them to survive once the inflammation is cleared and survival signals are withdrawn. Furthermore, the oxidation of fatty acids seems to be of special importance for memory T cells (Pearce et al., 2009). Metabolic capacities of $CD4^+$ and $CD8^+$ T cells have so far not been directly compared and it can therefore not be excluded that their different functional properties and the differential antigen dependency observed here are correlated with distinct metabolic properties.

In order to determine whether the metabolic capacities of $CD4^+$ and $CD8^+$ T cells differ, the mitochondrial stress response of both subsets was analyzed following in vitro stimulation. In agreement with the above studies, T cell activation increased glycolysis and to a smaller extent also oxidative phosphorylation in stimulated T cells. Stimulated $CD8^+$ T cells displayed an increased spare respiratory capacity (SRC) compared with their $CD4^+$ counterparts. Following adoptive transfer into antigen-free mice, $CD4^+$ and $CD8^+$ T cells are not only confronted with the withdrawal of TCR- and costimulatory signals but also with a strong reduction in nutrient- and oxygen concentrations compared to the cell culture system (Pearce et al., 2013). It can be hypothesized that in analogy to memory T cells, their enhanced metabolic stress resistance might allow $CD8^+$ T cell to continue proliferation following TCR stimulus withdrawal, whereas $CD4^+$ T cells, due to the lack of this metabolic property, are unable to continue proliferation following transient TCR stimulation.

In the future, metabolic characterization of naïve $CD4^+$ and $CD8^+$ T cells (both TCR tg and polyclonal), as well as transiently and persistently stimulated $CD4^+$ and $CD8^+$ T cells sorted from recipient mice may extend the validity of these findings. A metabolic disparity of $CD4^+$ and $CD8^+$ T cells may be correlated with their differential functions in the adaptive immune response.

6.4 Differential antigen dependency in the context of the immune response

In this study, the prolonged presentation of MHC II presented $MCC_{93\text{-}103}$ but not MHC I presented $OVA_{257\text{-}264}$ peptide on the surface of activated DCs has been shown in vivo, in agreement with previous studies (Cella et al., 1997; Obst et al., 2007). In a recent study, similar observations were made on human monocyte-derived DCs, using stable isotope labeling of nonessential amino acids with heavy water (Robert Busch, London, personal communication).

Differential stability of MHC molecules may not be the only cause of differential presentation kinetics of pMHC complexes to $CD4^+$ and $CD8^+$ T cells. When DC subsets were

first characterized, they were shown to display distinct antigen presentation capacities. Whereas $CD8^+$ DCs were specialized in crosspresentation, $CD8^-$ DCs displayed a pronounced ability to present peptides in the context of MHC II (den Haan et al., 2000; Dudziak et al., 2007; Pooley et al., 2001), indicating that $CD4^+$ and $CD8^+$ T cells may be primed by different DC subtypes. This hypothesis is supported by a recent study that observed the transcription factor interferon regulatory factor 4 (IRF4), on which $CD8^-$ DCs rely for their development, to be responsible for the expression of components of the MHC II pathway in these cells (Vander Lugt et al., 2013).

Furthermore, the sources of peptide presentation by MHC I and MHC II molecules are distinct: MHC I molecules are loaded with peptides derived from proteasomal proteolysis of cytoplasmic proteins in the endoplasmic reticulum. MHC II loading takes place in late endosomes. Peptides generated by the lysosomal degradation of proteins that have been taken up by phagocytosis, macropinocytosis or endocytosis are the main source of MHC II antigen presentation (see also section 3.2). Additionally, MHC II presented peptides can also derive from cytoplasmic proteins if these are degraded by autophagy and extracellular protein derived peptides can be loaded on MHC I molecules by a process called crosspresentation (Blum et al., 2013). It has been suggested that MHC I presented peptides derive from incompletely translated or misfolded proteins that are quickly degraded and are therefore called defective ribosomal products (DRiPs (Yewdell et al., 1996)). Even though there is growing evidence for DRiPs as a major source of MHC I presented peptides (Yewdell, 2011), some studies show that this is not an exclusive pathway as MHC I molecules can also present peptides derived from mature proteins (Colbert et al., 2013; Farfán-Arribas et al., 2012).

Mackay et al. addressed this question in a comparative manner. They used an in vitro-system of doxycycline-dependent antigen expression in EBV transformed human B cell lines, where they could precisely terminate mRNA transcription and monitor protein stability. They observed a clear correlation between active gene transcription and the presentation of pMHC I complexes as measured by the response of cognate $CD8^+$ T cell clones. On the other hand, the presentation of peptides in the context of MHC II correlated with the stability of mature protein (Mackay et al., 2009), thus supporting the concept of MHC I presented peptides being derived from active gene expression whereas MHC II peptides originate from mature proteins. This concept implies that the antigen presentation to $CD4^+$ and $CD8^+$ T cells display intrinsically different kinetics.

Such a kinetic difference has in turn been reported to influence T cell responses in agreement with data presented here: In an in vivo system of antigen-targeting to DCs by mAbs, a strong $CD4^+$ T cell mediated antibody production was elicited by a targeting-mAb with a long serum half-life in the absence of adjuvants (Lahoud et al., 2011). This finding indicates that DCs can initiate T cell responses even in the absence of "danger signals" and challenges the current concept of their role in the mediation of tolerance and immunity (Caminschi and Shortman, 2012).

Increasing the complexity of this issue further, $CD4^+$ T cells have been observed to decrease the expression of MHC II on DCs following cognate interaction. This phenomenon might be related to cross-linking induced MHC II degradation (Furuta et al., 2012) or trogocytosis (the uptake of cell membrane patches containing the TCR-ligated MHC II), a process that could in turn lead to prolonged TCR signaling (Osborne and Wetzel, 2012).

Taken together, the differential antigen dependency of $CD4^+$ and $CD8^+$ T cell proliferation observed in this study seems to correlate with the differential kinetics of antigen presentation to the subsets. Even though the stabilization of pMHC II complexes following DC activation observed here might not be universally transferable to all APCs and antigenic epitopes, it can by hypothesized that $CD4^+$ T cells experience prolonged antigenic stimulation in the context of an infection due to the stabilization of pMHC II on the surface of activated DCs and the stability of mature proteins as the source of peptides. On the other hand, the presentation of peptides in the context of MHC I may be limited both by the turnover of pMHC I and the dependence on gene transcription for peptide generation.

The differential proliferation patterns observed here seem to be consistent with the distinct functions of $CD4^+$ and $CD8^+$ T cells during adaptive immune responses. $CD4^+$ T cells orchestrate adaptive immunity and possess the potential to differentiate into a variety of subsets characterized by specific effector and immune regulatory functions (see also section 3.4.2.1). These subsets display a certain plasticity (O'Shea and Paul, 2010), a potential that might require strict regulation in order to prevent dysfunction. The differentiation spectrum of $CD8^+$ T cells, on the other hand, appears to be much less diverse with cytotoxicity as the key function ((Kaech and Cui, 2012), see also section 3.4.2.2). In order to fulfill their main task, the elimination of virus-infected or neoplastic cells, strong expansion of uniform $CD8^+$ T cells is sufficient for clearance. Strict control of $CD4^+$ T cell proliferation via antigen presentation could thus provide a rheostat to prevent overshooting and deregulated responses, a measure that might not be equally important for $CD8^+$ T cells.

Additionally, the differential antigen dependency of both subsets might allow for a negative feedback of adaptive immune responses, as cytotoxic $CD8^+$ T cells were suggested to eliminate antigen presenting DCs (Hermans et al., 2000; Ritchie et al., 2000; Wong and Pamer, 2003b) and might thus terminate the antigen-dependent proliferation of $CD4^+$ T cells (Ma et al., 2012b), preventing in turn further T cell help to $CD8^+$ T cells. Thus, an antigen-independent expansion phase of $CD8^+$ T cells could contribute to the prevention of overshooting immune responses.

Recently, even antigen surveillance strategies have been reported to differ between $CD4^+$ and $CD8^+$ T cells. Naïve $CD4^+$ T cells were observed to stay in a given lymph node for shorter periods of time than $CD8^+$ T cells (~12 h versus ~21 h) and made contact with fewer DCs during that time (~160-200 DCs) than their $CD8^+$ counterparts (~300 DCs) (Mandl et al., 2012). Thus, $CD4^+$ T cells would scan more lymph nodes than $CD8^+$ T cells in a given time period but in a less rigorous manner. Interestingly, only for $CD4^+$ T cells was the lymph node transit time in the steady state dependent on MHC presence. Additionally, also tissue resident $CD4^+$ and $CD8^+$ memory T cells show different migration behaviors: while $CD4^+$ memory T cells migrate more freely through tissues after the infections is cleared, $CD8^+$ memory T cells seem to be retained at the site of infection in an antigen-independent way (Gebhardt et al., 2011; Mackay et al., 2012). These findings suggest that $CD4^+$ T cell responses are more dependent on MHC/TCR interactions than $CD8^+$ T cell responses, which operate in a more programmed way.

In summary, the primary expansion of $CD4^+$ T cells has been shown to be more dependent on antigen persistence than that of $CD8^+$ T cells in the present study. This finding is consistent with the differential kinetics of antigen presentation to both subsets and the distinct sources of antigenic peptides. The differential antigen dependency of $CD4^+$ and $CD8^+$ T cells may enhance the efficiency of adaptive immune responses and might also be important for the regulation of T cell responses.

6.5 Outlook

In the study presented here, differential antigen dependency of $CD4^+$ and $CD8^+$ T cells has been shown to be cell intrinsic and correlated with differential proliferation kinetics and partial metabolic disparity. Further and more detailed studies on the proliferation kinetics of both subsets could expand the findings. A deeper analysis of $CD4^+$ and $CD8^+$ T cell metabolism, including further time points and experimental conditions (naïve polyclonal and

TCR tg T cells as well as T cells subjected to transient and persistent TCR stimulation), target processes (e.g. glycolysis, fatty acid metabolism) and experimental approaches (monitoring of the metabolism of $CD4^+$ and $CD8^+$ T cells during the priming phase in vitro (Gubser et al., 2013)) may provide a deeper understanding of the metabolic requirements of both subsets. The potential influence of the RTK-inhibitor *Spry1* and the genotoxic stress-associated genes *Cables1*, *Gadd45b* and *Gadd45b* on differential antigen dependency of $CD4^+$ and $CD8^+$ T cells could be addressed by using T cells deficient in (either from knock-out mice or generated by retroviral knock-down) or overexpressing (by retroviral overexpression) the respective genes. Even though the existence of one transcriptional master regulator for the phenomenon described here is not anticipated from the gene expression data generated in the present study, these experiments could allow further insight into the underlying mechanism.

The background expression of $OVA_{257-264}$ in dtg-O mice could be abrogated by the introduction of a tetracycline-controllable transrepressor, which has been shown to result in tight expression of doxycycline regulated gene expression (Jellison et al., 2012). Following this improvement, the mouse model of doxycycline-dependent antigen expression could be utilized to extend the comparison of $CD4^+$ and $CD8^+$ T cell behavior to the fields of memory differentiation and function, T cell exhaustion and tolerance induction. In all of these fields, direct comparisons of both subsets have thus far rarely been done despite sustained interest in the mechanisms governing the respective differentiation processes. For example, $CD8^+$ memory T cells have recently been observed to be sensitive to bystander activation during a heterologous secondary infection (Chu et al., 2013). The transferability of these findings to the $CD4^+$ T cell compartment could be addressed in dtg mice following the above-described modification.

7 References

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Lebenslauf

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Publikationen

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Eidesstattliche Erklärung

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Hiermit erkläre ich an Eides statt,
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